ALLELIC CHARACTERIZATION AND NOVEL FUNCTIONS OF THE OUTER MEMBRANE PORIN U IN *VIBRIO CHOLERAE*

by

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B.S. Khulna University, 2014
M.S. Khulna University, 2018

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
in the Burnett School of Biomedical Sciences
in the College of Medicine
at the University of Central Florida
Orlando, Florida

Summer Term
2019

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ABSTRACT

Vibrio cholerae is the etiological agent of the severe diarrheal disease cholera. The bacterium is a natural inhabitant of brackish and estuarine waters . To date, only a subset of *V. cholerae* strains, those belonging to the pandemic group (PG), can cause cholera in humans while the rest (environmental group, EG) cannot cause the disease. Recently, we discovered that *V. cholerae* PG contains allelic variations in core genes that confer preadaptation to virulence, which we termed Virulence Adaptive Polymorphisms (VAPs). We identified nine core genes that encode potential VAPs, one of which encodes the outer membrane porin U (OmpU). OmpU provides tolerance to bile and acidic pH, resistance to antimicrobials and facilitates biofilm formation. In this study, several alleles of *ompU* were analyzed to determine whether these VAPs encode different functional properties. We performed multiple phenotypic assays and observed increased survival for strains encoding the PG-like alleles in the presence of bile, organic acid, anionic detergents and the antimicrobial peptide P2. On the other hand, EG-like alleles only showed increased biofilm formation. Interestingly, tests for motility and tolerance of inorganic acid, polymyxin B and protamine sulphate showed no differences in survival for strains encoding either alleles indicating that some of the properties conferred by OmpU are allelic independent. We have also discovered that *V. cholerae* OmpU shows resistance against Rifamycin, EDTA and Trifluoperazine and interestingly, Rifamycin has been found to be PG-allele dependent. Our findings provide further evidence that genetic

variations in core genes lead to the emergence of virulence adaptive traits in pathogenic *V. cholerae* and can be extrapolated to other bacterial pathogens.

ACKNOWLEDGEMENTS

First, I would like to thank my thesis advisor, Dr. Salvador Almagro-Moreno, for his precious advices, guidance, support and for being an invaluable mentor throughout my graduate research career at UCF. I would also like to acknowledge all the members of the Moreno Lab, particularly Geethika Reddi, Trudy-Ann Grant and Jane Maureen Jayakumar for their continuous assistance and support; I am also grateful to my thesis committee, Dr. Sean Moore and Dr. Herve Roy with their presence as my committee members and for providing me insightful suggestions and advice in preparation of this manuscript. Finally, I want to give my special appreciations to my family members and friends for encouraging me every step of the way.

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OmpU- Outer membrane porin U

CT – Cholera toxin

TCP – Toxin coregulated pilus

PG – Pandemic group

EG – Environmental group

VAPs- Virulence adaptive polymorphisms

SDS – Sodium dodecyl sulphate

AMP- Antimicrobial peptide

LB-Luria-Bertani

IF-Inoculating fluid

CHAPTER ONE: INTRODUCTION

1.1 Cholera

Cholera is a major scourge in countries with poor sanitation systems, limited access to clean drinking water or hygienically handled and stored food and where the infrastructure of sewerage system has been devastated due to political unrest or natural disasters (1–3). The disease cholera has been classified as one of the two class A infectious diseases according to CDC alongside plague. Patients with cholera suffer from acute and secretory diarrhea, severe dehydration, abdominal cramps, vomiting that often leads to death if not treated immediately (4–6) It is a major public health concern as there are a rough estimate of 1.3-4 million cholera cases and 21,000-1,43,000 deaths due to cholera worldwide every year (7). Seven pandemics of cholera have been documented so far spanning Indian continent, Middle East, North and South America, Europe and Africa (1,8). Recently, one of the biggest cholera epidemic took place in Haiti due to the massive earthquake in 2010 and according to CDC, nearly 700000 cholera cases and almost 8500 deaths were reported (9–13). In recent times, the largest epidemic outbreak of cholera is taking place in Yemen where there are over 1,000,000 suspected cholera cases (14).

1.2 *Vibrio cholerae*

Vibrio cholerae is the etiological agent of the severe diarrheal disease cholera. *V. cholerae* is one of the most widely studied member of the family *Vibrionaceae* (13,15).

The bacterium is a facultative pathogen that is naturally found in brackish and estuarine waterbodies (16).

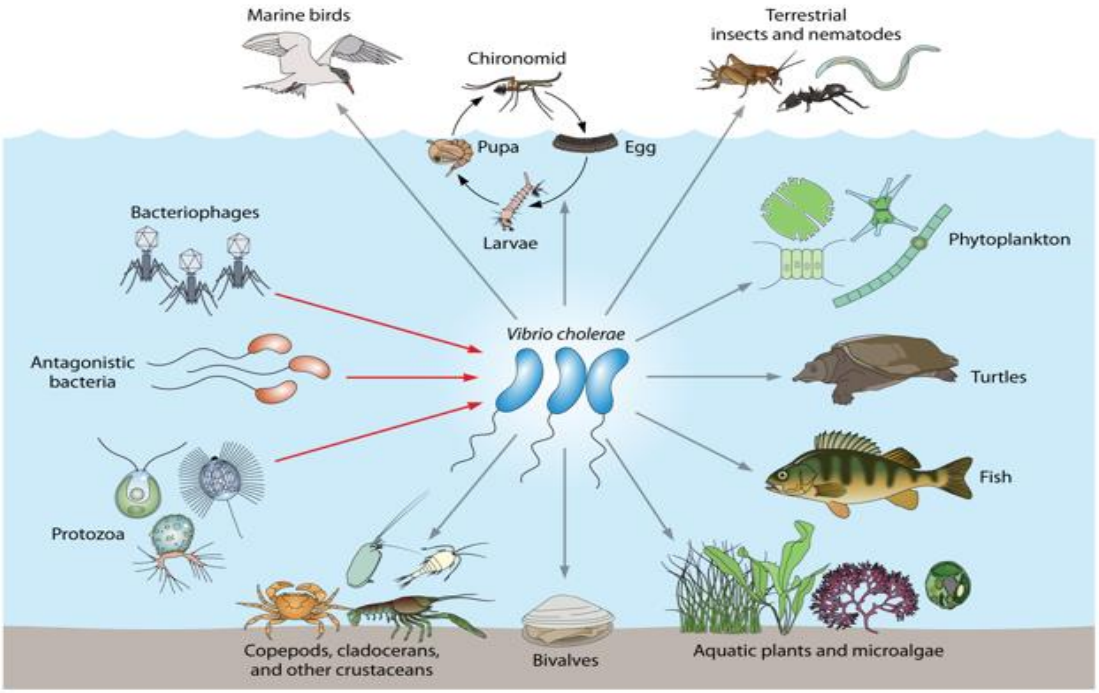

Figure 1: *Vibrio cholerae* interactions in its natural environment

The associations of *V. cholerae* with reservoirs and antagonistic organisms that shape its virulence potential are shown. Gray arrows indicate reservoirs, such as crustaceans, copepods, chironomid egg masses, phytoplankton, fish, turtles, aquatic birds, shellfish, and protozoa. Red arrows indicate antagonistic relationships with protists, bacteriophages, and predatory bacteria (16).

Even though microbes of the *Vibrionaceae* family are natural inhabitants of the aquatic environment, some of them have emerged as human pathogens such as *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* (13,17). *V. cholerae* is found frequently associated with numerous organisms in its natural habitat (Fig. 1) (16,18–20). *V. cholerae* strains are grouped into two defined biotypes: El Tor and classical and over 200 serotypes (21,22). The El Tor and classical biotypes are differentiated based on biochemical properties and phage sensitivity, whereas serogroup differentiation is based on O-antigen structure (22). It has been reported that strains with serogroup O1 and O139 belongs to the Pandemic group (PG) that can cause the disease cholera (17,23). Most nonpathogenic, environmental isolates belongs to the serogroup non-O1 and/or non-O139. A number of reports have demonstrated that some strains of these environmental group (EG) can cause diarrheal diseases or local infections, but do not have the ability to cause epidemic outbreaks (17,24). Besides the toxigenic O1 and O139 serogroups, *V. cholerae* strains belonging to more than 200 serogroups are wide spread in aquatic environments (25).

It is quite striking that only a handful strains of *V. cholerae*, only those belonging to the PG group, have the ability to cause the disease cholera while the vast majority of the strains (EG group) cannot cause cholera (23,26). Several lines of evidence indicate that major virulence factors such as cholera toxin (CT) and toxin coregulated pilus (TCP) are not an exclusive feature to choleragenic strains of *V. cholerae* PG as they are also encoded by many non-choleragenic strains (21,23,27,28). Moreover, it is evident through numerous sampling data that many pathogenic and non-pathogenic strains co-exist in the

same geographic area (e.g. Bay of Bengal) and indicates that no sympatric speciation is occurring among these strains (26,29). Recently, Shapiro et al. reported that *V. cholerae* PG contains a unique genomic background that renders them susceptible to become pathogenic (17). Absence of these genomic features limits the possibility of pandemic clones from emerging within a bacterial population (17). These allelic variations confer different functional properties for intestinal colonization and facilitates pathogenesis of *V. cholerae* (17).

1.3. Virulence adaptive polymorphisms

Recently, we have discovered that allelic variations in the core gene of pandemic *V. cholerae* strains confers preadaptations to virulence which we termed Virulence adaptive polymorphisms (VAPs) that enhance the potential of the bacterium to give rise to a pandemic clone (17). VAPs exist and are also encoded by the environmental population of *V. cholerae*. VAPs confer preadaptations to virulence besides the acquisition of virulence genes such as cholera toxin (CT) or toxin-coregulated pilus (TCP) (17). We reported that, VAPs must be present in the genomic background of *V. cholerae* before it can emerge as a successful pathogenic clone. The authors proposed a scenario in which VAPs circulate in the natural environment, get selected and enriched under different ecological conditions and a genomic background containing several VAPs acquires virulence factors that allows for its emergence as a pathogenic clone (17). The authors reported nine such core genes that encode potential VAPs. For example, allelic variations in the core gene (*ompU*) encoding OmpU confers preadaptation to virulence in the human

host (17). Because of their diverse functions both in the environment and host, VAP alleles of *ompU* can be used to study how *V. cholerae* evolve in their natural environment, gain prolonged ecological fitness, and ultimately emerge as a pandemic clone.

1.4. The outer membrane porin U

OmpU is considered as the principal outer membrane porin of *V. cholerae* (30,31). *ompU* (*V*C0633) is a protein coding core gene located on the chromosome 1 of *V. cholerae* N16961 El Tor. The nucleotide sequence of the outer membrane protein is 1053 base pairs long and there are 350 amino acid residues that forms the structure of the porin (32–34) OmpU is a porin embedded in the outer membrane and regulated by the transmembrane transcriptional activator and virulence regulator ToxR (35,36). It has been shown that bile stimulates the expression of ToxR mediated expression of OmpU. *ompU* expressing cells are more resistant to bile, anionic detergents, organic pH, antibiotics and antimicrobial peptides (31,32,37). It is crucial to understand the role of OmpU porin of *V. cholerae* to comprehend their role in intestinal colonization, environmental persistence and survival.

OmpU is a pore forming protein of *V. cholerae*. The porin activity of the ompU protein has been shown by the liposome swelling assay (38). OmpU porin allows the selective diffusion of hydrophilic molecules across the outer membrane. OmpU is also involved in adhesion of *V. cholerae* during intestinal infection (38). Recent studies on the X-ray crystallography of OmpU porin has revealed the structural features of the OmpU porin (39). In brief, the first gate is a highly positively charged gate and is constituted by several

residues: Arg61, Arg74, Arg76, Arg116, Arg164, Asp163, Lys158, Asn153, and Tyr150. Among these, the first five arginine residues (Arg61, Arg74, Arg76, Arg116, Arg164) are responsible for building the lining of the gate while Arg57, Arg318 and Arg347 are some of the other residues that are buried towards the periplasm (39). The second gate inside the porin is highly negatively charged. The presence of a unique and non-canonical N-terminal coil is reported in the pore lumen of OmpU porin that is almost half of the circumference of the second gate. The residues in this coil have hydrophilic properties. The N-terminal coil needs the constriction loop L3 to form the smaller pore/second gate inside the protomer barrel porin. The residues that take part in forming the small circular pore with N-terminal coil are Asn34, Asp38, Glu96, Tyr117, Asp135, Lys181, Gly139 and Asp143. Arginine residues dominates the lining of the pore of OmpU and It has been reported that these residues are responsible for the electrostatic properties of the pore. Their role may also be associated with the ion selectivity and channel conductance of the porin. There are eight loops in each of the protomer of OmpU with different features and these loops are designated as L1, L2, L3, L4, L5, L6, L7 and L8. Among these, loop 4 or L4 is the longest loop that protrudes in the extracellular space of the membrane. This loop contains a β-hairpin motif with it which is constructed by two short and antiparallel β-sheets connected by thinner loops. Loop L4 is highly mobile and have been reported to confer resistance against phage predation. Two other loops in this porin along with L4 has been reported to have role in phage predation which are L3 and L8. This Loop (L4) is highly mobile which is also applicable in case of two other loops of this porin namely L6 and L8 (39). Beside the loops, there is a noncanonical N-terminal coil of the OmpU

porin. It differentiates the two gates inside the pore lumen and protrudes towards the periplasmic side of the bacterium (39,40).

1.5. Functions of the outer membrane porin U

OmpU is a virulence factor in *V. cholerae* and plays a role in bile resistance, acidic pH resistance, antimicrobial resistance and phage and amoeba predation resistance. Overall, PG alleles of OmpU conferred efficient intestinal colonization in the host intestine (5,41). Few of the important roles of the OmpU porin has been described in the following sections.

1.5.1. Bile and detergent tolerance

Bile is a biogenic detergent like molecule that restricts the activity of *V. cholerae* in the host intestine (42). It is previously shown that OmpU porin of *V. cholerae* confers bile resistance (37,43). It has been demonstrated that mutants with a deletion of the gene encoding *ompU* have retarded survival percentage compared with the wild type strains of *V. cholerae* when tested in the presence of the bile (0.4%), and deoxycholate (DC), a bile component. (37). Numerous studies have shown the effects of bile and the resistance mechanisms adopted by *V. cholerae* associated with this antimicrobial compound (44). Unlike other pathogens such as *E. coli* or *Salmonella*, expression of *ompU* upon bile exposure is a unique characteristics of *V. cholerae*. Resistance to bactericidal bile exposure in *V. cholerae* has been found associated with several mechanisms (44). The role the outer membrane porin, OmpU and OmpT has been shown to be critical for bile tolerance (37,45). In the presence of bile, ToxR induces the expression of OmpU and

represses the expression of OmpT. The reason for OmpU to be resistant to bile is the anion selective property of the porin. OmpU restricts the passage of anions or negatively charged molecules. Bile is a complex mixture of negatively charged molecules such as deoxycholate. These components cannot passage through OmpU and thus makes the bacteria resistant to bile. Besides the OmpU mediated bile tolerance, several other mechanisms have been shown to be associated with bile tolerance. For example, AcrAB-TolC system, BreAB, VexAB and VprAB are some of the efflux pumps that take part in bile tolerance mechanism (46–49) These pumps are essential to colonize and establish infection in the intestine when *V. cholerae* is under constant exposure to bile. Recently, the role of a transcriptional regulator LeuO has been shown to play a protective role against this antimicrobial compound (50). ToxR regulates the activity of LeuO and the authors hypothesized that the periplasmic signaling domain of ToxR is associated with the specific response (50). Although the specific role of LeuO in bile tolerance is yet to be elucidated.

Provenzano et al. (37) also studied the role of OmpU in detergent tolerance to evaluate whether the detergent tolerance property is restricted within the anionic type of detergent tolerance by OmpU. They found that, ectopic expression of OmpU was able to confer resistance against large anionic molecule containing detergents such as Sodium Dodecyl Sulphate (SDS). OmpU is an anion selective porin and it restricts the passage of large anionic molecules such as molecules present in loryl sulphate SDS. Provenzano et al. showed that in a toxR-negative strain, the upregulated expression of OmpU was able to

show sublethal SDS tolerance where in a toxR-positive strain, the ectopic expression of OmpT was incapable to show similar growth in the presence of SDS (37).

1.5.2. Acidic pH tolerance

V. cholerae can survive both organic and inorganic acid shocks at pH 4.5 after getting adapted to a mildly acidic pH (pH 5.7). These cells can colonize in both suckling and adult mice after the shock (32). Merrel and camilli showed the upregulation and downregulation of 60 and 50 different genes respectively upon exposure to organic acid challenge (51). However, the role of the outer membrane porin OmpU has been investigated extensively because of the high abundance of this porin in the membrane (~60%). Merrell and Camilli hypothesized two possible models that best signifies the role of the outer membrane porin in mediating organic acid tolerance response (32). In the first model the authors showed that the presence of OmpU alone mediates resistance to organic acids because these molecules cannot pass through OmpU porin and reach to the periplasm upon organic acid challenge. The alteration of such porin activity has been evaluated in a delta *toxR* mutant where both *ompU* and *ompT* is unable to express. In a *ΔtoxR* strain, the co-expression of *ompU* and *ompT* was able to bypass organic acid tolerance response and regained the resistance. However, individual ectopic expression of both of these porins were an exclusive observation in this experiment as the ectopic expression of *ompU* was able to regain the resistance but *ompT* was unable to do so. This means the expression of OmpU is important in this resistance mechanism among the two porins regulated by ToxR**.** The role of OmpT in regulating cellular integrity around organic acidic environment was also tested by overexpressing *ompT* in a wild type strain of *V. cholerae*. Result

indicated that overexpression of *ompT* behaved like the wild-type strain which proves that OmpT is a less important factor in comparison with OmpU to consider it as organic acid responsive (32,52). It has been reported according to another model of Merrell and Camilli that in the presence of organic acid, the loss of OmpU porin leads to the disintegration of the outer membrane (32). OmpU protein compose up to 60% of the outer membrane of *V. cholerae* and one of the most vital membrane components that ensures membrane integrity. The loss of OmpU thus leads to the permeation of organic acids through the disintegrated membrane and reaches to the periplasm. It has been reported by Jubair et al that loss of OmpU from the flagella has resulted in severely attenuated integrity of the flagella which consolidates the fact that OmpU is required to ensure membrane permeability properties (53). Due to alteration of the membrane property and absence of OmpU, organic acid molecule passage through the membrane and generates the bactericidal effect in an elevated organic acidic environment. It is important to mention that, such disintegration of membrane can occur due to the absence of OmpU in the acidic environment. However, a few other factors such as *V. cholerae* GshB and HepA factors have been identified as required for acid tolerance response (54). Expression of cadBA operon by CadC, cadC by AphB has also been shown to provide response in organic acid challenge (51).

