

MEKA, APARNA, M.S. Analysis of a Possible Multienzyme Complex Encoded by Mother Cell Metabolic Gene (*mmg*) Operon of *Bacillus subtilis* Strain 168. (2010)
Directed by Dr. Jason J. Reddick. pp.72.

Bacillus subtilis is a rod shaped, aerobic, endospore forming gram-positive bacterium. *B. subtilis* is used as a model organism to study cell differentiation during sporulation in prokaryotes. When there is inadequate supply of carbon resources, *B. subtilis* undergoes a process of sporulation. The mother cell metabolic gene (*mmg*) operon is expressed at early stages of sporulation. It has six open reading frames *mmgABCDE* and *yqiQ*. The products of these genes *mmg A, B, C, D, E* and *yqiQ* in *mmg* operon share sequence homology with enzymes involved in fatty acid metabolism and methyl citric acid cycle. The genes *mmgABCD* were successfully cloned, over expressed and purified. The fatty acid degradation enzymes exist, as a complex in *E.coli* and our hypothesis is that the fatty acid degradation proteins may function as a complex as well. We tested this hypothesis by cloning and coexpressing all four *mmg ABCD* in a single strain of *E.coli*. This was accomplished by cloning *mmgA* with a His-tag for Ni-NTA affinity chromatography. If the four proteins formed a complex, the remaining *mmgBCD* would co-purify with the His-tagged *mmgA*. We observed a ~26 kDa protein coeluting with *mmgA* (which may be *mmgB*) and also 40-50 kDa protein. (*mmgC* - 40710 Da and *mmgD*- 41946 Da)

ANALYSIS OF A POSSIBLE MULTIENZYME COMPLEX ENCODED BY
MOTHER CELL METABOLIC GENE (MMG) OPERON
OF *BACILLUS SUBTILIS* STRAIN 168

by

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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

2010

Approved by

Committee Chair

To My Parents

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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ACKNOWLEDGEMENTS

I would like to thank my research advisor, Dr. Jason Reddick, for his time, patience, support and for much appreciated guidance throughout my graduate years. It is not often that one finds an advisor who takes time for listening to the little problems and roadblocks that unavoidably crop up in the course of performing research. His technical and editorial advice was essential to the completion of this thesis

I would also like to thank my committee members, Dr. Raner who is my graduate advisor, and Dr. Alice Haddy for their assistance and valuable suggestions and sharing their knowledge in the classroom.

I thank my parents, Bala Bhaskar and Madhavi, for their unlimited support and inculcating in me the dedication and discipline to do well, whatever I undertake.

We gratefully acknowledge generous financial support of this research from the National Science Foundation (Award # 0817793)

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CHAPTER I

INTRODUCTION

I.A Sporulation in *Bacillus subtilis*¹

Bacillus subtilis is an aerobic, gram-positive, endospore forming, rod shaped bacterium. It secretes enzymes that are of commercial importance in various industries. Hence this organism is extensively studied. The genome of this bacterium has around 4000 protein coding sequences, which include 87% of the genome sequence.

Because of its ability to use different carbohydrates, the glycolytic pathway along with the TCA cycle is utilized in this organism. It also can grow in anaerobic conditions using nitrogen as the electron acceptor. Under dense population conditions or during carbon and nitrogen starvation, *B. subtilis* undergoes a developmental pathway where it produces spores. Spores are different from vegetative cells as they are more resistant to high temperatures and organic solvents. This is the only process that occurs in the stationary phase of the *B.subtilis* culture growth. At the end of exponential phase and the beginning of stationary phase morphological changes take place which leads to the formation

of dormant endospore and this transition takes place by the involvement of the enzymes in the Krebs's cycle.²

Sporulation is a complex process that involves the sequential activation and regulation of the different σ subunits in RNA polymerase (RNAP) resulting in the formation of a larger mother cell and a smaller forespore. The RNAP is a large molecule. The core enzyme has 6 different subunits, $\alpha_2\beta_2\sigma\omega$. The core of RNA polymerase is itself capable of synthesizing RNA, but it recognizes the specific promoter regions with the binding of the σ subunit.³ Sequential activation of the different σ -subunits of RNA polymerase occurs during sporulation. Each σ factor is responsible for the gene recognition by RNA polymerase. Morphological changes occur in sporulation due to the transcription of particular genes as shown in figure 3.

Spo0A, which is a key regulatory protein, has to be phosphorylated by a phosphorelay cascade for spore formation. Phosphorelay is a multistage process, which involves the movement of phosphoryl groups of histidine kinases in the bacterial signal transduction. The decision to sporulate is taken when there is a signal due to nutrient depletion, cell density and DNA damage. These signals are used to induce phosphorylation in Spo0A. There are around 3 histidine kinases involved in the phosphorylation of Spo0A. These kinases transfer the phosphate

group to relay protein Spo0F, from there to Spo0B and then finally to Spo0A.

Phosphorylation of the Asp residue in the N-terminal domain activates the regulator protein Spo0A. Accumulation of this Spo0A ~P is needed to activate the transcription of genes that help for the sporulation. This Spo0A ~P activates the transcription by binding to the promoters of these genes and operons, which acts in conjunction with the σ^A factor of RNA polymerase in *spoIIIE* and *spoIIIG* and σ^H in *spoIIA*.⁴

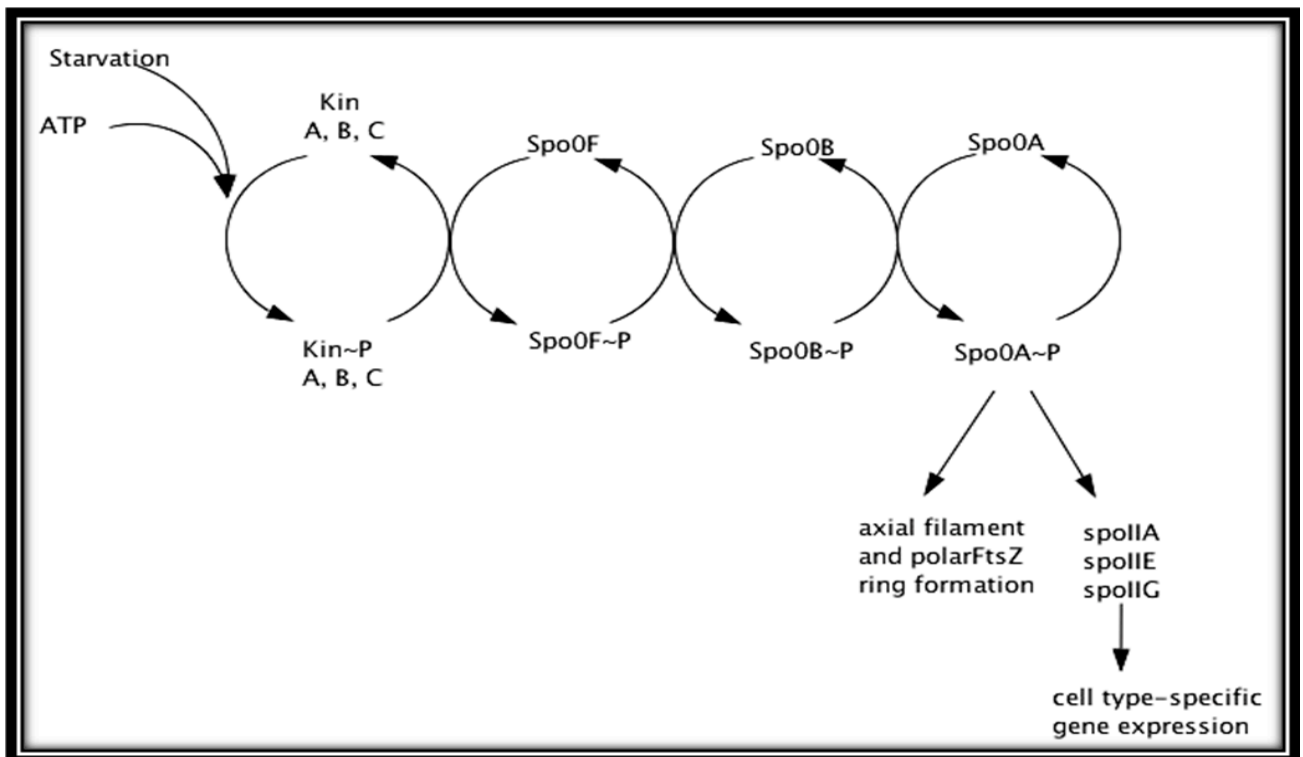


Figure 1: Phosphorylation and formation of Spo0A~P

I.B Stages involved in the Sporulation:

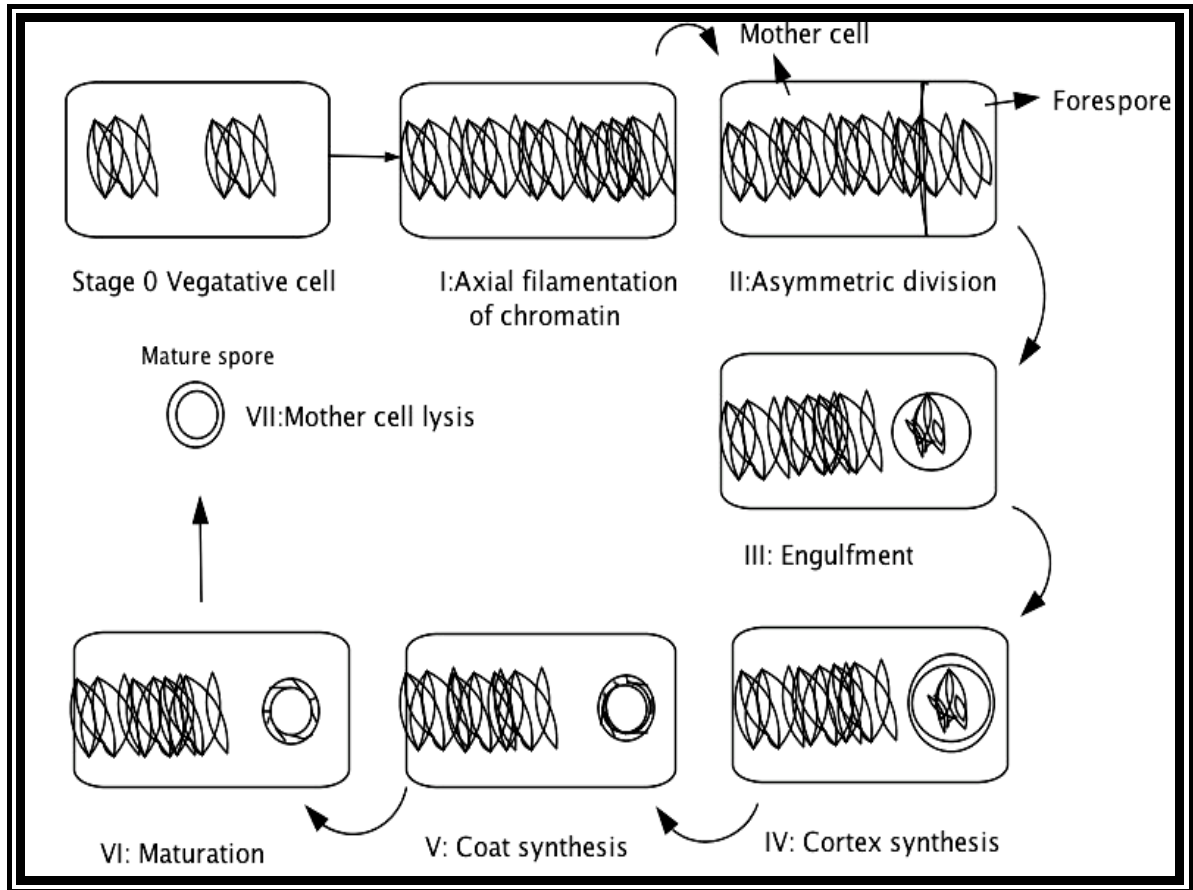


Figure 2: Stages involved in Spore Formation⁶

The vegetative cells before entering the sporulation stage are said to be in the stage 0.⁷ DNA replication, axial filament and polar FtsZ ring formation occur in this stage.

Many genes are involved in the sporulation process and are named “spo” genes. Activation of the Spo0A is needed for the initiation of the sporulation.

Septation of the polar FtsZ rings occurs in stage II. A larger mother cell and a small forespore are formed. σ^A is present in the growing cells in this stage. σ^H directs the transcription of genes whose products are needed in the septum formation and in the chromosomal division where one copy of it goes to the mother cell and other to the forespore. These σ^A and σ^H transcribe operons that have σ^E and σ^F respectively. These are dormant until the polar septum is formed. σ^F becomes active in the forespore and σ^E in the mother cell. After the septum is formed, chromosomes are translocated across the septum into the forespore. In stage III, a phagocytosis like process occurs where the mother cell engulfs the forespore. The septal peptidoglycan starts to degrade from the center to the periphery. The rigid structure in between the cells start to dissolve resulting in a bulging of the forespore into the mother cell, leading to the migration of membrane from the edges to the forespore, forming a double membrane with opposite polarity. σ^F and σ^E controls the whole process.

The formation of the spore cortex occurs in Stage IV. Once the septum is formed σ^F transcribes σ^G in the forespore and σ^E transcribes σ^K in the mother cell. σ^G is activated in the engulfed forespore. σ^F and σ^G in the forespore govern the σ^E and σ^K of the mother cell. σ^E generates signals that help for the synthesis of σ^G in the forespore and its activation. σ^G and σ^K are involved in the formation of cortex and coat layers in the mother cell to encase the forespore.

Coat formation and maturation of the spore occurs in Stage V and VI. The inner spore coat protein deposition occurs in the stage V and outer spore coat protein deposition in the stage VI. The inner spore coat is multilayered and complex. Once the formation of spore coat is completed, the spore develops resistance to chemicals and adverse conditions. The spore finally matures in Stage VI and is released from the mother cell.

LC Role of Sigma factors in Sporulation:

The transcription of the genes, to form products that are responsible for the morphological changes in sporulation is controlled by the σ factors in the RNA polymerase. This occurs via a complex regulatory network.⁸ The Spo0A~P is important in the regulation. When higher levels of Spo0A~P is accumulated, σ^A that is present in higher amounts in the cell and σ^H present in minor amounts are activated. These help for the partition of the chromosomes to the mother and forespore and also the septum formation. σ^H RNAP transcribes the genes for σ^F and σ^A for σ^E . Only after the formation of the septum, the σ^F is active in the forespore and σ^E in the mother cell. The products of both these genes are used for the engulfment process where the forespore with opposite polarity in the membrane is formed. The σ^F is held inactive by the anti- σ factor SpoIIAB. This inhibition is reversed by anti-anti- σ factor SpoIIAA, as it binds to the SpoIIAB-

σ^F and releases the σ^F by steric displacement.⁹ The anti-anti- σ factor SpoIIAA is inactive in the phosphorylated state. This is inactivated by SpoIIAB and activated by SpoIIE by dephosphorylation. Thus the activation of σ^F and σ^G involves the release of anti- σ factor SpoIIAB. The pro-protein precursors have to be proteolytically processed to activate the sigma factors in the mother cells. σ^F and σ^G controls the pro- σ^E and pro- σ^K respectively. Once the σ^F is activated this causes the activation of σ^E . The SpoIIR signal from the forespore activates the pro- σ^E via the protease SpoIIGA. Both SpoIIGA and pro- σ^E are found to be expressed in the mother cell. SpoIIGA cleaves the N-terminal pro-sequence of the pro- σ^E activating it to σ^E in to the mother cell.¹⁰

σ^F RNAP transcribes for σ^G and σ^E RNAP for σ^K . The products of SpoIIIA and SpoIIIJ in the prespore are involved in the activation of σ^G . σ^G turns out to be active in the forespore that is engulfed. Transcription of the genes that cause the protein to condense are activated and also the chromosome is protected here so that the spore can be germinated when there are enough nutrients. σ^K is active in the mother cell. σ^E and σ^K are needed for the formation of the cortex and coat layers in the mother cell to surround the forespore. This σ^K also directs the transcription of genes that help for the lysis of the mother cell and mature spore.

