# ABSTRACT

Title of Thesis:
GENETIC MODIFICATION
OF PROBIOTIC *ESCHERICHIA COLI* TO
PRODUCE OMEGA-3 FATTY ACIDS

Authors:
Julianna Greenberg,
Neha Kalla, Roja Kambhampati,
Erin Murphy, Aasheen Qadri,
Mateo Reveiz

Mentored By:
Dr. Debabrata Biswas, PhD
Department of Animal
and Avian Science

Omega-3 ($\Omega$-3) fatty acids are an essential component of the human diet that have been linked to reduced risk of both neurological and cardiovascular diseases in humans; however, $\Omega$-3 fatty acids are far underconsumed in the typical American diet. The expense of fatty fish such as salmon makes obtaining $\Omega$-3 fatty acids difficult for much of the population and plant alternatives have proven to be insufficient sources of this vital nutrient. To address aforementioned issues, probiotic bacteria will be modulated to produce $\Omega$-3 for the potential application in fermented food products like yogurt. This new application would not only increase access to $\Omega$-3, but also provide a significant environmental benefit through reduced overfishing and ocean contamination currently associated with the mass production of fish products for their $\Omega$-3 benefits.

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By

Team OMEGA


Julianna Greenberg, Neha Kalla, Roja Kambhampati, Erin Murphy, Aasheen
Qadri, Mateo Reveiz

Mentor: Dr. Debabrata Biswas

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Advisory Committee:
Dr. Debabrata Biswas, Faculty Mentor
Dr. Muhammed Shafeekh Muyyarikkandy
Dr. Mengfei Peng
Dr. Shaik Rahaman
Ms. Zajeba Tabashsum

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## Chapter 1: Introduction

Omega-3 ($\Omega$-3) fatty acids are a class of polyunsaturated fatty acids (PUFA) that are essential to human health, yet are currently far under-consumed by the American population (Bell et al., 1986; Papanikolaou et. al., 2014). Although $\Omega$-3 fatty acids are not produced naturally by the human body, they are vital to organ development, disease prevention, and neurological and cardiovascular wellness (Mazza et al., 2007). Humans obtain the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) $\Omega$-3 fatty acids through the consumption of fatty fish such as anchovies, bluefish, herring, mackerel, salmon, sardines, sturgeon, lake trout, and tuna (Covington et al., 2004). However, there are issues with sustainably sourcing fatty fish including overfishing of wild sources and poor management of farm fisheries which produce harmful contaminants that contribute to ocean acidification and climate change (Lee et al., 2009). As an alternative, humans can also obtain $\Omega$-3 fatty acids from sources such as olives, flax seeds or fish oil supplements; however, these sources are often insufficient because they either do not provide significant amounts of EPA or DHA to produce the aforementioned health benefits or they are not readily incorporated into the body (Neale et al., 2013; Simopoulos et al., 2016). To combat this issue, innovative and environmentally-conscious methods to introduce new sources of $\Omega$-3 fatty acids into the American diet are currently under exploration.

Team OMEGA will research if $\Omega$-3 fatty acid production can be induced in microorganisms with applications in food production, such as probiotic bacteria, in order to enrich foods naturally lacking $\Omega$-3 fatty acids. We will utilize the gene cluster responsible for the production of $\Omega$-3 fatty acids within the genome of *Shewanella*

*baltica,* a marine bacterium found in the gut of some fatty fish, for example, salmon. *Escherichia coli (E. coli)* Nissle, a bacterium employed as a probiotic in European supplements, will be genetically engineered to produce EPA and DHA. *E. coli* Nissle is not yet approved by the Food and Drug Administration (FDA) however it is available in some European countries (Henker, 2017) .

This project builds upon previous research conducted by groups looking to modify bacteria to produce Ω-3 fatty acids using the gene cluster from *S. baltica.* In 2010, the *pfaABCDE* gene cluster, responsible for the production of EPA and DHA in *S. baltica* was isolated and integrated in to *E. coli (*Amiri-Jami and Griffiths, 2010). Soon after, in 2016 the same gene was integrated into our target bacteria, *E. coli Nissle* (Amiri-Jami et al., 2016). However, in both cases, antibiotic resistant genes were not removed from the bacteria after transformation, meaning that the bacteria can not be utilized in food production. In addition, in neither case was the *pfaABCDE* gene integrated in to the genome of the *E. coli* strain. Through the course of our research we hope to remedy both of these limitations and work to remove the antibiotic resistance markers from the gene cluster and encourage the integration of the gene cluster into the chromosomal DNA of *E. coli.*

**Research Questions**

This research aims to investigate the following questions in regards to the Ω-3 fatty acid production in probiotic *E. coli* Nissle:

1.  Can *E. coli* Nissle be genetically modified to produce Ω-3 fatty acids using the *pfaABCDE* gene cluster isolated from *Shewanella baltica*?

2.  Does the EPA productivity of the genetically engineered *E. coli* Nissle compare to the productivity of the wild type strain, *S. baltica*?

**Hypothesis**

If the *pfaABCDE* gene cluster from *S. baltica* is successfully transferred into *E. coli* Nissle, then it will be possible to culture the transgenic microorganism in fermented foods like yogurt to increase their Ω-3 content and provide a novel source of Ω-3.

**Objectives**

The objectives for the project were addressed as follows:

I.  Isolation the *pfaABCDE* Ω-3-fatty-acid-producing gene cluster from the DNA of *S. baltica*.

II.  Ligation of the isolated gene cluster to the pCR™ - 2.1 TOPO™ cloning vector and transformation of cloning bacteria strains, *E. coli* DH5α and DH10β.

III.  Preparation for measurement of the EPA output in the transformed *E. coli* DH5α and DH10β bacteria.

IV.  Isolation of target bacterial strain, *E. coli* Nissle in preparation for transformation.

## Chapter 2: Literature Review

### Introduction

Omega-3 ($\Omega$-3) fatty acids are naturally occurring compounds that have been shown to improve several neurological and cardiovascular conditions (Mazza et al., 2007). For example, those with higher levels of $\Omega$-3 fatty acids in their diet have improved cognitive development and reduced arterial inflammation (Deckelbaum et al., 2012). Furthermore, studies have revealed that a rich diet of $\Omega$-3 fatty acids can reduce cardiovascular-related mortality by 36% and total mortality by 17% (Mozaffarian and Rimm, 2006).

Despite its numerous health benefits, $\Omega$-3 cannot be produced by the human body. Instead, $\Omega$-3 fatty acids must be obtained from a well-rounded diet. One primary source of $\Omega$-3 includes certain fatty fish like anchovies, bluefish, herring, mackerel, salmon, sardines, sturgeon, lake trout, and tuna (Covington et al., 2004). In addition to fatty fish and marine products, some plant products such as olives, flaxseeds, flaxseed oil, chia seeds, walnuts and leafy greens also produce $\Omega$-3 fatty acids (Williams and Burdge, 2006).

Therefore, considering the nutritional importance of $\Omega$-3 fatty acids, in addition to the fact that it is not produced by the human body, it is vital for human health to obtain sufficient amounts of $\Omega$-3 fatty acids from external sources. However, studies have shown that a rising number of American adults are not meeting the recommended daily values of $\Omega$-3 fatty acids of 300mg per day, or 2 meals of fish per week (Papanikolaou et al., 2014; Venegas-Calerón et al., 2010). Due to the significant role that $\Omega$-3 fatty acids play in organ development, neurological and cardiovascular wellness, and in preventing many chronic diseases, the availability of this essential component is an urgent issue that

must be addressed to improve public health (Deckelbaum et al., 2012; Mozaffarian and Rimm, 2006).

As previously mentioned, fatty fish are a significant source of $\Omega$-3 fatty acids (Covington et al., 2004).  However, environmental concerns such as overfishing and ocean pollution threaten the viability of fatty fish as a sustainable source of $\Omega$-3 fatty acids in the future (Venegas-Calerón et al., 2010).  One such area of interest to address the issues of $\Omega$-3 intake and sustainable production of $\Omega$-3 is genetically engineered organisms (GMOs).  Through genetic engineering, bacteria previously incapable of producing  $\Omega$-3 fatty acids, such as lactic acid bacteria, would be transformed to produce $\Omega$-3 fatty acids and used in food applications such as fermentation.  Thus, the purpose of this research project is to determine whether probiotic bacteria such as *E. coli* Nissle can be genetically modified to produce $\Omega$-3 fatty acids using the *pfaABCDE* gene cluster isolated from the marine bacteria *S. baltica*. The *pfaABCDE* gene cluster encodes five essential enzymes in the polyketide synthase (PKS) pathway responsible for biosynthesis of EPA and DHA (Orikasa et al., 2006). The goal of Team OMEGA's research project is to create a novel and sustainable source of $\Omega$-3 fatty acids that may address the issue of inadequate $\Omega$-3 intake in the American population.

**Health benefits of $\Omega$-3 fatty acids**

The health benefits of $\Omega$-3 fatty acids are diverse, ranging from improving cognition and mental health to preventing and reducing the effects of illnesses such as cardiovascular disease and autoimmune disorders.  Deficiency of $\Omega$-3 fatty acids has also been shown to be associated with Major Depressive Disorder (Song et al., 2016).  In a

recent study, subjects who received increasing daily levels of Ω-3 fatty acids for eight weeks scored lower on the Hamilton Depression Rating Scale than prior to Ω-3 fatty acid intake adjustment (Song et al., 2016).  Similarly, Ω-3 fatty acid intake levels can serve as predictors of suicidal behavior, with high intake levels positively correlating with lower suicide risk and attempt (Sublette et al., 2006).

One of the most widely known benefits of Ω-3 fatty acids is reduced arterial inflammation, which results from an increased presence of anti-inflammatory biomarkers and lower amounts of cholesterol delivered to certain arterial walls through the blockage of lipid binding (Deckelbaum et al., 2012).  Subsequently, anti-inflammatory responses, in combination with other noted effects of high Ω-3 intake such as lower blood pressure, reduced platelet reactivity, and increased influence on membrane-ion channels, lead to lower chances of coronary heart disease (Lee et al., 2013).  Increased anti-inflammation can also decrease the risk of rheumatoid arthritis and other autoimmune diseases (Simopoulos et al., 2002).

**Current issues and recommended solutions of Ω-3 fatty acid intake**

The three types of Ω-3 fatty acids are alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA).  Ω-3s can be consumed by humans through a variety of dietary sources.  ALA, a short stranded Ω-3 fatty acid, can be absorbed through the consumption of various fats and oils, nuts and seeds, and vegetables, while uptake of long stranded PUFAs, EPA, and DHA is mostly derived from fish, marine sources, and fish oils (Figure 1).

In regards to human health, ALA is inferior to both EPA and DHA. ALA is difficult to incorporate into human plasma and tissue lipids even when large amounts of the fatty acid are consumed (Nettleton et al., 1991; Rodríguez-Leyva et al., 2010). This is because in order for ALA to provide the health benefits associated with $\Omega$-3 fatty acids, the human body must expend energy to convert ALA into EPA and DHA (Figure 1). This transformation process is highly inefficient for the body, so much so that less than 1% of ALA is converted to physiologically effective levels of EPA and DHA (University of Maryland Medical Center et al., 2014). Additionally, the human body tends to oxidize ALA to produce energy rather than incorporating it into human tissue (Nettleton et al., 1991). Furthermore, it has been found that about 4 g of ALA is equivalent to only 0.3 g of EPA and DHA, thus corroborating the inefficiency of ALA in the body (Rodríguez-Leyva et al., 2010).

Although studies have shown that ALA does provide some cardiovascular benefit, the effects are not as well documented as those of combined EPA and DHA (Rodríguez-Leyva et al., 2010). Thus, food sources with high levels of EPA and DHA PUFAs, as opposed to those with only ALA, are more beneficial and effective sources $\Omega$-3 fatty acids.

