The Role of the NhaP1, NhaP2, and NhaP3 Antiporters in the Acid Tolerance of *Vibrio cholerae.*

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ABSTRACT

Vibrio cholerae (Vc), the etiological agent of cholera, has developed many mechanisms to combat the great fluctuations in pH, ion content and osmotic conditions of the vastly differing environments it encounters through its infections of the human host. A series of cation antiporters are found in the Vc membrane to assist the organism to combat these varying conditions. An estimated twelve antiporters reside in the bacterial membrane including NhaP1, NhaP2, and NhaP3. These three proteins have been identified as K+(Na+)/H+ antiporters specifically active in low pH conditions. We tested the hypothesis that these three antiporters are required for the survival of *Vibrio cholerae* in low pH environments. In this study the "6x6 drop plate method" by Chen et al. (10) was used to compare the survival of *Vibrio cholerae* O395N1 (wild type) to Vc-O395N1 *ΔnhaP1ΔnhaP2ΔnhaP3* (the triple mutant) in acidic conditions over a period of 90 minutes. At 15 minutes in pH 3.5 potassium-modified Luria broth, the wild type had a statistically significant higher percentage of survival than the triple mutant in comparison to non-acid treated cells (2.08% compared to 0.0223%, $P<0.0001$). When comparing complemented *nhaP1, nhaP2,* or *nhaP3* to the wild type strain, complemented *nhaP3* was able to restore the wild type phenotype ($P=0.1251$) while complemented *nhaP1* or *nhaP2* showed reduced survivability/culturability at 15 minutes at pH 3.5 ($P=0.0003$ and $P<0.001$, respectively). This study supports the hypothesis that *nhaP1, nhaP2,* and *nhaP3* intact are beneficial for the organism under extremely acidic conditions such as the human stomach but are not necessary for survival in other conditions.

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1. INTRODUCTION

Vibrio cholerae is the microorganism responsible for the human disease, Cholera. Virulent strains of this organism may cause life-threatening secretory diarrhea that can be so extreme that it may lead to hypovolemic shock or acidosis and subsequent death (1). In endemic countries there has been an estimated 1.4 to 4.3 million cases per year with 28,000 to 142,000 of these cases leading to death (2). Outbreaks of cholera occur in regions with inadequate water sanitation and crowded housing conditions (1). The spread of this organism can be rapid as 80% of its human hosts can remain asymptomatic. Although having no symptoms, these asymptomatic patients are still capable of shedding the microorganism through feces one to 10 days after infection (3).

In order for cholera to manifest in the host, the organism, *Vibrio cholerae,* must travel from contaminated food or water, through the acidic conditions of the stomach to reach the small intestine of its host (4). The acidity of the stomach of a healthy individual can be as low as pH 1.5 (5). This pH can play such a strong role in susceptibility to infection that it is even recommended to be wary of using antacids, histamine receptor blockers or proton pump inhibitors as these drugs affect gastric acidity and therefore may increase chances of infection (6). Within the small intestine, *Vibrio cholerae* releases an enterotoxin that leads to the leaking of Na+ and Cl- by the epithelial cells. This ion imbalance causes rapid dehydration of the cells lining the lumen leading to potentially deadly watery diarrhea (7).

As the organism transitions from the outside environment to the host environment, it encounters a variety of pH conditions. A key factor in their physiology, as well as in most

other bacteria, is the active use of cation antiporters. Antiporters are membrane proteins that exchange cations across cell membranes. In the case of *Vibrio cholerae*, sodium (Na+) and hydrogen (H+) antiporters are essential for the control of internal pH, volume homeostasis and regulation of intracellular sodium and potassium concentrations (8). In addition to sodium, these transporters are capable of exchanging protons for lithium, potassium, rubidium and calcium(9). In a study performed by Resch et al. (9), NhaP2 was characterized as an antiporter that swaps sodium or potassium for hydrogen in environments of neutral or slightly acidic pH. Additionally, the protein has a high affinity for potassium transport and is more active in environments containing high levels of potassium (9). As suggested by Quinn et al. (7), the NhaP1 antiporter also trades potassium or sodium for hydrogen and is essential for survival in low pH. Both of these antiporters are highly important for cellular pH homeostasis in a high potassium environment (7).

In summation, *Vibrio cholerae* is very versatile to survive in a wide variety of environmental conditions including bodies of water and the acidic human intestinal system. In this study we will model the acidic gastric conditions of the human gut to better understand *Vibrio cholerae's* ability to evade such a harsh environment. We hypothesize that the three genes, *nhaP1, nhaP2* and *nhaP3,* in combination, are required for the survival of *Vibrio cholerae* in the low pH milieu of the human gastrointestinal tract.

