The goal of this research is to provide a greater chemical understanding of the mechanisms by which *Bacillus subtilis* transforms itself into a spore during times of nutrient deprivation. The area of interest of this study lies in the mother cell metabolic gene (mmg) operon, which is activated by a sigma-E transcription factor, during the early stages of sporulation. This operon is proposed to play roles in fatty acid metabolism, the methyl citric acid cycle, and the citric acid cycle during the intermediate stages of sporulation. The genes within this operon: *mmgA, mmgB, mmgC, mmgD, mmgE*, and *yqiQ* encode for proteins that have proposed biochemical roles to promote the above activities. The goal of this thesis is to biochemically confirm the activity of the protein encoded by the *yqiQ* gene. Our hypothesis is that this gene encodes for a 2-methylisocitrate lyase, the last enzyme of the methylcitric acid cycle. Methylisocitrate lyases catabolize 2-methylisocitrate into pyruvate and succinate, interconnecting the activities of the methylcitric acid cycle and the citric acid cycle of the bacterium. This pathway ultimately allows for the entrance of propionate from the metabolism of odd-chain fatty acids into the bacteria’s citric acid cycle to promote ATP production.

From work done in this lab previously, *yqiQ* has been cloned, and its protein (YQIQ) was isolated and purified. In this work we optimized the yield of this purification producing approximately 2.17 milligrams per liter of culture. We are studying the “reverse” reaction by utilizing an assay which uses equimolar concentrations of pyruvate and succinate to produce 2-methylisocitrate (1). The reverse reaction is being conducted due to the lack of commercial
availability of 2-methylisocitrate. Results have been able to provide partial evidence of this conversion over time. In order to investigate the proposed “forward” reaction mechanism, we are also working on the chemical synthesis of 2-methylisocitrate.
CHARACTERIZATION OF THE BIOCHEMICAL ACTIVITY OF THE OPEN READING FRAME

“YIQ” OF BACILLUS SUBTILIS

by

William T. Booth, II

A Thesis Submitted to
the Faculty of The Graduate School at
The University of North Carolina at Greensboro
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of the Requirements for the Degree
Master of Science

Greensboro
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Approved by

______________________________
Committee Chair
To all those who have influenced and supported me through this process as I continue to transcend academically, I thank you from the bottom of my heart.
APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Chair ________________________________

Committee Members ________________________________

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Date of Acceptance by Committee

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Date of Final Oral Examination

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CHAPTER I

BACILLUS SUBTILIS AND THE PROCESS OF SPORULATION

I.A Background Information on Bacillus Subtilis

Bacillus subtilis, also known as hay bacillus, is a Gram positive, non pathogenic bacteria that is commonly found in soil, marine and freshwater sediments, certain foods, and cases of food poisoning (7). This organism is also utilized in the commercial production of antibiotics and surfactants (4). The bacterium is also one of the more dominant forms of bacteria found in seawater in polluted saltwater costal sites (7). B. subtilis is commonly chosen as a model organism because it is highly accessible to genetic manipulation, and in 1997 it was the first Gram positive bacterium to have a completed genome sequence (5). At publication, the genome was said to contain 4,214,810 base pairs and 4100 protein coding genes (5). This organism is considered “robust” and will grow readily in most media, and can be easily isolated for study (7).

I.B Explanation of the Process of Sporulation

One of B. subtilis’s most significant characteristics is its ability to sporulate. The bacterium has been a model organism for the study of sporulation for the past 62 years (7). The process of sporulation generally occurs during times of starvation and/or high bacterial density in which the bacterium produces a dormant, heat and chemical resistant spore (8). When conditions return back to a state in which nutrients are plentiful, the bacterium returns back to its vegetative state (7).
In general terms, the morphological process of sporulation initiates at the end of the exponential (vegetative) growth phase into a state known as the “stationary phase”, the phase at which there is a near halt in the reproduction of the bacterium by binary fission (2). Along with factors of nutrient deprivation and bacterial cell density, regulatory signals from the Kreb's cycle, DNA synthesis, as well as DNA damage also can play a part in the initiation of sporulation (9).

*B. subtilis* undergoes a series of morphological changes during the formation of a dormant endospore. The metamorphosis is generally described in eight stages (2):

Stage zero, describes the vegetative stage, the stage at which the bacterium is actively replicating its DNA (10).

Stage one describes the stage at which the sporulation signal originates, and the DNA is stretched across the length of the cell by synthesized chromatin (10).

At Stage two, the beginnings of what is known as the asymmetric septum, a semi-permeable membrane that develops toward one end of the cell, develops to house one of the copies of DNA transcribed at the zero stage (10). At the start of the second stage, the septum only houses approximately 30 percent of the replicated DNA, but before transition into the next stage the remaining 70 percent is assisted across the septum with the help of transport protein SPOIIIE (The abbreviaton “spo” describes genes utilized during the sporulation process, and “SPO” describes proteins) (10).

Stage three describes the process known as “engulfment” which is when the septum eventually encases the transported genome (10). Under fluorescence microscopy, the engulfment event looks like a small cell within a larger cell. This smaller cell within the larger cell
is referred to as the endospore (10). The larger portion of this cell within which the endospore is housed is referred to as the “mother” cell (10).