1.5.3. Antimicrobial resistance

P2 is a bioactive and cytotoxic peptide that has been shown to induce cytotoxicity in *V. cholerae* and thus acts as an antimicrobial. P2 permeabilizes the outer membrane of the

bacteria and perturb the inner membrane which impairs the electron flow. Previously, studies on the P2 resistance of bacteria such as *Salmonella* spp. and *Escherichia coli* revealed mechanisms which portrays interesting defensive strategies adopted by bacteria to survive in the intestine .

Mathur and Waldor reported for the first time that *V. cholerae* can survive sublethal P2 challenge, and OmpU confers this protection by inducing the stress response pathway (55). Unlike in *E. coli* and *Salmonella* spp., P2 resistance is not mediated by *bipA* in *V. cholerae* (55). Mathur and Waldor proposed two models where they have demonstrated the protective mechanism in sublethal P2 challenge. When *V. cholerae* cells detects the presence of P2 in the surrounding environment, the OmpU activates its YDF motif which is generally buried in the C-terminal end of the protein. The YDF motif activates DegS, a periplasmic protease which in turn activates the main factor that induces stress response pathway, σ^E. σ^E stress response pathway has been reported to confer protection of cell envelope integrity in *V. cholerae*. It has been reported that the bacterial envelope stress activates the alternative sigma factor or σ^E which induces the expression of factors that contributes to preserve and/or restore the cell envelope integrity. Davis and Waldor have analyzed spontaneously arising suppressor mutations on *rpoE*, the gene that encodes sigma(E) factor of *V. cholerae* and found that more than one-third of the rpoE mutants contain suppressor mutations which reduce the production of OmpU. This means that OmpU acts as the key factor for the initiation of sigma(E) production in *V. cholerae*. Such single factor dependency is not common in case of other enteropathogenic bacteria, such

as E. coli, where numerous factors contributes for the expression of the rpoE which encodes the sigma(E) factor (56).

1.5.4. Motility

Motility aids *V. cholerae* during infection and thus considered as a virulence factor of *V. cholerae* (57). The flagella of *V. cholerae* is thicker compared to other gram-negative bacteria such as *Psedomonas aeruginosa* and *Salmonella enterica* serover typhimurium. The loss of motility reduces virulence capacity of *V. cholerae* (30). Although the role of different outer membrane proteins such as FlgO and FlgP has been shown to be involved in *V. cholerae* motility (58), the importance of OmpU in the monotrichus flagella of *V. cholerae* needs further elucidation. OmpU is an integral component of *V. cholerae* flagellum which led Bari et al. to peruse and analyze the role of this protein in the structural integrity and organization of flagellar sheath in *V. cholerae*. Both El tor C6706 and 0395 biotypes of *V. cholerae* possesses OmpU in their flagellar sheath along with other core flagellar proteins. They reported that OmpU and OmpT has role in the structural integrity and flagellar rotation of V. cholerae which might facilitate their passage through the intestine (30).

1.5.5. Phage resistance

Seed et. al. reported that, vibriophages prey on *V. cholerae* population and shapes their load in the intestine (13). They showed that, ICP2, a type of vibriophage that is capable of eliminating *V. cholerae* in the gut, failed to lyse almost 100% of the *V. cholerae* cells in the gut. Particularly, patients sample from the recent Haiti outbreak showed resistant to phage attack in a very high magnitude of almost 100%. While a few of the *V. cholerae*

population was sensitive to the invasion of ICP2, almost all the bacterial isolates showed resistance to the phage attack. Whole genome sequencing data and molecular analysis showed that mutations were prevalent in a gene that encodes a major porin of the outer membrane of *V. cholerae* – *ompU. ompU* encodes the outer membrane porin (OmpU) of *V. cholerae* which is the receptor for vibriophages such as ICP2. Phages failed to infect the bacteria because of mutations in the *ompU* alleles. This is the first report that illustrates the phage/bacterial antagonistic interaction or predator-prey relationship in human gut and the reason of why *V. cholerae* cells have the ability to resist phage lysis at the molecular level. Seed et. al. consolidated these findings by evaluating 54 clinical samples of *V. cholerae* from Bangladeshi patients collected at different time points from 2001 to 2011 (13). They have reported that 15% of *V. cholerae* cells has mutations in *ompU* alleles that makes them resistant to phage penetration. Mutations in the *toxR* gene that encodes OmpU has also been found to be defective for phage to efficiently lyse *V. cholerae* cells. Analysis *V. cholerae* cells were collected from the stool sample of a Bangladeshi patient and found to be resistant to the vibriophage, ICP2 in 22% of the *V. cholerae* cells. Previously, *in vitro* experiments demonstrate the influence of phage attack on bacteria and the fact that mutations in the surface exposed membrane protein can resist the invasion of bacteriophages (59). Gehring et al. reported that mutations in *E. coli*. externally exposed membrane proteins interferes the ability of the phage lambda viruses to infect the bacterial population (60). They showed *E. coli* mutants with mutated surface exposed protein showed resistance to phage invasion (13).

1.5.6. Biofilm formation

One of the reported adaptive role of OmpU in the environment is the formation of Biofilm (20,61). *V. cholerae* forms biofilm on the exoskeletons of crustaceans and abiotic environmental surfaces (20). It has been shown that, ompU mutants form robust biofilm on the surface of abiotic environmental surfaces (62). Although the detailed mechanism is currently under investigation, it has been reported that the robust biofilm formation of ompU deletion mutants is corelated with the activity of the virulence regulator ToxR (62). It has also been shown that the deletion mutant of OmpU and EG-like OmpU were maladaptive for intestinal colonization but formed robust biofilm on the abiotic surface (62).

1.5.7. Nutrient uptake

Several experiments has shown that, mutations in OmpU interferes the ability of the porin to transfer nutrients to the periplasm. Previously, Pagel et al. phenotypically characterized the OmpU mutants and examined several phenotypes including their growth on large sugars (63). Carbohydrates such as glucose, maltotetraose, maltohexaose and cyclodextrin were used as sole carbon source and evaluated their permeation . They reported that, mutations in arginine residues affects the pore properties of OmpU such as consuming and growing in the presence of these large carbohydrates or carbon sources (63). Mey et al. have reported that, addition of a combination of specific amino acids (Serine, arginine, asparagine and glutamate) upregulated the expression of ToxR protein which in turn promotes the production of OmpU porin in minimal media (64). These

findings indicate that OmpU has function to facilitate nutrient uptake and growth in *V. cholerae*.

1.5.8. Other functions

OmpU is known to be the most abundant and principal porin of the outer membrane of *V. cholerae* and they are involved in numerous functions involved in stress relief, nutrient uptake and resistance to toxic molecules (56). It has been shown that the outer membrane porin, OmpU is prevalent up to 60% in the outer membrane out of the total 2% protein in the outer membrane based on the enrichment of media. The presence of OmpU is conserved throughout Vibrio. Other members of the *Vibrionaceae* family contains OmpU in the outer membrane but the expression, regulation and physiological functions of OmpU varies from species to species (56). For example, the expression of *ompU* in *Vibrio fischeri* is independent of ToxR while ToxR reciprocally regulates the expression of ompU and ompT in *V. cholerae*. This is an indicator of the evolution of ompU that is divergent and reshaped based on their lifestyle and activity (65).

Given that OmpU is involved in regulating functions associated with virulence, there is a strong possibility that this porin might be involved in unknown functions that, if revealed, might shed light on the virulence properties of *V. cholerae.* There might be other functions of OmpU that might render them susceptible to virulence. The functions might vary with allelic variation that can either be adaptive or maladaptive to virulence. For example, bile tolerance is a property that confers preadaptation to virulence. We have shown that also shown that bile resistance provided by OmpU in *V. cholerae* is dependent on alleles as some of the environmental alleles (*ompU* $^{GBE\ 428}$ and *ompU* $^{GBE\ 658}$) were almost equally

resistant to 0.4% bile as the PG-wild type. On the other hand, another allele (*ompU* $^{GBE\ 1114}$) was incapable to provide resistance and survived similarly as the deletion mutant of *ompU*. Furthermore, resistance to polymyxin B is not associated with any specific *ompU* alleles. We have also shown that the presence of all the three mutant alleles (*ompU* $^{GBE\ 1114}$, *ompU* $^{GBE\ 428}$, *ompU* $^{GBE\ 658}$) do not interfere polymyxin B resistance as all of the three alleles survived similarly as the PG-wild type.

In *V. cholerae* biology, the virulence properties of *ompU* and their functions that provide resistance to toxic molecules are well studied. But the properties related to niche adaptation and host colonization needs to be elucidated further (2).

1.6. Hypothesis and specific aims

Since our preliminary studies regarding the allelic characterization and phenotypic analysis using isogenic mutants encoding either the PG or EG-like allele revealed significant differences in survivability among strains encoding either allele, and PG-allele is the one that is likely to emerge as a successful pathogen, **we hypothesize that, PG-like allele of the outer membrane porin U confers virulence associated properties in *Vibrio cholerae.*** In this study, we aim to determine the PG-allele dependency of some known virulence associated functions of OmpU and identify some novel functions of OmpU which we will achieve by pursuing the following aims.

Aim 1: Determine whether the functions of OmpU are dependent on PG allele.

In this aim, several alleles of *ompU (ompU*^{GBE1114}*, ompU*^{GBE428,} *ompU*^{GBE658}*)* will be analyzed to determine whether the functions of OmpU are dependent on the PG allele

and whether these VAPs encode different functional properties. We will perform multiple phenotypic assays and observe survival for strains encoding the PG, PG-like and EG-like alleles in the presence of bile, organic and inorganic acid, antimicrobial peptides and anionic detergents. We will also phenotypically characterize these alleles to observe the biofilm formation and motility pattern of *V. cholerae.* We will be able to identify whether the functions are allele dependent or independent and also, specifically, whether these functions are PG-allele dependent.

Aim 2: Identify novel functions of OmpU in *Vibrio cholerae*.

In this study, we will identify some novel functions of OmpU that will corroborate the fact that OmpU is involved in regulating functions associated with virulence. Previously we have shown that significant difference in virulence and survival exist encoding either PG or EG-like alleles of *ompU* in *V. cholerae*. We will expand our analysis by utilizing BiOLOG assays to identify and test new compounds which may indicate differences between the alleles. Our goal is to quantitatively measure a wide array of cellular phenotypes related to carbon utilization, sensitivity to variations in pH and osmolarity and examine chemical compounds such as antibiotics conferred by the *ompU* alleles of different virulence potential and background. The finding will also reveal the correlation between genotypic variation and phenotypic expression and the metabolic sensitivity of *V. cholerae*.

1.7. Conclusion

Our findings will provide further evidence that genetic variations in core genes lead to the emergence of virulence adaptive traits in pathogenic *V. cholerae.* Study of the functions

of OmpU with allelic variation will give us a holistic view regarding the regulations of OmpU in varying host environment. We will be able to forecast the traits of the emergent facultative pathogen *V. cholerae* and this knowledge can be extrapolated to other human pathogens.

CHAPTER TWO: MATERIALS AND METHODS

2.1. Bacterial strains and media.

V. cholerae N16961 was used as wild-type (WT) strain of El Tor biotype. Knockout mutant of *ompU (⊿ompU)*, and strains with PG-like allele of *ompU* (*ompU*$^{GBE\ 428}$ and ompU$^{GBE\ 0658}$) and EG-like allele of *ompU* (*ompU*$^{GBE\ 1114}$) cloned into the background of *V. cholerae* N16961 strain were used in the overall study. Strains cultivated on solid medium were grown on LB (Luria-Bertani) agar plates; strains in liquid media were grown in LB broth at 37°C unless otherwise stated. Compounds used for the in-vitro assays such as bile, antimicrobial peptides or acids were obtained from different companies and mentioned in respective sections of materials and methods.

2.2. Survival assays.

Survival assays were conducted to compare the functionality of *ompU* alleles using isogenic mutants encoding either the PG or EG-like allele. The survival analyses of these alleles in the presence of various antimicrobial compounds, bile, pH or anionic detergents revealed that significant differences in survivability exist among strains encoding either allele.

2.2.1. Survival assay to determine bile tolerance response of *ompU* alleles

Survival assay in whole bile was performed according to the study presented by Provenzano et. al. (37) with minor modifications based on the requirement of the study.

2.2.1.1. Survival assay in the presence of whole bile

Inoculated *V. cholerae* cells of the WT strain and strains with *ompU* alleles (mentioned in section 2.1) were cultured overnight in aerated conditions in a 37°C rotary shaker. 1:100 dilution of each overnight cultures of specific strain were prepared. The diluted cultures were then grown in a 37° incubator with rotary shaker until they reach to the mid-log phase or an optical density (600 nm) of ~0.50. It takes ~2 hours to grow the cells to reach to the midlog phase. 1 ml of these cells from specific *ompU* alleles were pelleted by centrifuging for 1 minute at 13.1 r.p.m and supernatants were removed by aspiration. Each centrifuge tube was washed two times with LB media to remove any carryover. Next, cells were resuspended in either LB broth or LB containing three different concentrations (0.4%, 0.8% and 1.2%) of whole bile (Sigma). Each centrifuge tube was then incubated in a 37°C incubator with rotary shaker for 1 hour. After incubation, cells were serially diluted (1:10) and the colony forming units per ml (c.f.u./ml) was calculated by plating dilutions in LB plates. Survival percentage was calculated by dividing the c.f.u./ml after the treatment by the c.f.u./ml before the treatment and then multiplying the value with 100 ($N \geq 4$). No samples were excluded.

2.2.1.2. Survival assay in the presence of bile salt

Survival assay in the presence of bile salt was conducted as described in the section (2.2.1.1.). Three different concentrations (0.4%, 0.8% and 1.2%) of bile salts (Oxoid) were added to LB to determine whether the strains can survive the exposure to the bile salt treatment. Experiments were conducted four times ($N \geq 4$). No samples were excluded.

2.2.2. Survival assay to determine Sodium dodecyl sulphate (SDS) resistance response

Survival assay in the presence of SDS was conducted as described above in the section (2.2.1.1.). with the following modifications. LB containing different concentrations (0.001%, .0025%, .005%, .0075%, .01%, .025%, .50%, .075%, .1%) of anionic detergent SDS (Fisher Chemical) was added to measure the survival of the strains. Survival was calculated by comparing the c.f.u./ml in LB plus SDS treatment versus LB only (N ≥ 4). No samples were excluded.

2.2.3. Survival assay to determine acidic pH tolerance

2.2.3.1. Inorganic acid stress tolerance response assay

Organic acid tolerance response assay was performed following the protocol developed by Merrel and Camilli (32) with minor modifications. In brief. each test strain were grown in LB broth in a 37° C rotary shaker overnight. Overnight cultures of each of the strains were diluted 1:100 into testubes containing 5 ml of LB media. The diluted cultures were then grown in a 37° incubator with rotary shaker until they reach to the midlog phase or an optical density (600 nm) of ~0.50. It takes ~2 hours to grow the cells to reach to the midlog phase. The optical density of these cells were measured in the spectrophotometer (Company name) at 600 nm. Next, 1 ml of each cultures were centrifuged and pelleted at 5000×*g* for 5 min at room temperature. The supernatants were discarded by aspiration and washed two times with LB media. At this point, cells were resuspended in 1 ml of LB broth of pH 7.0. Next, 10% (100μl) and 90% (900μl) of the cells were placed into two individual centrifuge tubes. Next, the cells were centrifuged and pelleted at 12000×*g* for 1 min at 25°C. The supernatants were discarded and the 10% cell pellet was resuspended

in 1 ml of LB broth, pH 7.0, and the 90% cell pellet in 1ml LB broth, pH 5.7, and these were subsequently grown at 37°C with aeration for 1 hour. Cultures grown at pH 7.0 are unadapted, while those at pH 5.7 are adapted. Next, one half of each of the adapted and unadapted cultures was pelleted at 12000×*g* for 1 min and the supernatants were removed by aspiration. Next, both adapted and unadapted cells were resuspended in LB broth of pH 4.5 (acid shock) in respectively labelled microcentrifuge tubes. Viability of cells was assayed upon resuspension (*t*=1) and subsequently at 30 and 60 minutes by making serial dilutions in LB broth of pH 7.0, and plating onto LB agar. Plates were incubated overnight at 37°C, and viable cells were enumerated. Percentage survival was calculated by dividing the total number of viable cells at each time point (30 and 60 minutes) by the initial number of viable cells at *t* = 1 minute and multiplying the value by100 (N≥ 4). No samples were excluded.

2.2.3.2. Organic acid stress tolerance response assay

Survival assay to determine the ability of cells to survive sublethal exposure to organic acid environments was performed following the protocol developed by Merrel and Camilli with minor modifications (32,51). The protocol is similar to the inorganic acid stress tolerance response assay (Section 2.2.3.1.) except the adaptation medium was LB supplemented with 0.075× organic acid cocktail and the pH adjusted to 5.7 using HCl. Acid shock medium was LB broth supplemented with 0.1× organic acid cocktail and the pH adjusted to 4.5 using HCl; 1× organic acid cocktail was 87 mM acetic acid (Thermo

Fisher), 25 mM butyric acid (Sigma-Aldrich) and 37 mM propionic acid (Acros Organics). Acid stress in this assay was organic in nature.

2.2.4. Survival assay to analyze antimicrobial resistance response of *ompU* alleles

2.2.4.1. Survival assay in the presence of polymyxin B

Survival assays in the presence of Polymyxin B was performed as described in the section (2.2.1.1.) except with the following modifications. LB containing different concentrations of polymyxin B (250 U/ml, 500 U/ml, 750 U/ml, 1000 U/ml, 1250 U/ml and 1500 U/ml). were used as the treatment group. Polymyxin B was purchased from Sigma. Survival was calculated by comparing the c.f.u./ml in LB plus polymyxin B treatment versus LB only ($N \geq 4$). No samples were excluded.

2.2.4.2. Survival assay in the presence of the antimicrobial peptide (AMP), P2

Survival assays in the presence of Polymyxin B was performed as described in the section (2.2.1.1.) except with the following modifications. Two different concentrations of AMP, P2 (80 ug/ml and 120 ug/ml) was added to LB to measure the survival. AMP, P2 was purchased from GenScript. Survival was calculated by comparing the c.f.u./ml in LB plus P2 treatment versus LB only ($N \geq 4$). No samples were excluded.

2.2.4.3. Survival assay in the presence of protamine sulphate

Survival assays in the presence of Polymyxin B was performed as described in the section (2.2.1.1.) with minor modifications, differing based on the assay. Different concentrations of protamine sulphate (0 ug/ml, 100 ug/ml, 250 ug/ml, 500 ug/ml, 750 ug/ml, 1000 ug/ml

and 1250 ug/ml) was added to LB to measure the survival. Protamine sulphate was purchased from Sigma-Aldrich. Survival was calculated by comparing the c.f.u./ml in LB plus P2 treatment versus LB only (N ≥ 4). No samples were excluded.

