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Identification of Genes Conferring Acid Resistance in Vibrio parahaemolyticus

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Identification of Genes Conferring Acid Resistance in *Vibrio parahaemolyticus*

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APPROVAL PAGE

Honors Thesis

Investigation into Possible Genes Conferring Acid Resistance in *Vibrio Parahaemolyticus*

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Abstract

Vibrio parahaemolyticus has the capability to be acid resistant due to known and unknown mechanisms. The goal of this project was to determine possible genes involved in conferring acid resistance of this bacterium. The vp1277 gene (designated as HA) and its downstream gene vp1999 were investigated for their role in acid resistance. Another potential gene that could contribute to acid resistance is vp0820 because of its similarity to the ToxR gene in *Vibrio cholera.* All three of these knockout strains and the wild type were grown in neutral LB and then tested with acidic LB's of pH 4.5, 5, and 5.5. Despite its similarities to cholera, the vp0820 knockout did not appear to lead to decreased bacterial growth when treated with acid. The HA and vp1999 knockout strains grew less when treated with acid especially at pH 4.5. Once vp1999 was identified as possibly being involved it was ligated into the pmmB207 plasmid and conjugated into sm10 *E. coli* to determine if this addition into the genome could be successfully completed. The vp1999 gene was determined to have been conjugated effectively by PCR and gel electrophoresis. Also, prior to this PCR amplification was run to amplify the vp1999 gene in the wild type and vp1999 knockouts. The wild type band appeared at approximately the length of the vp1999 gene and the gene was unable to be amplified in the knockout confirming the knockout and that the primers designed work. Overall, both vp1277 and vp1999 may be involved in acid resistance of *V. parahaemolyticus.*

Key words: *Vibrio parahaemolyticus,* acid resistance, vp1999, vp0820, HA

Introduction

Antibiotic resistance is becoming an increasing problem in our world. In 2013 the CDC reported that every year in the US a minimum of 2 million people contracted antibiotic resistant bacteria and of these people, 23,000 die.[1] When antibiotics are taken properly the body eliminates the few antibiotic resistant strains that develop, but if not, these strains will grow and replicate into a larger resistant colony. The antibiotic resistant bacteria can now be spread to other people. On the contrary, antibiotics are also sometimes taken when they are not needed and this overuse also has led to resistance. In fact, 30% of prescribed antibiotics are not needed by patients.[1]

Antibiotic resistance is especially a problem in Gram negative bacteria because they possess β-lactamases that break the rings of beta lactam antibiotics; the most common class of antibiotics used to treat these bacteria. β-lactam antibiotics disrupt the synthesis of bacterial cells walls and when they can no longer do this they are ineffective.[2] Carbapenem resistant Enterobacteriaceae pose one of the biggest threats to humans in regards to antibiotic resistant bacteria because they are resistant to all β-lactam antibiotics.[2] It is very possible that more types of bacteria will become resistant to these antibiotics in the coming years.

Vibrio parahaemolyticus is a gram negative enteric bacterium that can infect humans if raw seafood and shellfish are consumed. The seaborne pathogen causes gastrointestinal infection by growing in the small intestine and disrupting the tight junctions of epithelial cells.[3] This was shown by Ritchie *et al.* in infant rabbits.[3] These effects were specifically observed in the lower portion of the small intestine and the disruption led to leaks, inflammation, and inflammation, and attachment and effacement lesions.[3] The bacteria also employs Type III secretion systems that secrete effectors into host cells that are necessary to cause infection.[3]

It is the most prevalent cause of seaborne diarrheal disease in the world.[4] *V. parahaemolyticus* is especially prevalent in third world countries specifically Asia.[3] However, there have also been outbreaks in US including in May-August 2013 when there were 104 cases in 13 states. Prior to 2012 the CDC was unlikely to see cases of vibrio coming from the Atlantic Ocean. In a study testing the antibiotic resistance of *V. parahaemolyticus* in isolates from 2000-2017 all of the samples were resistant to two antibiotics and 86% were classified as multidrug resistant.[5] These statistics in combination are a cause for concern of who vibrio will affect in the future.