1.1 Expected Outcomes and Impact

The overreaching goal of this research is to further understand the role of the NhaP proteins in the infectious process. The NhaP antiporters are found in many bacterial species including the human pathogens *Yersinia pestis* and *Pseudomonas aeruginosa* that are also being investigated but are not reported in this study (12). The work developed here can serve as a launch pad for future investigations of ion homeostasis in these bacteria and knowledge for these antiporter's possible function in other organisms. A large hurdle for the organism to overcome within the host body is the acidic environment of the human stomach. Further understanding of this mechanism could bring insight into the possible development of novel intervention therapies to treat human disease (13).

Keywords:

Vibrio cholerae, 6x6 Drop Plate Method, NhaP antiporter

2. MATERIALS &METHODS

2.1 Bacterial Strains, Plasmids and Media

The strains used in this study are listed in Table 1 and stored in 15% glycerol at -80°C. The strains were cultured in potassium-modified Luria broth (LBK) (10 grams tryptone, 5 grams yeast extract and 10 grams KCl per liter, pH ~7.0) and were plated onto 1.5% LBK agar. For acid treatment, LBK adjusted to 100mM KCl was used and the pH was adjusted with 1 M hydrochloric acid or sodium hydroxide, respectively. When appropriate, ampicillin (100 μg/mL) and 1mM arabinose was added.

Table 1: Bacterial Strains. The following strains were used in the acid assays of this study. All but one, the complement P1, came from Hase lab freezer stocks. Complement P1 was created through electrotransformation as described in section 2.2. In this table (^{r}) indicates resistance and ara is abbreviated for arabinose.

Strain or Plasmid	Description	Source
V. cholerae		
Wild type	O395 N1 strep^r	Häse Lab Stocks
Wild type with empty plasmid	0395 N1 pBAD24, amp^r, strep^r, ara inducible	Häse Lab Stocks
Triple mutant	0395 N1 ΔnhaP1 ΔnhaP2 ΔnhaP3, strep^r	Häse Lab Stocks
P1 Donor	0395N1 Δ*nhaP3* <pBAD24::*nhaP1*> amp^r, strep^r	Häse Lab Stocks
Complement P1	0395 N1 ΔnhaP1 ΔnhaP2 ΔnhaP3 <pBAD24::nhaP1>, amp^r, strep^r, ara inducible	This Study
Complement P2	0395 N1 ΔnhaP1 ΔnhaP2 ΔnhaP3 <pBAD24::nhaP2>, amp^r, strep^r, ara inducible	Häse Lab Stocks
Complement P3	0395 N1 ΔnhaP1 ΔnhaP2 ΔnhaP3 <pBAD24::nhaP3>, amp^r, strep^r, ara inducible	Häse Lab Stocks

2.2 Complement Construction

Vibrio cholerae O395N1 Δ*nhaP1* Δ*nhaP2 ΔnhaP3*<pBAD24::*nhaP1*> (Complement P1) was not available upon start of this project therefore a plasmid containing *nhaP1* was harvested from Vc 0395N1ΔnhaP3<pBAD24::nhaP1> using the Qiagen plasmid miniprep kit (QIAGEN, USA) using the protocol provided by the manufacturer. For transformation of this plasmid into the triple mutant, one colony was incubated in LBK at 37°C with shaking overnight. 200 microliters (μl) of the triple mutant was seeded into eight 10mL LBK aliquots and incubated at 37°C with shaking for three hours. Once the recipient strain reached an optical density at 600 nanometers (OD_{600}) of 0.6 to 0.8, the cultures were immediately chilled on ice for 30 minutes. Electrocompetency was then achieved by washing the cells twice in ice-cold distilled water and then twice in 10% glycerol concentrating the cells at least 100-fold.

80uL of electrocompetent cells and ~100 nanograms of plasmid DNA were added to two pre-chilled electrotransformation cuvettes. The cells were pulsed using a GenePulser Xcell (Bio-Rad Laboratories Inc., Hercules, CA) with 2.5 kilovolts and 25 microfarads resulting in a time of 4.7 milliseconds in a 1 mm cuvette. Immediately after electrotransformation, super optimal broth (SOB; 5g yeast extract, 20g tryptone, 0.584g NaCl, 0.186g KCl, 2.4g $MgSO_4$ per liter) was added to the electrocuvettes, the cells recovered for one hour at 37°C with shaking and subsequently plated on pre-warmed LBK with ampicillin for overnight incubation at 37°C. Resulting transformants were cultured overnight in five mL LBK with ampicillin at 37°C with shaking, sequenced, and stocked in 15% glycerol at -80°C.

