5-2020

Fatty acid mediated alterations in biofilm formation with Vibrio cholerae

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Departmental Honors Thesis

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Examination Date: 3 April 2020

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Abstract

Vibrio cholerae is a Gram-negative bacterium known as a waterborne pathogen. It is often found in marine conditions and can infect humans through ingestion. *V. cholerae* is responsible for cholera, a disease characterized by profuse diarrhea, vomiting, and other dehydration symptoms. Ultimately, severe cases can cause extreme electrolyte imbalances, shock, and acute renal failure which lead to death in a short period of time. Annually, *V. cholerae* causes approximately 3 million cases of cholera and 100,000 deaths worldwide. *V. cholerae* can persist in motile and biofilm lifestyles under aquatic and host conditions, a unique biphasic lifestyle that contributes to *Vibrio*'s survival and pathogenicity. Previous research has shown that *V. cholerae* responds to various fatty acids by altering behavior associated with virulence (biofilm formation, motility, and antibiotic resistance). This study aimed to identify *V. cholerae* genes that participate in fatty acid-mediated formation of biofilms, which are communities of bacteria formed for survival, persistence, and pathogenicity. Using a transposon library, bioinformatically selected mutants were evaluated for their ability to form biofilms in distinct physiological conditions with or without three polyunsaturated fatty acids (PUFAs). The candidate mutants were subjected to 3 physiologically relevant PUFAs (alpha-linoleic acid [18:3α], arachidonic acid [20:4], and docosahexaenoic acid [22:6]). Each mutant's growth characteristics and biofilm formation were assessed under marine (30°C, pH 8.1, 600mM NaCl) and human host (37°C, pH 7.1, 150mM NaCl) conditions. As indicated in preliminary research, temperature and salt induced changes in biofilm formation and growth among the selected mutants. Interestingly, transposon mutant VCA0785 displayed a decrease in biofilm production in marine and host conditions, indicating an importance of this gene in biofilm formation. VC1348 produced biofilm mimicking wild-type under marine conditions, but showed striking amounts of biofilm when PUFAs were available under host conditions. Interestingly 18:3α elicited almost 10 times the amount of biofilm in host versus marine conditions. Under marine conditions, VC1710 displayed a stark decrease in biofilm formation in the presence of each fatty acid, with 22:6 causing opposite effects between conditions. Collectively, this study identified environment-specific alteration to fatty acid-mediated biofilm formation, as well as implicating some potential methyl-accepting chemotaxis proteins involved in signaling biofilm production in *V. cholerae*.

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Chapter 1: Introduction

1.1 Gram-Negative Membranes

Gram-negative bacteria can be differentiated microscopically from Gram-positive bacteria by their inability to retain crystal violet (CV) following the staining procedure developed by Hans Christian Gram.[1] Whereas, Gram-positive bacteria are able to retain the CV and thus appear purple under light microscopy after staining. This retention of CV is primarily due to the thick peptidoglycan layer that is found on the outside of the single lipid bilayer

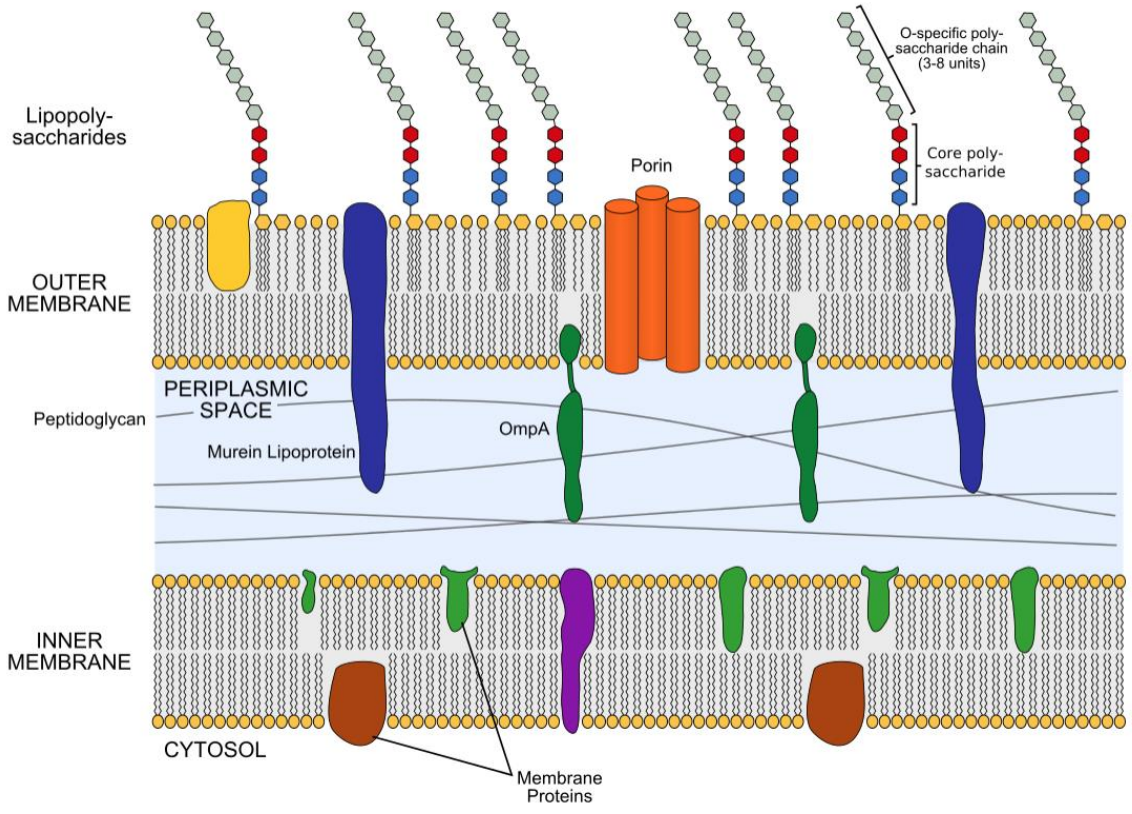

Figure 1.1 Structural model of a gram-negative cell envelope[2]

membrane.[3] As displayed in Figure 1.1, a thin layer of peptidoglycan can be found in the periplasmic space in Gram-negative bacteria.

In Gram-negative bacteria the envelope, comprised of an inner membrane and outer membrane, provides a platform for lipopolysaccharides (LPSs) and phospholipids (PLs), as well as membrane proteins involved in processes such as transport, adhesion, cellular interaction, and environmental sensing. Pertinent to this study are membrane proteins involved in the transport and assimilation of exogenous fatty acids (FAs), as well as sensing proteins that modify bacterial behavior by initiating signaling cascades depending on small molecules detected in the environment.[4] This study involves methyl-accepting chemotaxis proteins (MCPs), which have been linked to critical involvement in cell regulation that impacts pathogenicity in bacteria.[5, 7, 10] MCPs are located in the inner membrane in a gram-negative bacterium typically as part of two-component regulatory systems capable of recognizing small molecules and activating proteins that will influence transcription of genes that will facilitate a response to the chemical stimuli.[8] These proteins have been shown to mediate chemical detection that results in motility; however, *V. cholerae* possesses an excess of homologues to MCPs, suggesting a wider range of behavioral modification. The goal of this project is to determine whether MCPs participate in regulating biofilm formation by responding to exogenous fatty acids. Previous studies have established the membrane-associated machinery responsible for uptake and incorporation of fatty acids.[7] It is thought that as a bacterial cell takes up a fatty acid, or other materials that aid in virulence, a metabolism pathway is engaged in allowing this molecule to reach the inner membrane and MCPs, where a signaling cascade is triggered, leading to a change in gene regulation that could change biofilm formation.[8, 9] An increased diversity of fatty acids (ranging in carbon number and level of unsaturation) have been used as candidates to demonstrate membrane phospholipid

remodeling with various bacteria including *V. cholerae*. [7, 10, 11, 14] These studies discovered a range of fatty acid-mediated effects on phenotypes associated with virulence, including motility and biofilm formation. This study attempts to uncover genes involved in sensing fatty acids for control of biofilm formation in *V. cholerae*.