In addition to antibiotic resistance, bacteria can also be acid resistant. Understanding the mechanisms of acid resistance in enteric bacteria is especially important because these bacteria travel through the gastrointestinal tract to infect their host exposing them to low pHs. Shigella and E.coli can survive in pH=2.5 for two hours which qualifies them as acid resistant according to Gorden *et al.*[6] In these bacteria it was growth phase that affected their acid resistance.[6] Shigella is not very acid resistant until it is in the stationary phase of growth. Shigella has also been found to have a low infectious dose which may be due to their entrance into the stationary phase right before they leave the colon.[6]

Weizhe *et al.* provides another explanation for acid resistance in enteric bacteria by discovering a chaperone dependent mechanism.[7] The possible chaperones involved are HdeA and HdeB and acid resistance is caused by a conformational change in these chaperones. Normally, when proteins are exposed to acid they misfold and aggregate, but these chaperones can help prevent this and then once a more neutral pH is restored they can help refold proteins.[7] In both of the cases described the presence of acid causes some change to then make the bacteria resistant to acid.

Although Vibrio bacteria are not as acid resistant as some of the other enteric bacteria acid resistance still may play a role in their ability to infect the small intestine.[8] One such pathway of acid resistance has been shown in *V. parahaemolyticus.* Tanaka *et al.*[8] demonstrated the lysine decarboxylase enzyme to facilitate acid resistance. This is similar to the glutamate decarboxylase system, but does not permit survival in quite as acidic environments.[7] The glutamate decarboxylase system allows bacteria to survive in pHs as low as 2. *Vibrio cholera* and *V. parahaemolyticus* both do not have this system.[7] In this paper the strains of vibrio were grown up at pH=7.5 and then inoculated into pH=4.[8] They were immediately removed and grown in pH=5.5.[8] The acid treated bacteria were able to grow more than the strains that were not previously exposed to acid.[8] This led to the conclusion that exposure to low pH may induce the bacteria to then be resistant to lower pHs than usual.[8] However, there may be other mechanisms and genes that contribute to acid resistance in these bacteria.

Hypothesis/Specific Aims

The goal of this project was to determine other genes responsible for acid resistance in *V. parahaemolyticus.* To do this, the bacteria were grown when three separate genes were knocked out. These genes include vp1999, vp1277, and vp0820. All of these genes are located on chromosome 1 of *V. parahaemolyticus*. Figure 1 describes the basic experimental design that was used to evaluate growth before and after acid treatment. The pH range of 4.5-5.5 was chosen because pH 4.5 is thought to be the lethal pH.[8] In the experiment pH 4 was also chosen to be tested to verify pH 4.5 as the lethal pH. The bacteria can be grown with carbenicillin since *V. parahaemolyticus* is resistant to this antibiotic.

