VIRULENCE GENE EXPRESSION OF *VIBRIO PARAHAEMOLYTICUS* IN THE VIABLE BUT NONCULTURABLE STATE

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ABSTRACT

Virulence gene expression of *Vibrio parahaemolyticus* in the viable but nonculturable state

Vibrio parahaemolyticus is a food-borne pathogen commonly associated with the consumption of raw or undercooked seafood resulting in primary infections of the human gastrointestinal tract. It is estimated to cause about 4500 illnesses each year in the United States. However, infection from this food-borne pathogen can be avoided if this organism is detected in the implicated food, prior to consumption. Current standard methods of detecting this organism are dependent on the culturability of the bacteria. Detection based on an organism's culturability may be problematic as *V. parahaemolyticus* has been known to exist in a viable but nonculturable (VBNC) state. Bacteria in the VBNC state are characterized by low levels of metabolic activity and the inability to be cultured by standard laboratory practices. When bacteria enter the VBNC state, their gene expression profile may be different than the culturable counterpart. We were interested in comparing the expression of two virulence-associated genes between VBNC and culturable cells of *V. parahaemolyticus*. *V. parahaemolyticus* RIMD2210633 was incubated at 4°C in modified Morita mineral salt solution supplemented with 0.5% NaCl (MMS) or trypticase soy broth supplemented with 2% NaCl (TSBS), which represented nutrient poor and rich conditions, respectively. The number of VBNC and culturable cells were determined by standard plate count and fluorescence microscopy. The expression levels of virulence-associated genes *tdh2* and *escU,* were measured relative to the housekeeping gene, *pvsA*, by qRT-PCR. Nutrient availability and temperatures exerted variable effects on the virulence gene expression. It is possible that VBNC *V. parahaemolyticus* cells may retain their pathogenicity potential.

Keywords: *Vibrio parahaemolyticus*, viable but nonculturable, virulence gene expression

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I. Introduction

Vibrio parahaemolyticus is a halophilic, Gram-negative bacterium. As a natural inhabitant of estuarine environments, it is frequently found in marine organisms, including plankton, fish, and shellfish (Lopatek et al., 2011; Yeung and Boor, 2004). *V. parahaemolyticus* is a prevalent food-borne pathogen in Japan, Taiwan, and other coastal countries (Yeung and Boor, 2004). It primarily causes disease through the consumption of raw, undercooked, or contaminated seafood resulting in primary infections of the human gastrointestinal tract (Yeung and Boor, 2004). Symptoms include watery diarrhea, abdominal pain, nausea, vomiting, fever, and chills (Levin, 2006; Pazhani et al., 2014; Yeung and Boor, 2004). These signs usually occur within 24 hours of consumption and the illness is self-limited within three days (Chowdhury et al., 2013; Yeung and Boor, 2004).

Since the first United States FoodNet surveillance began in 1996, the incidence of *Vibrio* infections (0.51 per 1000,000 population) has continued to rise in 2013 as compared to 2010-2012 and 2006-2008 (Crim et al., 2014). Among the infections associated with the genus *Vibrio*, the majority was caused by *V. parahaemolyticus* (Crim et al., 2014). While most foodborne pathogens (i.e., *Listeria, Campylobacter,* and *Escherichia coli* O157) have shown a decrease in incidence from 1998 to 2008, *V. parahaemolyticus* has increased 47% within this time period (Banerjee et al., 2014). Additionally, many cases likely go unreported due to limitations of foodborne disease investigation and data acquisition (Scallan et al, 2011). Underreporting can be attributed by the lack of awareness of the disease, the self-limiting nature of the infection, and the lack of urgency to visit physicians (Banerjee et al., 2014).

Conventional isolation and detection methods of *V. parahaemolyticus* involve cultivating the bacteria followed by biochemical and molecular tests. These methods were used to determine the total number of *V. parahaemolyticus*, which were then used to estimate the numbers of the pathogenic subtype (Di et al., 2015; Malcolm et al., 2015). Molecular techniques such as gene-specific probe and PCR are increasingly used to detect pathogenic *V. parahaemolyticus* defined by the presence of certain virulence markers (Malcolm et al., 2015). A loop-mediated isothermal amplification method has been recently developed and has since been coupled with other molecular techniques (Di et al., 2015; Malcolm et al., 2015; Notomi et al., 2015). These newer DNA-based methods, in conjunction with the conventional biochemical tests often require the bacterial cells to be cultured on typical growth media first. Detection based on an organism's culturability can overlook a significant portion of *V. parahaemolyticus* cells as they have been found to exist in the viable but nonculturable (VBNC) state under some conditions.

Viable but Nonculturable (VBNC)

Bacteria in the VBNC state are viable but exhibit very low levels of metabolic activity (Jones et al., 2008; Roszak et al., 1984; Shleeva et al., 2004). VBNC, known as "conditionally viable environmental cells (CVEC)" in some literature for *V. cholerae*, do not form colonies in common laboratory growth media since they are incapable of undergoing sustained cellular division, and hence, cannot be propagated using routine laboratory practices employing culturing techniques (Baffone et al., 2003; Jones et al., 2012a; Jones et al., 2012b; Oliver and Bockian, 1995; Vora et al., 2005). Under unfavorable conditions such as low temperature and low nutrients, Gram-negative bacteria were found to persist in the VBNC state, possibly as a survival mechanism

(Colwell et al., 1996). The leading hypothesis suggests that this may be an adaptive strategy for long-term survival of bacteria under unfavorable environmental conditions. For example, some bacteria in the VBNC state form novel starvation and cold shock proteins (Oliver, 2005). VBNC *V. parahaemolyticus* cells are more resistant to acidity allowing them to survive in lower pH conditions (Wong and Want, 2004). Various Gram-negative bacteria have also been shown to express virulence genes in the VBNC state (Fischer-Le Saux et al., 2002; Nilsson et al., 2002; Yaron and Matthews, 2002). Yaron and Matthews (2002) found that a variety of genes, including the virulence gene *stx1* (encodes Shiga toxin 1), were expressed in VBNC *E. coli* O157:H7. Since virulence gene expression had been detected in other VBNC Gram-negative bacteria, it is therefore likely that *V. parahaemolyticus* could express its major virulence gene in the VBNC state. On the other hand, VBNC cells exist in a state typically perceived to have low metabolic activity; thus, it is intriguing that VBNC cells would express energetically expensive virulence genes. To reconcile this difference, we speculated that some virulence genes may be essential for survival *ex vivo*.

VBNC induction: environmental factors

The VBNC state was initially discovered in *Salmonella* Enteritidis in 1984 by Roszak (Coutard et al., 2005; Roszak et al., 1984). Since then, over 60 species were confirmed to have the ability to enter the VBNC state, including a large number of human pathogens (Oliver, 2005). Many *Vibrio* species were found to enter the VBNC state as a response to stresses related to temperature, salinity, and nutrient. For instance, *V. harveyi* SF1 entered into the VBNC state upon incubation in seawater at 4°C for 60 days (Jia et al., 2013). Ramaiah et al (2002) reported that *V. harveyi* ATCC 14126 and *V. fischeri* UM1373 could enter the VBNC state in response to both low nutrient (artificial

sea water, ASW, to impose carbon starvation condition) and different salinities (35 ppt and 10 ppt) at 22°C. Other studies showed that low temperature alone was the main factor of VBNC induction for *V. parahaemolyticus* (Jiang and Chai, 1996; Mizunoe et al., 2000; Wong et al., 2004). However, Coutard et al (2005) showed both decreased temperature and low nutrient availability were needed. In this previous study, *V. parahaemolyticus* Vp4 could not reach the VBNC state when cells were maintained in ASW at room temperature for over 21 days, but VBNC cells were induced when downshifting the temperature to 4°C for 42 days.

Virulence genes in *Vibrio parahaemolyticus*

Known and putative virulence factors are identified for *V. parahaemolyticus*. The widely recognized virulence factor, thermostable direct hemolysin (TDH), is almost exclusively found in clinical isolates (Miyamoto et al., 1969; Wong et al., 2004; Yeung and Boor, 2004). Thus, it is believed to be a major contributor to the pathogenesis (Makino et al., 2003). TDH is a pore-forming toxin composed of 165 amino acid residues (Park et al., 2004). It alters ion flux on intestinal cell membranes and ultimately results in diarrhea (Takahashi et al., 2000; Yeung and Boor, 2004). TDH is an enterotoxin whose action is mediated by intracellular calcium (Raimondi et al., 2000). This toxin has been demonstrated to increase fluid accumulation in rabbit ileal mucosa, which can result in secretory diarrhea in the intestine (Nishibuchi et al., 1992). Rabbit ileal mucosal tissue inoculated with TDH-positive strains stimulated a gradual production of enterotoxins measured in Ussing chambers 20 to 80 minutes post-inoculation as compared to those inoculated with a TDH-negative strain (Nishibuchi and Kaper, 1995). TDH is encoded by *tdh* gene(s), which are embedded in the pathogenicity island on one of the two chromosomes (chromosome 2) in *V. parahaemolyticus* RIMD2210633 (Makino et al.,

2003). There are five variants of the *tdh* gene (*tdh1* to *tdh5*) sharing >97% similarity (Baba et al., 1991). Of those genes, only *tdh1* and *tdh2* appear to be responsible for the beta-hemolysis on Wagatsuma blood agar, known as the Kanagawa phenomenon (KP) (Nishibuchi et al., 1992; Nishibuchi and Kaper, 1995). The *tdh2* gene accounted for >90% of the total TDH protein production and the *tdh1* gene accounted for about 9% (Nishibuchi and Kaper, 1995). The high expression level of *tdh2* relative to *tdh1* (and the other *tdh* genes) is due to differences in the basal-level production of mRNA and in the degree of transcriptional activation by ToxRS, a global regulator of many *V. parahaemolyticus* genes (Nishibuchi and Kaper, 1995).

The other putative virulence factor is the type III secretion system (T3SS), which was discovered from a pathogenic *V. parahaemolyticus* O3:K6 strain following genome sequencing (Makino et al., 2003). T3SS is thought to be triggered when the bacteria come in close contact with the host. Effector proteins, such as VopT (involved in cytotoxicity) and VopZ (enables intestinal colonization and diarrheagenesis) are injected directly into host cells via its needle-like structures spanning the bacterial inner and outer membrane (Kumar et al., 2014; Zhou et al., 2013). T3SS is found in both chromosomes of *V. parahaemolyticus*: abbreviated as T3SS1 (on chromosome 1) and T3SS2 (on chromosome 2) (Makino et al., 2003). T3SS1 is found in many *V. parahaemolyticus* strains, while T3SS2 is exclusively present in the TDH-producing strains (Makino et al., 2003; Park et al., 2004). Therefore the presence of T3SS2 is strongly related to the pathogenicity of *V. parahaemolyticus* to humans. T3SS2 promotes bacterial cell invasion, inactivation of the host immune pathway, and disruption of the gut epithelial barrier (Ritchie et al., 2012; Zhang et al., 2012; Zhou et al., 2013), resulting in enterotoxicity in infant rabbit models (Ritchie et al., 2012). T3SS1 is associated with killing host cells through autophagy, membrane blebbing, and finally cell lysis (Burdette

et al., 2008). *V. parahaemolyticus* mutants lacking T3SS2 exhibited poorer survival in the presence of the predator, marine bacterivorous protists, than mutants lacking TDH or T3SS1 (Matz et al., 2011). This implied T3SS2 plays a key role in environmental fitness of *V. parahaemolyticus*. The seven protist species exhibited positive growth in the presence of T3SS1 or T3SS2 defective mutants. On the other hand, the wild types kill 50% of the initial protist population in as few as 12±1.6 hours (Matz et al., 2011). Therefore, some virulence genes of *V. parahaemolyticus*, particularly T3SS, likely play a role in the environmental fitness (Jones et al., 2012a).

