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Bacillus subtilis is widely used as a model organism for the study of sporulation. While its genome has been sequenced, the function of many encoded proteins have not been investigated. The mother cell metabolic gene (*mmg*) operon is an example that contains *mmgA*, *mmgB*, and *mmgC* which all encode for proteins in a fatty acid degradation pathway, however the pathway is incomplete. *yhaR* is a candidate gene which encodes for a putative hydratase that may help complete the *mmg* fatty acid degradation pathway. The *yhaR* gene was cloned in *Escherichia coli* and the protein was overproduced and purified to a yield of 8 mg per L of culture. The activity of the purified enzyme was tested by high performance liquid chromatography and liquid chromatography coupled to mass spectrometry analysis, using butenoyl-CoA as the substrate. These experiments revealed that the enzyme was active in producing 3-hydroxybutyryl-CoA, with a pH optimum of 6.5. LC-MS data for the reaction products displayed the expected 852 m/z for the 3-hydroxybutyryl-CoA product. Kinetics experiments revealed that at 37 ^{o}C and pH 6.5 the enzyme has a k_{cat} of 6.1 X 10^{-3} ± 9.2 X 10^{-4} s^{-1}. Butenoyl-CoA substrate was found to have a K_M of 0.14 ± 0.03 mM.

PURIFICATION AND CHARACTERIZATION OF AN ENOYL-COA HYDRATASE ENCODED BY THE *YHAR* GENE FROM *BACILLUS SUBTILIS* STRAIN 168

by

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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.

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I would also like to thank my family for their overwhelming support of my work and efforts.

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CHAPTER I

BACILLUS SUBTILIS AND FATTY ACID BETA-OXIDATION

I.A Background of *Bacillus Subtilis*

Bacillus subtilis is a rod shaped, aerobic, endospore forming bacteria that is the most characterized Gram positive bacterial species. *Bacillus subtilis* is commonly found in soil, water and some associated plants.[1] *Bacillus* strains are commonly used for their ability to create large amounts of proteins.[1] *Bacillus subtilis* specifically is widely used as a model for sporulation and for the production of biosurfactants.[2,3,4] An increased understanding of the metabolic processes undergone during sporulation will increase the utility of *B. subtilis* as a model organism and its usefulness for biotechnological applications such as biosurfactant production.[4]

Although the complete genome of *B. subtilis* has been sequenced many of the protein functions have not been investigated.[1] The *B. subtilis* genome consists of 4,214,810 base pairs of which 87% comprise 4,100 protein coding sequences.[1] Only about 58% of the *B. subtilis* gene products have been assigned activity based on sequence homology alone.[1] For a more complete understanding of specific metabolic processes for this organism these gene products must be further investigated.

B. subtilis undergoes sporulation when under nutritional limitations, specifically when there is not sufficient amounts of carbon, nitrogen, or phosphorus in the growth

environment.[3] Initiation of sporulation causes the disruption of the normal cell vegetation cycle and the asymmetrical division of the bacterial cell in which the larger portion is designated the mother cell and the smaller portion the forespore.[1,5] After this onset state the two portions are separated by a septum which begins the commitment stage of sporulation.[5,6] The forespore becomes engulfed by the mother cell and becomes a free floating endospore.[3,5,7] At this point the endospore matures and is protected with a spore coat and layers of protein after which the mother cell is lysed releasing the matured spore into the environment.[3,6,7]

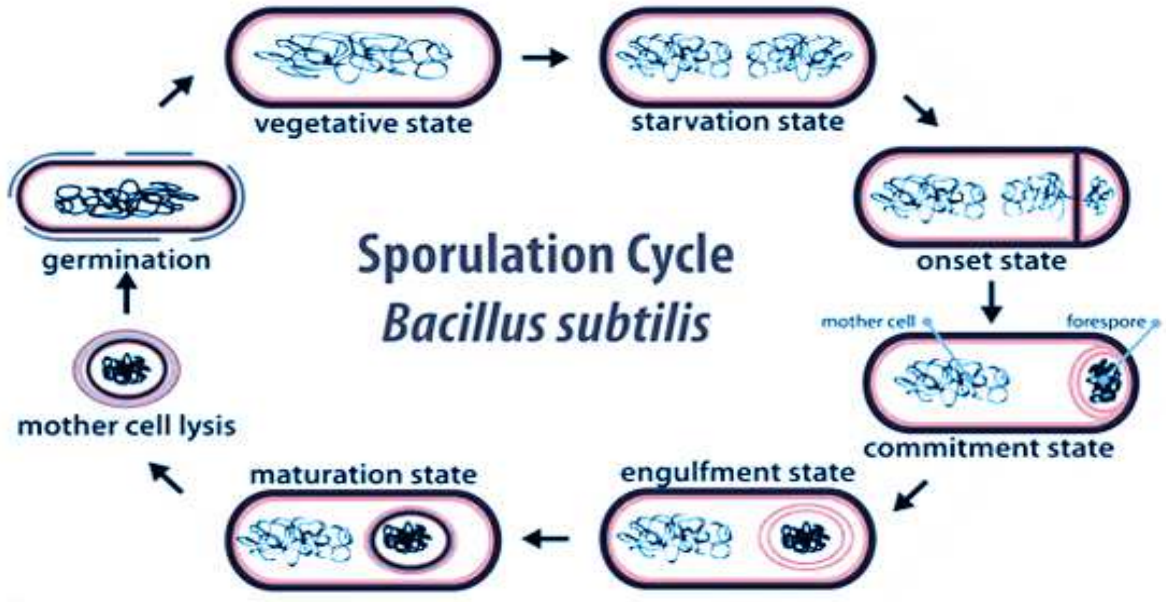

Figure 1. Sporulation Cycle of *Bacillus Subtilis*.[8]

Nutritional stress initiates signals that activate the key transcriptional regulator Spo0A, which in turn activates the sigma factor E (σ^E) of RNA polymerase during the onset state of sporulation.[9] It is σ^E that promotes the expression of several genes in the

mother cell including the mother cell metabolic gene (*mmg*) operon.[9] The *mmg* operon contains six open reading frames in the sequence of *mmgA, mmgB, mmgC, mmgD, mmgE* and *yqiQ* (Figure 2).[9,10] Bryan et al discovered *mmgA* through part of *mmgE* and gave the genes their name. The genome sequence later showed *yqiQ* as part of the same operon, however since it was not named before and was not studied prior to sequencing it received the "y" designation for unstudied genes. Transcription of these genes are regulated by catabolite control protein A (CcpA). In the presence of glucose CcpA becomes active and can bind *mmgO*, a catabolite response element, and represses expression of the *mmg* operon.[9] The expression of the *mmg* operon during sporulation in the absence of glucose suggest that the derivative proteins are involved with an alternative energy source.

Figure 2. Mother Cell Metabolic Gene (*mmg*) Operon.[2]

I.B Fatty Acid Beta Oxidation

Previous work has shown that the mother cell metabolic gene (*mmg*) operon encodes for proteins involved in the breakdown of fatty acids and the methylcitric acid cycle during sporulation. The genes *mmgA*, *mmgB,* and *mmgC* are involved with a fatty acid beta-oxidative pathway and the genes *mmgD, mmgE* and *yqiQ* are involved in the methylcitric acid pathway.[2,11] While our laboratory has shown that the *mmg* operon

encodes for these pathways this operon lacks an activity necessary to complete the fatty acid beta-oxidative pathway.

Beta-oxidation begins with the oxidation of a fatty acid by an acyl-CoA dehydrogenase forming an enoyl-CoA, where electrons are transferred to either an FAD or FMN cofactor. The enoyl-CoA is then hydrated by an enoyl-CoA hydratase to produce a 3-hydroxyacyl-CoA. Another oxidation step follows where the 3-hydroxyacyl-CoA is acted upon by a dehydrogenase forming a 3-ketoacyl-CoA. This step is facilitated by either a NAD^+ or $NADP^+$ cofactor. The last step involves a thiolase activity with CoA-SH producing acetyl-CoA and an acyl-CoA shorter by two carbons.

