EXPLORING THE PHYSIOLOGICAL ROLE OF *VIBRIO FISCHERI* PEPN

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Sally L. Cello

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COMMITTEE MEMBERSHIP

TITLE:	Exploring the physiological role of *Vibrio fischeri* PepN
AUTHOR:	Sally L. Cello
DATE SUBMITTED:	April 2015

COMMITTEE CHAIR:	Pat Fidopiastis, Ph.D. Associate Professor of Biological Sciences
COMMITTEE MEMBER:	Chris Kitts, Ph.D. Department Chair of Biological Sciences Professor of Biological Sciences
COMMITTEE MEMBER:	Candace Winstead, Ph.D. Associate Professor of Biological Sciences

ABSTRACT

Exploring the physiological role of *Vibrio fischeri* PepN

Sally L. Cello

The primary contributor to *Vibrio fischeri* aminopeptidase activity is aminopeptidase N, PepN. Colonization assays revealed the *pepN* mutant strain to be deficient at forming dense aggregates and populating the host's light organ compared to wildtype within the first 12 hours of colonization; however the mutant competed normally at 24 hours. To address the role of PepN in colonization initiation and establish additional phenotypes for the *pepN* mutant strain, stress response and other physiological assays were employed. Marked differences were found between *pepN* mutant and wildtype strain in response to salinity, acidity, and antibiotic tolerance. This study has provided a foundation for future work on identifying a putative role for *V. fischeri* PepN in regulating stress response.

ACKNOWLEDGMENTS

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I. Introduction

Vibrio fischeri, a marine bioluminescent bacterium, is the sole symbiont of *Euprymna scolopes*, the Hawaiian bobtail squid[1]. This highly specific relationship culminates with the colonization of the squid's light organ by *V. fischeri*. To ensure the success of its symbiont, *E. scolopes* implements a series of physiological and mechanical barriers that inhibit the establishment of nonsymbionts during colonization. Initially, ciliated external appendages capture planktonic bacteria in peptide-rich mucus matrices promoting biofilm formation in tight aggregates. Some bacteria then regain motility and disperse from the matrices to access the pores of the ducts. The ducts, filled with nitric oxide, promote oxidative stress, selecting against ill adapted bacteria. Following the ducts are the deep crypts which are protected by hypochlorous acid produced by halide peroxidases in host tissue, in addition to being lined with hemocytes, innate immune cells that recognize microbe-associated molecular patterns, such as lipopolysaccharide [2]. In the deep crypts *V. fischeri* then successfully outcompetes remaining nonsymbionts to form a monoculture[1]. The nutrient rich environment promotes density-dependent luminescence, which in turn appears to be utilized by *E. scolopes* in an anti-predatory behavior dubbed "counterillumination" [1,3].

This distinct symbiosis has been the focus of many studies fixated on identifying factors fundamental to the ability of *V. fischeri* to establish colonization. A study by Fidopiastis et al. (2012) sought to investigate the role of proteases in the colonization process. Aminopeptidase N, PepN, was identified as the protease that produces the majority of cell-associated aminopeptidase activity. *V. fischeri* PepN displays characteristic, conserved peptide motifs HExxH and GAMEN, and shares 75% identity with *E. coli* PepN in the first 400 amino acids [4,5]. *V. fischeri* PepN is a broad spectrum metalloprotease with a preference for basic and hydrophobic amino acids at the N-terminal and utilizes Zn^{2+} as a cofactor, consistent with PepN homologs in other bacteria [4,6]. A PepN mutant strain was constructed and used in squid colonization assays. The PepN deficient

strain was outcompeted during the first 12 hours of colonization by wildtype and displayed smaller population densities, but achieved wildtype colonization levels at 24 hours, and thus competed evenly. Notably, the PepN deficient strain formed lower cell density aggregates lending a reason for the initial colonization delay [5].

Aminopeptidase N has been identified in many organisms including fungi, mammals, archaea, and various genera of bacteria [5,7]. Hypothesized roles for PepN include functions in intracellular protein turnover, exogenous peptide metabolism, abnormal protein elimination, and as a regulator in response to external stressors [4,6,7]. For example, *E. coli pepN* expression was 4 times greater under phosphate limiting conditions and anaerobiosis [6]. Moreover, *E. coli* strains lacking PepN outgrew the wild type in the presence of sodium salicylate, an external stress inducer [7]. These results may suggest that PepN is a negative regulator of stress response pathways.

This study aimed to define the physiological role of PepN for *V. fischeri* and its symbiosis. Specifically, we sought to define this role by analyzing expression of *pepN* and utilizing a PepN deficient strain in physiological assays and phenotype microarrays designed to address its role in stress response and aggregate formation. An array of conditions known to effect *pepN* expression in other bacteria, as well as PepN function, including stimuli novel to the host, *E. scolopes*, were tested. We expected to find supporting evidence that illuminated the putative role of PepN in colonization initiation, and that *V. fischeri* PepN would have a similar role to *E. coli* PepN in stress-inducing environments.

II. Materials & Methods

i. Strains and Growth Conditions

Vibrio fischeri wild type (ES114), a *pepN* mutant ($PepN^-$; Fidopiastis et al., 2012), and the *pepN* complemented strain ($PepN^+$; Fidopiastis et al., 2012) were utilized in all experiments. In addition, the following strains were utilized in sodium salicylate MIC assays: ES114+ vector only (pEVS105; Dunn et al., 2006), ES114 with PepN expressed *in trans* (pDDG5, Fidopiastis et al., 2012), and $PepN^-$ + vector only (pEVS105). Strains from freezer stocks were grown on LBS plates overnight at 25°C. A single colony was selected to inoculate 3 ml of LBS broth incubated in a shaker overnight at 25°C for all experiments unless otherwise specified.

ii. Assessing the Potential Role of PepN in Symbiosis-related Activities

a. Growth & luminescence curves in LBS Medium

To determine if PepN influences growth rate and luminescence, ES114, $PepN^-$, and $PepN^+$ strains were measured for light emission per cell and growth rate in LBS, a nutrient rich medium. Optical density at 600 nm (OD_{600}) was measured using a spectrophotometer. Approximately 20 μl of overnight culture was used to inoculate flasks to achieve an OD_{600} of .005 in 10 ml LBS broth for each strain. As a proxy for symbiosis conditions, one flask per strain received autoinducer at a final concentration of 1 μg/μl N-3-oxo-hexanoyl homoserine lactone for luminescence enhancement. OD_{600} and luminescence were measured periodically, increasing in frequency once log phase was reached. Sampling concluded nearing 1.000 OD_{600}.

b. Growth and luminescence curves in Seawater Tryptone Medium

Sea water tryptone broth is similar to the salinity composition of the natural environment of *V. fischeri* [5]. This was used to further determine the role of PepN in growth and luminescence. OD_{600} was measured using a spectrophotometer. Approximately 30 μl of overnight

culture was used to inoculate flasks to achieve an OD_{600} of .005 in 15 ml SWT broth for each strain. One flask per strain received autoinducer at a final concentration of 0.6 μg/μl N-3-oxo-hexanoyl homoserine lactone for luminescence enhancement. OD_{600} and luminescence were measured periodically, increasing in frequency once log phase was reached. Sampling concluded at 360 minutes. This experiment was performed in quadruplicate.

c. Mucin growth curve

Mucin, a common substance *V. fischeri* encounter upon entry into the light organ, was used to assess if PepN was necessary to thrive in such an environment. Wildtype and $PepN^-$ were grown overnight on LBS plates from freezer stock at 24°C. A single colony was used to inoculate 5 ml LBS broth for overnight growth. Overnight LBS broth cultures were used to inoculate flasks of 15 ml Mucin (Porcine mucin, Sigma Chemical Co.) broth to an OD_{600} of 0.05 [5]. Serial dilutions up to 10^{-8} were performed at time points 0, 120, 240, 300, 360, 420, 480, and 540 minutes. Two LBS spread plates were made per dilution using 100 μl aliquots. Plates were incubated at 24°C for 24 hours then colonies were counted. This experiment was performed in duplicate.

d. Biofilms

This experiment investigated the role of PepN in biofilm production, which is essential to the primary steps of colonization and might be necessary for persistence in the light organ. An overnight culture was used to inoculate 6 mini test tubes for an initial OD_{600} of .005 per strain. Test tubes received 500 μl of biofilm media that consists of (per liter) 500 ml 2X-artificial seawater, 461.25 ml deionized water, 11.9g HEPES, 5 ml 20% NH_4Cl ,30 ml 10% casamino acids stock, 3.75 ml 80% glycerol stock, 10 ml 0.001M Ferric SO_4 stock, and 0.2 ml glucose . Two tubes per strain incubated for 24 hours at 25°C then OD_{600} was measured. Cultures were then discarded and tubes were rinsed with deionized water. Tubes were dyed with 500 μl of

Calcofluor for 30 minutes or Crystal Violet for 5 minutes. Tubes dyed with Calcofluor were qualitatively assessed using a UV box. Crystal Violet dyed tubes were qualitatively assessed upon drying. This process was repeated at 48 hours and 72 hours. The study was repeated using LBS media as well. This experiment was performed in triplicate.