1.2 Biofilms

Biofilms are clusters of bacteria anchored to a surface and held together by an extracellular matrix, primarily composed of polymeric substances such as polysaccharides. [6, 7, 12, 14] Biofilm is considered an important factor in environmental survival for *V. cholerae*; as it is a known phenotype related to bacterial persistence and survival in various environments.[7, 14] In addition to being problematic in medical settings (catheters, ventilators), biofilms are ubiquitous in nature and can be found in industrial, aquatic, and other environmental water systems. Biofilm forming microorganisms display a distinct genetic phenotype that allows for attachment, biofilm cellular formation, and detachment unlike other microorganisms.[12] *V. cholerae* is known to create biofilms because it is often in turbid intertidal environments, where a capsular adaptation is essential for survival and persistence for the bacteria.[15] *V. cholerae* is prominent due to its biphasic lifestyle; sessile and motile. This adaptability in lifestyle allows *V. cholerae* to take advantage of multiple environments, like the mammalian intestines, which are nutrient rich, as well as estuarine aquatic environment where more scarce nutrient sources such as copepods, chironomids, cyanobacteria, fish and even water birds are available as reservoirs.[16, 17] Transitioning through these phases allows *V. cholerae* the ability to maintain pathogenicity. However, this is only accomplished because *V. cholerae* has various virulence factors to withstand environmental stressors encountered such as: nutrient limitations, physical, chemical, extreme temperature, bacteriophage predations, oxidative stress, and protozoan grazing in the

marine environment.[18, 19] In the human intestinal environment *V. cholerae* must overcome additional stressors such as: iron limitations, bile acids, low pH, antimicrobial peptides, and periods of nutrient deprivation.[20] Under these various stressors *V. cholerae* can employ its sessile lifestyle to allow attachment and formation of a biofilm as a means of survival.[18, 19] *V. cholerae*'s ability to survive in stressful environments is integral to its pathogenicity since it is hypervirulent in the motile stage immediately after excretion from a human mammalian intestine (primarily due to the abundance of nutrients it experiences in the host intestines), thus has the best chance at furthering the infectious cycle.[21]

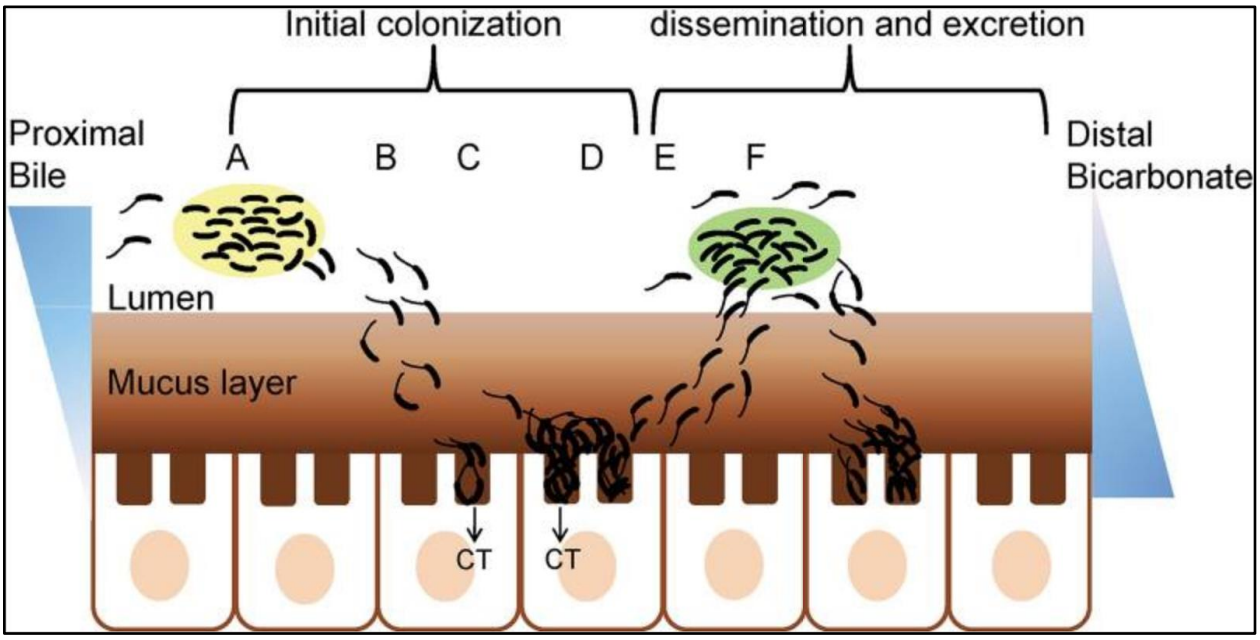

Figure 1.2 Diagram of biofilm formation in human intestines, displays motile and sessile life phases of V. cholerae.[15]

While the specific signaling pathway involved in initiation of significant transcriptomic changes resulting in biofilm formation is unknown, much has been identified about the pathways involved in signaling cascades utilized once cells are in a biofilm matrix.[15] Signaling cascades that are utilized by the bacteria to regulate biofilm formation once it reaches the level of gene

activation include, the HapR regulator and VpsT or VpsR-dependent antirepression cascade. HapR is a quorum sensing regulator that allows *V. cholerae* to enhance expression of a factor involved in the stationary phase, *V. cholerae* also has FadL which all contribute to signaling that allows *V. cholerae* to change and express different phenotypes such as motility and biofilm.[15] This study attempts to investigate possible genes involved in the initiation of attachment as the initial stage of biofilm formation, through the use of *V. cholerae* MCP transposon mutant library.

Many studies note the membrane remodeling *V. cholerae* is capable of and its dependence on environmental conditions including the presence of fatty acids.[7, 13, 14] This remodeling has been linked to signaling pathways that are triggered based on the environment a bacteria experiences.[4, 15] *V. cholerae* is known, among other characteristics, for its substantial amount of MCPs present in its genome, these have been cited as possibly involved in more signaling systems than chemotaxis alone.[13] Intriguingly, a study by Lambert et al. correlated biofilm formation with several MCP genes, which further justified the current study. [29]

1.3 Phospholipids and Fatty Acids

The PUFAs used in this study include linoleic acid (18:2), α-linolenic acid (18:3α), arachidonic acid (20:4), and docosahexaenoic acid (22:6). These FAs are shown in Figures 1.3 through 1.5. The original plan was to use 18:2, 20:4, and 22:6 based on their relevance in *V. cholerae*'s known environments. Additionally, these PUFAs are chemically diverse with regard to carbon length and level of unsaturation. Initially the PUFAs 20:4 and 18:2 were chosen because they are prevalent within the human host, with 18:2 being a major fatty acid in the intestinal mucosa and 20:4 being a common cellular membrane fatty acid and signaling molecule during inflammation. Both fatty acids are also commonly components of bile, which is secreted into the small intestine to aid in digestion. [10, 12, 22] The PUFA 22:6, a component of fish oil and

was selected for its prevalence in aquatic environments, which *V. cholerae* is also known to live in.[15, 23] Furthermore, previous research has shown that 18:2, 20:4, and 22:6 are incorporated into *V. cholerae*'s membrane when it is exposed to these long-chain PUFAs. However, due to experimental error the vial containing 18:3α was selected, in place of the 18:2 vial. Fatty acid 18:3α has been seen in *V. cholerae* species after exposure to selected bile concentrations.[24]

The ability of bacteria, such as *V. cholerae*, to acquire fatty acids is essential to this study because *V. cholerae* must process the necessary machinery to allow the PUFAs to reach the MCPs on the inner membrane, which we posit are part of a signaling pathway that initiates biofilm formation. Since MCPs are known to sense chemicals composed of double bonded carbons, it would also be possible that they could also sense small molecular fatty acids, which are made up of double bonded carbons much like the aforementioned chemical.

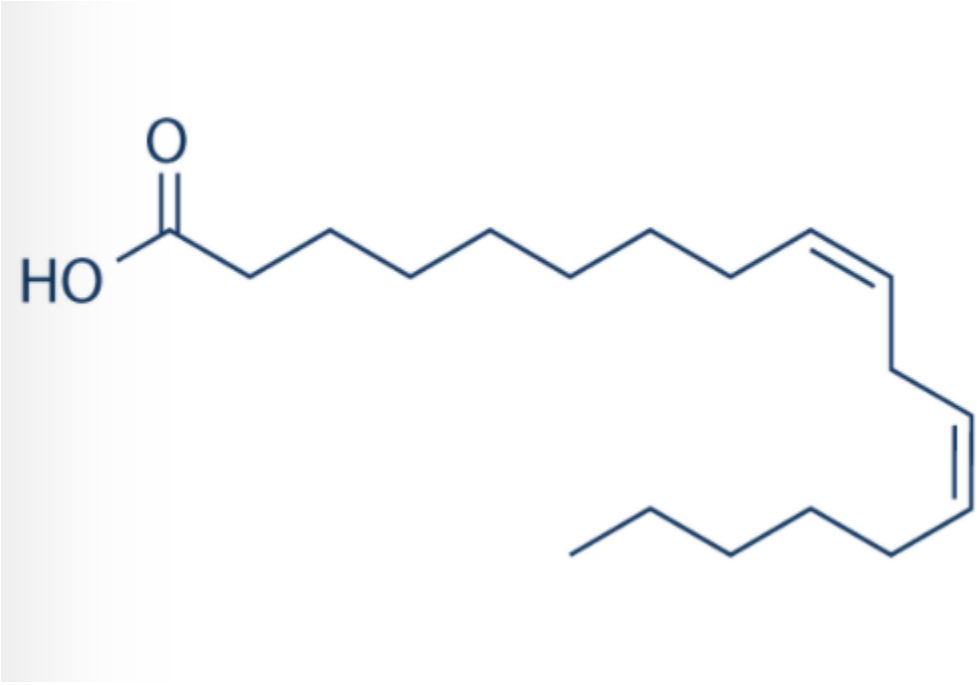

Figure 1.1 linoleic acid (18:2)

