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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Genetic characterization of omega-3 polyunsaturated fatty acid synthesis in
the psychrophilic bacterium *Colwellia psychrerythraea*

A thesis submitted in partial satisfaction of the requirements for the degree
Master of Science

in

Biology

by

Brittany Jean Fernandez

Committee in charge:

Professor Eric E. Allen, Chair
Professor Douglas H. Bartlett
Professor Emily Troemel

2011

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University of California, San Diego

2011

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ABSTRACT OF THE THESIS

Genetic characterization of omega-3 polyunsaturated fatty acid synthesis in the psychrophilic bacterium *Colwellia psychrerythraea*

by

Brittany Jean Fernandez

Master of Science in Biology

University of California, San Diego, 2011

Professor Eric E. Allen, Chair

Bacterial fatty acid biosynthesis can occur utilizing one of three “types” of biosynthesis systems. This thesis focuses on the genetic characterization of the *pfa* gene cluster (*pfaA-E*) involved in the synthesis of the omega-3 polyunsaturated fatty acid (PUFA) docohexaenoic acid (DHA, C22:6*n*-3) in the marine psychrophilic bacterium *Colwellia psychrerythraea* strain 34H. The

research presented is an attempt to interrogate bacterial DHA biosynthesis via molecular genetic methods so as to advance our understanding of the physiological significance and biotechnological potential of bacterial omega-3 PUFA biosynthesis.

Through the combined use of Gas Chromatography-Mass Spectrometer (GC-MS) analysis, total fatty acid production was compared in *C.psychrerythraea* cultivated at: 4°C, 8°C, and 16° C. When compared to fatty acid profiles of the mesophile *Escherichia coli*, the profiles of *C.psychrerythraea* do not show evidence of thermal compensation. This was apparent by the minimal production of *cis*-vaccenic acid (C18:1*n*-7), a result of FabF activity, at all temperatures examined.

To examine the functional role of the Pfa Synthase system, a mutant defective in DHA synthesis is currently in the process of being engineered. Specifically, a 516 bp internal fragment of the Ketoacyl Reductase domain of *pfaA* is being used to disrupt the *pfa* operon in *C.psychrerythraea*. Conjugal transfer of the suicide vector pEE3 into wild-type *C.psychrerythraea* and selection for kanamycin resistant exconjugants will allow, for the first time, an assessment of the functional significance of DHA biosynthesis in the growth and fitness of an obligate psychrophilic bacterium.

Chapter 1:

INTRODUCTION:

1.1 A Brief History of Omega-3 PUFA Production in Microbial Lineages

Omega-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (C₂₀:5 n -3; EPA) and docosahexaenoic acid (C₂₂:6 n -3; DHA) (Figure 1.1) are essential components of eukaryotic cells where they are critical in numerous basal metabolic and physiological processes, including providing fluidity to the membrane and lowering the amount of cholesterol and triglycerides circulating in the bloodstream (Christiansen, E, 1991; Siscovick, 1995). Because mammals lack the ability for *de novo* synthesis of omega-3 PUFAs, consumption of a diet rich in these very long chain fatty acids has been shown to promote proper eye and brain development while lowering cardiovascular disease and cancer risks (Siscovick, 1995; Sioen et al, 2006).

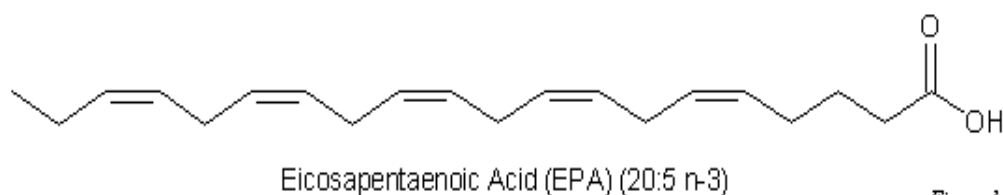


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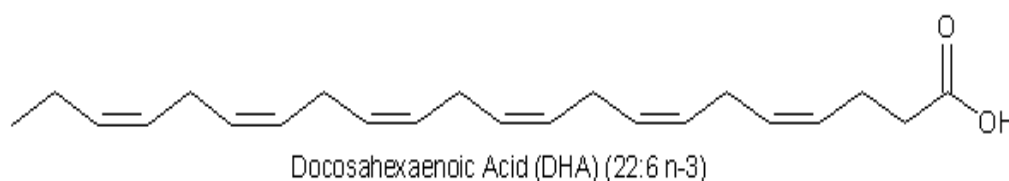


Figure by RMB

Figure 1.1 Chemical structures of the omega-3 polyunsaturated fatty acids eicosapentaenoic acid and docosahexaenoic acid.

In animals, long-chain omega-3 PUFAs such as EPA and DHA are synthesized from the plant-based essential fatty acid (EFA) precursor molecules, linoleic acid (C18:2*n*-6) and α -linolenic acid (C18:3*n*-3), through a series of acyl desaturation and elongation reactions. Specifically, linoleic acid is metabolized to arachidonic acid (C20:4*n*-6) whereas α -linolenic acid is metabolized to EPA and DHA (Cunnane SC., 2003). In general, *de novo* synthesis of PUFAs up to 18 carbons in length are synthesized by plants, while PUFAs that consist of 20+ carbons in length are synthesized by microorganisms, including fungi, eukaryotic phytoplankton species and marine bacteria (Erwin and Blotch, 1964, Cunnane SC., 2003).

In addition to the elongation and desaturation of plant-derived PUFA products, a primary source of omega-3 PUFAs in the human diet derives from marine products such as fish species. The ultimate source of these lipids in marine metazoans however derives from primary production of PUFAs by marine phytoplankton and other single cell algae. Concentration of these lipids through the marine food web results in significant quantities of omega-3 PUFAs in higher trophic levels such as commercial fish species (Mattson and Grundy, 1985; Simopoulos, 2000). Hence, dietary omega-3 PUFAs can be acquired via the direct consumption of fatty fish species, such as tuna and salmon that have fed on PUFA-synthesizing microorganisms, or in the form of fish oil extracts.

Augmenting the paradigm of microbial (phytoplankton)-based PUFA synthesis in the marine environment is the relatively recent discovery that

certain marine bacteria also produce omega-3 PUFAs such as EPA and DHA (Russell and Nichols, 1999). Consequently, while algal contributions likely represent the primary base of the aquatic food web in terms of PUFA lipid input, the contribution of bacterial production of these lipid molecules may also represent a significant source (Randall et al., 1990, Russell and Nichols 1999, Schweizer and Hofmann, 2004). However, the relative contribution of bacterial PUFA production into the aquatic food web has yet to be determined quantitatively.

1.2 Bacterial Fatty Acid Biosynthesis

Three distinct mechanisms of fatty acid biosynthesis are recognized with the domain Bacteria (Figure 1.2). The first type, and most common in bacteria, is the Type II Fatty Acid Synthase (FAS) (Cunnane, 2003, Schweizer and Hofmann, 2004, White *et al.*, 2005). Type II FAS organization involves independent proteins encoded by the fatty acid biosynthesis, or *fab*, genes and is responsible for the production of cellular fatty acids typically containing between 12 and 18 carbons (Schweizer and Hofmann, 2004). The involvement of the FabF enzyme has been analyzed in the mesophilic species *Escherichia coli* (Heath and Rock, 1996) and the piezophilic species *Photobacterium profundum* SS9 (Allen and Bartlett, 2000) and is responsible for temperature (or pressure)-induced alterations in membrane fatty acid composition, specifically the increased production of monounsaturated fatty

acids at low temperatures (or elevated hydrostatic pressure in piezophilic species).

Another type of FAS, although significantly less frequent in bacteria, is the Type I FAS system. The Type I FAS is the archetypical system found in eukaryotic cells however some coryneform bacteria of the order Actinomycetales also possess these systems (Lynen, 1980, Rangan and Smith 2003, Scheweizer and Hofmann, 2004). Bacterial Type I FASs consist of a large, multifunctional biosynthetic complex containing all enzymatic domains necessary for acyl chain elongation and functional derivatization (Kollattukudy *et al.*, 2008, Scheweizer and Hofmann, 2004).

The third recognized mechanism of bacterial fatty acid biosynthesis consists of a multi-enzymatic domain complex similar to Type I FAS and Polyketide Synthase (PKS) arrangements. These FAS/ PKS systems, herein referred to as the “*Pfa Synthase*” (Allen and Bartlett, 2002), are involved in the *de novo* biosynthesis of very-long-chain PUFAs (consisting of 20 carbons or more), such as EPA and DHA, and coexists with the aforementioned Type II FAS in certain species of marine Gammaproteobacteria (Metz *et al.*, 2001, Nichols and McMeekin, 2002, Nichols, 2003). It has been proposed that the connection between polyketide synthases (PKS) and fatty acid synthases (FAS) provides insight into the evolution of lipid diversification in numerous organisms (Allen and Bartlett, 2002, Cunnane, 2003, Scheweizer and Hofmann, 2004).

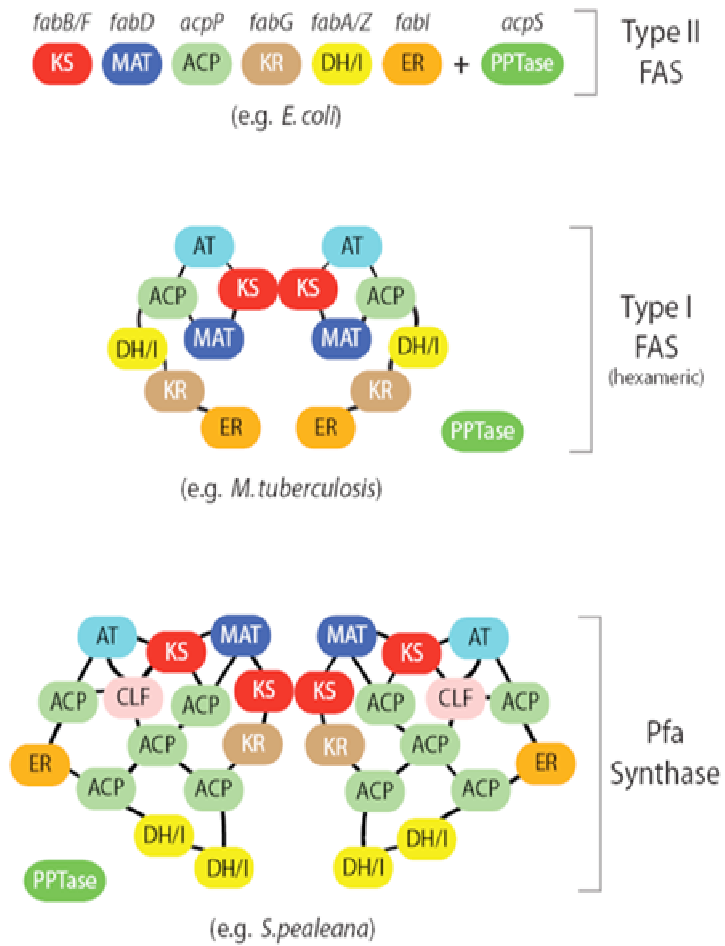


Figure 1.2 Illustration of the three types of FAS found in Bacteria.

The genes responsible for the synthesis of bacterial omega-3 PUFAs are *pfaA*, *pfaB*, *pfaC*, *pfaD*, and *pfaE*. The proteins encoded by the *Pfa* Synthase catalyze the *de novo* synthesis of the PUFA product from acetyl-CoA (2 carbon starter) via the following enzymatic activities: 1) Ketoacyl synthase [KS] catalyzes a condensing function responsible for chain-elongation; 2) Ketoacyl reductase [KR] catalyzes the reduction of the carbonyl group to a hydroxyl group; 3) Dehydratase/Isomerase [DH/I] catalyzes the dehydration of the β -hydroxyacyl intermediate generated by KR to a trans-2-enoyl derivative and the subsequent isomerization from the trans-2 to the cis-3 configuration; and lastly 4) Enoyl reductase [ER] catalyzes the reduction of double bond generated by DH to complete the chain elongation process. Other essential activities include acyl carrier protein [ACP] function which tethers the growing fatty acyl chain as a thioester as it is acted upon by other enzyme activities and phosphopantetheinyl transferase [PPTase] activity which converts ACP products from the inactive apo- form to the active holo-form via the posttranslational addition of a 4'-phosphopantetheine prosthetic group from acetyl coenzyme A. Acyltransferases [AT] catalyze the general transfer of a nascent acyl substrate from acyl-CoA to ACP for elongation of the fatty acyl chain. Malonyl-CoA:ACP transacylase [MAT] is a type of acyltransferase that specifically catalyzes the transfer of a 2C malonyl moiety from malonyl-CoA to ACP (Shulse and Allen, 2011). The ordering of the genes in the cluster is highly conserved, with the occasional exception of *pfaE*, which may be located elsewhere on the genome and encodes the PPTase

necessary for post-translational activation of ACP domains (Okuyama et al, 2007).

In addition to what is known about the *Pfa* Synthase gene cluster involved in omega-3 PUFA synthesis in marine bacteria, 20 additional types of *Pfa*-like Synthases have been identified in 86 bacterial species representing 10 phyla (Shulse and Allen, 2011). Of these 20 pathways identified, 14 represent novel gene clusters (with probable novel long-chain fatty acid products) and the other six have known products, including EPA and DHA (Shulse and Allen, 2011). The domain order of these novel type *Pfa* synthase gene clusters is fairly conserved; among the enzymatic activities encoded on these genes are the KS-MAT-ACT-KR (*pfaA*); ER (*pfaD*), and the PPTase (*pfaE*), while *pfaB* and *pfaC* show slight variations (Shulse and Allen, 2011). These lipid products are collectively termed “*secondary lipids*” to distinguish these products from those synthesized by primary (core) fatty acid biosynthetic mechanisms. The importance of secondary lipid molecules in cell physiology and growth remains to be determined.

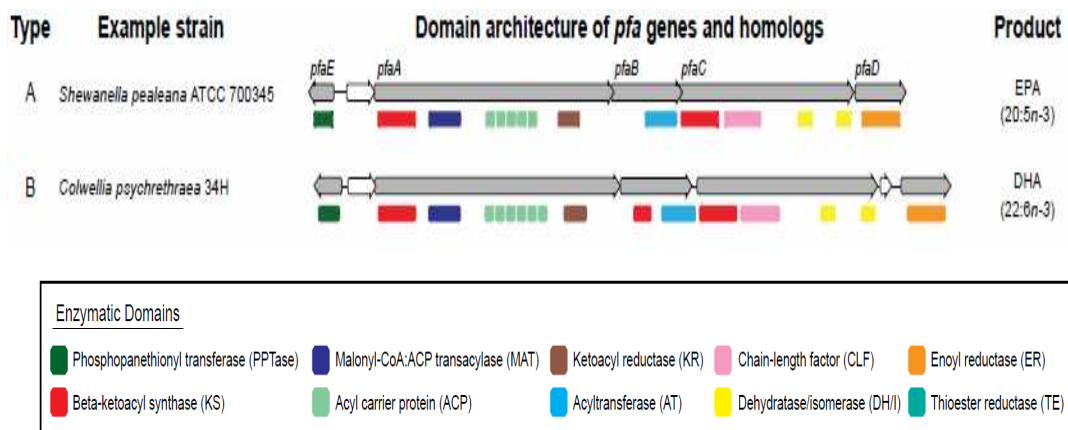


Figure 1.3 A depiction of the *Pfa* Synthase gene cluster in EPA (C20:5n-3) and DHA (C22:6n-3) producing marine bacteria. Included is the domain architecture of the *pfa* genes and description of the component enzymatic domains.

1.3 Bacterial Omega-3 PUFA Biosynthesis

It was previously concluded that bacteria do not produce PUFAs (Erwin and Bloch, 1964). However, the discovery of long-chain omega-3 PUFA production in deep-sea bacteria refuted these prior claims (DeLong and Yayanos, 1986) and subsequent work has shown widespread diversity in bacterial synthesis of mono- and polyenoic fatty acid molecules (Shulse and Allen, 2011). The production of very-long-chain PUFAs in microorganisms permits for the speculation that PUFA synthesis is a vital adaptation to manage various pressure and temperature differences in aquatic environments. In general, PUFA incorporation into phospholipid membranes shares an indirect relationship with the increase in temperature and a direct relationship with the increase in pressure (Allen and Bartlett, 2002). However, for *Photobacterium profundum* strain SS9, growth was independent of the preceding relationship (Allen, 1999).

Based on the computational analysis and research carried out on the genomes of various PUFA-producing bacteria, it became evident that DHA producing marine microorganisms, such as *Colwellia* and *Moritella*, include an additional KS domain in *pfaB* when compared with the genomes of EPA-producers. Recent experiments demonstrate that the heterologous expression of *pfaB* from DHA-producing organisms in conjunction with the expression of *pfaA*, *pfaC*, *pfaD* and *pfaE* from an EPA-producer results in the production of both EPA and DHA (Okuyama, 2007). Moreover, additional ACP domains

have been shown to increase the amount of polyunsaturated fatty acid product (Jiang et al., 2008).

It was previously discovered that cerulenin treatment, a fungal antibiotic, is advantageous to enhance intracellular PUFA production in PUFA-producing bacteria (Allen and Bartlett, 1999; Morita, 2005). It has also been noted that bacterial fatty acid components are susceptible to oxygen and reactive oxygen species (ROS) due to their location in the plasma membrane (Halliwell and Gutteridge, 1985). When an EPA-producing *E.coli* strain was engineered through the transformation of the *pfa* gene cluster from the marine bacterium *Shewanella pneumatophori* SCRC-2738, it was observed that EPA protected cells from the effects of exogenous peroxidase (H_2O_2) and other oxidative challenges; these effects included growth inhibition and rupture of the cell structure (Nishida et al., 2006 and Okuyama et al., 2008).

Potential future applications of bacterial secondary lipid synthase products include the production of biofuels and industrial chemical co-applications, the engineering of optimized “designer” lipids as dietary nutraceuticals, and lastly, the application of secondary lipids as medicinal chemical agents in the treatment of inflammatory disease.

