VIBRIO VULNIFICUS TYPE 6 SECRETION SYSTEM EXHIBITS INFLUENCE IN MARINE AGGREGATE COLONIZATION

by

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A Thesis
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Master of Science
Biology

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A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at George Mason University

by

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Bachelor of Science
George Mason University, 2019

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Fall Semester 2022
George Mason University
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DEDICATION

This is dedicated to my mother for always being a pillar of support.

ACKNOWLEDGEMENTS

Thank you to all my committee members, especially to Dr. Froelich for his continued guidance throughout this research.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF EQUATIONS

LIST OF SYMBOLS

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ABSTRACT

VIBRIO VULNIFICUS TYPE 6 SECRETION SYSTEM EXHIBITS INFLUENCE IN MARINE AGGREGATE COLONIZATION

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George Mason University, 2022

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Oysters are among the most economically beneficial species with regards to aquaculture, the process of farming aquatic species. As such, in order to ensure that oyster farming remains profitable, it is necessary to maintain a product that is safe for consumption. As oysters are oftentimes consumed raw, they have been known to cause seafood-based disease, particularly from bacteria in the genus *Vibrio*. *Vibrio* infections can be costly to treat and depending on the severity of disease, can even be fatal. With sea water temperatures rising due to global climate change, infection rates have also been on the rise. There is therefore a need to rapidly develop therapies that will prevent a further number of clinical cases. Gaining a better understanding of *Vibrio* ecology would aid with doing so, however, data examining this is limited. Previous studies have examined higher oyster uptake levels when bacteria are integrated onto marine aggregates. This project aims to observe the relationship between three different strains of pathogenic *Vibrio vulnificus* species both on artificially generated marine aggregates and within oyster matrices.

This study found that within both aggregate and oyster competitions the three Vibrio vulnificus strains initially displayed an “rock-paper-scissors" effect. In competition between two Environmental (E) genotype strains VV JY1305 pVSV102 (hereby dubbed VV5-102) and SREL 106 pVSV208 (VV8-208), VV5-102 was outcompeted. Strain VV5-102 was able to outcompete the Clinical (C) genotype strain VV C7184 pVSV208 (VV2-208). Unexpectedly, VV2-208 was found to be more abundant than strain SREL 106 pVSV102 (VV8-102). However, this was later understood to be the occurring due to the presence of the plasmid. There was a novel discovery made that established the type 6 secretion system plays a role in integration of *V. vulnificus* into natural marine aggregates. These results expand upon our understanding of *Vibrio* interactions as well as highlight the need for more uptake experimentation in order to create viable treatments.

CHAPTER ONE: INTRODUCTION

The Importance of Aquaculture

Aquaculture, or aquafarming, is an essential food production system that allows for the farming of organisms such as aquatic plants, fish, and mollusks. It is a billion-dollar industry, bringing in around $1.5 billion worth of seafood in 2017, with among the most profitable species being oysters, clams, and salmon (By the Numbers | FishWatch, n.d.). Oysters, specifically, are not only a good source of nutrients such as niacin, vitamin A, and vitamin B12 but also play a role in providing multiple types of ecosystem services (Venugopal & Gopakumar, 2017). such as the creation of habitats for fish, environmental nitrogen regulation, phytoplankton & sediment filtering, and protecting estuarine environments from shoreline erosion (Beseres Pollack et al., 2013; Grabowski et al., 2012).

Oysters

Oysters belong to the bivalve family Ostreidae, which include around 75 species, of which around 18 are consumed by humans (Salvi et al., 2014, Ruesink et al., 2005). One of the most commonly consumed and economically important species is *Crassostrea virginica* (the Eastern oyster), that generated an estimated $193 million in 2021 (HOME, n.d.). They reside in estuaries along the coasts of the Atlantic Ocean, in depths of over 5 meters (Ruesink et al., 2005). *C. virginica* are crucial as they help provide the creation of hard-substrate biogenic reefs, which become habitats for many invertebrates including

sponges, corals, crabs, shrimp, and for various fish species (Ruesink et al., 2005; Beseres Pollack et al., 2013). This is accomplished through large-scale filter feeding, where sediments composed of feces are formed from particles that oysters separate out of the water (Ruesink et al., 2005). The reefs in turn provide a physical barrier that prevents soil erosion (Grabowski et al., 2012, Ruesink et al., 2005). Oyster reefs have also been shown to improve overall water quality through the reduction of nitrogen levels. *C. virginica* specifically maintain high levels of nitrogen removal when compared to other bivalves (Beseres Pollack et al., 2013). Oysters do this by placing nitrogen onto sediments, thereby increasing denitrification rates (Beseres Pollack et al., 2013, Grabowski et al., 2012). Nitrogen is also absorbed into both the tissue and shell, and subsequently removed once oysters have been harvested (Beseres Pollack et al., 2013). Despite all the perks that oysters provide, there is a large risk that comes with human consumption as they can often contain within them large amounts of the bacteria known as *Vibrio*.

CHAPTER TWO

Vibrio

Vibrio spp. are gram-negative, rod-shaped bacteria that belong to the family Vibrionaceae. There are more than 100 species, consisting of several that are pathogenic to both humans and marine organisms. *Vibrio* live in marine environments and are typically found in higher concentrations during times when the water temperature is warmer. In general, *Vibrio* are found at low densities at temperatures below 20^{o}C and at high densities between 20-30^{o}C (Tantillo et al., 2004). Pathogenic *Vibrio* bacteria can pose a significant health concern to individuals who consume raw or undercooked seafood, especially shellfish. Among the most common pathogenic species are *Vibrio vulnificus* and *V. parahaemolyticus*. It is estimated that annually in the US there are over 52,000 cases of foodborne illnesses from pathogenic *Vibrio* species (Scallan et al., 2011). *Vibrio vulnificus* specifically is responsible for 95% of seaborne deaths, with 96% of cases caused from the consumption of raw oysters (Oliver, 2013). The number of *Vibrio* infections have also been on the rise since 2007, more than doubling in incidence (CDC, 2018). Recent evidence also suggests increases in water temperatures due to climate change will cause the incidence of infection to become greater (Burge et al. 2014 and Vezzuli et al., 2012). Oysters are the main reservoirs of infection as they are normally consumed raw. Therefore, conducting further research on *Vibrio* uptake and interactions within contaminated oysters can help to create future therapies for eradicating pathogenic species and aid in reducing the number of future cases in humans.