The beta oxidative pathway encoded for by the *mmg* operon (Figure 3) begins with the gene *mmgC* encoding for an FAD-dependent acyl-CoA dehydrogenase.[11] The next activity required by the pathway is missing from the operon; this activity is an enoyl-CoA hydratase (crotonase) which would provide the 3-hydroxyacyl-CoA substrate required for the third enzyme in the pathway *mmgB*. The *mmgB* gene encodes for an NADP-dependent 3-hydroxyacyl-CoA dehydrogenase.[1,12] The final activity is provided by a protein encoded for by *mmgA*, an acetoacetyl-CoA thiolase.[2]

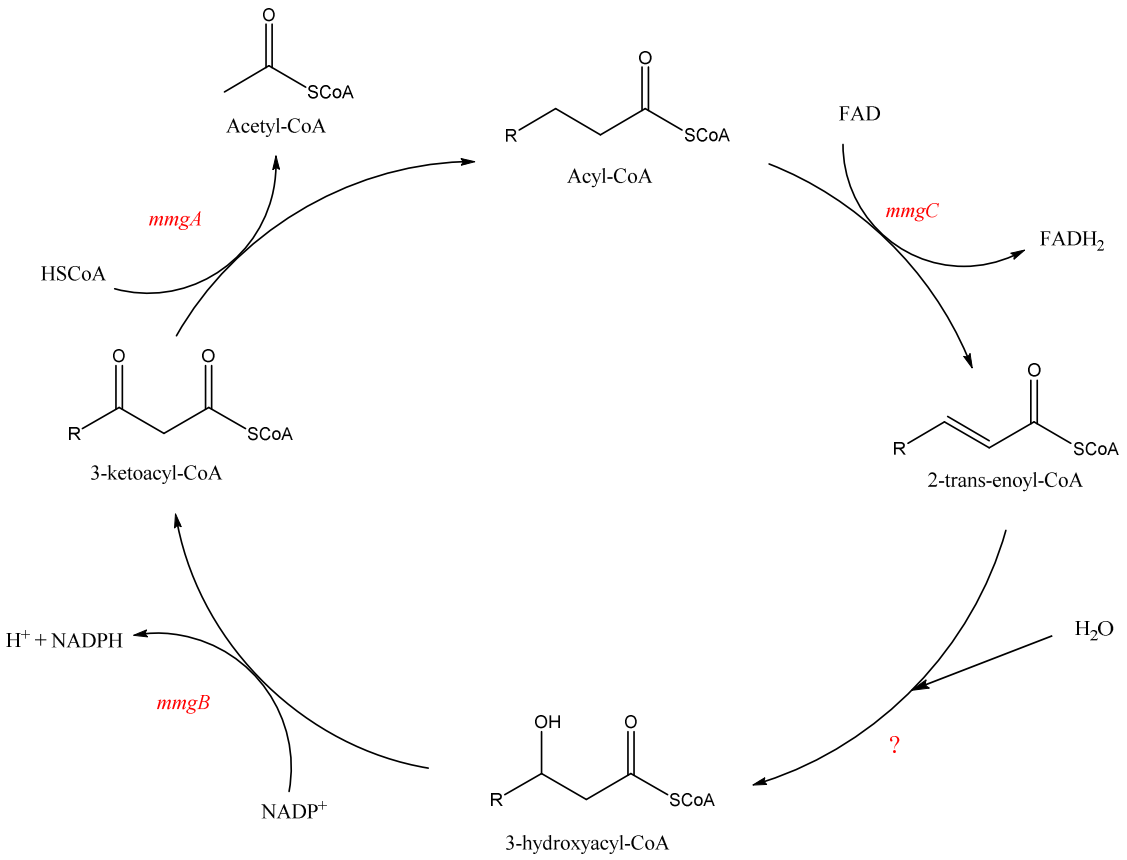

Figure 3. Proposed Fatty Acid Beta-Oxidative Pathway. Enzymes found in red text.

I.C Overview of *yhaR* and Goal

To determine the gene responsible for the missing enoyl-CoA hydratase activity a basic sequence alignment tool (BLAST) was utilized to compare *B. subtilis* coded proteins to a known enoyl-CoA hydratase found in *Escherichia coli*. The top four BLAST results were *yngF, fadB, menB,* and *yhaR*.[13] Of these results only *yngF* and *yhaR* are expressed during the onset state of sporulation in which the *mmg* operon is also expressed. Work previously done in the Reddick lab by Jeffery Smith has shown that *yngF* is involved with the formation of HMG-CoA as seen in Figure 4. This is a similar

reaction however not part of fatty acid metabolism. This leaves *yhaR* as the next most likely responsible gene and the focus of study for this thesis.

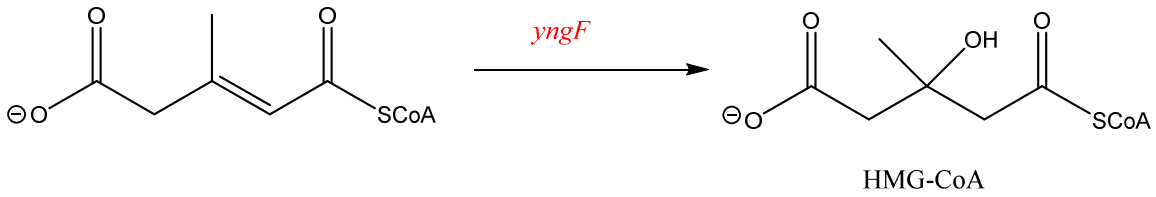

Figure 4. Observed Activity of the *yngF* Enzyme.

The overall objective of this thesis is to identify an enzyme that is able to supply the enoyl-CoA hydratase activity that is missing from the *mmg* beta-oxidation pathway. The central hypothesis of this objective is that *yhaR* encodes for the missing activity found in the *mmg* operon fatty acid beta-oxidative pathway. The rationale that underlies the research proposed here is that knowledge of metabolic pathways during sporulation will allow new investigations of *B. subtilis* sporulation which is important because of its widespread use as a model for sporulation.

CHAPTER II

EXPERIMENTAL PROCEDURES

II.A DNA Amplification Through PCR

Polymerase chain reactions (PCR) were conducted using *yhaR* primers and *Bacillus subtilis* 168 (BS 168) template DNA. Primers were designed as follows, down primer 5'-CTGTTACGCTTGAAACTTCGGCTCC-3' and up primer 5'-CACCATGGAGTTTGTTCAATATGCATGTAATGG-3'. Template DNA was gathered from a 5 mL overnight culture of *B. subtilis* 168 bacteria following the Wizard® Genomic DNA Purification Kit protocol. PCR mixtures contained 1 µL of purified template DNA, 1 µL each of up and down primers, 1 µL of deoxyribonucleotide mixture, 10 µL of 5X Phusion high fidelity buffer solution, 0.5 µL of Phusion polymerase, and 35.5 µL of sterile distilled deionized water (ddH_2O) for a final solution volume of 50 µL. Negative controls were also conducted in which the template DNA was removed and replaced with sterile water to maintain the final volume of 50 µL. Reactions were conducted using the protocol of 1 cycle of 98.0 ^oC for 30 seconds; 30 cycles of 98 ^oC for 5 seconds, 59 ^oC for 10 seconds, 72 ^oC for 30 seconds; and lastly one cycle of 72 ^oC for 10 minutes followed by a storage setting of 4 ^oC.

Successful DNA amplification was verified using an agarose gel. Gels were prepared using 30 mL of 1x TAE buffer, approximately 0.3 g of agarose, and 20 µL of

1% ethidium bromide for visualization. Gels were loaded with 16 µL of PCR product and 4 µL of 6x gel loading blue dye. A 2-log DNA ladder was used and loaded following the NEB ladder loading protocol. Gels were electrophoresed in TAE buffer at 120 V for approximately one hour. After confirmation of initially successful PCR, subsequent PCR products were purified using the Qiagen® QIAquick PCR Purification Kit, as outlined in the QIAquick PCR Purification Kit quick-start protocol. Unpurified PCR product and purified PCR product were loaded on agarose gel to verify DNA amplification.

II.B Vector Cloning and Cell Transformation

Purified PCR product was cloned into the pET-200 vector following the protocol for the Champion™ pET Directional TOPO® Expression Kit user manual. The TOPO® cloning reaction contained 4 µL of purified PCR product, 1 µL of salt solution, and 1 µL of TOPO® vector; the salt solution and vector were provided in the kit. The reaction mixture was incubated at room temperature for 5 minutes then immediately used for the transformation of One Shot® TOP10 cells. Transformation was carried out following the chemical transformation protocol provided in the Champion™ pET Directional TOPO® Expression Kit user manual. Transformed cells were plated on Luria-Bertani (LB) agar plates containing 30 µg/mL kanamycin and incubated overnight at 37 ºC. Colonies were restreaked and 5 mL cultures containing 30 µg/mL kanamycin were made from the subsequent colonies. Each overnight culture was centrifuged and LB supernatant removed. Plasmid DNA from each source colony was isolated using the Qiagen QIAprep® Miniprep kit. The protocol using a microcentrifuge was followed. Plasmid products were then analyzed through PCR using the protocol outlined previously.

Purified plasmid was used to transform competent *E. coli* strain BL21(DE3) bacteria. The *E. coli* cells were made competent by growing one colony of cells in 5 mL of LB broth until slightly cloudy. The culture was then centrifuged for 5 min at 4 ^{o}C and the supernatant discarded. Cells were resuspended in 3 mL of cold 50 mM $CaCl_2$ by pipetting, then vortexed briefly. Then cells were incubated for 30 minutes on ice, which was followed by another centrifuge step with the supernatant being discarded. Finally the cell pellet was resuspended in 1 mL of cold 50 mM $CaCl_2$ by pipetting. Competent *E. coli* cells (100 µL) was then mixed with 2 µL purified plasmid. The mixture was briefly vortexed then incubated at 37 ^{o}C for 2 minutes followed by an incubation at room temperature for 10 minutes. LB broth (1 mL) was added and the solution was allowed to incubate for 1 hour at 37 ^{o}C. Cells were then spun down at 8000 rpm for 3 minutes, 1 mL of supernatant was discarded then the cell pellet was resuspended in the remaining solution. The transformed cells (100 µL) were spread onto LB agar plates containing 30 µg/mL kanamycin and incubated overnight at 37 ^{o}C. Successful transformation was confirmed upon colony growth on the selective medium. The transformed strain was stored at -80 ^{o}C in 10% glycerol. Subsequent samples were gained from fresh plates inoculated with the stored strain.