2.3 Acid Tolerance Assays

One colony of each strain was inoculated in 35mL of sterile LBK and incubated overnight at 37°C with shaking until an OD_{600} of 0.6 to 0.8 is reached (log phase). This optical density is the range when cells are in logarithmic phase. The cells were pelleted in a bucket centrifuge at 3600 x g for five minutes at 4°C. Supernatant was removed and the pellets were re-suspended in one milliliter of LBK and divided into two Eppendorf tubes. The cells were then re-pelleted in a tabletop centrifuge at 4,000 x g for one minute. The supernatant was removed once more by centrifugation and one pellet underwent acid treatment with one milliliter of acidic LBK and the other was re-suspended in neutral pH LBK to act as the negative control. Incubation was done at room temperature on the bench. At 15, 60, and 90 minutes, the cells were washed to remove the acidic media and the surviving cells as well as the untreated control were counted using the plating technique developed by Chen et al. (14).

2.4 6x6 Drop Plate Method

For the dilutions, all but the first column of wells of a 96-well plate was filled with 180μl of LBK (Sigma-Aldrich Co. LLC., St. Louis, MO). Then, into the first column 20μl of the bacterial suspension was added resulting in a 1:10 dilution. After thorough mixing by pipette (10 times), 20μl of the first column was transferred to the second column resulting in a 1:10 dilution, continuing throughout the rest of the plate (Figure 1). When this serial dilution was done, 10μl of each well was drop-plated onto a pre-dried LBK plate with neutral agar (Figure 2). The plates were incubated at 37°C overnight and resulting colonies were counted the next day. Cell survival was calculated and analyzed with GraphPad PRISM.

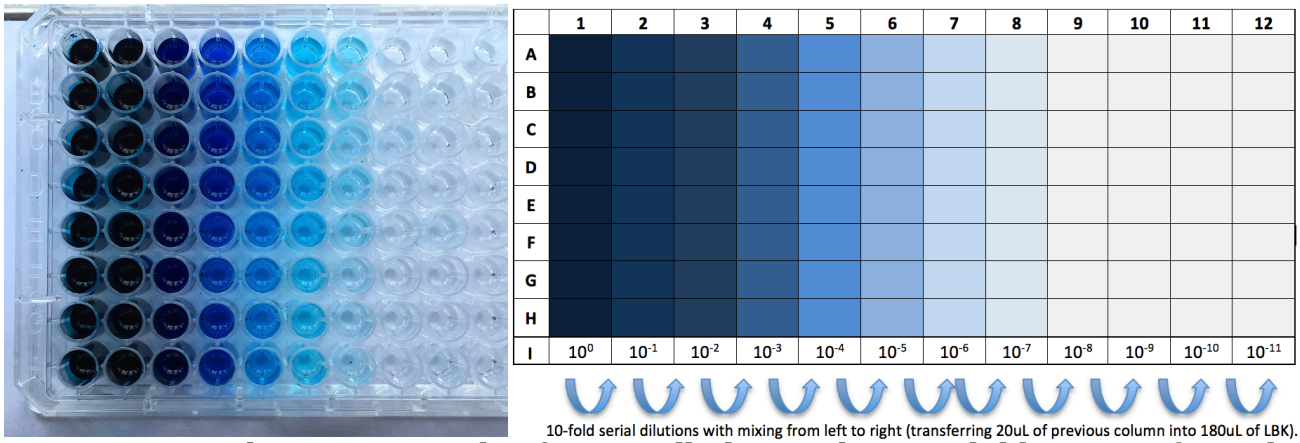

Figure 1: Panel A is an example of a 96-well plate with a serial dilution performed using dye and water. Panel B is a schematic summarizing the serial dilution. For a given time point, 50µl samples were taken directly from the cell solution and added to empty wells in column 1. From here, we diluted by transferring 20µl of the first column to 180µl of LBK broth in the second column to make a 1:10 dilution before applying onto an agar plate. Each column (1-12) represents increasing dilutions of cells denoted as a column dilution factor (cDF: 10^{0} – 10^{-11}), 10^{0} representing undiluted sample in column 1 and 10^{-11} being the most diluted sample in column 12. Rows A-C and D-F, respectively, contained triplicate samples of different strains. Row G and H were usually empty.

