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Bacillus subtilis has been extensively studied and serves as a model organism for Gram-positive bacteria which can be attributed to its capacity to be genetically manipulated, form endospores, and a sequenced genome. *B. subtilis* undergoes a disproportionate cellular division during a process known as sporulation, which has been widely used to study more complex phenomena, such as cellular differentiation. In addition, *B. subtilis* is widely used by industry and a greater understanding of its metabolic pathways would be a valuable contribution to the genomic annotation in this model organism.

A long-term goal of our research is to provide a more complete description of the biochemical pathways that are encoded by the mother cell metabolic gene (*mmg*) operon within *B. subtilis*. One specific objective of this research is to compare the 2-methylcitrate stereoisomer produced by 2-methylcitrate synthase, the PrpC enzyme, in *Escherichia coli* to the 2-methylcitrate stereoisomer produced by the homologous enzyme, MmgD, in *B. subtilis*. In addition, we wish to further investigate the subsequent dehydration reaction catalyzed by homologous enzymes PrpD and MmgE, which both have been found to produce (*Z*)-2-methylcitrate. The *prpC and prpD* gene products were cloned, overexpressed, and purified using a culture of *E. coli* strain BL21(DE3) via methods established by researchers of the Reddick Lab. The presence of the desired *prpC* and *prpD* gene products were verified by SDS-PAGE analysis and activity assays.

To compare the 2-methylcitrate synthase activity of PrpC to MmgD, the reaction product was assessed by standardization with commercially available 2-methylcitrate racemate. Activity assays with PrpD and cross-species experiments were prepared in the same manner. Using this methodology, it was found that PrpC and MmgD produce either (2*S*, 3*R*)-2-methylcitrate or (3*S*, 2*R*)-2-methylcitrate. In addition, by confirming the substrates for these enzymes, we demonstrated the substrate for PrpD is not (2*S*, 3*S*)-2-methylcitrate and, therefore, PrpD does not exhibit a *syn*-elimination mechanism to produce (*Z*)-2-methylcitrate.

INVESTIGATION AND COMPARISON OF STEREOISOMER PRODUCTS OF 2-METHYLCITRATE SYNTHASE AND 2-METHYLCITRATE DEHYDRATASE IN *ESCHERICHIA COLI* STRAIN K12 AND *BACILLUS STUBTILIS* STRAIN 168

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

1.1 Review of Literature

1.1.1 Overview and Applications of *Bacillus subtilis*

As a Gram-positive, rod-shaped endospore forming bacterium, *Bacillus subtilis* has been extensively studied and serves as a model organism which can be attributed to its capacity to be genetically manipulated.[1] Although, *B. subtilis* can be found in a wide range of environments, growth is associated with the presence of decomposing organic matter, such as that found in soil and the gastrointestinal tract of certain animals.[2] In 1997, *B. subtilis* became the first Gram-positive bacterium to have its genome sequenced and consists of 4,100 protein-coding genes.[3] *B. subtilis* has historically been classified as a strict aerobe, but there is evidence of anaerobic growth in the presence of nitrate.[4]

As the first Gram-positive bacterium to have its genome sequenced, *B. subtilis* has proven to be a versatile and valuable model organism in a number of capacities including, food safety, vaccine development, and enzyme production.[3] The bacterium has been classified as "Generally Recognized as Safe" by the Food and Drug Administration.[5] It is readily used in food safety techniques, such as involvement in food fermentation and post-harvest produce washing.[5] The industrial enzymes, Carbohydrase and protease produced by *B. subtilis* have been deemed by the FDA to be nonpathogenic and nontoxicogenic.[6] Enzymes that *B. subtilis* produces, such as proteases and amylases,

have also been found to be useful in industry.[7] The wide use of *B. subtilis* in both industry and academia points to the importance of elucidating metabolic pathways and genetic strategies of this model organism.

1.1.2 Summary of Sporulation

Under strenuous conditions that are not conducive with vegetative life, such as the absence of a carbon or nitrogen source, *B. subtilis* will undergo sporulation, which is an uneven cellular division, in which the size and composition of the cells differ.[8] The process of sporulation initially produces a mother cell and a forespore.[9] Once sporulation is completed, a dormant and resilient structure, known as a spore, is produced that has been estimated to survive for up to thousands of years.[9] The initiation of this asymmetrical division is regulated by the SpoOA master transcription factor that becomes phosphorylated as a result of harsh environmental conditions and initiates sporulation-specific gene expression.[9] Along with kinase events, SpoOA plays an integral role in the decision to undergo sporulation.[9] Once the septum has been formed, the first compartment-specific transcription factor activated is σ^F and is responsible for the subsequent activations of other transcription factors, including σ^E, σ^G, and σ^K.[9] The first mother-cell-specific transcription factor is σ^E, which upon activation leads to the expression of genes within the mother metabolic gene (*mmg*) operon.[10] This gene cluster has been characterized by the work conducted within the laboratory of Dr. Jason Reddick.[11]

The asymmetric division that *B. subtilis* undergoes during sporulation has been used to study more complex phenomena, such as, cellular differentiation.[12] Also, it has

been observed that sporulation-specific proteins may have homologous activity to proteins present during normal growth.[9] Sporulation is a conserved feature amongst other species including *Bacillus anthracis*.[13] Although the morphological features of sporulation have been studied extensively, there are still many poorly understood biochemical pathways that occur during sporulation.

1.1.3 Genetic Regulation of the Methylcitric Acid Cycle

The metabolism of propionate, a short chain odd-carbon fatty acid, via the methylcitric acid cycle (MCC) was first identified in *Yallowia lipolytica*.[14] The propionate (*prp*) operon, which codes for five enzymes involved in the MCC, has been studied in other species including, *Salmonella typhimurium* LT2 and *Escherichia coli*.[15,16] The *prp* locus consists of two transcriptional units involved in propionate degradation.[15] The portion known as, *prpR* has been shown to initiate transcription of the other portion of the *prp* locus, *prpBCDE*.[15] In addition, the σ^E dependent *mmg* operon found in *B. subtilis* contains fatty-acid metabolizing enzymes and *prp* homologs, specifically those involved in the MCC.[10,11] The *prp* operon in *E. coli* compared to the *mmg* operon found in *B. subtilis* is pictured in Figure 1. The proteins encoded by *prpC* from *E. coli* and *mmgD* from *B. subtilis* share an amino acid sequence identity of 34% and 75% overall similarity. Homology of *prpD* in *E. coli* and *mmgE* in *B. subtilis* has previously been reported; these enzymes share a 61% amino acid sequence identity and 75% overall sequence similarity.[17]

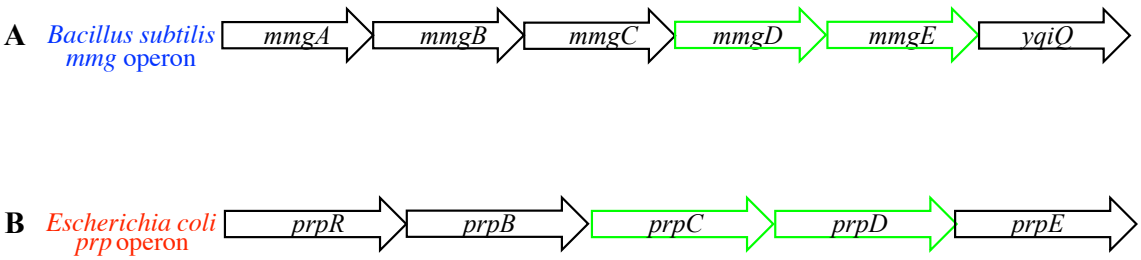


Figure 1. Comparison of Propionate Operon and Mother Cell Metabolic Gene Operon. A: Illustration of *mmg* operon from *B. subtilis* responsible for fatty-acid degradation. **B:** Scheme of the *prp* operon from *E. coli* involved in propionate oxidation.