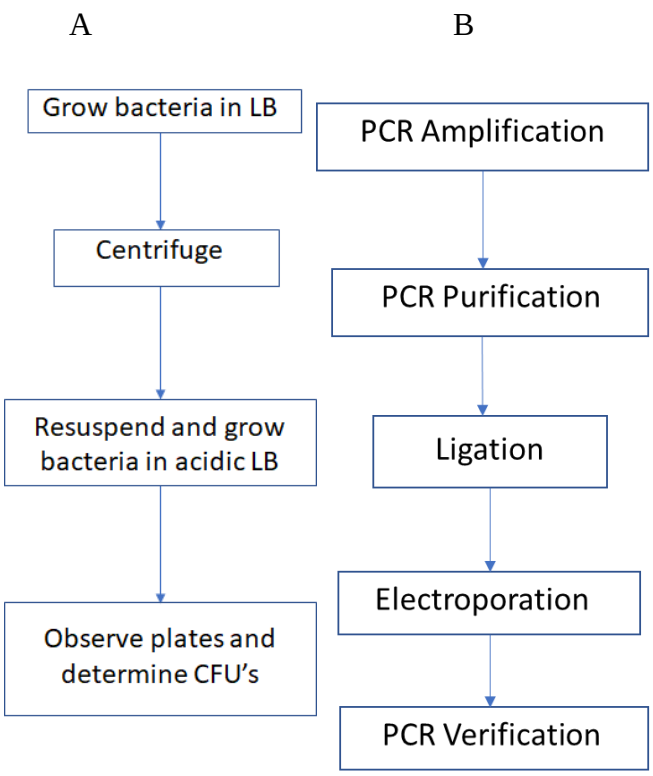


Figure 1. Basic experimental designs. A. Experiments performed on wild type, Δvp1999, ΔHA, and Δ0820. B. Experiment done on Δvp1999 and sm10 *E. coli* conjugated with pmmB207-1999.

In Dr. Zhou's lab it has been found that the vp1276 and vp1277 genes make up a histidine kinase/response regulator pair that can activate β-lactamases when the bacterium senses the use of β-lactam antibiotics.[4] The histidine kinase (VbrK) is phosphorylated when β-lactams are sensed.[4] It was also discovered that the HA gene plays a role in acid resistance. Furthermore, the HA strain regulates the vp1999 gene and due to its regulatory role in acid resistance it is possible that vp1999 also facilitates acid resistance. Vp1999 is called the hypothetical protein on NCBI[9] so researchers are not sure on its exact function. Identical protein groups to it are efflux RND transporter periplasmic adaptor subunits so the vp1999 proteins may help to efflux protons in acidic conditions.

In *Vibrio cholera*, the ToxR gene can sense pH and contributes to acid resistance.[10] A similar ToxR gene (also called 0820) is present in *V. parahaemolyticus*. *V. cholera* and *V.*

parahaemolyticus are of the same genus so this gene may have a similar function in both types of bacteria. The vp-ToxRS was found to be 50-60% similar to vc-ToxRS through sequence analysis.[11] ToxR is needed for organic ATR (acid tolerance resistance) in *V. cholera* and OmpU is a porin that may be involved in this resistance.[10] It is also important to note that *V. cholera* was found to be more virulent when grown in a lower pH than when it was grown in a neutral pH. These mechanisms may also be true for *V. parahaemolyticus.* In addition, vp0820 has been classified as an ABC transporter substrate-binding protein by NCBI[9]. This function could allow it to facilitate the export of protons when presented with acidic conditions.

Overall, the vp1999 gene should be amplified to approximately 1134 base pairs[12] when using the designed primers while the knockout should not. The vp1999, HA and vp0820 knockout strains are expected to grow less than the wild type strain when treated with LB of pH 4.5. Finally, upon electroporation of the sm10 *E. coli* and plasmid containing the vp1999 gene this bacteria strain is expected to be shorter when amplified than the wild type strain.

Materials and Methods

Strains and Plasmids The wild type *Vibrio parahaemolyticus* RIMD2210633 from the Zhou laboratory at UConn was used throughout the experiments. The knockout strains were created in this lab by conjugation with a pdm4 plasmid prior to these experiments.

Strain	Description	Source
V. Parahaemolyticus RIMD 2210633	Clinical isolate	Zhou
Sm10 E. coli	contains a chromosomally integrated RP4 plasmid for conjugation	Zhou
pmmB207-1999	Vp1999 sequence cloned into pmmB207	This work
V. Parahaemolyticus RIMD 2210633 Δvp1999	Vp1999 knockout	Zhou
V. Parahaemolyticus RIMD 2210633 ΔHA	HA (vp1277) knockout	Zhou
V. Parahaemolyticus RIMD 2210633 Δ0820	0820 (toxR) knockout	Zhou

Table 1. A description of the strains and plasmids used and their sources.