1.4 Heterologous Expression of Secondary Lipids

The goal of this research focuses on classical and contemporary methods for the cloning and heterologous expression of secondary lipid synthase gene clusters from the three bacterial strains: *Dinoroseobacter*

shibae, *Renibacterium salmonarium*, and *Colwellia psychrerythraea*. The remainder of this introduction discusses the culture dependent approach that was attempted to unveil the fatty acid products of certain bacterial strains predicted to produce novel lipid products based on the identification of novel *Pfa* Synthase gene clusters. These strains display diverse physiologies as well as optimal growth conditions, and therefore provide the opportunity to investigate secondary lipid product diversity. *C.psychrerythraea* is of marine origin and grows optimally at 8°C, while *D.shibae* expresses optimal growth at 33°C, and *R.salmonarium* grows best, albeit slowly, in 15°-18°C environment s. Each bacterium possesses novel Pfa-like gene clusters predicted to produce PUFAs and/or novel lipid products that may or may not be necessary for optimal growth at various temperatures (Russell and Nichols, 1999, Allen and Bartlett, 2002, Methe et al., 2005, Biebl et al, 2005, Peikarski et al. 2009).

A primary fatty analysis of the three strains was first carried out, in order to determine the fatty acid production potential of each strain. This consisted of fatty acyl methyl ester (FAME) derivatization followed by Gas Chromatography-Mass Spectrometer (GCMS) analysis.

The bacterial strains were then cultured in their appropriate media, mini-prepped, and through transfection and/or the contemporary method of a one step isothermal reaction, the Pfa-like gene clusters of interest were ligated into a vector; the process of transformation into the *E.coli* host, where PUFA production would be heterologously expressed, is currently in the process of completion. A FAMEs derivatization and GCMS analysis will follow.

Successful recombinant production of PUFAs (or otherwise “exotic” fatty acid products) would verify the genetic basis required for secondary lipid synthesis and may ultimately provide a cost effective alternative route for producing PUFAs for human and animal consumption. To interrogate products synthesized by these three strains, heterologous expression of the secondary lipid synthase gene clusters in recombinant *E. coli* was attempted. The utilization of various techniques helped to determine the most efficient means of cloning these large fragments of DNA (>20 kbp) into *E.coli*. The techniques applied in the recombinant engineering experiments included the construction of a fosmid library and/or a one-step isothermal *in vitro* assembly method. Because the final reaction product was known for *C.psychrerythraea* (DHA), this was the only strain utilized for development of the one-step isothermal in vitro synthesis method. *C.psychrerythraea* was also the model organism used for all RT-PCR analyses and mutational analyses described below.

RT-PCR allowed for transcriptional analysis of the *pfa* genes, specifically investigation of the transcriptional regulation under various temperature conditions. The extent to which DHA production affects the growth of *C.psycherythraea* was examined by the engineering of a mutant strain defective in DHA synthesis. Briefly, the suicide vector pEE3 was used to clone a 516 bp region of the KR domain encoded by *pfaA* gene from *C.psycherythraea*. Growth was observed at three different temperatures,

4°C, 8°C, and 16°C; the extent to which PUFA synthesis influences growth and survival will be evaluated by comparing wild-type versus mutant strains.

Chapter 2:

Materials and Methods

2.1 Bacterial Strains and Isolating DNA

Before beginning, it was required to examine and comprehend literature of *Dinoroseobacter shibae*, *Renibacterium salmonarium*, and *Colwellia psychrerythraea*. The samples were purchased from DSMZ and arrived as a freeze-dried pellet. The samples were re-hydrated for 30 minutes, streaked onto plates, and grown up in their respective medium and under their required temperature conditions. When their optical densities (O.D.) were approximately between 0.7-0.8, a glycerol stock was made using 50% glycerol and stored in the -80°C.

DNA isolation consisted of setting up a 1:100 dilution of a 25ml culture for each bacterial strain. A required O.D. of 0.7-0.8 is required before moving forward; this is when fatty acid production is at its peak. 25ml of cells were harvested at 5000 x g for 8 minutes. The cells were resuspended in Buffer A (see Appendix for buffer contents). 2ml of molten 1.3% LMP agarose was mixed into the cell suspension. The cell-agarose suspension was drawn into a 1ml syringe and allowed to solidify at 4°C. The plugs were extruded into 9 plug volumes of Buffer B. Detergents were added. Lysozyme was added to a final concentration of 1mg/ml. The solution was incubated at 37°C overnight. The plugs were rinsed in 50ml sterile TE buffer, and 9 plug volumes of Buffer C was added, followed by the addition of necessary detergents. Proteinase K was added to a final concentration of 1mg/ml, and incubated in a 50°C water bath 24-48 hours. The plugs were washed 4 times in 40 ml TE buffer at room

temperature (RT) for 2 hours. The plugs were stored in 4°C. Note: for long-term storage (> 2 weeks), plugs need to be kept in 40ml of TE10 buffer.

2.2 Construction of Fosmid Libraries (Epicentre protocol)

The copy control fosmid protocol allows for clone stability afforded by single copy cloning as well as the benefit of obtaining high copy number DNA “on demand” through the use of the DNA induction solution on individual clones.

Before beginning, the genomic DNA (in 1% agarose plugs underwent an equilibration reaction in TE buffer to a final concentration of 0.5ug/μl, and a *GEL*ased reaction followed.

A supplied glycerol stock of EPI300 *E.coli* cells were struck on a plate in the absence of antibiotics, and placed in the 37°C overnight. The plate was then placed in the 4°C for storage.

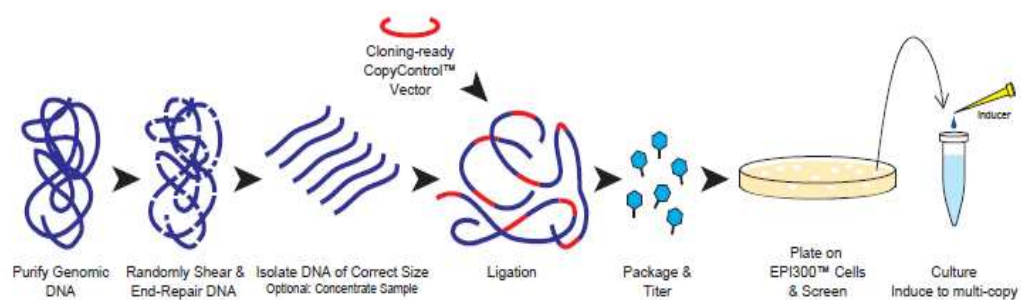


Figure 2.1 Overview of the CopyControl Fosmid Library Production process. The isolate DNA represents the *pfa* gene cluster of *C.psychrerythraea*.

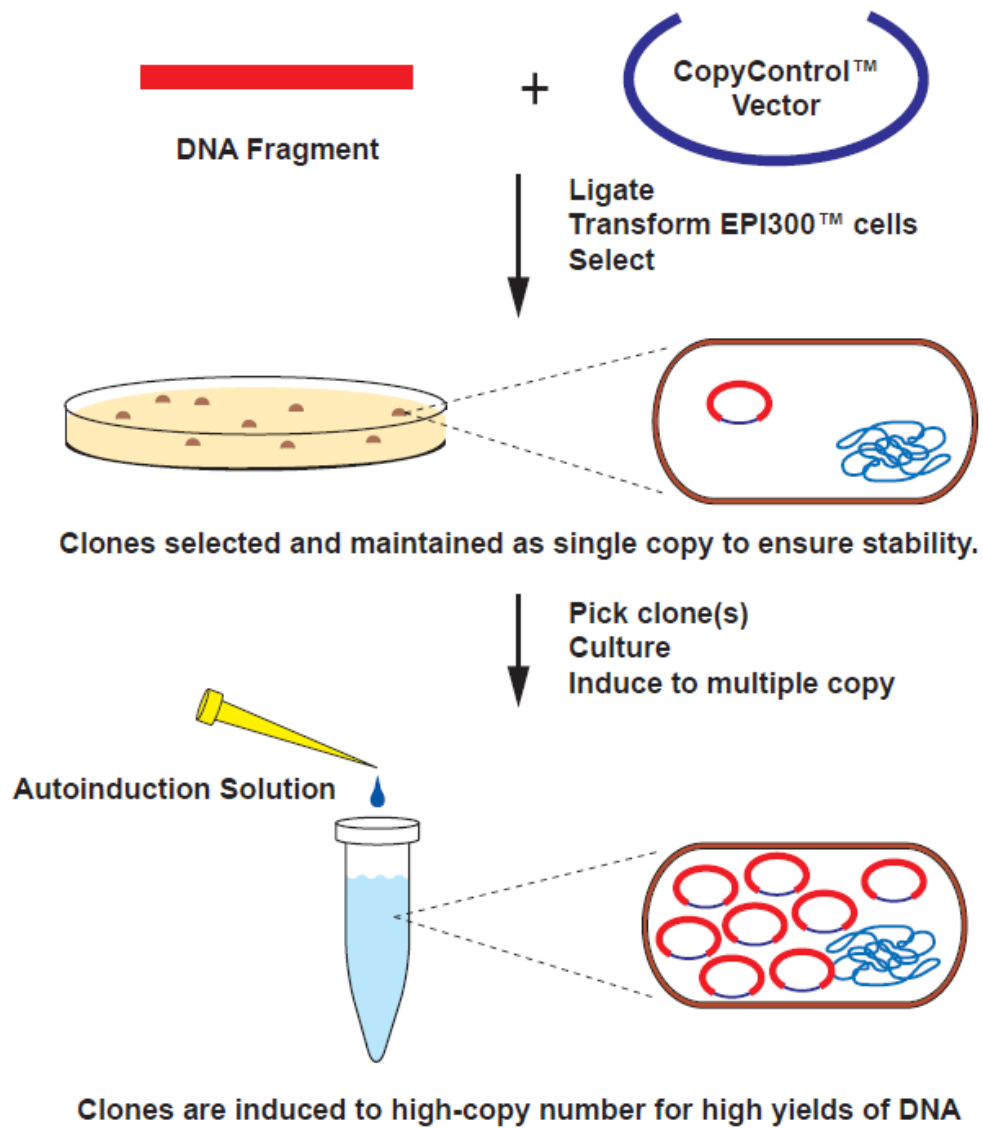


Figure 2.2 A demonstration of how to select a clone of interest (*E. coli* containing the *C. psychrerythraea pfa* gene cluster ligated into the pCC2FOS vector).

2.2.1 Shearing the DNA

Shearing the DNA allows for the random creation of 40kb sizes from genomic DNA. Through the generation of random sizing, the DNA is not limited or constricted to a biased digestion, as it is during restriction digests. Shearing the DNA involves passing 2.5ug (500ng/μl) of genomic DNA through a 200μl pipette tip, and aspirating and expelling the DNA 50-100 times. However, in this experiment, the 1.3% agarose plug dialyzed in the respective restriction digest buffer, and an in vitro restriction digest was executed. A previous bioinformatics analysis was required to determine the size of digests produced. Once it was verified that the sheared DNA is the same size as the desired insert, the entire DNA sample was end repaired and left to run through a Pulse Field Gel Electrophoresis (PFGE).

2.2.2 End Repair DNA

The kit components in this step include a(n): End repair enzyme mix, 10X Buffer, dNTPs, ATP. The contents were combined according to the recommendation by Epicentre's Copy Control Fosmid Library Production Kit and brought to a total reaction volume of 80μl. The contents incubated at room temperature for 60 minutes, gel loading buffer was added, and the reaction underwent inactivation by incubation at 70°C for 10 minutes. Samples were stored at -80°C.

2.2.3 Size Selection of End Repaired DNA

Size selection allows for the genomic DNA to be fractionated and selected by low melting point (LMP) agarose gel electrophoresis in TAE buffer. End repaired DNA was inserted into the Pulse Field Gel Electrophoresis (PFGE). PFGE allows for a slow separation of the DNA ranging from 10-100kb and does not require the addition of Ethidium Bromide in the gel or running buffer. A voltage recommended by the manufacturer was set, and the gel ran overnight. Separation was observed by staining the gel after the run was complete. The desired size of 40Kb was excised from the gel.

2.2.4 Recovery of the Size-Fractionated DNA

The weights of the gel sizes were determined, and the *GEL*ased 50X Buffer was warmed to 45°C. LMP-containing agarose plugs were melted in a heat block set at 70°C for 15 minutes. The tubes were transferred to 45°C and the warmed *GEL*ased 50X Buffer was added to the solution to a final concentration of 1X. 1U of *GEL*ased *Enzyme* was added for each 100µl of melted agarose. An overnight incubation was carried out.

By transferring the sample to 70°C for 10 minutes, inactivation of the *GEL*ased enzyme was accomplished. 500µl aliquots were chilled, centrifuged to remove any unwanted insoluble oligosaccharides, and the upper 90% of the supernatant was transferred to a sterile tube. DNA precipitation took place,

and the DNA was resuspended in TE Buffer and stored in the -20°C. A Nanodrop apparatus was used to determine the DNA concentration.

2.2.5 Ligation Reaction

The number of CopyControl Fosmid clones needed was determined through the Appendix of Epicentre-CopyControl Fosmid Library Production Kit. The following reagents were combined in sequence and mixed thoroughly. 1µl 10X Fast-Link Buffer, 1µl 10mM ATP, 1µl CopyControl pCC2Fos Vector (0.5ug/µl), 7 µl of concentrated insert DNA, 1ul Fast-Link DNA Ligase. Sterile water was excluded. An incubation at room temperature was carried out for 2 hours, and the reaction was inactivated at 70°C for 10 minutes.

2.2.6 Packaging the CopyControl Fosmid Clones

The day of the reaction, 50ml of LB broth + 10mM MgSO₄ was inoculated with 5ml of the EPI300-T1 overnight culture made during the preparation step. The culture incubated at 37°C until the O.D. reached 0.8-1.0. The cells may be stored in 4°C for 72 hours if desired. 1 tube of MaxPlax Lambda Packaging Extracts was thawed on ice for every ligation reaction performed in the previous step. 25µl was immediately transferred to a second tube and placed on ice. The remaining MaxPlax Packaging Extract was stored in -70°C for later use. 10 µl of the ligation reaction was added to each 25µl of thawed extracts. An incubation step was executed for 90 minutes at 30°C. The remaining 25µl of the MaxPlax Packaging Extract which was

stored in -70°C , was added to the reactions, and a second 90 minute incubation was carried out at 30°C . Phage Dilution Buffer was added to a final volume of 1ml in each tube. $25\mu\text{l}$ of chloroform was added to each mix as well.

2.2.7 Titering the Packages CopyControl Fosmid Clones

Titering aids in determining the number of plates and dilutions to make to obtain a library that meets the needs of the user. Serial dilutions were made and $10\mu\text{l}$ of each dilution was individually added to $100\mu\text{l}$ of the prepared EPI300-T1 host cells. The reaction was incubated for 20 minutes at 37°C . The infected EPI300-T1 cells were spread on a LB plate + $12.5\mu\text{g/ml}$ chloramphenicol and incubated at 37°C overnight. The colonies were counted and the titer of the packaged phage particles was calculated based on a standard calculation (see Appendix).

2.2.8 Plating and Selecting the CopyControl Fosmid Library

Based on the titer of the packages CopyControl Fosmid clones and the estimated number of clones required, a calculation of the volume of the packaged fosmid clones that will be needed to prepare the CopyControl Fosmid Library was established. It was necessary to dilute the phage particles to obtain the desired number of clones and clone density on the plate. The diluted phage particles were mixed with EPI300-T1 cells in the ration of $100\mu\text{l}$ of cells for every $10\mu\text{l}$ of diluted phage particles. The culture adsorbed at 37°C

for 20 minutes and spread onto LB+12.5µg/ml chloramphenicol plates. An overnight incubation was carried out at 37°C to select for fosmid clones.

2.2.9 Preparation of the Clone Induction Inocula and Copy Number Amplification

5ml of LB + 12.5 µg/ml chloramphenicol were added to 15 tubes to be induced to high copy number. The media was inoculated with individual clones and grown overnight at 37°C with shaking. Appropriate volumes of fresh LB + chloramphenicol were combined with the overnight culture and the CopyControl Induction Solution (See Appendix). Due to the importance of aeration during this step, the samples shook for 5 hours at 37°C. The cells were centrifuged, and the clones were purified and mini-prepped. Verification of inserted bacterial DNA was confirmed via Polymerase Chain Reaction (PCR) (See Appendix for Primer Sets Used).

2.3 One Step Isothermal Reaction – *in vitro* pathway synthesis

The One Step Isothermal Reaction allows for an innovative approach to provide a faster, more efficient assembly of a desired insert up to 40 kilobase pairs in size. The marine bacterium, *Colwellia psychrerythraea*, was used due to its harboring a complete *pfa* gene cluster, as well as the previous analysis and certainty of its PUFA product, DHA. The one step assembly of the ~24 kb *pfa* gene cluster will permit the incorporation of the preferred insert into a high copy number vector in minimal time and will allocate for straightforward

transformation and heterologous expression of the plasmid into the *E.coli* host strain.

