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# Phospholipid remodeling via exogenous polyunsaturated fatty acid uptake modulates stress resistance in Vibrio cholerae

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# Phospholipid Remodeling via Exogenous Polyunsaturated Fatty Acid Uptake modulates Stress Resistance in *Vibrio cholerae*

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# Abstract

Antibiotic-resistant pathogens represent an escalating threat to public health worldwide, substantially increasing the burden of healthcare and community-acquired infections. Several factors contribute to the emergence and spread of this threat, including but not limited to improper antibiotic use and prescriptions in health-care settings and the community, increasing global travel and migration from countries that have higher levels of antibiotic-resistant pathogens, and a lack of new antibiotics under development. According to the World Health Organization, rising rates of resistance among Gram-negative bacteria (such as *Vibrio cholerae*) are of particular concern. These bacteria have evolved a number of endogenous membrane remodeling strategies to sense and adapt to their environment. However, another membrane remodeling strategy employed by these bacteria, the uptake and assimilation of exogenous long-chain fatty acids, remains largely unexplored. Here, we seek to address this knowledge gap by determining the extent to which phospholipid remodeling through uptake of exogenous PUFAs impacts antimicrobial resistance in *V. cholerae.* As expected, resistance to polymyxin B is substantially lower for mutants lacking the ability to modify LPS compared to the wild-type (El Tor) strain. More interestingly, PMB resistance varies when cells are grown in the presence of long-chain fatty acids. This difference is most noticeable for long-chain fatty acids abundant in the human intestines.

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## Introduction

*Vibrio cholerae* is a Gram-negative comma shaped bacterium that is associated with the intestinal disease cholera. Each year there are an estimated 1.3 to 4.0 million cases of cholera, and 21,000 to 143,000 of these cases end in death. The main treatment of cholera is immediate administration of oral rehydration solution and antibiotics. There is also a traveler's vaccine for cholera that reports a 2-year protection [1]. Cholera is characterized with intense watery diarrhea and severe dehydration. *V. cholerae* is found in brackish water and contaminated food. Therefore, areas along the coast or those that without access to clean water are susceptible to cholera outbreaks. Outbreaks are relatively rare in the industrialized world due to the advances in public health and basic health care availability [2]. However, natural disasters can temporarily disable these systems and create an environment where *V. cholerae* (*Vc*) can spread unchecked. Outside of a human host, this bacterium can survive in bodies of water and marine animals, such as oysters and crabs.

Because *Vc* is capable of surviving in drastically different environments, it can adapt to its environment using numerous strategies. Perhaps the most effective of these is membrane modification, wherein the bacterium will alter its membrane composition in response to a stimulus, such as antimicrobial substances, immune system responses, or pH. Fatty acids present in bile are involved in motility and virulence regulation as well as membrane modification. Left untreated, *V. cholerae* will make its way to the small intestine. There it will interact with fatty acids and antimicrobial peptides present in the small intestine [1]. In order to remain in the small intestine, *Vibrio* will stick to the epithelial wall by forming a biofilm. Biofilm is an extracellular matrix that bacteria use to stick to surfaces. Once the bacteria colony reaches a quorum, the colony begins to produce cholera toxin (CT). Cholera toxin is comprised of A and B subunits.

The B subunit binds to the epithelial cell via a GM1 receptor. The A subunit can then enter the cell and enzymatically interact with G proteins. Multiple active G proteins lead to increased cAMP levels and adenylyl cyclase activation [3, 4]. This series of events leads to a substantial efflux of salt, water, and chlorine. This can very quickly spiral into dehydration and weakening of the immune system.

*V. cholerae* outbreaks are well documented in history. These outbreaks were extremely deadly due to a lack of sanitation. The more recent epidemics of *Vc* are thought to be spread by a particular biotype: O1 El Tor. This strain is well adapted to survive in marine environments by utilizing chitin as a carbon source [5]. The El Tor biotype differs by producing varying amounts of cholera toxin and is capable of surviving in more stressful environments when compared to the classical biotype. Also, this strain is more resistant to certain antibiotics. We hypothesize that this resistance involves phospholipid modification, which would involve the inner leaflet of the outer membrane, or the outer leaflet of the inner membrane. The aim of this project is to better understand the effect of phospholipid modification on antimicrobial resistance.

Gram-negative bacteria possess two distinct membranes: an asymmetric outer membrane composed of lipopolysaccharide (LPS) and phospholipids, and a symmetric inner membrane of phospholipids. Conventional research focuses on outer membrane LPS as the critical barrier against the environment. The LPS structure is composed of a bioactive lipid-A anchor, a core oligosaccharide, and a long O-antigen chain. Another remodeling strategy employed by *Vc*, the remodeling of phospholipid leaflets through the uptake and assimilation of long-chain fatty acids, may also be important but remains poorly understood. This remodeling strategy is not unique to *Vc*. Other pathogenic bacteria such as *Acenitobacter baumanii*, *Klebsiella pnuemoniae*, and *Pseudomonas aeruginosa* have been shown to utilize exogenous long-chain fatty acids for

membrane fortification [6-8]. However, *Vc* can incorporate larger fatty acids (20:5 and 22:6) while other organisms, such as *E. coli,* struggle to incorporate fatty acids larger than 20 carbon chains [9]. Previous research has assumed that the inner membrane functions primarily as a passive homeostasis regulator of the cell and is not important for adapting to environmental stressors. It has been demonstrated that *Vc* uptakes exogeneous long-chain fatty acids and assimilates them into its membrane phospholipids. [4].