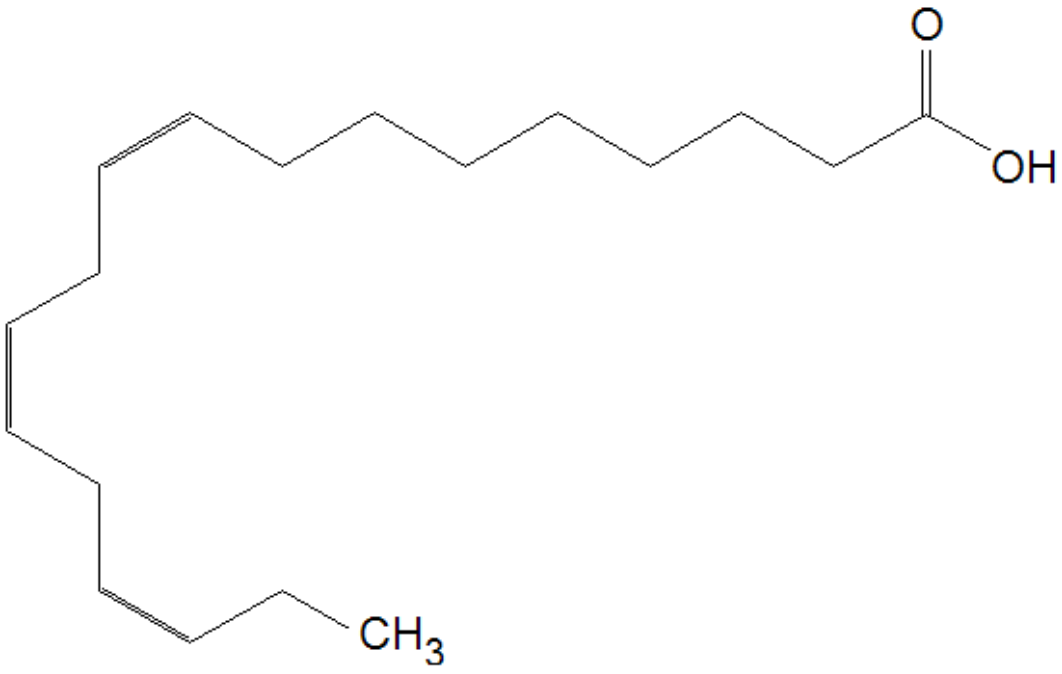

Figure 1.2 a-linolenic acid (18:3a)

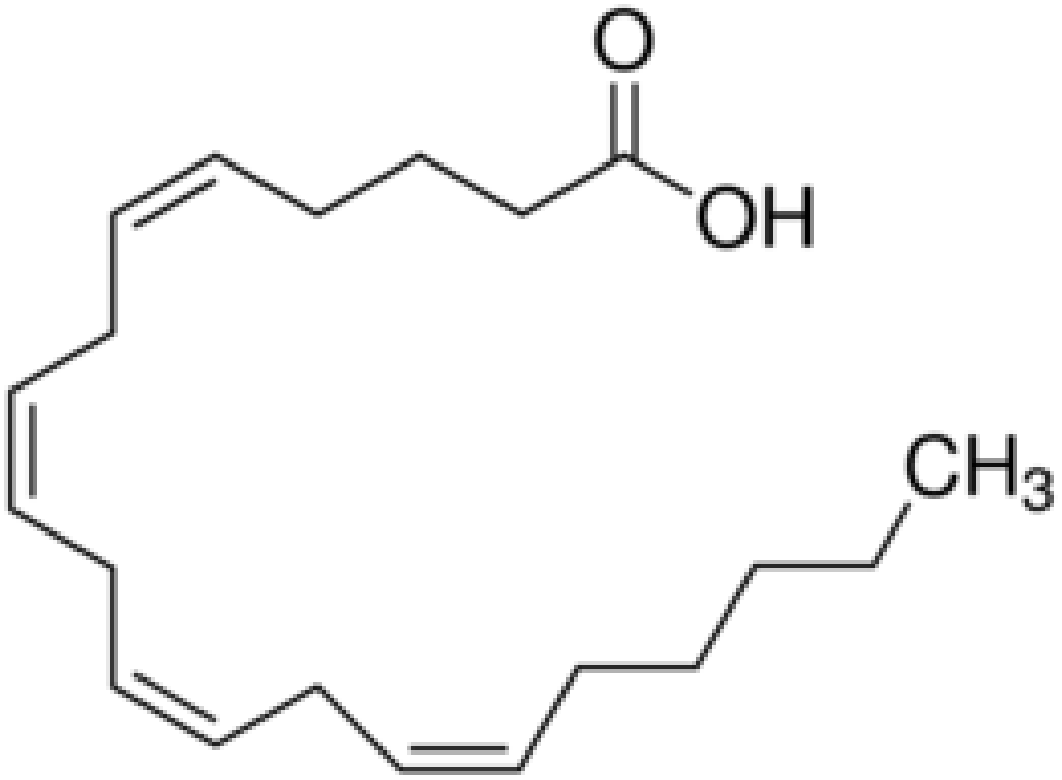

Figure 1.3 arachidonic acid (20:4)

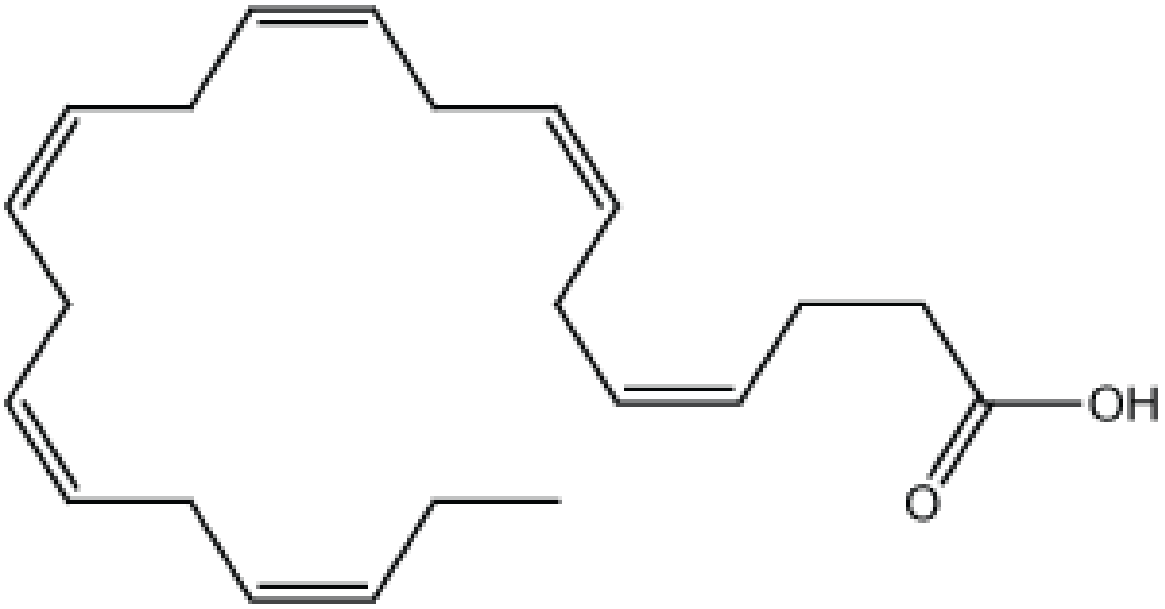

Figure 1.4 docosahexaenoic acid (22:6)

1.4 Previous Research

Previous research has shown various gram-negative bacterial species altering membrane constitution and biofilm formation, as well as other virulence factors, in response to exogenous FAs. The current treatment of cholera includes rehydration therapy, antibiotic treatment, and zinc treatment (cases with minors).[26] These treatment courses are effective for mild cases of cholera. However with antibiotic resistance of *V. cholerae* on the rise there is a need for new treatment options.[7, 27] The diarrheal disease, cholera, is mainly contracted through contaminated water or food that often has cholera bacteria in it from improper handling of fecal matter.[26] Due to the continued prevalence of *V. cholerae*, there have been many studies on prevention and treatment.

