12-2017

Sub-cloning Genes of the Mevalonate Pathway from Enterococcus faecalis into pDUET and pET28

Madison Anne Harris
Abilene Christian University

Sub-cloning Genes of the Mevalonate Pathway from *Enterococcus faecalis* into pDUET and pET28

An Honors College Project Thesis

Presented to

The Department of Chemistry and Biochemistry

Abilene Christian University

In Partial Fulfillment

of the Requirements for

Honors Scholar

by

Madison Anne Harris

December 2017

This Project Thesis, directed and approved by the candidate's committee, has been accepted by the Honors College of Abilene Christian University in partial fulfillment of the requirements for the distinction

HONORS SCHOLAR

Dr. Jason Morris, Dean of the Honors College

Nov 17, 2017

Date

Advisory Committee

Dr. Autumn Sutherlin, Committee Chair

Dr. Sarah Lee, Committee Member

Dr. Qiang Xu, Committee Member

Dr. Kim Pamplin, Department Head

ABSTRACT

All organisms are capable of synthesizing isopentenyl pyrophosphate (IPP), a precursor to many different biomolecules such as ubiquinone Q8, cholesterol, and β-carotene. Two pathways, the mevalonate and non-mevalonate pathway, synthesize IPP. Unlike most eubacteria that use the non-mevalonate pathway, low-G+C gram-positive cocci bacteria solely use the mevalonate pathway. This pathway differs from mammals at the rate-limiting step of HMG-CoA reductase and is a potential target for future antibiotics against nosocomial infections caused by *Enterococcus faecalis*. Unique to enterococci is a fusion protein (encoded by *mvaE*) made of the first enzyme (acetoacetyl-CoA thiolase) and the third enzyme (3-hydroxy-3-methylglutaryl-CoA reductase) of the pathway. This research focuses on the first three enzymes of the pathway and relies on a series of sub-cloning. First, to isolate thiolase from the fusion protein, sub-cloning *mvaC*, the gene for thiolase, into pET28 was attempted. Secondly, sub-cloning of the gene for the fusion protein (*mvaE)* and gene for HMG-CoA synthase (*mvaS)*, which is the second enzyme in the pathway, into pDUET was attempted. Each sub-cloning reaction employed a double restriction digest, gel electrophoresis, DNA purification, ligation of insert and vector, transformation, and mini-preparation of plasmid DNA. Despite repeated attempts and troubleshooting, results were inconclusive. This research has led to the refining of the sub-cloning protocol using the pDUET vector, and future research is needed to understand more about the fusion protein in *Enterococcus faecalis.*

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INTRODUCTION

Enterococcus faecalis is a gram-positive bacterium that causes nosocomial infections and is problematic as some strains are antibiotic resistant (1). New methods to target *E. faecalis* are needed. One possible target is the bacteria's biosynthesis of isopentenyl diphosphate (IPP), a five-carbon precursor to many different molecules such as steroids, cholesterol, and coenzyme Q (1, 2).

IPP is synthesized in one of two ways, the mevalonate pathway (Fig. 1) or the non-mevalonate pathway (1, 2). Unlike most bacteria, which synthesize IPP by the non-mevalonate pathway, low-G+C gram-positive cocci such as the Enterococcus species, Staphylococcus species, and Streptococcus species synthesize IPP by the mevalonate pathway (2). This is similar to eukaryotes, archaea, and fungi (2). Previous studies comparing DNA sequences suggest that low-G+C gram-positive cocci acquired the mevalonate pathway through horizontal gene transfer with primitive eukaryotes (2).

The mevalonate pathway begins with acetyl-CoA and produces IPP after several enzymatic reactions. The first three enzymes of the pathway, acetoacetyl-CoA thiolase, 3-hydroxy-3-methylglutyral-CoA (HMG-CoA) synthase, and HMG-CoA reductase are of particular interest. The rate-limiting step for this pathway is at HMG-CoA reductase. Previous studies have determined two different classes of HMG-CoA reductase, based on the kinetic properties and structure of the enzyme (2). While eukaryotes employ class I HMG-CoA reductase, *E. faecalis*, *S. aureus*, and *S. pneumonia* all use class II. Class I HMG-CoA reductase is more sensitive to statin drugs than class II reductase (2). This is a key point in developing a new antibiotic that can

target HMG-CoA reductase class II in gram-positive cocci and not target HMG-CoA class I in mammals.