2.5. Biofilm formation assay

Overnight cultures of the each strain were prepared by using LB media and incubating at 30°C. Cultures were diluted to 1:100 and 100 µl of these diluted cultures were placed in 96-well plate (Company name) and labeled appropriately. Only LB was added in the last lane of the microtiter plate as blank media. Plates were incubated at three different temperatures in a 30°C incubator, 37°C incubator and room temperature (22°C) on bench for 24 hours. After 24 hours, liquid contents were discarded from the plates, and washed gently with LB media twice. A 200 µl volume of 0.01% crystal violet was added to stain the wells and incubated at room temperature for 5 minutes. Liquid contents were discarded and plates were washed extensively with low flow of double distilled water (ddH_2O) to avoid biofilm detachment. After the plates were dry, 150 µl of 50% acetic acid were added to each well by using a multichannel pipette. Next, liquid contents were transferred to a flat-bottomed 96-well plate (Falcon) and quantitated in a microtiter plate reader at OD_{550} (66). Values of three independent experiments were plotted using Prism software ($N = 15$).

2.6. Motility test

Motility of *V. cholerae* strains was determined using swarm/motility agar plates. The experiment was performed with cells grown in LB broth. Each strain were grown in LB

agar plates and incubated overnight at 37° incubator. Next morning, an inoculating wire was used to swab a single colony from each respective plates and then stabbed into the middle site of the motility agar plate. The swarm/motility agar plates were incubated in a 37° incubator for 15 hours. Zones of migration of bacterial strains around the inoculating site were measured by a scale every 2 hours starting from the first hour. Data from each independent experiment (N=4) were plotted in prism software to generate motility graph.

2.7. Biolog phenotypic microarray assay

2.7.1. Biolog phenotypic microarray for phenotype testing by using PM1, 2A (Carbon Utilization Plates) and PM 9-20 (Osmolarity, pH and Chemical compounds) plates

PM (Phenotypic microarray) plates 1 and 2A are designed to test the Carbon utilization capabilities of a microorganism. The wells contain carbon sources such as Glucose-6-Phosphate, Fructose, Sorbitol and others in PM1 and Gelatin, Lysine, Arginine and others in PM2A. A total of 190 types of carbon sources are available in these microplates to test the metabolic capabilities and carbon utilization efficiency of the microorganism. Additionally we also have screened PM plates 9-20 those are designed to assay sensitivity to variations in osmolarity, pH and different chemical compounds. PM plates 11–20 contain 240 different chemicals such as polymyxin B, EDTA, Trifluoperazine, Rifamycin and others each at four differing concentrations**.**

Methods and materials for Biolog phenotypic microarray experiment was obtained from BiOLOG Inc. and the protocol of Mackie et al (67,68) In brief, overnight culture plates of the respective strains of *V. cholerae* were grown in LB agar plates at 37° incubator. Cells

were sub-cultured in LB agar plates as it is recommended to subculture the cells if they are removed from the -80° stock. Next, 12.5 mL of Biolog IF-0a media (Minimal media) and 2. 5 mL sterile double distilled water was pipetted and mixed into a sterile capped test tube. 5 mL of this mixture was removed from the test tube and reserved in another test tube to use for sample dilution later if necessary. After that, a sterile pipette tip was used to swab several colonies from the agar plate. Colonies were dipped and transferred in the tube that contains 10 mL Biolog IF-0a media and water mixture. Turbidity of this suspension was checked frequently by using the Biolog turbidimeter. 42% transmittance was achieved when the carbon utilization efficiency test was performed and 85% transmittance was achieved when the pH, osmolarity and chemical sensitivity of the cells was tested. The suspension of 42% transmittance and 85% transmittance was placed to one side and 'IF-0a plus dye mix' was prepared. 'IF-0a plus dye mix' is a minimal media that contains 15.25 mL Biolog IF-0a minimal media, 0.22 mL Biolog redox dye mix and 2.83 mL sterile double distilled water. After preparation of the dye mix media, 1:5 dilution of cells were prepared by adding 3.7 mL of cell suspension with 42% transmittance that was prepared before to the dye mix. After thorough and gentle mixing, the turbidity of the final cell suspension was checked again. This cell suspension will have a 85% transmittance in the turbidimeter or a OD value of 0.07 at 600 nm. Such procedure to prepare the dye mix is applicable when testing the carbon utilization efficiency of the strains (PM plates 1 and 2A). The preparation of dye mix media is slightly different when testing the osmolarity, pH and chemical sensitivity of the cells (PM plates 9-20). Enriched media such as Biolog IF-10 is used in place of Biolog IF-0a media to prepare the "IF-10

plus dye" mix when PM plates 9-20 was evaluated. 'IF-10 plus dye mix' is an enriched media that contains Biolog IF-10 media, Biolog redox dye and sterile double distilled water. During the experiment with PM 9 and 10, 'IF-10 plus dye mix' contains 18.3 mL of Biolog IF-10 media, 0.22 mL Biolog redox dye and 3.37 mL sterile double distilled water. After preparation of the dye mix media, 1:200 dilution of cells were prepared by adding 0.11 mL of cell suspension with 85% transmittance that was prepared before to the dye mix. During the experiment with PM 11-20, 'IF-10 plus dye mix' contains 8.33 mL of Biolog IF-10 media, 0.1 mL Biolog redox dye and 1.52 mL sterile double distilled water. After preparation of the dye mix media, 1:200 dilution of cells were prepared by adding 0.5 mL of cell suspension with 85% transmittance that was prepared before to the dye mix. 100 µL of the respective final cell suspension was inoculated into each wells of PM plates 1 and 2A, 9-20. Multichannel pipette and sterile reservoirs were used to transfer the cell suspension to the PM microplates. The plates were incubated for 48 hours at 37° under shaking conditions. Optical density (O.D.) values of each well were taken every 1 hour for 48 hours. After normalization, values were plotted in Prism software to generate the growth curves which is an indicator of respiration of respective strains. Area under the curve of each growth curve was measured to analyze the respiration efficiency of each strains. Every experiment was conducted twice to achieve statistical significance. The following table shows the required volume of media, dye, water and final cell suspension (67,68).

Table 1: Biolog phenotypic microarray assay setup for indicated PM microplates

Materials	**PM 1 and 2**	**PM 9–20**
IF-0a (1.2×)	15.25	-
IF-10 (1.2×)	-	8.33
Dye mix (100×)	0.22	.1
H_2O	2.83	1.53
42 % T cell suspension	3.7	-
85 % T cell suspension	-	.05
Final volume	22	10

All volumes are in mL. Final volume amounts are sufficient to inoculate a single microplate in the given series using 100 µL per well.

2.8. *In vitro* Competition assay to analyze the novel carbon source

2.8.1. *In vitro* Competition assay in the presence of propionic acid

In vitro competition assays were performed following the protocol of Almagro-Moreno and Boyd (69). In brief, stationary-phase cells of respective *V. cholerae* strains mentioned in section 2.1 and an O1 N16961 Δ*lacZ* strain were prepared by overnight growth at 37°C rotary shaker incubator. For the in vitro competition assay, 1:1,000 dilutions from the respective were mixed 1:1 with the lacZ-negative strain. 100 µl of the mixed culture was added to 5 ml of freshly prepared minimal media plus 2mM/ml propanoic acid solution. 100 ul of the mixed culture was also added to the LB medium that is to be used as the positive control. Primary inoculum were plated onto LB supplemented with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Fisher Chemicals) to count the input ratio. After that, the cultures were grown overnight at 37°C with aeration. Next morning, serial dilutions were plated on LB supplemented with X-Gal to recover the output ratio. In the X-gal supplemented LB plate, the mutants appear as the blue colored colony and the WT colonies appear as white because of the lacZ-negative phenotype. The input ratio and the output ratio was compared and calculated to generate the in vitro competitive index (70). Four independent experiments were performed to achieve the statistical significance.

2.9. Survival assay to analyze novel compounds

Survival assay in the presence of the novel compounds (Rifamycin SV, EDTA and Trifluoperazine) were performed following the protocol mentioned in the section 2.2.1.1. As these compounds are prospective to show novel functions of OmpU, a range of

different physiological concentrations of these compounds were tested from the point of vigorous growth to the point of no survival in the LB medium. The following modifications in the concentration were introduced based on the compound tested in the assay: 0 ug/ml, 5 ug/ml, 10 ug/ml, 15 ug/ml, 20 ug/ml and 25 ug/ml of Rifamycin SV (MP Biomedicals, LLC); 0 ug/ml, 50 ug/ml, 100 ug/ml, 250 ug/ml, 500 ug/ml, 1000 ug/ml and 1500 ug/ml of EDTA (Fisher Chemicals); 0 ug/ml, 50 ug/ml, 60 ug/ml, 70 ug/ml, 80 ug/ml, 90 ug/ml and 100 ug/ml of Trifluoperazine (Sigma-Aldrich) was added to LB to measure the survival of the strains. Experiments with each individual compounds were conducted four times (N ≥ 4). No samples were excluded.

2.10. Statistical analysis

All experiments were repeated with at least four (4) times as biological replicates and data were represented as mean ± SD. Students t-test were used to analyze statistical significance of all experiments. *p* value less than 0.05 were used to define significance of all experiments.

CHAPTER THREE : RESULTS

3.1. Aim 1: Determine whether the functions of OmpU are dependent on PG- allele

3.1.1. Bile tolerance is dependent on the PG-allele

To determine whether the PG-allele itself is the one that confers bile tolerance and also whether the bile tolerance phenotype is dependent on allelic variation, we performed survival assay in the presence of whole bile in three different concentrations (0.4%,0.8% and 1.2%).

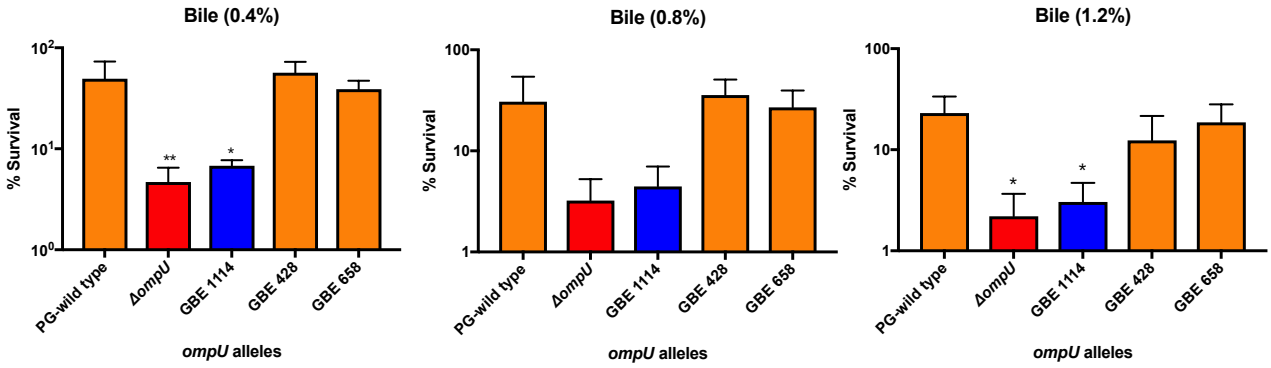

Figure 2: Survial of *ompU* mutants in the presence of whole bile

The figure depicts survival of *ompU* mutants in the presence of whole bile at three (0.4%, 0.8% and 1.2%) different concentrations (n=4). Student's t-test was used to make statistical comparisons. *P < 0.05; **P < 0.01

Our experimental finding is consistent with the results provided by Provenzano and Klose (2000) that absence of OmpU porin makes the strains susceptible to bile (37). According

to our results, the mutant strain encoding the EG-like *ompU* allele from the strain GBE1114 (*ompU*^{GBE1114}) showed similar survival defect as the deletion mutant in

the presence of 0.4% bile (Fig. 1). In contrast, strains encoding the PG-like *ompU* allele (*ompU*$^{GBE\ 428}$ and *ompU*$^{GBE\ 658\ )}$ show similar survival as the PG-wild type in the presence of 0.4% bile (Fig. 2). We also examined the survival of these strains in the presence of 0.8% and 1.2% bile, as it has previously been shown that *V. cholerae* encounters and can tolerate up to 1.2% bile in the host intestine (45). We also wanted to observe whether the allelic dependency of bile tolerance varies in a dose-dependent manner and affects the potential of the PG-allele to confer bile tolerance. Results indicate that EG-like *ompU* allele reduces the ability of the clinical strain upon bile treatment regardless of the concentration tested in this experiment (Fig. 2). This experiment also indicate that strains encoding PG-like alleles (*ompU*$^{GBE\ 428}$ and *ompU*$^{GBE\ 658}$) confer properties beneficial for virulence as they have retained the bile resistance function of OmpU porin similar to the PG-wild type.

Whole bile is a complex mixture of different components such as cholate, deoxycholate, cholesterol and a few other byproduct compounds secreted from the liver (42).

Previously, Provenzano et al. has determined the role of OmpU porin to confer bile tolerance in the presence of the bile component deoxycholate (DC) and found that strains with deletion in *ompU* is sensitive to deoxycholate treatment (31,37). Therefore, we wanted to examine whether the bile tolerance function of OmpU and allelic variation

observed in the presence of whole bile differs from the pattern of bile salt tolerance and whether the PG allele itself shows bile salt tolerance.

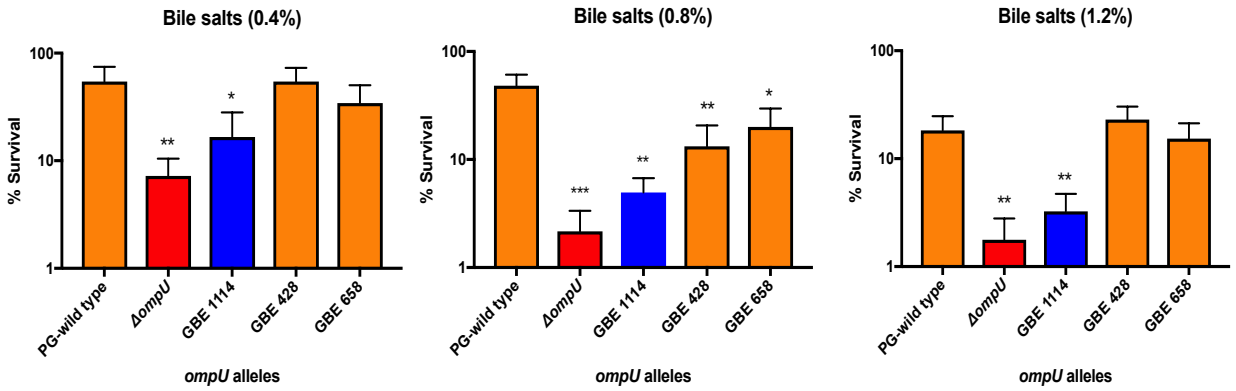

Figure 3: Survival of *ompU* mutants in the presence of bile salt

The figure shows survival of *ompU* mutants in the presence of bile salt at three different concentrations (0.4%, 0.8% and 1.2%) of bile salts (n=4). Student's t-test was used to make statistical comparisons. *P < 0.05; **P < 0.01 *** P < 0.001

Our data is showing that the effect of the whole bile is slightly more bactericidal in case of all the mutants tested in the experiment (Fig. 3). Despite that, the whole bile and DC provide similar and retarded survival in different concentration (0.4%,0.8% and 1.2%) (Fig. 3). It is also evident that, the effect of 1.2% whole bile and DC is more bactericidal than the 0.4% and 0.8% whole bile and DC treatment. As shown in the figure, the PG wild type shows similar survival as the strain encoding *ompU* $^{GBE\ 428}$ and *ompU* $^{GBE\ 658}$, while

survival percentage of the strain encoding *ompU* $^{GBE\ 1114}$ is closely similar to the survival percentage of the *⊿ompU* mutant. Interestingly, the survival of strain encoding *ompU* $^{GBE\ 428}$ shows decreased survival in only 0.8% bile salt in comparison with the strain encoding *ompU* $^{GBE\ 658}$ strain but higher survival percentage in case of 0.4% and 1.2% bile salt (Fig. 3). This data indicates that, bile salts are the primary components those are inducing bile stress and the PG-allele of OmpU confers resistance to bile salts.

3.1.2. Sodium dodecyl sulfate (SDS) tolerance is PG-allele dependent

Previously, Provenzano et al. reported that OmpU confers resistance to the anionic detergent Sodium dodecyl sulfate (SDS). In accordance to our aim to determine whether the PG allele of *ompU* confers SDS or anionic detergent tolerance, we performed survival assay of the strains encoding PG and EG-like *ompU alleles* in the presence of different concentration of SDS. We selected a range of concentrations starting from the minimum concentration (0% SDS) where all the strains survive similarly to the point of highest concentration (0.1% SDS) where no survival was observed. We found that, the ability to tolerate this detergent appears to be dependent on allelic variation and most importantly the resistance is PG-allele dependent. The strain encoding the *ompU*^{GBE1114} showed attenuated survival as the strain with the deletion mutant of *ompU* at 0.025% and 0.050% SDS concentrations (Fig. 4).

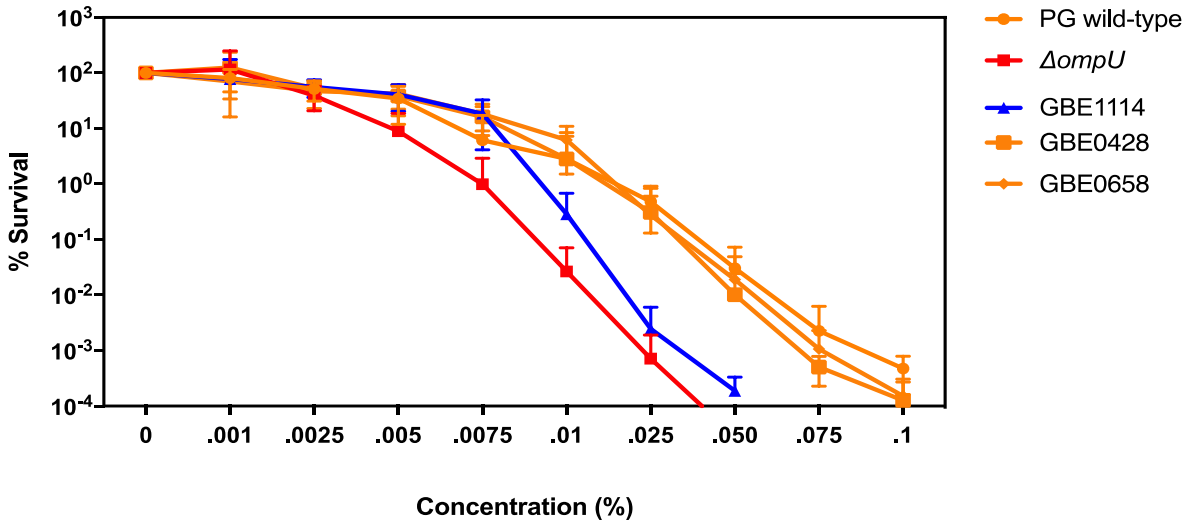

Figure 4: Survival of *ompU* mutants in the presence of SDS

The figure depicts survival of *ompU* mutants in the presence of SDS at various concentrations (n=4). Student's t-test was used to make statistical comparisons. *P < 0.05; **P < 0.01 *** P < 0.001

Two other strains encoding PG-like alleles of *ompU* (*ompU*$^{GBE\ 428}$ and *ompU*$^{GBE\ 658}$) had a fairly similar survival percentage as the PG-wild type (Fig. 4). The result of this experiments show that, the ability to tolerate SDS by OmpU porin is PG-allele dependent

as the strains with PG-like *ompU* alleles retain the ability of the wild type clinical strain to confer SDS tolerance.