Stage four describes a thickening of the newly formed endospore coat surrounding the genome. Peptidoglycan, a common component of all bacterial cell walls, forms within the coat which reinforces it into what is referred to as the “endospore cortex” (10).

In Stage five, the coat continues to thicken from protein deposits received from the mother cell known as “small, acid-soluble spore proteins” or SASP proteins. The aggregation of these proteins on the endospore cortex is what makes the spore resilient in harsh environments (10).

Stage six describes the stage of “spore maturation”. This means that the spore is at the end of its developmental stage, and is ready to be released from the mother cell (10).

Stage seven describes the apoptotic activity of the mother cell, and the mature spore’s eventual release into the non-nutritive environment. The spore will return back to the vegetative state (Stage zero) when the environment surrounding the spore provides enough nutritive materials for growth and replication (10).

I.C Molecular Genetics Overview of Transcription Factors Involved in Sporulation

At the start of sporulation (stage zero to stage one) Krebs cycle regulatory signals along with transcription factors “sigma A and H” initiate a series of phosphate group transfers known as the phosphorelay (4,8). The relay includes the activation of five consecutive kinases, known as kinases A through E, that allow for the passage of a phosphate group from kinase A to the integral sporulation activation protein, SPO 0A (9). Upon phosphorylation the transition from stage one to stage two begins with the activation of transcription factors sigma-E and sigma-F.
Both factors are considered to be in an inactive state until the formation of the asymmetric septum (8).

During Stage two, Sigma-F relegates itself to the smaller portion of the asymmetrical split, while Sigma-E is produced in the mother cell in an inactive form known as “pro-sigma E” (7,8). Once Sigma F is activated it promotes the transcription of the gene spoIIR to produce the protein “SPOIIR”. SPOIIR binds to and activates a septum membrane protein known as “SPOIIGA” (8). Upon activation, the removal of a 27-amino acid peptide strand from pro-sigma E converts it to sigma E. The Sigma F/E interaction plays a major role in the formation of the septum as well as providing direction for the replicated DNA to move toward its endospore, which is supplemented by SPOIIIE as stated in the previous paragraph.

As the transition from stages two to three begin, sigma E activates another sporulation transcription factor known as sigma G (8). Sigma G is found in the newly developed endospore, and only becomes active when engulfment is complete. Sigma G activates the transcription of the spoIVB gene to express for the SPOIVB protein (8). This protein binds to an endospore surface protein complex composed of proteins BofA, SpolVFB, and SpolVFA, and provides a means for the activation of the mother cell transcription factor, sigma K (8). Sigma-K, the last transcription factor in this cascade, has similar properties to sigma E in that sigma K converts from an inactive form “pro-sigma K” until it is activated by the surface complex (8).

This allows for transition into the latter stages of sporulation, maturation stages four through six (9). Sigma K activates what is known as the sigma K regulon, which is a series of genes involved in the formation of the spore coat as well as maturation of the spore (8).

At the conclusion of sporulation, stage 7, the process of apoptotic activation occurs, which is poorly understood (6).
CHAPTER II
EXPLANATION OF THE RESEARCH

II.A Introduction to the σ^E Dependent, *Mmg Operon*, and Current Findings

This research project is based upon the discovery of the sigma E-dependent mother cell metabolic gene operon (*mmg operon*) by Edward Bryan et al. (4). The objective is to gain a clearer chemical understanding of the biochemical activity of the *mmg operon* during the intermediate stages of sporulation. The proteins encoded by the six open reading frames (ORF’s) of this operon are similar to genes that encode for proteins involved in fatty acid metabolism (*mmgA-C*), as well as proteins of the methyl-citric acid cycle(*mmgD,E, and yqiQ*) (2).

Currently, the Reddick group is working towards characterizing each of the *mmg* open reading frames to biochemically define their activity. So far, ORF *mmgA* was successfully cloned, purified, and characterized as an acetoacetyl-CoA thiolase (4). *MmgB* has been cloned and purified, but no activity has been identified (11). *MmgC* has been cloned and purified, and has been shown to have acyl-CoA dehydrogenase activity (11). *MmgD* has been cloned, purified, and characterized as a citrate and methylcitrate synthase by Rejwi Acharya (1). *MmgE* has been cloned, purified, and is currently being characterized by Grant Hardesty. *YqiQ* has been cloned and purified, but only partly characterized. Figure 1 provides a pictorial representation of the organization of the *mmg operon.*
**II.B General Biochemical Explanation of Activity of the Mmg Operon and Current Findings**

Chemically, the *mmg operon* is proposed to have a function in the integration of activity between the citric acid cycle, methyl citric acid cycle, and the process of fatty acid metabolism, all producing elements that lead to the electron transport chain which promotes the formation of adenosine triphosphate (ATP). ATP is a necessary molecule in the progression of almost all biological processes. The proposed chemical mechanism of action of each gene within the operon is described in Figure 2. The diagram describes the proposed progression of propionate through the three cycles. It starts from its liberation from the odd chained fatty acid and its transfer into the methyl citric acid cycle. At the conclusion of the methyl citric acid cycle, *yqiQ* is projected to act as a 2-methylisocitrate lyase (2-methylisocitrate is eventually synthesized from the propionyl group) which cleaves 2-methylisocitrate into pyruvate and succinate. These catabolites are components of the citric acid cycle necessary for energy production during the intermediate stages of sporulation. The net result is the oxidation of propionyl CoA to pyruvate.

