# Cloning, Expression and Purification of the Antimicrobial Targets *etfB* and *etfDh* of *Burkholderia cenocepacia*

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**Abstract**

*Burkholderia cenocepacia is a pathogenic bacterium that causes life-threatening infections in cystic fibrosis patients. Through a conditional growth mutant library, two essential genes of this bacterium, etfB and etfDh, encoding an electron transfer flavoprotein unit and an etf dehydrogenase respectively, have been identified. While the essential role of these proteins in B. cenocepacia is unknown, protein characterization and interaction analysis will aid in the development of their capacity as antimicrobial targets. The experimental goal of this study was to clone, express, and purify etfB and etfDh. Both genes were separately cloned into the bacterial plasmid pE-SUMO and transformed into E. coli BL21-DE3 GOLD. Protein expression of etfB and etfDh was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG), and the soluble and insoluble protein fractions were analyzed. etfB was found in the soluble fraction, which is expected as ETFs are cytoplasmic proteins. etfDh was mainly present in the insoluble fraction suggesting that the B. cenocepacia etfDh is a membrane-bound dehydrogenase. The protein purification process has resulted in a highly pure etfB and etfDh samples, which can be used to raise antibodies. Consequently, antibodies raised against these proteins will allow immunofluorescence and coimmunoprecipitation studies of etfB and etfDh. The results of the current work provide the tools to address the hypothesis of an intracellular interaction between cytoplasmic etfB and membrane-bound etfDh to further understand their role and essentiality in B. cenocepacia.*

**Keywords:** Antimicrobial Resistance, *Burkholderia cenocepacia*, Cystic Fibrosis, Electron Transfer Flavoprotein (ETF), Essential Genes

## 1. Introduction

*Burkholderia cenocepacia* is a Gram-negative bacterium belonging to the *Burkholderia cepacia* complex (Bcc), which is a group of Gram-negative $\beta$-proteobacteria (Shommu, Vogel, & Storey, 2015). Many species belonging to the Burkholderia genus are found in natural environments and are not pathogenic in non-immunocompromised humans (Lipuma, 2010). However, *B. cenocepacia* is a life-threatening pathogen in cystic fibrosis patients (Shommu et al., 2015). The lungs of these patients have an alteration in liquid present in the airways, which renders patients susceptible to infection (Lipuma, 2010). These infections cause continuous inflammation of high intensity and permanent damage to the airways and lungs (Cantin, Hartl, Konstan, & Chmiel, 2015). Chronic infections can lead to progressive lung disease and pulmonary failure, which is the primary cause of death in cystic fibrosis patients (Lipuma, 2010).

*B. cenocepacia* has been found to be resistant to multiple antibiotics including polymyxins, aminoglycosides, trimethoprim, chloramphenicol, quinolones and $\beta$-lactams (Shommu et al., 2015), making it difficult to eradicate when present in the lungs (Coutinho, de Carvalho, Madeira, Pinto-de Oliveira, & Sa-Correia, 2011).

These existing antibiotics target a narrow set of genes involved in essential bacterial functions, which for most, resistant strains have already emerged. Therefore, the study of new essential genes of *B. cenocepacia* as novel antimicrobial targets is critical in finding new treatment options for infected cystic fibrosis patients.

To identify essential genes of *B. cenocepacia*, a conditional growth mutant library was developed. Conditional growth mutants with rhamnose-inducible promoters were developed and used to assess the essentiality of these mutated genes (Bloodworth, Gislason, & Cardona, 2013). The essential genes discovered are defined as genes required for bacterial growth in rich, undefined media (Bloodworth et al., 2013). This development has allowed investigation of many of these genes and their encoded products as antimicrobial targets. Two essential genes, an electron transfer flavoprotein, subunit B *(etfB)* and etf dehydrogenase (*etfDh*) identified through this process, were the focus of this experiment. Although the genotype of *B. cenocepacia* has been investigated and essential genes have been identified, the delineation of the phenotype corresponding to these genes is lacking (Bloodworth, Zlitni, Brown, & Cardona, 2015). The current project is a critical step in the exploration of the specific functions of two essential genes involved in the electron transport chain in *B. cenocepacia*.

ETFs are a very broad group of proteins found in all kingdoms of life (Toogood, Leys, & Scrutton, 2007). The primary function of ETFs is to act as electron carriers between a variety of flavoprotein dehydrogenases (Toogood et al., 2007). For example, interactions between trimethylamine dehydrogenase and its ETF partner have been shown in *Methylophilus methylotrophus* (Shi, Mersfelder, & Hille, 2005). This type of interaction has also been identified in the mitochondrial respiratory chain, where ETF-QO transfers electrons from 11 dehydrogenases, to subsequently form energy (Watmough & Frerman, 2010).

While there is a great deal of diversity of ETFs, some distantly-related organisms can have very similar ETF conformations. This is illustrated by the crystal ETF structure of *Paracoccus denitrificans* being folded identical to the human ETF, except for a single loop region (Roberts, Salazar, Fulmer, Frerman, & Kim, 1999). Furthermore, in many organisms ETFs are found to be non-essential proteins. For example, *S. cerevisiae* ETF knockout strains were able to survive and grow, despite the lack of ETFs (Wanduragala, Sanyal, Liang, & Becker, 2010). This raises the need to further understand why *etfB* and *etfDh* are essential in *B. cenocepacia*.

*etfB* is an electron transfer flavoprotein containing FAD, allowing this protein to transfer electrons from different membrane-bound dehydrogenases to the respiratory chain (Winsor et al., 2008). *B. cenocepacia* cells depleted of *etfB* are not viable and lack redox potential (Bloodworth et al., 2015). The *etfDh* protein is the putative membrane-bound dehydrogenase and potential *etfB* partner (Bloodworth et al., 2015). It has also been shown that *B. cenocepacia* cells depleted of *etfDh* are not viable (Bloodworth et al., 2015).

The hypothesis of this study is that there is an intracellular interaction between cytoplasmic *etfB* and membrane-bound *etfDh* which is essential to *B. cenocepacia* survival. The experimental goal of this project is to clone, express, and purify the essential genes *etfB* and *etfDh*. Both genes of interest were separately cloned into pE-SUMO, then transformed into E. *coli* BL21-DE3 GOLD via heat shock therapy. Protein expression of *etfB* and *etfDh* was induced by IPTG, and the soluble and insoluble fractions of this induction were analyzed. *etfB* and *etfDh* were then purified via a Ni-NTA Fast Start column. In the future, the work of this paper will provide the tools for the study of protein function and interaction of *etfB* and *etfDh*.