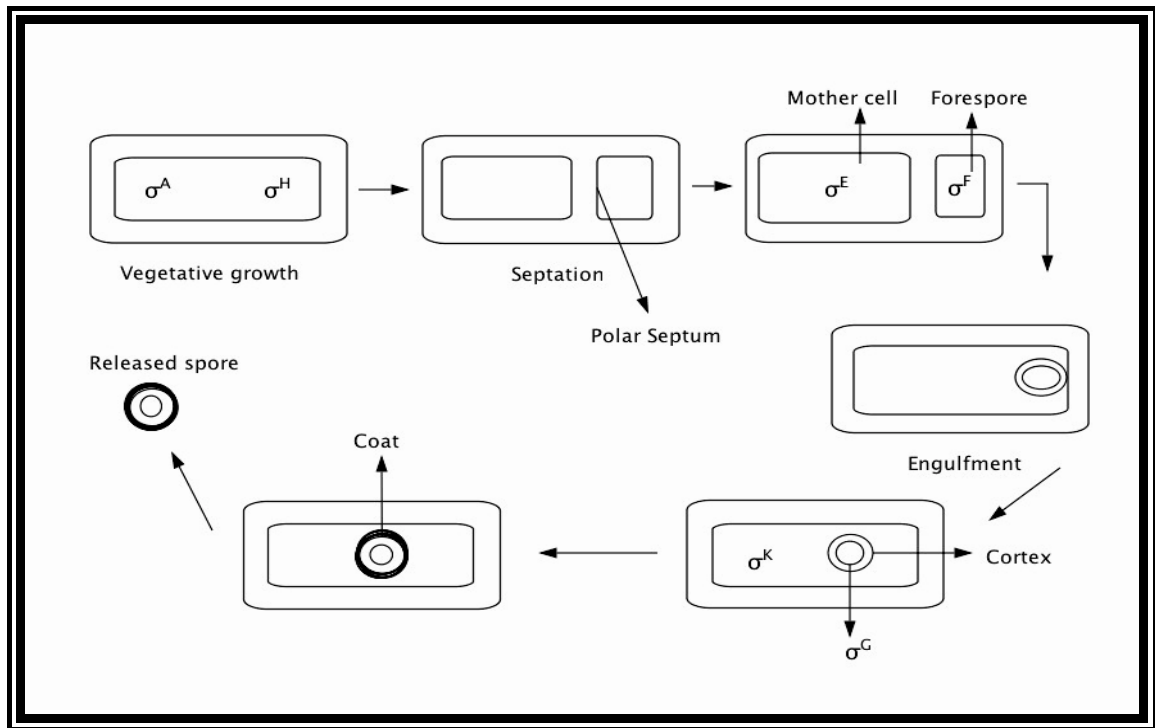


Figure 3: Sigma factors at different stages of Sporulation

1.D The Mother cell Metabolic Gene (mmg) Operon:

The σ^E dependent promoters were screened to identify the genes involved in the intermediate stages of sporulation. It was found that one of these promoters controlled at least 5 open reading frames (ORF). These ORFs were named *mmgA* (393a.a), *mmgB* (287a.a), *mmgC* (379a.a), *mmgD* (371a.a), and *mmgE*. Later, the complete genome sequence revealed that a 6th ORF, *yqiQ*, was found. The products of the first three ORF were similar by sequence to the enzymes involved in the fatty acid metabolism and that of the fourth ORF is similar to citrate

synthase. The mother cell sigma factor σ^E is involved in the transcription of these genes and hence the name *mmg* (mother cell metabolic genes) operon.

I.D.1 Transcriptional regulation:

A 14 base pair sequence *mmgO*, which is located 22 bps downstream of the transcriptional start site of the *mmg* operon, is found similar to catabolite responsive elements (CRE) in other promoter regions. CcpA protein when bound to CRE in the presence of glucose, causes a decrease in transcription from specific promoters. It was shown that the *mmgO* and CcpA are needed for the glucose repression of *mmg* promoter activity in the σ^E -dependent promoters like *mmg* promoter. Thus the *mmg* operon is activated by σ^E but can be repressed by glucose.

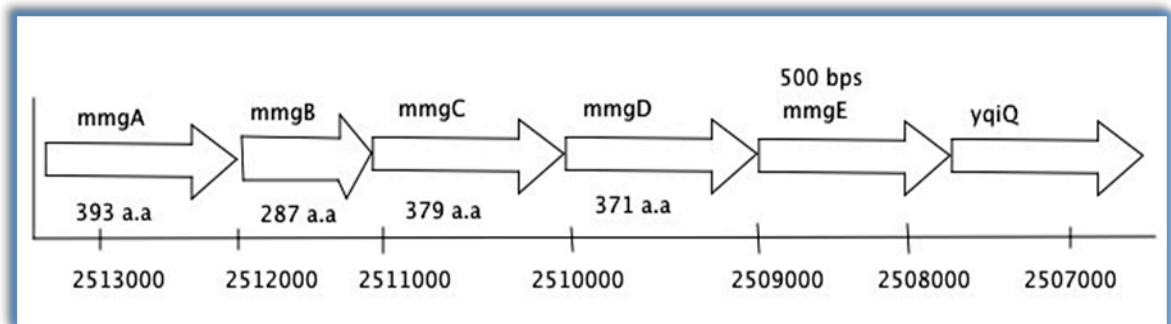


Figure 4: The Mother Cell Metabolic Gene (*mmg*) Operon.¹¹

I.D.2 Functions of different genes:

The *mmgA* gene product has 68% similarity and 49% identity to acetyl coenzyme A acetyl transferase (thiolase). That of *mmgB* has 55% identity and 66% similarity to 3-hydroxybutyryl-CoA dehydrogenase⁹ and the product of *mmgC* is 52% identical and 68% similar to acyl-CoA dehydrogenase. The citrate synthase of *Bacillus coagulans* is 80% similar and 66% identical to that of the *mmgD* product. The sequence of *mmgE* is found to be similar to 2-methylcitrate dehydratase, and *yqiQ* to isocitrate lyase^{9,13}. This shows that the genes *mmgABC* are homologs of thiolase, 3-hydroxyacyl CoA dehydrogenase and acyl CoA dehydrogenase respectively i.e. they are similar to the enzymes in the fatty acid metabolism.

Based on sequence homology, *mmgD*, *mmgE* and *yqiQ* are similar to the enzymes involved in propionate metabolism. The *mmgDE* and *yqiQ* functions as the enzymes in methyl citrate cycle, where propionyl-CoA is involved. One of the important regulators of the Krebs's cycle is citrate synthase.

I.D.3 Proposed reactions for different genes:

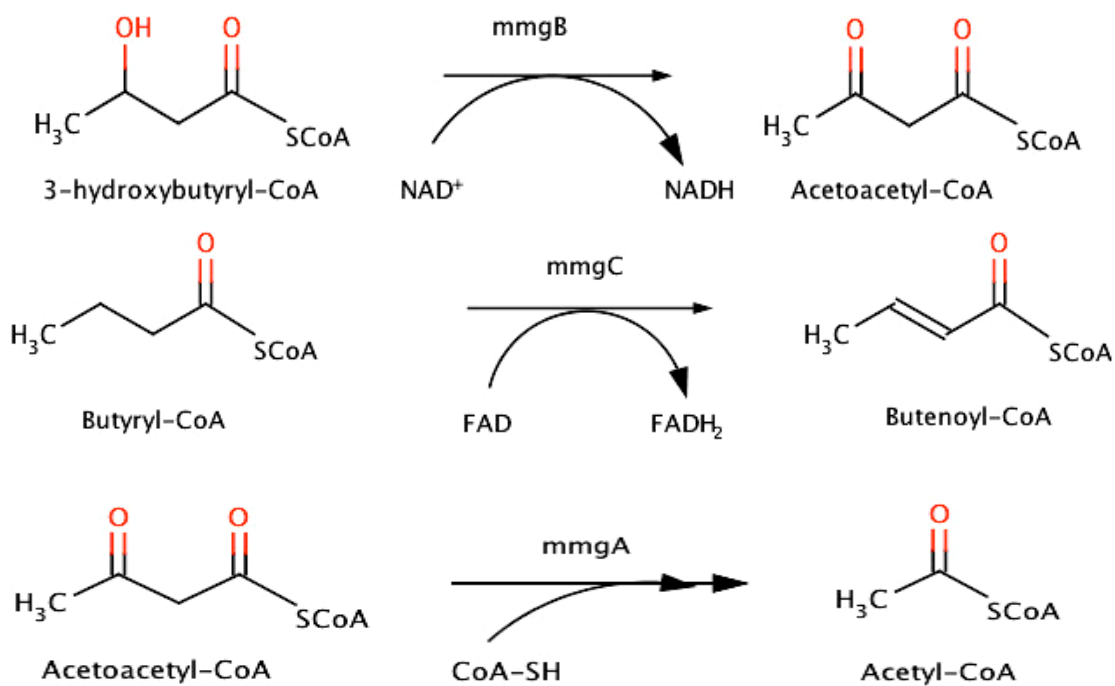


Figure 5: Proposed reactions of the genes mmgA, B and C

In the citric acid cycle, citrate synthase catalyses the reaction, involving acetyl CoA and oxaloacetate with citrate as the product. In the methyl-citric acid cycle, propionyl CoA and oxaloacetate are involved resulting in methyl citrate as the product. It was found that mmgD-like enzymes in *Pseudomonas aeruginosa* and *Salmonella typhimurium* are bi-functional.¹²

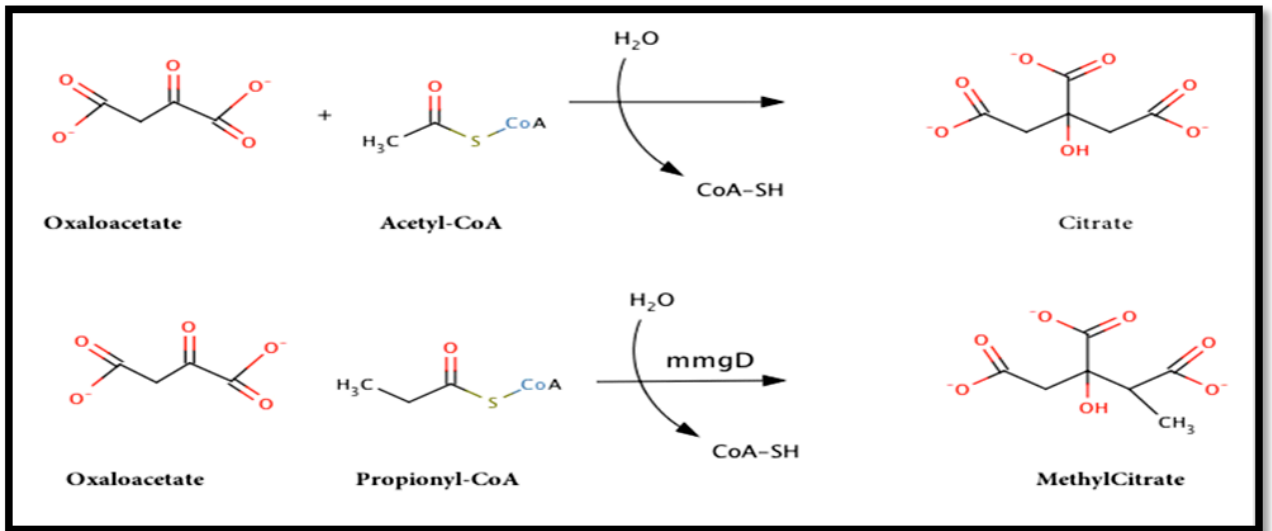
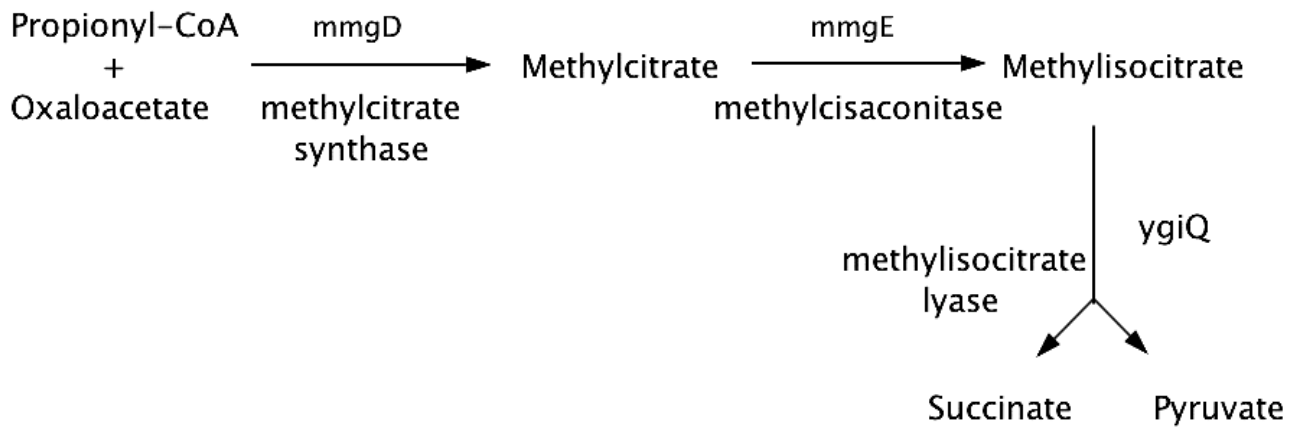


Figure 6: Condensation of Oxaloacetate and Acetyl-CoA to Yield Citrate; Condensation of Oxaloacetate and Propionyl-CoA to yield Methyl citrate

I.D.3.A Putative pathway suggested for *Bacillus subtilis*¹³



I.E Fatty acid metabolism:

In bacteria, phospholipids are the most commonly occurring class of lipids forming the major constituent of the cell membranes.¹⁴ Phospholipids contain a diacylglycerol, a phosphate group and a simple organic molecule like choline. Two hydroxy groups of glycerol are linked to fatty acids via esters, whereas the third hydroxy group is linked to a phosphate ester. Hence the properties of the phospholipid depend on the distribution of fatty acids in the C1 and C2 positions and the nature of the phosphate derivative in the 3rd position.

