SIRKISOON, SHERONA R., M.S. Characterization of *CitB* in the Methylcitric Acid Cycle of *Bacillus subtilis* 168 and Characterization of Antimicrobial activity in the Mucosal Epithelial Layer and Gill Tissue of Largemouth Bass (*Micropterus salmoides*). (2014)
Directed by Dr. Jason J. Reddick. 59 pp.

Bacillus subtilis 168 is a Gram-positive, aerobic, rod shaped bacterial species that has the ability to undergo sporulation when under nutritional stress. There is an incomplete understanding of the metabolic pathways in *B. subtilis* 168 during sporulation. Characterizing the metabolic pathways of *B. subtilis* is important for understanding the sporulation process in pathogenic bacteria including *B. anthracis* (anthrax), for understanding cell differentiation of stem cells, and for maximizing biotechnological applications of *B. subtilis* 168. In this study, we hypothesize that *citB,* the only known aconitase in *B. subtilis* 168, can convert 2-methylaconitate to 2-methylisocitrate in the methylcitric acid cycle. This is an important step missing from the methylcitric acid cycle encoded by the mother cell metabolic gene (*mmg*) operon. In this study we utilized an overall approach involving purified *citB* protein, HPLC coupled to UV-VIS, and LC-MS to show that the *citB* protein can convert 2-methylaconitate to 2-methylisocitrate. We have also shown that a previously uncharacterized 2-methylisocitrate lyase (*yqiQ*) from *B. subtilis* 168 can convert the *citB* product 2-methylisocitrate to succinate and pyruvate, thus completing the methylcitric acid cycle. This study confirmed our hypotheses regarding *citB* and *yqiQ* and also achieved the first *in vitro* reconstitution of a complete methylcitric acid cycle from *B. subtilis*.

There is little known about the biochemistry of largemouth bass (*Micropterus salmoides*), especially the antimicrobial properties that may be present in the outer mucosal layer of the skin and gills as an initial defense against bacteria, protozoa and fungi. Understanding the antimicrobial properties of the small antimicrobial peptides in largemouth bass is important for the development of new antibiotics for potential use in humans and for decreasing the mortality rates of largemouth bass handled in recreational and tournament fishing, as well as those captured and released for studies by state wildlife management agencies. It is hypothesized that the skin secretions and gills of largemouth bass contain a small, cationic, amphipathic peptide that prevents initial infection from microorganisms present in the environment. In this study, we have tested the hypothesis with *in vitro* experiments through the use of disc diffusion assays on *Escherichia coli* K12 and *Bacillus subtilis* 168 bacteria and liquid growth assays using *Staphylococcus aureus*. Results of this study show that there is antimicrobial activity in the gills of largemouth bass. We will also report ongoing efforts toward isolating and characterizing the components responsible for this antimicrobial activity.

CHARACTERIZATION OF *CITB* IN THE METHYLCITRIC ACID CYCLE OF

BACILLUS SUBTILIS 168 AND CHARACTERIZATION OF

ANTIMICROBIAL ACTIVITY IN THE MUCOSAL

EPITHELIAL LAYER AND GILL TISSUE OF

LARGEMOUTH BASS

(*MICROPTERUS*

SALMOIDES)

by

Sherona R. Sirkisoon

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

Greensboro
2014

Approved by

_____
Committee Chair

To each and every person who has continually guided and supported me on my journey

for knowledge, I sincerely thank you.

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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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 METHYLCITRIC ACID CYCLE

I.A Background of *Bacillus Subtilis*

B. subtilis is a Gram-positive, aerobic, rod shaped bacterial species that has the ability to undergo sporulation when under nutritional stress, similar to *B. anthracis* (anthrax), a close relative.[1] Not only is *B. subtilis* a model organism for the study of sporulation, it is also used to remove sulfur components from coal and has biotechnological applications such as a biosurfactant production and as a source for antibiotic lipopeptides.[2,3,4] Although the complete genome has been sequenced, many of the genes remain unstudied and all of the metabolic pathways encoded by these genes are not fully understood, including the methylcitric acid cycle.[5] Characterizing the metabolic pathways of *B. subtilis* and the genes that play a role in each pathway is important for understanding the sporulation process in pathogenic bacteria including *B. anthracis*, for understanding cell differentiation of stem cells, and for maximizing biotechnological applications of *B. subtilis*. Also, a better understanding of key regulatory points of sporulation in *B. subtilis* will lead to a better understanding and treatment of pathogenic bacteria such as *B. anthracis*. For example, a comparison between *mmgE*, a gene expressed during sporulation, from *B. subtilis* and the anthrax genome showed 76% identical and 24% similar amino acid sequence for 29 different regions of the anthrax

genome suggesting that regulatory points of sporulation *B. subtilis* may be applied to regulating sporulation of anthrax.[6]

I.B The Methylcitric Acid Cycle

B. subtilis undergoes sporulation when there is a depletion of carbon, nitrogen or phosphorous.[7] Sporulation is the result of an asymmetric division of a cell into two separate compartments. The larger portion of the dividing cell is termed the mother cell, and the smaller portion, which later becomes the spore, is termed the prespore. The mother cell aids in the development of the spore and lyses to release the environmentally resilient spore.[8] Environmental stress leads to downstream signaling which turns on the key transcription regulator, Spo0A, by phosphorylation. Once Spo0A is activated, the RNA polymerase sigma factor E (σ^E) is turned on in the mother cell, which controls the expression of several genes, including the *mmg* (mother cell metabolic genes) operon.[9] The *mmg* operon encodes six genes, *mmgABCDE* and *yqiQ* (Figure 1), three of which are homologs of enzymes from other organisms that play a role in the methylcitric acid cycle, *mmgDE* and *yqiQ*.[4,10]

Figure 1. Mother Cell Metabolic Gene (mmg) Operon. Proposed genes involved in the methylcitric acid cycle, *mmgD* (methylcitrate synthase), *mmgE* (2-methylcitrate dehydratase) and *yqiQ* (isocitrate lyase).[4,8]

Nutritional stress triggers the expression of the methylcitric acid cycle, which is believed to be an emergency pathway in bacteria and fungi.[11,12] The methylcitric acid

cycle for the metabolism of odd-carbon n-alkanes or propionyl-CoA using seven-carbon

tricarboxylic acids in yeast is indicated by the bold arrows in Figure 2.[13] In *E. coli*, the

first step in the methylcitric acid cycle involves propionyl-CoA, oxaloacetate, and 2-

methylcitrate synthase, PrpC, to form 2-methylcitrate.[10] The next step includes the

dehydration of 2-methylcitrate to 2-methyl-*cis*-aconitate by 2-methylcitrate dehydratase,

PrpD. The third step involves the rehydration of 2-methyl-*cis*-aconitate to form 2-

methylisocitrate. In *E. coli*, the rehydration is done by AcnB, which is one of two

aconitases encoded in the *E. coli* genome. The fourth step involves the 2-methylisocitrate

lyase, PrpB in *E. coli*, to produce pyruvate and succinate. The enzymes from the mmg

operon, *mmgDE* and *yqiQ*, are homologs of the *E. coli* enzymes that play a role in the

methylcitric acid pathway. The homolog in *B. subtilis* 168 of PrpC, 2-methylcitrate

synthase, is *mmgD*. The homolog in *B. subtilis* 168 of PrpD, 2-methylcitrate dehydratase,

is *mmgE* and for PrpB, 2-methylisocitrate lyase, it is *yqiQ*. In some species, an mmgE

homolog does both steps, whereas in other species, mmgE catalyzes the dehydration step

and an aconitase comes in to catalyze the rehydration step.[10] However, reactions with

mmgE stop at the 2-methyl-*cis*-aconitate product and does not go further to produce 2-

methylisocitrate, the next step in the methylcitric acid pathway.[14] This suggests that there

is a step missing in the methylcitric acid pathway that is not encoded by the mmg operon,

the conversion of 2-methyl-*cis*-aconitate to 2-methylisocitrate. *CitB* is the only known

aconitase in *B. subtilis* 168, but its function in the methylcitric acid cycle has not been

characterized. Previous members of the Reddick lab have characterized the forward

reactions of *mmgD* and *mmgE* enzymes. The reverse reaction of *yqiQ* was characterized

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previously as well, however, the forward reaction has not been characterized since there

is no commercially available standard of 2-methylisocitrate, the substrate for *yqiQ*.
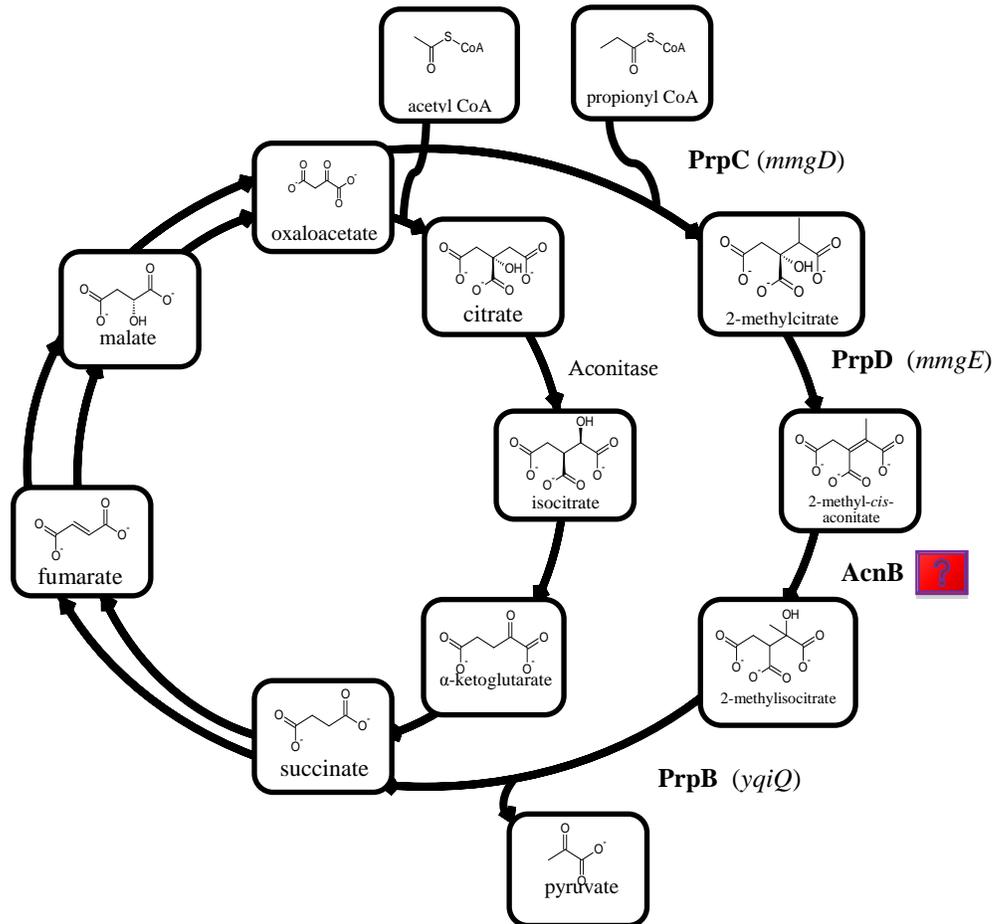
Figure 2. Citric Acid Cycle and Methylcitric Acid Cycle Pathway. *E. coli* enzymes in bold and proposed *B. subtilis* enzymes in parentheses for the methylcitric acid cycle. [4,8]

I.C Overview of *CitB* and Goal

CitB is a gene that codes for the bifunctional enzyme aconitase in *Bacillus subtilis*. The *citB* enzyme catalyzes the reversible conversion of cis-aconitate to isocitrate. In the *citB* null mutant of *B. subtilis*, citrate accumulates and chelates Mn^{2+} and $Fe^{2+.}$ This prevents phosphorylation of Spo0A, the main transcription factor for early sporulation-specific genes that is activated by phosphorylation. The sporulation defect is partially overcome upon the addition of excess Mn^{2+} and Fe^{2+} ions. Researchers suggested that while citric acid accumulation causes the stage 0 block in sporulation, an additional function of *citB* is vital to sporulation at later stages. First, the *citB* protein has sequence similarity to the eukaryotic iron regulatory protein 1 (IRP-1), which is known to bind RNA. Expressing yeast mitochondrial aconitase in a *citB* null *B. subtilis* mutant restored the mutant's ability to isomerize citrate to isocitrate. However, this mutant expressing the yeast aconitase, which does not possess RNA binding activity, only underwent partial sporulation.[11,7] It was determined that the RNA binding activity of *citB* missing in the null mutant is essential for sporulation.

CitB function is dictated by the iron-sulfur cluster cofactor necessary for catalytic activity. The cofactor is a 4Fe-4S cluster that interacts directly with citrate, aconitate, or isocitrate, therefore, the cluster is exposed to solvent and is susceptible to oxidation. Under low oxidation conditions, only one Fe atom from the 4Fe-4S cluster may be lost leaving a 3Fe-4S cluster and a catalytically inactive enzyme. Eventually the other three iron atoms are lost resulting in the apo form of the protein. Once the protein is in the apo form, it behaves like IRP-1, binding RNA and functioning as a posttranscriptional

regulator. The switch in activity, between the enzymatic function and the RNA binding function, is mainly due to the amount of citrate present since citrate is a chelator of iron and is also cotransported with iron. Therefore, at high citrate concentrations, iron is taken from iron-containing proteins such as aconitase, resulting in the RNA binding form of aconitase. The rationale is that excess citrate levels require excess aconitase levels in order to metabolize the citrate.

The overall goal of this project is to discover and characterize the 2-methylaconitate hydratase needed by the methylcitric acid cycle pathway of *B. subtilis*. The hypothesis being tested is that *citB*, the only known aconitase in *B. subtilis*, can convert 2-methylaconitate to 2-methylisocitrate. This is an important step missing from the methylcitric acid cycle encoded by the mother cell metabolic gene (*mmg*) operon. The *citB* enzyme has been shown to carry out the reversible reaction of converting citrate to isocitrate in the "normal" citrate cycle.[11] To further characterize *citB*, we will test whether the expected reaction product of *citB*, 2-methylisocitrate, will be converted to pyruvate and succinate when given the proposed 2-methylisocitrate lyase, *yqiQ*, from *B. subtilis*.

CHAPTER II

EXPERIMENTAL PROCEDURES

II.A Bacteria and Culture Methods for *CitB*

B. subtilis strain AWS198 was cultured on Difco sporulation medium (DSM) plates with 2.5 µg/ml chloramphenicol.[11] DSM plates were prepared by combining approximately 8 g Bacto nutrient broth, 10 g KCl, 12 g $MgSO_4 \cdot 7H_2O$, 1 mL of 1 M $Ca(NO_3)_2$, 1 mL of 0.01 M $MnCl_2 \cdot 6H_2O$ and 1 mL of 0.001 M $FeSO_4 \cdot 7H_2O$ with water for a total volume of 1 L. To 500 mL of DSM 7.5 g of agar was added and autoclaved for 20 minutes. Prior to pouring plates, 500 µL of chloramphenicol at 2.5 mg/mL was added to each 500 mL aliquot of DSM for a final concentration of 2.5 µg/mL. Bacteria was streaked onto plates using standard procedures and incubated overnight at 37 °C. A starter culture was prepared by adding 1 colony of bacteria to 5 mL of DSM with 2.5 µg/mL chloramphenicol and incubated overnight at 37 °C while shaking.[11]

II.B *CitB* Purification

Prior to harvesting, 1 mL of the starter culture was added to 1 L of DSM until an OD_{600} of approximately 1 was reached for two separate cultures. Cells were then centrifuged at 6,500 rpm using a JA-10 rotor for 30 minutes at 4 °C. Pellets were washed with 20 mM Tris-citrate buffer at pH 7.35 then centrifuged again at the same conditions. The supernatant was discarded, and pellets were combined and stored at -80 °C until use. Cells were thawed to room temperature and resuspended in 20 mL of buffer containing

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200 mM KCl, 50 mM Tris-HCl, 10% Triton X-100, 10% glycerol, 1 mM PMSF, 0.2 mM

EDTA, and 0.5 mM dithiothreitol. The resuspended cell mixture was sonicated for 3

minutes with 30 second resting intervals and centrifuged for 30 minutes at 11,500 rpm in

a JA-20 rotor at 4 °C. The supernatant was dialyzed for 1 hour at 4 °C in 4 liters of buffer

containing 200 mM KCl, 50 mM Tris-HCl, 10% v/v Triton X-100, 10% v/v glycerol, and

1 mM PMSF with a final pH of 7.5. The cobalt column was prepared by packing 4 mL of

a 50% mixture of Ni-NTA and Agarose by gravity. Once packed, 12 mL of 1x strip

buffer containing 0.5 M NaCl, 100 mM EDTA, and 20 mM Tris-HCl (pH 7.9) was added

to remove the nickel, then rinsed with 6 mL of water. The column was recharged with

cobalt (II) with 10 mL of buffer containing 0.1 M cobalt (II) sulfate heptahydrate. The

column was conditioned with 6 mL of a binding buffer containing 10 mM imidazole, 200

mM KCl, 50 mM Tris-HCl, 10% Triton X-100 and 10% glycerol with a final pH of 7.5.

