An investigation into the multiple Type VI Secretion Systems of *Enterobacter cloacae*

A Thesis
Presented to
The Academic Faculty

by

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In Partial Fulfillment
Of the Requirements for the Degree
Bachelor of Science with Research Option
In the School of Biological Sciences
Georgia Institute of Technology

Georgia Institute of Technology
May 2021

An investigation into the multiple Type VI Secretion Systems of *Enterobacter cloacae*

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Date Approved: May 2021

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Acknowledgements

I would like to thank Dr. Hammer for giving me this wonderful opportunity to do research in his lab and for the continual support in writing this paper, as well as others during my time here. I've learned an incredible amount that I will take with me into my future careers. I'd also like to thank Dr. Ratcliff for his continued support in this project. I'd like to thank my graduate mentor, Siu Lung Ng, for all his daily guidance throughout this project, as well as always patiently answering what feels like my never-ending questions. I'd like to thank the entire Hammer lab for making my research experience so enjoyable – it will be truly missed. Lastly, I'd like to thank former lab members, Jessica Mulligan, Mansi Gupta and Vishnu Raghuram, whose contributions to this project were foundational.

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Abstract

Enterobacter cloacae is a Gram-negative, opportunistic bacterial pathogen that is commonly acquired by patients in hospitals. The Type VI Secretion System (T6SS) is a harpoon-like apparatus that injects toxins into the cell envelop of neighboring bacteria to defend or compete for resources**.** It's commonly found in a range of bacteria including *Vibrio cholerae*, *Pseudomonas aeruginosa*, and *Escherichia coli*. Previous research in this lab has shown that *E. cloacae* kills in a contact dependent manner and bioinformatic analysis found three *vask* genes, which encode for a protein in the Type VI apparatus. We created three single mutant strains (Δ*vask1*, Δ*vask2*, Δ*vask3*) as well a double mutant (Δ*vask1*Δ*vask3*). These strains were tested in competition assays with target WT *E. coli*, with the survival of the target being indicative of *E. cloacae* killing ability. We show here that Δ*vask2* had no change in killing ability, Δ*vask1* and Δ*vask3* had some reduction in killing ability, and Δ*vask1*Δ*vask3* had a complete reduction in killing ability. This initial result suggests *E. cloacae in vitro* killing ability is dependent on two T6SSs.

Introduction

Enterobacter cloacae is a Gram-negative, opportunistic bacterial pathogen that is commonly found in hospitals where it colonizes surgical equipment and forms biofilms on devices.[1] While up to 80% of the population can have *E. cloacae* living benignly in the gastrointestinal (GI) tract, it becomes dangerous in immunocompromised individuals.[2] As *E. cloacae* continues to rise in prevalence at hospitals around the country, research has been increasingly focused on potential virulence factors.[3] *E. cloacae* has the ability to adhere to other cells, produce an iron chelating agent called aerobactin, and encodes several secretion systems.[2,4] The ability to adhere to cells is a necessary property in order to form biofilms, which are attached polymicrobial communities of cells that communicate and cooperate with each other.

There are seven types secretion systems (T1SS-T7SS) found within the bacterial world, each unique in its structure and function.[10] Specifically, *E. cloacae* contains multiple Type VI Secretion Systems (T6SS) which are encoded on separate loci. T6SS is a harpoon-like apparatus commonly found in Gram-negative bacteria that injects toxins into the cell envelope of neighboring bacteria to defend or compete for resources.[5] *E. cloacae* is a complex pathogen with many internal mechanisms to uncover. Our research focuses specifically on the T6SS in *E. cloacae* and ultimately how it contributes to competition and pathogenicity.

While T6SS has been widely studied in a variety of pathogens such as *Vibrio cholerae* and *Pseudomonas aeruginosa*, little research has been conducted on the T6SSs in *E. cloacae*. To date, only one paper has been published that describes the role of each T6SS in the clinical *E. cloacae* reference strain ATCC13047. Our study investigates the *E. cloacae* environmental

isolate ZOR0014 taken from a zebrafish by the Parthasarathy lab at the University of Oregon, which has not been studied before in this context.

Previous research in this lab done by M.S. student Vishnu Raghuram established *E. cloacae* ZOR0014's virulence against *V. cholerae* and other pathogens (Fig. 1), including *E. coli* (not shown in Fig. 1). He was also able to show that when *E. cloacae* ZOR0014 was separated from its target strain with filters, it was unable to kill that target (Fig. 1). This established contact dependent killing which is indicative of a Type VI secretion system.