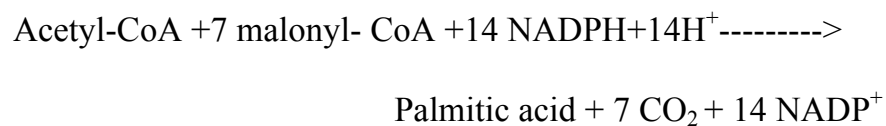
Lysylphosphatidylglycerol are synthesized in high amounts in *B.subtilis*. The fatty acid components adjust the fluidity of phospholipid bilayer. The fatty acids involved in the lipid synthesis are divided into 2 categories; Saturated and Unsaturated fatty acids. *B. subtilis* has no poly-unsaturated fatty acids.

Saturated fatty acids are divided into straight and branched chain fatty acids. Palmitic, stearic, linoleic and linolenic acids are the common straight carbon chain fatty acids in higher plants and animals and less common in *Bacillus*. The fatty acids in plants and animals are mostly polyunsaturated but the ones in bacteria are mostly branched, hydroxylated and rarely poly-unsaturated.

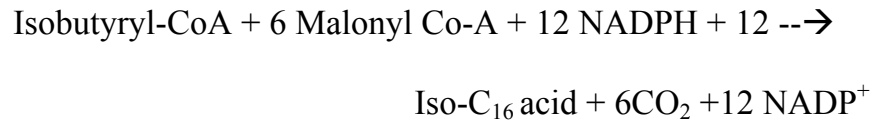
In *Bacillus subtilis*, the terminally branched fatty acids like 13-methyl tetradeconic acid (iso- C₁₅) and 15-methyl hexadeconic acids (iso- C₁₇),

14-methyl pentadeconic acid (iso-C₁₆) and in species of *Sarcina* the 12-methyltetradeconic acids (anteiso-C₁₅) were seen. The branched chain fatty acids are further divided into even numbered iso, odd numbered iso and anteiso depending on their biosynthetic origins. The iso and anteiso constitute about 55-95% of the total fatty acids in *B.subtilis*. The anteiso-C15 is more abundant. *Iso*-Methyl branched fatty acids have the branch point on the ω-2 carbon (penultimate carbon), while *anteiso*-methyl-branched fatty acids have the branch point on the ω-3 carbon (ante-penultimate carbon atom).

The *de novo* synthesis of fatty acids, in most of the organisms, occurs by the repeated condensation of malonyl-coenzyme A with acetyl-CoA in the presence of the palmitic acid synthetases. The end product is palmitic acid and NADP is oxidized.



But in *Bacillus subtilis*, the acyl Co-A esters like isobutyryl, isovaleryl or 2-methylbutyryl are the chain initiators; malonyl-CoA act as the chain extenders and NADPH is the hydrogen donor.



I.E.1 β -Oxidation of fatty acids:

Two carbon units are sequentially removed by the oxidative degradation of saturated fatty acids from the molecule in each turn of the cycle to form acetyl CoA, which enters the citric acid cycle. The β oxidation occurs initially by activation of the fatty acids by coenzyme A to form a fatty acyl thioester, which is followed by four repetitive steps, dehydrogenation, hydration, oxidation and thiolysis.

Each round of the cycle results in the formation of acetyl-CoA and an acyl-CoA, which is 2 carbons shorter than the starting acyl CoA. The cycle is repeated until the entire molecule is completely degraded into acetyl CoA products.

Dehydrogenation:

The β oxidation initiates when two hydrogens are removed from the C-2 and C-3 by acyl-CoA dehydrogenase to yield an α,β unsaturated acyl CoA. An enoyl CoA is formed which transfers the electrons to FAD, which is reduced to FADH₂.

Hydration:

The α,β unsaturated acyl CoA reacts with water in this step. The water acts as a nucleophile, which adds to the β carbon of the double bond forming an intermediate thioester enolate. In the presence of the enzyme enoyl-CoA hydratase, 3- hydroxyacyl Co-A is formed.

Alcohol Oxidation:

The 3- hydroxyacyl Co-A is oxidized to β keto acyl Co-A. This reaction is catalyzed by 3- hydroxyacyl Co-A dehydrogenase. This reaction needs NAD^+ , which is reduced to NADH/H^+ as the by-product.

Chain cleavage/ Thiolytic:

This reaction is catalyzed by β ketoacylCoAThiolase. This reaction takes place initially by the nucleophilic addition of the Cys-SH on the enzyme reacting with the keto group of β keto acyl-CoA followed by the cleavage of the C2-C3 bond resulting in an acetyl CoA enolate ion. This is protonated giving acetyl CoA. The attached acyl CoA undergoes nucleophilic acyl substitution with Coenzyme A resulting in acyl CoA. This enters another round of β oxidation for further degradation.

If the starting fatty acid had an even number of carbons for example four carbons, at the end of the first round of β oxidation 2 acetyl-CoA. Molecules are found. If there are more carbons than four the reaction continues until the whole

molecule is converted to acetyl CoA. Similar is the case with the fatty acid having odd number of carbons in the chain, which would result in several acetyl Co-A but also one propionyl Co-A.

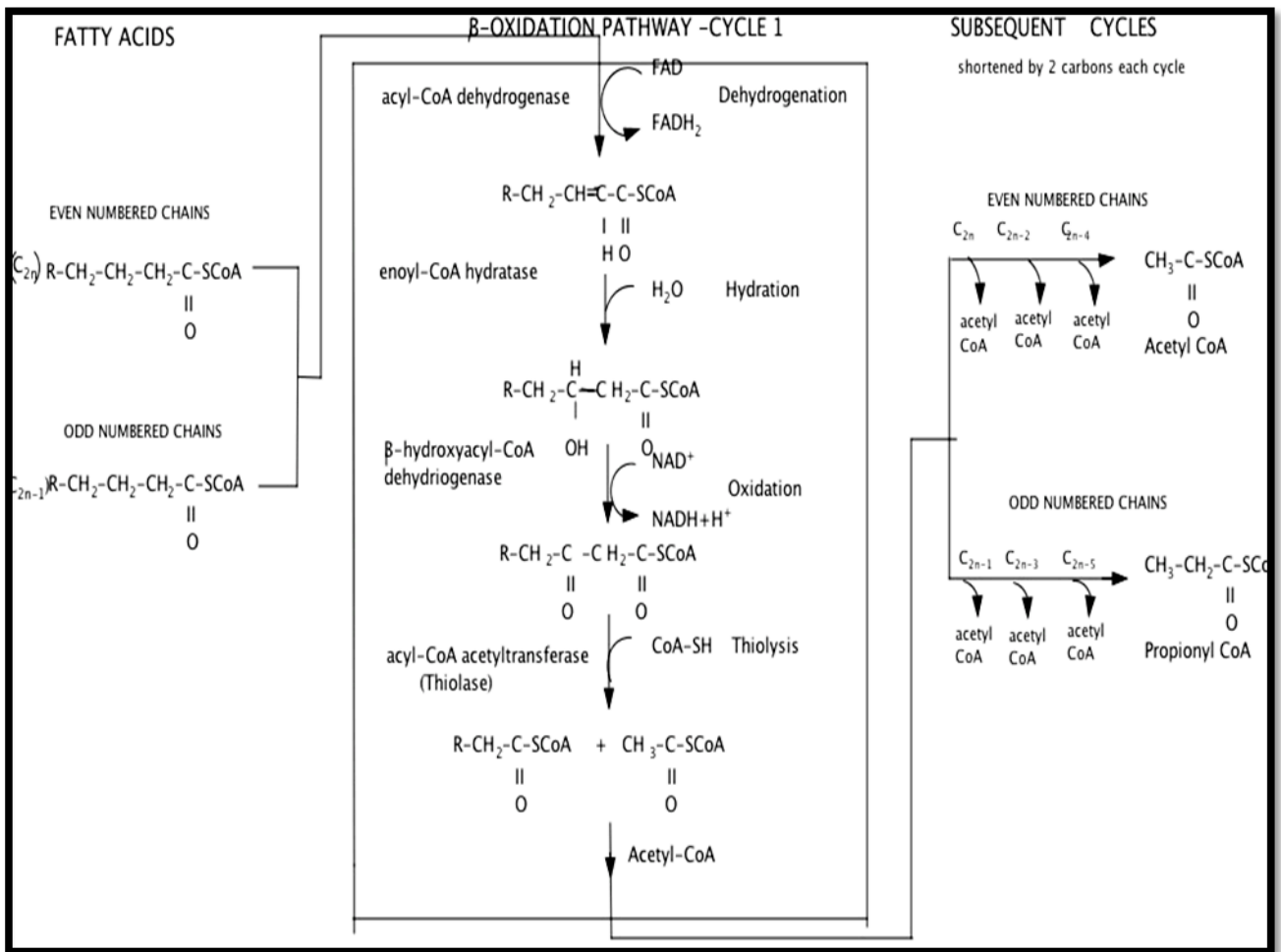


Figure 7: β-Oxidation of Fatty acids¹⁵

I.E.2 Fatty acid degradation enzyme complex

In *E.coli*, five enzymes are involved in the long chain fatty acid β -Oxidation pathway. They are acylCoA synthetases, acyl CoA dehydrogenase, enoyl CoA hydratase, 3-hydroxy acyl dehydrogenase and keto acyl-CoA thiolase^{16, 17}

These enzymes are also involved in the long chain fatty acid degradation in *E.coli* but the short chain Fatty acid catabolism requires three enzymes, crotonase, 3-hydroxyacylCoA dehydrogenase and acyl CoA thiolase.¹⁶ The catabolism of β keto short chain fatty acids requires 2 more enzymes, acetyl CoA transferase and acetoacetylCoA thiolase. In *E.coli*, acetoacetylCoA thiolase and β ketoacylCoA thiolase were heat resistant. The molecular mass of acetoacetyl CoA thiolase in SDS-PAGE was 41,500 Da.

It is known that the enzymes exist as a multi enzyme complex in *E.coli* with a molecular weight of 260, 000 Da. When analyzed by SDS PAGE, two types of subunits with the molecular weights 78 kDa and 42 kDa were seen by Schulz and co workers. The purified complex of fatty acid oxidation in *E.coli* was found to have the 3- hydroxyacyl-coenzyme A epimerase and enoyl CoA isomerase in addition to the enoyl CoA hydratase, 3-hydroxy acyl Co A dehydrogenase and 3-keto acyl CoA thiolase activities.^{18,19} The genes that code for these enzymes form an *fad* operon. All these three enzymes co-purified as a result of heat treatment.

These proteins co-eluted from Sepharose 6B gel filtration column, which shows that they associate as a single multi protein complex. However, the acyl CoA synthetases and acyl CoA dehydrogenase were not seen in the complex after heat treatment.

In *E.coli*, the fatty acid β -oxidation pathway is encoded by the *fad* (fatty acid degradation) regulon.²⁰ A tetrameric complex containing 2 copies of FadB and FadA catalyses the other steps in β oxidation. FadB has the enoyl CoA hydratase and 3-hydroxy acyl CoA dehydrogenase activity, whereas FadA has 3-keto acyl CoA thiolase activity.

The activities of all these complexes were tested with the appropriate substrates. The advantages of the β -Oxidation enzymes existing as a complex is the greater kinetic efficiency due to the channeling of Fatty acid oxidation intermediates from one active site to the next without equilibrating the medium and also to prevent accumulation of intermediates.

Similar complexes were also seen in other organisms like *Pseudomonas fragi*²¹ and, *Caulobacter crescentus*.²⁰

In *Pseudomonas fragi*, the complex exhibited enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, 3-oxoacyl-CoA thiolase, enoyl-CoA isomerase, and 3-hydroxy acyl-CoA epimerase.²¹

In *Caulobacter crescentus*, the complex exhibited acyl coenzyme A synthetases, acyl CoA dehydrogenase, enoyl CoA hydratase, 3-hydroxy acyl dehydrogenase and keto acyl-CoA thiolase.²⁰

The *mmgA* protein sequence in *B.subtilis* has 33% identity and 51% similarity to *fadA* of *E.coli* . Both *mmgA* and *fadA* have thiolase activity.

mmgB protein sequence has 37% identity and 52% similarity to the *fadB* of *E.coli*. Both *mmgB* and *fadB* have 3-hydroxy acyl CoA dehydrogenase activity.

Clustal W was used as a multiple sequence alignment computer program²², here, to identify the conserved sequence regions in *mmgA* and *B* of *B.subtilis* and *FadA* and *FadB* of *E.coli*.

mmgA of *B.subtilis* with FadA of *E.coli*

CLUSTAL 2.0.12 multiple sequence alignment

```

B.subtilis MRKTIVIVSAARTPPG-KFGGVLKEVKAELGGIVMKEALQQAGVSGDDVEGNVMGMVVQA 59
E.coli      MEQVVIVDAIRTPMGRSKGGAFRNVRAEDLSAHLMRTLLARNPALEAAAALDDIYWGCVQQ 60
          *.:.***.* **:* . **.:***:* :*.. !*! * : . . :! **

B.subtilis GSGQ--IPSRQAARLAGMPWSVPSSETLNKVCASGLRAVTLCDQMIRAQDADILVAGGMES 117
E.coli      TLEQGFNIARNAALLAEVPHSVPVAVTVNRLCGSSMQALEHDAARMIMTGDAQACLVGVEH 120
          * :*:** ** :* **:* :*:**.*.:** : . :** : ** : :.***:

B.subtilis MSNIPYAVPAGRWGARMGDGELRDLMVYDGLTCAFDEVHMAVHGNTAAKEYAISRREQDE 177
E.coli      MGHVPMH-----GVDFHPGLSRNVAKAAGMMGLTAEMLARMHG-----ISREMQDA 167
          *.:!* : * . : * *! : * : * : :** ***. **

B.subtilis WALRSHARAAKAADEGKPFQDEIVPVNWIGRKGKPNVVDKDEAIRRDTSLDQLAKLAPIYA 237
E.coli      FAARSHARAWAATQSAAPKNEIIPGTHDADGVVKQFNIDEVIRPETTVEALATLRPAFD 227
          !* ***** *!.. *!***:.. . .* : .! **. ** !:!! **.* * :

B.subtilis S-DGSITAGNAPGVNDGAGAFVLMSEEKAAELGKRPLATILGFSTTGMPAHELAAAPGFA 296
E.coli      PVNGMVTAGTSSALSDGAAAMLVMSERAHGLKPRVRVSMVAVGCDPSIMGYGPVPA 287
          . !* !**.:...:***.*:!!***.!* ** !* * : .!..* . !. .* *

B.subtilis INKLLKKNGLTVQDIDLFEVNEAFASVVLTCCKIVGFDL---EKVNVNGGAIALGHPIGA 353
E.coli      SKLALKKAGLSASDIGVFEMNEAFAPAAQILPCIKDLGLIEQIDEKINLNGGAIALGHPLGC 347
          ! ** **!..**.*!***:***! !*.* * !* : **!*!*****!*,

B.subtilis SGARILMTLVYELKRRGGGLGVAaicsgaaqgdavlvqvH 393
E.coli      SGARISTLLNLMERKDVQFGLATMCIGLGGIATVFERV 387
          ***** **! :!:. !*:*!* * .* ** *.:!
  
