MECHANISMS OF *ESCHERICHIA COLI* AND *VIBRIO CHOLERAE* FITNESS WHEN GROWN IN CO-CULTURE

by

Candace Longoria, B.S.

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Committee Members:

Robert J.C. McLean, Chair

Karl Klose

Kavida Kakirde

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DEDICATION

I dedicate my thesis to my amazing grandparents, who have always told me “Be the best you can be”. And my best friend and partner in crime, Lady Iphigenia (Wee Pig, Little Pig, and/or Pig).

ACKNOWLEDGEMENTS

I would like to acknowledge, firstly, Dr. McLean and his dedication to not only myself, but all of his research students. I would also like to acknowledge the faculty and staff at Texas State University for the excellent education I have received during my time here, the various opportunities I have been presented with, and the knowledge I will take with me. I want to thank all of the science teachers throughout my entire academic career who contributed their own unique quirks to my love for nature, both the macro-sciences and the micro-sciences. A special thank you to Dr. Sean Colloms from the University of Glasgow for providing the peptidase strains used for this experiment. Lastly, I would like to acknowledge the "Slime Gang" for the wonderful friendships we created that will last a lifetime, and the camaraderie we developed through our love for science and our desire for learning.

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ABSTRACT

Vibrio cholerae is a gram negative bacillus that possesses a single flagellum and is commonly known to have two toxigenic strains: serogroups O1 and O139; both are causes of epidemics. *V. cholerae* normally lives in brackish aquatic environments that have varying conditions that include temperature, salinity, and pH. *Escherichia coli* is normally found in the intestinal tract and *E. coli* K-12 is a commensal non-virulent strain used in many laboratory settings. *E. coli* and *V.* cholerae were observed in planktonic and biofilm mixed cultures and *V.* cholerae was seen to have a reduced fitness in the biofilm environment. To test which *E. coli* genes are essential for growth with *V. cholerae*, we used the *E. coli* KEIO knockout collection of specific K-12 genes and tested the ability of various knockouts to grow with *V. cholerae*. Once the initial screening was complete we saw the deletion of peptidase activity, most so with *pepA*-, had visible changes in fitness and growth in both planktonic and biofilm mixed culture. We also used various *pepA* strains and plasmids with altered DNA-binding and peptidase activity and observed the growth in mixed culture over a forty-right hour period. Based on our data, biofilm mixed culture and the lack of peptidase activity may affect the growth and fitness of both *E. coli* and *V. cholerae*.

I. INTRODUCTION

A significant number of microorganisms are found in biofilms and mixed culture environments both within the human body and throughout nature. Biofilms, surface-attached populations, differ from free-floating planktonic bacteria by the generation of an extracellular polymeric substance (EPS) matrix, a reduction in growth rates, and specific gene regulation. Biofilms are formed on environmentally exposed or contained locations on either living or nonliving surfaces. Attachment to and detachment from surfaces is achieved through the use of numerous pili, controlled by gene pathways (Donlan, 2002). Biofilm formation occurs in cyclic stages: free-floating planktonic bacteria, reversible attachment, irreversible attachment, maturation, and then the release of bacteria into a free-floating environment (Pratt and Kolter, 1998) (Sauer et al., 2002). *Escherichia coli*, including the K-12 strain, forms biofilms with other commensal microbes within the gastrointestinal tract (Macfarlane and Dillon, 2007). In the GI environment, motility, but not chemotaxis, plays a critical role in *E. coli* K-12 biofilm formation; this suggests that motility is important for surface interactions and movement along surfaces (Pratt and Kolter, 1998). *E. coli* biofilms are a source of major urinary tract infections (UTIs) within hospital and healthcare environments, typically with catheters and other enclosed objects that transfer liquids from individuals (Ferrieres et. al. 2007). Secondary infections in the young and in the elderly stemming from UTIs are one of the major causes of bacteriuria (Nicolle, 2014). Other bacterial species that interact with *E. coli* within the catheter (i.e. *Proteus* species) produce urease, which hydrolyzes urea to ammonia and consequently raise the pH of the urine and leads to encrustations. Catheter encrustation, which is the

development of crystals on the catheter, and antibiotic resistant bacteria can make treatment for biofilm formation difficult (Nickel and Costerton, 1992).

Vibrio cholerae forms biofilms and controls virulence factors using quorum signaling pathways and different levels of intracellular cyclic-diguanylate (c-di-GMP) through extracellular signal molecules called autoinducers (Chouhan et al., 2016). This quorum-signaling pathway regulates the transcription of specific gene pathway circuits that control the production of *Vibrio* extracellular polysaccharides (Vps) and biofilm formation in varying cell densities (Berk et. al. 2012). These circuits not only control the formation of biofilms, but also control functions like motility, conjugation, and virulence (Hammer and Bassler, 2003). Matrix proteins *rbmBCDEF* and *bap1* have been identified as significant contributors to the ability to form biofilms on solid surfaces (Fong and Yildiz, 2007). It is known that the marine bacterium *Vibrio harveyi* has two quorum sensing systems (1 and 2). *V. cholerae* has been found to have a system similar to *V. harveyi*'s System 2 and possesses multiple parallel signaling channels that aid in controlling virulence factors (Miller et. al 2002).

V. cholerae is a gram negative bacillus that possesses a single flagellum, thrives in brackish water, and is commonly known to have two toxigenic strains: serogroups O1 and O139, which are both causes of epidemics. Symptoms of cholera infections include watery diarrhea, vomiting, fatigue, fever, and, if left untreated, can be fatal. Children are more susceptible to *V. cholerae* infection than adults and retain a higher mortality rate (CDC, 2014). A majority of *V. cholerae* do not colonize in humans and are considered nonpathogenic (Todar, 2008). O1 has two biotypes: classical and El Tor, and the latter has become the more dominant and virulent of the two strains (CDC, 2014). The first six

cholera pandemics occurred between 1817 and 1961 and were identified as classical biotype strains. The El Tor biotype was discovered in 1961 in Indonesia and since it's discovery has become the seventh and ongoing devastating pandemic (Ping et. al. 2014). The El Tor biotype is highly adaptable and, since it's discovery, has out-competed all classical strains in combined environments whether occupying a host or in the microbial ecosystem (Pradhan et. al. 2010). Serogroup O139 had its first outbreak in 1992 in Indonesia and is hypothesized to have originated from an El Tor biotype strain of O1 (Calia et. al. 1994). O139 has also been shown to be more resistant to antibiotics than O1 strains, increasing the virulence of O139 for infection and treatment (Calia et. al. 1994).