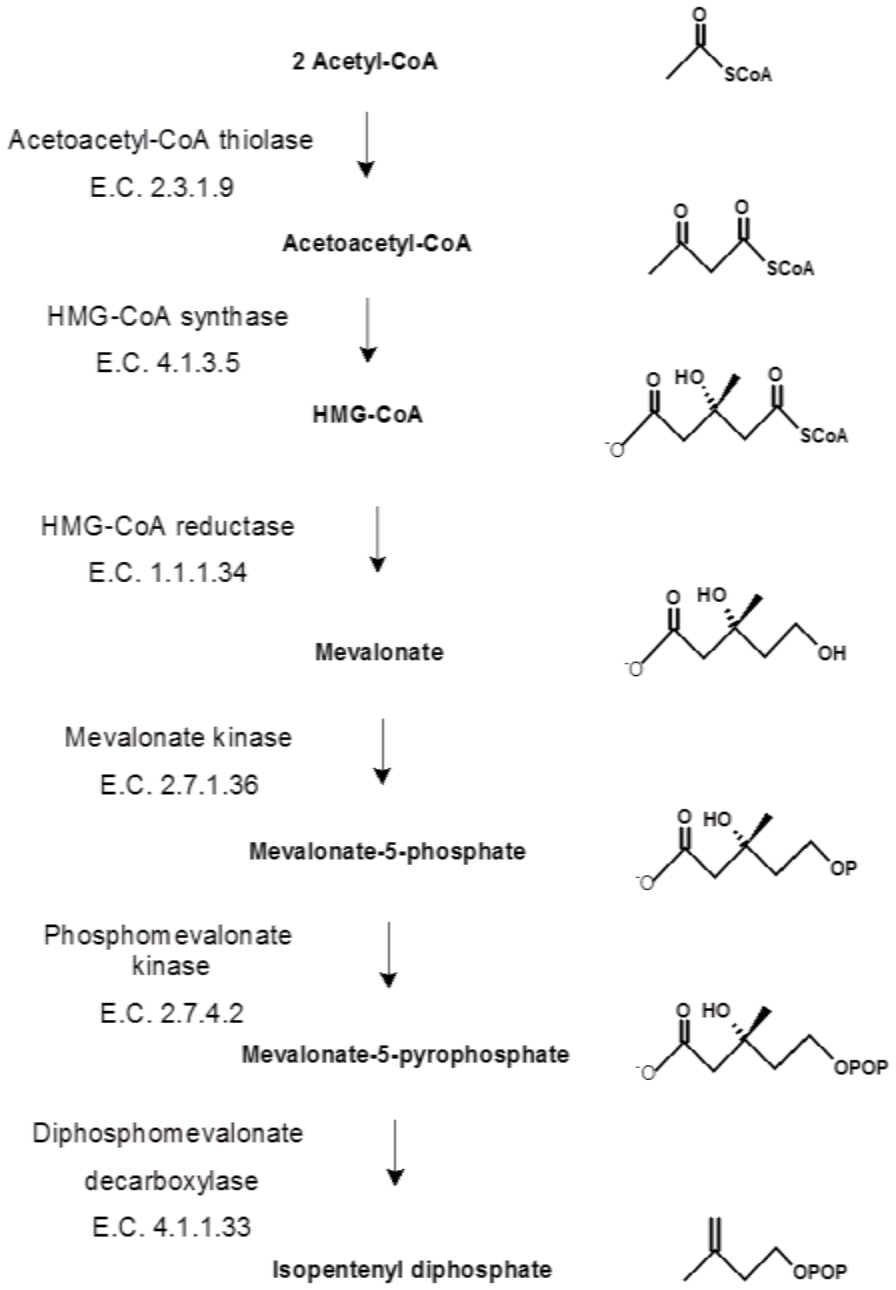

Figure 1: The Mevalonate Pathway (4).

Importantly, the mevalonate pathway is essential for gram-positive cocci bacteria to live, as determined in a gene-knockout study with *S. pneumonia* (2). Bactericidal effects were observed when the gene for HMG-CoA reductase (*mvaA*) was knocked out and a supply of mevalonate was not provided. *E. faecalis* is inferred to behave in the same manner because it is a gram-positive cocci bacterium and solely uses the mevalonate pathway (2). Combining the fact that the mevalonate pathway is believed to be essential to *E. faecalis* and differs from that in mammals, it is a potential target for new antibiotics.

Unique to Enterococci is a fusion protein comprising the first and third enzymes of its mevalonate pathway, thiolase (encoded by *mvaC*) and reductase (encoded by *mvaA*) (2). The two enzymes are encoded on the same RNA transcript, with one start codon and one stop codon. Their transcript is translated into one large fusion protein, encoded by *mvaE* (Fig. 2). Little is known about the fusion protein, so it is of interest to research. Previously, reductase was isolated from *Enterococcus faecalis* and it was determined to be a dimer (4). However, isolated reductase presented different kinetic properties than when part of the fusion protein (4). Thiolase has yet to be isolated from the fusion protein, and the proper folding of the fused protein in *E. faecalis* is of interest to research.

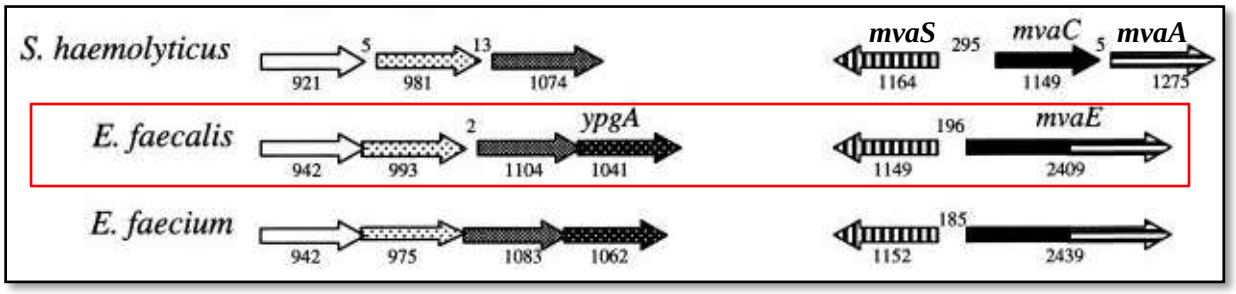

Figure 2. Depiction of the fusion protein in *Enterococcus faecalis* that is encoded by the gene *mvaE*. *mvaE* contains the genes for thiolase (*mvaC)* and reductase (*mvaA)*. The gene that encodes synthase is *mvaS* (2).

To investigate the fusion protein in *E. faecalis*, this research attempts to isolate thiolase and sub-clone *mvaC* into pET28 (Fig. 3). Additionally, HMG-CoA synthase, the second enzyme of the mevalonate pathway and not part of the fusion protein is utilized to learn more about the fusion protein. By expressing synthase (encoded by *mvaS)* along with the fusion protein, there is hope that synthase will help the fusion protein fold correctly. Thus, this research attempts to sub-clone *mvaE* and *mvaS* into a single plasmid, pDUET (Fig. 4).