Primer Design Development All of the primers needed for the experiments were available in the Zhou laboratory except one for Vp1999 so this primer was designed and ordered from Biosupply at UConn. The primer was designed using the Oligonucleotide Calculator from Northwestern (Figure 2). Approximately 20 nucleotides in the beginning and end of the sequence were chosen to create the primers. The list of primers used in this study can be found in Table 2.

VP1999

atgacgaaacgttggcttgtctccgctgtgagcattgcgctgctcggcggtggtacgtat
ttctatctccaatctgccgctcaaccagaattgctgcctacattgattgtggaaaaaggc
acgattgaaaaacaagctgtggctgttggtaaaattgtccctgcacactctgtgtctatt
aagtcacaaatcgacggcatcgtcggtgaaatttacgcgaaagttggtgaaaaggtgaag
caaggccagccgctgatcaaagtgcgtccaaacccaaccccccaagcattaacggatgca
tcggcagaactcatgcgtagcgaagccgatctggaatccgccaaacaaaaactgtccaac
ttagaaagcttggtcaagcaggacattattccaagtaactacgacgaatacgtaagtgcg
cgctcggcagtcaaatctgcgcaagcggacgtattgcaaaagcgccagaatctcgaactc
atccgcagtggtgaggcgtctatcggtgatgcgcgtttaacgtcgacgatctacgcgcca
attgatggcacggtactcaaccagaaagttgaggtcggagagccgatcatctcgactcaa
tcaagccaagccgcgactgaaatgatgtcattggccgacatgaacagtctgatttttaaa
ggtagcgtcagtgagcatgatgctgcgcagttgtctccgggaatgcccgttatgttgacg
gttgcgccttatcctgatgtggcgatttctggtgtgctaaccaaagtggcgattcaatct
gagaaccttaactcaccagagggcaatgcttctgcaaaaagttttgataacggttttgag
gttgaagtgggagaactcaagatcccacaagatgtgttgcttcgttctggtttttcatcg
acggctcaaatcattctgaagaagtctgaaaacgtgctcactctaccggagcgcgctctg
caatttgatggtgacgcaccgaatgttctgatccccgacagctcagaacaagggtttcat
aaacaacctgtgaaactcggtctgtcagatggcatcaatgtcgaagtgcttgacggcgtg
gagcttgatgaagaggttatcgacaacagcatgatgggagcggcgcatggttaa

*yellow highlighted sequence= sequences used to create primers

Forward Primer

GGTACCCGGGGATCCTCTAGATGTAAGGAGGTAGGATAATAATGACGAAACGTTGGCTTGTC

Vp1999_pmmB_RBS_F

Reverse Primer

TCCGCCAAAACAGCCAAGCTTTAATGGTGGTGGTGATGATGACCATGCGCCGCTCCC

Vp1999_pmmB_His_R

Blue= plasmid pmmB

Purple= RBS

Green= designed primer (complementary to parental strand)

Black= stop codon

Pink= his tag

Figure 2. VP1999 nucleotide sequence from Kyoto Encyclopedia of Genes and Genomes with outlined primer creation using the Oligonucleotide Properties Calculator.[13]

Primer Name	Sequence 5'→3'
Vp1999_pmmB_RBS_F	GGTACCCGGGGATCCTCTAGATGTAAGGAGGTAG GATAATAATGACGAAACGTTGGCTTGTC
Vp1999_pmmB_His_R	TCCGCCAAAACAGCCAAGCTTTAATGGTGGTGGTG ATGATGACCATGCGCCGCTCCC
pmmB207_F	AGGCTCGAGCAGACTGGAGGTGGCAAC
pmmB207_R	AGGAAGCTTTGTTTCCTGTGTGAAATTGTTATC

Table 2. Primers Used in this Experiment

PCR Verification PCR verification was performed to prove that the knockout of vp1999 being used for the rest of the experiments was a knockout. A sample of liquid wild type DNA and a colony of the knockout both had the vp1999 gene amplified and gel electrophoresis was used to view the respective banding patterns. HA and 0820 were previously tested and these samples were inherited, so PCR verification was not repeated.