The dialyzed supernatant was syringe filtered using a 0.45 μm filter, loaded onto the

column, and flowed by gravity. The column was washed with 20 mL of binding buffer,

then 12 mL of a 60 mM imidazole buffer containing 200 mM KCl, 50 mM Tris-HCl,

10% Triton X-100, and 10% glycerol (pH 7.5). Lastly, the column was loaded with 12

mL of the elution buffer and collected in 1 mL fractions. The elution buffer contained

300 mM imidazole, 200 mM KCl, 50 mM Tris-HCl, 10% Triton X-100, and 10%

glycerol (pH 7.5).[11]

Each fraction was tested for the presence of aconitase by running a 10% SDS-

PAGE gel containing 4.1 mL H_2O, 2.5 mL of 1.5 M Tris-HCl (pH 8.8), 3.3 mL

acrylamide, 50 μL of 20% SDS, 120 μL 10% APS, and 15 μL TEMED. Fractions

showing a ~100 kDa band were dialyzed overnight at 4 °C in 4 liters of a dialysis buffer containing 50 mM KCl, 20 mM Tris-HCl, and 10% glycerol at pH 7.5.

II.C *CitB* Activity Assays

Prolonged exposure to air causes the Fe-S clusters in the CitB aconitase to become oxidized leaving the enzyme inactive. An activation buffer, freshly prepared, containing 50 mM Tris-Base, 8 mM dithiothreitol, and 0.8 mM $Fe(NH_4)_2(SO_4)_2$ was used to restore the Fe-S clusters. To reactivate the enzyme, 60 μL activation buffer was added to 40 μL enzyme and was kept at room temperature for 10 minutes before use. To test for activity, a 500 μl sample was prepared by adding 10 μL active enzyme to 20 mM D,L isocitrate and 20 mM Tris-HCl (pH 7.5) and monitored over 30 minutes at 240 nm using a UV-Visible spectrophotometer. HPLC, coupled with a UV-Vis detector, was utilized to further verify that the enzyme was active. Reverse phase chromatography was done using a Synergi, hydro-RP, 250 x 4.60 mm, 4 micron, 80 Å, column by Phenomenex.[15] An isocratic method, at a 0.7 mL/min flow rate, was done using 20 mM sodium phosphate buffer (pH 2.9) as the mobile phase with detection at 240 nm.[11]

II.D *CitB* Activity in the Methylcitric Acid Cycle

To test for the 2-methylaconitate hydratase activity of the *citB* enzyme, a 1 mL reaction was prepared containing 895 μL of 20 mM Tris-HCl (pH 7.5), 0.9 mM 2-methylcitrate, and 20 μL of *mmgE*. After 2 hours at room temperature, 40 μL of activated *citB* protein was added. The reaction was left at room temperature for 3 hours, and then quenched with 100 μL of 1 M sodium phosphate (pH 2.9). A 0.9 mM standard of 2-

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methylcitrate was prepared as well as a control containing 0.9 mM 2-methylcitrate and 20 μL of *mmgE* for 5 hours at room temperature.

II.E Further Characterization of *CitB* in the Methylcitric Acid Cycle

To further characterize the activity of *citB*, a reaction containing the *mmgD*-synthesized 2-methylcitrate was utilized. Before experiments could begin, *mmgD* had to be purified.

II.E.1 Bacteria and Culture Methods of *mmgD*

E. coli strain BL21(DE3) replicating the *mmgD*-pET-28a plasmid was cultured on LB agar plates containing 30 μg/mL kanamycin.[16] LB agar plates were prepared by combining approximately 11 g of Bacto Tryptone, 11 g NaCl, and 5 g yeast extract into 1 L of water. A 500 mL aliquot of LB was removed for starter cultures and approximately 8 g of agar was added to the remaining 500 mL of media. Both aliquots were autoclaved for 20 minutes. Prior to pouring plates, 500 μL of 30 mg/mL kanamycin was added to the 500 mL aliquot containing agar for a final concentration of 30 μg/mL kanamycin. Bacteria was streaked onto plates using standard procedures and incubated overnight at 37 °C. In order to recover more purified mmgD, the bacteria growth was doubled. Two starter cultures were prepared by adding 1 colony of bacteria and 5 μL of 30 mg/mL kanamycin to two 5 mL aliquots of LB for a final concentration of 30 μg/mL. The starter cultures were incubated overnight at 37 °C while shaking at 220 rpm.

The following day, 2 μL of each starter culture was added to two separate 1 L flasks of LB containing 30 μg/mL kanamycin and incubated at 37 °C while shaking at 245 rpm until an OD_{595} of approximately 0.4 was reached. The cultures were then kept at

room temperature 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 each culture and incubated overnight at 18 °C while shaking at 220 rpm. 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 stored at -80 °C.

II.E.2 Purification of *mmgD* Protein

Cell pellets were thawed to room temperature and resuspended in 40 mL of a 1x binding buffer containing 0.5 M NaCl, 80 mM Tris buffer, and 5 mM imidazole (pH 8). Approximately 0.6 g of lysozyme was added to the resuspended cells while stirring gently for 15 minutes and then sonicated on ice for six 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 8 mL of a 50% mixture of Ni-NTA and Agarose by gravity for a final column volume of 4 mL. Since the bacterial culture was doubled, the column volume needed to doubled as well. Once packed, the cell lysate was loaded and allowed to flow by gravity and 40 mL of binding buffer containing 80 mM Tris-HCl, 0.5 M NaCl, and 5 mM imidazole (pH 8). Next, 24 mL of wash buffer containing 60 mM imidazole was loaded and allowed to flow by gravity. Lastly, 24 mL of elution buffer containing 1 M imidazole was added and collected in 1 mL fractions. The 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

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presence of protein, the red/brown reagent changes color to bright blue. Eight fractions showed a bright blue color change and were pooled and dialyzed overnight at 4 °C in buffer containing 25 mM Tris-HCl (pH 8). Once dialyzed, approximately 10 mL of protein was recovered. To this, sterile glycerol was added for a final concentration of 10% and was stored at -80 °C in 100 μL aliqots until use.

The purity of *mmgD* protein was tested using a 12% SDS-PAGE gel containing 3.4 mL of H_2O, 4 mL of 30% 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. An expected band around 40 kDa was observed, indicating purified mmgD.

II.E.3 Reactions with *mmgD* Protein

A 1 mL reaction was prepared containing 820 μL 20 mM Tris-HCl (pH 7.5), 0.4 mM propionyl-CoA, 0.2 mM oxaloacetate, 10 μL *mmgD* protein, and 100 μL *mmgE* protein. After 3 hours at room temperature, 40 μL of *citB* protein was added and left at room temperature for 2 hours. The reaction was quenched with 100 μL of 1 M sodium phosphate (pH 2.9). Several control reactions and standards were also prepared to verify the results. A control of the *mmgD* reaction was prepared by combining 960 μL 20mM Tris-HCl (pH 7.5), 0.4 mM propionyl-CoA, 0.2 mM oxaloacetate, and 10 μL *mmgD* protein at room temperature for 5 hours. A control of the *mmgE* reaction was prepared by combining 935 μL of 20 mM Tris-HCl (pH 7.5), 0.9 mM 2-methylcitrate, and 20 μL of *mmgE* protein at room temperature for 5 hours. A 0.9 mM standard of 2-methylcitrate was prepared as well as a 0.4 mM standard of propionyl-CoA, and a 0.2 mM standard of oxaloacetate. Standards of 2-methyl-*cis*-aconitate and 2-methylisocitrate were not

prepared because they are currently commercially unavailable. All reactions and controls were analyzed using HPLC coupled to a UV-Vis detector. Reverse phase chromatography was done using the same column mentioned above. The chromatography was done using 20 mM sodium phosphate buffer (pH 2.9) with a linear increase in methanol as the mobile phase and absorbance was at 240 nm.[11] The gradient used in this method starts with 100% sodium phosphate (pH 2.9) and increases linearly to 15% methanol for 20 minutes. From 20 to 25 minutes the methanol concentration continues to increase linearly to 40% and is held at 40% for 10 minutes. In the next five minutes there is a linear decrease of methanol from 40% to 0%. The sodium phosphate (pH 2.9) solvent is held at 100% for 10 minutes completing the 50 minute method.

The same reactions and control samples were prepared for analysis on the LC/MS. The mobile phases used were 0.1% formic acid and methanol using the same gradient mentioned above for sodium phosphate and methanol. Reactions were quenched using 100 µl of 0.01% formic acid.

II.F Conversion of 2-Methylisocitrate to Succinate and Pyruvate

II.F.1 Bacteria and Culture Methods of *yqiQ*

Culture and purification methods were followed as outlined in William Booth.[17] *E. coli* strain BL21(DE3) replicating the *yqiQ*-pET-28a plasmid was cultured on LB agar plates containing 30 µg/mL kanamycin. LB agar plates were prepared by combining approximately 11 g of Bacto Trypotone, 11 g NaCl, and 5 g yeast extract into 1 L of water. A 500 mL aliquot of LB was removed for starter cultures and approximately 8 g of agar was added to the remaining 500 mL of media. Both aliquots were autoclaved for 20

minutes. Prior to pouring plates, 500 µL of 30 mg/mL kanamycin was added to the 500 mL aliquot containing agar for a final concentration of 30 µg/mL kanamycin. Bacteria was 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 5 mL of LB for a final concentration of 30 µg/mL. The starter culture was incubated overnight at 37 °C while shaking at 220 rpm.

The following day, 2 µL of starter culture was added to 1 L of LB containing 30 µg/mL kanamycin and incubated at 37 °C while shaking at 245 rpm until an OD_{595} of approximately 0.4 was reached. The culture was then kept at room temperature 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 and incubated overnight at 18 °C while shaking at 220 rpm. 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 stored at -80 °C.

II.F.2 Purification of *yqiQ* Protein

Cell pellets were thawed to room temperature and resuspended in 20 mL of a 1x binding buffer containing 0.5 M NaCl, 80 mM Tris buffer, and 5 mM imidazole (pH 8). Approximately 0.3 g of lysozyme was added to the resuspended cells while stirring gently for 15 minutes and then sonicated on ice for six 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 4 mL of a

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50% mixture of Ni-NTA and Agarose by gravity for a final column volume of 2 mL. Once packed, the cell lysate was loaded and allowed to flow by gravity and 20 mL of binding buffer containing 80 mM Tris-HCl, 0.5 M NaCl, and 5 mM imidazole (pH 8). Next, 12 mL of wash buffer containing 60 mM imidazole was loaded and allowed to flow by gravity. Lastly, 12 mL of elution buffer containing 1 M imidazole was added and collected in 1 mL fractions. The 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. Fractions 2, 3, and 4 showed a bright blue color change, therefore, these fractions were pooled and dialyzed overnight at 4 °C in buffer containing 25 mM Tris-HCl (pH 8). After dialysis, approximately 2.8 mL of protein was recovered. To this, 400 μL of 80% glycerol was added for a 10% final concentration of glycerol and was stored at -80 °C in 100 μL aliqots until use.

The purity of *yqiQ* protein was tested using a 12% SDS-PAGE gel containing 3.4 ml of H_2O, 4 ml of 30% 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. An expected band around 30 kDa was observed, indicating that yqiQ was successfully purified.

II.F.3 Reactions with *yqiQ* Protein

A complete 1 mL reaction was prepared by combining 520 μL of 20 mM Tris-HCl (pH 7.5), 0.4 mM propionyl-CoA, 0.2 mM oxaloacetate, 2 mM DTT, 2 mM MgCl₂, and 10 μL of *mmgD* protein, and 100 μL of *mmgE* protein. After three hours at room temperature, 40 μL of activated *citB* protein and .05 mg of *yqiQ* protein were added. The

reaction was left at room temperature overnight. Control reactions of *mmgD*, *mmgD* + *mmgE*, and *mmgD* + *mmgE* + *citB* were prepared similarly and were left at room temperature overnight. Standards of 0.2 mM oxaloacetate, 0.4 mM propionyl-CoA, 0.9 mM 2-methylcitrate, 0.4 mM succinate, 0.4 mM pyruvate, and 2 mM DTT were also prepared. All reactions, controls, and standards were quenched with either 100 μL of 1 M sodium phosphate (pH 2.9) or 100 μL 0.01% formic acid and analyzed on the HPLC UV-Vis and on the LC/MS.

CHAPTER III

RESULTS AND DISCUSSION

III.A *CitB* SDS-PAGE Results

CitB has a molecular weight of 100 kDa. A band is observed around ~100 kDa indicating that citB was successfully purified. To test for activity, a reverse reaction containing isocitrate and *citB* protein was carried out using the UV-Vis spectrophotometer.
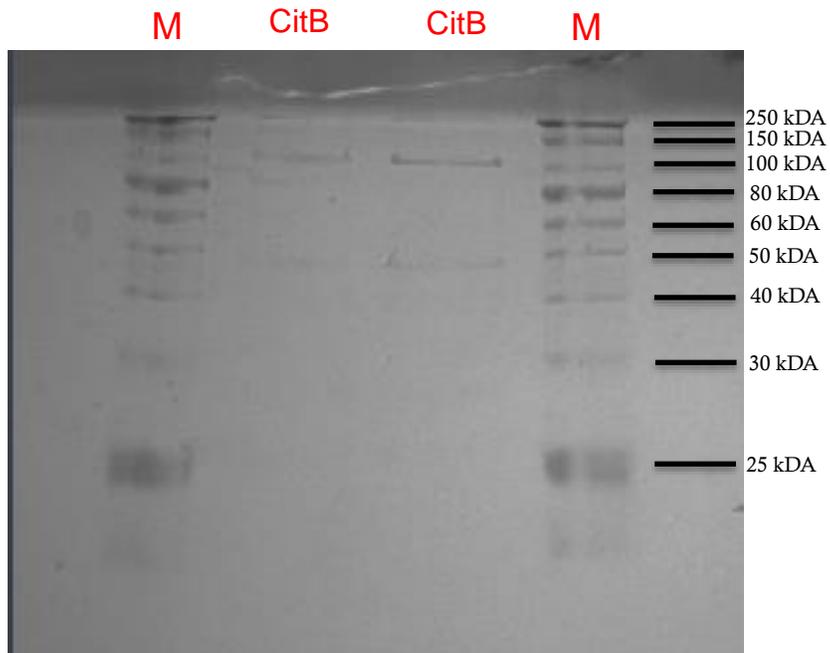
Figure 3. 10% SDS-PAGE Gel of *citB* Protein. Lanes labeled M contain 5 μl molecular weight ladder. Lanes labeled citB contain 20 μl purified protein.