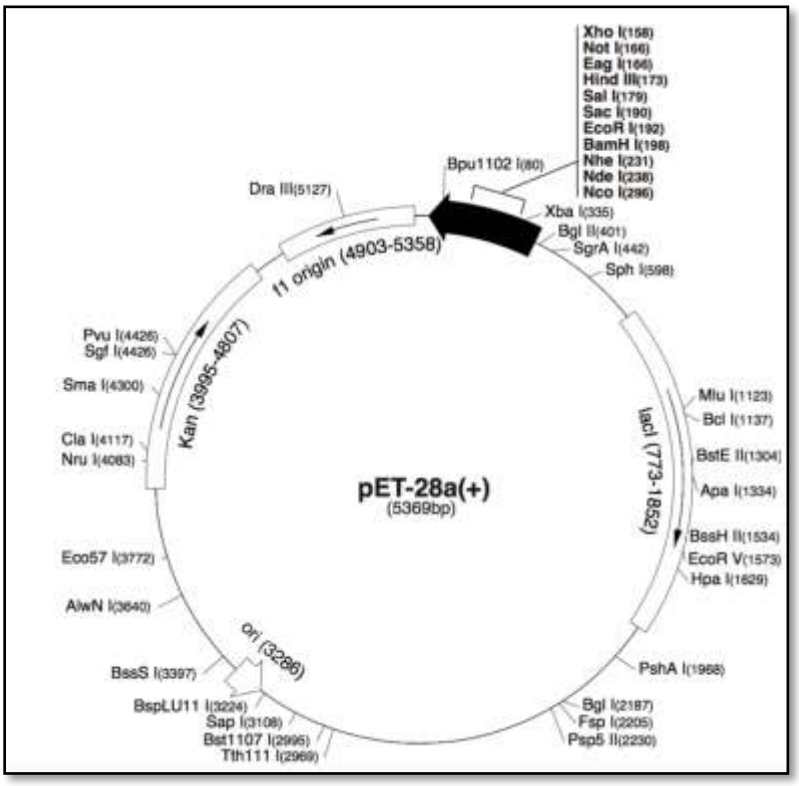

Figure 3. Plasmid map for pET28. To cut the gene encoding the fusion protein *(mvaE)*, restriction enzymes Nco I and BamHI were used, and to cut the gene encoding only thiolase (*mvaC)*, restriction enzymes Nco I and Nde I were used. To cut the gene encoding synthase (*mvaS)* the restriction enzymes Nde I and Xho I were used (New England Biolabs Inc.).

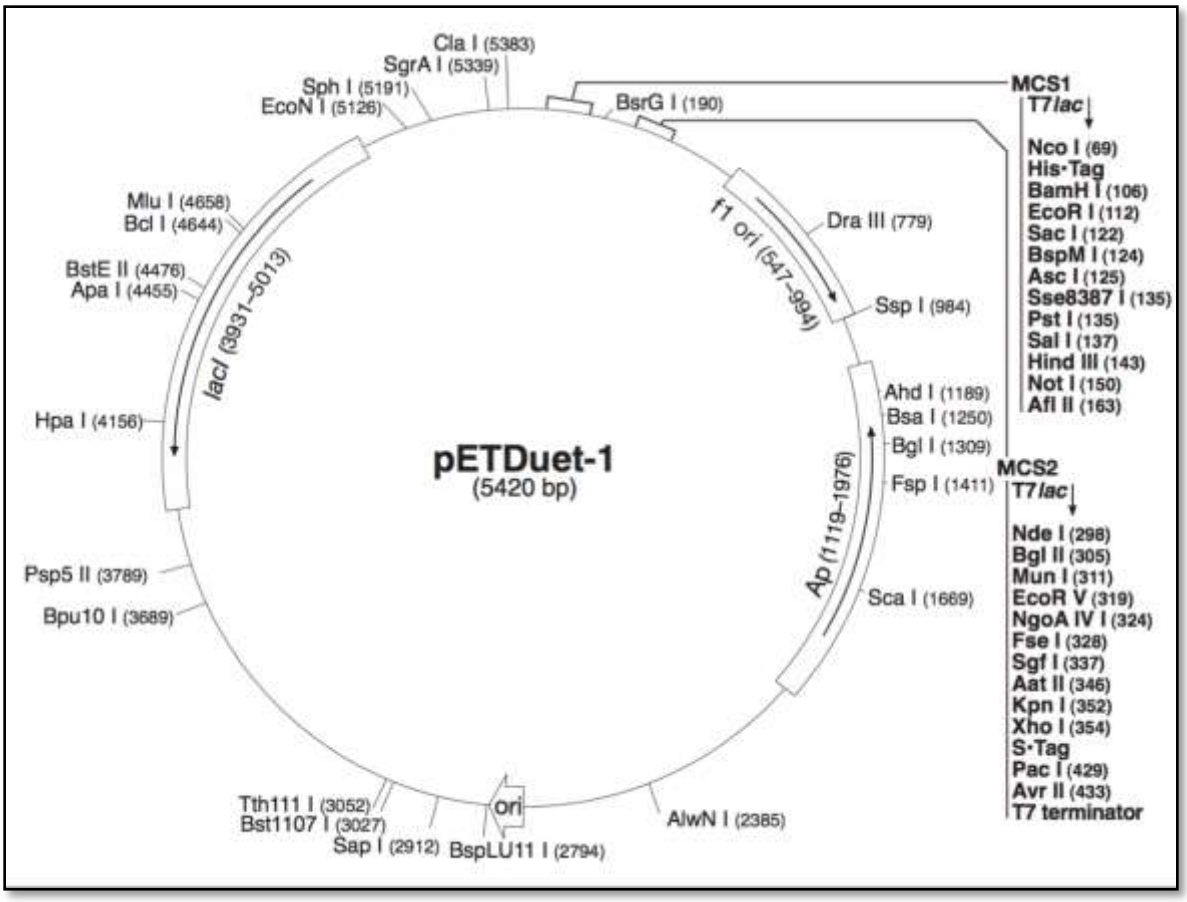

Figure 4. The plasmid map for pDUET used to sub-clone both *mvaE* and *mvaS* (New England Biolabs Inc.).