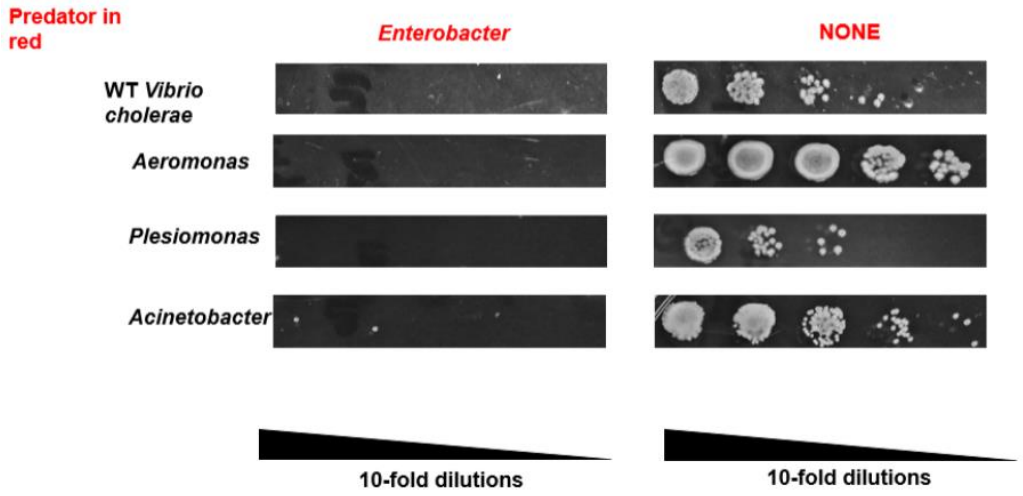

Figure 1. Preliminary research established that *E. cloacae* was able to kill a variety of other bacterial species. *E. cloacae* ZOR0014 was co-cultured in competition assays with *V. cholerae*, *aeromonas*, *plesiomonas*, and *acinetobacter* in a 1:10 ratio (*E. cloacae* ZOR0014: target strain). A negative control in which the target strains were cultured without *E. cloacae* is on the right for comparison. (Figure from Vishnu Raghuram)

Next, M.S. students Mansi Gupta and Jessica Mulligan set out to establish through bioinformatic analysis whether *E. cloacae* ZOR0014 contained a T6SS loci, how many and where they were located. They were able to find three *vask* genes, which encode for a protein in the T6SS. Two of these are confirmed to be located in large clusters with other T6SS genes while

the third is in a smaller cluster. It is still unknown if the third *vask* is part of a T6SS loci or independent.

Our first goal is to confirm the presence of these T6SSs and discern to what degree each contributes to interbacterial competition between *E. cloacae* ZOR0014 and the model bacterium *E. coli* in vitro. Following our study, these strains will be used by our collaborators in the Parthasarathy lab for in vivo experiments in the zebrafish host to study the contribution of T6SS activity in a relevant animal model. Zebrafish are unique model organisms due to their ability to stay sterile for the first 14 days and their transparency allows for direct visualization of internal mechanisms as a result of colonization by fluorescently-labeled microbes including *E. cloacae.*[6] Understanding precisely how T6SS affects *E. cloacae's* ability to interact, compete, and colonize in a live host is crucial to making progress in combating this pathogen.

Literature Review

E. cloacae is a Gram-negative, opportunistic bacterial pathogen. It is commonly found as a benign commensal gut bacterium in up to 80% of human gastrointestinal (GI) tracts but becomes problematic in immunocompromised patients.[2] *E. cloacae* is often found in biofilms on surgical equipment or intravenous devices, which can cause infections.[1] This bacterium causes many hospital-acquired infections such as urinary tract infections, osteomyelitis, cholecystitis, meningitis, bacteremia, and lower respiratory tract infections.[7] This pathogen is increasingly important to study as its prevalence in hospitals continues to rise, currently accounting for 7% of all nosocomial infections within the United States.[3] Concerningly, *E. cloacae* is also inherently resistant to several antibiotics, including ampicillin, amoxicillin–clavulanic acid, and cephalothin.[1] In fact, *E. cloacae* is the second most common carbapenem-resistant

Enterobacteriaceae (CRE) in the United States.[8] However, there are multiple strains of *E. cloacae* isolated from both environmental and clinical sources. Research conducted on one strain is not always applicable to others. For example, there are certain strains of *E. cloacae* which are not inherently resistant to ampicillin, which underscores the value of studying more than a single reference strain of a bacterial species.

Due to *E. cloacae's* increasing prevalence, understanding its virulence properties has become a point of interest for researchers over the past couple decades. Initial studies found that *E. cloacae* has the ability to produce aerobactin, an iron chelating agent, and adhere to other cells. Aerobactin is a known virulence factor in *E. coli* due to its ability to sequester iron.[2] Iron is an essential nutrient for hosts and when deprived of it, the host's condition deteriorates. Cell adherence is a common measure of virulence because this allows cells to form biofilms which can cause infections. More recent studies have shown that *E. cloacae's* pathogenicity is dependent on a variety of other things as well, such as outer membrane proteins, efflux pumps and secretion systems.[4, 9]