Vibrio infection from consumption of contaminated or raw seafood can result in either gastroenteritis and/or septicemia (Tantillo et al., 2004). Gastroenteritis, or inflammation of the intestines can occur in infections from both *V. vulnificus* and *V. parahaemolyticus* (Baker-Austin et al., 2018). Symptoms include diarrhea, vomiting, cramps, and in severe cases it can bring about fever, chills, and bloody stools (Tantillo et al., 2004). There is limited mortality associated with gastroenteritis infection alone (Daniels and Shafaie, 2000). Septicemia occurs when bacteria enter the bloodstream through the portal vein or the intestinal lymphatic system (Tantillo et al., 2004). The main symptoms associated with the condition are abdominal pain, nausea, vomiting, and diarrhea (Tacket et al., 1984). Around 70% of patients also develop characteristic bullous skin lesions in lower regions due to the transfer of fluid and proteins from blood to tissues (Heelan, 2001). In humans, *V. vulnificus* can cause primary sepsis with mortality rates reported to be at 50% (Heng et al., 2017).

V. vulnificus species are broken up into three biotypes depending on their biochemical and serological properties. Biotype 1 is the most common, normally found in brackish water while biotypes 2 & 3 are more rare (Heng et al., 2017). Unlike other *Vibrio* species, *V. vulnificus* is an opportunistic pathogen; usually affecting patients with liver impairment and weaker immune systems (Baker-Austin et al., 2018). Around 80% of individuals that become infected have liver disease resulting in increased levels of serum iron (Baker-Austin et al., 2018). *V. vulnificus* is also found to be more lethal in association with elevated iron levels; being thought to collect it from transferrin proteins (Drake et al., 2007).

Shellfish oftentimes harbor great amounts of pathogenic *Vibrio* bacteria within them. They push seawater through their gills to filter feed and to harvest oxygen, and as consequence collect many microorganisms, such as *Vibrio* species that are present, at concentrations up to 100 times that of overlying water (Jaksic et al., 2002; Drake et al., 2007). Shellfish carrying *V. vulnificus* show no difference in taste, odor, or appearance compared to uncontaminated ones (Horseman and Surani, 2011). As a result, there is no clear way of determining whether an organism is contaminated with the pathogen.

The risk of *Vibrio* infection is also increasing as water temperatures around the world continue to rise. Around 85% of infections occur between the months of May through October, when the water is warmest (Oliver, 2013). However, the prime infection period is broadening as temperatures remain higher for longer into the year. Outbreaks of *Vibrio* infection have spread further than before as has been recently documented in northern Europe, where there has been an increase in abnormally warm summer weather (Baker-Austin et al., 2018). For instance, in 1994, the first cases of infection were identified along the Baltic Sea and in 2014, 89 cases were found in Sweden & Finland (Burge et al., 2014; Baker-Austin et al., 2017). Climate change also induces an uptick in natural disasters like hurricanes and floods which in turn cause *Vibrio* to be spread across much further than it normally would (Burge et al., 2014). As such, there is also the risk of bacterial blooms, given as *Vibrio* has a generation time of up to 20 minutes (Froelich & Daines, 2020). With infection becoming more common, it is essential to focus on the risk of transmission via consumption of shellfish.

The price for treating *Vibrio* infections is very high, with the annual cost in the US being $350 million in those exposed to *V. vulnificus*, *V. parahaemolyticus*, and *V. alginolyticus* (Heng et al., 2017). Current procedures that allow for seafood to be safely consumed (i.e. flash freezing & pasteurization) are fatal to shellfish and therefore will hurt the aquafarming industry (Muth et al., 2002). Focusing on methods to clear pathogenic *Vibrio* from organisms such as oysters will make them safer for consumption; aiding in reducing the number of occurrences and time spent on treatment in humans. However, our understanding of pathogenic *Vibrio* colonization activity within oysters is still largely unexplored and will need to be better understood in the hopes of developing potential therapies.

Earlier studies examining oyster uptake would add bacteria directly to water and would find lower efficiency rates than present in the environment (Froelich et al., 2013). *Vibrio* is known to attach itself to particulates within the water. These particles are known as marine snow or marine aggregates and are formed together via Brownian motion. They are longer than 500 µm in length, and are composed of fecal pellets, larvacean houses, phytoplankton, and other various inorganic debris (Alldredge & Silver, 1988). Marine aggregates harbor bacteria in higher concentrations (2-5 orders of magnitude) compared to surrounding seawater (Alldredge & Silver, 1988). For *C. virginica* specifically, particulates need to be between 5 to 7 µm in diameter to ensure successful uptake, whereas the typical *Vibrio* bacterium is only around 2 µm (Froelich et al., 2013). However, given their fragile nature marine aggregates are unable to be collected. Therefore, artificial aggregates must be generated.

There are various factors that affect aggregate attachment and subsequent oyster colonization, one of which being genotypes. Two genotypes are associated with *Vibrio*, E-type (of which come from environmental isolates) and C-type (from clinical isolates). C-genotype strains are associated with infection, being found in 93% of clinical cases (Rosche et al., 2005). They have been found to have more resistance to human serum and are more likely to retain their capsular polysaccharide major virulence factor (Rosche et al., 2010). Despite E and C-types existing in similar amounts within surrounding ocean water, E-types are found to be the majority within oysters, consisting of around 85% of *Vibrio* collected (Rosche et al., 2010). Previous competitions on marine aggregates have found that E-genotypes outcompete C-genotypes (Froelich et al., 2013).