So far, confirmed activity of these processes were identified in 2008 by Spencer Russell, from the Reddick lab, in which he was able to provide evidence to support that *MMGB* and *C* played a role in the metabolism of fatty acids (9). In a paper published from the same lab in 2008, Jamye Williams was also able to provide evidence that *mmgA* encodes for an enzyme that

![Figure 1. Pictorial representation of the *mmg operon* (4)](image-url)
acts as a β-ketoacyl thiolase, which likely plays a role in the formation of propionyl CoA from the metabolism of odd chained fatty acids (8).

![Diagram of Fatty Acid Metabolism and Methyl Citric Acid Cycle](image)

**Figure 2.** The proposed biochemical pathway encoded by the *mmg* operon

It was concluded that *MMGA* plays a role in the entrance of propionyl CoA into the bacteria’s methyl citric acid cycle (8). In 2009, Rejwi Acharya, found evidence that *mmgD* encodes for a protein that performs a dual role and supports the activities of the methyl citric acid and citric acid cycles. It was shown that the enzyme facilitates the first step of the methyl citric acid cycle, the formation of methylcitrate utilizing propionyl-CoA and oxaloacetate as substrates. *MMGD*’s secondary role is in the synthesis of citrate, a component of the citric acid cycle, using acetyl-CoA and oxaloacetate as substrates. *MMGE* is proposed to play a role in the
conversion of 2-methylcitrate into 2-methyl aconitase by an elimination of water. This gene has recently been cloned and over-expressed in our lab. The enzyme CITB is the only aconitase found in Bacillus subtilis and is considered to play a role in the rehydration of 2-methylaconitate to form 2-methyl isocitrate. The research done on the enzyme yqiQ, the focus of this thesis, has only been able to provide partial evidence of its catabolic activity.

In comparison to the mmg operon, the prp operons found in microorganisms like E. coli and Salmonella species, have been found to have DNA sequence homology and are both considered to encode for the methylcitric acid cycle(3). The PRPB protein also acts as a 2-methylisocitrate lyase in E.coli, and has sequence homology with yqiQ at approximately 43 percent (2, 15). We have utilized these similarities as a guide to aid us in experimental setup.
CHAPTER III

EXPERIMENTAL SECTION

III.A Cloning of yqiQ Gene (Overview)

The original cloning and isolation of the yqiQ enzyme was conducted by Amy Quattlebaum and Suzette Mills in 2008. The process was conducted by designing DNA primers for polymerase chain reaction (PCR) amplification of yqiQ. This product was ligated into a pET-28a, a commercial plasmid, in-frame with a series of nucleotides that encoded for a long series of histidine residues also known as a “His-tag” (This His-tag will be described in the next section which focuses on the isolation and purification). In order to clone the gene of interest, sequence-specific enzymes known as restriction enzymes are utilized to “cut” the desired section of DNA from the PCR product in order to transfer the desired gene into the plasmid. The newly synthesized DNA and plasmid were connected together utilizing DNA ligase. Upon ligation, the plasmid was transformed into a commercial strain of BL21(DE3) Escherichia coli (E. coli), a genetically modified strain of E. coli that will not destroy foreign DNA. The E. coli was placed on Luria-Bertani agar (LB agar) plates infused with Kanamycin (an antibiotic), and incubated. Upon the growth of transformed bacterial cell colonies, a colony was removed and placed in liquid LB/Kanamycin media for the purpose of creating a culture of the E.coli. This particular bacteria strain was used as a vehicle for increased over-expression of the yqiQ protein, which will be explained in greater detail in the protein expression and isolation section.
III.B The pET-28a Plasmid System

The plasmid system, pET-28a (shown in Figure 3), is a vector used for the purpose of protein expression in *E. coli*. As the diagram illustrates, the system is divided into four major areas: the “origin of replication” (ori-3286) ensures that the plasmid is copied by the dividing *E. coli*. The next major area is referred to as the “lac repressor” (lacI 773-1852) which encodes for a repressor protein that prevents the expression of the new genetic materials and controls transcription of the third major site known as the multi-cloning site (Bpu 1102). This site is the location in which the *yqiq* gene was inserted. The fourth major section of this plasmid is known as the Kanamycin resistance gene (Kan 3995-4807). This section aids in ensuring that only transformed BL21-DE3 *E. coli* will grow.