III.B Confirmation of *CitB* Activity in the Citric Acid Cycle

Results from the UV-Vis spectrophotometer of the reverse reaction of citB and isocitrate showed an increase of absorbance over the time, indicating an active enzyme. The positive slope from the complete reaction compared to various negative-control reactions suggests that the aconitate product was being produced by citB. To further characterize functional *citB*, reactions and controls were confirmed using HPLC with ultraviolet detection
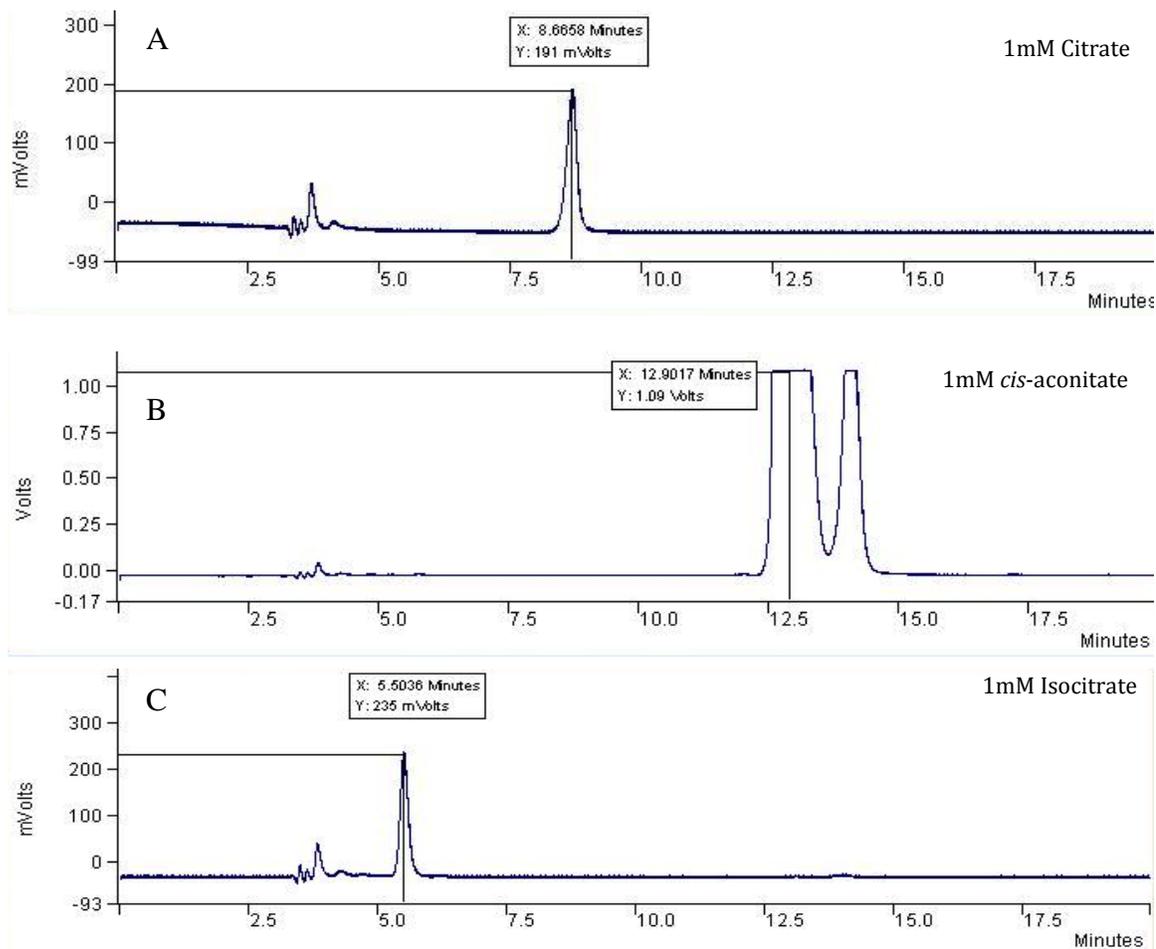
Figure 4. HPLC Chromatograms of Citric Acid Cycle Standards. A, B, and C are 1mM standards of citrate, *cis*-aconitate, and isocitrate, respectively.

Results of the HPLC chromatograms (Figure 4) illustrate that commercially available citrate standard comes off of the column around 8 minutes. The commercially available standard for *cis*-aconitate comes off around 12 minutes and the standard for isocitrate comes out around 5 minutes. The standards for citrate and isocitrate are distinguishable since they come off of the column with different retention times. This trend of isocitrate eluting from the column before citrate will be applied analogously to

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our analyses for 2-methylcitrate and 2-methylisocitrate for reactions in the Methylcitric

acid cycle where most products are not commercially available.
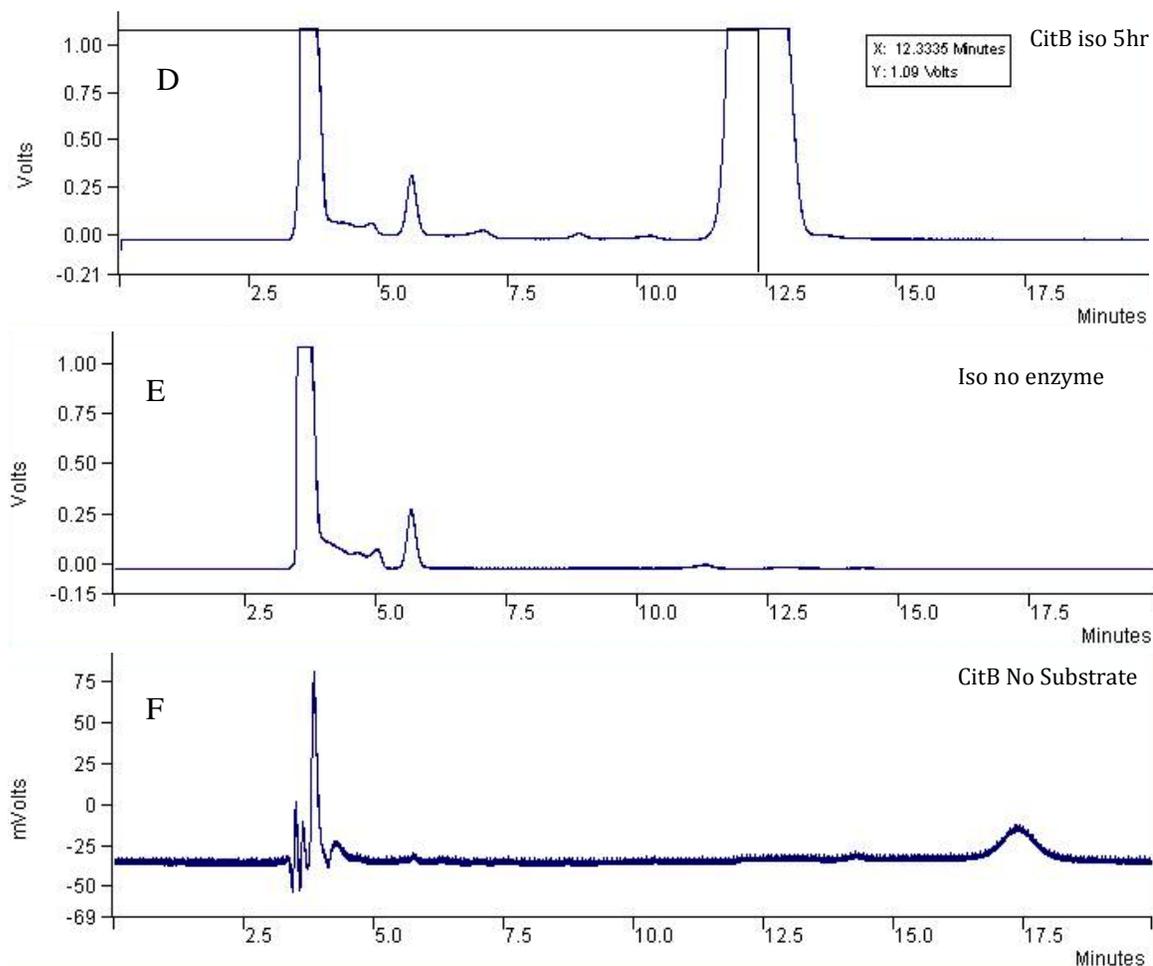
Figure 5. HPLC Chromatograms of Isocitrate and *citB* Enzyme with Positive and Negative Controls. D shows the reaction of CitB with 1 mM isocitrate for 5 hours which has an additional peak that lines up with *cis*-aconitate. E has 1 mM isocitrate with no enzyme. F shows the active enzyme with no substrate.

The reverse reaction of isocitrate and *citB* should produce *cis*-aconitate. As

revealed in Figure 5D, where a reaction of isocitrate and *citB* was carried out, a

characteristic peak of *cis*-aconitate, around 12 minutes, is observed. A control incubated

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in the same way, but lacking enzyme was done by combining isocitrate and the activation buffer used for *citB* as shown in Figure 5E. There is no peak around 12 minutes confirming that no *cis*-aconitate is being made. In Figure 5F, a control containing active *citB* but no isocitrate, is shown. There are no peaks around 5 minutes or 12 minutes, which further demonstrates that no reaction is occurring without the substrate present.
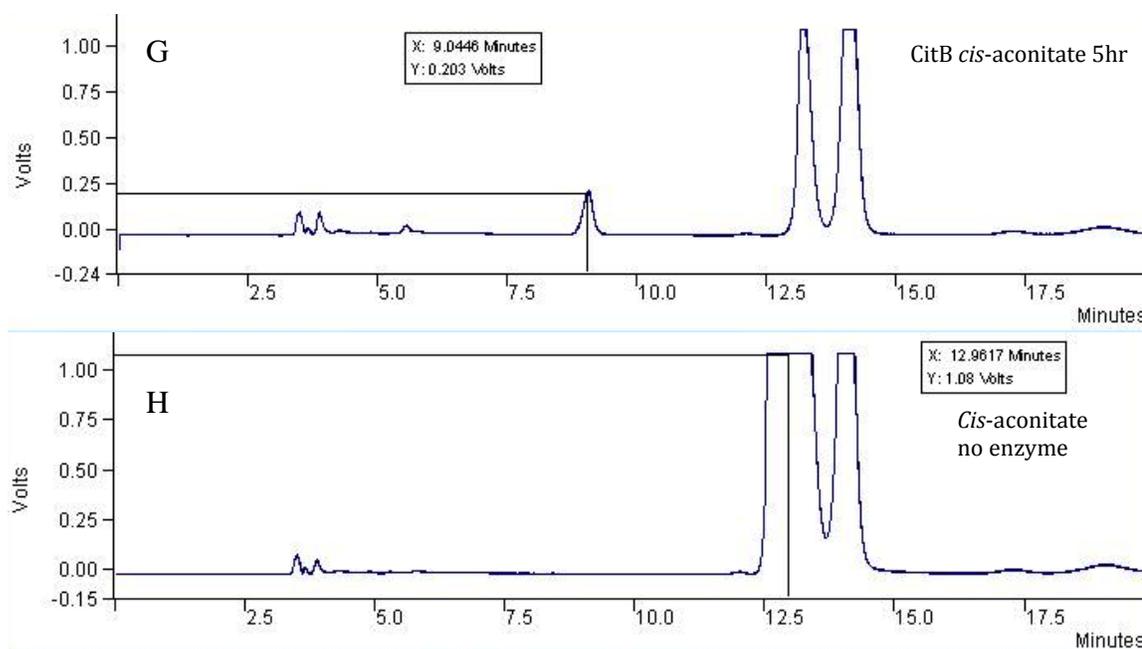
Figure 6. HPLC Chromatograms of *Cis*-aconitate and *citB* Enzyme with Negative Control. G contains a reaction of 1 mM *cis*-aconitate with CitB for 5 hours and has an additional peak that lines up with citrate. H shows 1 mM *cis*-aconitate with no enzyme.

In Figure 6G, the HPLC chromatogram shows a reaction containing 1 mM *cis*-aconitate and active *citB* for 5 hours. A peak around 9 minutes is observed, similar to that of citrate, which has a retention time around 8.6 minutes. This suggests that *citB* is rehydrating the *cis*-aconitate to form citrate, the next step in the reverse direction of the citric acid cycle. A control containing 1 mM *cis*-aconitate and activation buffer without

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enzyme was performed and no peak is observed around 9 minutes proving that a reaction

is not occurring without *citB* present.
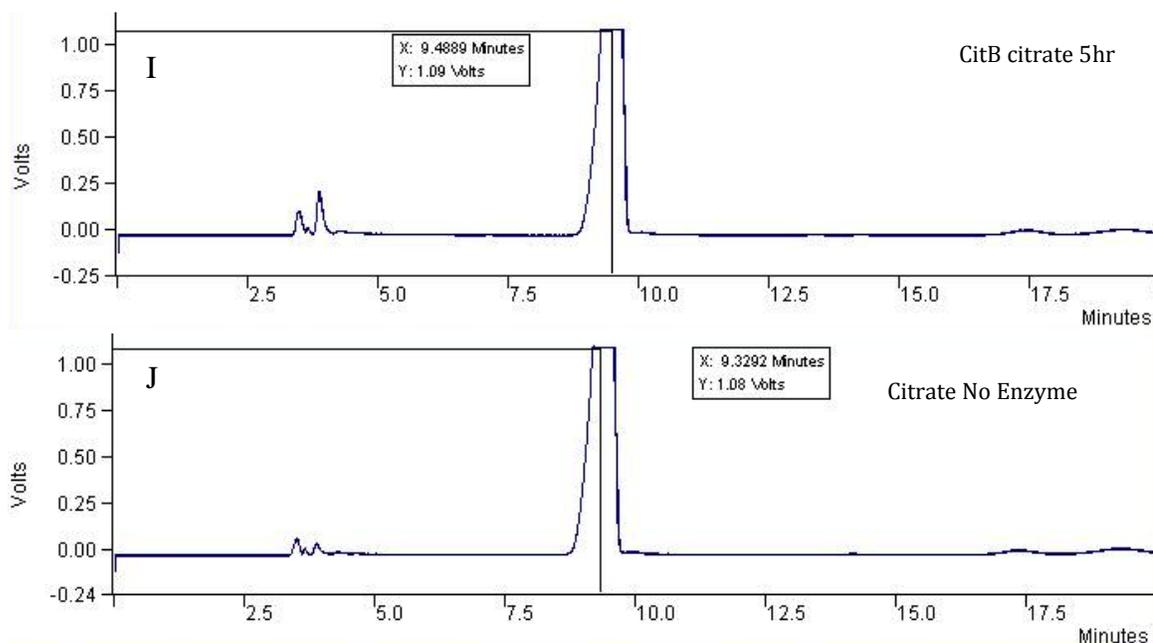
Figure 7. HPLC Chromatograms of Citrate and *CitB* with Negative Control. I shows the reaction of CitB
with 1 mM citrate for 5 hours. J shows 1 mM citrate with no enzyme.

The forward reaction containing 1 mM citrate and active *citB* is shown in Figure

7I. A control containing 1 mM citrate and activation buffer without enzyme is shown in

Figure 7J. Both HPLC chromatograms look similar with an intense peak around 9

minutes, which is characteristic of citrate. This suggests that the forward reaction of *citB*

is not as favorable as the reverse reaction.[18] The reaction is possibly driven in the forward

direction by the overall forward direction of the citric acid cycle.

Results from the HPLC UV-VIS show that functional *citB* was isolated and can

work in reverse taking isocitrate and converting it to *cis*-aconitate (Figure 5) as is

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expected. When treated with 1 mM of D,L isocitrate or 1 mM cis-aconitate over 30 minutes, 1 hour, 2 hours, 5 hours, or overnight, an expected cis-aconitate peak was observed confirming the aconitase is active in the citric acid cycle. Therefore, *citB* can carry out two steps in reverse; the first is taking isocitrate and dehydrating it to form *cis*-aconitate and the second is taking *cis*-aconitate and rehydrating it to form citrate. Although this activity of the *citB* enzyme has been previously characterized, this is the first time the Reddick group has been able to show this.