3.1.3. Organic acid tolerance response is dependent on PG-allele

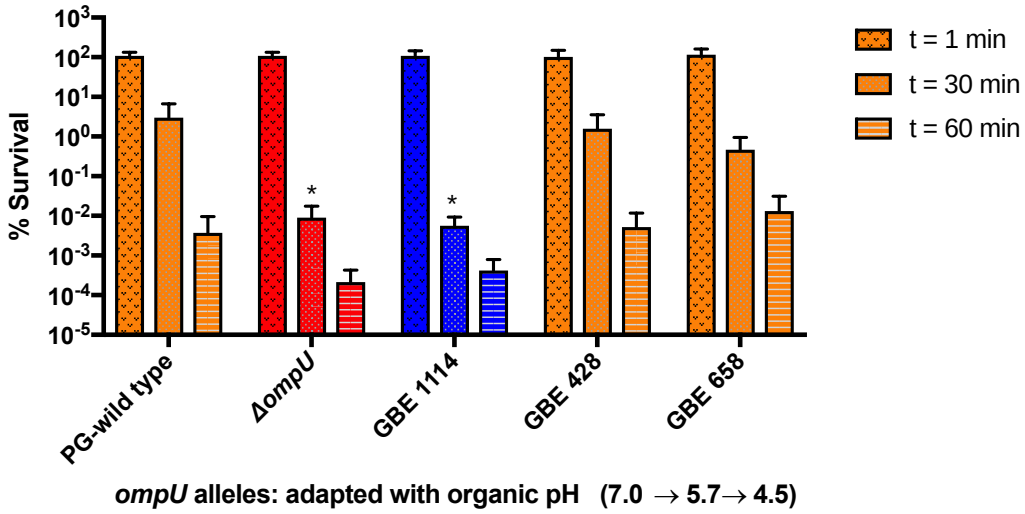

Figure 5: Survival of *ompU* mutants in adapted organic acid

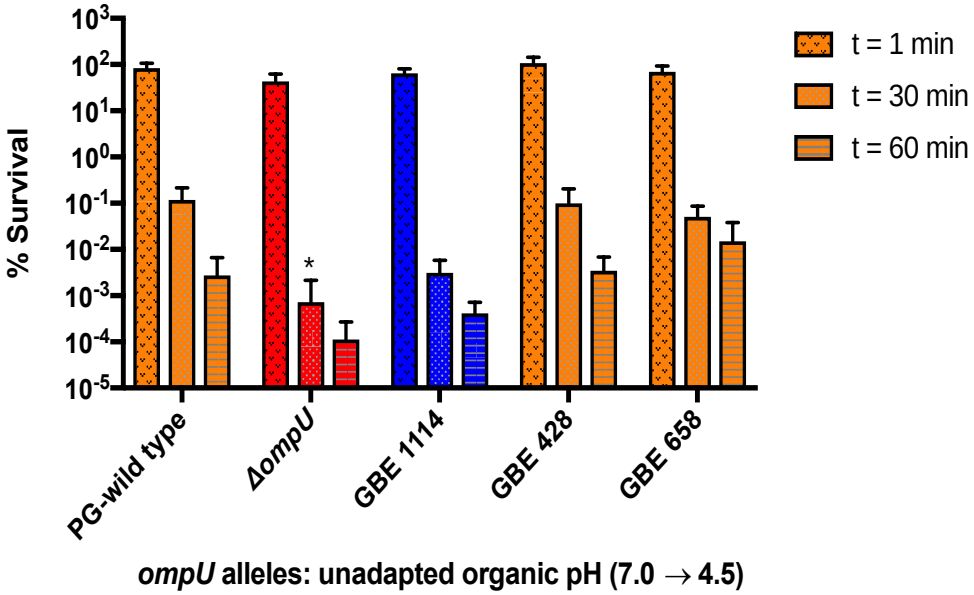

Figure 6: Survival of *ompU* mutants in unadapted organic acid

Previously, Merrell and Camilli showed the role of OmpU porin in conferring organic acid tolerance in *V. cholerae* (32). In accordance with our aim, we wanted to examine whetherthe organic acid tolerance is dependenton PG-allele or this function is an allele dependent function, we performed organic acid tolerance assay at three different exposure time points (1 minute, 30 minutes and 60 minutes). Results indicate that organic acid tolerance in *V. cholerae* is an allelic dependent property. Our data is consistent with the findings of Merrell and Camilli that organic acid tolerence response requires OmpU as we have a three log difference in survival between the *⊿ompU* and the PG-wild type strain. At 1 minute and 1 hour time exposure, there was no detectable and significant difference among the strains (Fig. 5 and 6).

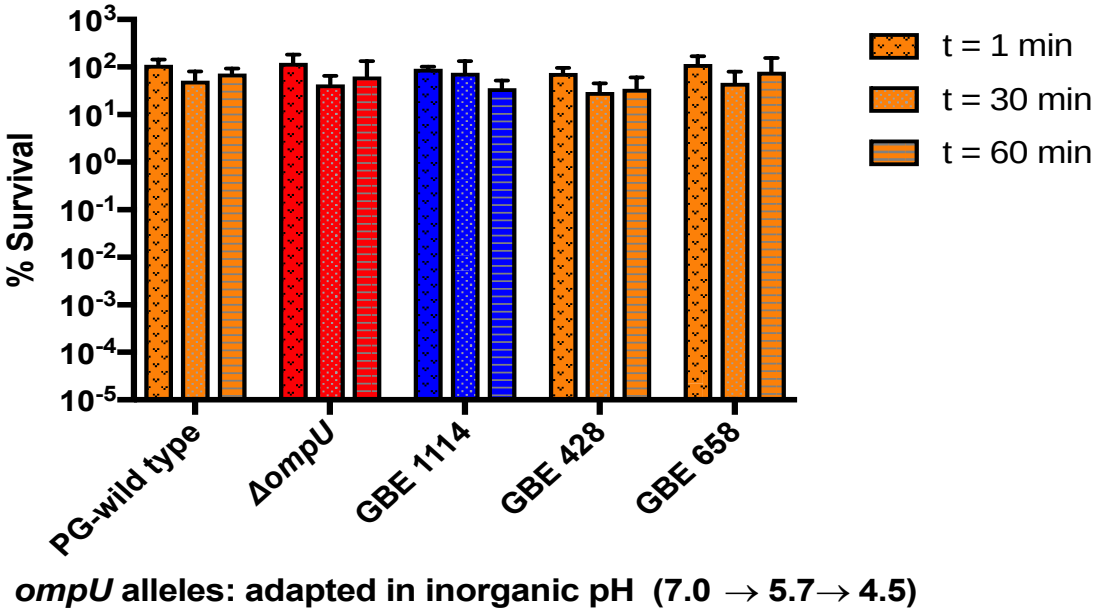

Figure 7: Survival of *ompU* mutants in adapted inorganic acid

After 30 minutes exposure, the difference among the PG-wild type and *ΔompU* was significant and so was the difference between PG-wild type and the strain encoding *ompU*^{GBE1114}. We found that, mutant strain that is encoding with *ompU*^{GBE1114} survived similarly to the *ΔompU* strain. In contrast, mutant strains with *ompU*^{GBE428} and *ompU*^{GBE658} survived similarly to the PG-wild type which indicates that these alleles retained the ability of the clinical strain to tolerate organic acid stress (Fig. 5 and 6).

We also have examined the role of OmpU in conferring inorganic acid tolerance even though Merrell and Camilli reported previously that OmpU has no role in inorganic acid tolerance (51). However, we wanted to verify whether the mutants encoding PG-like and EG-like alleles shows any survival variation in inorganic acidic environment at different time points. Moreover, the inorganic acid HCl is secreted from the stomach of human host and is a barrier for *V. cholerae* to passage through the stomach upon ingestion.

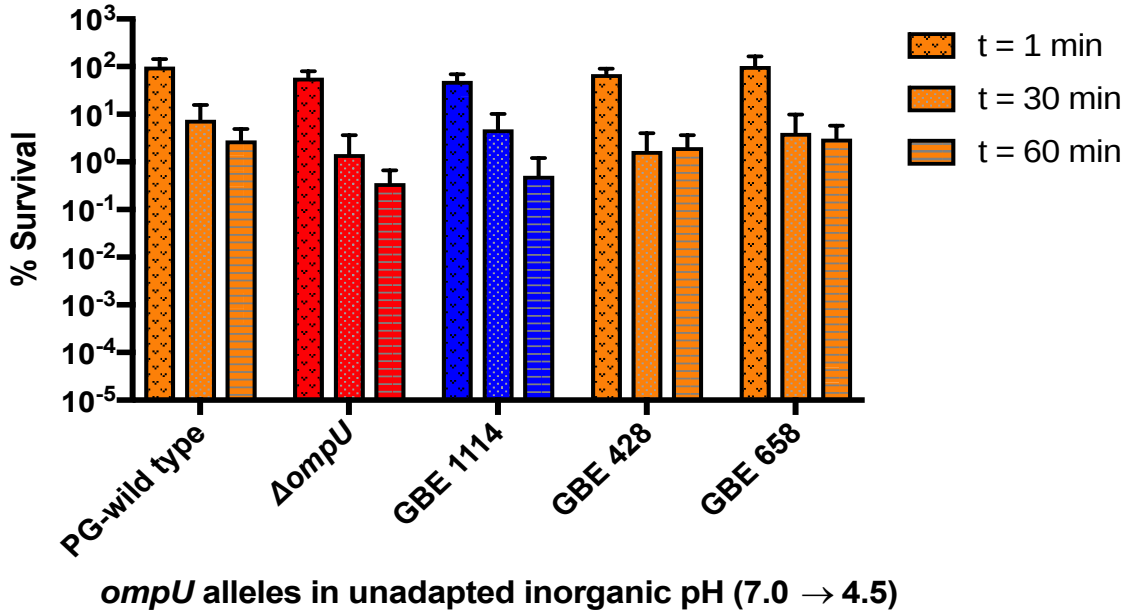

Figure 8: Survival of *ompU* mutants in unadapted inorganic acid

Therefore, we performed inorganic acid HCl tolerance response assay where we treated our test strains (PG-wild type, *⊿ompU,* GBE 1114, GBE 428 and GBE 658) with HCl to identify any possible effect of the strains encoding different versions of the OmpU protein. Results showed no variation in the survival percentage of these strains across various time points (Fig. 7 and 8). The result from this experiment consolidates the finding that inorganic acid tolerance of *V. cholerae* is OmpU independent.

3.1.4. Antimicrobial resistance shows variation in PG-allele dependency

3.1.4.1. BPI (Bactericidal/Permeability increasing protein) derived antimicrobial peptide, P2 resistance is PG allele dependent

P2 is a bioactive and cytotoxic peptide that has been shown to induce cytotoxicity to *V. cholerae*. Mathur and Waldor reported that OmpU induces sigmaE stress response pathway to protect and repair the outer membrane of *V. cholerae* upon encountering BPI (Bactericidal/Permeability increasing protein) derived peptide P2 challenge (55).

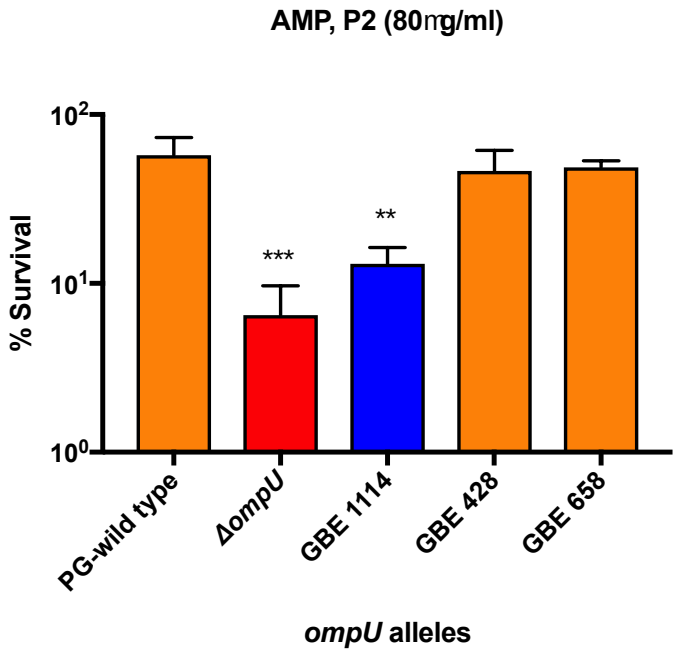

Figure 9: Survival of *ompU* mutants in the presence of AMP, P2

Since we wanted to determine if this resistance is due to the PG-allele, we performed survival assay in the presence of the antimicrobial peptide P2 and examined our test strains (PG-wild type, *ΔompU,* GBE 428, GBE 658, GBE 1114) at a concentration of 80 μg/ml of P2. Data indicates that, the resistance to P2 is OmpU dependent because there is a two log difference between the PG-wild type and *ΔompU* in the survival percentage (Fig. 9).

Interestingly, strain encoding the *ompU* allele of the GBE 1114 strain had a one log difference in the survival percentage at 80 μg/ml when compared with the PG-wild type, which indicates that the function of OmpU porin is PG-allele dependent. We also have found that, PG-like strains GBE 428 and GBE 658 showed similar survival to the PG-wild type in the presence of 80μg/ml of P2 indicating that these two alleles have the ability to retain the virulence of the clinical strain.

3.1.4.2. Polymyxin B resistance is PG allele independent

Polymyxin B is a cationic peptide that perturbs the membrane of bacteria and causes cell death. Previously, Mathur and Waldor showed that OmpU has a protective role to tolerate the effects of this antimicrobial compound (55). As our goal was to identify whether PG allele confers Polymyxin B resistance, we determined the survival of our test strains in the presence of a two different concentration (1000Uml-1 and 1500Uml-1) of Polymyxin B because allelic dependency may differ based on the concentration.

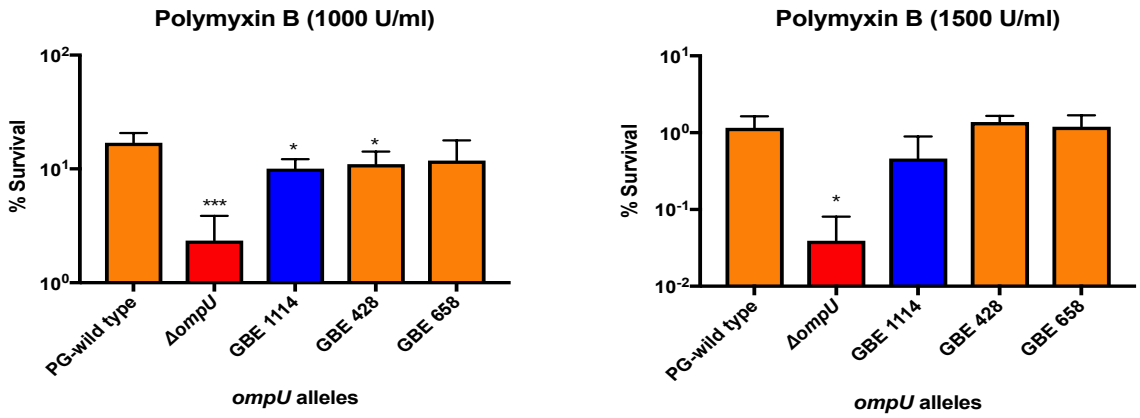

Figure 10: Survival of *ompU* mutants in the presence of polymyxin B

The figure depicts the survival of *ompU* mutants in the presence of polymyxin B at two (1000 U/ml and 1500 U/ml) different concentrations (n=4). Student's t-test was used to make statistical comparisons. *P < 0.05; *** P < 0.001

Our results suggest that polymyxin B resistance in *V. cholerae* is *ompU* dependent with almost a two log difference in the survival percentage between the PG-wild type and the *ΔompU* at 1500U/ml of polymyxin B. However, the survival in this antimicrobial peptide is not allelic dependent as the strains encoding the $ompU^{GBE\ 114}$, $ompU^{GBE\ 428}$, and $ompU^{GBE\ 658}$ survived similarly as the PG-wild type as indicated in the graph (Fig. 10).

Specifically, 1000U/ml polymyxin B treatment on *ΔompU* strain showed a drastic difference in survival compared with the PG wild-type (Fig. 10). However, no significant difference was observed across other concentrations tested in our experiment suggesting

that strains encoding either PG-like or EG-like alleles are resistant to polymyxin B treatment. The results from this experiment indicates that polymyxin B resistance is not PG-allele dependent.

3.1.4.3. Protamine sulphate resistance is PG allele independent

Protamine sulphate is an antimicrobial peptide that has been found in salmon sperm in the aquatic environment. Ruby et al showed that OmpU porin of *Vibrio fischeri* (A member of the *Vibrionaceae* family) was required in order to show resistance against the antimicrobial peptide protamine sulphate. Moreover, the *ompU* sequence of *V. fischeri* has been reported to have a significance sequence similarity with the *ompU* sequence of *V. cholerae* (65). Since we wanted to determine whether the PG allele itself is the one that confers antimicrobial resistance, we examined our test strains in the presence of different concentrations of protamine sulphate (Fig. 11).