V. cholerae aggregation and colonization in the intestines is caused by toxin co-regulating pilus (TCP) and cholera toxin (CT) by using a lysing bacteriophage **(**Hammer and Bassler, 2003). The control of CT and TCP expression is regulated by the transcriptional activator protein ToxT (Webber et al., 2014). The ToxR regulon, which regulates transcription of *toxT* and other important genes, is controlled by quorum sensing and various environmental factors (Weber and Klose, 2011). Cholera infections are more prominent in underdeveloped or overly populated countries with insufficient water sanitation resources. Other sources of infection include consumption of contaminated water and bivalves, specifically undercooked shellfish. There are no known animal hosts, but the bacteria can easily attach to chitin containing surfaces in marine bivalves (CDC, 2014).

E. coli is a gram negative bacillus that is a natural flora of the human body and acts as a protective agent by preventing colonization of foreign pathogens in the intestines (CDC, 2014). *E. coli* is normally found in the intestinal tract and K-12 is a

commensal non-virulent strain used in many laboratory settings. Because *E. coli* can cause fatal bacterial infections when found outside the human intestinal tract, it may be considered an opportunistic pathogen. The KEIO collection used for the screening of this experiment uses the K-12 strain and deletions of 3985 nonessential genes using a kanamycin cassette with a FLP recognition target (FRT) inserted by process of homologous recombination. The FLP recombinase will excise the inserted cassette and create an in-frame deletion (Baba et. al. 2006).

Peptidase, also referred to as a proteinase or a protease, is an enzyme that performs proteolysis (Rawlings et. al. 2016). During proteolysis, proteins are broken down and degraded into polypeptides or amino acids by cleaving a peptide bond. Proteinase K, a type of peptidase, cleaves at the peptide bond adjacent to the carboxyl group of aliphatic and aromatic amino acids, and is commonly used due to its broad spectrum specificity (Sigma-Aldrich, 2016). Proteinase K can be used to remove bound endotoxins and nucleases, and in the case of our experiment, was used to compliment the removed peptidase activity of the KEIO knockout *pepA*-. Multifunctional Aminopeptidase A (PepA) is a hexametric aminopeptidase that is involved in DNA-binding activity and works in transcription control and plasmid dimer resolution (Charlier et. al. 2000) (Colloms, 2013). PepA is involved in controlling two distinct DNA processed: transcriptional repression of the carAB operon that encodes carbamoyl phosphate synthase and sit-specific resolution of ColE1-type plasmid multimers (Minh et al., 2009). PepA is believed to have varying regulatory functions that have remained unchanged by evolution to control numerous transcriptional regulators that are involved in both catalytic and regulatory properties in *E. coli* (Charlier et. al. 2000).

This experiment was designed to observe the growth of *E. coli* and *V. cholerae* in a mixed culture and biofilm environment and potentially identifying specific *E. coli* genes that positively or negatively affect the combined growth, specifically the function of the *pepA*- knockout and the deletion of peptidase activity.

II. MATERIALS AND METHODS

Strains and Culture Conditions

The bacterial strains used were *V. cholerae* C6707 provided by Dr. Karl Klose, *E. coli* BW25113, the parent strain for the KEIO mutant collection, the KEIO mutant *E coli* collection, and *pepA*- strains and plasmids provided by Dr. Sean Colloms (University of Glasgow). Broth cultures were grown in Luria Bertani (LB) at 37°C for 16-22 hours while shaking at 150 RPM. *V. cholerae* C6707 has an antibiotic resistance for streptomycin inserted into the genome, and was isolated on LB and 50µg/mL streptomycin for all observations of mixed culture growth. The KEIO mutant knockout collection has antibiotic resistance to kanamycin and is selective for LB and 30µg/mL kanamycin. All KEIO mutants were used from a replicated freezer stock of the original collection and kept in 96-well plates at -80°C.

Establishment of Competition

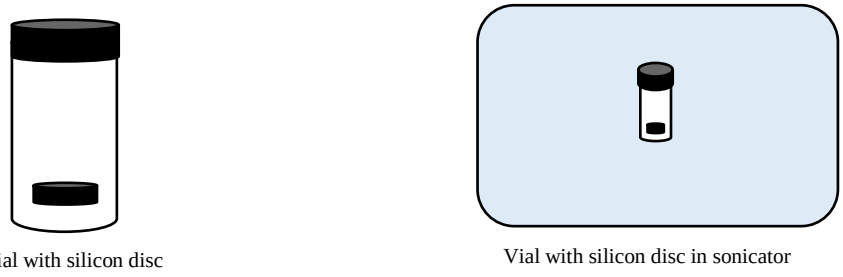

Figure 1. Biofilm extraction. Vials were filled with sterilized deionized water and a sterile silicon disc, then sonicated to remove biofilms.

A mixed culture serial dilution was performed to observe the growth of both *V. cholerae* and *E. coli* together in planktonic and biofilm environments by serially diluting

mixed cultures and plating them on LB and the *V. cholerae* on LB with 50µg/mL streptomycin. Bacterial cultures were grown in tubes of LB broth for 16-22 hours at 37°C for flask inoculation. Inoculum volumes for mixed culture planktonic and biofilm growth flasks were 0.5 mL and all OD_{600} values were 0.15± 0.05. Cultures were grown in 50mL LB broth in a 125 mL flask at 37°C shaking incubation at 150 RPM for 16-22 hours. Serial dilutions were performed by removing 0.5mL of culture inoculum and diluting in 4.5mL sterile deionized water. Planktonic competition was established by plating serial dilutions on LB and LB with streptomycin. The difference in colony counts between the LB and LB with streptomycin was used to determine the amount of *V. cholerae* within the mixed culture. Biofilm competition was observed with *V. cholerae* and *E.coli* by inserting sterile silicon discs into 50mL LB in 125mL flasks grown for 16-22 hours at 37°C shaking at 150 RPM. Discs were removed using a sterile syringe and dipped in 10mL sterile deionized water to remove excess LB broth. Discs were then individually placed in a vial with 10mL of sterile deionized water and sonicated for 1 minute (Figure 1). The deionized water was serially diluted in tubes of 4.5mL deionized water by removing 0.5 mL of inoculum from the sonicated vial and plated on both LB and LB with streptomycin to observe mixed culture biofilm growth. The difference between the LB and LB with streptomycin colony counts was used to establish the amount of biofilm growth that was *V. cholerae*.

Screening

The screening process was used for all KEIO mutants grown together with both the *E. coli* parent strain as a control and *V. cholerae.* The procedure for screening began

with growing the *E. coli* parent strain and *V. cholerae* in LB broth at 37°C overnight in LB broth tubes. In a 96-well plate, 200 µL of LB broth was added to each well using a multi-channel pipette. A 96-well replicator was used to transfer KEIO mutants from freezer stock 96-well plates to the experimental 96-well plate. After KEIO inoculation, 100µL of either *E. coli* or *V. cholerae* were added and the 96-well plate was incubated at 37°C for 16-22 hours. After the growth period, 5µL of the mixed culture inoculum was plated on LB agar, LB agar with streptomycin, and LB agar with kanamycin using a multi-channel pipette. Four columns were plated from the 96-well plate onto LB agar at a time (Table 1).