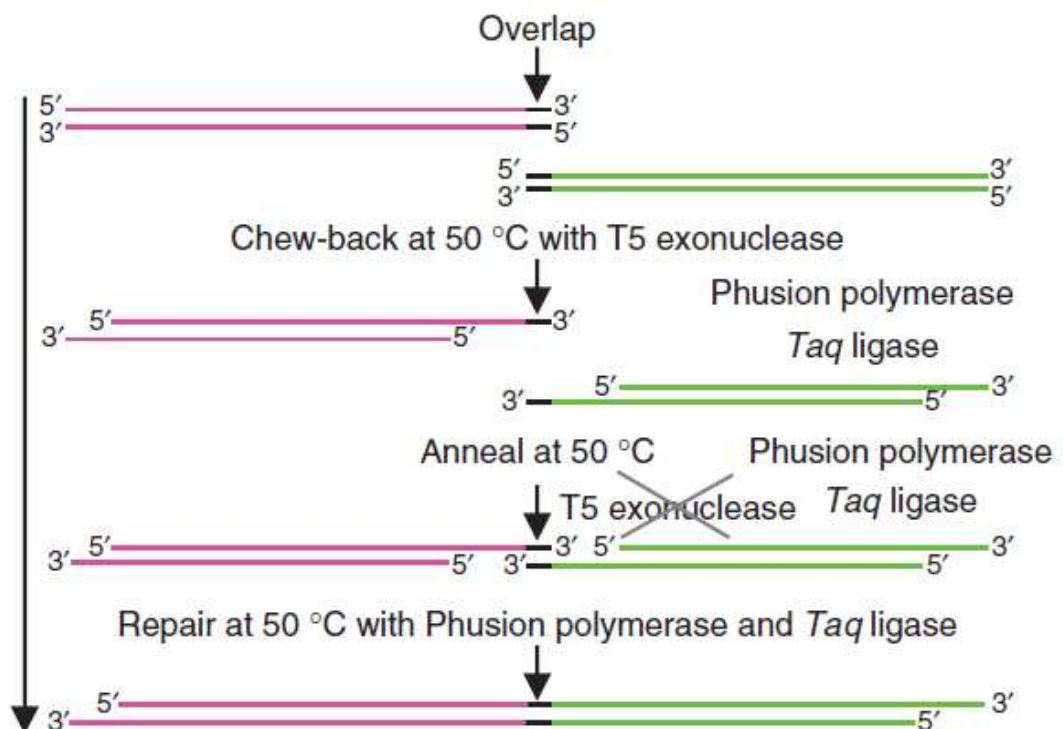


Figure 2.3 One step isothermal *in vitro* recombination. For simplicity, two adjacent DNA fragments (magenta and green) sharing terminal sequence overlaps (black) were joined into a covalently sealed molecule in a one-step isothermal reaction. T5 exonuclease removed nucleotides from the 5' ends of the double-stranded DNA molecules, complementary single-stranded DNA overhangs annealed, Phusion DNA polymerase filled the gaps and Taq DNA ligase sealed the nicks. T5 exonuclease is heat-labile and is inactivated during the 50°C incubation. [Gibson et al., 2009]

2.3.1 Selecting Primers and Preparation of DNA Molecules for *in vitro* Assembly

It was vital that primer selection provided primers with compatible melting temperatures, overhang lengths and extension times. (See Appendix for Primers, length of amplified product and base pair overhang length).

Pfu ultra II polymerase was used during the initial amplification of the desired PCR *C.psychrerythraea* product fragments 1-5 due to its 19kb target length, faster Cycling, higher yield for longer PCR lengths, and accuracy over Phusion/Proof and Taq DNA polymerase.

Table 2.1: Lists the PCR components for amplification of the five *C.psychrerythraea* fragments that, when ligated, yield the *Pfa* Synthase gene cluster

Component	Amount per Reaction (1x)	Amount per Reaction (6.5x) 5 Fragments + 1 NTC
Distilled Water	40.5µl	251.16µl
10X Pfu µltra II Reaction Buffer	5.0µl	32.5µl
dNTP	25mM each (1.25µl 10mM)	8.125µl
DNA template (100ng/µl) *47.40ng/µl	2.11µl	2.11µl
Forward Primer (10uM)	1µl	1.5 of each primer was added to each PCR tube as needed
Reverse Primer (10uM)	1µl	
Pfu µltra II fusion HS DNA Polymerase	1µl	6.5µl
Total	50µl	50µl/tube

Table 2.2 PCR parameters needed to amplify the individual fragments based on their size.

Segment	# of Cycles	Temperature (°C)	Duration	Cpsy 1 *Cpsy1 pfu (~3.8 kb)	Cpsy 2 *Cpsy4 pfu (~4.0 kb)	Cpsy 3 *Cpsy-58 (~6.0 kb)	Cpsy 4 *Cpsy4 pfu (~5.0 kb)	Cpsy 5 *Cpsy-58 (~6.1 kb)
1	1	95°C	2 min	2 min	2 min	2 min	2 min	2 min
2	30	95°C	20 sec	20 sec	20 sec	20 sec	20 sec	20 sec
		PrimerT-5°C	20 sec	43.4°C 20 sec	59°C 20 sec	58°C 20 sec	59°C 20 sec	50°C 20 sec
		72°C	15sec/kb	3kb 0:45	4kb (1:00) 1:15	6kb 1:30	5kb 1:15	6kb 1:30
3	1	72°C	3 min	3 min	3 min	3 min	3 min	3 min

Note: *C.psychrerythraea* is designated *C.psy*. (*) indicates thermocycler protocol.

2.3.2 T5 Exonuclease Dilution Buffer

Before beginning, T5 exonuclease was diluted 1:50 from 10U/ μ l in its stored buffer (50% glycerol, 50mM Tris-HCl pH 7.5, 0.1mM EDTA, 1mM DTT, 0.1M NaCl and 0.1% Triton X-100) due to the overlap size of <150bp. For overlap sizes larger than 150bp, 1.0U/ μ l T5 exonuclease is used.

2.3.3 5X Isothermal Reaction Buffer

5X reaction buffer was a vital component of the 1 step isothermal reaction. 25% PEG-800, 500mM Tris-HCl pH 7.5, 50mM MgCl₂, 50mM DTT, 1mM each of the four dNTPs and 5mM NAD were combined to equal a final volume of 1ml. The 5X isothermal reaction buffer was used as a component of the one-step DNA assembly.

2.3.4 1 Step Isothermal Assembly Protocol

The five *C.psychrerythraea* DNA fragments, along with a restriction digested pCC2Fos vector (BstXI), were assembled in 20 μ l reactions, half the amount of what the Gibson Protocol suggested. The one step isothermal reaction mixture consisted of 4 μ l 5X isothermal buffer, 0.4 μ l of 0.4U/ μ l T5 exonuclease-concentration of [0.08], 2 μ l of 40U/ μ l *Taq* DNA ligase and 0.25 μ l of 2U/ μ l Phusion DNA polymerase. 100ng/ μ l of DNA (*Colwellia psychrerythraea* fragments1-5 and pCC2Fos digested plasmid) was aliquoted

and added to the mixture according to Nanodrop concentrations and sterile water was added to a final volume of 20 μ l.

A second one step isothermal reaction was carried out using 4.4 μ l of the 5X reaction buffer, which included 0.4 μ l (0.4U/ μ l T5 exonuclease), 2 μ l (40/ μ l) *Taq* ligase, 0.2 μ l Phusion DNA polymerase, 100ng/ μ l of each DNA fragment-5 *Colwellia psychrerythraea* fragments and pCC2Fos digested plasmid, and sterile water up to a final volume of 30 μ l.

The contents were mixed and placed into a PCR tube. The tube was incubated in a thermocycler and 2 separate reactions were carried out: one for 30 minutes at 50 $^{\circ}$ C and the other for 50 minutes at 50 $^{\circ}$ C.

2.3.5 Transformation and Verification of Heterologous Expression of DHA Producing *E.coli*

Electroporation involved electroporating 1 μ l of the 1 step isothermal reaction product into 50 μ l of electrocompetent cells. Electroporation conditions were 1.8 kV, 25 μ F capacitance, 200 Ω resistance.

Chemical transformation consisted of the addition of 1 μ l of the 1 step isothermal reaction product into 50 μ l of chemically competent DH5 α cells. The product was iced for 30 minutes, incubated in a 42 $^{\circ}$ C water bath for 20 seconds, and iced for 2 minutes.

950 μ l of SOC were added to the transformed cells, incubated for 1 hour at 37 $^{\circ}$ C, and 100 μ l of both transformations were plated on LB +12.5 μ g/ml chloramphenicol+XGAL+IPTG plates.

The colonies were grown in a 5ml culture, mini-prepped and verified for insertion of the correct plasmid using internal *Colwellia psychrerythraea* primers (*C.psychrerythraea* fragment 4) as well as those encoding overlapping fragments (*C.psychrerythraea* 1 and 5) of *C.psychrerythraea* and pCC2Fos.

2.4 Transcriptional Regulation of *Pfa* Synthase System

To determine transcriptional control of *Colwellia psychrerythraea* under three temperatures: 4°C, 8°C, and 16°C, the cultures were harvested when they reached mid-log phase (O.D. of 0.6-0.8). RNase-Away was used to clean all surfaces before beginning. Only certified RNase/ DNase free tips/tubes/etc were used during this experiment. Centrifugation was carried out at 8000rpm for 10 minutes. The medium was quickly decanted, and the pellet was resuspended in 4ml Trizol reagent (Invitrogen) to stabilize the RNA. The mixture was transferred to a new 15ml Falcon tube, and 800µl of chloroform was added. The tube was mixed by inversion for 20 seconds and then incubated on ice for 5 minutes. The sample was centrifuged at 9000rpm for 15 minutes, and 2ml of the top aqueous layer was removed and transferred to a new 15ml Falcon tube. 2ml of isopropanol was added and mixed. An incubation at room temperature followed for 10 minutes. The sample was centrifuged at 9000rpm for 5 minutes, and the remaining pellet was resuspended in 4ml 75% ethanol.

To clean the sample, it was necessary to perform a centrifugation step at 8000rpm for 5 minutes and air-dry the pellets until the pellet was completely

dry. The pellet was resuspended in 175µl DEPC-treated water and 5µl was run on a 1.5% agarose gel to verify the presence of RNA.

2.4.1 RT-PCR

Designated primers were used on each sample (*C.psychrerythraea* 4°C, 8°C, 16°C) at the Protocol for SuperScript III 1st Strand Synthesis System was used for RT-PCR. 3.5µl of RNA was used along with 1ul of Primer (50ng/µl Random Hexamers were used to convert all RNA to DNA), 1ul 10mM dNTP mix, and 4.5µl of DEPC-treated water for a final volume of 10µl. The sample was incubated at 65°C for 5 minutes, and then placed on ice for 1 minute.

2.4.2 cDNA Synthesis Mixture

9ul of 10X RT buffer, 18ul of 25mM MgCl₂, 9ul of 0.1M DTT, 4.5µl RNaseOUT (40U/µl), and 4ul of SuperScript III RT (200U/µl) were combined to carry out 4.5 reactions. 10µl of cDNA Synthesis was added to each RNA/primer mixture, mixed gently, and collected by a brief centrifugation. Incubation occurred for 10 minutes at 25°C, followed by 50 minutes at 50°C. The reactions were terminated at 85°C for 5 minutes then set to chill on ice. 1ul of RNase H was added to each tube and incubated for 20 minutes at 37°C. Each cDNA synthesis reaction was immediately used for PCR.

2.4.3 RT-PCR Protocol

Taq 2X Master Mix was used to amplify the cDNA area of interest- 231bp. 12.5 μ l of *Taq* 2X Master Mix was used, along with 1 μ l of each primer (10mM), 50ng/ μ l DNA, and DEPC-treated water, totaling a 25 μ l PCR reaction.

Table 2.3 RT-PCR parameters used to examine expression of *pfa* genes in *C.psychrerythraea*.

Segment	# of Cycles	Temperature (°C)	Duration
1	1	95°C	30 seconds
2	30	95°C	30 seconds
		Primer T _m -5°C (T _m =58.4°C)	30 seconds
		68°C	30 seconds (1minute/Kb DNA)
3	1	68°C	5 minutes

2.5 Temperature-dependent Growth and Fatty Acid Phenotype of *Colwellia psychrerythraea*

Fatty Acid Analysis of *C.psychrerythraea* was done in order to determine how PUFA (DHA) production is influenced by temperature (4°C, 8°C and 16°C). Under these growth conditions, 100ml of *C.psychrerythraea* was grown until their optical density reached 0.7-0.8. The cells were harvested via centrifugation at 5000 X g for 25 minutes at 4°C. The cells were then washed in an equal volume of 50% artificial seawater, transferred to 14ml Falcon tubes, and centrifuged again. The remaining cells were kept in the -80°C and left to freeze overnight. The cells were set to lyophilize the following day, and they were left overnight. Fatty Acid Methyl Ester (FAME) derivatization consisted of reacting 10mg of lyophilized cell sample with 5% H₂SO₄ in anhydrous methanol at 90°C for 90 minutes. Water will inhibit this reaction. The samples were left to cool, and the FAMEs were extracted twice with hexane, mixed for 10 minutes, allowed to separate, and the aqueous (lower) layer was extracted and discarded. The vial was filled with 10% NaCl, mixed for 5 minutes, and allowed to settle. The sample was centrifuged at a low speed for 1 minute, and the organic (upper) layer was transferred to a new tube. All but 25µl were evaporated under nitrogen, and the samples were stored at -20°C.

2.5.1 Fatty Acid Analysis

All fatty acid analysis samples were carried out on Gas Chromatography Mass Spectrometer. An Agilent Technology's HP-5ms column was used, and the analytical program, Bandit, allowed for the integration and comparison of PUFA production at different temperatures. The parameters used for fatty acid analysis involved a 100°C oven start for 5 minutes, followed by an increase in 4°C/minute until the column reached a temperature of 280°C. There was a hold at 280°C for 8 minutes. The total run time was 53.5 minutes per sample. A "split" mode was utilized and the pressure was maintained at 13.743 psi.

2.6 Engineering of a DHA-defective Mutant in *Colwellia psychrerythraea*

Due to the establishment of DHA as the PUFA product in *Colwellia psychrerythraea*, this strain was selected to examine the specific physiological role of DHA production. As noted previously, previous mutational studies have been conducted in EPA-producing strains however no analogous experiments have been performed in DHA-producing strains. Insertional inactivation of the KR domain region of *pfaA* was chosen as the targeted region for genetic disruption. Engineering of the knockout mutant consisted of PCR amplification of a 516 bp portion of the KR domain using *Taq* 2X Master Mix (See Appendix for Primers). The 516 bp product was then ligated into the linearized PCR4 vector, transformed into TOP10 cells and plated on LB-Kan (50ug/ml) plates.

A 5ml overnight culture of the TOP10 product was mini-prepped and PCR4 was digested with EcoR1. The pEE3 vector was digested with EcoR1, and both the 516 bp insert as well as the pEE3 vector were gel purified, ligated, and transformed into DH5 α cells and plated on LB-Kan (50ug/ml) plates.

A 5ml overnight culture was mini-prepped and the insert was verified into the pEE3 vector via Polymerase Chain Reaction using *Taq* 2X Master Mix (See Appendix for Primers). Transformation followed, combining pEE3+516bp into BB1 chemical competent cells.

Although yet to be completed, the next steps is a bi-parental conjugation between a rifampin resistant *C.psychrerythraea* strain (Rif-100) and the BB1 containing pEE3+516bp insert followed by confirmation of single-crossover insertion of the complete pEE3 construct into the chromosome of *C. psychrerythraea* at the *pfaA* locus.

Following successful engineering of a DHA-defective mutant, growth measurements will be compared between the mutant and parental genotypes at varying temperatures (4°C, 8°C, 16°C, and 24°C). Fatty acid analysis of the mutant versus parental genotype will also be performed to analyze the fatty acid phenotype of these strains at these temperatures. These experiments are still in progress.

Results and Discussion:

Chapter 3

3.1 Isolating and Storing Bacterial DNA

Table 3.1 Bacterial growth requirements for subsequent fatty acid analysis

Bacterial Strain	Media	Optimal Growth Temperature (°C)	Optical Density (600 nm)
<i>Dinoroseobacter shibae</i>	2216	33	0.75
<i>Renibacterium salmonarium</i>	KDM-2 (in the presence of Calf Bovine Serum)	15	0.85
<i>Colwellia psychrerythraea</i>	2216	8	0.8

The optical density of 0.7-0.8 is indicative of mid-log growth, when the strains are ready for harvesting. After inoculating each culture, it was necessary to incorporate a dynamic environment in their respective medium so that the bacteria had optimal aeration for growth. Preparing a glycerol stock required combining 1ml of bacteria in the mid-log phase along with 1ml of a sterile stock of 50% glycerol. The acquired optical densities of the three strains of bacteria were obtained throughout the experiment to maintain consistency. When comparing VLC-PUFA production of *Colwellia psychrerythraea* at 3 distinctive temperatures, 4°C, 8°C, and 16°C, it was necessary to isolate each culture of *C.psychrerythraea* DNA in mid-log phase, although the 16°C sample took longer to reach the required O.D. The longer propagation time for the 16°C sample is due to environmental conditions, initially thought to result from low levels of cis-vaccenic acid (C18:1*n*-7) assembly, and consequently, low levels of FabF production.

3.2 Genetic and Fatty acid analysis of *Dinoroseobacter shibae*

After isolating DNA, ligating the desired *Pfa* Synthase insert into pCC2Fos and transfecting the DNA into the *E.coli* host strain, via Epicentre's CopyControl Production Kit protocol, the verification of the presence of DNA through a successful boil prep DNA extraction was necessary before anticipating subsequent analysis.

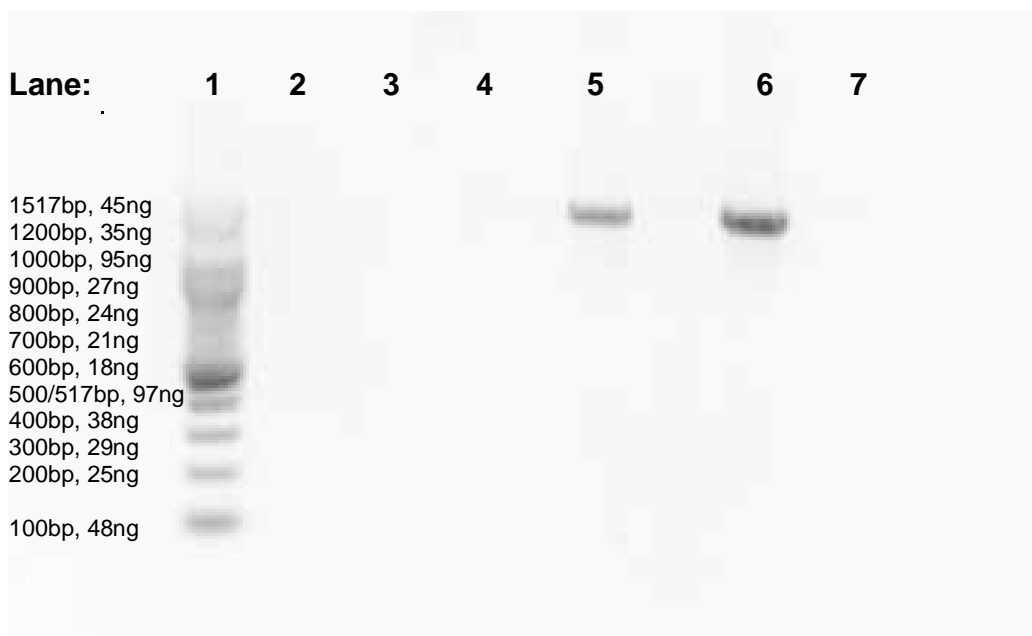


Figure 3.1 Lane 1-100bp ladder, lane 2-Sample #1 (D.Shib at 15°C) 1963 primers with D.Shibae DNA, lane 3- Sample #2 (D.Shib at 30°C) 19 63 primers with D.Shibae DNA, lane 4- No Template Control (NTC), lane 5-Sample #1 with universal 16S primers (BAC27F and BAC 1552R), lane 6-Sample #2 with universal 16S primers, lane 7-NTC.