II.C Bacteria and Culture Methods of *yhaR*

E. coli strain BL21(DE3) replicating the *yhaR* plasmid was cultured on Luria-Bertani (LB) agar plates containing 30 µg/mL kanamycin. Plates were made by combining 10 g of Bacto Tryptone, 10 g of NaCl, 5 g of yeast, and 15 g of agar in 1 L of water. The mixture was adjusted to pH 7.5, then autoclaved for 45 minutes, 25.75 µL of

200 mM kanamycin was added to a final concentration of 30 μg/mL before pouring the plates. Bacteria were streaked onto plates using standard procedures and incubated overnight at 37 °C. A starter culture was prepared by adding 1 colony of bacteria and 5 μL of 30 mg/mL kanamycin to a 5 mL aliquots of LB for a final concentration of 30 μg/mL kanamycin. The starter cultures were incubated overnight at 37 °C while shaking at 220 rpm.

The next day 1 L of LB broth was prepared by combining 10 g Bacto Tryptone, 10 g NaCl, and 5 g yeast extract in 1 L of water. The mixture was adjusted to pH 7.5 then autoclaved, and kanamycin was added as described for the previous broth. The starter culture (2 mL) was added to the 1 L flask of LB containing 30 μg/mL kanamycin and incubated at 37 °C while shaking at 220 rpm. The culture was allowed to incubate until an OD_{595} of approximately 0.5 was reached, then 0.2383 g (1 mM) of isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture and incubated overnight at 37 °C while shaking at 220 rpm. The cells were then centrifuged using a JA-10 rotor at 6500 rpm for 30 minutes at 4 °C. The supernatant was discarded and cell pellets were either immediately resuspended for lysis or stored at -80 °C.

II.D Purification of *yhaR* Protein

Room temperature cell pellets were resuspended in 20 mL of binding buffer consisting of 0.5 M NaCl, 20 mM Tris buffer, and 5 mM imidazole (pH 8). Resuspended cell pellets were sonicated on ice for ten 30 second intervals with 30 second rest intervals in between. The cell lysate was centrifuged using a JA-20 rotor at 11,000 rpm for 30 minutes at 4 °C. The supernatant was syringe-filtered using a 0.45 μm filter to remove

cell debris and loaded onto a Ni-NTA nickel affinity column. Prior to loading the column with the cell lysate, the column was prepared by packing approximately 4 mL of a 50% mixture of Ni-NTA and Agarose by gravity for a final column volume of 2 mL. Once packed the column was washed with 6 mL of water followed by 6 mL of binding buffer for equilibration. The cell lysate was loaded and allowed to flow by gravity, followed by 20 mL of binding buffer. Next, 12 mL of wash buffer (0.5 mM NaCl, 20 mM Tris, and 60 mM imidazole, pH 8) was loaded and allowed to flow by gravity. Lastly, 12 mL of elution buffer (0.5 mM NaCl, 20 mM Tris, and 1 M imidazole, pH 8) was added and collected in 1 mL fractions. The entire chromatography was conducted at 4 ^{o}C. Bradford reagent was used to test each fraction for protein by combining 33 µL of the fraction with 1 mL of Bradford reagent. In the presence of protein the red/brown reagent changes color to bright blue. On average four fractions were found to contain protein of significant concentration, signified by an intense blue color change. Protein-containing fractions were pooled and dialyzed overnight at 4 ^{o}C in 25 mM Tris-HCl buffer (pH 8.0). Once dialyzed, approximately 4 mL of protein was recovered. To this, sterile glycerol was added to a final concentration of 10%. The protein was stored at -80 °C in 100µL aliquots until use.

The purity of *yhaR* protein was tested using a 12% SDS-PAGE gel containing 3.4 mL of H_2O, 4 mL of 40% Acrylamide, 2.5 mL of 1.5 M Tris-HCl (pH 8.0), 50 µL of 20% SDS, 15 µL TEMED, and 120 µL of 10% APS.

II.E Reactions with *yhaR* Protein

Initial enzyme activity assays were conducted using 500 µL reactions containing 50 µL of purified protein for a final concentration of 0.014 mg/mL, 0.1 mM of butenoyl-coA, and 44.5 mM potassium phosphate buffer (pH 8.0). The reverse dehydration reaction was also analyzed using 50 µL of purified protein for a final concentration of 0.014 mg/mL, 0.1 mM of 3-hydroxybutyryl-CoA, and 44.98 mM potassium phosphate buffer (pH 8.0). Reactions were analyzed using UV-Vis spectrometry measuring absorbance at 263 nm.[14] Clear product formation was not observed from spectrographs. The reactions were then analyzed through HPLC with UV detection utilizing a reverse phase chromatography column. Reverse phase chromatography was done using a Synergi, hydro-RP, 250 x 4.60 mm, 4 micron, 80 Å, column by Phenomenex.[15] Chromatography was performed using 20 mM sodium phosphate buffer (pH 2.9) and methanol as mobile phase with a constant flow rate of 0.7 mL/min and detection at 220 nm. The method began with 20% methanol that increased linearly to 40% during the first 20 minutes. Then, methanol was increased linearly from 40% to 60% for 5 minutes, which was held constant for 10 minutes. Methanol was then decreased linearly from 60% to 20% over 5 minutes after which the flow was held constant at 20% methanol for 10 minutes to re-equilibrate the column prior to the next sample injection. To analyze each sample 50 µL were injected onto the column. Standards of the reaction substrate and product were analyzed containing 0.2 mM butenoyl-CoA in 50 mM potassium phosphate buffer (pH 8.0) and 0.2 mM 3-hydroxybutyryl-CoA in 50 mM potassium phosphate buffer (pH 8.0) respectively.

Enzyme activity was then tested using 1 mL reaction volumes. The 1 mL reactions were prepared containing 880 µL 50 mM potassium phosphate buffer (pH 8.0), 0.2 mM butenoyl-CoA, and 100 µL of *yhaR* protein for a final concentration of 0.027 mg/mL. Reactions were quenched with 100 µL of 1 M sodium phosphate monobasic (pH 2.8).

During the weeks following the first experiments that successfully demonstrated activity, the protein from the same purification batch lost its activity, perhaps through precipitation or aggregation. Precipitation was limited through adjustments to the next trials of purification. The Ni-NTA column was conducted at room temperature and the imidazole concentration in the elution buffer was decreased from 1 M to 0.2 M. The overnight dialysis was also performed at room temperature instead of 4 ºC. Mercaptoethanol was added to dialyzed protein to a final concentration of 5 mM along with the sterile glycerol at a 10% final concentration. These steps prolonged enzyme activity when samples were frozen immediately after collection.

Even after these precautions, the reaction samples injected onto the HPLC quickly clogged the HPLC guard column. To prevent collection of precipitated protein on the HPLC column the reaction quenching procedure was altered and reactions were scaled down to conserve material. The 1 mL reactions were scaled down to 200 µL reactions prepared with 176 µL 50 mM potassium phosphate buffer (pH 6.5), 0.2 mM butenoyl-CoA, and 20 µL of *yhaR* protein for a final concentration of 0.225 mg/mL. Reactions were then quenched with 20 µL of 1 M sodium phosphate monobasic (pH 2.8) followed by 20 µL of 30% perchloric acid. Quenched reaction samples were snap-frozen in an

acetone dry-ice slurry then thawed to precipitate protein. Samples were then centrifuged at 13,200 rpm for 5 min in an Eppendorf Centrifuge 5415D. The resulting supernatants were analyzed by the HPLC method described above. Control reactions and samples were similarly scaled and quenched.

Protein activity was then analyzed at various pH, ranging from pH 6.5 to pH 10. The pH was adjusted by using 50 mM potassium phosphate buffers of varying pH. All reactions were allowed to incubate for 2 hours at room temperature before quenching.

Reactions were also analyzed using LC-MS to confirm the formation of the desired 3-hydroxybutyryl-CoA product. The chromatography was run using the previously utilized Synergi column. For compatibility with mass spectroscopy the mobile phases used were changed to 0.1% formic acid and methanol. The method started with 100% 0.1% formic acid and increased linearly to 15% methanol for 20 minutes. From 20 to 25 minutes the methanol concentration continued to increase linearly to 40% and is held at 40% for 10 minutes. In the next five minutes there was a linear decrease of methanol from 40% to 0%. The 0.1% formic acid solvent was held at 100% for 10 minutes completing the 50 minute method. Flow rate was maintained at 0.7 mL/min throughout the entire method program. Reactions were allowed to run for 2 hours and were quenched with 0.01% formic acid.

II.F SNAC Analogue Activity

Enzyme activity with the truncated analogue *S*-crotonyl-*N*-acetylcysteamine (butenoyl-SNAC) was analyzed. Reactions with 200 µL total volumes were prepared containing 176 µL 50 mM potassium phosphate buffer (pH 6.5), 1 mM butenoyl-SNAC,

and 20 µL of *yhaR* protein for a final concentration of 0.225 mg/mL. Reactions were then quenched with 20 µL of 1 M sodium phosphate monobasic (pH 2.8) followed by 20 µL of 30% perchloric acid as done previously.