Secretion systems are very common in bacterial pathogens. To date, there have been seven systems discovered (T1SS-T7SS). Each system is unique and provides different functions, but they all contribute to bacterial growth and virulence in some way.[10] These secretion systems are encoded on gene clusters in bacterial chromosomes. Genomic analysis of various *E. cloacae* strains has indicated that they contain multiple Type VI Secretion Systems (T6SS).[11] T6SS is a harpoon-like apparatus commonly found in Gram-negative bacteria that injects toxins into the cell envelope of neighboring bacteria to defend or compete for resources. T6SS was first discovered by Mougous, et al. in *Pseudomonas aeruginosa*. *P. aeruginosa* is a commonly found bacterium in the lungs of cystic fibrosis (CF) patients. The researchers found components of the

T6SS apparatus in pulmonary secretions of these patients, indicating that T6SS might contribute to *P. aeruginosa* pathogenicity and ability to colonize CF lungs.[5] T6SS has since been shown to be present in up to 25% of all Gram-negative bacteria, including *Escherichia coli*, *Vibrio cholerae*, and *Enterobacter cloacae*. [11,12,13,14]

The T6SS apparatus consists of at least 13 subunits that are encoded by anywhere from 12-20 genes. T6SS is composed of three sections: the proteins forming the membrane, the tail complex and the baseplate complex.[12] The T6SS has an inner tube, or "harpoon", topped with a conical protein containing toxins, surrounded by an outer membrane. The harpoon is fired into neighboring cells, piercing their membranes and injecting the toxins, causing lysis and death of the cell. Most species have one or two T6SS clusters each encoding for an apparatus, but certain species can have up to six.[15]

Besides its initial discovery in *P. aeruginosa*, T6SS has been shown to contribute to virulence in other bacteria as well. When introduced into live zebrafish, *V. cholerae* was shown to induce gut contractions, or peristalsis, which expelled resident bacteria into the water supply in order to infect more hosts. Researchers disrupted crucial T6SS genes in *V. cholerae* and found that the bacteria had harder time colonizing the host zebrafish and expelling itself.[13] Studies conducted in vivo have shown that mutants lacking critical T6SS genes have significantly lower ability to kill neighboring bacteria, such as *E. coli*. Similar in vivo results have been observed for studies of the T6SS various species of enterobacteria.[16]

Compared to other bacteria, *E. cloacae's* Type VI secretion systems have been understudied. Recently, a paper published by Soria-Bustos, et al. described the role both T6SSs play within *E. cloacae.*[11] They discovered that one of the T6SS contributes to interbacterial

competition and biofilm formation in vitro, the second contributes to cell adherence in vitro and both are necessary for in vivo colonization using mouse models.[11] However, much is still unknown about T6SS in *E. cloacae* and the role it plays in interbacterial competition with a wide variety of bacteria. This is also the only paper covering T6SSs role in *E. cloacae* specifically.

Results

After finding the locations of the *vask* genes, myself and Jessica Mulligan worked on disrupting them under the guidance of graduate student Siu Lung Ng. To access the effectiveness of the multiple T6SS's in *E. cloacae*, we disrupted (*vask1* and *vask3*) or deleted (*vask2*) the *vask* genes, which encode for a protein in the T6SS.

We established an appropriate ratio for competing *E. cloacae* against *E. coli*. Various ratios of killer to target were tested, including 1:12, 1:10, 1:8, 1:4, and 1:2 (Fig. 2A) before deciding the 1:2 ratio was ideal for visualizing the differences between mutants (Fig. 2B). This allowed for up to 3 log differences to be clearly seen between the *E. cloacae* strains.

The phenotypes were assessed through competition assays, first with spot plating and then quantified with colony forming units (CFUs). Each of the single mutant strains (*Δvask1, Δvask2, Δvask3* – Fig. 3, blue bars) and the double mutant (*Δvask1Δvask3* – Fig. 3B, green bar) were co-cultured with WT *E. coli*. For reference, WT *E. cloacae* ZOR0014 as a positive control (Fig. 3B, orange bar) and WT *E. cloacae* ZOR0014 + filter as a negative control (Fig. 3B, brown bar) were also co-cultured with *E. coli*. The filter blocks any contact between the two strains, allowing uninhibited *E. coli* growth.

E. cloacae's killing ability was measured through *E. coli* survival – the higher *E. coli* survival, the worse *E. cloacae's* relative ability to kill was. The Δ*vask1* mutation showed a partial reduction in killing by about one log (Fig. 3B). *E. coli* survival was between that of the WT *E. cloacae* ZOR0014 but not completely reduced to the level of the negative control. The Δ*vask2* mutant showed no reduction in killing ability. It displayed the same level of *E. coli* survival as the original wildtype *E. cloacae* ZOR0014 (Fig. 3B). The Δ*vask3* mutant showed reduced killing ability by half a log when competed against *E. coli* (Fig. 3B). The double *Δvask1Δvask3* mutant showed a complete reduction in killing ability, with *E. coli* survival increasing by about 3 logs (Fig. 3B). The *E. coli* survival against the double mutant was nearly exactly the same as the double filter negative control.