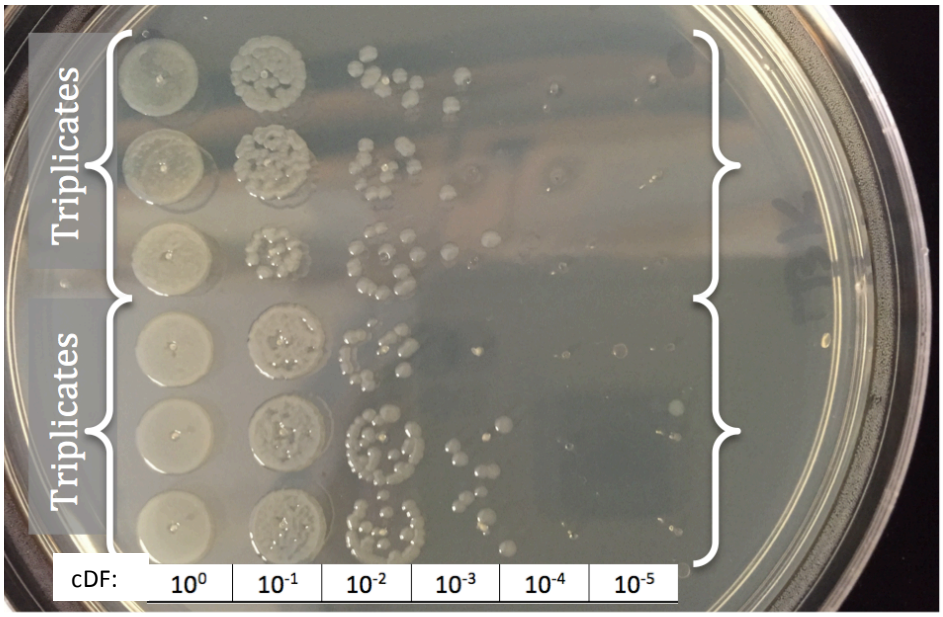

Figure 2: 6x6 Drop Plate with dilutions of *Vibrio cholerae.* The example agar plate has dilutions of *Vibrio cholerae* in droplets in a 6x6 format. The bottom values indicate the column dilution factor (cDF) 10^{0} to 10^{-5}.

2.5 Calculation of Cellular Concentration

To determine the percentage of cell survival at each time point, cellular concentration at t=0, was used as a baseline for comparison and as the negative control in this study. 10μl droplets that grew confluent due to high cell numbers were considered too numerous to count (TNTC). Those with distinct colonies were counted and multiplied by the appropriate column dilution factor (cDF) as well as the plating dilution factor (pDF). The pDF was 10^2 representing 10μl (rather than 1mL) of cell suspension in the form of a droplet was plated. The dilution factor is the level of dilution required to reach a countable range.

Figure 3 is a schematic of a drop plate of the experiment. The numbers indicate solitary colonies in each 10μL drop. The upper three rows in this example are representing the colonies counted at the zero time point (rows A-C; Figure 1) and the lower three rows (rows D-F, Figure 1) contain the number of colonies counted at 15 minutes within each droplet. The formula used was (# of colonies at t=x) multiplied by (cDF at t=x) multiplied by (pDF) then divided by (# of colonies at t=0) multiplied by (cDF at t=0) multiplied by (pDF) (Figure 3). In the following calculation, "t=x" would represent 15 minutes, the cDF of this column is 10^4, and the DF from plating is 10^2. In this example, the final percent of cell survival reported would be 66.7 percent.

