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Influence of environmental conditions on fatty acid-induced changes in Vibrio cholerae persistence and pathogenicity

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ABSTRACT

Vibrio cholerae, a Gram-negative bacterium, is responsible for the acute intestinal infection known as cholera. This illness is due in part to *V. cholerae*'s ability to sense and adapt to changing environments as it is ingested into the human body from brackish environments. It was shown in recent studies that this bacteria has the ability to uptake exogenous fatty acids, resulting in changes to *V. cholerae* persistence and pathogenicity [5]. The aim of this research is to determine the extent to which these additional exogenous UFAs influence the persistence and pathogenicity of *V. cholerae* throughout its transitional period from brackish environments to human host conditions. Our hypothesis is that environmental conditions (e.g., temperature, pH, salinity) direct fatty acid-induced changes in *V. cholerae*'s capacity to spread disease by triggering changes in the bacterium's virulence factors such as: biofilm formation, antibiotic resistance, and cholera toxin production. Initial work focused on identifying the environmental conditions that trigger fatty acid-induced changes in *V. cholerae* persistence and pathogenicity using a design of experiments approach. The final work of this project focused on literary analysis of effect of the exogenous fatty acids on *V. cholerae*'s genetic regulatory pathways.

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Chapter 1. Scope of Work

1.1 Initial Focus

As originally proposed, this work was to experimentally determine key environmental variables that influence *Vibrio cholerae* persistence and pathogenicity. Previous studies have shown that *V. cholerae* has the ability to uptake exogenous unsaturated fatty acids (UFAs) into its cellular membranes. It is theorized that these fatty acids then act to repress or enhance gene expression within the bacteria [1]. By using two UFAs found in the human body and two associated with marine environments, environmental influence can be measured quantitatively by observing both persistent and pathogenetic traits. Persistent traits are measured through antibiotic resistance while pathogenicity is measured through toxin production and biofilm formation. Our hypothesis is that environmental conditions (e.g., temperature, pH, salinity) direct fatty acid-induced changes in *V. cholerae*'s ability to survive and spread disease. This was to be done using a design of experiments approach, seen below in figure 1.

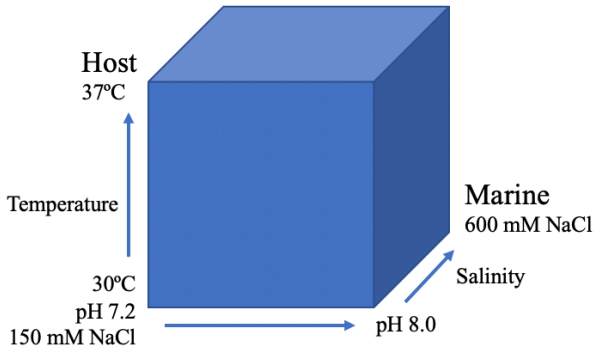

Figure 1. Experimental Approach for Variable Isolation

Initially measuring antibiotic resistance and biofilm formation in the bacterium's two environments, human host and brackish waters, allows for further isolation of each

environmental variable. By using these two environmental conditions as base cases for comparison, one variable such as pH can be varied while holding all others static, allowing for determination of key environmental triggers. These extremes would be similarly used for comparison to a transition from marine to human host, mimicking the ingestion of the bacterium.

1.2 Deviations in Experimental Data and Protocols

In order to collect this data, protocols were researched and established, many of which were customized from procedures used in different labs. This was necessary to adapt each assay to our bacterium, media, fatty acid utilization, and environmental conditions. However, upon changing these methods, variations in results were apparent. Due to the variation in protocols from previous studies and the addition of fatty acids, there was no mean for data comparison with published studies. After isolating and resolving several suspected causes of the inconsistencies with no change in results, the work began to include and eventually solely focus on perfecting protocols and further exploration of *V. cholerae*'s regulatory networks through literature research.

1.3 Final Focus

Upon awarding of a CEACSE (Center of Excellence in Applied Computational Science and Engineering) award, data collection was to be carried out in a microbiology lab, as specified within the grant, for access to more materials. Due to this, a hold was placed on current variable laboratory experiments until this change of location took place. Similarly, an increased focus on grant writing required literature research on experimental niches. The CAREER Award, granted by the National Science Foundation, is given to junior faculty integrating education and research. In order to apply for this, it was essential to find an unexplored niche regarding *V. cholerae*'s regulatory cascade. While studies have been published regarding individual genes within *V.*

cholerae, there is little known about the overall hierarchy of the operons controlling persistence and pathogenicity. The final focus of this thesis is the development of protocols and literature research regarding the understanding of *V. cholerae*'s regulatory pathways.

Chapter 2. Introduction

2.1 Infectious Diseases and their Transmission

Infectious diseases kill approximately 17 million a year worldwide, translating to as many as 47 thousand individuals a day or 19 hundred an hour. Emerging infections are defined as infections that have either not yet occurred in humans, have only infected a small amount of people, or have been only recently recognized. Examples of these include HIV/Aids, Lyme disease, and Ebola. Reemerging infections are diseases that were at once a significant health issue for a large population, disappeared, and have since begun infecting populations again. This type of infection includes tuberculosis, rabies, and malaria [2, 3]. According to the World Health Organization 2007 report, these types of infections are occurring at an increasingly large rate. Since 1970, approximately 40 new diseases have been identified and, in the last 5 years, there were 1,100 identified outbreaks across the world [4].

Virulence, the severity of a disease, can be further enhanced by the mode of transmission. The World Health Organization estimates that food- and waterborne pathogens kill approximately 2 million people a year. Waterborne illnesses can occur via ingestion of water contaminated with microorganisms, often introduced by feces. Outbreaks predominately occur in third world countries due to lower sanitation levels; public health measures detect and filter bacterial pathogens found in water supplies and the lack thereof allows them to progress unregulated. To date, roughly 1.1 billion people across the world live without safe water and 2.6 billion live without proper sanitation [4]. Additional risk factors for waterborne bacterial pathogens include droughts, climate change and high temperatures, and increased populations, all of which are found frequently in developing countries [3]. Droughts increase the bacterial concentration in the depleted water levels, while climate change can increase precipitation events

causing the flooding of water and sewage treatment facilities, and larger populations allow for increased transmission.

Higher oceanic temperatures further provide preferable growth conditions for bacteria [5]. According to the EPA, sea temperature is increasing at a rate of 0.13ºF per decade [6]. Not only do warmer temperatures promote bacterial growth but they also promote algae growth and copepod survival. Studies show these not only support the bacterium but also provide a degree of resilience to pH and temperature variability [7]. With increased algae and copepod populations, bacteria populations grow and become more apt to survive varying environmental conditions.

V. cholerae, the waterborne pathogen responsible for the acute intestinal illness known as cholera, is heavily influenced by these environmental triggers. Likewise, *V. cholerae* associates with extant organisms such as algae, copepod, and fish populations as reservoirs for survival during dormancy, using carbon and nitrogen from the chitin present [8, 9]. As populations grow and migrate to cities, human interaction increases, along with the likelihood of spreading infectious diseases. Globalization, the interaction of governments and people around the world, also poses an increased likelihood of disease development. As people travel and countries trade goods, the possibility of infected individuals or contaminated goods transmitting disease becomes more distinct. In 1991, Latin America had its first cholera epidemic in a century. What began with a single case in January, thought to have been carried via contaminated seafood from Peru, spread rapidly and by mid-April a hospital in Lima was receiving approximately 162 cholera cases a day. By the end of the year, there were 400,000 reported cases and 4,000 deaths in Latin America. By 1995, there were 1 million cases reported and 10,000 dead in these regions [4, 10]. This efficient model of infectious disease dissemination can be seen in many other bacterial pathogens.

2.2 *Vibrio cholerae*

Vibrio cholerae is a comma shaped, Gram-negative bacterium with pili and a single flagellum [11]. Figure 2 below shows a transmission electron microscopy (TEM) image of *V. cholerae* at a magnification of 6,000 at 35mm in size.

Figure 2. TEM image of V. cholerae [12]

Current studies report the bacterium results in as many as 143,000 deaths a year, with over 4 million cases reported a year [13]. Like most waterborne pathogens, it is found chiefly in developing nations where bathing and drinking water is unpurified. This water has often been contaminated with the feces of an infected individual, where the bacterium can grow and reproduce.

Originally living either attached to copepod, algae, and fish populations or as free-living cells, *V. cholerae* remains dormant and non-virulent. Upon ingestion, the bacterium surviving the acidity of the stomach must adhere to and colonize the epithelial cells of the duodenum, the first portion of small intestine immediately after the stomach. In order to do this, the flagellum allows *V. cholerae* to penetrate the outer mucus layer of the duodenum so that the pili can further aid in colonization of the ileum, a later portion of the small intestine. Upon colonization via biofilm formation, the bacterium produces cholera toxin (CTX), the protein complex responsible for the characteristic diarrhea of the disease. Upon return to the environment, *V. cholerae* exists again as free-living cells in reservoirs as previously mentioned [14].

Over time, *V. cholerae* has evolved and adapted to survive two conditions: an aquatic environment and the human host. In the ocean, the bacteria may exist in a free-standing dormant state or it may inhabit various marine organisms. Here it must resist phagocytosis, low temperatures, higher salt concentrations, and starvation. In the human host, the bacteria must contend with stomach acidity, digestive secretions (e.g. bile), and immune system defenses. *V. cholerae* commonly remains in a dormant state in aquatic environments, but upon ingestion, will colonize the small intestine, forming biofilms and secreting an enterotoxin. The host then suffers from severe, watery diarrhea and, if left untreated, may die [15, 16] Most serotypes of *V. cholerae* are differentiated based on the presence or absence of the O antigen on the lipopolysaccharide of the outer membrane. The two major O serogroups, O1 and O139, produce cholera toxin and are the most common pathogenic strains [17]. These strains have virulence factors attributing to the pathogenicity and persistence of the bacterium.