Biofilm production is known to be highly reliant on polysaccharides mainly exopolysaccharides (EPS). EPSs are regulated and thus can be viewed as a selective quality of biofilms in *V. cholerae.* Most researchers agree that the regulators of *V. cholerae* are

transcriptional regulators, most commonly, quorum sensing regulators and two-component signal transduction.[28]

By 2016, researchers identified the importance of fatty acids as more than carbon sources that provide energy to bacterial cells. Fatty acids came to be seen as key nutrients influencing virulence cascades.[14] Previous research has significantly addressed the connections of fatty acids and many other phenotypic qualities of *V. cholerae*. PUFAs, not synthesized *de novo* by *V. cholerae*, could represent environment-specific signals for this pathogen which oscillates between marine and host niches.[29] Due to this, researchers have been investigating the impact of fatty acids on biofilm formation and other phenotypes associated with virulence and survival.

Biofilms are a phenotype shared among bacteria. They provide a method for survival and transmission of bacteria in diverse environments, because biofilms are an enclosed matrix that forms a surface community. As one article states, biofilm formation is the preferred mode of growth because it provides enhanced growth & survival due to protection from antimicrobial compounds and access to nutrients.[28] An interesting feature of *V. cholerae* is that it encodes a significantly larger number of methyl-accepting chemotaxis proteins (MCPs) than other related bacteria.[30, 31] *V. Cholerae* is also primarily found in environments, both aquatic and host, that have a high diversity of fatty acids, some have posited that the abundance of MCPs could be attributed to their role in control of biofilm formation. [11, 32] Lambert et al's study provides a compiled list of genes to begin testing in my research due to the inclusion of indicated gene expression (up-regulation and down-regulation).[30]

1.5 *Vibrio cholerae*

Vibrio cholerae is a gram-negative bacterium discovered in 1871 when the first pandemic was recorded in India.[33] *V. cholerae* is known to cause infection in human hosts, which is called

cholera. This disease is characterized by vomiting, diarrhea, dehydration, electrolyte imbalances, and acute renal failure. In severe cases this can lead to death in a relatively short period of time.[26] Fish can also be infected by this disease and act as carriers to the human host. According to the CDC cholera is commonly contracted via shellfish and contaminated water. [13, 28]

V. cholerae has a very high infectious dose and is hyper infectious upon exiting its host. *V. cholerae* is a flagellated bacterium that inhabits the lower intestines. Despite low tolerance to acidity of the stomach, the bacteria possesses a virulence cascade that is initiated upon entry into the small intestine.[7, 13, 14] The ensuing pathogenesis allows *V. cholerae* to colonize the intestinal epithelial cell layer and ultimately express cholera toxin, the key virulence factor that causes cellular water loss and diarrhea, the main symptom of cholera.[14] The biphasic lifestyle is unique to *V. cholerae* and allows this bacterium to live in both marine and host conditions. *V. cholerae* also has the ability to produce a biofilm when under stress. Some literature indicates that various fatty acids could be linked to sensing and signaling proteins in *V. cholerae.*[30] The initiation of attachment for the formation of biofilm is thought to be linked to MCPs sensing fatty acids once inside the outer membrane.[11, 14]

Currently, treatment for *V. cholerae* includes rehydration therapy, antibiotics protocols, and zinc treatments.[33] However since this bacterium is more virulent after growth in a host it is important to note sanitation must be taken into account for disease control. Additionally, antibiotic resistance is on the rise.[7, 28, 36] Thus, this project sought to, collectively, focus on i) the identification of genes likely to participate in fatty acid-mediated biofilm formation using previous literature and bioinformatics and ii) utilizing the *V. cholerae* nonredundant transposon library to test candidate genes for their potential roles in fatty acid control of biofilm formation, in the aim to identify possible genes indicated in the regulation of biofilm formation in *V.*

cholerae.[36] Identification of genes involved in mediated biofilm formation would contribute to our understanding of environmental sensing in *V. cholerae*. Central to the hypothesis of this project is that some of the MCPs may be involved in sensing and responding to exogenous fatty acids. To simulate *V. cholerae*'s biphasic environments, where fatty acids can be found, this study used two designed environmental conditions, marine and host. **Our study aimed to identify possible genes involved in *Vibrio cholerae* biofilm formation influenced by physiological fatty acids ([18:3α], [20:4], & [22:6]) as a way of targeting biofilm regulation in *V. cholerae*.**

Chapter 2: Materials and Methods

2.1 Growth Procedure and Inocula Preparation

Vibrio cholerae O1 El Tor C6706 was the wild-type strain used in this study. The transposon mutants were acquired by duplicating the transposon library previously published.[34] The bacterial cultures used in this study were routinely maintained in Luria broth and agar media. Overnight cultures were incubated shaking for approximately 16 hours in Luria broth. For many experiments, cultures were pelleted, washed, and resuspended in M9 minimal media supplemented with 0.4% casamino acids (0.2% glucose, 2mM MgSO4, 0.1mM CaCl2). Bacterial inocula was prepared at an OD_{600} of 0.1 for biofilm experiments. The *V. cholerae* cultures used in growth curve analysis were grown in CM9 minimal medium incubated at 37°C for 24 hours with inocula starting at OD_{600} of 0.05, with readings taken every hour.[32]

2.2 Media preparation

The initial screening tests were conducted using CM9 media, however after evaluation, based on observed pH changes the media was converted to CM9 minimal media buffered with HEPES (0.1M) at 7.1pH (host) or 8.1pH (marine) and 150mM NaCl concentration (host) or 600mM NaCl concentration (marine). HEPES was prepared using a HEPES stock concentration of 0.25M. The desired pH was obtained through addition of sodium hydroxide pellets while monitoring pH. After this procedural change, all mutants were tested using the HEPES media and CM9 was discontinued.

Each fatty acid was prepared using 1ml of media such that the final concentration would be 300uM in each well containing 200ul. In a biosafety hood the inocula and FAs were pipetted into a 96 well microtiter plate; the fatty acid solutions were added to their respective wells in 30

microliters per well and the incocula were added in 170 microliters per each well using a multipipettor.[37]

2.3 Growth Curve Analysis

A traditional growth curve was conducted on initial *V. cholerae* transposon mutants to establish growth characteristics and a baseline for comparison in bioassay growth analysis. At one-hour intervals over the course of 10 hours, each culture was assessed for growth spectrophotometrically at an absorbance of 600nm. The cultures of *V. cholerae* were grown in CM9 minimal media without fatty acids at 37℃ for 12 hours. Each culture was inoculated at a starting OD_{600} of 0.05 and growth was assessed by measuring the absorbance (OD_{600}) of the cultures once every hour for 10 hours.

2.4 Biofilm Formation Assay Procedure and Incubation

To assess the effects of FAs 18:2, 18:3α, 20:4, and 22:6 on biofilm formation in *V. cholerae*, transposon mutant cultures were exposed to two main environmental designs, marine and physiological, to gauge response in biofilm formation assays. Both conditions held incubation time at 24 hours static incubation, as the indicated standard via various studies, and utilized CM9 and HEPES minimal media as the base nutrient source.[4, 38, 39] Additionally, both conditions had a control consisting of wild type *V. cholerae* (C6706) and no fatty acid. Each assay also included the 7 other *V. cholerae* mutants being tested and three FAs in triplicate form. These assays were then examined using crystal violet staining methods to measure biofilm growth.[36] The physiological design consisted of the above experimental procedure and main conditions with the distinction of supplementing 150mM NaCl and incubating at 37℃, while the marine design had 600mM NaCl and 30℃ incubation temperature. All incubations were

performed under static conditions in an incubator with a humid environment (water trays beneath microtiter plates).

2.5 Spectrophotometric Quantification

Spectrophotometry uses absorbances to measure the amount of a substance by measuring the number of photons absorbed after the selected light wavelength passes through the substance. In this project UV-Vis at 590nm is used to quantify the amount of biofilm formed. The absorbance of crystal violet indicates the amount of biofilm formed per microtiter well, which is measured by staining the polysaccharides that make up the biofilm, and then resuspending them in acetic acid.[41] A Biotek Synergy 2 instrument was used for all the readings and produced an excel document containing the absorbance values per well.

