2021

Role of RelA in Dormancy and ToxR Proteolysis in Vibrio cholerae

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ROLE OF RELA IN DORMANCY AND TOXR PROTEOLYSIS IN *VIBRIO CHOLERAE*

by

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A thesis submitted in partial fulfillment of the requirements
for the Honors in the Major program in Biomedical Sciences
in the College of Medicine
and in the Burnett Honors College
at the University of Central Florida
Orlando, Florida

Spring Term
2021

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ABSTRACT

Vibrio cholerae, the etiological agent of the severe diarrheal disease cholera, is an enteric pathogen that can be found in aquatic ecosystems when not colonizing the human gastrointestinal tract. Under adverse environmental conditions, *V. cholerae* is capable of entering dormant states that increase its survival during these ecological fluctuations. In these states, *V. cholerae* slows its metabolic activity and exhibits drastically altered gene expression and morphology. Stressors that lead to entry into these states vary from nutrient limitation, suboptimal pH, or antimicrobials. Cells in these dormant states are highly resistant to antimicrobials and cannot be detected using standard microbiological techniques which poses major public health challenges such as food or water contamination. In *V. cholerae*, proteolysis of virulence regulator ToxR has been identified to be required for entry into a dormant state called viable but nonculturable (VBNC) under nutrient limitation and alkaline pH mediated by the sigma-E stress response. However, the mechanisms that lead to the initiation of this cascade remain unknown. The stringent response is another mechanism involved in mediating bacterial survival during late stationary phase. The stringent response involves the alarmone (p)ppGpp, which acts at the level of transcription to inhibit cellular processes that consume significant resources and activate genes responsible for biosynthetic processes. RelA is one enzyme responsible for the synthesis of (p)ppGpp, which in turn activates transcription of RpoE, suggesting a potential connection with ToxR proteolysis. Therefore, the aim of this study is to define the role of RelA in dormancy and ToxR proteolysis in *V. cholerae*. Our results show that RelA alone is not sufficient to control dormancy and ToxR proteolysis in *V. cholerae*. Nonetheless, another regulator (SpoT) is also associated with (p)ppGpp synthesis,

indicating that other stringent response-associated mechanisms might be involved in ToxR proteolysis.

ACKNOWLEDGEMENTS

Thank you to Dr. Moreno, Dr. Borgon, and Dr. Ambivero for being on my thesis committee and making this all possible. I would also like to say a huge thank you to everyone in the Moreno Lab, especially Deepak, for guiding me through this process.

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INTRODUCTION

Microorganisms have survived on Earth for billions of years in a vast array of environments. To survive fluctuating conditions, microorganisms must adapt quickly and efficiently. While it is still not fully understood, one of the most effective modes of adaptation to ensure survival is entering a state of dormancy [1]. Many bacteria spend most of their lives in a state of low metabolic activity with little or no growth but remain ready to divide when their environmental conditions become favorable [2]. Although dormancy has traditionally been viewed as a robust and definitive response to the environment in sporulating bacteria, many nonsporulating species (such as *Vibrio cholerae*) enter a dormant state in a more progressive process that changes with time and can be stochastic or a result of environmental cues [1]. Sporulation occurs predominantly in gram-positive bacteria in response to nutritional deprivation. Spore formation begins with a smaller cell forming inside a mother cell, which eventually gets enclosed by a multilayered structure—an inner membrane, peptidoglycan cell wall, peptidoglycan cortex, outer membrane, and rigid spore coat—to protect the spore from extreme temperatures, dehydration, chemicals, and other stressors. However, many gram-negative bacteria are not capable of forming spores, so they enter other states of dormancy. One such state is called the viable but nonculturable (VBNC) state, which was first discovered in *V. cholerae* and *Escherichia coli* [3]. The VBNC state can be defined as a subpopulation of cells that remain alive (viable) but cannot be cultured using standard laboratory techniques [3]. The VBNC state is also associated with changes in morphology and cell envelope, modified fatty acid composition of the cell membrane, altered gene expression, and changes in DNA replication [4-6]. Despite these changes, VBNC cells still maintain an active metabolism, membrane integrity, and can produce proteins [7, 8].