Bacteria Plating Bacteria were grown in Lysogeny broth (LB) containing **3%** agar with 0.5% sodium chloride. ~2mL of LB was added to each tube along with 1ul carbenicllin per 1 mL LB in a rotating incubator. The bacterial strains were plated on agar containing carbenicillin and 0.5mL of 50% glycerol/1 mL of culture were added to the remaining samples which were then saved in the freezer. The bacteria from 9/14/17 were plated from the freezer on carbenicillin plates and placed in an 37℃ incubator for approximately 24 hours on 1/31/18 and these were used for the remainder of the experiments.

Acidic LB Creation HCl was added dropwise to LB and a pH meter was used to measure pH. LB's of pH 4.5, 5, and 5.5 were created. In order to sterilize these solutions, they were passed through a filter into new tubes.

Acid Treatment Bacterial colonies from plates were grown in Lysogeny broth (LB) tubes containing 1ul carbenicllin per 1 mL LB in a rotating incubator for ~3 hours. After 3 hours the growth was checked and if the bacteria did not grow enough then the samples were left in the incubator and monitored for longer. HA tends to grow slower than the other cultures so more colonies were typically added (ie. 2-3). 1 mL of each bacteria culture was transferred to a centrifuge tube and spun down. The supernatant was poured off and the pellets were resuspended in LB liquids of varying pH. Titrations of WT, 1999, HA, and 0820 were performed to 6 dilutions before the acid treatment. The dilutions included 1/10, 1/100, 1/1000, 1/10000,

1/100000, and 1/1000000. 5ul of each titration was plated onto an LB plate for each of the gene knockouts and wild type. This plate was then incubated overnight at 37 C or at room temperature for 3 days. The same procedure was repeated for the samples in acid and the plates before and after acid treatment were then compared.

PCR Amplification and Purification The vp1999 gene was amplified in wild type *V. parahaemolyticus* DNA through PCR and the amplification was verified through gel electrophoresis. Then, the PCR product was purified by adding 600µL of binding buffer to the sample and centrifuging for 1 minute. Next, 750 µL of wash buffer was added and centrifuged two times through. Finally, the filter was placed in a new tube and 30 µL of water was added and the tube was centrifuged one final time. The concentration of the DNA was measured on the nanodrop.

Ligation and Diolysis A pre-made ligation mixture was mixed with the pmmB207 plasmid with HindIII and XhoI and the purified wild type DNA with the amplified vp1999 sequence. This was incubated at 50°C for 90 minutes in the thermocycler. The product was diolyzed in deionized water for 30 minutes to remove any ions from the sample and placed in a new tube.

Electroporation and Insertion of Gene of Interest into Plasmid Sm10 *E. coli* was used as a competent cell and electroporated with the ligation mixture. After electroporation, 1mL of LB was immediately added and mixed with the electroporated cells. The tube was then incubated at 37C for 1 hour. The cells were centrifuged and then left in 100-200ul of LB. To verify that the gene of interest (vp1999) was inserted into the sm10 *E. coli*, this solution was then plated on an LB plate containing chloramphenicol because the pmmB207 plasmid is resistant to this antibiotic**.** To verify this further, PCR was used with the pmmb207 forward and reverse primers.