Virulence gene expression in VBNC state

Entering into the VBNC state may represent an adaptive response of the bacteria that are under conditions when metabolism is significantly compromised (Shleeva et al., 2004). Bacteria in the VBNC state are reported to have a lower metabolic rate. VBNC cells of *Micrococcus luteus* showed weak fluorescence signal after staining with Rhodamine-123, indicating poor membrane energization (Kaprelyants and Kell, 1992). The endogenous respiratory rate of VBNC *Mycobacterium smegmatis* was negligible when measured polarographically (Shleeva et al., 2004). Despite exhibiting low metabolic activity, some VBNC Gram-negative bacteria have been shown to express virulence factors. VBNC *Helicobacter pylori* expressed key toxins, VacA (vacuolating cytoxin A) and UreA (urease A), when cells became VBNC after starvation for >28 months (Nilsson et al., 2002). Temperature (4^{o}C) stressed VBNC *E. coli* expressed shiga-like toxin 1 gene, *stx1*, as detected by RT-PCR (Yaron and Matthews, 2002). VBNC *V. vulnificus* expressed its cytotoxin-hemolysin virulence gene, *vvhA* (Fischer-Le Saux et al., 2002; Vora et al., 2005). Since these Gram-negative bacteria have been shown to express virulence markers or genes when the cells are in VBNC state, VBNC

V. parahaemolyticus would also likely express virulence-associated genes. Current knowledge regarding virulence gene expression of VBNC *V. parahaemolyticus* is limited and contradictory. The most recognized virulence gene, *tdh*, was not detected via RT-PCR by Coutard et al (2005), but was detected via microarray by Vora et al (2005). Most studies investigating VBNC *V. parahaemolyticus* cells and their virulence tend to focus on gene expression after resuscitation, i.e., after VBNC cells return to culturable state, instead of during the VBNC state. Not surprisingly, VBNC cells may resuscitate and regain virulence under suitable conditions in the human gastrointestinal tract (Oliver and Bockian, 1995). *V. harveyi* was shown to retain pathogenic potential following a lethal inoculation (2.8×10^4 CFU/mL) of VBNC cells in zebra fish after 7 days (Sun et al., 2008). Oliver and Bockian (1995) demonstrated that VBNC *V. vulnificus* retained its pathogenicity after resuscitation within 24 hours in the peritoneal cavity of mice resulting in lethality. VBNC *V. parahaemolyticus* and *V. alginolyticus* cells were inoculated intragastrically and resuscitated in rat ileal loop assays, where the cells were able to reactivate their pathogenic potential within 48 hours and to cause disease (Baffone et al., 2003). With much of the focus on virulence gene expression after resuscitation, *V. parahaemolyticus* virulence gene expression in the VBNC state remains insufficiently understood.

It is expected that virulence genes are normally expressed when the pathogen resides in conditions resembling the host environment. Stronger *tdh* expression in *V. parahaemolyticus* was detected at 37°C compared to 28°C (Mahoney et al., 2010). *V. parahaemolyticus* also displayed greater cytotoxicity to CaCo-2 cells at 37°C than 28°C (Mahoney et al., 2010). Although the expression of virulence genes by culturable *V. parahaemolyticus* under normal conditions has been well studied, there is conflicting

data on the virulence of *V. parahaemolyticus* in the VBNC state as mentioned above. In a study by Coutard et al (2005), neither virulence gene, *tdh1* nor *tdh2,* was detected by RT-PCR in *V. parahaemolyticus* Vp4 when it reached VBNC state after 42 days maintained in ASW at 4°C. The cells were monitored over time and the VBNC state was reached when viable cells, confirmed with fluorescence microscopy, yielded less than one CFU on heart infusion (0.5% NaCl) agar from 20 mL of ASW cell suspension. In contrast, Vora et al (2005) demonstrated expression of virulence genes, *tdh* and VP1696 (*yscC*; a T3SS1 marker), in VBNC *V. parahaemolyticus* O3:K6 (F5828). They used temperature stress (4°C for 76 days) in ASW (1% salinity) to induce the cells into VBNC. They defined the cells were in the VBNC state when <0.3 CFU/mL was yielded on tryptic soy agar. To detect viability and virulence, they combined an oligonucleotide microarray with a modified amplification protocol to target mRNA. They acknowledged a contradiction with Coutard et al (2005), attributing the discrepancies to experimental factors (e.g., assay sensitivity, VBNC induction period) and biological variations (strain, gene copy number, regulation of expression). Interestingly, Coutard et al (2007b) conducted another study using a different strain, Vp5, and detected the presence (but not upregulation) of virulence genes, *tdh2* and *escU.* The latter gene encodes inner membrane proteins of T3SS2. Interestingly, the VBNC state was reached after merely 15 days following inoculation at 4°C in ASW (Coutard et al., 2007b). This newer study, however, focused on recovery or resuscitation of VBNC cells. Resuscitation of bacteria require the cells to be reintroduced to their favorable conditions, where stress is no longer a factor, thus altering their gene expression profiles. Taken together, there are very limited studies on virulence gene expression of *V. parahaemolyticus* in the VBNC

state, in which cells are under stressful conditions. The virulence gene expression profile likely shows a marked difference than culturable cells or cells after resuscitation.

Hypothesis and Objectives

Many Gram-negative bacterial cells enter the VBNC state when they are stressed. Similarly, the food-borne pathogen, *V. parahaemolyticus*, enters the VBNC state upon encountering low temperatures and low nutrients. VBNC cells are thought to exhibit low metabolic activity; hence, genes that are nonessential for basic survival are expected to be downregulated or not expressed at all (Oliver, 2005). Although previous studies showed some virulence genes were expressed in the VBNC cells of other Gram negative bacteria, expression levels of TDH and T3SS of *V. parahaemolyticus* was found to be temperature-dependent (Matz et al., 2011). As virulence gene expression can be energetically expensive, we hypothesized that VBNC *V. parahaemolyticus* cells – usually maintained at low temperature – decrease expression for *tdh* and T3SS-related genes. Specifically for this study, VBNC *V. parahaemolyticus* cells were predicted to decrease *tdh2* and *escU* expression, when they were subjected to stressful conditions of cold temperatures and limited nutrient availability. The objectives of this study were to induce *V. parahaemolyticus* into VBNC state, and to compare the relative expression of these two genes between VBNC and culturable *V. parahaemolyticus*. To this end, *V. parahaemolyticus* cells were subjected to different temperature and growth media for varying amount of time. Enumeration methods were optimized to quantify viable cells – culturable and nonculturable. Total and nascent RNA was extracted from cells subjected to different treatments, followed by conversion to cDNA, and then qRT-PCR. A housekeeping gene, *pvsA*, was used to normalize qRT-PCR data. The expression level of *tdh2* and *escU* was compared to *pvsA*, yielding relative expression ratio (RER). In

addition to comparing VBNC with culturable cells, our experiments also shed light on the effects of environmental factors on expression of these genes in culturable cells.

II. Methods

Cultures and Media

A TDH-positive, *V. parahaemolyticus* serotype O3:K6 strain was isolated at the Kansai International Airport quarantine station in 1996 from a patient with traveler's diarrhea (Makino et al., 2003). The strain was named RIMD2210633. We acquired the strain from the Research Institute for Microbial Diseases, Osaka University. A complete genome sequence of this strain is available, showing this strain harbors *tdh2* and *escU*. The frozen stock, which was kept at -80°C, was subcultured on trypticase soy broth or agar supplemented with 2% NaCl (TSBS and TSAS, respectively) at least three times prior to all experiments. Both solutions were made with a TSB stock concentration of 30 g/L. Some cultures were also incubated in a defined medium named modified Morita mineral salt solution (MMS-0.5% NaCl). The composition was described by Jiang and Chai (1996), which consisted of 5 g NaCl, 0.8 g KCl, 5.6 g $MgCl_2$-$6H_2O$, 7.6 g $MgSO_4$-$7H_2O$, 0.9 mg $FeSO_4$-$7H_2O$, 1.54 g $CaCl_2$-$2H_2O$, 0.1 g Na_2HPO_4, 1.21 g Tris buffer (pH 7.8) in one liter of deionized water.

Experimental treatments

V. parahaemolyticus was simultaneously exposed to an array of culture and temperature conditions (Table 1). Cultures were subjected to combinations of nutrient conditions (TSBS or MMS-0.5% NaCl), temperature conditions (4, 25, or 37°C), and incubation times (1, 2, 7, or ≥14 days). Five separate trials were conducted.

Table 1. Media and incubation conditions in the experimental treatments.

Treatment	Medium	Temperature (°C)	Time
# 1	TSBS	4	≥14 days
# 2	TSBS	25	7 days
# 3	TSBS	37	7 days
# 4	MMS-0.5%NaCl	4	≥21 days
# 5	MMS-0.5%NaCl	25	7 days
# 6	MMS-0.5%NaCl	37	7 days
# 7	TSBS	25	2 days
# 8	TSBS → MMS-0.5%NaCl	37 → 4	1 day → 1 hr

Enumeration of viable cells – culturable or nonculturable

At various times, cultures were monitored by standard plate count (SPC) and viability stain followed by direct microscopic count (DMC). Viability was evaluated based on cell membrane integrity, which is a well-accepted criterion for distinguishing viable cells from dead cells. The LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit L7012 (Invitrogen, Grand Island, NY, USA) was used to determine cell viability (Figure 1). *V. parahaemolyticus* cells were considered to be in the VBNC state when <1 CFU was detected upon plating 100 μL on a nonselective medium (TSAS) while >99.99% cells are viable based on DMC method.

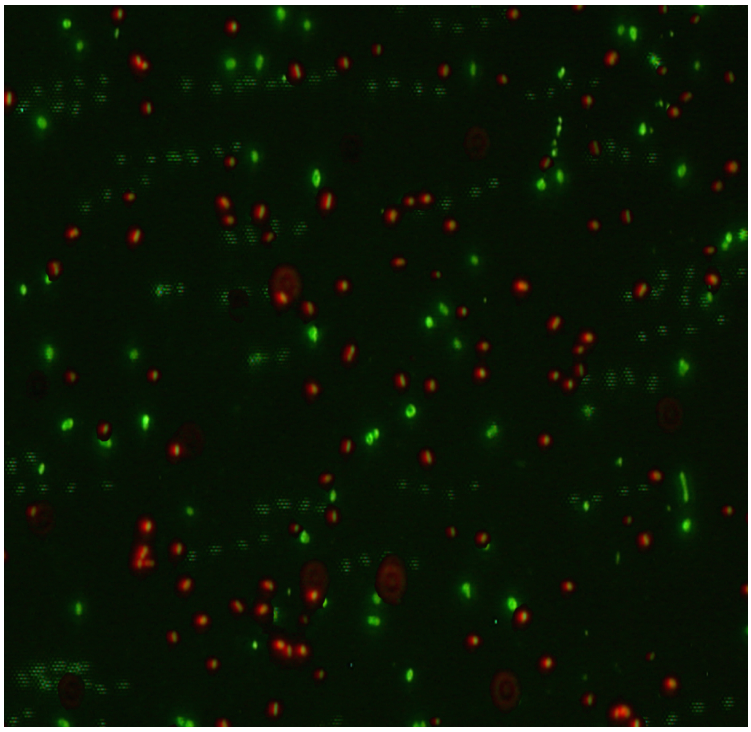

Figure 1. Fluorescent images of *V. parahaemolyticus* at 400X. The LIVE/DEAD® BacLight™ bacterial viability kit uses two fluorescent dyes based on the integrity of the bacterial membrane. Syto9 has excitation/emission maxima of 480/500 nm and stains all bacteria green. Propidium iodide has excitation/emission maxima of 490/635 nm and penetrates bacteria with damaged membranes. Longpass and dual emission filters were used for simultaneous viewing of both stains. The green cells represent those with an intact cell membrane, considered viable. Red or red-yellow cells represent those with an injured or compromised cell membrane, considered nonviable.

LIVE/DEAD® BacLight™ bacterial viability kit detects live cells with an intact membrane stained green by SYTO9 and dead cells with a damaged membrane stained red by propidium iodide. Using known amount of live and dead cells, our preliminary work showed that staining with a 1:1 ratio of Syto9:propidium iodide, as suggested by manufacturing protocols, yielded an overestimate of live cells. A 1:3 ratio of Syto9:propidium iodide was found to yield more accurate results. After 30 minutes of incubation in the dark with AntiQuench at room temperature, the stained samples were spread with 5 µL of 80% glycerol onto a 0.22 µm-pore size polycarbonate black filter overlaid with a 22 mm^2 cover slip. The samples were viewed at 400X in a dark room. A

filter cube encompassing the excitation/emission maxima of both stains was used to visualize both green and red cells simultaneously. Staining of the cells with Syto9 and propidium iodide simultaneously allows for the visualization of viable and nonviable cells based on fluorescent emissions. Green cells represent those with an intact cell membrane, considered viable. Red or red-yellow cells represent those with an injured or compromised cell membrane, considered nonviable. The viable and nonviable cells were counted from at least ten different fields of vision. The DMC/mL of viable and nonviable cells was determined by the following equation:

$$average\ number\ of\ cells\ per\ field\ of\ vision \times Microscopic\ Factor \times \frac{1}{dilution\ factor}$$

The Microscopic Factor (MF) was determined by dividing the area of the stain (22 mm^2) by the area of the field of vision (0.0043225 mm^2) and then multiplying it with the reciprocal of the volume of the stain (0.010 mL). Trial 1 and parts of Trial 2 contained 3 μL of BacLight dyes in 1 mL of sample, and was not further diluted in slide preparation. Thus, the dilution factor was 1000/1003. Parts of Trial 2, and all of Trials 3, 4, and 5 contained 3 μL of BacLight dye and 5 μL AntiQuench in 1 mL of sample. When spreading onto the membrane filter, 5 μL of sample was diluted further with 5 μL glycerol. Thus, the dilution factor was $\frac{125}{252}$.