Reactions and standards of the butenoyl-SNAC substrate and 3-hydroxybutyryl-SNAC were analyzed using HPLC with UV detection. Chromatography was done with a Nova-Pak C-18 150 x 3.9 mm, 4 micron, 60 Å, column by Waters with a two solvent system.[16] The polar mobile phase was deionized water with 0.1% trifluoroactic acid (solvent A) and the non-polar mobile phase was acetonitrile with 0.1% trifluoroactic acid (solvent B). The method profile used for this separation started at 95% solvent A and 5% solvent B. The percent of solvent B was increased linearly to 45% for 25 minutes. The second gradient occurred over a five minute interval and linearly increased solvent B from 45% to 80%. The percentage of B was held constant for five minutes then linearly decreased to 5% B over five minutes. Finally solvent B was held at 5% for five minutes before completion of the HLPC method.

II.G Michaelis Menten Kinetics

Quantification by HPLC analysis was achieved through the introduction of crotonoic acid as an internal standard. Crotonoic acid was introduced to the 1 mM sodium phosphate quenching solution to a final concentration of 1.4 mM. A butenoyl-CoA calibration plot was made comparing varying concentrations of butenoyl-CoA to their percent relative integration when compared to the integration value of the intenal standard crotonoic acid. A fit for the 3-hydroxybutyryl-CoA product was made following

the same method used for the substrate fit. Each concentration tested was run in triplicate for both the substrate and product fits.

Initial rates of reaction were gathered for reactions containing butenoyl-CoA concentrations of 0.05 mM, 0.1 mM, 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM, and 1.0 mM. Reactions were conducted with an 850 µL total volume containing 85 µL of *yhaR* enzyme for a final concentration of 0.225 mg/mL, varying volumes of 10 mM butenoyl-CoA to reach the desired concentration, and 50 mM potassium phosphate buffer added to fulfill the 850 µL total volume. Reactions were allowed to run for a total of 20 minutes with 200 µL aliquots removed and quenched at the 0, 5, 10 and 20 minute intervals. Each 200 µL aliquot was quenched and analyzed as previously described. Each reaction was run in triplicate where 0.4 mM, 0.6 mM, and 0.8 mM reactions were run four times.

CHAPTER III

RESULTS AND DISCUSSION

III.A PCR Results

The gene *yhaR* consists of 768 bp. Visualization of the agarose gel displayed the expected band at the position between 700-800 base pairs for the positive PCR reaction and no band for the negative reaction, as seen in Figure 5. Visualized agarose gels analyzed after PCR product purification displayed the expected bands between 700-800 base pairs for both the purified and unpurified PCR products. Purified PCR product was carried forward to TOPO® cloning. PCR with the *yhaR* cloning primers was conducted with the plasmid DNA isolated from TOPO10 cells and analyzed with agarose gel electrophoresis. Five colonies were tested to confirm the presence of the *yhaR* insert within the plasmid. As seen in Figure 6, only two colonies showed presence of recombinant plasmid. Purified plasmid from colony 3 (lane 6 in Figure 6), found to contain the *yhaR*-plasmid, was used to transform competent *E. coli* strain BL21(DE3) bacteria.

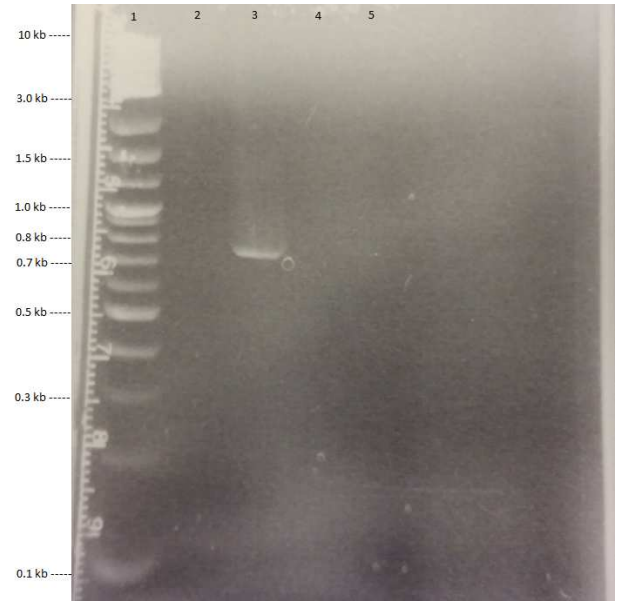

Figure 5. Visualization of Crude PCR Product. Lane three contains PCR product band at approximately 768 base pairs. Negative control in lane 5 showed no signs of DNA amplification.

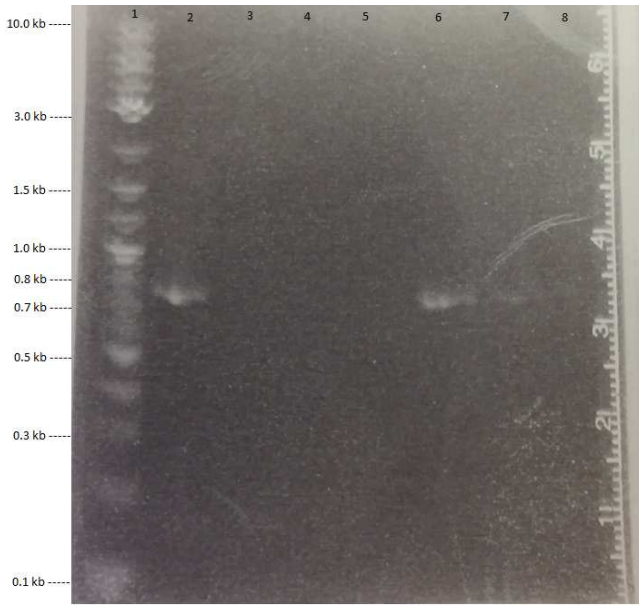

Figure 6. Agarose Gel to Check for Successful Ttransformation of TOPO10 Cells. Lanes left to right: ladder, genomic BS 168 DNA, negative control, and then colonies 1 through 5. Bands in lanes 6 and 7 suggest successful plasmid formation in colonies 3 and 4.

III.B *yhaR* SDS-PAGE Results

The transformed BL21 (DE3) *E. coli* cells were grown in LB and overexpression of the *yhaR* enzyme induced by the introduction of IPTG. The protein was purified by Ni-NTA affinity chromatography and the subsequent protein samples were through SDS-PAGE. The His-tagged fusion protein of *yhaR* has a molecular weight of 31.6 kDa. A band is observed around ~31 kDa indicating purified *yhaR*. The broad band seen in Figure 7 indicates significant overexpression of the *yhaR* enzyme.

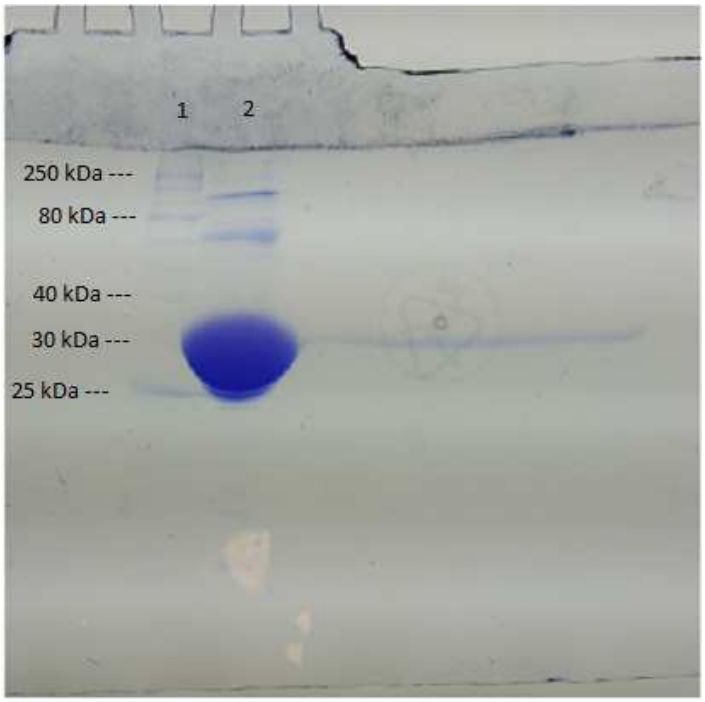

Figure 7. 12% SDS-PAGE Gel of Purified *yhaR*. Ladder and purified *yhaR* found in lanes 1 and 2 respectively. Gel displays clear overexpression of protein with a mass around 30 kDa, the expected mass of the *yhaR* enzyme.

III.C Enoyl-CoA Hydratase Activity of *yhaR* Protein

Results of the HPLC chromatograms (Figure 8) show that the commercially available butenoyl-CoA and 3-hydroxybutyryl-CoA have retention times of approximately 18.1 minutes and 9.6 minutes respectively.