Several different solutions have been proposed in order to ameliorate the issue of insufficient EPA and DHA $\Omega$-3 fatty acid consumption in the American population. One of the most common methods of supplementing $\Omega$-3 fatty acid consumption is by taking fish oil capsules or concentrated $\Omega$-3 fatty acid pills. While this may be a more cost effective option, $\Omega$-3 fatty acids from capsules have been shown to be less effectively incorporated into lipids in the cells than $\Omega$-3 fatty acid from fish (Visioli et al., 2003). A

study on adult obesity also found that consuming fish oil supplements had a different effect on the body than consuming fish itself. An increase in high molecular weight adiponectin was found when fish was consumed but when fish oil supplements were consumed, the levels of high molecular weight adiponectin dropped (Neale et al., 2013). High molecular weight adiponectin is a hormone that decreases inflammation in the body; $\Omega$-3 fatty acid consumption increases the levels of this hormone, but more effectively through consumption of fish than through fish oil pills (Neale et al., 2013).

Another method researchers are implementing to increase $\Omega$-3 consumption in the American diet is the modification of plants to synthesize ALA to produce long-chain PUFAs, which include EPA, DHA, and the $\Omega$-6 fatty acids linoleic acid (LA), and arachidonic acid (AA) (Venegas-Calerón et al., 2010). To genetically-engineer plants to produce long-chain PUFAs and have the ability to synthesize these fatty acids, at least three non-native, energy intensive, enzymatic reactions must occur (Graham et al., 2007). While this has been successfully accomplished, these fatty acids cannot be used as a substitute for fish consumption due to the fact that the levels of EPA and DHA produced are not comparable to those from marine sources (Venegas-Calerón et al., 2010). Proponents of genetically modified crops (GMC) argue that toxins in the oceans and mercury levels in fish have made $\Omega$-3 fatty acid producing fish less safe to eat (Venegas-Calerón et al., 2010; Graham et al., 2007). However, these claims have not been able to be substantiated in recent years.

Ultimately, the reason that these GMC's are not able to provide consumers with sufficient amounts of $\Omega$-3 fatty acids is due to the production of $\Omega$-6 and the dangerous ratio of $\Omega$-6 to $\Omega$-3 produced in these genetically modified plants (Venegas-Calerón et

al., 2010).  In the current North American diet, the ratio of $\Omega$-6 to $\Omega$-3 ranges from 6:1 to 20:1.  This is a large increase from the estimated 1:1 ratio thought to exist during pre-modern times, from 4 million BCE to the beginning of agricultural revolution (Simopoulos et al., 2016).  The problem with this higher ratio of $\Omega$-6 to $\Omega$-3 is that excessive amounts of $\Omega$-6 promote the progression of many diseases, including cardiovascular disease, cancer and inflammatory and autoimmune diseases.  On the other hand, increasing levels of $\Omega$-3 and maintaining a low ratio of $\Omega$-6 to $\Omega$-3 help fight these same diseases (Simopoulos et al., 2002).  Thus, until GMCs can solely elevate the levels of EPA and DHA in amounts comparable to fish and not elevate levels of $\Omega$-6, they are not a healthy source of $\Omega$-3 fatty acids.

In North America, the majority of consumers get their EPA and DHA from fish or fish oils, since other food sources such as nuts, vegetables and meats produce small amounts of these fatty acids (Figure 1).  Fish oil pills and GMCs are also unsatisfactory sources for humans to obtain $\Omega$-3 fatty acids from due to the aforementioned negative health effects (Neale et al., 2013; Simopoulos et al., 2016).  Due to these factors, it is beneficial for humans to consume fish derived $\Omega$-3 fatty acids in order to integrate high levels of EPA and DHA in their diets.

Current research indicates that an increasing number of American adults who consume typical Western diets do not meet the recommended amounts of fish derived $\Omega$-3 fatty acids in their daily diet (Papanikolaou et al., 2014).  The average intake of $\Omega$-3 fatty acids that American adults typically consume from fish is around 150 mg per day, which is comparable to one meal of fish per week (Surette et al., 2008).  However, the International Society for the Study of Fatty Acids and Lipids recommends a daily intake

of at least 500 milligrams of EPA and DHA (Surette et al., 2008). Furthermore, the American Heart Association suggests that people without coronary heart disease consume at least two fish meals each week, which is equivalent to a daily intake of about 300 mg of $\Omega$-3 fatty acid (Surette et al., 2008). Meanwhile, patients who suffer from coronary heart disease are recommended to consume 1000 mg of EPA and DHA daily (Surette et al., 2008).

As seen in prior research, $\Omega$-3 fatty acids provide necessary health benefits such as reducing the risk for cardiovascular disease and autoimmune disorders as well as improving mental health and cognition (Deckelbaum et al., 2012). However, these health problems and disorders are becoming more prevalent as an increasing number of Americans are not reaching the recommended daily intake of $\Omega$-3 fatty acids.

**Environmental impacts of overfishing**

Currently, it is estimated that over one-third of all fish stocks are overexploited and face the threat of collapse. Of the 1,835 billion fish caught per year, on average, 725 billion are used in fish meal and fish oil to create a product to provide $\Omega$-3 content (FAO et al., 2018). This means that almost half of all fish caught are used in connection to their $\Omega$-3 content. A reduction in the amount of fish farmed specifically for $\Omega$-3 could make a significant positive impact on the problem of overfishing. Successfully applying transformed bacteria to different foods and producing comparable $\Omega$-3 levels could reduce the overfishing of fatty fish for the food industry and could also replace fish oil supplements and $\Omega$-3 pills.

**Metabolic pathways of Ω-3 fatty acid production**

Since the human body cannot produce Ω-3 fatty acids, they must be obtained from external sources (Ji et al., 2015). While fish oils are the major dietary source of Ω-3 fatty acids, fish do not actually produced these compounds (Monroig et al., 2013). Rather, the major synthesizers of Ω-3 fatty acids are autotrophic marine organisms such as protists, algae, and some bacteria (Monroig et al., 2013). From these organisms, fish are able to incorporate the PUFA into their bodily oils, the consumption of which is one mechanism through which humans obtain their dietary values of Ω-3 fatty acid (Ji et al., 2015).

Many marine bacteria living in the gut of cold-water fish are able to produce both EPA and DHA (Ji et al., 2015). For example, *Shewanella* is one such genus of marine bacteria that has been well studied (Hirota et al., 2015). Although both EPA and DHA are traditionally believed to be produced solely through two aerobic pathways--either the Δ-6 desaturase pathway or the Δ-9 pathway--recent studies have shown that they can be produced through an anaerobic polyketide synthase (PKS) pathway as well (Xue et al., 2013; Monroig et al., 2013). It is believed that marine bacteria such as *Shewanella* must use the aerobic pathway to produce EPA or DHA, but are capable of using the anaerobic PKS pathway as well (Monroig et al., 2013).

Generally, the aerobic pathway involves the synthesis of the unsaturated fatty acid from the starting saturated fatty acid reagent; by definition, this is achieved by creating double bonds from single bonds (Monroig et al., 2013). First, the Δ-9 and Δ-12 desaturase enzymes produce linoleic acid, which is an Ω-6 PUFA (Monroig et al., 2013). Δ-15 desaturase then adds another double bond on carbon-15, creating the Ω-3 alpha-

linoleic acid (Monroig et al., 2013).  Finally, Δ-6 desaturase, Δ-5 desaturase, and Δ-4

desaturase act on the other end of the ALA, placing two additional double bonds in

conjugation with the double bonds already placed by Δ-9 and Δ-12 desaturase (Monroig

et al., 2013).  Elongase acts in between the desaturates to add two additional carbons onto

the polyunsaturated chain, thus converting ALA to EPA (Monroig et al., 2013).  DHA

can be produced by the addition of two more carbons and a final degree of unsaturation

(Monroig et al., 2013).

Other than the absence of oxygen, the anaerobic PKS pathway differs from the

aerobic pathway in that the former involves adding alkenes onto the chain, while the

latter involves the addition of double bonds onto the preexisting alkane (Monroig et al.,

2013).  Additionally, the anaerobic PKS pathway uses different enzymes, including 3-

ketoacyl synthase (KS) and enoyl reductase (ER)  (Monroig et al., 2013).
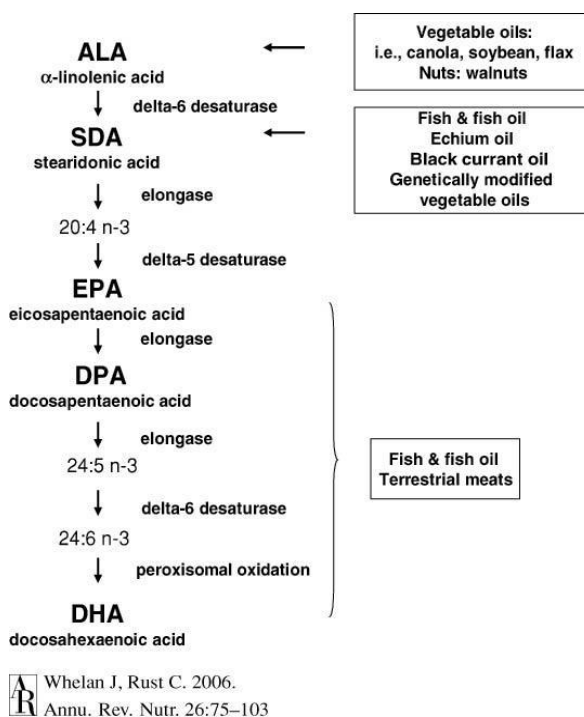
Figure 1: The metabolic pathway of Ω-3 fatty acids and the traditional food sources of Ω-3 in the American diet (Whelan & Rust el al., 2006)

**Evaluation of relevant prior research and its limitations**

In order to address this widespread issue of Ω-3 deficiency, past researchers have attempted to genetically modify organisms to produce or increase the production of Ω-3 fatty acids. Multiple organisms, including algae, fungi and bacteria, have been used to create Ω-3 fatty acids (Barclay et al., 1994).

As previously mentioned, fish such as salmon and mackerel contain high levels of Ω-3 fatty acids, which are produced by internal bacteria. The most notable marine bacteria found to produce Ω-3 are related to the *Shewanella* and *Colwellia* families (Okuyama et al., 2007). Specifically, *Shewanella putrefaciens* and *S. baltica* have been used in several studies related to the production of Ω-3 fatty acid (Gentile et al., 2003; Yazawa et al., 1996). In these studies, the EPA/DHA gene cluster consisting of *pfaA* (type 1 polyketide synthase), *pfaB* (permase), *pfaC* (beta-hydroxyacyl-ACP dehydratase), *pfaD* (2-nitropropane-dioxygenase), and *pfaE* (4' phosphopantetheinyl transferase) was identified (Amiri-Jami et al., 2010; Okuyama et al., 2007).

According to a study conducted by Amiri-Jami, choosing the microbe *S. baltica* MAC1 from which to isolate the Ω-3 fatty acid producing genes strengthened their research by allowing for the isolation of all five genes, *pfaABCDE*, which are crucial in the synthesis of both EPA and DHA (Amiri-Jami et al., 2010). In other studies, researchers isolated the genes *pfaABCDE* in different forms of marine bacteria, such as *Shewanella pneumatophori*, and found that the *pfaB* gene codes for the essential enzyme that determines the final product in the synthesis of both DHA and EPA (Orikasa et al., 2009). Additionally, it was determined that without the presence of the *pfaE* gene, recombinant production of EPA and DHA was not possible as the product of this gene is

required in the post-translational modification of the proteins synthesized by *pfaA* (Allen et al., 2002; Metz et al., 2001; Orikasa et al., 2006).  This information aids the project as it specifies that using *S. baltica* as the source of Ω-3 fatty acid genes will allow for the isolation of all five genes responsible for Ω-3 fatty acid production of both  DHA and EPA.

Other organisms commonly researched for their viability in Ω-3 fatty acid genetic recombination are marine diatoms and marine fungi protists (Hamilton et al., 2015; Barclay et al., 1994; Yongmanitchai & Ward et al., 1991).  For example, the diatom *Phaeodactylum tricornutum* has been genetically modified by overexpressing genes related to the LC-PUFA biosynthetic pathway to be able to store high levels of Ω-3 fatty acids (DHA and DPA) (Hamilton et al., 2015).  In that particular study, *Phaeodactylum tricornutum* was successfully used in photobioreactors to industrially produce Ω-3.  This study shows that multiple organisms could potentially be modified in order to increase the production of Ω-3 fatty acids, thus serving as viable alternatives if issues arise with *S. baltica* MAC1.