```

Figure 8: The multiple sequence alignment of mmgA of *B.subtilis* with FadA of *E.coli*. In the figure, * refers to identical or conserved residues in all sequences in the alignment, “:” to conserved substitutions, “.” Semi-conserved substitutions and “-” indicates a gap inserted by the algorithm

mmgB of *B.subtilis* with FadB of *E.coli*

CLUSTAL 2.0.12 multiple sequence alignment

```

-----
B.subtilis      IGLVDCVVKAEKLVGAKAVLRQAINGDLDWKAKRQPKLEPLKLSKIEATMSFTIAKGMV 240
E.coli

-----
B.subtilis      AQTAGKHYFPAPITAVKTI EAAARFGREEALNLENKSFVPLAHTNEARALVGIFLNDQYVK 300
E.coli

-----
B.subtilis      -----MKRLKKQL 0
E.coli          GKAKKLTEDVETPKQAAVL GAGIMGCGGIAYQSAWKGVVPMKDINDKSLTLG MTEAAKLL 360
                                     *.. **

-----
B.subtilis      ARDAEKGKRTETEVKSVINRISISQTL EEAHADIVIEAIAENMAAKTEMPKTLDRICPP 68
E.coli          NKQLERCKIDGLKLAGVISTIHPTLDYAGFDRVDIVVEAVVENPKVKAVLAETE QKVRQ 420
      :: **::  :: **, *  :  ::,***:**;. ** .*. ::  ::

-----
B.subtilis      HTILASNTSSLPITEIAAVTNRPQRVIGMHFMNPVPMKLV EIRGLATSEETALDVMAL 128
E.coli          DTVLASNTSTIPISELANALERPENFCGMHFFNPVHRMPLVEIIRGEKSSDETI AKVVAV 480
      .;*****;:**;:* . :**;. . ***** * **;*** :;* ** .;*

-----
B.subtilis      AEKMGKTAVEVNDFFPCFVSNRVLLPMINEAIYCVYECVRTEAIDEVMKLG MHPMGPLAL 188
E.coli          ASKMGKTPIVVNDPCGFFVNRVLPFPYFAGFSQLLRD GADFRKIDKVMKQFGWPMGPAYL 540
      *.*****. : ** ** , **;* :  : |* . **;*** |. **** *

-----
B.subtilis      ADFIGLDTCLSIMEVLHSGLDGSKYRPCP-LLRKYVKAGWLK KSGRGFYDYEEKTS--- 244
E.coli          LDVVGIDTAHEAQAVMAAGFPQRMQKDYRDAIDALFDANRFGQKNLGFWR YKEDSKGKP 600
      *.|** . * :| : | :  : ..* .|**.* ** |* .|

-----
B.subtilis      -----
E.coli          KKEEDAAVEDLLAEV SQPKRDFSEEEIIARMMIPMVNEVVRCLEEGIIATPAEADMALVY 660

-----
B.subtilis      -----
E.coli          CLGFFFFBOCAFRLD TLGSAKYLDMAQQYQH LGPLYEVPEGLRNKARHNEPYYPVEPA 720

-----
B.subtilis      -----
E.coli          RPVGDLKTA 729

```

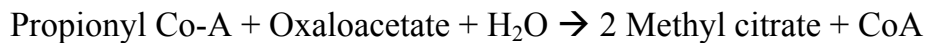
Figure 9: The multiple sequence alignment of mmgB of *B.subtilis* with FadB of *E.coli*. In the figure, * refers to identical or conserved residues in all sequences in the alignment, “:” to conserved substitutions, “.” Semi-conserved substitutions and “-” indicates a gap inserted by the algorithm.

I.F Methyl citric acid cycle :

The acetyl Co-A formed during the β oxidation pathway enter the citric acid cycle in combination with oxaloacetate forming citric acid, as shown below.



But when propionyl CoA is formed as a result of the β oxidation of odd number of carbon chains, it undergoes a different pathway called the methyl citric acid cycle where it combines with oxaloacetate forming methyl citrate.^{23,24}



Methyl citrate synthase, which also have the activity of citrate synthase, catalyses this reaction. The methyl citrate so produced isomerizes from cis-2-methylaconitate to methyl isocitrate followed by cleavage to pyruvate and succinate. Succinate is a citric acid cycle intermediate and is converted to oxaloacetate. Pyruvate can be converted to acetyl CoA. Thus the methyl citrate cycle catalyses the α oxidation of propionate to pyruvate.

This whole pathway is found to involve 5 enzymes in *E.coli*.²⁵ Of these only methyl citrate synthase and 2-methylisocitrate lyase are characterized.

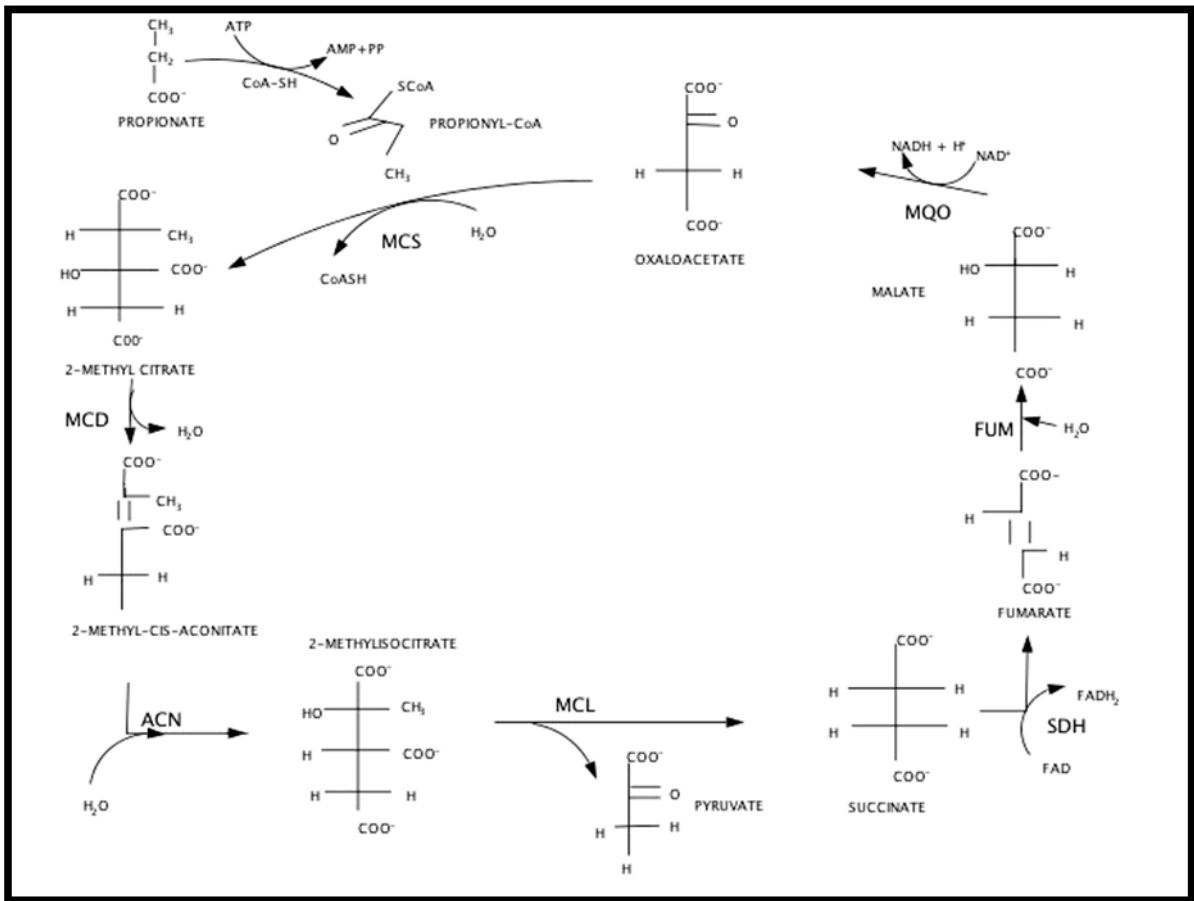


Figure 10: Methyl-Citrate Cycle :²⁶ MCS- methyl citrate synthase, MCD- methyl citrate dehydratase, ACN- Aconitase, MCL- methyl isocitrate lyase, SDH- Succinate dehydrogenase, FUM- Fumarase, MQO, malate-quinone oxidoreductase.

I.G. Overview Of The Previously Done Work

Previously in our lab, work was done to determine the activities of the proteins encoded by the genes *mmg ABCD*. As shown in the table, *MmgA*¹¹, *MmgC*²⁷ and *MmgD*²⁸ were found to be active. However, our group has had difficulty in demonstrating the enzymatic activity of *MmgB*. *MmgA*, *B*, *C* and *D*

were cloned into a pET-28a vector separately, purified by Ni affinity chromatography and enzymatic activities were tested. There is an ongoing work in the lab on testing the enzymatic activity of *yqiQ* and also to establish a system for study of *mmgE*

GENE	CLONED	PURIFIED	DEMONSTRATED ACTIVITY
<i>mmgA</i>	YES	YES	YES ¹¹
<i>mmgB</i>	YES	YES	? ²⁷
<i>mmgC</i>	YES	YES	YES ²⁷
<i>mmgD</i>	YES	YES	YES ²⁸
<i>mmgE</i>	NO	NO	NO
<i>yqiQ</i>	YES	YES	PARTLY ²⁹

Table 1: Summary of the work done in Reddick's lab

A major problem we are approaching in this thesis is with *mmgB*.

Russell Spencer encountered difficulty inconsistently observing the catalytic activity of *MmgB* towards D or L- β -hydroxybutyryl CoA.

We have stated a few hypotheses as to why our *mmgB* system provides inconsistent enzymatic activity.

- 1) The presence of C-terminal histidine tag on the *mmgB* might be interfering with the activity.
- 2) May be 3-hydroxybutyrylCoA is a wrong substrate
- 3) Some of the fatty acid degradation enzymes are known to exist as a complex. Likewise, MmgB may also require such a complex to be fully functional.

In this work we are beginning an approach to study the 3rd hypothesis. Towards this end we first need to test another hypothesis that Mmg proteins exist as a multisubunit complex.

I.G.1 Goals for this research to test the hypothesis:

- 1) To clone the *mmgABCD* gene into the pETDuet expression system for co expression.
- 2) To test whether or not the *mmg* fatty acid degradation enzymes form a multi subunit complex.

CHAPTER II
OVER EXPRESSION AND PURIFICATION OF *MMG* PROTEIN
COMPLEX FROM *Bacillus subtilis*

II.A Introduction to Molecular Cloning

Molecular cloning is a procedure where the gene of interest is isolated and amplified.

1) Isolation of the gene of interest

Isolation of the gene of interest is done by polymerase chain reaction. Initially primers are designed and a PCR reaction is conducted. After the successful PCR, the isolated gene is inserted into the host vector. Duet vectors pETDuet-1 and pACYCDuet are chosen for this work. The genes of interest were *mmg A, B, C* and *D*. The *mmgA* was isolated such that the gene will align with the sequence in the plasmid that codes for a C-terminal His-Tag. This allows us to purify the protein using a Nickel-Nitrilotriacetic Acid (Ni-NTA) column.

2) Transformation

The ligated product is transformed into a cloning strain of *E.coli* and the mixture is grown on agar plates with a selected antibiotic. Then the individual colonies are picked and screened for the plasmid of interest.

3) Screening

Identifying the cells replicating the gene is important. This is done by DNA sequencing and PCR.

II.A.1 pETDuet System

The pET system is a commercially available system for cloning and expression of recombinant protein. The genes of interest are put under the control of bacteriophage T7 transcription and translation signals. When the T7 RNA polymerase is active and begins to work, all the cell resources are devoted to the translation of the desired gene.

II.A.2 Duet Expression System

All the Duet vectors have the T7 promoters for the expression of the target genes by IPTG induction. It is designed in such a way that 2 different target proteins can be co expressed in the *E.coli*. The vectors pETDuet-1 and pACYC Duet are used in my work. These two vectors can be replicated and co-expressed in the same host.

pETDuet-1 has 2 Multiple Cloning Sites (MCS). In the beginning of each MCS, there is a T7 promoter/ lac operator and a ribosome-binding site (rbs). The plasmid also has pBR 322-derived ColE1 replicon, LacI gene and *bla* gene for

ampicillin resistance. pACYCDuet-1 vector has a similar arrangement but has the P15A replicon and *cat* gene for chloramphenicol resistance.

The recombinant plasmid so produced is transferred to a host *E.coli* strain BL21 (DE3), a strain that has the T7 RNA polymerase. “DE3” indicates that the host is a lysogen of λ bacteriophage DE3. The *E.coli* genome and the pETDuet plasmid have the *lacI* gene, which encodes the *lac* repressor protein. This protein prevents the binding of any RNA polymerase to the *lac* promoter region and hence the transcription of the T7 gene doesn't occur. Once the IPTG is added, the *lac* repressor protein is removed, allowing the *E.coli* RNA polymerase to transcribe the T7 RNA polymerase. The T7 RNA polymerase transcribes the target gene in the plasmids in the promoter region, resulting in the production of protein.

pETDuet-1 vector includes two multiple cloning sites (MCS). Each MCS has a T7 promoter, *lac* operator and ribosome-binding site for the expression of the target gene. The promoter is followed by the ribosome binding site and MCS. The presence of an optimized ribosome binding sequence on the mRNA aids in translation of a protein.