While these were the initial results, statistical analysis of CFU counts showed slightly different results. While the reduction in killing for the Δ*vask1* mutant is statistically significant from the wildtype, the Δ*vask2* mutant and Δ*vask3* mutant was not (Fig. 3B). The difference in *E. coli* survival between the WT *E. cloacae* ZOR0014 and the *Δvask1Δvask3* mutant was significant (Fig. 3B). Additionally, there was no significant difference between the negative control (WT + filter) and the double mutant (Fig. 3B).

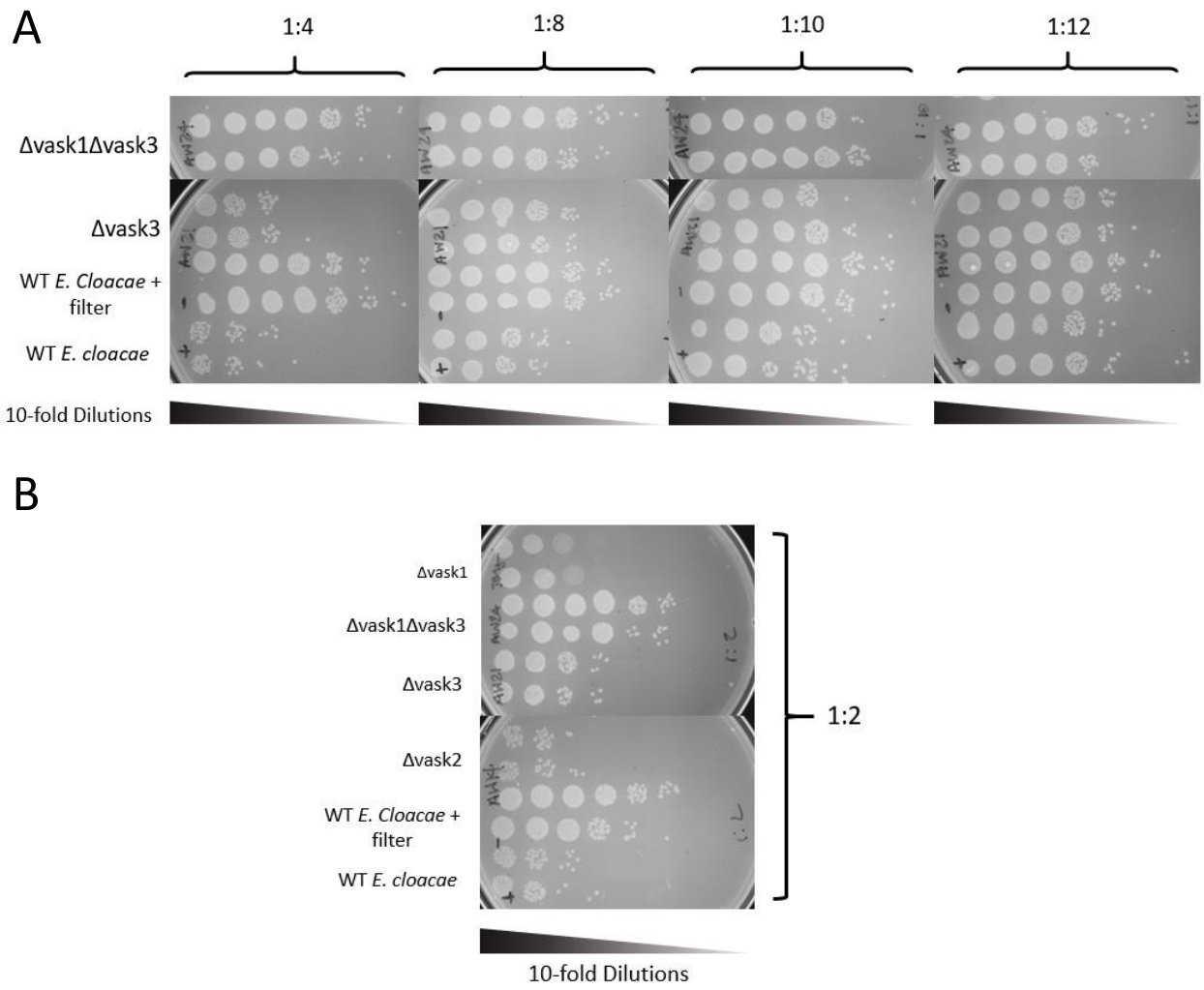

Figure 2. 1:2 ratio of *E. cloacae* strains to WT *E. coli* produces the best results for visualizing the differences between strains. (A) WT *E. cloacae* ZOR0014, WT *E. cloacae* ZOR0014 + filter, and a sample of the mutants (*Δvask3* and *Δvask1Δvask3*) were co-cultured in a competition assay with WT *E. coli* in four different ratios: 1:4, 1:8, 1:10, and 1:12 (*E. cloacae: E. coli*). (B) Additionally, a 1:2 ratio was tested with all three single mutants and the double mutant *E. cloacae* strains. Duplicates of each combination are shown here.