The EPA and DHA gene cluster has been successfully expressed in other organisms as well, such as *Escherichia coli* (*E. coli*) and *Lactococcus lactis* (Amiri-Jami & Griffiths et al., 2010; Amiri-Jami et al., 2014; Yazawa et al., 1996).  This serves as the proof of concept that genetic transformation with the EPA/DHA gene cluster can be accomplished.  In particular, in the study performed by Amiri-Jami and his colleagues, it was found that the choice of the host bacteria also affected the production of EPA and DHA (Amiri-Jami et al., 2010).  However, the significance of their methodology was

weakened because they were unable to determine which specific factors of the host bacteria contribute to the success of its bacterial transformation.

**Isolation, amplification, and confirmation of *pfaABCDE* gene cluster**

According to the literature referenced above, the first step in engineering *E. coli* to produce Ω-3 fatty acid involves the isolation of the five target genes located in the wild type strain, *S. baltica,* which are responsible for EPA and DHA production. These specific genes, named *pfa*A, *pfa*B, *pfa*C, *pfa*D, and *pfa*E, are collectively 20 kilobases (kb) in length and have consistently been found as common genes in the bacteria of different Ω-3 EPA and DHA producing fish. The functions and locations of these genes have been previously defined through genome sequencing of multiple Ω-3 producing bacteria, including the *S. baltica* wild type strain (Amiri-Jami et al., 2010).

Polymerase Chain Reaction (PCR) will be used to amplify the *pfaABCDE* gene cluster after it has been successfully isolated from *S. baltica.* Primers must be designed specifically for this PCR to produce results that amplify the target genes. Primers with high efficiency must be used because the gene cluster is approximately 20 kilobases (kb) in length, which is a relatively large strand to amplify. When designing primers several factors must be addressed, such as primer length, primer melting and annealing temperatures, guanine cytosine (GC) base content, and the formation of primer secondary structures (hairpins and dimers) (PREMIER Biosoft). If necessary, computer programs that can identify optimal primers for PCR will be used to produce the most accurate PCR results. Based on previous research, primers in open reading frames 5 and 8, where our

primers are found, have proven successful in the amplification of the transferred *pfaABCDE* genes (Amiri-Jami et al., 2010).

Nanodrop analysis will be employed to confirm isolation of DNA molecules. The Nanodrop conducts fluorescent analysis of the provided sample to identify the concentration of nucleic acids in that sample. Nanodrop machines offer the distinct benefit of requiring only 1-2 µL of liquid to perform an accurate analysis, far less than many other technologies. It is able to utilize such a small sample by exploiting natural surface tension properties of the small sample, capturing it and holding it in place (Desjardins & Conklin et al., 2010).

**Specifications of host bacteria: *Lactobacillus casei***

*Lactobacillus casei* (*L. casei)* is a bacterial probiotic species that has been extensively studied and used in food products such as cheese, yogurt, and other fermented milk products. There are two main strains of *L. casei* that are used in food processing which are Shirota and DN-114001. Like all L. casei strains, *L. casei* Shirota is a rod-shaped gram-positive bacteria that has a length of one to two microns and a width of 0.4 to 0.8 microns (Yakult et al., 2019). This strain of *L. casei* can survive in anaerobic and aerobic conditions and at temperatures between 15 and 41 degrees Celsius with 37 degrees Celsius being the optimal temperature (Yakult et al., 2018). On its own, *L. casei* Shirota has many beneficial effects which include improving digestion and cholesterol levels (Douillard et al., 2013). *L. casei* DN-114001 is another strain that is used in food products. Specifically, it is in the probiotic-marketed drinkable yogurt, Actimel that is produced by Danone (Douillard et al., 2013). *L. casei* DN-114001 has

been studied in several animal and human studies for its probiotic properties. Studies have also shown that the *L. casei* strain LcA is present in Actimel and when isolated, was shown to be identical to the seed culture strain DN-114001 (Douillard et al., 2013). Since both of these strains of *L. casei* bacteria are already used in food products, they make good choices of host bacteria for our project since we plan to transform bacteria that would go into food products to increase their $\Omega$-3 content.

**Probiotic Bacteria**

Probiotics are live microorganisms known as "health friendly bacteria" commonly found in fermented foods and cultured milk (Shi, 2016). Probiotics have many beneficial health properties such as preventing bowel diseases, improving the immune system, and having antihypercholesterolemic and antihypertensive effects (Shi, 2016). Researchers in the past have argued that some effects of probiotics are caused by cellular components which does not require living organisms for colonization in the gut (Wassenaar, 2016). The World Health Organization's definition of probiotics include that they must "remain viable and stable after culture, manipulation and storage before consumption (and) have to survive gastric acid and biliary and pancreatic digestion" (Wassenaar, 2016). These authors argue that because the host's pattern recognition receptors (PRRs) are essential in probiotic applications, it does not matter if the bacteria is dead or alive (Wassenaar, 2016). Probiotic products based on living organisms are also more difficult to produce, store and quality control compared to cellular components (Wassenaar, 2016). However, there is not much data to support either claim about whether living organisms are more effective than dead ones (Wassenaar, 2016).

**Specifications of host bacteria: *Escherichia coli* (DH5α, DH10β, Nissle)**

*Escherichia coli* (*E. coli*) is a gram-negative, non-sporulating facultative anaerobe bacteria which inhabits the intestines and feces of warm-blooded animals and reptiles (Tenaillon et al., 2010).  Similarly, *E. coli* is predominantly found in the gut microbiota (Tenaillon et al., 2010).  Commensal strains of *E. coli* within the digestive tract are located in the large intestine, specifically in the caecum and colon (Tenaillon et al., 2010).  There are three strains of *E. coli* that we plan to use throughout our research methodology including *E. coli* Nissle, DH5α, and DH10β.

*E. coli* Nissle is a well-studied non-pathogenic, gram-negative probiotic bacterial strain (Amiri-Jami et al., 2015; Scaldaferri et al., 2016).  *E. coli* Nissle has been commonly used as a probiotic and to treat a wide variety of inflammatory diseases such as diarrhea, uncomplicated diverticular disease and inflammatory bowel disease (Hafez et al., 2009; Scaldaferri et al., 2016). Similarly, *E. coli* Nissle has several mechanisms of action including immunomodulatory properties, reinforcing the intestinal barrier, and inhibiting the effects of pathogenic *E. coli* (Scaldaferri et al., 2016). Although lactic acid bacteria has been the primary choice of targeted delivery in probiotics, prior research has demonstrated that in certain situations, such as when persistent colonization is necessary, *E. coli* Nissle has been advantageous compared to lactic acid bacteria (Behnsen, 2013). Similarly, lactic acid bacteria only transiently colonizes gut bacteria (Behnsen, 2013). As a result, understanding the way *E. coli* Nissle colonizes in the gut can be essential in improving the efficacy as well as developing other Gram-negative bacteria for probiotic use (Behnsen, 2013). Similarly, *E.coli* Nissle has been shown to provide colonization resistance to mucosal pathogens (Behnsen, 2013). Its biosafety and probiotic effect has

been highlighted in various trials and previous research (Behnsen, 2013). In addition,

*E.coli* Nissle is found in the drug Mutaflor which is commonly used to treat infectious

diarrheal diseases and inflammatory bowel disease (Behnsen, 2013). *E.coli* Nissle has

also been successful in preventing the colonization of multidrug-resistant pathogens in

neonate digestive tracts (Behnsen, 2013).

DH5α is one of the most commonly used laboratory strains of *E. coli* (Anton &

Raleigh et al., 2016). It has a vast array of properties which make the strain highly

suitable for many different cloning applications (Anton & Raleigh et al., 2016). DH5α

has a high transformation efficiency and yields high-quality plasmid DNA (Anton &

Raleigh et al., 2016). Similarly, DH10β is a high efficiency strain of *E. coli* and is ideal

for cloning large plasmids (Durfee et al., 2008).

**Microbial culture conditions and modulation**

The culture conditions are a key factor for the proper development of bacteria.

All bacteria have optimum growth conditions, and these conditions can be related to

temperature, pH, and general nutrition, water, and oxygen requirements. Furthermore,

the culture media also plays an important role in bacterial growth and must be selected

according to the bacteria's specific requirements.

For *Shewanella sp.*, it was reported that the production of Ω-3 fatty acid was

dependent on carbon sources and temperature, as low temperatures successfully increased

the proportion of unsaturated fatty acids up to about 74% (Gentile et al., 2003). Marine

Broth 2216 medium was used for *S. baltica* (Amiri-Jami & Griffiths et al., 2010; Gentile

et al., 2003). Special consideration will have to be given to these variables in the culture conditions in the methodology, in order to maximize the production of $\Omega$-3 fatty acids.

A study showed that a newly discovered strain of marine bacteria, SRCR-2738, which is a relative of *S. baltica*, produced the maximum content of EPA between 15-20 degrees Celsius, decreased EPA production at 25 degrees Celsius, and ceased production after 30 degrees Celsius (Yazawa et al.,1996).

Another study corroborated Yazawa's findings by expressing the gene cluster in *E. coli DH5a* and *DH10β* at multiple temperatures and detecting a maximum production at around 15 degrees Celsius (13.4 and 11.3 % of total fatty acids respectively) (Amiri-Jami & Griffiths et al., 2010). This methodology is strengthened because it tested how the rate of EPA production changed with growth conditions, specifically with temperature. Information from these previous studies can be used in the project to determine the optimal conditions not only for bacterial growth, but also for maximum $\Omega$-3 fatty acid production. Upregulation in our bacterial strains would allow for increased production with respect to *S. baltica*.

**Methods of increasing rate of $\Omega$-3 production**

Other research has been performed pertaining to increasing the overall $\Omega$-3 fatty acid production of bacteria once it had been transformed. A study conducted by Amiri-Jami and his colleagues used Tn5 transposons to mutate $\Omega$-3 fatty acid producing *S. baltica* bacteria to increase the rate of $\Omega$-3 fatty acid production (Amiri-Jami et al., 2006). They found that these isolated mutants produced three to five times as much EPA than the wild type bacteria.

The strengths in the Amiri-Jami study stem from the use of a suicide vector, the pUT plasmid, to insert the random mutations into the new cell, allowing for the expression of chromosomal DNA without replication of the plasmid itself (Amiri-Jami et al., 2006). The method of using a Tn5 transposon to mutate the DNA also did not affect the capability of the transformed bacteria to produce $\Omega$-3 fatty acid, making it a potential method of either inserting $\Omega$-3 fatty acid producing genes into the host bacteria or mutations that will increase the $\Omega$-3 fatty acid production of the transformed bacteria (Amiri-Jami et al., 2006). However, a weakness of this methodology is that the insertions were random and produced a small number (3 out of 60 random samples) of mutants with increased production of $\Omega$-3 fatty acid (Amiri-Jami et al., 2006). Considering both this study and the limited time of the project, it can be concluded that it is more beneficial to change the conditions of the growth media or culture in order to increase $\Omega$-3 fatty acid production.

**Quantification and measurement of $\Omega$-3 production**

Several studies use the Bligh and Dyer Method in order to extract fatty acids from tissues (Bligh & Dyer et al., 1959). However, because the plan is to extract fatty acids from unicellular organisms, a modified version must be used (Lewis et al., 2000) (See Appendix D). The recovered amounts increased by 30% by adding solvents to the biomass in order of increasing polarity. These results show that the efficiency of lipid recovery from different organisms can vary substantially between extraction techniques. The extraction protocols will be standardized in order to compare the production of $\Omega$-3 fatty acids by modified bacteria and the wild type.

Another method to extract the fatty acids is cell disruption by sonoporation. In this case, the bacterial cells will be resuspended in PBS and subject to ultrasonication three times (Biswas et al., 2016a). Finally, a third technique could involve freezing bacteria at -20°C and thawing with gentle mixing. Cellular debris is removed by centrifugation at 3,000 rpm for 15 min after repeating the freezing/thawing cycle three times (Biswas et al., 2016a). Multiple alternatives have been proposed in case some methods fail or yield poor results.