2.3 Pathogenicity and Persistence

Pathogenicity is a bacteria's ability to spread disease. This is key to bacterial survival because it grants the opportunity for a pathogen to grow, multiply, and be released back to a reservoir for new infections. This trait can be measured via virulence factors. *V. cholerae*, a primary pathogen, has strains with both little to no virulence factors and strains with several that have been selected for over time. Pathogenic strains O1 and O139 use mechanisms like toxin production, a toxin-coregulated pilus, and biofilm formation to efficiently colonize and cause disease within a host.

Cholera toxin, in short, causes the epithelial cells to lyse, secreting electrolytes and water into the intestinal lumen. As CTX is produced, it binds to the external receptors of enterocytes to trigger endocytosis. Once inside the cell, it locks into place, resulting in the overproduction

cAMP. This activates the chloride channel proteins, causing an efflux of chloride ions from enterocytes while disabling the intestine from reabsorbing them. This buildup of ions results in the watery diarrhea characteristic of the disease [18].

Pili are typically used for mobility, adherence, and biofilm formation. The toxin-coregulated pilus (TCP) is not a direct cause of cholera symptoms, but instead is necessary for colonization of the small intestine. This is done by holding the bacterial cells together to form microcolonies. It is also theorized that the TCP aids in adhesion to host epithelial cells.

A biofilm is an aggregate of microorganisms with the ability to attach to surfaces or other microorganisms. Biofilm formation allows *V. cholerae* to adhere to the epithelial lining of the small intestine, reproduce, and eventually cause disease. Without this extracellular matrix, the bacterium would simply pass through the digestive tract of an infected individual, unable to cause disease. However, *V. cholerae* produces robust biofilms, allowing it to be studied as a mode of pathogenesis. Furthermore, *V. cholerae* also produces biofilms in the aquatic environment as a means of persistence.

Bacterial persistence is a phenotypic characteristic in which bacteria are able to survive environmental stressors. Bacteria that exhibit these traits are often the more fit of the population, using slower growth to acquire genetic mutations that are passed down to future offspring. In marine environments, this can be characterized by the viable but nonculturable (VBNC) state. Within this state, bacteria can become dormant while remaining metabolically active. These bacteria are also smaller and cannot be grown on growth media. This allows *V. cholerae* to withstand stress-inducing situations like high salinity or nutrient deprivation, common in marine conditions [19, 20].

In the host, this can be characterized by measuring antibiotic resistance in a culture. As antibiotics are developed through modern medicine, bacteria have begun to evolve in response. Any bacteria that survive treatment with antibiotics are then able to pass on this resistance to offspring. Through this evolution, bacteria become more fit. By exposing *V. cholerae* to antibiotics and measuring growth over time, the persistence of the bacteria can be quantified.

2.4. Environmental Cues

As previously discussed, *V. cholerae* survives two environmental conditions: brackish waters and the human host. In the marine environment, the bacteria withstand a temperature of 30ºC, a pH of 8.0, and a salinity of 600mM. In the human body, the bacteria are then able to survive a temperature of 37ºC, a pH of 7.2, and a salinity of 150mM. Recent studies have shown that *V. cholerae* actually uses these environmental stimuli to adapt to its changing surroundings, along with sodium bicarbonate and bile. Environmental conditions are responsible for bacteria entering the VBNC state. Low nutrient levels, higher osmolarities, and lower temperatures can all induce this dormancy. According to previous research, bacteria in this state can be resuscitated back to a culturable state after being cocultured with eukaryotic cells, meaning upon ingestion, these dormant bacteria can become active again [19, 20].

It has been shown that free-living *V. cholerae* are highly sensitive to low pH, while *V. cholerae* encompassed in biofilm are more acid resistant, meaning this resistance to acid could promote survival during passage through the stomach and small intestine [21]. Similarly, bile, an acidic fluid present in the small intestine, enhances motility but represses other virulence factors like CTX and biofilm production. When first ingested and bile is present, CTX production and biofilm formation is potentially repressed. At this point, there is no need for toxin secretion as it would be a waste of energy and would have no effect on the host. At this same time, motility is

increased. At these early stages of infection, motility is crucial for initial penetration into the epithelial cells and biofilm formation would inhibit this from occurring. However, once within the epithelial cells, *V. cholerae* must begin to form microcolonies through the use of biofilm and secrete cholera toxin, both of which are expressed in the absence of bile [22].

Bile acts as a natural effector in *V. cholerae* due to the unsaturated fatty acids present within, namely linoleic and arachidonic acids. Previous studies have shown that this bacterium has the ability to uptake exogenous fatty acids through its cell envelope, structurally altering its phospholipids [22, 23]. In addition to membrane remodeling, these long-chain UFAs can be used for energy production, via β-oxidation, or possibly as signaling molecules controlling the virulence cascade. If used for membrane remodeling, the bacteria become more or less fit for stress resistance. This can be quantified via antibiotic resistance. If used as a signaling molecule, this triggers changes in the aforementioned virulence factors, namely motility, biofilm formation, and toxin production.

2.5 Unsaturated Fatty Acids

According to emerging studies, several external factors assist *V. cholerae* in persistence and pathogenicity throughout its life cycle. *V. cholerae* has the ability to uptake exogenous unsaturated fatty acids into its cellular membranes to a larger degree than most bacteria. These fatty acids then repress or enhance virulence factors like biofilm formation, motility, and antibiotic resistance [24]. At early stages of infection, many virulence genes are repressed. At this stage, the bacteria have not yet colonized, and virulence genes are not yet needed. At this same stage, however, motility is increased, allowing the bacteria to more efficiently penetrate into the epithelial cells [22]. Upon further examination, the presence of specific unsaturated fatty acids (UFAs) was shown to play a role in these changes. The UFAs were identified as

arachidonic acid, linoleic acid, and oleic acid. It was shown that, while each inhibited CT production, linoleic acid did so by over 30-fold [23].

While the effect of specific fatty acids to virulence factor alterations has been previously studied, these effects throughout the marine to host transition have not. Therefore, this project aims to identify how exogenous UFAs influence the persistence and pathogenicity of *V. cholerae* in real-time during the transition from a marine environment to the human host. The UFAs studied in this project were: linoleic (18:2) and arachidonic (20:4) acids (host), and eicosapentaenoic (20:5) and docosahexaenoic (22:6) acids (marine). Linoleic and arachidonic acids were chosen to represent the human host as they are found in bile and intestinal mucosa. Eicosapentaenoic and docosahexaenoic acids were chosen to represent brackish waters as they are omega-3 fatty acids found in fish oils.

Chapter 3. Protocols and Assays[1]

3.1 Media

Growth medium is designed to support cell growth and there are different types for each type of cell, assay, etc. Three types of media were used throughout this project: Lysogeny Broth (LB), M9 minimal media, and HEPES. LB is commonly used for bacterial growth and is nutritionally rich. Rich media is preferred when trying to overproduce a characteristic; the goal is a high product yield without concern as to what the bacteria is being fed. M9 media contains only the bare basics necessary for cell growth, nothing more. Due to this, it is typically supplemented with nutrients. Minimal media is typically used in experimental analyses. Without excess nutrients, any observed data trends can be accounted to the independent variable tested. Finally, HEPES is a pH buffered media. When culturing bacteria, pH fluctuates due to carbon dioxide and other byproducts released during cellular respiration. As this project aims to rule out various environmental factors such as pH as causative agents, it's essential to keep this constant while testing other variables.

In every media, additives are used as supplemental nutrients. Glucose is added as the primary food source, calcium chloride as a sodium source, magnesium sulfate for DNA replication, and casamino acids provide a nitrogen source for amino acid production. Per previous protocols, 20% w/v glucose and casamino acids are added, while 1M magnesium sulfate and calcium chloride are used. UFAs are added at a concentration of 300μM, based on previous research [24].

[1] Full protocols are available in the Appendix.

3.2 Growth Curves

Growth curves are performed in order to mark the four main phases of a bacteria's growth. Cells initially enter into a lag phase in which they begin to grow and reproduce. Here, growth may be slow due to low initial population density or poor physiological adaptation to the growth medium. They then go through an exponential phase in which growth and replication vastly outweigh cell death. Stationary phase, the third phase, occurs when the rate of new cell formation roughly equals the rate of cell death. Finally, death phase occurs when either all nutrients within the media have been used or waste accumulation has become toxic to the cells. Growth curves must be done initially to understand the growth patterns of a specific bacteria. Once this is understood, further assays can be tailored to these bacteria based upon their growth phases.

Growth curves are performed by growing bacteria in a flask or bioreactor and taking optical density readings at 600nm (OD_{600}) at preset time increments. The OD_{600} value is a measure of the concentration of bacteria present in the culture by passing a wavelength through a sample of the culture in a cuvette to measure the turbidity of the sample. These OD_{600} values are recorded every 30 minutes and plotted to visualize the growth phases of the bacteria. Typically, lag phase occurs up to an OD_{600} of 0.2, while exponential growth typically occurs around 0.2 – 2.5. Most growth curves are performed to define this range for assay development; culture sampling is done in the exponential phase so bacteria will continue to grow and multiply to produce the phenotypic traits being sampled for. Stationary phase typically occurs at 2.5 where bacteria are no longer fit for sampling due to increased cell death and nutrient depletion. Death phase occurs similarly occurs once the OD_{600} begins to drop from 2.5 at the stationary phase at and the culture is no longer fit for study.

3.3 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is an assay typically used to detect proteins, peptides, and antibodies, and can be used to detect cholera toxin. In this assay, *V. cholerae* cultures are grown in hydrophobically-coated 96-well plates that have been coated to block all non-specific binding sites. Figure 3 below shows the antibody interaction used.