In addition to the VBNC state, persister cell formation is another prevalent dormancy state that was originally found to arise after antibiotic treatment and may contribute to relapsing infections [9]. Persister cells share many of the same characteristics with VBNC cells. Both VBNC and persister cell formation can be induced by many environmental stressors, varying from starvation, suboptimal pH, or antibiotics [1]. Most importantly, VBNC and persister cells can withstand a variety of stressors, including but not limited to starvation, hot or cold temperature, decreased oxygen levels, suboptimal salinity, suboptimal pH, and antimicrobials [1]. Both VBNC and persister cells can also be formed stochastically in unstressed environments, indicating these dormancy mechanisms could be used as a bet-hedging strategy to ensure survival from future, unknown stressors [10]. This can effectively facilitate the survival of bacterial populations in a wide range of conditions. VBNC cells have been associated with chronic infections [11, 12], antibiotic resistance [13, 14], and food contamination due to their prolonged survival and difficulty to detect [15, 16]. For example, the dose of the antibiotic vancomycin that is needed to be effective on VBNC cells of *E. faecalis* is over 500 times the dose needed for culturable cells [17]. The ability of pathogens to evolve to survive in the presence of antimicrobials is of great concern as it could lead to antibiotic failure and persistent infections, which is arguably one of the most important challenges human health and modern medicine will face in the next decades [18]. Ultimately, dormant states like the VBNC and persistent state present a major public health threat, warranting further study into the mechanisms causing these phenomena.

Over 115 different species are known to enter a dormant VBNC state, including over 70 human pathogenic bacteria [16, 19, 20]. This study will focus on *V. cholerae*, the etiological agent of the severe diarrheal disease cholera. According to the World Health Organization, it is estimated that there are 1.3 million to 4 million cases of cholera each year, with 21,000 to 143,000 deaths

worldwide. In the environment, *V. cholerae* lives in brackish rivers and coastal waters. Being an enteric pathogen, *V. cholerae* switches between aquatic ecosystems where it is found as free living or attached to biotic or abiotic surfaces, and human hosts where it colonizes intestinal epithelium [21,22]. Cholera transmission usually occurs by drinking water or ingesting food that has been contaminated by feces from a person infected with the bacterium [23]. Cholera is most likely to occur in places with inadequate water treatment or poor sanitation [23]. In areas where cholera is prevalent, aquatic ecosystems serve as reservoirs for *V. cholerae* VBNC cells, facilitating survival upon exit from the human host [24-27]. Due to their prolonged survival and undetectability, ingestion of VBNC cells from contaminated water sources likely acts as a major mode of cholera transmission [3, 24]. Therefore, identifying the processes controlling VBNC formation and similar dormant states is crucial in understanding and mitigating the spread of cholera, as well as many other diseases [28-30].

At the molecular level, little is known about the factors regulating dormant states like the VBNC state [31-33]. In *V. cholerae*, proteolysis of ToxR has been identified to be required for entry into a dormant state resembling VBNC during late stationary phase [28, 33]. ToxR is a virulence regulator that has been shown to influence the expression of more than 150 genes in *V. cholerae* [34]. ToxR, along with TcpP, is required to activate the transcription of the master virulence regulator, ToxT [35-38]. ToxR and TcpP are both transcriptional regulators localized to the inner membrane [35, 37]. ToxT directly activates the expression of *V. cholerae's* two major virulence factors—the toxin co-regulated pilus (TCP), an essential intestinal colonization factor, and the cholera toxin (CT), which is responsible for the diarrhea associated with the disease [39-42]. In addition to virulence, ToxR regulates the expression of genes involved in cellular transport, energy metabolism, motility, and iron uptake [33]. One example is ToxR's role in regulating the

expression of two outer membrane porins, OmpU and OmpT, in response to the nutritional status of the cell [43, 44]. OmpU is activated by ToxR in nutrient rich environments and plays a role in resistance to organic acids, bile, and antimicrobial peptides [45-48]. On the other hand, OmpT, whose role is not well understood, is derepressed in nutrient limiting environments [44, 49].