The primary tool used to quantify and measure the levels of $\Omega$-3 fatty acid is gas chromatography with a flame ionization detector and mass spectrometry (Gentile et al., 2003). These methods are able to determine if recombinant production of fatty acids is made from the transgenic host bacteria containing the EPA/DHA gene cluster (Amiri-Jami & Griffiths et al., 2010). In gas chromatography, this is accomplished by comparing the relative retention times to a standard which contains pure samples of EPA and DHA (Amiri-Jami et al., 2014). In a past study, analysis conditions for $\Omega$-3 fatty acids were "210°C column temperature, 250°C injection and detector temperatures, helium as carrier gas, and pentanoic acid (C15:1) as internal standard" which are the same analysis conditions that the team will use due to the fact that the same chemical compounds will be analyzed (Yongmanitchai & Ward et al., 1991). Mass spectrometry is a chemical analysis technique that can be used to determine the masses of distinct functional groups within the compound based on the mass-to-charge ratio (Amiri-Jami et al., 2014). With mass spectrometry, the amount of $\Omega$-3 fatty acids produced can be measured due to its distinctive functional groups.

Other inexpensive and less laborious methods of quantifying fatty acids exist.

Previous researchers have developed spectrophotometric assays for the detection of free

fatty acids using the Acyl-CoA synthase and Acyl-CoA Oxidase enzymes (Hosaka,

Kikuchi, Mitsuhida, Kawaguchi, 1981). The assay indirectly measures the quantity of

free fatty acids by the production of $H_2O_2$ from the reaction of Acyl-CoA synthase and

Acyl-CoA Oxidase enzymes with the target free fatty acid (Hosaka et al., 1981). The

$H_2O_2$ in the presence of catalase and a coloring agent is detected using a

spectrophotometer set to an absorbance of 550 nm and the assay has been shown to

exhibit broad specificity to long-chain fatty acids including ALA (Hosaka et al., 1981).

Additionally, tests with the assay using palmic acid indicated there no effect of the

presence or absence of *E. coli* molecular debris on the assay's sensitivity (Hosaka et al.,

1981). As such, similar methods may present viable alternatives to measuring the Ω-3

fatty acid output of the transformed target bacteria.

Regardless of the method selected for quantification, it is necessary to

differentiate the intracellular and extracellular (media) fatty acid concentration. Samples

from both environments have to be taken and compared to a negative control or baseline

consisting of non-transformed bacteria.

**Integration of modified bacteria into food grade products**

Although researchers have been able to successfully transform lactic acid

bacterial strains to produce Ω-3 fatty acids, the transfer of the modified bacteria into an

actual food product has yet to be accomplished (Amiri-Jami et al., 2014).  In Amiri-

Jami's methodology, the *E. coli* containing the *pfaA*, *pfaB*, *pfaC*, *pfaD*, and *pfaE* genes

was grown in a broth supplemented with 12.5 μg/ml of the antibiotic chloramphenicol (Amiri-Jami et al., 2010, 2014).  The issue with transferring these antibiotic-resistant bacteria into food grade products is that transfer of resistance to other bacteria inhabiting the human intestinal tract. This transfer of DNA may affect the efficiency of antibiotics that people take for illness (Landers et al., 2012).  Thus, the team plans to overcome this obstacle by creating a model where the antibiotics used to grow *E. coli* are removed and applying another method of selective pressure that does not rely on antibiotics.  Without the antibiotics,  the *L. casei* containing the Ω-3 gene cluster can be safely transferred to food products.

In order for our transformed target bacteria to be put into food products such as yogurt and vegetables, the antibiotic resistance marker needs to be removed from the plasmid. Antibiotic resistance in bacteria is a huge problem globally so our product should not contribute to the issue (World Health Organization, 2018). Through an extensive literature search, it was determined that the *alr* gene system for antibiotic marker removal would be a sensible option to explore in the future to make our bacteria food-grade. *alr* is a gene that encodes for the alanine racemase enzyme which catalyzes the conversion of L-alanine to D-alanine. D-alanine is involved for the cross-linking of cell wall peptidoglycan layers in many bacteria which is essential for cell growth. The *alr* gene is native to *E. coli* strains which makes it an ideal candidate for the replacement of the antibiotic resistance marker. This antibiotic removal system works by cutting out the antibiotic resistance marker from the plasmid through restriction enzyme digestion and then ligating the *alr* gene to the plasmid in the place of the antibiotic resistance marker.

The selection pressure that keeps the plasmid inside the target bacteria is still maintained since the *alr* gene is essential for the growth of the bacteria itself.

**Conclusion**

The numerous health benefits of $\Omega$-3 fatty acids and the necessity of greater access to $\Omega$-3 makes the genetic engineering of *E. coli* Nissle an important and justifiable area of study. Although solutions to address this problem, such as $\Omega$-3 capsules or the genetic engineering of plants to create $\Omega$-3, have already been proposed in both the marketplace and the scientific community (Pikes Place et al., 2016), these methods are limited by efficacy and health effects (Neale et al., 2013; Simopoulos et al., 2016). Furthermore, previous studies that conducted similar experiments support the proposed methodology: the creation of a *S. baltica* plasmid, the insertion of this plasmid into *E. coli* Nissle, and the confirmation of $\Omega$-3 production by spectrometric and chromatographic methods (Amiri-Jami et al., 2014). Therefore, the use of genetically engineered *E. coli* Nissle to supplement food products in order to increase their $\Omega$-3 content is a viable research endeavor that has the possibility to benefit many.

## Chapter 3: Methodology

### Approach

In order to achieve the goal of successful transforming *E. coli* to produce $\Omega$-3 fatty acids, four objectives were performed. The first objective was to isolate the *pfaABCDE* gene cluster from, *S. baltica*, a strain of marine bacteria that naturally produces $\Omega$-3 fatty acids. The second objective was to insert the isolate *pfaABCDE* gene cluster into the cloning *E. coli* bacterial strains using the pCR™ - 2.1 TOPO™vector and the third objective was to re-isolate the transform. The fourth objective was to quantify the production of EPA in the transformed *E. coli* using gas chromatography - mass spectroscopy. Significance was assessed by comparing *E. coli* $\Omega$-3 production with an EPA standard. Objectives I and II were undertaken in parallel with members of Dr. Biswas's lab, including from the Department of Animal and Avian Sciences, University of Maryland, College Park.

### Approach to achieve objective I:

To grow *E. coli DH5a*, *DH10β* cloning strains and to isolate the pfa*ABCDE* gene cluster from *S. baltica* using PCR.

### 1A. Description of growth conditions.

In order to create stocks of bacteria, we needed to culture the *S. baltica* (ATCC BAA-1091D-5) and *E. coli* (ATCC 68233) using specific growth conditions. The *S. baltica* bacteria grow best in aerobic conditions, but are able to grow in anaerobic conditions as well. They were allowed to grow in LB Broth Lennox at 30℃ for 24 hours. *S. baltica* is a gram-negative bacteria. The *E. coli* strain was grown in LB Broth at 30℃

overnight.  After growing, the *E. coli*  was plated and then subcultured.  Growth of the desired *E. coli* was visually confirmed on our plates by the presence of shiny, round light yellow colonies characteristic of the bacteria.

## 1B. Restriction sites of *pfaABCDE* gene cluster and primer design for PCR.

Due to the length of the gene cluster being isolated, restriction sites for primers that would allow the primer to be divided into two fragments, to be ligated together again after isolation, were selected.  By reducing the length of the gene cluster, from 20 kb to two pieces of approximately 10 kb each, transformation efficacy was increased and total length of PCR was decreased.  Primers that bound to BamHI and XhoI restriction sites were selected for use in our PCR.  The BamHI restriction site was appended on both sides of the target *pfaABCDE* gene cluster.  The internal primers overlap with a Xho1 restriction site that falls between genes *pfaA* and *pfaB,* effectively dividing the cluster in half.  The following primers were designed for use in isolation of the gene cluster: "BamHI forward" (5'-GGATCCTTAAAAACGATTCAGCGG-3'), "BamHI reverse" (5'-GGATCCGCCGTTAATAATCGGCAAGTA-3'), "XhoI forward" (5'-GTTTTACCACCTCGAGCACTAGATAG-3'), and "XhoI reverse" (5'-CTATCTAGTGCTCGAGGTGGTAAAAC-3').  One concern with the use of these primers was their low GC content.  GC bonds in a primer should be between 40% and 60% of the primer's total length to promote stable binding with DNA.  The above primers range from 45.83% GC content to 48.15% GC.
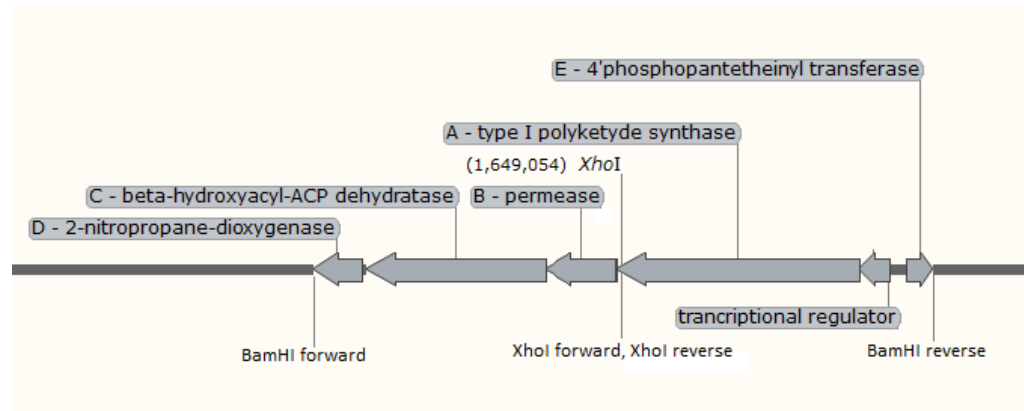
Figure 2: Binding sites of BamHI and XhoI primers on the *S. baltica* genome with respect to other genes in the genome.
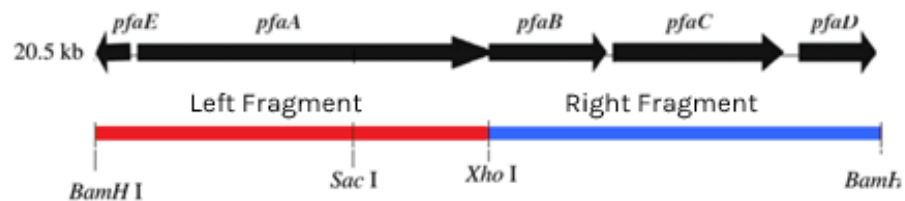


Figure 3: "Left fragment" and "right fragment" of the *pfaABCDE* gene cluster.

The gene cluster was cleaved at the sites labeled BamHI to remove the cluster from the rest of the genome and at XhoI to divide the cluster into two 10kbp pieces.

**1C. Isolation and amplification of *pfaABCDE* gene cluster in *S. baltica***

After the *S. baltica* was successfully cultured, the DNA was isolated so that the *pfaABCDE* gene cluster could be amplified. In order to isolate the DNA, a sample of the bacteria that had been growing on the plate was suspended in 100 μL of DNA free water. Following suspension, the bacteria was run through a PCR incubation protocol where the bacteria was heated to 99°C for 10 minutes and brought back down to room temperature.

The solution was then centrifuged at 1300 rpm for 10 minutes. After centrifugation, the bacteria was prepared for a second PCR to amplify the left and right fragments.

The above procedure was applied to two seperate tubes, one of which would receive primers corresponding to the left fragment ("BamHI forward" and "XhoI reverse") of *pfaABCDE* and the other would receive the primers for the right half of *pfaABCDE* ("BamHI reverse" and "XhoI forward"). To prep for PCR, the protocol put forth for fragments greater than 10 kb in the 2x PCR Master Mix Solution [i-MAX II], including their suggested cycling parameters, was followed. Following the PCR, the products were purified using an Invitrogen PureLink Quick Gel Extraction and PCR purification combo kit. After the purification was completed, the product was run through gel electrophoresis on an agarose gel at 85 V for 30 minutes with a GeneRuler DNA Ladder Mix to confirm the size of the isolated fragments.

Following confirmation of PCR product using gel electrophoresis, samples were run through a NanoDrop machine (Thermo Scientific NanoDrop products) to confirm the purity of the isolated DNA. If the sample on the NanoDrop had a A260/280 value of greater than 1.8 it is considered to be uncontaminated DNA of suitable quality to work with for future analysis. If the A260/280 value was less than 1.8, there is potential that the sample has been contaminated with foreign proteins and should not be used further.