Table 1. Plate breakdown for microtiter to agar transfer

A1/A5/A9	A2/A6/A10	A3/A7/A11	A4/A8/A12
B1/B5/B9	B2/B6/B10	B3/B7/B11	B4/B8/B12
C1/C5/C9	C2/C6/C10	C3/C7/C11	C4/C8/C12
D1/D5/D9	D2/D6/D10	D3/D7/D11	D4/D8/D12
E1/E5/E9	E2/E6/E10	E3/E7/E11	E4/E8/E12
F1/F5/F9	F2/F6/F10	F3/F7/F11	F4/F8/F12
G1/G5/G9	G2/G6/G10	G3/G7/G11	G4/G8/G12
H1/H5/H9	H2/G6/G19	H3/H7/H11	H4/H8/H12

Plates were incubated for 16-22 hours at 37°C and observed for absence, weak, or heavy culture growth. This procedure was used for all 89 plates of the KEIO mutant collection as well as the replications if changes in growth were seen.

Selected Gene Competition

The *pepA*-, *pepB*-, *pepE*-, and *pepQ*- knockouts were grown in mixed culture with *V. cholerae* to observe mixed planktonic culture growth. A mixed culture serial dilution to observe the growth of both *V. cholerae* and selected gene was performed by serially diluting mixed cultures and plating them on LB and the *V. cholerae* on LB with 50µg/mL streptomycin. Bacterial cultures were grown in tubes of LB broth for 16-22 hours at 37°C for flask inoculation. Inoculum volumes for mixed culture planktonic and biofilm growth flasks were 0.5 mL and all OD_{600} values were 0.15± 0.05. Culture inoculums were grown in 50mL LB broth in a 125 mL flask at 37°C shaking incubation at 150 RPM for 16-22 hours. Serial dilutions were performed by removing 0.5mL of culture inoculum and diluting in 4.5mL sterile deionized water. Mixed culture competition was established by plating serial dilutions on LB and LB with streptomycin. The difference in colony counts between the LB and LB with streptomycin was used to determine the amount of *V. cholerae* within the mixed culture. Biofilm competition was observed with *V. cholerae* and *pepA*- by inserting sterile silicon discs into 50mL LB in 125mL flasks grown for 16-22 hours at 37°C shaking at 150 RPM. Bacterial cultures were grown in tubes of LB broth for 16-22 hours at 37°C for flask inoculation. Inoculum volumes for mixed culture planktonic and biofilm growth flasks were 0.5 mL and all OD_{600} values were 0.15± 0.05. Discs were removed using a sterile syringe and dipped in 10mL sterile deionized water to remove excess LB broth. Discs were then individually placed in a vial with 10mL of sterile deionized water and sonicated for 1 minute. The deionized water was serially diluted in tubes of 4.5mL deionized water by removing 0.5 mL of inoculum from the

sonicated vial and plated on both LB and LB with streptomycin to observe mixed culture biofilm growth. The difference between the LB and LB with streptomycin colony counts was used to establish the amount of biofilm growth that was *V. cholerae*.

Growth Curve Protocol

Protocol for spent media growth curves was provided by Dr. Robert McLean and Shelly Pringle (Pringle et. al. In Press). Growth curves were performed in 96-well microtiter plates over a forty-eight hour period and set up in the format provided in Table 2 and the example of growth can be seen in Figure 2.

Table 2. Template for growth curve experiments

	1	2	3	4	5	6	7	8	9	10&11	12
A	i-blank	ii-blank	iii-blank		i-blank	ii-blank	iv-blank		LB-blank		LB-a
B	i-a	ii-a	iii-a		i-a	ii-a	iv-a		LB-blank		LB-a
C	i-a	ii-a	iii-a		i-a	ii-a	iv-a		LB-blank		LB-b
D	i-b	ii-b	iii-b		i-b	ii-b	iv-b		LB-blank		LB-b
E	i-b	ii-b	iii-b		i-b	ii-b	iv-b		LB-blank		LB-c
F	i-c	ii-c	iii-c		i-d	ii-d	iv-d		LB-blank		LB-c
G	i-c	ii-c	iii-c		i-d	ii-d	iv-d		LB-blank		LB-d
H	i-c	ii-c	iii-c		i-d	ii-d	iv-d		LB-blank		LB-d

i: BW supernatent + filter sterilize (+proteinase K)
ii: VC supernatent + filter sterilize (+ proteinase K)
iii: *pepA*- strain/plasmid supernatent + filter sterilize
iv: *pepA*- strain/plasmid supernatent + filter sterilize
a: BW25113
b: VC
c: *pepA*- strain/plasmid
d: *pepA*- strain/plasmid

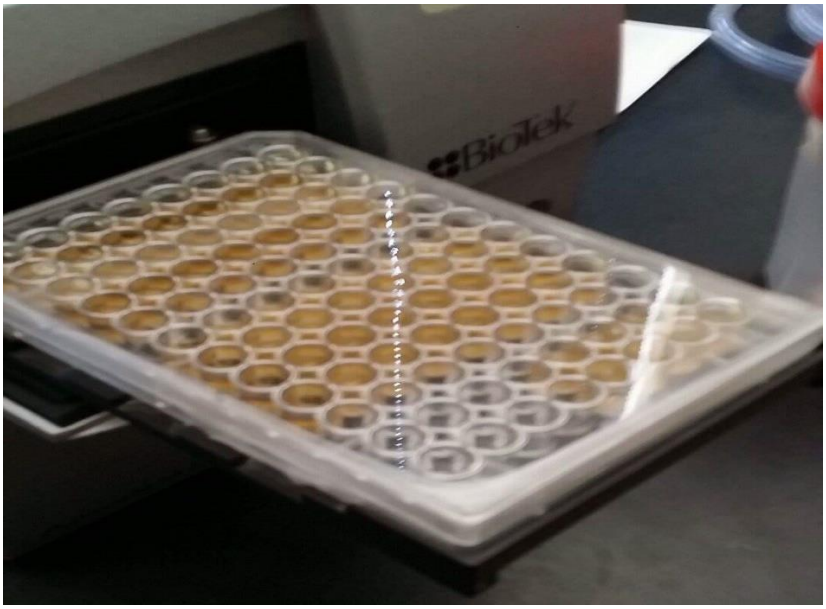

Figure 2. Microtiter plate with bacterial growth.