MATERIALS AND METHODS

Materials. Vectors used include pDUET and pET28 (New England Biolabs Inc.). *E. coli* DH5 alpha competent cells (New England Biolabs Inc.) were used. Enzymes used include Nco I, Nde I, Bam HI, Xho I, T4 bacteriophage ligase, RNAase (New England Biolabs Inc). Buffers used include buffer 3.1, CutSmart buffer, 6x loading dye, and ligase buffer (New England Biolabs Inc.), as well as freshly prepared 0.5 X TBE (Sigma-Aldrich). Other material included the Sigma GenElute Gel Extraction kit (Sigma-Aldrich), DNA ladder (GoldBio), Luria-Bertani media (Fischer), agar (Fischer), and 1% ethidium bromide (Fischer-BioReagents).

Double Restriction Digest. For *mvaE*, buffer 3.1 and restriction enzymes Nco I and Bam HI were used to cut pDUET and pET28 with *mvaE.* Restriction enzymes Nde I and Xho I were used to cut pDUET and pET28 with *mvaS,* and the cutsmart buffer was used. To cut pET28 and *mvaC* from the fusion protein, Nde I, Nco I, and buffer 3.1 were used. The total volume of each double restriction digest was 50 µL. Each double restriction digest incubated at 37 ˚C from 15 minutes to 4 hours. Typical run time was 1 hour (9).

Gel Electrophoresis. Gel electrophoresis was used to obtain the desired fragments of cut DNA following the restriction digest. A 0.7 % agarose gel was used with 0.5x TBE buffer and a 1 % solution of ethidium bromide. Loading dye was added to each sample to a final concentration of 1X. Gel wells were individually loaded with the DNA ladder and the maximum amount of sample. Electrophoresis ran typically at 100 volts for 1 hour. Bands were viewed with UV light. Desired DNA fragments were excised (10).

DNA Purification. Excised bands were weighed and then purified from the gel with the Sigma GenElute Gel Extraction kit. The kit provided the Column Preparation Solution, Gel Solubilization Solution, Wash Solution Concentrate G, Elution Solution, GenElute binding columns, and collection tubes for the experiment. The procedure was followed exactly as described by the kit (5).

Phenol/Chloroform Extraction. If gel electrophoresis was omitted for the vectors, a phenol chloroform extraction was used to remove the protein from the nucleic acids. The protocol for this was obtained from the Molecular Cloning Laboratory Manual (8). ACS grade Chloroform was used, as well as a 1:1 ratio of Phenol:Chloroform in TB. The aqueous layer was removed three total times.

Dephosphorylation. Dephosphorylation of cut pDUET was carried out following the procedure from the Molecular Cloning Laboratory Manual (7). Calf intestinal alkaline phosphatase (CIP) was used to remove the 5' phosphate group along with a 10x CIP buffer, and incubated for 30 minutes at 37 ˚C.

Ligation. The insert (*mvaS, mvaE,* or *mvaC*) and corresponding vector (pDUET or pET28) were ligated together. The quantity of vector and insert used was determined by the concentration and the New England Biolabs online generator for 2:1, 3:1, and 5:1 ratios of insert:vector. T4 DNA bacteriophage ligase and ligase buffer were used. Ligation conditions included incubation at room temperature for four hours, 37 ˚C for one hour, or stored in the refrigerator overnight. The most frequent ligation condition was at 37 ˚C for one hour (11).

Transformation. *E. coli* DH5 alpha competent cells were transformed with ligation samples, and were plated onto Luria-Bertani (LB) media with the necessary antibiotic. Ampicillin plates (50 μg/mL) were used for experiments with pDUET, and kanamycin plates (10 μg/mL) were used for pET28. Incubated overnight (about 17 hours) at 37 ˚C.

Isolation of ligated plasmid. Transformed colonies that grew overnight were stabbed at random and grown in liquid LB media with the appropriate antibiotic. Three to five colonies from each plate were stabbed to account for mutations. The colonies were grown overnight with shaking at 37^{o}C in LB with 50 μg/mL of ampicillin for pDUET, and 10 μg/mL of kanamycin for pET28.

Mini-preparation of DNA. After incubating at 37 ˚C overnight, DNA for each sample was mini-prepped to remove the DNA from the competent *E. coli* cells. Protocol for the small-scale preparation of plasmid DNA was obtained from the Molecular Cloning Laboratory Manual (6). Concentrations to make solutions 1-3 were provided in the lab procedure, with solution 2 prepared fresh for each mini-preparation. The final DNA was redissolved in a TE buffer of pH 8.0, and RNAase (0.5 mg/ml) was added to remove any RNA from the sample.

Nanodrop. To check the DNA concentration after a mini-preparation as well as a purification of DNA, the Nanodrop was used. DNA was noted by a significant peak at 260 nm and was differentiated from protein by looking at the 260:280 ratio. A peak at any other wavelength indicated a contamination.

RESULTS

Genes cut from pET28. Each gene was successfully cut out of the plasmid pET28 (Fig. 3). Restriction enzymes Nco I and Bam HI successfully cut *mvaE* from pET28. The 2,500 base pair *mvaE* was seen on the gel. Nde I and Nco I successfully cut *mvaC*, about 1,500 base pairs, and Nde I and Xho I successfully cut *mvaS*, about 1,100 base pairs (Fig. 5).