**Approach to achieve objective II:**

To ligate the isolated left and right fragments of the *pfaABCDE* gene cluster to the pCR™ - 2.1 TOPO™ cloning vector and transform target bacterial strains, *E. coli* DH5α and DH10β.

## 2A. Ligation of the *pfaABCDE* gene cluster fragments to the pCR™ - 2.1 TOPO™ cloning vector.

Once the *pfaABCDE* gene cluster from *S. baltica* was isolated, segmented into left and right fragments, and amplified, the next step was to transfer the fragments of the isolated gene into a vector. The vector employed was the Invitrogen pCR™ 2.1 - TOPO™ (3.5 kbp). Instructions for ligation were taken and modified from the TOPO™ - TA Cloning™ kit supplied with the vector (see Appendix B). The left and right fragments were ligated to the vector using the BamH1 and Xho1 restriction sites included in the fragments during primer design, creating two plasmids of 13.8 kbp each. Due to limitations in the size of the gene insert for the pCR™ 2.1 - TOPO™ vector and to facilitate the transformation with such large inserts, left and right fragments of the *pfaABCDE* gene were inserted into two separate plasmids and recombined in a process detailed below in section 2D.
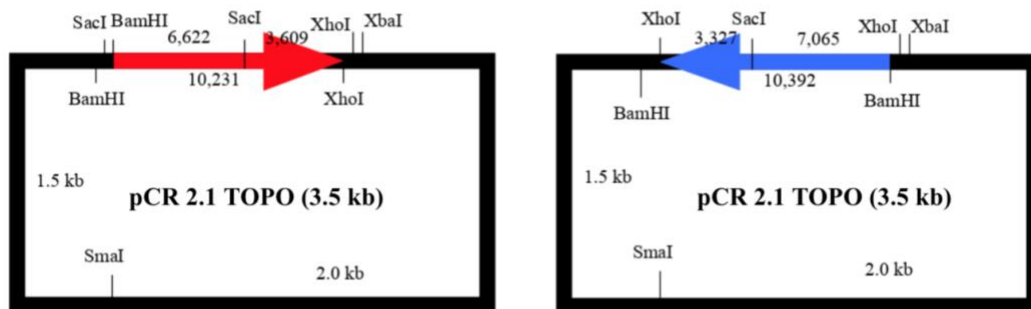


Figure 4: Right and left fragments ligated to the pCR™ 2.1 TOPO™.

## 2B. Transformation of the target bacterial strains and antibiotic selection of transformed bacteria.

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The two pCR™ - 2.1 TOPO™ vectors with the left and right fragment
inserts were then used to perform a transformation reaction on the target bacterial strains,
*E. coli* DH5α and DH10β.  Figure 5 illustrates the heat shock transformation method.
Specific instructions for preparing competent target bacteria as well as quantities of
reagents and procedures for the heat shock transformation method are outlined in
Appendices A and B.  The pCR™ 2.1 - TOPO™ vector contained both ampicillin (AMP)
and kanamycin (KM) selection markers.  As such, the transformed bacteria were plated
on Luria-Bertani (LB) agar plates containing 50 μg/mL KM and 100 μg/mL AMP and
allowed to grow at 37° Celsius overnight to select for positive transformants.
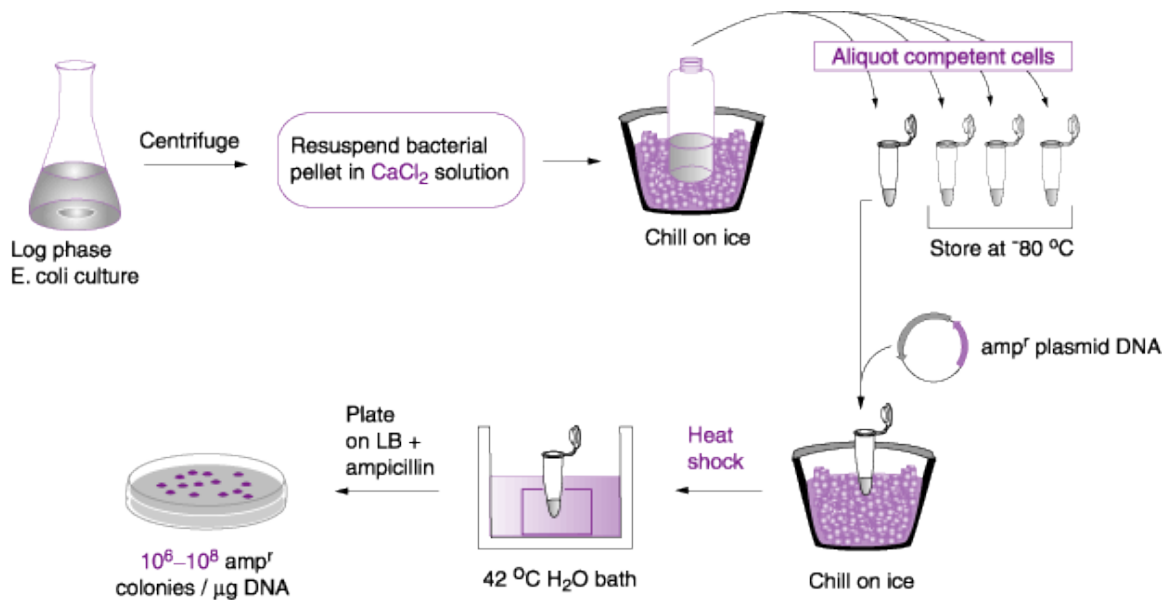


Figure 5: The heat shock method of bacterial transformation is the most simple method of
transformation. It is very straightforward and cost effective, but has slightly lower
transformation rates than other methods (Miesfeld, 2000).

**2C. Confirmation of transformed bacteria using colony cracking.**

Confirmation of successful transformation and identification of transformants with the PCR insert were assessed using colony cracking. Colony cracking is a simple and quick method of screening for transformants predicated on the difference in electrophoretic mobility of DNA with and without the insert in agarose gel. Plasmids with the insert will travel slower and appear as higher bands on an agarose gel than plasmids with the insert. Following colony cracking, plasmids with the insert were run through a gel with a DNA ladder to assess the correct size of the insert.

To perform colony cracking, positive transformants were incubated overnight in LB broth then centrifuged at 13000 rpm for one minute to condense the suspended cells into a pellet. The pellet was then resuspended in 42°C lysis buffer and centrifuged again at 13000 rpm for 10 minutes to separate the cellular components from the isolated plasmid DNA in the supernatant. Isolated plasmid DNA were separated and visualized through gel electrophoresis. Further information regarding the cracking protocol can be found in Appendix C. After successfully confirming transformants, the left and right fragments of the *pfaABCDE* gene cluster were recombined in a process detailed below.

**2D. Recombination of left and right *pfaABCDE* gene fragments into single plasmid and confirmation using restriction enzyme digestion.**

Recombination of the left and right *pfaABCDE* gene cluster fragments was accomplished by the team working in tandem on this project in Dr. Biswas's lab through restriction enzyme digestion at the XhoI and BamHI and restriction sites followed by ligation of fragments to a single plasmid. The right fragment was cut and excised from

the plasmid at the BamHI restriction site and ligated to the plasmid containing the left

fragment at the XhoI restriction site to create a plasmid of 24.1 kb. Combination and

proper orientation of the left and right fragments were assessed through restriction

enzyme digestion.
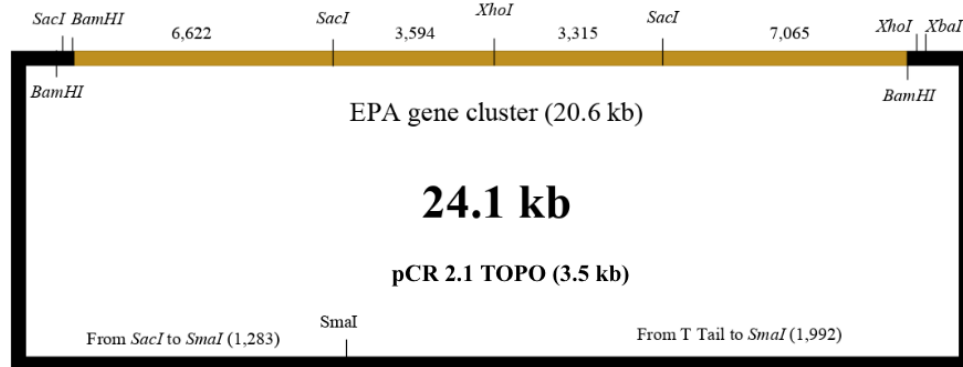


Figure 6: Plasmid containing both the left and right fragments of the *pfaABCDE* gene cluster in the correct orientation.

**Approach to achieve objective III:**

To measure EPA output in the transformed *E. coli* DH5α, DH10β, using gas

chromatography - mass spectroscopy (GC-MS) and compare to pure EPA standard.

**3A. Growth curve to assess growth stages of transformed *E. coli* DH5α and DH10β.**

A growth curve was obtained by counting colony-forming units (cfu) on serial

dilutions and by measuring the optical density (OD $\lambda$=600nm) with the Perkin Elmer

Lambda Bio + spectrophotometer in 4 to 24 hour intervals over the course of 72 hours.

The maximum growth prior to the stationary phase was recorded and used to culture

bacteria. This time allows the bacteria to accumulate fatty acids which can then be further analyzed by GC-MS methods.

**Approach to achieve objective IV:**

To isolate the target bacteria, *E. coli* Nissle in preparation for transformation with the pCR™ 2.1 TOPO™ vector containing both the left and right fragments of the *pfaABCDE* gene cluster.

**4A. Isolation of *E. coli* Nissle and confirmation through PCR.**

Cultures of *E. coli* Nissle were isolated from the Mutaflor® supplement using MacConkey's agar to select for enteric bacteria and grow overnight at 37℃. Confirmation of the isolation of *E. coli* Nissle was obtained with PCR detection methods that employ Muta 1-10 primers to determine the presence of pMUT1 and pMUT2 plasmids unique to the *E. coli* Nissle 1917 strain isolated from our supplement (Blum-Oehler et al., 2003).

Table 1. Primers for PCR detection of *E. coli* Nissle 1917

| Primers | DNA Region | Fragment length (bp) |
|---|---|---|
| Muta 1 & 2 | *fim*A | 253 |
| Muta 3 & 4 | *foc*A | 441 |
| Muta 5 & 6 | Plasmid pMUT1 | 361 |
| Muta 7 & 8 | Plasmid pMUT2 | 427 |
| Muta 9 & 10 | Plasmid pMUT2 | 313 |

**4B. Re-isolation of vector with combined *pfaABCDE* gene cluster.**

In preparation of the transformation of *E. coli* Nissle, the pCR™ 2.1 TOPO™ plasmid containing the recombined *pfaABCDE* gene cluster was isolated from a stock of *E. coli* DH5α kindly provided by the other members of Dr. Biswas's lab. Plasmid isolation and purification was accomplished using the Intron Technologies DNA-Spin Plasmid Purification kit.