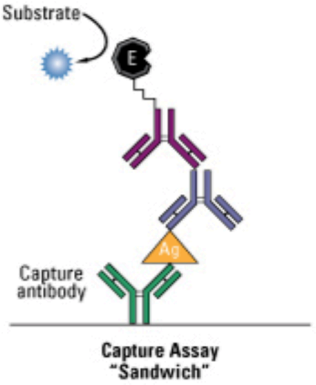

Figure 3. Substrate complex utilized in an ELISA assay [25]

The plate is then coated with a 'capture antibody', pictured in green, that allows for specific binding with CTX. The culture is then added and any CTX present, yellow, can bind to the capture antibody. The plate is then washed to rid it of any unbound antigen. Typically, a specific antibody, blue, is added that binds to CTX, sandwiching it, and then a detection antibody, purple, is added that binds to the specific antibody. Lastly, a substrate is added that reacts with the detection antibody to change colors. After approximately 20 minutes, the color-changing reaction is stopped with sulfuric acid. The color vibrancy is then measured in a plate reader to quantitatively measure the amount of CTX produced. The deeper the color, the more CTX available for antibodies to bind with [25]. The plate configuration can be seen below in Figure 4.

	PBS	OD 0.6	OD 0.6	OD 0.6	OD 0.6	OD 0.6	OD 0.6	CTX 100 ng/ml	CTX 10.0 ng/ml	CTX 1.00 ng/ml	CTX 100 pg/ml	PBS
PBS	Media	●	●	●	●	●	●	●	●	●	●	Media
Control	●	●	●	●	●	●	●	●	●	●	●	●
18:2	●	●	●	●	●	●	●	●	●	●	●	●
20:4	●	●	●	●	●	●	●	●	●	●	●	●
20:5	●	●	●	●	●	●	●	●	●	●	●	●
22:6	●	●	●	●	●	●	●	●	●	●	●	●
PBS	Media	●	●	●	●	●	●	●	●	●	●	Media
--	○	○	○	○	○	○	○	○	○	○	○	○

Figure 4. 96-well ELISA plate layout

In the assay developed, overnight cultures are grown for 18 hours at the chosen environmental condition. The plate used is coated in Superblock Blocking Buffer which is an optimized PBS and TBS solution that contains a blocking protein. This is done to inhibit non-specific binding and minimize signal noise. GM1, a known binding receptor for CTX, is then added for capture [26]. A horseradish peroxidase (HRP)-conjugated CTX antibody is then added to bind to any CTX present, with the HRP enzyme being necessary for catalyzing the color change once TMB is added. This TMB-facilitated color change is stopped with sulfuric acid after 20 minutes. The plate is then read at both 550 and 540 nm and the final absorbance reading is the difference between them, in order to account for optical imperfections in the microtiter plate.

The culture added in columns 2 – 7 is taken at an OD_{600} of 0.6 and contains an unknown amount of cholera toxin produced. Rows 8 – 11 contain a known concentration of toxin that is added to develop a calibration curve. By graphing these known concentrations against the absorbance readings obtained, an equation can be developed to back calculate the unknown toxin concentrations produced by the six rows with no added CTX. To test the effects of UFAs on toxin production, the second row contains culture with no exogenous UFAs present and the next

four rows each contain one UFA of interest. Media is plated in the four corners of the plate to measure for any contamination that could have occurred throughout the experiment. If growth is seen in any of the four wells, the data can be deemed unreliable. PBS is added in the same rows and columns as the media to reduce the edge effect. This effect occurs when some of the media or culture in the perimeter of a plate evaporates during incubation. This results in unreliable data due to varying concentrations and volumes, leading to erratic absorbance readings.

3.4 Biofilm Formation

Biofilm formation can be measured quantitatively by growing bacteria in a 96-well plate in the presence or absence of UFAs. Overnights are grown in the chosen media at the desired conditions (human host or marine environment). After overnights have grown for 18 hours, they are removed from the shaking incubator and centrifuged to separate the bacterial cells (the pellet) from the media (the supernatant). Five microcentrifuge tubes of the resuspended pellet culture and media are all started at an OD_{600} of 0.2. One UFA is added to each tube while one tube with no fatty acid serves as a control. These five tubes are used to inoculate a 96-well plate with 200uL in each well as shown below. PBS and media are used around the perimeter as described previously.

	PBS	Culture	Culture	Culture	Culture	Culture	Culture	Culture	Culture	PBS		
PBS	Media	●	●	●	●	●	●	●	●	Media	○	○
Control	●	●	●	●	●	●	●	●	●	●	○	○
18:2	●	●	●	●	●	●	●	●	●	●	○	○
20:4	●	●	●	●	●	●	●	●	●	●	○	○
20:5	●	●	●	●	●	●	●	●	●	●	○	○
22:6	●	●	●	●	●	●	●	●	●	●	○	○
PBS	Media	●	●	●	●	●	●	●	●	Media	○	○
--	○	○	○	○	○	○	○	○	○	○	○	○

Figure 5. 96-well biofilm plate layout

After the culture has been plated, it is allowed to grow for 24 hours at the desired conditions. The culture is then removed from the plate and the plate is washed to remove any remaining planktonic cells. After washing, only the biofilm formed by the culture within the 24-hour period will be left along the sides of each well. This is then stained with crystal violet, emptied and washed, and allowed to dry for another 24 hours. Lastly, the stained biofilm is solubilized with 30% (w/v) acetic acid, transferred to a fresh microtiter plate, and read using a plate reader. The higher the absorbance, the more biofilm that was formed.

3.5 Antibiotic Resistance

Overnight cultures for the antibiotic resistance assay are grown and pelleted similarly to the biofilm assay. The inoculum is be started at an OD_{600} of 0.1 to obtain cells in the beginning of the exponential phase. Instead of plating them immediately, test tubes are incubated in the presence of one UFA each with one negative control to see the potential membrane remodeling that occurs with UFA uptake and the subsequent effect on antibiotic resistance. The antibiotic administered in this assay, polymyxin B, is commonly used to treat infections involving Gram-

negative bacteria and interferes with the cytoplasmic membrane of the cell. This is added to the plate, shown below, in two-fold increasing amounts in order to find the minimum inhibitory concentration, the lowest amount of antibiotic that inhibits bacterial growth.

Antibiotic concentration	PBS	0 µg/ml	10 µg/ml	20 µg/ml	40 µg/ml	80 µg/ml	160 µg/ml	320 µg/ml	640 µg/ml	1280 µg/ml	2560 µg/ml	PBS
PBS	Media	●	●	●	●	●	●	●	●	●	●	Media
Control	●	●	●	●	●	●	●	●	●	●	●	●
18:2	●	●	●	●	●	●	●	●	●	●	●	●
20:4	●	●	●	●	●	●	●	●	●	●	●	●
20:5	●	●	●	●	●	●	●	●	●	●	●	●
22:6	●	●	●	●	●	●	●	●	●	●	●	●
PBS	Media	●	●	●	●	●	●	●	●	●	●	Media
———	○	○	○	○	○	○	○	○	○	○	○	○

Figure 6. 96-well antibiotic resistance plate layout

In the plate, 30uL of each antibiotic concentration is added along with the appropriate UFA (or none) and 170uL of culture. This is allowed to grow for 20 hours in the shaking incubator at the desired conditions and then the plate is read at 600nm, for a typical OD_{600} growth reading. By comparing each fatty acid row with the control group, the effect of each UFA on antibiotic resistance can be seen. The higher the growth in a well, the more antibiotic resistance the culture in that well has acquired, potentially due to membrane remodeling via UFA uptake.

Chapter 4. Preliminary Experimental Data

4.1 Growth Curve

4.1.1 Human Host, 37ºC

As *V. cholerae* is largely dormant in oceanic conditions, a growth curve was only performed at human host conditions. This data can be seen below in Figure 7.

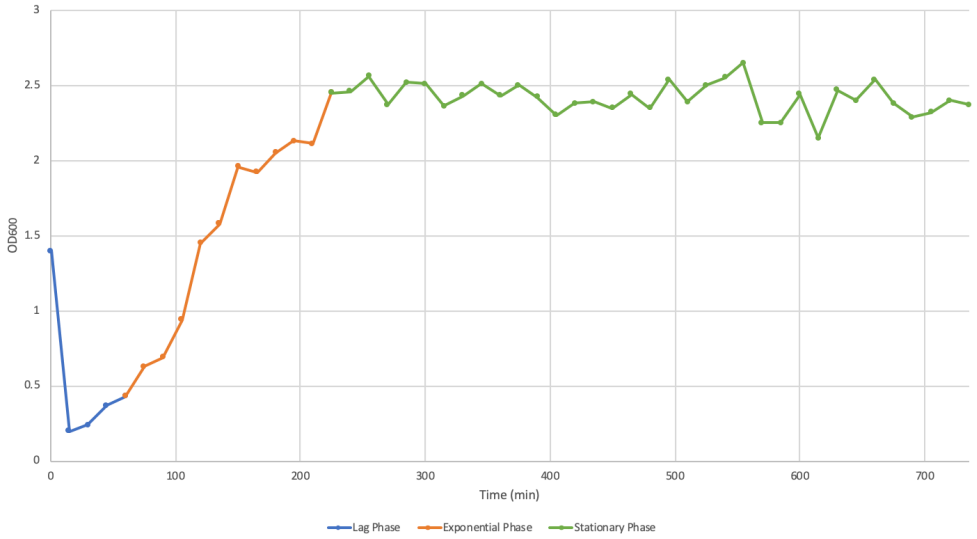

Figure 7. *V. cholerae* growth curve

This growth curve shows the first three phases of cell growth: lag, exponential, and stationary. The initial optical density of 1.4 is the inoculum culture. The absorbance then significantly lowers as the bacteria become accustomed to the new media. The growth curve experiment was performed in order to verify that temperature and pH could be monitored and controlled in the bioreactor, and to determine the optical density range corresponding to exponential growth for subsequent assays. For example, the test tubes in the antibiotic resistance assay needed to be started at the beginning of the exponential phase for optimal UFA uptake. After performing a growth curve, this was determined to be 0.1. Similarly, this curve validated that an OD of 0.6 is still in exponential phase, used in the assay for toxin production.