The standard plate count was conducted to determine the culturability of cells. Ten-fold serial dilutions were made, followed by plating on TSAS. The CFU/mL was determined by dividing the average number of CFU per plate by the dilution factor of the plate. In addition, 0.1 mL of the undiluted sample was streaked on TSAS. The number of VBNC cells was the difference between culturable cell count (i.e., CFU/mL) and viable cell count (i.e., DMC/mL).

Preparation of template for qRT-PCR

RNA was extracted from all treatments using a TRIzol method with PureLink RNA Mini Kit (Invitrogen, Grand Island, NY, USA). One volume of DNase (10 μL DNase stock in 190 μL RDD buffer) was added to 1 volume of RNA extract, followed by DNase inactivation with 0.5 mM EDTA heat-treated for 10 minutes at 65°C. RNA and/or DNA were quantified spectrophotometrically.

One μg RNA extract was reverse transcribed to cDNA using 50 ng/μL random nonspecific hexamers from the commercial kit – SuperScript II First Strand Synthesis System for RT-PCR or SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Life Technologies, Carlsbad, CA, USA). RNase H was added to remove the RNA from the cDNA:RNA hybrid. RNA and ssDNA were quantified by NanoPhotometer (Implen GmbH, Muchen, Germany) to determine the purity of the cDNA.

Nucleic acid labeling

To confirm RNA was synthesized by viable cells, nascent RNA was labeled using the Click-iT® Nascent RNA Capture Kit prior to RNA extraction. Four milliliters overnight cultures and 4 mL VBNC cells (obtained from treatments #1-TSBS/4°C and #4-MMS/4°C) each were incubated overnight at 25°C with 5-ethynyl uridine (EU) at a final

concentration of 0.2 mM. EU was incorporated into the nascent RNA. Total labeled RNA was extracted with the same TRIzol method described above. The extract was then biotinylated to an azide-modified biotin, bound to Dynabeads® MyOne™ Streptavidin T1 magnetic beads, and washed thoroughly. These transcripts were quantified and used to construct cDNA as described above.

Primers and qRT-PCR conditions

Primers for the housekeeping and virulence-associated genes were selected according to Coutard et al (2007a) (Table 2). The gene, *pvsA*, was reported to have the most stable transcriptional expression levels among four housekeeping gene candidates (*rpoS*, *pvsA*, *fur*, and *pvuA*) in RT-PCR (Coutard et al., 2007b). Analysis for the stability of gene expression was evaluated with by geNorm software, resulting with *pvsA* and *pvuA* having the most stable transcriptional expression genes and a high level of regulation of the *rpoS* gene. Involved in the iron uptake pathway, *pvsA*, is responsible for the biosynthesis and transport of siderophore vibrioferrin in *V. parahaemolyticus* (Tanabe et al., 2003). Used as a positive control for the reverse transcription process and qRT-PCR, 1 ng of MS2 RNA (Roche Diagnostics, Indianapolis, Indiana) was included in most qRT-PCR runs to amplify *oco* gene (Coutard et al., 2007b). The two virulence genes, *tdh2* and *escU*, were the target genes.

Table 2. Primers used in this study (Coutard et al., 2007a)

Gene	Primer sequence (5' to 3')	Primer length (base)	G+C content (%)	Melt temp (°C)	Amplicon length (bp)
pvsA	F2-pvsA: CTC CTT CAT CCA ACA CGA T	19	47.4	58.3	104
	R2-pvsA: GGG CGA GAT AAT CCT TGT	18	50.0	58.4	
tdh2	F-tdh2: CAA CTT TTA ATA CCA ATG CAC	21	33.3	55.6	129
	R2-tdh2: GCC ATT TAG TAC CTG ACG	18	50.0	58.4	
escU	F1-escU: TAA CCC GAC ACA TAT TCT GG	20	45.0	58.4	163
	R-escU: CAT GGC TCT TGC TAA CGG	18	55.6	60.7	
MS2	Oco-1: GCT CTG AGA GCG GCT CTA TTG	21	57.1	65.3	69
RNA	Oco-2: CGT TAT AGC GGA CGT	18	61.1	63.0	

In most trials, RNA extract or cDNA was normalized to similar concentration prior to qRT-PCR. The cDNA template and primer set was mixed with reagents from a commercial kit, DyNAmo ColorFlash SYBR Green qPCR kit (Thermo Fisher Scientific, Waltham, WA, USA). cDNA template and 0.3 µM of each primer were added to the kit's master mix which contained hot-start version of a modified *Thermus brockianus* DNA polymerase, SYBR® Green I, PCR buffer, $MgCl_2$ (2.5 mM final conc) and dNTP mix. Each qRT-PCR reaction was performed in 20 µL running on 7300 Real-Time PCR system (Applied Biosystems). The thermal cycling conditions were adapted from (Coutard et al., 2007b): a denaturation program (95°C for 10 min), an amplification program repeated 40 times (95°C for 15 s and 60°C for 1 min), and a melting-curve program (55°C to 95°C). Positive control (MS2) and negative controls (no templates and RNA from treatment cells) were included in each run. All standards, controls, and samples were run in triplicate wells in each microplate. Standard curves for each gene (*pvsA, tdh2, escU*) were constructed using DNA extract of 10-mL overnight *V. parahaemolyticus* cultures. A 5-fold dilution was performed, with the highest DNA concentration adjusted to be 200 ng/µL. The standard curve slopes ranged from -3.1 to -3.5, which corresponded to 90-110% PCR efficiency.

Relative quantification and statistical analysis of virulence genes

The expression of *tdh2* and *escU* was determined relative to that of *pvsA* according to (Pfaffl, 2001). The following relative expression ratio (RER) equation was calculated for each gene of interest by using a mathematical model as described by (Pfaffl, 2001). This method is commonly used to investigate the physiological changes in gene expression (Coutard et al., 2007b; Delcenserie et al., 2012).

$$Relative\ expression\ ratio\ = \frac{(E_{target})^{\Delta C_{T(target)}}}{(E_{ref})^{\Delta C_{T(ref)}}}$$

Real-time PCR efficiency (E) was calculated as $10^{(-1/slope)}$. E_{ref} was the reference, or housekeeping, gene, *pvsA*. E_{target} was either of the target genes, *tdh2* and *escU*. The slope is derived from the standard curve. $\Delta C_{T(target)}$ is the difference between two treatments of a single virulence gene. $\Delta C_{T(reference)}$ is the difference between two treatments of the reference (i.e., housekeeping) gene. Thus, RER indicated up- or down-regulation of virulence gene in certain treatment (such as #1 or #4 that yielded VBNC) relative to control (such as #8 that yielded culturable cells).

III. Results

Conditions to obtain VBNC cells

Bacterial cells from all eight treatments were enumerated on the initial and final days of treatment. On the initial days of treatment, SPC and DMC (viability stain) were performed. On the final days of treatment, both enumeration methods and RNA extraction were performed. In all treatments, virtually 100% of cells were culturable on the day of the experiment (Table 3). As described in Chapter II, viability of the cells was assessed by using membrane permeable (Syto9) and impermeable (PI) stains. The injured cells appeared as red or reddish yellow, thus were counted as nonviable cells. Indeed, viable cells consist of VBNC cells, as well as injured cells that are often nonculturable on selective media (Ducret et al. 2014). It was expected that injured cells would be formed following exposure to some treatments. These cells, however, should grow on non-selective media at typical incubating temperature. In our experiments, CFU results were obtained by plating all samples on non-selective, complex medium TSAS followed by incubation at 35^{o}C for ~48 h. These plates were further incubated at room temperature for additional 3-5 days before they were disposed of. The *V. parahaemolyticus* strain used in this study can grow at both temperatures. We did not detect additional CFU after the incubation period at room temperature. Specifically, no CFU was observed from treatments that yielded VBNC cells. Therefore, the presence of injured cells was insignificant and gene expression of these cells, if any, was negligible.

Table 3. Means and SDs of SPC and DMC of viable and nonviable cells on Day=0 and on the final day (i.e., day of RNA extraction). Five replicates were conducted per treatment.

Treatment	Day 0		Final Day	
	CFU/mL	DMC/mL of viable cells	CFU/mL	DMC/mL of viable cells
#1 trials 1 to 3 (TSBS, 4°C, 14 d)*	(5.1 ± 1.6) x10^8	(4.1 ± 1.3) x10^8	(8.3 ± 1.4) x10^7	(4.6 ± 2.2) x10^8
#1 trials 4 and 5 (TSBS, 4°C, 25 d)	(8.2 ± 3.6) x10^8	(2.9 ± 2.6) x10^8	<10	(1.3 ± 0.3) x10^8
#2 (TSBS, 25°C, 7 d)	(2.9 ± 2.2) x10^8	(3.1 ± 2.2) x10^8	(6.0 ± 1.2) x10^6	(3.8 ± 3.2) x10^8
#3 (TSBS, 37°C, 7 d)	(4.3 ± 1.4) x10^8	(4.0 ± 1.2) x10^8	(1.0 ± 0.6) x10^7	(6.0 ± 4.2) x10^8
#4 (MMS, 4°C, ≥21 d)	(5.2 ± 1.7) x10^8	(4.7 ± 1.3) x10^8	<10	(4.5 ± 2.2) x10^8
#5 (MMS, 25°C, 7 d)	(3.9 ± 2.8) x10^8	(4.1 ± 1.2) x10^8	(8.4 ± 6.9) x10^6	(4.9 ± 4.4) x10^8
#6 (MMS, 37°C, 7 d)	(4.7 ± 1.9) x10^8	(2.7 ± 1.8) x10^8	(9.7 ± 1.3) x10^6	(3.8 ± 2.7) x10^8
#7 (TSBS, 25°C, 2 d)	(4.7 ± 4.1) x10^8	(2.7 ± 0.6) x10^8	(7.9 ± 7.3) x10^7	(4.9 ± 0.9) x10^8
#8 (TSBS, 37°C, 1 d → MMS, 4°C, 1 h)	(6.6 ± 3.8) x10^8	(3.5 ± 1.8) x10^8	(3.7 ± 2.3) x10^7	(1.7 ± 1.7) x10^8

*The first three trials of treatment #1 did not yield VBNC cells.

As shown in Table 3, a loss of culturability was observed over time, especially when cells were incubated at 4°C. *V. parahaemolyticus* cells were defined to have reached VBNC state when no CFU was obtained after 100 μL was inoculated on TSAS, and thus <10 CFU/mL was culturable, whereas >99.99% of the cell population exhibited green fluorescence. Among the five trials in treatment #1 (TSBS at 4°C), the first three did not yield VBNC cells following 14 days of incubation. After maintaining the cells in the medium for 25 days, VBNC cells were obtained in trials 4 and 5. On the other hand, all trials in treatment #4 (MMS at 4°C) yielded VBNC cells. Therefore, we successfully obtained >99.99±0.68% VBNC cells by maintaining *V. parahaemolyticus* in MMS (all trials in treatment #4) or TSBS (two trials in treatment #1) at 4°C for ≥21 days (Figure 2).

The number of viable cells as detected by DMC was fairly consistent between the initial and the final day of treatment.

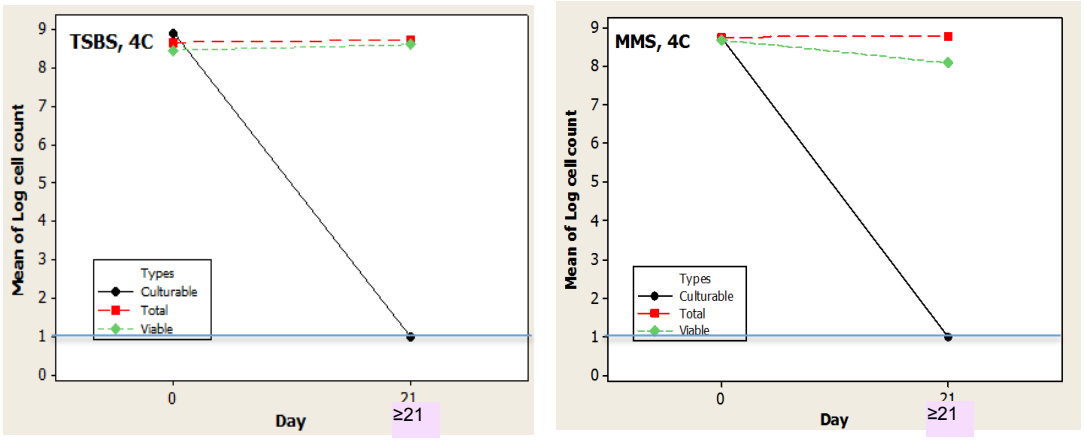

Figure 2. Entry of *V. parahaemolyticus* into the VBNC state in different media maintained at 4°C. The culturable cells (•) were enumerated by standard plate count on TSAS. Viable (♦) and nonviable cells were enumerated by direct microscopic method following viability staining. Total count (■) includes viable and nonviable cells. The blue solid line represents the detection limit of the SPC method (10 cfu/mL).