iii. Assessing the Potential Role of PepN in Stress Response

a. Sodium salicylate MIC

Sodium salicylate (NaSal), a general stress response inducer, was used to determine if PepN is vital to a stress response as demonstrated in other bacterial species containing a PepN homolog [7]. Six strain variations of *V. fischeri* ES114 were grown from freezer stock on LBS plates overnight. Strains were as follows: ES114, $PepN^-$, $PepN^+$, ES114+ vector (pEVS105; Dunn et al., 2006), ES114 with PepN expressed *in trans* (pDDG5, Fidopiastis et al., 2012), $PepN^-$ + vector only (pEVS105)[8]. A colony from each strain was single line streaked onto LBS plates containing final concentrations of 0, 8, 10, 12, or 16 mM NaSal from a 1M stock solution. Growth was assessed 24-120 hours later.

b. Phenotype microarray

The ability of *V. fischeri* to respire in the presence of a multitude of bacterial stress-inducing compounds was tested using phenotype microarray plates (Biolog) in order to uncover subtle physiological differences between wildtype and *pepN* mutant strains. $PepN^-$ freezer stock was grown overnight at 25°C on a TSA 1.4% (final concentration) NaCl plate. Culture was used to prepare the inoculation fluid consisting of 1.8 mL Redox Dye D mix and 23.2 mL 9% sterile saline to an OD_{600} of .073. 100 µl of inoculation fluid was added to 96 wells per phenotype microarray plate. Plates 9-20 were selected to compare to previously collected data on wildtype *Vibrio fischeri*. Respiration was tested in response to osmolyte variation in plate 9, pH tolerance in plate 10, and chemical sensitivities in plates 11-20. Inoculated plates were incubated at 25°C

for 24 hours in a humidifier box. Respiration was assessed after 24 hours. Data from assays in which wildtype did not respire, but the *pepN* mutant did were grouped into the following 8 subjective categories: osmolytes, pH, non-macrolide antibiotics, macrolides, chemicals, antifungals, drugs, and ion channel blockers (Appendix 4).

c. Phosphate starvation growth and luminescence curves

Previous studies using *E. coli* found an increase in *pepN* expression under phosphate starvation conditions [6], providing impetus for a similar investigation in *V. fischeri*. Growth and luminescence curves were performed using HEPES minimal media (HMM) with and without the addition of K_2HPO_4, a phosphate source. OD_{600} was measured using a spectrophotometer. Approximately 45 µl of overnight culture was used to inoculate six flasks to achieve an OD_{600} of .005 in 15 ml HMM+K_2HPO_4 or HMM broth for each strain. One flask per strain received autoinducer at a final concentration of 0.6 µg/µl N-3-oxo-hexanoyl homoserine lactone for luminescence enhancement. OD_{600} and luminescence time points were taken periodically, increasing in frequency once log phase was reached. Sampling concluded at 1.000 OD_{600}.

d. Nitric oxide challenge

To determine the role of PepN in stress response, nitric oxide was selected as a stress inducer due to its presence in *E. scolopes* ducts of the light organ. This protocol was adapted from Y. Wang et al. (2010) with a few alterations. Overnight cultures of wildtype and $PepN^-$ grown on LBS agar were used to inoculate 3 mL of LBS broth shaking at 250 rpm, 28°C to achieve an OD_{600} of 0.8. 20 µl of culture was used to inoculate 20 mL LBS in 125 mL Erlenmeyer flasks. Flasks receiving the NO challenge had DEA-NONOate (Cayman Chemicals), a NO donor, added to a final concentration of 100 µM. Flasks with a pretreatment received a final concentration 40 µM DEA-NONOate 45 minutes before NO challenge. Using a spectrophotometer, growth was monitored for all cultures until an OD_{600} of 2.000 was reached.

iv. Determining the Effects of Selected Environmental Conditions on *pepN* Gene Expression

a. Phosphate starvation

E. coli pepN had a four-fold expression increase under phosphate starvation prompting a similar study with *V. fischeri* PepN to determine a role in stress response [6]. Wildtype freezer stock was grown overnight at 24°C on LBS agar. Single colonies were used to inoculate 3 test tubes of 5 mL HMM+K_2HPO_4 and 3 test tubes of 5mL HMM stored at 24°C overnight. Total RNA was harvested from cells using Ambion PureLink RNA Mini Kit. A NanoDrop (Thermo Scientific) was used to quantify RNA. Applied Biosystems High-Capacity RNA-to-cDNA Kit was used to obtain cDNA using 0.30 μg RNA. qRT-PCR was performed in triplicate on Applied Biosystems 7300 Real Time PCR System using 30 ng cDNA/reaction of Taqman Probe Mastermix. Primers were designed to target an internal fragment (~103-122 bp) of *pepN* or an endogenous control gene as described in Y. Wang et al. (2010) (DNA polymerase I, *VF_0074*) [10]. Relative expression levels were calculated using ΔΔCt method (Applied Biosystems-*Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR*, 2008).

b. Anaerobiosis

Anaerobic conditions were found to increase *E. coli pepN* expression suggesting a role in stress response thus leading to a similar investigation in the *V. fischeri* PepN homolog[6]. Wildtype freezer stock was grown overnight at 24°C on LBS agar. Single colonies were used to inoculate 6 test tubes of 5 mL LBS. Three test tubes were stored aerobically, shaking and three test tubes were housed in anaerobic, stagnant conditions at 24°C overnight. Total RNA was harvested from cells using Ambion PureLink RNA Mini Kit. A NanoDrop (Thermo Scientific) was used to quantify RNA. Applied Biosystems High-Capacity RNA-to-cDNA Kit was used to obtain cDNA using 0.25 μg RNA. qRT-PCR was performed in triplicate using an Applied

Biosystems 7300 Real Time PCR System with 25 ng cDNA/reaction of Taqman Probe Mastermix. Primer design and relative expression level calculations were as previously described.

III. Results

i. Loss of PepN does not affect growth, luminescence, or biofilm production.

General assays were performed to identify changes in basic physiological functions of the *pepN* mutant. The *pepN* mutant did not differ in growth rate, luminescence emission per cell in culture during log phase, and biofilm formation on various media. Though not significant, in SWT broth, a lower nutrient medium than LBS, luminescence per-cell by the PepN mutant was greater than wildtype (p-value= 0.0689, Fig. 1). LBS broth, a nutrient-rich medium, did not elicit any differences in growth rate or luminescence per cell in the PepN mutant (Fig. 2). PepN mutant and wildtype grew similarly in mucin broth and formed comparable cellulosic and polysaccharide biofilms (Appendix 1, Appendix 2).

ii. Loss of PepN does not affect growth under phosphate starvation or in the presence of stress inducers.

The absence of a phosphate source and the presence of sodium salicylate are stress conditions found to induce *pepN* expression in *E. coli*, or show PepN to be a negative regulator of the stress response, respectively [6,7]. Compared to wildtype, *V. fischeri pepN* mutant did not exhibit any difference in tolerance to NaSal during MIC assays and assays from phenotype microarray plate 17 utilizing NaSal. Both wildtype and PepN mutant grew similarly in the presence of increasing concentrations of NaSal (Fig. 3). $PepN^{+}$ and WT + vector (pEVS105) exhibited lower tolerance to NaSal as concentration increased compared to other strains possibly due to slowed growth by carrying a multi-copy number plasmid exacerbated by a selective environment (Dr. E.V. Stabb, University of Georgia, Personal Communication, Fig. 3c). Growth rates of wildtype and *pepN* mutant strains were higher in medium containing phosphate than medium without (Fig. 4). No differences in growth rate or luminescence per cell were found between strains in either condition. *V. fischeri* strains grown in medium containing nitric oxide,

an antimicrobial agent present in the light organ of *E. scolopes* during colonization, did not exhibit growth rate differences (Fig. 5). Both strains had a decreased growth rate in the presence of DEA-NONOate.

iii. PepN-deficient cells of *V. fischeri* have greater tolerance to an array of chemical environments.

Out of 432 biochemical assays tested in Biolog phenotype microarray plates 9-20, the *pepN* mutant grew in 328 compared to 254 by wildtype (Fig. 6). This tolerance difference amounted to 4 conditions in which wildtype respired, but *pepN* mutant did not compared to 78 conditions in which *pepN* mutant respired, but wildtype did not. Wildtype *V. fischeri* demonstrated a greater tolerance for higher salinity environments than the *pepN* mutant, respiring in 4% NaCl and 6% potassium chloride. Wildtype *V. fischeri* also respired in greater concentrations of cupric chloride and was tolerant of thioridazine, a presumed ion channel inhibitor. The *pepN* mutant was found to respire in 78 assays that wildtype did not. These assays were grouped into the previously described 8 categories (Fig. 7). Notable differences between wildtype and mutant included that the *pepN* mutant had an increased tolerance to urea, sodium lactate, and acidity compared to wildtype.

iv. Expression of *pepN* is not effected by phosphate starvation or anoxic conditions.