Bacteria were grown in various combinations of spent media, filter sterilization, proteinase K, and *pepA*- plasmids or strains (Table 3).

Table 3. Growth curve experimental plasmid and strain combinations

Bacteria/strains/plasmids	Spent media	Filter Sterilization	Proteinase K
E. coli, V. cholerae, pepA-	Yes	Yes	Yes
E. coli, V. cholerae, CD10	Yes	Yes	No
E.coli, V. cholerae, DSC941	Yes	Yes	No
E. coli, V. cholerae, PSC9	Yes	Yes	No
E. coli, V. cholerae, K6E	Yes	Yes	No
E. coli, V. cholerae, SDC1	Yes	Yes	No
E.coli, V. cholerae, PSC126	Yes	Yes	No
E.coli, V. cholerae, PRM40	Yes	Yes	No
E. coli, V. cholerae, SDC7	Yes	Yes	No

Eight different *pepA-* plasmids and strains were used for varying growth curves, and each had a different combination of properties including the presence of absence of DNA binding and/or peptidase activity.

Graphing and Plotting Data

Growth curves were recorded and exported using a BioTek plate reader and Gen5 software. All graphs and plotted growth curve data was formatted using SigmaPlot 11.0

III. RESULTS

Screening

Since this method was created specifically for this experiment, the known inhibition of *E. coli* in mixed culture with *Pseudomonas aeruginosa* was used to test the screening protocol (Chu et. al., 2012). After the known forty-eight hour growth period, the transfer method using the 96-well replicator to transfer KEIO mutants from freezer stock microtiter to experimental microtiter plates was successful.

Establishment of Competition

Initial competition between *V. cholerae* and *E. coli* was established using equal inoculum amounts and serially diluting mixed and pure planktonic cultures to calculate CFUs. Figure 3 shows the CFUs of *V. cholerae* and *E. coli* in pure and mixed planktonic cultures. When in mixed planktonic culture, 43% is made up of *V. cholerae* and 57% is made up of *E. coli* (Figure 3). This procedure was also done to observe growth in a mixed culture biofilm setting. Figure 4 shows CFUs in pure and mixed biofilm culture, with 17% of the biofilm bacteria made up of *V. cholerae* and 83% made up of *E. coli*. Comparison of the CFU values between pure and mixed culture at twenty-four hours shows the *E. coli* outcompetes the *V. cholerae* in mixed culture planktonic growth and significantly outcompetes it in a biofilm environment.

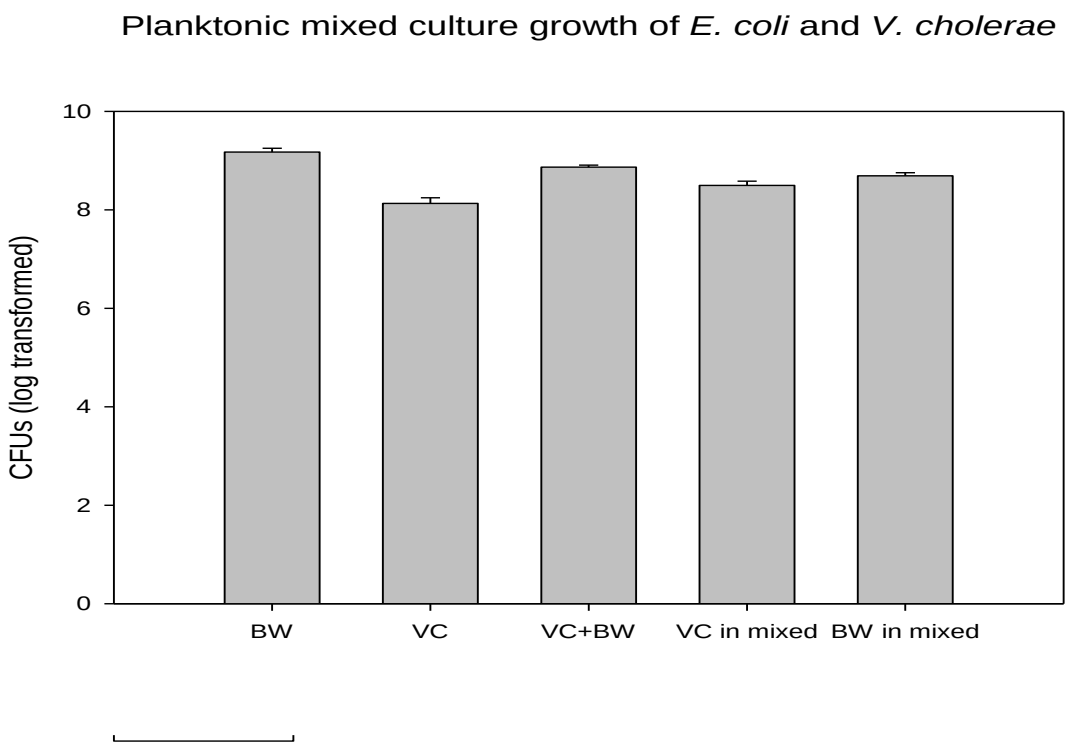

Figure 3. Planktonic mixed culture CFUs of *E. coli* and *V. cholerae*. 43% of mixed culture CFUs were *V. cholerae*, and 57% of mixed culture CFUs were *E. coli*. Error bars in all CFU figures represent one standard error within the data.