As the other treatments did not yield sufficient amount of VBNC cells, qRT-PCR data from these treatments were not used to determine gene expression of VBNC cells. Nevertheless, they were used to examine the effects of temperature and nutrient availability on gene expression of culturable cells.

qRT-PCR using EU-labeled RNA

As cells in the VBNC state exhibit low metabolic activity and thus are believed to transcribe only genes necessary for immediate survival, we wanted to confirm that the virulence genes detected were *de novo*. Therefore, we focused on the two treatments (#1 and #4) that resulted in cells successfully reaching the VBNC state (i.e., TSBS at 4°C and MMS at 4°C) to test for newly synthesized RNA. After confirming cells are in

VBNC state, samples (10^9 cells) were incubated with an analog of uridine, EU, which was incorporated into the newly synthesized RNA. This EU-labeled RNA was then isolated and quantified spectrophotometrically. The mean±SD RNA concentration for treatment #1 (TSBS) and treatment #4 (MMS) was 27.5±1.7 ng/μL and 27.7±7.0 ng/μL, respectively (*n*=3). The purity was good because A260/A280 of these six samples of labeled-RNA extracts was no lower than 1.8. In comparison, a culture grown in TSBS at 25°C for 24 h had an RNA concentration of 49 ng/μL and A260/A280 of ~2.0. A negative control using only dead cells was unfortunately not included in this analysis. These results tentatively suggest that VBNC cells were active in expressing RNA at about half the rate of overnight culturable cells. The next step was to convert these labeled-RNA extracts into cDNA, normalized to ~200 ng/μL, prior to qPCR.

As shown in Figure 3, MMS-induced VBNC cells yielded Ct values of 38.6±0.3, 36.3±0.1, and 36.4±1.2 for *pvsA, tdh2,* and *escU,* respectively. TSBS-induced VBNC cells yielded Ct values of 37.2±0.3, 35.8±1.2, and 36.1±1.5 for *pvsA, tdh2,* and *escU,* respectively. The two negative controls (no templates and no RT) were both undetected (i.e., no amplification). These preliminary results suggested that VBNC cells expressed these genes, if any, in small quantities.

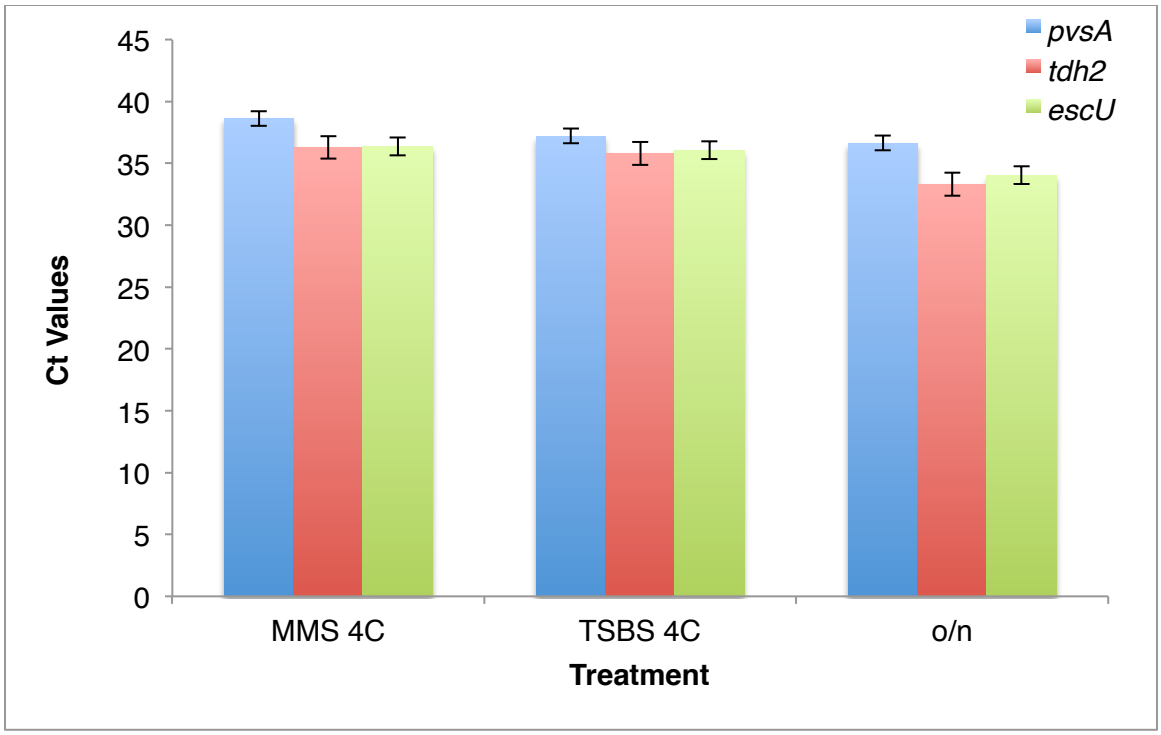

Figure 3. Mean Ct values of nascent RNA from VBNC cells and overnight cultures. VBNC cells were induced after maintaining *V. parahaemolyticus* in MMS or TSBS at 4°C for ≥21 days. Overnight (o/n) cultures were prepared by incubating the same strain in TSBS at 25°C for 24 h. Newly synthesized RNA was used in the qRT-PCR to detect for the expression of two virulence markers (*tdh2* and *escU)* and a housekeeping gene (*pvsA)*.

On the other hand, after comparing the Ct values between different types of culturable cells, it appeared that labeled RNA was not a good template for qRT-PCR. Overnight cultures yielded Ct values of 36.6±0.3, 33.3±0.2, and 34.1±0.2 for *pvsA, tdh2,* and *escU,* respectively. The overnight cultures used in this qRT-PCR were 24 h-old cultures, which yielded good amount of growth as reflected by the turbidity of the broth. The Ct values from these cultures, however, were much higher than those obtained using total RNA extracts of similar cultures grown for 48 h instead of 24 h (treatment #7). These 48-h old cultures yielded Ct values of 24.6±4.0, 23.3±3.9, and 24.7±6.9 for *pvsA, tdh2,* and *escU,* respectively (Fig 5).

The experimental melting temperatures for the *pvsA*, *tdh2*, and *escU* amplicons showed target melting temperatures at 79.2, 77.0, and 78.5°C, respectively. Using the uMELT[SM] Melting Curve Predictions Software (https://www.dna.utah.edu/umelt/umelt.html#), the predicted melting temperatures for these genes are 80.0, 78.5, and 79.5°C. The prediction was based on the assumption that the concentration of monovalent ions in the PCR reaction was 20 mM, which might be different than the actual concentration.

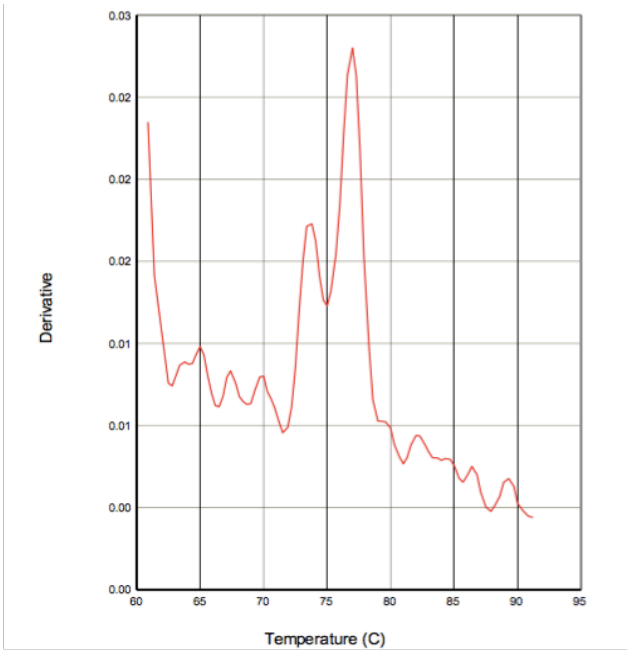

Figure 4. Derivative melting curve for *tdh2* using EU-labeled RNA. The profile was obtained using TSBS-induced VBNC cells of *V. parahaemolyticus*. The peaks to the left of 65°C are likely associated with primer-dimers or other non-specific amplification as they were present in all melting curve analyses. The peak at ~77°C was expected for *tdh2*. The additional peak at ~74°C was only seen in qRT-PCR using labeled RNA.

However, unlike qRT-PCR using total RNA extracts, the derivative melting curve using nascent RNA sometimes showed additional peaks (Figure 4) suggesting that 1) there might be non-specific amplification, or 2) the labeled RNA affected the cDNA products resulting in a different melting profile. Together with higher than expected Ct

values, the EU-labeled RNA likely was not a good template for qRT-PCR. Consequently, total RNA extracts were used in further qRT-PCR experiments.

Expression of virulence factors in VBNC cells

Owing to questionable template quality of the EU-labeled RNA, total RNA of VBNC and culturable cells were extracted to determine the expression levels of virulence associated genes relative to the housekeeping gene under various conditions. Total RNA may contain residual RNA in addition to nascent RNA (Table 4). RNA was not quantified in the first trials. Concentrations of the cDNA template were normalized in the last two trials.

Table 4. Concentration and purity of nucleic acid from all treatment.

	Trial 2*		Trial 3*		Trial 4**		Trial 5**	
	ng/μL	A260/ A280	ng/μL	A260/ A280	ng/μL	A260/ A280	ng/μL	A260/ A280
#1 (TSBS, 4°C, 25 d)	290	2.0	190	2.0	201	1.9	201	1.9
#2 (TSBS, 25°C, 7 d)	547	2.0	492	1.9	203	1.8	199	1.9
#3 (TSBS, 37°C, 7 d)	188	1.8	165	2.0	196	1.9	201	1.9
#4 (MMS, 4°C, ≥21 d)	254	2.0	278	1.9	203	1.9	206	1.9
#5 (MMS, 25°C, 7 d)	74.5	1.9	51	1.9	203	1.9	206	1.9
#6 (MMS, 37°C, 7 d)	78.4	2.0	70.6	1.9	201	1.8	206	1.9
#7 (TSBS, 25°C, 2 d)	171	2.0	406	2.0	203	1.8	199	1.8
#8 (TSBS, 37°C, 1 d → MMS, 4°C, 1 h)	457	2.0	751	1.9	199	1.9	206	1.9

* Concentrations of trials 2 and 3 are for RNA.
** Concentrations of trials 4 and 5 are for cDNA.

Checking for data quality

The means and standard deviations of Ct for all genes were shown in Figure 5. All qPCR runs (15 total) included a positive control and two negative controls, which are no template and RNA as template (Appendix B). These controls helped us determine the qPCR run performance and if there was a contamination issue. There was an occasion where one microplate was likely contaminated due to the Ct values of negative controls being slightly lower than Ct values of the genes. These Ct values were eliminated prior to RER analyses. In most of the qPCR runs, the triplicate wells returned similar Ct values, which were expected. In a few occasions, one well returned "undetermined" which was likely due to pipetting error. To verify that elimination of this replicate well would not affect the conclusion, we compared results from RER analyses that did or did not include this undetermined Ct. For the analysis that included this well, a Ct of 45 was assumed to enable calculation. The presumed number was chosen because some negative controls yielded Ct of ~45. The RER results were either no change or only differed minimally between these analyses. In a few instances the RER varied but the overall conclusion remained unchanged. For example, when comparing treatment #6 vs #5 (MMS, 37^{o}C vs 25^{o}C), *tdh2* was upregulated 20.8 fold at 37^{o}C when the RER analysis included the assumed number of 45. Eliminating the undetermined Ct value from the analysis (i.e., did not use assumption) yielded an upregulation of 5.2 fold instead.

Ideally the initial template concentrations should be similar across all qPCR runs. Otherwise, a treatment that generates higher amount of cells (and thus initial template) would likely produce a lower Ct value for the amplicon than a treatment that has a lower template yield, even when in fact the former treated cells do not upregulate the

expression of the target gene. In this study, cDNA was normalized for the last two trials of all treatments. After closely examining all data, no particular trend was identified between these two trials and other trials using varying amount of templates. On the other hand, it appeared that expression of all genes was affected by the treatments (Figure 5).