A generic representation of propionate degradation via the MCC involving either *prp* or *mmg* operon encoded enzymes is shown in Figure 2. This metabolic pathway begins with the gene product of *prpE*, propionyl-CoA synthetase, which catalyzes the reaction using substrates propionate, coenzyme A, and adenosine triphosphate to produce propionyl-CoA.[15] The following step is catalyzed by the gene product *prpC*, 2-methycitrate synthase, which is the condensation of propionyl-CoA with oxaloacetate producing 2-methylcitrate.[15] The sequential step involves the dehydration of 2-methylcitrate by 2-methylcitrate dehydratase, the gene product of *prpD*, to form (*Z*)-2-methylaconitate. The following reaction is catalyzed by an aconitase that converts 2-methylcitrate to 2-methylisocitrate.[17] The final step in the MCC involves 2 methylcitrate lyase, the gene product *prpB*, that produces succinate and pyruvate.[15]

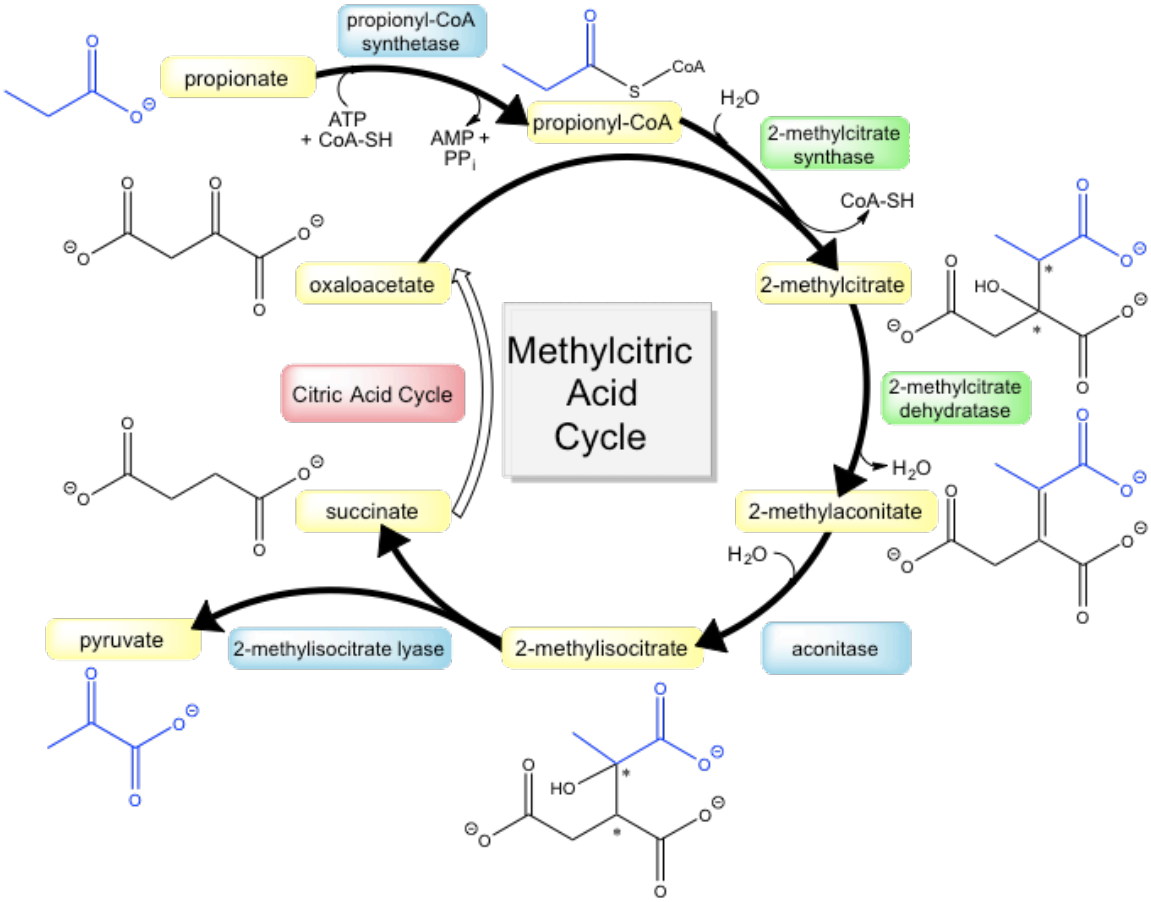


Figure 2. The Degradation of Propionate Via the Methylcitric Acid Cycle.

1.1.4 Previous MCC Results Obtained from *B. subtilis*

The genes encoding the enzymes responsible for carrying out the MCC have been characterized, enzyme activity investigated, and homologous genes determined within other species.[18] The MCC has most recently been studied in *B. subtilis*.[11] *B. subtilis* has exhibited homologous gene products that have been previously identified in *S. typhimurium* and *E. coli*.[17] Despite reports of homology between *B. subtilis* and *E. coli*, there does appear to be a discrepancy between the products and substrates of two specific steps within the MCC. It was reported that the gene product of *prpC*, 2-methylcitrate

synthase in *E. coli* catalyzed a condensation reaction that produced (2*S*, 3*S*)-2-methylcitrate, which was "verified" by enantioselective multidimensional capillary gas chromatography.[17] Unfortunately, there was no data reported with this claim. Also, the stereo assignment of (2*S*, 3*S*)-2-methylcitrate as the substrate for the gene product of *prpD*, 2-methylcitrate dehydratase, was reinforced by the observed maximum activity. Listed was substrate specificity for a variety of compounds, but there was no data listed for the activity of PrpD using (2*SR*, 3*RS*)-2-methylcitrate.[17] In addition, there were no activity values reported for authentic (*Z*)-2-methylaconitate, due to unavailability reported by the author. It has been previously stated that PrpD demonstrates substrate specificity for (2*S*, 3*S*)-2-methylcitrate only.[17] The reaction of this particular enzyme would therefore exhibit a *syn-elimination* mechanism, which is peculiar.[17] Illustrated by a Newman projection shown in Figure 3, is the eclipsed conformation that would be necessary for the reaction to proceed through *syn-elimination*.[17] If in fact PrpD underwent a *syn-elimination* mechanism, it would be considered a novel dehydratase.

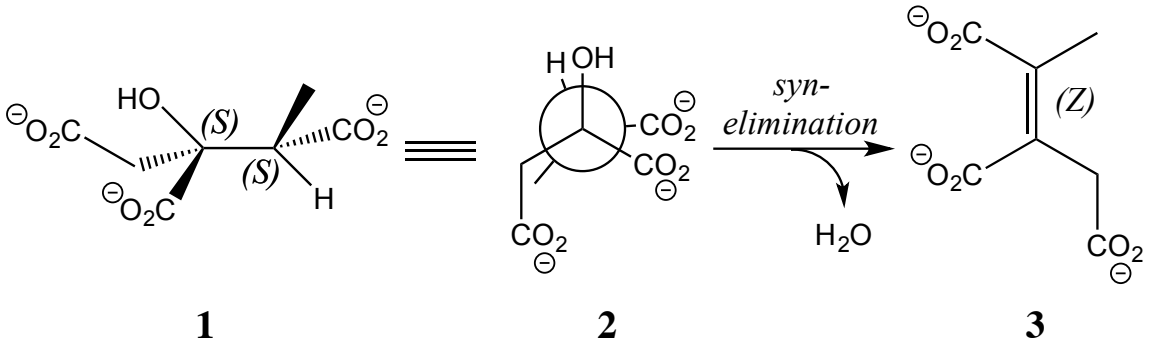


Figure 3. The Previously Reported *Syn-elimination* Mechanism of PrpD, 2-Methylcitrate Dehydratase. The proposed substrate of PrpD (2*S*, 3*S*)-2-methylcitrate **(1)**, also portrayed as a Newman projection **(2)**, producing exclusively (*Z*)-2-methylaconitate **(3)**.