III.C *CitB* Activity in the Methylcitric Acid Cycle

Experiments demonstrating the known enzyme activity (aconitase) of the purified *citB* demonstrate that the enzyme is active and fully functional in our hands. Therefore, the role of *citB* in the methylcitric acid pathway can be examined
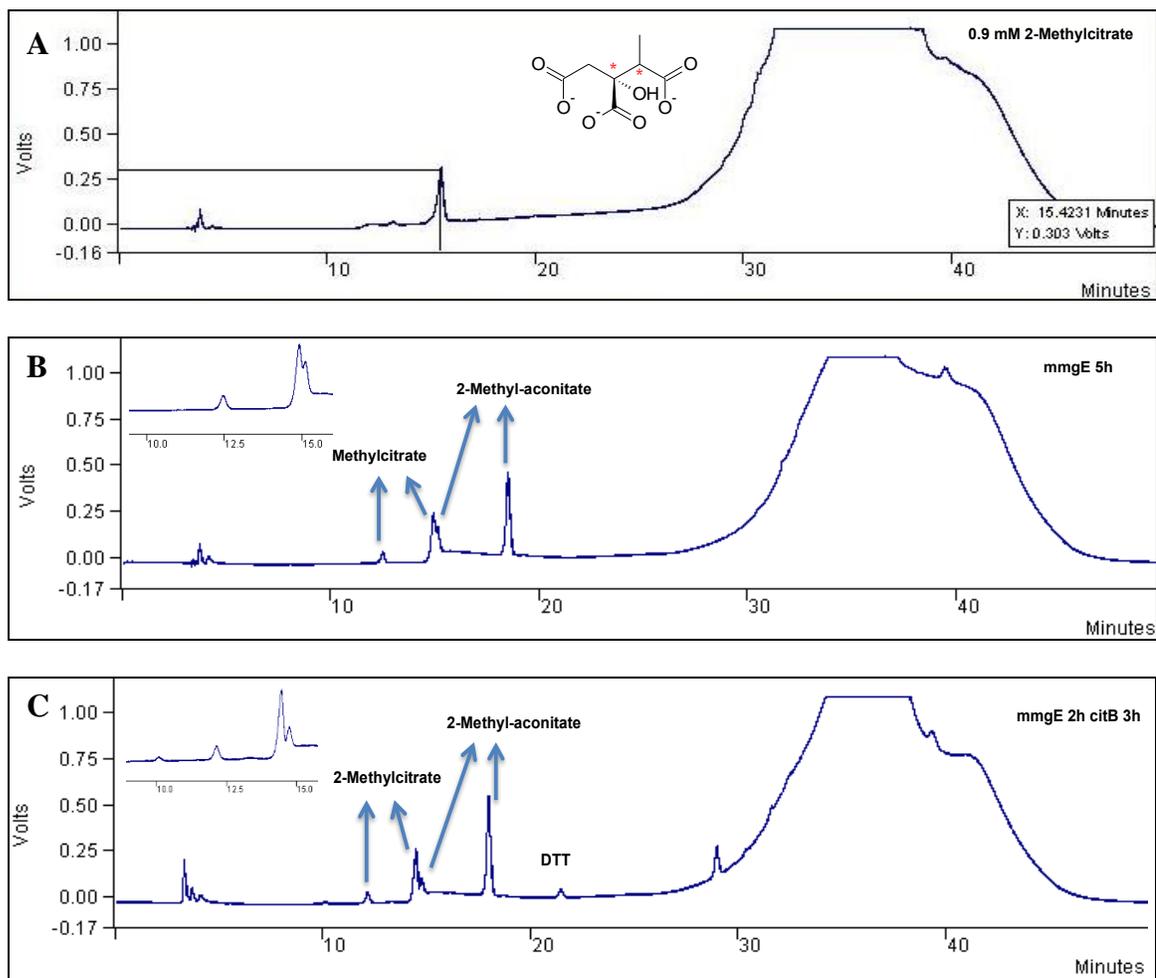
Figure 8. HPLC Chromatograms of 2-Methylcitrate, *mmgE* and *citB* Enzymes with Negative Control. A shows the commercial standard of 0.9 mM 2-Methylcitrate. The structure is also shown with asterisks next to the two chiral centers. B shows the 5 hour reaction of 0.9 mM 2-Methylcitrate with *mmgE* as a negative control. C shows the reaction of 0.9 mM 2-Methylcitrate and *mmgE* for 2 hours then citB for an additional 3 hours. Peaks are labeled respectively. In the upper left corner of B and C, the 10 to 15 minute region of the chromatogram is zoomed in.

The HPLC chromatogram in Figure 8A shows the commercial 0.9 mM 2-methylcitrate which has a peak with a retention time around 15 minutes and a smaller peak around 11 minutes. There are 2 chiral centers in 2-methylcitrate, giving rise to four diastereomers, with each set of enantiomers showing up as two peaks on the

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chromatogram. A reaction mixture containing 0.9 mM 2-methylcitrate and mmgE (Figure 8B) has a peak around 11 minutes and 15 minutes, characteristic peaks of 2-methylcitrate. The peak around 15 minutes is split and there is an additional peak around 18 minutes indicating that the product, 2-methylaconitate, is being made. The separate peaks suggest that both the *cis* and *trans* isomers are present. The chromatogram shown in Figure 8C depicts a reaction containing 0.9 mM 2-methylcitrate and mmgE for 2 hours then activated *citB* for and additional 3 hours. Additionally, there is a hump around the 10 minute region of the chromatogram which is zoomed in and shown in the upper left corner of Figure 8C. Results demonstrate that *citB* is taking the 2-methyl-*cis*-aconitate and converting it to 2-methylisocitrate. Note that the 2-methylisocitrate peak is slightly before the two peaks from 2-methylcitrate. This is consistent with the characteristics of the commercially available standards of citrate and isocitrate. We expect these methyl analogs of citrate and isocitrate to behave with a similar trend in the chromatography. These experiments have shown a new peak that is not only *citB* dependent, but is also dependent on the conversion of 2-methylcitrate by *mmgE*. However, at this point we only have UV-detected peaks showing the activity of the *citB* enzyme in the methylcitric acid cycle, therefore, more detailed characterization was done using MS.
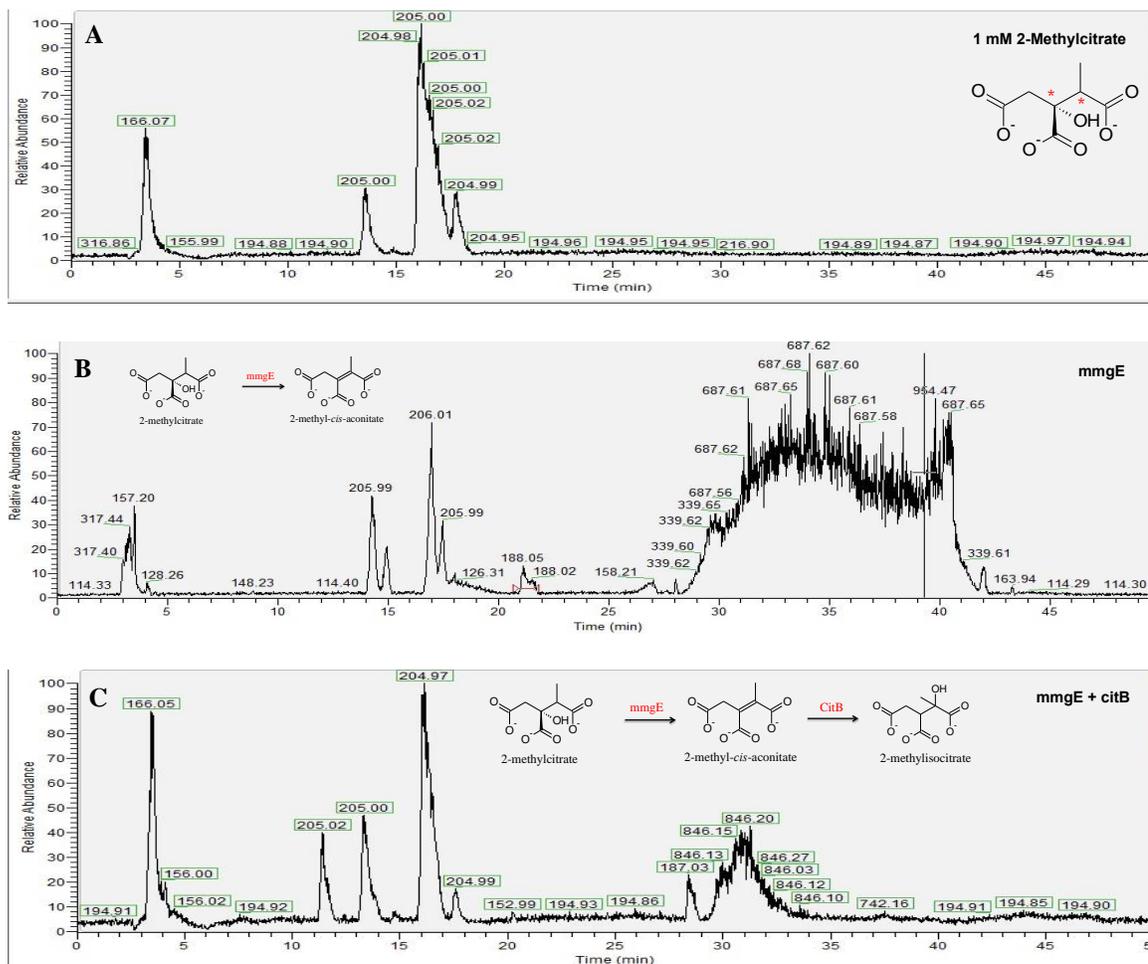
Figure 9. LC/MS Chromatograms of 2-Methylcitrate, *mmgE* and *citB* Enzymes with Negative Control. A shows the commercial standard of 0.9 mM 2-Methylcitrate. B shows the 5 hour reaction of 0.9 mM 2-methylcitrate with *mmgE* as a negative control. Lastly, C shows the reaction of 0.9 mM 2-methylcitrate and *mmgE* for 2 hours then *citB* for an additional 3 hours. Reaction schematics are shown in B and C respectively.

Confirmation of the HPLC results was carried out using LC/MS, which requires different chromatography conditions. The LC/MS requires a volatile solvent, therefore, 0.1% Formic acid and methanol were used as the two mobile phases instead of the 20 mM Sodium Phosphate buffer. Similar controls and reactions were prepared, under the same conditions used in Figure 8, for analysis by LC/MS in order to confirm results from

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the HPLC. Figure 9A shows the LC/MS chromatogram of the commercial 2-methylcitrate, which has a relative mass to charge ratio of 205. Two peaks are observed, as seen in the HPLC UV-Vis chromatograms, for the mixture of 85% (2SR, 3SR)- and 15% (2RS, 3SR)-stereoisomers.[19] The major peak, with a retention time around 16 minutes, is the (2S, 3S)- and (2R, 3R)-stereoisomers and the minor peak around 14 minutes is the (2S, 3R)- and (2R, 3S)-stereoisomers. The chromatogram in Figure 9B represents a reaction containing 2-methylcitrate and *mmgE* that was recently done by Natalie Hage, an undergraduate student in the Reddick lab, using a method with a higher methanol gradient. In the LC/MS method used for all the other reactions, the 2-methylaconitate peak does not show up suggesting that it does not come off of the column, or does not ionize for mass detection as easily as the other analytes in the mixture. In case the less polar 2-methylaconitate products were too tightly retained on the column, the concentration and time of the second mobile phase, methanol, was increased in order to remove the 2-methylaconitate from the column. Evidence of the *mmgE* product, 2-methyl-*cis*-aconitate, is seen in Figure 9B where a peak with the 2-methylaconitate mass to charge ratio of 188 is observed around 22 minutes. In Figure 9C, the chromatogram represents a reaction containing 2-methylcitrate, *mmgE* and *citB*. There is an additional peak around 11 minutes, which is slightly before the first peak of 2-methylcitrate, with a mass to charge ratio of 205 signifying that 2-methylisocitrate is being made.

III.D Further Characterization of *CitB* in the Methylcitric Acid Cycle

CitB was further characterized by using *mmgD* to produce 2-methylcitrate instead of the commercially available standard. Reactions were analyzed via HPLC with UV detection and LC/MS.
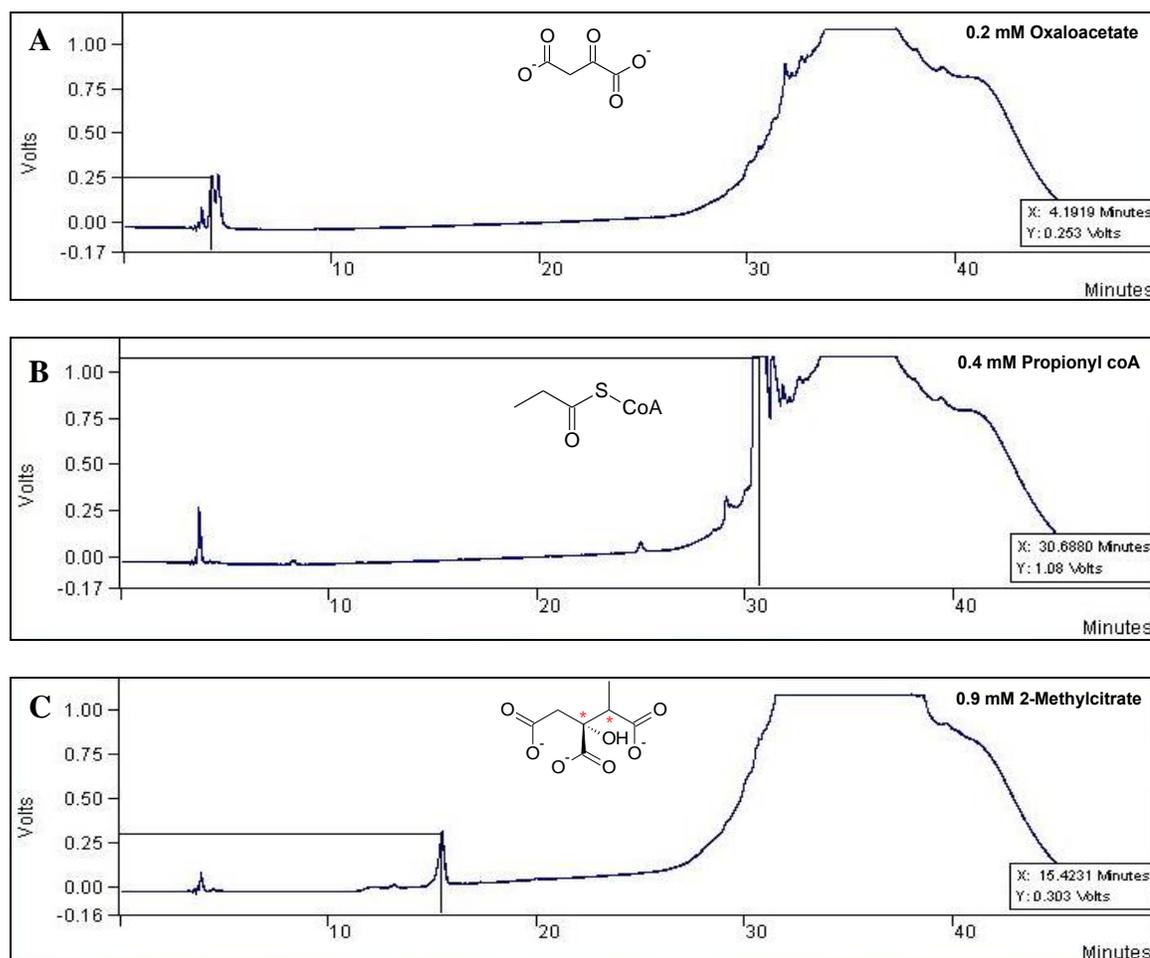

Figure 10. HPLC Chromatograms of Standard Controls. A shows the commercial standard of 0.2 mM Oxaloacetate. B shows the commercial standard of 0.4 mM Propionyl-CoA. C shows the commercial standard of 0.9 mM 2-Methylcitrate. Structures for each standard are shown in A, B, and C respectively.