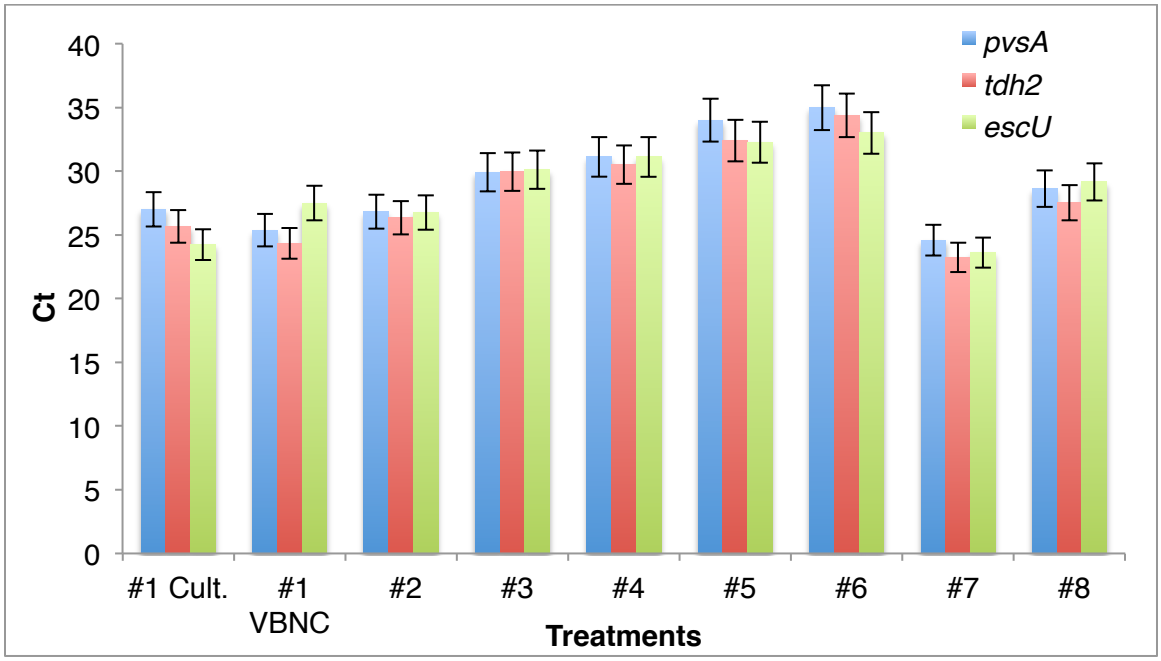

Figure 5. Mean Ct values of the housekeeping gene, *pvsA*, and virulence genes, *tdh2* and *escU*, from the various treatments. Treatment #1(TSBS, 4°C, 14-25d) is divided into trials 1 to 3, and 4 to 5, because the first three trials did not yield VBNC cells. Treatment #2 (TSBS, 25°C, 7d). Treatment #3 (TSBS, 37°C, 7d). Treatment #4 (MMS, 4°C, ≥21d). Treatment #5 (MMS, 25°C, 7d). Treatment #6 (MMS, 37°C, 7d). Treatment #7 (TSBS, 25°C, 2d). Treatment #8 (TSBS, 37°C, 24h → MMS, 4°C, 1h).

In addition to Ct values, specific amplification of the genes was indicated by melting curve analysis that was performed in each qPCR run (Figure 6). Amplification of a single target gene usually exhibits one peak in a derivative melting curve. As mentioned in the previous section, the empirical melting temperatures for the *pvsA*, *tdh2*,

and *escU* amplicons were found to be 79.2, 77.0, and 78.5°C, respectively. These numbers are very close to the predicted ones.

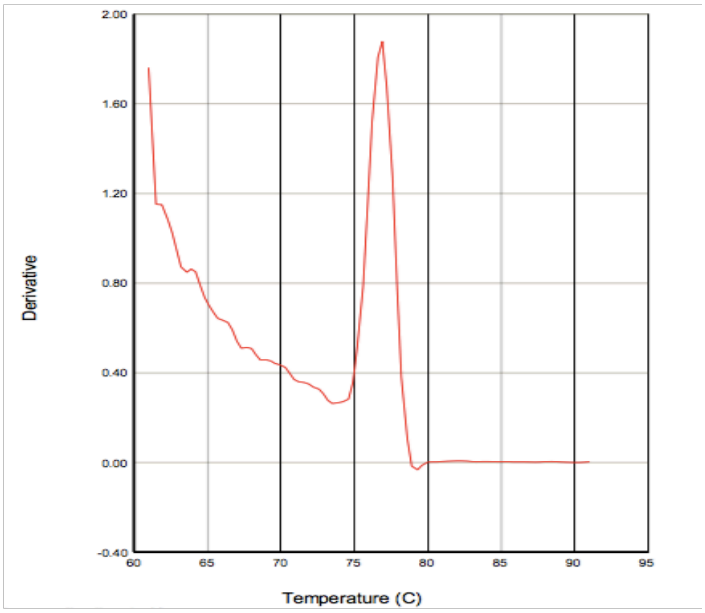

Figure 6. A representative derivative melting curve of *tdh2* using total RNA.

A standard curve was included in each qPCR run using DNA from overnight cultures of *V. parahaemolyticus*. The average slope of *pvsA*, *tdh2*, and *escU* was -3.17, -3.19, and -3.31. The range was -3.6 to -3.1 representing 90-110% amplification efficiency. The average R^2 of *pvsA*, *tdh2*, and *escU* was 0.93, 0.91, and 0.97, respectively (Appendixes A and C). The R^2 values show how well the standard curve fits its measured data therefore the reliability of the assay. With the values of both the slope and R^2 within the ideal range, the amplification efficiency of our qPCR assays was good.

Establishing a reference treatment or baseline

The mean RERs of the gene of interest, *tdh2* and *escU*, were calculated for each treatment using total RNA. The reference gene, *pvsA*, was used to normalize the raw

expression levels of the genes of interest. Therefore, each RER shows the expression levels of *tdh2* or *escU* relative to *pvsA* between two treatments. We were interested in finding the expression levels of *tdh2* and *escU* in VBNC cells relative to culturable cells. Therefore, the mean RER for these genes from VBNC cells obtained from treatment #1 (TSBS/4°C) and #4 (MMS/4°C) was compared against the culturable cells obtained from treatment #8 (TSBS/37°C → MMS/4°C). It is important to have a legitimate control that provides a more representative baseline. Typical overnight cultures in TSBS at 25 or 37°C may not be a good control in VBNC experiment because they negate the effect of temperature shock experienced by the treated cells. Similarly, typical overnight cultures also do not take into account the nutrient shock experienced by cells such as those in treatment #4.

Table 5. RERs of virulence markers in VBNC cells compared to different overnight cultures.

	Reference treatment: cells grown in TSBS at 25°C for 48 h (treatment #7)		Reference treatment: cells grown in TSBS at 37°C for 24 h then switched to MMS at 4°C for 1 h (treatment #8)	
	TSBS-induced VBNC cells (treatment #1)	MMS-induced VBNC cells (treatment #4)	TSBS-induced VBNC cells (treatment #1)	MMS-induced VBNC cells (treatment #4)
tdh2	0.48	0.49	1.05	0.85
escU	13.02	11.72	1.19	2.08

Evaluating the RER obtained using different overnight controls may result in vastly different values that can lead to overreaching conclusions of virulence expression. The RER obtained from overnight cultures accounting for environmental variations led to a more conservative conclusion than using cultures that had not experienced the variations (Table 5). Since RNA expression is activated upon changes in the environment, we reasoned that some RNA species produced by VBNC cells were in

response to temperature and nutrient shock which might not be specific to the VBNC state. For example, the two recently discovered T6SSs in *V. parahaemolyticus* expressed under different environmental conditions. T6SS2 was expressed at all tested temperatures (23°C, 30°C, and 37°C) by Salomon et al. (2013). In low (0.5%) salt conditions, T6SS2 was most active at cold and warm temperatures. In contrast, T6SS1 was active under marine-like conditions (23 and 30°C, but not 37°C; higher expression in 3% salt conditions). In *E. coli* O157:H7, low doses of glucose (0.1 to 0.5%) were able to downregulate genes involved in the production of Shiga toxin (Delcenserie et al., 2012). Therefore, *V. parahaemolyticus* cells obtained from treatment #8, which experienced similar environmental variations as the VBNC cells, would serve a better control treatment in our RER analyses.

RER analyses

RER was obtained by comparing target gene expression with reference gene expression within the treatment, and then comparing the test treatment with the control treatment. In this study, the gene expression of the two virulence genes, *tdh2* and *escU*, were calculated relative to that of the housekeeping gene, *pvsA*. Encompassed by this analysis was the change in Ct, which was obtained by comparing the treatment of interest (#1 or #4) to the control (#8). For example, to get the RER of *tdh2* in TSBS-induced VBNC cells from treatment #1, the calculation would be $(E_{tdh2})^{\Delta CT(\#8-\#1)}$ / $(E_{pvsA})^{\Delta CT(\#8-\#1)}$. Our results suggested that VBNC cells expressed *tdh2* and *escU* at a similar or higher level than culturable cells (Figure 7). This is a tentative conclusion because total RNA was used. Analysis of gene expression between VBNC (treatment #4) and culturable (treatment #8) cells suggests that *escU* was significantly upregulated (> 2-fold change) in MMS-induced VBNC cells. The expression level of *escU* was lower

in TSBS-induced VBNC cells (1.19-fold). The mean RER of *tdh2* in MMS-induced and TSBS-induced VBNC cells was 0.85 and 1.05, respectively, indicating a similar level of expression to the culturable cells. On the other hand, when comparing the first three vs the last two trials of treatment #1, the RER in VBNC of *tdh2* was 0.80 and *escU* was 0.03, indicating similar relative expression level of *tdh2* between VBNC and culturable cells but significantly downregulation of *escU* in VBNC cells.

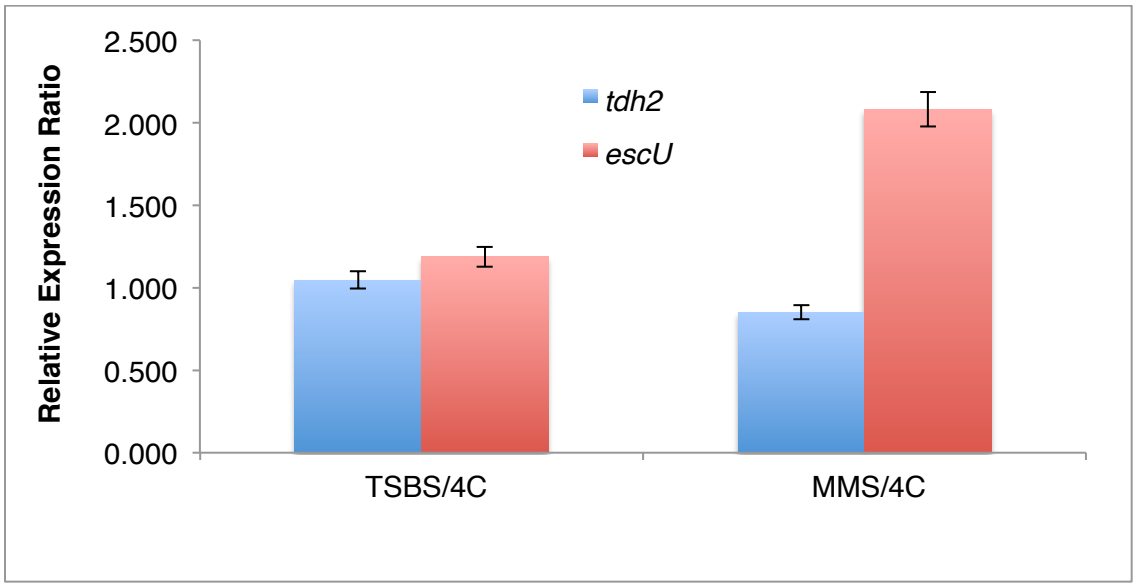

Figure 7. Mean RER of *tdh2* and *escU* in TSBS- or MMS-induced VBNC cells compared to culturable cells. Culturable cells were initially grown in TSBS at 37°C for 24 h, then switched to MMS at 4°C for one hour (treatment #8). VBNC cells were maintained in TSBS at 4°C for ≥21 days (treatment #1) or in MMS at 4°C for ≥25 days (treatment #4).

Effect of temperature and media on virulence gene expression in culturable cells

The relative virulence gene expression of *escU* in VBNC cells varied depending on media or nutrient availability (Figure 7). Expression levels of *escU* increased when cells were in MMS. Relative expression of *escU* in TSBS, and *tdh2* in MMS and TSBS, were similar to that for culturable cells. It appears that, VBNC cells in nutrient rich

conditions (TSBS) showed little RER fluctuation, whereas VBNC cells in nutrient poor conditions (MMS) showed greater RER variation.

To determine if gene expression in culturable cells also varied depending on environmental conditions, qRT-PCR data from other treatments (#2, 3, 5, 6) that yielded culturable cells were analyzed. In these treatments, cells were incubated in either TSBS or MMS at 25 or 37°C. When examining the effects on culturable cells, *escU* had overall higher expression levels than *tdh2* at these temperatures. Relative expression of *escU* was 3.6- and 8.6-fold higher in TSBS than MMS at 25 and 37 °C, respectively (Figure 8, Appendix D3). This observation was consistent using VBNC cells that *escU* expressions tend to be higher in TSBS.