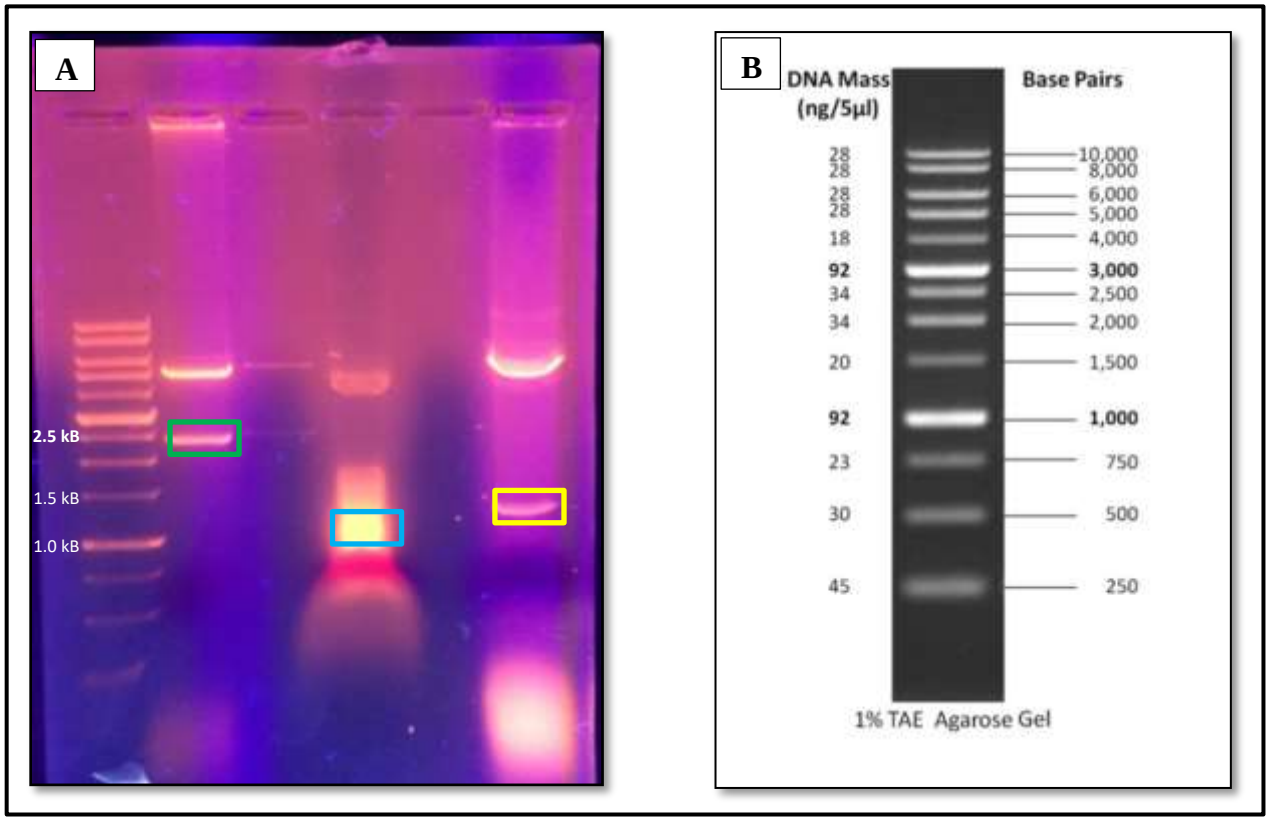

Figure 5. A) Double Restriction Digest to cut inserts from pET28. The green box highlights *mvaE*; the blue box highlights *mvaS*, and the yellow box highlights *mvaC*. B) DNA ladder used to identify to correct DNA fragment by looking at base pair length (12). The two brightest bands are 3,000 bps and 1,000 bps.

The restriction digest was typically run for an hour at 37 ˚C. A short (15 minutes) and long (1 hour) restriction digest were originally attempted, with the short digest cutting the insert equally as well as the long digest. Thereafter, Nco I began to have problems cutting the DNA, so new enzyme was ordered from New England Biolabs Inc. Each subsequent restriction digest ran for an hour and successfully cut the appropriate gene from pET28 as described above.

Vectors pDUET and pET28. Unlike the successful restriction digests with each gene, cutting plain plasmid, pDUET and pET28, were not always cut with both enzymes. Sometimes the restriction digest with the two appropriate enzymes for pDUET and pET28 was successful. However, this was not consistent.

Sub-cloning *mvaC* into pET28. To isolate thiolase from the fusion protein, *mvaE* was cut with Nco I (at the beginning of the sequence) and Nde I, which was in the middle of the sequence (Fig. 6). As previously mentioned, *mvaC* was successfully cut from the fusion protein. pET28 appeared to be cut by the restriction enzymes; however, they have not been successfully ligated together.

The results of the ligation of *mvaC* with cut pET28 showed about 5-10 colonies for each insert:vector ratio, 2:1, 3:1, and 5:1 (Fig. 7). Some colonies grew on the control plate, although fewer than the experimental plates. After growing the colonies overnight, mini-prepping the DNA, and running a diagnostic restriction digest, it was determined that the colonies that grew did not contain the *mvaC* insert (Fig. 8).