## 2. Materials and Methods

### 2.1. Gene Cloning of *etfB* and *etfDh*

For cloning and expression of *etfB* and *etfDh* genes, the SUMOpro Gene Fusion Technology Kit, Kanamycin resistance (LifeSensors Inc.)

was used. The pE-SUMO vector is designed for expression of a protein of interest, where expression of the protein can be controlled by the T7 RNA polymerase system. pE-SUMO contains a Kanamycin (Km) cassette for selectivity.

#### 2.1.1 Preparation of *B. cenocepacia* K56-2 Genome

2 ml of overnight culture of *B. cenocepacia* K56-2 was pelleted and the supernatant discarded. Next, 500 $\mu$l of 0.85% saline was used to wash cells, then cells were pelleted and supernatant discarded. Cells were washed with 500 $\mu$l TES, pelleted and supernatant discarded. Cells were then re-suspended in 250 $\mu$l of T10E25. Furthermore, 50 $\mu$l of lysozyme solution (1 $\mu$l RNase) was added, cells were then incubated for 15 minutes at 37 °C. 60 $\mu$l of sarcosyl-protease was added and the culture was incubated overnight at 37 °C. To isolate the genome, 361 $\mu$l chloroform/isoamyl was added and the Eppendorf tube was gently inverted for 10 minutes. The mixture was spun at 10,000 rpm for 10 minutes and the top layer was transferred to a new tube, while avoiding the white middle layer. The above 2 steps were repeated 1 to 2 times, or until there is no more white precipitate. Then, 0.54 volumes of isopropanol, and 1/10 volume of 3M NH4OAc were added and the tube was gently inverted and left at room temperature for 30 minutes. The mixture was centrifuged at 13,200 rpm for 10 minutes at 4°C and supernatant was removed without disturbing the pellet. 1000 $\mu$l of ice cold 70% EtOH was added and the mixture was again centrifuged at 13,200 rpm for 10 minutes at 4°C and the supernatant was removed. Samples were then allowed to dry for approximately 15 minutes, until no visible ethanol remained in the tube. 100 $\mu$l of nuclease-free H2O was used to re-suspend the DNA.

#### 2.1.2 Empty Vector Extraction.

The pE-SUMO vector carrying Km resistance cassette was extracted from an overnight culture of E. *coli* BL21 using QIAprep Spin Miniprep kit (Qiagen) as recommended by the manufacturer. Briefly, 4 ml of the overnight culture was pelleted by centrifugation at 8,000 rpm for 3 minutes at room temperature. Pellet was then re-suspended in 250 $\mu$l Buffer P1 and transferred to a microcentrifuge tube. 250 $\mu$l Buffer P2 was added and mixed thoroughly by inverting tube 6 times until solution was clear. 350 $\mu$l Buffer N3 was immediately added and mixed by inverting the tube 6 times. Tube was then spun at 13,000 rpm for 10 minutes. The supernatant was decanted off of the pellet and applied to the QIAprep spin column and spun at 13,000 rpm for 1 minute. Flow through was discarded. To wash, 500 $\mu$l Buffer PB was added to the spin column and spun at 13,000 rpm for 1 minute, then flow through was discarded. The column was washed with 750 $\mu$l Buffer PE. The column was spun twice at 13,000 rpm for 1 minute: first, to discard flow through and second, to remove residual wash buffer. The *QIAprep column* was then placed into a 1.5 ml Eppendorf tube. To elute the DNA, 50 $\mu$l nuclease-free water was placed on the center of the column and was left at room temperature for 3 minutes. The tube was then spun at 13,000 rpm for 1 minute and the flow through was collected.

#### 2.1.3 Primer Design for *etfB* and *etfDh* Amplification.

Primers to amplify BCAL2935 and BCAL1468 genes of *B. cenocepacia* K56-2 (Table 1), which encode *etfB* and *etfDh* respectively, were designed using Geneious Software 8.5.1 and were purchased from Integrated DNA Technologies (IDT Inc). *BsaI* and *XbaI* restriction sites were included in forward and reverse primer sequences, respectively (Table 2). To ensure the primers did not bind to unspecific regions of the *B. cenocepacia* genome, we analyzed their homology by BLAST, which confirmed primer specificity to their appropriate binding sites, either BCAL2935 or BCAL1468 (Table 2).

**Table 1.** Bacterial Strains and Plasmids used in this study

| Strains and Plasmids | Features | Source |
|---|---|---|
| Strains | | |
| *B. cenocepacia* K56-2 | Cystic fibrosis clinical isolate | Dr. Eshwar Mahenthiralingam, Cardiff University (Mahenthiralingam et al. 2000) |
| *E. coli* BL21-DE3 GOLD | *E. coli* B F$^-$ *ompT hsdS*($r_B^-$ $m_B^-$) *dcm*$^+$ Tet$^r$ gal λ(DE3) *endA* Hte | Dr. Brian Marks, University of Manitoba |
| Plasmids | | |
| pE-SUMO | Circular dsDNA, Kan$^R$, T7 promotor and terminator, $His_6$-SUMO protein gene, MCS | SUMO pro Life Sensors Inc. |
| pAB1 | pE-SUMO derivative, *etfB* insert | This study |
| pAB2 | pE-SUMO derivative, *etfDh* insert | This study |

**Table 2.** Primer Sequences and Annealing Locations

| Primer Name | Sequence | Restriction Site | Annealing Location |
|---|---|---|---|
| Primer 671 | TATGGTCTCAAGGTATGAAAATCTTGGTGCCAGTGA | *BsaI* | BCAL2935 |
| Primer 673 | CATTCTAGATTACAGCACCTTGGCTTCGG | *XbaI* | BCAL2935 |
| Primer 699 | TATGGTCTCAAGGTATGACCCCCGCAAGCCTCAT | *BsaI* | BCAL1468 |
| Primer 700 | CATTCTAGATTACATGTTCGGGTAATTCGGCC | *XbaI* | BCAL1468 |
| Primer 156 | TAATACGACTCACTATAGG | None | T7 Promotor |
| Primer 155 | GCTAGTTATTGCTCAGCGG | None | T7 Terminator |
| Primer 691 | AGCACCTTCAGGATCTTCGC | None | BCAL2935 |
| Primer 692 | CCTGAAGGAAGCGGGTGTC | None | BCAL2935 |

#### 2.1.4 Amplification of *etfB* and *etfDh* genes.

The *etfB* and *etfDh* genes of *B. cenocepacia* K56-2 were amplified by PCR using Hotstar HiFidelity PCR Kit (Qiagen). The reaction mix for the amplification included water, 5x Hotstar buffer, HiFi Taq and primers (671 and 673 for *etfB* and 699 and 700 for *etfDh*). Due to the high guanine-cytosine (GC) content of *B. cenocepacia* genome, a parallel reaction adding Q buffer to the mix was carried out for both genes, for comparison with the no-Q condition as a negative control. The Q buffer aids in the optimization of gene amplification of high GC-content genomes. The optimal PCR conditions were first established by a temperature gradient for both pairs of primer. The PCR cycle used was as follows: 5 minutes at 95 °C for the initial activation step followed by 30 cycles of: 15 seconds of denaturation at 94 °C, annealing stage for 1 minute run on a temperature gradient, 1 minute at 72 °C for the extension stage, and a final extension at 72 °C for 10 minutes.