Another factor that is potentially aiding some *Vibrio* strains in colonization is the type 6 secretion system (T6SS). The T6SS consists of 13 proteins that form a "syringe-like" structure used to transport cytotoxic effectors into an adjacent cell (Bingle et al., 2008; Church et al., 2016; Hubert & Michell, 2020). Recently, it has been observed that *V. vulnificus* strains can contain two T6SSs, T6SS1 and T6SS2 (Church et al., 2016). T6SS2 is present within all Vibrio strains while T6SS1 is only found in some (Church et al., 2016). Both *V. vulnificus* T6SSs have been shown to be involved in both interspecies and intraspecies killing as opposed to in other bacteria which just secrete anti-eukaryotic effectors (Church et al., 2016; Hubert & Michell, 2020).

A recently discovered, non-pathogenic, *Vibrio fluvialis* strain has been found to kill *V. vulnificus* and *V. parahaemolyticus in vitro*. However, it has not been tested to observe if this phenomenon also occurs within oysters. If viable, this killer strain could be used as

a therapy in oysters with the potential to reduce both the number of cases and the cost of treatment as it would stop the spread of infection.

This study aimed to compete three different strains of *Vibrio vulnificus* with one another to measure the colonization activity on both marine aggregates (i) to examine initial integration and then within oyster matrices (ii) to observe if the same patterns continue. The T6SS would be examined (iii) to determine its role in aggregate competitions. Finally, *V. vulnificus* was integrated onto aggregates in a competitive coculture alongside *V. fluvialis* (iv) to measure whether there is a reduction in pathogenic cells *in viv*o as was previously observed *in vitro*.

CHAPTER THREE: MATERIALS & METHODS

Bacterial strains and growth conditions

V. vulnificus strain C7184 pVSV208 (VV2-208) is a C-type strain with chloramphenicol resistance. Strains SREL 106 pVSV208 (VV8-208) and JY1305 pVSV102 (VV5-102) are E-type strains with chloramphenicol and kanamycin resistance respectively. The strains with reverse primers are C7184 pVSV102 (VV2-102), SREL 106 pVSV102 (VV8-102), and JY1305 pVSV208 (VV5-208). Other strains used were VV5-208 T6SS Knockout (KO) and NCTC11327 (*V. fluvialis*). Antibiotic-resistant strains were grown on brain heart infusion agar with the addition of 1 g liter−1 kanamycin or 0.3 g liter−1 chloramphenicol. Vibrio vulnificus strains had kanamycin or chloramphenicol resistance to differentiate them from marine bacteria within both aggregation and oyster uptake experiments.

For competition experiments, CHROMagar™ Vibrio (Kanto Chemical Co, JP) was used as the medium, with 74.7 g of powder base used per liter of deionized water. Broth was microwaved until clear and then supplemented with either 1 g liter^{-1} kanamycin or 0.3 g liter^{-1} chloramphenicol. *Vibrio vulnificus* appeared as blue colonies on plates.

Oyster Collection

Oysters local to the eastern coast/Virginia were acquired (*Crassostrea virginica*) by purchasing through a commercial aquaculture facility. A total number of 50 oysters were transported to the laboratory at George Mason University. The oysters were split into in half and kept in separate tanks within the lab to be monitored and fed daily.

Oyster Care in Aquaria

Before being placed in the tanks, any unclean oyster shells were cleaned using filtered ocean water and a scrub brush. The aquariums were equipped with airline tubing, an aerator, and air stones. The tanks had artificial sea water (ASW; Instant Ocean, Blacksburg, VA) and were kept at 23° C. Water was passed through a 0.45µm filter (Tetra, Blacksburg, VA) and retained 16‰ salinity using deionized water. Oysters were fed a mixture of fish meal, spirulina, and kelp (Kent Marine, Franklin WI). Any deceased oyster was removed immediately.

Marine Aggregate Generation

Laboratory-grown marine aggregates were generated using the method of Sharks and Emmerson (1989) with modifications used by Ward and Kach (2009), as done in Froelich et al. (2013). Chosen bacterial strains were normalized to an OD600 of 1.0 in 2.5 mL. Next, 25 µL of each strain was added to natural seawater (NSW: collected from the coast of Yorktown, VA) of at least 18‰ salinity in 250 ml roller bottles. 10 µg/liter of hyaluronic acid was added to aid in aggregation. The bottles were placed on a roller machine for 24 hours at maximum rotation. Static bottles placed in the same orientation served as non-aggregated controls. Bacterial incorporation was measured by placing all bottles at a 45-degree angle for 20 minutes to allow for aggregates to settle, making sure to invert the control bottles three times before doing so. Next, 750 µl of sample containing aggregates/non-aggregated particles was collected, vortexed to disrupt the aggregates, and spread on appropriate antibiotic plates. Each experiment had 3 rolled bottles and 3 static bottles. In uptake experiments, 5 oysters would be selected and placed into experimentation

tanks with 4 L of artificial seawater and airline tubing. Bottles with the formed aggregates were inverted three times before being poured over the oysters during their feeding time. Time zero results were gathered with oysters that did not receive any bacterial treatment.

Oyster Dissection

After 24 hours, oysters were removed from experimentation tanks. They were rinsed with 70% ethanol to remove external bacteria, patted dry, and shucked with a flame-sterilized oyster knife. Oyster meat was placed in a sterile 50-ml conical tube and weighed. 20 ml of phosphate buffer saline (PBS) was added to each tube before homogenizing in sterile blending cups (Waring, Torrington, CT). Blending was done 3 times for 15 s with 5 s of pause in between.