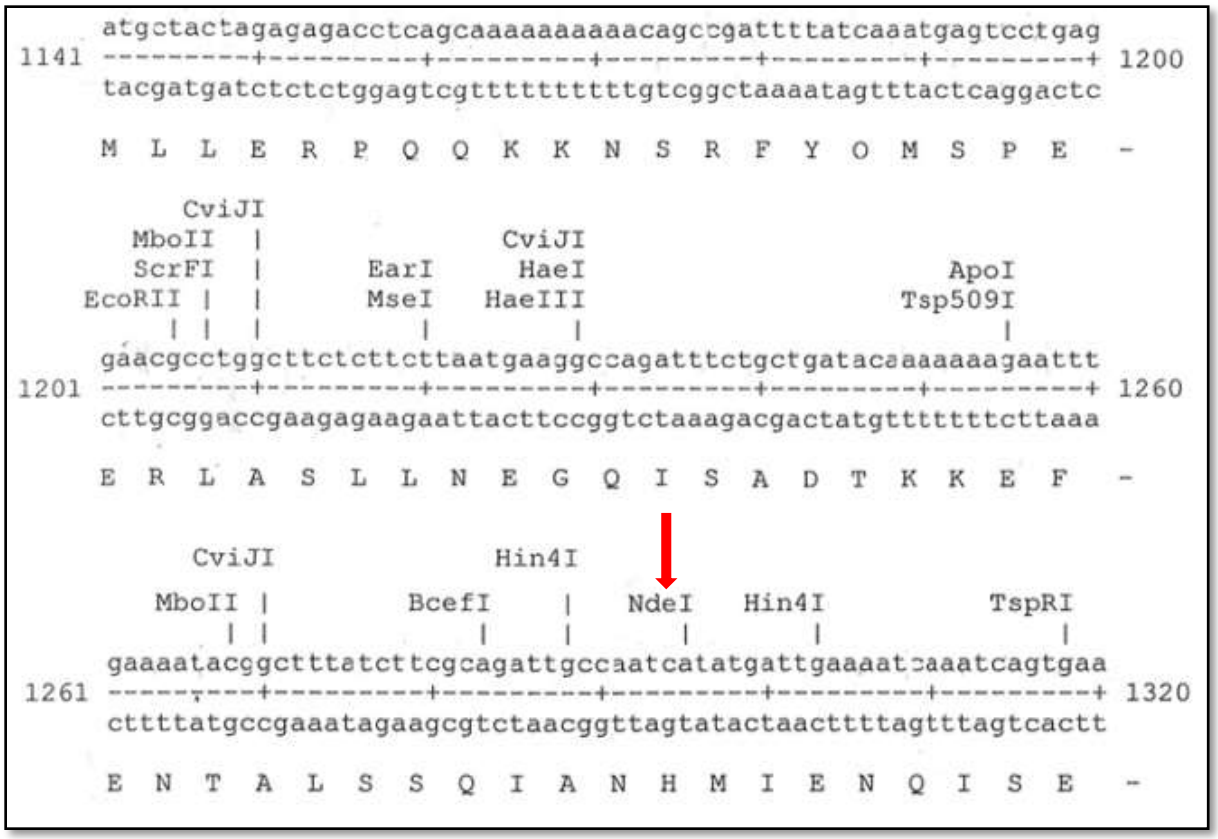

Figure 6. A part of the DNA sequence for *mvaE*. The red arrow points to the cut site half way through the fusion protein that was utilized to isolate the thiolase portion.

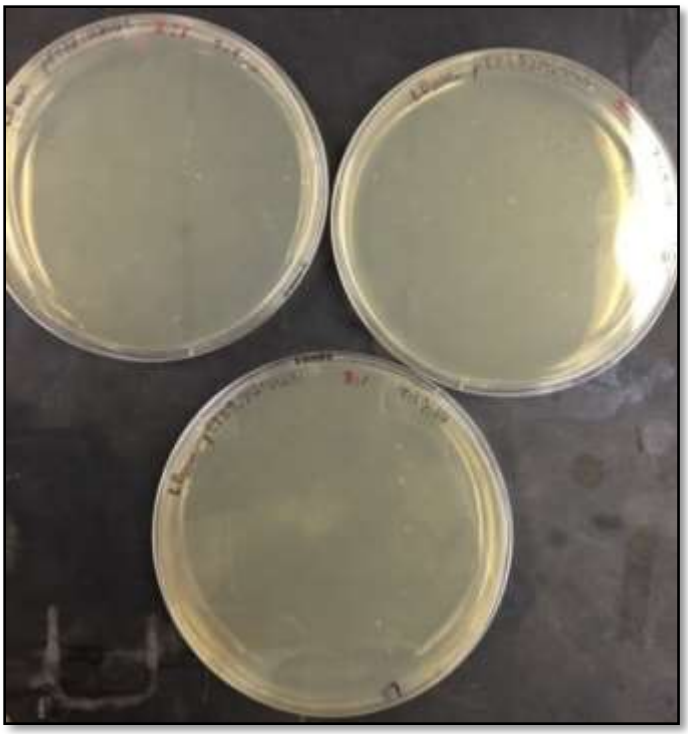

Figure 7. Sub-cloning *mvaC* into pET28. Ligation insert:vector ratios 2:1 (top left), 3:1 (bottom center), and 5:1 (top right). There were 5-10 colonies on each plate (LB Kanamycin).

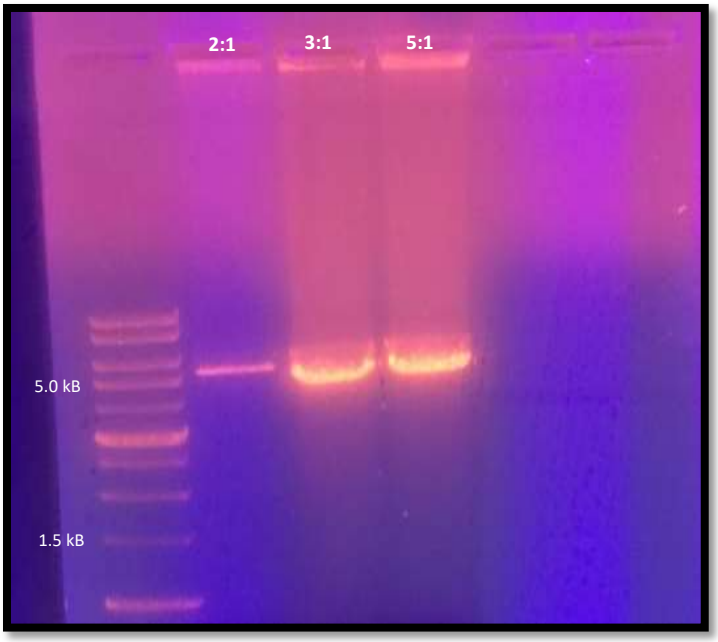

Figure 8. Agarose gel of *mvaC*/pET28. No band for *mvaC* near 1,500 bps. For each ligation ratio (from left to right: 2:1, 3:1, and 5:1) the only band present was pET28 around 5,000 bps.