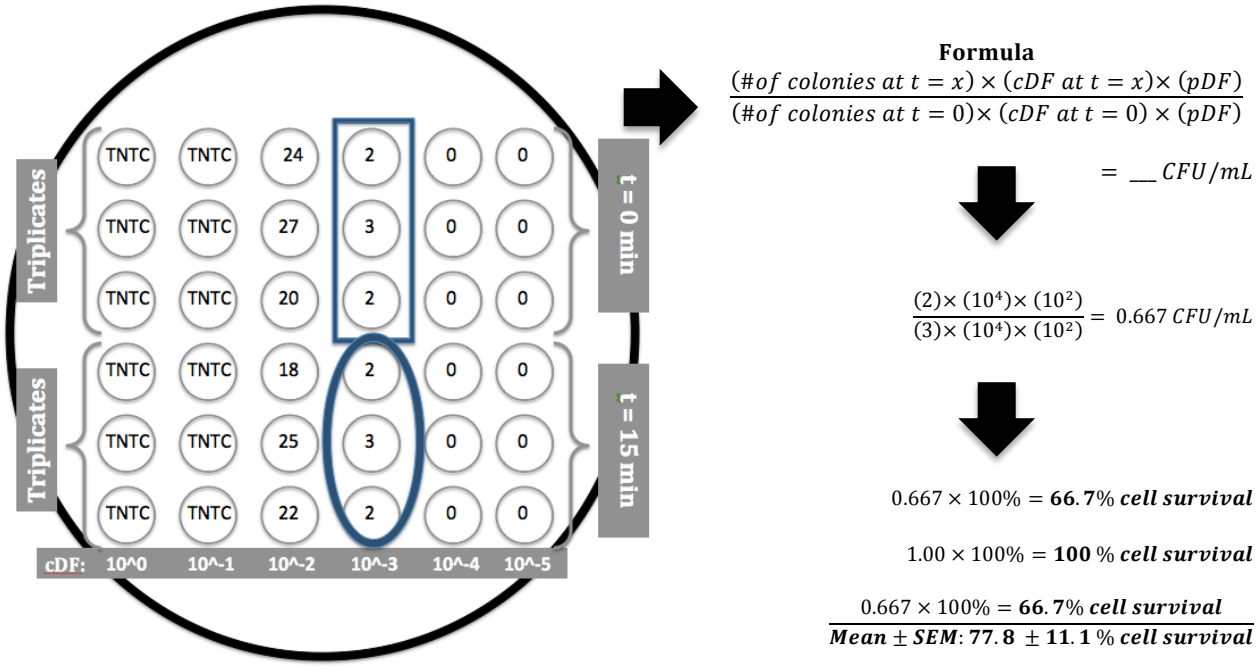

Figure 3: Summary of the method of calculation of percent cell survival. In this example, the upper three rows (triplicates) are the cell forming units (CFU) at t=0 minutes and the lower three rows (triplicates) are CFUs at t=15 minutes. The values within the box are averaged and plugged into the denominator of the formula to create a base value against which later time points are compared. This is also the cellular concentration at t=0 in cell forming units per milliliter (CFU/mL). The values within the oval are plugged into the numerator of the formula to determine the number of cells present at 15 minutes (CFU/mL). The number of colonies is multiplied by a cDF to account for the dilutions that were performed to reach a countable range. In this example, the cells were diluted three times in the 96-well plate (1:1,000) thus requiring the multiplication by 10^4 CFU.

2.6 Statistical Analysis

GraphPad Prism Version 6.07 was used for all statistical analysis. A two-way ANOVA and a subsequent Sidak's multiple-comparison test was used to evaluate the results. For all strains, three biological replicates with each three technical replicates were analyzed unless otherwise noted. The threshold for significance was $P< 0.05$.

3.0 RESULTS

3.1 Comparison of the Triple Mutant to the Wild Type

In the acid assay with pH 3.0, no cellular growth occurred at 15 minutes for either strain. In Figure 4 panel A, in exception to the other pHs, full bars at time-point zero have been added to show that the cells were viable before encountering acidic media. Time points 15, 60 and 90 minutes always represent percent survival in comparison to non-acid treated cells (t=0 min, representing 100 percent).

For pH 3.5, at 15 minutes, the wild type had a higher percentage of cell survival by 2.06 percent compared to the triple mutant which makes it statistically significant ($P<0.0001$). At 60 minutes, the wild type had a higher survival of 1.42×10^{-4} percent and the triple mutant had 1.4×10^{-6} % survival, but this difference was not statistically significant ($P>0.999$). At 90 minutes there was statistically no growth difference (no wild type growth while the mutant had 5.0×10^{-6} % survival; $P>0.999$) (Supplemental Data 1). Under these conditions, the triple mutant dies at a higher rate than the wild type, this trend being statistically significant at 15 minutes (Figure 4).

In pH 4.0, although not statistically significant, the wild type survives better than the triple mutant at all time points. At 15, 60, and 90 minutes, the wild type had a higher average percentage of survival by 11.5, 8.0, and 11.55 percent, respectively, compared to the triple mutant ($P=0.500$, $P=0.758$, $P=0.506$) (Supplemental Data 1).

For pH 4.5, again all differences failed to be statistically significant but a trend can be observed where the wild type survives slightly better than the triple mutant with 15 minutes being 79.8 percent and 69.1 percent, respectively (P=0.905). At 60 and 90 minutes, the wild type survived better by 24.0 and 11.4 percent respectively (P=0.4432, P=0.895) (Supplemental Data 1).

Summarized, at all four pH conditions and over a period of 90 minutes, the wild type survived better than the triple mutants. There was no growth at 15 minutes in pH 3.0 by either strain. There was statistical significance between the wild type and triple mutant at 15 minutes in pH 3.5. At pH 4.0 and 4.5 and at all three time points the trend was noted that the triple mutant had a greater reduced rate of culturability in acidic treatment as compared to the wild type.