In Figure 10, HPLC chromatograms of commercially available standards from the methylcitric acid pathway are shown. A standard of 0.2 mM oxaloacetate is presented in Figure 10A with a peak around 4 minutes. A second standard containing 0.4 mM propionyl-CoA is displayed in Figure 10B with a peak around 30 minutes. The third standard containing 0.9 mM 2-methylcitrate (Figure 10C) has a peak around 15 minutes.
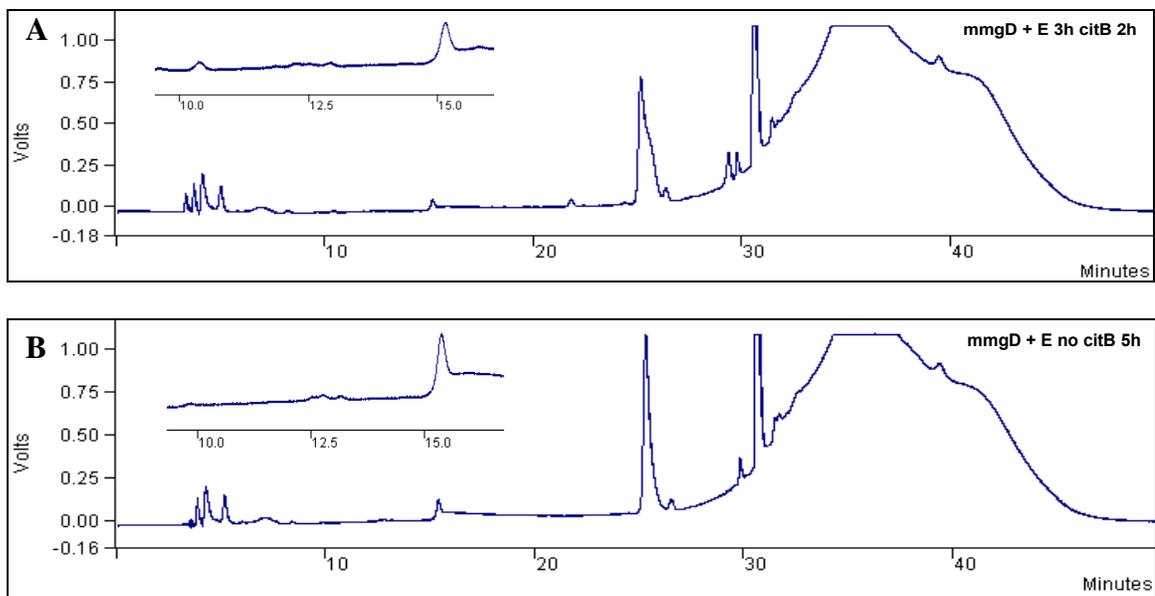
Figure 11. HPLC Chromatograms of the *mmgD*, *mmgE* and *citB* reaction and Negative Control. A shows a reaction containing 0.2 mM Oxaloacetate, 0.4 mM Propionyl coA, mmgD and mmgE for 3 hours then citB for 2 hours. B depicts the negative control containing everything except citB. In the upper left corner of A and B the 10-15 minute region of the chromatogram is zoomed in.

Results from Figure 11A, a reaction containing propionyl-CoA, oxaloacetate, *mmgD*, *mmgE*, and *citB*, shows a peak around 10 minutes similar to that in the reaction with the commercial 2-methylcitrate. This is consistent with *citB* making 2-methylisocitrate from the naturally synthesized 2-methylcitrate by *mmgD*. A control without *citB* lacked this peak around the 10 minute region (Figure 11B). To confirm the

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results from the HPLC UV-Vis the same standards, reaction, and control was done using

LC/MS.
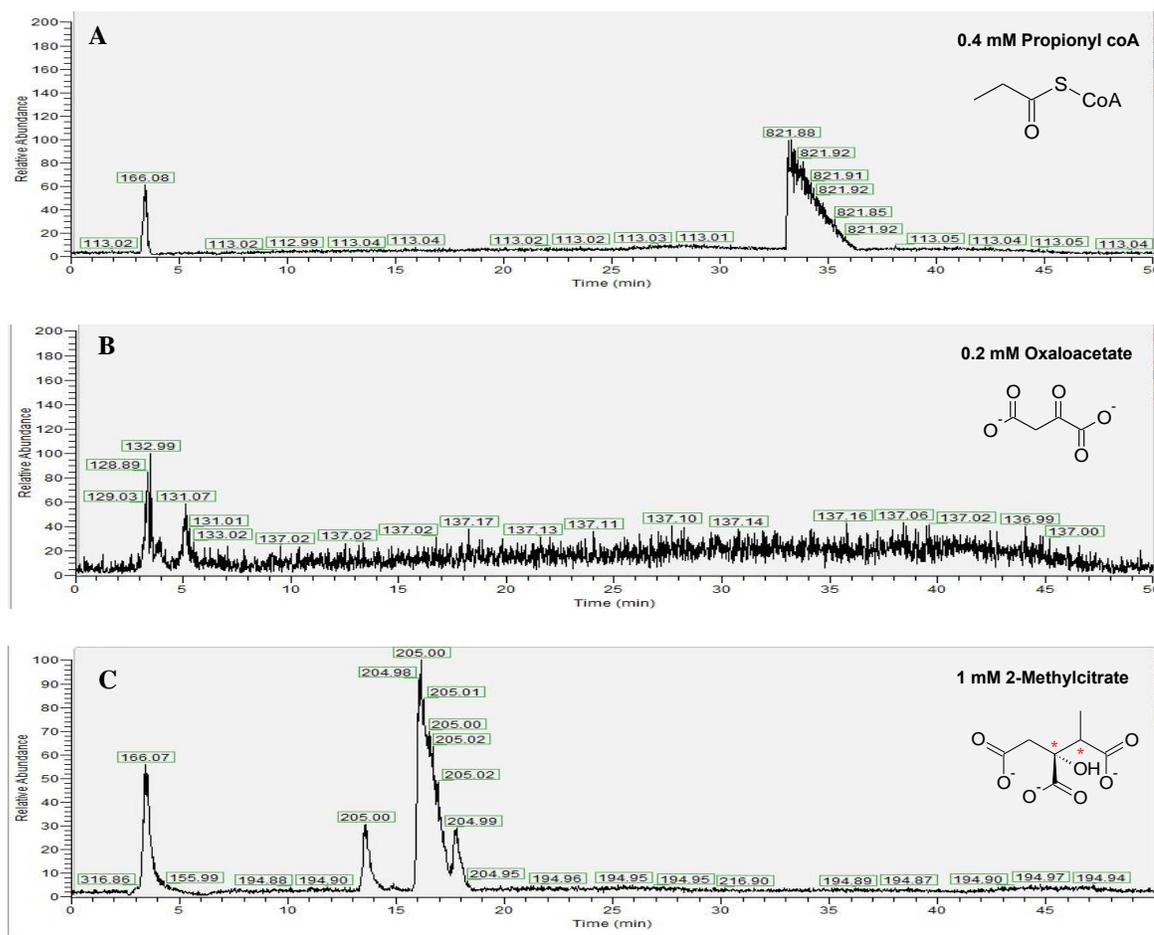
Figure 12. LC/MS Chromatograms of Methylcitric Acid Cycle Standards. A shows the commercial standard of 0.2 mM Oxaloacetate. B shows the commercial standard of 0.4 mM Propionyl coA. C shows the commercial standard of 0.9 mM 2-Methylcitrate. Structures for each standard are shown in A, B, and C respectively.

The commercially available standard of propionyl-CoA has a relative mass to charge ratio of 821 with a retention time around 33 minutes as seen in Figure 12A. Oxaloacetate has a relative mass to charge ratio of 132 and a retention time around 4 minutes (Figure 12B). As observed previously, 2-methylcitrate has a relative mass to charge ratio of 205 and a peak around 14 and 16 minutes for the mixture of stereoisomers.
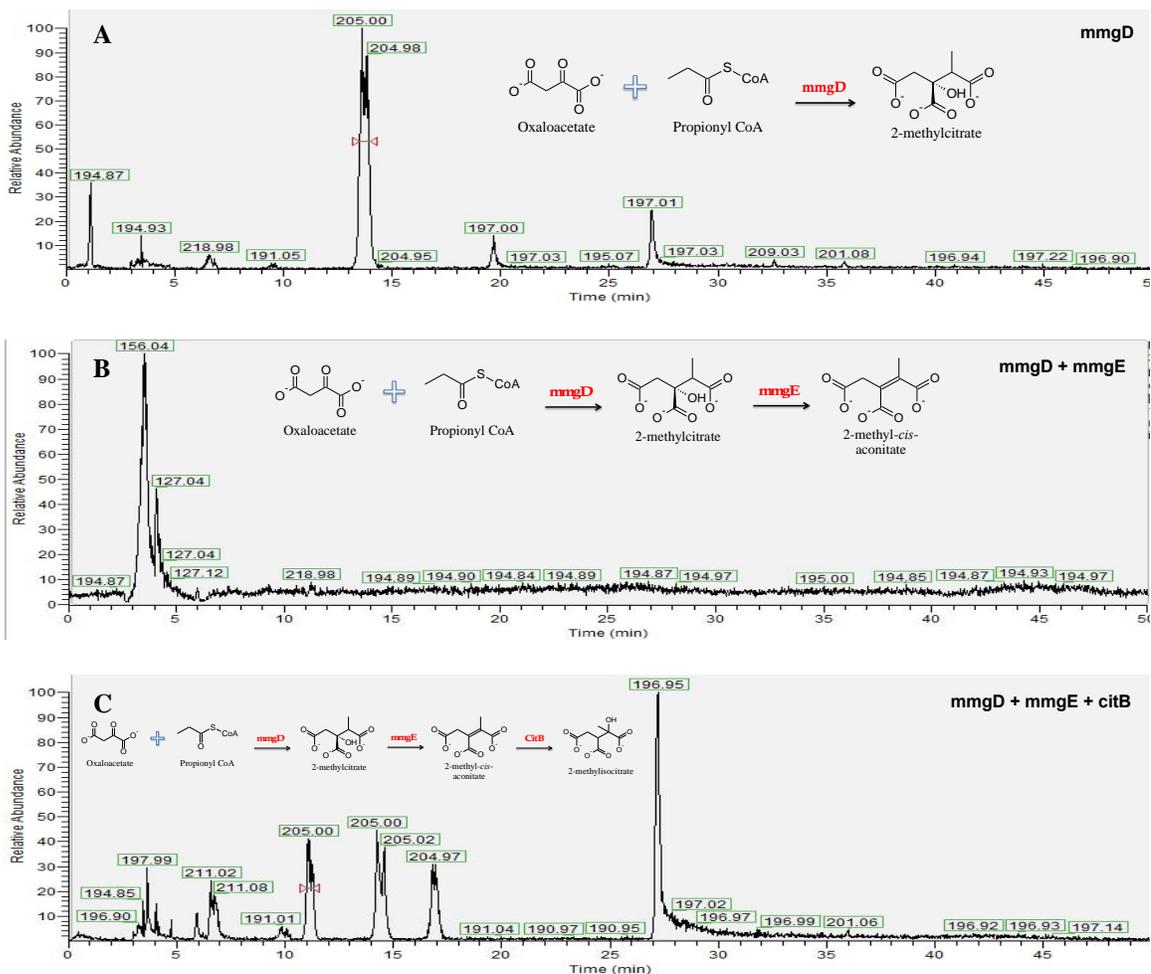
Figure 13. LC/MS Chromatograms of the *mmgD*, *mmgE*, and *citB* Reactions. A shows a reaction containing 0.2 mM Oxaloacetate, 0.4 mM Propionyl coA, and mmgD overnight. B shows a reaction containing 0.2 mM Oxaloacetate, 0.4 mM Propionyl coA, mmgD, and mmgE overnight. C shows a reaction containing 0.2 mM Oxaloacetate, 0.4 mM Propionyl coA, mmgD, mmgE for 3 hours then citB overnight.

A reaction containing oxaloacetate, propionyl-CoA and *mmgD* is shown in figure 13A. A single peak with a mass to charge ratio of 205 around 14 minutes is observed in the LC/MS chromatogram which is characteristic of 2-methylcitrate. However, the second peak around 16 minutes is not observed. This is due to the stereoselectivity of *mmgD*, which only produces either the (2R, 3S)- or (2S, 3R)-stereoisomer, which

together elute at 14 minutes. In the following chromatogram (Figure 13B) a reaction was prepared with oxaloacetate, propionyl-CoA, *mmgD* and *mmgE*. The characteristic peak of 2-methylcitrate disappears suggesting that it is being converted to the next product in the methylcitric acid pathway, 2-methyl-*cis*-aconitate, by *mmgE*. As previously stated, the method gradient used for these experiments was not optimized to get the 2-methylaconitate product by LC/MS. Lastly, the third chromatogram (Figure 13C) represents a reaction containing oxaloacetate, propionyl-CoA, *mmgD*, *mmgE*, and *citB*. The characteristic peak of 2-methylisocitrate is observed around 11 minutes (the same observed when starting with the commercial 2-methylcitrate, shown in Figure 12C), confirming that *citB* carries out the missing step in the methylcitric acid cycle.

III.E Conversion of 2-Methylisocitrate to Succinate and Pyruvate

Since there is no commercially available standard for 2-methylisocitrate, the product of *citB* cannot be confirmed by comparison to an authentic standard of 2-methylisocitrate. Therefore, *yqiQ* was utilized to carry out the next step in the methylcitric acid pathway, converting 2-methylisocitrate to succinate and pyruvate, which are commercially available.
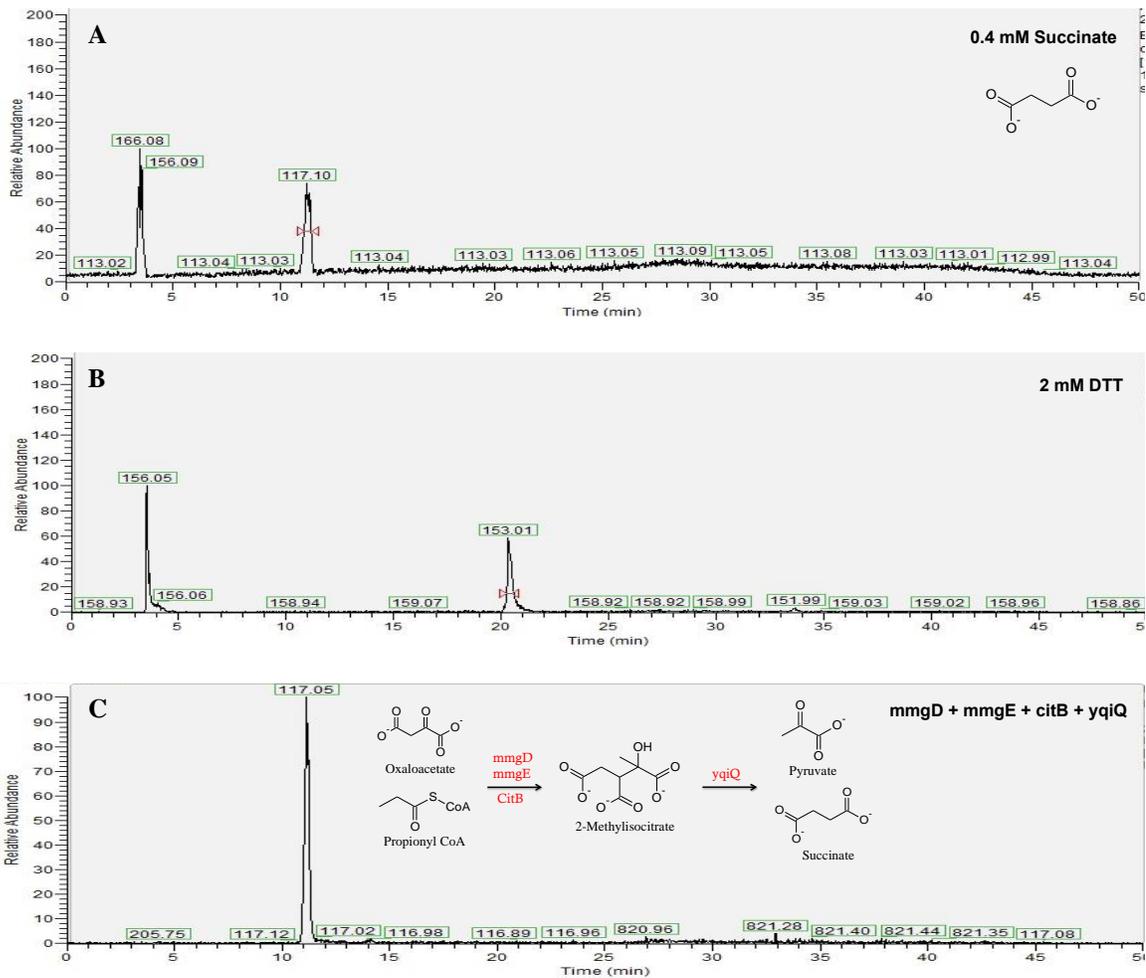
Figure 14. LC/MS Chromatograms of the *mmgD*, *mmgE*, *citB*, and *yqiQ* Reaction and Controls. A shows the commercial standard of 0.4 mM Succinate. B shows a control containing 2 mM DTT. Lastly, C shows the full reaction containing 0.2 mM Oxaloacetate, 0.4 mM Propionyl coA, 2 mM DTT, 2 mM MgCl₂, *mmgD*, and *mmgE* for 3 hours then citB and yqiQ overnight.