Sub-cloning *mvaE* and *mvaS* into pDUET. pDUET was used in the reaction with *mvaE* and *mvaS,* as it has multiple cloning sites. For both experiments with pDUET, the ligation was not successful.

mvaE. To sub-clone *mvaE* into pDUET, the plasmid was cut with Nco I and Bam HI. As described above, a new supply of Nco I was ordered, and the reaction was allowed to progress for 1 hour rather than 15 minutes. Under these conditions, *mvaE* was consistently cut from pET28, while results for the restriction digest with pDUET were inconsistent. Purifying *mvaE* and what appeared to be cut pDUET, were ligated together for a short time (1 hour at 37 ˚Celsius) and long time (4 hours at room temperature). Colonies grew for both the short and long ligation, and there was an absence of growth on the cut control (Fig. 9). However, a diagnostic restriction digest revealed that *mvaE* failed to insert into pDUET (Fig. 10).

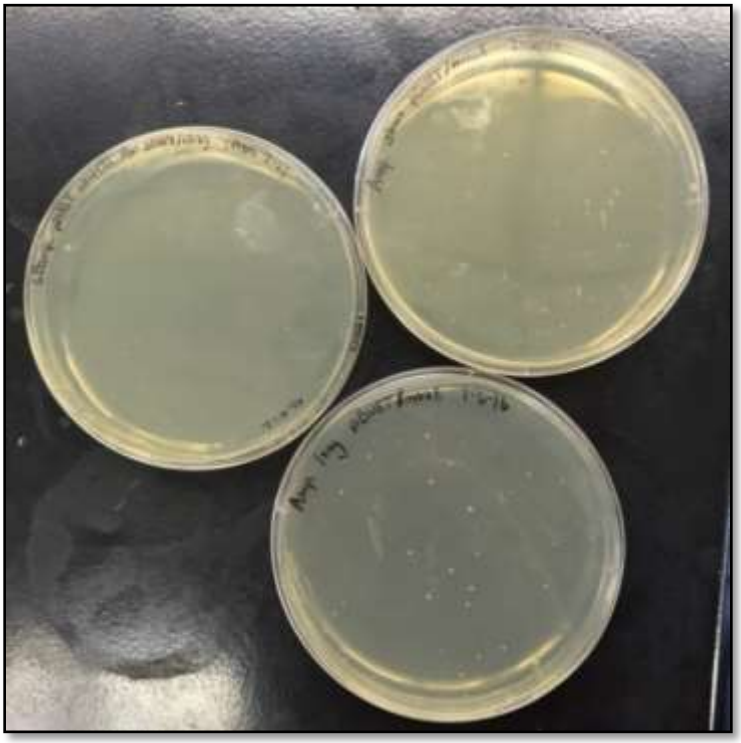

Figure 9. LB Ampicillin plates with sub-cloned *mvaE* in pDUET. No growth on cut control (top left). Colonies grew for both short ligation time (top right), and the long ligation (bottom center).

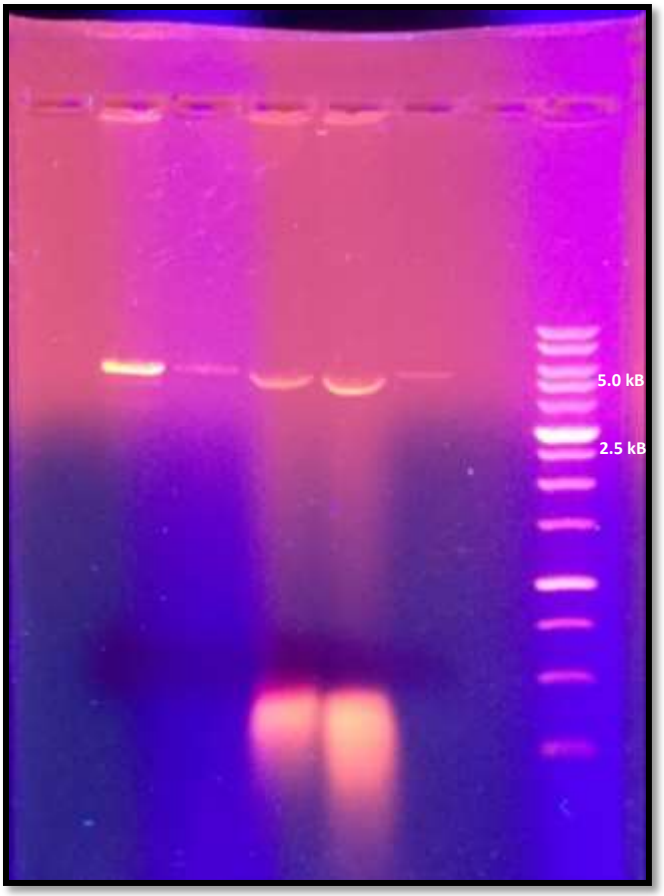

Figure 10. Agarose gel of short ligation time for *mvaE*/pDUET plasmids. No band for the insert gene, *mvaE* at 2,500 bps. pDUET fragment is shown at 5,000 base pairs.