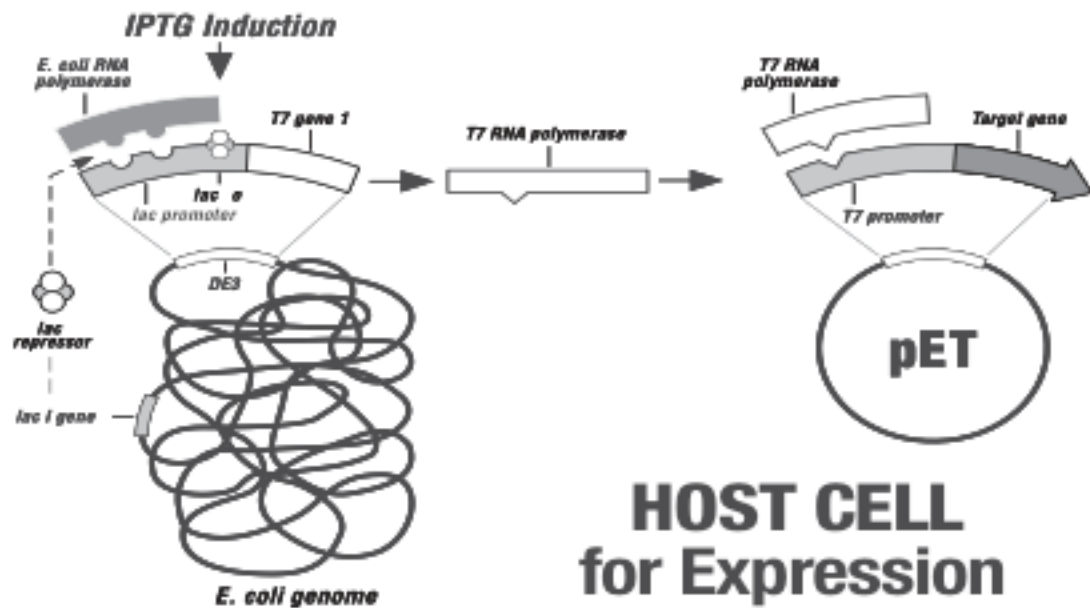


Figure 11: Over-expression System²⁸

II.A.3 Protein Purification:

The purification of the His-tag protein is done with nickel nitrilotriacetic acid (Ni-NTA) metal affinity chromatography. NTA is a tetradentate chelating absorbent. It has 6 ligand binding sites. Of which nickel was bound on the four-ligand binding sites and a 6xHis tag occupies the remaining two. The His-tag securely holds the protein on to the metal chelating surface.²⁸

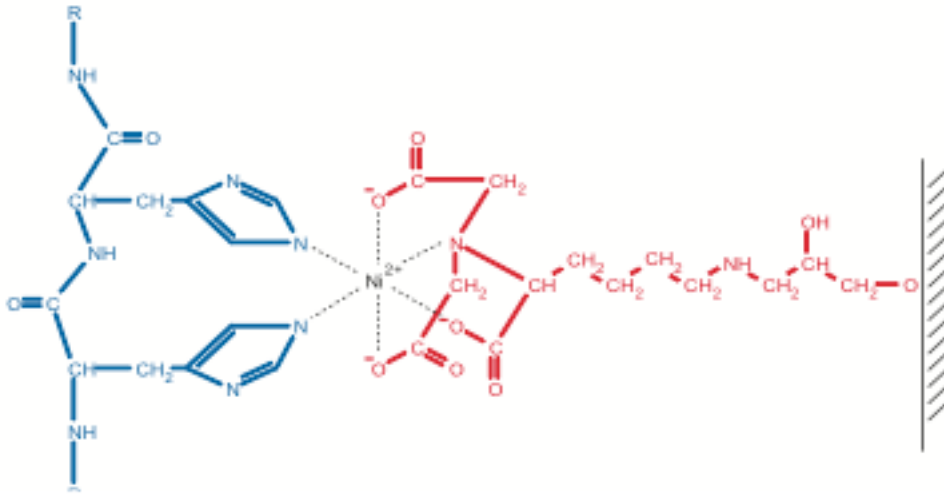


Figure 12: The interaction of the His-tag and Ni-NTA column²⁸

The protein as it moves down the Ni-NTA column, binds to the matrix. The column is rinsed with low concentration of imidazole to remove unwanted proteins in the wash step. The final elution buffer that is added has a high concentration of imidazole to elute the desired His-Tagged protein. Due to structural similarity, imidazole competes with the histidine of 6xHis tag protein and binds to the Ni-NTA on the column.

CHAPTER III

RESULTS AND DISCUSSION

III.A Amplification of *mmgA* and *mmgB*

Mmg A and *mmgB* were successfully amplified from the *Bacillus subtilis* strain 168 genome by the polymerase chain reaction. In the agarose gel pictures, it can be seen that the amplified gene of *mmgA* falls between 1.0Kb and 1.2 Kb. This is consistent with the expected size of 1179bps.

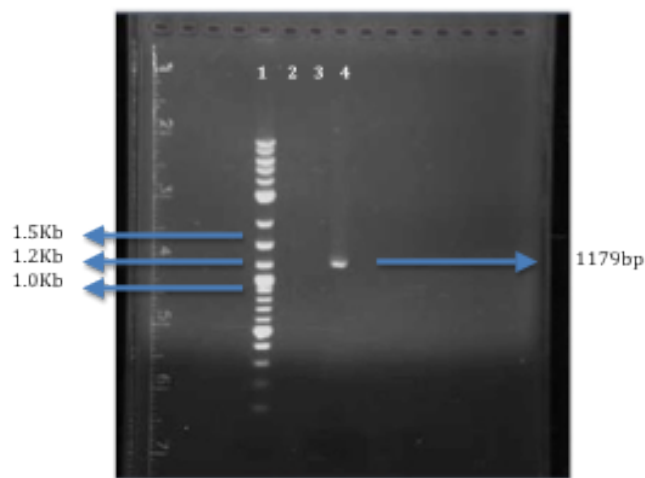


Figure 13: Agarose gel of the PCR reaction product of BS 168. Lane 1: Standards. Lane 2: negative control (without the template) of the PCR product. Lane 3: PCR product. The expected size of *mmgA* is 1179 bps

As shown in figure 12, the PCR product of *mmgB* is seen between 0.7 kb and 0.8 kb and is consistent with its gene length 732bps.

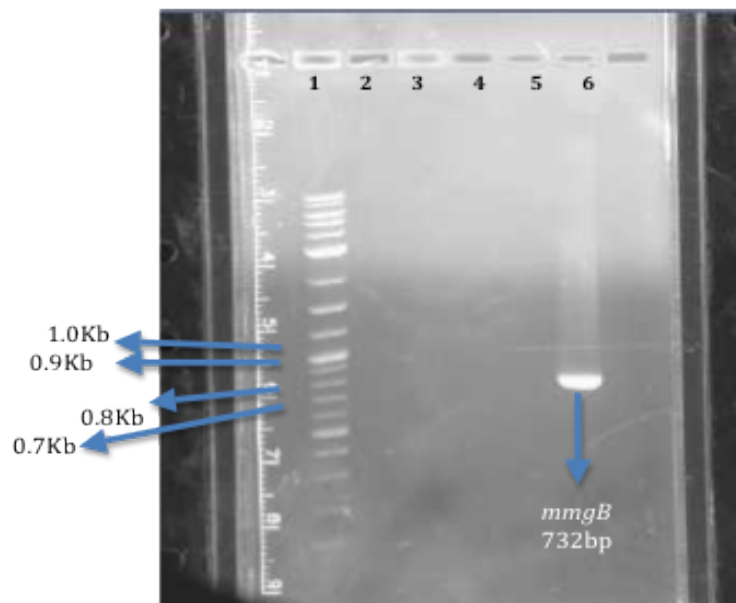


Figure 14: Agarose gel of the PCR of BS 168 genome.
Lane 1: Standards. Lane 6: PCR product. The expected size of *mmgB* is 732 bps.

III.A.1 Cloning *mmgA* into pETDuet-1 vector

Restriction digestion and Ligation:

MmgA and pETDuet-1 were cut with the restriction enzymes BamHI and Sall and purified on 1% agarose gel (Figure 13). Each bands was gel extracted and using T4-DNA ligase, *mmgA* was ligated into the vector. The ligation mixture was transformed into competent *E. coli*. Individual colonies were screened for the

presence of the plasmid with the insert by PCR. A plasmid that tested positive for the insert was transformed into DH5 α , sent for sequencing and retransformed into *E.coli* BL21 (DE3).

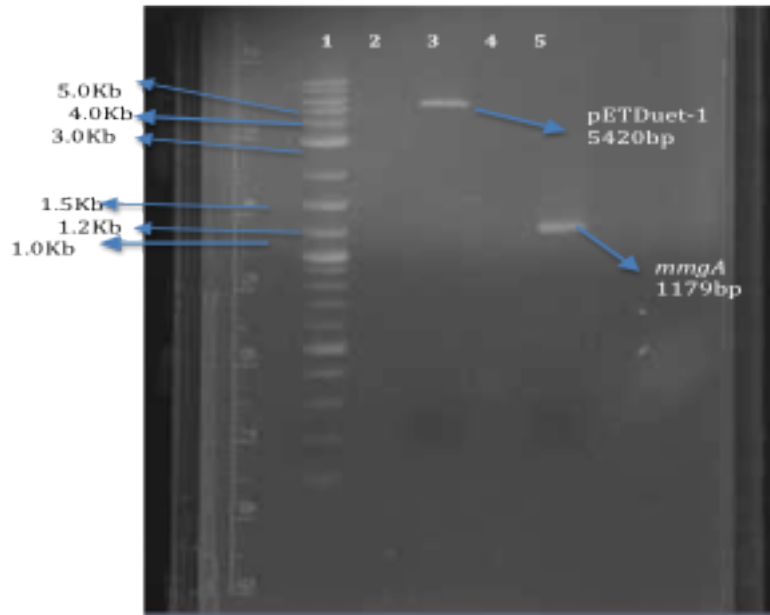


Figure 15: Agarose gel of the restriction digested plasmid and *mmgA*. Lane 1: Standards. Lane 3: Restriction digested plasmid Lane 5: Restriction digested insert. The length of the plasmid vector is 5420 bp and that of the insert *mmgA* is 1179 bp.

After ligation, individual colonies were picked up and screened for the plasmid that tested positive for the insert. Here, three individual colonies were picked up and tested. The sample that gave the brighter band (in figure 14, the one in the fourth lane) was selected for further retransformation and sequence submission.

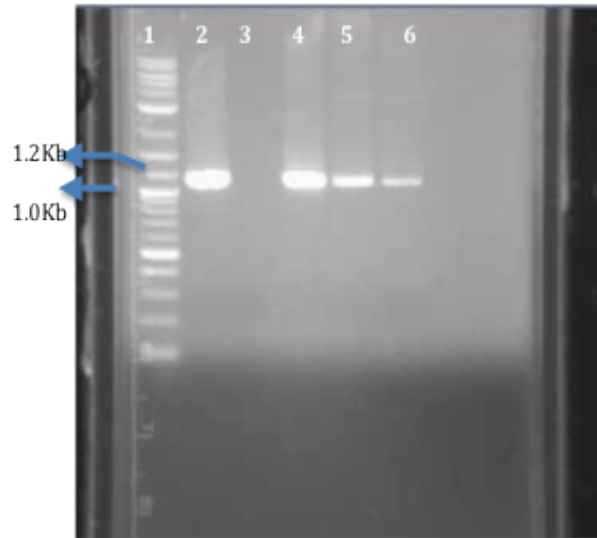


Figure 16: Agarose gel showing the screened plasmid samples testing for the insert *mmgA*, by PCR. Lane 1: Standards. Lanes 2: positive control with BS168 genomic DNA. Lane 3: negative control. Lane 4, 5, 6: Positive Transformants.

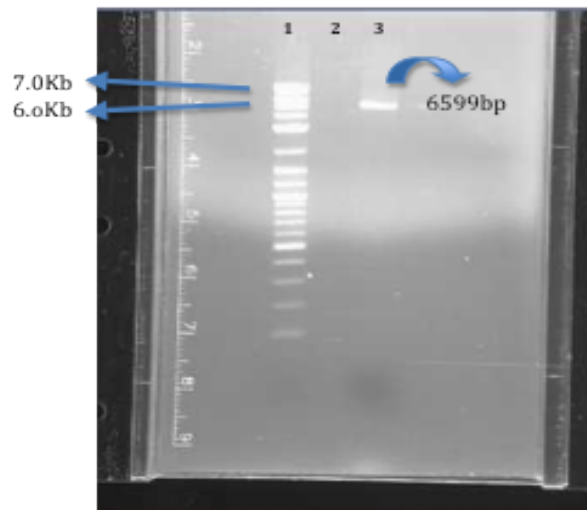


Figure 17: Agarose gel showing plasmid with the insert. Lane1: Standards. Lane2: Negative control of the PCR product. Lane 3: Plasmid with the insert. The expected length of the vector with the *mmgA* insert is 6599 bp.

III.A.2 Cloning *mmgB* into pETDuet-1 with *mmgA*

Next *mmgB* gene was inserted into this vector that already has the *mmgA* gene. To accomplish this, the first step was to cut both *mmgB* and pETDuet-1/*mmgA* with the restriction enzymes NdeI and XhoI. Then they were purified by a 1% agarose gel. As shown in figures 16 and 17. Those bands were gel extracted and by using T4-DNA ligase the *mmgB* gene was ligated into the vector.

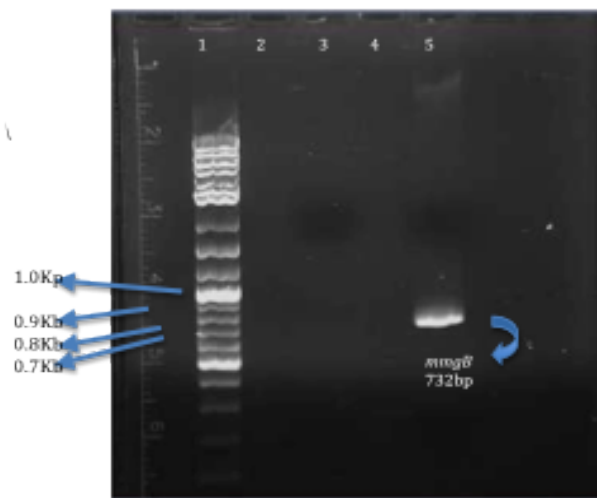


Figure 18: Agarose gel picture showing the insert after restriction digestion. Lane1: Standards Lane5: Insert after restriction digestion.

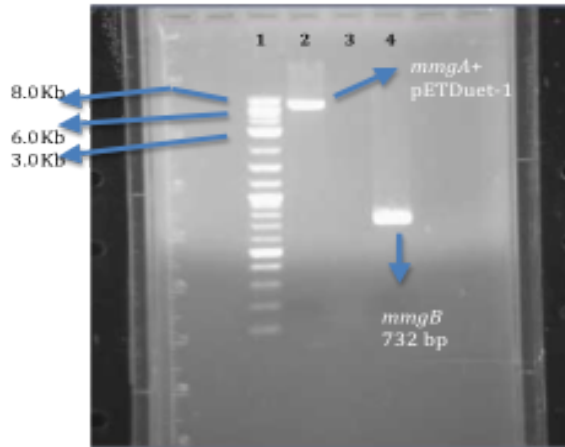


Figure 19: Agarose gel picture showing the restriction digested product of the pETDuet/*mmgA*. Lane 1: Standards Lane 2: pETDuet-1/*mmgA*. Lane 4: amplified *mmgB*. The expected length of the plasmid with the insert *mmgA* is 6599 bp and *mmgB* is 732 bps

The individual transformants obtained from a mixture were screened for the presence of the plasmid with the insert by PCR. Five colonies were picked to test for the insert and all of them gave a positive result. The sample in the 3rd lane was transformed into DH5 α , sent for sequencing and retransformed into *E.coli* BL21 (DE3).