Of the range of PUFAs that *Vc* has demonstrated ability to uptake, we chose to focus on those found in the human intestine or commonly associated with marine life. This study will involve 4 total fatty acids, two of which are found in human intestines (18:2 and 20:4) and two that are found in marine environments (20:5 and 22:6). Fatty acids are long carbon chains with a carboxyl group at one end. Fatty acids present in these environments have been previously linked to membrane phospholipid remodeling, permeability, and cascades related to fitness[10, 11]. *Vc* is capable of transporting exogenous fatty acids through its outer membrane via FadL and then transporting them through the periplasmic layer through unknown means [12]. The human intestine is also rich in bile salts and antimicrobial peptides that are not present in marine life or oceanic environments [4]. Current research links long-chain fatty acid uptake with changes in cell permeability and membrane composition. Long-chain fatty acids are known to be heavily involved in membrane modification and virulence regulation [3]. Virulence regulation is thought to be linked to ToxT production. Certain long-chain fatty acids, such as 18:2, have been shown to affect DNA binding of ToxT, which would result in lowering virulence and pathogenicity [13]. Fatty acid concentrations are relatively high in bile and more specifically in the duodenal lumen. ToxT production is significantly lower at these conditions. However, ToxT production increases as long-chain fatty acid concentration decreases, which is similar to the conditions of

the epithelial wall. *Vc* at these conditions produces biofilm, CT, and becomes pathogenic. What is not well understood is the impact of long-chain fatty acid uptake on triggering certain stress resistant cascades and if these modifications are derived from LPS modification or inner membrane modification. Additionally, *Vc.* has been shown to break down these fatty acids using acyl-CoA, a coenzyme for fatty acid metabolism. Acyl-CoA attaches to the long-chain fatty acids to form fatty acyl-CoA esters which are then targeted for β-oxidation, phospholipid synthesis through phosphatidic acid production, or transcriptional regulation [11, 12, 14]. We aim to see if *Vc* is capable of using these long-chain fatty acids as an environmental marker for membrane modification that results in antimicrobial peptide resistance among other relevant phenotypes. This could help explain *Vc's* proficiency in cycling between drastically different environments and adapting to changes in pH, temperature, and salinity.

Cation antimicrobial peptides (CAMPs) are positively charged chains of amino acids that are capable of binding to the LPS of Gram-negative bacteria. Polymyxin B is a highly selective CAMP that can bind to the LPS at very specific places in order to disrupt the outer membrane. Polymyxin B (PMB) bonds tightly to the LPS with orders of magnitude tighter than the intramolecular forces that bind the lipid-A core together. Therefore, the ionic charge of the LPS is displaced and this disrupts assembly of the outer membrane. PMB is also capable of crossing the outer membrane and interfacing with the inner membrane. Here, PMB interacts with the inner membrane to inhibit cellular energization which damages DNA replications and can result in cell death [15]. *V. cholerae* El Tor is unique because of a recently discovered glycine modification that is capable of reducing the overall negative charge of the outer membrane, thus increasing its resistance to outer membrane disrupting CAMPs [16, 17]. A proposed model of polymyxin B interaction is shown below in Figure 1 [15]:

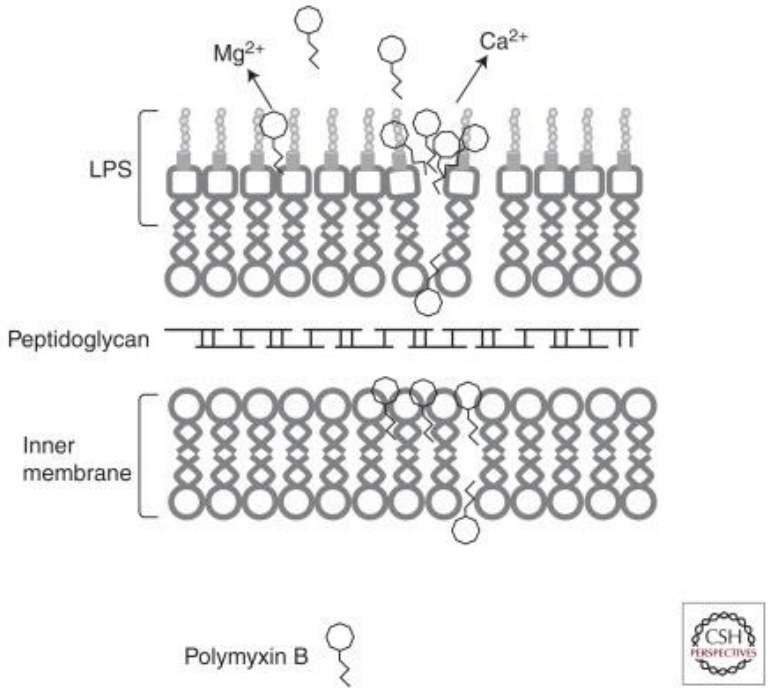


Figure 6: Polymyxin B Interaction with Inner and Outer Membrane

Polymyxin B is a cyclic cationic peptide that is soluble in aqueous environments. It was chosen for this project due to its ability to disrupt the inner and outer membranes of Gram-negative bacteria such as *Vc.*

Mutant strains of *V. cholerae* El Tor 1577 (AlmG) were provided by Dr. Giles from the biology, geology, and environmental sciences department. The mutant strain used for this project is unable to use the Alm-EFG operon for LPS modification. AlmF is transported across the inner membrane to the outer bioactive LPS. Here it serves as a donor for AlmG to add a glycine or diglycine group to LPS. This operon is not found in the classical biotype but is found in El Tor [18]. Therefore, the mutant strain used in the below experiments (*Vc* 1577) is incapable of adding a glycine or diglycine group to the LPS, which should therefore decrease resistance to CAMPs. Many other operons are suggested to be involved in inner membrane modification or antibiotic resistance, such as EptA and LpxR. However, the Alm-EFG variant was chosen due to

its reliance on inner membrane modification and the parameters of the chosen experiments. A suggested pathway for Alm-EFG outer membrane modification is shown below in Figure 7 [3, 19]:

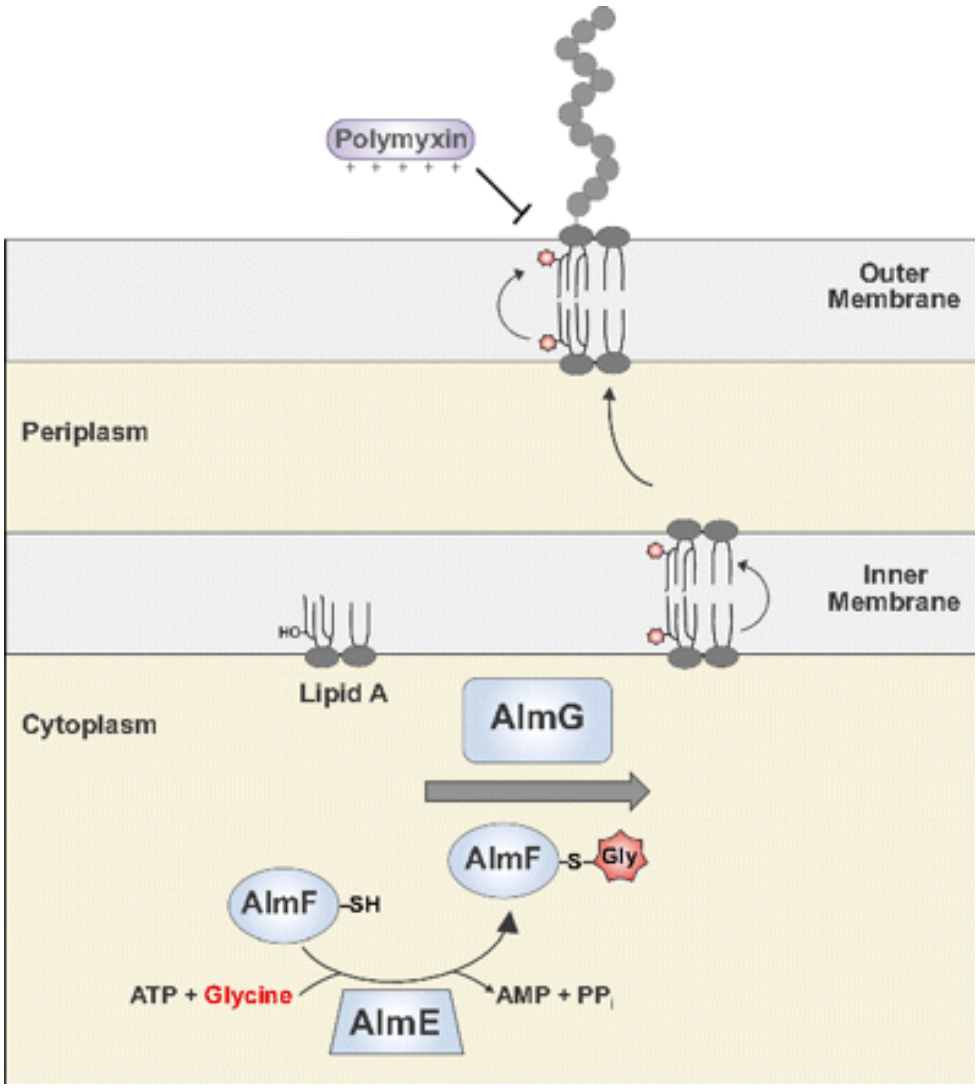


Figure 7: Diglycine Modification of LPS Structure via Alm-EFG Operon

This research will advance our scientific understanding of how bacterial pathogens like *Vc* use environmental markers to sense and adapt to stressors while cycling between host and marine reservoirs. This could lead to novel yet simplistic preventative measures for dealing with

bacterial pathogens, particularly those with increased resistance to antibiotics. It is possible that this research could lead to the identification of new treatment methods and preventative measures for *Vc* in addition to other pathogens that may rely on phospholipid membrane fortification for stress response.

**Methods**

*Vibrio cholerae* C6706 El Tor and the mutant strain *V. cholerae* El Tor 1577 (AlmG) were used throughout the experiments.

For liquid Luria broth (LB), 25 g of granulated LB was dissolved in 1 L of sterile DI water. For LB agar plates, 25 g of granulated LB and 15 g agar are dissolved in 1 L of sterile DI water. After autoclave sterilization, the solution is distributed among 50mm diameter Petri dishes.

For M9 minimal media, (28.2) g of M9 salts and 20.67 g of NaCl are dissolved in sterile DI water to make a 5x stock. Filter sterilized stock solutions stored include: 20% (w/v) glucose, 1M $MgSO_4$, 1M $CaCl_2$, and 20% casamino acids (CAAs).

For CM9 media, dilute 9ml of 5x M9 stock in 36ml of sterile DI water in a 50ml sterile conical. Then add 450ul of previously made CAA, 900ul glucose, 4.5ul $CaCl_2$, and 90ul of $MgSO_4$.

Antibiotic Resistance Assay

Antibiotic stock is made by dissolving 30 mg of PMB powder in 1 ml of sterile DI water.

1) Create 2 50 mL conicals of CM9 (*you will use a little more than 50mL per experiment day.)

a) Use the sterile graduated cylinder to measure 9 mL of the 5x M9 + NaCl into two 50mL conicals and add 36 mL of sterile water in each
    i) This creates a total of 45mL 1x M9 with 150mM (37°C) or 300mM (30°C) NaCl
b) Add 450μL filtered CAA to both conicals
c) Add 900μL filtered glucose to both conicals
d) Add 9μL filtered calcium chloride to both conicals
e) Add 90μL filtered magnesium sulfate to both conicals

2) Use a blank with 1000$uL$ of DI Water to set the reference point, then remove overnight cultures and record the ODs.
3) Pour 3 of the overnights into 2 15 mL conicals labeled ‘A1’ and ‘A2’ and spin for 10 mins at 4500 rpm.
    a) While spinning, label 5 test tubes ‘-’, ‘18:2’, ’20:4’, ’20:5’, ’22:6’ and put 5 mL of the CM9 (made in step 1) into each
    4.) Once done spinning, pour off the supernatant and fill the 15 mL conicals with 1 mL CM9 and spin for 5 minutes at 4500 rpm
    b) While spinning, retrieve FAs from black freezer downstairs and put 300mM in the corresponding test tube

| **FATTY ACID** | **VOLUME (μL)** |
|---|---|
| **18:2** | 0.54 |
| **20:4** | 1.83 |
| **20:5** | 1.83 |
| **22:6** | 1.97 |

4) Once done spinning, pour off supernatants and re-suspend again in 1 mL in CM9
5) Break up the pellets and then take the OD of the two 15mL conicals.
6) Start the 5 labeled test tubes at an OD of 0.2 with the following equation:

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

*remember to calculate culture from OD machine with an order of magnitude of 1.