The presence of *E.coli* clones that appeared after plating, to some extent, denoted the *E.coli* host strain as a PUFA producing organism, in the event that the *Pfa* Synthase gene cluster was involved in this heterologous

expression. However, the *D. shibae* specific primers (1963 primers), which are involved in the amplification of the *pfaA* region, did not amplify. The fact that the universal 16S primers (Figure 3.1 lanes 5 and 6) provided a 1.5kb amplification product of *E.coli* DNA illustrates a successful boil prep procedure, and confirmed the absence of any possible contaminants. The *D.shibae* DNA isolation appeared to be successful despite lacking the amplification of the *pfaA* region.

The next step involved troubleshooting by means of new boil prepped samples along with new sets of *D.shibae*-specific primers. Amplification of the boil prepped *E.coli* host strains from *D.shibae*-specific primers was thought to confirm *D.shibae* DNA incorporation into the vector; thereby being responsible for the heterologous expression of PUFA production. Verification of the primers using NCBI and *Artemis* confirmed that *Dinoroseobacter shibae* 1952F and 1952R (See Appendix for Primer Sequence) should yield a ~700bp product. The new primers were used to verify a direct *GEL*ased sample of both the 15°C and 30°C cultures, as well as a boil prepped sample of *E.coli* transfected with *D.shibae* DNA obtained at both temperatures. Again, amplification was not observed on any of the agarose gel photographs (Figure similar to that of 3.1); however the universal 16S primers continued to yield a 1.5kb product.

When comparing the 16S primer-amplified DNA of the *D.shibae* LMP plugs and the transfected *E.coli* host strains, DNA appeared to be present in both samples (Figure 3.2). The fact that 16S primers worked, but verification

of the *Pfa* Synthase gene cluster did not, may be an indication of the possibility that the 25Kb piece of DNA necessary for PUFA production was not included in the GELased slice analyzed on the pulse field gel. The restriction digest may have carried on too long, and the chance that the *Pfa* synthase gene cluster was digested into smaller fragments than necessary is probable, however unlikely. Also, there is a slight chance that the *D.shibae* bacterial strain DF-12 may be producing VLC-PUFAs, such as EPA and DHA, via a novel mechanism and/or receiving PUFAs from its external environment, much like human acquisition. Further analysis obtained from the GC-MS, is still remaining to be completed.

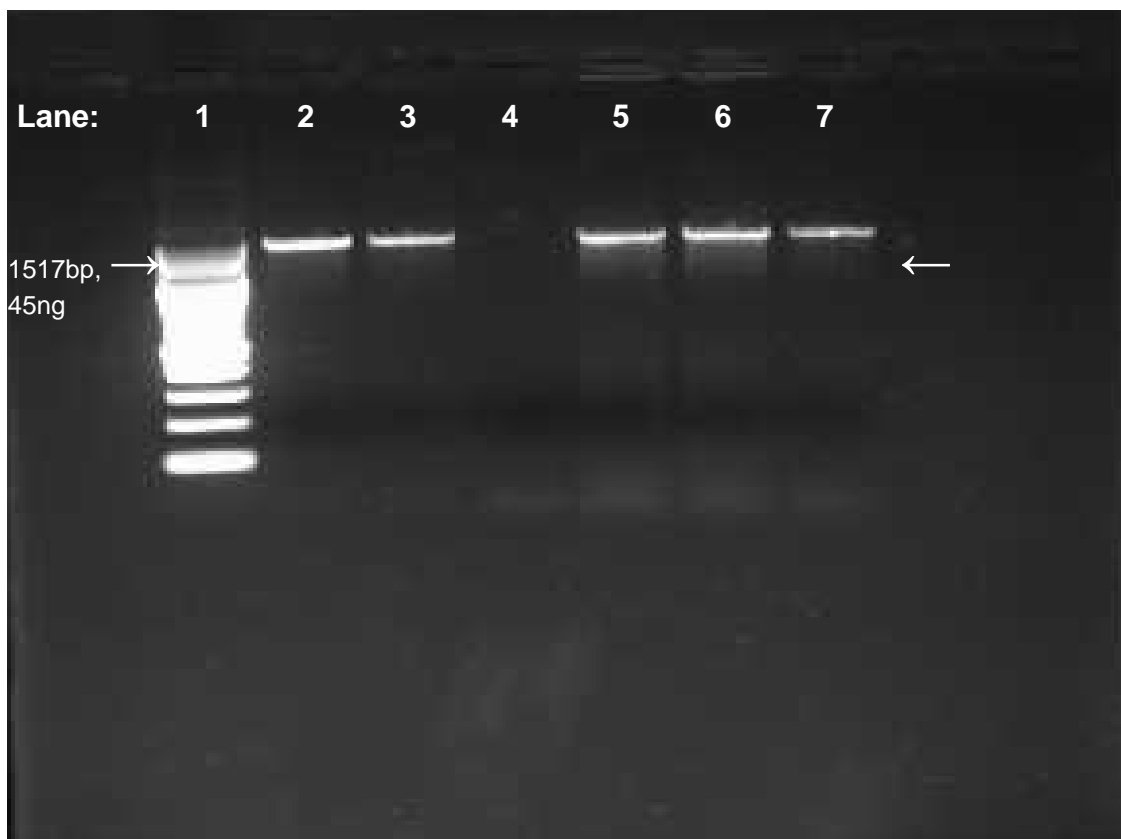


Figure 3.2 Lane 1-100bp ladder, lane 2-Sample #1 (D.Shib at 15°C) GELased Plug, lane 3-Sample #2 (D.Shib at 35°C) GELased Plug, lane 4-NTC, lane 5-Sample #1-Boil Prepped, lane 6-Sample #2-Boil Prepped, lane 7-Sample #1-Boil Prepped by C. Shulse*

*This was done to rule out technical problems that may have occurred.

, → indicates ~1.5Kb

3.3 One Step Isothermal Assembly of the *Colwellia psychrerythraea* pfa gene cluster

The primary steps in creating an *E.coli* strain producing DHA involves the amplification of 5 fragments that, when ligated, overlap in such way as to reassemble the Pfa Synthase gene cluster and total 25,068bp in length.

The digestion of pCC2Fos using EcoR1, created a linear product with 3' overhangs, 5'AATC3'. *C.psychrerythraea* fragments 1 and 5 (end fragments that would flank the digested vector) contained 20bp overhangs corresponding to the digested pCC2Fos vector. Fragment 1 forward primer included the first 20 base pairs upstream of the pCC2Fos restriction site, while fragment 5 reverse primer contained the 20 base pairs downstream from the pCC2Fos digestion site.

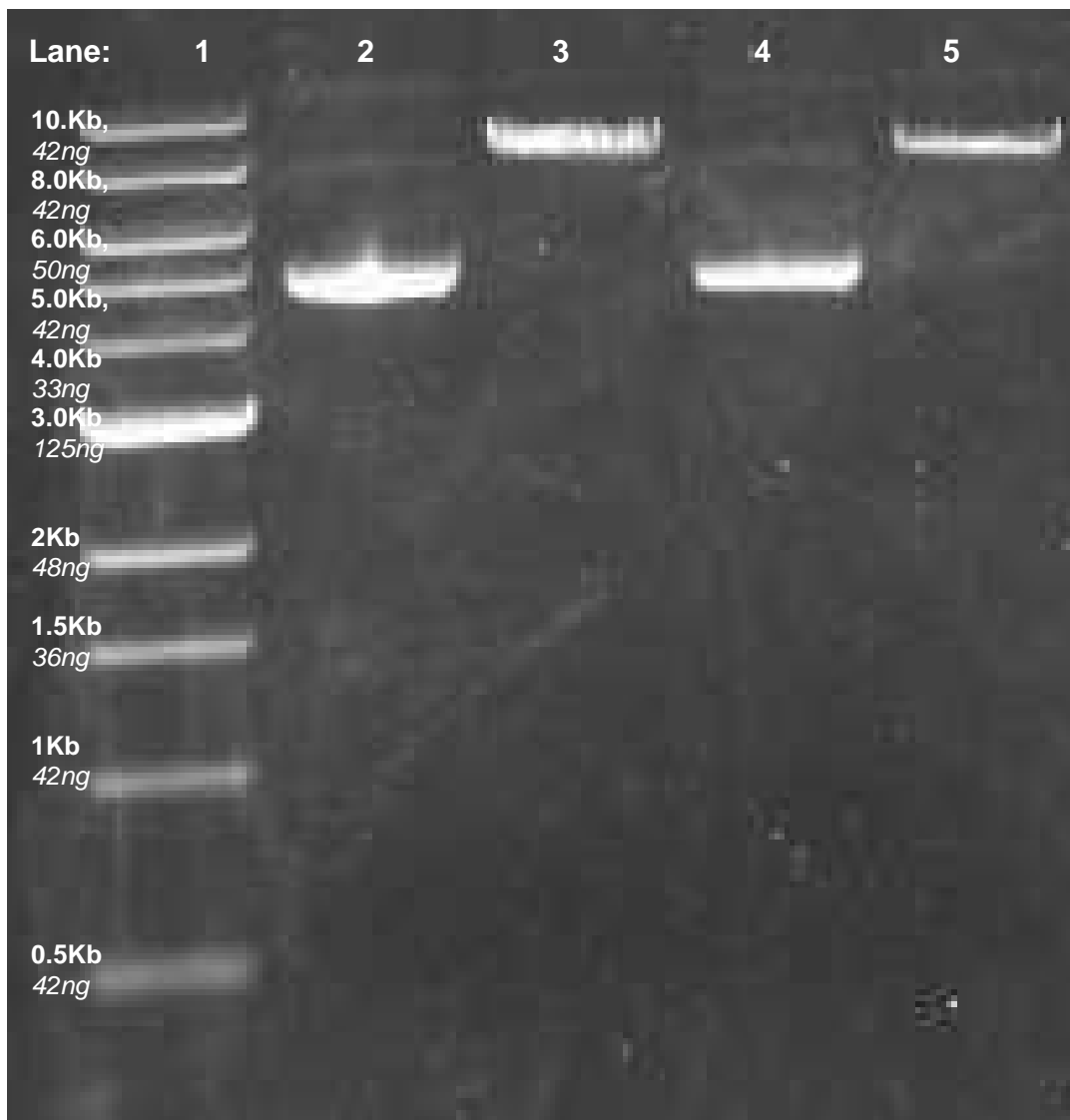


Figure 3.3 Lane 1-1Kb ladder, lanes 2 and 4-digested pCC2Fos DNA, lanes 3 and 5-undigested pCC2Fos DNA

Because only a single band is observed, it was not necessary to gel purify the digested pCC2Fos vector (Figure 3.3). Unlike, the Gibson *et al.* protocol, pCC2Fos was not amplified prior to one-step enzymatic assembly. It was not necessary to follow up the generation of pCC2Fos with a PCR

amplification due to the high copy number induction protocol used to obtain the DNA. An undigested pCC2Fos vector was previously obtained from a “blue colony” of *E.coli* which was transformed with the undigested vector. 5ml of LB was inoculated with the colony, the culture was harvested in the presence of induction solution, and the vector was obtained through mini-preparation.

Universal 16S rRNA gene primers, BAC27 forward and BAC1522 reverse were used to verify the presence of DNA, and a preliminary attempt to amplify such a large fragment of DNA, ~6Kb, was carried out using Phusion Ultra II Taq DNA Polymerase.

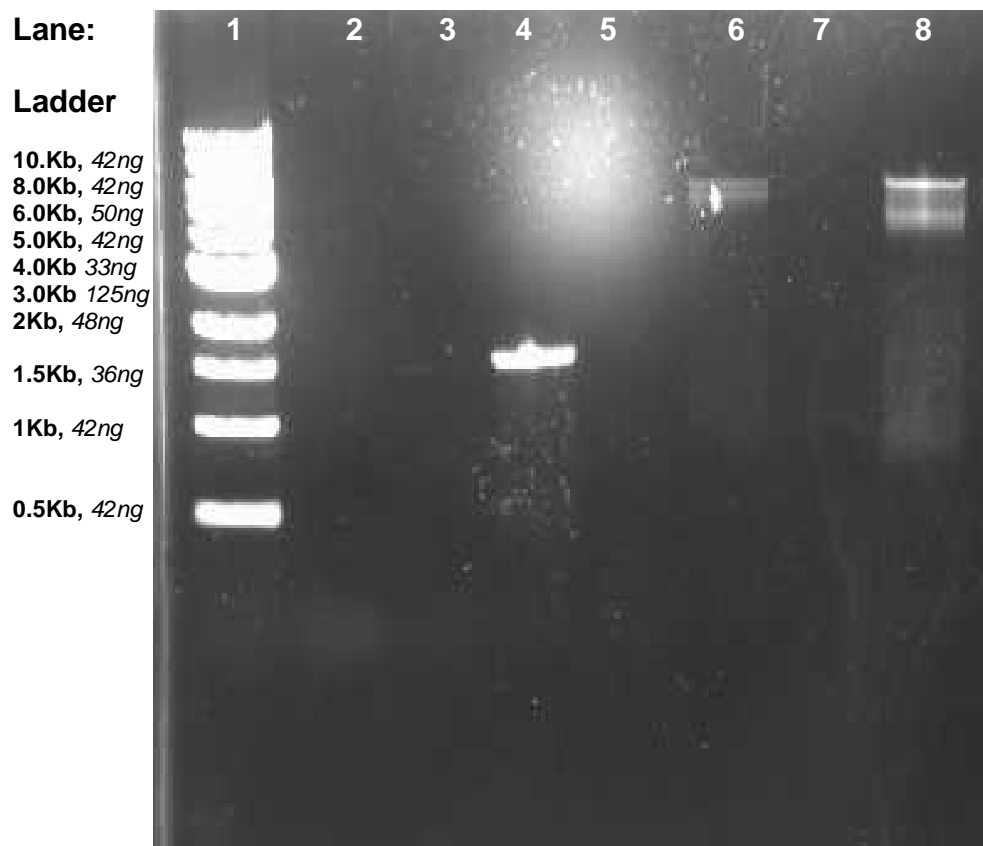


Figure 3.4 Lane 1-1Kb ladder, lane 2-16S Primers on *C. psychrerythraea* DNA isolated 10/5/10, lane 3- NTC, lane 4-16S Primers on *C. psychrerythraea* DNA isolated 10/6/10, lane 5-NTC, lane 6-6Kb (*C. psychrerythraea* fragment 3) using PCR protocol for samples <10Kb, lane 7-NTC (for lanes 6 and 8), lane 8-6Kb (*C. psychrerythraea* fragment 3) using PCR protocol for samples >10Kb

Because the 16S primers did not have an amplification product for the *C.psychrerythraea* DNA isolated 10/5/10, but an accurate product for the sample isolated 10/6/10, the DNA isolated on 10/6/10 was used for the remainder of the experiments involving *C.psychrerythraea* DNA (Figure 3.4, lanes 2 and 4, respectively). Two PCR protocols were available for use when amplifying DNA with Pfu Ultra II Polymerase: one protocol for the amplification of desired fragments <10kb, and one protocol for fragments >10kb. Since a

6kb product was the largest fragment size to consider, it was determined that the >10kb protocol would provide better separation between multiple products of *C.psychrerythraea* as well as a more cohesive amplified product (Figure 3.4, lanes 6 and 8). Gel purification was carried out on amplified products that produced multiple bands (Figure 3.5 lanes 3 and 5). The top band was always chosen during gel excision. The remaining *C. psychrerythraea* fragments successfully amplified using the Pfu Ultra II Phusion polymerase; although the inner fragments were more easily amplified than the outer fragments: 1 and 5.