Sampling

Homogenized samples were serially diluted in sterile PBS and plated on the appropriate antibiotic media. The total CFU/g of oyster tissue was also calculated. Equation 1: Raw Count*10((Oyster Weight + PBS Volume)/Oyster Weight).

Statistics

Data was compared either with an unpaired T-test, a one-way analysis of variance (ANOVA) with Tukey's multiple comparisons post hoc test, or a Kruskal-Wallis test with Dunn's multiple comparisons. Data was analyzed using GraphPad Prism version 9.4.1.

Scope

Cells were grown in an overnight culture, normalized, and mixed in a 1:1 ratio. 10 µl of each competition was spotted on BHI agar in a 12-well plate and grown in 37° C for 24

hours. Green and red fluorescence was observed using a Cytation 5 Cell Imaging Multimode Reader (BioTek, Winooski, VT).

CHAPTER FOUR: RESULTS

Marine aggregates generated significantly higher bacterial uptake

Marine aggregates were generated in bottles that were placed on the roller machine but not in static bottles. When strains were placed in a competitive coculture there was significant difference ($P < 0.0001$, Figure 1) seen in bacterial between aggregated and non-aggregated cells. However, there was no such difference observed in cells within non-aggregated bottles (Figure 1). Similar results were concluded in a study by Froelich et al (2013).

Competitions between V. vulnificus strains initially demonstrated a "rock-paper-scissors" pattern of integration into aggregates

VV5-102 vs VV8-208

Initial uptake experiments were conducted by adding 2.5 ml of each bacterial strain to the aggregates. Although no significant difference was observed (Figure S1), VV8-208 did appear to be present in larger amounts. To prevent bacterial overload all future aggregate competitions bacterial was reduced to 25 µl per strain. When done so, VV8-208 was found to outcompete VV5-102 (Figure 2A, ANOVA; Tukey's multiple comparisons: $P < 0.0001$).

VV5-102 vs VV2-208

There was a statistically significant difference in integration between VV5-102 and VV2-208 with VV2-208 showing less aggregate affinity (Tukey's: $P < 0.05$, Figure 2B).

VV8-102 vs VV2-208

Given that VV8-102 is an E-genotype strain it was expected to outcompete the C-type strain VV2-208. However, the opposite was found to be true, with VV8-102 having significantly less integration (Tukey's: $P < 0.05$, Figure 2C).

Scope pictures and Oyster Competitions corroborated aggregate results

Bacterial strains were integrated onto aggregates and poured over oyster tanks for uptake and sampled after 24 hours of incubation. Uptake experiments found the same patterns of bacterial concentrations as observed in the aggregate experiments. Higher uptake was found for VV8-208 (in competition between VV5-102 & VV8-208, Tukey's: $P < 0.01$, Figure 3A), VV5-102 (VV5-102 & VV2-208, Tukey's: $P < 0.05$, Figure 3B), and VV2-208 (VV8-102 and VV2-208, Tukey's: $P < 0.05$, Figure 3C). Strains that were outcompeted also had no statistical difference when compared to time zero results.

The three competitive co-cultures were spotted onto BHI, incubated for a day, and placed under a microscope to observe fluorescence activity. Again, the same pattern was observed (Figure 4).

Aggregate competitions using the opposite plasmids yielded differing results

To account for any variances in growth and potential integration onto aggregates the same competitions were run with the opposite plasmids.

<u>VV5-208 vs VV8-102</u>

Interestingly, there was no significant difference noted between cells from aggregated bottles (Figure 5A). The overall pattern of integration was switched, with VV5-208 displaying higher levels of integration on average compared to VV8-102.

<u>VV5-208 vs VV2-102</u>

Although there was no statistically significant difference between cells on aggregates, it followed the same pattern seen previously with VV5 being present in higher amounts compared to VV8 (Figure 5B).

VV8-208 vs VV2-102

Here it was observed that VV8-208 significantly outcompeted VV2-102 (Tukey's: $P < 0.05$, Figure 5C). This contradicted previous results and called into question the effectiveness of the plasmids on VV8.

VV8-102 was likely being affected by the addition of the plasmid

Results of all previous competitions were combined to observe the pattern of aggregate integration. There was a significant difference observed between VV5/VV2 (Tukey's: $P < 0.05$, Figure 6B), but none was seen between VV5/VV8 (Figure 6A) and VV2/VV8 (Figure 6C). With the common denominator of both being VV8, it is probable that the plasmids, specifically in VV8-102 were negatively impacting growth. Competitions between VV2/VV8-102 and VV2/VV8-208 displayed similar results (Figure S3).

T6SS1 Knockout reduced integration of VV5 onto aggregates

Aggregate competitions between VV5 and VV2 found that VV5 was more highly concentrated regardless of plasmid. As VV5 has two T6SSs, it was necessary to examine whether T6SS1 was affecting colonization via intraspecies killing. VV5-208 T6SS knockout (KO) strain was competed with VV2-102. Despite not observing significant results between aggregates there was a reduction in the amount of VV5 compared to VV2, both in aggregated and non-aggregated bottles (Figure 7).

V. fluvialis was not found to reduce pathogenic Vibrio when integrated on aggregates

V. fluvialis has been found to have killing properties against pathogenic *Vibrio in vitro*. *V. fluvialis* was placed in a competitive coculture with VV8-208 and rolled to form aggregates. Initial experimentation found no significant difference in the concentration of pathogenic *Vibrio* (Figure 8A). The concentration of *fluvialis* was then doubled, however there was still no statistically significant difference (Figure 8B). The salinity of ocean water then was concentrated to 5 ‰ to replicate conditions found on plates. Despite this, VV8-208 showed no reduction (Figure 8C).