Ex. Read 0.120 *apply order of magnitude of 1 gives* 1.20

7) Once solved for the equation add the calculated amount of culture to each of the 5 test tubes.
8) Place the 5 test tubes in the incubator shaking at 250 rpm at experimental temperature until the OD has reached 6-8 (approx. 1.5 hours)

Antibiotic Resistance: Part 2

1) At ≈1.5 hours, take OD of control (should be 0.6-0.8)
2) Create the PMB dilutions in 1.5mL microcentrifuge low-bind tubes as follows:

| **Number** | **Dilution** | **Volume of PMB stock (µL)** | **Volume of CM9 (µL)** |
|---|---|---|---|
| **1** | 0 | 0 | 500 |
| **2** | 40 | 4.5 | 495.5 |
| **3** | 80 | 9 | 491 |
| **4** | 160 | 18 | 482 |
| **5** | 320 | 35.5 | 464.5 |
| **6** | 640 | 71 | 429 |
| **7** | 1280 | 142 | 358 |

| | | | |
|---|---|---|---|
| **8** | 2560 | 284.5 | 215.5 |

3) Label the polypropylene 96-well microtiter plate as shown in the *Appendix*.
4) Add 30 μL of each PMB dilution to the corresponding wells
    a) Do this near the end of the growing period
5) Pour the 5 test tubes into 5 labeled 15mL conicals, balance the centrifuge, and spin for 10 minutes at 4500 rpm
    a) While spinning, pipette 3.5 mL of CM9 into another set of 5 15mL conicals
6) Once done spinning, pour off the supernatant and fill the culture filled 15mL conicals with growth with 1 mL CM9 and spin for 5 minutes at 4500 rpm
    a) While spinning, retrieve FAs stored at -20°C and add 300 mM to the corresponding 15 mL conical

| **Fatty acid** | **Volume (μL)** |
|---|---|
| **18:2** | 0.574 |
| **20:4** | 1.935 |
| **20:5** | 1.935 |
| **22:6** | 2.093 |

7) Once done spinning, pour off supernatant and resuspend in 1 mL of CM9
8) Break up the pellets and take the OD of the conicals

9) Calculate the amount of culture needed to bring the conicals to an OD of 0.117/0.235(?) according to the equation in step 7 of the antibiotic resistance part 1 procedure
    a) Bring the total volume to 4.5 mL by subtracting this value from 1000 and adding this amount of CM9 to each conical, using the table in the appendix to keep track of volumes
10) Add 170 μL of each conical to the corresponding rows
11) Place the plate in the incubator, taped down and on top of a rack, shaking at 250 rpm

Reading the Antibiotic Plate

1) Remove the plate from the shaking incubator after 20 hours and use the microtiter plate reader to read the plate at 600 nm
2) Ensure that no buildup has formed in the center of the well; if so, use a sterile pipette tip to stir the contents of the well.

## CFU Dilution Plate Assay

Antibiotic solutions are made by dissolving polymyxin B in CM9. The 10x stock concentrations are as follows: Low (10ug/ml), Mid (100ug/ml) and High (200ug/ml).

CFU Dilution Assay:

1. Inoculate 2 tubes of 5ml LB and shake overnight at 250rpm and 37ºC.
2. After 16-20 hours, transfer the overnight culture into 15ml conical tubes and centrifuge for 10 minutes at 4500 rpm.
3. Pour off the supernatant, being sure to not disturb the pelleted solids, and add 1ml of CM9 to the two conical tubes.

4. Put the conical tubes back in the centrifuge again for 10 minutes at 4500 rpm.
    a. While spinning, label 4 test tubes '-', 'Low', 'Mid', 'High', and put 4.5 mL of CM9 into each tube.
    b. For fatty trials, add 300mM of the desired fatty acids to each test tube
5. Pour off the supernatant and suspend in 1ml of CM9
6. Using the spectrophotometer, take the OD of 1000ul of DI water for a reference point.
7. Take the OD of each sample
8. Start the 4 labeled test tubes at an OD of 0.2 with the following equation:

$$culture\ volume\ (ul) = \frac{Desired\ OD * Total\ volume\ (ul)}{Culture\ OD} * 1000$$

9. Add the calculated amount of culture to each test tube.
10. Add 500ul of 10ug/ml antibiotic stock to the test tube labelled 'Low', 500ul of the 100ug/ml antibiotic stock to the test tube labeled "Mid", and 500ul of the 200ug/ml antibiotic stock to the test tube labeled "High". Add 500ul of CM9 to the test tube labelled '-'. This will create the desired antibiotic concentrations of 1ug/ml, 10ug/ml, and 20ug/ml for testing.
11. Place the 4 test tubes in the incubator shaking at 250 rpm at until the OD has reached 0.5-0.7 (approx. 2 hours)

12. Set up 2ul microcentrifuge tubes and label them as shown below

| | | | | | |
|---|---|---|---|---|---|
| -1 | -2 | -3 | -4 | -5 | -6 |
| L1 | L2 | L3 | L4 | L5 | L6 |
| M1 | M2 | M3 | M4 | M5 | M6 |
| H1 | H2 | H3 | H4 | H5 | H6 |

13. Fill each microcentrifuge tubes with 900ul of sterile DI water
14. Pipette 100ul of "-" culture and add to the microcentrifuge tube labeled "-1"
15. Shake the microcentrifuge tube until well mixed, and then pipette 100ul of the diluted mixture from "-1" into "-2". Repeat this dilution 6 times, and for the 3 other cultures with antibiotics.
16. Label two agar plates for each dilution (A and B).
17. Pipette 100ul of each dilution onto the appropriate two plates.
18. Submerge the cell spreader in 70% ethanol, flame it, and hold near the flame to dry.
19. Once cooled, apply a small amount of pressure and slowly spin the plate until an even layer of culture is visible.
20. Repeat step 17 and 18 for each plate for all dilutions.
21. Wrap each plate in parafilm
22. Incubate for 24 hrs at 37 ºC.
23. Count the number of colonies on each plate. The plate is not relevant if the number of colonies is below 30 or above 300.
24. Determine the number of colony forming units (CFU) by using the following formula

$$CFU = (\#\ of\ colonies)(0.1) * (10^n)$$

Where n is the dilution number.