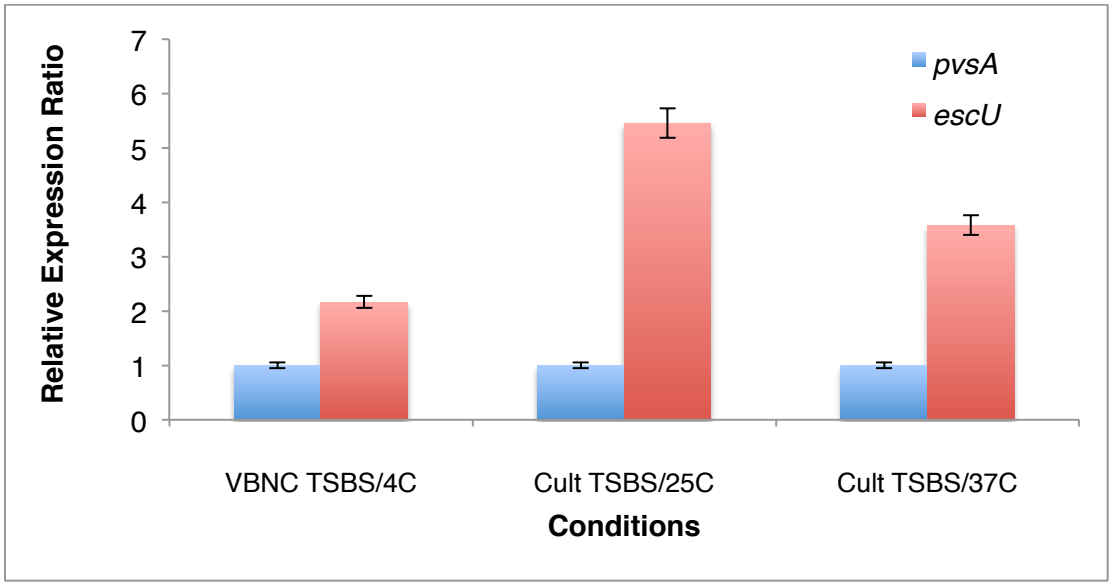

Figure 8. Mean RER of virulence genes in VBNC or culturabe cells of *V. parahaemolyticus* incubated in different media. The RER of *escU* was calculated by comparing expression in TSBS relative to MMS at three different temperatures. The housekeeping gene (*pvsA*) expression was normalized to RER of 1.

The effect of temperature of gene expression was also determined for culturable cells. Relative expressions of *tdh2* were consistently higher at 37°C regardless of media (Figure 9, Appendix D2). .

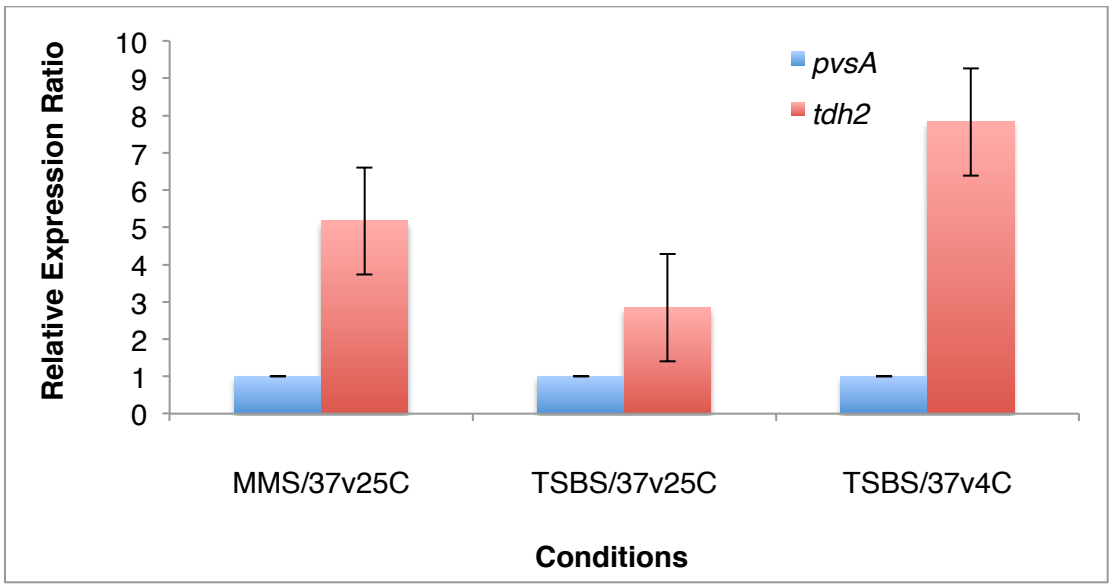

Figure 9. Mean RER of virulence genes in culturabe cells of *V. parahaemolyticus* incubated at different temperatures. RER of *tdh2* was calculated by comparing expression at 37°C relative to 25 or 4°C in TSBS or MMS. The housekeeping gene (*pvsA*) expression was normalized to RER of 1.

IV. Discussion

Determination of VBNC state

By definition, cells in the VBNC state are non-culturable in standard growth media, yet still viable. This phenomenon was observed in this study – *V. parahaemolyticus* cells were culturable at the beginning for all treatments but became non-culturable after extended incubation at 4°C in either nutrient rich or poor environments (Figure 2). Viable cells becoming nonculturable at 4°C in both nutrient conditions show that low temperature in conjunction with long incubation period is sufficient in inducing *V. parahaemolyticus* to enter the VBNC state. This is in agreement with some previous studies, which showed that a decrease in temperature was the main factor to induce VBNC cells formation provided that cold temperatures were held for at least 12 to 49 days (Jiang and Chai, 1996; Mizunoe et al., 2000; Wong et al., 2004). It appears that nutrient starvation alone was not sufficient to activate VBNC state. Coutard et al (2005) demonstrated the VBNC state of *V. parahaemolyticus* could only be reached when cells were maintained in ASW (carbon starvation) at 4°C, but not at room temperature. Our treatments #5 and #6 (MMS at 25 or 37^{o}C for 7 d) also did not yield sufficient VBNC cells.

In this study, VBNC cells were only stained with Syto 9 because viable cells were impermeable to PI. Therefore these cells exhibited green fluorescence under fluorescence microscopy. On the other hand, nonviable cells would fluoresce red due to compromised membranes allowing diffusion of PI. The use of these two nucleic acid stains is a common method for assessing the viability of bacterial cells. Rao et al., (2014) used similar microscopic method, with Syto9, to check for viability of VBNC *V. vulnificus* cells following treatments with both low nutrient (ASW) and low temperature

(4°C). Mishra et al. (2012) confirmed cell viability of *V. cholerae* by using the same commercial kit (LIVE/DEAD® *Bac*Light™) after the cells were induced into the VBNC state under low nutrient (sterilized lake water) and low temperature (4°C).

It appears that LIVE/DEAD® *Bac*Light™ is among the most common methods to confirm cell viability. There are other methods also based on cell membrane integrity. The use of 6-CFDA (6-carboxyfluorescein diacetate) assesses esterase activity indicating viability (Na et al., 2006). Live cells with intact cell membranes can be somewhat quantified by measuring the intracellular esterase activity. A direct microscopic count method was used to detect viable cells by their abilities to enlarge in the presence of nalidixic acid (Kogure et al., 1979). Other methods detect for respiratory activities such as the reduction of INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride) (Zimmermann et al., 1978) or CTC (5-cyano-2,3-ditolyl tetrazolium chloride) (Rodriguez et al., 1992). As VBNC cells are metabolically active, and thus have electron transport chain activity. Both INT and CTC are soluble tetrazolium salts that compete with oxygen as the final electron acceptor, which are then reduced to insoluble formazan in metabolically active cells. Thus, the accumulation of formazan in viable cells would cause the cells to appear dark red under microscopy (Zimmermann et al., 1978). Most of the above methods, however, are more time consuming and potential less sensitive. After preliminary trials with 6-CFDA and the LIVE/DEAD® *Bac*Light™ kit, we selected the latter as it was easier to call the results, less time consuming, and appeared to give more consistent results.

Rationale of test and control treatments

In *V. cholerae,* ToxT, a transcriptional activator protein of virulence factors for cholera toxin and toxin-coregulated pilus, was shown to be controlled by temperature-sensing RNA sequences located in the 5' UTR of mRNA (Weber et al., 2014). Under lower temperature of 25°C, this region folds into a structure to prevent access of the ribosome, thus reducing translation of *toxT* by ~150% as measured by β-galactosidase levels and qRT-PCR (Weber et al., 2014). As temperature increases the structure unfolds by a zipper mechanism, allowing for ToxT translation and subsequent virulence factor expression at 37°C (Weber et al., 2014). These studies demonstrate the significant impact different environmental conditions can have on gene expression within a relatively short amount of time.

Treatment #8, which accounted for the potential confounding effects of temperature and media, was used as the control to investigate gene expression specific to VBNC state. In this control treatment, cells were initially grown in TSBS at 37°C for 24 hours and then switched to MMS at 4°C for one hour. If typical culturable cells were used as control, such as treatment #7, relative expression of *tdh2* would be off ~2-fold and that of *escU* would be off ~10 (Table 5). The large discrepancy observed, especially for *escU*, showed that environmental conditions exert a great impact on gene expression. Thus, our control (treatment #8) was likely a more legitimate control than typical overnight cultures used in previous VBNC studies. Though the cultures were shifted to MMS at 4°C for only one hour, it accounted for partial expression in response to environmental changes, which ultimately leads to a more accurate conclusion. In future experiments, extending the transitional time from one hour to a few more hours might be better as previous studies noted that RNA expression profiles changed after

exposing cells to the experimental conditions for a few hours. For instance, a low concentration of glucose (0.1 and 0.5%) downregulated various virulence genes of *E. coli* O157:H7 (expression ratios of -3.27 and -2.66, respectively) after 4 hours of incubation, including those involved in attaching effacing lesions (Delcenserie et al., 2012).

Evaluation of EU-labeled RNA

Results from the preliminary experiment extracting EU-labeled RNA suggested that overnight cultures incubated in TSBS at 25°C produced double the amount of nascent RNA than VBNC cells. However, in order to verify, additional controls using only dead cells and mixture of viable and dead cells must be included. Ct values using EU-labeled RNA of VBNC and culturable cells were unexpectedly high. For VBNC cells, it might mean that they did not express the target genes. However, the culturable cells should have expressed the genes, as shown by the results obtained from treatment #7. This suggests the labeled RNA was not a good template to be converted to cDNA. Inhibitors may be inadvertently included in the qPCR run when reagents from the Click-iT® RNA labeling/isolation was transferred to the template.

Despite the high Ct values, the target genes were apparently amplified after examining the melting curve analyses more closely. However, non-specific amplification might also occur. Several peaks were observed in most of the derivative melting curves; these were likely due to inter- and intra-primers annealing. It is important to note that although some derivative melting curves showed two distinct peaks, it is not necessarily the result of multiple products. Generally, DNA exists in two states, ssDNA and dsDNA. When temperature slowly increases, the two strands of the amplicon separate into

ssDNA, and the melting curves show this transitional change. An amplicon with varying concentrations of G-C rich regions could result in more than one melting temperatures.

Future studies, therefore, should confirm the presence of amplicon by running the PCR products on agarose gels followed by staining with ethidium bromide and visualized by UV transillumination. To confirm the presence of RNA before qPCR, Northern Blot using specific probe targets can be performed. Furthermore, different primer sets may be designed to confirm the results. As of early 2015, a newer version of Click-It Nascent RNA Capture Kit was available that appeared to be compatible with qRT-PCR. In summary, though we have some evidences to tentatively show VBNC cells synthesized new RNA and expressed the target genes, the level of expression was not known.

Impact of nascent and residual RNA on virulence gene expression in VBNC cells

Ideally, newly transcribed mRNA are used in all gene expression analyses, as this would be a bona fide measurement of metabolic activity in VBNC cells. However our labeled nascent RNA was not a reliable template and hence total RNA was used in all subsequent qRT-PCR. Therefore, gene expression could be a result of a combination of nascent and residual RNA in the template. To interpret the results, it is necessary to assume the two extreme scenarios: the template contained either exclusively 1) nascent RNA from VBNC cells, or 2) residual RNA from previously culturable cells.

Scenario 1: Nascent RNA

In this scenario, virulence and housekeeping gene expression of VBNC *V. parahaemolyticus* cells was due to the result of *de novo* mRNA synthesis. This was possible because we had observed the predicted melting temperatures of the three genes using EU-labeled RNA. A serendipitous finding that treatment #1 yielded both

VBNC and culturable cells allowed us to further speculate VBNC cells continued to express *tdh2*. In particular, VBNC cells (after 25 d of incubation) expressed similar level (RER = 0.8) of *tdh2* compared to culturable cells (after 14 d of incubation). On the other hand, *escU* expression was drastically reduced in 25-d old cells, reaching RER of 0.03. These results must be interpreted carefully, however, due to the smaller sample size. The half-life of *tdh2* and *escU* RNA is also different (Coutard et al., 2007b).