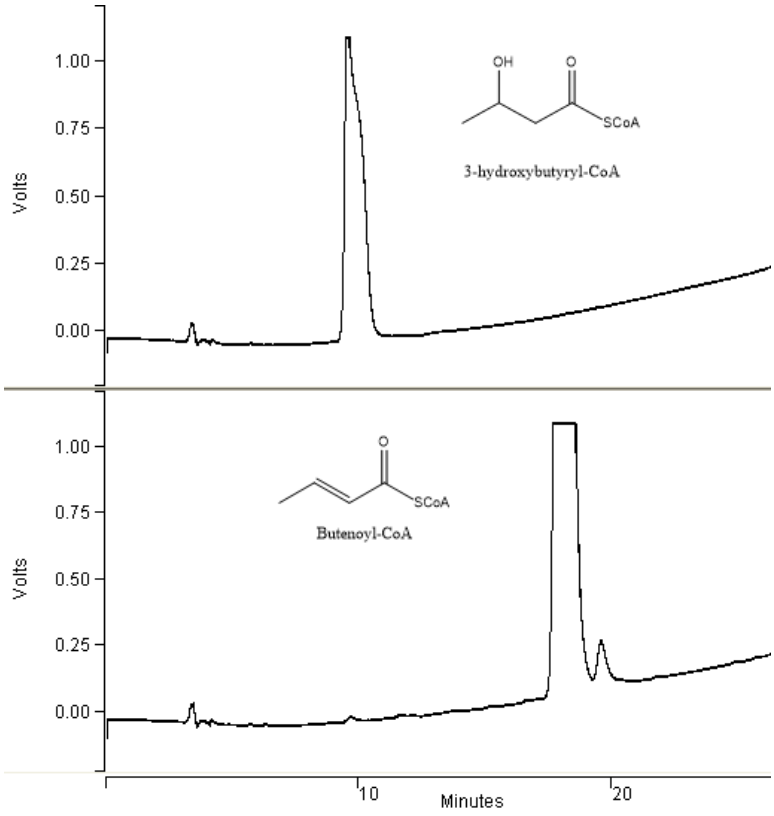

Figure 8. HPLC Chromatogram of 3-Hydroxybutyryl-CoA and Butenoyl-CoA Standards.

Chromatograms for reactions run for 30, 60, 90, and 120 minutes containing 0.027 mg/mL of *yhaR* enzyme, 0.2 mM butenoyl-CoA, and 44 mM potassium phosphate buffer are shown in Figure 9. These chromatograms show the growth of the product peak

(~10 minute retention time) as time progresses, indicating the successful conversion of the butenoyl-CoA substrate into the 3-hydroxybutyryl-CoA product.

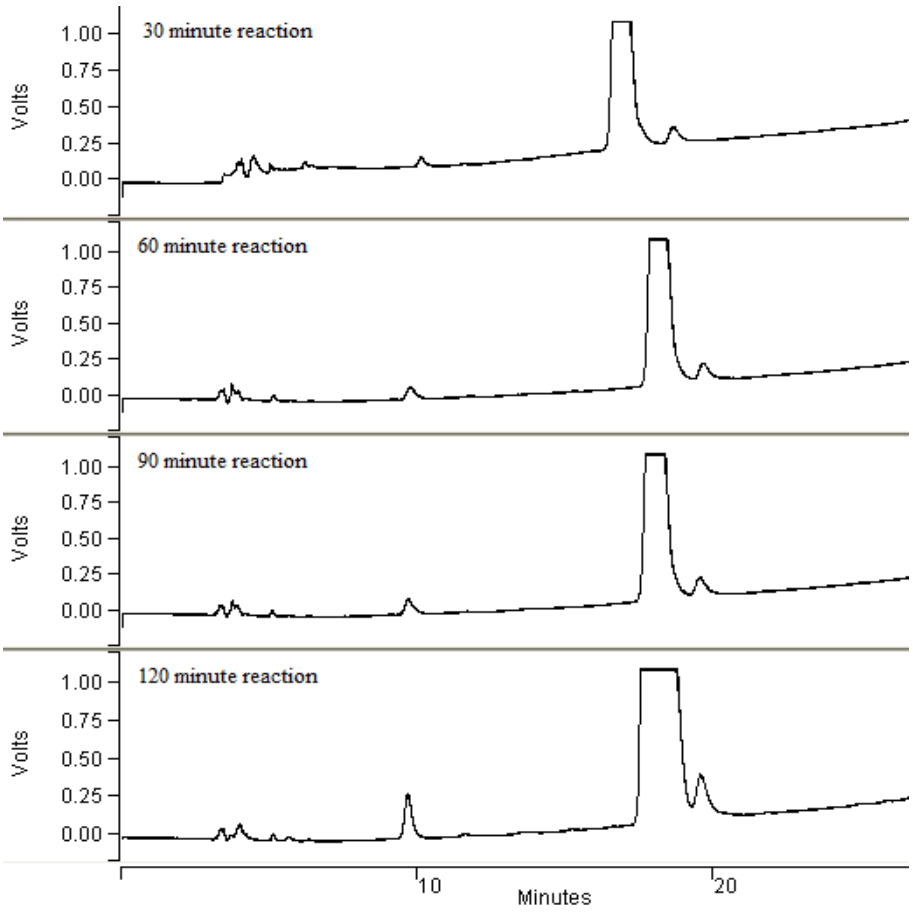

Figure 9. HPLC Chromatograms of *yhaR* Reactions Carried Out for 30, 60, 90, and 120 Minutes. Reactions showed production of the expected product with continued production over time.

One month after purifying *yhaR* protein the stored samples began to lose activity. The observed loss of activity was attributed to the aggregation and precipitation of the protein. Subsequent protein isolations demonstrated precipitation during the purification process and quickly lost the activity observed immediately after isolation. Enzyme activity was preserved through the addition of mercaptoethanol functioning to minimize

disulfide bonds. The addition of mercaptoethanol was found not to interfere with the retention time of butenoyl-CoA or 3-hydroxybutyryl-CoA however it did cause side reactions under certain conditions.

Enzyme activity was explored at varying pH in order to determine the optimal pH for the enzyme. At pH 10 it was found that butenoyl-CoA could be hydrolyzed in the absence of enzyme so it was not a viable pH to test enzyme activity further. Enzyme activity in pH 6.5, 7.0, 7.5, 8.0, and 8.5 can be seen in Figure 10. pH 6.5 was found to provide the maximum enzymatic activity while minimizing possible side reactions with mercaptoethanol. Mercaptoethanol is present in each chromatogram with a retention time of ~6 minutes, while the major side reaction peak has a retention time of approximately 5.3 minutes.

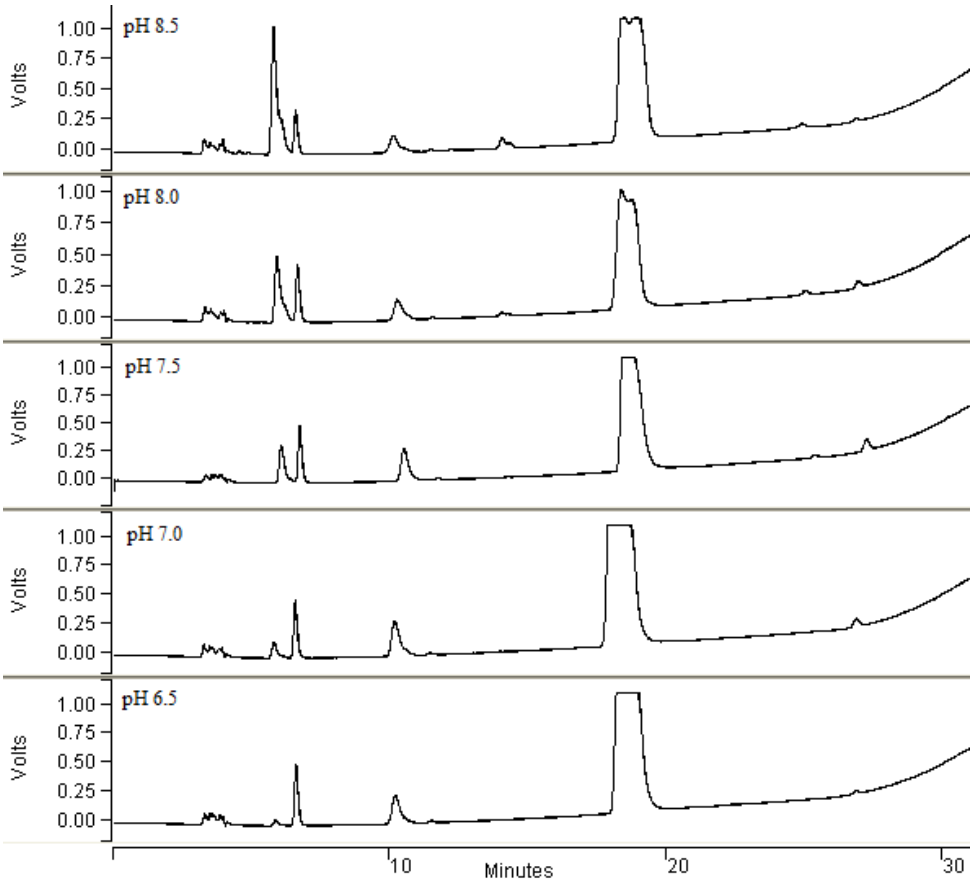

Figure 10. HPLC Chromatograms of *yhaR* Enzyme Reactions at Various pH. All reactions were run for two hours at room temperature. Trend shows enzyme pH optimum around 7.0 to 6.5 pH, while pH 6.5 minimizes potential side reactions with mercaptoethanol.