CHAPTER FIVE: DISCUSSION

The ecology surrounding *V. vulnificus* colonization of marine aggregates and oysters is essential to understanding how to prevent the further spread of disease. In this study, it was initially discovered that C-genotype VV2-208 outcompeted E-genotype VV8-102 in both marine aggregates and oysters, however it was later shown that it was the addition of the plasmid that was affecting results. Given previous studies which demonstrate greater concentrations of E-genotypes in aggregates (Froelich et al., 2013) it is more likely that the plasmid pVSV102 (Green Fluorescence Protein, GFP and Kan^r) is responsible. Dunn et al. (2006) observed no discernable difference between *V. fischeri* cells with pVSV102 or pVSV208 and wild-type parent strains. However, fluorescent proteins can have a negative impact by lowering fitness among other gram-negative bacteria. The doubling time of multiple bacterial species increased proportionally to greater GFP expression (Rang et al., 2003). This is possibly due to the presence of the protein being a greater energy expenditure for the cell (Allison & Sattenstall, 2007). Rekecki et al. (2012) noted a growth delay for GFP-containing strains *in vivo* compared to *in vitro*, citing the likely cause to be a change in nutrient availability. Other species of *Vibrio* which contained GFP had a reduction in swimming motility, but otherwise showed no changes in virulence (Wang et al., 2021). Bacterial flagella have been shown to assist in attachment and biofilm formation within marine aggregates and oysters (Lyons et al., 2007, Kiørboe et al., 2003, Duan et al., 2013). Therefore, it is possible that GFP is causing either one or both of these effects in VV8 but seemingly not in VV5 and VV2. This phenomenon should be further

investigated, and future studies should take into account the negative effects that come with the protein.

E-genotype strain VV5 was found to outcompete the C-genotype strain VV2. This pattern of genotype colonization has been documented before (Rosche et al. 2010, Froelich et al., 2013, Lydon et al., 2021). Further testing VV2 against VV5 with a T6SS1 Knockout found that while it was not significant there was a decrease in VV5 compared with previous competitions, indicating that there was no longer killing occurring. This is the first case in which it is distinctly seen that the T6SS is a driving factor in intraspecies competition between C- and E-genotype strains in natural marine snow. However, given that the reduction seen was not significant it suggests that other factors have more of an impact with regards to integration onto aggregates. While the exact reasoning for higher concentrations of E-genotypes is still not fully understood, it has been linked to differences in motility, quorum sensing, and expression type IV pilin attachment genes (Froelich et al., 2013, Lyons et al., 2007, Phippen & Oliver, 2015). In *Vibrio* and within other bacterial species the type IV pilin has been found to aid in adherence and biofilm formation in both biotic and abiotic surfaces (Paranjpye et al., 2007, Phippen & Oliver, 2015, Pombo et al., 2022). Specifically, the *pilA*, *pilD*, and *mshA* genes are responsible for increased attachment; with E-genotypes displaying higher expression levels of these genes compared to C-genotypes (Paranjpye et al., 2007, Phippen & Oliver, 2015). Within marine aggregates, chitin is a major polymer that *V. vulnificus* tends to attach to (Froelich et al., 2013, Phippen & Oliver, 2015). C-genotypes in response to increased stressors have been shown to increase production of autoinducer-2 (AI-2) molecules (used in quorum sensing),

leading to more detachment from chitin (Phippen & Oliver, 2015). It is likely then, that it is a combination of attachment genes, reduced ability to detach, and within some strains the presence of T6SS1 in tandem allow for greater colonization of E-genotypes compared to C-genotypes.

Finally, while *V. fluvialis* previously demonstrated killing on plates, the same effect was not observed on aggregates. It is possible that *V. fluvialis*' killing ability is inhibited due to background bacteria present in natural seawater, decreased binding affinity, or differences in salnitity (5‰ on plates vs 18‰ in seawater). Further testing will be needed to determine the cause.

Due to the low number of competitions and strains examined, future studies will be necessary to establish more definitively the results presented in this study. The adverse effect that fluorescent proteins had on aggregate colonization in VV8 are likely to be found in other strains as well. A possible solution is analyzing the competition between wild-type strains in addition to the strains with plasmids. Hubert & Michell (2020) developed a methodology using artificial marine snow, which was found to be easier to control bacterial integration. Using some form of artificial marine snow could also be used alongside sterile ocean water that therefore lacks the background bacteria present in natural sea water that makes it impossible to examine strains without the use of a plasmid. Competing strains would then be plated on appropriate differential media such as mannitol. Artificial marine snow could also be useful in examining the killing ability of *V. fluvialis*. More work will also need to be done to examine the role of T6SS1 by using knockout strains. In conclusion,

this research expands upon the previously established role of the T6SS within *Vibrio vulnificus* strains on marine aggregates.

CHAPTER SIX: TABLES & FIGURES

Table 1 Strain Names

Strain Abbreviation	Strain Name	Clinical/Environmental Isolate
VV2	VV C7184	Clinical
VV5	VV JY1305	Environmental
VV8	VV SREL 106	Environmental
V fluv	NCTC11327	Killer strain