Coutard et al (2005) observed expression of both housekeeping 16S-23S rDNA and *rpoS* genes, but not virulence gene, *tdh1*, via RT-PCR in the VBNC *V. parahaemolyticus* Vp4, indicating that there is still active transcriptional activity in the VBNC state. Other previous studies used mRNA half-life information as a marker for viability (Lleo et al, 2000; Fisher-Le Saux et al., 2002; Yaron and Matthews, 2002; and Coutard et al 2005). Coutard et al (2007b) quantified mRNA decay following the addition of rifampin to halt new RNA synthesis of *V. parahaemolyticus* cells in ASW at 4°C. They found that half-lives of mRNA were 5.1±0.54, 3.3±0.62, and 2.1±0.04 days for *pvsA*, *tdh2*, and *escU*, respectively. Fischer-Le Saux et al. (2002) could still detect mRNA of the cytotoxin hemolysin (*vvhA*) in VBNC *V. vulnificus* after maintaining the cells in ASW at 4^{o}C for 133 days. They concluded that mRNA was synthesized by viable cells because no RT-PCR band was detected after boiling the cells for 10 min. On the other hand, amplification was detected using the total RNA extracted before the heat treatment, and interesting, after boiling these purified RNA extracts. Thus, this previous study showed that VBNC *V. vulnificus* cells actively transcribe virulence genes. In another study, Dinu and Bach (2011) detected the presence of Vero (Shiga) toxins 1 (Stx1) and 2 (Stx2) in VBNC *E. coli* O157:H7 in the phyllosphere of lettuce. The verotoxin proteins were detected for 3 days in samples that contained 10^4 to 10^5 VBNC

cells using Ridascreen verotoxin enzyme immunoassay kit. This enzyme assay had a high detection limit. Therefore the authors concluded that *E. coli* O157:H7 were induced on lettuce plants and the production of virulence factor by these VBNC cells had food safety implications.

Under the assumption that gene amplification was a result of *de novo* mRNA synthesis, our results show that VBNC *V. parahaemolyticus* cells express virulence genes, some maybe even upregulated. If this is true, this suggests that some virulence factors may be essential for survival in conditions that do not supporting growth. The virulence gene involved in T3SS, *escU*, exhibited upregulation in the MMS-induced VBNC cells compared to the culturable cells. The elevated level of *escU* may be correlated to the expression of other genes involved in T3SS apparatus, which overall suggests T3SS may be required for fitness in addition to virulence. Further experiments are needed to determine the expression of other T3SS genes including those encoding the effectors secreted through T3SS. On the other hand, TSBS-induced VBNC cells showed similar *escU* expression to culturable cells. These results corroborate a previous study by Coutard et al (2007b) in which *escU* was detected by RT-PCR, but was not significantly upregulated in VBNC *V. parahaemolyticus*. However, this Coutard study induced VBNC formation by maintaining the cells in ASW at 4^{o}C which was considered a nutrient poor condition similar to MMS. It is well known that environmental conditions, such as temperature and nutrient availability, serve as triggers to alter virulence gene expression (Delcenserie et al., 2012; Mahoney et al., 2010; Salomon et al., 2013a). Hence, it is not surprising that *V. parahaemolyticus* cells exposed to different conditions may have varying effects on virulence gene expression.

Expression levels of *tdh2,* which encode the thermostable direct hemolysin, were similar between the VBNC and culturable state, regardless of the type of media to induce VBNC formation. These results are consistent with Vora et al (2005) in which the *16S-23S* rDNA housekeeping gene and *tdh* gene were examined by microarray-based assay. The Vora study considered the cells of *V. parahaemolyticus* F5052 had reached the VBNC state when <0.3 CFU/mL could be detected, which were attained by maintaining the cells in ASW at 4°C for 76 days. Similarly, Coutard et al. (2007b) found that *tdh2* was expressed but not upregulated. On the contrary, Coutard et al. (2005) detected the expression of two housekeeping genes (*16S-23S* rDNA and *rpoS*) using RT-PCR, but not *tdh1* and *tdh2*. The Coutard et al. (2005) study considered the cells to have reached the VBNC state when ≤0.05 CFU/mL could be detected, which were attained by maintaining the cells in ASW at 4°C for 42 days. The conflicting results between Vora et al (2005) and Coutard et al (2005) could be due to different strains (F5052 vs IF Vp18), VBNC induction periods (76 vs 42 days), and the sensitivity of the assays (microarray vs RT-PCR).

VBNC cells that actively transcribe virulence genes can have implications in pathogenesis because they pose a human health risk. Expression levels of *tdh2* in VBNC cells are comparable to those in culturable cells, suggesting that the TDH proteins may be translated at similar levels. In addition, the upregulation of *escU* in MMS-induced VBNC suggests that VBNC cells may have enhanced virulence. Currently, there are very few studies examining the role of *escU* in *V. parahaemolyticus* pathogenesis. More is known regarding other T3SS associated genes. Many T3SS1 and T3SS2 associated genes (translocator genes and their chaperones, regulators, structural, and effector proteins) showed substantial increases over the course of

infection in HeLa cells (Hiyoshi et al., 2010; Nydam et al., 2014; Zhou et al., 2009). T3SS1 appeared to be responsible for the cytotoxicity to several mammalian cell lines (Burdette et al., 2008; Hiyoshi et al., 2010; Nydam et al., 2014; Zhou et al., 2009) and mortality in a mouse model (Hiyoshi et al., 2010; Pineyro et al., 2010).

Scenario 2: Residual RNA

In this scenario, virulence and housekeeping gene expression of VBNC cells are entirely due to the presence of residual mRNA, which were synthesized by previously culturable cells (e.g., before 14 day of incubation). These residual RNA could be protected from nuclease degradation and thus remained in the sample (Yaron and Matthews, 2002). It is plausible that VBNC cells do not actively synthesize mRNA pf certain genes because gene expression is energetically expensive. These cells would express only those necessary for survival until they are once again in appropriate conditions. In this scenario, VBNC cells would resemble a "spore-like" state, in which they strive to survive stressful conditions until they are exposed to a more favorable environmental, such as the gastrointestinal tract of human. Once in favorable conditions, VBNC *V. parahaemolyticus* cells resuscitate and regain the culturability.

MMS-induced VBNC cells of *V. parahaemolyticus* were shown to resuscitate after an upshift in temperature (Wong et al., 2004). Nalidixic acid was added to inhibit bacterial cell multiplication. Therefore, the authors concluded that the presence of culturable cells after the temperatures shift was due to resuscitation of VBNC cells instead of regrowth of a few culturable cells that may have not been detected. VBNC cells have also been shown to regain virulence after resuscitation in the human gastrointestinal tract (Sun et al., 2008; Oliver and Bockian, 1995). Baffone et al. (2003) confirmed the resuscitation of VBNC *V. parahaemolyticus* cells in rat ileal loop assays.

Following two consecutive passages of various strains of VBNC *V. parahaemolyticus* in the ileal loops, accumulation of fluid in rat ileal loops was noted. Several assays were performed by Baffone et al. (2003), including hemolysis on Wagatsuma agar (indicate TDH production), adhesion assay using Hep-2 cells, and cytotoxicity assay using CHO, to verify the reactivation of virulence characteristics of the VBNC strains. Colwell et al. (1996) also showed that pathogenic VBNC *V. cholerae* O1 resuscitated in the human intestine following oral administration by volunteers. In this Colwell study, an attenuated recombinant vaccine strain CVD 101, which did not express cholera toxin, was maintained at VBNC cells for 3 and 7 weeks before the human study. The subject's stool samples were evaluated. Culturable cells of *V. parahaemolyticus* were recovered on the selective medium, TCBS, in subject infected with 3-week old VBNC cells, indicating resuscitation in the gut. On the other hand, only VBNC cells were recovered in subjects infected with 7-week old VBNC cells. These results show that the age of VBNC cells affect their resuscitation ability.

The above studies show that VBNC cells, though unable to transcribe virulence genes, still pose a threat due to their ability to resuscitate to culturable state under appropriate conditions and become pathogenic (Roszak et al., 1984; Bates and Oliver, 2004; Colwell et al. 1996; Oliver and Bockian 1995).

In this study, RER of *tdh2* and *escU* in VBNC cells range from 0.85 to 2.08 compared to a control culture that experienced a temperature and nutrient shift. The range is 0.48 to 13.02 if the control culture is a 2-day old culture grown at 25^{o}C. This suggests the presence of a fairly significant amount of residual RNA in VBNC cells, which can be translated into proteins if there is no active degradation by RNAse or other means of mRNA decay. Virulence proteins can be detected and semi-quantified by

Western Blot analysis. Unfortunately, antibodies against TDH and EscU are not commercially available and will require expensive custom production.

Effects of environmental factors on virulence gene expression

The virulence of a pathogen is intricately linked to the environmental factors, especially in stressful conditions. Expression of virulence genes had been shown to be influenced by environmental conditions (Delcenserie et al., 2012; Mahoney et al., 2010; Salomon et al., 2013b). In *E. coli* O157:H7, a low concentration of glucose (decreased from 20% to 0.1-1%) downregulated various virulence genes, including those involved in attaching effacing lesions (Delcenserie et al., 2012). Salomon et al (2013) showed that the expression of T6SS1 and T6SS2 was activated in different conditions (in seawater versus inside a marine animal). Therefore, it is a challenge to determine changes in gene expression between VBNC and culturable state solely, without taking environmental factors into consideration.

As mentioned in previous sections, the control treatment (#8) helped reduce the effects caused by these potential confounding factors. With the partial background expression levels corrected for, and assuming the presence of nascent RNA in VBNC cells, *escU* appeared to be upregulated in MMS compared to TSBS. Expression of *tdh2* in VBNC did not appear to be influenced by the media type.

We were able to determine the effects of temperature on gene expression in culturable cells. Consistent with previous studies, *tdh2* expression was higher at 37^{o}C. In contrast to VBNC cells, culturable cells exhibited higher *escU* expression when cells were grown in TSBS than MMS. Interestingly, consistently increased levels of *escU* expression were observed in culturable cells grown in TSBS across all temperatures.

Thus, future research direction may examine if T3SS is activated without interaction with the host, and the specific role of T3SS inducing and maintaining VBNC cells.

In general, *escU* expression in culturable cells is higher in nutrient rich media, whereas *tdh2* expression is higher at warmer temperatures. These genes are under multiple and overlapping transcriptional controls. The expression of T3SS2-related genes and *tdh* are regulated by VtrA (VPA 1332) and VtrB (VPA 1348) (Kodama et al., 2010). Immunoblotting of *vtrA*- and/or *vtrB*-deleted mutant of *V. parahaemolyticus* RIMD2210633 showed a marked decrease in T3SS2-related proteins and TDH production (Kodama et al., 2010). Furthermore, the VtrB expression is directly controlled by VtrA (Gotoh et al., 2010; Kodama et al., 2010). Although the exact molecular control is not yet elucidated, these previous studies suggest VtrB and VtrA regulate expression of genes found in the pathogenicity island region of *V. parahaemolyticus* in a highly specific manner. Understanding the components involved in activation and regulation of both TDH and T3SS is critical to understand the expression of these genes in culturable and VBNC cells.

V. Conclusions and Significance

In summary, we found that temperature and time appeared to be the most important factors to induce *V. parahaemolyticus* cells into the VBNC state. Nutrient availability has variable effects on the degree of virulence gene expression in both VBNC and culturable cells. VBNC cells of *V. parahaemolyticus* induced under nutrient poor condition appeared to upregulate *escU*, a gene involved in T3SS apparatus. VBNC cells also expressed (but not upregulate) similar levels of *tdh2* than culturable cells. Nevertheless, these interpretations are preliminary because residual RNA might be present in the RNA template. Since VBNC cells of *V. parahaemolyticus* may actively transcribe virulence genes, and have shown to resuscitate in human host, they pose a risk for human health. Consequently, there is a need to enumerate the pathogenic *V. parahaemolyticus*, whether it is culturable or VBNC. However, the number would be underestimated using the conventional methods relying on culturing techniques. Hence, culture-independent methods must be incorporated to screen for the presence of pathogenic subtypes of *V. parahaemolyticus* in food products and environmental samples.