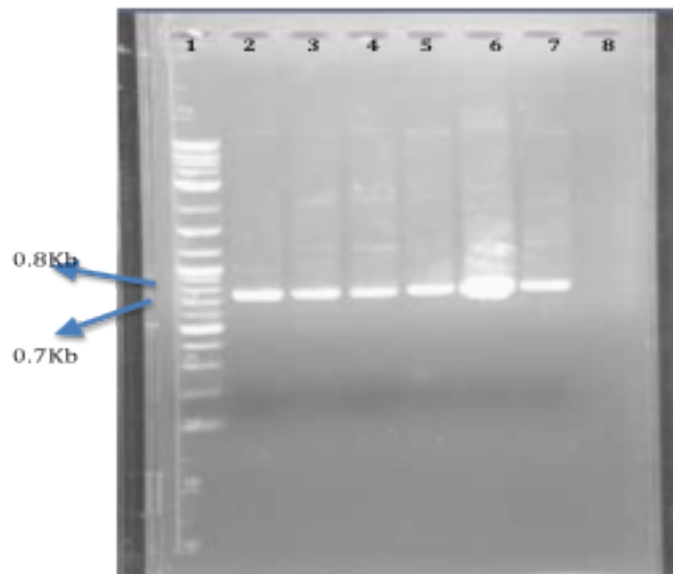


Figure 20: Agarose gel showing the screened plasmid samples testing for the insert *mmgB* using PCR. Lane1: Standards
Lanes 2: positive control. Lane 8: negative control. Lanes 3, 4, 5, 6, 7: Transformants.

III.B Amplification of *mmgC* and *mmgD*

III.B.1 Cloning *mmgC* into pACYCDuet vector

Restriction digestion and ligation

MmgC and pACYCDuet were cut with the restriction enzymes PciI and NcoI and purified by 1% agarose gel as shown in the figure 19. Those bands were gel extracted, and using T4-DNA ligase, *mmgC* was ligated into the vector.

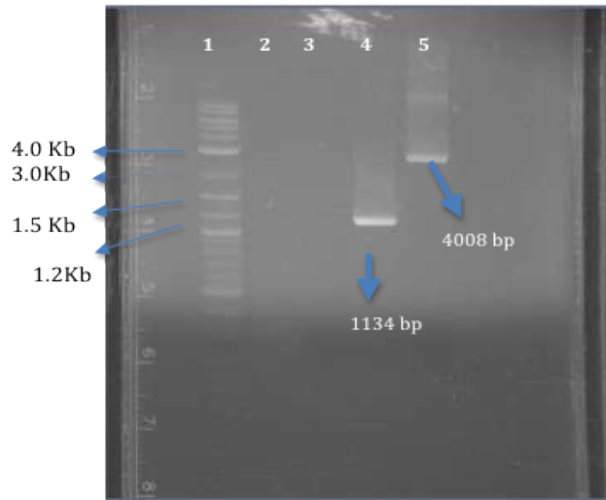


Figure 21: Agarose gel showing PCR product of *mmgC* and the plasmid pACYCDuet after restriction digestion. Lane 1: Standards. Lane 4: PCR product (insert) Lane 5: Plasmid after restriction digestion. Expected length of the PCR product *mmgC* is 1134 bp and that of pACYCDuet vector is 4008bps.

Four individual colonies were selected and screened for the presence of the plasmid with the insert by PCR as shown in the figure 20. The sample in the 4th lane was taken and transformed into DH5 α .

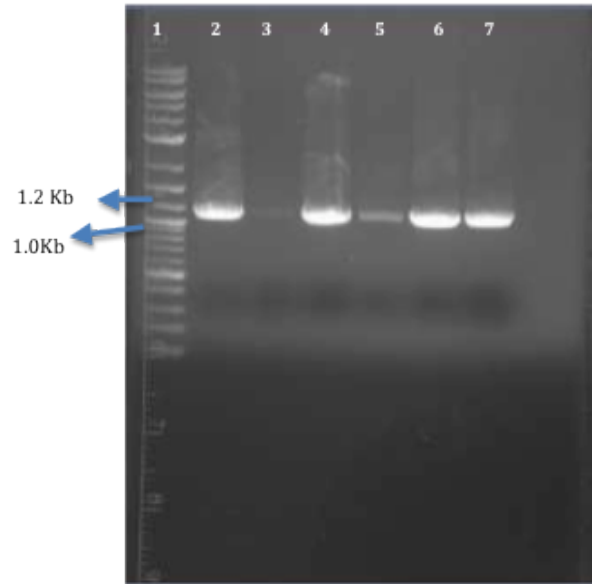


Figure 22: Agarose gel showing the screened plasmids of pACYCDuet for the insert by PCR. Lane1: Ladder Lanes 2: positive control Lane 3: negative control Lanes 4, 5, 6, 7: Transformants

After transformation into *E. coli* we now have the pACYC vector with *mmgC* gene. This sample was sent for the DNA sequencing and retransformed into the *E. coli* BL21 (DE3).

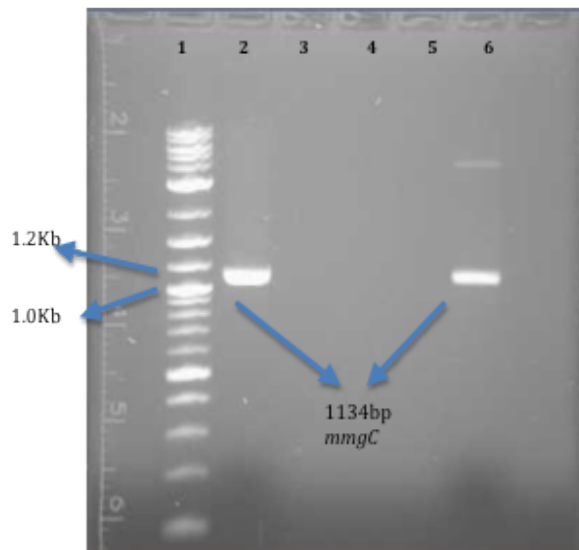


Figure 23: Agarose gel showing the PCR product before sequencing. Lane 1: Standards. Lane 2 and 6: PCR product of the sample to be sequenced. The expected size of *mmgC* is 1134 bps.

III.B.2 Cloning *mmgD* into pACYCDuet vector containing *mmgC*

Restriction digestion and ligation

We have the *mmgC* cloned into the vector pACYCDuet. The next step was to insert *mmgD* into the same vector containing *mmgC*.

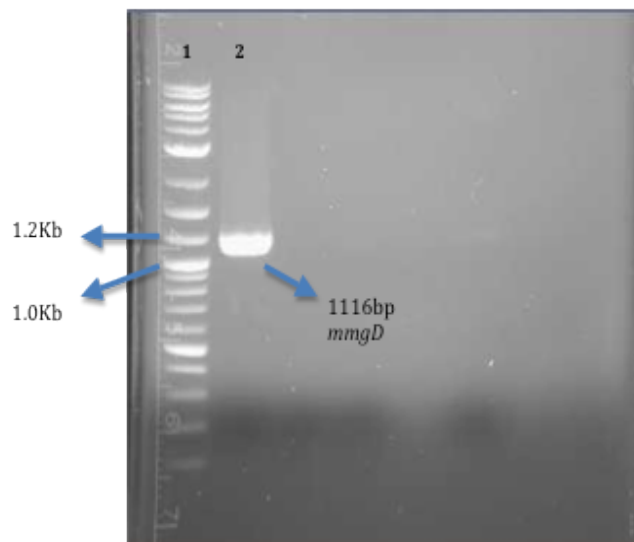


Figure 24: Agarose gel showing the amplified PCR product of *mmgD*. Lane 1: Ladder Lane 2: amplified PCR product. Lane 3: Negative control of the insert. The expected length of *mmgD* is 1116 bps.

mmgD and pACYC were cut with the restriction enzymes NdeI and XhoI and purified on a 1% agarose gel as shown in the figure 23.

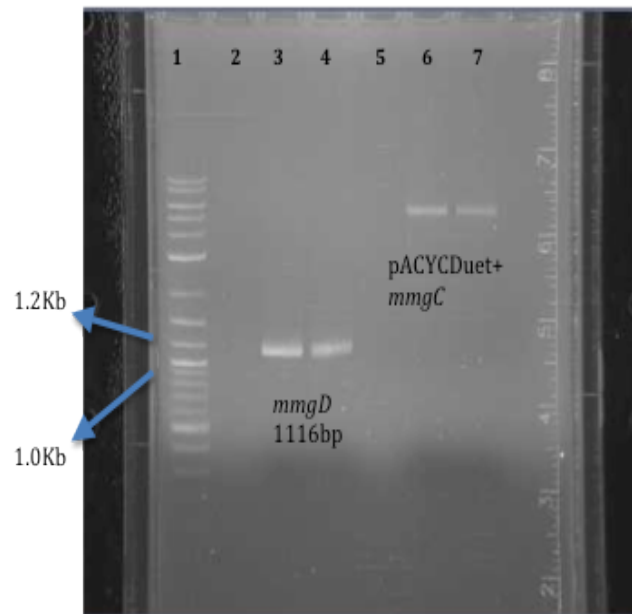


Figure 25: Agarose gel showing the insert and plasmid after restriction digestion. Lane 1: Standards Lane 3, 4: the Insert Lanes 6 and 7: Plasmid. Expected length of the insert *mmgD* is 1116bp and that of the pACYCDuet/*mmgC* is 5142bps.

The bands obtained from 1% agarose gel were gel extracted and ligated with T4-DNA ligase. Three individual colonies were picked up and screened for the presence of the plasmid that tested positive for the insert. All the three gave a positive result as shown in the figure 26.

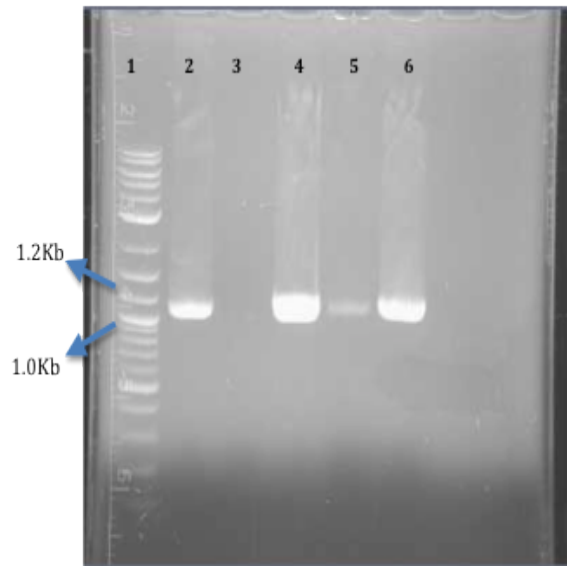


Figure 26: Agarose gel showing the screened plasmid samples for *mmgD*. Lane 1: Standards Lane 2: Positive control Lane 3: negative control Lanes: 4, 5, 6: Transformants.

The colony in the 4th lane was taken as it gave the brightest band and transformed into BL21 (DE3). A single colony was picked and tested with PCR before sending it for sequencing. It is shown in the figure 27.

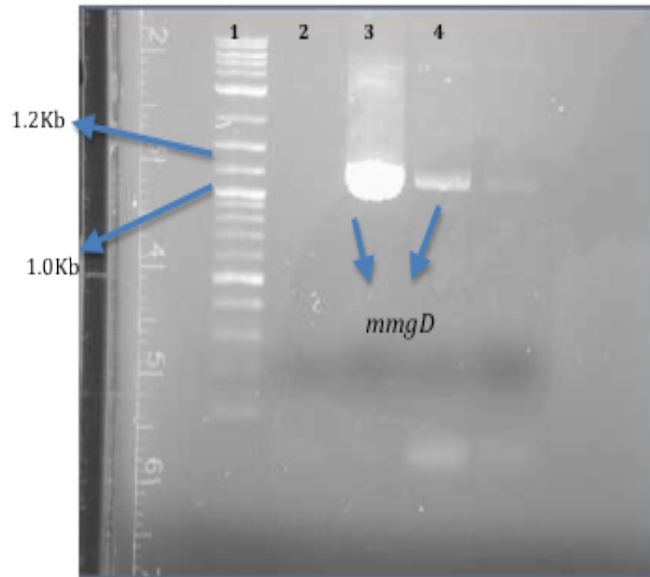


Figure 27: Agarose gel showing the PCR product before sequencing. Lane 1: Standards. Lanes 3, 4: *mmgD* PCR products.

Gene	100 % Sequence alignment	No .of base pairs in each gene
pETDuet- <i>mmg A</i>	Yes	1179
pETDuet- <i>mmg B</i>	Yes	732
pACYCDeut- <i>mmg C</i>	Yes	1134
pACYCDuet- <i>mmgD</i>	Yes	1116

Table 2: The results of the DNA sequencing of each gene.

III.C Mmg ABCD Purification:

MmgAB/pETDuet was transformed into BL21 (DE3). Then a culture of BL21(DE3) /*mmgAB*/pETDuet was made competent and transformed with

pACYCDuet/*mmgCD*. This resulted in a single strain of *E. coli* BL21 (DE3) containing both plasmids.

After successful transformation into *E. coli* BL21 (DE3), the *mmg ABCD* was over expressed in liquid culture. We incubated the induced culture overnight at 37 °C and 25 °C. At 37 °C, SDS-PAGE showed that large amounts of protein was produced but couldn't be purified due to inclusion body formation. This was observed by running an SDS gel of the whole cell extract, which couldn't give the appropriate protein band after purification. So in an effort to decrease the inclusion body formation and increase in the amount of protein production, overnight incubation was done at 18 °C.

To solve this problem, we also tried inducing with lower concentrations, (0.05 mM, 0.2 mM, 1 mM) of IPTG. Samples were collected at different stages of the preparation of the protein sample. The expected molecular weights of *mmgA* with the His-Tag is 41,019 Da, *mmgB* is 26,919 Da, *mmgC* is 40,710 Da and that of *mmgD* is 41,946 Da.

III.C.1. 10% SDS PAGE gel of BL21 (DE3) ABCD protein sample with 0.05 mM IPTG

The samples were collected at each stage in the preparation of the protein sample. In figure 28, a prominent band was seen at 26 KDa until the sample was

sonicated and centrifuged. After the centrifugation some of the bands were not observed. This could be because of the formation of inclusion bodies.. However, bands were seen around 40 KDa.

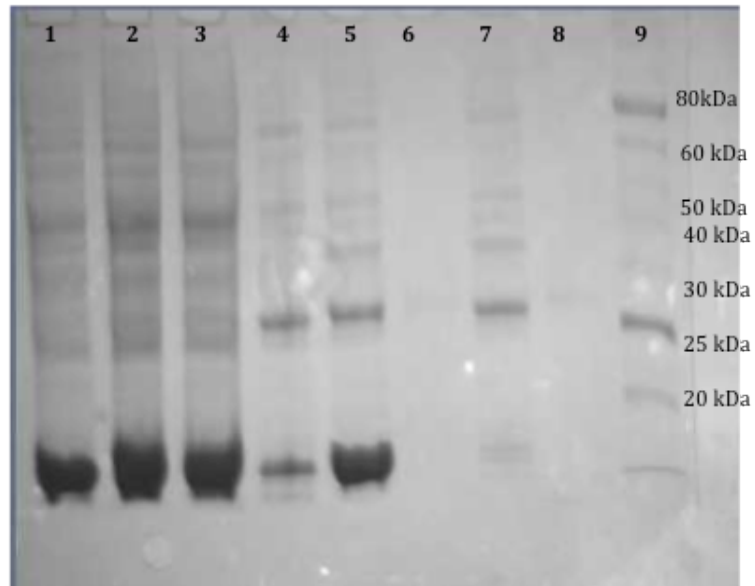


Figure 28: 10% SDS PAGE gel of BL21 (DE3) ABCD protein sample with 0.05 mM IPTG at 18 °C

(From Right to left) Lane 9: Protein ladder Lane 8: after overnight growth with IPTG Lane 7: After centrifugation Lane 6: Supernatant Lane 5: After adding Lysozyme Lane 4: After sonication Lane 3: before syringe filter Lane 2: crude extract. Lane 1: Column flow through of the crude extract.