![Figure 3](image.png)

*Figure 3. The pET-28a plasmid expression system (13)*
III.C Culture and Over-expression of yqiQ

Upon growth of transformed *E. coli* on the Kanamycin agar plate, the new colonies were cultured in liquid LB media to increase the volume of *E. coli* growth. A colony is initially added to 5 ml of liquid LB media, along with an added 0.50 µl of 200 mM kanamycin, and was shaken overnight at 37° Celsius. On the same day, a liter liquid LB media was made which was composed of 10 grams of sodium chloride, 5 g of yeast extract, 10 g of tryptone, and 1 L of nanopure water. The pH of the solution was brought to 7.5 using 1 M solution of sodium hydroxide and continuous agitation. After attaining the desired pH, the solution was autoclaved. Upon cooling to 25° Celsius, 1 ml of 200 mM Kanamycin is added to the solution for selection during the growth of the culture.

After overnight incubation at 18° Celsius, 2 ml from the 5 ml culture was added to the 1 L solution of LB media and allowed to incubate at 37 ° Celsius with shaking at 245 revolutions per minute (rpms). Periodic spectrophotometric tests to determine the rate of bacterial growth was conducted at 595 nm for approximately three hours. Upon attaining an absorbance result of approximately 0.5, the solution was removed from the incubator to prepare for the over-expression phase.

The over-expression of the yqiQ gene was conducted by utilizing a synthetic analog of galactose known as Isopropyl β-D thiogalactopyranoside (IPTG). IPTG aids in the removal of the lac repressor to promote over expression of the yqiQ gene. IPTG was added to the liter LB culture to give total solution concentration of approximately 1.00 mM, and was placed in an incubator, overnight, at 18° Celsius. This solution was rotated at 225 rpms and was tested the following day for the presence of the YQI/Q protein.
III.D Purification of YQIQ

After overnight incubation of the bacterial culture preparations were made to separate the over-expressed protein from the bacterial cell in order to isolate it. The incubated culture was centrifuged at 7500 g at a temperature of 4 ° Celsius, for thirty minutes. After this first stage of centrifugation the supernatant was removed, and the remaining cell pellet was prepared for cell lysis and sonication. This was initiated by the addition of 20 ml of the binding buffer (a solution of 0.5 M NaCl, 80 mM Tris buffer, and 5 mM imidazole at pH 8) to the pellet along with the addition of approximately 300 mg of lysozyme. The suspension was stirred for fifteen minutes. The suspension was sonicated for six, thirty second periods, allowing a thirty second rest interval between periods. At the completion of these periods, the solution was centrifuged for thirty minutes at 14,600 g, also at 4° Celsius. After centrifugation the supernatant was filtered through a 0.45 µm syringe filter and loaded on a Ni-NTA nickel affinity column.

III.E The Nickel Affinity Column

The nickel affinity column, shown in Figure 4, is used to purify protein by utilizing a collection of agarose beads with nickel nitrilotriacetic acid bound in a uniform fashion around the surface of the bead. Imidazole washes, in increasing concentrations, helps to remove bound protein from the bead and allows for filtration through the column. The filtered supernatant from the cell lysis was placed in the compartment above the column and allowed to filter through by gravity. The passage of the supernatant through the column creates interactions between the coordinated nickel sphere of the nickel nitrilotriacetic acid and the Histidine-tags (His-tag®) of the over-expressed protein. The nickel has an affinity for the nitrogens on the ring of the histidine residues of the His-tag®, and keeps the desired protein within the column. All non- His-Tagged proteins are more likely to filter through the column. All eluted fractions are
collected separately and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

**Figure 4.** The anatomy of the Nickel affinity column and picture of the chemical structure of imidazole

Imidazole, also shown in Figure 4, has the same ring structure as the ring on the histidine residue, and is used in a series of washes to first remove undesired proteins and ultimately the protein of interest. The use of imidazole promotes competition between the nitrogens of the imidazole ring and the histidine residues of the protein for the Nickel atom. Imidazole was allowed to filter through the nickel column in a series of washes at the following concentrations: 5 mM described as “binding buffer”, 60 mM the “wash buffer”, and the final
wash at 1 M concentration, the “eluate buffer”. After the eluate wash was conducted, Bradford-Coomasie-Blue Protein Assay indicator was used to determine the presence of protein in the eluate fractions. Upon indication for the blue color change, the eluate fraction was saved, and allowed to dialyze overnight in a solution of 25 mM Tris buffer (pH 8.0) to remove excess imidazole. After the dialysis bag was removed the next day, a small portion of the purified fraction is denatured, centrifuged to remove impurities, and analyzed using SDS-PAGE to confirm isolation of the desired protein.

III.F SDS-PAGE Preparation

To prepare the 12% gel, a solution of: 5.9 milliliters of nanopure water, 5 ml of 30% acrylamide, 3.8 ml of 1.5 molar Tris buffer (pH 8.8), 0.15 ml of 10% SDS, 0.15 ml of Ammonium persulfate, and 0.006 ml of tetramethylethylenediamine (TEMED) was made and added to the gel apparatus. After solidification of the gel, a “stacking gel” was added on top of the 10% gel to improve the quality of protein separation. A plastic sample-well comb was placed into the stacking gel to create testing troughs for placement of the protein control ladder and samples of protein from each collection from the nickel column.