Confirmation of the HPLC results was carried out using LC/MS. A reaction containing 0.1 mM butenoyl-CoA was incubated for 2 hours then quenched and analyzed, Figures 11 and 12 show the resulting chromatograms. Chromatograms display two peaks with retention times of 31.70 minutes and 38.26 minutes. The peak with retention time of 31.70 minutes has a most abundant mass/charge ratio of 852.09 m/z corresponding to what would be expected for the product 3-hydroxybutyryl-CoA (Figure 11) while the

peak with retention time of 38.26 has a most abundant mass charge of 834.07 m/z corresponding to what would be expected for the substrate butenoyl-CoA (Figure 12).

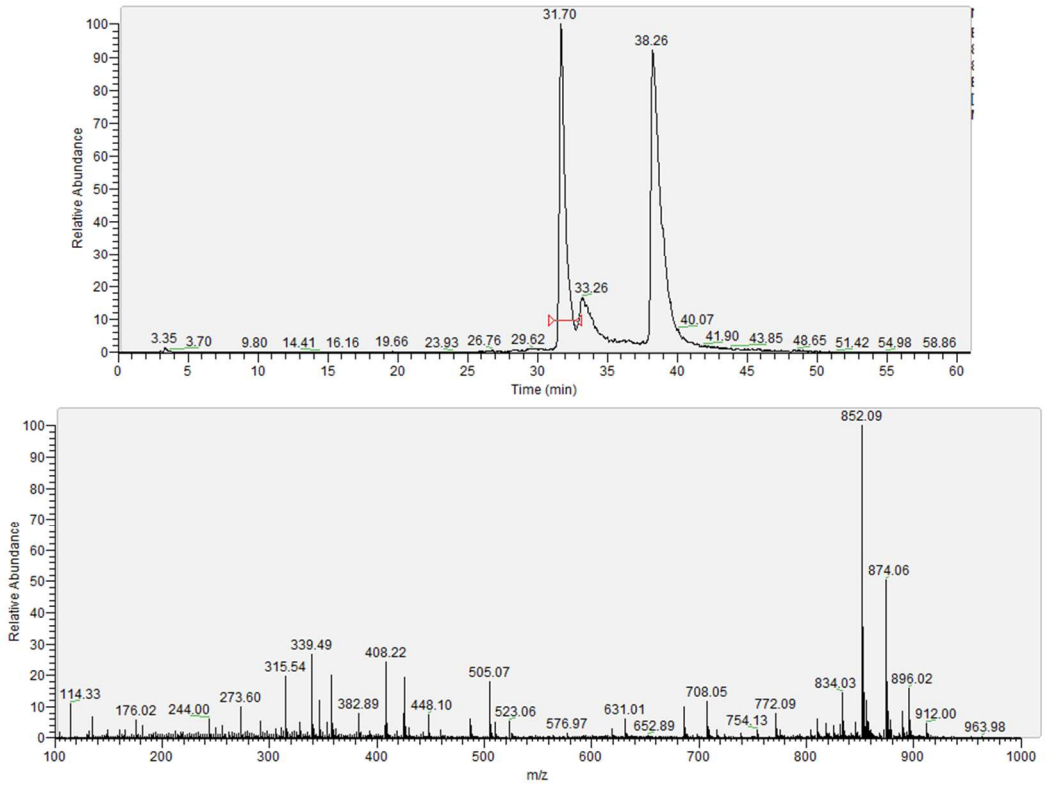

Figure 11. LC/MS Chromatogram of *yhaR* Reaction Showing Most Abundant m/z Corresponding to 3-Hydroxybutyryl-CoA. Retention time of 31.70 minutes.

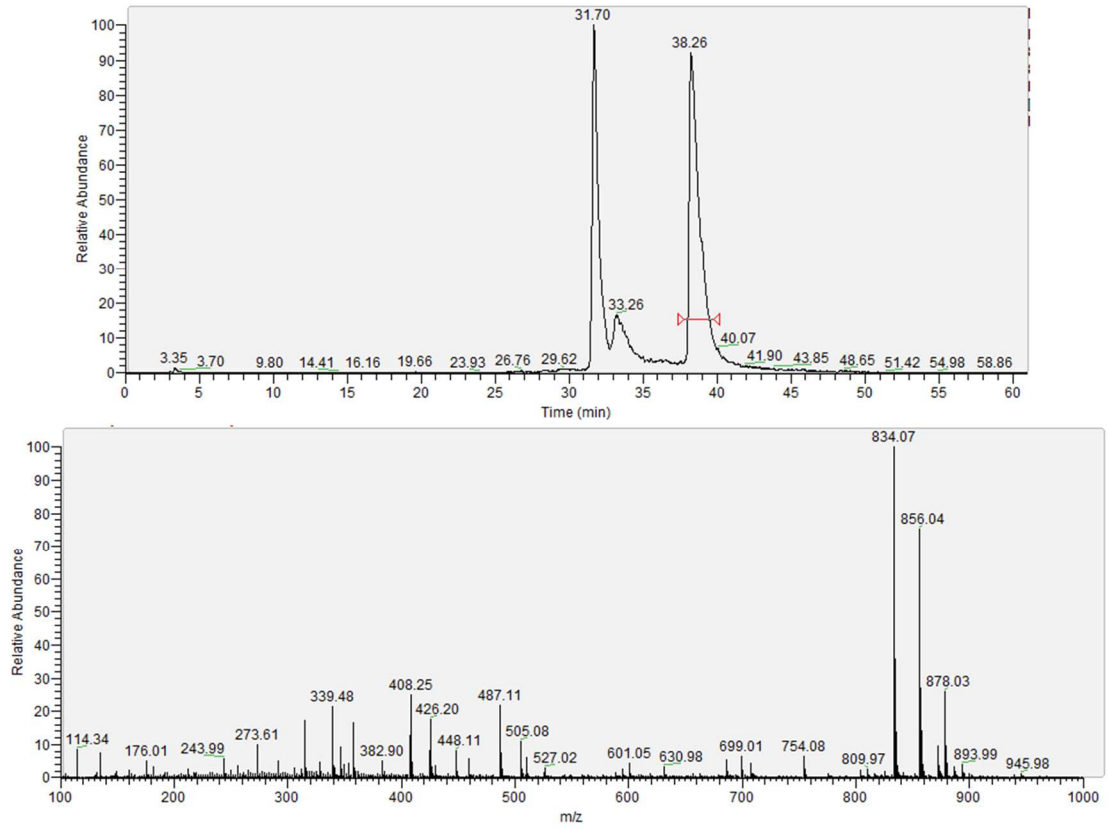

Figure 12. LC/MS Chromatogram of *yhaR* Reaction Showing Most Abundant m/z Corresponding to Butenoyl-CoA. Retention time of 38.26 minutes.

III.D SNAC Analogue Activity

To this point the *yhaR* enzyme has been shown to be active with a straight chain acyl-CoA derived from a straight-chain fatty acid, one the major types of fatty acid in *B. subtilis*.[17] The most abundant type of fatty acid is the branched-chain fatty acid, which consists of approximately 85% of fatty acids in *B. subtilis*.[17-19] Given the occurrence of branched-chain fatty acids in *B. subtilis, yhaR* activity with methyl-branched acyl-CoA should be explored. The methylated version of butenoyl-CoA is not available commercially and the sensitivity of the CoA complicates efficient synthesis. More easily

synthesized truncated analogues have been used to investigate activity when the natural substrate is unavailable. One example of this is the truncated analogue N-acetylcysteamine (SNAC) and its high activity with polyketide synthase.[21] Enzyme activity with the truncated substrate analogue butenoyl-SNAC was analyzed with HPLC coupled to UV detection. The butenoyl-SNAC substrate and 3-hydroxybutyryl-SNAC product standards were synthesized by Jennifer Doyle, an undergraduate in the Reddick lab, and the resultant chromatograms can be seen in Figures 13 and 14 respectively. Butenoyl-SNAC was found to have a retention time of approximately 11.9 minutes while the 3-hydroxybutyryl-SNAC was found to have multiple peaks suggesting some contamination of the sample.

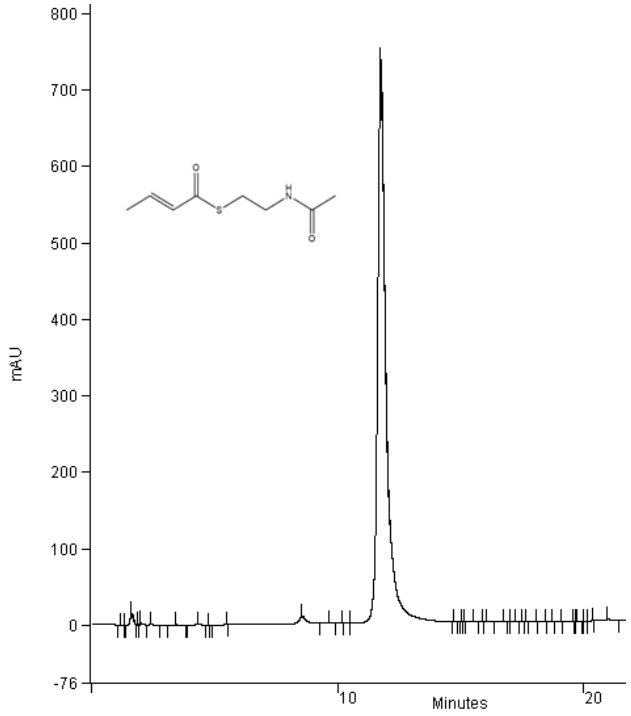

Figure 13. Chromatogram of Butenoyl-SNAC Standard.