A standard of 0.4 mM succinate is shown in the LC/MS chromatogram in Figure 14A, which has a mass to charge ratio of 117 and retention time around 11 minutes. Another standard containing 2 mM DTT is shown in Figure 14B. DTT was used to reduce sulfur groups present in *citB* and *yqiQ*. Figure 14C shows the complete reaction containing 0.2 mM oxaloacetate, 0.4 mM propionyl-CoA, *mmgD*, *mmgE*, *citB*, and *yqiQ*.

A peak around 11 minutes with a mass to charge ratio of 117 is observed which is characteristic of succinate. This confirms that *citB* is making 2-methylisocitrate and that *yqiQ* can go in the forward direction to make succinate and pyruvate. The standard for pyruvate and the pyruvate product from the complete reaction is not being shown in these experiments. Pyruvate has a relative mass to charge ratio of 87 and the lowest mass detection of the MS was set to 100, therefore, peaks for pyruvate cannot be observed. However, experiments on the HPLC show that pyruvate is being made when *yqiQ* is added to the reaction. This further confirms the product of citB and the function of *yqiQ* in the forward direction.

CHAPTER IV

CONCLUSION

In conclusion, we have confirmed that *citB* can carry out the reverse reaction in the citric acid cycle of converting isocitrate to *cis*-aconitate and can also convert *cis*-aconitate to citrate using HPLC coupled to a UV-Vis detector. We have shown that *citB* acts as a 2-methyl-*cis*-aconitate rehydratase by converting 2-methyl-*cis*-aconitate to 2-methylisocitrate, completing the missing step in the methylcitric acid cycle encoded by the *mmg* operon. We have also shown the forward reaction of *yqiQ* as 2-methylisocitrate lyase, converting 2-methylisocitrate to succinate and pyruvate using HPLC and LC-MS. In the process we have achieved the first reconstitution of the full methylcitric acid in *B. subtilis*. The methylcitric acid cycle is a metabolic pathway dedicated to the metabolism of propionate, a capability which has so far been unknown in this important model organism. Further aims of this project include repeating these reactions and controls on the LC/MS using an optimized method to show the 2-methyl-*cis*-aconitate peak and to verify reproducibility of results.

CHAPTER V

ANTIMICROBIAL PEPTIDES AND LARGEMOUTH BASS

V.A Fishing Statistics and Largemouth Bass

There is little known about the biochemistry of largemouth bass (*Micropterus salmoides*), especially the antimicrobial properties that may be present in the outer mucosal layer of the skin as an initial defense against bacteria, protozoa and fungi. The American Sport fishing Association and the 2011 National Survey of Fishing, Hunting and Wildlife-Associated Recreation reported that there are approximately 60 million anglers in the United States (this is more than two times the number of people attending all of the NFL games in 2011 combined). Retail sales from anglers have generated approximately 46 billion dollars, and more than 828,000 jobs. Of the 27.1 million freshwater anglers, 10.6 million fish for largemouth bass.[20] The mortality rates of largemouth bass during tournament fishing can be as low as 0-24% or as high as 85% often due to infection of mishandled fish.[21] There is a problem with current overuse of antibiotics, which facilitate mutant resistant strains of bacteria for humans and with the potential for high mortality rates of the popular sport fish, largemouth bass, during tournament fishing. Understanding the antimicrobial defense system of largemouth bass is important for the development of new antibiotics for potential use in humans and for decreasing the mortality rates of largemouth bass handled in recreational and tournament

fishing, as well as those captured and released for studies by state wildlife management agencies.

V.B Background of Largemouth Bass

Largemouth bass (*Micropterus salmoides*) are freshwater fish that are apex predators in southeastern waters. Many anglers target these fish because of their enthusiastic fight when captured.[22] During fishing tournaments, catch and release is used to prevent over fishing. However, lesions appear at sites where the mucosal layer is removed from handling and weighing of the fish, allowing bacterial infections that lead to fish mortality.[21] This suggests that the largemouth bass possess some type of antimicrobial defense that is degraded upon a loss of the outer epithelial mucosal layer.

Fish inhabit an aqueous environment that is filled with many pathogens and microorganisms, which are in continuous contact with the gastrointestinal tract, gills, and epithelial layer of the fish. To overcome the constant threat of infection, an initial defense system is needed. Antimicrobial peptides (AMPs) in a wide variety of organisms, including many aquatic species, play an important role in the initial defense against a variety of microorganisms including bacteria, parasites, viruses and fungi and are present in many aquatic species.[23] They are typically less than 100 amino acids long, have a net positive charge at physiological pH, are amphipathic, and have a linear a-helical structure.[24,25]

There are several different ways that AMPs kill bacteria such as targeting cytoplasmic components, interfering with their metabolism, and disruption of the membrane.[24] The proposed mechanism for killing bacteria by fish AMPs is that the

cationic antimicrobial peptide binds to the anionic surface of the bacteria and causes the cells to lyse, by a mechanism similar to that of detergents.[26]

Recently, several AMPs have been discovered in fish, specifically from their initial protective barriers including the mucosal epithelial layer, intestinal mucus, and gills. Examples of AMPs isolated from the mucosal epithelial layer of fish include pleurocidin from winter flounder (*Pleuronectes americanus*), parasin I from catfish (*Parasilurus asotus*), pardaxin from moses sole fish (*Pardachirus marmoratus*), and pelteobagrin from yellow catfish (*Pelteobagrus fulvidraco*).[27,28] AMPs have also been isolated from the gills of fish which include chrysophsin from red sea bream (*Chrysophyrs major*) and piscidin 4 from hybrid striped bass (*Morone chrysops x Morone saxatilis*).[23] This list includes both fresh and saltwater fish.

V.C Expected Significance and Goal

With the emergence of mutant bacterial strains becoming resistant to all known forms of antibiotics and the overuse of antibiotics by humans, an alternative form of treatment is necessary. AMPs can also be more effective in certain physiological environments where antibiotics are not useful. For example, patients with cystic fibrosis have high concentrations of NaCl in their pulmonary mucosa, which prevented the immune system from killing bacteria, allowing the development of lung diseases. Pleurocidin, an AMP isolated from the outer mucosal layer of winter flounder, was found to have bactericidal properties at high NaCl concentrations and could potentially aid in the antibacterial treatment in patients with cystic fibrosis.[26]

The rationale that underlies the research is that the inadvertent removal of the mucosal layer from the skin of largemouth bass can result in bacterial infections that are often fatal. Furthermore, fish gills are not only continuously exposed to microbes but also have a rich blood supply with only a thin epithelial layer as protection from pathogens entering the circulatory system.[25] This research will allow new investigations of the antimicrobial properties present in the mucosal layer, which is important because we may be able to lower the mortality rates during tournament fishing, wildlife management studies, and possibly lead to the development of a new antibiotic for human use.

The overall goal of this project is to purify and characterize antimicrobial activity from the skin or gills of largemouth bass. The hypothesis is that largemouth bass contain an antimicrobial peptide in the outer mucosal layer or the gill tissue that should inhibit bacterial growth when treated with concentrated fish sample. This hypothesis is supported by the fact that small antimicrobial peptides were isolated from several freshwater and saltwater fish species. In this study, we have tested the hypothesis with *in vitro* experiments through the use of disc diffusion assays on *Escherichia coli* K12 and *Bacillus subtilis* 168 and liquid growth assays using *Staphylococcus aureus*.

CHAPTER VI

EXPERIMENTAL PROCEDURES

VI.A Bacteria and Culture Methods

B. subtilis strain 168 or *E. coli* strain K-12 were streaked on Luria Bertani (LB) agar plates and incubated overnight at 37 °C. *B. subtilis* strain 168 is a tryptophan auxotroph, therefore, 0.02 mg/ml tryptophan was supplied for every culture of *B. subtilis*. A starter culture was prepared by adding 1 colony of bacteria to 5 mL of LB media and was incubated overnight at 37 °C while shaking. LB media was prepared by adding approximately 10 g of Bacto Tryptone, 5 g of yeast extract, and 10 g of NaCl to one liter of water which was then brought to a pH of 7.5, using HCl, and autoclaved. At times a M9 minimal medium was used instead of LB for the disc diffusion assay. A stock solution of M9 salts was prepared with 33.9 g Na_2HPO_4, 15.1 g KH_2PO_4, 2.5 g NaCl, and 5.1 g NH_4Cl per 1 liter. The M9 minimal medium was prepared by combining 140 mL M9 salts, 1.4 mL of 1 M $MgSO_4$, and 70 µL of 1 M $CaCl_2$ with water for a final volume of 700 mL. For agar plates, 600 mL of media (LB or M9 minimal) was mixed with 9 g of agar and autoclaved. For top agar, 100 mL of media was mixed with 1 g of agar and autoclaved. To the 600 mL of sterile medium was added 12 mL of sterile 20% glucose and to the 100 mL of medium 2 mL of sterile 20% glucose was added. Just before use the sterile top agar was microwaved until it was fully melted.

VI.B Antimicrobial Disc Diffusion Assay

To carry out the disc diffusion assay, 200 µL of an overnight starter culture was added to 9 mL top agar, (1% agar in LB or M9 minimal medium). The bacteria and top agar mixture was poured onto a pre warmed LB or M9 minimal medium plate. Discs were placed on different parts of the plate and were treated with different concentrations of the fish sample (see below). Unless stated otherwise, a disc treated with 2 µL of Kanamycin (30 mg/mL) was used as a positive control on each plate. Plates were incubated overnight at 37 °C.

VI.C Fish Extraction Methods

VI.C.1 From Outer Mucosal Coating

Wild largemouth bass (with the minimum legal size of 14 inches in length; individuals in this study were between 14-22 inches) were captured and the mucosal coating was scraped from the fish using a plastic spatula while avoiding the area around the anal vent to prevent contamination.[26] The material was stored at 4 °C until use. To this 1 mL of 50 mM Tris-HCl (pH 8.0) was added and the sample was centrifuged at 13,000 rpm for five minutes. Either 10, 30, or 50 µL of sample was added to individual discs in the diffusion assays. However, there appeared to be no antimicrobial activity in the outer mucosal coating of the fish. Due to the low sample volume, the fish sample was concentrated by placing 500 µL of sample into a SpeedVac overnight then reconstituting in 50 µL of water. A disc diffusion assay was carried out on both LB agar plates and M9 minimal medium plates. Another sample preparation approach was taken by homogenizing the fish skin and mucosal layer.

VI.C.2 From Intact Skin Tissue

Wild largemouth bass (with the minimum legal size of 14 inches in length; individuals in this study were between 14-22 inches) were filleted with the removal of intact skin by conventional means.

VI.C.2.a Solid Phase Skin Extraction

Just prior to homogenization and extraction, the skin was chopped into small ~ 1 square inch pieces before adding approximately 150 mL of 0.2 M Sodium Acetate and 0.2% Triton X-100. Once homogenized, the sample was centrifuged at 4 °C using a JA-10 rotor at 13,500 rpm for 20 minutes. The supernatant was stored at -20 °C until use. Reversed phase solid phase extraction was done using a Sep-Pak Vac 3cc (200 mg) solid phase extraction C_{18} column. The column was conditioned and equilibrated with 2 mL of acetonitrile, then 2 mL 0.1% trifluoroacetic (TFA), and then 2 mL of 0.2 M sodium acetate containing 0.2% Triton X-100 and allowed to flow by gravity. Once equilibrated, 2 mL of fish supernatant was added and the column was washed with 2 mL of 0.1% TFA. Sample was eluted with 500 μL of 60% acetonitrile and 0.1% TFA. Another elution step was carried out using 500 μL of the acetonitrile and TFA mixture.[26] After each filtration step the extract was collected and tested by doing a disc diffusion assay using 50 μL of each extract.

VI.C.2.b Concentrated Skin

Supernatant from the intact skin tissue was concentrated by placing 1 mL aliquots into 10 microcentrifuge tubes in the SpeedVac overnight. The sample was reconstituted in approximately 1.1 mL of methanol, centrifuged at 13,000 rpm for five minutes and

stored at -20 °C. As an additional control, 10 ml of the 0.2 M sodium acetate and 0.2% Triton X-100 buffer was also concentrated overnight and reconstituted in 1.1 mL of methanol. A disc diffusion assay was conducted using 30 μL of the concentrated fish sample, 30 μL of the concentrated buffer, 30 μL methanol and 2 μL of kanamycin (30 mg/mL).

VI.C.2.c Skin Preparation without Triton X-100

Due to the effects of the Triton on *B. subtilis* strain 168, a sample preparation without Triton X-100 was done. The intact skin tissue was acidified by boiling for five minutes in 1% acetic acid at a 1:4 dilution. The fish skin was homogenized and centrifuged at 11,500 rpm using a JA-20 rotor for 20 minutes. The supernatant was concentrated, reconstituted in 1 mL of methanol, and stored at -20 °C.[29]

VI.C.2.d Solid Phase Skin Extraction with PMSF

Another attempt was made by following the procedures stated above for preparing the intact skin tissue, however, 1 mM phenylmethylsulfonyl fluoride (PMSF), a protein inhibitor, was added to the 0.2 M sodium acetate and 0.2% Triton X-100 buffer prior to homogenization. Reverse solid phase extraction was done using a C_{18} column as stated previously with changes made only to the elution step. The sample was eluted off of the column with 5 mL of 60% acetonitrile and 0.1% TFA in water for a total of 9 different fractions. Another elution step was done using 100% acetonitrile. After each filtration step the extract was collected and 2 mL of each was concentrated then reconstituted in 100 μL of 50 mM Tris-HCl (pH 7.5).

VI.D Liquid Growth Assay

The liquid growth assay, completed by Joseph Egan from the Cech lab, was done against *Staphylococcus aureus* wild type bacteria. Berberine, derived from many plants including goldenseal, was used as a positive antibacterial control. All test samples and controls were done in triplicate in 96 well plates. The plates were incubated at 37 °C for 12 hours then the absorbance was read at 600 nm at the end of the 12 hour incubation period.

VI.D.1 Liquid Assay of Mucosal Layer

The mucosal sample was prepared as outlined previously. The sample was syringe filtered and 1 mL of 50 mM Tris-HCl (pH 8) was added then centrifuged at 13,000 rpm for 5 minutes. The sample was stored at 4 °C until use. A 2 ml aliquot of the fish slime was lyophilized overnight then reconstituted in 500 µL of methanol. Similarly, 2 mL of 50 mM Tris-HCl (pH 8) was lyophilized overnight and reconstituted in 500 µL of methanol, which was used as a vehicle control.

VI.D.1 Liquid Assay of Gill Tissue

The gill arches were removed from wild largemouth bass (with the minimum legal size of 14 inches in length) and the lamellae were trimmed then placed in 1% boiling acetic acid at a 1:4 dilution for five minutes. The sample was immediately placed on ice and homogenized on ice. Next, the tissue was centrifuged at 15,000 x g for 45 minutes at 4 °C. Once centrifuged, 5 mL of sample was lyophilized overnight and were reconstituted in methanol or hexanes. A liquid growth assay was done using *S. aureus* and various amounts of the extracts.