#### 2.1.5 PCR Product Purification of *etfB* and *etfDh*.

250 $\mu$l Buffer PB was added to 50 $\mu$l PCR product and the mixture was transferred to a *QIAquick column* (Qiagen). The mixture was centrifuged at 13,000 rpm for 1 minute and the supernatant was discarded. 750 $\mu$l Buffer PE was then added to the column, spun at 13,000 rpm for 1 minute and the flow through was discarded. The column was spun at 13,000 rpm for 1 minute to remove any residual wash buffer. The *QIAquick column* was moved to a collection tube and 50 $\mu$l nuclease-free water was added to the center of the column and left at room temperature for 3 minutes. The column was spun at 13,000 rpm for 1 minute and the flow through was collected.

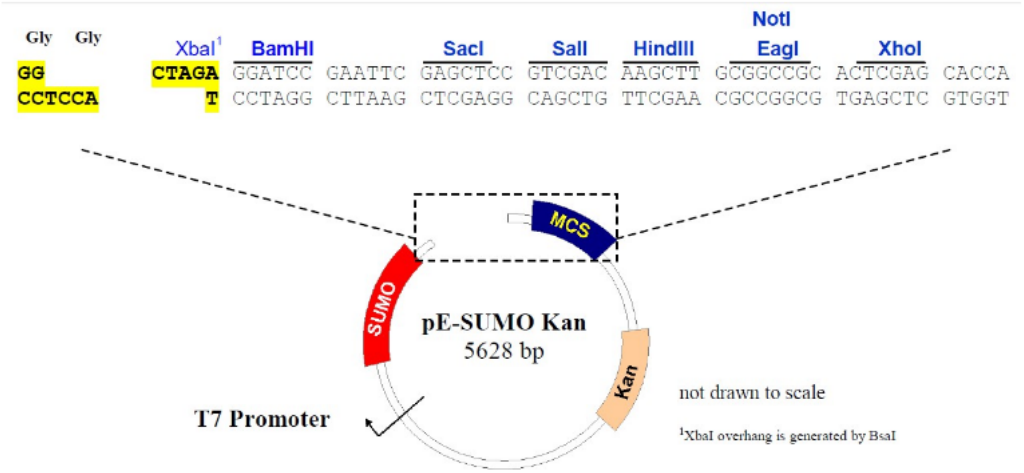


**Figure 1:** *pE-SUMO vector was used in this study, as etfB and EtfDh were inserted within, forming pAB1 and pAB2, respectively. pE-SUMO carries a Kanamycin selection marker. It also contains a Histidine6-SUMO protein fusion tag. pE-SUMO contains T7 Promoter and expression is controlled by T7 RNA polymerase produced from the host cell. Figure by LifeSensors Inc.*

#### 2.1.6 Restriction Digestion of Inserts and Vector.

The restriction digest reaction mixture for both inserts was 30 $\mu$l of insert PCR product, 5 $\mu$l 10x Cutsmart buffer, 13 $\mu$l water, 2 $\mu$l *BsaI* and 2 $\mu$l *XbaI* (New England Biolabs Inc). The pE-SUMO vector restriction digest mixture included 30 $\mu$l of vector DNA, 5 $\mu$l 10x Cutsmart buffer, 11 $\mu$l water and 2 $\mu$l *BsaI* enzyme, which recognizes non-palindromic sequences in pE-SUMO, leading to the production of both *BsaI* and *XbaI* restriction overhangs (Figure 1). In all reaction mixtures, the restriction enzymes were added last. All tubes were incubated in a water bath at 37 °C for 2 hours, and subsequently cleaned via the QIAquick Kit, previously mentioned.

#### 2.1.7 Ligation of pE-SUMO Vector and Gene Insert.

Digested inserts (*etfB* and *etfDh*) were ligated into digested pE-SUMO vector using T4 DNA ligase (New England BioLab Inc). Three specific volume ratios of vector to insert were prepared, 1:1, 1:2 and 1:3 at a final volume reaction of 20 $\mu$l. 2 $\mu$l 10x T4 Buffer and 1 $\mu$l T4 DNA ligase were added to each reaction mixture. A negative control of each ratio was also prepared by removing a small aliquot of reaction mixture before T4 DNA ligase was added. All reactions were incubated overnight at 16 °C. The results were confirmed by 1% agarose gel electrophoresis.

#### 2.1.8 Transformation into competent E. *coli* BL21-DE3 GOLD strain.

8 $\mu$l of ligation reaction of each product, pE-SUMO containing *etfB* and pE-SUMO containing *etfDh*, at each volume ratio (1:1, 1:2, and 1:3) were mixed with 100 $\mu$l ice cold E. *coli* BL21-DE3 cells (previously prepared). 8 $\mu$l of water was used in place of the ligation reaction in a separate reaction as a negative control. The mixtures were exposed to heat shock therapy to induce transformation. Briefly, the samples were kept on ice for 15 minutes, then placed in the 42 °C water bath for 45 seconds. Samples were then returned to ice for 2 minutes. Afterwards, 1 mL SOC medium was added and the tubes were incubated on the shaker for 90 min-

utes at 37 °C. 100 $\mu$l of each sample was then plated on pre-warmed LB plates containing 35 $\mu$l/mL of Km.

#### 2.1.9 Colony PCR verifying Positive Clones.

Colonies that grew on LB plates with Km following 16 hours of incubation at 37 °C were picked and plated with a numbering system. Colony PCR was performed on 15 colonies of each constructed plasmid. The PCR mixture consisted of 6.3 $\mu$l water, 1 $\mu$l 10x Buffer, 0.2 $\mu$l dNTPs, 0.2 $\mu$l Primer 155, 0.2 $\mu$l Primer 156, 0.1 $\mu$l Taq polymerase and 2 $\mu$l of re-suspended (in water) colony. The PCR cycle used was as follows: 5 minutes at 95 °C for the initial activation step followed by 25 cycles of: 30 seconds of denaturation at 95 °C, annealing stage for 30 seconds at 50 °C, 2 minutes at 72 °C for the extension stage, and a final extension at 72 °C for 10 minutes.