The gel is placed within an electrophoresis instrument. SDS-PAGE running buffer (25 mM Tris buffer, 192 mM glycine, 0.1% SDS at pH 8.3) is added to the instrument. Next, 5.0 microliters of protein control ladder is added to the first trough, and then the remaining samples are added consecutively in separate troughs. After the addition of the last sample, the instrument is covered and connected to a power source set at 200 V, 300 mA, and 300 W. The electrophoresis was allowed to run until the dye in the SDS loading buffer can show movement across approximately three-fourths of the gel. When the desired point is reached, the gel is removed and prepared for staining.
Staining of the gel is conducted by immersing the electrophoresed gel in Coomassie Stain (a solution of methanol, water, Glacial acetic acid, and Coomassie Brilliant Blue) for thirty minutes. At the end of the thirty minute period, the gel is rinsed in distilled water, and de-stained in a solution of water, ethanol, and glacial acetic acid for two fifteen minute periods. After de-staining, the gel was ready for analysis.

III.G Determination of Enzyme Conversion of Succinate and Pyruvate to 2-Methylisocitrate

III.G.1 Spectrophometric Determination of Pyruvate Concentration

The hypothesis for this research is that YQI/Q protein acts as a 2-methylisocitrate lyase that converts 2-methylisocitrate into pyruvate and succinate, as shown in Figure 5. The goal of this study was to determine whether or not the activity of the enzyme correlated with our hypothesis. A particular dilemma that slowed the progress of this study was the fact that 2-methylisocitrate is not commercially available. However, if conditions are kinetically favorable, some enzyme catalysts have the ability to operate in the reverse of their proposed reactions (1). With that knowledge, we conducted an experiment to test the ability of the 2-methylisocitrate lyase to convert pyruvate and succinate into 2-methylisocitrate, the reverse of the proposed reaction. This had precedent in a paper by Brock et al. which provided evidence to support this activity, and produced 2-methylisocitrate in low yield (less than 10 %) (1).

![Diagram of enzymatic reaction]

**Figure 5.** Proposed activity of YQI/Q as a 2-methylisocitrate lyase
To determine the conversion of pyruvate and succinate into 2-methylisocitrate we utilized the reaction assay solution described in the Brock paper (2) that made use of a 10 ml mixture of 0.4 mM of Nicotinamide adenine dinucleotide (NADH), 36 U of lactic dehydrogenase, and 20 mM of HEPES buffer. The role of NADH and lactic dehydrogenase in the assay mixture was to aid in the determination of pyruvate concentration in the reaction mixture over time. Lactic dehydrogenase and NADH combined with the reaction mixture would promote a hydride transfer from NADH to pyruvate and would convert all pyruvate into lactate. NADH concentration can be determined spectrophotometrically at a wavelength of 340 nm. If the reaction mixture reacted with the assay solution and absorbance of each timed sample increased, it can be concluded that there was a decrease in the amount of available pyruvate over time. Experimentally, less pyruvate in the reaction would promote an increased absorbance, due to the fact that more NADH would be found in solution. A schematic of the assay is shown in Figure 6.

![Chemical reaction](image_url)

**Figure 6.** Assay for determination of pyruvate concentration
III.G.2 Determination of Pyruvate Standard Curve

In order to prepare a standard curve to determine optimal activity for LDH, an assay was conducted to utilize one milliliter solutions of pyruvate, HEPES buffer, and magnesium chloride (MgCl₂). The concentrations of pyruvate in each 1.50 milliliter container equaled: 0 mM, 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM, and 0.7 mM respectively. Four hundred microliters of pyruvate solution was mixed with 500 microliters of LDH solution (0.4 mM NADH / 36 U LDH) to give experimental solutions with pyruvate concentrations of 0.04 mM, 0.08 mM, 0.12 mM, 0.16 mM, 0.20 mM, 0.24 mM, and 0.28 mM, respectively. Each experimental solution was measured by UV spectroscopy at 340 nanometers.

III.G.3 YQIQ Reverse Reaction Assay

A 5 ml reaction mixture consisted of 400 µl of 3.75 mM pyruvate, 400 µl of 3.75 mM succinate, 500 µl of 2.0 mM magnesium chloride, 400 µl of 1.3 mM dithiothreitol, and 3300 µl of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (“HEPES” buffer at pH 7.0 (2). At the initiation of testing, 100 µl of the yqiQ enzyme (0.31 mg/ml concentration) was added to the reaction mixture and was tested over a twelve minute interval, as well as daily (up to ninety-six hours).

The assay was conducted by removing a sample of the reaction mixture at timed intervals and mixing with the assay solution. This procedure was initiated directly after the introduction of YQIQ to the reaction mixture. After mixing, the removal of 400 microliters of yqiQ reaction mixture was removed, and incubated at 90° Celsius for five minutes to denature the enzyme. Afterwards, the sample was removed and allowed to cool. Each minute, up to twelve minutes, a sample was removed in the same fashion. All samples were conducted in triplicate. The reaction was also tested in triplicate at 24, 72, and 96 hours. Prior to
spectrophotometric testing, the denatured samples were centrifuged to remove protein from the solution. The 400 µl reaction solution was mixed with 500 µl of the NADH reaction assay solution and measured spectrophotometrically at 340 nm over a thirty second time frame. The lowest absorbance value was recorded to reflect the actual amount of available pyruvate left in solution.