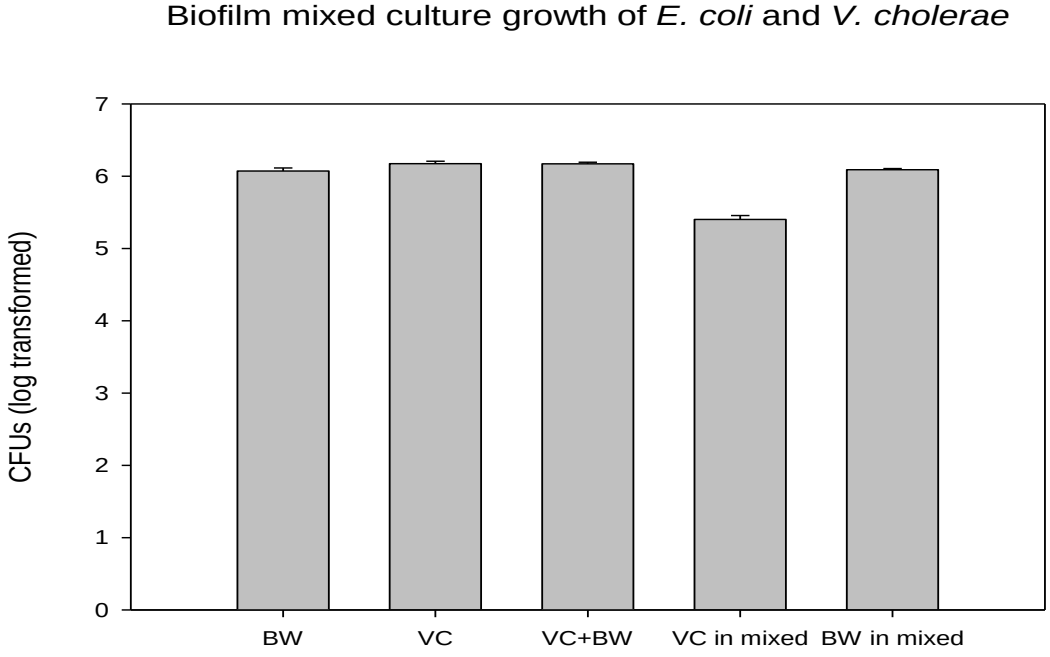

Figure 4. Mixed culture CFU growth of *E. coli* and *V. cholerae* in biofilm settings. In biofilm mixed culture, 17% of CFUs were *V. cholerae*, and 83% of CFUs were *E. coli*.

Selected Gene Competition

After the initial screening was performed and select mutants were re-screened, knockouts *pepA*-, *pepB*-, *pepE*-, and *pepQ*- demonstrated multiple replicates of altered growth (Figure 4 and Figure 5).

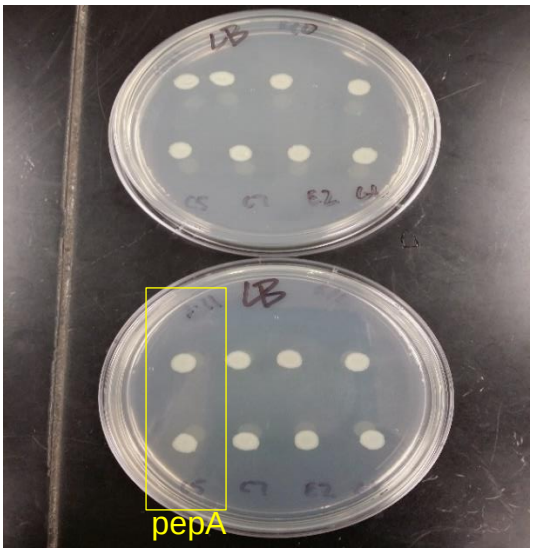

Figure 5. *Pep*- knockouts and *V. cholerae* in mixed culture on LB

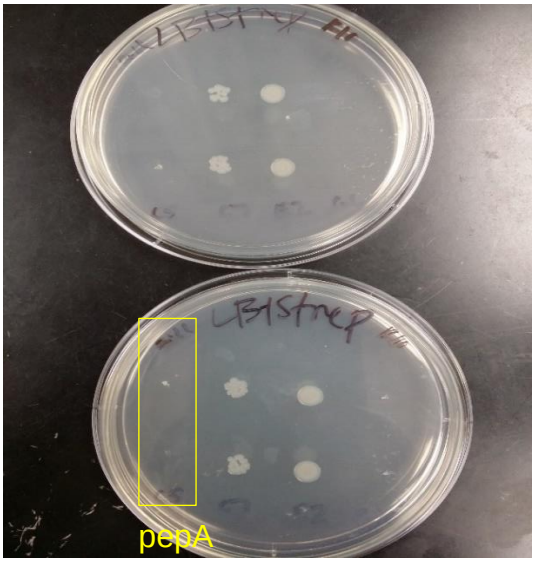

Figure 6. *Pep*- knockouts and *V. cholerae* in mixed culture on LB agar with streptomycin

These collective genes have mutations in the location that codes for peptidase activity and were grown individually in mixed culture with *V. cholerae* for twenty-four hours and serially diluted. After twenty-four hours of growth, all of the mixed cultures were plated on LB and selective media to observe CFUs for each bacteria. All four of the selected genes outcompeted the *V. cholerae* in planktonic mixed culture growth (Figure 6-9) and *pepA*- showed the most fitness and was selected to continue observation of mixed culture growth (Table 4). Biofilm growth was observed between *pepA*- and *V. cholerae* and CFUs were counted using LB and LB with selective media (Figure 10)

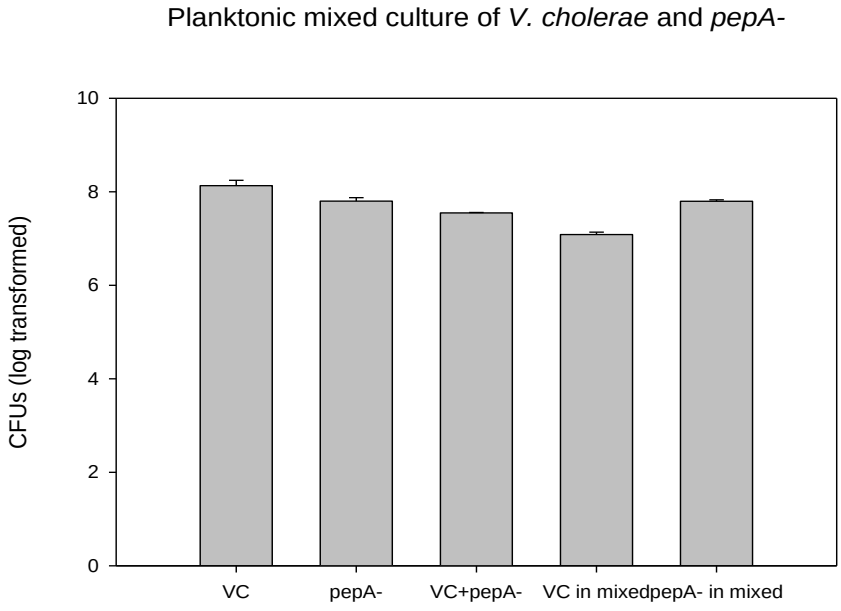

Figure 7. Planktonic mixed culture CFUs of *V. cholerae* and *pepA*-. 16% of mixed culture CFUs were *V. cholerae*, and 84% of mixed culture CFUs were *pepA*-.