#### 2.1.10 Sequence Confirmation of Plasmids.

After confirmation of the presence of insert, the plasmids were renamed as: pAB1 (pE-SUMO carrying *etfB*) and pAB2 (pE-SUMO carrying *etfDh*). Plasmids pAB1 and pAB2 were extracted via the QIAprep Spin Miniprep Kit (Qiagen). Nanodrop analysis was performed on all the samples. Subsequently, 4 samples of *etfB*-containing plasmid were concentrated into one tube by desiccation. The concentrated sample was sent, along with internal primers (Primers 691 and 692) and T7 primers (Primers 155 and 156), for sequencing.

### 2.2. Expression of *etfB* and *etfDh* proteins

#### 2.2.1 Total Protein Sample Analysis E. *coli* BL21-DE3 GOLD Cells containing pAB1.

Two overnight cultures of each pAB1 clones 2 and 3 were grown at 37 °C in LB broth plus Km 35 $\mu$l/mL and sub-cultured to an OD600 of 0.6. Sample 1 was 5 ml pAB1 clone 2 induced with 1 mM IPTG. Sample 2 was 5 ml pAB1 clone 2 control, with no added IPTG. Sample 3 was 5 ml pAB1 clone 3 induced with 1 mM IPTG. Sample 4 was 5 ml pAB1 clone 3 control, with no added IPTG. All 4 samples were incubated for 3 hours at a temperature of 28 °C. After incubation, 0.5 ml of each sample was transferred separately to a new tube and frozen at -80 °C over 72 hours. After 72 hours, the 4 samples were thawed and spun down for 5 minutes at 13,000 rpm. Pellets were kept and re-suspended in 1x SDS Loading Buffer to a final concentration of 10 $\mu$l/mg of bacterial cells. Samples were then boiled for 10 minutes at 95 °C and spun down for 1 minute at 13,000 rpm. Finally, 5 $\mu$l from the top of the supernatant was loaded into wells of 12% SDS PAGE gel stained in Coomassie Blue dye.

#### 2.2.2 Mass Spectrometry Analysis of *etfB*.

A sample of the experimentally expressed *etfB* was sent to Dr. Lynda Donald, of the Department of Physics and Astronomy at the University of Manitoba, for mass spectrometry analysis for protein identity confirmation. The *etfB* sample was digested with trypsin and analyzed by MALDI mass spectrometry. The translation of the *etfB* reading frame was evaluated by the calculated m/z values of ions expected from a tryptic digest of *etfB*. These values were then compared to the ions present in the MALDI spectrum and the matching sequences were recorded.

#### 2.2.3 Soluble and Insoluble Protein Sample Analysis of *etfB* and *etfDh*.

For the induction and analysis of *etfB*, 3 samples of pAB1 clone 2 were grown overnight at 37 °C in LB broth plus Km 35 $\mu$l/mL, sub-cultured, and grown to an OD600 of 0.6. IPTG was added to a final concentration of either 0.5 mM or 1 mM to 2 of the 3 experimental tubes, while the third tube acted as a control (no IPTG added). All 3 tubes were incubated for 3 hours at 28 °C. The samples were spun down at 6,000 rpm for 3 minutes and the pellets were stored

at -20 °C. 500 $\mu$l TNG buffer was added to each pellet, being careful not to disturb the pellet. All samples were kept on ice and broken by 3 cycles of sonication, where each cycle involved 6 sonication pulses and a spin down for 1 minute at 13,000 rpm. Afterwards, all 3 samples were centrifuged for 20 minutes at 13,000 rpm at 4 °C. The supernatant of each sample was removed and kept as the soluble protein fraction, while the 3 pellets were re-suspended in 500 $\mu$l TNG buffer and named the insoluble protein fraction. Finally, all 6 samples were measure by Nanodrop (see Table S2) to allow proper amount of samples loaded in 12% SDS PAGE gel. A second procedure was carried out for pAB2 clone 1 to induce *etfDh* expression, following the same protocol outlined above.

## 2.3. Purification of *etfB* and *etfDh*

### 2.3.1 Purification of *etfB* under Native Conditions.

The purification of soluble *etfB* was performed by QIAexpress Ni-NTA Fast Start kit, which is used for purification of recombinant 6xHis-tagged proteins (Qiagen). Briefly, an overnight of pAB1-containing E. *coli* BL21-GOLD cells were subcultured into 250 ml LB Km35 until an OD600 of 0.6. The sample was then induced to with 1 mM IPTG and incubated at 28 °C for 4 hours. Cells were then harvested by centrifugation at 8000 rpm for 20 minutes. The pellet was stored overnight at -80 °C. Then, the pellet was thawed on ice for 15 minutes then resuspended in 10 ml native Lysis Buffer and incubated on ice for 30 minutes with gentle swirling 2 – 3 times. The sample was then centrifuged at 11,000 rpm at 4 °C for 30 minutes to pellet the cellular debris, and the supernatant was retained. In our first attempt of *etfB* purification, we followed the exact protocol from the QIAexpress Ni-NTA Fast Start kit, which commenced with the application of cell lysate to the Ni-NTA column and collection of flow-through. Then, we washed the column two times with 4 ml of native Wash Buffer. Finally, we eluted our protein of interest with two 1 ml aliquots of Native Elution Buffer. Our elution samples contained co-eluted proteins; therefore we made alterations in the protocol. Once the supernatant was retained (as stated above), the supernatant was applied to the column. Next, the column was washed with 4 ml of our Lysis Buffer (50mM Tris pH 8.0, 1M NaCl, 0.1 mM PMSF). A second wash was applied of which consisted of 4 ml Lysis Buffer (50mM Tris pH 8.0, 1M NaCl, 0.1 mM PMSF) plus 25 mM imidazole. Then, a third 4 ml wash was applied, consisting of Lysis Buffer (50mM Tris pH 8.0, 1M NaCl, 0.1 mM PMSF) plus 50 mM imidazole. Finally, we eluted our protein of interest with two 1 ml aliquots of Lysis Buffer (50mM Tris pH 8.0, 1M NaCl, 0.1 mM PMSF) plus 250 mM imidazole. Dialysis was performed on the purified elution samples against 1 L of 50 mM Tris pH 8.0, 150 mM NaCl, 2 mM DTT overnight at 4 °C. The next day, dialysis was performed against 1 L of 50 mM Tris pH 8.0, 150 mM NaCl, 2 mM DTT, 30% glycerol for ~4 hours at 4 °C. Purified elution samples of *etfB* were then stored at -20 °C.