Unlike *E. coli* PepN [6], *pepN* expression in *V. fischeri* was not increased under phosphate limited or anoxic conditions. When wildtype *V.fischeri* was grown in HMM without K_2HPO_4, *pepN* was induced 0.60-1.90 fold compared to HMM with K_2HPO_4 as measured by quantitative real-time PCR (qRT-PCR). In addition, wildtype *V. fischeri* was grown in anoxic conditions that prompted a 0.67-1.48 fold increase in *pepN* expression compared to aerobic conditions. Neither experiment demonstrated a significant change in *pepN* expression (Appendix 3).

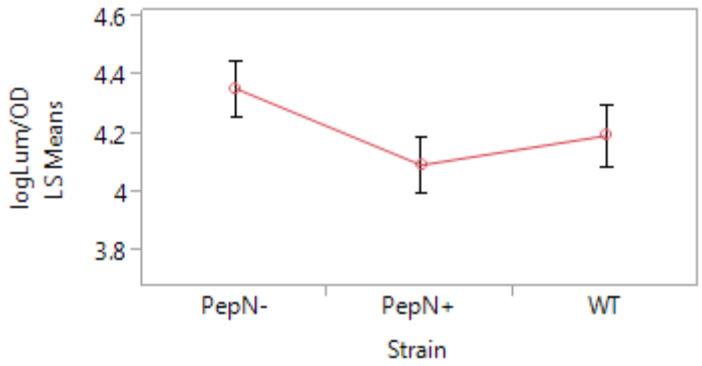

Figure 1. Log luminescence per cell in SWT broth (Least squares means plot with standard error bars).

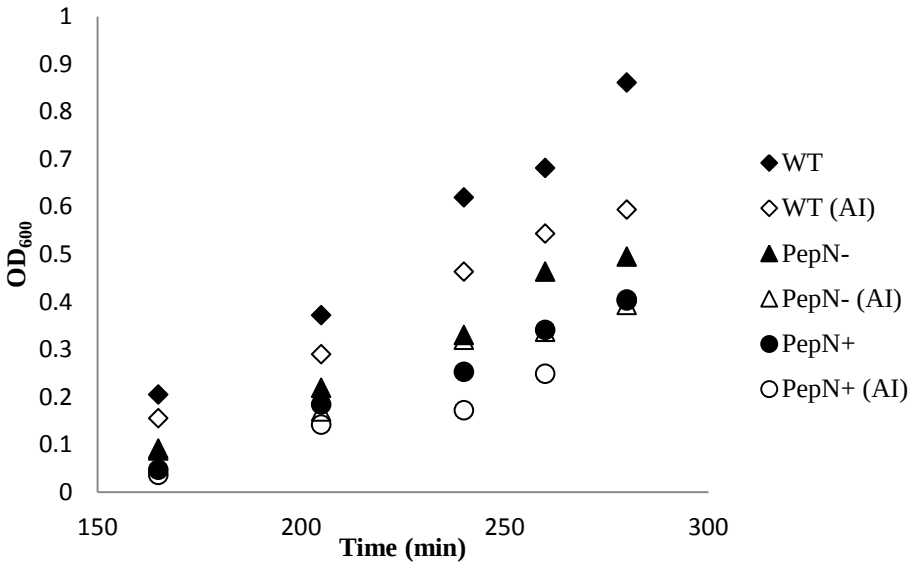

Figure 2. Growth curve during log phase of *V. fischeri* strains in LBS broth. Wildtype vs. $PepN^-$ p-value is 0.302 from a student's two-tailed t-test indicating difference of growth rate in a nutrient-rich environment was not significant. Cells were grown shaking in LBS at 25°C and 250rpm. N-3-oxo-hexanoyl homoserine lactone, autoinducer, was added to cultures indicated with "AI". The doubling times are as follows (minutes): WT- 62, WT (AI)- 75, $PepN^-$ - 54, $PepN^-$ (AI)- 55, $PepN^+$ - 50, $PepN^+$ (AI)- 54.

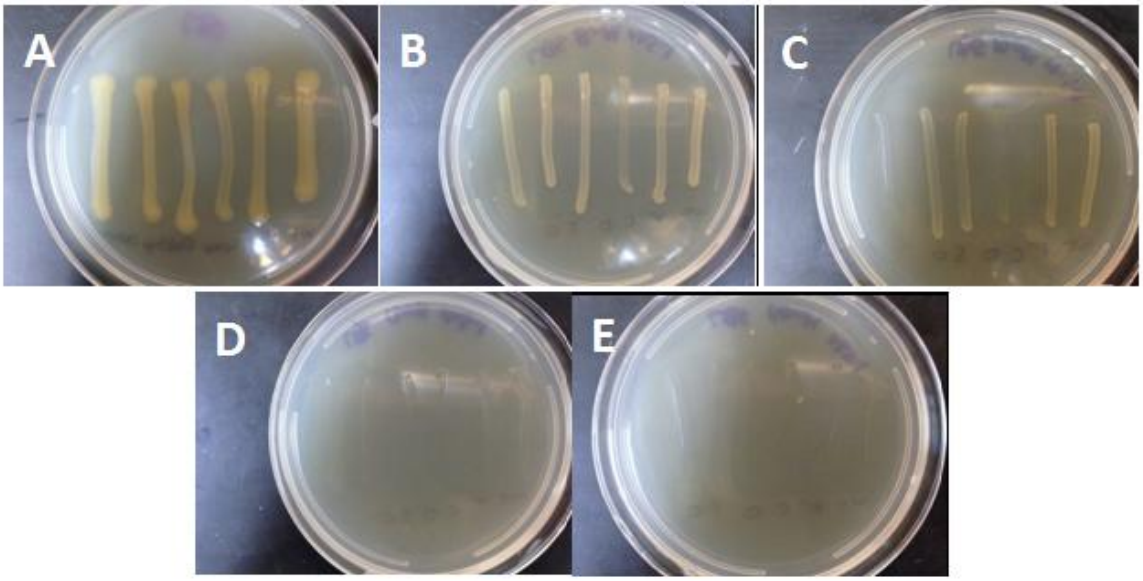

Figure 3. MIC plates with final concentrations of 0, 8, 10, 12, or 16 mM NaSal (A-E). From left to right, strain order is as follows: ES114+ vector only (pEVS105), ES114 with PepN expressed *in trans* (pDDG5), $PepN^-$ + vector only (pEVS105), $PepN^+$, $PepN^-$, and wildtype.

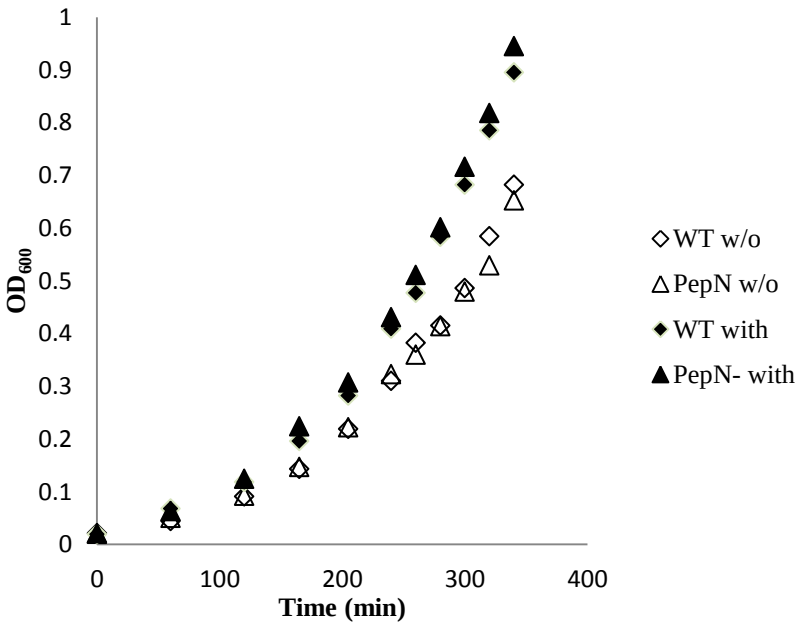

Figure 4. Growth curve of *V. fischeri* wildtype and *pepN* mutant strains in HMM with and without addition of K_2HPO_4.