This experiment was repeated with a phenol:chloroform extraction of the vector as well as dephosphorylation of the 5' end of pDUET, as described in the methods section. Following these changes, *mvaE* still did not ligate with pDUET.

mvaS. Similar problems were experienced with sub-cloning *mvaS* into pDUET. Purified *mvaS* and cut pET28 with restriction enzymes Nde I and Xho I failed to ligate together. Colonies never grew for this set of experiments.

DISCUSSION

Currently, *Enterococcus faecalis*, a low-G+C gram-positive cocci bacteria that depends on the mevalonate pathway to produce IPP, is an antibiotic resistant bacteria causing nosocomial infections. New methods to target these bacteria are essential for the lives of those infected.

This research looked at the unique fusion protein encoding the first and third enzymes of the mevalonate pathway, acetoacetyl-CoA thiolase and HMG-CoA reductase respectively, as well as the second enzyme, HMG-CoA synthase. Little is known about the fusion protein, and to investigate it, a series of sub-cloning experiments were conducted. First, isolation of thiolase from the fusion protein was attempted through a double restriction digest and sub-cloning *mvaC* into pET28. Secondly, *mvaE* and *mvaS* were sub-cloned into pDUET with the ultimate goal of simultaneously expressing synthase and the fusion protein in the hopes that synthase would help the fusion protein fold properly.

From this research, each gene insert (*mvaC, mvaE,* and *mvaS)* was successfully removed from pET28 and purified for the ligation. Unfortunately, problems were faced with cutting the vectors, pDUET and pET28, and as a result, the insert genes were unable to ligate with the vectors.

One possible explanation for the absence of insert in each corresponding vector is that cut vector (pDUET or pET28) ligated with another cut vector. It is possible for two of the same vectors to ligate with themselves due to the same sticky ends. Results did not improve, however, after increasing the insert to vector ratios for the ligation, indicating that this scenario is not likely the problem.

Specifically for the pDUET/*mvaE* experiment, it is possible that the vector pDUET re-ligated with itself. Due to very similar, sticky ends cut by Nco I and Bam HI, it is possible that one pDUET vector re-ligated (Fig. 11). The sticky ends were the same length, and when ligated together have only two mismatched base pairs. Prevention of this problem was attempted by dephosphorylating the 5' end of pDUET; however, this did not improve the results. Additional trials of this research are needed to understand if the sticky ends of pDUET are ligating with themselves.

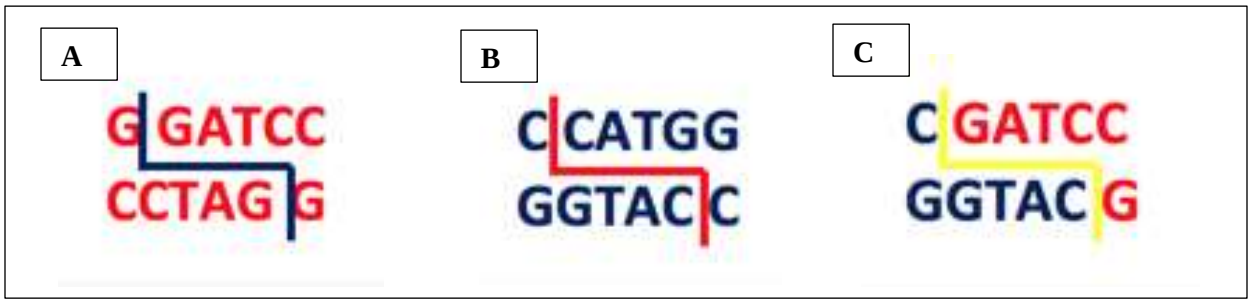

Figure 11. Restriction sites for the restriction enzyme BamHI (A) and Nco I (B), and the possibility of the sticky ends of pDUET re-ligating with itself without the insert gene (C).

Because of the difficulty reproducing our results of cutting each plasmid (pET28 and pDUET), another explanation is that the restriction enzymes did not initially cut the vectors, as they should. For pET28, there was some growth (about 5 colonies) on the cut control plate suggesting that it is likely both enzymes failed to cut pET28. The same problem could explain the results for pDUET. After running a four-hour long double restriction digest for each sub-cloning experiment (pDUET/*mvaE,* pDUET/*mvaS,* and pET28/*mvaC*), enzymes Nco I and/or Nde I seemed to be having trouble cutting the plasmids.

Additional restriction digests with fresh enzymes and various digest conditions are necessary to improve the cutting of pDUET and pET28. With successful restriction digests, there is hope to sub-clone *mvaE* and *mvaS* into pDUET and *mvaC* into pET28. A second idea to express *mvaS* and *mvaE* simultaneously is to sub-clone *mvaS* into pET21 and *mvaE* in pET28 to grow together in a transformation. The two vector plasmids, pET21 and pET28 have different antibiotic resistance, so the only cells that should grow will have genes for both synthase and the fusion protein. After each gene has been sub-cloned into its corresponding vector, future research is needed on the expression of the genes to study the structure and function of the fusion protein as well as thiolase alone. More understanding of the structure and function of thiolase and the fusion protein may provide useful knowledge for future drug targets on the mevalonate pathway in *E. faecalis*.

ACKNOWLEDGMENTS

From the Welch Foundation, I am thankful for their funding of this research (Grant No. R-0021). I would also like to thank the Department of Chemistry and Biochemistry at Abilene Christian University for providing the Summer Research Internship that initiated my research project, as well as the resources, technology, and means to conducting this research up to the present time. A special thank you to Dr. Sutherlin, for allowing me the opportunity to work alongside her in her research, teaching me, and pushing me to develop a scientific mind. Finally yet importantly, I would like to thank my advisory committee Dr. Sarah Lee, Dr. Qiang Xu, and Dr. Kim Pamplin, and the Honors College for the opportunity and guidance in writing an honors thesis.

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