The way in which stressor signals are relayed throughout the cell to produce physiological changes prompts further investigation. One common mechanism that both prokaryotes and eukaryotes use to respond to extracellular stress is regulated intramembrane proteolysis (RIP) [50, 51]. The most widely studied example of RIP involves the sigma-E envelope stress response [52, 53]. In *V. cholerae*, ToxR has been shown to undergo RIP during late stationary phase at nutrient limitation and alkaline pH, mediating entry into a dormant state via the sigma-E stress response [28]. This process involves RseA, a membrane localized anti-sigma factor that sequesters RpoE (sigma factor) under standard growth conditions. In response to outer membrane stress, RseA undergoes RIP in which DegS (site-1 serine protease) cleaves a periplasmic portion, and RseP (site-2 protease) cleaves an intramembrane portion; other proteases may be involved as well, consequently releasing RpoE into the cytosol to act as a transcriptional regulator by interacting with RNA polymerase [54-56]. While the site-2 protease RseP has been shown to be directly involved in the proteolysis of ToxR, other unknown proteases are also involved. RpoE likely activates the transcription of other proteases involved in the proteolytic cascade of ToxR [33].

While we know that ToxR proteolysis is associated with the entry of *V. cholerae* into a dormant, nonculturable state, the mechanisms that initiate this cascade remain unknown. In addition to the sigma-E envelope stress response, the stringent response is another mechanism involved in mediating entry into a dormant, nonculturable state during late stationary phase [28].

The stringent response is a widely conserved mechanism by which cells detect amino acid starvation and respond by slowing growth and increasing amino acid synthesis [1]. This is accomplished through production of the alarmone guanosine penta-or tetra-phosphate [(p)ppGpp]. (p)ppGpp is a second messenger that alters gene expression by binding RNA polymerase; it inhibits cellular processes that consume significant resources, such as cell division, transcription, and translation, and increases the transcription of genes necessary for biosynthetic processes [57]. RelA-SpoT Homologs (RSH) comprise a family of proteins responsible for synthesizing and hydrolyzing (p)ppGpp [58]. RelA and SpoT are stringent response regulators generally present in gram-negative bacteria. Upon amino acid starvation, deacylated tRNAs accumulate, as they cannot keep up with protein synthesis; RelA interacts with an uncharged tRNA in a vacant ribosomal A-site, activating (p)ppGpp synthesis from GTP or GDP [59]. While RelA only plays a role in synthesizing (p)ppGpp, SpoT is a bifunctional enzyme that can synthesize and hydrolyze (p)ppGpp. Additionally, RelV, while not as widely conserved as RelA or SpoT, has also been found to synthesize (p)ppGpp in *V. cholerae* [60]. In relation to dormancy, persister cells have been found to require the alarmone (p)ppGpp [61]. RelA has been shown to be upregulated in the VBNC state in *V. cholerae* [62]. Additionally, excess production of the alarmone (p)ppGpp has been found to be necessary for entry into the VBNC state [63], while mutants devoid of (p)ppGpp have shown a significant reduction in VBNC formation [64].