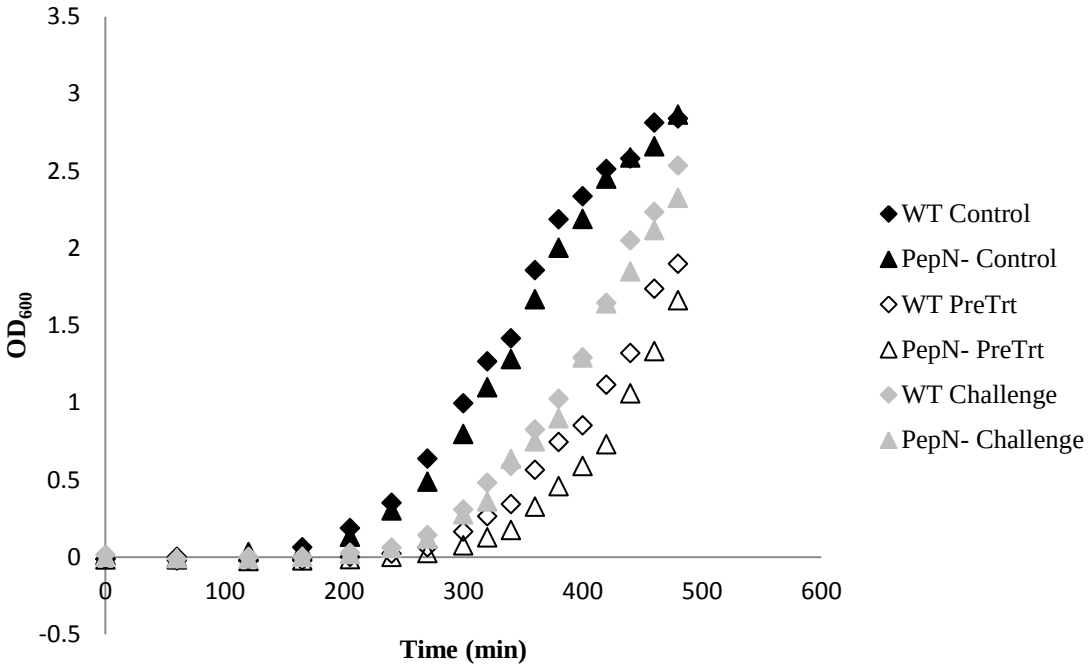

Figure 5. *V. fischeri* nitric oxide challenge growth curve. Cultures were either unconditioned (control), pre-treated with 40 µM (final concentration) DEA-NONOate before challenge with 100 µM (final concentration) DEA-NONOate, or initially challenged with 100 µM (final concentration) DEA-NONOate.

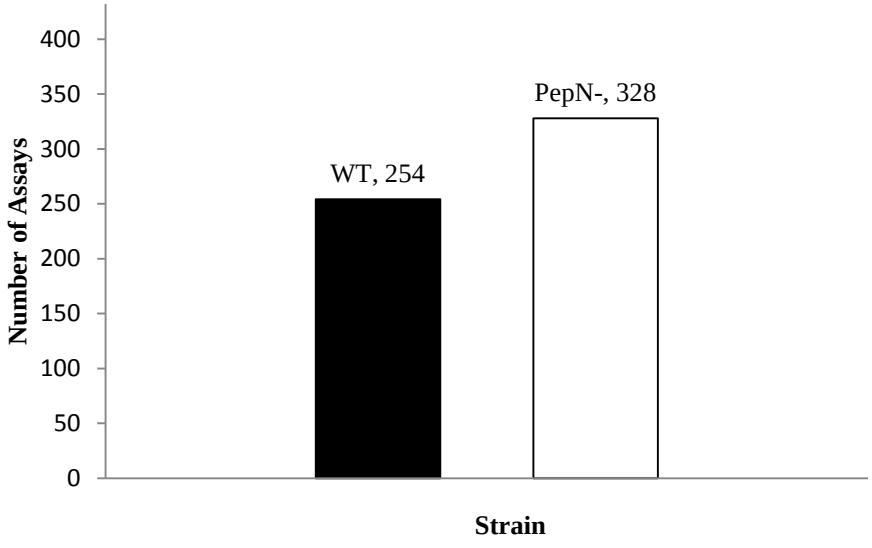

Figure 6. Biolog phenotype microarray assays (PM 9-20) resulting in respiration.

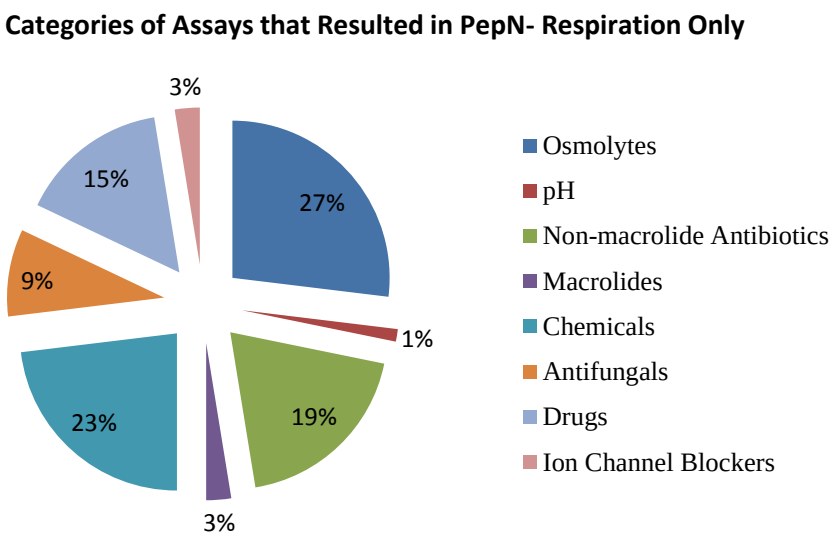

Figure 7. Categories of the 78 Biolog phenotype microarray assays in which *pepN* mutant respired but wildtype did not.

IV. Discussion

PepN is the major contributor of aminopeptidase activity in *V. fischeri* and contributes to the initiation of colonization of *E. scolopes* [5]. In addition to the *pepN* mutant strain being outcompeted and growth deficient compared to wildtype during the first 12 hours of colonization, it was found to form less dense aggregates on the mucin-rich, exterior surface of the light organ prior to migration [5]. Based on these observations three hypotheses were proposed: 1) PepN activity might be required to breakdown the peptide-rich mucus matrices for nutrition and to facilitate detachment, 2) PepN activity could affect biofilm formation, and consequently, the ability of cells to form dense aggregates, and 3), the *pepN* mutant might be more susceptible to inherent environmental stressors propagated by *E. scolopes* during colonization. This study tested these hypotheses through a number of physiological assays in various media and stress conditions, in addition to gene expression analysis by qRT-PCR, with the goal of further elucidating the role of PepN in *V. fischeri*.

PepN does not influence growth rate in mucin-rich media, suggesting that PepN is probably not required for mucin degradation within aggregates. This is further corroborated by a previous study, in which mucin hydrolysis between wildtype *V. fischeri* and the *pepN* mutant were similar (Dr. P.M. Fidopiastis, CPSU- San Luis Obispo, Personal Communication). Furthermore, the loss of PepN did not alter biofilm production, suggesting that PepN activity does not directly affect the ability of cells to aggregate. Being that assays were performed *in vitro* in hopes of mimicking conditions of the host, a combination of additional unknown factors may contribute to the decreased ability of the *pepN* mutant to form aggregates and grow within the first 12 hours of colonization. The chemical environment of mucin is complex, containing proteins, polysaccharides, antimicrobials, nitric oxide vesicles, and a low pH [18]. Though our results do not support a direct role for PepN in mucin degradation it is known that there are zinc proteases capable of degrading mucin and are speculated to be important for detachment [26]. In

addition, the necessity of hexosaminidases and chitinases in biofilm degradation is well documented [19]. *V. fischeri* dispersal utilizing an as yet unrecognized protease and/or the aforementioned carbohydrate-degrading enzymes may be a focus for future studies on mucin degradation. In addition, a study investigating PepN influence on chemotaxis, another important colonization step present during dispersal and migration into the pores, might be worthwhile [18, 20]. Notably, *V. fischeri* has been found to utilize different metabolic pathways during symbiotic and free-living lifestyles, varying its response to the external environment [8]. Further efforts to identify the unique chemical composition of the mucus matrices of *E. scolopes* should be evaluated and peptide utilization assays performed with purified PepN may be valuable as well.

To investigate the role of PepN in stress response, as demonstrated with other Gram negative bacteria, known microbial stressors were employed [6, 7, 9]. First, growth in SWT broth, a nutrient-deplete medium, showed a trend of increased luminescence by the *pepN* mutant compared to wildtype. This could support a role for PepN in stress response, as increased luminescence is induced under stressful conditions like anoxia [14]. The *pepN* mutant should be further studied in other nutrient-deficient media to see if this trend is repeated. Second, in the presence of two stress-inducing compounds, sodium salicylate (NaSal) and nitric oxide, wildtype *V. fischeri* and the *pepN* mutant strain had similar growth patterns with no significant deviations. A previous study had similar findings with a gene expression microarray that was performed using wildtype *V. fischeri* cells after exposure to nitric oxide, these data showed no change in *pepN* expression [11]. Based on the data provided here and past experiments, PepN does not appear to aid in regulating stress response to nitric oxide and this squid defense is not likely to have caused the slowed early colonization by the PepN mutant. Contrary to expected results, the *pepN* mutant did not have increased tolerance to NaSal exposure as demonstrated with an *E. coli pepN* mutant [7]. In *E. coli*, the addition of PepN resulted in inhibited growth on NaSal plates suggesting PepN negatively regulates NaSal-induced stress response. A follow-up study found *E.*

coli pepN expression did not increase in the presence of NaSal, but further studies are necessary to understand the mechanism by which PepN regulates NaSal stress-response [9]. However, this same study found that during nutritional downshift and high temperature (NDHT) stress, PepN activity is necessary for growth. Specifically, wildtype had a significantly higher growth rate than the *pepN* mutant and *pepN* mutant growth rate was restored upon ectopic expression of catalytically active PepN. In addition, *pepN* mutant growth rate was also restored by the exogenous addition of casamino acid residues. The necessity of PepN under these conditions is attributed to its known role in protein turnover, increasing the availability of intracellular amino acids/peptides for *E. coli* adaptation and growth in response to NDHT stresses [9,4]. Similar results were obtained with a *Salmonella typhimurium* PepN mutant subjected to NDHT stress, highlighting the role of PepN in stress response particularly during starvation conditions [13,12]. A study by Graf et al. (1998) found the extracellular fluid of the light organ of *E. scolopes* to provide at least 9 amino acids necessary for promoting the proliferation of *V. fischeri* [27]. These amino acids may not be readily supplied in the mucus, and thus, may explain the pattern for delayed colonization by the *pepN* mutant. It would be valuable to address this hypothesis in a follow up study.