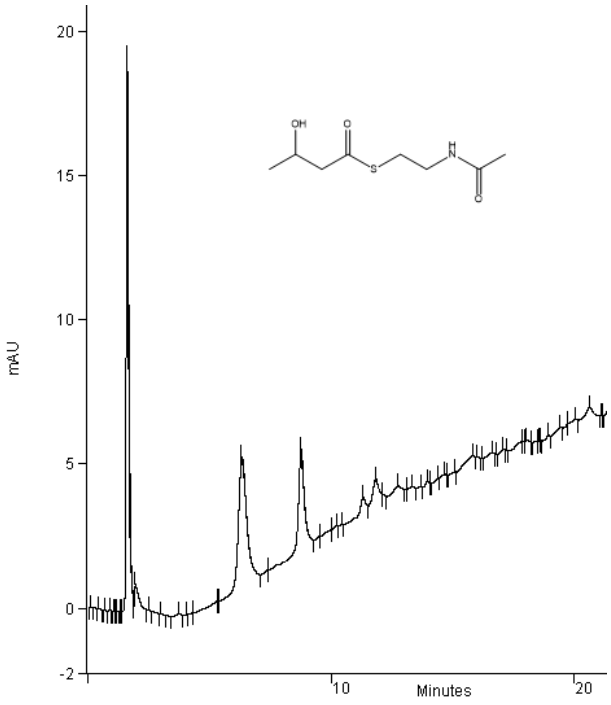

Figure 14. Chromatogram of 3-HydroxyButyryl-SNAC Standard.

In Figure 15, the HPLC chromatogram shows a reaction containing 1mM butenoyl-SNAC and *yhaR* incubated for 2 hours. A peak at approximately 10 minutes is observed that is not seen in the butenoyl-SNAC standard chromatogram indicating potential product formation, however Figure 16 displays the chromatogram from a control reaction with all components except the *yhaR* enzyme in which the peak is still present. This suggested that the observed peak was some side reaction potentially with mercaptoethanol.

At this point the lack of any observable *yhaR* activity with the SNAC analogue and the apparent slight lability of this analogue under these conditions led us to move back to investigating the natural CoA-derived substrate.

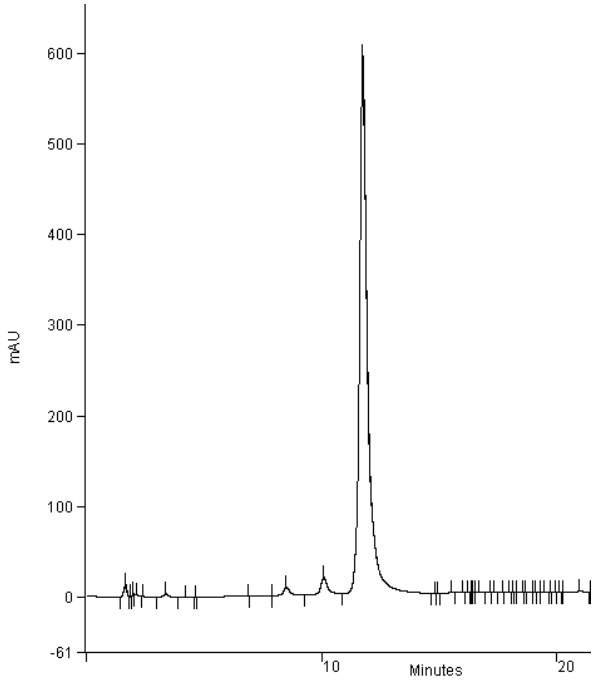

Figure 15. Chromatogram of 1.0 mM Butenoyl-SNAC Reaction with *yhaR*.

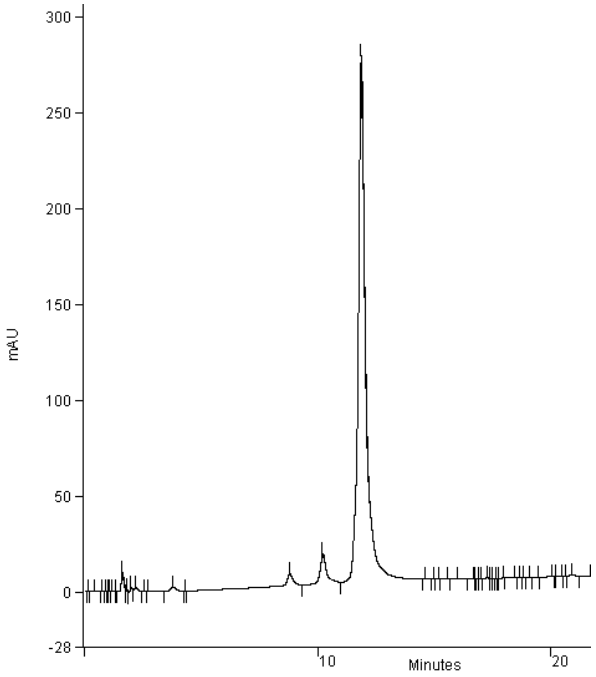

Figure 16. Chromatogram of 1.0 mM Butenoyl-SNAC Control without *yhaR*.

III.E Michaelis Menten Kinetics

Crotonoic acid was added to quenched reactions as an internal standard to allow for quantification by HPLC analysis. Figures 17 and 18 demonstrates the retention time of crotonoic acid relative to butenoyl-CoA and 3-hydroxybutyryl-CoA respectively where crotonoic acid has a retention time of approximately 13 minutes.

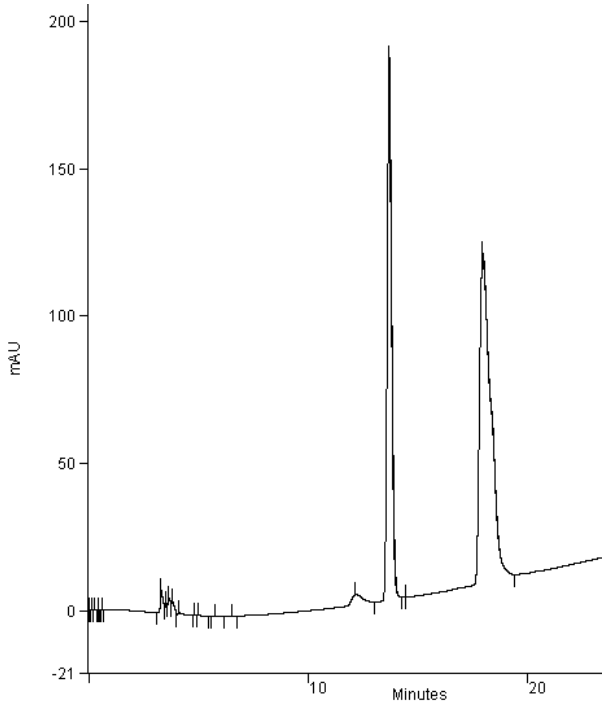

Figure 17. Chromatogram Containing Crotonoic Acid and Butenoyl-CoA. Crotonoic acid and butenoyl-CoA have retention times of 13 and 18 minutes respectively.

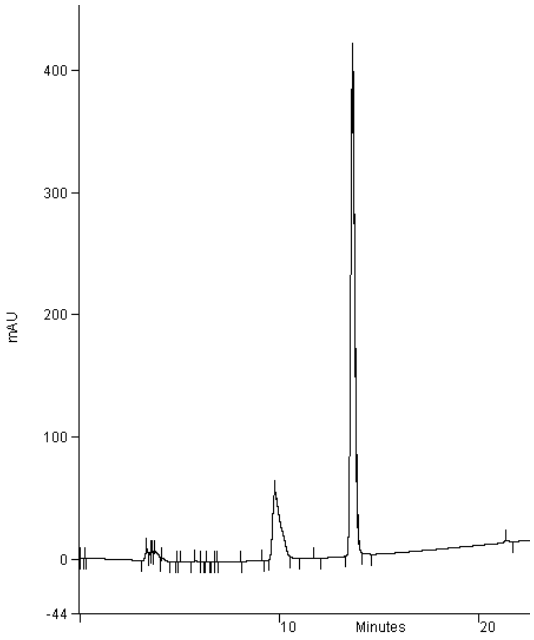

Figure 18. Chromatogram Containing Crotonoic Acid and 3-Hydroxybutyryl-CoA. Crotonoic acid and 3-hydroxybutyryl-CoA have respective retention times of 13 and 10 minutes.

The calibration plots of concentration of butenoyl-CoA vs percent relative integration and concentration of 3-hydroxybutyryl-CoA versus percent relative integration can be found in Figures 19 and 20 respectively. Both fits suggest that utilizing crotonoic acid as an internal standard was a viable method that can provide an accurate quantification of either substrate or product.