Although the virulence regulator ToxR and the stringent response regulator RelA appear to repress and facilitate the formation of dormant states respectively, their potential relationship has not been studied [65]. Of particular interest is the ability of (p)ppGpp to activate transcription of RpoE [66]. While the sigma-E envelope stress response and the stringent response are two distinct pathways, our overall hypothesis is that both converge towards RpoE activating the

transcription of proteases involved in ToxR proteolysis, ultimately leading to dormancy. Specifically, the aim of this study is to define the role of RelA in dormancy and ToxR proteolysis in *V. cholerae* under ToxR proteolysis inducing conditions (TPI conditions). As shown in Figure 1, during late stationary phase, RelA leads to the accumulation of (p)ppGpp; accumulation of (p)ppGpp activates transcription of RpoE [66]. RpoE can then go on to activate transcription of proteases involved in the proteolysis of ToxR. To our knowledge, RelA and the stringent response has not been linked to ToxR proteolysis. Although RelA is not the only enzyme responsible for (p)ppGpp production in *V. cholerae*, it was chosen as the focus of this study as it only functions as a synthetase and is generally found in other bacteria. While a Δ*relA* mutant would be expected to produce lower levels of (p)ppGpp and potentially increase ToxR stability, our results show that RelA alone is not sufficient to control dormancy and ToxR proteolysis in *V. cholerae*, prompting further study into the role of other stringent response regulators in ToxR proteolysis.

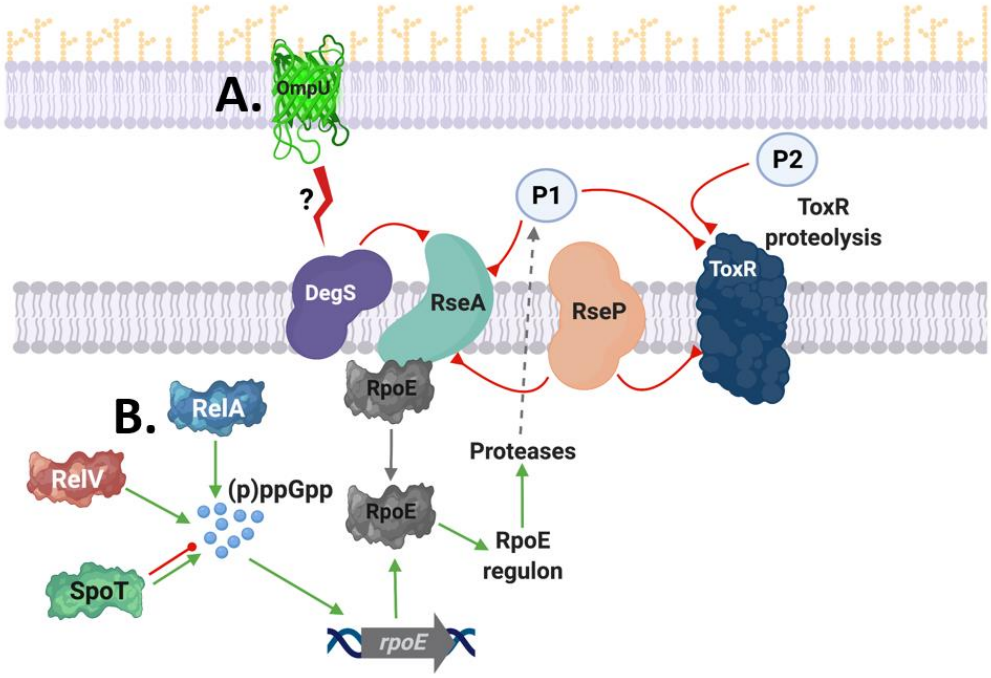

Figure 1: Proposed role of RelA in the proteolytic cascade of ToxR. During the late stages of colonization, as nutrients get depleted and the environment becomes alkalinized, ToxR is proteolyzed. **(A)** Outer membrane stress (sensed by OmpU) triggers activation of the sigma-E envelope stress response, which promotes degradation of RseA by DegS, RseP, and other proteases, resulting in the release of the sigma factor RpoE, which may go on to activate transcription of proteases involved in the proteolysis of ToxR. **(B)** We hypothesize that the stringent response regulator RelA feeds into this pathway by producing the alarmone (p)ppGpp, which activates transcription of RpoE, which in turn proceeds to activate transcription of proteases to assist in the proteolysis of ToxR. SpoT can synthesize and hydrolyze (p)ppGpp; RelV has also been shown to synthesize (p)ppGpp.