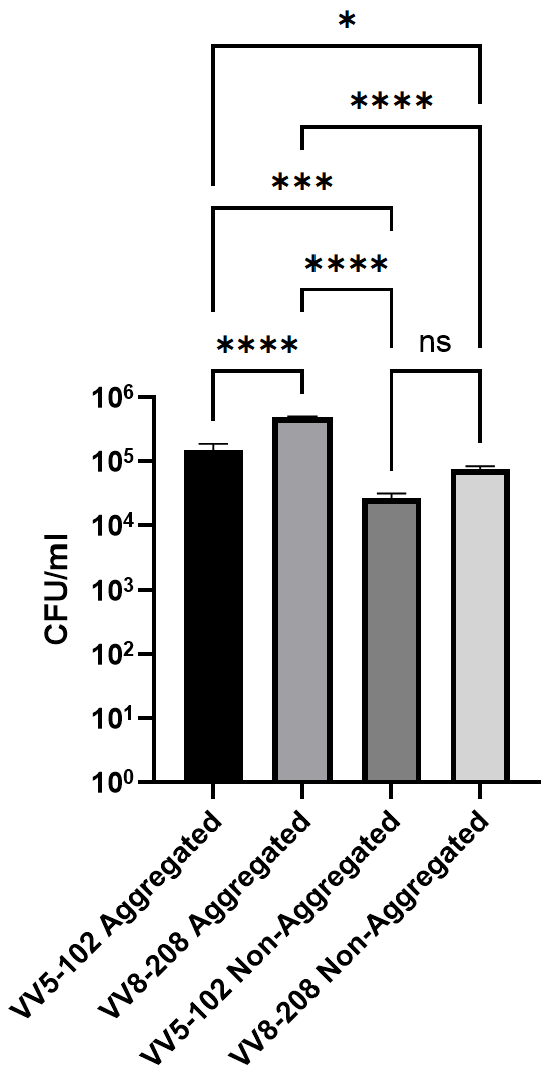

Figure 1 *V. vulnificus* uptake after incubation in competitive coculture in bottles that were rolled (aggregated) or static (non-aggregated). Significant difference (ANOVA; Tukey's: $P < 0.0001$) noted between aggregated and non-aggregated bottles but not between non-aggregates.

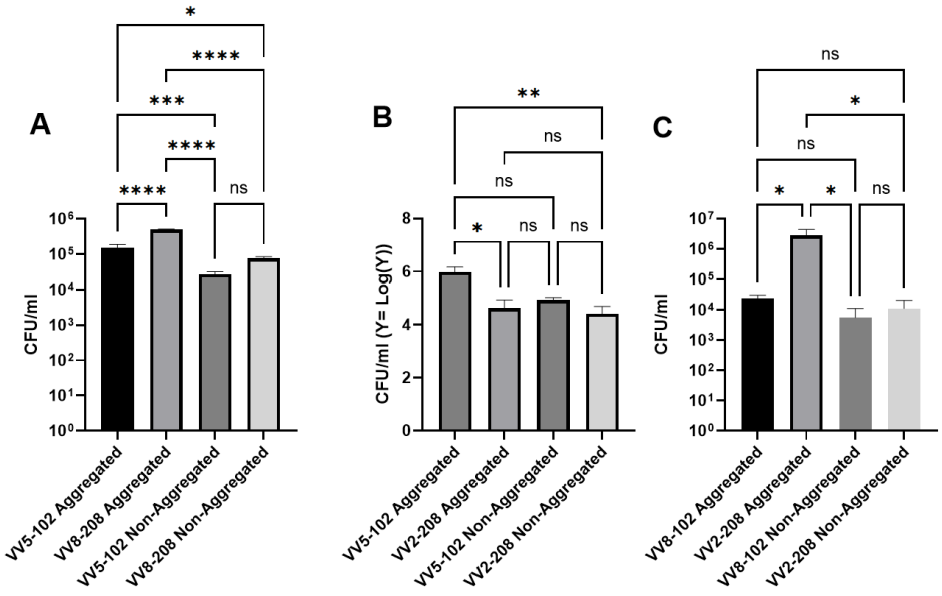

Figure 2 *V. vulnificus* uptake after incubation in competitive coculture onto aggregates, significant difference (P < 0.05) noted between aggregated cells but not between non-aggregated. (A) Competition between VV5-102 and VV8-208 (ANOVA; Tukey's: P < 0.0001). (B) Competition between VV5-102 and VV2-208 (Tukey's: P <0.05). (C) Competition between VV8-102 and VV2-208 (Tukey's: P < 0.05).

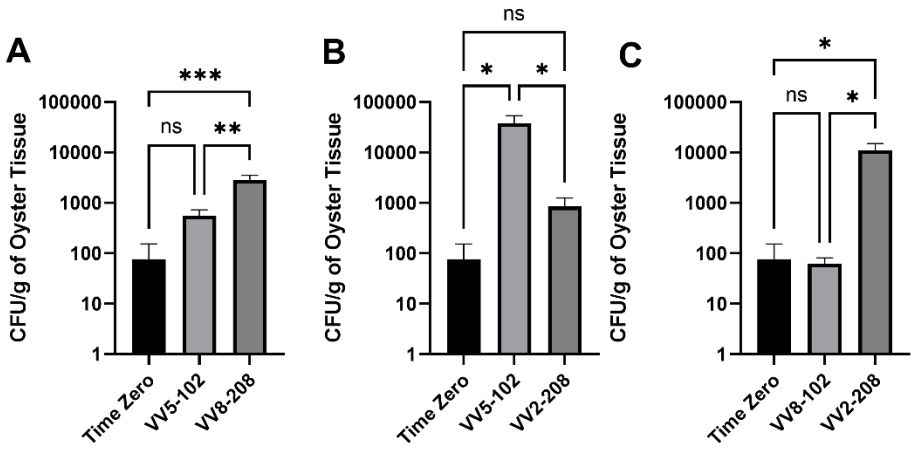

Figure 3 *V. vulnificus* uptake in Oysters fed with competitive coculture on aggregates, with significance noted between aggregates. (A) Competition between VV5-102 and VV8-208 (Tukey's: $P < 0.01$). (B) Competition between VV5-102 and VV2-208 (Tukey's: $P < 0.05$). (C) Competition between VV8-102 and VV2-208 (Tukey's: $P < 0.05$).