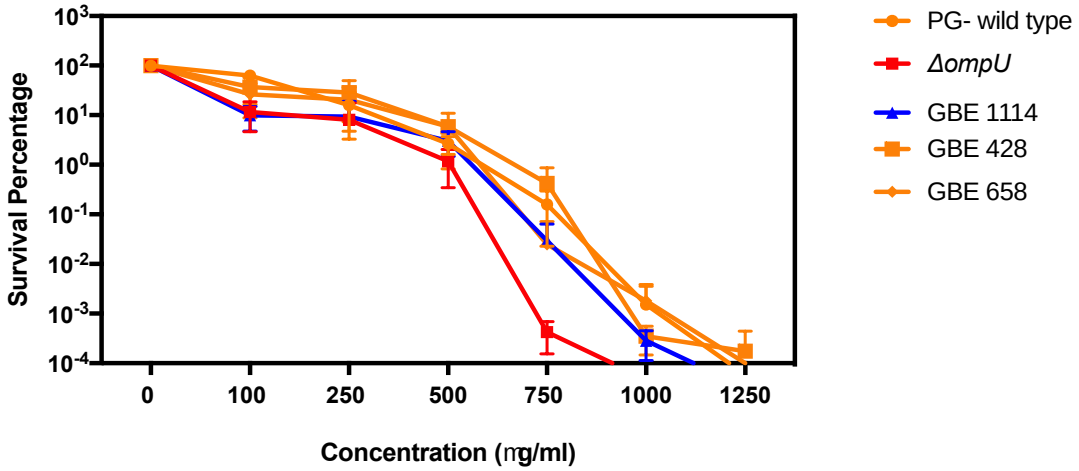

Figure 11: Survival of *ompU* mutants in the presence of protamine sulphate

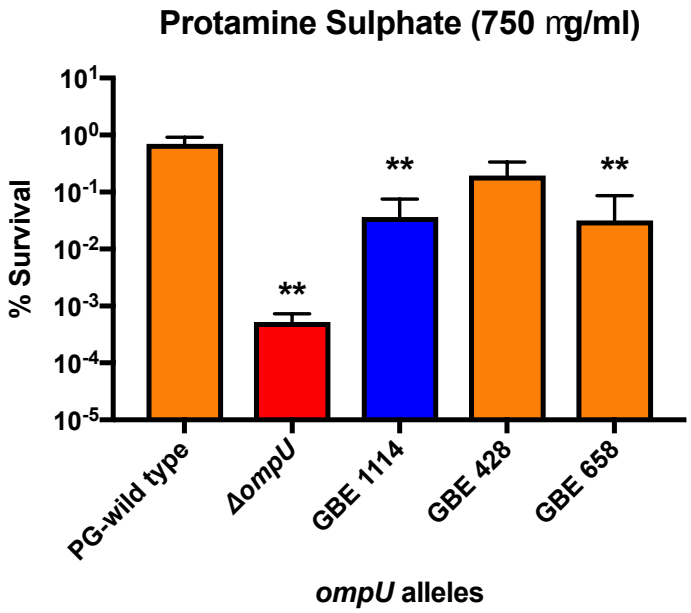

Figure 12: Survival of *ompU* mutants in 750 µg/ml protamine sulphate.

We found that, upon 750 µl/ml concentration of protamine sulphate treatment, there is almost around four log survival difference between the PG-wild type strain and the *ΔompU* strain which indicates that, similar to *V. fischeri*, protamine sulphate resistance of *V. cholerae* is dependent on OmpU (Fig. 12). However, the survival percentage of the other test strains in the presence of this antimicrobial peptide indicates that the resistance is not allelic dependent as the strains encoding the PG-like and EG-like *ompU* alleles (*ompU*$^{GBE\ 428}$, *ompU*$^{GBE\ 114}$ and o*mpU*$^{GBE\ 658}$) survived almost similarly as the PG-wild type strain as indicated in the graph (Fig. 12).

3.1.5. Biofilm formation at varying temperature is allelic dependent

It has been shown that, *V. cholerae* strains with deletions in OmpU form significantly robust biofilm on abiotic surfaces in comparison with the wild type strains. In addition, Shapiro et al showed the adaptive role of OmpU alleles in the environment by performing the biofilm assay. They have shown that biofilm formation on abiotic surfaces are allelic dependent at 30°C. Additionally, it has been reported that temperatures have a substantial effect on the dynamics of biofilm formation. Since we wanted to verify whether the allelic dependency has any variation at different temperatures, we evaluated the biofilm formation experiment at three different temperatures (22°C, 30°C and 37°C).

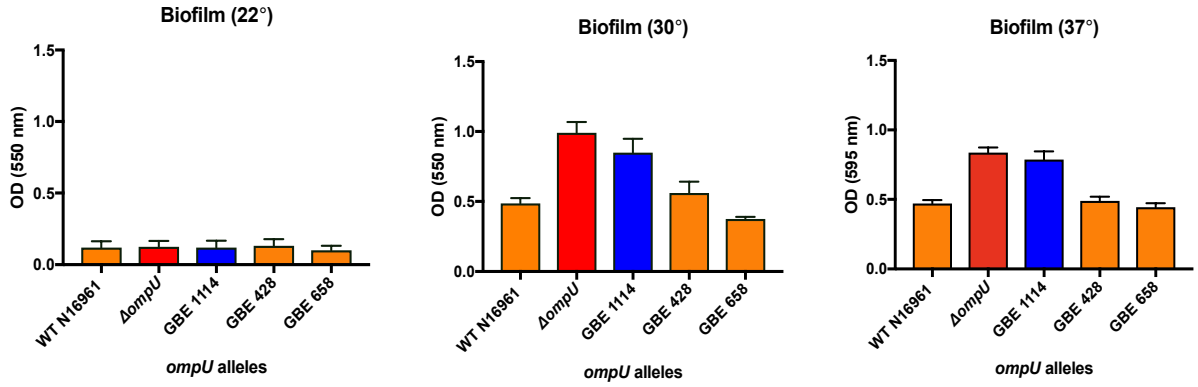

Figure 13: Formation of biofilm on abiotic surface by *ompU* mutants

Biofilm formation of PG and EG-like *ompU* mutant strains at three different (22°, 30°, 37°) temperatures on an abiotic surface (n=15). Student's t-test was used to make statistical comparisons. *P < 0.05; **P < 0.01 *** P < 0.001

We compared our test strains with the PG wild type and found that, strain encoding *ompU*$^{GBE\ 1114}$ forms similar biofilm like the deletion mutant of *ompU* in case of both 30°C and 37°C. Specifically, the optical density (0.8) of GBE 1114 nearly resemblance the optical density of *⊿ompU* (1.0) at 37°C (Fig. 13).

On the other hand, strains encoding PG-like alleles of *ompU* (*ompU*$^{GBE\ 428}$ and *ompU*$^{GBE\ 658}$) encoded OmpU similar to the PG-wild type OmpU as their optical density is almost identical to the PG-wild type strain. This finding was consistent when we included temperature variation in our experiments. However, no biofilm accumulated when we performed our experiment at 22°C (Fig. 13) indicating that biofilm formation on abiotic surfaces is limited at lower temperature such as 22°C or below this temperature gradient. The results of this experiment suggests that, biofilm formation is dependent on allelic variation in different alleles of *ompU* from the environmental groups.

3.1.6. Motility of different strains is independent of PG-allele

Motility of *V. cholerae* depends on the outer membrane porins (OmpU and OmpT) as these porins has been shown to confer structural integrity of the flagellum. It has been shown that strains with deletions in *ompU* shows attenuated motility on motility agar LB plates. In order to identify whether motility is dependent on allelic variation of *ompU* and more specifically, whether the PG-allele orchestrates motility in *V. cholerae*, we performed motility assay. We found that, motility pattern of the strains encoding both PG-like or EG-like alleles shows motility similar to the PG-wild type strain (Fig. 14).

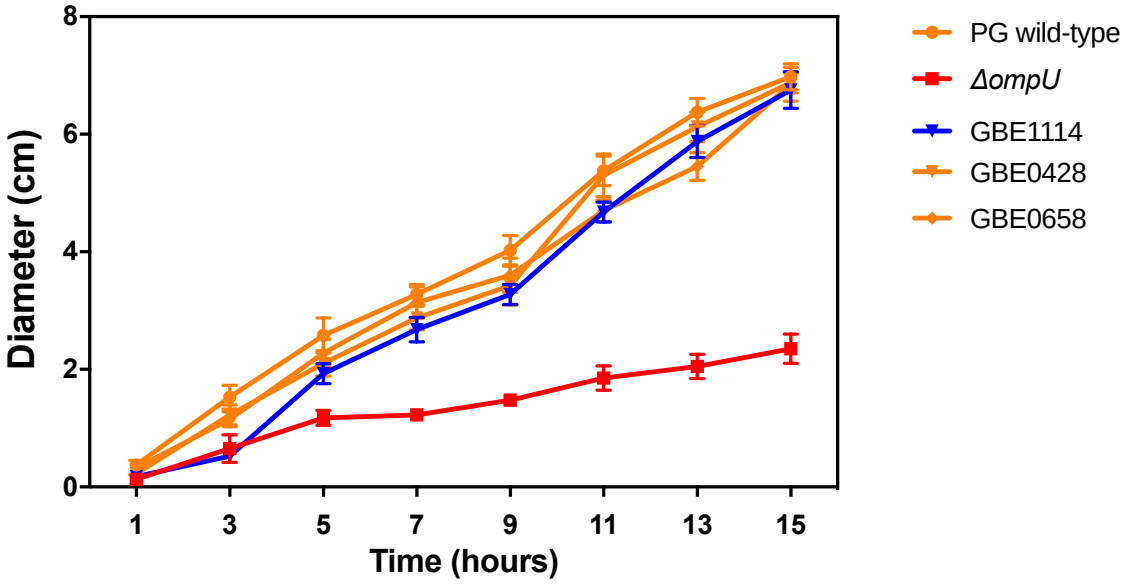

Figure 14: Motility of *ompU* mutants in motility agar media.

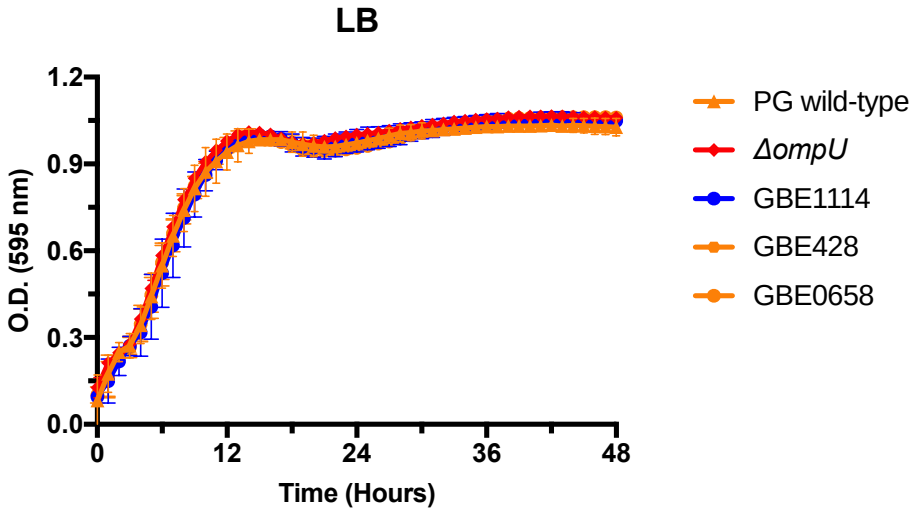

Figure 15: Growth of *ompU* mutants in LB media for 48 hours

Even though the strain encoding the *ompU* allele of GBE 1114 had similar motility to the *ΔompU* strain at the first three hours, it followed the motility pattern of the rest of the alleles including the PG *ompU* and other PG-like *ompU* alleles (Fig. 14). To confirm whether motility is affecting the growth of our strains, we performed growth assay. All the strains were grown in the LB medium as a control. All the strains grew similarly which shows that motility did not affect growth (Fig. 15). This result suggests that, strains with PG and EG-like *ompU* alleles did not lose the structural integrity of their flagellum due to the variation and nature of the OmpU porin embedded on their flagellum.

3.2. Aim 2: Identify novel functions of OmpU in *Vibrio cholerae*

3.2.1. High throughput screening of carbon sources to identify carbon utilization

In order to identify the novel function conferred by OmpU, We used Biolog phenotypic microarray technology. First, we measured the ability of PG-wild type and *⊿ompU* strain to utilize a diverse range of carbon sources as it has been reported that mutation in the residues of OmpU porin intereferes its ability. We used this high throughput screening test to identify candidate carbon sources those are regulated by OmpU in *Vibrio cholerae*. 190 types of different carbon sources were evaluated by using PM1 and PM 2A, and based on their respiration and growth kinetics data, we found that, a total of 63 different metabolites were used by both of strains (Table 1 and Fig. 14). According to the data set, the following table was prepared to depict the utilization efficiency of 190 different carbon sources by PG-wild type and *⊿ompU* . The response of PG-wild type and Δ*ompU* mutant was divided into three categories: Responsive, differentially responsive and no response based on their carbon utilization efficiency. The carbons those were differentially responsive were selected for further investigation through *in-vitro* competition assay.

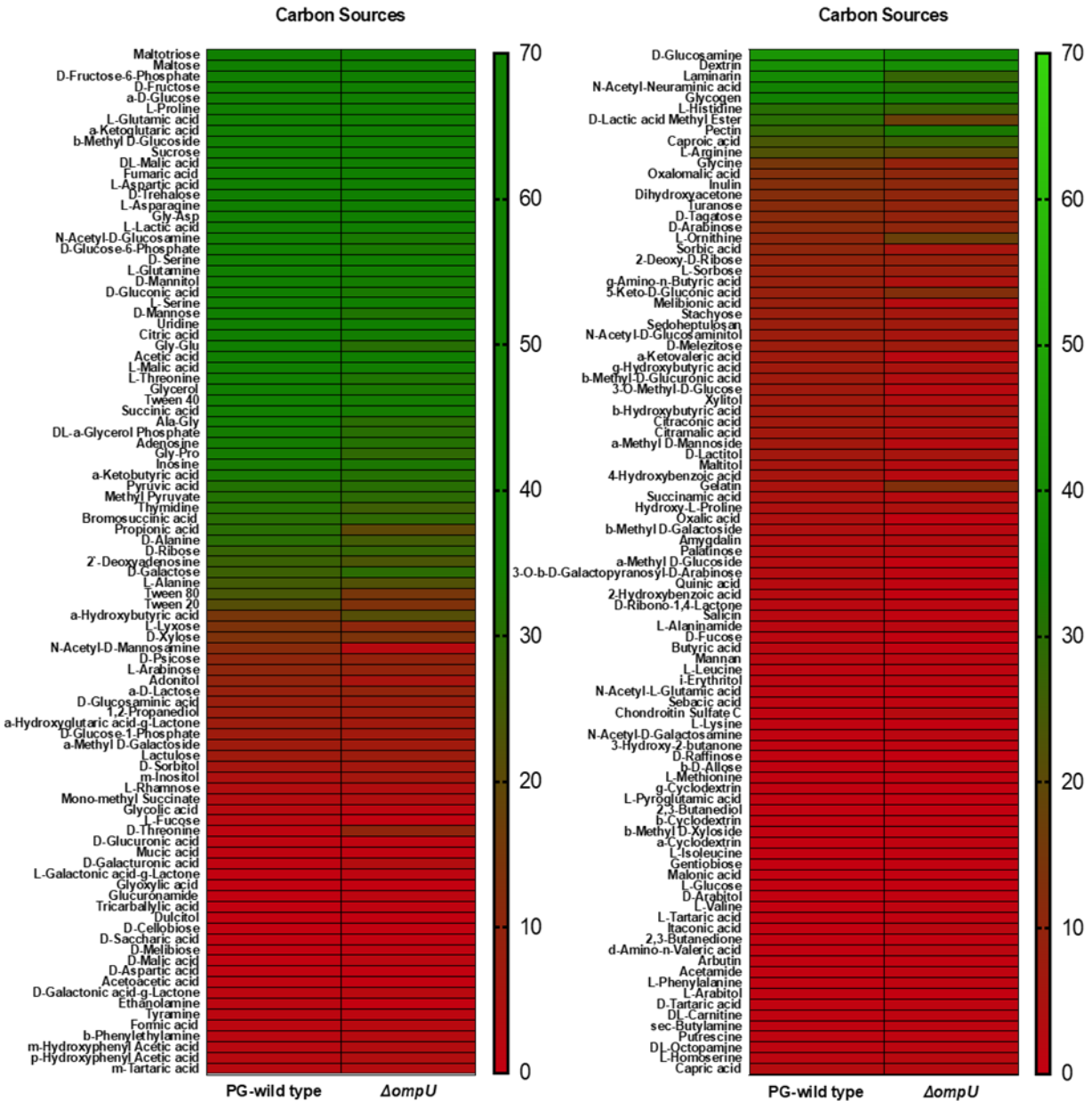

Figure 16: Heat Maps of carbon microplates PM1 and PM2A

Table 2: Response of the PG-wild type and Δ*ompU* strains in the presence of 190 different carbon sources.

Responsive			**Differentially responsive**	**Negative response**		
N-acetyl D-Gucosamine	α- D Glucose	D-Mannitol	Propionic Acid	D-Saccharic Acid	D-Glucose-1-Phosphate	α-Methyl-D-Galactoside
Succinic acid	Maltose	L-Glutamic Acid	D-Fructose	Dulcitol	α-hydroxy Glutaric Acid-γ-Lactone	α-D-Lactose
D-Galactose	Glycyl-L-Aspartic Acid	Citric Acid	β-Methyl-D-Glucosidase	D-Sorbitol	Adonitol	Lactulose
L-Aspartic Acid	L-Asparagine	D,L-Malic Acid	L-Lactic Acid	L-Fucose	m-Inositol	m-Tartaric Acid
L-Proline	Tween 40	D-Ribose	D-Mannose	D-Glucuronic Acid	D-Threonine	Tyramine
D-Alanine	α-Keto-Glutaric Acid	Tween 20	L-Glutamic Acid	Formic Acid	Mucic Acid	L-Lyxose
D-Trehalose	α-Keto-Butyric Acid	Inosine	Uridine	D-Galactonic Acid-γ-Lactone	Glycolic Acid	L-Galactonic Acid-γ-Lactone
L-Serine	Fumaric acid	Acetic Acid	Thymidine	L-Rhamnose	Glyoxylic Acid	Mono Methyl Succinate
Glycyl-L-Glutamic Acid	Glycyl-L-Aspartic Acid	Glycyl-L-Proline	Sucrose	D-Melibiose	D-Cellobiose	D-Malic acid
Glycerol	L-Alanyl-Glycine	Pyruvic Acid	D-Glucose-6-Phosphate	D-Aspartic Acid	Tricarballylic Acid	p-Hydroxy Phenyl Acetic Acid
D-Gluconic Acid	D-Fructose-6-Phosphate	Methyl Pyruvate	D-Serine	D-Glucosaminic Acid	Acetoacetic Acid	m-Hydroxy Phenyl Acetic Acid
D,L,-α-Glycerol Phosphate	α-Hydroxy Butyric Acid	L-Malic Acid	L-Glutamine	1,2-Propanediol	N-Acetyl-β-D-Mannosamine	Phenylethyl-amine
D-xylose	Maltotriose	L-Serine		Glucuronamide	D-Psicose	D-Galacturonic Acid
	2-Deoxy-Adenosine	L-Threonine		2-aminothanol		
Citric Acid	Adenosine	L-Alanine				
Fumaric acid	Bromo Succinic Acid					

3.2.1.1. Strains show no competition defect in minimal media supplemented with propionic acid

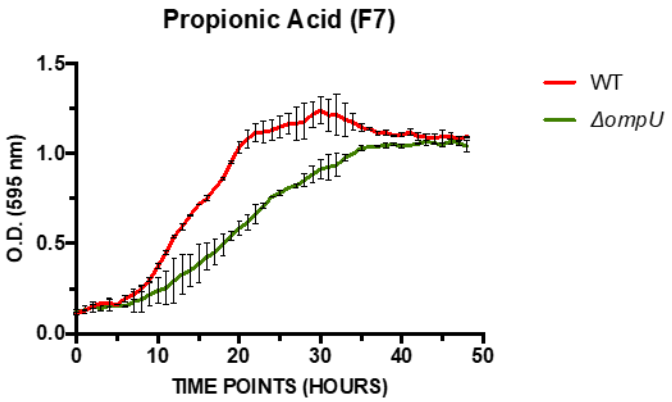

Figure 17: Respiration kinetics of PG-wild type and *ΔompU* mutant in the presence of propionic acid

Out of the carbon sources those were differentially responsive (See Table 1), propionic acid was selected for in-vitro competition assay because of the significant difference observed in the kinetics data (Figure 16). We wanted to observe if there is any competition for colonization exists among our test strains for the use of propionic acid as a carbon source. Results from this assay indicates no significant difference in competition for propionic acid (2mM/mL) in minimal media (Fig. 17).