4.2 ELISA

4.2.1 Human Host, 37ºC

Preliminary data for CTX production in M9 was gathered for qualitative analysis. Initially, in the four columns with no added CTX, culture was pulled and used at ODs of 0.2, 0.4, and ~ 0.6 in three columns. This was done to confirm the optimum cell density as 0.6 for measurable, consistent toxin production data. In this trial, three different antibody concentrations were used as well to establish the necessary dilution for optimum binding. A dilution of 1:500 was ultimately used based upon published protocols. Similarly, it gave the most consistent absorbance readings. This preliminary data can be seen below in Figure 8.

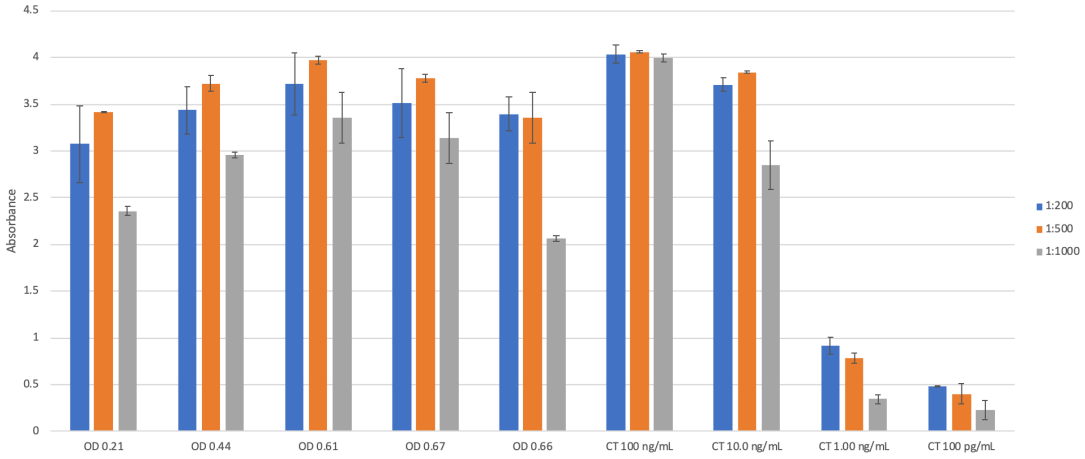

Figure 8. Preliminary ELISA data

This data was not used to calculate the amount of CTX produced, but simply to demonstrate the feasibility of the assay in our hands and ensure that CTX production could be observed in M9. Data on CTX production in the literature was only reported under virulence-inducing conditions. These assays were used to show the protocol was viable, but as the process took around 13 hours, it was determined that quantitative data for this assay needed to be gathered at a later date.

4.3 Biofilm Formation

4.3.1 Human Host, 37ºC

Tests for biofilm formation were conducted in CM9 and HEPES. This shows the effect of additives on biofilm formation as well as the significance of pH control. Preliminary data gathered for biofilm formation in CM9 can be seen below in Figure 9.

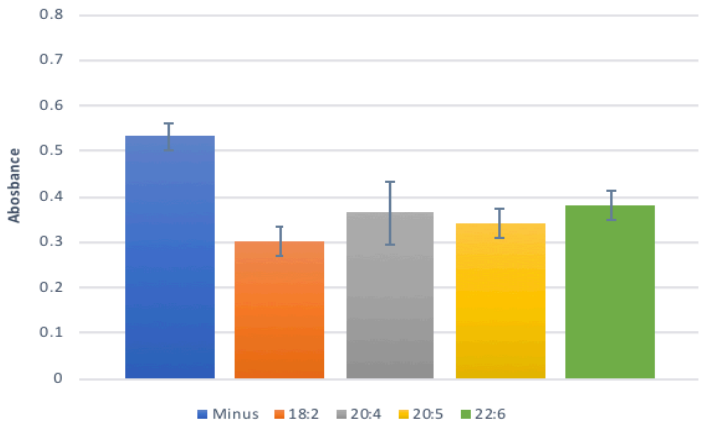

Figure 9. Biofilm Formation in CM9 at 37ºC

At 37ºC and 150mM NaCl, UFAs lowered the amount of biofilm produced in *V. cholerae*. Biofilm formation is reduced more significantly, by half, in the presence of 18:2, which agrees with previous studies [24].

Biofilm formation at the same conditions, but at a constant pH of 7.2 using HEPES, was significantly different. This could suggest that pH has a significant effect of biofilm production. This can be seen below in Figure 10.

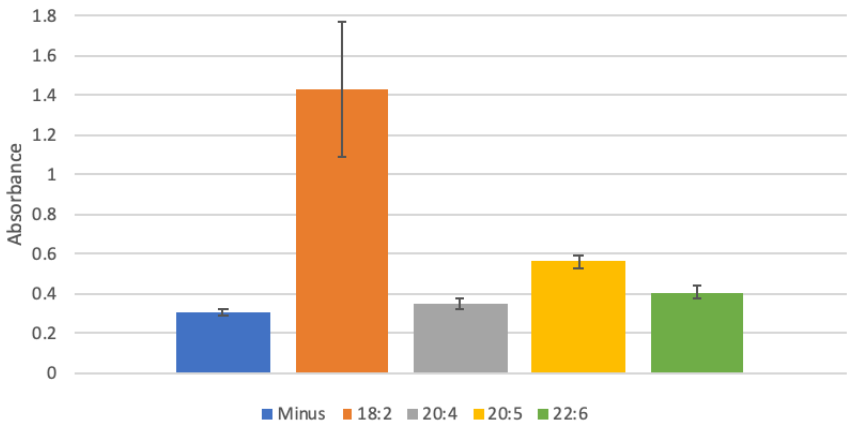

Figure 10. Biofilm Formation in HEPES at 37ºC

This preliminary data shows that 18:2 nearly quintuples biofilm formation, while 20:5 does so less significantly. This trend suggests that a lower pH increases biofilm formation, which also agrees with literature. Similarly, biofilm formation is important in the human host for adherence to the small intestine, where pH ranges from 6.0-7.2. This data suggests that environment-specific fatty acids and pH act synergistically to trigger the virulence cascade.

4.3.2 Oceanic Conditions, 30ºC

Preliminary data for biofilm formation in marine conditions can be seen below in Figure 11.

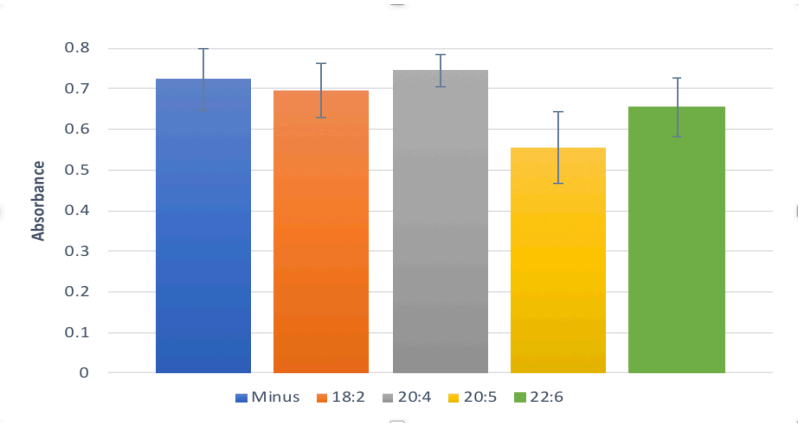

Figure 11. Biofilm Formation in CM9 at 30ºC

From this data, 18:2, 20:4, and 22:6 seem to have no effect on biofilm production at 30ºC and 300mM NaCl while 20:5 lowered production. This could suggest that 20:5 is constitutively

diffused through *V. cholerae*'s membrane to behave as a signaling molecule as it causes a change in biofilm formation at each environmental condition and in each media.

4.4 Antibiotic Resistance

4.4.1 Human Host, 37ºC

Minimum inhibitory concentrations (MIC) for polymixin B were determined in CM9. The preliminary data below shows the antibiotic resistance at various antibiotic concentrations at 37ºC and 150mM.

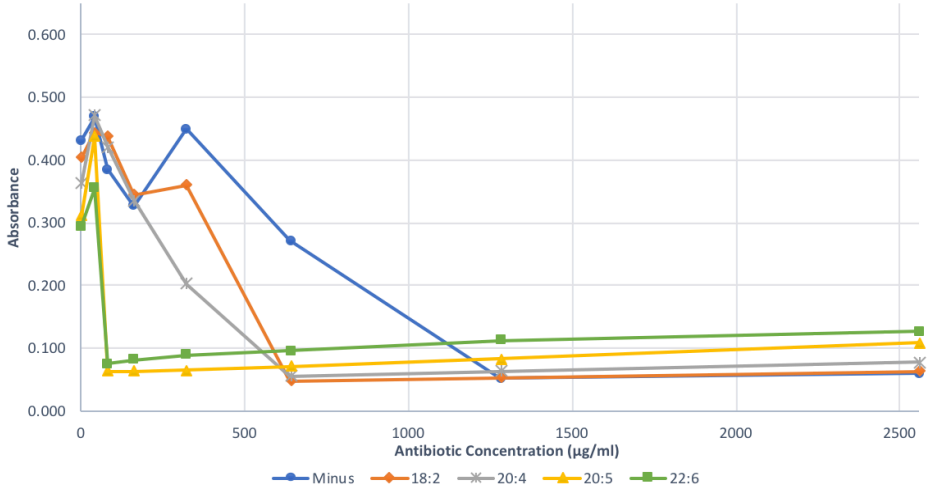

Figure 12. Antibiotic Resistance at 37ºC

Table 1 below gives the minimum inhibitory concentration of polymixin B for *V. cholerae* growth at these conditions.