### 2.3.2 Purification of *etfDh* under Denaturing Conditions.

The purification of insoluble *etfDh* followed the same procedure as *etfB*, with a few adjustments. An overnight of pAB2-containing E. *coli* BL21-GOLD cells were subcultured into 250 ml LB Km35 until an OD600 of 0.6. The sample was then induced to with 1 mM IPTG, incubated at 28 °C for 4 hours, harvested cells by centrifugation at 8000 rpm for 20 minutes and then the pellet was stored overnight at -80 °C. To commence the purification process, the pellet was resuspended in 10 ml native Denaturing Lysis Buffer and incubated at room temperature for 60 minutes with gentle swirling 2 – 3 times. The sample was then centrifuged at 11,000 rpm at room temperature for 30 minutes to pellet the cellular debris, and the supernatant was retained. The supernatant was retained and applied to the Ni-NTA column.

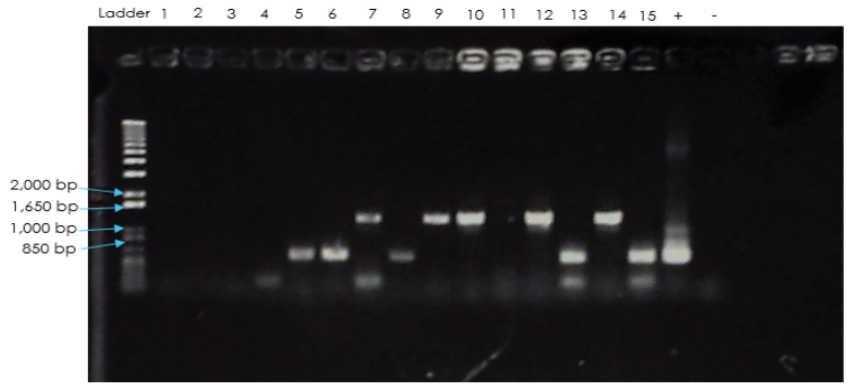


**Figure 2:** *Results of Colony PCR of E. coli BL21DE3 GOLD to analyze clones containing pAB1. PCR performed with T7 primers and T7 enzyme. Lanes 1 – 15 represent clones able to survive overnight on LBKm35. Lane 16 is signified as our positive control, as it represents the pE-SUMO vector with no etfB insert. Lane 17 is signified as our negative control, as it represents water added to the T7 primer mixture. Colonies from lane 7, 9, 10, 12 and 14 were identified as clones containing pAB1, as the band is present at ~1.3 kb, which is the expected band of etfB plus the restriction digest overhangs and T7 amplified plasmid area. The experiment moved forward with colonies from lane 9, 10, 12 and 14.*

## 3. Results

### 3.1. Cloning of *etfB* and *etfDh*

The optimal annealing temperature for the *etfB* amplification, using primers 671 and 673, was determined as 55 °C. The *etfDh* gene was successfully amplified using primers 699 and 700 with annealing temperature of 54 °C. In both sets of primers above, the forward primer contains the *BsaI* restriction site while the reverse primer contains the *XbaI* restriction site. During the transcription and translation of the two genes encoding our proteins of interest, we knew the proper reading frame must be retained. This was a factor in our design of these primers. A spacing of five bases between the *BsaI* restriction site and start codon ATG was on the forward primer and the *XbaI* restriction site on the reverse primer was designed to be immediately adjacent to the complementary stop codon TTA. In this way, we retained the reading frame needed for proper translation. We successfully amplified *etfB* and *etfDh* in the Q solution condition, while *etfB* and *etfDh* were not amplified in the no-Q condition, as these conditions are not optimized for amplification of genes with high GC content (see Figure S1). The restriction digestions of *etfB*, *etfDh* and pE-SUMO showed good concentration and both the *etfB* insert and the *etfDh* insert were separately and successfully ligated into the pE-SUMO vector and transformed into competent E. *coli* BL-21 DE3 GOLD cells. We recovered numerous colonies on the LB Km plates and performed colony PCR on these colonies to check for the presence of either *etfB* or *etfDh* within the plasmid.

The PCR of colonies 1 to 5 of each ligation ratio was negative for the presence of *etfB* gene. Therefore, we performed a second colony PCR on colonies 6 to 10 of each ligation ratio. Four colonies showed evidence of the presence of *etfB* insert. In Figure 2, the ~1.3 kb band seen on the gel is representative of *etfB*, the restriction digest overhangs and the T7 plasmid amplified area.

The PCR of colonies 1 to 5 of each ligation ratio was checked for the presence of *etfDh*. As shown in Figure 3, the presence of a strong band at ~2.3 kb, representing *etfDh*, was observed in one colony (Figure 3).

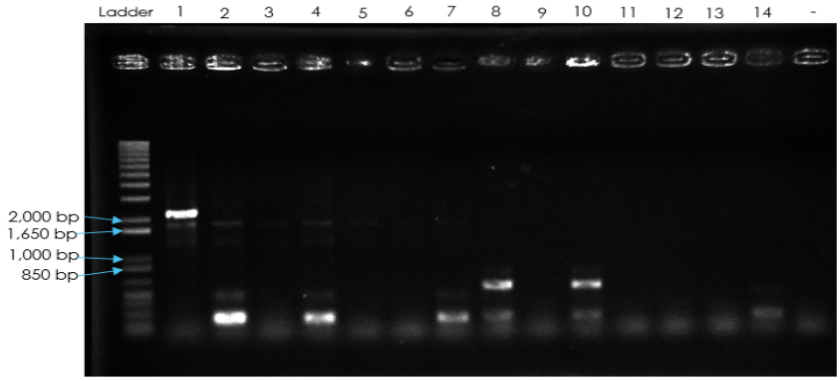


**Figure 3:** *Results of Colony PCR of E. coli BL21-DE3 GOLD to analyze clones containing pAB2. PCR performed with T7 primers and T7 enzyme. Lanes 1 – 14 represent clones able to grow overnight on LBKm35. Lane 15 is the negative control, as it represents water added to the T7 primer mixture. Colony 1 of the 1:1 ligation ratio found in lane 1 was identified as the clone containing pAB2, as the band is present at ~2.3 kb, which is the expected band of etfDh plus the restriction digest overhangs and T7 amplified plasmid area*

We extracted the plasmids within the identified positive clones and renamed the sample of extracted plasmids containing the *etfB* insert as pAB1. Colony 9 of the 1:2 ligation ratio is pAB1 clone 1, colony 10 of the 1:2 ligation ratio is pAB1 clone 2, colony 7 of 1:3 the ligation ratio is pAB1 clone 3 and colony 9 of the 1:3 ligation ratio is pAB1 clone 4. The sample of the extracted plasmid containing the *etfDh* insert in colony 1 of the 1:1 ligation ratio was renamed pAB2 (see Table S1).