### III.G.4 Determination of Optimum pH for Enzymatic Activity

An important goal in this experiment was to find optimum conditions for enzyme activity. We therefore tested for enzymatic activity at increasing pH, and at increased incubation times. The assay was designed in the same manner as stated in the previous experiment. The only variable that changed was the pH of the 20 mM of HEPES buffer in the reaction mixture. The pH values tested were: 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0.

### III.G.5 Determination of Enzymatic Activity at Increased Pyruvate and Succinate Concentrations

With the knowledge gained from the previous two experiments, it was confirmed that the concentration of pyruvate had decreased over time. It was also recognized that the process was highly inefficient. Attempts were made to increase the concentration of available pyruvate and succinate to 200 mM molar and 100 mM quantities, respectively in order to increase the mass of the product formed. Conducting an assay of this type could also provide a greater concentration of 2-methylisocitrate for confirmation by nuclear magnetic resonance spectroscopy (NMR). The tests were conducted using the same experimental setup as described in the first assay. At the point of reaction mixture setup, the concentrations of pyruvate and succinate were brought to 200 mM and 100 mM, respectively. At that point, yqiQ was added, and collection was conducted in the same manner as mentioned previously, except that the
collection times were every fifteen minutes for an hour. The maximal assay concentration of pyruvate, according to our standard curve, was 0.12 mM pyruvate in the reaction mixture, so after centrifugation, the sample was diluted by taking 2.5 microliters of the timed samples and diluting them using 997.5 microliters of HEPES buffer (pH 7.5).

III.H Synthesis of 2-Methylisocitrate

It was anticipated that the 9 percent (0.35 mg) theoretical yield of synthesized product would have been too small for analysis by NMR. There was an even lower measured yield (~0.13 percent/ 0.0053 mg) when the substrate concentration was increased. In order to determine the mass of synthesized product, mass spectrometry could have been used, but it was decided to forgo spectral analysis of the reaction, and synthesize the starting substrate to begin testing the forward reaction. The next goal was to synthesize this compound following a procedure from Brock et al. (2001). Through the synthesis of 2-methylisocitrate, our assumption is that upon interaction with the yqiQ protein we will be able to produce pyruvate and succinate in greater amounts for confirmation by NMR. Currently, work is still being done to synthesize 2-methylisocitrate. The synthesis of the compound is a six step process that produces five intermediate compounds (Figure 7): Cis-epoxymethylsuccinate disodium salt (3a, 3b, 3c, 3d), Cis-epoxymethylsuccinic acid (4a, 4b, 4c, 4d), Dimethyl cis-epoxymethylsuccinate (5a, 5b, 5c, 5d), 2-methyl-5-oxo-2,3,4 tricarboxymethoxytetrahydrofuran (6a,6b, 6c, 6d), and Erythro 2-methylisocitrate lactone(7a, 7b, 7c, 7d) (1). The synthesis of the target compound would be composed of a racemic mixture of a total of 4 stereoisomers of 2-methylisocitrate initiated from the starting compounds citriconic acid and mesaconic acid. In the reaction mechanism described in Figure 7, citriconic acid will lead to the formation of the cis-epoxides (S,R / R,S  enantiomeric
pairs), and mesaconic acid will lead to the formation of the trans epoxides (R,R/ S,S enantiomeric pairs).

\[ \text{Figure 7. Reaction mechanism for the synthesis of the 2-methylisocitrate} \]

III.H.1 Synthesis of Cis-epoxymethylsuccinate Disodium Salt

The synthesis of cis-epoxymethyl succinate disodium salt (3a-d) was conducted utilizing a procedure from the Brock paper. In a round bottom flask, at 0° Celsius, 15.23 ml (19.0 g/ 170 mmol) of citric anhydride and water (60 ml) were added and allowed to mix utilizing a magnetic stirrer (2). Sodium hydroxide (10 g dissolved in 15 ml water) was then added while monitoring the temperature to ensure that it did not exceed 65° Celsius (2). Upon the complete mixture of the sodium hydroxide, dry sodium tungstate (1.70 g/ 5.0 mmol) was added to the
solution and was dissolved until completion (2). After dissolving the sodium tungstate, hydrogen peroxide (20 ml/176 mmol) was slowly added to the solution, still monitoring the temperature (2). After the addition of peroxide the solution was mixed for five minutes in the ice bath to ensure proper mixing and prevent side reactions. After the five minute mixing, the solution was allowed to come to room temperature (22-25 °C). Once the solution reached room temp, the solution was placed in a 65 °Celsius oil bath and stirred for 90 minutes (2). At the conclusion of incubation, excess water was removed in vacuo until the solution was approximately half its original volume (2). At the conclusion of evaporation, the remaining solution was added to an excess of acetone and allowed to develop a crystalline precipitate over forty-eight hours (2). The excess acetone was filtered away and the remaining white-crystalline precipitate was weighed at 37.52 g. The precipitate was analyzed by NMR in solution with deuterium oxide.