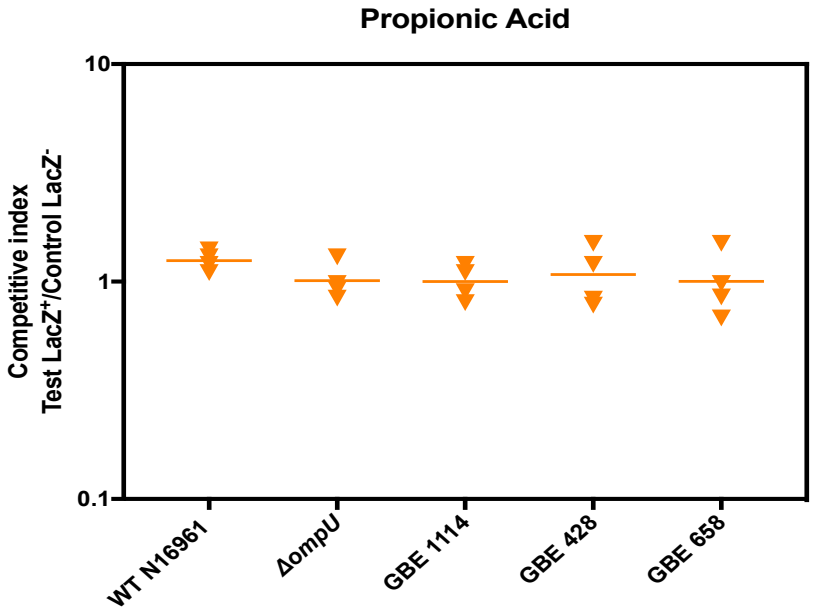

Figure 18: In-vitro competition for colonization of *ompU* mutants in minimal media supplemented with propionic acid

PG-wild type, *ΔompU* mutant and the strains encoding three different *ompU* alleles (*ompU*$^{GBE\ 114}$ *ompU*$^{GBE\ 428}$ and *ompU*$^{GBE\ 658}$), all colonized similarly which indicates that, competition for colonization in the presence of propionic acid is not dependent on OmpU as well as allelic variation.

3.2.2. High throughput screening for chemical sensitivity of the *ΔompU* mutant

In order to identify the novel function of OmpU, a total of 60 different chemical compounds with four different concentrations were evaluated through BIOLOG phenotypic microarray experiment to observe the difference in respiration of two strains: PG-wild type and *ΔompU* mutant in the presence of these compounds. We found significant difference in the respiration ability of the *ΔompU* mutant when compared the PG-wild type in the

presence of a few of these compounds (Table 3 and Fig. 19, 20 and 21). Our goal was to identify the antimicrobial compounds where OmpU shows a protective role to tolerate their bactericidal effects. Among these compounds, significant difference was observed in case of three compounds that showed significant difference: Rifamycin SV, EDTA and Trifluoperazine. These result indicates that, strains without the OmpU porin has a respiration defect in the presence of these compounds. To confirm our findings, rifamycin SV and EDTA were selected for further investigation to observe whether or not the resistance is PG-allele dependent.

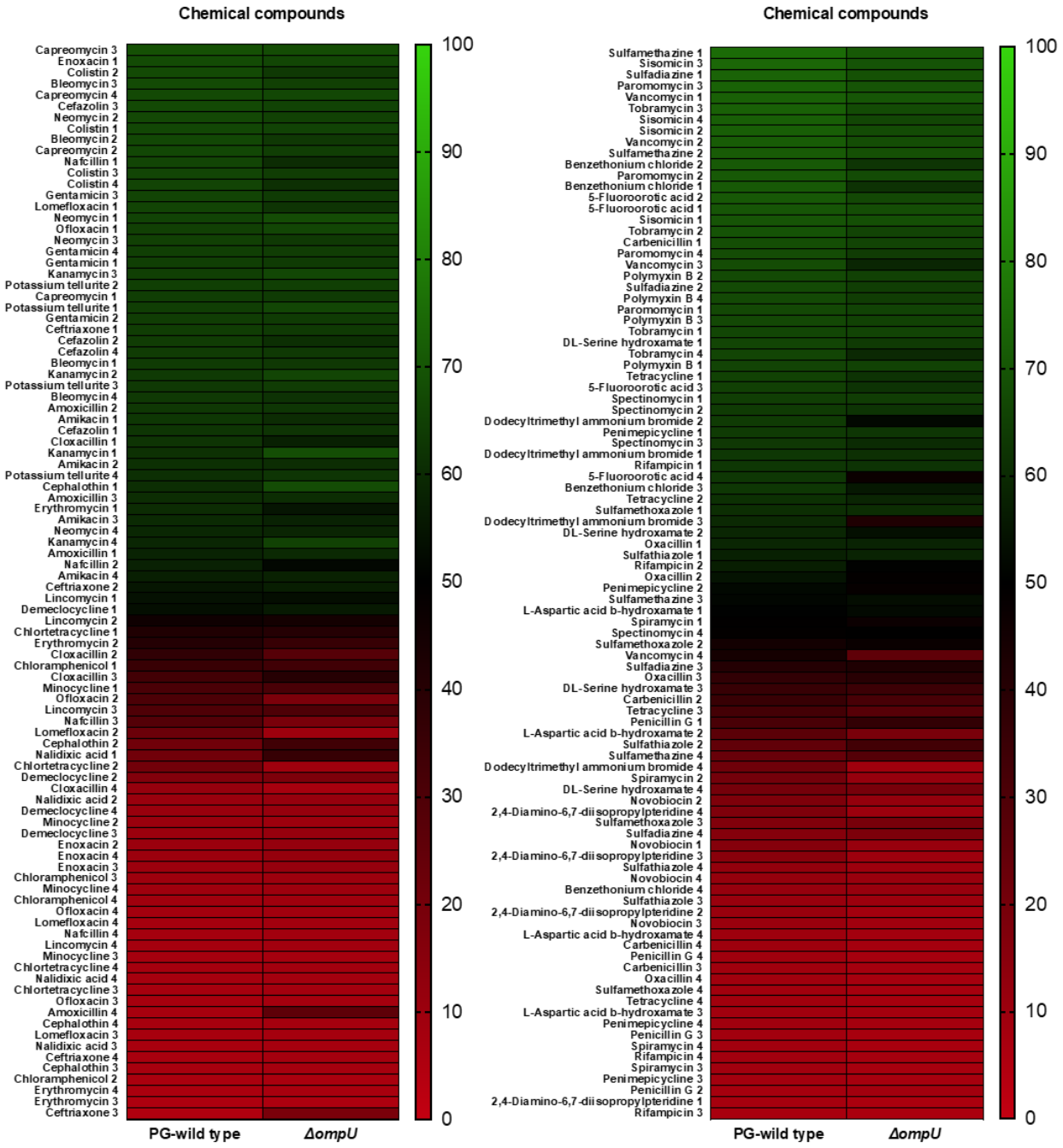

Figure 19: Heat Maps of carbon microplates PM 11 and PM 12

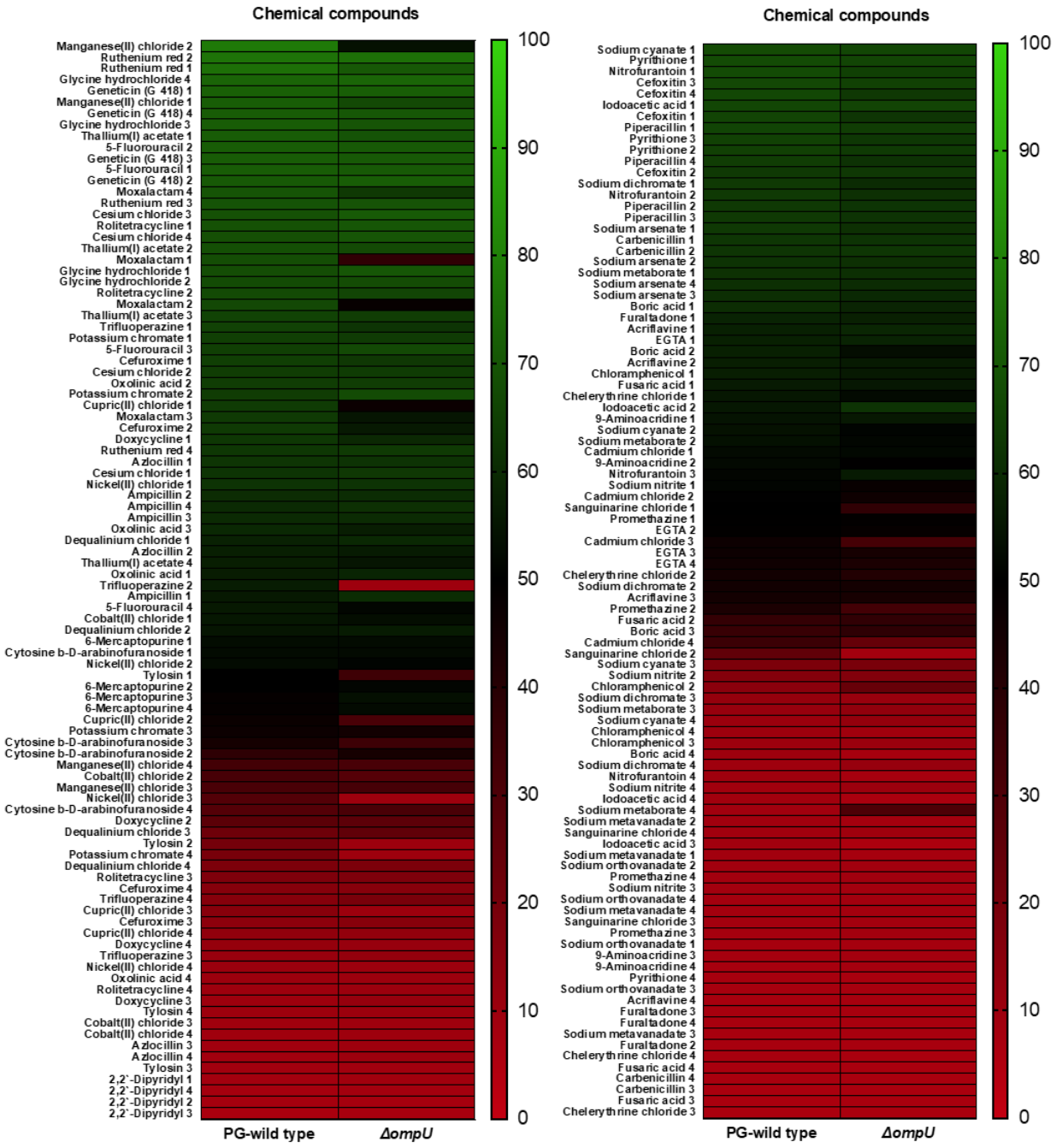

Figure 20: Heat Maps of carbon microplates PM 13 and PM 14

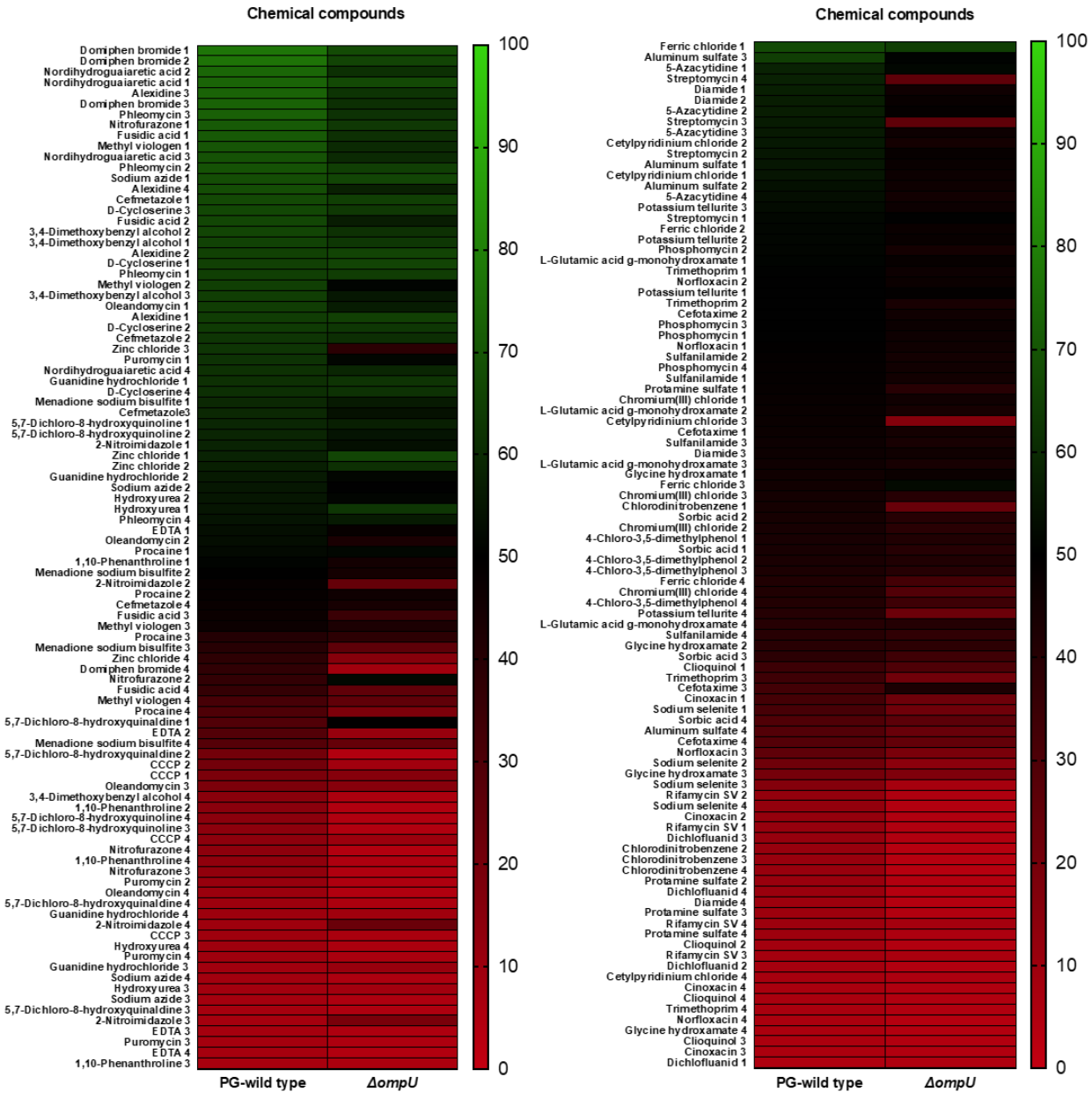

Figure 21: Heat Maps of carbon microplates PM 15 and PM 16

Table 3: Chemical compounds for further investigation

PM 11	PM 13	PM 14	PM 15	PM 16	PM 17	PM 19	PM 20
Ceftriaxone	Manganese chloride	Sangui-narine	Phleomycin	Dichlofluanid	Niaproof	Lauryl sulfobrtaine	Amitriptyline
Cephalothin	Nickel chloride	Chloram-phenicol	Procaine	Sodium selenite	Chlorambucil	Harmane	3,5-Dinitrobenzoic acid
Cloxacillin	Trifluoperazine	Nitro-furantoin	EDTA	Rifamycin SV	Sulfamono-methoxine	Cinnamic acid	Proflavine
-	-	-	-	-	Sulfachloro-pyridazine	-	Crystal violet
-	-	-	-	-	-	-	4-Hydroxycoumarin
-	-	-	-	-	-	-	Tolylfluanid
-	-	-	-	-	-	-	Ciprofloxacin
-	-	-	-	-	-	-	Troleandomycin

3.2.2.1. Rifamycin SV resistance is OmpU and PG-allele dependent

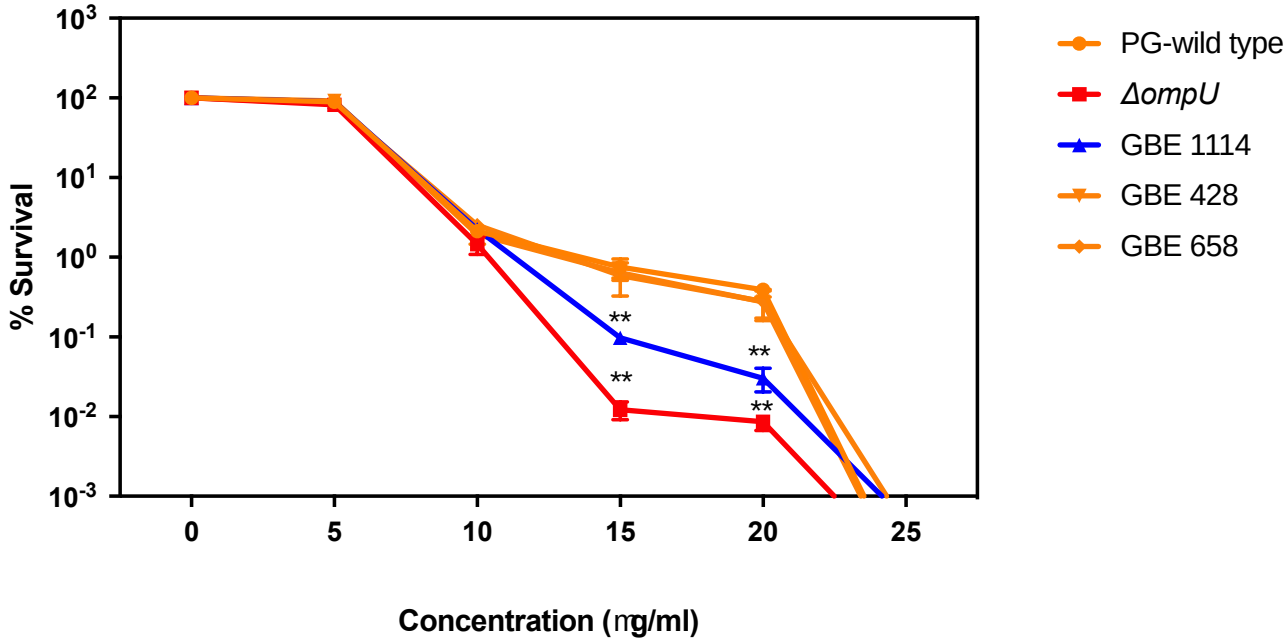

Figure 22: Survival of *ompU* mutants in the presence of of Rifamycin SV

Survival of ompU mutants in the presence of different concentrations of Rifamycin SV (n=4). Student's t-test was used to make statistical comparisons. **P < 0.01

Rifamycin B belongs to the family of Rifamycin antibiotics those are used for antimicrobial and antitubercular agent. It blocks the RNA-polymerase mediated transcription of DNA in Bacteria. As our goal was to identify whether PG allele confers Rifamycin SV resistance, we determined the survival of our test strains in the presence of a range of different concentration (0μg/mL, 5μg/mL, 10μg/mL, 15μg/mL and 20μg/mL) of

Rifamycin SV because allelic dependency may differ based on the concentration. Our results suggest that Rifamycin SV tolerance in *V. cholerae* is *ompU* dependent with a two

log difference in the survival percentage between the PG-wild type and the *⊿ompU* when treated with 15μg/mL rifamycin SV (Fig. 20). Similarly, 20μl/ml of rifamycin SV treatment on *⊿ompU* strain showed a drastic difference in survival compared with the PG wild-type (Fig. 20). On the other hand, *ompU*$^{GBE\ 428}$ and *ompU*$^{GBE\ 658}$ survived similarly as the PG-wild type as indicated in the graph (Figure 20). Interestingly, the survival in the presence of rifamycin SV is allelic dependent as the strains encoding the *ompU*$^{GBE\ 114}$ had a survival defect similar to the *⊿ompU* mutant. However, no significant difference was observed across other concentrations tested in our experiment. The results of this experiment suggests that rifamycin SV resistance is OmpU dependent and only strains encoding PG and PG-like alleles of *ompU* shows resistance to rifamycin SV.