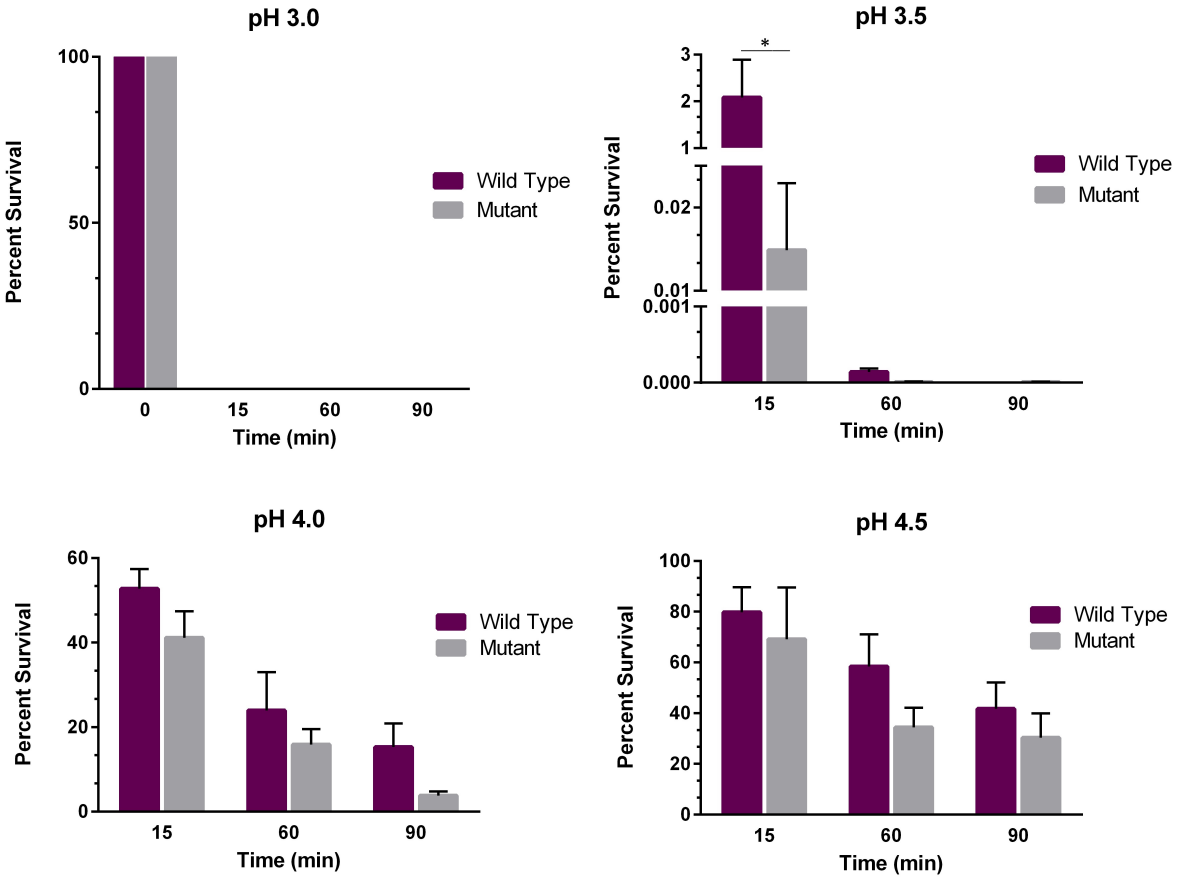

Figure 4: Comparison of the percent survival of the triple mutant to the wild type strain over time under varying acidic conditions. In all four experiments, percent survival of *Vibrio cholerae* O395N1 *ΔnhaP1ΔnhaP2ΔnhaP3* was compared to *Vibrio cholerae* O395N1. Both strains were incubated in pH 3.0, 3.5, 4.0, and 4.5 LBK broth without shaking at room temperature for 90 minutes. At 15, 60, and 90 minutes samples of the cell solution were diluted and plated using Chen's 6x6 drop plate method (13). Cellular concentration was calculated and compared to the zero time point for determination of percent survival over time.

3.2 Survival of the Complement Strains under pH 3.5 Exposure

When incubated in acidic broth at pH 3.5, at 15 minutes, as expected, the wild type strain containing the empty plasmid pBAD24 had the highest rate of survival with 9.99 percent surviving. The triple mutant having *nhaP3* complimented was the next highest survivor with 6.16 percent surviving. The triple mutant with *nhaP1* complimented followed with 1.62 percent survival and *nhaP2* complimented had 0.164 percent surviving (Supplemental Data 2).

A two-way ANOVA was performed on this data set. The only statistical significant difference in this experiment was with between both *nhaP1* and *nhaP2* compared to the wild type at 15 minutes (P=0.0003 and P<0.001 respectively). *nhaP3* complemented strain restored the wild type phenotype (P=0.1251) and it was significantly different from *nhaP2* at 15 minutes (P=0.0175) (Figure 5).

At 60 minutes, the wild type strain with the empty vector and the complimented *nhaP1, nhaP2,* or *nhaP3* had survival rates of 0.000263, 0.000972, 0.000341, or 0.000597 percent respectively. All *p*-values were greater than or equal to 0.999. At 90 minutes, the triple mutant with *nhaP1* was the only strain with survival of 0.000245 percent (Supplemental Data 2) with *p*-values being greater than 0.999.

Summarized, when all four strains were incubated in pH 3.5 LBK, at 15 minutes, the wild type strain with the empty vector had the highest survival as compared to complement P1, complement P2 and complement P3. Compliment P1 and complement P2 had greatly

reduced survival with complement P3 surviving at a rate comparable to the wild type strain. At 60 minutes, survival of all four strains was not statistically different, with minimal survival at 90 minutes (Figure 5).