25. To calculate percent survivability

$$Percent\ survivability = \frac{CFU\ experimental\ group}{CFU\ control\ group} * 100\%$$

Where control group is defined as the group without exogenous fatty acids or polymyxin B present.

$OD_{600}$

**Results and Discussion**

All collected data was done with the help of Devin Martin and Konner Glass, fellow undergraduate researchers working under Dr. Harris. The first method of testing polymyxin involves serial dilutions across a 96 well plate. Sample data is shown below in Figure 8:

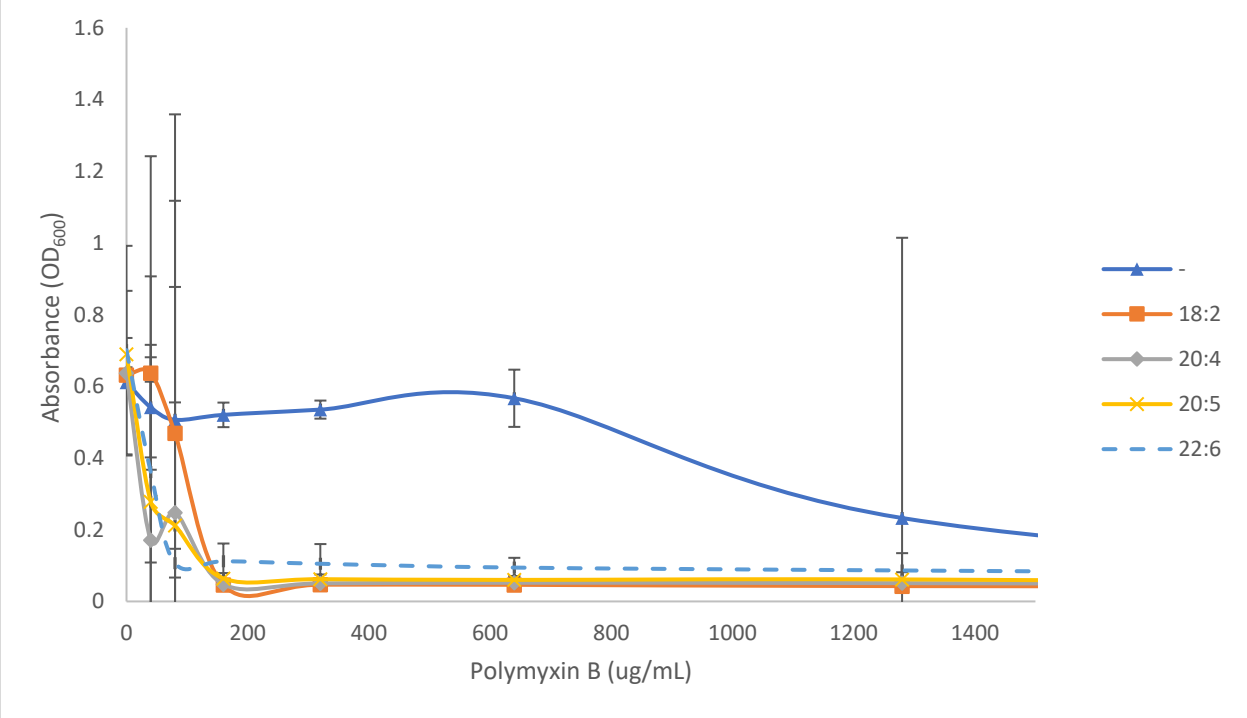


Figure 8: Absorbance vs. Polymyxin B across Long-chain Fatty Acids

Error bars represent a 95% confidence interval based on the Student's t distribution. Low and high concentrations (0-80 ug/ml and 1200 ug/ml) of polymyxin B resulted in large error bars when compared to the 200-600ug/ml range. Although this method is capable of testing a large range of antibiotic concentrations, we were unable to reproduce data consistently. Therefore, we made the decision to pursue a different assay.

A CFU plate dilution was chosen to determine growth of *Vc* under these conditions. Samples of *Vc* were grown with or without fatty acids. After an incubation period, aliquots of *Vc* were plated using a cell spreader. After an overnight incubation, cells were counted, and percent survivability was calculated. Percent survivability is a statistic that is based on the control, which for our purposes was chosen to be the trial with no fatty acids or antibiotics present. Therefore, percent survivability for the control will always be at 100%. A trial corresponding to a percent survivability below 100% means that the trial grew less than a control trial. A trial corresponding to a percent survivability above 100% means that the trial grew more than a control trial. It should be noted that each trial was performed only once, in duplicate, due to time constraints. Colony counts were averaged and then a CFU calculation was performed. Results for the wild type (*Vc* El Tor) are shown below in  Figure 9 and 5:

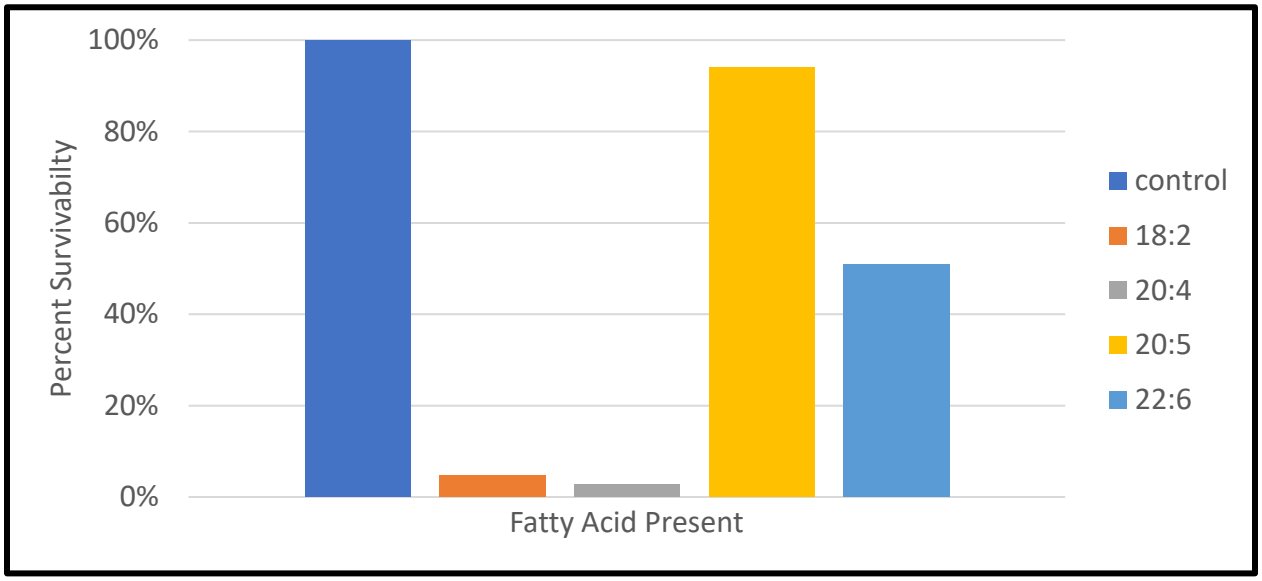


Figure 9: Percent Survivability of *Vc* El Tor Wild-Type, without Polymyxin B added

Percent survivability of the 18:2, 20:4, and 22:6 trials were significantly lower than the control or 20:5 trial. 18:2 and 20:4 are found in the human intestine. Therefore, it can be interpreted that fatty acids present in the human intestine hinder *Vc*'s ability to adapt to environmental stress through an as yet unknown mechanism. *Vc* is capable of cycling between drastically different environments. Therefore, long-chain fatty acids unique to each environment may be involved in signaling cascades to combat stress. As previously mentioned, this could be due to a ToxT operon interaction with these fatty acids. *Vc* has been shown to remodel its phospholipid layer to combat membrane stress [6, 8]. Meanwhile, the reduction in survivability was noticeably less pronounced for 20:5 and 22:6, which are omega-3 fatty acids commonly associated with marine life. Taken collectively, these data can be construed to suggest that long-chain fatty acids play a vital role in *Vc*'s ability to cycle between environmental niches.

Antibiotics by their very nature are expected to inhibit growth. Minimum inhibitory concentration (MIC) is the lowest concentration at which the antibiotic reduces growth of the bacteria by at least 1 log. Polymyxin B at multiple concentrations (1ug/ml, 10ug/ml, 20ug/ml) were used throughout the experimentation.

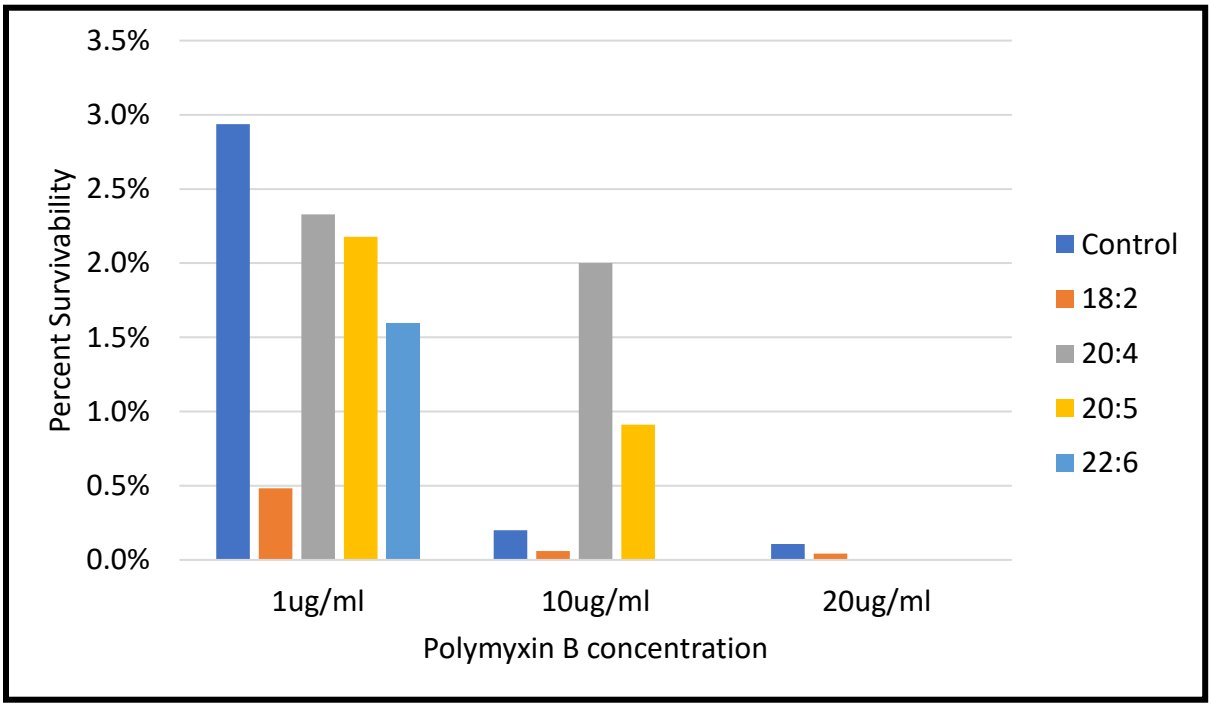


Figure 10: Percent Survivability as a Function of PMB for *Vc* El Tor Wild-Type

Percent survivability is calculated by finding the CFU of a trial with an antibiotic, and dividing by the control CFU (no antibiotic, no fatty acids present). In general, the fatty acid trials had reduced resistance to antibiotics. 20:4 and 20:5 showed increased survivability for the 10ug/ml trial and similar survivability for the 1ug/ml polymyxin B trial when compared to the control group. This is interesting when compared to drastically initial growth of 20:4 as shown previously in Figure 9. 20:5 also exhibited higher resistance to the 10ug/ml polymyxin B trial when compared to the control group. Generally, 18:2 and 22:6 exhibited less growth and less

resistance to antibiotic. The high antibiotic trial (20ug/ml polymyxin B) produced low percent survivability across all trials.