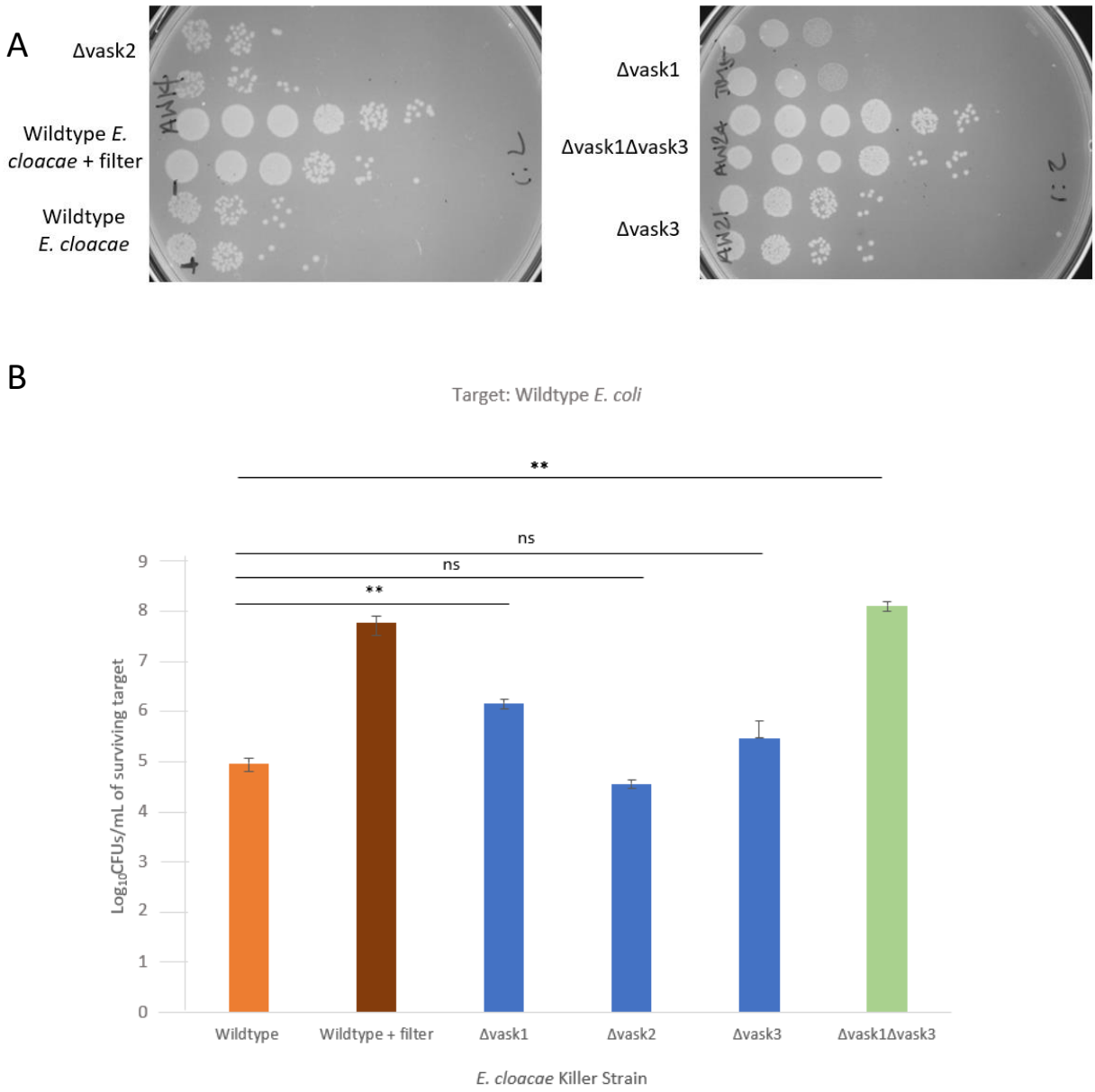

Figure 3. *E. cloacae* has two functioning Type VI Secretion Systems that contribute to contact dependent killing *in vitro*. (A) Preliminary results from a competition assay (B) This was quantified by measuring CFUs. The WT *E. cloacae* ZOR0014 strain (orange bar), WT *E. cloacae* ZOR0014 + filter (brown bar), three single *vask* mutants (*Δvask1, Δvask2, Δvask3* - blue bars) and the double mutant (*Δvask1Δvask3* – green bar) were co-cultured with WT *E. coli*. Data shown is the mean survival of triplicates +/- standard deviation. A one-way ANOVA test with Tukey-Kramer post hoc test was used to determine significance. ** $p < 0.05$, ns $p > 0.05$.