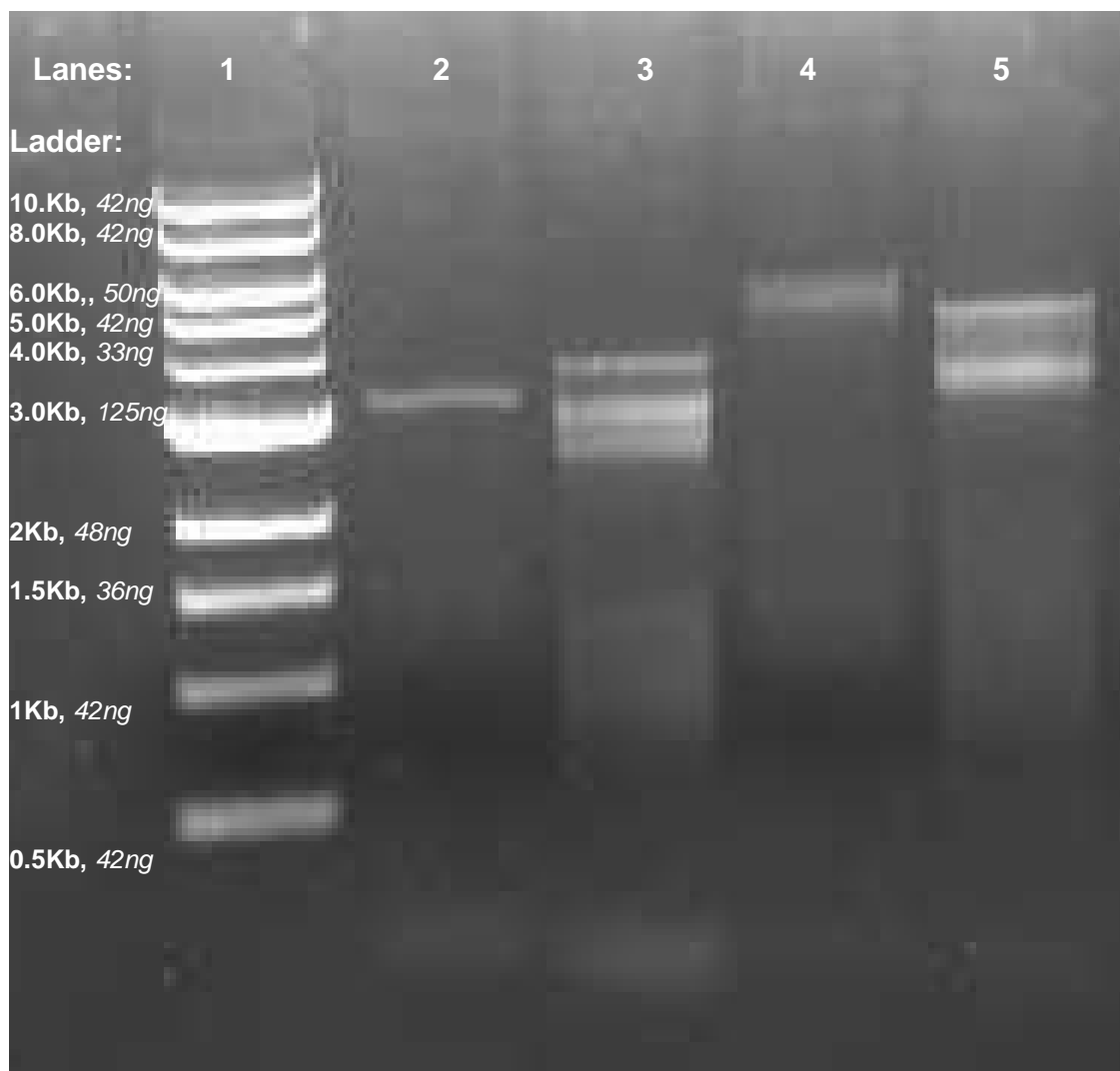


Figure 3.5 Lane 1-1Kb ladder, lane 2- *C.psychrerythraea* Fragment 1 (3833bp), lane 3- *C.psychrerythraea* Fragment 2 (4061bp), lane 4- *C.psychrerythraea* Fragment 3 (6026bp), lane 5- *C.psychrerythraea* Fragment 4 (5036bp)

Due to the production of multiple bands of fragments 2 and 4 (figure 3.5 lanes 3 and 5), and since they were run in the same thermocycler and under the same conditions (see Table 2.2), the parameters were altered and the annealing temperature was tested and set accordingly.

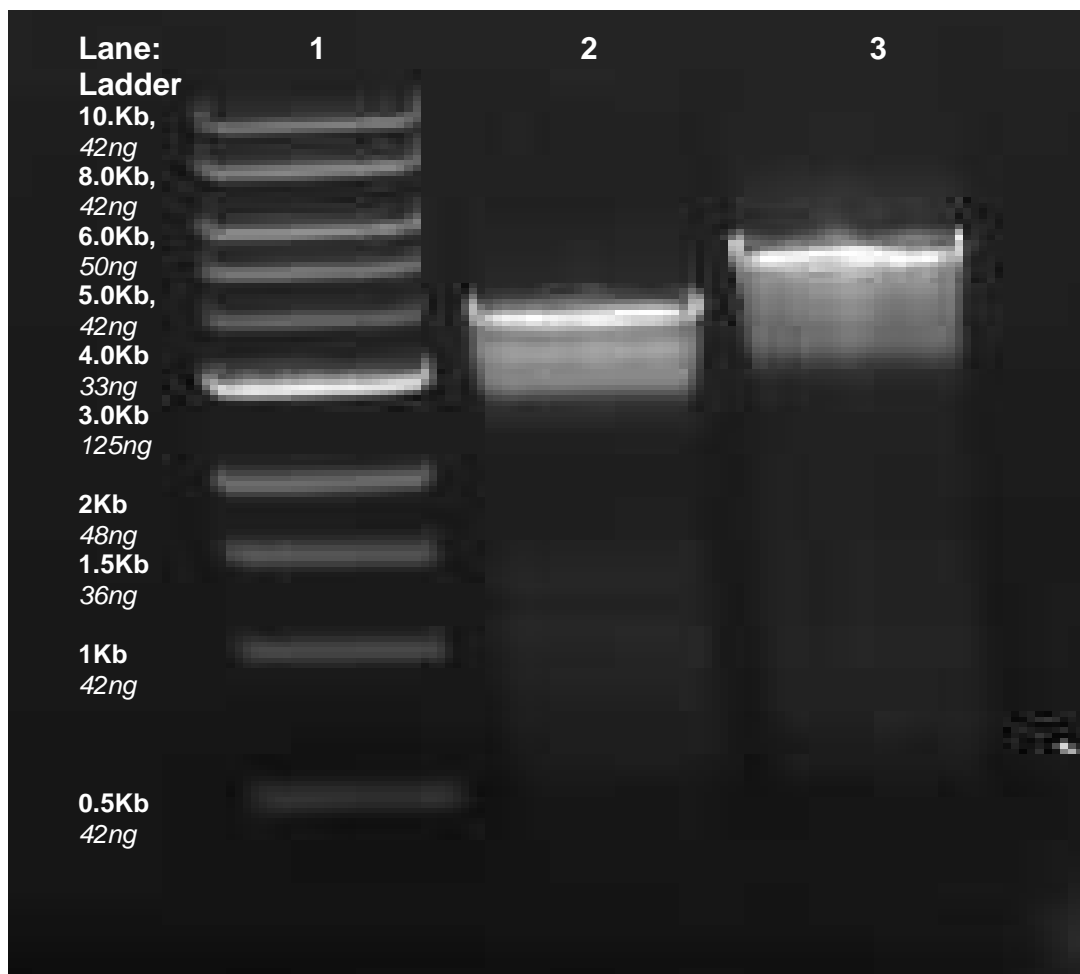


Figure 3.6 Lane 1-1Kb ladder, lane 2- *C.psychrerythraea* Fragment 2 (4061bp), lane 3- *C.psychrerythraea* Fragment 4 (5036bp). The PCR for both fragments ran in the same thermocycler with the annealing temperature set to 59°C.

Based on gel analysis, it was determined that an annealing temperature of 59°C generates the most cohesive and greatest amount of PCR product for both fragments (Figure 3.6). Like the other fragments, a gel excision and purification step was required.

Following the amplification of all five fragments, a preliminary one step isothermal reaction was carried out between *C.psychrerythraea* fragments 1 and 2, due to their length in size and amount of amplified product (Figure 3.7 lanes 2 and 3).

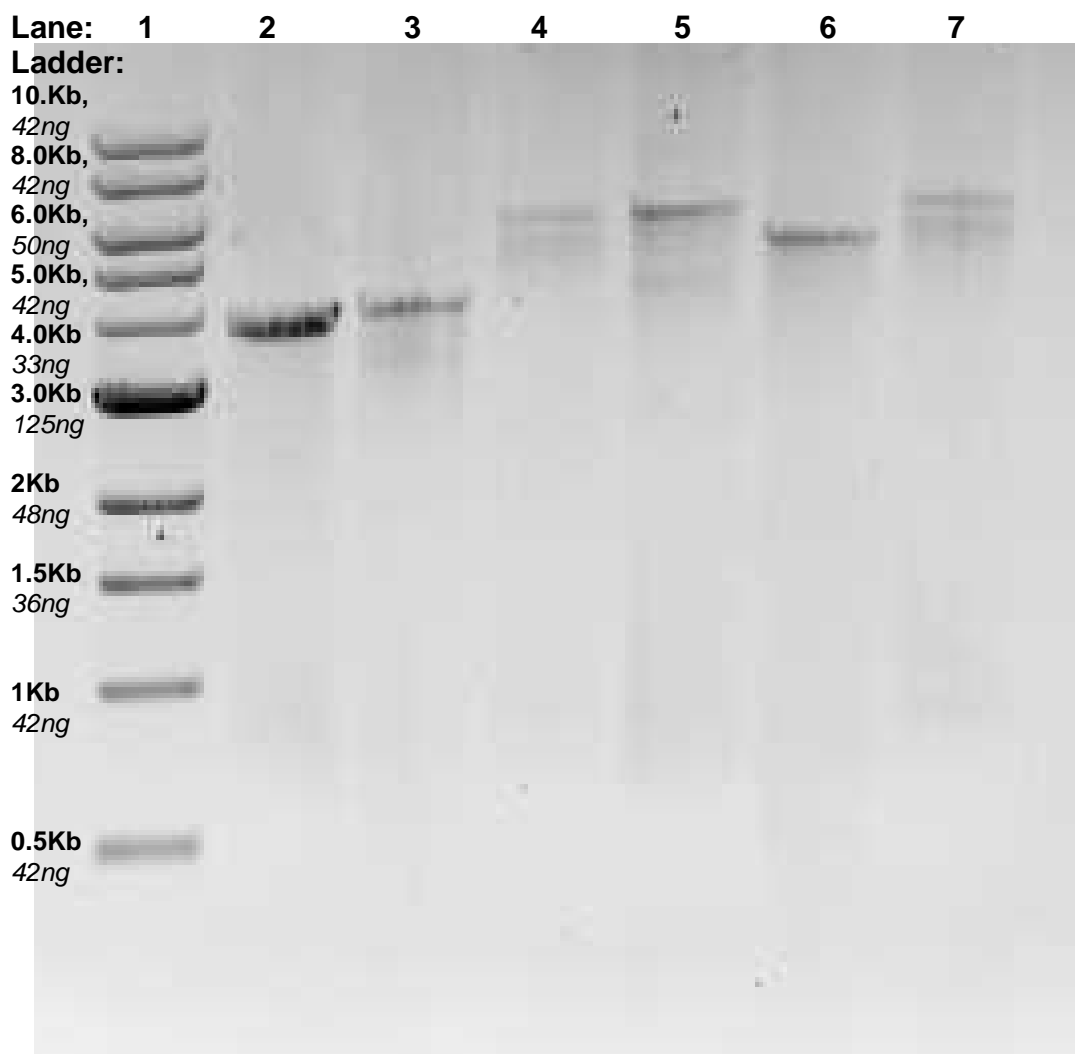


Figure 3.7 Lane 1-1Kb ladder, lane 2- *C.psychrerythraea* Fragment 1 (3822bp), lane 3- *C.psychrerythraea* Fragment 2 (4061bp), lanes 4 and 7- *C.psychrerythraea* Fragment 5 (6112bp), lane 5- *C.psychrerythraea* Fragment 3 (6026bp), lane 6- *C.psychrerythraea* Fragment 4 (5036bp).

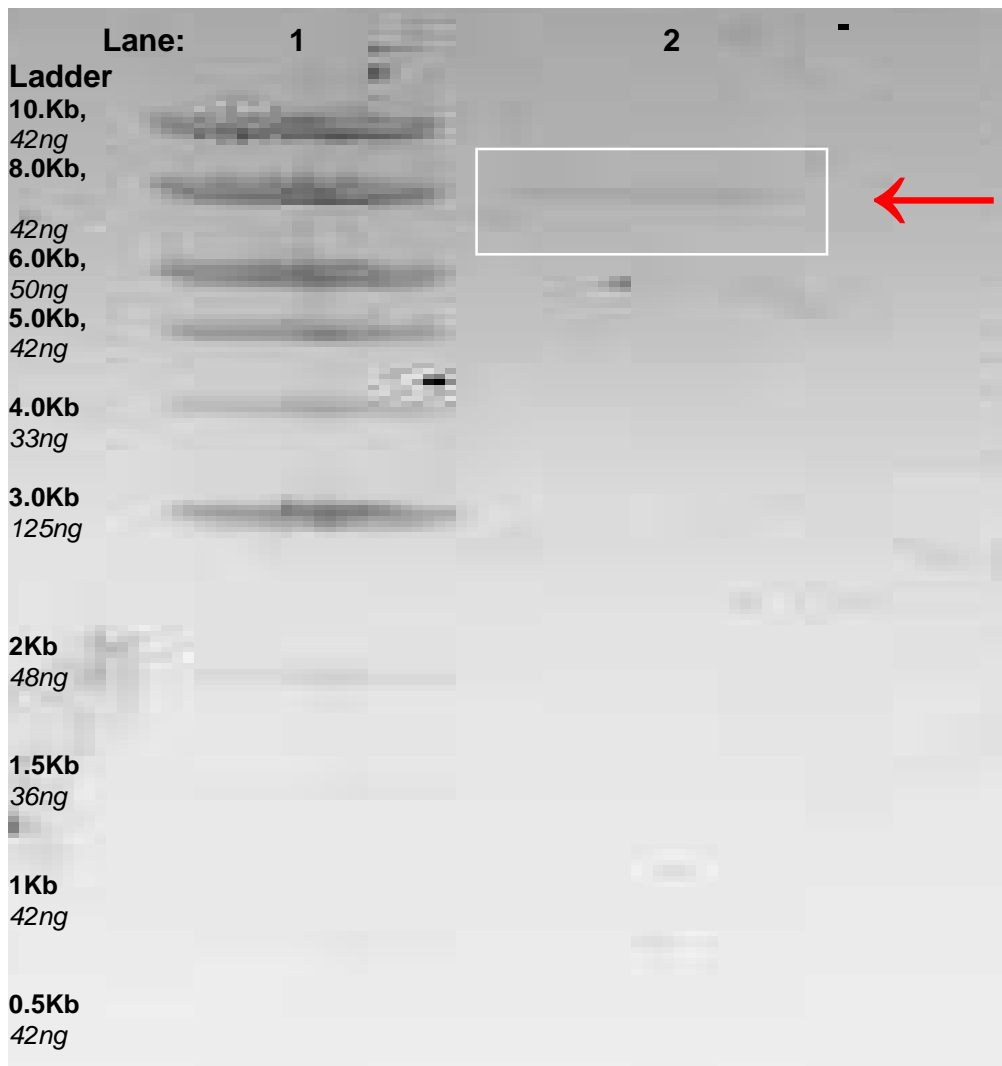


Figure 3.8 Lane 1-1Kb ladder, lane 2-Combined *C.psychrerythraea* Fragments 1 (3833bp) and 2 (4061bp) via one step isothermal reaction: total fragment length 7894bp indicated by the arrow.

The preliminary reaction (a one step isothermal reaction of *C.psychrerythraea* fragments 1 and 2) was carried out for one hour. (Figure 3.8 red arrow), illustrates successful exonuclease activity of the T5 enzyme, pairing and extension of the two harmonizing fragments of interest. Due to the

success of the one step isothermal reaction, all six fragments were combined and set for assembly in the thermocycler. The fragments include the five *C.psychrerythraea* fragments of the *Pfa* Synthase gene cluster, as well as the digested pCC2Fos vector.

The ligation was carried out at 50°C for 30 minutes as well as for 60 minutes. After chemical transformation, the obtained clones were mid-prepped and, through a polymerase chain reaction, verified for the correct orientation and insertion of the *Pfa* Synthase gene cluster into the pCC2Fos vector (Figure 3.9).

Of the obtained clones, none produced a correct insert. Using a forward primer that annealed to the pCC2Fos vector-*C.psychrerythraea* attachment on the 5' end along with a reverse internal primer and vice versa on the opposite end, (See Appendix for Primer sets), it was evident that the clones of interest were not produced. In the absence of an insert, the pCC2Fos primers selected would yield a ~280bp product; the attachment of *C.psychrerythraea* fragment primers 1 and 5, reverse and forward respectively, would cease to occur and annealing would only take place on the vector itself-as observed in the (-) control (Figure 3.9 lane 7).

As for the 100bp fragments produced by the pCC2Fos primers flanking *C.psychrerythraea* "clone" DNA, this may be due to the non-specific activity of the primers binding elsewhere on the vector. It is evident that the negative control of the pCC2Fos flanking primers produced a much more intense band when no insert was cloned into the vector (Figure 3.9 lane 7). This indicates

the amount of amplified pCC2Fos DNA was due to the absence of the insert; the reason that a ~280bp fragment was amplified when using the 5' *C.psychrerythraea* forward primer with pCC2Fos flanking reverse primer was due to imprecise binding of the forward primer (Figure 3.9 lane 5).

Subsequent ligation of the five *Colwellia* fragments along with the pCC2Fos vector was unachievable as shown in Figure 3.10.