The microtiter plate is then processed and read after the 24-hour incubation. By using the established technique for staining biofilms, the microtiter plate is first dumped and then gently washed by submersion in deionized water 4-5 times. Then 0.01% crystal violet (CV) solution is multipipetted into each well in 225 microliter amounts. The plate then sits for 15 minutes with the CV. After the 15 minutes the plate is submerged in 4-5 more washes of deionized water until the water is clear. The plate was then placed upside down to air dry for a minimum of 30 minutes. At the conclusion of this drying time each well received 225 microliters of acetic acid, which then sat in the wells for 15 minutes. After the 15 minutes, the contents of each well were transferred to a new labeled plate, to avoid coloration interference. The plates were then scanned in a mass spectrometer to assess the amount of biofilm formation in the solution.

The data gathered from the biofilm processing was compiled into excel documents and then analyzed. Two biological replicates were obtained for each assay, and each experiment was performed in triplicate. The data was transferred to Excel for generation of graphs and

calculation of averages and standard deviations. All results in this study were interpreted based on biofilm response of the wild-type control; therefore, evaluation is described by relative differences as opposed to calculations of statistical significance. Measures of significance are based on the degree of change observed in the mutant when compared to the experiment control (C6706 and no PUFA exposure), indicating notable findings -- not statistical significance due to the extensive amount of variables.

Chapter 3: Results

3.1 Introduction

This project sought to identify deviations from wild-type trends in biofilm formation among 30 *V. cholerae* transposon mutants when exposed to PUFAs under marine and host conditions. The initial steps of this project included screenings of 14 indicated mutants and development of two separate assay conditions to analyze *V. cholerae*'s biphasic lifestyle in biofilm formation. After identifying particular trends further testing was conducted; expanding the screening to over 30 MCPs available in the aforementioned transposon library. Unfortunately, due to restrictions from the COVID-19 pandemic screening was unable to be completed.

3.2 Growth Curve Results

Figure 3.1 shows the growth curve performed on transposon mutants selected from the initial grouping of test mutants. This growth curve was conducted in CM9 minimal media using cultures that had been grown overnight at 37°C in CM9 media with a starting OD_{600} of 0.05. Absorbance measures were taken every hour for ten hours. These results show that two transposon mutants (VCA0658 and VCA0785) had significantly higher growth when compared to the wildtype mutant (C6706) and the other mutants. This is thought to indicate that VCA0658 and VCA0785 are more planktonic growers, thus VCA0658 and VCA0785 are both characterized as low biofilm producers. This confirms the results of the assays despite the presence of a true blank.

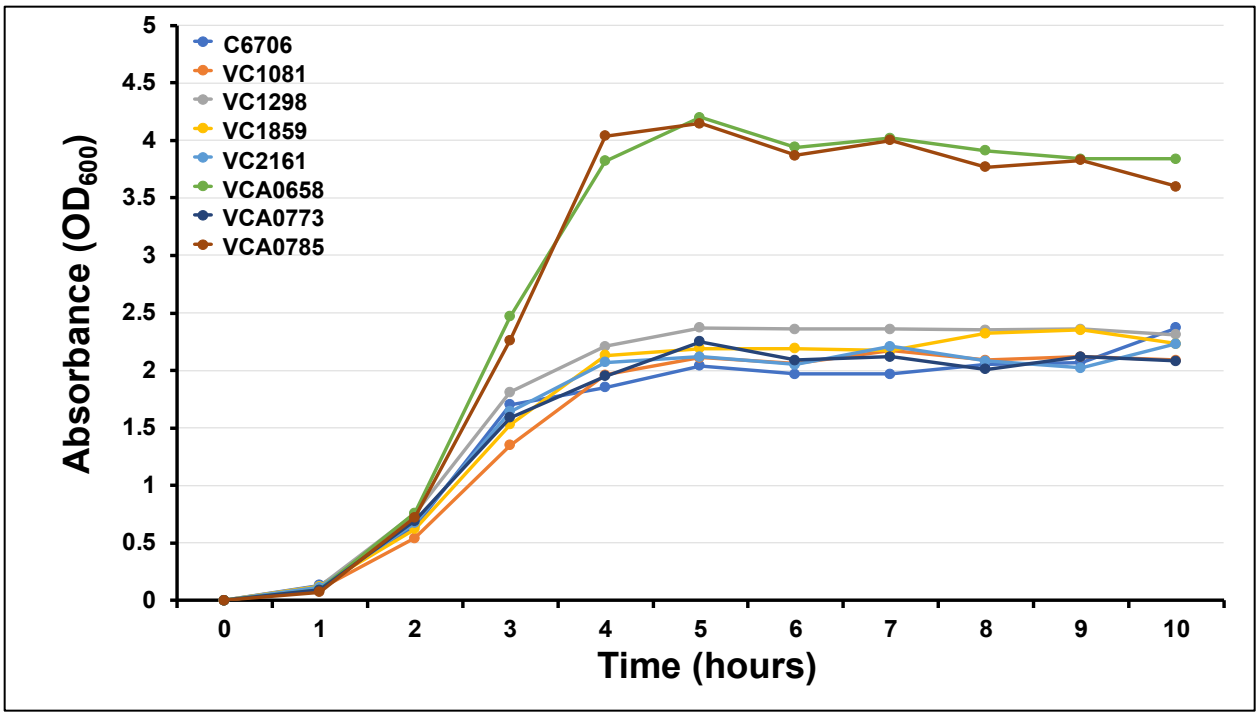

Figure 3.1 Growth curve results including wildtype and 7 transposon mutants of V. cholerae

3.3 Initial Screening Bioassay Samples

Biofilm formation in *V. cholerae* in the presence or absence of one of the three PUFAs was tested, all in 300µM amounts, using the established. These biofilms were stained and measured according to the above procedure. Figure 3.2 shows biofilm formation of Group B transposon mutants in initial testing conditions. This bioassay shows six transposon *V. cholerae* mutants that display a trend with no notable deviations from the control sample, thus eliminating them from further study.

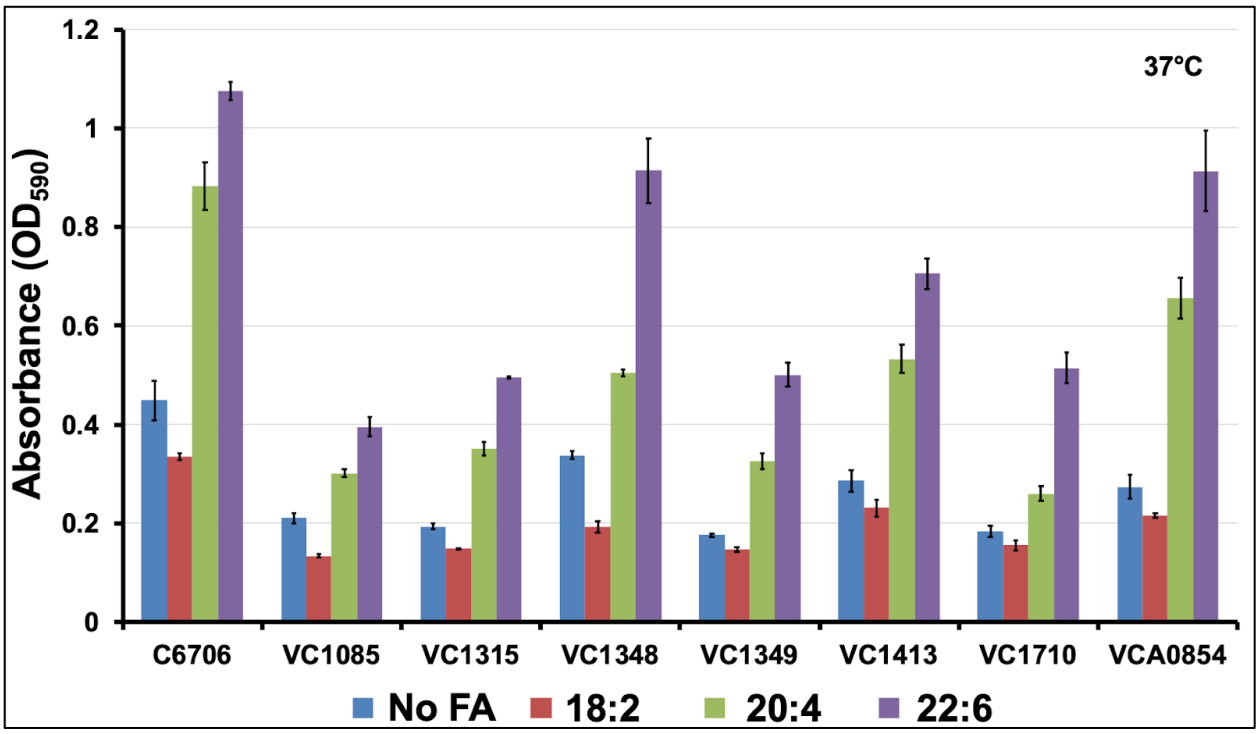

Figure 3.2 Biofilm formation of V. cholerae in the presence of alpha-linoleic acid, arachidonic acid, and docosahexaenoic acid with CM9 media incubated at 37°C for 24 hours. No notable deviation from control (C6706).