3.2.3.2. EDTA resistance is OmpU dependent but PG-allele independent

EDTA is a cationic peptide that disrupts the membrane of bacteria and causes cell death. As our goal was to identify whether PG allele confers EDTA resistance, we determined the survival of our test strains in the presence of a range of different concentrations (0μg/mL, 50μg/mL, 100μg/mL, 250 μg/mL, 500μg/mL, 1000μg/mL, 1500μg/mL and 2000μg/mL) of EDTA because allelic dependency may differ based on the concentration.

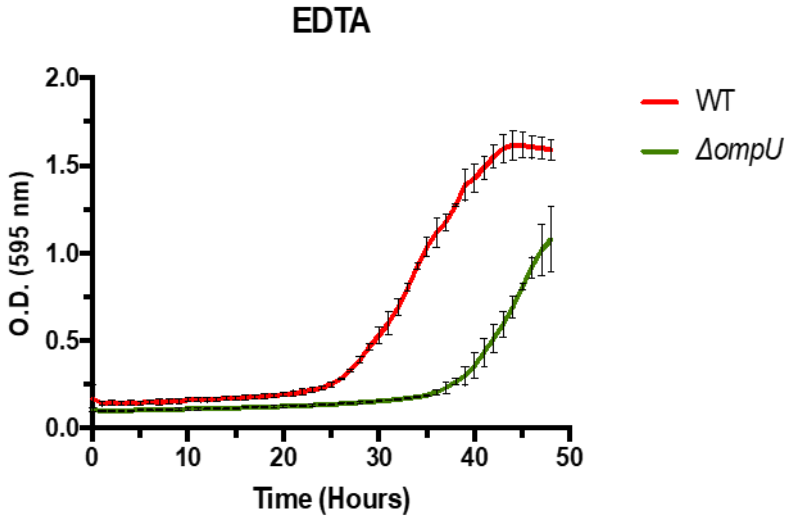

Figure 23: Respiration kinetics of PG-wild type and *⊿ompU* mutant in EDTA

Our results suggest that EDTA resistance in *V. cholerae* is *ompU* dependent with a log difference in the survival percentage between the PG-wild type and the *⊿ompU*. However, the survival in this antimicrobial peptide is not allelic dependent as the strains encoding the *ompU*$^{GBE\ 114}$, *ompU*$^{GBE\ 428}$, and *ompU*$^{GBE\ 658}$ survived similarly as the PG-wild type as indicated in the graph (Figure 21 and 22). Specifically, 250μg/mL 500μg/mL and 1000μg/mL of EDTA treatment on *⊿ompU* strain showed a drastic difference in survival compared with the PG wild-type.

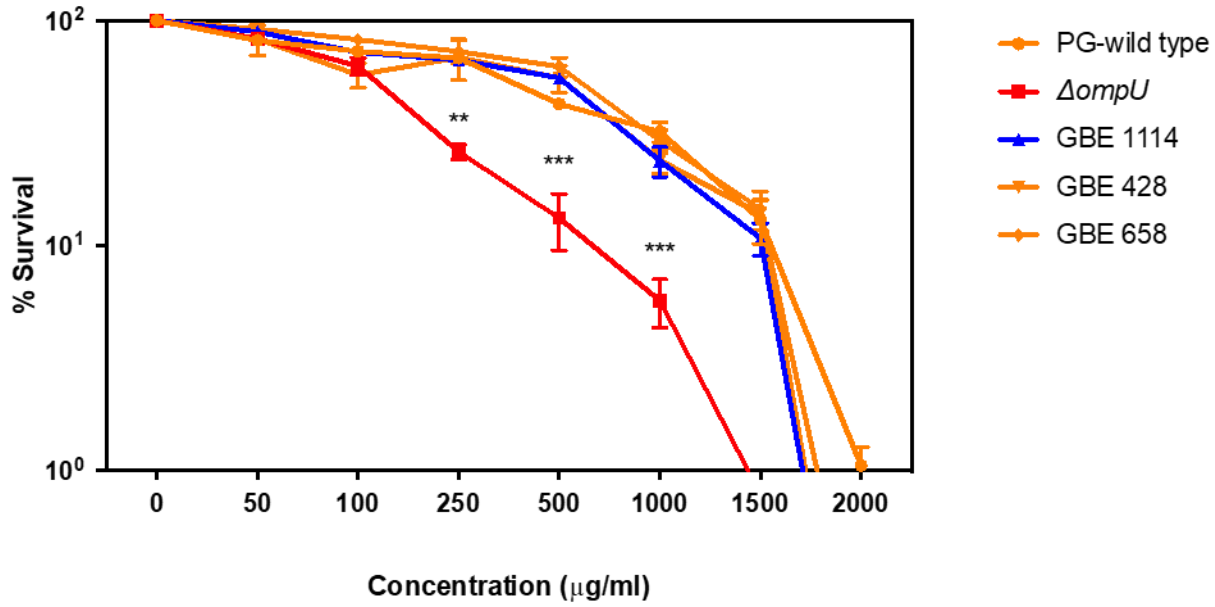

Figure 24: Survival of *ompU* mutants in the presence of EDTA

The figure depicts the survival of *ompU* mutants in the presence of different concentrations of EDTA (n=4). Student's t-test was used to make statistical comparisons. **P < 0.01; *** P < 0.001

However, no significant difference was observed across other concentrations tested in our experiment suggesting that strains encoding either PG-like or EG-like alleles are resistant to EDTA treatment. The results from this experiment indicates that EDTA resistance is not PG-allele dependent.

3.2.3.3. Trifluoperazine resistance is OmpU dependent but PG-allele independent

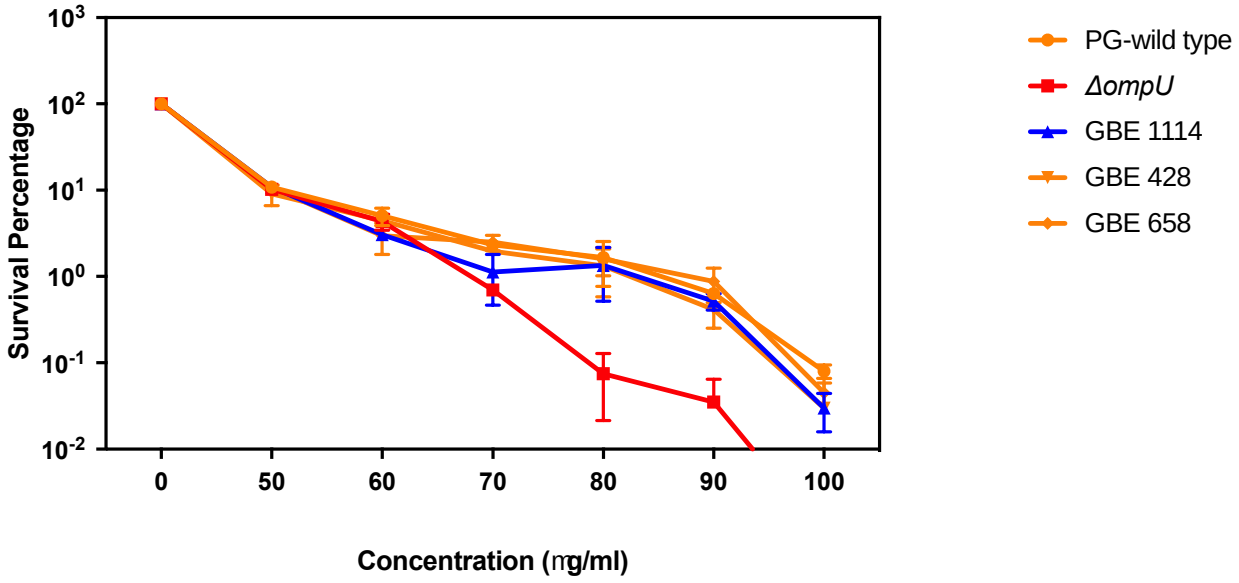

Figure 25: Survival of *ompU* mutants in the presence of Trifluoperazine

The figure depicts the survival of *ompU* mutants in the presence of different concentrations of Trifluoperazine (n=2). Student's t-test was used to make statistical comparisons. **P < 0.01; *** P < 0.001

Trifluoperazine is an anti-psychotic drug that is mainly used to treat brain disorders such as schizophrenia (71). It mediates conformational change in the $Ca2^{+}$ dependent signaling in eukaryotic cells which affects different physiological processes (71). As our

goal was to identify whether PG allele confers trifluoperazine resistance, we determined the survival of our test strains in the presence of a range of different concentrations (0μg/mL, 50μg/mL, 60 μg/mL, 70 μg/mL, 80 μg/mL, 90 μg/mL, and 100 μg/mL) of trifluoperazine because trifluoperazine is a novel compound in terms of its effect on *ompU* mutant and also, allelic dependency may differ based on the concentration. Our results indicates that there is a log difference in the survival percentage between the PG-wild type and the *ΔompU* at 80 μg/mL and 90 μg/mL. However, the survival in the presence of trifluoperazine is not allelic dependent as the strains encoding the *ompU*$^{GBE\ 114}$, *ompU*$^{GBE\ 428}$, and *ompU*$^{GBE\ 658}$ survived similarly as the PG-wild type as indicated in the graph (Fig. 25). The results from this experiment suggests that strains encoding either PG-like or EG-like alleles are resistant to trifluoperazine treatment the resistance is not PG-allele dependent.

CHAPTER FOUR: DISCUSSION

V. cholerae has developed strategies to overcome host's barriers and successfully colonize the gut and establish and infection. The bacterium encounters bile, acidic pH, competitive commensal bacteria, reactive oxygen species, antimicrobials and other stressors that could otherwise hinder their activity inside the host. Our study shows that the ability to withstand some of these stressors is dependent on the PG allele of the outer membrane porin U (*ompU*). These *ompU* alleles of *V. cholerae* confer critical preadaptations that mediate virulence associated properties such as bile tolerance, acidic pH tolerance, antimicrobial peptide resistance, motility and biofilm formation. We determined that PG-allele itself is the one that confers most of the virulence associated properties those are crucial for intestinal infection. Allelic characterization of PG and EG-like *ompU* alleles has enabled us to dissect the PG allele dependency and independency of virulence associated properties in *V. cholerae*. Our findings indicate that due to the presence of the PG-allele and PG-like allele, *V. cholerae* can maximize its pathogenic potential. For example, bile resistance is dependent on the PG-allele of *ompU*. It has been previously shown that OmpU role in bile resistance is due to the anion selectivity of the porin. OmpU has been reported to restrict the influx of negatively charged molecules as those molecules present in bile and other anionic detergents such as SDS (37). As we have found that, the strains that encode *ompU* allele from GBE 1114 cannot confer bile tolerance, we concluded that bile tolerance is PG-allele dependent. One possible reason to restrict bile influx by OmpU porin could be the presence of non-canonical N-terminal

coil in the pore lumen. Structural comparison from our study between PG and EG-like OmpU porins, show that OmpU $^{GBE\ 1114}$ does not contain a major portion of the N-terminal coil. The coil might form a smaller pore with the constrict loop L3 within the OmpU barrel porin. This coil also serves as the barrier between the two gates inside the porin those are highly positively charged and negatively charged Future studies on the structure and functional characterization of OmpU alleles can shed light and differentiate between the bile and detergent resistance mechanism of PG alleles and EG alleles.

We have also shown that PG-allele confers organic acid tolerance and antimicrobial tolerance which is crucial for intestinal colonization. It remains to be determined in the future that why *V. cholerae* strain encoding a EG-like *ompU* allele cannot confer the organic acid tolerance and the strains with PG-like *ompU* alleles retains the ability of the clinical strain to remain organic acid tolerant. According to Merell and Camilli, the loss of OmpU porin from the membrane facilitates the passage of organic acid molecules into the periplasm and causes cell death. Therefore, it is possible that the structural integrity of the membrane is compromised in low acidic environment for the strain encoding EG-like ompU allele (*ompU*^{GBE1114}) and the passage of organic acid molecules is fluent to the degree that mediates cell death. Increased cell death thus shows *⊿ompU*-type phenotype in the clinical strain that encodes EG-like *ompU* allele. However, The details mechanism remains to be elucidated in the future of why strains encoding the EG-like *ompU* allele cannot retain organic acid tolerance.

Another interesting finding from our study is that the strain encoding the EG-like *ompU*

allele (*ompU*^{GBE1114}) show variation in PG-allele dependency in the presence of antimicrobial peptides P2 and polymyxin B. Strain encoding EG-like *ompU* allele is polymyxin B resistant but shows significant sensitivity upon P2 challenge. This finding leads to a natural question – are the adopting different mechanisms in the presence of these two antimicrobial peptides? Previously, Mathur and Waldor demonstrated the protective mechanism of the outer membrane of *V. cholerae* in sublethal P2 challenge (55,72). According to their model, when *V. cholerae* cells detects the presence of P2 in the surrounding environment, the OmpU exposes its YDF motif, which is generally buried in the C-terminal end of the protein. The YDF motif activates DegS , a periplasmic protease which in turn activates the main factor that induces stress response pathway by releasing RpoE after proteolysis of the anti-sigma factor RseA σ^E. σ^E stress response pathway has been reported to confer protection of cell envelope integrity in *V. cholerae* (73). It is possible that P2 cannot expose the YDF motif of OmpU ^{GBE1114} which activates σ^E stress response pathway while the YDF motif is exposed upon polymyxin B challenge. Therefore, strains encoding the *ompU* allele of GBE 1114 is susceptible to P2 challenge but remains resistant in sublethal polymyxin B exposure. Bioinformatics based study of the structure and function of the alleles of *ompU* and gene expression analysis in the presence of these antimicrobials may shed light in the future to elucidate their mechanisms.

Our study also add knowledge that El tor N16961 of *V. cholerae* strains requires OmpU in its flagella for efficient motility. As shown by the growth assay of all the tested strains in LB medium, loss of motility does not affect the growth. Although, it has previously

been shown that combined activity of the outer membrane porins (OmpU and OmpT) have significant control over motility in *V. cholerae,* we have shown that *ompT* expression alone cannot restore the wild-type motility phenotype or functional integrity of the flagella**.** Loss of OmpU from the flagella affects the flagellar rotation and the structural integrity of *V. cholerae* flagellum. The possible change of the structural integrity or flagellar rotation of the mutant strains needs further investigation to identify the role of different alleles of OmpU in motility and concomitant virulence mechanism. For future studies, analysis of the protein content culture supernatants of PG-wild type, deletion mutant of *ompU* allele might shed light on the role of the alleles in motility.

We have also identified the range of temperature (22°C-37°C) within which biofilm is formed on abiotic surfaces. Our finding is consistent with the findings reported by Shapiro et al. that EG-like allele mediates biofilm formation (17). Here we report that, based on temperature, this trait can be different as we have shown that, no biofilm accumulates at 22°C and similar biofilm forms at 30°C and 37°C. The possible reason of efficient biofilm formation by the Δ*ompU* strain can be linked with their compromised motility. It has been shown that Δ*ompU* mutant is nearly non-motile because it loses the structural integrity of the flagellum. Previously, Watnick et al. showed that absence of flagellar integrity and cessation of flagellar activity stimulates exopolysaccharide (EPS) synthesis which is required for efficient three-dimensional biofilm formation in *V. cholerae* (74,75). Additionally, similar behavior of motility, exopolysaccharide secretion and biofilm formation has been shown in case of several other bacteria (75). It would be interesting to examine why is the strain encoding the EG-like *ompU* allele concomitantly confers

efficient motility and forms robust biofilm on abiotic surface even though these two phenotypic traits are contrary in case of other strains? It needs to be investigated in the future through advanced microscopy and biofilm assays to determine the EG-allele dependency of biofilm formation and what could be some other factors besides temperature that might be involved in OmpU and biofilm formation in *V. cholerae*.