Results

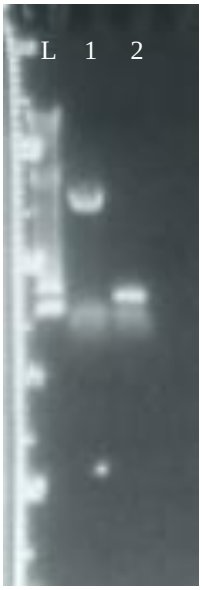
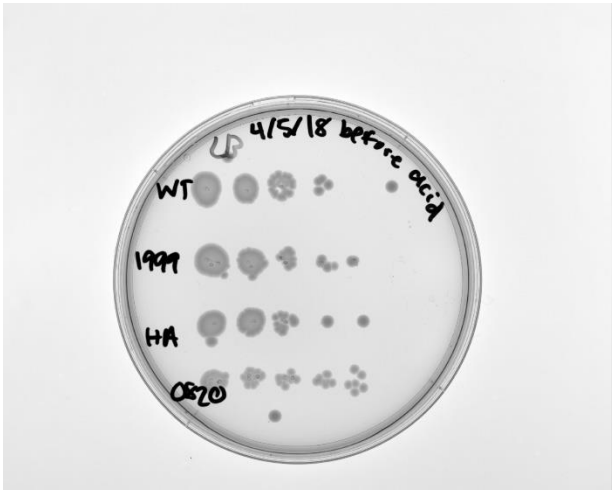


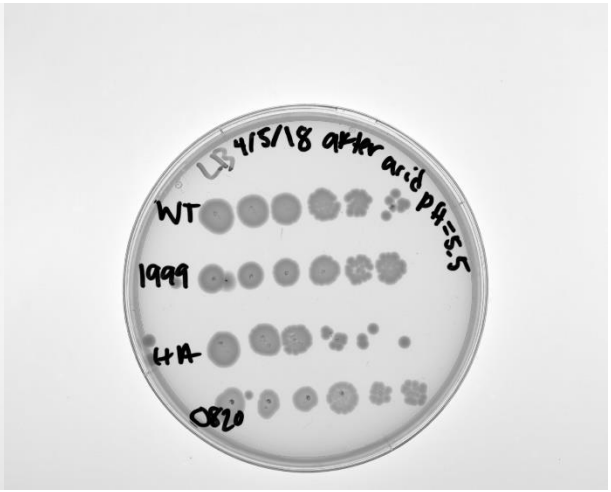
Figure 3. Electrophoresis gel ran at 200V for 15 minutes using the Invitrogen 1Kb Plus DNA ladder as a reference. The first lane (L) is the ladder, 1 is the wild type and 2 is the vp1999 knockout. The vp1999 forward and reverse primers described in Figure 1 were used.

The vp1999 gene is shown to be knocked out because the band appears much lower in the PCR electrophoresis results. The vp1999 gene is 1134 base pairs and in the wild type sample the band is shown to be approximately this length using the primers designed for this experiment. Then, the acid treatments show approximately equal colony formation for all strains at pH of 5.5. At a pH of 4.5 these two knockouts grew less than the wild type and also grew less than they did before acid treatment. There are less colonies at the lower concentrations for the HA and Δvp1999 strains and at the final titration there are 2 colonies present. At a pH of 4 none of the strains of bacteria grew including the wild type control. At a pH of 5.5 all of the strains were able to grow further once treated with acid for an hour. The results indicated that vp1277 and vp1999 are the two genes that confer resistance to acid in *Vibrio parahaemolyticus.*