Figure 29 is a continuation of figure 28. The samples from the column with different buffers were collected and analyzed on the SDS gel. In figure 29, the bands were not clear in any of the samples. We concluded that 0.05 mM of IPTG

is not best for the preparation of protein sample. So, we tried a different concentration, 1 mM IPTG.

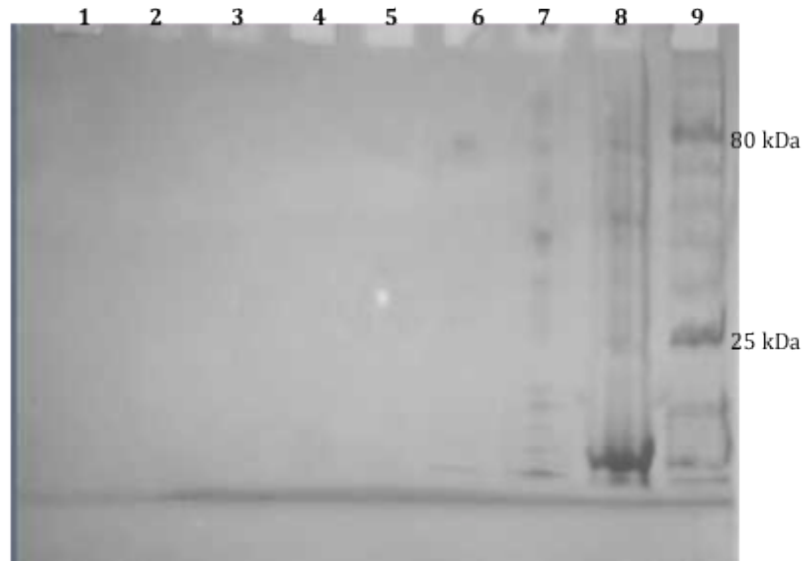


Figure 29: 10% SDS PAGE gel of BL21 (DE3) ABCD protein sample with 0.05 mM IPTG at 18 °C.(From Right to left) Lane 9: Protein Ladder. Lane 8: 5 mM imidazole. Lane 7: 60 mM imidazole. Lanes 3, 4, 5, 6: 1 M imidazole.

III.C.2. 10% SDS PAGE gel of BL21 (DE3) ABCD protein sample with 1 mM IPTG

As shown in figures 30, the crude extract had interacting proteins especially on the molecular weight range for mmg B. Those bands were not observed after the centrifugation step. In figure 31, the bands from the 1 M imidazole were not

clear, which could be because of the formation of inclusion bodies and being lost due to 1 mM IPTG.

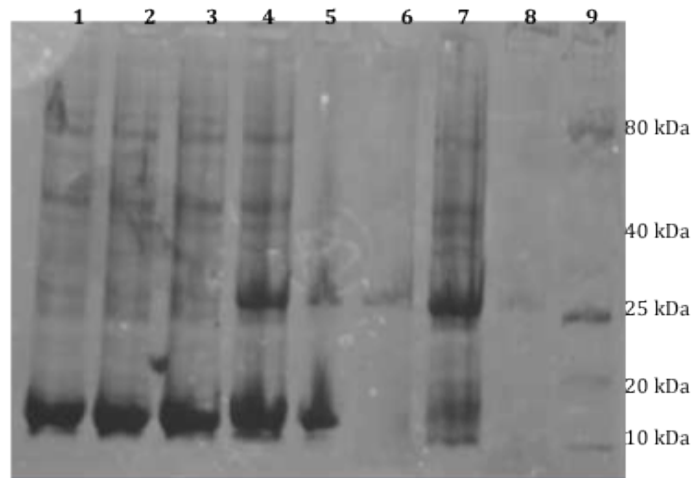


Figure 30: 10 % SDS PAGE gel of BL21(DE3) ABCD protein sample with 1 mM IPTG at 18 °C.(From Right to left) Lane 9: Protein ladder Lane 8: after overnight growth with IPTG Lane 7: After centrifugation Lane 6: Supernatant Lane 5: After adding lysozyme. Lane 4: After sonication Lane 3: before syringe filter. Lane 2: crude extract. Lane 1: Column flow through of the crude extract

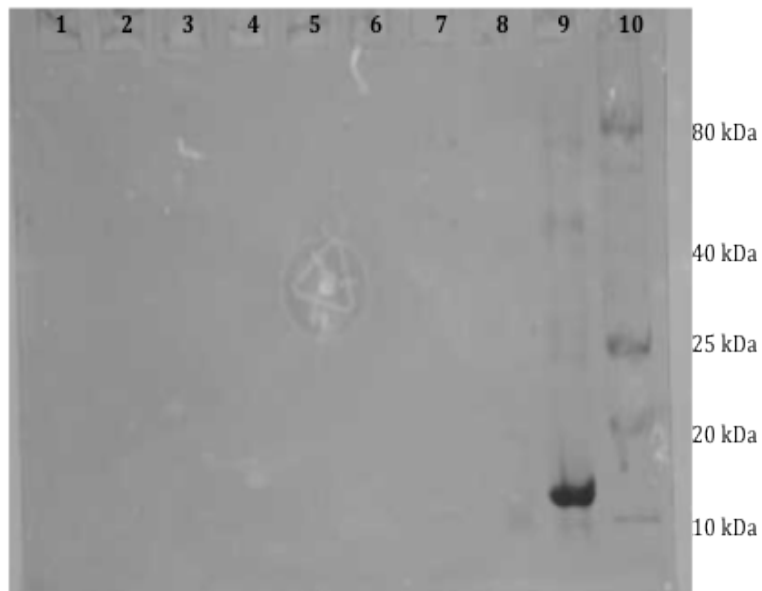


Figure 31: 10% SDS PAGE gel of BL21 (DE3) ABCD protein sample with 1 mM IPTG at 18 °C.

(From Right to left) Lane 10: Protein Ladder. Lane 9: 5 mM imidazole. sample
Lane 8: 60 mM imidazole. Lanes 4, 5, 6, 7:
1 M imidazole.

III.C.3. 10% SDS PAGE gel of BL21 (DE3) ABCD protein sample with 0.2

mM IPTG

In figure 32, we see 26 kDa band in all the samples. Bands around 40 kDa were seen after sonication in the crude extract and in the column flow through of the crude extract.

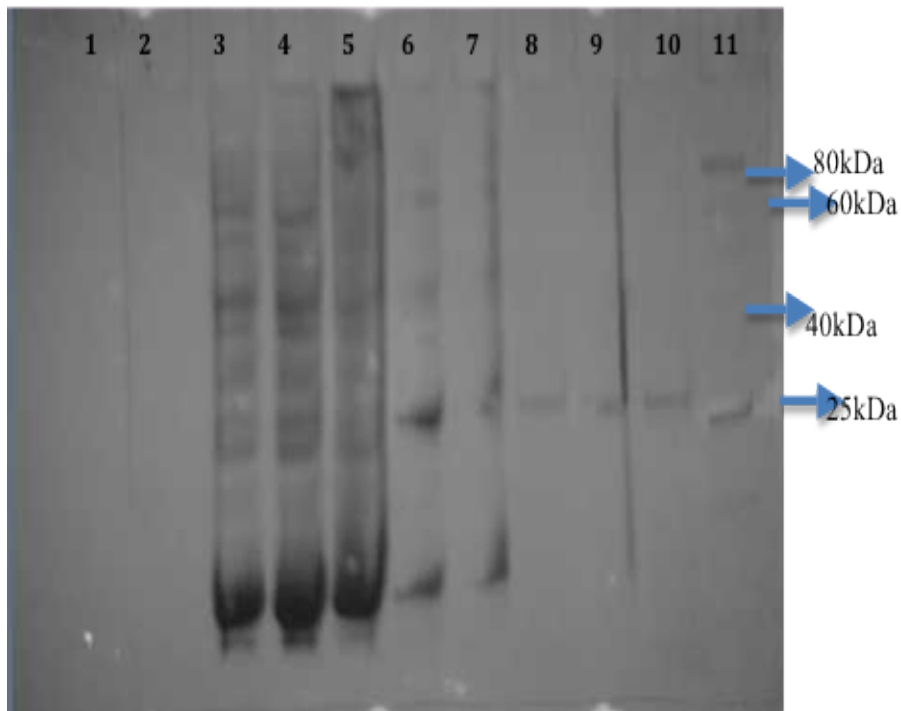


Figure 32: 10% SDS PAGE gel of BL21 (DE3) mmg ABCD protein sample with 0.2 mM IPTG at 18 °C
 (From Right to left) Lane 11: Protein ladder Lane 10: after overnight growth with IPTG. Lane 9: After centrifugation. Lane 8: Supernatant. Lane 7: After adding Lysozyme. Lane 6: After sonication but before centrifugation. Lane 5: before syringe filter. Lane 4: crude extract. Lane 3: Column flow through from loading crude extract.

In the figure 33, the column flow through of the 5 mM Imidazole and 60mM imidazole, bands were seen at 26 kDa and also around 40 kDa. One of the bands that was seen with 5 mM imidazole disappeared with 60 mM IPTG. In the first and second ml of the 1 M imidazole, bands were seen at the 26kDa and also 40kDa.

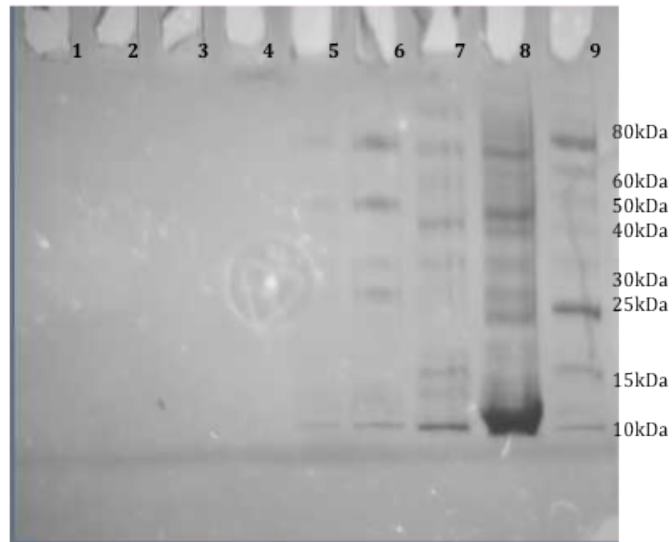


Figure 33: 10% SDS PAGE gel of BL21 (DE3) ABCD protein sample with 0.2 mM IPTG at 18 °C.(From Right to left) Lane 9: Protein Ladder Lane 8: 5mM imidazole Lane 7: 60 mM imidazole Lanes 3, 4, 5, 6: 1mM imidazole.

0.2 mM IPTG was used in the further experiments as it gave good and consistent results when compared to that of 0.05 mM and 1 M IPTG.

III.C.4. 10% SDS PAGE gel of BL21 (DE3) AB protein sample with 0.2 mM IPTG

In figure 34, it can be seen that the sample with only AB was run on the SDS gel. In the lane 8 we observed a band around 40 kDa. And also in figure 35 we observed faint bands around 40 kDa, with 1 M imidazole samples.

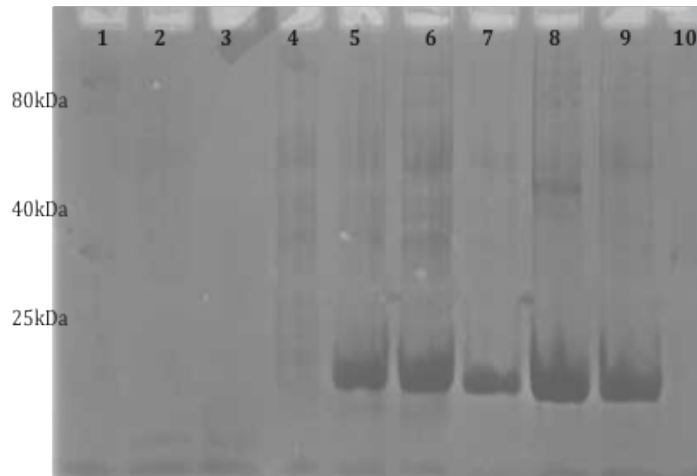


Figure 34: 10% SDS PAGE gel of BL21 (DE3) AB protein sample with 0.2 mM IPTG at 18 °C. Lane 1: Ladder. Lane 2: After overnight growth with IPTG. Lane3: Supernatant after centrifugation. Lane 4: Before adding Lysozyme. Lane 5: After adding Lysozyme. Lane 6: After sonication. Lane 7: Crude extract before column flow through. Lane 8: Column flow through of crude extract. Lane 9: 5 mM imidazole.



Figure 35: 10% SDS PAGE gel of BL21(DE3) AB protein sample with 0.2mM IPTG and at 18 °C. Lane 1: Ladder. Lane 2: 60 mM imidazole, Lane 3, 4, 5, 6: 1 M imidazole

III.C.5. 10% SDS PAGE gel of BL21 (DE3) CD protein sample with 0.2 mM

IPTG

In figure 34, we see the gel containing the protein sample of only mmg CD. We were able to observe bands in the crude extract after running through the column. Protein bands were not seen in the lanes containing 5 mM , 60 mM. A band around 25 kDa was seen in the 4th ml of the eluent ie; 1 M imidazole.