Conflicting chromatography results obtained from *B. subtilis* are illustrated in Figure 4. The chromatograph of a commercially available racemic mixture of (2*SR*, 3*RS*)-2-methylcitrate and (2*SR*, 3*SR*)-2-methylcitrate is shown in Figure 4A. Depicted in Figure 4B, MmgD from *B. subtilis* was shown to catalyze the condensation of propionyl-CoA reacting with oxaloacetate to form (2*S*, 3*R*)-2-methylcitrate or (2*R*, 3*S*)-2-methylcitrate.[11] The 2-methylcitrate dehydratase in *E. coli*, PrpC was reported to produce (2*S*, 3*S*)-2-methylcitrate, which corresponds to the product peak at 16.2 minutes.[17] This is the preceding step to the dehydration reaction catalyzed by the gene product of *mmgE* from *B. subtilis*. As shown in the chromatogram in Figure 4C, it was observed that MmgE, using the mixture of diastereomers of 2-methylcitrate standard as its substrate, catalyzed the elimination of water resulting in the formation of two products (*Z*)-2-

methylaconitate and (*E*)-2-methylaconitate.[11] The active peaks at 13.6 and 16.3 minutes indicate unreacted starting material.

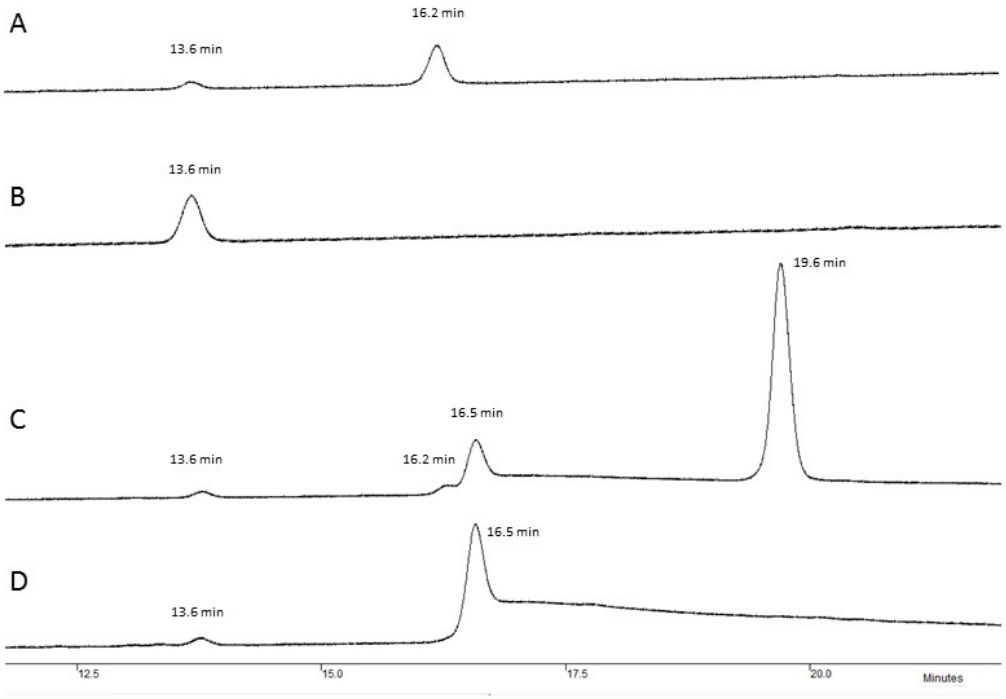


Figure 4. HPLC Chromatograms from *Bacillus subtilis* Enzymes, MmgD and MmgE. A: HPLC chromatogram of 2-methylcitrate racemate, including four stereoisomers, (2*SR*, 3*RS*) as the minor product and (2*SR*, 3*SR*) as the major product. **B:** Chromatography of substrates propionyl-CoA and oxaloacetate being supplied to MmgD. **C:** The chromatogram of the full MmgE reaction with the commercial 2-methylcitrate as the substrate source. The new product peaks at 16.5 and 19.6 minutes correspond to (*Z*)-2-methylaconitate and (*E*)-2-methylaconitate respectively. **D:** Chromatogram of full MmgD and MmgE reaction with propionyl-CoA and oxalacetate only as the substrates displaying one new product peak at 16.5 minutes.

The sequence identities of *mmgE* and *prpD* support the conclusion that these proteins would have similar enzymatic behavior. Based on this reasoning, either the stereochemical conclusion for *B. subtilis* has been incorrectly assigned or the stereochemistry provided by the literature for *E. coli* has been misassigned.

1.2 Central Hypothesis and Objectives

Despite the precedence within the literature and the previous results observed with *B. subtilis*, the central hypothesis of this project is *mmgD and mmgE* in *Bacillus subtilis* and *prpC and prpD* in *Escherichia coli* are genes that encode for homologous enzymes that produce the same stereoisomer products.

One objective of our current work is to compare the stereoselectivity of 2-methylcitrate synthase from *B. subtilis* and *E. coli* using the same methodology as in our laboratory's work with *B. subtilis*. In this specific objective, the gene sequence of interest, *prpC*, was isolated from the *prp* operon in *E. coli*. This gene has exhibited similarities to methylcitrate synthases in other species including *S. enterica* and *B. subtilis.* PrpC was acquired through recombinant DNA methods. The reaction products of PrpC from *E. coli* and MmgD from *B. subtilis* were compared using HPLC methods already developed in our laboratory.

In addition, we have been interested in investigating 2-methylcitrate dyhydratase from *B. subtilis* and *E. coli*. The subsequent step to the condensation of propionyl-CoA in the MCC is the dehydration of 2-methylcitrate. The substrate of the gene product of *prpD*, 2-methylcitrate dehydrates, in *E. coli* is 2-methylcitrate. PrpD from *E. coli* was obtained through cellular transformation and protein purification. To reveal whether

MmgD from *B. subtilis* and PrpC from *E. coli* are producing the same stereoisomer, HPLC was used to determine the reaction product of PrpD when supplied with 2-methylcitrate produced by PrpC. Cross-species experiments were used to confirm whether these enzymes are interchangeable and producing the same compounds.

CHAPTER II

EXPERIMENTAL

2.1 *prpC* Gene Cloning

2.1.1 Primer Design for *prpC*

Primers for the gene *prpC* were designed using the genomic sequence of *E. coli* strain K12 (ECK12) and Bioedit Software. All primers had a T_m of approximately 60 °C and ranged from 21 to 34 base pairs in length. The oligomers designed for the gene *prpC* consisted of the following sequences: *prpC* forward: 5'-CAC CAT GAG CGA CAC AAC GAT CC-3', and *prpC* reverse: 5'-TTA CTG GCG CTT ATC CAG CGC-3'. The CACC base pair sequence is added to the forward primer to enable the overhang of the pET-200 vector to invade the PCR product and propagate the proper insertion of the sequence.

2.1.2 DNA Amplification of *prpC* via PCR

Lysogeny Broth (LB), 5 mL, was inoculated with ECK12 and grown at 37 °C overnight. The genomic DNA from ECK12 was isolated using the Wizard® Genomic DNA Purification Kit procedure, according to the manufacturer's instructions. The gene *prpC* was then amplified using PCR, the mixture included 2 µL of purified genomic DNA, 3 µL of each *prpC* forward and reverse primer, 17 µL of nanopure water, and 25 µL of Phusion® High-Fidelity PCR MM w/HF-Buffer, which brought the total volume of the reaction to 50 µL. Thermocycler settings were adjusted to the following conditions:

one cycle consisting of 98 °C for 30 seconds, 30 cycles of 98 °C for 5 seconds, 58 °C for 10 seconds, 72 °C for 75 seconds, an additional cycle at 72 °C for 10 seconds, and concluding with a temperature setting at 4 °C. The fresh PCR product was purified by the Qiagen® QIAquick PCR Purification Kit, according to the manufacturer's instructions.

DNA amplification was confirmed by 1% agarose gel electrophoresis in 1X TAE buffer with visualization by ethidium bromide. To prepare samples, 4 µL of Gel Loading Dye (6X), 4 µL of nanopure H_2O, and 16 µL of each sample were combined. The standard ladder sample was prepared with 1 µL of 2-Log DNA Ladder, 4 µL of dye, and 4 µL of nanopure H_2O. The gel was carried out at 120V in 1X TAE buffer.