III.H.2 Synthesis of Cis-epoxymethylsuccinic Acid

The synthesis of cis-epoxymethyl succinic acid (4a-d) describes only the protonation of the carboxyl groups of the cis-epoxy methyl succinate disodium salt to produce cis-epoxymethylsuccinic acid. Eleven grams of 3a-d (58 mmol) was added to 20 ml of water, and allowed to dissolve (2). The solution was added to 300 ml of diethyl ether and stirred continuously using a magnetic stirrer (2). Next, concentrated sulfuric acid (6.5 ml dissolved in 70 ml diethyl ether) was added slowly to the solution and allowed to stir at room temperature for three hours (2). After the three hour incubation period, the solution was placed into a separatory funnel and allowed to separate into a water layer and an ether layer (2). The water layer was allowed to drain and was placed in a separate flask; the ether layer was drained as well and placed in a separate flask (2). The water layer was reintroduced to the separatory funnel along with 100 ml of diethyl ether, mixed vigorously, and is drained and separated in the
same containers that were used previously (2). After completion of the repeated processes, anhydrous magnesium sulfate was introduced to the combined ether layers to remove any other residual water from the solution, the solution is then filtered, and the anhydrous ether solution was collected (2). This ether solution was placed under vacuum pressure and all excess ether in the solution is removed (2). Upon removal of the ether, a clear oil (6.73 g) is left behind which is considered to be the desired acidic end-product. The chemical structure of the resulting oil is identified by NMR in a solution of deuterated chloroform.

III.H.3 Synthesis of Dimethyl Cis-epoxymethylsuccinate

The synthesis of Dimethyl cis-epoxymethylsuccinate (5a-d) describes the methylation of the protonated carboxyl groups of 3a-d. In order to synthesize this product 14.4 g (100 mmol) of 4a-d was reacted with a solution of 7.50 ml (100 mmol) of distilled thionyl chloride and 100 ml of dry methanol, at 0°Celsius (2). The solution was removed from the ice bath and allowed to incubate at 25°Celsius overnight (approximately 12 hours). After the overnight incubation, the solvent was evaporated, and the remaining product was added to an excess of ether (100 ml), and was washed with 100 ml of sodium bicarbonate (1 M) in a separatory funnel (2). After mixing, the water layer was removed and the ether layer was dried using anhydrous magnesium sulfate (2). The magnesium sulfate was filtered off leaving an anhydrous ether solution that was evaporated to prepare for flash column chromatography (2). A 3:2 hexanes to ethyl acetate solution produced an Rf of approximately 0.35 and was used to run the column. The contents of collection tubes, verified by thin layer chromatography that contained the purified product were evaporated and the remaining 5a-d (11.21 g) was confirmed by NMR in a solution deuterated chloroform (2).
CHAPTER IV

RESULTS SECTION

IV.A Isolation and Purification of YQI\(\text{Q}\)

IV.A.1 SDS-PAGE Results

During the first purification, seven eluate collections were kept and tested against Bradford indicator, and of the seven, two tested a positive for protein. All fractions that provided evidence of protein were saved and placed in a dialysis bag for overnight dialysis in a solution of 25 mM Tris Buffer (pH 8.0) for removal of imidazole. Upon removal of the bag the next day all collected crude filtrates were analyzed by SDS-PAGE to confirm protein retrieval and isolation of the \textit{yqiQ} protein (results shown in Figure 8).

![SDS-PAGE Image]

**Figure 8.** SDS-PAGE supporting the isolation of \textit{yqiQ}
yqiQ has a theoretical molecular weight of 34 kiloDaltons. The SDS-PAGE showed a band slightly above the 30 kiloDalton protein of the control ladder, which provided evidence to support the isolation of the yqiQ enzyme.

**IV.A.2 Optimization of YQ/Q Retrieval**

The procedure for the isolation of the yqiQ was further optimized to increase the yield of the enzyme. The process was improved by decreasing the amount of imidazole in the eluate buffer down to 200 mM. This decrease prevented the rapid precipitation of the target protein from the column, which resulted in approximately 2.17 mg/L of yqiQ (see Figure 9).

![Image](image.png)

**Figure 9.** SDS-PAGE supporting increased output of pure yqiQ at 200 mM imidazole eluate wash concentration
IV.B  YQIQ Reverse Reaction Assay Results

IV.B.1  Lactic Dehydrogenase(LDH) Assay/Pyruvate Standard Curve

The results from the pyruvate standard curve provide evidence to support that 0.12 mM is the optimum pyruvate concentration for activity. From this knowledge, we were able to design the reverse reaction assay that ensured the pyruvate concentration within the reaction mixture allowed for optimal LDH activity. The pyruvate standard curve is shown in Figure 10.

![Pyruvate Standard Curve](image)

**Figure 10.** Standard curve for pyruvate

IV.B.2  YQIQ Overnight Incubation Results

After some repetition and optimization, the spectrophotometric results provided evidence that the presence of the YQIQ enzyme promoted a decrease in available pyruvate
concentration in the reaction mixture. Results for the 24, 72, and 96 hour incubation provided support for the decreasing amounts of pyruvate over time. Results for the twelve minute time interval provided inconsistent results and are not shown. The conclusion was that the turnover correlated well to the Brock paper, graphically determining an approximately 7% yield in desired product. This finding led us to conclude that the YQIQ enzyme was converting pyruvate and succinate into minute concentrations of 2-methylisocitrate.