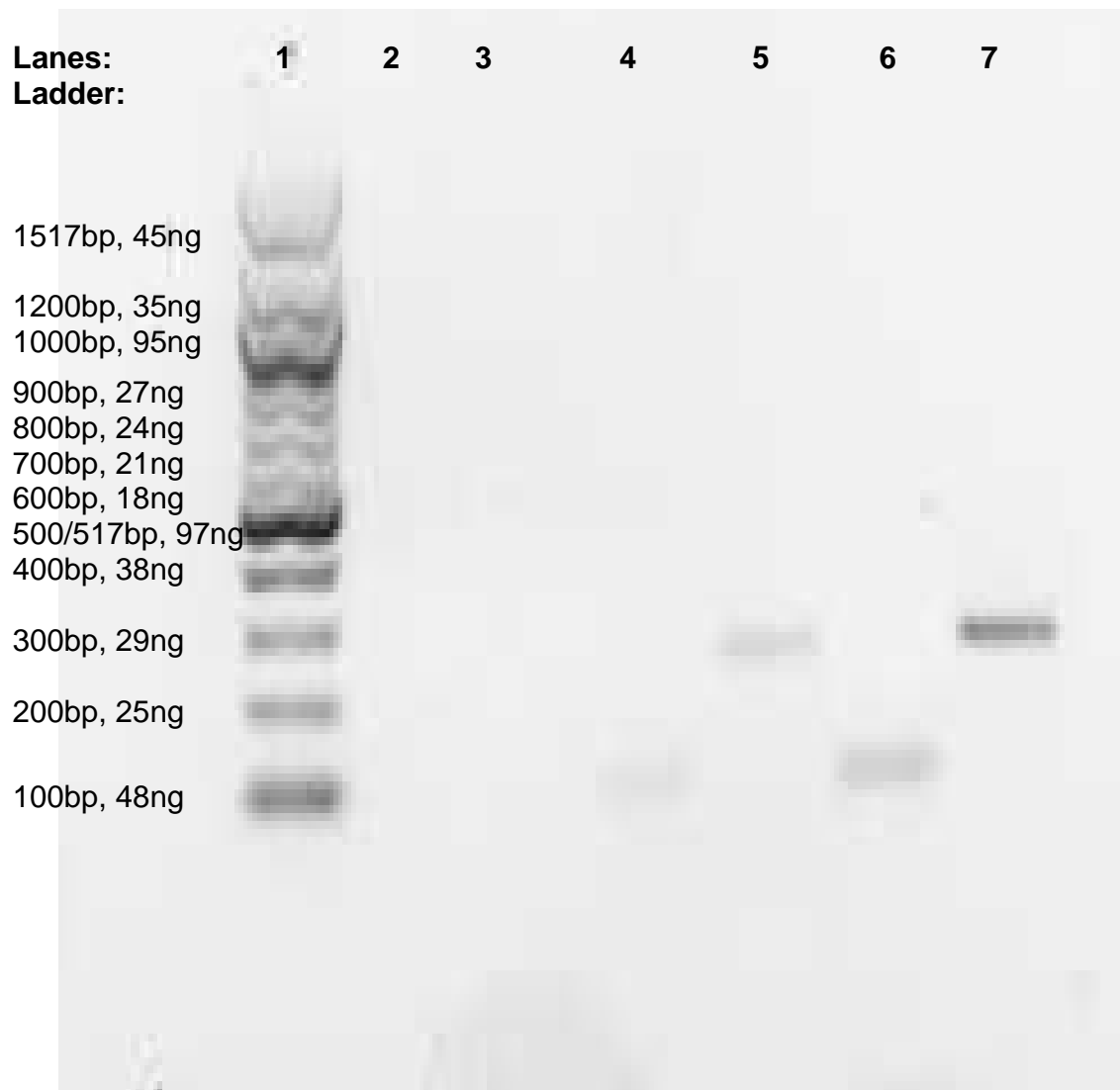


Figure 3.9 Lane 1- 100bp ladder, lane 2-Forward and Reverse primers flanking the 5' end of the pCC2fos vector with the insert, lane 3- Forward and Reverse primers flanking the 5' end of the pCC2fos vector without an insert, lane 4- Forward and Reverse primers flanking the 3' end of the pCC2fos vector with the insert, lane 5- Forward and Reverse primers flanking the 3' end of the pCC2fos vector without the insert, lane 6- Negative Control: Forward and Reverse primers flanking the 5' end of the pCC2fos vector without the insert, lane 7-Negative Control: Forward and Reverse primers selective for the pCC2Fos vector without an insert.

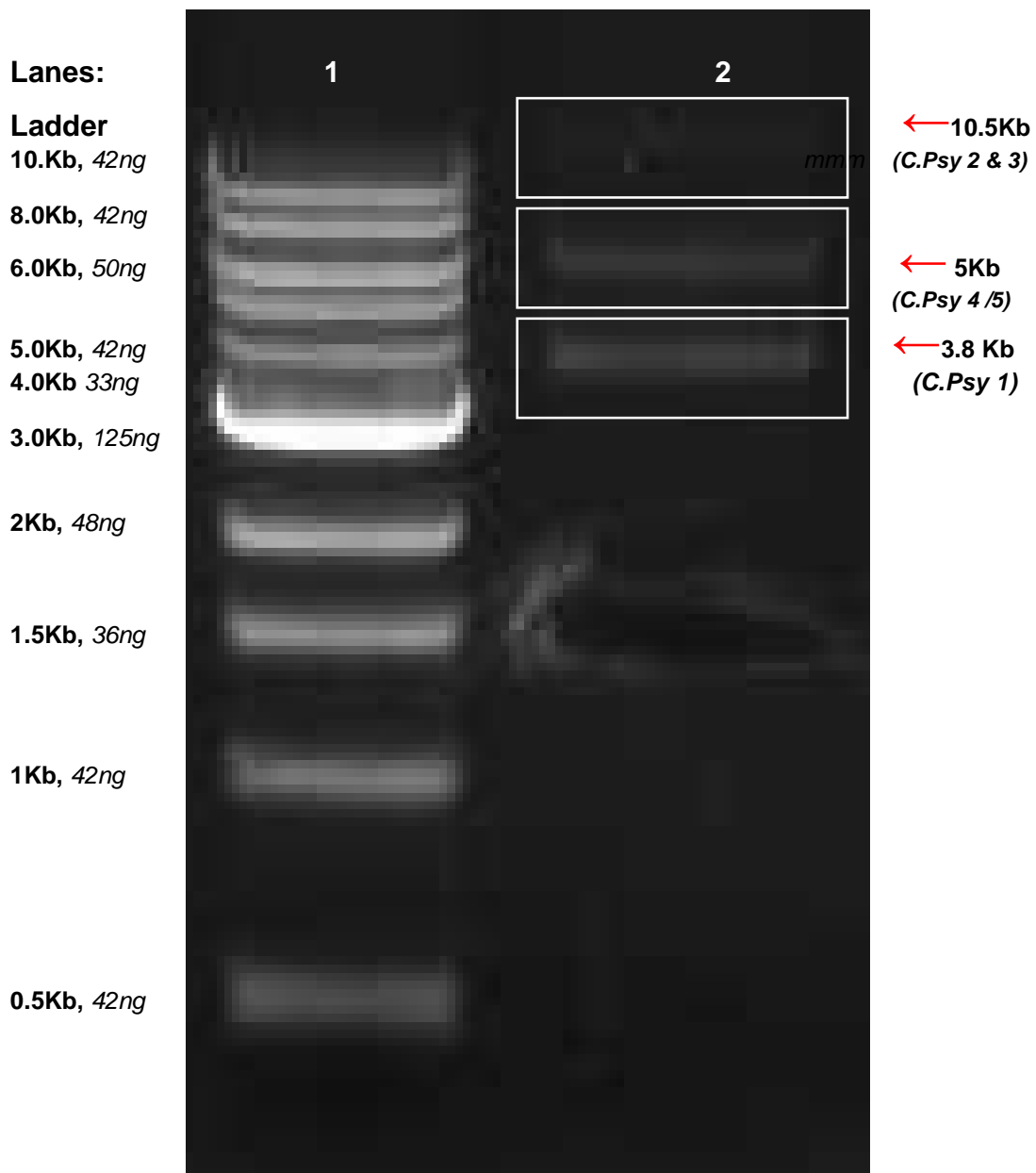


Figure 3.10 Lane 1- 1 Kb ladder, lane 2-5 *C.psychrerythraea* fragments.
* indicates and/or

As demonstrated through the preliminary ligation reaction using *Colwellia psychrerythraea* fragments 1 and 2, the one step isothermal reaction appeared to be successful, however when all 6 fragments undergo the one

step isothermal reaction, annealing is prevented by, what may be due to the presence of the T4 Gene 32 Protein in the reaction, used when troubleshooting the reaction. Most DNA is observed as *C.psychrerythraea* fragment 1, and the only ligation that could be deduced was the presence of an 10.5Kb band that may be the result of the unification of *C.psychrerythraea* fragments 2 and 3 (Figure 3.10).

3.3.1 Optimizing PCR Conditions for Pathway Synthesis

T4 Gene 32 Protein is used to stabilize single stranded DNA, which is produced in a matter of minutes during this reaction and remains on the outermost fragments of *C.psychrerythraea* DNA, ready to anneal to the pCC2Fos Vector. Albeit, T4 Gene 32 Protein typically aides in better stabilization and increased amplification product in soil sediments, it was worth ruling out the possibility of obtaining better stabilization of *C.psychrerythraea* overhangs 1 and 5 for later attachment to the pCC2Fos vector. The fact that the one step isothermal reaction did not anneal may be due to the inference of T4 Gene 32 Protein on the single stranded DNA, preventing Phusion polymerase from filling in the gaps (Figure 3.10).

3.3.2 Benefits of One Step Isothermal Assembly

The one step isothermal reaction provides a fast and easy way to achieve recombination effects of various isolate forms of DNA. A one step assembly also allows for the organization of bacterial products in the presence

of yeast, which has yet been achieved with *Colwellia psychrerythraea*. If it is one day possible to compile an arrangement of the most successfully and efficiently DHA and EPA producing, yeast, alternative forms of nutraceuticals would be an option, along with alternative sources of energy production. Society would be able to promote a better, healthier lifestyle through additives in everyday foods, and the dread of taking a giant PUFA pill would no longer be an issue. Cheaper alternatives of energy could exist, being that bacteria can be easily harvested and low maintenance.

3.4 Transcriptional Regulation of DHA biosynthetic genes in *C.psychrerythraea*

In order to determine if the *pfa* gene cluster was under transcriptional control, RT-PCR analysis was conducted on a 231 bp fragment of the *pfaA* gene in *C.psychrerythraea* cultivated under different temperature regimes (Figure 3.11).

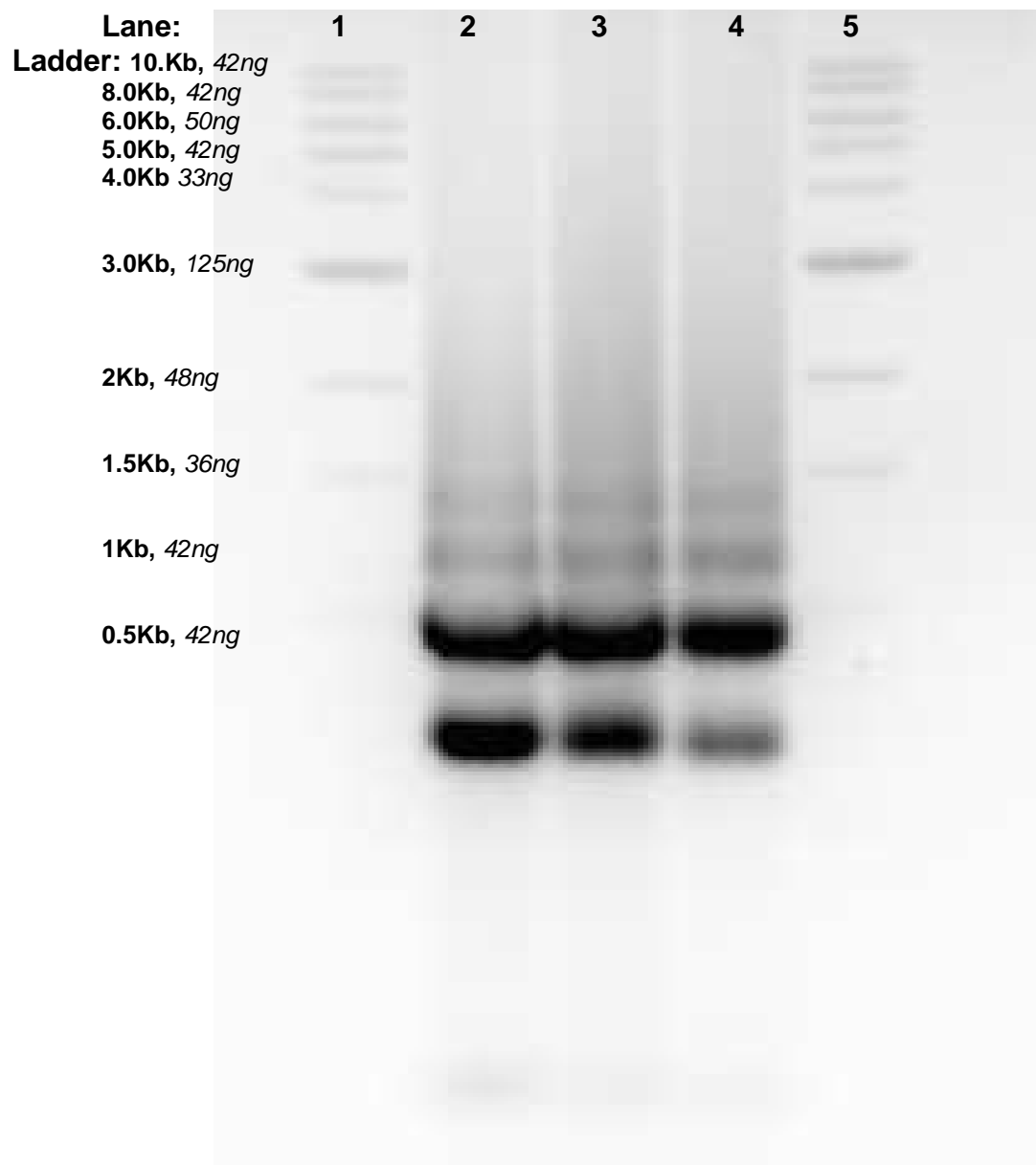


Figure 3.11 Lane 1- 1 Kb ladder, lane 2- *C.psychrerythraea* RNA 4°C, lane 3- *C.psychrerythraea* RNA 8°C , lane 4- *C.psychrerythraea* RNA 16°C, lane5- 1Kb Ladder

Based on the agarose gel results, it is evident that *C.psychrerythraea* RNA was successfully isolated from *Colwellia psychrerythraea* (Figure 3.11). Consequently, Reverse Transcriptase (RT) PCR was carried out for analysis.

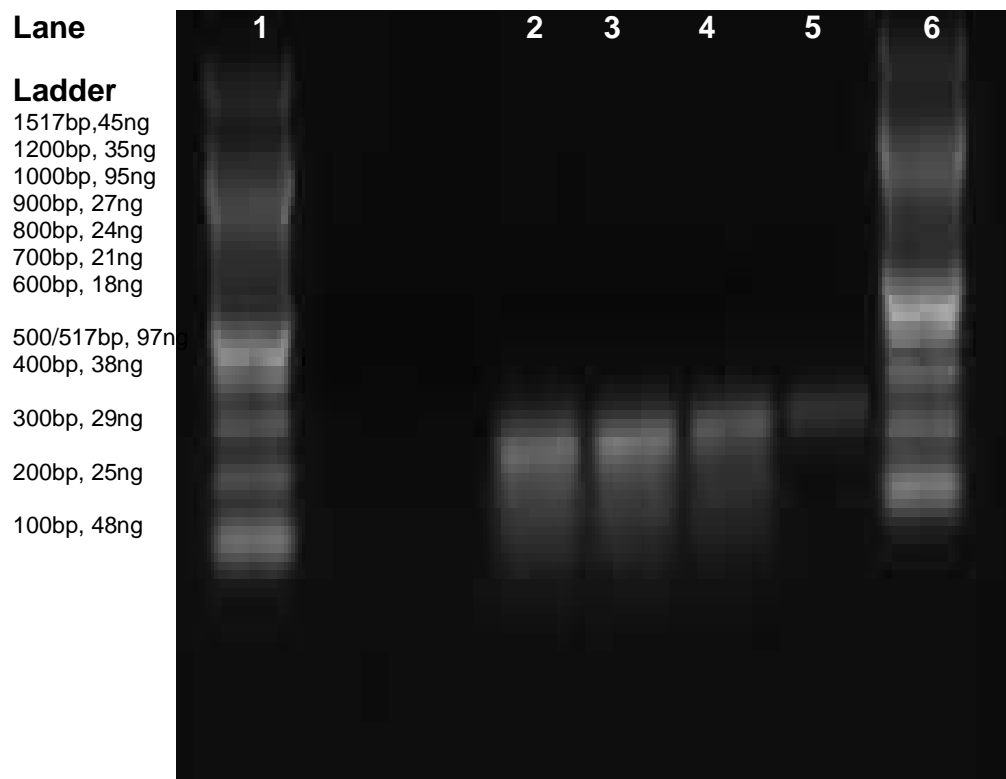


Figure 3.12 Lane 1- 100bp ladder, lane 2- *C.psychrerythraea* RNA 4°C, lane 3- *C.psychrerythraea* RNA 8°C, lane 4- *C.psychrerythraea* RNA 16°C, lane 5- *C.psychrerythraea* DNA (+) Control, lane 6- 100bp ladder

The presence of cDNA amplification (231bp) (Figure 3.12, lanes 2-4) demonstrate active expression of the *pfa* gene cluster all temperature conditions examined. The 8°C band appears to be the strongest and most cohesive, followed by the 4°C band. Not surprisingly, the 16°C band appears the least intense, suggesting the possibility of transcriptional regulation of the *pfa* genes at temperatures above the optimal temperature of 8°C.

Further analysis by qRT-PCR is in progress to quantify preliminary results.

Given the obtained results, it was decided to explore DHA production of *Colwellia psychrerythraea* under the three temperature conditions. It was expected that total DHA production would have an indirect effect to increasing temperature conditions, but further growth and fatty acid production analysis was required. The influence of temperature on DHA production was examined.

3.5 Influence of Temperature on DHA Production in *C.psychrerythraea*

Cultures of *C.psychrerythraea* were harvested at 4°C, 8°C, and 16° C. All three cultures were grown until an optical density of 0.8 was reached. Based on the fatty acid analysis completed by the GCMS run at certain parameters (see materials and methods 2.5.1), it appears that the presence of docosahexaenoic acid varies with the temperature in which it is produced.