2.1.3 Vector and Cell Transformation of *prpC*

The *prpC* TOPO® Cloning Reaction was performed using the following volumes of reagents: 3.35 µL of sterile H_2O, 1 µL of salt solution, 0.65 µL of fresh PCR product, and 1 µL of TOPO® vector, and all were combined which brought the final volume to 6 µL. The reaction was incubated at ambient temperature for 5 minutes. The One Shot® Top10 Chemical Transformation Protocol was followed by adding 3 µL of the TOPO® Cloning Reaction into a thawed vial of One Shot® Top10 Chemically Competent *E. coli* and incubated for 30 minutes on ice. The procedure was carried out in accordance to the manual with the exception of the entire vial being plated onto a prewarmed selective plate instead of only 100-200 µL of transformation being plated. After an overnight incubation at 37 °C, 5 colonies were selected from the plated One Shot® Top10 Chemically Competent *E. coli* and grown in 5 mL of LB broth and 5 µL of kanamycin (0.5mg/mL). The starter cultures were centrifuged and the supernatant was discarded. In order to

screen for successful clones, the Qiagen QIAprep® Miniprep Kit protocol was followed and the purified plasmid products were analyzed. An additional PCR was carried out as stated before, with the exception of one plasmid specific primer with one gene specific primer (T7 and *prpC*) in place of the gene specific primers. An electrophoresis gel was carried out to identify plasmids containing the *prpC* gene sequence. Plasmids indicating gene insertion by gel electrophoresis results were sent to be sequenced to verify proper reading frame position.

2.2 *prpD* Gene Cloning

2.2.1 Primer Design for *prpD*

The PCR oligomers for the gene *prpD* were designed using the following sequence: *prpD* reverse: 5'-TTA AAT GAC GTA CAG GTC GAG ATA CTC ATT GAC-3', *prpD* forward: 5'-CAC CAT GTC AGC TCA AAT CAA CAT CC-3'

2.2.2 DNA Amplification of *prpD* via PCR

The same procedure mentioned previously was followed with the exception of using the *prpD* forward and reverse primers in place of the *prpC* primers for the PCR.

2.2.3 Vector and Cell Transformation of *prpD*

Following the manufacturer's protocol, the PCR product was cloned into pET-200 using topoisomerase based cloning methodology. The manufacturer's instructions provided a range for the concentration of fresh PCR product to add to the TOPO® Cloning Reaction. To obtain a proper ratio of PCR product to vector, two molar ratios of purified PCR product were used to TOPO® Clone into the pET-200 TOPO® vector. The 1:1 molar ratio of PCR product to TOPO® vector contained: 3 μL of H_2O, 1 μL of salt

solution supplied in the kit, 1 μL of purified PCR product, and 1 μL of vector. To prepare the 1:2 molar ratio sample, 2 μL H_2O was added to 1 μL of salt, followed by the addition of 2 μL of diluted purified PCR product, and lastly 1 μL of vector was added. The only successful *prpD* TOPO® Cloning Reaction was accomplished by obtaining the concentration of the *prpD* PCR product and calculating the volume needed to fall within the desired range of 0.5:1 to 2:1 molar ratio of PCR product: TOPO® vector One Shot®. The TOPO® Cloning Reaction was performed using the following quantities of reagents: 3.5 μL of nanopure H_2O, 1 μL of Salt Solution, 0.5 μL of fresh *prpD* PCR product, and 1 μL of TOPO vector. The reaction was allowed to incubate at ambient temperature for 5 minutes. TOP10 Chemical Transformation Protocol was then followed by adding 3 μL of the different molar ratio TOPO® Cloning Reactions to separate vials of One Shot® TOP10 Chemically Competent *E. coli* and mixed gently with a pipette tip. The protocol was completed by spreading the entire transformation reaction onto a prewarmed LB agar plate containing (0.5 mg/mL) and incubated overnight.

To screen for successful transformation, several colonies were selected from each plate to inoculate a mixture of 5 mL of LB broth and 5 μL of kanamycin (0.5 mg/mL), which were shaken and grown overnight at 37 °C. The starter cultures were centrifuged and the supernatant was discarded. The pellet was resuspended followed by the protocol of the Qiagen QIAprep® Miniprep kit and the purified plasmid products were analyzed. Similar to the protocol followed with *prpC* transformants, the plasmids containing the *prpD* gene were identified with electrophoresis gels of the PCR products using vector and *prpD* gene specific primers.

2.3 Protein Overexpression and Purification

2.3.1 PrpC Protein Expression and Purification

To overexpress PrpC, BL21 Star™ (DE3) One Shot® Cells were used as the host strain. As instructed by the manufacturer's protocol, 1 µL of the purified plasmid was added to the cells and the remaining procedure carried out. The entire transformation reaction was plated onto a prewarmed LB agar plate containing kanamycin (0.5 mg/mL). One colony was selected and used to inoculate a starter culture made up of 5 mL of LB broth and 5 µL of kanamycin (0.5 mg/mL). A large culture flask was prepared with 1 L of LB broth, 1000 µL of kanamycin (0.5 mg/mL), and 2 µL of the starter culture. This culture was allowed to grow at 37 °C until an absorption value of 0.526 at 595 nm was reached. The large culture was then induced with 0.238 g of IPTG and allowed to grow for an additional 3 hours at 37 °C. Using a JA-10 rotor, the sample was centrifuged at 6500 rpm for 30 minutes at 4°C. After the supernatatant was discarded by decanting, the cell pellet was stored at -80 °C.

One cell pellet was resuspended in 10 mL of 1X binding buffer consisting of 0.5 M NaCl, 20 mM Tris buffer, and 5 mM imidazole (pH=8). The cell suspension was sonicated on ice for 3 minutes total, cycling between 30 second intervals of sonication and rest. The cell lysate was centrifuged using a JA-20 rotor at 11,500 rpm for 30 minutes at 4 °C . Using a syringe filter, the supernatant was filtered and loaded onto an Ni-NTA affinity column equilibrated in binding buffer. To ensure proper binding of PrpC protein to the column, 20 mL of 1X binding buffer was loaded onto the column, followed by 12 mL of wash buffer (0.5 M NaCl, 20 mM Tris Buffer, and 60 mM imidazole, pH 8). To

elute, 12 mL of elution buffer (0.5 M NaCl, 20 mM Tris Buffer, and 200 mM imidiazole, pH 8) was loaded onto the column and seven 1 mL fractions were collected. After the fractions have been collected, 1 μL of Bradford reagent was added to 33 μL of each fraction to test for the presence of protein in the fractions. The fractions that contained protein indicated by the Bradford reagent changing from a reddish/brown color to a brilliant blue were then combined and dialyzed in 4 L of nanopure water and 12.14 g of Trizma Base, pH 8.5 overnight at 6 °C. After dialysis, 2.2 mL of solution was collected and protein aliquots were prepared containing 10% glycerol and stored at -80 °C. Using a Bradford Standard (BSA) method, the concentration of the collected protein was 0.7134 mg/mL with a total of 1.6 mg of protein being collected per 500 mL of total culture .

2.3.2 PrpD Protein Expression and Purification

After confirming a positive clone via sequencing data, 1 μL of the isolated pure plasmid DNA was used in the protocol Transforming BL21 Star™ (DE3) One Shot® Cells. Starter cultures were inoculated with the same protocol as previously mentioned, which were then shaken and grown overnight at 37 °C. The large 1L culture was prepared as stated previously. Several growth parameters were implicated. The large culture was allowed to grow at 37 °C until the optical density reached 0.3 and then the temperature was lowered to 18 °C until an absorption reading between 0.5 and 0.6 at 595 nm was reached. The large culture was then induced with 0.238 g of IPTG and allowed to grow overnight at 18 °C. Several parameters of growth conditions were altered, and the highest and purest concentration of PrpD protein was achieved when the large culture was grown at a constant 37 °C until its optical density at 595 nm reached 0.511, at which point

0.238g of IPTG was added and the culture was permitted to shake at 200 rpm and grow overnight. Using a JA-10 rotor, the sample was centrifuged at 6,500 rpm for 30 minutes at 4 °C. The supernatant was disposed of by decanting and the cell pellet was stored in the -80 °C.