Sequencing of two clones of pAB1, with internal primers (Primers 691 and 692) and T7 primers (Primers 155 and 156), revealed that both pAB1 clones contained *etfB* in the correct orientation and there were no mutations in the gene itself, reassuring the translational coding frame will be correct. Sequencing of pAB2 clone 1 is underway.

## 3.2. Expression of *etfB* and *etfDh* proteins

We performed total protein extraction of the E. *coli* BL21-DE3 GOLD cells containing pAB1 (Figure 4). The band of interest is 41.6 – 46.6 kDa, which represents the 26.6 kDa *etfB* protein with its attached SUMO protein tag, which is 15 – 20 kDa. We can deduce that in the IPTG induction conditions, pAB1 was transcribed and translated at a higher amount than other proteins of the cell. This is also emphasized when comparing to the negative control, where the expression of *etfB* was not induced, and therefore there is not a strong band at 41.6 – 46.6 kDa. As both pAB1 clone 2 and 3 show near identical results by IPTG induction, moving forward we worked solely with pAB1 clone 2. The results of mass spectrometry analysis have confirmed we have expressed *etfB* as our protein of interest (Figure 5).

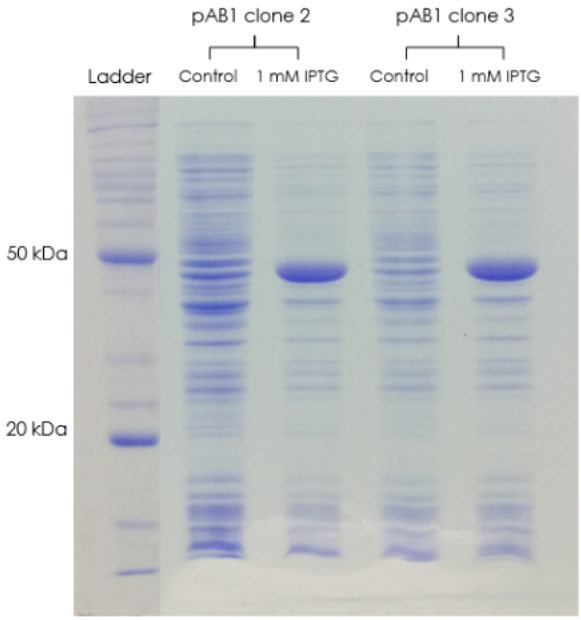


**Figure 4:** *12% SDS PAGE gel of total protein extraction and analysis of EtfB protein of B. cenocepacia K56-2 transformed and expressed in competent E. coli BL21-DE3 GOLD cells. Cells were induced by 1 mM IPTG (with negative controls to compare) at 28 °C. The size of the EtfB protein (26.6 kDa) with attached SUMO protein tag (15-20 kDa) is a total of 41.6 – 46.6 kDa.*

MKILVPVKRV VDYNVKVRVK SDNTGVDIAN VKMSMNPFDE IAVEEAVRLK
EAGVATEVIA VSVGVTQAQE TLRTALAIGA DRAILVESND SVEPLAVAKI
LKVLVDKEQP QLVILGKQAI DDDSNQTGQM LAALAGLPQA TFASKVTVAD
GKATVAREVD GGAETLSLTL PAVVTTDLRL NEPRYVTLPN IMKAKKKPLE
TVKPEDLGVD VAPRLKTLKV VEPPKRAAGV KVPDVKTLVE KLKTEAKVL

**Figure 5:** *Translation of EtfB Reading Frame by Mass Spectrometry. m/z values of ions expected from a tryptic digest of EtfB were calculated and compared to the ions present in the MALDI spectrum. Underlined sequences above are the matching sequences between the EtfB protein and the ions present in the MALDI spectrum. Performed by Lynda Donald in the Department of Physics and Astronomy of the University of Manitoba.*

We then analyzed the soluble and insoluble fractions of *etfB* and *etfDh*, in regards to the IPTG induced samples versus our negative control samples. In both cases of either *etfB* or *etfDh* induction, we determined the IPTG was successful in the induction of a large amount of protein. As shown in Figure 6, the soluble and insoluble fraction of the IPTG induced *etfB* samples contain a large quantity of *etfB*, with a size of 41.6 – 46.6 kDa, including the protein and SUMO protein tag. Analyzing specifically the soluble and insoluble fraction of the IPTG induced *etfDh* samples, we saw that the insoluble fraction contains a very high quantity of protein. The band is found at 75.6 – 80.6 kDa, when including the 60.6 kDa protein and the 15 – 20 kDa SUMO protein tag. *etfDh* was absent in the soluble fraction.

## 3.3. Purification of *etfB* and *etfDh*

The purification of *etfB* protein of *B. cenocepacia* K56-2 is seen in Figure 7. The size of *etfB* protein with attached SUMO protein tag is 41.6 – 46.6 kDa. The non-induced and IPTG induced samples serve as negative and positive controls, respectively. The supernatant illustrates an ample amount of *etfB* was found in the cell

lysate during the purification protocol. Examining the supernatant flow-through, we can see that a small portion of *etfB* did not bind to the column when the supernatant was applied, most likely due to the saturation of the column. The three washing steps show minimal loss of *etfB* while many contaminating proteins were washed off the column, which is illustrated by the purity of the two elution samples, in particular the second elution sample.