A complete chromatogram of the three samples appeared as follows:

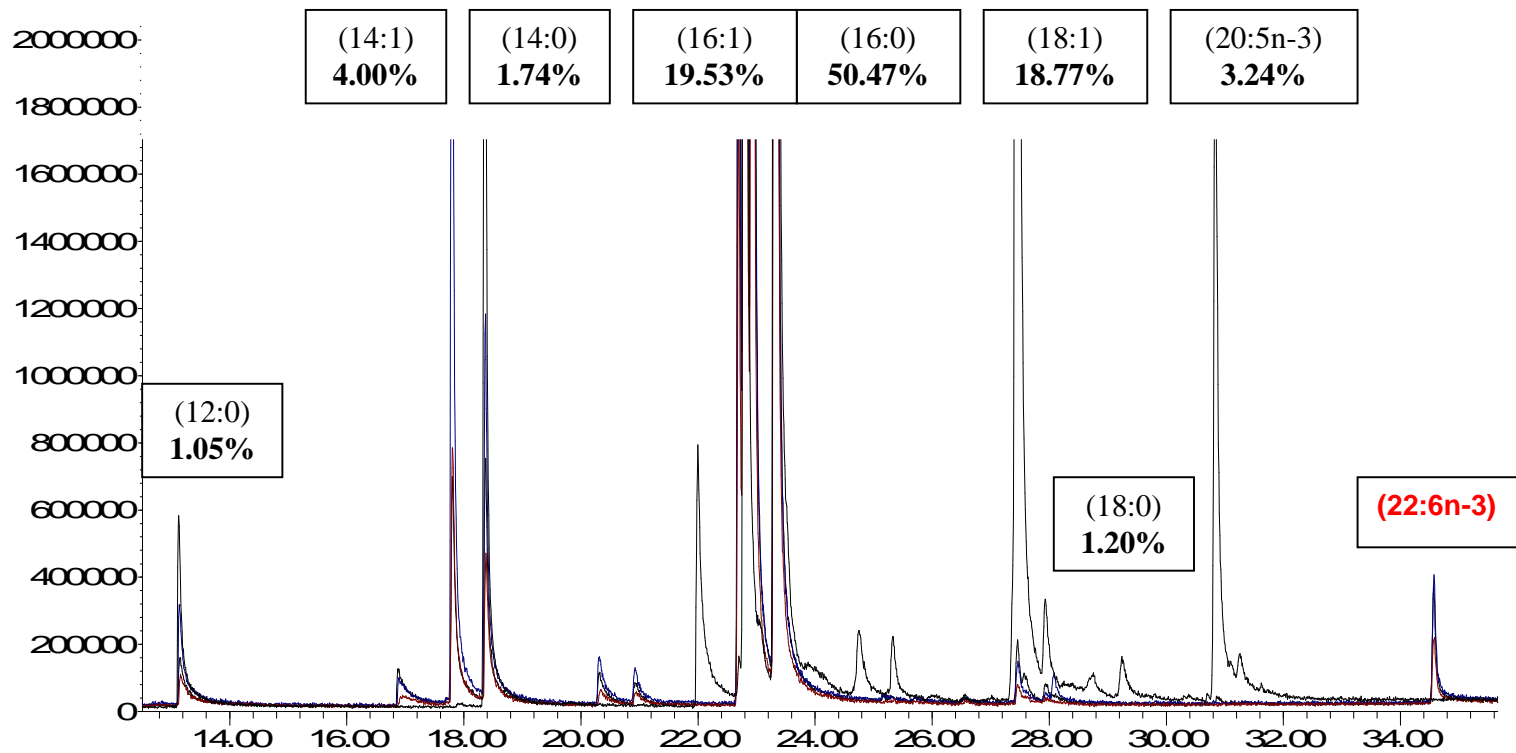


Figure 3.13 Overlaying fatty acid analysis of EPA producing *E.coli* (black peaks) (strain 1F12) and *Colwellia psychrerythraea* (blue and red peaks) grown at 4°C, 8°C, and 16° C. *E.coli* fatty acid production and relative percentage is indicated by black peaks and boxes. *C.psychrerythraea* DHA production indicated in red.

When comparing the recombinant EPA-producing *E.coli* strain 1F12 to the DHA producing *C.psychrerythraea*, it is evident that all three *C.psychrerythraea* cultures are in fact producing DHA (3.13).

Docosahexaenoic acid elutes off the column after eicosapentaenoic acid due to its additional 2 carbons, and consequently, warmer column necessary to elute the compound at a higher temperature and later elution time. All other fatty acids are present in both bacterial strains; however when comparing PUFA production in *E.coli* versus those produced by *C.psychrerythraea* it is evident that EPA production is responsible for four times the amount of DHA production in the obligate psychrophile. Because *C.psychrerythraea* was harvested at the three temperatures: 4°C, 8°C, and 16°C, a GC-MS analysis of each sample was an important element when determining the amount PUFAs produced under certain conditions. A fatty acid of interest is cis-vaccenic acid (C18:1*n*-7), given its direct relationship to FabF as well as the bacterial response to temperature dependent alterations.

Table 3.2: Comparing relative fatty acid abundance of a recombinant EPA producing *E.coli* and DHA producing *C.psychrerythraea*

Bacterial Strain- Growth Conditions	12:0 (%)	14:1n-5 (%)	14:0 (%)	15:0 (%)	16:1n-7 (%)	16:0 (%)	18:1n-7 (%)	18:0 (%)	20:5n-3 (%)	22:6n-3 (%)
<i>E.coli</i>-16°C	1.05	4.00	1.74		19.53	50.47	18.77	1.20	3.24	
<i>C.psychrerythraea</i>- 4°C	0.85	6.17	2.49	0.67	55.89	32.59	0.33	0.27		0.74
<i>C.psychrerythraea</i>- 8°C	0.63	3.44	2.11	0.42	52.71	39.52	0.30			0.88
<i>C.psychrerythraea</i>- 16°C	0.49	1.96	2.05	0.70	50.95	42.34	0.48	0.24		0.80

Focusing on dodecanoic acid, all three samples of *Colwellia psychrerythraea* appear to produce a higher volume of dodecanoic acid (C12:0) relative to their total fatty acid production as temperature decreases (Ouattara, 2000). The increase in production of dodecanoic acid at lower growth temperatures may be due to the requirement to use dodecanoic acid as a precursor for VLC-PUFAs as well as membrane components, allowing for the production of a permeable structure at lower temperatures (Table 3.2, Supplemental figures 3.17a, 3.18a, 3.19a).

Tetradecanoic acid (14:0) and (14:1) increases according to the amount of dodecanoic acid produced. The direct relationship between dodecanoic acid and tetradecanoic acid is evident in the *E.coli* 1F12 sample as well; giving rise to the conclusion that bacterial (*C.psychrerythraea*) production of subsequent fatty acids is directly dependent on precursors undergoing dehydration and elongation reactions.

Comparing the 1F12 *E.coli* sample (Figure 3.13) to *C.psychrerythraea* 4°C, 8°C, and 16°C (Supplemental figures 3.17a, 3.18a, 3.19a, respectively) illustrates a much higher production of *cis*-vaccenic acid: 18.77% versus an average 0.33%, indicating the requirement for (18:1) fatty acids and their relationship to growth ability. The FabF gene in bacteria helps to sustain growth, however, in the absence or mutation of FabF, which may occur while the bacteria experiences uncharacteristic growth conditions (Table 3.2, and Supplemental figures 3.17a and 3.19a), increases in *cis*-vaccenic acid is observed.

It is interesting to notice that the 18:0 fatty acid, octadecanoic acid, is most prominently observed in *C. psychrerythraea* cultures grown in 4°C and 16°C, out of the three samples (Table 3.2). The presence of cis-vaccenic acid (18:1) in *C. psychrerythraea* grown in an 8°C environment is smallest in quantity, and the presence of octadecanoic acid (18:0) is minimal, almost absent. As previously noted, *Colwellia psychrerythraea* grows optimally at 8°C; this may be due to the fact that the production of fatty acids, and FabF, which aide in the production of obligatory temperature alterations is not necessary in *Colwellia psychrerythraea* (Table 3.2).

It was anticipated that DHA production would exhibit temperature-dependent synthesis in the following order: 8°C, 4°C, and 16°C. As expected, DHA production is highest when grown in an 8°C environment; however, DHA production in the 16°C sample was higher than observed in the 4°C sample (Table 3.2 and Supplemental figures 3.17a, 3.18a, 3.19a). This observation may be due to the appearance of more hexadecanoic acid production in the 16°C sample as a result of the loss of environmental maintenance of the 4°C environment.

3.6 Progress Towards Engineering a mutant defective in DHA synthesis

After inoculating a 5ml culture of 2216 with 200ug/ml kanamycin as well as plating *Colwellia psychrerythraea* onto 2216-100ug/ml rifampin plates, it was evident that 200ug/ml kanamycin prevented *C.psychrerythraea* growth and a rifampin mutant was produced.

A 516bp fragment of the KR region in the pfaA domain was amplified from *Colwellia psychrerythraea* DNA and ligated into a pcr4 vector (Figure 3.14).

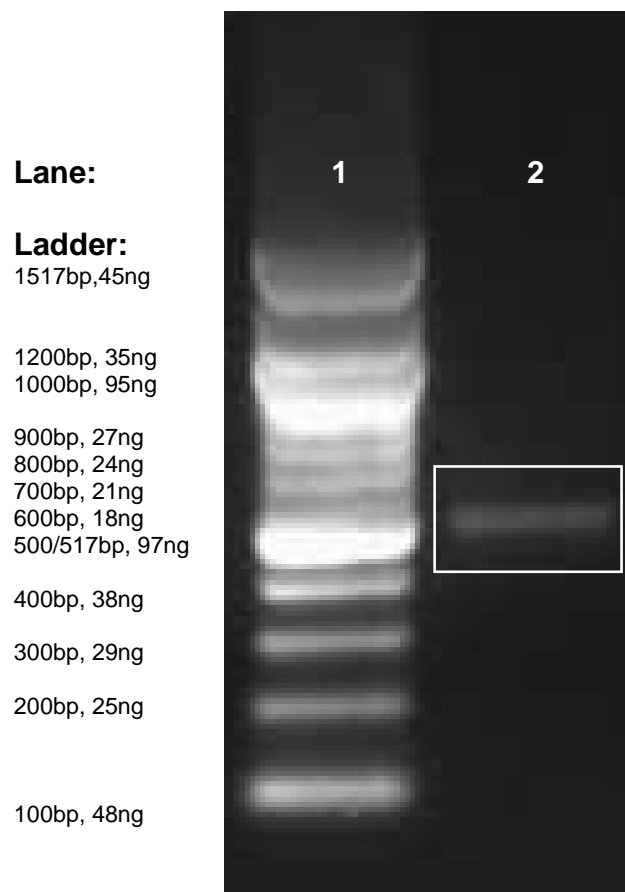


Figure 3.14 Lane1- 100bp ladder, Lane2- Amplified 516bp Rif target DNA

The amplified 516bp fragment of *C.psychrerythraea* is now ready for insertion into the digested pcr4 vector. After a ligation into the vector, the fragment was digested with EcoR1, creating a ~550bp product (Figure 3.15, lane 3).

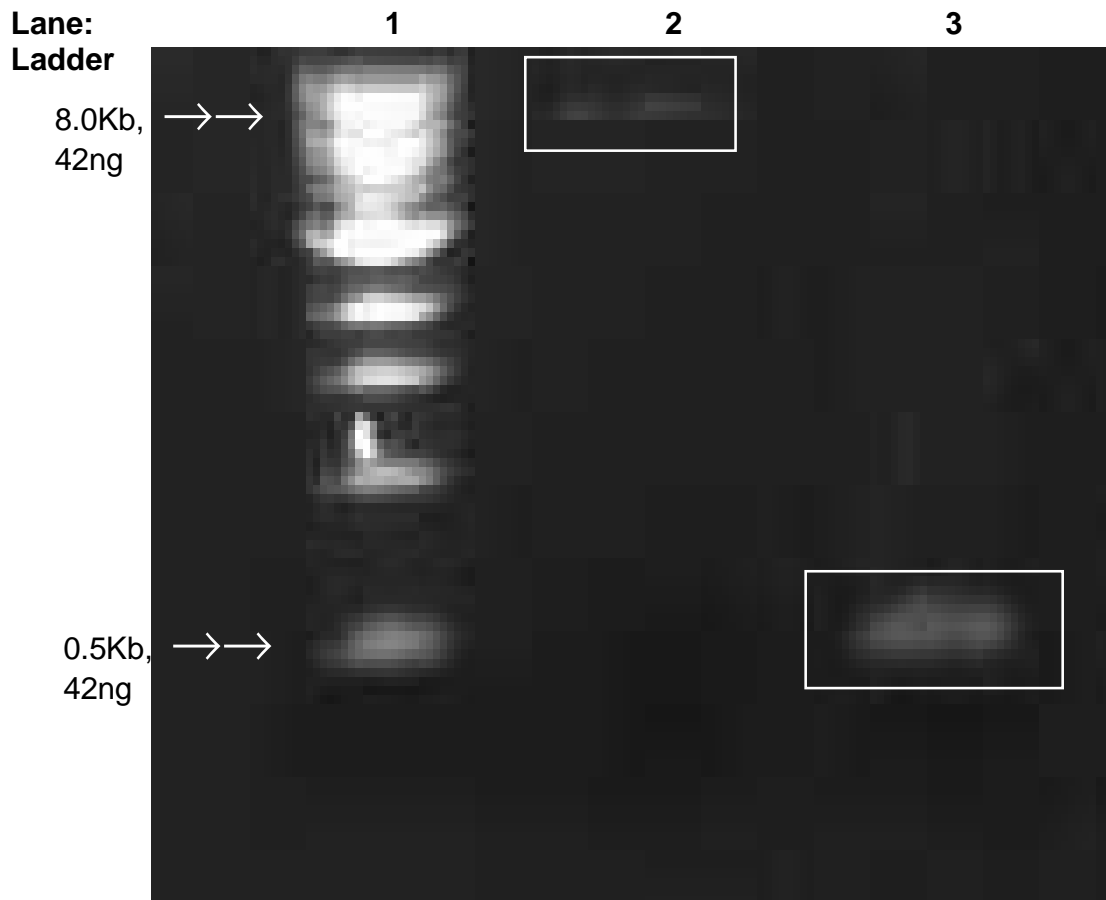


Figure 3.15 Lane 1- 1Kb ladder, lane 2- digested pEE3 (EcoR1), lane 3- amplified KO target of the KR domain.

pEE3 was also digested with EcoR1, linearizing the vector and creating complement ends to the purified 516bp fragment. A double digest of the insert required to complete the KO of the KR domain was necessary due to the inability to ligate and clone directly into the pEE3 suicide vector.

With the help of an (online) ligation calculation, the 3:1 insert to vector ratio was composed by combining 8.5ng/ μ l of insert to 11ng/ μ l of pEE3 vector. After transformation of 1ul mini-prepped DNA, (pEE3 containing 516 KO target), into 50 μ l of chemically competent DH5 α , a successful amplification of the 785bp product using the M13 primers was accomplished (Figure 3.16).

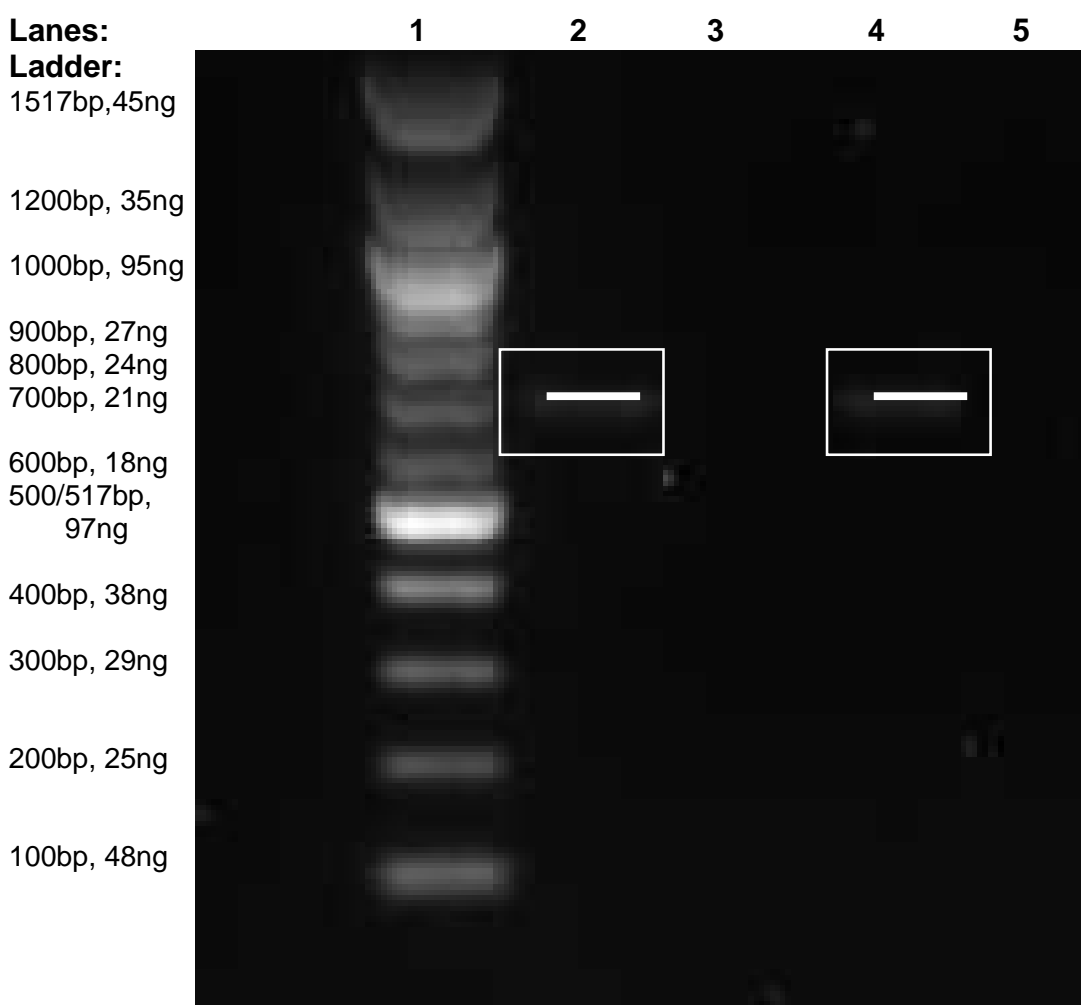


Figure 3.16 Lane1- 100bp ladder, lane2-MP pEE3 + 516bp*, lane 3-NTC, lane 4- MP pEE3 + 516bp*, lane 5- NTC
*using the M13 Forward and Reverse primers yielded a ~700bp product which included the 516 target DNA.