The pellet was thawed and resuspended in 10 mL of 1X binding buffer. The sample was then sonicated on ice for a total of 5 minutes by alternating cycles of 30 seconds on and 30 seconds of rest. The lysate was then centrifuged using a JA-20 rotor at 11,500 rpm for 30 minutes. The supernatant was then syringe filtered using a 0.45 micron filter and loaded onto a 2 mL Ni-NTA Agarose column for purification. After the supernatant was allowed to pass through the column, 20 mL of 1X binding buffer was applied to the column followed by 12 mL of wash buffer, and approximately 10 mL of 200mM elution buffer. The fractions were collected 1 mL at a time and 2 of the 8 factions exhibited a brilliant blue color upon addition of the Bradford reagent. The two fractions were combined and dialyzed overnight using SnakeSkin™ Dialysis Tubing in a 4 L solution of 25 mM Tris-HCl buffer at a pH of 8.25 at 6 °C. Aliquots were prepared with 10% glycerol and stored at -80 °C until further analysis.

2.4 Activity Assays

2.4.1 Activity Assays with Purified 2-methylcitrate Synthase and 2-methylcitrate Dehydratase

To determine the stereochemical outcome of the gene product of PrpC and PrpD, High Performance Liquid Chromatography with UV detection (HPLC-UV) experiments were conducted with a 250 x 4.6 mm Synergi 4m Hydro-RP 80A column using a Varian

Prostar HPLC. *Analytical Liquid Chromatography - High performance liquid chromatography with UV detection (HPLC-UV)*. HPLC with UV detection was conducted on a Varian Prostar HPLC with a 250 x 4.6 mm Synergi 4m Hydro-RP 80A column (Phenomenex, Torrence, CA). The chromatography solvents were as follows: Solvent A: 20 mM sodium phosphate in water, pH 2.91 and Solvent B: methanol. A gradient elution was employed as follows: after injection, Solvent B (methanol) was increased from 0-15% over 20 minutes. All of the products eluted in this time, but unreacted acyl-CoA substrates were retained on the column and were eluted with a methanol flush involving a linear increase to 60% Solvent B from 20-25 minutes, held at 60% from 25-35 minutes, followed by a linear decrease to 0% Solvent B from 35-40 minutes, which was held for another 10 minutes at 100% Solvent A until the next injection. Prior to injecting the samples onto the HPLC column, they were quenched by the addition of 1 M sodium phosphate buffer at pH 2.9 (100 mL per 1 mL of sample). The addition of this phosphate buffer was critical to achieving consistent separation and peak-shape during the HPLC elution. The identity of reaction mixture constituents were assigned using retention times of standards of CoA, acetyl CoA, propionyl CoA, oxaloacetate, citric acid, *cis*-aconitate, *trans*-aconitate, succinate, pyruvate, and 2-methylcitric acid (a commercial mixture of diasteromers as described above). Unfortunately standards of 2-methylaconitate or 2-methylisocitrate were not commercially available.

The method for all activity assays contained the following chromatography solvents: Solvent A: 20 mM sodium phosphate, pH 2.91 and Solvent B: HPLC grade

methanol. Several methods and various buffers were tried in order to optimize HPLC conditions. Depicted in Table 1 are various reaction conditions that were implemented in order to obtain clear reproducible chromatography.

Table 1. Various Reaction Conditions Implemented to Analyze Enzyme Activity.

	First Condition	Second Condition	Third Condition	Fourth Condition
Reaction Buffer	200 mM Tris-HCl pH = 7.4	10 mM Tris-HCl pH = 7.4	20 mM Sodium Phosphate pH = 7.4	10 mM HEPES pH = 7.2
Final Propionyl-CoA Concentration	5 mM	10 mM	10 mM	10 mM
Final Oxaloacetate Concentration	20 mM	20 mM	20 mM	20 mM
Final 2-methylcitrate Concentration	5 mM	1 mM	1 mM	1 mM
Enzyme Volume	50 µL	50 µL	75 µL	75 µL
Reaction Quenching Solution	20 mM Sodium Phosphate pH = 2.95	20 mM Sodium Phosphate pH = 2.95	100 mM Sodium Phosphate pH = 2.95	1 M Sodium Phosphate pH = 2.95
Incubation Duration	3 Hours	Overnight	Overnight	Overnight

To investigate the activity of 2-methylcitrate synthase, PrpC, four separate reactions were carried out, a complete PrpC reaction, a complete PrpC reaction minus oxaloacetate, a complete PrpC reaction minus propionyl-CoA, and 1mM 2-methylcitrate standard. The substrates and buffers in the complete PrpC reaction were combined to reach the following final concentrations: 0.25 mM propionyl-CoA, 2 mM oxaloacetate, 10 mM HEPES buffer, and 75 µL of PrpC protein. Negative controls were carried out to ensure the observed peaks were not due to contaminants. The volume of propionyl-CoA

and oxaloacetate was replaced with 10 mM HEPES buffer in the PrpC reaction minus propionyl-CoA and PrpC reaction minus oxaloacetate respectively. Neither of these reactions displayed any new peaks between the 10-25 minute time range. The 2-methylcitrate standard was prepared by combining 50 μL of commercially provided 20 mM 2-methylcitrate to 950 μL of 10 mM HEPES buffer to obtain a final concentration of 1 mM 2-methylcitrate. All reactions, with the exception of the 2-methylcitrate standard, which was prepared and quenched just before injection, were incubated overnight and quenched using 1M sodium phosphate. The reactions were centrifuged at 13,000 rpm for 5 minutes before injection.

To further investigate the stereochemistry of the product of PrpC, also the substrate for PrpD in the subsequent step, a reaction with PrpD and the commercially obtained 2-methylcitrate as the only substrate was carried out.

2.4.2 "Cross-species" Activity Assays with MCC Enzymes from ECK12 and BS168

To compare the products of 2-methylcitrate synthase in ECK12 (PrpC) to the homologous enzyme in BS168 (MmgD), interspecies experiments were conducted with the subsequent enzymes in the MCC pathway, specifically PrpD from ECK12 and MmgE from BS168. The PrpC/MmgE reaction was prepared with the following final concentrations of enzymes, substrates, and buffer: 0.25 mM propionyl-CoA, 2 mM oxaloacetate, 10 mM HEPES buffer, and 75 μL of each enzyme. The reaction was incubated overnight, followed by the addition of 100 μL of 1M sodium phosphate buffer with a pH=2.9 and centrifugation for 5 minutes before injection into the HPLC. To provide further evidence of homology of these particular enzymes within the MCC

pathway, a 1mL reaction was prepared which contained 75µL of each enzyme (MmgD and PrpD), 25 µL of 10 mM propionyl-CoA, 100 µL of 20 mM oxaloacetate, and the remaining volume made up by 10 mM HEPES buffer with a pH= 7.2. After overnight incubation, the reaction was quenched with 100 µL of 1M sodium phosphate buffer (pH-2.9) and centrifuged for 5 minutes before analysis.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Electrophoresis Gel Results for Genes and Transformants

3.1.1 Electrophoresis Gel of ECK12 Genomic DNA with *prpC* Primers

Pictured in Figure 5 is an electrophoresis gel of the PCR product using the previously described *prpC* primers and isolated genomic DNA from ECK12. Due to the length of the gene sequence of *prpC* and *prpD*, the annealing period was extended from 45 to 75 seconds to optimize PCR settings. The gel displayed a strong band between 1.2 and 1.0 kilobasepairs, which correlated with the expected length of the *prpC* sequence length, 1.17 kilobasepairs.

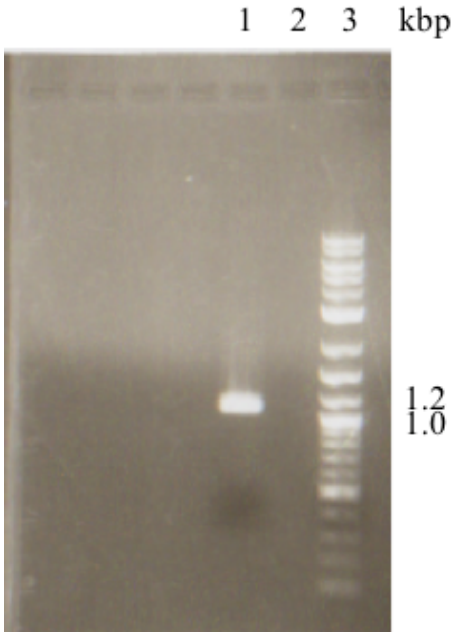


Figure 5. Electrophoresis Gel of *prpC* Gene Sequence Product in ECK12. Lane 1: PCR product with *prpC* forward and reverse primers; Lane 3: 2-Log DNA Ladder with corresponding lengths of the fragments in kilobasepairs.