**Figure 11.** Pyruvate unreacted after 24-, 72-, and 96-hour incubation
IV.B.3 Determination of Optimal pH for Activity of YQ/Q

Results from this experiment allowed us to determine that the YQ/Q enzyme had an optimal pH of 7.5 in the synthesis of 2-methylisocitrate (Figure 12). From this result, all successive experiments with the enzyme were kept at a pH of 7.5.

![Figure 12. Absorbance results for pH assay](image)

IV.B.4 Determination of YQ/Q Activity at Increased Substrate Concentration

As mentioned previously, the goal of this study was to increase the output of 2-methylisocitrate by increasing the reaction mixture concentrations to 200 mM pyruvate and 100 mM succinate. The results for this assay did not correlate with the desired Michaelis-Menten kinetics, and were not as promising as the previous results. Over an hour period, absorbance values averaged at slightly under 0.0100 to signifying that the amount of available pyruvate was
still high in solution, and the conversion into 2-methylisocitrate had not occurred. It was concluded that in this experiment enzymatic activity provided no measureable effect as a result of increased substrate concentration. Results are shown in Figure 13.

![Concentrated Substrate Assay](image)

**Figure 13.** Assay results at 200 mM pyruvate and 100 mM succinate

**IV.C Synthesis of 2-Methylisocitrate Results**

**IV.C.1 Results for Synthesis of Cis-epoxymethylsuccinate Disodium Salt**

One dimensional proton nuclear magnetic resonance results provide evidence to support the presence of the disodium salt with chemical shift peaks as follows: $^1$H-NMR (500Mhz, D$_2$O): $\delta = 1.38$ ( 3H, s, CH$_3$), $3.24$ ( 1H, s, OCH); $^{13}$C-NMR (125 MHz) : $\delta = 19.57$, 61.93, 63.62, 174.00, 175.47 ppms. Other major peaks in these spectra describe impurities from acetone and water from the experimental work-up. Spectra are shown in Figures 14 and 15 and correlate with previously reported data.
Figure 14. 1-D proton spectrum of cis-epoxymethylsuccinate disodium salt
Figure 15. 1-D Carbon NMR of cis-epoxymethylsuccinate disodium salt
IV.C.2 Results for Synthesis of Cis-epoxymethylsuccinic Acid

One-dimensional proton NMR results provides evidence to support the presence of cis-epoxymethylsuccinic acid with results as follows: $^1$H-NMR (500 MHz) $\delta = 1.56$ (3H, s, CH$_3$), 3.57 (1H, s, OCH); $^{13}$C-NMR (125 MHz, CDCl$_3$) $\delta = 18.40$, 58.30, 60.22, 167.26, 168.52. Other major peaks in these spectra describe impurities from acetone and ether from the experimental work-up. Spectra are shown in Figures 16 and 17.

![Figure 16](image_url)

**Figure 16.** Proton NMR results supporting the synthesis of cis-epoxymethylsuccinic acid
Figure 17. Carbon NMR results supporting the synthesis of cis-epoxymethylsuccinic acid
IV.C.3 Results for Synthesis of Dimethyl Cis-epoxymethylsuccinate

One-dimensional proton NMR results provide evidence to support the synthesis of dimethyl cis-epoxymethylsuccinate with results as follows: $^1$H-NMR (500 MHz) $\delta = 1.53$ (3H, s, CH$_3$), 3.80 (3H, s, CH$_3$), 3.81(3H, s, CH$_3$) 4.58 (1H, s, OCH); $^{13}$C-NMR (125 MHz, CDCl$_3$) $\delta = 23.23$, 52.80, 53.39, 53.61, 61.81, 167.17, 173.29. Other major peaks in these spectra describe impurities from ethyl acetate, hexanes, and chloroform from the experimental work-up. Proton and carbon NMR results are pictured in Figures 18 and 19.

![Figure 18](image-url)  
*Figure 18. Proton NMR results supporting the synthesis of dimethyl cis-epoxymethylsuccinate*
Figure 19. Carbon NMR results supporting the synthesis of dimethyl cis-epoxymethylsuccinate
CHAPTER V

CONCLUSION

So far, we have been able to provide partial evidence to support the proposed reaction mechanism of \( yqiQ \), 2-methyl isocitrate lyase. Results from this research support that in a solution of pyruvate and succinate, the presence of \( YQI/Q \) promotes a decrease in the concentration of pyruvate over time. This provides an increase in absorbance spectrophotometrically, due to the fact that lactic dehydrogenase has a lesser concentration of pyruvate to convert into lactate.

Successful synthesis of the first three intermediates of five to eventually produce 2-methylisocitrate has been conducted. The future goal of this project is to eventually complete the synthesis of 2-methylisocitrate in order to test the compound in solution with \( YQI/Q \) to attempt to confirm the forward reaction. Kinetic studies will follow to characterize the rate of conversion of the enzyme.
BIBLIOGRAPHY