Next steps currently in development involve the bi-parental conjugation of the rifampin resistant *C.psychrerythraea* and the pEE3 vector+516bp insert construct in *E. coli* strain BB1. After conjugation, selection of Kan + Rif resistant exconjugants will be verified for pEE3 insertion into the chromosome of *C. psychrerythraea* at the *pfaA* locus. .

Expectations include the observation of enhanced growth of the *C.psychrerythraea* parental strain compared to the *pfa* knockout mutant. Fatty acid production of the parental strain versus the *pfa* mutant will be analyzed via GCMS analysis of FAME derivatives as described previously. A closer look into *cis*-vaccenic acid (C18:1) dynamics will be of particularly interest. Are compensatory increases in monounsaturated fatty acids observed as a result of polyunsaturated fatty acid depletion? Clearly, much remains to be learned about the physiological function and ecological significance of bacterial omega-3 polyunsaturated fatty acid biosynthesis.

Appendix

Table. 1a High molecular weight DNA preparation in LMP agarose plugs

Buffer A		100 ml
10 mM Tris-Cl, pH 8		1 ml - 1 M Tris-Cl, pH 8
100 mM EDTA, pH 8		20 ml - 0.5 M EDTA, pH 8
500 mM NaCl		10 ml - 5 M NaCl
		69 ml - ddH ₂ O

Detergents		50 ml
20% Na-Deoxycholate		10 g
10% Brij-58		5 g
20% Sarkosyl		10 g
<i>*Filter sterilize – DO NOT AUTOCLAVE!</i>		

Buffer B		100 ml
10 mM Tris-Cl, pH 8		1 ml - 1 M Tris-Cl, pH 8
100 mM EDTA, pH 8		20 ml - 0.5 M EDTA, pH 8
250 mM NaCl		5 ml - 5 M NaCl
		74 ml - ddH ₂ O
<i>*add detergents just prior to use!</i>	<i>ADD PER ML:</i>	
0.2% Na-Deoxycholate		10 µl 20% Na-Deoxycholate
0.5% Brij-58		30 µl 10% Brij-58
1% Sarkosyl		25 µl 20% Sarkosyl

TE Buffer		100 ml
10 mM Tris-Cl, pH 8		1 ml - 1 M Tris-Cl, pH 8
1 mM EDTA, pH 8		0.2 ml - 0.5 M EDTA, pH 8
		98.8 ml - ddH ₂ O

Storage Buffer (TE10)		100 ml
10 mM Tris-Cl, pH 8		1 ml - 1 M Tris-Cl, pH 8
10 mM EDTA, pH 8		2 ml - 0.5 M EDTA, pH 8
		97 ml - ddH ₂ O

Buffer C		100 ml
10 mM Tris-Cl, pH 8		1 ml - 1 M Tris-Cl, pH 8
500 mM EDTA, pH 8		100 ml - 0.5 M EDTA, pH 8
<i>*add detergents just prior to use!</i>	<i>ADD PER ML:</i>	
1% Sarkosyl		25 µl 20% Sarkosyl

Titer calculation from Epicentre

$$\frac{(\# \text{ of colonies}) (\text{dilution factor}) (1000\mu\text{l/ml})}{(\text{volume of phage plated } [\mu\text{l}])}$$

Table 2a Induction Solution Preparation

Total volume of clone induction culture	Volume of fresh LB + chloramphenicol (12.5ug/ml)	Volume of overnight 5ml culture	Volume of 1000X CopyControl Induction Solution*
1ml	800 μ l	200 μ l	1 μ l
5ml	4.5ml	500 μ l	5 μ l
50ml	45ml	5ml	50 μ l

*Mix thoroughly after thawing

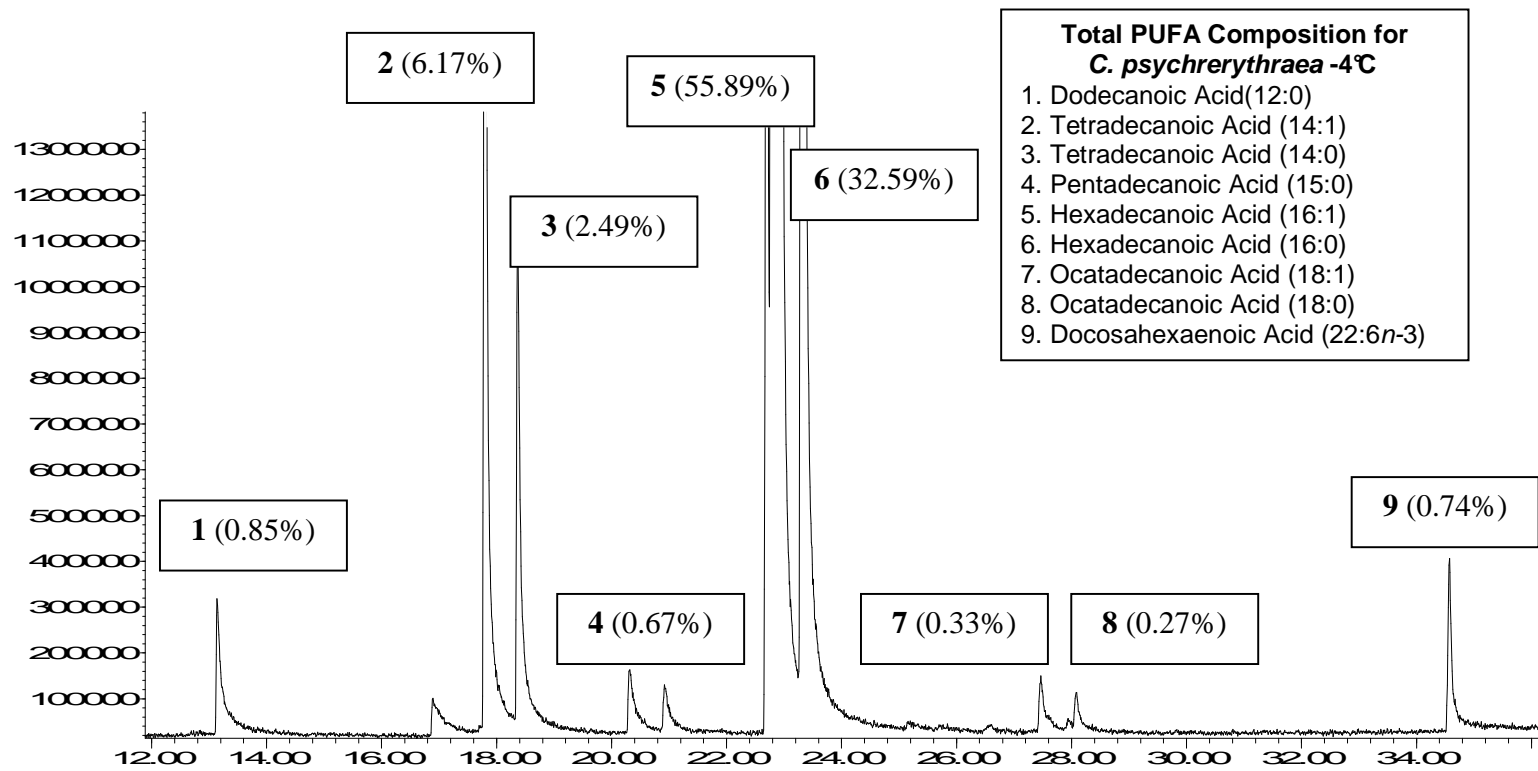


Figure 3.17a GCMS fatty acid analysis of *C. psychrerythraea* at 4°C.

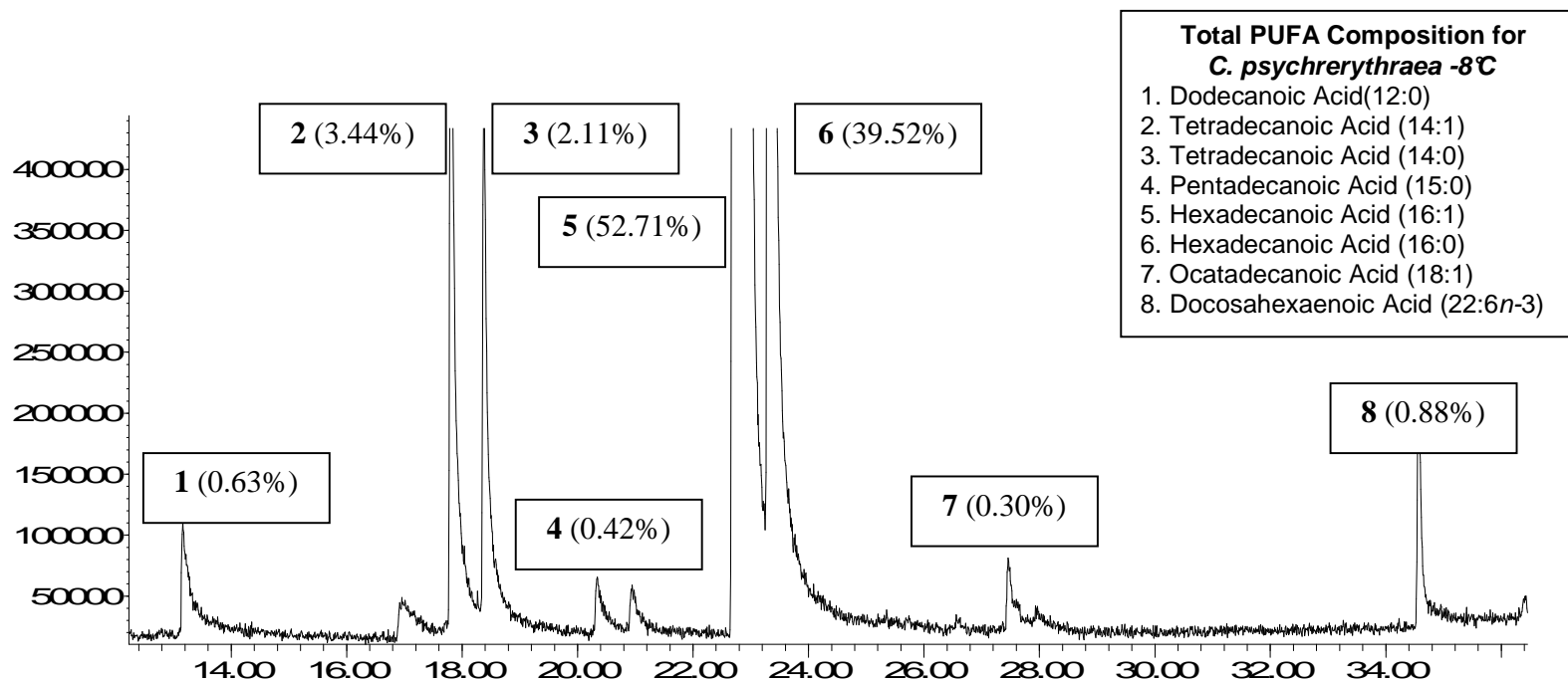


Figure 3.18a GCMS fatty acid analysis of *C. psychrerythraea* at 8°C.

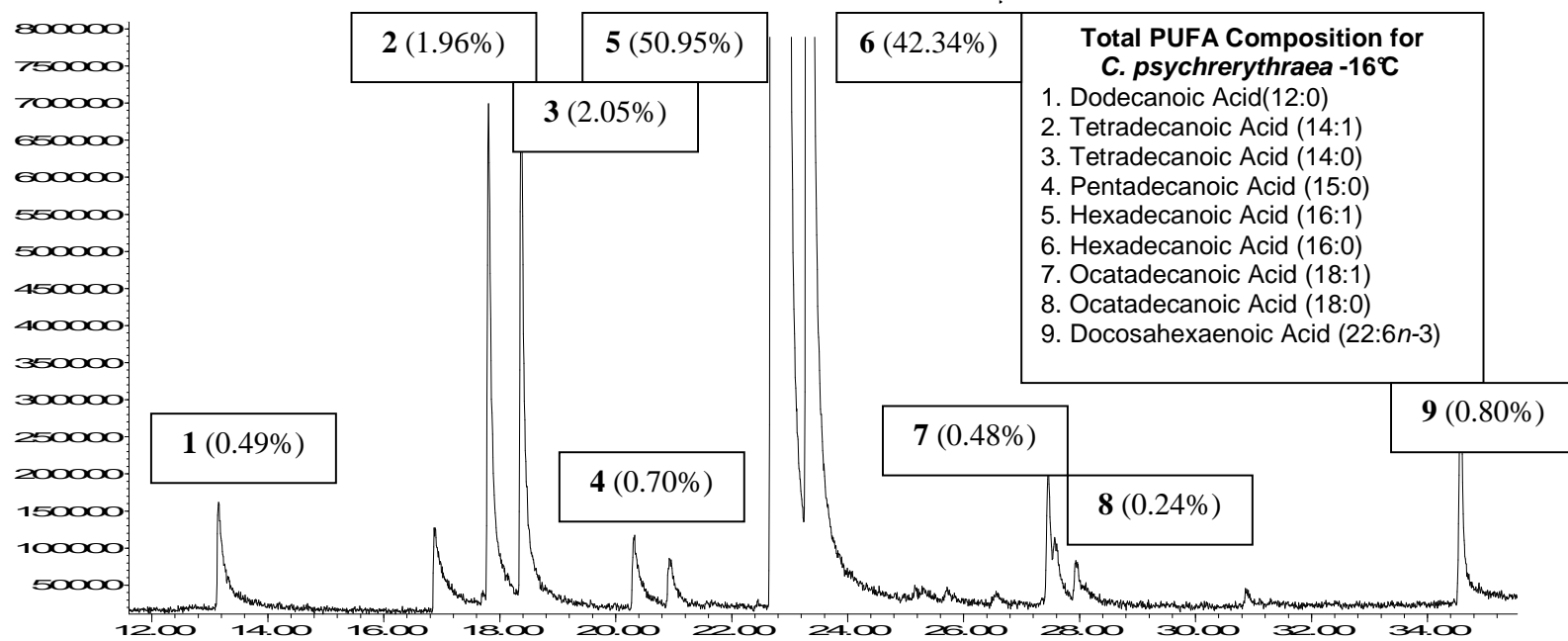


Figure 3.19a GCMS fatty acid analysis of *C. psychrerythraea* at 16°C.

Table.3a.Primer Sets

<u>Primer Name</u>	<u>Sequence (5'→3')</u>	<u>Usage</u>
1963F	CTTCCAGAACCCCTTTGATT	To verify <i>D.Shibae</i> insert in CopyControl Fosmid Library
1963R	TCGGCAAAGACCTTAGACAG	
BAC27F	AGAGTTTGATCMTGGCTCAG	Universal 16S primers used to PCR bacterial inserts- <i>D.shibae</i> and <i>C.psychrerythraea</i>
BAC27R	AAGGAGGTGATCCARCCGCA	
1952F	TAGGCATCGCCTCGATCTAT	<i>D.shibae</i> primers to verify insert in CopyControl Fosmid Library
1952R	GCTTTTTCAGGATCGACAGG	
CPsy-1F	CATCATCGAGGACAATTAACCTGTG	Fragment 1 (1 step isothermal reaction using <i>C.psychrerythraea</i>) -Fragment Length= 3833bp -Overhang length with Fragment 2= 108 bp
CPsy-1R	TTCTTCCGCAACCTTGATGG	

Note: Primers labeled CPsy is short-hand for *C.psychrerythraea

Table.3a.Primer Sets

Primer Name	Sequence (5'→3')	Usage
CPsy-2F	ACCGCCAGCCATGGTAGTGC	Fragment 2 (1 step isothermal reaction using <i>C.psychrerythraea</i>) -Fragment Length= 4061bp -Overhang length with Fragment 3= 187bp
CPsy-2R	GCGGCGCACAAATGCCAACA	
CPsy-3F	AATGGCGAGCGGCTCACGTG	Fragment 3 (1 step isothermal reaction using <i>C.psychrerythraea</i>) -Fragment Length= 6026bp -Overhang length with Fragment 4= 151 bp
CPsy-3R	ATTAAAGCGGCAGGCGGGCA	
CPsy-4F	CCGCTAAGACACCCGCACCA	Fragment 4 (1 step isothermal reaction using <i>C.psychrerythraea</i>) -Fragment Length= 5036bp -Overhang length with Fragment 5= 284bp
CPsy-4R	GGGCCGCGAGCTTGCTTGTA	
CPsy-5F	CAAAGCGCGGTTAATTCACC	Fragment 5 (1 step isothermal reaction using <i>C.psychrerythraea</i>) -Fragment Length= 6112bp
CPsy-5R	TCAATTTCCCAACACGATCAG	

***Note:** Primers labeled CPsy is short-hand for *C.psychrerythraea*

Table.3a.Primer Sets

<u>Primer Name</u>	<u>Sequence (5'→3')</u>	<u>Usage</u>
Fwd.1.pCC2Fos_Insert	CGACGGCCAGTGAATTGTAA	To verify <i>C.psychrerythraea</i> insert into pCC2Fos Vector at the 5' end
Rev.1.pCC2Fos_Insert	TGAAGAAGCGAACTTAGCGC	
Fwd.2.pCC2Fos_Insert	TGAGCTTAACCAATGCCAGC	To verify <i>C.psychrerythraea</i> insert into pCC2Fos Vector at the 3' end
Rev.2.pCC2Fos_Insert	CACCCCAGGCTTTACACTTT	
CPsy-516 Forward	TACCATGCCACCATCCCAAG	To amplify a 516bp insert of the KR domain necessary for KO of <i>Pfa</i> Synthase System
CPsy-RNA Reverse	GCAAGCGGCGAAAAACCAAC	
CPsy-RNA Forward	ACCCGCACCATGAATGATGC	To determine and measure the transcriptional regulation of <i>C.psychrerythraea Pfa</i> Synthase System
CPsy-RNA Reverse	GCAAGCGGCGAAAAACCAAC	
M13 Forward	GTAAAACGACGGCCAG	To verify 516bp insert in pEE3 vector
M13 Reverse	CAGGAAACAGCTATGAC	

Note: Primers labeled CPsy is short-hand for *C.psychrerythraea

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