Table 1. MIC of polymixin B at host conditions

CULTURE	MIC (µG/ML)
CONTROL	1280
18:2	640
20:4	640
20:5	160
22:6	160

The presence of any fatty acid lowers the MIC with respect to the control. In these conditions, 18:2 and 20:4 result in a two-fold decrease, while 20:5 and 22:6 result in a six-fold decrease in MIC.

4.4.2 Oceanic Conditions, 30ºC

Figure 13 below shows antibiotic resistance in *V. cholerae* at 30ºC and 300mM NaCl. This assay was done at an osmolarity of 300mM, not 600mM, based upon old data. Although this data cannot be used to quantify brackish water, it could be used as an intermediary osmolarity and to verify the protocol.

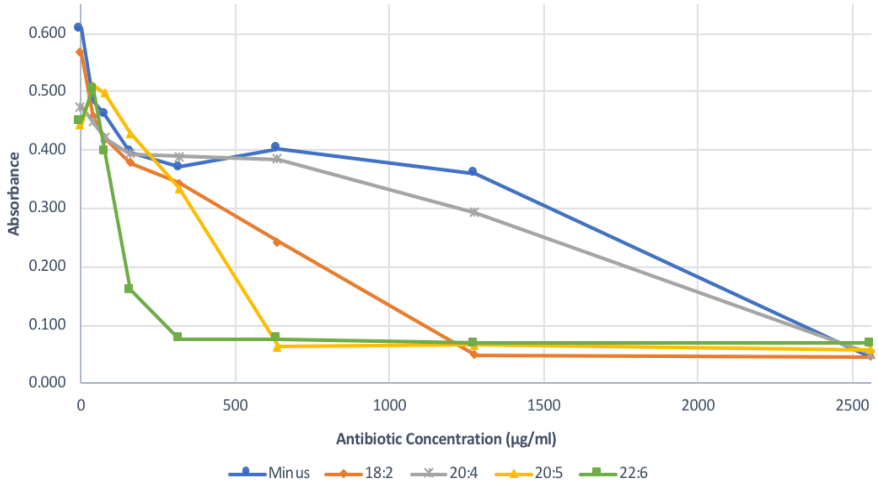

Figure 13. Antibiotic Resistance at 30ºC

Table 2 below shows the minimum inhibitory concentrations of polymixin B for *V. cholerae* growth at oceanic conditions.

Table 2. MIC of polymixin B at marine conditions

CULTURE	MIC (μG/ML)
CONTROL	2560
18:2	1280
20:4	2560
20:5	640
22:6	320

The MIC is noticeably lowered by the presence of each fatty acid at aquatic conditions of 30℃ and 300mM salinity, except 18:2 and 20:4. 22:6 has the highest inhibitory effect on the minimum inhibitory concentration for PMB with a six-fold decrease, while 20:4 has a minimal effect with no change in MIC. 18:2 resulted in a two-fold decrease and 20:5 gave a four-fold decrease in MIC. Because 18:2 and 20:4 had little to no effect in saltwater conditions but lowered MIC in host conditions, *V. cholerae* may only uptake these UFAs at host conditions or there is another variable controlling the use of these fatty acids. As 20:5 and 22:6 lower antibiotic resistance in both environmental conditions, *V. cholerae* could constitutively use them to modulate antibiotic resistance.

Chapter 5. Regulatory Systems

5.1 Background Information

Gene regulation allows bacteria to maintain biochemical flexibility while optimizing energy efficiency. While bacteria carry all of the genetic information for synthesizing many proteins, only a subset of this information is expressed at any time. An operon, shown below in Figure 14, is a group of bacterial structural genes that are transcribed as a group to optimize protein synthesis. Essentially, an operon controls the conversion of other genes into a product to be expressed.

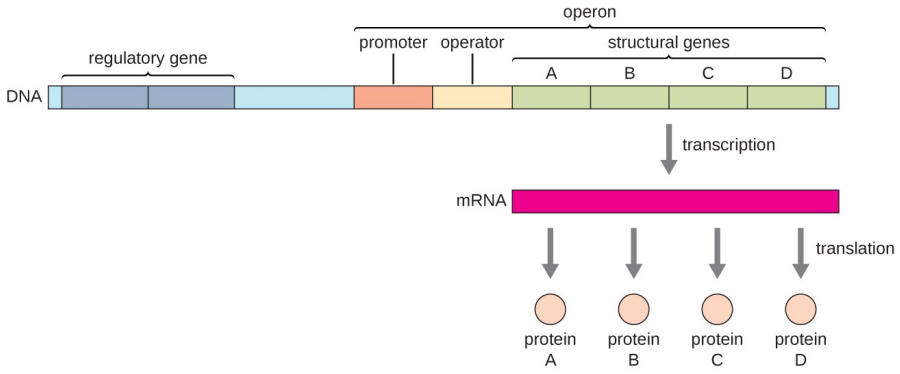

Figure 14. General operon schematic [27]

The regulator gene helps control the expression of structural genes of the operon by increasing or decreasing their transcription but is not considered part of the operon. The promoter is the region of DNA where RNA Polymerase binds to begin transcription. The operator is a region preceding the operon, overlapping the promoter, where regulatory can protein bind to affect transcription. The structural genes code for various proteins. This is essential to a cell in order to respond to environment-specific needs, instead of constantly producing these proteins, even when unnecessary.

Transcriptional regulation can be positive or negative. In positive control, a regulatory protein is an activator that stimulates transcription. In negative control, a regulatory protein acts as a repressor, binding to DNA and stopping transcription. By binding to the operator, the promoter is blocked by the repressor, RNA polymerase is unable to bind to the DNA, and no genetic information is transcribed. Likewise, expression of a gene or protein can be constitutive, or always expressed.

Gene expression can also be controlled by the available nutrients. Catabolite repression is the process in which genes that participate in the metabolism of other sugars are turned off when glucose is present. This occurs because glucose requires less energy to metabolize; therefore, when available, glucose is preferentially used as an energy source. The catabolite activator protein complexes with the nucleotide cAMP and together they stimulate transcription. The concentration of cAMP is regulated within the cell so that it is inversely proportional to the available glucose. Thus, when glucose is available, there is less cAMP available to complex and minimal transcription occurs. This further allows bacteria to respond to the surrounding environment.

5.2 Gene Expression in *Vibrio cholerae*

Gene expression in *V. cholerae* can be seen below in Figure 15. Ovals represent proteins and porins while pentagons represent genes. Table 3 below shows what each protein regulates and what each gene codes for.

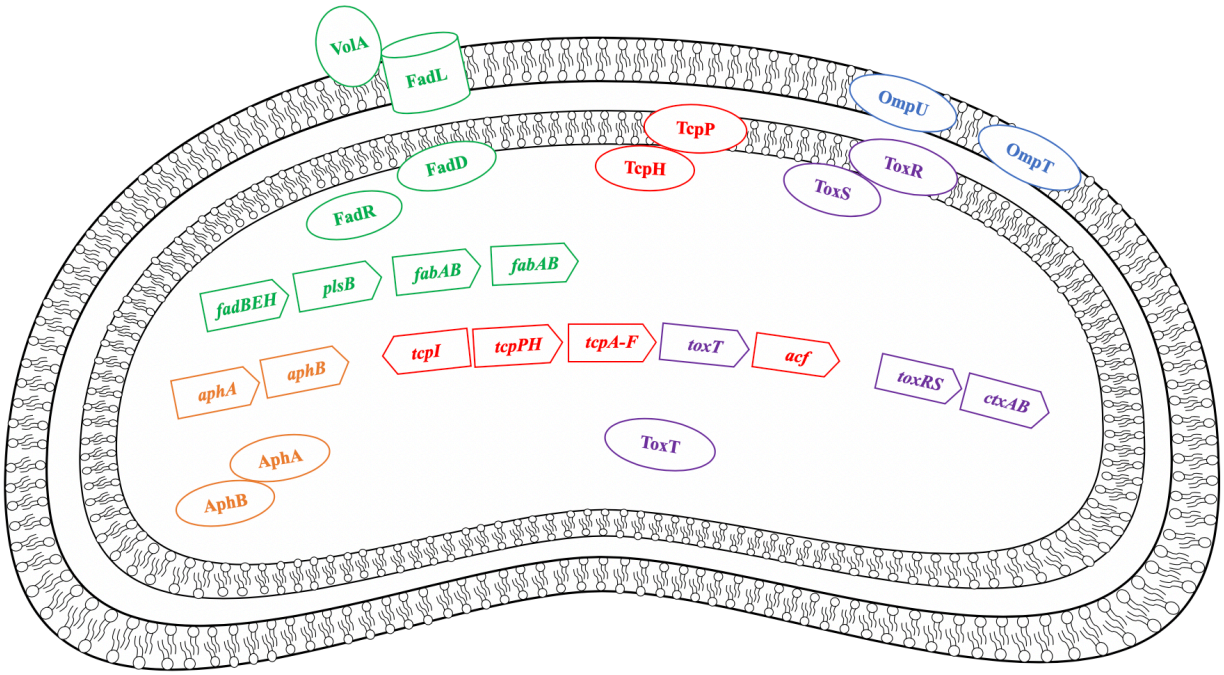

Figure 15. *V. cholerae* regulatory networks

Table 3. *V. cholerae* regulatory factors

Transcriptional Regulatory proteins	
ToxRS	Cholera toxin master regulator
TcpPH	Toxin coregulated pilus master regulator
AphAB	TcpPH regulator
OmpU	Outer membrane porin
OmpT	Outer membrane porin
ToxT	Cholera toxin regulator
VolA	Outer surface lipoprotein
FadL	Fatty acid transport
FadD	Fatty acid activator
FadR	Fatty acid regulator

Structural Genes	
toxRS	ToxRS proteins
tcpPH	TcpPH proteins
aphAB	AphAB proteins
tcpI	TcpPH inner membrane localization
tcpA-F	TCP precursors
acf	Accessory colonization factors
toxT	ToxT protein
ctxAB	Cholera toxin
fadBEH	Fatty acid degradation
plsB	Phospholipid biosynthesis
fabAB	UFA biosynthesis
fabHF	SFA biosynthesis

V. cholerae has two major types: pathogenic and non-pathogenic. It is theorized that the pathogenic strains evolved from their aquatic, non-pathogenic counterparts through the progressive acquisition of virulent traits. This evolution eventually gave them the ability to colonize the human host while simultaneously retaining marine fitness. The acquisition crucial to the evolution of virulence was the cluster of genes stretching from tcpI to acf called the Vibrio pathogenicity island (VPI). *V. cholerae* was able to attain the VPI via horizontal gene transfer, which is the transfer of genetic material from cellular organisms of different species, not from parent to offspring. These genes were attained by the bacteria before many of the proteins shown in figure 15 [28].