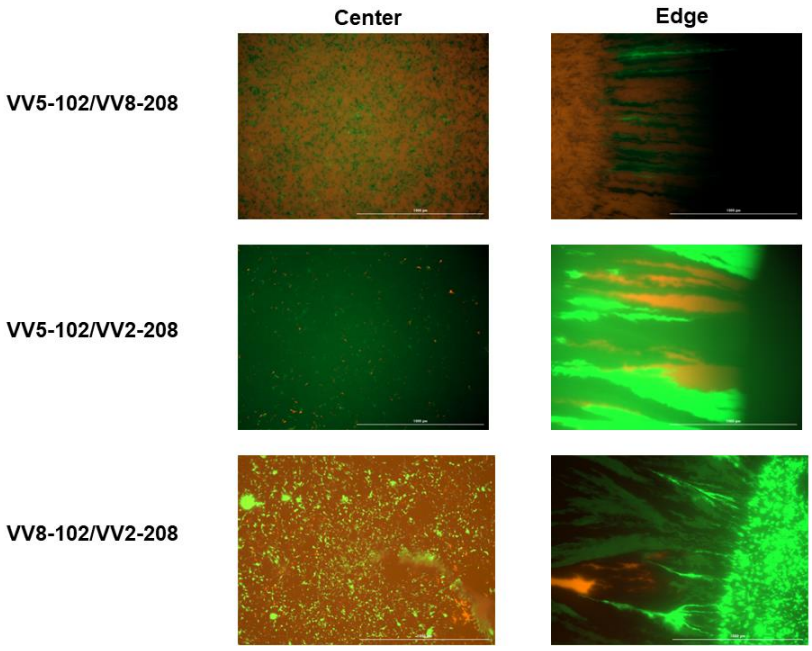

Figure 4 Green and Red Fluorescent protein expression in *V. vulnificus* competitions: center and edge view. (A) Competition between VV5-102 (green) and VV8-208 (red). (B) Competition between VV5-102 (green) and VV2-208 (red). (C) Competition between VV8-102 (green) and VV2-208 (red).

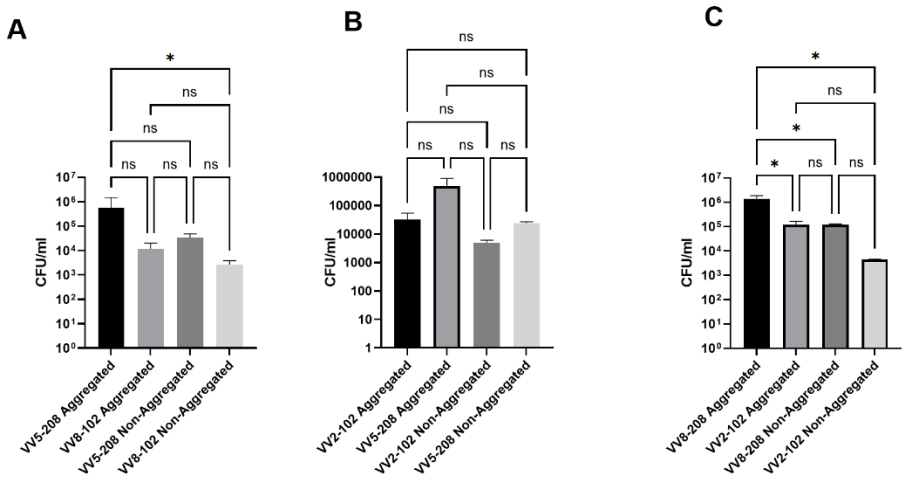

Figure 5 *V. vulnificus* uptake into aggregates using the reverse primers (A) Competition between VV5-208 and VV8-208. (B) Competition between VV5-208 and VV2-102. (C) Competition between VV8-208 and VV2-102 (Tukey's: $P < 0.05$).

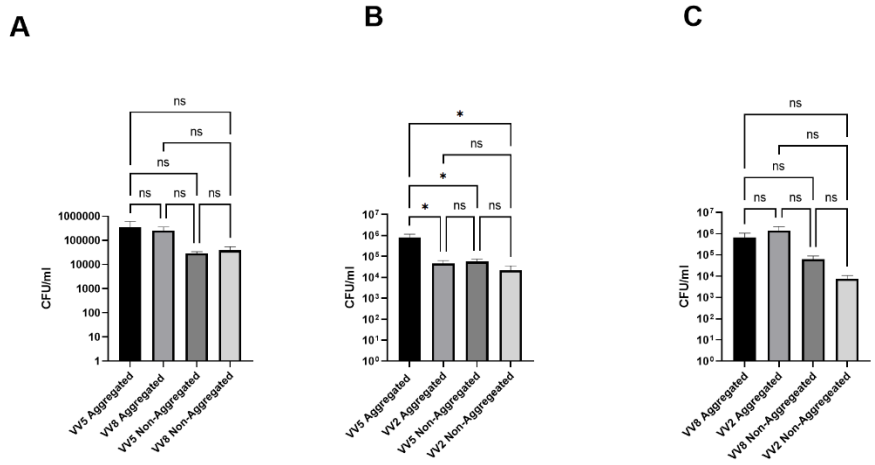

Figure 6 Combined plasmid results aggregate competitions. (A) Competition between VV5 and VV8. (B) Competition between VV5 and VV2 ($P < 0.05$). (C) Competition between VV8 and VV2.