Two stressors known to induce expression of *pepN* in *E. coli* are phosphate starvation and anaerobiosis, yet *V. fischeri* did not display enhanced *pepN* expression in either condition [6]. Unlike, in *E. coli*, which showed a 4-fold increase in *pepN* expression during anaerobiosis, *V. fischeri* did not have a significant change compared to aerobic conditions [6]. Suggesting growth under oxygen-limited conditions in the light organ is not affected by PepN. Researchers who studied *E. coli pepN* expression under anaerobic conditions suggested their results are indicative of several pathways for anaerobic regulation of gene expression besides the well-known FNR protein [6, 15]. Furthermore, *pepN* expression in *V. fischeri* was not influenced by phosphate levels. In *E. coli*, *pepN* expression was greater under phosphate starvation compared to

phosphate supplemented conditions [6]. Using a *pepN-lacZ* fusion, Gharbi et al. (1985) demonstrated a 4-fold difference in *pepN* expression under phosphate starvation and found this expression was not attributed to the *pho* regulon, which is responsible for regulating genes participating in phosphate metabolism, and thus, modulation of many cellular processes [6, 28]. Conversely, a previous *E. coli* study by McCaman et al. (1982) failed to find an effect from the loss of PepN on growth rate and wildtype PepN levels remained constant within various conditions including phosphate starvation. It was concluded that *pepN* expression is constitutive and is not significantly affected by the cell's external environment, consistent with its proposed role in intracellular protein turnover and degradation [12]. Our findings for PepN in *V. fischeri* are similar to those reported by McCaman et. al (1982).

In an effort to identify stress-related processes and pathways which may involve PepN activity, stress-response-related Biolog phenotype microarray assays were performed. Marked strain differences from this experiment included the ability of wildtype to grow in higher salinity environments and in the presence of thioridazine, a known efflux pump inhibitor. Conversely, the PepN mutant showed increased resistance to antibiotics, acidity, and urea. Reminiscent of results found by Chandu et al. (2003), a wildtype strain of *E. coli* experienced greater susceptibility to stressors compared to the PepN mutant [7]. Our findings lend further support to the role of PepN as a negative regulator in certain stress response pathways as demonstrated by increased tolerance to stressors by the PepN mutant.

Notably, the *pepN* mutant had increased resistance to 17 antibiotics, only two of which were macrolides, whose resistance could be attributed to the erythromycin resistance marker insertion in our *pepN* mutant. However, ribosome modification may have occurred by the methyltransferase properties of the erythromycin marker leading to the inconclusive resistance of 9 of these other antibiotics whose mechanisms of action involve ribosome inhibition (Dr. P.M. Fidopiastis, CPSU- San Luis Obispo, Personal Communication). The mechanisms of action for

cloxacillin, nalidixic acid, oxacillin, rifampicin, and sulfisoxazole are not directly associated with ribosome function. An increase in resistance to other antibiotics might be due to the role of PepN activity in activating the antibiotics' lethal mechanisms [4]. For example, PepN activates antimicrobial activity for albomycin in *E. coli* and *Salmonella typhimurium* [16]. Conversely, the presence of metalloprotease activity in *S. aureus by* BlaR, a zinc-dependent transmembrane protease signal-transducer, increases β-lactam resistance by causing the derepression of □-lactamase and penicillin-binding protein 2a [23]. Metallopeptidases are becoming potential targets for new antibiotics due to their purported role in key cellular functions like protein turnover [21]. For example, bacitracin belongs to a particular class of antibiotics called "metalloantibiotics" that inhibit metalloproteases [22]. A follow up Biolog study should be performed with a complemented *pepN* strain to observe if the addition of PepN restores the wildtype pattern of antibiotic sensitivity.

In *E. coli*, there was no increase in PepN production when cells were grown in urea-containing media [6]. However, in *V. fischeri*, the *pepN* mutant displayed increased tolerance to urea at concentrations up to 7% compared to the 2% tolerance see in wildtype. The role of PepN in urea tolerance should be further studied due to its importance in marine animal tissue as an osmolyte and major waste product from the squid's diet. An important colonization condition modified by the squid host is pH. Kremer et al. (2013) found that a slight difference in acidity between the mucus matrix (~6.4) and pores (5.9) influenced important symbiotic host protein activity. It is tempting to speculate that the loss of PepN results in greater acidity tolerance due to an increased pH stress response echoing the proposed role of PepN as a negative regulator in stress response by Chandu et al. (2003) [7]. The *pepN* mutant may allocate greater amounts of energy resources to the over activation of proton pumps to stabilize pH creating the initial lag in colonization similar to results found with NDHT stress [9]. Additionally, the ability of wildtype to grow in higher salinity environments coupled with the ability to resist thioridazine may suggest

a greater role for PepN in cytoplasmic membrane regulation, in particular, pathways that involve efflux pump functions. Metalloproteases have been identified in *Halobacillus sp.* LY6 and *Bacillus cereus* as important to salinity tolerance, exhibiting high activity at increased salinity [24, 25]. In *Mycobacterium tuberculosis* thioridazine inhibited efflux pumps and their expression [17]. It is possible that this inhibition in tandem with the loss of function by PepN is responsible for the lack of respiration by the *pepN* mutant. *V. fischeri* PepN activity was found to be cell membrane-associated [5]. Subsequent assays should be performed to further evaluate the role of PepN with respect to salinity tolerance and thioridazine resistance.

To date, the best-supported role for bacterial PepN is in intracellular protein degradation and turnover [4,6,7,9,12,13]. Though this study uncovered stress conditions that may affect PepN activity, gene expression regulators have yet to be defined. Assays utilizing *V. fischeri* pepN-lacZ fusion constructs may be useful in identifying assays for follow-up testing on stress conditions identified by the phenotype microarrays and an NDHT conditions prior to executing qRT-PCR. A timed *E. scolopes in vivo* assay for symbiotic *V. fischeri pepN* expression detected by qRT-PCR would be valuable as well, to help address discrepancies in metabolic shifts between free-living and symbiont lifecycles. Differences in the physiological pathways between symbiont and free-living *V. fischeri* and unidentified factors may contribute to the difficulty in uncovering the influence of PepN in aggregate formation and colonization rate within the initial steps of light organ colonization.