3.4 Marine Condition Bioassay Samples

As previously mentioned, biofilm formation in *V. cholerae* in the presence or absence of one of the three PUFAs was tested, all in 300µM amounts, using the established procedure explained in Chapter 2 of this work. These biofilms were stained and measured according to the above procedure. Figure 3.3 shows biofilm formation results for Group D mutants, see table 1.1 in supplemental material. Interesting findings include the upregulation in the presence of all three PUFAs (particularly 20:4 and 22:6) that is observed in VC1348. VC1710 and VCA0785 mutants show no biofilm production in the presence of the three PUFAs, this could indicate that these mutants are vital in biofilm formation when exposed to marine conditions.

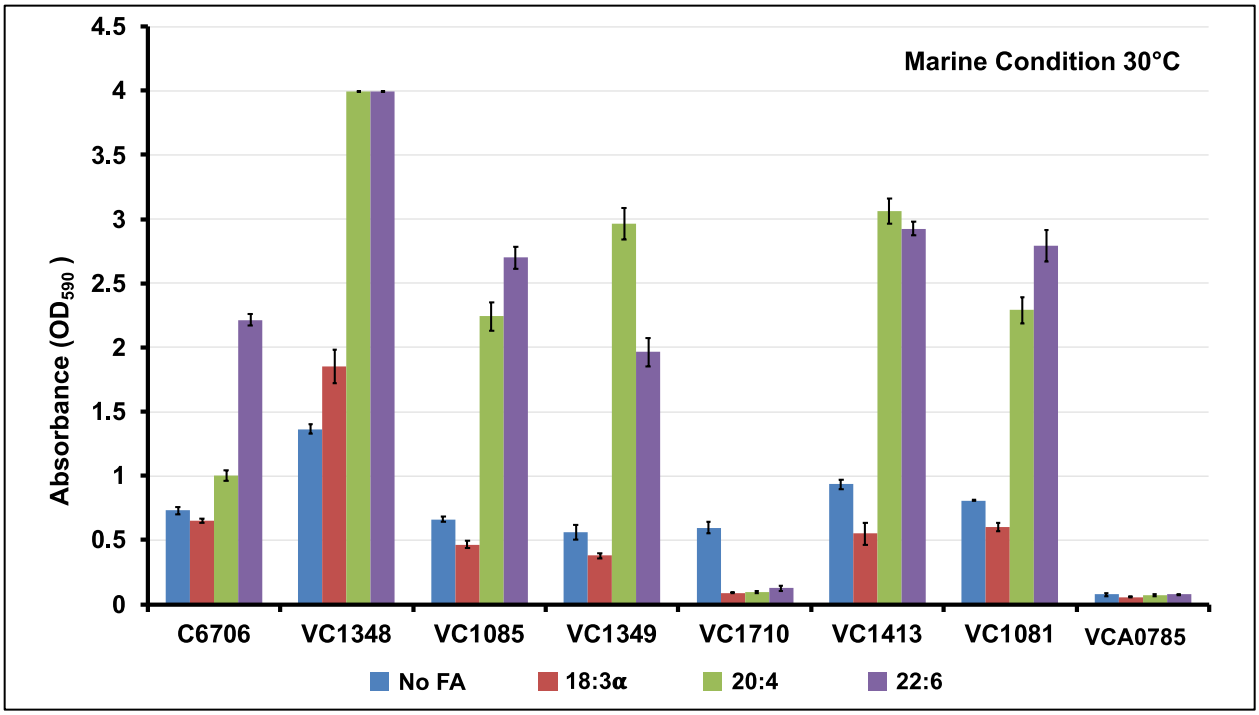

Figure 3.3 Biofilm formation of V. cholerae transposon mutants Group D in the presence of alpha-linolenic acid, arachidonic acid, and docosahexaenoic acid with HEPES pH 8.1 NaCl 600mM incubated at 30°C.

Figure 3.4 displays identical testing conditions as used in the bioassay for Figure 3.3 with the exception of the transposon mutant group, which is now Group E according to Table 1.1 in the supplemental material section. These biofilm formation results show an increase in biofilm formation in the presence of PUFAs 20:4 and 22:6 compared to the control sample, excluding VC1898 where biofilm production in presence of 20:4 is not greater than control. In VC1405 and VC1403 biofilm formation is decreased compared to control in the presence of PUFA 18:3α.

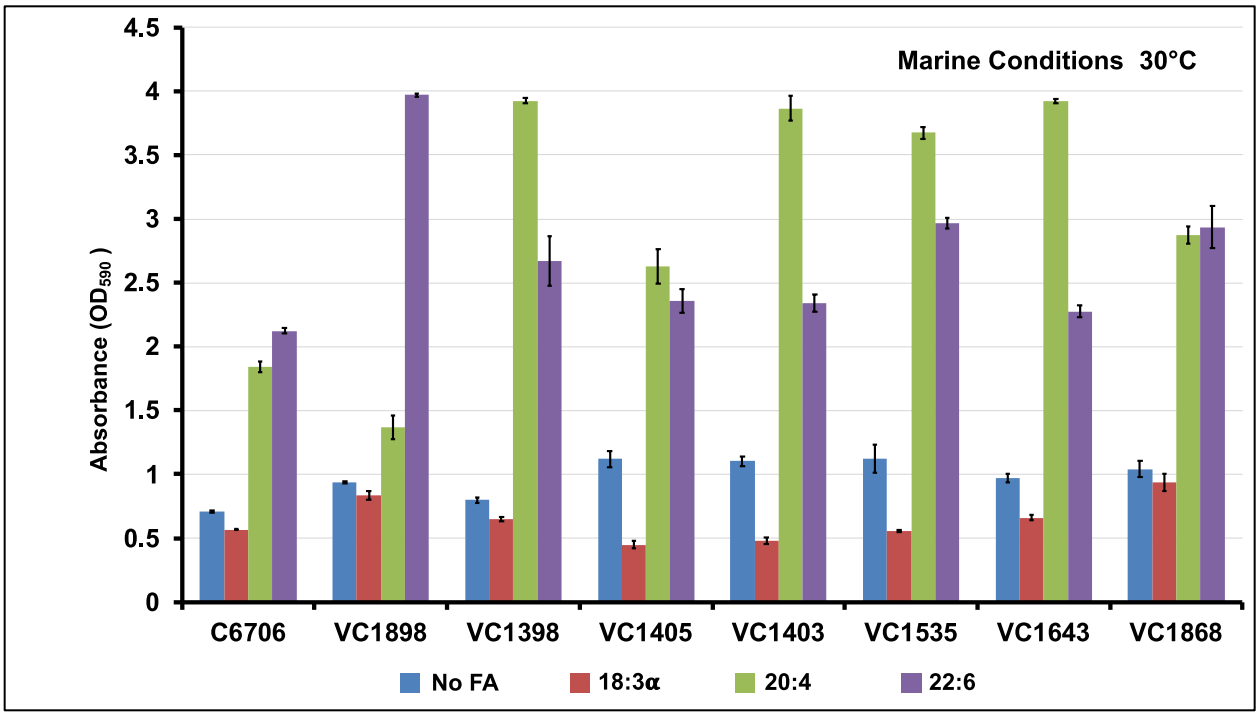

Figure 3.4 Biofilm formation of V. cholerae transposon mutants Group E in the presence of alpha-linolenic acid, arachidonic acid, and docosahexaenoic acid with HEPES pH 8.1 NaCl 600mM incubated at 30℃.

Figure 3.5 also uses identical testing conditions as both assays in Figure 3.3 and Figure 3.4, but with *V. cholerae* transposon mutants Group F according to Table 1.1. Figure 3.5 results display notable increases in biofilm production in the presence of 20:4 and 22:6 in VC2439, VC1967, VCA0068, VCA0176, VCA0663, VCA0008. In all mutants, excluding VC2439 and VC1967, biofilm formation was decreased in the presence of 18:3α, this suggests these mutants are important for biofilm formation under marine conditions.