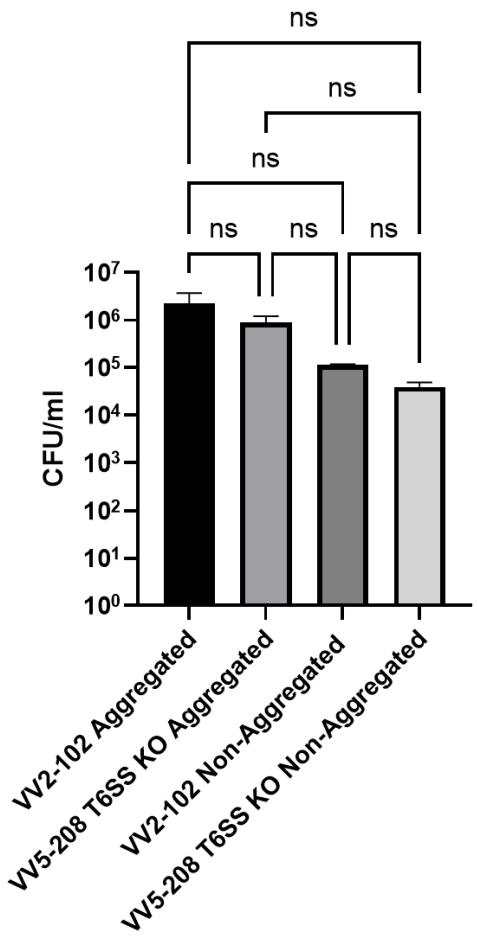

Figure 7 *V. vulnificus* integration into aggregates: VV2-102 vs VV5-208 T6SS KO. No significance observed between aggregated bacteria (Kruskal-Wallis; Dunn's: $P > 0.9999$) but killing effect of VV5 was not observed.

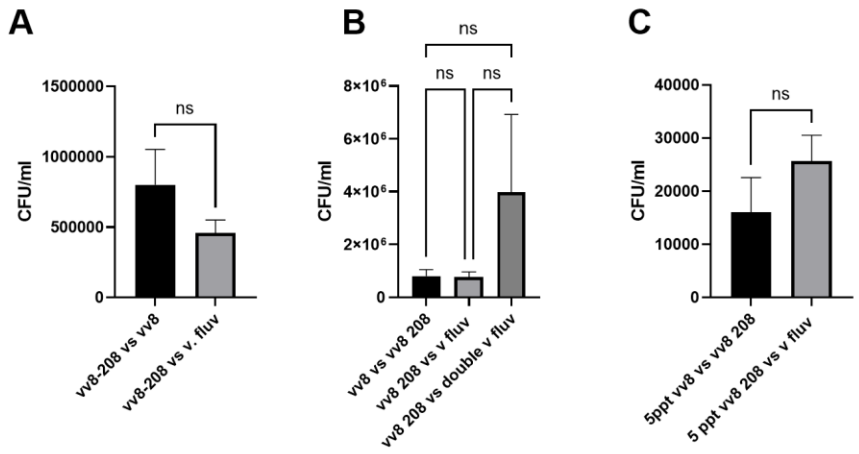

Figure 8 Competition in aggregates between VV8-208 and *V. fluvialis*. (A) Competition between VV8 and *V. fluvialis*. (B) Competition between VV8 and *V. fluvialis* in a 1:2 ratio. (C) Competition between VV8 and *V. fluvialis* at 5‰.

CHAPTER SEVEN: SUPPLEMENTAL TABLES & FIGURES

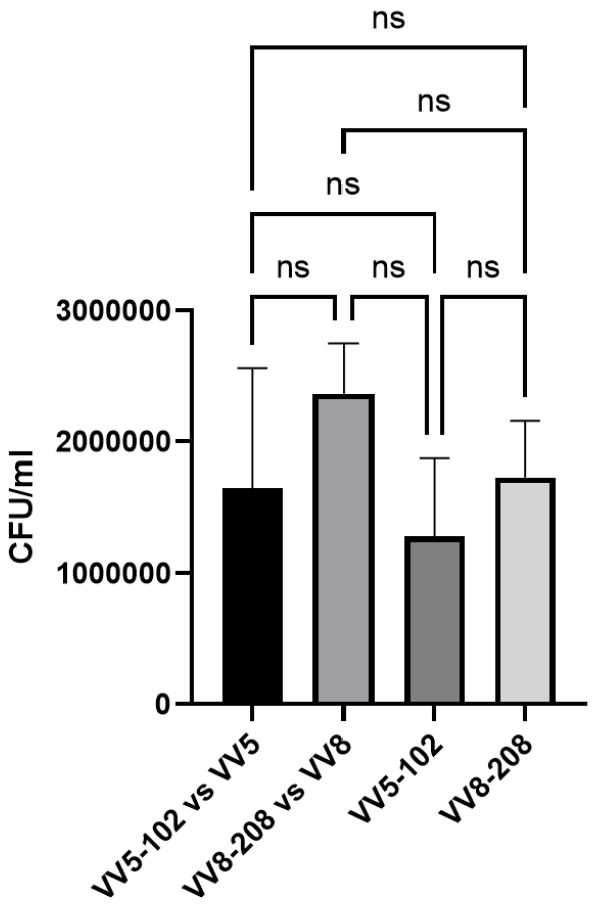

Figure 9 S1 *V. vulnificus* uptake onto aggregates (2.5 ml).

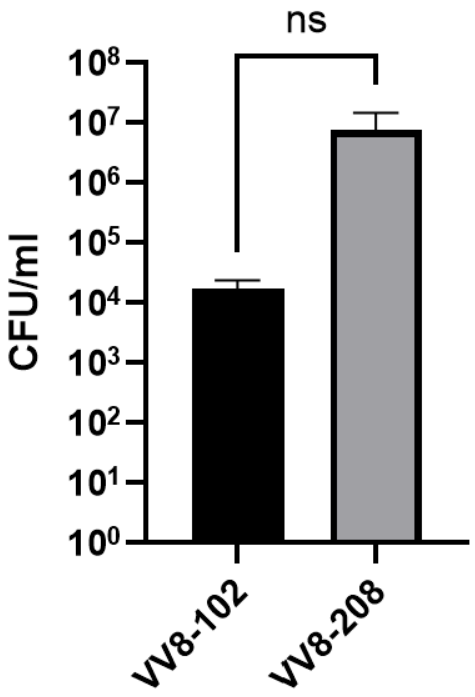

Figure 10 S2 Aggregate competition between VV2/VV8-102 and VV2/VV8-208

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BIOGRAPHY

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