Virulence in *V. cholerae* is controlled mainly by two transcriptional activators, ToxR and TcpP, via the upregulation or downregulation of ToxT. ToxR, the main regulatory protein, was the first positive regulator discovered in *V. cholerae* and spans the inner membrane. ToxS, a secondary regulatory protein, is crucial for the function of ToxR, although it is unknown why;

however, it is hypothesized that ToxS may help stabilize ToxR. ToxRS regulate OmpU and OmpT, two outer membrane porins that provide stress resistance when triggered by certain environmental cues [29, 30].

TcpP, the regulatory protein that enables the gene for the toxin coregulated pilus, is also a transmembrane protein that requires a secondary transcriptional activator, TcpH. This is theorized to be used in the same way as ToxS. Without TcpH, TcpP is degraded. While ToxR and TcpP are both necessary for the activation of ToxT, ToxR serves as an enhancer to enable TcpP to bind with RNA Polymerase to activate transcription. Membrane localization of ToxR is mandatory for its interaction with TcpP. While the ToxRS proteins are found in all strains of *V. cholerae* and are constitutively expressed, TcpPH are found only in pathogenic strains, further verifying that TcpP is the primary transcriptional activator of ToxT [30-32]. TcpPH are regulated by activators AphA and AphB. AphB is a secondary effector similar to ToxS and TcpH. AphAB bind to TcpP and activate the protein. AphA is also used for quorum sensing, which allows a bacterium to detect the surrounding population density and vary virulence accordingly. In this regard, it upregulates biofilm formation [21, 30].

Cholera toxin, encoded by the *ctxAB* gene, is regulated by ToxT. This cytoplasmic protein is essentially at the end of the regulatory cascade responsible for *V. cholerae* virulence. ToxT binds to the promoter upstream of *ctxAB*, *tcpA-F* and *acf.* The regulatory pathway of these genes can be seen below in Figure 16, where an arrowhead represents upregulation and a dash represents downregulation.

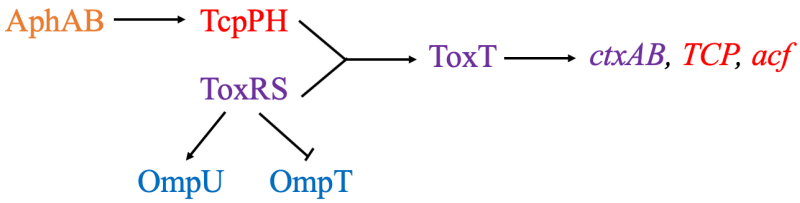

Figure 16. ToxR regulatory cascade

This is the regulatory cascade of *V. cholerae* with no external effects. Due to the bacterium's ability to uptake exogeneous fatty acids and responds to environmental cues, this changes between environments. This will be discussed in the next section.

ToxR is required for the expression of OmpU, while ToxR represses OmpT. It has been shown that OmpU may also serve as an adhesin, binding to fibronectin and arginine-glycine-asparagine (RGD) tripeptide. Fibronectin can serve in eukaryotic extracellular matrices, such as epithelial cells, as a bridge to extracellular proteins, like porins in *V. cholerae*. The RGD tripeptide is the key factor that mediates cell-to-cell adhesion and interaction via integrins, ligands, and fibronectin in the extracellular matrix. The selective binding of OmpU to these factors suggests that this protein assists in *V. cholerae* binding to eukaryotic cells for infection. Furthermore, this coincides with the upregulation of this protein according to ToxRS; *V. cholerae* must be attached to host cells to cause infection with the expression of virulence factors, also upregulated by ToxRS. OmpT, however, is repressed by ToxRS. The variation in regulation of OmpU and OmpT is due to their different selectivities; OmpT facilitates passage of negatively charged cations, such as bile or other detergents. With unregulated influx, these cations would disrupt the cytoplasmic membrane and lower growth rates. OmpU is much more selective in passage, explaining why this porin is upregulated via the virulence cascade while OmpT is repressed [33, 34].

Recent *V. cholerae* genomic studies have revolved around the fatty acid degradation cascade including FadL, FadD, and FadR. FadL is a long-chain fatty acid transporter spanning the outer membrane and is coexpressed with VolA, which aids in uptake of exogenous lysophosphatidylcholines. FadD is a long chain fatty acyl-CoA ligase, which activates long chain fatty acids to be used for phospholipid synthesis. FadR is the master regulator of fatty acids, controlling FA biosynthesis, degradation, and phospholipid biosynthesis [35, 36]. Figure 17 shows this regulation.

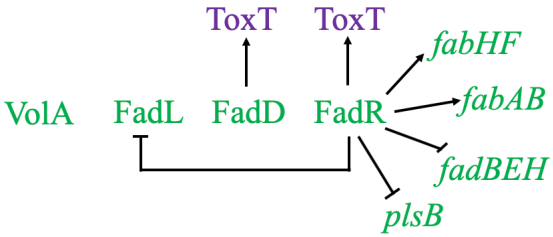

Figure 17. Fatty acid regulatory cascade

Without environmental interaction, FadR downregulates phospholipid biosynthesis and fatty acid degradation while stimulating saturated and unsaturated fatty acid biosynthesis. Interaction with long chain fatty acids (LCFA) disrupts this regulation. Therefore, as FadR is only active when no LCFA has been imported, it is imperative that the bacteria be able to synthesize its own fatty acids and not degrade them. These fatty acids are used to synthesize the phospholipids necessary for the cell membrane. FadR regulates these genes by binding directly to their promoters. FadR and FadD both upregulate ToxT but do so independently of one another [36]. This is not done via ToxR but through another unknown mechanism. Although FadD does not alter ToxR or TcpP production, it is necessary for TcpP membrane localization. The absence of FadD induces the extracytoplasmic stress response (ESR), which is one way in which bacteria sense the changing environment. The ESR responds to changes in the cell membrane, such as the

absence of a particular membrane protein, FadD. This response ultimately activates an integral membrane protease that degrades TcpP, which in turn decreases other virulence genes [32, 37].

Lipopolysaccharides, comprising the outer leaflet of the outer membrane, successfully allow for the diffusion of short chain fatty acids while blocking long chain fatty acids in bacteria. Therefore, the presence of FadL is required for transport into the bacterial cell [38]. In the absence of external factors, FadR represses FadL. As FadR upregulates FA synthesis and downregulates FA degradation, expression of FadL is unnecessary since fatty acids are already being synthesized *de novo*.

5.3 Environmental Cues

In response to a changing environment, *V. cholerae* must adapt in the presence of various cues. These markers include pH, temperature, salinity, and fatty acids. In the main virulence cascade, the ToxR regulon, exogenous unsaturated fatty acids act as configurational repressor by binding to ToxT in the place of ToxR, disabling the structural genes encoding toxin, the pilus, and other colonization factors [29]. This is shown below.

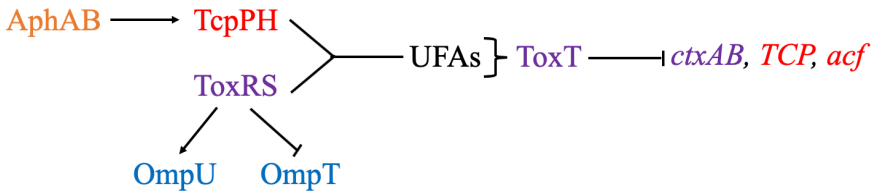

Figure 18. Change in the ToxR regulon with UFAs

Commonly, these UFAs are introduced in the human host within bile. As previously discussed, bile represses biofilm formation and toxin production. By repressing ToxT during the early stages of infection, *V. cholerae* can maximize energy efficiency in gene expression by repressing factors unnecessary to initial epithelial penetration. In the presence of bile, the repression of OmpT is critical. If OmpT were not repressed, excess amounts of bile would be transported into

the cytoplasmic membrane, stopping growth by simultaneously competing with nutrient entry and damaging the inner membrane. However, with OmpT repressed and OmpU expressed, bile acids are selectively excluded from membrane diffusion, allowing bacteria to survive entrance into the small intestine. It has been shown that strains expressing OmpU are more bile-resistant, pathogenic, and better able to colonize the small intestine than those expressing OmpT. This regulation increases antibiotic resistance simply as less pores are open for antibiotic diffusion. [33, 34].

Unsaturated fatty acids are introduced in the marine environment within the reservoirs used by *V. cholerae*. The repression of virulence via these marine UFAs is similarly important; production of virulence genes is a waste of energy as there is no host to infect at this point. Likewise, the repression of OmpT is critical due to the high salinity of the ocean. If OmpT were to be open, the cell would ultimately become hypotonic and lyse due to the constant influx of sodium chloride resulting in a high osmotic pressure.