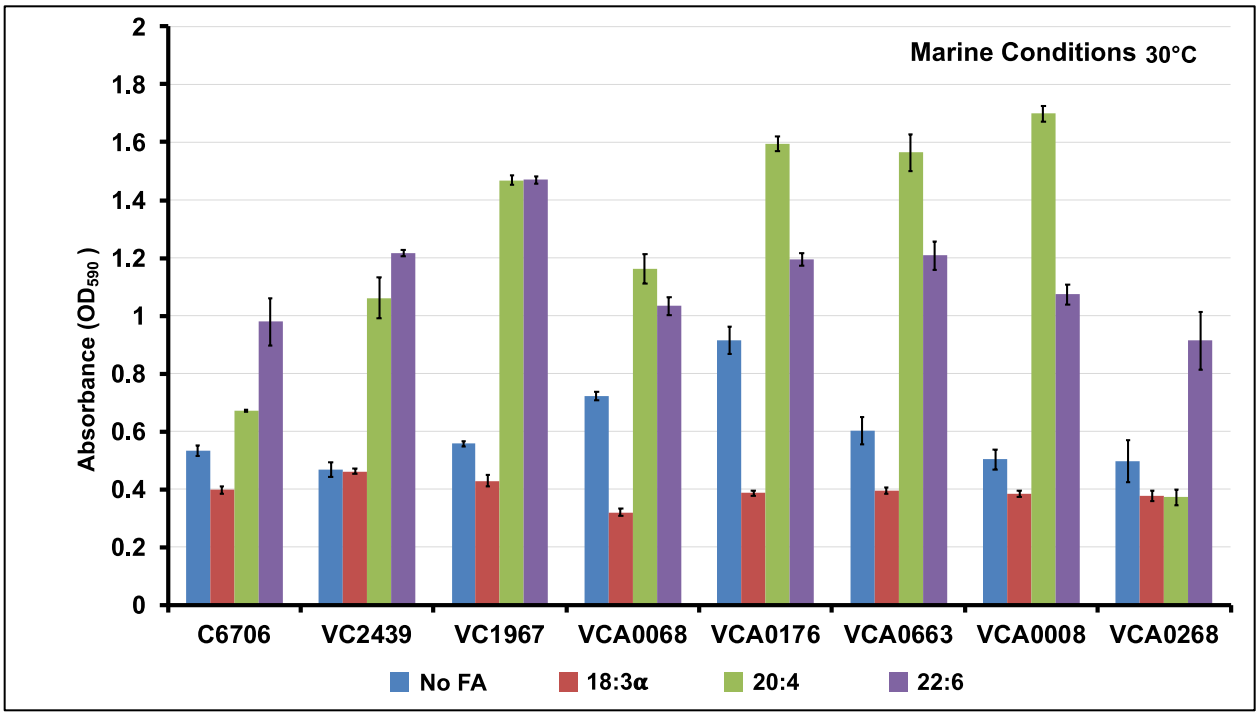

Figure 3.5 Biofilm formation of V. cholerae transposon mutants Group F in the presence of alpha-linolenic acid, arachidonic acid, and docosahexaenoic acid with HEPES pH 8.1 NaCl 600mM incubated at 30℃.

3.5 Host Condition Bioassay Samples

Biofilm production in *V. cholerae* transposon mutants in the presence of the three selected PUFAs was also tested under host conditions to accommodate *V. cholerae*'s biphasic lifestyle and provide comparative data. Figure 3.6 displays biofilm production in Group D mutants, similar to Figure 3.3. This data displays an interesting increase in biofilm formation in the presence of PUFA 22:6 for mutant VC1348 and a decrease in all the other mutants when compared to the control sample. This suggests these mutants could be regulators of biofilm production under host conditions when exposed to FA 22:6. Interestingly, VCA0785 shows a decrease in biofilm production in all exposures when compared to the control sample, this indicates VCA0785 is an important gene in biofilm formation under host conditions.

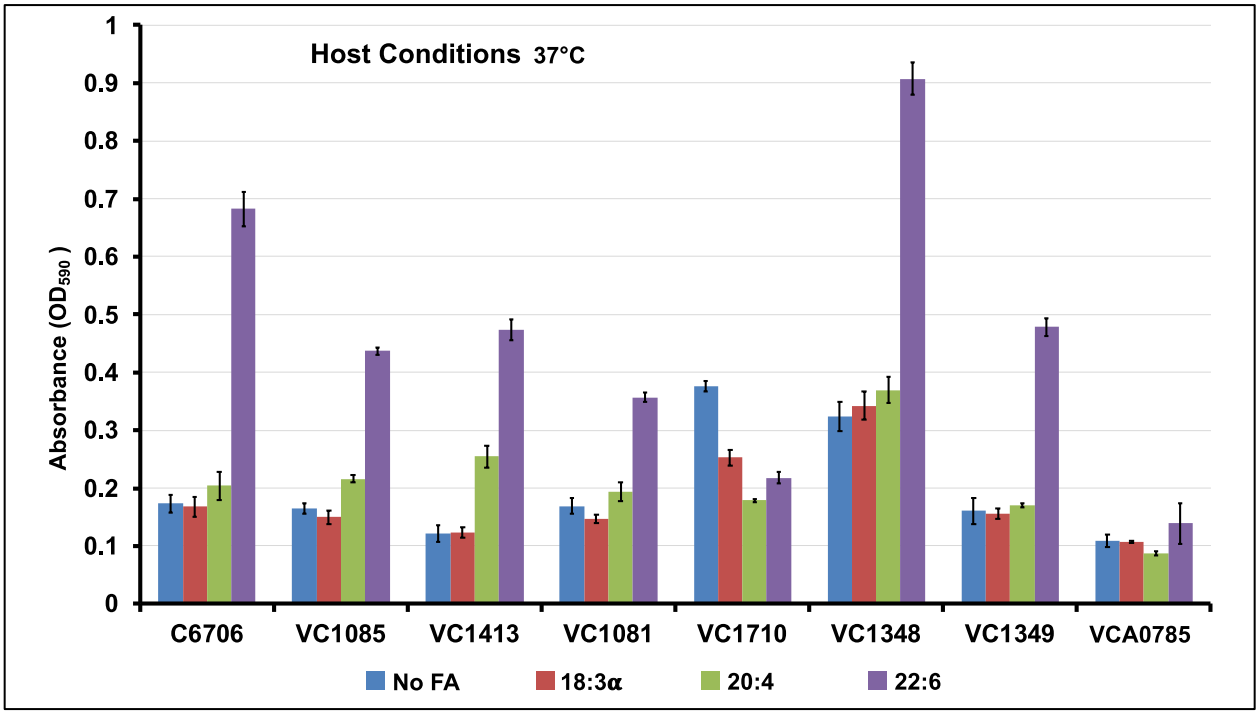

Figure 3.6 Biofilm formation of V. cholerae Group D transposon mutants, in the presence of alpha-linolenic acid, arachidonic acid, and docosahexaenoic acid with HEPES pH7.1 NaCl 150mM incubated at 37℃.

Figure 3.7 displays Group F mutants, similar to Figure 3.5, except Figure 3.7 shows the results from host conditions. This data shows all of the mutants, excluding VCA0176 and VCA0268, behave as the control sample in biofilm formation. VCA0176 shows similar amounts of biofilm production in the presence of 18:2 and 20:4 as observed in no fatty acid. VCA0268 shows abnormal trends when considering no fatty acid and 18:2 in comparison to the control sample. All mutants in Figure 3.7 show decreased biofilm production in all PUFAs conditions when compared to the control sample (C6706).