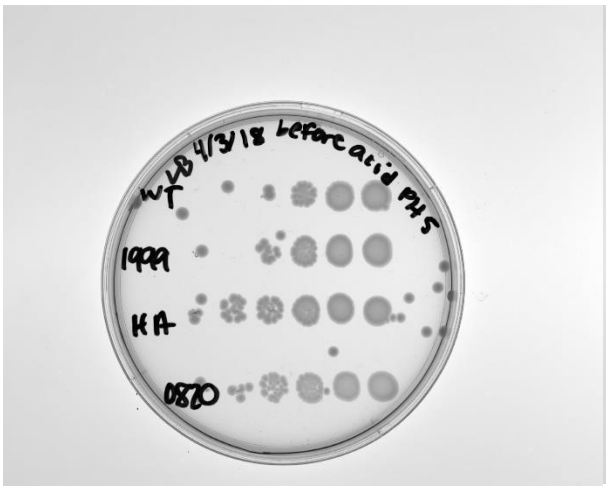
A


LB 4/5/18 before acid
WT
1999
HA
0820

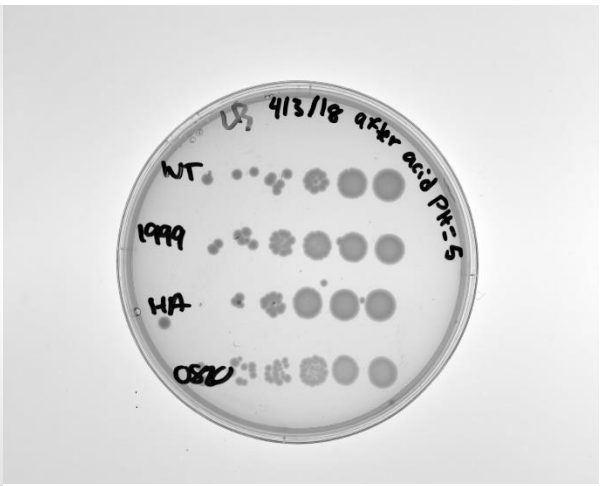
B


LB 4/5/18 after acid pH=5.5
WT
1999
HA
0820

C


LB 4/3/18 before acid PH 5
WT
1999
HA
0820

D


LB 4/3/18 after acid PH=5
WT
1999
HA
0820

E F

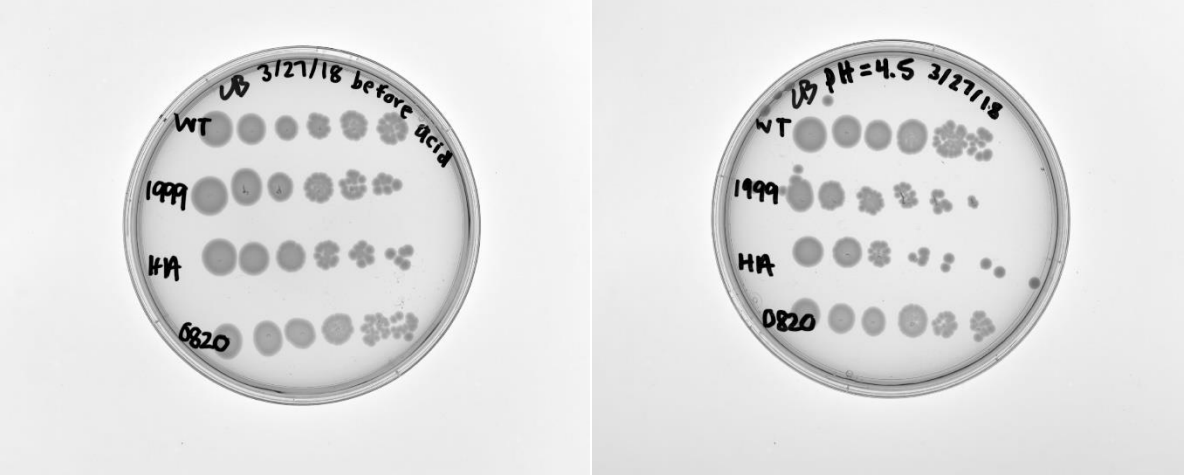


Figure 4. A,C,E. Wild type control, Δvp1999, ΔHA, and Δ0820 after being grown in LB with carb for three hours at 37C. B, D, F Wild type control, Δvp1999, ΔHA, and Δ0820 after being grown in acidic LB for one hour at 37C.

pH=4.5						
Strain	CFUs					
	1	2	3	4	5	6
Wild type	Tmtc	Tmtc	Tmtc	Tmtc	Tmtc	Tmtc, 12
Δvp1999	Tmtc	Tmtc	Tmtc	Tmtc, 12	Tmtc, 4	6, 2
ΔHA	Tmtc	Tmtc	Tmtc	Tmtc, 4	7, 2	5, 2
Δ0820	Tmtc	Tmtc	Tmtc	Tmtc	Tmtc	Tmtc, 9

Table 3. CFUs before and after treated with LB pH=4.5. The number of colonies before treatment is listed and after the comma the number of colonies after treatment was listed. If there were too many colonies to count accurately, tmtc was written.