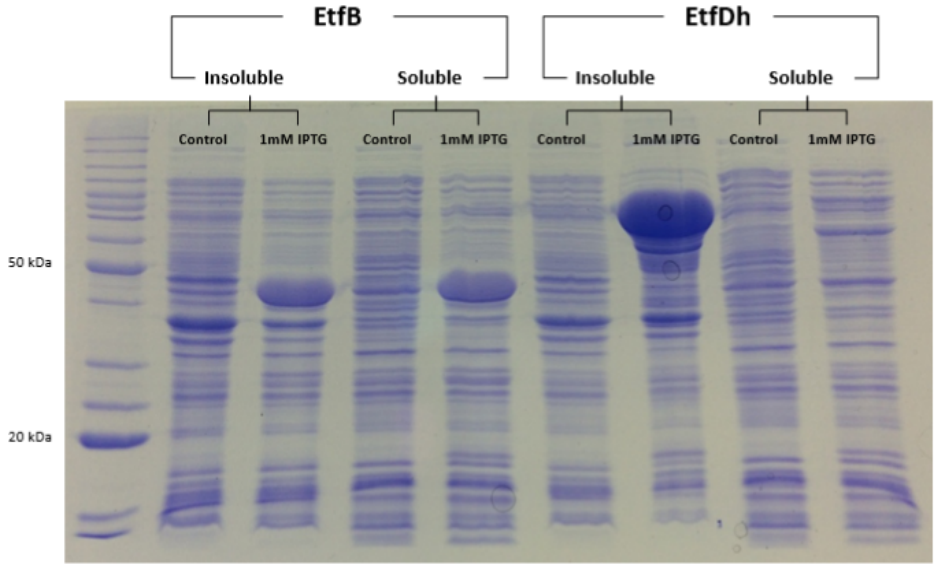


**Figure 6:** *12% SDS PAGE gel of Soluble and Insoluble fraction analysis of EtfB and EtfDh proteins of B. cenocepacia K56-2 transformed and expressed in competent E. coli BL21-DE3 GOLD cells. Competent cells were induced by 1 mM IPTG (with negative controls to compare) at 28 °C and broken via sonication. The size of EtfB protein with attached SUMO protein tag is 41.6 – 46.6 kDa. The size of EtfDh protein (60.6 kDa) with attached SUMO protein tag is 75.6 – 80.6 kDa.*

The purification of *etfDh* protein of *B. cenocepacia* K56-2 is seen in Figure 8. The size of *etfDh* protein with attached SUMO protein tag is 75.6 – 80.6 kDa. The IPTG induced sample serves as a positive control. The supernatant illustrates an ample amount of *etfDh* found in the cell lysate. As seen in the supernatant flow-through lane, a large amount of *etfDh* did not bind to the column when the supernatant was applied. The three washing steps show minimal loss of *etfDh* while many contaminating proteins were washed off the column. The three elutions show high purity of *etfDh* protein, especially in the second and third elutions.

## 4. Discussion

The analysis of the soluble and insoluble expression of *etfB* and *etfDh* revealed a large amount of *etfB* found in the soluble fraction at the corresponding band size (Figure 4). This result is consistent with literature showing ETFs as soluble proteins found in the cytoplasm of the cell (Winsor et al., 2008). However, a similar, if not equal amount of *etfB* was recovered in the insoluble fraction. Potential explanations for this include that the optimization conditions for protein expression were not optimal. Alternatively, if the induction temperature or the concentration of IPTG were too high and overbearing to the cells, a high amount of protein would be produced, leading to the misfolding of *etfB*. This misfolding would render the proteins into the insoluble fraction. It can also be hypothesized that *etfB* is found in the insoluble fraction because this ETF is interacting with a membrane-bound partner, like the dehydrogenase protein *etfDh*. Because of this inter-

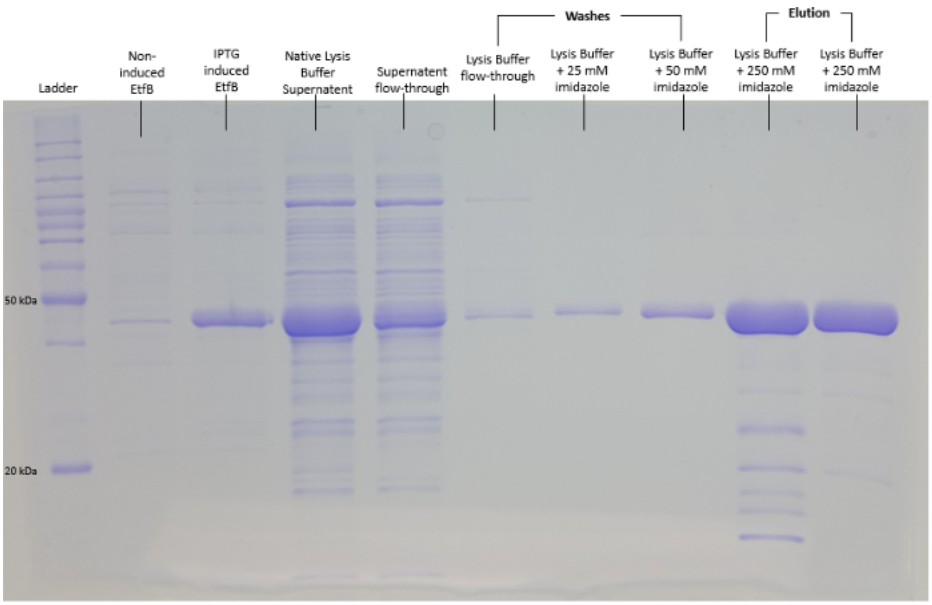


**Figure 7:** *12% SDS PAGE gel of the Purification of EtfB protein of B. cenocepacia K56-2 which was transformed and expressed in competent E. coli BL21-DE3 GOLD cells. Protein purification was performed through a Ni-NTA column of QIAexpress Fast Start protocol with adjusted wash and elution buffers (discussed in Materials and Methods). The size of EtfB protein with attached SUMO protein tag is 41.6 – 46.6 kDa.*

action, when the cells were broken apart and centrifuged, *etfB* was bound and subsequently trapped in the cell membrane and cell debris, therefore appearing in the insoluble fraction. However, there is still an ample amount of protein in the soluble fraction, allowing *etfB* protein purification to be performed on the soluble fraction.

The results of *etfDh*'s soluble and insoluble fraction (Figure 6) reveal a high quantity of *etfDh* in the insoluble fraction, consistent with literature, indicating that this protein is a membrane-bound dehydrogenase (Winsor et al., 2008). In comparison, the soluble fraction does not contain *etfDh*, again confirming there is no *etfDh* in the cytoplasm and that the protein is found in the cell membrane.