3.1.2 Electrophoresis Gel of ECK12 Genomic DNA with *prpD* Primers

The gel depicted in Figure 6 shows a strong band at approximately 1.5 kilobasepairs, which correlated with the expected length of the *prpD* gene sequence, 1.452 kilobasepairs.

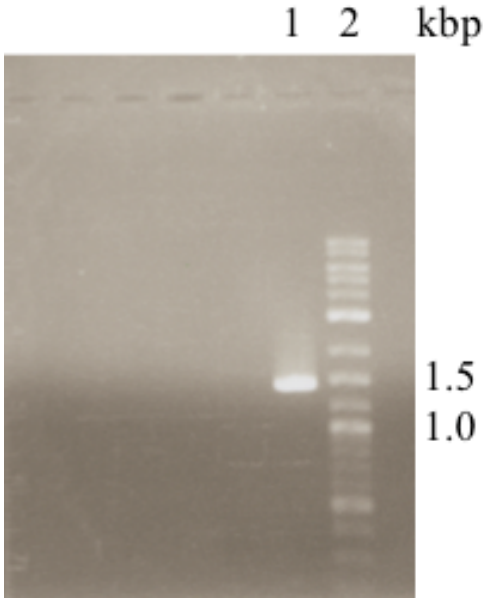


Figure 6. Electrophoresis Gel of *prpD* Gene Sequence Product in ECK12. Lane 1: PCR product with *prpD* forward/reverse primers; Lane 2: 2-Log DNA Ladder with corresponding lengths of fragments in kilobasepairs.

3.1.3 Cloning: Challenges and Solutions

The manufacturer's instructions provide a molar ratio of PCR product to TOPO vector for successful transformation. In order to quantify the yield of the *prpC* PCR product, intensities between the PCR product and the 2-Log DNA ladder were measured. A 1:1 molar ratio was achieved by measuring band intensities using gel camera software with comparison to a DNA ladder having known quantities of each standard.

Transformants were initially screened using the gene sequence primers, *prpC*/*prpD* forward and reverse. Due to the primers being non-specific to the gene ligated into the vector, the primers returned false positive results from a contaminating sequence or genomic DNA in the reactions. Introducing the T7 primer, in addition to one of the

gene primers, provided a more successful screening method. Illustrated in Figure 7, is a diagram of the pET-200 TOPO® vector that displays the location of the TOPO reaction and gene insertion, as well as the T7 primer binding locations.

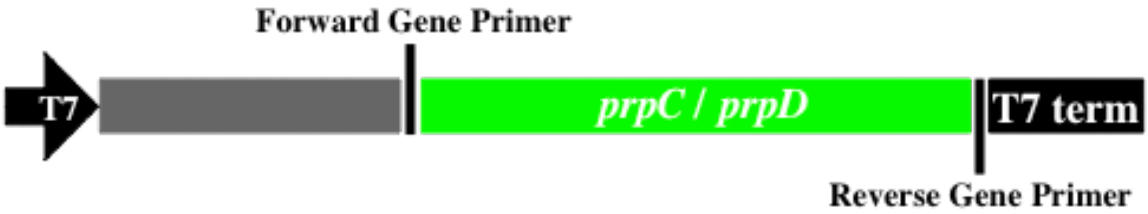


Figure 7. A Depiction of the pET200/D-TOPO Vector with *prpC* or *prpD* Gene Insertion. Note location of T7 and T7 terminal primers.[19]

The T7 reverse primer and *prpC* forward primer were used to detect successful gene insertion into the plasmid, which is displayed in Figure 8. In addition, Figure 9 displays the electrophoresis gel obtained using T7 forward and *prpC* reverse primers were used to revalidate the results obtained in Figure 8. The electrophoresis gel depicted in Figure 9 confirms a successful insertion of the gene *prpC* into the vector containing antibiotic resistance, pET-200 plasmid. The proper insertion of the gene into the plasmid was confirmed by sequencing data obtained from Eurofins Genomics.

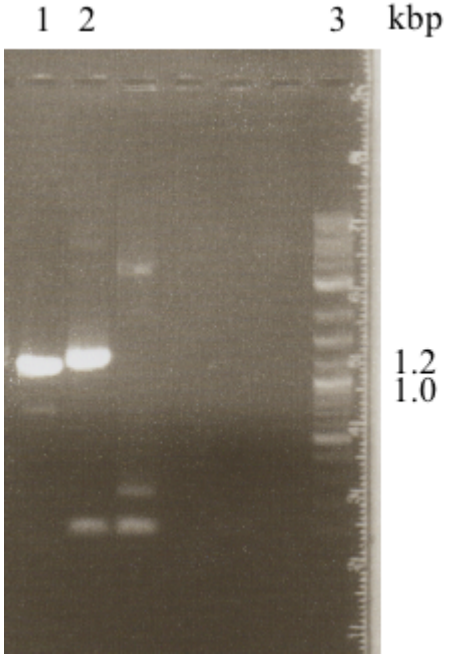


Figure 8. Electrophoresis Gel of *prpC* Transformant. Lane 1: PCR product with *prpC* forward/reverse primers and isolated genomic DNA; Lane 2: *prpC* transformant with *prpC* forward and T7 reverse primers; Lane 3: 2-Log DNA Ladder with corresponding lengths of fragments.

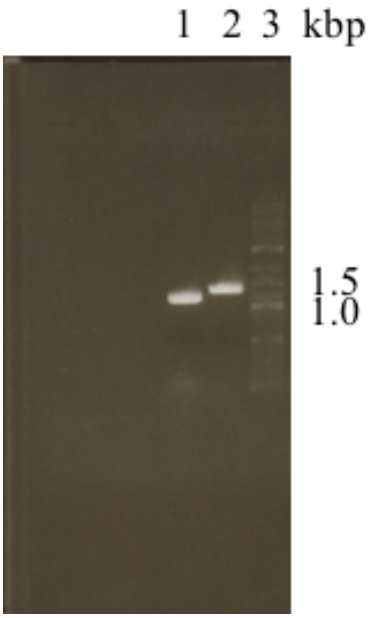


Figure 9. Electrophoresis Gel of *prpC* Transformant Screened with Forward T7 and Reverse Gene Primer. Lane 1: PCR product with *prpC* forward/reverse primers and isolated genomic DNA; Lane 2: *prpC* transformant with *prpC* reverse and T7 forward primers; Lane 3: 2-Log DNA Ladder with corresponding lengths of fragments in kilobasepairs.

To determine successful ligation, PCR products were obtained using T7 forward and *prpD* reverse primers on the purified plasmid DNA shown in Figure 10. Purified plasmid samples 1, 2, and 4 exhibited positive results for the desired clone using the plasmid specific primers and were then sequenced. Based on the sequencing results generated by Eurofins Genomics, it was confirmed that only purified plasmid sample # 4 contained the *prpD* gene sequence, in addition to the His-tag and proper frame configuration.

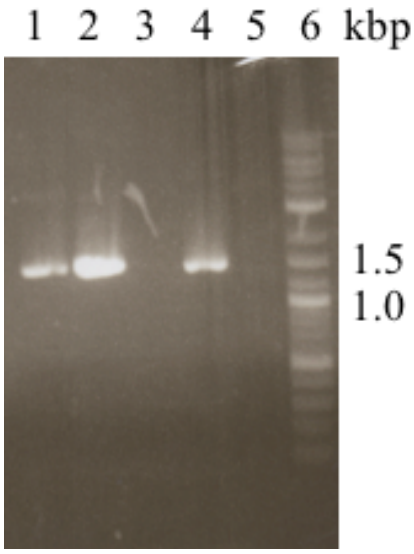
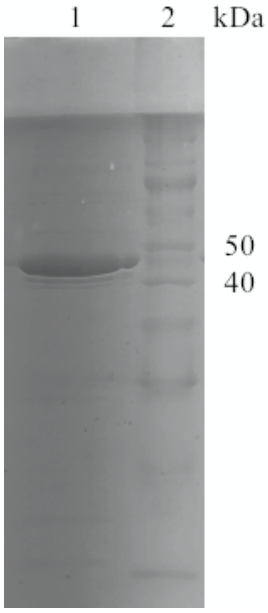


Figure 10. Electrophoresis Gel of *prpD* Transformant with T7 Forward and *prpD* Reverse Primers. Lane 1: Purified plasmid # 1 PCR product with T7 forward and *prpD* reverse primers; Lane 2: Purified plasmid # 2 PCR product with T7 forward and *prpD* reverse primers; Lane 4: Purified plasmid # 4 PCR product with T7 forward and *prpD* reverse primers; Lane 6: 2-Log DNA Ladder with corresponding kilobasepairs.