Figure 5. Electrophoresis gel ran at 200V for 15 minutes using the Invitrogen 1Kb Plus DNA ladder as a reference. The wild type vp1999 segment of the genome was amplified through PCR using the primers shown in Figure 2. The PCR product was purified and the concentration was measured to be 54.5 ng/µL on the nanodrop.

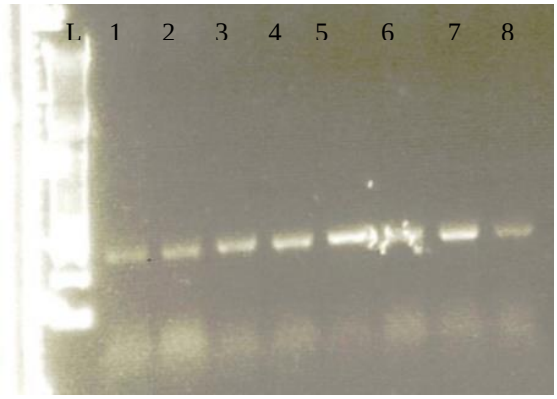


Figure 6. Electrophoresis gel ran at 200V for 15 minutes using the Invitrogen 1Kb Plus DNA ladder as a reference. Eight colonies were chosen from the plate of the sm10 conjugated to the pmmB207-vp1999 plasmid. They were amplified through PCR using the pmmB207 forward and reverse primers in Table 2.

The wild type band seen in Figure 5 is longer than the bands seen in Figure 6 because the plasmid primers amplify just the plasmid and not the plasmid with the gene inserted. The colonies that grew on the cm plate contain the plasmid because only the bacteria that took up the

plasmid would be resistant to this antibiotic. The PCR results further confirm the appropriate conjugation.

Discussion

The vp1999 primer development was successful which was shown by the results of the amplification of this gene in the wild type *V. parahaemolyticus.* The vp1999 knockout strain was confirmed to be a knockout because this gene was unable to be amplified in this strain. The conjugation of the ligated pmmB207 plasmid with the wild type amplified vp1999 DNA and the sm10 *E. coli* competent cell also proved to be successful. This was shown through a much shorter PCR product than the wild type. The plasmid was confirmed to be present in all eight of the colonies that PCR was run on.

This thesis project shows that vp1999 is possibly involved in the acid resistance mechanism of *V. parahaemolyticus.* The plate assays indicate that the HA and vp1999 knockouts grow less than the wild type when treated with pH 4.5 LB. At a pH of 4.5 these two knockouts and also grew less than they did before acid treatment. However, despite its role in cholera, the toxR gene does not appear to induce acid resistance in *V. parahaemolyticus* based on these experiments. When treated with the LB pH 4.5 the 0820-knockout strain grew about the same amount as the wild type strain and did not grow less than in the normal LB conditions.

Despite being of the same family of bacteria *V. parahaemolyticus* and *V. cholera* have been shown to have many differences such as in the way they cause infection. *V. parahaemolyticus* causes inflammatory diarrhea while *V. cholera* causes non-inflammatory diarrhea.[14] These differences could mean that their genes, despite similarity to have different functions. The Vc-ToxR and Vp-ToxR genes have been shown to have a similar enterotoxin function[11], but it has not been shown that they both are involved in acid resistance. Further

experimentation would be necessary to determine if vp0820 has any involvement in acid resistance. The vp1999 gene appears to be more promising and if further research is done confirming vp1999's and/or other genes involvement in acid resistance inhibiting this gene in conjunction with antibiotics could prove to be a beneficial treatment of this bacteria.

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