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APPENDICES

Appendix A: Standard Curves

Table A1. Standard curve construction for trial 1 (n=3)

Gene	cDNA (ng/μL)	Avg Ct	StdDev Ct
pvsA	182.5	14.59	0
	36.5	16.03	0.13
	7.3	18.44	0.26
	1.46	20.93	0.13
	0.292	23.00	0.13
	0.0584	25.46	0.12
	0.01168	27.96	0.14
tdh2	182.5	14.30	0.11
	36.5	15.76	0.18
	7.3	17.97	0.09
	1.46	20.41	0.06
	0.292	22.97	0.03
	0.0584	25.07	0.15
	0.01168	27.92	0.11
escU	182.5	15.25	0.10
	36.5	17.11	0.09
	7.3	19.34	0.25
	1.46	22.03	0.10
	0.292	24.65	0.11
	0.0584	26.88	0.03
	0.01168	28.60	0.25

Table A2. Standard curve construction for trial 2 (n=3)

Gene	cDNA (ng/μL)	Avg Ct	StdDev Ct
pvsA	1.668	19.97	0.05
	0.002668	31.03	0.08
	0.005337	34.38	1.53
	0.00010675	37.44	1.22
	0.0000213504	35.72	2.00
tdh2	41.7	14.11	0.01
	0.0026688	27.08	0.01
	0.0053376	29.66	0.42
	0.0000213504	35.67	2.10
escU	41.7	14.40	0.04
	1.668	17.22	0.05
	0.06672	23.02	0.07
	0.0026688	27.93	0.07

Table A3. Standard curve construction for trial 3 (n=3)

Gene	cDNA (ng/μL)	Avg Ct	StdDev Ct
pvsA	8.34	18.58	0.43
	0.013344	30.13	0.07
	0.000106752	36.20	0.27
	0.0000213504	34.25	0.24
tdh2	8.34	18.21	0.09
	0.06672	27.87	0.38
	0.0053376	34.92	0.37
	0.0000213504	35.39	0.58
escU	41.7	13.85	0
	1.668	15.30	0.09
	0.013344	24.32	0.20
	0.000106752	31.47	1.28

Table A4. Standard curve construction for trial 4 (n=3)

Gene	cDNA (μL/mL)	Avg Ct	StdDev Ct
pvsA	235	13.93	0.01
	1.88	19.15	0.07
	0.0752	24.59	0.05
	0.0006016	32.45	0.41
	0.000024064	35.90	0.17
tdh2	235	13.77	0.01
	1.88	17.51	0.06
	0.0752	22.99	0.14
	0.0006016	31.57	0.68
	0.000024064	Undetermined	-
escU	235	18.11	0.95
	1.88	25.39	0.37
	0.0752	30.50	0.16
	0.0006016	38.67	0.47
	0.000024064	39.21	0.04

Table A5. Standard curve construction for trial 5 (n=3)

Gene	cDNA (ng/μL)	Avg Ct	StdDev Ct
pvsA	275	16.20	0.57
	2.2	22.24	0.33
	0.0176	30.26	0.07
	0.0001408	39.93	0
	0.000005632	37.27	0.57
tdh2	275	14.8	0.33
	2.2	20.93	0.13
	0.0176	29.18	0.10
	0.0001408	39.57	0.40
	0.000005632	36.33	0.56
escU	275	14.28	0
	2.2	19.58	0.06
	0.0176	28.34	0.41
	0.0001408	37.47	0.60
	0.000005632	36.98	1.05

Appendix B: Ct Values

The two negative controls were NTC and RT (no reverse transcriptase). The positive control was MS2. “Und." means undetermined.

Table B1. Individual Ct for each treatment in triplicate for gene, pvsA.

	#1	#2	#3	#4	#5	#6	#7	#8	(-) NTC	(-) RT	(+) MS2
Trial 1	23.1	20.9	27.8	24.9	29.7	29.9	19.2	24.4	33.7	Und.	-
	23.2	21.4	27.5	25.1	29.5	30.2	19.3	24.2	33.1	18.1	-
	23.2	21.1	27.4	25.1	29.4	30.2	19.2	24.2			
Trial 2	28.0	26.9	31.1	32.3	34.9	37.5	24.2	27.3	Und.	Und.	32.9
	28.1	27.0	31.4	32.5	35.2	38.4	24.5	27.6	Und.	Und.	33.1
	28.2	27.1	31.3	33.1	34.5	Und.	24.4	27.7			
Trial 3	29.6	27.6	35.4	36.1	32.8	37.1	24.7	36.9	36.9	Und.	35.6
	30.1	27.7	36.0	35.4	32.9	38.4	24.9	36.0	33.4	Und.	35.9
	29.6	28.0	36.0	36.7	32.7	35.9	24.9	36.2			
Trial 4	23.6	27.1	24.8	27.2	33.5	32.1	23.3	25.1	36.2	Und.	13.5
	23.6	27.2	25.2	27.4	34.6	31.7	23.2	25.1	Und.	Und.	13.5
	23.7	27.4	24.9	27.2	34.2	31.4	23.2	25.1			13.5
Trial 5	27.1	30.9	29.8	34.8	39.3	39.1	31.1	29.9	Und.	Und.	16.4
	27.1	30.7	30.1	34.9	39.1	Und.	31.1	29.9	Und.	Und.	17.3
	27.1	31.0	29.9	34.4	37.9	39.2	31.8	29.7			

Table B2. Individual Ct for each treatment in triplicate for gene, *tdh2.*

	#1	#2	#3	#4	#5	#6	#7	#8	(-) NTC	(-) RT	(+) MS2
Trial 1	22.1	21.6	28.9	24.7	28.5	29.1	18.4	24.0	34.1	Und.	-
	22.3	21.3	27.1	24.5	29.1	29.7	18.3	24.3	31.6	26.3	-
	22.1	21.3	26.9	24.4	28.3	29.6	18.3	24.4			
Trial 2	25.1	24.6	31.5	32.6	30.6	44.3	22.0	24.3	Und.	Und.	44.4
	25.1	25.1	31.5	31.5	32.2	Und.	21.8	24.8	44.2	Und.	35.7
	25.2	24.7	33.4	30.6	32.1	34.2	22.1	24.7			
Trial 3	29.6	29.0	35.8	35.5	32.8	36.1	25.4	36.0	37.7	Und.	35.8
	29.9	29.0	35.9	38.7	32.3	35.7	25.6	35.6	37.8	Und.	35.1
	29.7	29.1	37.1	38.8	32.5	36.6	25.5	35.5			
Trial 4	22.3	26.0	24.2	26.1	31.9	30.9	21.3	24.1	Und.	39.5	13.3
	22.1	26.5	24.2	26.3	32.2	30.8	21.2	24.2	Und.	39.2	13.4
	22.3	26.3	24.2	26.0	Und.	Und.	21.4	24.3			
Trial 5	26.3	30.3	29.6	34.9	37.1	35.9	29.1	28.8	39.3	Und.	14.5
	26.5	30.2	29.4	35.0	37.1	35.7	29.3	29.0	38.4	Und.	14.6
	26.4	30.1	29.4	34.4	38.8	36.2	29.0	28.6			14.4

Table B3. Individual Ct for each treatment in triplicate for gene, *escU.*

	#1	#2	#3	#4	#5	#6	#7	#8	(-) NTC	(-) RT	(+) MS2
Trial 1	25.0	23.5	29.2	26.9	29.8	30.4	20.5	26.8	29.7	Und.	-
	24.9	23.5	29.3	26.8	30.1	30.3	20.3	*26.5*	30.3	Und.	-
	24.6	23.8	29.2	27.2	30.5	30.3	20.8	26.8			
Trial 2	27.6	27.6	31.7	31.7	33.4	31.8	24.3	28.1	35.4	Und.	31.6
	27.6	27.6	33.1	32.4	32.5	34.3	24.4	28.2	31.6	Und.	32.4
	27.7	27.7	32.8	31.9	32.4	33.9	24.6	28.3			
Trial 3	20.1	19.3	28.9	31.1	23.9	30.1	16.2	30.1	33.3	Und.	25.9
	20.0	19.3	28.4	31.7	23.7	31.5	16.1	Und.	Und.	44.8	27.2
	20.3	19.5	29.6	31.4	24.1	31.1	16.4	32.5			
Trial 4	30.3	34.3	31.5	33.2	39.1	37.3	29.1	32.1	Und.	Und.	15.7
	29.8	34.6	31.4	32.8	39.5	36.9	28.4	32.1	Und.	Und.	15.6
	29.8	34.2	31.2	32.3	37.5	32.5	Und.				15.7
Trial 5	24.9	29.3	28.5	32.4	36.2	34.2	28.1	27.6	Und.	Und.	14.1
	25.1	29.2	28.7	32.3	35.6	35.7	28.3	*28.2*	39.6	Und.	14.1
	25.1	29.1	28.3	32.4	36.0	Und.	28.1	*27.4*			14.1

Appendix C: qRT-PCR Reaction Efficiencies

Table C. qRT-PCR reaction efficiencies for all reference and virulence genes per trial

	Slope	R^2
Trial 1 *escU*	-3.316586	0.995883
Trial 1 *pvsA*	-3.31789	0.996829
Trial 1 *tdh2*	-3.331476	0.994968
Trial 2 *escU*	-3.321648	0.982943
Trial 2 *pvsA*	-3.442745	0.855803
Trial 2 *tdh2*	-3.409674	0.954814
Trial 3 *escU*	-3.326275	0.968216
Trial 3 *pvsA*	-3.023706	0.925927
Trial 3 *tdh2*	-3.149312	0.83158
Trial 4 *escU*	-3.255825	0.969571
Trial 4 *pvsA*	-3.321061	0.991446
Trial 4 *tdh2*	-3.32172	0.962236
Trial 5 *escU*	-3.332287	0.954455
Trial 5 *pvsA*	-2.901851	0.946757
Trial 5 *tdh2*	-3.102449	0.923261
Click it Trial 1 *escU*	-2.508219	0.947679
Click it Trial 1 *pvsA*	-2.600221	0.954532
Click it Trial 1 *tdh2*	-3.121318	0.992153
Click it Trial 2&3 *escU*	-3.3	0.977556
Click it Trial 2&3 *pvsA*	-3.317447	0.97257
Click it Trial 2&3 *tdh2*	-3.319398	0.975202

Appendix D: RER Analysis of Culturable Cells

Table D1. RER of virulence genes in VBNC cells compared to culturable cells (treatment #8) in each trial.

	Genes	VBNC (TSBS 4°C) vs Culturable Cells	VBNC (MMS 4°C) vs Culturable Cells
Trial 1	*tdh2*	-	1.384
	escU	-	1.415
Trial 2	*tdh2*	-	0.274
	escU	-	2.175
Trial 3	*tdh2*	-	-
	escU	-	-
Trial 4	*tdh2*	1.414	1.176
	escU	1.635	2.803
Trial 5	*tdh2*	0.679	0.567
	escU	0.738	1.935
Overall mean	***tdh2***	**1.05 ± 0.54**	**0.85 ± 0.52**
	escU	**1.19 ± 0.60**	**2.08 ± 0.58**

The RER means of each trial was provided. The first three trials in treatment #1 (TSBS 4°C) did not yield VBNC cells, and thus were excluded from the VBNC analysis. Trial 3 was not included in the analysis due to contamination issues.

Table D2. RER of virulence genes at 37°C compared to 4°C or 25°C in culturable cells grown in TSBS or MMS.

	Genes	37°C vs 4°C in TSBS (#3 vs #1)	37°C vs 25°C in TSBS (#3 vs #2)	37°C vs 25°C in MMS (#6 vs #5)
Trial 1	*tdh2*	2.06	0.86	1.20
	escU	1.00	0.57	0.17
Trial 2	*tdh2*	13.59	8.16	79.50
	escU	3.59	1.72	0.19
Trial 3	*tdh2*	-	-	-
	escU	-	-	-
Trial 4	*tdh2*	1.55	1.15	2.25
	escU	1.07	0.58	0.56
Trial 5	*tdh2*	1.03	1.22	0.31
	escU	1.16	1.29	0.37
Overall mean	***tdh2***	**7.83 ± 8.15**	**2.84 ± 3.54**	**20.81 ± 39.13**
	escU	**2.30 ± 1.83**	**1.04 ± 0.57**	**0.33 ± 0.18**

The RER means of each trial was provided for culturable cells at 37°C relative to 25°C and 4°C in both nutrient conditions, TSBS and MMS. In the 4°C analysis, only culturable cells were used (first three trials of treatment #1). Trial 3 was not included in the analysis due to contamination issues.

Table D3. RER of virulence genes in TSBS compared to MMS at different temperatures.

	Gene	4°C TSBS vs MMS (#1 vs #4)	25°C TSBS vs MMS (#2 vs #5)	37°C TSBS vs MMS (#3 vs #6)
Trial 1	*tdh2*	0.71	2.29	1.63
	escU	0.83	3.66	2.71
Trial 2	*tdh2*	0.27	1.91	0.20
	escU	1.01	5.49	50.17
Trial 3	*tdh2*	-	3.58	-
	escU	-	2.00	-
Trial 4	*tdh2*	0.83	2.12	1.09
	escU	1.71	5.45	5.59
Trial 5	*tdh2*	0.84	2.07	12.35
	escU	2.62	1.29	17.40
Overall mean	***tdh2***	**0.83 ± 0.00**	**2.39 ± 0.67**	**0.97 ± 0.73**
	escU	**2.17 ± 0.64**	**3.58 ± 1.93**	**8.57 ± 7.79**

The cells were VBNC at 4°C. The RER means of each trial was provided for VBNC cells (4^{o}C) and culturable cells (25 and 37^{o}C). Trial 3 was not included in the analysis due to contamination issues. Expression of *tdh2* was similar or higher in TSBS relative to MMS across all temperatures. Expression of *escU* was significantly upregulated in TSBS relative to MMS across all temperatures.