Discussion

Bioinformatic analysis of the *E. cloacae* genome has indicated that there are three *vask* loci, two large and one small cluster. Previously, it was unknown whether these loci were active and contributed to T6SS mediated killing. Our work so far has disrupted/deleted all three of the *vask* loci, named *vask1*, *vask2* and *vask3*, which correlate to T6SS1, T6SS2, and T6SS3, respectively. *E. cloacae* Δ*vask1* was competed against wildtype *E. coli* to assess changes in killing ability. It was found that *E. cloacae* Δ*vask1* displayed partial decreased killing ability towards *E. coli*, indicating that T6SS1 was functional and active in T6SS mediated killing. However, since *E. coli* survival was still lower than the negative control, T6SS1 could not have been entirely responsible for all of *E. cloacae* T6SS mediated killing. The same experiment was performed for *E. cloacae* Δ*vask2* and it was found to have no effect on *E. coli* survival. This indicated that either T6SS2 was not involved in *E. cloacae* ZOR0014 T6SS mediated killing or that this *vask2* gene was not part of a Type VI cluster. Since *vask2* was found in the smaller cluster and it is unknown whether other necessary genes for T6SS are included in this cluster, it seems feasible that *vask2* might be independent of a T6SS.

The third mutant, Δ*vask3*, showed a reduced capacity for killing but not to the level Δ*vask1*. While Δ*vask1* experienced one log of increased *E. coli* survival, Δ*vask3* only had a half log increase. Again, this indicated that *vask3* is functional and active and T6SS3 contributes to T6SS mediated killing. However, when statistical analysis was applied to the differences between all three single mutants and the WT *E. cloacae* ZOR0014, it was found that there was not a statistically significant difference for the Δ*vask2* and Δ*vask3* mutants but there was for Δ*vask1* (Fig. 4). Disrupting T6SS3 on its own does not significantly impair *E. cloacae*'s ability to kill its predator, nor does disrupting T6SS2. We wondered what effect disrupting both the

T6SS1 and T6SS3 would have. Based on our preliminary results, it seemed likely that both T6SS1 and T6SS3 together were responsible for the *E. cloacae's* ability to kill.

The next step was to test the double mutant (*Δvask1Δvask3*) to observe the effect that knocking out both T6SS's will have on *E. cloacae's* killing ability. The double mutant showed a complete reduction in contact dependent killing ability, with about three logs increase in *E. coli* survival, which compared to the wildtype survival was statistically significant. This likely indicates the T6SS1 and T6SS3 are both necessary for contact dependent killing. Additionally, there was no statistically significant difference between the negative control (WT + filter) and the double mutant (Fig. 3). Moving forward, the double mutant can be used as an effective replacement for the double filter method to obtain a negative control.

Several bacteria, like *Pseudomonas aeruginosa* PA01, encode multiple T6SSs (H1-T6SS, H2-T6SS, and H3-T6SS) that play distinct roles in different conditions[17]. For example, in *P. aeruginosa* the H1-T6SS display anti-prokaryotic activity. By contrast, the H2-T6SS and H3-T6SS show both anti-prokaryotic and anti-eukaryotic activity that appear to contribute to epithelial cell invasion *in vivo*. As a result, moving forward, a triple mutant disrupting all three *vask* genes needs to be made. Initial results indicated that Δ*vask2* did not impair *E. cloacae* killing ability at all *in vitro*. However, considering that both the single Δ*vask1* and Δ*vask3* had small increases in *E. coli* survival, but when both genes were disrupted together exhibited a much significantly larger increase in survival, it's necessary to make a triple mutant to rule out the possibly of Δ*vask2* having an effect in conjunction with Δ*vask1* and Δ*vask3.* Additionally, these strains will be sent back to our collaborators in the Parthasarathy lab to be tested in vivo. While the Δ*vask2* mutant may show no difference in vitro, it could potentially behave very different in a live zebrafish model, as could Δ*vask1,* Δ*vask3,* and Δ*vask1*Δ*vask3.*

Conclusion

Our research will help expand on the role of T6SS in *E. cloacae,* particularly as it pertains to virulence. While studies done on other *E. cloacae* strains, such as the standard clinical strain ATCC13047, have elucidated a lot of information, this is not always applicable to other strains. Specifically, the strain used in our research (ZOR0014) is a unique environmental isolate taken from the intestines of a zebrafish and to date, no studies have been published with this particular strain. This strain is valuable because of its ability to be studied in zebrafish models. Zebrafish models are uniquely valuable model organisms due to their ability to stay sterile in the first 14 days and their transparency, allowing for full visualization of internal mechanisms. This technique allows for a high degree of control, excellent visualization and a more precise understanding of how *E. cloacae* interacts in vivo in regard to T6SS.