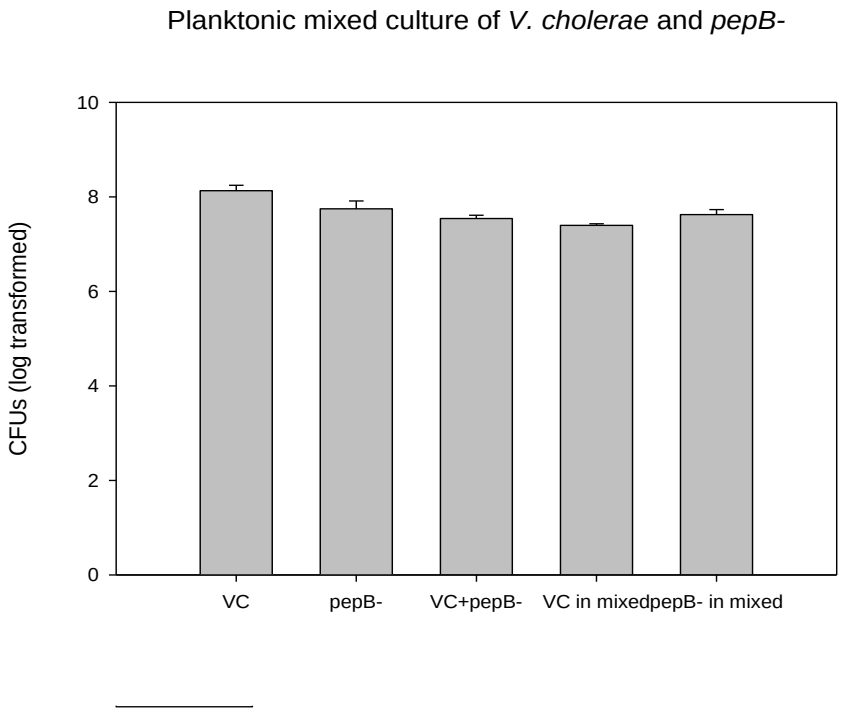

Figure 8. Planktonic mixed culture CFUs of *V. cholerae* and *pepB*-. 37% of mixed culture CFUs were *V. cholerae*, and 63% of mixed culture CFUs were *pepB*-.

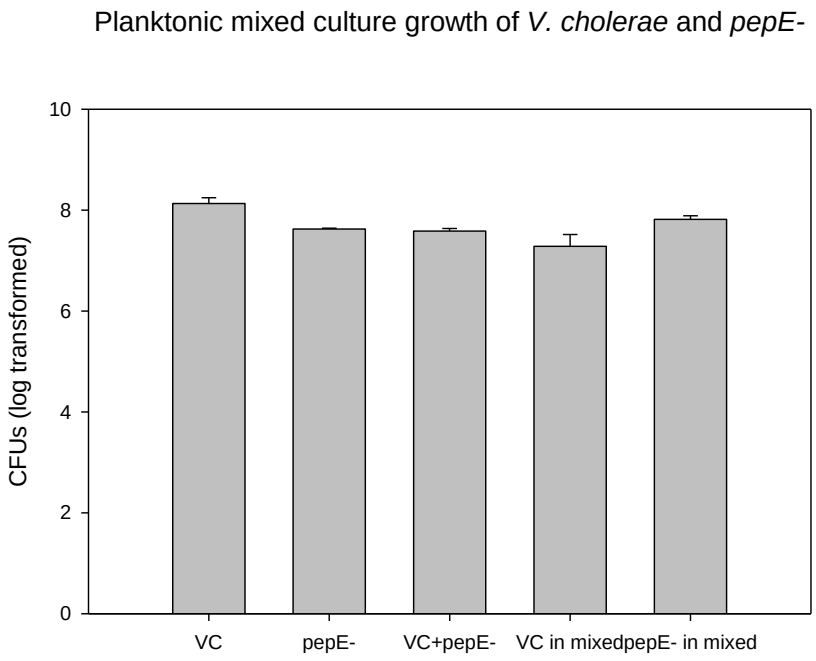

Figure 9. Planktonic mixed culture CFUs of *V. cholerae* and *pepE-*. 24% of mixed culture CFUs were *V. cholerae*, and 76% of mixed culture CFUs were *pepE-*.

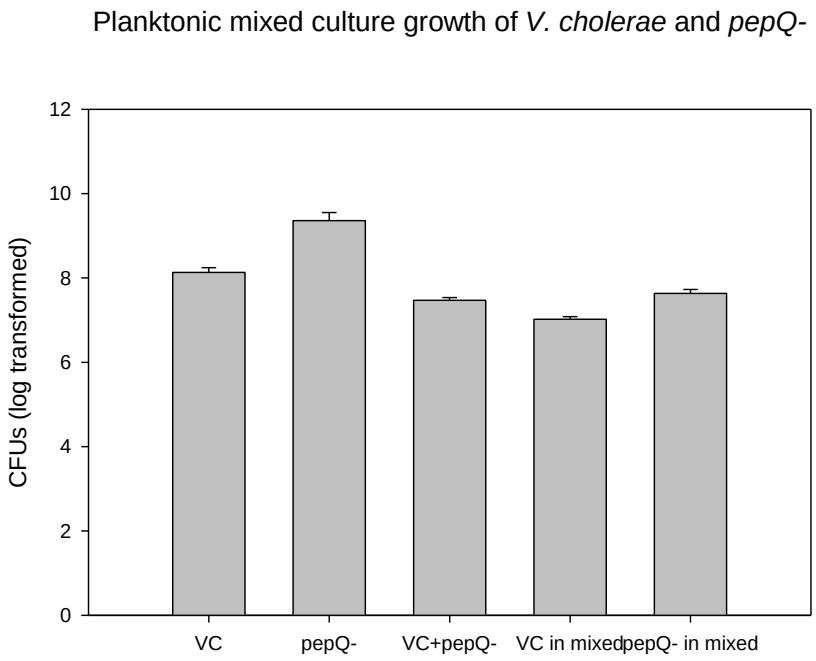

Figure 10. Planktonic mixed culture CFUs of *V. cholerae* and *pepQ-*. 20% of mixed culture CFUs were *V. cholerae*, and 80% of mixed culture CFUs were *pepQ-*.