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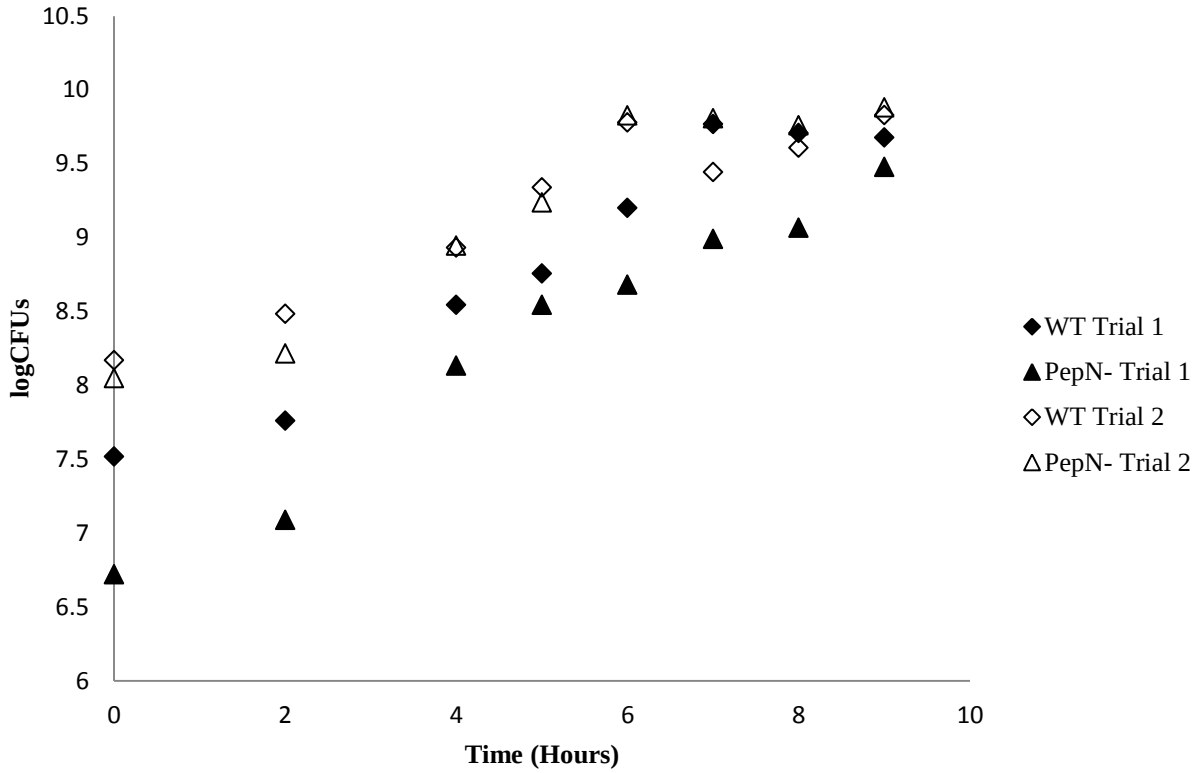
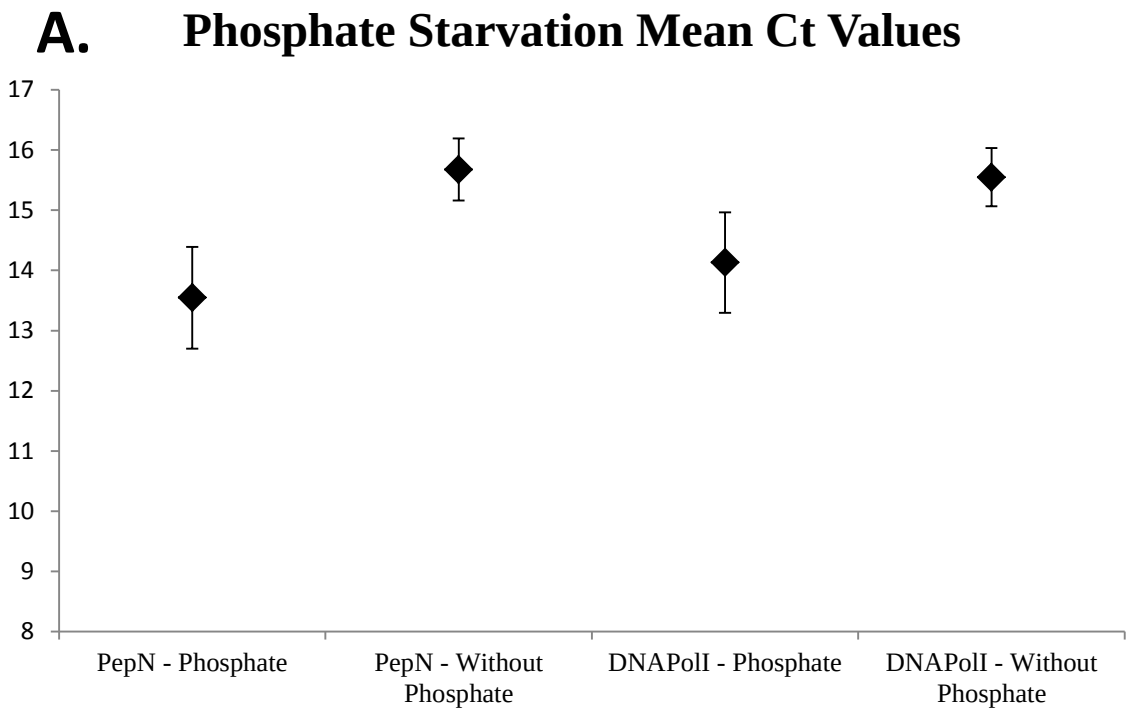
Appendix of Supplemental Data & Figures

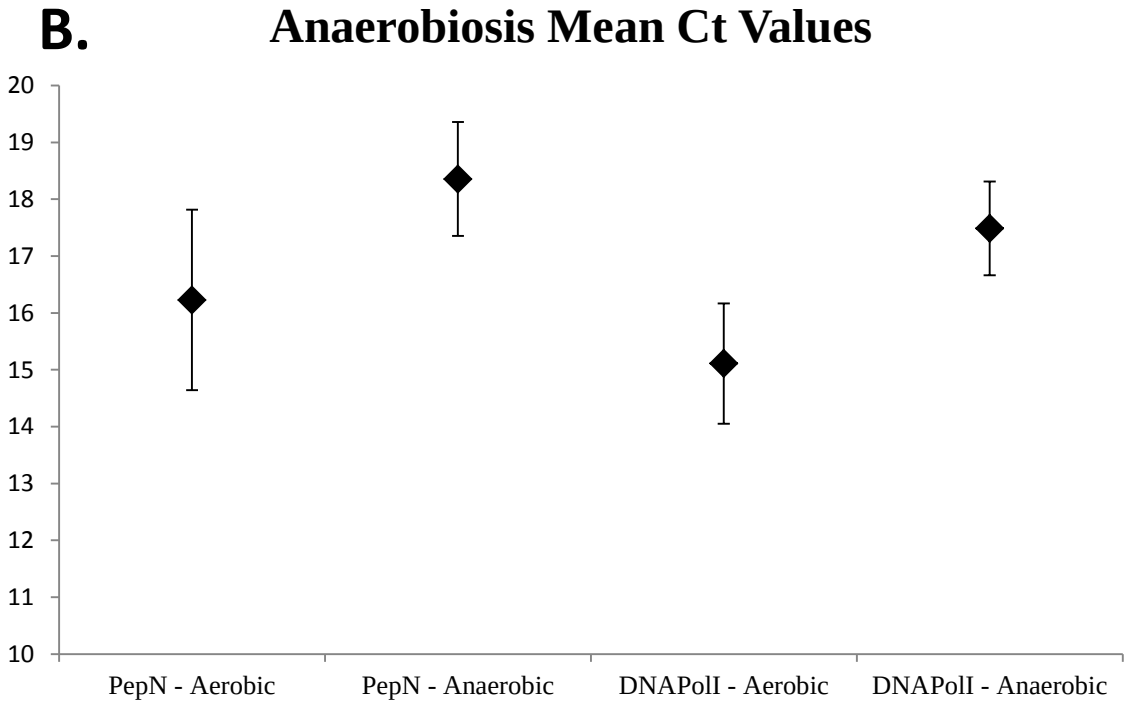


Appendix 1. Log CFUs of *V. fischeri pepN* mutant and wildtype strains grown in mucin broth.

	OD600					
	Crystal Violet			Calcofluor		
Time (hr)	WT	PepN-	PepN+	WT	PepN-	PepN+
24	1.059	1.13	1.361	1.377	1.523	1.645
48	1.357	1.488	1.795	1.5	1.643	1.765
72	1.462	1.636	1.934	1.741	1.873	1.897

Appendix 2. Raw data of *V. fischeri* wildtype, *pepN* mutant, and complemented strains grown in biofilm media. Cell density was measured quantitatively using OD_{600} spectraphotometric readings and biofilm production was measured qualitatively by observing biofilm ring formations. Representative data from three trials. Calcofluor dyed biolfilms were not visible. Crystal violet dyed biofilms corresponded to cell densities and were comparable between strains.





Appendix 3. *Vibrio fischeri* ES114 *pepN* and *DNApolI* mean critical threshold values with standard deviations from phosphate starvation (A) and anoxic conditions (B) qRT-PCR assays.

	Compound	WT	PepN-	Plate
1	NaCl 1%	x	x	**9**
2	NaCl 2%	x	x	
3	NaCl 3%	x	x	
4	NaCl 4%	x	o	
5	NaCl 5%	o	o	
6	NaCl 5.5%	o	o	
7	NaCl 6.5%	o	o	
8	NaCl 6%	o	o	
9	NaCl 7%	o	o	
10	NaCl 8%	o	o	
11	NaCl 9%	o	o	
12	NaCl 10%	o	o	
13	NaCl 6%	o	o	
14	NaCl 6% + Betaine	o	o	
15	NaCl 6% + N-N Dimethyl Glycine	o	o	
16	NaCl 6% + Sarcosine	o	o	
17	NaCl 6% + Dimethyl sulphonyl propionate	o	o	
18	NaCl 6% + MOPS	o	o	
19	NaCl 6% + Ectoine	o	o	
20	NaCl 6% + Choline	o	o	
21	NaCl 6% + Phosphoryl Choline	o	o	
22	NaCl 6% + Creatine	o	o	
23	NaCl 6% + Creatinine	o	o	
24	NaCl 6% + L- Carnitine	o	o	
25	NaCl 6% + KCl	o	o	
26	NaCl 6% + L-Proline	o	o	
27	NaCl 6% + N-Acetyl L-Glutamine	o	o	
28	NaCl 6% + β-Glutamic Acid	o	o	
29	NaCl 6% + γ-Amino-N-Butyric Acid	o	o	
30	NaCl 6% + Glutathione	o	o	
31	NaCl 6% + Glycerol	o	o	
32	NaCl 6% + Trehalose	o	o	
33	NaCl 6% + Trimethylamine-N-oxide	o	o	
34	NaCl 6% + Trimethylamine	o	o	
35	NaCl 6% + Octopine	o	o	
36	NaCl 6% + Trigonelline	o	o	
37	Potassium chloride 3%	x	x	
38	Potassium chloride 4%	x	x	
39	Potassium chloride 5%	x	x	
40	Potassium chloride 6%	x	o	