Our research to date has shown that single Δ*vask1* and Δ*vask3 E. cloacae* mutants show a partial reduction in killing ability in comparison to the WT *E. cloacae*. However, the differences were only significant for Δ*vask1*. When both of these genes are disrupted together in a double *Δvask1Δvask3* mutant, there is a significant reduction in killing ability. Initial results indicated that the Δ*vask2* mutant doesn't play a role in *E. cloacae's* T6SS mediated killing *in vitro*. However, Δ*vask2* may behave very differently *in vivo* where it is exposed to other factors, such as anti-eukaryotic effectors in a fish host. Additionally, developing and testing a triple mutant (Δ*vask1*Δ*vask2*Δ*vask3*) along with the rest of these strains in a live zebrafish model will provide more information about how these mutants are affected in a more natural *in vivo E. cloacae* environment.

The T6SS plays a big role in the ability to compete and defend for resources in bacteria like *E. cloacae*. *E. cloacae* is a relatively understudied bacterium despite its many problematic characteristics, especially as it pertains to human health. Investigating and understanding a major system that *E. cloacae* uses to potentially harm its host is invaluable. The work done in this project is foundational and can easily be built upon in the future.

Methods and Techniques

Bacterial Strains and Growth Conditions

Enterobacter strain ZOR0014 used in this study was an environmental isolate obtained from zebrafish commensals sent by collaborators at the University of Oregon.[18] *E. coli* MG1655 CmR was used as prey in the killing assays. All strains were grown with liquid lysogenic broth (LB) medium or on LB agar at 37 °C overnight with appropriate antibiotics.

The LB medium was made by adding 10 g tryptone, 5 g sodium chloride, and 10 g yeast per liter (in the case of plates, 15 g of agar was added to the mixture) and the mixture was sterilized.

Kanamycin and ampicillin were added at 1 µg/mL, gentamycin at 0.33 µg/mL and diaminopimelic acid (DAP) at 2 µg/mL.

Disrupting ZOR0014 *vask1* and *vask2*

A Δ*vask*2 of ZOR0014 was constructed prior with assistance from Siu Lung Ng and not described here. A *dap* auxotrophic *E. coli* lambda-pir donor strain was used to deliver suicide vectors into ZOR0014 by conjugation. Growth of the *dap* auxotroph requires the addition of DAP (diaminopimelic acid) to agar plates, and its omission prevents donor growth. The pKAS-

*vask*1 vector encodes kanamycin resistance, the pKAS-*vask*3 vector encodes gentamycin resistance. Each suicide vector has an R6K origin of replication that restricts its replication to the *E. coli* donor strain carrying the Pir protein on its chromosome. Each suicide vector was constructed by Siu Lung Ng by PCR amplification of a 1000 bp DNA sequence of a *vask* gene that was cloned into the vector backbone by Gibson assembly, as described in Crisan, 2019. Once delivered into ZOR0014, neither vector can replicate. However, one can select for a rare recombination event of the vector into the chromosome by plating on agar containing antibiotic (kanamycin for pKAS-*vask*1, or gentamycin for pKAS-*vask3*).

Liquid overnight cultures of each *E. coli* donor were mixed with a liquid culture of the ZOR0014 isolate in a 1:8 ratio. 50 µl of each mixture was plated in a small patch on LB agar supplemented with DAP to ensure donor growth, and incubated overnight at 37°C. The following day, cells were scraped from the patch and placed into 1 mL of liquid LB. Ten-fold serial dilutions were performed. 50 µl of each dilution was plated on agar lacking DAP but supplemented with kanamycin (for *vask*1) or gentamycin (for *vask*3). Overnight incubation at 37°C selected for ZOR0014 recipients with integrated vectors and eliminated the *E. coli dap* auxotrophic donor. Multiple surviving colonies from each of the plates were selected and re-streaked on agar plates with antibiotic for the next two days. Frozen stocks of the ZOR0014 candidate mutants were also made (50% overnight grown in 5 mL liquid LB with antibiotic and 50% glycerol).

Confirming *vasK* disruptions

Genomic DNA purification was done on two candidate mutants (prepared using the methods described above) along with ZOR0014 as a control. The purification was conducted using QIA

Spin MiniPrep Kit in accordance with those specific instructions. After DNA purification, PCR was performed on the DNA using 5xTaq PCR protocol. An annealing temperature of 55°C and an elongation time of 40 minutes was used. The PCR product was run on a 1% agarose gel at 100 V for 60 minutes. The primers (GT3202/GT3203) used for the PCR annealed in positions that produced a PCR product of ~1 kb from template DNA derived from ZOR0014. By contrast, each *vasK* disruption was confirmed by a PCR product that was 10 kb due to integration of the suicide vector into the chromosome.