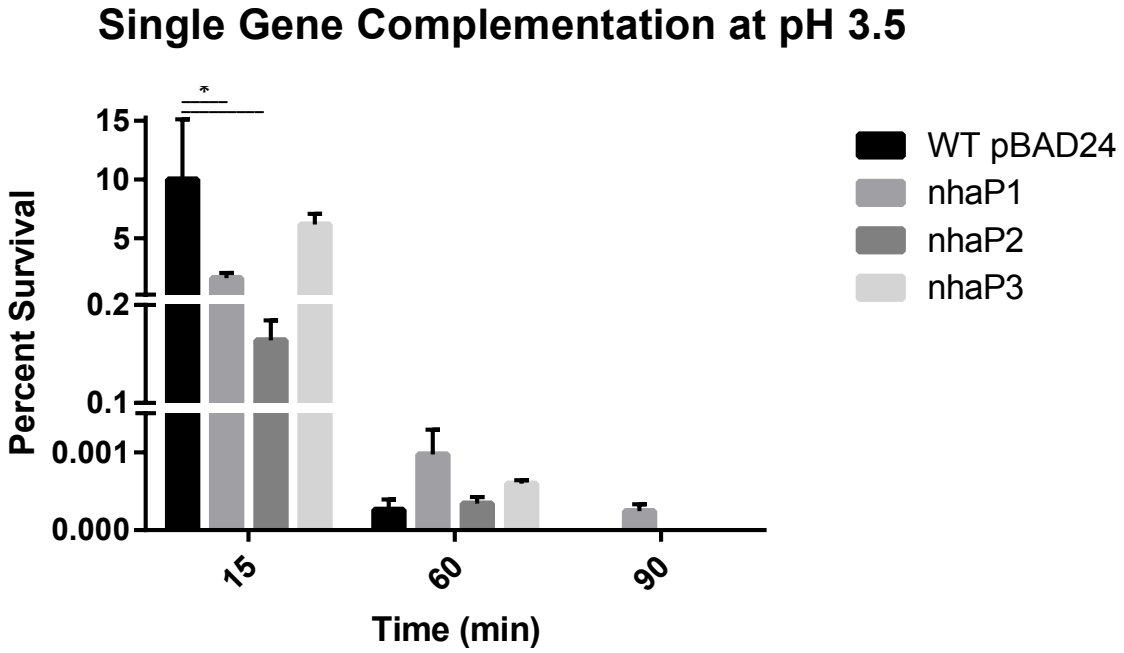

Figure 5: Comparison of the three complemented strains to the wild type strain containing an empty pBAD24 vector over time in pH 3.5 media. In this experiment, percent survival of Vc with an empty pBAD24, Complement P1, Complement P2 and Complement P3 were compared at pH 3.5 for 90 minutes. The strains were incubated for 90 minutes without shaking. Samples were taken at 15, 60, and 90 minutes, diluted and plated via the 6x6 drop plate method. Cellular concentration was calculated and compared to the zero time point.

4.0 DISCUSSION

In this study we wanted to simulate the acidic conditions of the human stomach in order to understand the role *nhaP1, nhaP2,* and *nhaP3* have in *Vibrio cholerae's* ability to survive this acidic environment. This was motivated by previous work describing *nhaP1* and *nhaP2* acting to function as a potassium and sodium ion antiporter in *Vibrio cholerae* under low pH conditions (7). Little is known about *nhaP3* currently. To assess survivability over time, the 6x6 drop plate method developed by Chen et al. was implemented (9). This method was useful as it reduced the amount of required materials and time spent compared to plating each dilution on a single plate. Additionally, this method allowed for clear visibility of culturability. With the concentration of cells decreasing so rapidly at each time point, it is difficult to estimate which dilution of cells to plate to get separate colonies. With the drop plate method, six dilutions and six replicates or several strains (6x6), respectively, can be plated out on the same plate. This greatly reduces the risk of missing the dilution entirely. Furthermore, this method involves serial dilutions in 96-well plates followed by applying diluted samples onto agar plates (13). In this study, each plate was able to contain two biological replicates including three technical replicates each. Using this technique, we found that triple deletion of *nhaP1, nhaP2* and *nhaP3* caused a slight decrease in cell survivability (culturability, respectively; see below) under extreme acidic conditions compared to the wild type. The difference was especially big at pH 3.5 with the wild type having a survival of 2.08 percent and the triple mutant having 0.0223 percent survival at 15 minutes ($P<0.0001$). This trend continued throughout the entire incubation period in experiments held in pH 4.0 and 4.5 (Figure 4). Visually, this trend is obvious but due to large standard error margins, statistical analysis determined these differences to not be

significant per a two-way ANOVA analysis. The data supported our hypothesis that *nhaP1, nhaP2* and *nhaP3* are beneficial for the organism under extremely acidic conditions but are not necessary for survival.