The same experiment was performed using the *Vc* 1577 strain, an AlmG deficient mutant. As previously stated, this strain is theoretically not capable of LPS modification that is relevant for polymyxin B resistance. The mutant strain control group data is shown below in Figure 11 and Figure *12*:

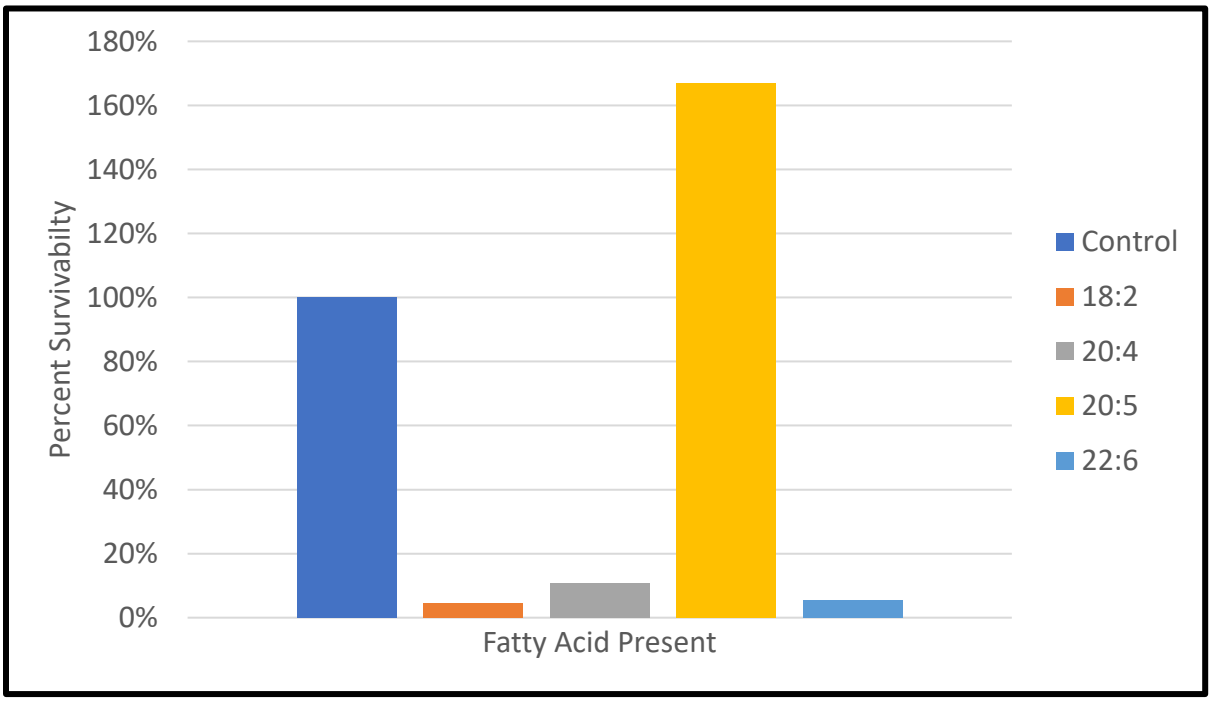


Figure 11: Percent Survivability for *Vc* 1577 (ΔAlmG), without PMB added

The 20:5 trial produced a 167% percent survivability, meaning that the trial grew more than the control group when polymyxin B is not present. It should be noted that the mutant control group had a significantly higher CFU count when compared to the wild-type trial (335500 vs. 197500). This could be due to the low amount of trials performed and the lack of

statistical analysis due to time constraints. When compared to the wild-type experiments, 18:2 and 20:4 exhibited similarly low percent survivability. 22:6 has a drastically lower percent survivability when compared to the wild-type experiments.

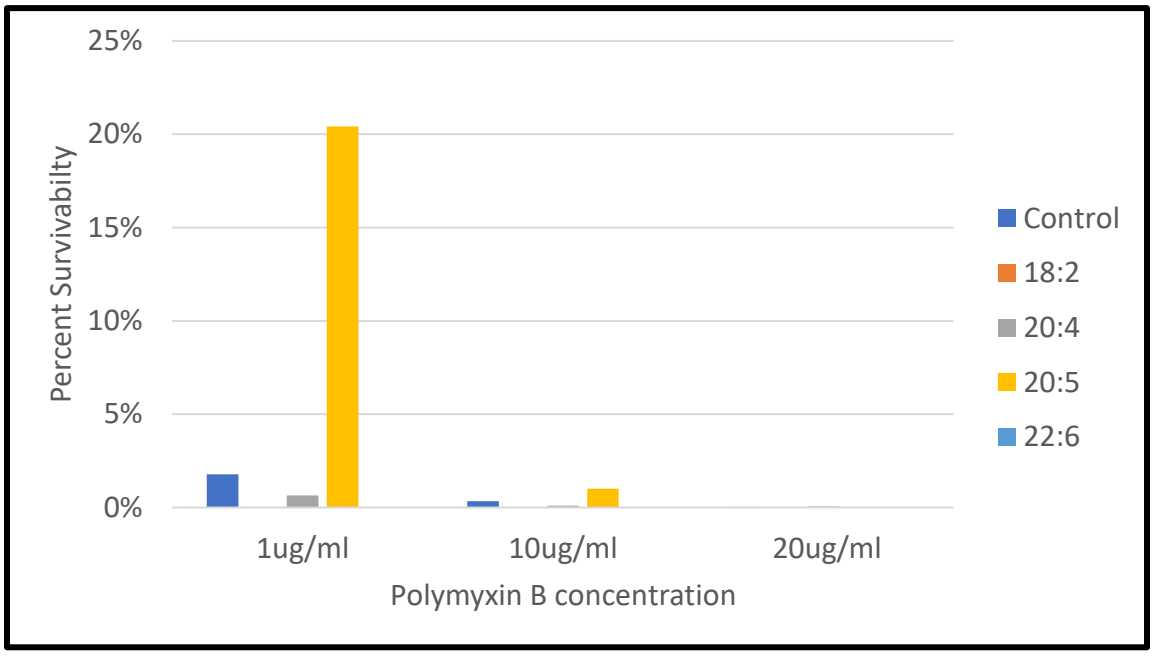


Figure 12: Percent Survivability as a Function of PMB for *Vc* 1577 (ΔAlmG)