CHAPTER VII

RESULTS AND DISCUSSION

VII.A Concentrated Mucosal Sample

A disc diffusion assay with the concentrated mucosal sample is shown in Figure

15. In region A, an area of no growth is observed around the disc treated with 2 μL of

kanamycin (30 mg/mL) as the positive control. However, discs treated with 10, 30, or 50

μL of concentrated mucosal sample show no growth inhibition. This may be due to the

way the sample was prepared or because of the small sample size. The sample was

reconstituted in water, which may have diluted the sample and did not completely

dissolve the hydrophobic regions of antimicrobial peptide. Therefore, methanol was used

to reconstitute concentrated fish samples in further experiments. Methanol is a polar

organic solvent, which may dissolve the amphipathic peptide.
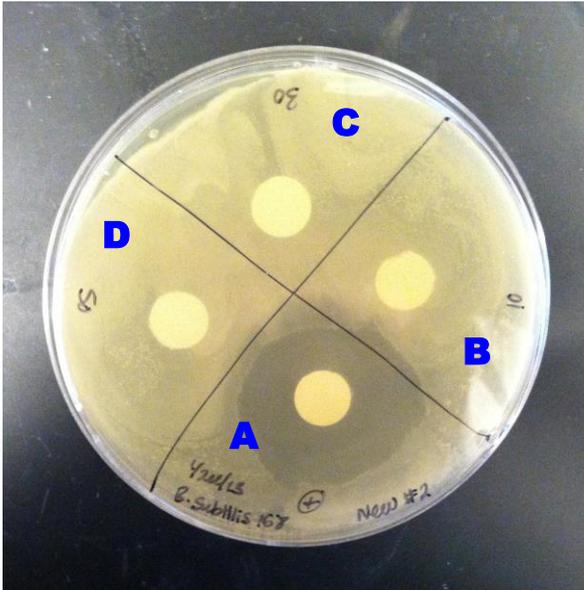
Figure 15. Concentrated Mucosal Sample. Region **A** contains 2 ul Kanamycin (30 mg/ml). Regions **B**, **C**, and **D** contains10, 30, or 50 ul of concentrated mucosal sample in water, respectively.

VII.B Solid Phase Skin Extraction

Results from the solid phase skin extraction tested using the disc diffusion assay, on *E. coli* k12, are shown in figure 16. Region A is a positive control, the disc was treated with 2 μL of Kanamycin (30 mg/ml). Region B contains the unextracted sample and region C shows the crude flow through. Region D shows the wash flow through and region E and F show the first and second eluent. Aside from the positive control, no growth inhibition is observed. Once again, this may be due to sample preparation and the small sample size or there may be no antimicrobial activity in this area of the fish.

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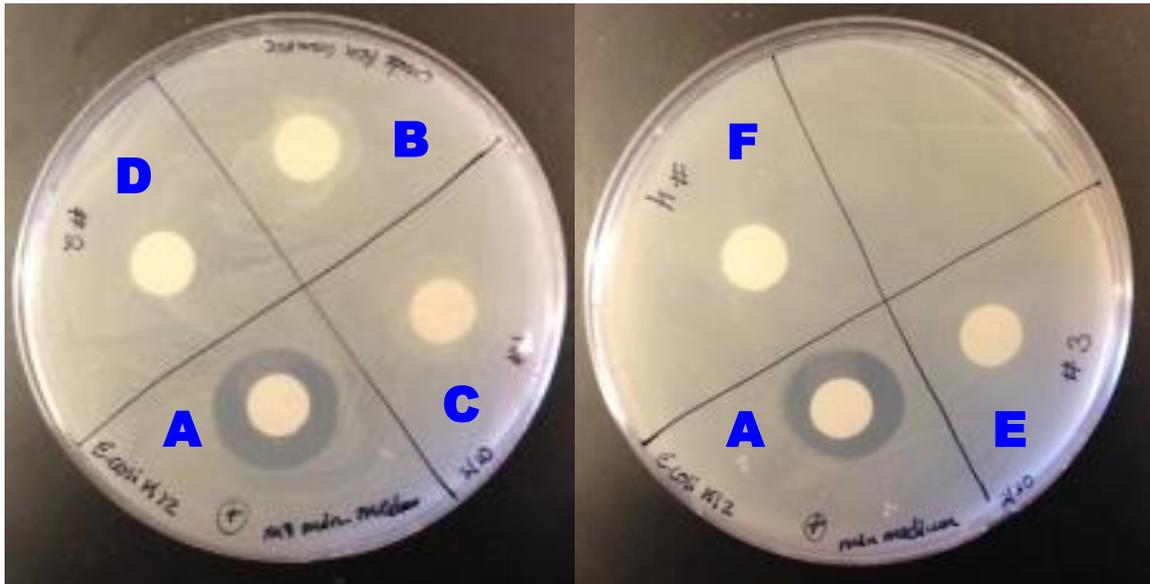
Figure 16. Fish Skin Extract. Region A contains 2 ul Kanamycin (30 mg/ml). Region B contains 50 ul unextracted Sample. Region C contains 50 ul of crude flow through. Region D contains 50 ul of the wash flow through. Region E contains 50 ul of the first eluent and region F contains 50 ul of the second eluent.

VII.C Concentrated Skin

Figure 17 shows the disc diffusion assay on *B. subtilis* 168 with concentrated fish skin samples. Region A contains the positive control of 2 μL kanamycin (30 mg/mL) and regions B, C, and D contain 10, 30, or 50 μL of concentrated fish skin reconstituted in methanol. Region E was treated with 30 μL of concentrated buffer containing 0.2 M sodium acetate and 0.2% Triton X-100. Region F contains 30 μL unconcentrated fish skin sample and region G shows 30 μL of methanol. An area of no growth was observed around the discs treated with the concentrated fish samples. However, an area of no growth was also observed around the disc treated with concentrated buffer. A search of the previous literature revealed that *B. subtilis* strain 168 is sensitive to Triton X-100 and will lyse in the presence of concentrated amounts of Triton.[30]
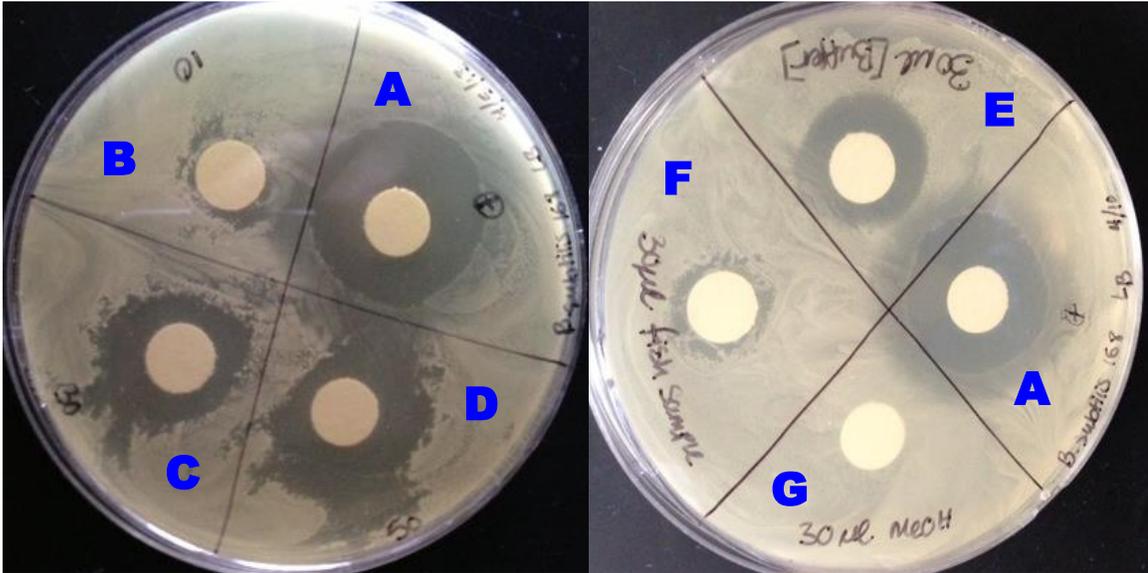
Figure 17. Concentrated Skin Sample. Region a contains 2 ul Kanamycin (30 mg/ml). Regions B, C, and D contains10, 30, and 50 ul concentrated skin sample in MeOH respectively. Region E contains 30 ul concentrated 0.2 M Sodium Acetate and 0.2% Triton. Region F contains 30 ul unconcentrated skin sample and region G contains 30 ul Methanol.

VII.D Skin Tissue without Triton X-100

A disc diffusion assay, with acidified skin tissue, is shown in Figure 18. Region A contains the positive control of 2 μL kanamycin (30 mg/ml). Region B and C contain either 30 or 50 μL of concentrated sample, reconstituted in methanol. There was no growth inhibition around any of the discs treated with the concentrated acidified fish sample.
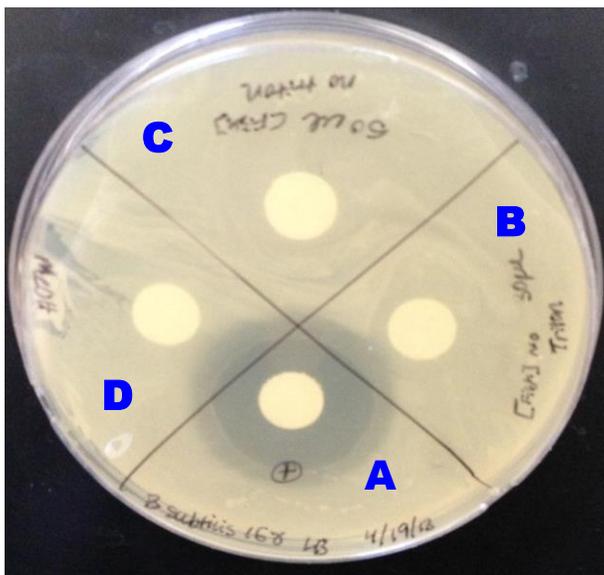
Figure 18. Concentrated Skin Tissue without Triton
X-100. Region A contains 2 ul Kanamycin (30 mg/ml).
Region B contains 30 ul concentrated skin sample in
methanol. Region C contains 50 ul concentrated skin
sample in methanol and region D contains 30 ul methanol.

VII.E Solid Phase Skin Extraction with PMSF

Figure 19 shows the disc diffusion assay with fish skin extract containing PMSF.

Region A is a positive control of 2 μL kanamycin (30 mg/mL). Region B and C shows

the concentrated crude flow through or the concentrated wash flow through reconstituted

in methanol. Regions D − L displays the concentrated 60% ACN eluent. Regions M − N

depicts the concentrated 100% ACN eluent and region O contains the concentrated

unextracted crude sample. Results show growth inhibition of *B. subtilis* 168 when treated

with the extracted concentrated fish sample, region D, and with the concentrated

unextracted sample, region O. However, the concentrated fish sample also contains 0.2%

of Triton X-100 which has been known to cause *B. subtilis* to lyse when concentrated.[30]

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Reverse phase solid phase extraction was done using a C_{18} column and the fraction showing activity was analyzed by mass spectrometry for the presence of triton. The MS results in Figure 20 shows that Triton X-100 was still present in the fraction showing activity. Therefore, *B. subtilis* growth inhibition was more than likely due to the presence of triton rather than an antimicrobial peptide.
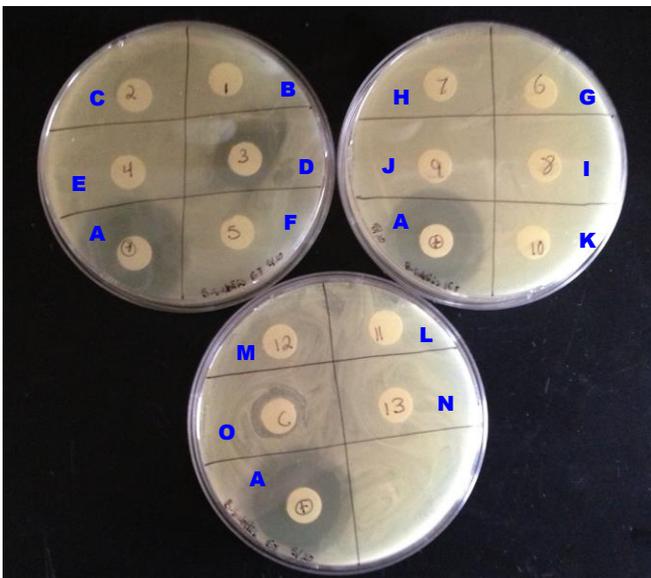

Figure 19. Fish Skin Extract with PMSF. Region A contains 2 ul Kanamycin (30 mg/ml). Region B contains concentrated crude flow through. Region C contains concentrated 0.1% TFA wash flow through. Regions D – L contains concentrated 60% ACN eluent. Regions M – N contains concentrated 100% ACN eluent and region O contains unextracted skin sample.
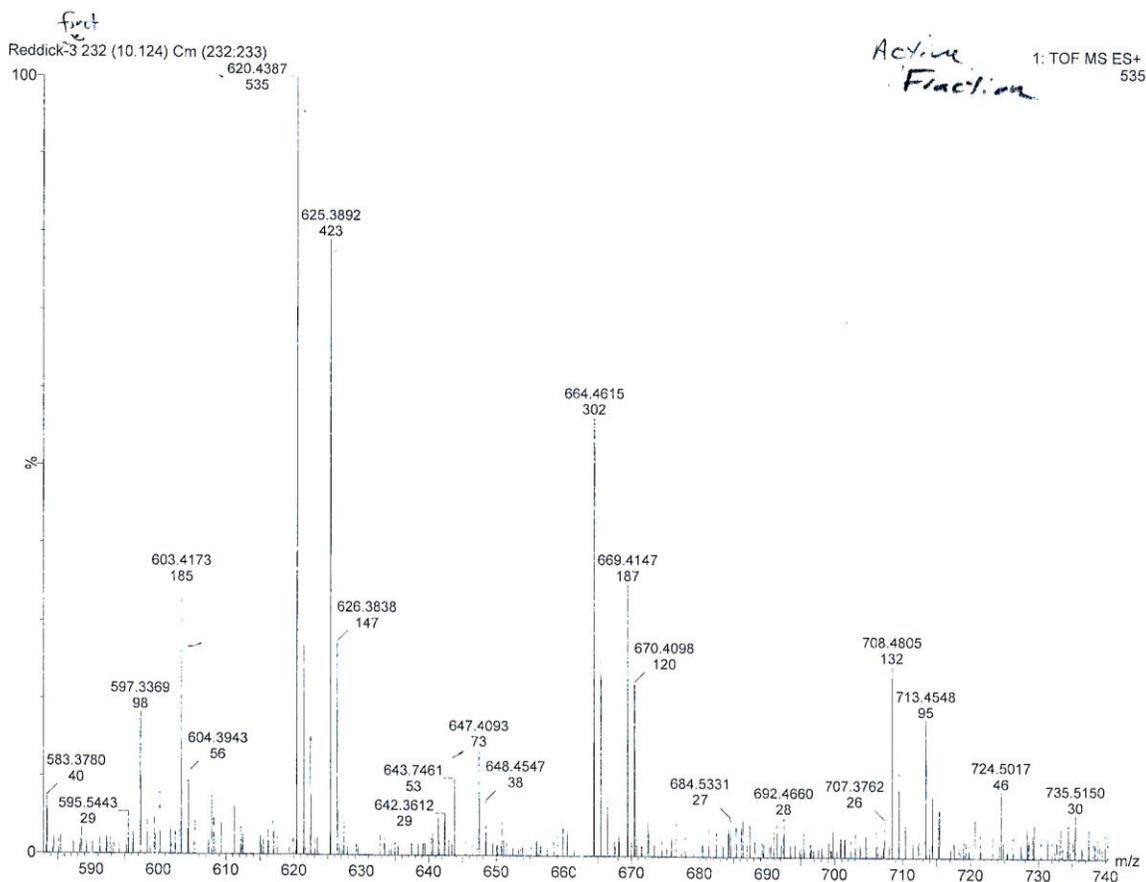
Figure 20. Spectrum of Concentrated Fraction 1 from Skin Extraction with PMSF (Labeled Region D in Figure 19). Fractions of Triton X-100 (647 g/mol) are observed throughout spectrum.