Table 4. Composition of *V. cholerae* in planktonic mixed culture with Pep- genes

Mixed cultures	Composition of *V. cholerae* in planktonic mixed culture
V. cholerae and *pepA-*	16%
V. cholerae and *pepB-*	37%
V. cholerae and *pepE-*	24%
V. cholerae and *pepQ-*	20%

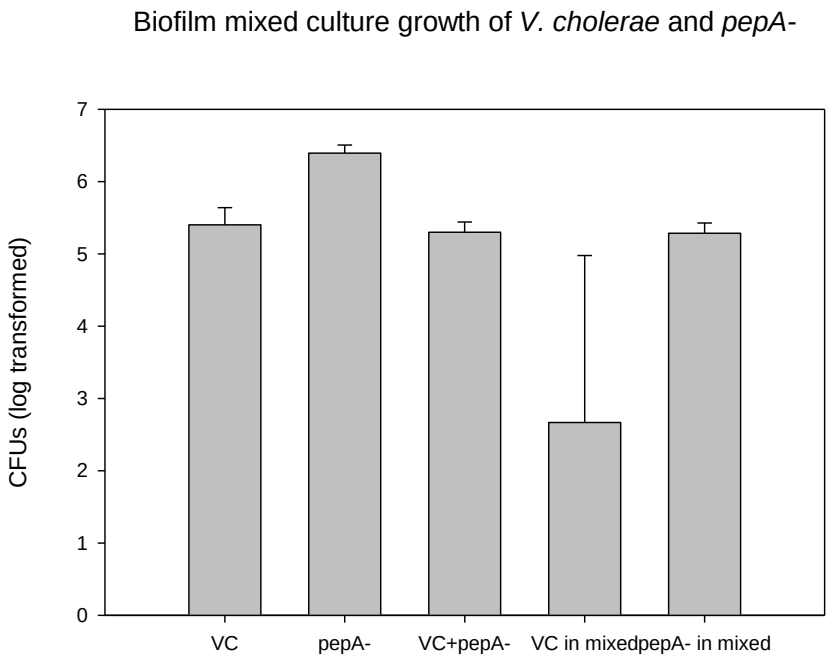

Figure 11. *V. cholerae* CFUs compared to *pepA*-. When CFUs were compared, 3.3% of biofilm mixed culture was made up of *V. cholerae.*

Table 5. Fitness of *V. cholerae* in planktonic mixed culture with Pep- genes

Mixed cultures	*V. cholerae* fitness
V. cholerae and *pepA*-	8.84%
V. cholerae and *pepB*-	18.04%
V. cholerae and *pepE*-	15.07%
V. cholerae and *pepQ*-	7.61%

Growth Curve Results

Observations of *E. coli*, *V. cholerae,* and *pepA*- were made over a forty-eight hour growth curve in pure and mixed culture to analyze the growth patterns. Growth curves were also performed with spent media from the bacteria that had been filter-sterilized to

remove larger by-products, and a combination of spent media from the bacteria, filter-sterilization, and proteinase K were observed over a forty-eight hour period of growth. Proteinase K was used to observe further peptidase activity among the mixed culture and help confirm the interaction with *pepA*-. When combined with the proteinase K, diauxic growth patterns were observed in the spent media with the *V. cholerae* (Chu and Barnes, 2015) (Figure 11).

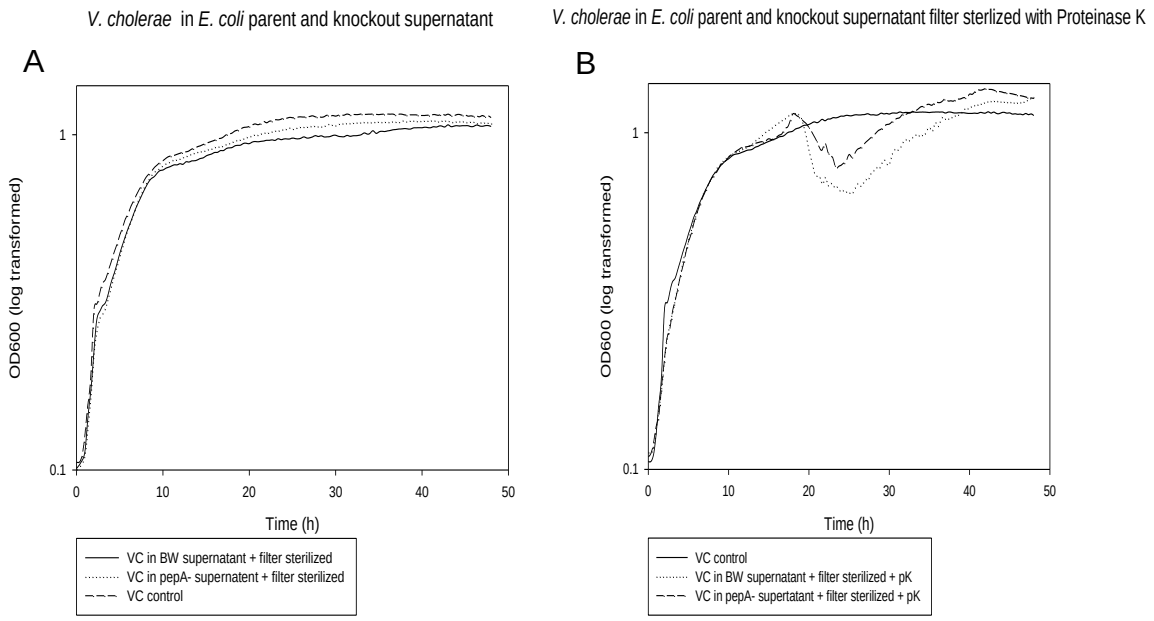

Figure 12. *V. cholerae* in spent culture with or without proteinase K. A. *V. cholerae* in filter sterilized supernatants of *E. coli* and *pepA*- over a forty-eight hour period. B. *V. cholerae* in filter sterilized supernatants of *E. coli* and *pepA*- with proteinase K added over a forty-eight hour period

Growth curves were also performed using *pepA*- plasmids and strains in mixed culture with *V. cholerae* (Table 3). Of these combinations, three different strains (K6E, SDC7, and SDC1) showed changes in the growth curves (Figure 12).