## Chapter 4: Results

**Objective I: Isolation the *pfaABCDE* Ω-3-fatty-acid-producing gene cluster from the**

**DNA of *S. baltica*.**
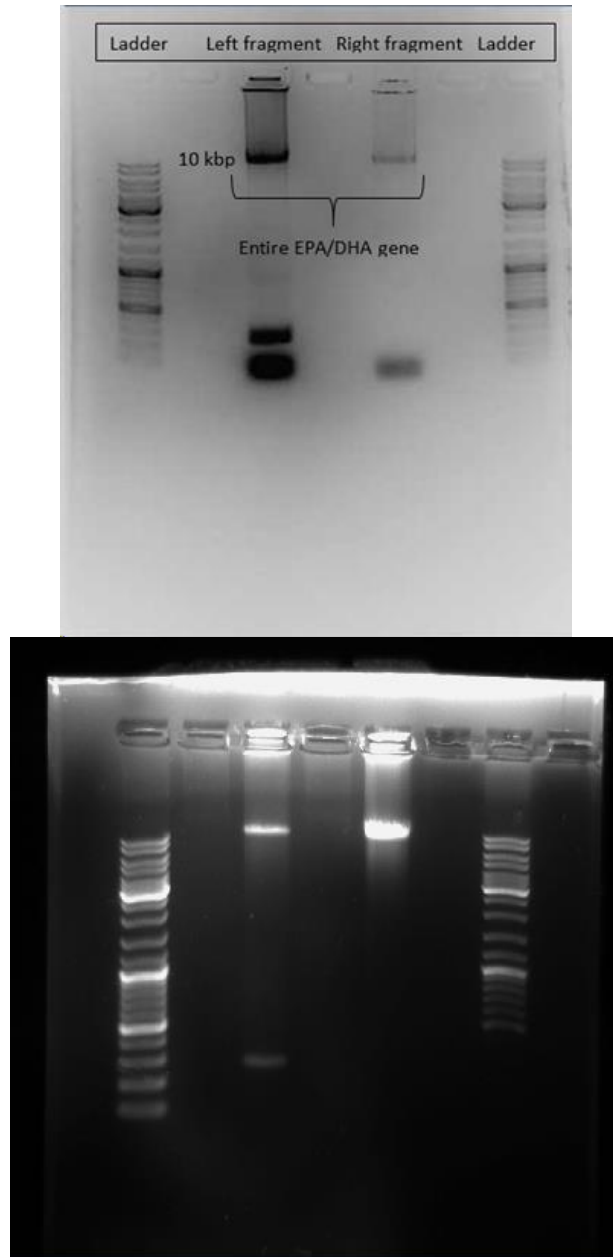


Figure 7: Gel from June 2017 showing successful isolation of the left and right fragments of the *pfaABCDE* gene cluster (left). Gel from September 2018 showing an second, independent isolation of the fragmented gene cluster (right).

In order to determine the size of the isolated DNA fragments, those bands were compared with a ThermoFisher Scientific GeneRuler DNA Ladder Mix which are shown in Figure 7 (on the left and right rows of each gel).  For each gel, a space was maintained between the samples for ease of read and clarity of imaging.  The left well contains the left fragment of the *pfaABCDE* gene cluster while the right well of the gel with the single band contains the right fragment of the gene cluster.  The GeneRuler DNA Ladder Mix ranges from 100 base pairs (bp) to 10,000 base pairs (10 kbp).  From comparing the sample bands to the ladder, it was determined that the lane with the left fragment has a two bands that have sizes of approximately 10 kbp and 500 bp.  For the right fragment, it is observed that there is a single band at approximately 10 kbp.

Tables 2 and 3 show the output from the NanoDrop machine used to assess the purity of the isolated DNA from *S. baltica*.  Table 2 shows the output for the left fragment of the *pfaABCDE* gene cluster and Table 3 shows the output for the right fragment.  On the left column A230 corresponds to the absorption, in optical density, of the sample at a wavelength of 230 nanometers.  A260 corresponds to absorption at 260 nm, A280 is absorption at 280 nm and A320 is absorption at 320 nm.  In both Table 2 and Table 3 we see that the A260/A280 ratio is > 1.80, indicating the purity of our samples.

Table 2: NanoDrop output for left fragment of *pfaABCDE* gene cluster (April 2018).

| Concentration | 149.0 µg/mL |
|---|---|
| Path length | 0.5 mm |
| A230 | 1.43 |
| A260 | 3.03 |
| A280 | 1.60 |
| A320 | 0.054 |
| A260/A280 | 1.828 |
| A260/A230 | 2.159 |

Table 3: NanoDrop output for the right fragment of the *pfaABCDE* gene cluster (April 2018).

| Concentration | 147.5 µg/mL |
|---|---|
| Path length | 0.5 mm |
| A230 | 1.45 |
| A260 | 3.04 |
| A280 | 1.70 |
| A320 | 0.089 |
| A260/A280 | 1.832 |
| A260/A230 | 2.169 |

**Objective II: Ligation of the isolated gene cluster to the pCR™ - 2.1 TOPO™ cloning vector and transformation of cloning bacteria strains, *E. coli* DH5α and DH10β.**

Figure 8 depicts 2 dark band in lanes 2 and 12 located proximally to the wells at the top of the gel and dark banding further along the lane towards the bottom of the gel.

The remaining 14 wells were also loaded with cracking solutions of unique 2 colonies

transformed with the plasmids containing the left or the right fragment. These wells

exhibit dark, uniform banding distal to the wells of the gel and the bands in lanes 2 and

12 as well as a faint band and a separate darker band closer to the beginning of the well.
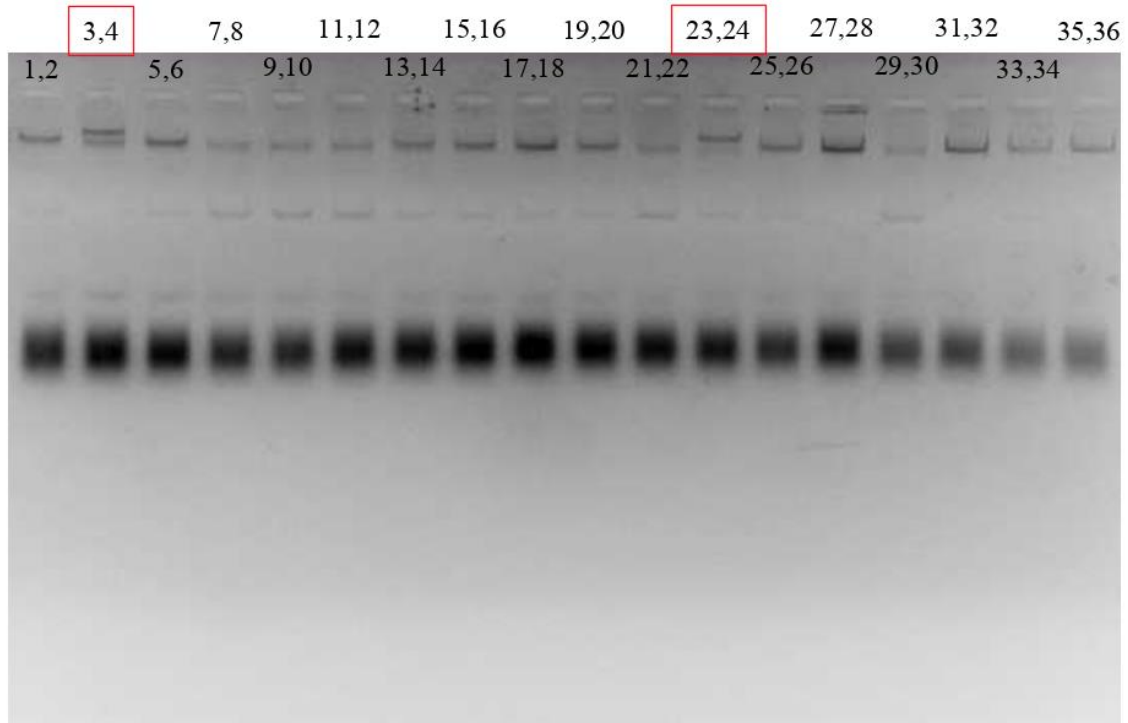


Figure 8: Colony cracking gel (colonies with left and right fragment combined in single well) provided by members of Dr. Biswas's lab (Department of Animal and Avian Sciences, University of Maryland, College Park) exhibiting confirmation of left and right positive transformants in lanes 2 and 12. There is no molecular standard present on this gel because we are comparing the relative length and number of fragments rather than their absolute length.

Figure 9 is two gel images of the isolated plasmid with both the left and right

fragments. In the image to the left, the first and third wells contain the Lambda

DNA/HindIII Marker, 2 gene ladder and the second and fourth wells contain the the

sample of isolated plasmid. Faint banding can be seen above the ladder marker for

approximately 20 kbp in the second and fourth wells. The image to the right depicts a separate isolation of the combined left and right fragment plasmid at a later date. In the right image, wells 1 and 5 contain the Lambda DNA/HindIII Marker, 2, wells 2 and 4 contain the Gene Ruler DNA Ladder mix, and well 3 contains the sample of isolated plasmid. When compared to the Lambda DNA/HindIII Marker, 2 ladder, the sample in well 4 appears to exhibit banding above approximately 20 kbp and 10 kbp when compared to the Gene Ruler DNA Ladder.
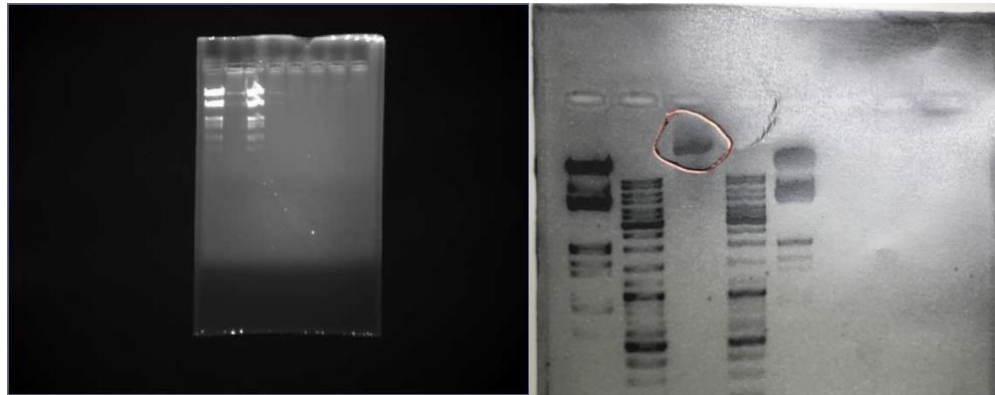


Figure 9: Gel images from 3/8/18 (left) and 2/18/19 (right) of isolated plasmid containing left and right fragments.

In the image to the left, Lane 2 exhibits banding at of the BamHI restriction digest products at approximately 20 kb and 3.5 kb while lane 3 which was loaded with the XhoI restriction digest products exhibits 2 bands around 10 kb. In the right image, there appears to be 5 products from the combined SacI and XhoI restriction digestion, with 2 products at approximately 7 kb and 3 products between 3 and 3.6 kb.
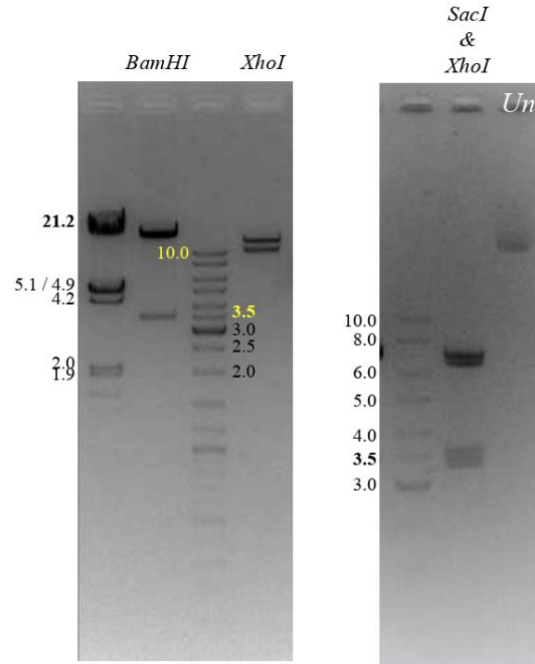
Figure 10: Gel of restriction enzyme digestion of the plasmid with combined right and left fragments provided by members of Dr. Biswas's lab (Department of Animal and Avian Sciences, University of Maryland, College Park).

Table 4 shows the output from the NanoDrop machine when a sample of isolated pCR™ 2.1 - TOPO™. The vector was inserted on March 7, 2019. The A260/A280 ratio is > 1.80. This indicates that the DNA from the vector is not contaminated by outside proteins and we have isolated pure DNA.