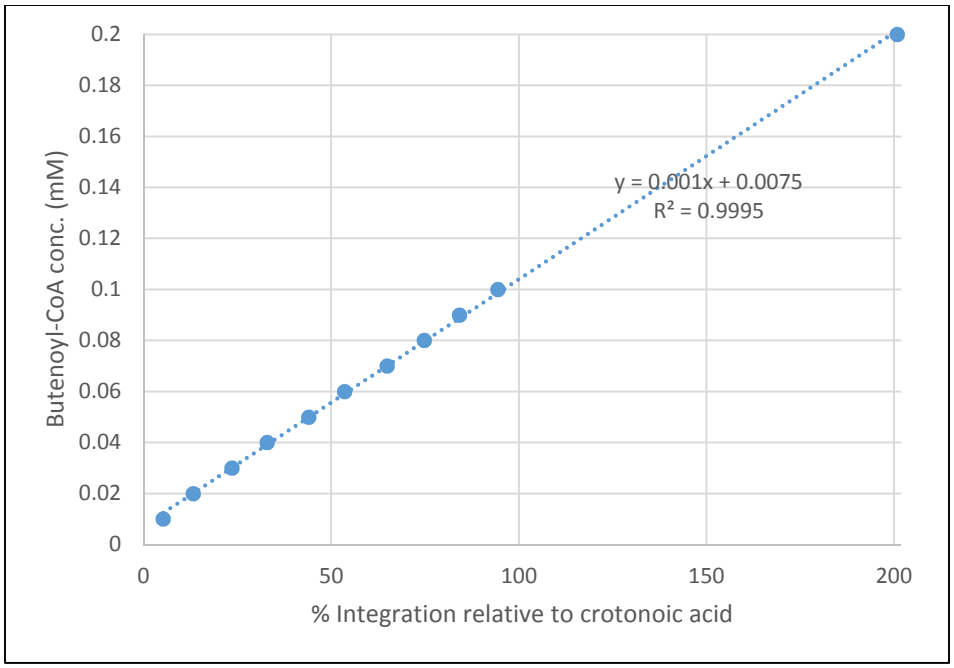

Figure 19. Butenoyl-CoA Calibration Plot.

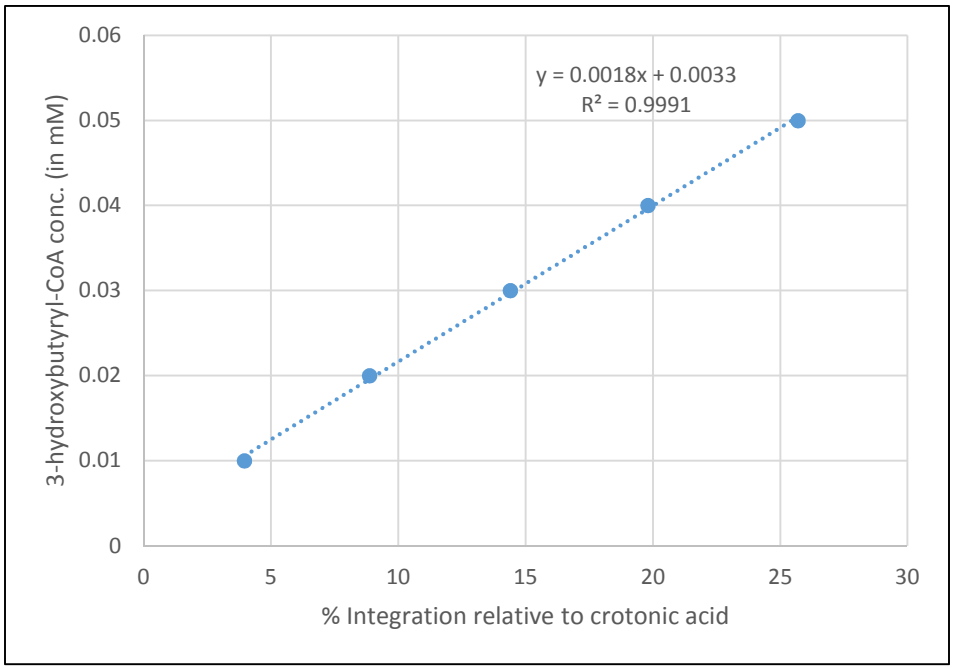

Figure 20. 3-Hydroxybutyryl-CoA Calibration Plot.

Results for the Michaelis Menten kinetics can be seen in Figure 21, were the fit was created using the SigmaPlot program. All reactions were run in pH 6.5 buffer and at 37 ºC. One anomaly observed for the Michaelis Menten curve was that initial rates for the 0.8 and 1.0 mM substrate concentration reactions did not match the expected trend. One possible reason for this is that baseline resolution of the UV detection was affected by the high concentrations. Analysis of the Michaelis Menten curve revealed that the enzyme has a V_{max} of 2.4 X 10^{-3} ± 2.0 X 10^{-4} mM/min and a K_m of 0.14 ± 0.03 mM under the given conditions. The k_{cat} was found to be 6.1 X 10^{-3} ± 9.2 X 10^{-4} s^{-1}. The low observed K_m suggest that butenoyl-CoA has a relatively high binding affinity with the *yhaR* enzyme, however binding affinity is lower than other crotonase derived from various organisms having K_m ranging from 20 to 70 μM.[14,20,22] Compared to other enoyl-CoA hydratase enzymes the observed V_{max} is relatively slow suggesting that conditions are not yet optimal for the reaction.

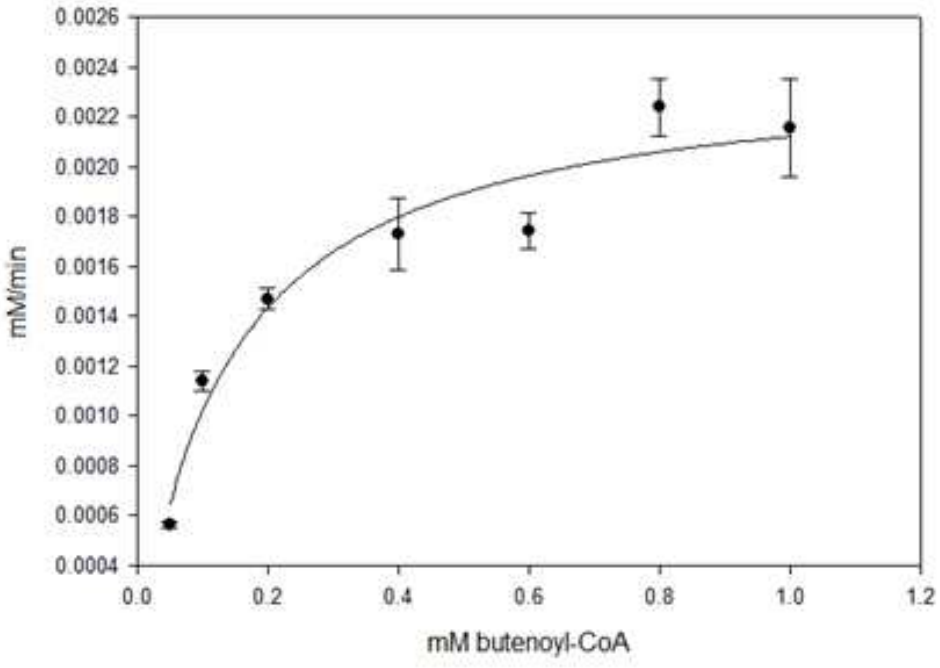

Figure 21. Michaelis Menten Curve of the *yhaR* Enzyme Activity on Butenoyl-CoA.

CHAPTER IV

CONCLUSION

In conclusion, we have confirmed that *yhaR* can carry out the enoyl-CoA hydratase activity missing in the fatty acid beta-oxidative pathway encoded for by the *mmg* operon. We have shown by using HPLC coupled to a UV detector that the *yhaR* enzyme can convert butenoyl-CoA into the hydratase product 3-hydroxybutyryl-CoA. Product formation was confirmed using LC-MS. Quantification of HPLC methods allowed for kinetics analysis. Michaelis Menten kinetics revealed that at 37 ^{o}C and pH 6.5 the enzyme has a k_{cat} of 6.1 X 10^{-3} ± 9.2 X 10^{-4} s^{-1}. Butenoyl-CoA substrate was found to have a K_m of 0.14 ± 0.03 mM. The low Km shows that butenoyl-CoA is a suitable substrate further suggesting that *yhaR* is a reasonable candidate for completing the beta-oxidative pathway encoded by the *mmg* operon during sporulation.

Future work to be done with the *yhaR* enzyme would include carrying through its reaction product to the *mmgB* protein, which is known to be a 3-hydroxybutyryl-CoA dehydrogenase, to demonstrate pathway compatibility. Further characterization would include continued investigations of substrate specificity. Branched chain fatty acids are common in *B. subtilis* so the ability to be active with methyl branched enoyl-CoA would be expected. Activity with methyl branched acyl-CoA beginning with *mmgC* following

through the pathway to *mmgA* would produce propionyl-CoA the substrate for *mmgD*, thereby leading the activity into the methycitric acid cycle.[11]

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