MATERIALS AND METHODS

Bacterial strains, mutants, and culture conditions

Vibrio cholerae C6706 was used as the wild-type strain in this study. Δ*toxR*, Δ*relA*, and Δ*toxR*Δ*relA* deletion mutants were made in the C6706 background. Unless otherwise indicated, cultures were plated from stocks on LB agar (10 g/L tryptone, 5 g/L yeast, 5 g/L NaCl, 15 g/L agar) and incubated at 37˚C for 16-18 hours, then inoculated in 5 ml of LB media to grow overnight (16-18 hours) on a rotary shaker at 37˚C.

ToxR proteolysis inducing (TPI) conditions

To induce ToxR proteolysis and dormancy, two different LB broths were used—LB starting pH 7 (unbuffered) and LB starting pH 9.3 (unbuffered). LB pH 7 buffered with 100mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was used as a control to prevent ToxR proteolysis and entry into dormancy. HCl and NaOH were added as necessary to set the pH. Unless otherwise stated, 50 µl of overnight culture was aliquoted into 5 ml of each condition (LB starting pH 7, LB starting pH 9.3, LB pH 7 buffered with 100 mM HEPES), and incubated on a shaker at 37˚C for the desired time (24, 48, or 72 hours). After 24 hours, LB starting pH 7 (unbuffered) reaches a pH close to 8. After 48 and 72 hours, LB starting pH 7 (unbuffered) maintains a pH between 9 and 10.

CFU counts

To determine the number of colony-forming units (CFU), 24-, 48-, and 72-hour samples were serially diluted by adding 50 µl of culture to 450 µl of sterile saline (0.85% NaCl) in 1.5 ml microcentrifuge tubes; the result was vortexed before aspirating and dispensing 50 µl into a new 450 µl aliquot of sterile saline. This process was repeated until reaching a dilution of 10^{-7}. 10 µl of each dilution was plated on LB agar and incubated overnight at 30˚C. Colonies were counted for each dilution. CFU/ml was calculated by dividing the colony count by the product of the dilution of the tube and the amount plated in milliliters (10^{-2}). Values were plotted and analyzed using GraphPad Prism 9 software.

Fluorescent microscopy

From each bacterial suspension, 100 µl was aliquoted and centrifuged at 13,000 rpm for two minutes. The supernatant was discarded, and the pellet was washed twice with saline; washing involves resuspending the pellet in saline, centrifuging, and discarding the supernatant. After two washes, 1,000 µl of sterile saline was added to the pellet to form a 1:10 dilution. 200 µl of this 1:10 dilution of bacterial cells was aliquoted into a new microcentrifuge tube to be stained with the fluorescence based LIVE/DEAD BacLight Bacterial Viability Kit (ThermoFisher, MA, USA). Equal amounts of SYTO9 and propidium iodide nucleic acid stain were mixed to form a 1:1 solution. 0.6 µl of this mixture was added per 200 µl of cells in saline. Samples incubated in the dark at room temperature for at least 30 minutes. Microscope slides were cleaned with 70% EtOH before mounting 10 µl of stained cells and adding coverslips. The slides were observed under the inverted microscope Axio Observer 7 (Carl Zeiss Microscopy, LLC, NY) at a 100x magnification;

immersion oil was added to the lens of the microscope. Images were analyzed using the Zen Pro software V2.3 (Carl Zeiss Microscopy). Dead or injured cells appear red, while live cells appear green. Additionally, culturable *V. cholerae* cells appear rod-shaped, while round, coccoid shaped cells are indicative of a dormant state.