For the purification process, a SUMO protein has been attached to the proteins of interest through transcription and translation using pAB1 and pAB2 as templates. This SUMO protein contains a 6-histidine tag, which electrostatically binds to the Ni-NTA columns provided by QIAexpress Ni-NTA Fast Start kit. Therefore the Ni-NTA resin will selectively purify both *etfB* and *etfDh*. Thus far, the purification of *etfB* illustrates an ample amount of protein recovered with minimal co-eluted proteins (Figure 7), which is the ideal scenario of a purified protein sample. The purification of *etfDh* was also successful in the purity of the elution samples, however, a smaller amount of protein was recovered due to the protein being lost in the supernatant flow-through (Figure 8). While this is an area for adjustment and optimization, the three elution samples achieved through the purification are useful, as these samples can be concentrated together. Following this purification process, the SUMO protein will be removed using SUMO protease I (LifeSensors Inc.), leaving solely the proteins of interest. The primary purpose of purifying our proteins of interest is to raise polyclonal antibodies against *etfB* and *etfDh* to subsequently track their intracellular localization via immunofluorescence and analyze their protein interactions via coimmunoprecipitation.

Immunofluorescence will be used to identify the *in vitro* localization of a protein of interest. This begins with polyclonal antibodies

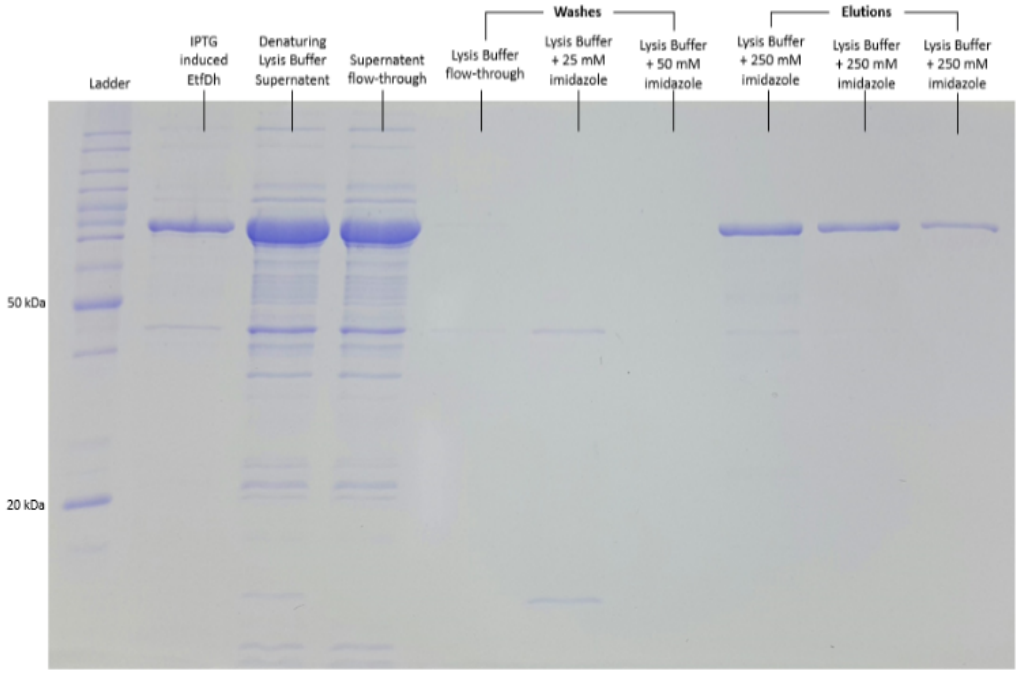


**Figure 8:** *12% SDS PAGE gel of the Purification of EtfDh protein of B. cenocepacia K56-2 which was transformed and expressed in competent E. coli BL21-DE3 GOLD cells. Protein purification was performed through a Ni-NTA column of QIAexpress Fast Start protocol with adjusted wash and elution buffers (discussed in Materials and Methods). The size of EtfDh protein with attached SUMO protein tag is 75.6 – 80.6 kDa.*

raised against a protein of interest. The cells containing the protein of interest are fixed and incubated with the polyclonal antibodies. After allowing the primary antibodies time to bind the protein of interest, the secondary fluorescent antibodies are added to the cell fixture, which are specific towards the primary applied antibodies. The goal of immunofluorescence is to identify intracellular localization and potentially protein-protein interactions between *etfB* and *etfDh*.

This technique was illustrated in a study performed on *Escherichia coli*, which was designed to investigate the localization and role of an essential glycosyltransferase MurG (Mohammadi et al., 2007). Antibodies were raised against MurG via a rabbit and the secondary antibody used was Cy3-conjugated (Mohammadi et al., 2007). MurG was found to localize in the lateral cell wall and the division site (Mohammadi et al., 2007). In this case, by immunofluorescence technique the authors confirmed the role of MurG in elongation and cell division (Mohammadi et al., 2007).

A further application of the polyclonal antibodies raised against both *etfB* and *etfDh* is coimmunoprecipitation technique. Coimmunoprecipitation is a useful method to analyze protein-protein interaction complexes through baiting one of the proteins in this complex with an antibody raised against it (Lee et al., 2013). This antibody is fixed to a bead surface, allowing the isolation of these protein-protein interaction complexes for further analysis (Lee et al., 2013). Coimmunoprecipitation would be very helpful in the current study to investigate the putative interaction of *etfB* and *etfDh* in *B. cenocepacia*, and also discover other proteins and molecules that could be interacting with them.

Identifying protein interactions of *etfB* and *etfDh* is instrumental in gaining insight as to their functional roles in *B. cenocepacia*. The importance of analyzing protein-protein interactions was highlighted by a study performed on *Clostridium perfringens*, focusing on the syn-

thesis of dipinic acid (DPA) (Orsburn, Melville, & Popham, 2010). By isolating the protein responsible for DPA synthesis, DPA synthase, an interaction between DPA synthase and EtfA was found, as EtfA was also discovered in the purified DPA synthase sample (Orsburn et al., 2010). Further studies showed EtfA is involved in catalyzing the formation of DPA, leading to the formation of endospores (Orsburn et al., 2010).

## 5. Conclusions

In conclusion, the current project's experimental goal of cloning, inducing expression of and purifying *etfB* and *etfDh* was achieved. The results support the hypothesis thus far, as *etfB* has been identified as being a cytoplasmic protein and *etfDh* has been identified as a membrane-bound protein. Furthermore, these results contribute to key goals of our laboratory, including deducing the localization and interaction of *etfB* and *etfDh*, assessing the reasoning behind their essentiality, and investigating their ability to become novel antimicrobial targets. Moving forward, questions to be addressed include: why are the genes *etfB* and *etfDh* essential in *B. cenocepacia*; what are their cellular functions and how do they interact; and can these two proteins be used as novel antimicrobial targets (Bloodworth et al., 2015). The future holds great promise as work continues to reveal the functions and interactions of *etfB* and *etfDh* in *B. cenocepacia* to exploit their potential as antimicrobial targets for treating B. *cepacia* complex infections in cystic fibrosis patients.

## 6. Acknowledgements

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