Following testing of the triple mutant, we tested the complement strain to confirm the phenotypes. This is done in many gene knockout studies as it confirms that the phenotype expressed when the genes are removed are indeed caused by the lack of the gene rather than an uncontrolled variable. In this study, single *nhaP* genes were complimented back into the triple mutant to see whether there is a difference in survival depending on which gene is present in *Vibrio cholerae.* We wanted to see which antiporter gene –*nhaP1, nhaP2* or *nhaP3*– specifically had a bigger role in the survival of the organism. To do this, the triple mutant strain was separately transformed with each individual gene on a plasmid (pBAD24) and tested at pH 3.5. This pH was chosen because in the previous experiment, this provided the largest difference in phenotype between the wild type and the triple mutant (Figure 4).

pBAD24 is a commonly used cloning expression vector. Its use is favorable as it contains the arabinose (*araBAD*) inducible promoter and an ampicillin resistance cassette. The addition of arabinose to the cell media turns the promoter on and therefore increases the expression of the gene cloned into this plasmid and thus our antiporter of interest. The addition of ampicillin to the experimental broth forces the organism to maintain the plasmid with the ampicillin resistance cassette and our gene of interest (15).

When individual antiporter genes complimented back in, complimented *nhaP1* and complimented *nhaP2* showed reduced survival compared to the wild type (P=0.0003 and P<0.001, respectively) while complementation of *nhaP3* restored the wild type phenotype (9.99% compared to 6.16% respectively, P=0.125) (Figure 5). Later time points (t=60 and t=90) were not different (P>0.9999). Therefore we conclude that they all act similarly at later times with restoration of *nhaP3* being most advantageous at 15 minutes.

The drop plate method used in this study has major advantages and some disadvantages. As described earlier, it saves a great deal of time, cost and materials. However, it is a very specific technique that requires training and great dexterity, allowing, for many potential opportunities for variability in the results, which might explain our large error bars (Figure 4). When transferring cell culture during dilutions, it is difficult to insure the correct amount of fluid is transferred each time. Additionally, the plates must be dried adequately or the droplets will run ruining the entire plate. Another factor to consider is the possible presence of viable but non-culturable (VBNC) cells. This is a state bacteria may enter where they lose culturability on routine agar due to exposure to stress. With this considered, there is the potential for our drop plates to not be fully representative of the concentration of live cells in our experiments. Flow cytometry using live/dead cell staining is typically the best method to insure all viable cells are counted to achieve the most accurate results, however those instruments are expensive and not always available in lab settings and thus it was not feasible in this study (16).

Potential future research could entail many of the other antiporters in *Vibrio cholerae* to see if one has a larger effect or whether it's a combination of all of the antiporters that allows for survival. Lastly, future studies to further confirm the role of *nhaP3* by assaying single and double mutants are recommended.

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6.0 SUPPLEMENTAL DATA

Supplemental Data 1: Calculated average percentage of survival of the wild type and triple mutant strains under the four different pH conditions. The wild type and triple mutant strains had been incubated in acidic LBK broth for 90 minutes at room temperature without shaking. At 0, 15, 60, and 90 minutes, samples were taken, washed, diluted, and drop plated onto an LBK agar plate in a 6x6 droplet format. The following percentages of cell survival were calculated per the method described in Figure 3. Bolded values indicate statistical significance ($P<0.05$).

Average Percentage of Survival in Comparison to Untreated								
Time	Wild Type				Triple Mutant			
(min)	pH 3.0	pH 3.5	pH 4.0	pH 4.5	pH 3.0	pH 3.5	pH 4.0	pH 4.5
15	0	**2.08**	52.7	79.8	0	**0.0223**	41.2	69.1
60	0	1.42×10^{-4}	23.9	58.4	0	1.4×10^{-6}	15.9	34.4
90	0	0	15.3	41.7	0	5.0×10^{-6}	3.75	30.3

Supplemental Data 2: Average percentage of survival of the wild type and triple mutant strains with single genes complimented at pH 3.5. Complemented strains having individual antiporter genes restored (*nhaP1, nhaP2 and nhaP3*) as well as the wild type containing empty vector were treated in acidic LBK for 90 minutes at room temperature without shaking. At 0, 15, 60, and 90 minutes, samples were taken, diluted and plated onto an LBK agar plate with ampicillin in a 6x6 droplet format. The following percentage of survival was calculated per the method described in Figure 3.

Average Percentage of Survival at pH 3.5				
Time (min)	Vc pBAD24	*nhaP1*	*nhaP2*	*nhaP3*
15	9.99	1.62	0.164	6.16
60	2.63×10^{-4}	9.72×10^{-4}	3.41×10^{-4}	5.97×10^{-4}
90	0	2.45×10^{-4}	0	0

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