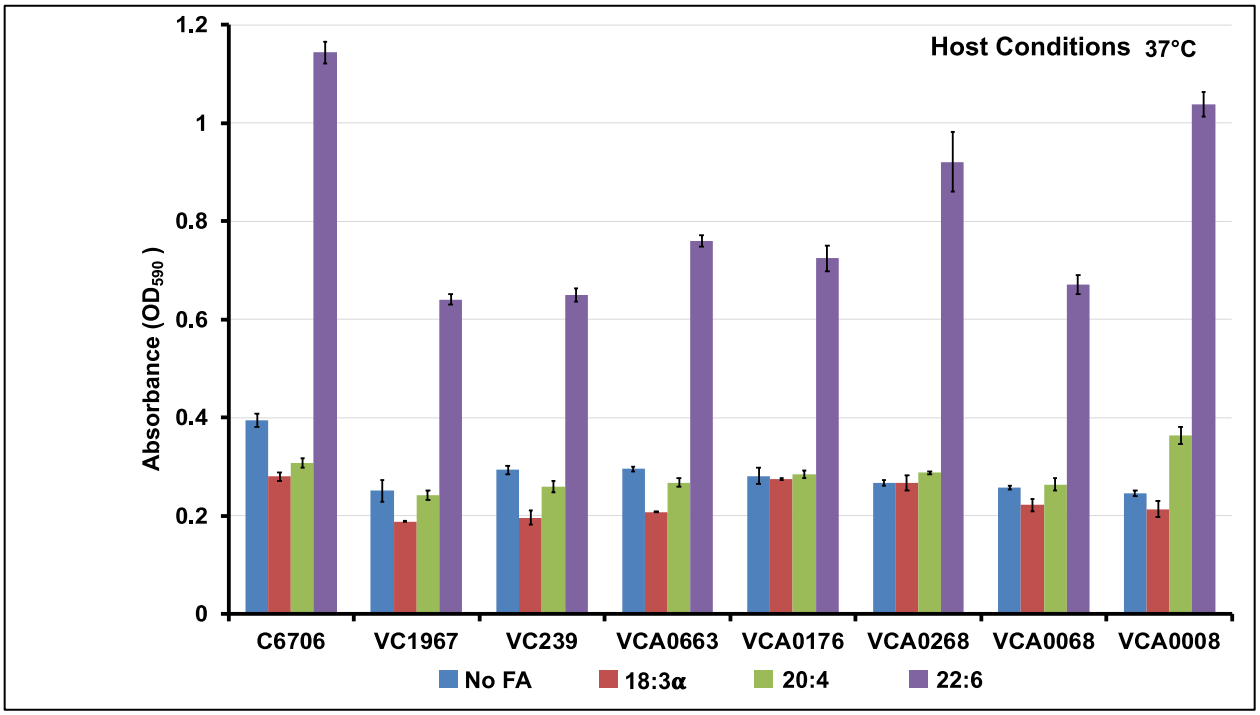

Figure 3.7 Biofilm formation of V. cholerae Group F transposon mutants, in the presence of alpha-linolenic acid, arachidonic acid, and docosahexaenoic acid with HEPES pH7.1 NaCl 150mM incubated at 37°C.

Chapter 4: Discussion

4.1 Discussion

V. cholerae is a gram-negative pathogen in humans that exhibits a biphasic lifestyle. This pathogen is responsible for cholerae infections worldwide every year. Antibiotics are the main course of treatment for, what in some cases is a life threatening disease.[13, 14, 25, 26] One of *V. cholerae*'s main virulence factors that allows it to be infectious is its ability to form a biofilm and resist antibiotic treatment.[27] Recently, researchers have seen an increase in antibiotic resistance in *V. cholerae,* thus novel treatments are being sought out. Once FAs reach the inner membrane MCPs have been known to transport them into the cell, which is thought to initiate signaling cascades that alter the bacterium's virulence genes.[42] Previous research on *V. cholerae* explored MCPs involvement in other physiological traits such as motility, membrane altering, and different genes involved in biofilm signaling pathways.[13, 14, 30] Additional research has shown gram-negative bacteria have the ability to sense PUFAs in their environment and alter their own membrane accordingly.[14] The purpose of this study was to examine the involvement of PUFAs in mediating biofilm formation via MCPs in various *V. cholerae* transposon mutants under conditions the bacteria would normally experience, such as marine and host.

The initial stages of this project focused on the screening of 14 *V. cholerae* transposon mutants indicated in biofilm formation signaling pathways, allowing confirmation of transposon mutants that displayed trends that diverged from the control sample during testing.[31, 36] This was done by assessing a representative sample of mutant growth characteristics and biofilm formation in the presence of CM9 media, to compare with the bioassays, using 3 physiologically relevant fatty acids (linoleic acid [18:2], arachidonic acid [20:4], and docosahexaenoic acid [22:6]). Biofilm formation of the wild-type strain (C6706) of *V. cholerae* in CM9 was

interestingly decreased in the presence of 18:2 and increased in the presence of 20:4 and 22:6. While growth patterns among the mutants remained similar, there were notable differences in capacity for biofilm formation using a microtiter plate-based crystal violet assay. Six of the tested mutants exhibited the wild-type pattern of biofilm formation as shown in Figure 3.2, suggesting that their respective gene products are not involved in the phenotype. There were no largely notable changes observed in the presence of 18:3α, suggesting further study would be needed to discover differences in biofilm production that are significant.

Ultimately, we found multiple mutant genes that indicate MCPs could participate in biofilm formation since these sensing molecules altered the *V. cholerae*'s ability to form biofilms under experimental conditions. Interestingly mutant VCA0785 lacks a functional methyl-accepting chemotaxis protein, but responds to all three fatty acids. VCA0785 displayed a decrease in biofilm production in the presence of all three PUFAs (18:3α, 20:4, 22:6); suggesting that *V. cholerae* might rely on this gene under both marine and host conditions. This result is unique because this gene is characterized by its GGDEF family protein, which is a signaling protein. This could mean that MCPs are involved supporting the hypothesis of MCP involvement in mediating biofilm formation. Future studies should explore the involvement of this gene in biofilm production for *V. cholerae* to further discover the mechanisms.

In comparison, VC1348 displayed an increase in biofilm production in the presence of all three tested PUFAs (18:3α, 20:4, 22:6): indicating that *V. cholerae* relies on this gene in both conditions for regulation of biofilm production. VC1348 has been identified as a response regulator through bioinformatics and thus should be considered more closely. Additionally, VC1710 displayed unharmonious biofilm production between the two conditions; showing an increase in biofilm production in the host conditions in the presence of PUFA 18:3α, and a

decrease in biofilm production under marine conditions in the presence of all tested PUFAs. This gene was chosen because it contains an EAL domain and is thus involved in diverse signaling. The above results suggest there could be complex signaling pathways at play, however further study is needed.

While initial testing in CM9 media provided proof of alterations in selected mutants under testing conditions, further testing was conducted to differentiate effects of temperature, pH, and salt concentration. Temperature changes were first examined remaining in CM9 minimal media, continuing use of 37℃ and adding 30℃ testing conditions, while performing the previously established experiment protocol.

Although initial testing did not show significant changes in media pH from the start of a bioassay compared to the end in CM9, a change in media to HEPES buffered CM9 was made to better control pH. HEPES is a pH buffered media and is better able to hold specific salt concentrations. As suspected, the results demonstrate different responses for biofilm formation between the two environmental conditions. This study found marine conditions elicit significantly higher biofilm production in the absence of fatty acids; while 22:6 yielded higher biofilm amounts in human conditions, 20:4 generated the most biofilm under marine conditions. This unique interaction could be due to various reasons since there are a large number of variables. Interestingly, some mutants exhibited divergent biofilm behavior; specifically, the VCA0658 (known for aerotaxis) mutant was only able to form biofilm in human conditions when 22:6 was supplemented. Collectively, we have identified intra- and inter-conditional dependencies among *V. cholerae* wild-type and MCP mutants that furthered our understanding of fatty acid-mediated biofilm formation for future work.

Possible sources of interference should be noted, as they cause significant deviation between experiments and thus limit the conclusiveness of the provided data. One interference source is the variance outside temperature, as every experiment was transported for a short period of time outdoors throughout various seasons and temperatures within the first minutes of exposure in the microtiter plate. While sterility was maintained, the effects of this short-term exposure on the bioassay are uncontrolled for and thus should be considered. This decision was due to testing facility necessity, and onsite construction. Furthermore, biofilm controls were included by staining control wells for biofilm production and quantified; they showed negligible absorbances (0.03).

4.2 Future Research

Future work to be carried out on this project includes completing the screening of MCPs using the available resources to test the MCP transposon mutants that were not tested due to the COVID-19 pandemic. Additionally, due to clerical error this study used alpha-linolenic acid thus, future studies should consider the use of fatty acid (linoleic acid [18:2]) since it is more prevalent in the human body, where *V. cholerae* can live. This study did not quantify the amount of fatty acid, of the 300µM concentration free fatty acid, consumed by the mutants during incubation. Future studies should consider the amount of fatty acid utilized by *V. cholerae* transposon mutants during a bioassay, which could be accomplished by conducting a titration to gauge sensitivity and assess physiologically relevant concentrations. A bioassay blank should also be included in future studies to provide a more concrete baseline measurement when determining absorbance for biofilm quantification. It would also be beneficial for future studies to conduct genetic complementation to confidently determine the transposon mutant effects. Finally, future work should examine the genomic characteristics hindered by the transposon

mutants to evaluate what signaling pathways might be involved in biofilm formation for *V. cholerae.*

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Supplemental Materials

Table 1.1 *V. cholerae* Transposon Mutant Grouping Key Independent of Media Type

Group A	Group B	Group C
VC1298 VC1413 VC1859 VC2161 VCA0658 VCA0773 VCA0854	VC1081 VC1085 VC1315 VC1348 VC1349 VC1710 VCA0785	VC1081 VC1298 VC1859 VC2161 VCA0658 VCA0773 VCA0785
Group D	Group E	Group F
VC1081 VC1085 VC1348 VC1349 VC1413 VC1710 VCA0785	VC1394 VC1403 VC1405 VC1535 VC1643 VC1868 VC1898	VC1967 VC2439 VCA0008 VCA0068 VCA0176 VCA0268 VCA0663
Group G		
VC1081 VC1085 VC1348 VC1349 VC1413 VC1710 VCA0785		