3.2 Protein Expression and Purification Optimization

In order to optimize large culture growth conditions, the temperature, quantity of IPTG, and duration of growth were all varied. The most successful PrpC and PrpD cultures were induced with 1 mM IPTG when the absorbance value rose between 0.5 to 0.6 range at 595 nm and the culture was allowed to grow overnight. PrpD did not exhibit any post column precipitation. In contrast, PrpC exhibited precipitation after purification using Ni-NTA affinity chromatography. This obstacle was overcome by altering the elution buffer from 1000 mM to 200 mM imidazole. Some precipitation was still

observed, but enough protein stayed in solution to be used in further analysis. To verify the identity and purity of the protein, an SDS-PAGE gel was conducted and pictured in Figure 11. The gel exhibits an intense band between the 50 and 40 kDa fragment in the protein ladder, which correlates with the expected protein size of PrpC, 43.102 kDa.



Firgure 11. 10 % Tris-glycine SDS-PAGE Gel of PrpC Protein. Lane 1: 20 µL of the sample containing: 50 µL of protein and 50 µL of Laemmli Sample Buffer with the corresponding molecular weights of the protein ladder; Lane 2: Unstained Protein Ladder, Broad Range (10-250 kDa).

The image shown in Figure 12, displays an intense band at the expected PrpD mass of 54.0 kDa.

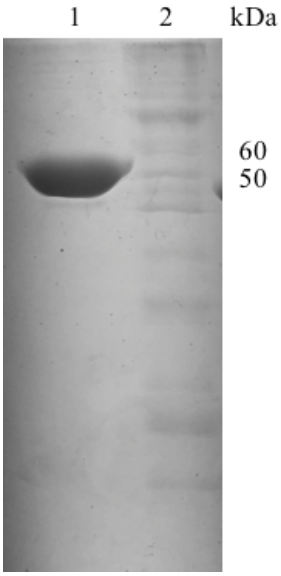


Figure 12. 10 % Tris-glycine SDS-PAGE Gel of PrpD Protein. Lane 1: 20 µL of the sample containing: 15 µL of protein and 85 µL of Laemmli Sample Buffer; Lane 2: Unstained Protein Ladder, Broad Range (10-250 kDa) with the corresponding molecular weights of the protein ladder.

3.3 Activity Reconstitution

Several HPLC reaction modifications played an integral role in obtaining successful assay results, such as extending the incubation period, increasing the volume of the enzyme used in the reaction, and changing the reaction buffer from Tris-HCl buffer to 10 mM HEPES buffer. The Tris-HCl buffer was found to react with oxaloacetate, which interfered with the production of the stereoisomers of interest.

Although the activity of PrpC and PrpD have been previously investigated, recent work with homologous enzymes in *B. subtilis* have revealed stereochemical discrepancies between the products catalyzed by these similar enzymes that share an amino acid

sequence and overall similarity. Previous results obtained in the Reddick laboratory characterized the activity of enzymes encoded within the *mmg* operon in *B. subtilis*. Specifically, MmgD demonstrated 2-methylcitrate synthase activity by producing (2*S*, 3*R*)-2-methylcitrate or (2*R*, 3*S*)-2-methylcitrate. MmgE exhibited dehydratase activity using one of the heterochiral stereoisomers to produce (*Z*)-2-methylaconitate. The same methodology used with the *B. subtilis* enzymes was applied to the *E. coli* enzymes to further explore the reaction products of 2-methylcitrate synthase and dehydratase.

Although HPLC is unable to distinguish between enantiomers, diastereomers exhibit different elution times. Results from the full PrpC reaction demonstrate that the product is one of the heterochiral (2*S*, 3*R*) or (2*R*, 3*S*)-2-methylcitrate diastereomers. Furthermore, there is no observable product peak at the elution time of the homochiral 2-methylcitrate products. These results are shown in Figure 13B. The injection of commercial 2-methylcitrate showed two major peaks at 12.4 and 14.7 minutes, which correlates with the precedent set forth in the literature and the Sigma-Aldrich stereochemical assignment with the heterochiral products, (2*R*, 3*S*)/(2*S*, 3*R*) set of diasteromers eluting at 12.4 minutes and the homochiral products, (2*R*, 3*R*)/(2*S*, 3*S*) set of diasteromers eluting at 14.7 minutes, as illustrated in Figure 13A. The full PrpC reaction exhibited only one new product peak at 12.5 minutes, therefore, PrpC is only producing one of the (2*R*, 3*S*)/(2*S*, 3*R*) diasteromers in these reaction conditions, as shown in Figure 13B.

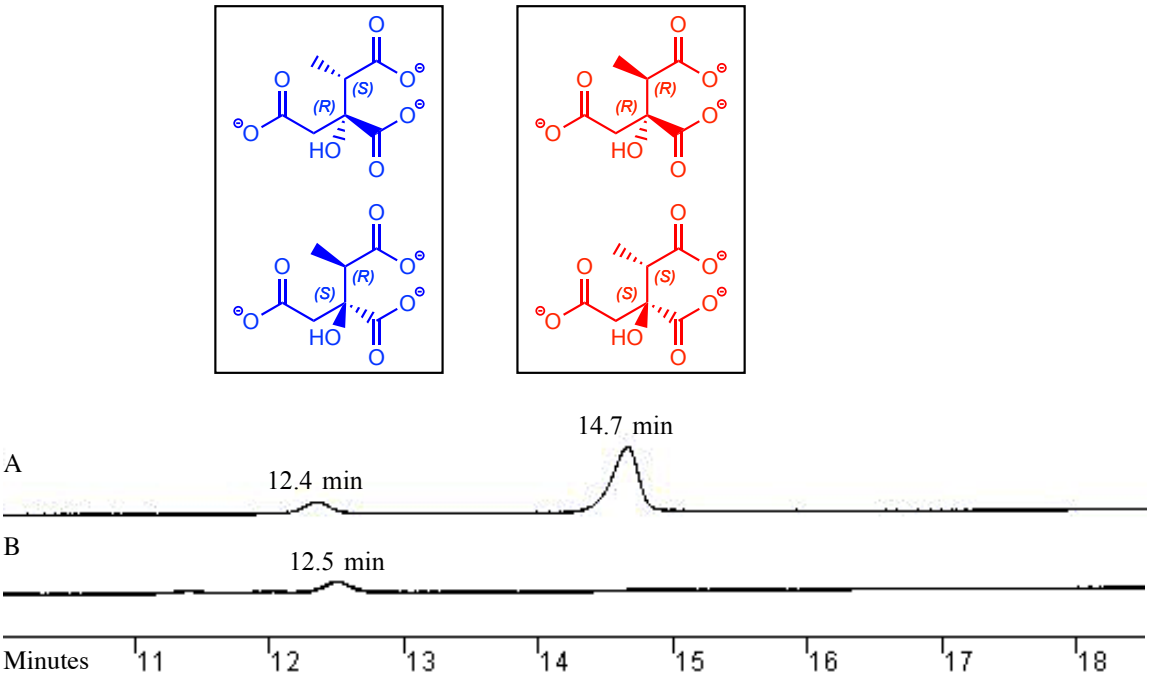


Figure 13. HPLC Chromatograms of 2-Methylcitrate Standard and PrpC. A: The commercially available 2-methylcitrate standard in a racemic mixture of 4 enantiomers, eluting as the heterochiral pair, (2*R*, 3*S*)/(2*S*, 3*R*), and the homochiral pair, (2*R*, 3*R*)/(2*S*, 3*S*). **B:** The chromatogram of the complete PrpC reaction with the substrates propionyl-CoA and oxaloacetate showing a new product peak eluting at 12.5 minutes.