The control group (no fatty acid present) has a percent survivability of 1.78% at 1ug/ml of polymyxin B, comparatively, the wild-type control group has a percent survivability of 2.93%. The 20:5 trial has a percent survivability of 20.41%, which suggests that *Vc* is capable of using 20:5 as a carbon source for reproduction and/or to modify its membrane to resist polymyxin B. The 10ug/ml polymyxin B trial has a 20:5 percent survivability of 1.01%, which is similar to the relevant wild-type trial. 18:2 and 22:6 exhibited almost no growth when exposed to 1ug/ml of polymyxin B, with percent survivabilities lower than 0.7%.

**Conclusion and Future Work**

*Vibrio cholerae* is a Gram-negative bacterium that is responsible for at least 21,000 deaths every year. Long-chain fatty acids present in human and marine environments interact with *Vc's* membrane and influence virulence, membrane composition, and antibiotic resistance [4, 12]. We suspect that *Vc* has evolved to uptake these long-chain fatty acids in order to cycle through different environments. This would involve adapting to changes in temperature, salinity, pH, and other stressors. Due to rising rates of antibiotic resistant pathogens, we chose to focus on the influence of fatty acid availability on antibiotic resistance. Our initial focus was to use a 96-well plate assay to test polymyxin B resistance for different fatty acids at a concentration of 350mM. However, due to unidentifiable issues, we were unable to reproduce data with acceptable error bars. Therefore, we made the decision to change our assay to a CFU plate assay which involves spreading serial dilutions on LB agar petri dishes, counting CFUs, and then calculating percent survivability. In addition to the wild-type strain, we tested a mutant strain (*Vc* 1577) which has the AlmG gene silenced. This renders *Vc* unable to fortify its outer membrane against CAMPs.

Interestingly, we found that the presence of 18:2 and 22:6 inhibited CAMP resistance for both strains of *Vc* tested. Additionally, these fatty acids reduced growth when polymyxin B was not present, while 20:4 and 20:5 had the opposite effect. They both had a neutral or positive effect on growth with or without polymyxin B present. This is interesting because 20:4 is a human fatty acid, and 20:5 is a marine environment fatty acid. Perhaps most surprising is that 22:6 had a negative effect on growth. This is a well-known carbon source for Gram-negative bacteria. It should be noted that plating 22:6 trials was an onerous endeavor, with many trials

ending in failure due to a lack of isolated colonies after overnight incubation. In light of this, there could be an interaction taking place with 22:6 causing an undercount of CFUs.

The motivation of this project is to find a new strategy to combat antibiotic-resistant microorganisms. The data collected throughout this project suggests that antibiotic resistance can be directly affected by environment cues such as fatty acid concentration. We suggest that this resistance is due in part to outer membrane modification through the uptake and utilization of long-chain fatty acids. The mutant *Vc* experiments suggest that Alm-EFG is involved in antibiotic resistance. However, the observed effect was not consistent across the range of fatty acids tested. We hypothesize that *Vc* utilizes 20:5 in particular to fortify the inner leaflet of its outer membrane, thereby conferring increased CAMP resistance. This hypothesis was formulated based on the percent survivability results shown in Figure 12. Other studies report a polymyxin B MIC of over 100ug/ml for *Vc* El Tor, and a MIC of 0.5-1.5 ug/ml for Alm-G mutants [3, 17]. Our data suggests a much lower MIC for *Vc* El Tor. Other studies rely on polymyxin B gradient test strips to assess MIC and not the plating method used in this project. A plating based assay was advantageous for this project due to the focus on comparing exogenous long-chain fatty acid uptake on antimicrobial resistance. The future work for this project would begin with performing each trial multiple times to attain statistical significance. It is unclear if any of the above data is relevant due to the low number of trials performed. This method could also be repeated using human defensins and other host-specific antimicrobial peptides. This would be vital for understanding how *Vc* adapts to the host immune response. Lastly, future work would also include evaluating a range of *Vc* mutants containing deletions to the outer membrane remodeling machinery.

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## Appendix

**Table 1: Wild-Type CFU vs. Fatty Acid Present**

| Fatty Acid Present | Colony Forming Units |
|---|---|
| Control | 197500 |
| 18:2 | 67000 |
| 20:4 | 5250 |
| 20:5 | 185500 |
| 22:6 | 100500 |

**Table 2: Wild-Type CFU vs. Fatty Acid Present and Polymyxin B Concentration**

| | Polymyxin B Concentration | | |
|---|---|---|---|
| **Fatty Acid Present** | **1ug/ml** | **10ug/ml** | **20ug/ml** |
| Control | 5800 | 395 | 210 |
| 18:2 | 955 | 117 | 86 |
| 20:4 | 4600 | 3950 | 0 |
| 20:5 | 4300 | 1800 | 0 |
| 22:6 | 3150 | 0 | 0 |

**Table 3: *Vc* 1577 CFU vs. Fatty Acid Present**

| Fatty Acid Present | Colony Forming Units |
|---|---|
| Control | 335500 |
| 18:2 | 15500 |
| 20:4 | 36500 |
| 20:5 | 560000 |
| 22:6 | 18650 |

**Table 4: *Vc* 1577 CFU vs. Fatty Acid Present and Polymyxin B Concentration**

| | Polymyxin B Concentration | | |
|---|---|---|---|
| **Fatty Acid Present** | **1ug/ml** | **10ug/ml** | **20ug/ml** |
| Control | 6000 | 1125 | 77.5 |
| 18:2 | 27 | 4.5 | 0 |
| 20:4 | 2200 | 330 | 235 |
| 20:5 | 68500 | 3400 | 0 |
| 22:6 | 44 | 0 | 0 |