Lysophosphatidylcholines (LPCs) are long chain chemical compounds with a choline head group and a variable fatty acid chain at one end. VolA, a lipoprotein located on the outer surface of the outer cell membrane that is coexpressed with FadL, cleaves the fatty acid chain off of these exogeneous LPCs. This allows FadL to transport this free fatty acid into the bacterial cell. This fatty acid must initially cross the periplasm, but must also reach FadD, located on the inner layer of the inner membrane's phospholipid bilayer. To do this, it must first be transported through the first leaflet. The mechanism by which this occurs is unknown [35, 39]. Once through the periplasm and the inner cell membrane, FadD activates this long chain fatty acid (LCFA) with an acyl-CoA, and this complex represses FadR [36]. This can be seen below in Figure 19.

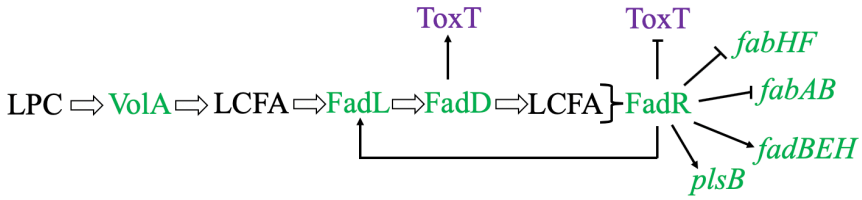

Figure 19. Change in fatty acid regulation with exogenous LCFA uptake

By repressing FadR, both short and long UFA synthesis is turned off, while phospholipid biosynthesis and fatty acid degradation is turned on. As this repression occurs when fatty acids have been imported into the cell, fatty acid biosynthesis is unnecessary and an energy waste. Opposingly, in the presence of these LCFAs, fatty acid degradation is turned on along with phospholipid synthesis. Thus, as these LCFAs are imported into the cell, *V. cholerae* can now degrade them for energy and use them for the phospholipid bilayer. As FadR is repressed, it's influence on ToxT is downregulated. Nonetheless, because ToxT is affected by FadR and FadD independently, it is still upregulated by FadD.

The uptake of these LPC-derived fatty acids is equally important in both environments. Within the human host, these chemical compounds can be found in the lumen of the small intestine. Due to the cleaving action of VolA, *V. cholerae* can use the newly freed fatty acids for membrane remodeling similarly to the action of unsaturated fatty acids. This membrane remodeling can allow the bacteria to survive subsequent membrane stress. In the aquatic environment, LPCs can be found in association with the bacteria's reservoirs. Here, they can similarly use the cleaved LCFA for membrane remodeling, but it has also been shown that *V. cholerae* can use LPCs as its sole carbon source. While in the brackish waters, nutrient deprivation is prevalent. Using LPCs as a carbon source allows the bacteria to become more fit and survive without entering the VBNC state [13, 20, 35].

Chapter 6. Conclusions and Future Work

6.1 Conclusions

This research demonstrates the viability of several protocols as well as preliminary insights into *Vibrio cholerae*'s phenotypic traits. Through the development of protocols, assays were tailored to *V. cholerae* using various medias, the addition of unsaturated fatty acids, and two environmental niches. A nutritionally rich medium was initially used for feasibility trials. To begin identifying key environmental triggers, assays were conducted using minimal media. The final media used, HEPES, offers pH stability throughout the growth of bacteria. Four UFAs were used: linoleic and arachidonic acids to represent the human host and eicosapentaenoic and docosahexaenoic acids to represent brackish waters.

It was shown that cholera toxin could be produced and quantified in non-virulence inducing conditions, whereas CTX production has been previously published only in virulent conditions. The protocol was tested to show that an OD_{600} of 0.6 was optimal for toxin production in these conditions and an antibody dilution of 1:500 was ideal for toxin capture.

Biofilm formation data was gathered in three conditions: with minimal media in both environmental niches and with HEPES in host conditions. It was shown that host conditions supplemented with 18:2 had the largest impact, while marine conditions had very little impact. This could potentially mean that *V. cholerae* only uptakes this UFA to use as a signaling molecule in the human host. As linoleic acid is found in human bile, this is a logical conclusion. Furthermore, the variation of 18:2-induced biofilm change with pH is vast. In minimal media, each UFA lowered biofilm formation, with 18:2 doing so by half. In HEPES, where pH is held constant, each UFA increased biofilm formation, with 18:2 doing so by five-fold. This could potentially signify that pH and unsaturated fatty acids synergistically alter the virulence cascade

within *V. cholerae*. According to published work, a lower pH increases biofilm formation, which agrees with this data. Therefore, *V. cholerae* is theoretically more adapted to the human host where pH ranges from 6.0 – 7.2, allowing it to use biofilm to both protect against membrane stress within the host and colonize the small intestine. In marine conditions, eicosapentaenoic acid (20:5) was the only unsaturated fatty acid that changed biofilm formation. 20:5 affected biofilm in both conditions, suggesting that it could diffuse through *V. cholerae*'s membrane to be used as a signaling molecule within the virulence cascade regardless of the surrounding environment.

By measuring the MIC of polymixin B, environment-specific effects relating to antibiotic activity could be evaluated. In host conditions, all unsaturated fatty acids lowered antibiotic resistance, with the 20:5 and 22:6 doing so by six-fold. In marine conditions, 20:4 had no effect, 18:2 and 20:5 had little effect, and 22:6 had a large effect on antibiotic resistance, again lowering the MIC by six-fold. As 18:2 and 20:4 had little to no effect in this environment but lowered MIC in host conditions, *V. cholerae* may only use these UFAs for membrane remodeling in the human body. Because 20:5 and 22:6 lowered antibiotic resistance in both environments, these may be constitutively used for membrane remodeling.

Bacteria are an ever-evolving domain, as evidenced by the *Vibrio* pathogenicity island. As we continue to develop means of fighting these infections, bacteria are able to adapt and evolve resistance against them. Bacterial antibiotic resistance is considered one of the greatest threats to human health. Beginning as a single mutation, a bacterium can rapidly develop mechanisms to survive a course of antibiotics; upon survival, it is able to reproduce and pass on this acquired resistance. As V. *cholerae* acquires these persistent traits, medicine begins falling behind. The WHO 2014 report stated "this serious threat is no longer a prediction for the future, it is

happening right now in every region of the world and has the potential to affect anyone, of any age, in any country. Antibiotic resistance—when bacteria change so antibiotics no longer work in people who need them to treat infections—is now a major threat to public health." In 2015 there were almost 700,000 infections were caused by antibiotic resistant bacteria [40]. Simply put, medicine isn't developing as fast as bacteria are.

The quantification of changes in antibiotic resistance poses an important potential benefit to modern medicine. By measuring the effects of unsaturated fatty acids on antibiotic resistance, they could potentially be incorporated within antibiotics. For example, in this research it was seen that docosahexaenoic acid lowers antibiotic resistance by six-fold in both brackish waters and the human host. Theoretically, this unsaturated fatty acid could be used synergistically with antibiotics, lowering resistance in the infecting bacteria. This application is not only germane to *V. cholerae*; it has been shown that several more bacteria possess the ability to uptake exogenous fatty acids [24]. This gives the potential to fight a whole class of resistant bacteria by a new means.

By understanding the regulatory networks of *V. cholerae*, specific proteins or genes can be identified as key points in the cascade. If virulence can be attributed to specific proteins, genetic recombination techniques could be developed to up or downregulate them. For example, it was shown that OmpT in *V. cholerae* is downregulated for selective diffusivity. If OmpT were upregulated, bile would flow in upon ingestion, resulting in cell death; infection would not occur. By studying bacterial genes and their regulatory networks, alternative treatments can be developed.

The Fad regulon has been identified in many bacteria and characterized in *E. coli*; however, *V. cholerae*'s ability to uptake long chain fatty acids to use for membrane remodeling has not

typically been seen in other Gram-negative bacteria. Although the Fad regulon has been classified in other bacteria, *V. cholerae* possesses multiple homologs of FadD and FadL [32]. This allows the bacteria to recognize and uptake a variety of fatty acids, ultimately allowing adaptation to multiple environments.

6.2 Future Work

Due to the inaccuracies of observed when assessing phenotypic traits, research will begin centering around proteomic and genomic studies. When testing phenotypes, only an end product is measured. Due to this, the independent variable could be affecting the expression of this physical characteristic at any point in the regulatory cascade. For example, when testing the effect of unsaturated fatty acids on toxin production, it is difficult to ascertain the true result in the assays developed; either AphA, ToxR, TcpP, or ToxT could be downregulated due to the introduction of UFAs. The only information available is the increase or decrease in CTX production. This prompted a shift in focus to genotypic studies.

With the awarding of a CEACSE grant, research will move towards the development of a computational framework to model *V. cholerae*'s fundamental pathogen dynamics. This will be backed with laboratory experiments to represent various ecological and environmental conditions. Combined molecular modeling will reveal information regarding the pathogenesis and transmission of waterborne pathogens.

Chapter 7. Materials and Methods

7.1 Bacterial Strain

Throughout these experiments, *Vibrio cholerae* strain C6707 was used. O1 El Tor classical biotype is an epidemic strain.

7.2 Growth Conditions

Granulated LB was obtained from Fisher Scientific. 25 g of this broth was dissolved in 1 L of sterile DI water.

M9 salts were similarly obtained from Fisher Scientific as a 5x. 28.2 g of M9 salts were dissolved in 500 mL sterile DI water and 20.67 g of NaCl added for a 0.75M solution (host) or 86.41 g is added for a 3.0M solution (marine). This was diluted to a 1x solution with sterile DI water to attain M9 with 150mM or 600mM salinity.

HEPES must be made at the correct pH and the temperature at which it was made must be accounted for. 200mL of 0.25M stock solution (5.55x) was stored. For host conditions, this was made by dissolving 11.915g of HEPES in 180mL of sterile DI water and titrated to a pH of 7.43 at room temperature of 25ºC. The volume was then brought to 200mL. At the experimental temperature of 37ºC, the pH held at 7.2. 9.74g of NaCl was added for a final diluted salinity of 150mM. The solution used is was 45mM HEPES solution made by diluting the stock with sterile DI water.