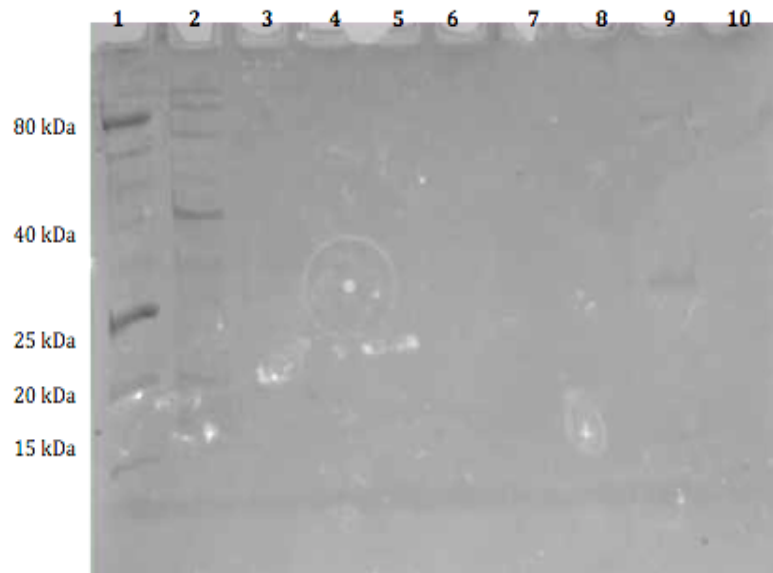


Figure 36: 10% SDS PAGE gel of BL21(DE3) CD protein sample with 0.2mM IPTG and at 18 °C. Lane 1: Ladder. Lane 2: Column flow through of the Crude extract. Lane 3: 5 mM imidazole. Lane 4: 60 mM imidazole. Lanes 5, 6, 7, 8, 9: 1 M imidazole

III.C.6 10% SDS PAGE GEL WITH BL21 (DE3) ABCD protein sample with 0.2mM IPTG .

Figure 37 shows the gel with bands of the BL21 (DE3) ABCD protein sample. We were able to see bands at 26 KDa and around 40 KDa in the crude extract and also the column flow through of the crude extract. The sample from the 1 M imidazole was not clearly seen but a faint band was seen around 40 KDa.

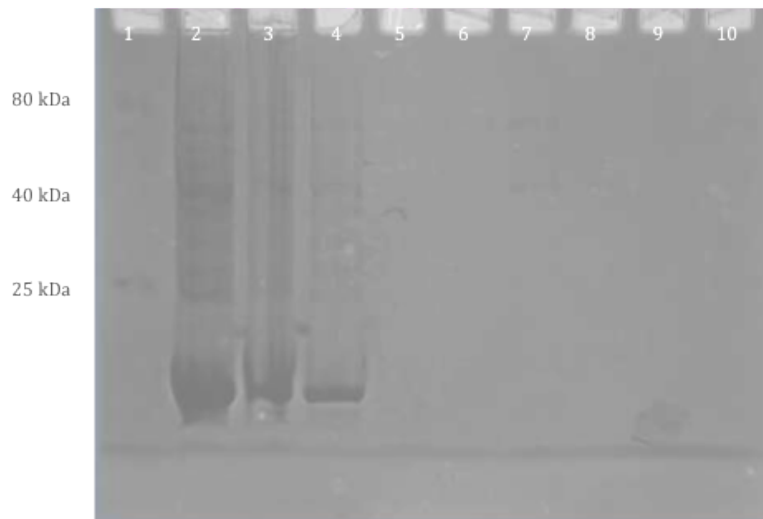


Figure 37: 10% SDS PAGE gel showing the BL21 (DE3) ABCD protein sample with 0.2mM IPTG and at 18 °C. Lane1: Protein Ladder Lane2: crude extract Lane 3: Column flow through of the crude extract Lane 4: Binding buffer. Lane 5: Wash buffer Lanes 6, 7, 8, 9: Eluent buffer.

Since we weren't able to clearly see the bands of the protein in the Eluent column flow through of BL21 (DE3) ABCD, the eluents of BL21 (DE3) AB, BL21 (CD), and BL21 (DE3) ABCD were concentrated using Centricon® Plus-20

Centrifugal Filter devices. These samples were analyzed on the 10 % SDS-PAGE gel as shown in the figure 38.

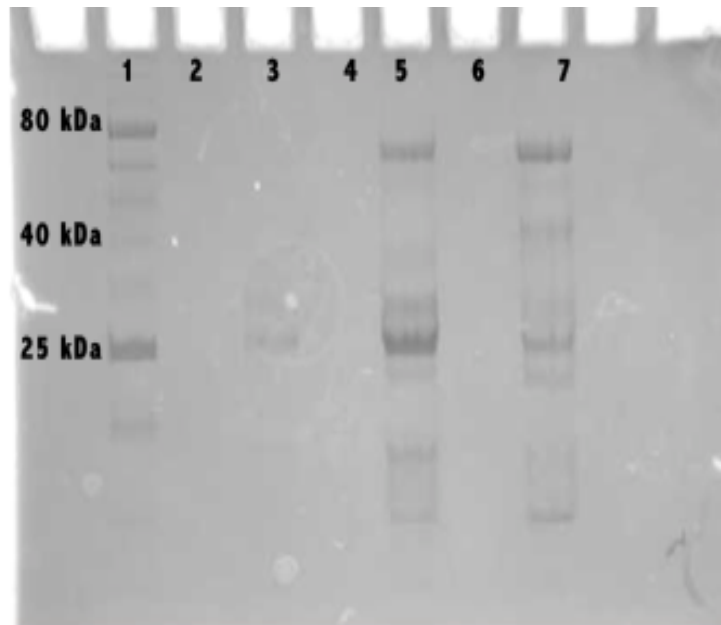


Figure 38: 10% SDS PAGE gel showing the concentrated Eluent column flow through of BL21 (DE3) ABCD protein sample. Lane 1: protein Ladder
Lane 3: Eluent of BL21 (DE3) CD protein sample, Lane 5: Eluent of BL21 (DE3) AB protein sample, Lane 7: Eluent of BL21 (De3) ABCD

In Figure 38, lane 3 has the BL21 (DE3) CD sample. We observed a band around 25 kDa, which we should not have seen since none of the proteins in the sample has a His-tag. This opens a point for future research. The lane 5 has the BL21 (DE3) AB sample. We observed a band around 25 kDa which is suggestive of MmgB, but we did not observe a band consistent with MmgA, although we did

observe a fuzzy band around 40 kDa. At this point we were expecting to see a clear band ~ 40 kDa because MmgA has a His-tag. Lane 7 has the BL21 (DE3) ABCD sample. We see a band at ~ 26 kDa which is suggestive of MmgB. We also observed a band around 40 kDa which could be MmgA or MmgC. We also observed a band at ~ 80 kDa. This protein likely originated from *E.coli*. MmgA is a close homolog of FadA. FadA is known to form a complex with FadB. Therefore it is possible that MmgA can likewise form a complex with FadB. Since MmgA has a His-tag, there are chances that the FadB protein from *E.coli* may have coeluted with MmgA.

CHAPTER IV
EXPERIMENTAL SECTION

IV.A Experimental Details:

IV.A.1: Cloning the mmgABCD genes.

Genomic DNA from *Bacillus subtilis* strain 168 (BS168) was isolated using the Wizard[®] Genomic DNA purification kit. This served as a template for the polymerase chain reaction experiments. Primers were designed to amplify the gene of interest. 10 µl of Phusion 5xHF buffer, 1 µl of 10mM dNTPs, 2 µl of the extracted BS168, 0.5 µl of Phusion polymerase and 1.25 µl each of upstream and downstream primers were used.

The thermo cycler was set up as 1X cycle of 98.0 °C for 1 min as the incubation temperature, followed by the 25X cycle primer extension temperatures of 98.0 °C for 0.05 mins as the denaturation temperature, 45°C - 70°C for 30 secs to 1 min as the annealing temperature, 72 °C for 30 sec to 1 min as the extension temperature for 30 cycles and 1x cycle of 72 °C for 10 mins and was held at 4°C. The annealing temperature depends on the T_m of the primers.

The PCR was setup for each gene with a difference in the annealing temperatures depending on the lowest T_m among the upstream and downstream

primers. The annealing temperature was 62 °C for mmgA and mmgD and 63°C for mmgB and mmgC

Primers:

mmgA:

- Upstream: 5' GAG GGG ATC CCA TGA GGA AAA CAG T 3'

BamHI -- restriction site enzyme.

T_m – 60.7 °C

- Downstream: 5'CAT GTC GAC ATG AAC CTG CAC TAA GA 3'

Sal I -- restriction site enzyme.

T_m – 59.3 °C

mmgB:

- Upstream: 5' CGG AGC ATA TGT TGA AAC GGC TGA AG 3'

XhoI -- restriction site enzyme.

T_m – 60.0 °C

- Downstream: 5' AAA CTC GAG TCA GGA AGT CTT CTC CT 3'

NdeI -- restriction site enzyme.

T_m – 60.2 °C

mmgC:

- Upstream: 5'GCG GAC ATG TAT GTA ACC CAA GAG C 3'

PciI -- restriction site enzyme.

T_m – 59.7 °C

- Downstream: 5' TTT TGT AAG CTT GGT TCC GCC AAG C 3'

NcoI -- restriction site enzyme.

T_m – 60.5 °C

mmgD:

- Upstream: 5' GTT GAG CAT ATG GAG GAG AAA CAG CA 3'

XhoI -- restriction site enzyme.

T_m – 59.1°C

- Downstream: 5' TAC CAG CTC GAG TCA TGA TTT TGT TT 3'

NdeI -- restriction site enzyme.

T_m – 59.8 °C

The PCR products were purified using QiaQuick[®] PCR Purification kit.

The PCR reactions were analyzed by 1% Agarose gel electrophoresis with ethidium bromide.

The desired restriction enzymes were added to both the target plasmid and PCR products. 1% Agarose gel was run to verify the products after the restriction digestion. Then the desired fragments were extracted from the gel using

QIAquick® Gel Extraction Kit and ligated using T4 DNA ligase, into the multiple cloning site of pETDuet, in a thermo cycler. The ligation temperatures were 37 °C for 30 mins, 23 °C for 2 hr 30 mins and held at 16 °C overnight.

The gene *mmgA* was cloned into the pETDuet vector. Then resultant ligated product was transformed into DH5 α *E.coli* competent cells and plated on LB agar containing ampicillin (50mg/ml). Individual colonies were picked and grown in LB medium containing ampicillin. Plasmids from each culture were tested for insert by PCR using the cloning primers followed by 1 % Agarose gel electrophoresis. Plasmids that tested positive for desired insert was retransformed into BL21 (DE3) strain and plated on the LB agar plate containing ampicillin. A colony was picked and grown in LB broth with ampicillin; plasmid purified and sent for sequencing. Also another colony was grown in LB containing ampicillin and stored at -80 °C in 10% glycerol.

The same procedure was followed to clone *mmgB* into the pETDuet vector containing *mmgA* (pETDuet/*mmgA*). Similarly *mmgC* was cloned in to the pACYC vector followed by *mmgD* into pACYC/*mmgC* vector, but chloramphenicol was used instead of ampicillin.

The resultant plasmid products are pETDuet-*mmgAB* and pACYCDuet-*mmgCD*.

All the four genes A, B, C, D (Duet system) are to be expressed in a single

host. To accomplish that, the BL21 (DE3)/pETDuet-mmgAB was streaked on to an LB plate containing ampicillin. A single colony was picked and grown on LB for 4-5 hrs. Centrifuged for 5 minutes at maximum speed. The supernatant was discarded and the pellet was dissolved in 3 ml of 50mM CaCl₂ and incubated on ice for 30 minutes. The culture was centrifuged and the pellet was redissolved in 1 ml of 50mM CaCl₂. For the transformation, 1-2 µl of pACYCDuet-mmgCD was added to 50 µl of competent cells. Incubated on ice for 30 mins followed by incubation at 37 °C for 2 mins and incubated at room temperature for 10 mins. One ml of LB was added and incubated at 37 °C for 1-2 hours. Then the cells are centrifuged at 8000 rpm for 3-5 mins, 1ml of the supernatant was discarded, and the cell pellet was resuspended in the remaining supernatant. Twenty µl of these cells were plated on the LB plates, containing both the antibiotics, chloramphenicol and ampicillin, and incubated at 37 °C overnight. Individual colonies of this strain BL21 (DE3)/pETDuet-mmgAB/pACYCDuet-mmgCD were picked for further cultures.

IV.A.2 Purifying the complex:

One colony (BL21 (DE3)/pETDuet-mmgAB/pACYCDuet-mmgCD) was inoculated into 5 ml LB containing 1µl of both the antibiotics, chloramphenicol (34mg/ml) and ampicillin (50mg/ml). The following day 2 ml of this starter

culture was inoculated into the 1liter LB containing ampicillin and chloramphenicol and allowed to grow at 37 °C. Samples were taken every 20 mins after 3-4 hrs and measured the absorbance until the optical density at 595 nm was 0.5-0.6. At this point Isopropyl-β-D-thiogalactopyranoside (IPTG) (final concentration of 1mM) was added and the culture was kept in the shaker at 18 °C overnight.

The cells from the overnight culture was centrifuged, the following day, at 6000 rpm for 30 mins at 4 °C. The pellet was washed with the 20ml of binding buffer (4M sodium chloride, 160 mM Tris, 40 mM imidazole) pH 7.9 and mixed with the magnetic stirrer and was transferred into a beaker and kept on ice. Lysozyme was be added and stirred for 15 mins and sonicated for 3 mins and the centrifuged at 11000 rpm for 30 mins at 4 °C. The resultant solution was filtered using a Corning[®] 0.45 micron, 26mm syringe.

After lysing the cells, the resultant solution was passed through a Ni-NTA column. We slowly increased the concentration of imidazole to remove the undesired proteins and to finally elute the mmgA along with the associated proteins with high concentration of imidazole.

We analyzed this elution mixture by SDS-PAGE. If the mmg proteins do not form a complex, then we expect to see only one band in the gel of the His-tagged mmgA (41019Da). If they form a complex, then we expect to see

additional bands matching the molecular weights of the other proteins (mmgB - 26919.2 Da, mmgC – 40710.9Da and mmgD – 41946.6 Da).

CHAPTER V

CONCLUSIONS AND FUTURE WORK

MmgAB were successfully cloned into pETDuet-1 and *mmgCD* were also cloned pACYCDuet-1. This was confirmed from by DNA sequencing data. All these plasmids were established in *E.coli* strain BL21 (DE3), to give a single strain, which can coexpress all four genes.

The transcription and translation was induced by the addition of IPTG to the culture. Because of the presence of an N-terminal His-tag on *mmgA*, a Ni-NTA column was used to purify the protein sample. After trying different concentrations of IPTG, 0.2 mM was found to be the best concentration for our experiments, as protein bands were seen clearly and consistently by SDS-PAGE when this concentration was used.

We were able to observe proteins with apparent molecular weights of 26 kDa and ~40 kDa, in the Elution buffer from cultures expressing all the genes. This suggests that *mmgB* may be interacting at least with *MmgA*.

In addition to the 26 kDa band (which may be *MmgB*), protein was also detectable in the 40-50 kDa range. Due to the low resolution of SDS-PAGE it is unclear whether this protein was comprised of only *MmgA* or additional proteins

with similar molecular weights (MmgC - 40710 Da, and MmgD- 41946 Da). At this stage, however, these results are suggestive that the *mmg* fatty acid degradative pathway enzymes indeed interact in a complex.

We will explore the composition of this protein mixture by MALDI-MS experiments that can also offer sequence data.

The experiments also reveal the identity of the ~80 kDa protein coeluting with the potential *mmg* complex. It is possible this ~80 kDa is the *E. coli* fatty acid metabolism enzyme. In the next steps, the identity of this protein in the coeluting with the complex is to be confirmed and measure the activity of the complex.

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