Table 4: Nanodrop output for the isolated *E. coli* plasmid

| Concentration | 69.5 µg/mL |
|---|---|
| Path length | 0.5 mm |
| A230 | 0.61 |
| A260 | 1.32 |
| A280 | 0.69 |
| A320 | -0.72 |
| A260/280 | 1.829 |
| A260/A230 | 2.044 |

**Objective III - A. Growth curve to assess growth stages of transformed *E. coli* DHα and DH10β.**

For the curve from the OD measurements, the maximum growth for all samples was observed at around 24 hours which corresponds to the late exponential phase of growth (Figure. 12). This time is consistent with previous research (Gentile et al., 2003; Vancanneyti et al., 1996). PCR-XL controls had a slightly higher OD when compared to the transformed DH5α and DH10β strains. Triplicates within each category showed great concordance.
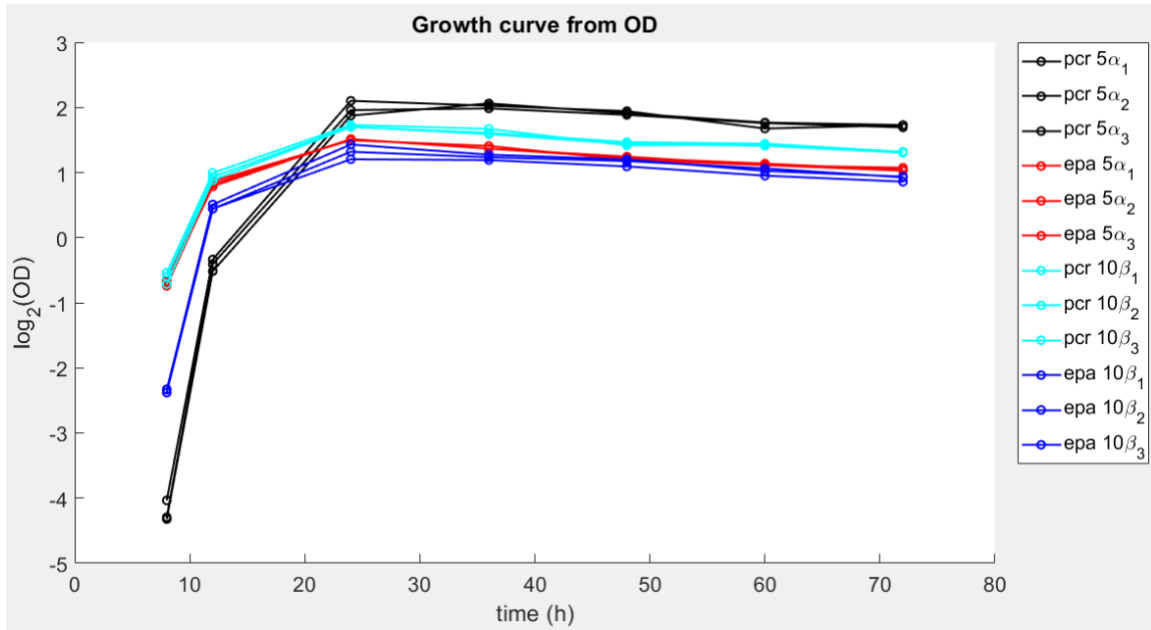
Figure 11: OD600 growth curve of *E. coli* DH5α (black and red), DH10β (cyan and blue) and controls triplicates.

The curve from CFU measurements had a maximum between 12 and 24 hours (See Figure. 13). *E. coli* DH10β in both LB and LB + KM shows a significantly faster decay when compared to other samples.
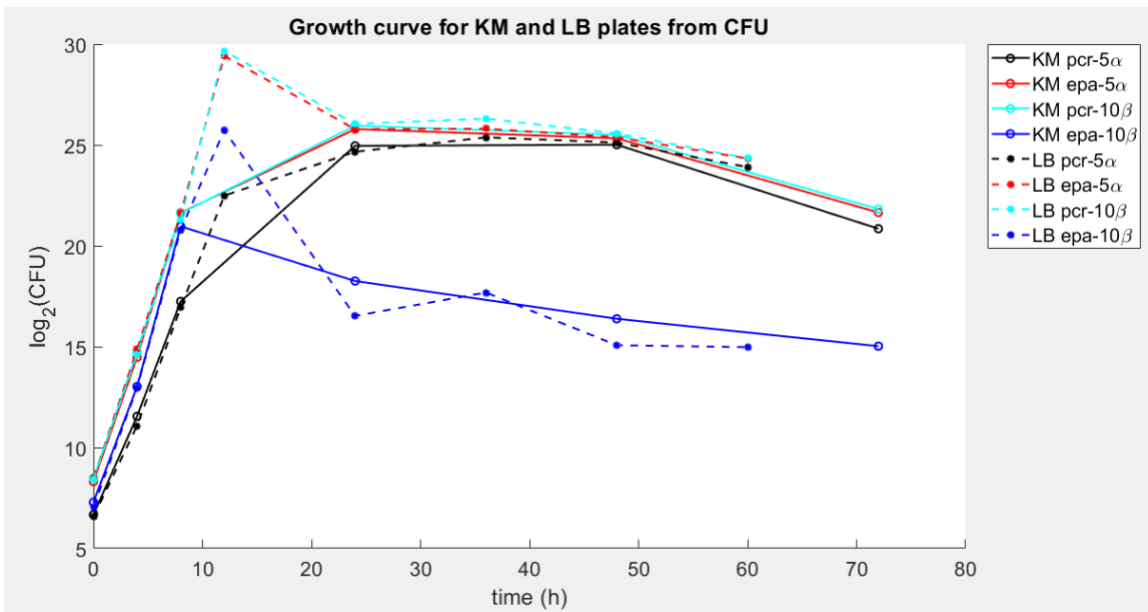


Figure 12: CFU growth curve of *E. coli* DH5α, DH10β and controls in LB+KM and LB agar plates.

**Objective IV: Isolation of target bacterial strain, *E. coli* Nissle.**
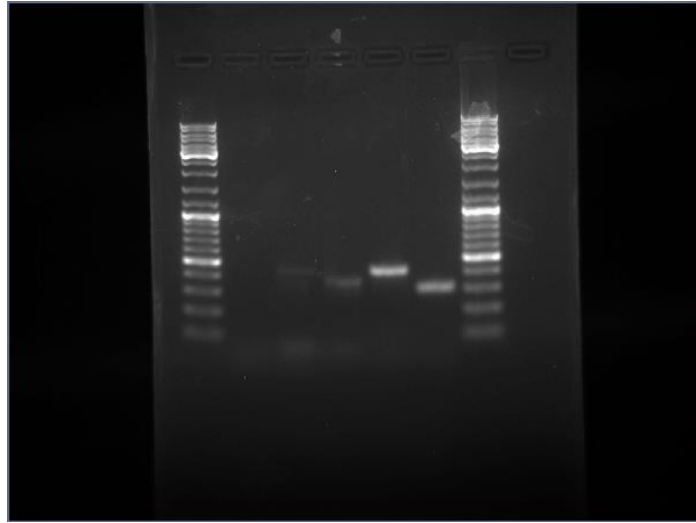


Figure 13: *E. coli* Nissle isolation confirmation through PCR.

Figure 11 depicts the isolation and amplification of four of the five genes specific to the target strain *E. coli* Nissle 1917 found on the pMUT1 and pMUT2 plasmids. Lanes 2 and 4 exhibit banding at approximately 500 bp while lanes 3 and 5 exhibit banding further along the gel closer to 300 bp.

## Chapter 5: Discussion

The end goal of this study was to obtain a transformed food-grade bacteria, containing the EPA/DHA cluster gene. Fatty acid production from this bacteria was expected to occur and reach significant concentrations when compared to other bacteria such as *S. baltica*, the original source of the EPA/DHA gene. Due to the complexity of the methodology, there were some challenges that were encountered which will be thoroughly explained in this section. In addition, the research project has multiple directions that can be explored in the future including antibiotic resistance marker removal which will be outlined as well.

**Methodology**

The first step to achieve this goal involved the successful isolation and amplification of the EPA/DHA cluster from *S. baltica* through PCR. Significant time was devoted to the amplification as the length of each fragment (10 kbp) far surpasses the usual length used in Taq-based PCR, which is estimated at around 5 kbp (ThermoFisher Scientific et al., 2012). However, the increased length should not affect the accuracy of the amplification as the error rate of Taq Polymerase is about $2.2 \cdot 10^{-5}$ errors per base pair per cycle, which results in 0.22 errors per cycle in our case. The left fragment yielded much higher amplification while the right fragment required multiple primer adjustments for the reverse BamHI primer used in the amplification process. The disparities between both fragments can be observed from the gels as evidenced by the difference in band contrast. As of currently, it has not been determined why the disparity between the fragments exists, but additional studies can be done in the future to understand the mechanism of this disparity.

The second objective involving the bacterial transformation was the most challenging step to perform in the methodology. Initially, the team was expecting to transform the gram-positive bacteria *L. casei*, because of its multiple food applications such as the fermentation of milk products like yogurt. However, after multiple attempts only the left fragment was successfully inserted and confirmed through cracking. This follows the trend of increased yield in the left fragment as compared to the right fragment.

While ligation rates can achieve around 50% success and fragment insertion rates remain modest, ranging between 10-50%, transformation is an intrinsically inefficient process (Biswas et al., 2016b). Some sources report rates as low as 0.01%, which translates into one transformed bacteria for every 5E4 in *E. coli* DH5α cells. This low transformation rate becomes even more problematic when dealing with gram-positive bacteria. Gram-positive organisms have a much thicker peptidoglycan layer (20-80 nm) as opposed to gram-negative bacteria (2-3 nm) (Sizar & Unakal et al., 2019), which makes it more difficult for the cell to uptake the plasmid. New methods have been described in the literature to improve the efficiency of gram-positive transformation, and *L. casei* specifically. These include the use protoplasts of the target bacteria combined with glass beads or the use of high efficiency electrotransformation (Rattanachaikunsopon & Phumkhachorn et al., 2009; Welker, Hughes, Steele, & Broadbent et al., 2015). In addition, when performing the transformation step, the heat shock method was employed. Even though this method is readily used for transformation, there are other methods of transformation that could have potentially worked better like electroporation. Electroporation uses an applied voltage to create

pores in the cell membrane to allow for the plasmid to go into the cell.  Further experiments using the electroporation method for transformation should be done in the future to determine if electroporation increases the transformation efficiency for the plasmid of interest.

Through collaboration with other members of Dr. Biswas's lab in the last few months, the recently obtained transformed bacteria (*E. coli* DH5α and DH10β), with both left and right fragments, was kindly donated to allow for the research to progress forward. The goal shifted in trying to transform *E. coli* Nissle with the plasmid from the donated transformed bacteria.  *E. coli* Nissle is a non-pathogenic and gram-negative bacterial strain which eliminates the issue associated with gram-positive bacteria while still being of food-grade.  With this bacteria, the whole methodology was started again.  We were unable to perform the final steps of ligation and transformation with this bacterial strain due to time limitations. However, these next steps can be done in the future if the research is continued.

**Limitations**

Limitations of the current approach include the potential for overexpression of EPA and DHA in future work. Overconsumption of EPA and DHA has been noted to increase low-density lipoprotein oxidation in the body which can lead to a range of negative health effects, including an increased risk in cardiovascular disease (Mata et al., 1996). This limitation can be minimized by performing the experiment through multiple iterations. Promoter regions near the gene can be suppressed or expressed as needed to achieve the desired level of EPA and DHA production. To increase EPA/DHA output,

the transformed bacteria can be incubated with isopropyl β-D-1-thiogalactopyranoside (IPTG) which is a metabolite that increases expression for the pCR™ - 2.1 TOPO™ vector that contains the lac promoter.  IPTG binds to the lac repressor and increases expression of the vector which in turn increases expression of the EPA/DHA gene.  This increased expression should lead to an increase in EPA/DHA output.

Another potential limitation of this work lies in the restriction enzyme digestion used to confirm the final orientation of the *pfaABCDE* gene cluster. There is the possibility that at some point during the experiment mutations in the *S. baltica* genome occurred that would modulate the expression of the gene in some way. The restriction enzyme digestion would likely not show a mutation unless it involved a major insertion or deletion. To ensure that a mutation did not occur, a complete sequencing should be performed on the transformed bacteria to ensure the correct sequence of the gene cluster. This sequencing was not accomplished over the course of this project and should be taken in to account by researchers looking to continue this work.