For a HEPES media appropriate for oceanic conditions, 11.915g of HEPES was dissolved in 180mL of sterile DI water and titrated to a pH of 8.07 at room temperature of 25ºC. This solution was brought to a total volume of 200mL and 38.96g of NaCl to give a 600mM solution upon dilution to the 45mM 1x HEPES solution.

In each media, supplemental nutrients were provided. Filter sterilized stock solutions stored include: 20% (w/v) $C_6H_{12}O_6$, 1M $MgSO_4$, 1M $CaCl_2$, and 20% CAA. These were then added to the media for growth. Final concentrations of each additive in each media were as follows: 2% $C_6H_{12}O_6$, 0.2% $MgSO_4$, 0.02% $CaCl_2$, and 1% CAA (v/v). The unsaturated fatty acids are added at a concentration of 300mM.

7.3 ELISA

7.3.1 ELISA Materials

The GM1 dilution in this assay was made by diluting 1mg of the ganglioside in 1mL of water. PBS/Tween was made as a dilution of 0.1% Tween, 100µL, in 100mL of PBS. 0.5mg of cholera toxin was diluted in 0.5mL of sterile DI water, made from a 2mg/mL vial of CTX. HRP was diluted with 0.1% BSA in PBS (w/v). 0.2M H_2SO_4 was used to stop the enzymatic reaction. The 96-well plates used were clear polystyrene coated in Immulon 1B for hydrophobic binding.

7.3.2 ELISA Protocol

1) Create 1 overnight culture to shake at 250rpm at the desired temperature
    a. Create 1 tube of media only to serve as a control for contamination
2) After 16-20 hours, use the overnight culture to inoculate 100mL of media in an Erlenmeyer flask
3) Record the initial OD_{600} and continue to shake the flask at the desired temperature
4) Sample the culture every 30 minutes until it has reached an OD_{600} of 0.6
5) Centrifuge the sample in a 15mL conical with a balance for 10 min at 6000rpm
6) Allow all reagents and plates to sit out to room temperature for at least 30 min
7) Label the UV sterilized coated 96-well microtiter plate

8) Add 150µL of blocking buffer to each well, cover plate, and allow to sit at room temperature for 1 hour
9) Wash the plate three times with PBS/Tween
10) Add 100µL of supernatant at OD 0.6 in rows 2 – 7
11) Add 100µL of CT control at all 4 concentrations in rows 8 - 11
12) Cover and incubate at the desired temperature for 1 hour
13) Wash the plate three times with PBS/Tween
14) Add 100µL of the Polyclonal Antibody at a concentration of 1:500 and incubate at the desired temperature for 1 hours
15) Wash the plate three times with PBS/Tween
16) Add 100µL of the TMB to each well
17) Incubate at room temperature until color appears in the positive controls (10-20min)
18) Stop the reaction with 50µL of 0.2M H_2SO_4.
19) Read the plate at 550nm and 450nm
20) Calculate the final absorbance as the difference of the two readings

7.4 Biofilm Formation

7.4.1 Biofilm Assay Materials

A 0.1% (w/v) crystal violet solution was used to stain the biofilm. A stock 5x solution was made by dissolving 5mg of CV powder in 5mL of water in a light-blocking container. The stained biofilm was solubilized with 30% acetic acid. The 96-well plate used was clear polystyrene.

7.4.2 Biofilm Formation Protocol[2]

1) Create 2 overnight cultures to shake at 250rpm at the desired temperature

[2] This protocol was adapted from O'Toole's biofilm assay [41]

   a. Create 1 tube of media only to serve as a control for contamination

2) After 16-20 hours, remove the overnight cultures and pour the overnights into a 15mL conical, place in centrifuge, balance, and spin for 10 minutes at 4500rpm
   a. Create five 2mL micro centrifuge tubes labeled '-', '18:2', '20:4', '20:5', and '22:6'
3) Once complete, pour off the supernatant and refill the tubes with 1 mL of media, carefully as to not disrupt the pellet, and spin for 10 minutes at 4500 rpm
   a. Replace these in the centrifuge with the pellet facing upward
   b. Add 300mM of each UFA to the corresponding microcentrifuge tube

FATTY ACID	VOLUME (µL)
18:2	0.248
20:4	0.843
20:5	0.843
22:6	0.909

4) Once complete, pour off supernatant and re-suspend in 1 mL media
   a. This is done by breaking up the pellet with the media and pipette
5) Measure the OD_{600} of the prepared inoculum
6) Calculate the volume of culture required to start the microcentrifuge tubes at an OD of 0.1, according to the equation below

$$(culture\ volume)(\mu L) = \frac{(desired\ OD)(total\ volume)}{(culture\ OD)} * 1000$$

7) Place a total volume of 1.8 mL in each microcentrifuge tube, culture added last, calculating the amount of media to be added as follows:

$$Media\ (\mu L) = 1800 - (culture\ volume) - (FA\ volume)$$

8) Label the UV sterilized uncoated 96-well microtiter plate
9) Plate 200µL of PBS in columns 1 and 7, with 200 µL of media in the four corners

10) Add 200µL of each microcentrifuge tube into its corresponding plate row
    a. Shake the microcentrifuge tube before adding its contents to the plate
11) Incubate the plate statically at the desired temperature for 24 hours

Staining Biofilm

12) Remove the plate from the incubator and measure OD_{600} values in the plate reader for growth comparison
13) Invert the wells and shake the plate carefully to remove the liquid contents
14) Wash the wells to remove excess medium and unattached cells with two boxes filled with DI water; blot the plate on a paper towel
    a. Repeat this step twice
15) Add 225µL of 0.1% CV solution to each well
16) Cover the plate with aluminum foil and incubate at room temperature for 10-15 minutes
17) Wash the plate 3-4 times in the same manner as before, until little CV is washed out
    a. If not done gently, the biofilm will be washed away as well
18) Place the plate upside down, tilted, and covered with aluminum foil to dry for 24 hours

Reading the Biofilm Plate

19) Add 225µL of 30% acetic acid solution to each well to solubilize the CV for transfer
20) Incubate the plate at room temperature for 15 minutes
21) Transfer the 225µL of solubilized CV to a new plate
22) Read the plate at 590nm using the microtiter plate reader

7.4 Antibiotic Resistance

7.4.1 Antibiotic Resistance Assay Materials

Polymixin B is used as the antibiotic in this assay as it is highly effective against Gram-negative bacteria. This is added in two-fold increasing concentrations, as seen in step 9 below. Clear, polystyrene 96-well microtiter plates are used similarly to the biofilm formation protocol.

7.4.2 Antibiotic Resistance Protocol

1) Create 2 overnight cultures to shake at 250rpm at the desired temperature
    a. Create 1 tube of media only to serve as a control for contamination
2) After 16-20 hours, remove the overnight cultures and pour the overnights into a 15mL conical, place in centrifuge, balance, and spin for 10 minutes at 4500rpm
    a. While centrifuging, create five 2mL test tubes labeled '-', '18:2', '20:4', '20:5', and '22:6'
3) Once complete, pour off the supernatant and refill the tubes with 1 mL of media, carefully as to not disrupt the pellet, and spin for 10 minutes at 4500 rpm
    a. Replace these in the centrifuge with the pellet facing upward
    b. Add 300mM of each UFA to the corresponding microcentrifuge tube

FATTY ACID	VOLUME (μL)
18:2	0.248
20:4	0.843
20:5	0.843
22:6	0.909

4) Once complete, pour off supernatant and re-suspend in 1 mL media
    a. This is done by breaking up the pellet with the media and pipette
5) Measure the OD_{600} of the prepared inoculum

6) Calculate the volume of culture required to start the microcentrifuge tubes at an OD_{600} of 0.1, according to the equation below

$$(culture\ volume)(\mu L) = \frac{(desired\ OD)(total\ volume)}{(culture\ OD)} * 1000$$

7) Put a total volume of 6mL in each microcentrifuge tube, culture added last, calculating the amount of media to be added as follows:

$$Media\ (\mu L) = 6000 - (culture\ volume) - (FA\ volume)$$

8) Place the 5 test tubes in the incubator shaking at 250rpm at experimental temperature until the OD_{600} has reached ~0.6 (approx. 2 hours)
9) Create the PMB dilutions in 1.5mL microcentrifuge tubes as follows:

NUMBER	DILUTION	VOLUME OF PMB STOCK (µL)	VOLUME OF MEDIA (µL)
1	0	0	1000
2	40	9	991
3	80	18	982
4	160	36	964
5	320	71	929
6	640	142	858
7	1280	284	716
8	2560	569	431

10) Label the UV sterilized uncoated 96-well microtiter plate
11) Add 30µL of each PMB dilution to the corresponding wells
    a. Do this near the end of the growing period
12) Pour the 5 test tubes into 5 labeled 15 mL conicals, balance the centrifuge, and centrifuge for 10 minutes at 4500 rpm
    a. Label five 15mL conicals '-', '18:2', '20:4', '20:5', '22:6'
13) Once done, pour off the supernatant and fill the tubes with 1 mL media and spin for 10 minutes at 4500rpm
    a. Add 353mM of each UFA to the corresponding microcentrifuge tube

FATTY ACID	VOLUME (µL)
18:2	0.648
20:4	2.205
20:5	2.205
22:6	2.377

14) Once done spinning, pour off supernatant and resuspend in 1mL of media
15) Take OD_{600} of the cultures
16) Add the amount of culture needed to bring the inoculum to an OD_{600} of 0.117 according to the equation in step 6
17) Bring the total volume to 4mL by adding media according to the below equation

$$Media\ (\mu L) = 4000 - (culture\ volume) - (FA\ volume) - (30\ \mu L\ dilution)$$

18) Add 170µL of each conical to the corresponding rows
19) Place the plate in the incubator, taped down and on top of a rack, shaking at 250rpm
20) Remove the plate from the shaking incubator after 24 hours and use the microtiter plate reader to read the plate at 600nm

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