VII.F Liquid Assay of Mucosal Layer

A liquid growth assay was used for the following experiments instead of the disc diffusion assay because and overnight incubation period was used for the disc diffusion assay, which may have been too long to observe low levels of growth inhibition. For example, if there was an early suppression of growth after several hours this was probably overcome by the bacteria overnight and falsely indicating no antimicrobial activity. The assay was carried out by Joseph Egan, an undergraduate student in Prof. Nadja Cech's laboratory at UNCG, with mucosal fish sample against *S. aureus* is shown

in Table 1. A decrease in absorbance indicates growth inhibition of the bacteria. As a control, 50 µl of the buffer containing 50 mM Tris-HCl (pH 8) was tested. The mucosal layer, 50 µl, shows an increase in *S. aureus* growth instead of inhibition. This may be due to carbohydrates or lipids in the mucosal layer, which may be promoting growth.

Table 1. Liquid Assay of Mucosal Layer.

Sample ID	Absorbance Values (OD 600)			Volume	Average Absorbance	% inhibition	Std Dev	Std Dev. %
Vehicle	0.254	0.228	0.123	50uL	0.202	25.031	0.07	6.94
Mucosal Layer	0.374	0.243	0.353	50uL	0.323	-60.331	0.07	7.04
Blank	0.245	0.264	0.298	0uL	0.269	0	0.03	2.69

VII.G Liquid Assay of Fish Gills

Results of the liquid growth assay, against *S. aureus*, with concentrated fish gill sample is shown in Figure 21. The gill sample was reconstituted in hexanes and methanol. A concentration of either 20 or 200 parts per million (ppm) was assayed by Joseph Egan. Growth inhibition is observed with gill tissue indicating antimicrobial activity. Although the gill tissue, reconstituted in hexanes, shows inhibition with the 200 ppm concentration, the standard deviation is too high to be considered repeatable. Therefore, further experiments with the concentrated gill sample are reconstituted in methanol.

Percent Inhibition against S. aureus
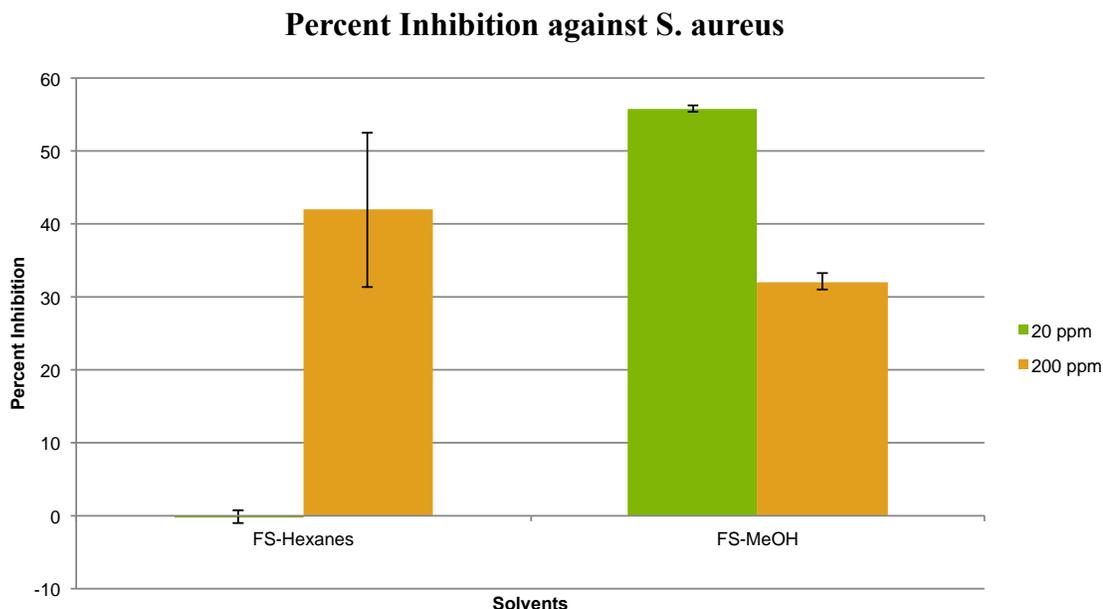
Figure 21. Liquid Assay with Fish Gills. Percent inhibition against S. aureus with concentrated fish gills reconstituted in hexanes or methanol. Assay was done using either 20 or 200 parts per million (ppm) of concentrated fish gill sample. Assay completed by Joseph Egan.

VII.H Dose Response Inhibition of *S. aureus*

To further characterize the antimicrobial activity observed from the gill tissue, an inhibition curve against *S. aureus*, completed by Joseph Egan, is shown in Figure 22. A standard curve of Berberine, an antibiotic from the golden seal plant, is shown as the green line in Figure 22. Concentrations of gill tissue ranging from 0 – 400 ppm is shown as the orange line in Figure 22 and a dose response inhibition is observed. However, at concentrations higher than 100 ppm, the absorbance increases instead of decreasing in a dose responsive manner. This was more than likely due to the turbidity of the gill tissue extract causing the increase in absorbance rather than an increase in bacterial growth.

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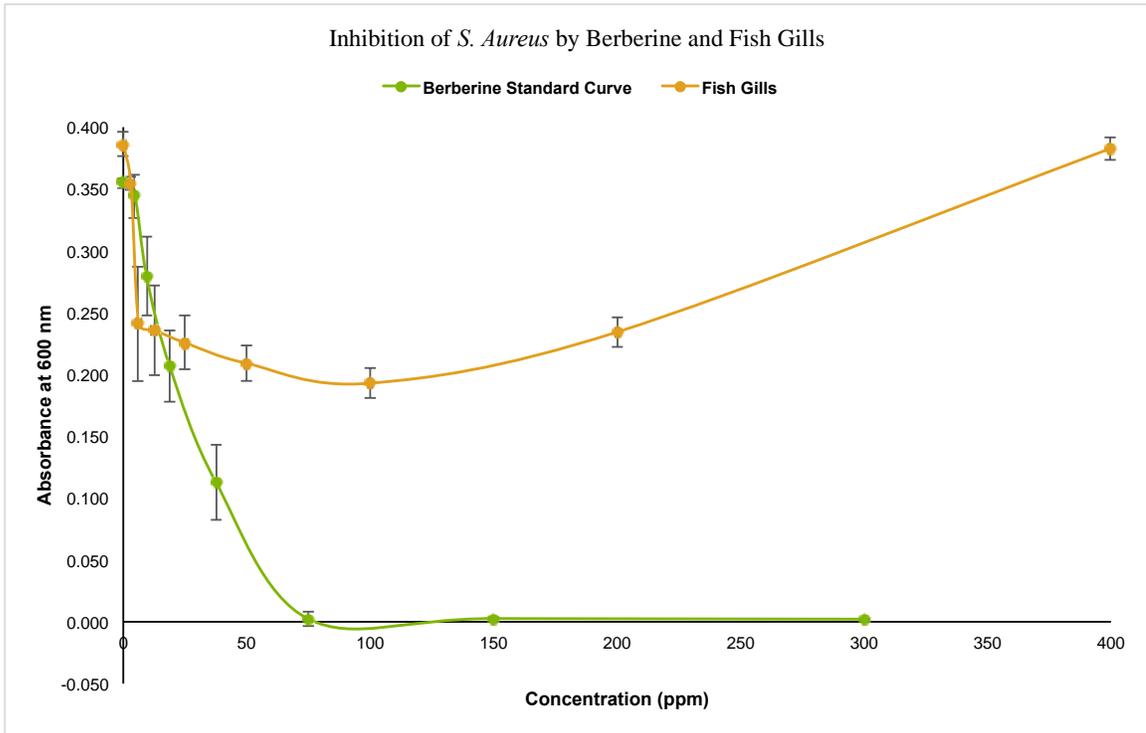
Figure 22. Liquid Assay Inhibition Curve of Fish Gills. A standard Berberine curve is shown in green. Concentrated fish gills were reconstituted in methanol and dose response inhibition was done using 0 – 400 ppm of fish gill sample. Assay completed by Joseph Egan.

CHAPTER VIII

CONCLUSION

In conclusion, we have shown that there is antimicrobial activity in the acidified gill tissue of largemouth bass. These experiments are an initial step in isolating and characterizing an AMP from largemouth bass. We will next fractionate this activity using reverse phase and cation exchange column chromatography, while selecting active fractions using the liquid growth assay. Sequencing of the expected AMP will be done using mass spectrometry and Edman degradation. Once successful, we will be in position to produce quantities of the peptide chemically or by biotechnological means for future studies and commercial applications.

BIBLIOGRAPHY

(1)   Richard Losick (Harvard) Part 1: Spore Formation in Bacillus Subtilis; 2010.

(2)   Abdel-Khalek, M. A.; El-Midany, A. A. Application of Bacillus Subtilis for Reducing Ash and Sulfur in Coal. Environ. Earth Sci. **2013**, 70, 753–760.

(3)   Gomaa, E. Z. Antimicrobial Activity of a Biosurfactant Produced by Bacillus Licheniformis Strain M104 Grown on Whey. Braz. Arch. Biol. Technol. **2013**, 56, 259–268.

(4)   Reddick, J. J.; Williams, J. K. The mmgA Gene from Bacillus Subtilis Encodes a Degradative Acetoacetyl-CoA Thiolase. Biotechnol. Lett. **2008**, 30, 1045–1050.

(5)   Kunst, F.; Ogasawara, N.; Moszer, I.; Albertini, A. M.; Alloni, G.; Azevedo, V.; Bertero, M. G.; Bessières, P.; Bolotin, A.; Borchert, S.; et al. The Complete Genome Sequence of the Gram-Positive Bacterium Bacillus Subtilis. Nature **1997**, 390, 249–256.

(6)   NCBI Blast:Protein Sequence (472 letters) http://www.ncbi.nlm.nih.gov/blast/Blast.cgi (accessed Sep 16, 2013).

(7)   Piggot, P. J.; Hilbert, D. W. Sporulation of Bacillus Subtilis. Curr. Opin. Microbiol. **2004**, 7, 579–586.

(8)   Hilbert DW, C. V., Piggot PJ. Contrasting Effects of sigmaE on Compartmentalization of sigmaF Activity during Sporulation of Bacillus Subtilis. J. Bacteriol. **2004**, 186, 1983–1990.

(9)   Bryan, E. M.; Beall, B. W.; Moran, C. P., Jr. A Sigma E Dependent Operon Subject to Catabolite Repression during Sporulation in Bacillus Subtilis. J. Bacteriol. **1996**, 178, 4778–4786.

(10)   Brock, M.; Maerker, C.; Schütz, A.; Völker, U.; Buckel, W. Oxidation of Propionate to Pyruvate in Escherichia Coli. Involvement of Methylcitrate Dehydratase and Aconitase. Eur. J. Biochem. FEBS **2002**, 269, 6184–6194.

(11)   Serio, A. W.; Pechter, K. B.; Sonenshein, A. L. Bacillus Subtilis Aconitase Is Required for Efficient Late-Sporulation Gene Expression. J. Bacteriol. **2006**, 188, 6396–6405.

(12)   Textor, S.; Wendisch, V. F.; De Graaf, A. A.; Müller, U.; Linder, M. I.; Linder, D.; Buckel, W. Propionate Oxidation in Escherichia Coli: Evidence for Operation of a Methylcitrate Cycle in Bacteria. Arch. Microbiol. **1997**, 168, 428–436.

(13)   Tabuchi, T.; Hara, S. Production of 2-Methylisocitric Acid from N-Paraffins by Mutants of Candida Lipolytica. Agric. Biol. Chem. **1974**, 38, 1105–1106.

(14)   Hardesty, Grant. The Characterization of MmgE from Bacillus Subtilis, University of North Carolina Greensboro, 2012.

(15)   HPLC Column | Synergi | Phenomenex http://www.phenomenex.com/products/detail/Synergi (accessed Mar 28, 2014).

(16)   APARNA, M. Analysis of a Possible Multienzyme Complex Encoded by Mother Cell Metabolic Gene (mmg) Operon of Bacillus Subtilis Strain 168, The University of North Carolina Greensboro, 2010.

(17)   BOOTH, W. Characterization of the Biochemical Activity of the Open Reading Frame "yqiQ" of Bacillus Subtilis, 2011.

(18)   Garrett, R.; Grisham, C. Biochemistry; Third Edition.; Physical Sciences, 2005.

(19)   Trisodium (2RS,3RS)-2-methylcitrate mixture of diastereomers, ≥85% (mixture of diastereomers, NMR) | Sigma-Aldrich http://www.sigmaaldrich.com/catalog/product/sigma/59464?lang=en&region=US (accessed Apr 18, 2014).

(20)   2011 National Survey of Fishing, Hunting, and Wildlife-Associated Recreation State Overview :: Documents http://digitalmedia.fws.gov/cdm/ref/collection/document/id/858 (accessed Sep 10, 2013).

(21)   Schramm, H. L.; Davis, J. G. Survival of Largemouth Bass from Populations Infected with Largemouth Bass Virus and Subjected to Simulated Tournament Conditions. North Am. J. Fish. Manag. **2006**, 26, 826–832.

(22)   Largemouth Bass. Wikipedia, the free encyclopedia, 2013.

(23)   Noga, E. J.; Silphaduang, U.; Park, N. G.; Seo, J. K.; Stephenson, J.; Kozlowicz, S. Piscidin 4, a Novel Member of the Piscidin Family of Antimicrobial Peptides. Comp. Biochem. Physiol. B Biochem. Mol. Biol. **2009**, 152, 299–305.

(24)   Arenas, G.; Guzmán, F.; Cárdenas, C.; Mercado, L.; Marshall, S. H. A Novel Antifungal Peptide Designed from the Primary Structure of a Natural Antimicrobial Peptide Purified from Argopecten Purpuratus Hemocytes. Peptides **2009**, 30, 1405–1411.

(25)   Lauth, X.; Shike, H.; Burns, J. C.; Westerman, M. E.; Ostland, V. E.; Carlberg, J. M.; Van Olst, J. C.; Nizet, V.; Taylor, S. W.; Shimizu, C.; et al. Discovery and Characterization of Two Isoforms of Moronecidin, a Novel Antimicrobial Peptide from Hybrid Striped Bass. J. Biol. Chem. **2002**, 277, 5030–5039.

(26)   Cole, A. M.; Weis, P.; Diamond, G. Isolation and Characterization of Pleurocidin, an Antimicrobial Peptide in the Skin Secretions of Winter Flounder. J. Biol. Chem. **1997**, 272, 12008–12013.

(27)   Iijima, N.; Tanimoto, N.; Emoto, Y.; Morita, Y.; Uematsu, K.; Murakami, T.; Nakai, T. Purification and Characterization of Three Isoforms of Chrysophsin, a Novel Antimicrobial Peptide in the Gills of the Red Sea Bream, Chrysophrys Major. Eur. J. Biochem. **2003**, 270, 675–686.

(28)   Su, Y. Isolation and Identification of Pelteobagrin, a Novel Antimicrobial Peptide from the Skin Mucus of Yellow Catfish (Pelteobagrus Fulvidraco). Comp. Biochem. Physiol. B Biochem. Mol. Biol. **2011**, 158, 149–154.

(29)   Seo, J.-K.; Lee, M. J.; Go, H.-J.; Park, T. H.; Park, N. G. Purification and Characterization of YFGAP, a GAPDH-Related Novel Antimicrobial Peptide, from the Skin of Yellowfin Tuna, Thunnus Albacares. Fish Shellfish Immunol. **2012**, 33, 743–752.

(30)   Tsuchido, T.; Svarachorn, A.; Soga, H.; Takano, M. Lysis and Aberrant Morphology of Bacillus Subtilis Cells Caused by Surfactants and Their Relation to Autolysin Activity. Antimicrob. Agents Chemother. **1990**, 34, 781–785.