Killing Assay Conditions

Both *E. coli* and ZOR0014 were grown overnight in liquid LB medium with shaking at 37°C. The strains were normalized to an OD_{600} of 1.0. Predator ZOR0014 (environmental and genetically modified, KanR) and prey *E. coli* were mixed at a ratio of 1:8 and 50 µl of each suspension was then spotted onto LB agar plates. The plates were incubated for 3 h at 37°C. The agar spot patches were removed with a sterilized tool and placed into Falcon tubes. The cells were removed from the agar by vortexing for 30 seconds in 5 mL of LB medium. The cells were serially diluted ten-fold and spot plated on LB agar. The plates were grown overnight at 37°C and results were recorded the following day (Fig. 4).

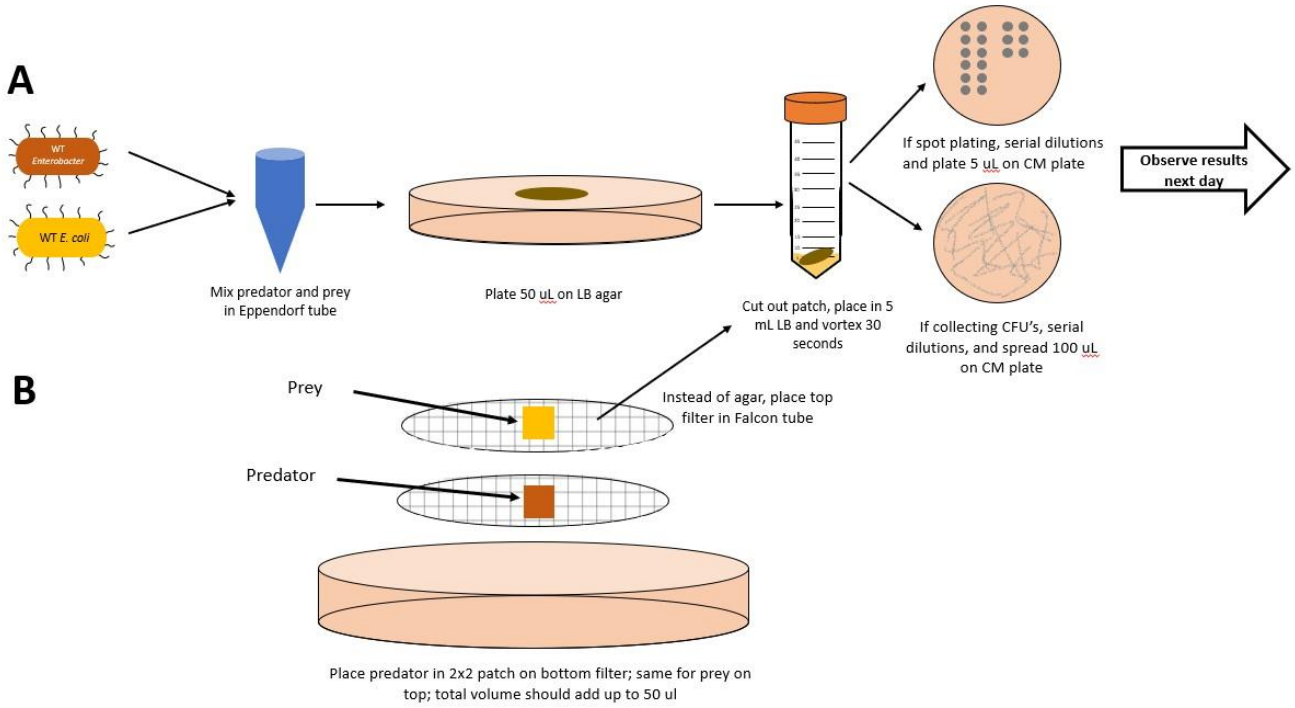

Figure 4 – Killing Assay Protocol to (A) allow and (B) prevent contact between competitors. (A) *E. coli* and ZOR0014 were mixed together in a 1:8 ratio after being normalized to OD_{600} of 1.0. 50 µl of the mixture was plated on LB agar and incubated for 3 hrs at 37°C. The agar patches were removed using a flame sterilized spatula and placed into 5 mL of liquid LB in a falcon tube and vortexed for 30 seconds. Ten-fold dilutions of each sample was made. If spot plating, 5 µl of every dilution for each sample was plated on agar + CM. If counting CFUs, for each sample 100 ul µl of one dilution (varies per sample) was spread on agar + CM using beads. (B) *E. coli* and ZOR0014 were normalize to OD_{600} of 1.0. A filter was placed directly onto an agar plate. 43.75 µl of ZOR0014 was placed in a 2x2 grid on top of the filter. After the patch was dry, another filter was laid directly on top of the first one. 6.25 µl of *E. coli* was placed in the same 2x2 patch on top of the second filter. The plates were incubated for 3 hrs at 37°C. The top filter was removed using a flame sterilized forceps and placed in a Falcon tube with 5 mL of LB and vortexed for 30 seconds. The rest of the procedure is the same as (A).

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