In this study, we have identified two novel functions of OmpU those are rifamycin SV resistance and EDTA resistance. First, we have shown that OmpU confers resistance to the antibiotic, rifamycin SV. Rifamycin SV is a derivative of a family of antibiotics that affect bacterial transcription (76). We found that *ΔompU* strains are sensitive to sublethal exposure of rifamycin (15μg/ml). Strains with PG allele are two logs more resistant than the strains encoding an EG allele or the deletion mutant. Given that, a strain with an EG-like allele (*ompU*^{GBE1114}) also shows sensitivity to sublethal rifamycin treatment, we concluded that rifamycin resistance is both OmpU and allele dependent. Although it is well documented that rifamycin inhibits the bacterial RNA polymerase dependent DNA transcription, the mechanism of entry through the bacterial membrane is unclear (76). Since OmpU is the most abundantly produced porin in *V. cholerae* it is possible that OmpU restricts the passage of such molecules into the periplasm mimicking a mechanism similar of bile tolerance. It has been recently shown that, another outer membrane porin, OmpA, is an integral part of the *V. cholerae* outer membrane. Unlike OmpU, OmpA has not been reported to restrict the passage of any antimicrobials. Rather, Matson et al. showed that OmpA is associated with the channeling of antimicrobials (Polymyxin B, LL-37) using the chaperone SipA, a stress induced protein (56). From here, we can speculate

that, rifamycin molecules are pumped into the periplasm through OmpA in the absence of OmpU and thus mediates the rifamycin sensitive phenotype of the *ΔompU* mutant. Having OmpU in the membrane substantially restrict the passage of these molecules which is detrimental upon rifamycin treatment when only OmpA porin is present. This notion leads to our hypothesis that, double deletion of OmpU and OmpA porins will restore the wild-type phenotype in *V. cholerae* in the presence of sublethal rifamycin treatment.

We also found that resistance of EDTA is OmpU-mediated. EDTA chelates the cations (Ca^{2+} and $Mg^{2+)}$ in the bacterial lipopolysaccharide and thus destabilizes the cell membrane of many gram-negative bacteria (77). Given that, the phenotype is allele independent, we can speculate that EDTA molecules may be involved in perturbing the cell membrane and inducing the pathway related to OmpU and stress response as has been hypothesized in case of the antimicrobial P2 and polymyxin B. Previously, Mathur and Waldor demonstrated that OmpU activates the σ^E stress response cascade by exposing its YDF motif to confer protection of cell envelope in *V. cholerae* (73). This notion leads to our hypothesis that EDTA molecules induces the OmpU mediated σ^E stress response pathway and repairs the cell membrane (Fig. 24). Gene expression analysis in the presence of EDTA will help us to determine the mechanism of OmpU mediated EDTA resistance.

The experiments from this study allowed us to determine the PG-allele dependent functions such as bile tolerance, organic acid tolerance, antimicrobial resistance a few other novel functions of OmpU. Clearly, these traits facilitates the passage of the

bacterium starting from the ingestion towards the successful colonization in the gut by overcoming critical barriers encountered in the host environment. Understanding this phenomenon will help us identify the molecular mechanisms that provide crucial host preadaptations. This will allow us to dissect the genetic basis of virulence and consolidate our understanding of how genetic variations may confer virulence associated properties to facultative pathogens such as *V. cholerae.*

APPENDIX: COPYRIGHT PERMISSION

REFERENCES

1. Reen FJ, Almagro-Moreno S, Ussery D, Boyd EF. The genomic code: Inferring Vibrionaceae niche specialization. Nat Rev Microbiol. 2006;4(9):697–704.

2. J. R, K.E. K. Vibrio cholerae and cholera: Out of the water and into the host. FEMS Microbiol Rev. 2002;26(2):125–39.

3. Manley G. Public Access NIH Public Access. 2013;71(2):233–6.

4. Azman AS, Rudolph KE, Cummings DAT, Lessler J. The incubation period of cholera : A systematic review. J Infect. 2013;66(5):432–8.

5. Almagro-Moreno S, Pruss K, Taylor RK. Intestinal Colonization Dynamics of Vibrio cholerae. PLoS Pathog. 2015;11(5):1–11.

6. Almagro-moreno S, Taylor RK. Cholera: Environmental Reservoirs and Impact on Disease Transmission. One Heal. 2014;149–65.

7. Ali M, Nelson AR, Lopez AL, Sack DA. Updated Global Burden of Cholera in Endemic Countries. 2015;1–13.

8. Lutz C, Erken M, Noorian P, Sun S, Mcdougald D. Environmental reservoirs and mechanisms of persistence of Vibrio cholerae. 2013;4(December):1–15.

9. No WV. Morbidity and Mortality Weekly Report Update on Cholera — Haiti , Dominican Republic , and Florida , 2010. 2010;59(50).

10. No WV. Morbidity and Mortality Weekly Report Human Paragonimiasis After Eating Raw or Undercooked Crayfish — Missouri , July 2006 − September 2010. 2010;59(48).

11. No WV. Morbidity and Mortality Weekly Report Update : Cholera Outbreak — Haiti , 2010. Vol. 59. 2010.

12. Dowell SF, Tappero JW, Frieden TR. Public Health in Haiti — Challenges and Progress. 2019;300–1.

13. Seed KD, Yen M, Jesse Shapiro B, Hilaire IJ, Charles RC, Teng JE, et al. Evolutionary consequences of intra-patient phage predation on microbial populations. Elife. 2014;3(August2014):1–10.

14. Weill FX, Domman D, Njamkepo E, Almesbahi AA, Naji M, Nasher SS, et al. Genomic insights into the 2016–2017 cholera epidemic in Yemen. Nature. 2019;565(7738):230–3.

15. Fraser CM, Heidelberg JF, Eisen JA, Nelson WC, Clayton RA, Gwinn ML, et al. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature. 2000;406(6795):477–83.

16. Sakib SN, Reddi G, Almagro-Moreno S. Environmental Role of Pathogenic Traits in Vibrio cholerae . J Bacteriol. 2018;200(15):1–12.

17. Shapiro BJ, Levade I, Kovacikova G, Taylor RK, Almagro-Moreno S. Origins of pandemic Vibrio cholerae from environmental gene pools. Nat Microbiol. 2016;2(3).

18. Halpern M, Izhaki I. Fish as Hosts of Vibrio cholerae. 2017;8(February):1–7.

19. Huq A, Huq SA, Grimes DJ, O'Brien M, Chu KH, Capuzzo JM, et al. Colonization of the gut of the blue crab (Callinectes sapidus) by Vibrio cholerae. Appl Environ Microbiol. 1986;52(3):586–8.

20. Abd H, Weintraub A, Sandström G. Intracellular survival and replication of Vibrio cholerae O139 in aquatic free-living amoebae. Environ Microbiol. 2005;7(7):1003–8.

21. Dziejman M, Balon E, Boyd D, Fraser CM, Heidelberg JF, Mekalanos JJ. Comparative genomic analysis of Vibrio cholerae: Genes that correlate with cholera endemic and pandemic disease. Proc Natl Acad Sci. 2002;99(3):1556–61.

22. Faruque SM, Albert MJ, Mekalanos JJ. Epidemiology, genetics, and ecology of toxigenic Vibrio cholerae. Microbiol Mol Biol Rev [Internet]. 1998;62(4):1301–14.

23. Mutreja A, Kim DW, Thomson NR, Connor TR, Lee JH, Kariuki S, et al. Evidence for several waves of global transmission in the seventh cholera pandemic. Nature. 2011 Aug 24;477:462.

24. Schuster BM. Microbial interactions with oysters from the Great Bay Estuary: Characterization of endemic Vibrio choleraeand oyster metagenetics. [Thesis]. 2010;

25. D. Scott Merrell*,†, Susan M. Butler*, Firdausi Qadri‡, Nadia A. Dolganov§, Ahsfaqul Alam‡, Mitchell B. Cohen‖, Stephen B. Calderwood¶, Gary K. Schoolnik§

and AC. Host-induced epidemic spread of the cholera bacterium. 2016;87(1):124–38.

26. Rivera ING, Chun J, Huq A, Sack RB, Icrobiol APPLENM. Genotypes Associated with Virulence in Environmental Isolates of Vibrio cholerae. 2001;67(6):2421–9.

27. Mukhopadhyay AK, Mitra R, Colwell RR, Shimada T, Takeda Y, Faruque SM, et al. Virulence Genes in Environmental Strains of Vibrio cholerae. Appl Environ Microbiol. 2002;66(9):4022–8.

28. Faruque SM, Chowdhury N, Kamruzzaman M, Dziejman M, Rahman MH, Sack DA, et al. Genetic diversity and virulence potential of environmental Vibrio cholerae population in a cholera-endemic area . Proc Natl Acad Sci. 2004;101(7):2123–8.

29. Constantin de Magny G, Colwell RR. Cholera and climate: a demonstrated relationship. Trans Am Clin Climatol Assoc [Internet]. 2009;120:119–28.

30. Bari W, Lee KM, Yoon SS. Structural and functional importance of outer membrane proteins in Vibrio cholerae flagellum. J Microbiol. 2012;50(4):631–7.

31. Provenzano D, Lauriano CM, Klose KE. Characterization of the role of the ToxR-modulated outer membrane porins OmpU and OmpT in Vibrio cholerae virulence. J Bacteriol. 2001;183(12):3652–62.

32. Scott Merrell D, Bailey C, Kaper JB, Camilli A. The ToxR-mediated organic acid tolerance response of vibrio cholerae requires OmpU. J Bacteriol. 2001;183(9):2746–54.

33. Chakrabarti SR, Chaudhuri K, Sen K, Das J. Porins of Vibrio cholerae: Purification and characterization of OmpU. J Bacteriol. 1996;178(2):524–30.

34. Sperandio V, Giron JA, Silveira WD, Kaper JB. The OmpU outer membrane protein, a potential adherence factor of Vibrio cholerae. Infect Immun. 1995;63(11):4433–8.

35. Crawford JA, Kaper JB, DiRita VJ. Analysis of ToxR-dependent transcription activation of ompU, the gene encoding a major envelope protein in Vibrio cholerae. Mol Microbiol. 1998;29(1):235–46.

36. Li CC, Crawford JA, DiRita VJ, Kaper JB. Molecular cloning and transcriptional regulation of ompT, a ToxR-repressed gene in Vibrio cholerae. Mol Microbiol. 2000;35(1):189–203.

37. Provenzano D, Klose KE. Altered expression of the ToxR-regulated porins OmpU and OmpT diminishes Vibrio cholerae bile resistance, virulence factor expression, and intestinal colonization. Proc Natl Acad Sci. 2000;97(18):10220–4.

38. Duret G, Delcour AH. Size and dynamics of the vibrio cholerae porins OmpU and OmpT probed by polymer exclusion. Biophys J. 2010;98(9):1820–9.

39. Li H, Zhang W, Dong C. Crystal structure of the outer membrane protein OmpU from Vibrio cholerae at 2.2 Å resolution . Acta Crystallogr Sect D Struct Biol. 2018;74(1):21–9.

40. Pathania M, Acosta-Gutierrez S, Bhamidimarri SP, Baslé A, Winterhalter M,

Ceccarelli M, et al. Unusual Constriction Zones in the Major Porins OmpU and OmpT from Vibrio cholerae. Structure. 2018;26(5):708-721.e4.

41. Yang JS, Jeon JH, Jang MS, Kang SS, Ahn KB, Song M, et al. Vibrio cholerae OmpU induces IL-8 expression in human intestinal epithelial cells. Mol Immunol [Internet]. 2018;93(November 2017):47–54.

42. Nishiyama SI, Takahashi Y, Yamamoto K, Suzuki D, Itoh Y, Sumita K, et al. Identification of a Vibrio cholerae chemoreceptor that senses taurine and amino acids as attractants. Sci Rep. 2016;6(January):1–11.

43. Bina JE, Mekalanos JJ. Vibrio cholerae tolC is required for bile resistance and colonization. Infect Immun. 2001;69(7):4681–5.

44. JR S, KP N, RB C, DA R, CS F. Survival of the Fittest: How Bacterial Pathogens Utilize Bile To Enhance Infection. Clin Microbiol Rev. 2016;29(4):819–36.

45. Wibbenmeyer JA, Provenzano D, Landry CF, Klose KE, Delcour AH. Vibrio cholerae OmpU and OmpT porins are differentially affected by bile. Infect Immun. 2002;70(1):121–6.

46. Bina JE, Provenzano D, Wang C, Bina XR, Mekalanos JJ. Characterization of the Vibrio cholerae vexAB and vexCD efflux systems. Arch Microbiol. 2006;186(3):171–81.

47. Cerda-Maira FA, Ringelberg CS, Taylor RK. The bile response repressor BreR regulates expression of the Vibrio cholerae breAB efflux system operon. J Bacteriol.

2008;190(22):7441–52.

48. Ante VM, Bina XR, Howard MF, Sayeed S, Taylor DL, Bina JE. and Contributes to Bile Resistance. 2015;197(22):3499–510.

49. Chaudhuri S, Chatterjee A, Chowdhury R, Saha G, Gupta S. Effect of Bile on the Cell Surface Permeability Barrier and Efflux System of Vibrio cholerae. J Bacteriol. 2004;186(20):6809–14.

50. Ante VM, Bina XR, Howard MF, Sayeed S, Taylor DL, Bina JE. Vibrio cholerae leuO Transcription Is Positively Regulated by ToxR and Contributes to Bile Resistance. 2015;197(22):3499–510.

51. Merrell DS, Camilli A. The cadA gene of Vibrio cholerae is induced during infection and plays a role in acid tolerance. Mol Microbiol. 1999;34(4):836–49.

52. Merrell DS, Camilli A. Acid tolerance of gastrointestinal pathogens. 1999;51–5.

53. Jubair M, Atanasova KR, Rahman M, Klose KE, Yasmin M, Yilmaz Ö, et al. Vibrio cholerae persisted in microcosm for 700 days inhibits motility but promotes biofilm formation in nutrient-poor lake water microcosms. PLoS One. 2014;9(3).

54. Merrell DS, Hava DL, Camilli A. Identification of novel factors involved in colonization and acid tolerance of Vibrio cholerae. 2002;43:1471–91.

55. Mathur J, Waldor MK. The Vibrio cholerae ToxR-regulated porin OmpU confers resistance to antimicrobial peptides. Infect Immun. 2004;72(6):3577–83.

56. Saul-McBeth J, Matson JS. A Periplasmic Antimicrobial Peptide-Binding Protein Is Required for Stress Survival in Vibrio cholerae. Front Microbiol. 2019;10(February):1–12.

57. Butler SM, Camilli A. Both chemotaxis and net motility greatly influence the infectivity of Vibrio cholerae. Proc Natl Acad Sci. 2004;101(14):5018–23.

58. Martinez RM, Dharmasena MN, Kirn TJ, Taylor RK. Characterization of two outer membrane proteins, FlgO and FlgP, that influence Vibrio cholerae motility. J Bacteriol. 2009;191(18):5669–79.

59. Seed KD, Bodi KL, Kropinski AM. Evidence of a Dominant Lineage of Vibrio cholerae -Specific Lytic. MBio. 2011;2(1):1–9.

60. Gehring K, Charbit A, Brissaud E, Hofnung M. Bacteriophage λ receptor site on the Escherichia coli K-12 LambB protein. J Bacteriol. 1987;169(5):2103–6.

61. Oppenheimer-Shaanan Y, Steinberg N, Kolodkin-Gal I. Small molecules are natural triggers for the disassembly of biofilms. Trends Microbiol [Internet]. 2013;21(11):594–601.

62. Valeru SP, Wai SN, Saeed A, Sandström G, Abd H. ToxR of Vibrio cholerae affects biofilm, rugosity and survival with Acanthamoeba castellanii. BMC Res Notes. 2012;5.

63. Ompu P, Pagel M, Li J, Lallemand M, Lauman B, Delcour AH. Phenotypic Characterization of Pore Mutants of the Vibrio cholerae. 2007;189(23):8593–600.

64. Mey AR, Butz HA, Payne SM. Vibrio cholerae CsrA Regulates ToxR Levels in Response to Amino Acids and Is Essential for Virulence. MBio. 2015;6(4):1–11.

65. Aeckersberg F, Lupp C, Feliciano B, Ruby EG. Vibrio fischeri outer membrane protein ompU plays a role in normal symbiotic colonization. J Bacteriol. 2001;183(22):6590–7.

66. Toole GAO. Microtiter Dish Biofilm Formation Assay. 2011;10–1.

67. Bochner BR. Global phenotypic characterization of bacteria. FEMS Microbiol Rev. 2009;33(1):191–205.

68. Mackie AM, Hassan KA, Paulsen IT, Tetu SG. Biolog Phenotype MicroArrays for Phenotypic Characterization of Microbial Cells. In: Paulsen IT, Holmes AJ, editors. Environmental Microbiology: Methods and Protocols. Totowa, NJ: Humana Press; 2014. p. 123–30.

69. Almagro-Moreno S, Boyd EF. Sialic acid catabolism confers a competitive advantage to pathogenic Vibrio cholerae in the mouse intestine. Infect Immun. 2009;77(9):3807–16.

70. McDonald ND, Lubin J-B, Chowdhury N, Boyd EF. Host-Derived Sialic Acids Are an Important Nutrient Source Required for Optimal Bacterial Fitness In Vivo . MBio. 2016;7(2):1–10.

71. Vandonselaar M, Hickie RA, Quail W, Delbaere LTJ. Trifluoperazine-induced conformational change in Ca2+-calmodulin. Nat Struct Biol. 1994;1(11):795–801.

72. Mathur J, Davis BM, Waldor MK. Antimicrobial peptides activate the Vibrio cholerae σE regulon through an OmpU-dependent signalling pathway. Mol Microbiol. 2007;63(3):848–58.

73. Haines-Menges B, Whitaker WB, Boyd EF. Alternative Sigma Factor RpoE Is Important for Vibrio parahaemolyticus Cell Envelope Stress Response and Intestinal Colonization. Infect Immun. 2014;82(9):3667–77.

74. Watnick PI, Lauriano CM, Klose KE, Croal L, Kolter R. The absence of a flagellum leads to altered colony morphology, biofilm development and virulence in Vibrio cholerae O139. Mol Microbiol. 2001;39(2):223–35.

75. Peterson KM. Vibrio cholerae Virulence Genes 29 Expression of Vibrio cholerae Virulence Genes in Response to Environmental Signals. Curr Issues Intest Microbiol. 2002;3:29–38.

76. Steffen R, Jiang ZD, Gracias Garcia ML, Araujo P, Stiess M, Nacak T, et al. Rifamycin SV-MMX® for treatment of travellers' diarrhea: equally effective as ciprofloxacin and not associated with the acquisition of multi-drug resistant bacteria. J Travel Med. 2018;25(1):1–11.

77. Schnaitman CA. Effect of ethylenediaminetetraacetic acid, Triton X-100, and lysozyme on the morphology and chemical composition of isolate cell walls of Escherichia coli. J Bacteriol. 1971;108(1):553–63.