**Future Directions**

There are various future directions for this research project that can be explored. Once a fully transformed *E. coli* Nissle bacteria is obtained, the EPA/DHA output will be measured using gas chromatography/mass spectrometry (GC/MS).  The percentage of EPA/DHA produced by the transformed bacteria will be compared to a threshold value found in literature to determine if the amount produced is comparable.  A total of three samples will be measured using the GC/MS method: a pure EPA standard, an EPA/DHA sample from *S. baltica*, and an EPA/DHA sample from the transformed bacteria.  The

pure EPA standard will aid in determining if there is any EPA in the other samples. At 25°C, *S. baltica* produces about 1.5 mg EPA/g of cell dry weight which was lower than those obtained at 15°C and 20°C (Amiri-Jami et al., 2010). This literature value would be the threshold EPA production value that the transformed bacteria should meet or exceed in order to safely assume that the transformed bacteria has a high EPA productivity.

The removal of the antibiotic marker in the plasmid could be a potential future step. As previously discussed, for food-grade products, there cannot be any antibiotics due to antibiotic resistance that can be built in those that uptake these foods. Therefore, antibiotic marker removal methods to be determined in order to make the plasmid safe for food-grade products. One such method is the insertion of the *alr* gene to replace the antibiotic marker. The *alr* gene encodes for the enzyme, alanine racemase, which is responsible for the conversion of L-alanine to D-alanine (Bron et al., 2002). D-alanine promotes the growth of gram-positive bacteria by allowing for cross-linking of the cell wall peptidoglycan layers. The deletion of the antibiotic marker and insertion of the *alr* gene is a safe genetic tool for antibiotic removal and allows for a food-grade complementation marker (Bron et al., 2002).

Another method is the use of a suicide vector to remove the antibiotic marker. With the insertion of a suicide vector into the bacteria through transformation, there is an allelic exchange in which there is a generation of mutants with gene knockouts (Oritz-Martin et al., 2006). A gene within the suicide vector is exchanged with the antibiotic marker in the other plasmid with the EPA/DHA gene and leads to the removal of that antibiotic marker. However, multiple suicide vectors would have to be created and tested

to determine which one would be the most effective which can be time-consuming (Oritz-Martin et al., 2006).

Once the transformation is completed, EPA/DHA output is measured, and the antibiotic marker is removed, the insertion of the bacteria into milk, yogurt, vegetables, or probiotic supplements (pills) should be explored. Through our research we were able to create a vector with the EPA/DHA-producing genes. Such vector may allow future researchers to insert the EPA/DHA genes into various strains of probiotic bacteria with established uses in food processing and food supplementation. Following appropriate modifications and measurement of $\Omega$-3 output, this library of bacteria with the EPA/DHA gene could then be applied to different food products to increase their $\Omega$-3 content with the aim of increasing the availability of $\Omega$-3 fatty acids.

## Chapter 6: Conclusion

The ultimate goal of the research project is to modulate the probiotic bacteria *E. coli* Nissle to produce Ω-3 fatty acids for application in fermented food products such as yogurt. The *pfaABCDE* gene cluster that produces EPA/DHA was isolated from *S. baltica* successfully through the use of PCR and restriction sites. From this step, the gene cluster was split into two fragments and each was then successfully ligated to a pCR2.1 cloning vector. Transformation of *E. coli* DH5α and DH10β strains was completed and confirmed through colony cracking. Finally, the target bacteria *E. coli* Nissle was isolated from a probiotic supplement and the isolation was confirmed through the PCR detection protocol. All of these steps in the methodology were completed successfully and inform the future direction of the project as described in the discussion. Overall, this work has provided a foundation for future researchers to build upon when working to modulate bacteria.

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# Appendices

### Appendix A: Chemical preparation of competent *E. coli* DH5α and DH10β cells

**Materials:**
- Single colony from target *E. coli* strains growth overnight at 37 ° Celsius
- ~100 mL LB broth
- 30 mL 0.1 M $CaCl_2$ (chilled)
- 6 mL 0.1 M $CaCl_2$ + 15% glycerol (chilled)

**Procedure:**
1. Inoculate colony into 2 mL of LB broth and place horizontally in 225 rpm shaking incubator at 37°C overnight.
2. Inoculate 1 mL overnight culture into 100 mL LB broth and incubate in 225 rpm shaking incubator at 37°C until $OD_{600}$ reads 0.25-0.30 (approximately 1.5 -2 hours).
3. Chill culture on ice for 15 mins then centrifuge cells for 10 mins at 4,000 rpm.
4. Discard medium and resuspend cell pellet in 30 mL of chilled 0.1 M $CaCl_2$.
5. Store cells on ice for 30 mins the centrifuge again for 10 mins at 4,000 rpm.
6. Remove supernatant and resuspend cell pellet in 6 mL of 0.1 M $CaCl_2$ + 15% glycerol solution.

**Appendix B:  Modified procedure for ligation of PCR insert to pCR™ 2.1 - TOPO™**

**and heat shock transformation of target bacteria.**

**Materials:**
- 4 μL of purified PCR product (concentration in 1:1 molar ratio to vector)
- 1 μL of pCR™ 2.1 - TOPO™  (10 ng/μL)
- 250 μL LB or S.O.C. medium
- 1 μL Salt solution (200 mM NaCl, 10 mM $MgCl_2$)
- LB plates with 50 μg/mL KM and 100 μg/mL AMP

**Procedure:**
1. Combine PCR product, TOPO™ vector, and salt solution to create TOPO™ reaction mixture.
2. Gently mix and then briefly (30 seconds) centrifuge TOPO™ reaction mixture.
3. Incubate mixture at room temperature for 30 minutes then store on ice.
4. Add 2 μL of mixture to 50 μL of competent cells and incubate on ice for 30 minutes.
5. Place cells to 42°C water bath for 30 seconds then transfer to ice and incubate for 2 minutes.
6. Add 250 μL of room temperature LB or S.O.C. medium and place horizontally in shaking incubator at 37°C and 225 rpm for 1 hour.
7. Spread 150 μL of cells on pre-warmed KM/AMP LB plate and incubate overnight at 37 ° C.

**Appendix C: Colony cracking for confirmation of positive transformants**

**Materials:**
- LB broth
- Lysis buffer (50 mM NaOH, 0.5% SDS, 5nM EDTA)
- 2X loading buffer
- Colonies of positive transformants
- 1% agarose gel

**Procedure:**
1. Inoculate separate 1 mL tubes of LB with colonies of positive transformants and incubate overnight in shaking incubator set to 225 rpm and 37°C.
2. Transfer 20 uL of cultured bacteria to eppendorf tubes and centrifuge at 13000 rpm for 1 minute then discard the supernatant.
3. Place lysis buffer in 42°C water bath for 10 mins then transfer 10 uL of buffer to tubes with pellet and resuspend cells.
4. Centrifuge resuspended cells for 10 mins at 13000 rpm.*
5. Add 10 uL of 2X loading buffer to tubes then transfer cell solutions to wells of 1% agarose gel.**
6. Run gel at 85 V for 30 mins and check for bands using UV-Transilluminator

*This step condenses the genomic DNA into the pellet and leaves the plasmid in the supernatant.

**This step was performed immediately prior electrophoresis to minimize degradation of bromophenol blue (loading dye) in alkaline lysis buffer solution.

**Appendix D: Modification of Bligh-Dyer Method for Lipid Extraction**

An efficient modification of the Bligh and Dyer method, given below, was proposed for the extraction of lipids from unicellular organisms. Several parameters were optimized to improve the fatty acid recovery (Lewis et al., 2000). Thus, it was shown that the total amount of recovered fatty acids increased by about 30% through the addition of solvents to the biomass in order of increasing, as opposed to decreasing, polarity.

Cells were harvested by centrifugation at high speed for 15 min. The supernatant was discarded, the cell pellet re-suspended in 100 ml 1.0% NaCl (w/v), and re-centrifuged. The second supernatant was discarded and the cell pellet frozen overnight at -30°C. Frozen biomass was freeze dried for 15 h and subsequently stored in a sealed glass container at -30°C.

A total of 114 ml of solvent was added to freeze-dried cells (about 100 mg) in the following sequence - chloroform, methanol, water - to achieve a final chloroform/ methanol to water ratio of 1/2/0.8 (v/v/v). Samples were shaken for 15 seconds immediately following the addition of each solvent, and allowed to stand for about 18 hours, with occasional shaking by hand.

Phase separation of the biomass-solvent mixtures in the separatory funnels was achieved by adding chloroform and water to obtain a final chloroform to methanol to water ratio of 1/1/0.9 (v/v/v). A known portion of each total lipid extract recovered from the lower chloroform phase was used for further analysis.

**Glossary of Terms**

*ALA*- Alpha-linolenic acid, a type of Ω-3 fatty acid

*Antibiotic selection marker -* DNA sequence in plasmid that encodes antibiotic resistance

and is used to select for transformed bacteria

*Bacterial Transformation -* the exchange of genetic material between strains of bacteria

by the transfer of a fragment of naked DNA from a donor cell to a recipient cell, followed

by recombination in the recipient chromosome.

*DHA -* docosahexaenoic acid, a type of Ω-3 fatty acid

*Electroporation -* a method of plasmid transfer in which the cells are shocked in order to

open the cell membrane and allow the shuttle vector to enter.

*EPA -* eicosapentaenoic acid, a type of Ω-3 fatty acid

*Fatty Fish -* a fish that naturally produces a significant amount of Omega-3 fatty acid;

includes fish such as anchovies, bluefish, herring, mackerel, salmon, sardines, sturgeon,

lake trout, and tuna (Covington et al., 2004).

*Gas chromatography (GC) -* chromatography in which the sample mixture is vaporized

and injected into a stream of carrier gas (as nitrogen or helium) moving through a column

containing a stationary phase composed of a liquid or a particulate solid and is separated

into its component compounds according to the affinity of the compounds for the

stationary phase

*Gel electrophoresis-* a basic biotechnology technique that separates macromolecules

according to their size and charge;  frequently used to analyze and manipulate samples of

DNA, RNA, or proteins (Carolina Biological Supply Company et al., 2005)

*Gut microflora* - the microorganisms that live in the intestines of a human or an animal

*High performance liquid chromatography (HPLC)* - liquid chromatography in which the degree of separation is increased by forcing a solvent under pressure through a densely packed adsorbent—abbreviation

*Mass spectrometry (MS)* - an instrumental method for identifying the chemical constitution of a substance by means of the separation of gaseous ions according to their differing mass and charge

*Merlangius merlangus* (*M. merlangus*) - a type of whiting fish that the wild type strain of *S. baltica* will be isolated from

*Omega-3 (Ω-3) fatty acids* - class of polyunsaturated fatty acids

*Polymerase chain reaction (PCR)* -  process used to amplify DNA/RNA

*pfaABCDE gene cluster* - the genes responsible for producing Ω-3 fatty acids in *Shewanella Baltica*

*Plasmid* - a circular strand of DNA that can replicate independently of the genomic chromosomes

*Primers* - segments of RNA that are recognized by DNA polymerase and used to transcribe new DNA fragments in PCR

*Real Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)* - PCR technology used to detect and measure the products of PCR

*Recombination* - The exchange of genetic material between two distinct organisms leading to a novel combination of genetic material in one organism

*Restriction site* - a sequence of DNA that is recognized by an endonuclease (see "Restriction Enzyme") as a site at which the DNA is to be cut; includes XhoI and BamHI restriction sites

*Restriction enzyme (Endonuclease)* - Enzyme that cuts DNA at restriction site; includes BamHI, SacI, and XhoI, restriction enzymes.

*Shuttle vector -* A DNA plasmid with both bacterial and eukaryotic replicative machinery, which allows it to"promiscuously" propagate within bacteria and yeasts. Recombinant DNA in shuttle vectors can be amplified in bacteria and expressed in eukaryotes. (shuttle vector. (n.d.); Farlex Partner Medical Dictionary et al., 2012); includes pMSP3535 vector

*Shewanella baltica* (*S. baltica*) **MAC1 -** bacterial strain isolated from the M. Merlangus whiting fish and includes the *pfaABCDE* genes responsible for producing $\Omega$-3 fatty acids

*Ultrasonication* - the irradiation of a liquid sample with ultrasonic (>20 kHz) waves resulting in agitation. Sound waves propagate into the liquid media result in alternating high-pressure (compression) and low-pressure (rarefaction) cycles. During rarefaction, high-intensity sonic waves create small vacuum bubbles or voids in the liquid, which then collapse violently (cavitation) during compression, creating very high local temperatures. (Royal Society of Chemistry et al., 2016)

*Vector -* a DNA molecule used to carry foreign genetic material from one cell to another