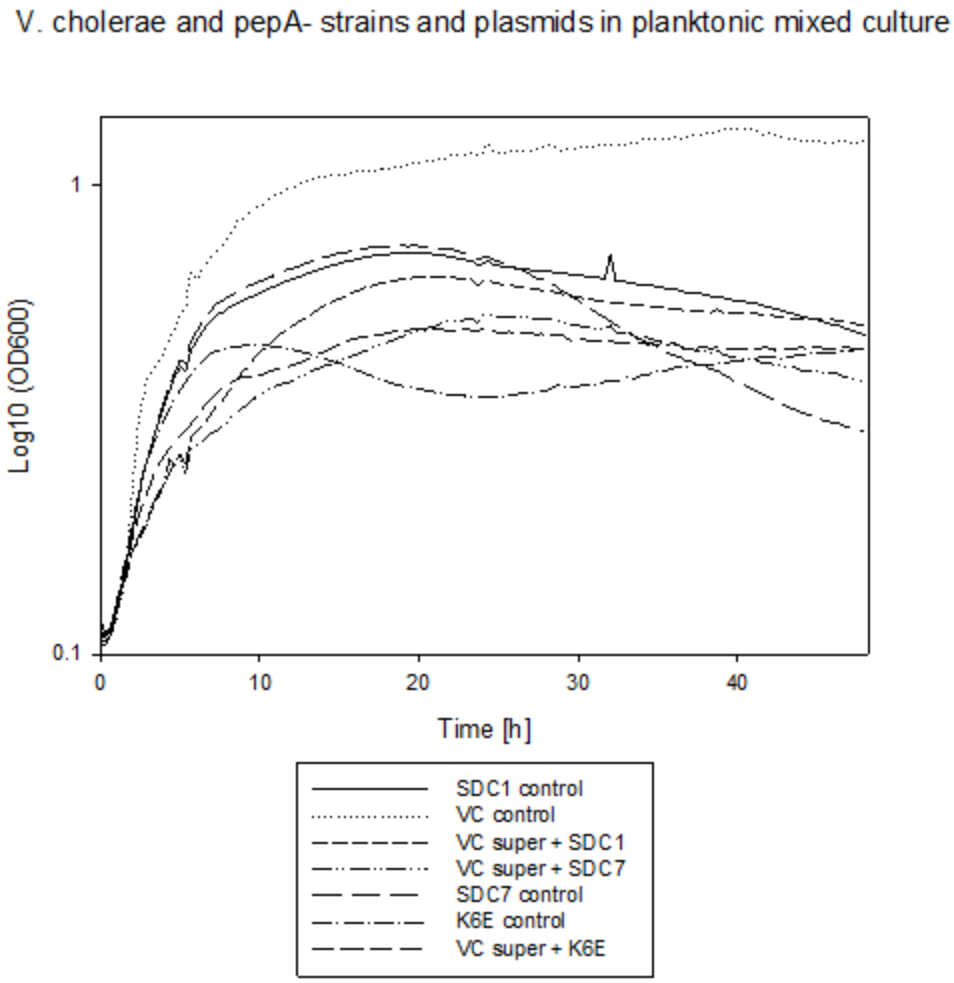

Figure 13. *V. cholerae* and K6E, SDC1, and SDC7 in mixed culture growth curve. All growth curves were read over a forty-right hour period.

Both the SDC1 and SDC7 strains have *pepA* replaced with antibiotic resistance genes, and the K6E plasmid possesses no DNA binding properties, but does possess peptidase activity. All other strains and plasmids provided by Dr. Colloms showed no changes in growth over a forty-eight hour period.

IV. DISCUSSION

The initial screening protocol and method developed for this experiment was successful for the detection of changes in growth of mixed culture bacteria. This developed method was also the first of its kind, designed specifically for transfer of the KEIO knockout mutant collection, and proved to be applicable to other bacteria in mixed culture aside from *V. cholerae* and *E. coli* in our experiment.

When grown in planktonic mixed culture, there is not a large difference in CFU count or competition between *V. cholerae* and *E. coli.* However, there is a significantly larger CFU and competition difference between *V. cholerae* and *E.* coli in during the production of a mixed culture biofilm. *E. coli* is known to produce and consume autoinducer-2 (AI-2) signals controlled through quorum sensing mechanisms that can affect the levels of cAMP/cGMP (Xavier and Bassler, 2005). *V. cholerae* quorum sensing receptors are known to use cGMP to regulate virulence factors and biofilm production, and there may be a potential connection between the signals released from *E.coli* and c-di-GMP levels. However, little is known as to how the AI-2 signal functions in mixed culture fitness (Hibbing et. al. 2010).

The screening process of the KEIO knockout collection found multiple *Pep*-genes affected the fitness of *V. cholerae* in planktonic mixed culture. The lack of peptidase activity in these mutants may affect downstream genes from these *Pep*-knockouts that increase the fitness of *E. coli* or decrease the fitness of *V. cholerae*. Peptidase and proteases in general can have a variety of functions and interactions with intracellular proteins and amino acids, and the absence of this cleaving may allow *E. coli* to increase certain signals like the AI-2 (Uniprot, 2016). After the fitness in the mixed

culture with the *V. cholerae* and *Pep-* genes was calculated, the *pepA-* knockout showed the largest change in *V. cholerae* growth. Homologous recombination in *E. coli* produces plasmid multimers. PepA is an assessory protein in Xer site specific DNA recombination, which is a system that resolves plasmid multimers (U. Leipzig, 2016). Although multimerization is seen to cause instability, the specific deletion of *pepA* in *E. coli* could possibly have an opposite effect on this instability (Summers and Sherratt, 1984). The forty-eight hour growth curves with the K6E plasmid and the SDC1 and SDC7 and their peptidase activity may provide clues as to how the deletion of peptidase activity either increases the fitness of *E. coli* or decreases the fitness of *V. cholerae.*

Our experiments show that in planktonic mixed culture, *E. coli* itself does not overly outcompete *V. cholerae*, but in biofilm environments *V. cholerae* has a significantly lower fitness compared to planktonic. The Pep knockout genes show decrease in fitness of *V. cholerae* in planktonic mixed culture. Of these knockouts, *pepA-* shows a significant reduction in *V. cholerae* fitness in both planktonic and biofilm mixed culture. Biofilm growth has been seen to be interrupted and reduced in *V. cholerae* O139 strain MO10 when in the presence of human non-specific antigen-specific secretory IgA (SIgA), indicating that virulence factors can be altered by human influences. Specifically, oligosaccharides containing mannose affected virulence factors in *V. cholerae* and biofilm formation without affecting the viability (Murthy et al., 2001). The *pepA-* mutant is known to have aminopeptidase activity, which means there is no longer cleavage at the N-terminus. ToxT has an N-terminus involved in dimerization and environmental sensing and a C-terminus that controls DNA binding. Since ToxT is known to control numerous virulence factors, there could be a link between a specific cleavage site no longer affected

by *pepA*- and the ToxT regulatory functions (Weber and Klose, 2011).

The deletion of peptidase activity and how it directly affects *V. cholerae* is not fully understood, but the results mimic growth similar to diauxic growth. These patterns of growth have been seen when a bacterial species is in the presence of two sugar sources and switches from one source to the second. Typical diauxic behavior has been shown to be a trade-off between a highly-sustained growth rate and adaptation from one resource to a different resource (Chu and Barnes, 2015). There are two hypotheses for this relatively unknown experiment. The first is the deletion of peptidase activity releases a metabolite unique to itself from the parent strain of *E. coli* or *V. cholerae.* The second is less plausible due to the diauxic behavior seen in the growth curves (Figure 11), but there may potentially be a signal or metabolite produced (or no longer produced) by the knockout that affects the growth of *V. cholerae.* There is also a possibility that instead of a small reduction in growth, the diauxic pattern seen may actually be a die-off of the bacteria, followed by a recovery. The application of a florescent molecule may be beneficial to observe changes in the ratio of *V. cholerae* to the added *E. coli* strains or knockouts.

More research will need to be done to understand how these changes in fitness occur when the peptidase activity is restored in a peptidase-free environment.

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