Experiments that contained the 2-methylcitrate racemate and PrpD were conducted to further validate the product of PrpC. The chromatogram produced from the reaction with PrpD and the commercial 2-methylcitrate racemate is shown in Figure 14B. To test the hypothesis that PrpC was only producing one of the heterochiral diastereomers, the full PrpC reaction, in which the only substrates included were propionyl-CoA and oaxaloacetate as mentioned previously, was combined with PrpD and

the activity was measured. There was only one observable new product peak at 15.0 minutes, which corresponded with (*Z*)-2-methylaconitate.

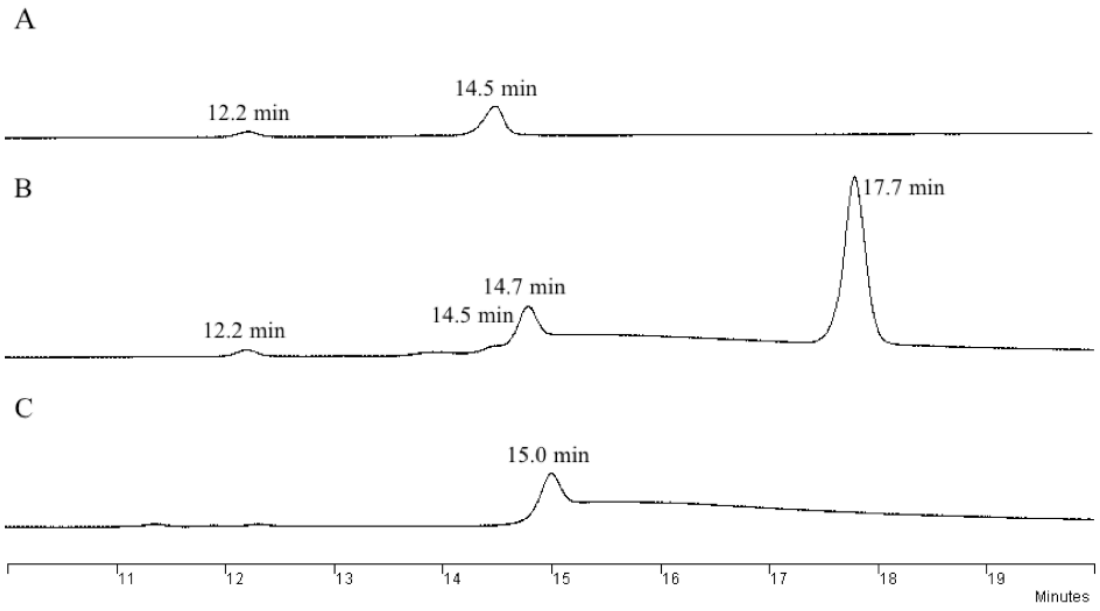


Figure 14. HPLC Chromatograms of 2-Methylcitrate, PrpC, and PrpD. A: The chromatogram of the commercially available 2-methylcitrate with elution times similar to those illustrated in Figure 13A. **B:** The chromatogram of the full PrpD reaction with the commercial 2-methylcitrate (2 sets of diastereomer pairs) as the only substrate source. The active peaks at 12.2 and 14.5 minutes indicate unreacted starting material. The new product peaks at 14.7 and 17.7 minutes correspond to the (*Z*)-2-methylaconitate and (*E*)-2-methylaconitate respectively. **C:** Chromatogram of full PrpC and PrpD reaction with propionyl-CoA and oxalacetate only as the substrates displaying one new product peak at 15.0 minutes.

Based on the chromatogram in Figure 14B, PrpD is not only capable of producing the (*Z*)-2-methylaconitate product, it can also produce the (*E*)-2-methylaconitate stereoisomer when other stereoisomers of 2-methylcitrate are available. This data points to the conclusion that PrpD does not possess rigid stereoselectivity in regards to substrates.

The chromatogram of a full PrpC and PrpD reaction is shown in Figure 14C, which displays a single product peak at the 15.0 minute elution time. This product peak coincides with (*Z*)-2-methylaconitate, which eluted at 14.7 minutes in a previous reaction containing commercially available 2-methylcitrate and PrpD. This data supports the notion that PrpC is only producing one of the stereoisomers; otherwise PrpD would be able to produce both (*Z*)-2-methylaconitate and (*E*)-2-methylaconitate if multiple substrate stereoisomers were supplied by PrpC, as seen in Figure 14B.

The cross-species experiments demonstrated the compatibility of homologous enzymes involved in the MCC. HPLC chromatograms displaying a 10-20 minute elution time scale are shown in Figure 15A and 15B and both illustrate a single product peak at 14.9 minutes, which correlates with the (*Z*)-2-methylaconitate stereoisomer. There were no additional product peaks, which would have been observed if PrpC had the capability of producing more than one 2-methylcitrate stereoisomer. The results obtained from these cross-species experiments further support the notion that PrpC from ECK12 and MmgD from BS168 are homologous in functionality and produce the same 2-methylcitrate stereoisomer, otherwise they would not demonstrate compatibility with the other species' 2-methylcitrate dehydratase (PrpD/ MmgE). The results of these cross-species

experiment using MmgD from BS168 and PrpD from ESK12 are illustrated in Figure 15B.

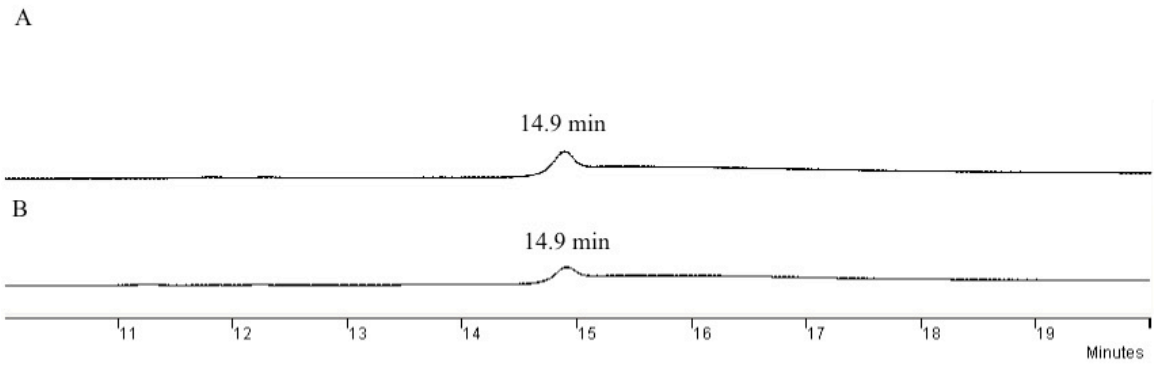


Figure 15. HPLC Chromatograms of "Cross-Species" Reactions with ECK12 and BS168 Enzymes. A: HPLC chromatogram of a "cross-species" reaction containing PrpC from *E. coli* and MmgE from *B. subtilis* displaying a single active peak at 14.9 minutes. **B:** The chromatogram of the "cross-species" reaction containing MmgD from *B. subtilis* and PrpD from *E. coli* with an active peak at 14.9 minutes.

3.4 Conclusion

Due to the lax specificity PrpD and MmgE exhibit, these two enzymes possess the ability to catalyze at least two stereoisomers of 2-methylcitrate. Therefore, if either of the 2-methylcitrate synthases (PrpC/MmgD) were producing a homochiral (2*SR*, 3*SR*)-2-methylcitrate product, a product peak would be observed where (*E*)-2-methylaconitate elutes, approximately 17.7 minutes. Based on this reasoning, MmgD nor PrpC are producing either version of the homochiral products. The possible reaction product of MmgD and PrpC can now be narrowed to either (2*S*, 3*R*) or (2*R*, 3*S*)-2-methylcitrate.

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