41	Sodium sulfate 2%	x	x
42	Sodium sulfate 3%	x	x
43	Sodium sulfate 4%	x	x
44	Sodium sulfate 5%	x	x
45	Ethylene glycol 5%	x	x
46	Ethylene glycol 10%	x	x
47	Ethylene glycol 15%	x	x
48	Ethylene glycol 20%	x	x
49	Sodium formate 1%	o	x
50	Sodium formate 2%	o	o
51	Sodium formate 3%	o	o
52	Sodium formate 4%	o	x
53	Sodium formate 5%	o	x
54	Sodium formate 6%	x	x
55	Urea 2%	x	x
56	Urea 3%	o	x
57	Urea 4%	o	x
58	Urea 5%	o	x
59	Urea 6%	o	x
60	Urea 7%	o	x
61	Sodium Lactate 1%	o	x
62	Sodium Lactate 2%	o	x
63	Sodium Lactate 3%	o	o
64	Sodium Lactate 4%	o	x
65	Sodium Lactate 5%	o	x
66	Sodium Lactate 6%	o	x
67	Sodium Lactate 7%	o	x
68	Sodium Lactate 8%	o	x
69	Sodium Lactate 9%	o	x
70	Sodium Lactate 10%	o	x
71	Sodium Lactate 11%	o	x
72	Sodium Lactate 12%	o	x
73	Sodium Phosphate pH 7 20mM	x	x
74	Sodium Phosphate pH 7 50mM	x	x
75	Sodium Phosphate pH 7 100mM	x	x
76	Sodium Phosphate pH 7 200mM	x	x
77	Sodium Benzoate pH 5.2 20mM	o	x
78	Sodium Benzoate pH 5.2 50mM	o	x
79	Sodium Benzoate pH 5.2 100mM	o	o
80	Sodium Benzoate pH 5.2 200mM	o	o
81	Ammonium sulfate pH 8 10mM	x	x

82	Ammonium sulfate pH 8 20mM	x	x	
83	Ammonium sulfate pH 8 50mM	x	x	
84	Ammonium sulfate pH 8 100mM	x	x	
85	Sodium Nitrate 10mM	x	x	
86	Sodium Nitrate 20mM	x	x	
87	Sodium Nitrate 40mM	x	x	
88	Sodium Nitrate 60mM	x	x	
89	Sodium Nitrate 80mM	x	x	
90	Sodium Nitrate 100mM	x	x	
91	Sodium Nitrite 10mM	x	x	
92	Sodium Nitrite 20mM	x	x	
93	Sodium Nitrite 40mM	x	x	
94	Sodium Nitrite 60mM	x	x	
95	Sodium Nitrite 80mM	x	x	
96	Sodium Nitrite 100mM	x	x	
97	pH 3.5	o	o	**10**
98	pH 4	o	o	
99	pH 4.5	o	o	
100	pH 5	o	x	
101	pH 5.5	x	x	
102	pH 6	x	x	
103	pH 7	x	x	
104	pH 8	x	x	
105	pH 8.5	x	x	
106	pH 9	x	x	
107	pH 9.5	x	x	
108	pH 10	x	x	
109	pH 4.5	o	o	
110	pH 4.5 + L-Alanine	o	o	
111	pH 4.5 + L-Arginine	o	o	
112	pH 4.5 + L-asparagine	o	o	
113	pH 4.5 + L-Aspartic Acid	o	o	
114	pH 4.5 + L-Glutamic Acid	o	o	
115	pH 4.5 + L-Glutamine	o	o	
116	pH 4.5 + Glycine	o	o	
117	pH 4.5 + L-Histidine	o	o	
118	pH 4.5 + L-Isoleucine	o	o	
119	pH 4.5 + L-Leucine	o	o	
120	pH 4.5 + L-Lysine	o	o	
121	pH 4.5 + L-Methionine	o	o	
122	pH 4.5 + L-Phenylalanine	o	o	

123	pH 4.5 + L-Proline	o	o
124	pH 4.5 + L-Serine	o	o
125	pH 4.5 + L-Threonine	o	o
126	pH 4.5 + L-Tryptophan	o	o
127	pH 4.5 + L-Citruline	o	o
128	pH 4.5 + L-Valine	o	o
129	pH 4.5 + Hydroxy-L-Proline	o	o
130	pH 4.5 + L-Ornithine	o	o
131	pH 4.5 + L-Homoarginine	o	o
132	pH 4.5 + L-Homoserine	o	o
133	pH 4.5 + Anthranilic Acid	o	o
134	pH 4.5 + L-Norleucine	o	o
135	pH 4.5 + L-Norvaline	o	o
136	pH 4.5 + α-Amino-N-Butyric Acid	o	o
137	pH 4.5 + ρ- Amino-Benzoic Acid	o	o
138	pH 4.5 + L-Cysteic Acid	o	o
139	pH 4.5 + D-Lysine	o	o
140	pH 4.5 + 5-Hydroxy Lysine	o	o
141	pH 4.5 + 5-Hydroxy Tryptophan	o	o
142	pH 4.5 + D,L- Diamino pimelic Acid	o	o
143	pH 4.5 + Trimethylamine-N-oxide	o	o
144	pH 4.5 + Urea	o	o
145	pH 9.5	x	x
146	pH 9.5 + L-Alanine	x	x
147	pH 9.5 + L-Arginine	x	x
148	pH 9.5 + L-asparagine	x	x
149	pH 9.5 + L-Aspartic Acid	x	x
150	pH 9.5 + L-Glutamic Acid	x	x
151	pH 9.5 + L-Glutamine	x	x
152	pH 9.5 + Glycine	x	x
153	pH 9.5 + L-Histidine	x	x
154	pH 9.5 + L-Isoleucine	x	x
155	pH 9.5 + L-Leucine	x	x
156	pH 9.5 + L-Lysine	x	x
157	pH 9.5 + L-Methionine	x	x
158	pH 9.5 + L-Phenylalanine	x	x
159	pH 9.5 + L-Proline	x	x
160	pH 9.5 + L-Serine	x	x
161	pH 9.5 + L-Threonine	x	x
162	pH 9.5 + L-Tryptophan	x	x
163	pH 9.5 + L-Tyrosine	x	x

164	pH 9.5 + L-Valine	x	x	
165	pH 9.5 + Hydroxy-L-Proline	x	x	
166	pH 9.5 + L-Ornithine	x	x	
167	pH 9.5 + L-Homoarginine	x	x	
168	pH 9.5 + L-Homoserine	x	x	
169	pH 9.5 + Anthranilic Acid	x	x	
170	pH 9.5 + L-Norleucine	x	x	
171	pH 9.5 + L-Norvaline	x	x	
172	pH 9.5 + Agmatine	x	x	
173	pH 9.5 + Cadaverine	x	x	
174	pH 9.5 + Putrescine	x	x	
175	pH 9.5 + Histamine	x	x	
176	pH 9.5 + Phenylethylamine	o	o	
177	pH 9.5 + Tyramine	o	o	
178	pH 9.5 + Creatine	x	x	
179	pH 9.5 + Trimethylamine-N-oxide	x	x	
180	pH 9.5 + Urea	x	x	
181	X-Caprylate	x	x	
182	X-α-D-Glucoside	x	x	
183	X-β-D-Glucoside	x	x	
184	X-α-D-Galactoside	x	x	
185	X-β-D-Galactoside	x	x	
186	X-α-D-Glucuronide	x	x	
187	X-β-D-Glucuronide	x	x	
188	X-β-D-Glucosaminide	x	?	
189	X-β-D-Galactosaminide	x	?	
190	X-α-D-Mannoside	x	x	
191	X-PO4	x	?	
192	X-SO4	x	x	
193	Amikacin	x	x	**11**
194	Chlortetracycline	o	o	
195	Lincomycin	o	x	
196	Amoxicillin	x	x	
197	Cloxacillin	o	x	
198	Lomefloxacin	x	x	
199	Bleomycin	x	x	
200	Colistin	x	x	
201	Minocycline	o	o	
202	Capreomycin	x	x	
203	Demeclocycline	o	x	
204	Nafcillin	x	x	

205	Cefazoline	x	x	
206	Enoxacin	x	x	
207	Nalidixic acid	o	x	
208	Chloramphenicol	o	x	
209	Erythromycin	o	x	
210	Neomycin	x	x	
211	Ceftriaxone	x	x	
212	Gentamicin	x	x	
213	Potassium tellurite	x	x	
214	Cephalothin	x	x	
215	Kanamycin	x	x	
216	Ofloxacin	x	x	
217	Penicillin G	x	x	**12**
218	Tetracycline	o	o	
219	Carbenicillin	x	x	
220	Oxacillin	o	x	
221	Penimepicycline	o	x	
222	Polymyxin B	x	x	
223	Paromomycin	x	x	
224	Vancomycin	x	x	
225	D,L-Serine hydroxamate	x	x	
226	Sisomicin	x	x	
227	Sulfamethazine	x	x	
228	Novobiocin	o	o	
229	2,4-Diamino-6,7-diisopropyl-pteridine	o	o	
230	Sulfadiazine	x	x	
231	Benzethonium chloride	x	x	
232	Tobramycin	x	x	
233	Sulfathiazole	x	x	
234	5-Fluoroorotic acid	x	x	
235	Spectinomycin	x	x	
236	Sulfa-methoxazole	x	x	
237	L-Aspartic-β-hydroxamate	o	x	
238	Spiramycin	x	x	
239	Rifampicin	o	x	
240	Dodecyltrimethyl ammonium bromide	x	x	
241	Ampicillin	x	x	**13**
242	Dequalinium chloride	x	x	
243	Nickel chloride	x	x	
244	Azlocillin	x	x	
245	2,2'-Dipyridyl	o	o	

246	Oxolinic acid	x	x	
247	6-Mercapto-purine	x	x	
248	Doxycycline	o	o	
249	Potassium chromate	o	o	
250	Cefuroxime	x	x	
251	5-Fluorouracil	x	x	
252	Rolitetracycline	o	x	
253	Cytosine-1-beta-D-arabino-furanoside	x	x	
254	Geneticin (G418)	x	x	
255	Ruthenium red	x	x	
256	Cesium chloride	x	x	
257	Glycine	x	x	
258	Thallium (I) acetate	x	x	
259	Cobalt chloride	o	o	
260	Manganese chloride	o	o	
261	Trifluoperazine	o	x	
262	Cupric chloride	x	o	
263	Moxalactam	x	x	
264	Tylosin	x	x	
265	Acriflavine	x	x	**14**
266	Furaltadone	o	x	
267	Sanguinarine	o	o	
268	9-Aminoacridine	x	x	
269	Fusaric acid	o	o	
270	Sodium arsenate	x	x	
271	Boric Acid	x	x	
272	1-Hydroxy-pyridine-2-thione	x	x	
273	Sodium cyanate	x	x	
274	Cadmium chloride	x	x	
275	Iodoacetate	o	o	
276	Sodium dichromate	x	x	
277	Cefoxitin	x	x	
278	Nitrofurantoin	x	x	
279	Sodium metaborate	x	x	
280	Chloramphenicol	o	x	
281	Piperacillin	x	x	
282	Sodium metavanadate	x	x	
283	Chelerythrine	x	x	
284	Carbenicillin	x	x	
285	Sodium nitrite	x	x	
286	EGTA	x	x	

287	Promethazine	o	x	
288	Sodium orthovanadate	x	x	
289	Procaine	x	x	**15**
290	Guanidine hydrochloride	o	x	
291	Cefmetazole	x	x	
292	D-Cycloserine	x	x	
293	EDTA	o	x	
294	5,7-Dichloro-8-hydroxy-quinaldine	o	x	
295	5,7-Dichloro-8-hydroxyquinoline	o	x	
296	Fusidic acid	x	x	
297	1,10-Phenanthroline	o	o	
298	Phleomycin	x	x	
299	Domiphen bromide	x	x	
300	Nordihydroguaia retic acid	x	x	
301	Alexidine	x	x	
302	5-Nitro-3-furaldehyde semicarbazone	o	x	
303	Methyl viologen	x	x	
304	3,4-Dimethoxy-benzyl alcohol	x	x	
305	Oleandomycin	x	x	
306	Puromycin	o	x	
307	CCCP	o	o	
308	Sodium azide	x	x	
309	Menadione	o	x	
310	2-Niroimidazole	o	x	
311	Hydroxyurea	o	x	
312	Zinc chloride	x	x	
313	Cefotaxime	x	x	**16**
314	Phosphomycin	x	x	
315	5-Chloro-7-iodo-8-hydroxy-quinoline	o	o	
316	Norfloxacin	x	x	
317	Sulfanilamide	x	x	
318	Trimethoprim	x	x	
319	Dichlofluanid	o	x	
320	Protamine sulfate	o	x	
321	Cetylpyridinium chloride	x	x	
322	1-Chloro-2,4-dinitrobenzene	o	x	
323	Diamide	x	x	
324	Cinoxacin	x	x	
325	Streptomycin	x	x	
326	5-Azacytidine	x	x	

327	Rifamycin SV	o	o	
328	Potassium tellurite	o	o	
329	Sodium selenite	o	x	
330	Aluminum sulfate	x	x	
331	Chromium chloride	x	x	
332	Ferric chlorade	x	x	
333	L-Glutamic-g-hydroxamate	o	x	
334	Glycine hydroxamate	o	x	
335	Chloroxylenol	o	x	
336	Sorbic acid	x	x	
337	D-Serine	x	x	**17**
338	β-chloro-L-alanine hydrochloride	x	x	
339	Thiosalicylic acid	x	x	
340	Sodium salicylate	o	o	
341	Hygromycin B	x	x	
342	Ethionamide	x	x	
343	4-Aminopyridine	x	x	
344	Sulfachloro-pyridazine	x	x	
345	Sulfamono-methoxine	x	x	
346	Oxycarboxin	o	x	
347	3-Amino-1,2,4-triazole	x	x	
348	Chlorpromazine	o	x	
349	Niaproof	x	x	
350	Compound 48-80	x	x	
351	Sodium tungstate	x	x	
352	Lithium chloride	x	x	
353	DL-Methionine hydroxamate	o	x	
354	Tannic acid	x	x	
355	Chlorambucil	o	x	
356	Cefamandole nafate	x	x	
357	Cefoperazone	x	x	
358	Cefsulodin	x	x	
359	Caffeine	x	x	
360	Phenylarsine oxide	x	x	
361	Ketoprofen	o	o	**18**
362	Sodium pyrophosphate decahydrate	x	x	
363	Thiamphenicol	o	x	
364	Trifluorothymidine	x	x	
365	Pipemidic Acid	x	x	
366	Azathioprine	x	x	

367	Poly-L-lysine	x	x	
368	Sulfisoxazole	o	x	
369	Pentachlorophenol	x	x	
370	Sodium m-arsenite	x	x	
371	Sodium bromate	x	x	
372	Lidocane	x	x	
373	Sodium metasilicate	x	x	
374	Sodium m-periodate	x	x	
375	Antimony (III) chloride	x	x	
376	Semicarbazide	x	x	
377	Tinidazole	o	x	
378	Aztreonam	x	x	
379	Triclosan	x	x	
380	3,5-Diamion-1,2,4-triazole (Guanazole)	o	x	
381	Myricetin	x	x	
382	5-fluror-5'-deoxyuridine	x	x	
383	2-Phenylphenol	x	x	
384	Plumbagin	x	x	
385	Josamycin	o	x	**19**
386	Gallic acid	o	x	
387	Coumarin	x	x	
388	Methyltrioctyl-ammonium chloride	x	x	
389	Harmane	x	x	
390	2,4-Dinitrophenol	o	x	
391	Chlorhexidine	x	x	
392	Umbelliferone	x	x	
393	Cinnamic acid	o	x	
394	Disulphiram	x	x	
395	Iodonitro Tetrazolium Violet	x	x	
396	Phenyl-methyl-sulfonyl-fluoride (PMSF)	x	x	
397	FCCP	o	o	
398	D,L-Thioctic Acid	o	x	
399	Lawsone	x	?	
400	Phenethicillin	x	x	
401	Blasticidin S	x	?	
402	Sodium caprylate	o	x	
403	Lauryl sulfobetaine	x	?	
404	Dihydro-streptomycin	x	x	
405	Hydroxylamine	x	x	
406	Hexammine cobalt (III) chloride	x	x	

407	Thioglycerol	x	x
408	Polymyxin B	x	x
409	Amitriptyline	o	x
410	Apramycin	x	x
411	Benserazide	x	x
412	Orphenadrine	o	x
413	D,L-Propranolol	x	x
414	Tetrazolium violet	o	o
415	Thioridazine	x	o
416	Atropine	x	x
417	Ornidazole	o	x
418	Proflavine	x	x
419	Ciprofloxacin	x	x
420	18-Crown-6 ether	x	x
421	Crystal violet	x	x
422	Dodine	x	x
423	Hexa-chlorophene	o	x
424	4-Hydroxy-coumarin	o	x
425	Oxytetracycline	o	x
426	Pridinol	o	x
427	Captan	o	x
428	3,5-Dinitro-benzene	o	x
429	8-Hydroxy-quinoline	x	x
430	Patulin	o	x
431	Tolylfluanid	o	x
432	Troleandomycin	x	x

Appendix 4. Respiration data of PepN$^-$ versus wildtype for phenotype microarray plates 9-20. The pepN mutant data was assessed qualitatively by the observance of well color compared to previously collected wildtype data assessed quantitatively through kinetic graphs produced by Biolog's Omnilog instrumentation. Respiration was considered negative if 3 or more of an assays replicates were colorless. Positive for respiration is represented by "x" and negative for respiration is represented by "o". Blue outlines designate assays in which only wildtype respired. Red outlines designate assays in which only the *pepN* mutant strain respirated. Background color of red outlined rows correspond to category colors from Figure 7. Rows with a gray background designate assays with an indeterminate result.