Protein electrophoresis and immunoblot

Whole cell protein extracts were prepared from cultures grown for various times under various conditions. Unless otherwise indicated, 60 µl of cells were aliquoted to 540 µl of LB (1:10 dilution) in cuvettes to measure the optical density (OD) at 600 nm in a spectrophotometer. After measuring the optical density, all samples were normalized to 1.0 OD by calculating the volume of cells necessary to obtain an OD of 1.0 per ml; this ensured equal amounts of protein were added to each lane of the gel. Samples were centrifuged at 14,000 rpm for two minutes to form a pellet. The supernatant was discarded, and the pellet was left to dry before resuspending in 100 µl of sample loading buffer dye (4% SDS, 12% of 1M Tris pH 7, 0.1 mg/ml bromophenol blue, 20% glycerol). SDS-PAGE was performed with 12% and 16% tris glycine gels (Invitrogen). The 1.0 OD samples were boiled for 10 minutes before loading the gel. Unless otherwise indicated, 5 µl of sample was added to each lane. Log-phase *V. cholerae* C6706 WT was used as a positive control, while log-phase *V. cholerae* C6706 Δ*toxR* was used as a negative control. 5 µl of BLUeye prestained protein ladder (FroggoBio) was loaded as the marker. SDS-PAGE was run at 150V for 60-90 minutes (depending on the gel). For gels not undergoing western transfer, gels were stained with Coomassie blue (10% acetic acid, 45% methanol, 0.25% Coomassie brilliant blue) for 5-10 minutes, then left to rock in low methanol destain (5% methanol, 7.5% glacial acetic acid) at room

temperature for at least two hours before imaging with ChemiDoc XRS+ (Bio-Rad). For gels undergoing western transfer, iBlot (Invitrogen) was used to transfer the protein to a nitrocellulose membrane. Blots were blocked in 5% nonfat milk in TBS-T (10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween20) at room temperature for one hour on a rocker. After the one-hour block, blots were washed three times (10 minutes each) with TBS-T. Then blots were incubated in anti-ToxR primary antibody (1:5,000 dilution in 1% BSA in TBS-T) for one hour on a rocker at room temperature. Then blots were washed three more times with TBS-T before incubating in secondary antibody (mouse anti-rabbit, GenScript, 1:10,000 dilution in 1% BSA in TBS-T) for 30 minutes on a rocker at room temperature. Finally, blots were washed three more times with TBS-T before visualizing. Blots were visualized with Pierce ECL western blotting substrate (Thermo Scientific); equal amounts of peroxide solution and luminol enhancer solution were mixed before adding to the membrane to detect HRP (horseradish peroxidase) via enhanced chemiluminescence with ChemiDoc (Bio-Rad). ImageJ software was used to obtain densitometry measurements of ToxR bands. The intensity of the bands was compared to the wild-type control and normalized against their loading control.

RESULTS

Effect of RelA on *V. cholerae* culturability under TPI conditions

Under conditions that are not conducive to active growth, *V. cholerae* can enter a dormant, nonculturable state to facilitate its survival [1, 22]. In response to nutrient limitation at alkaline pH, *V. cholerae* is known to enter VBNC mediated by the proteolysis of ToxR [33]. In other words, the presence of ToxR represses dormancy, while the absence of ToxR promotes dormancy and survival. The stringent response is a mechanism involved in facilitating entry into dormancy through the production of the alarmone (p)ppGpp [58]. RelA is one enzyme capable of synthesizing (p)ppGpp in *V. cholerae*. In contrast to ToxR, the presence of RelA helps promote dormancy.

CFU counts of *V. cholerae* C6706 Δ*toxR*, Δ*relA*, and Δ*toxR*Δ*relA* mutants were obtained at 24, 48, and 72 hours and compared to wild-type. To induce dormancy and ToxR proteolysis, two different culture conditions were used—LB starting pH 7 (unbuffered) and LB starting pH 9.3 (unbuffered). As cells are in stationary phase, the media alkalinizes, resulting in a higher pH. After 24 hours of growth, LB pH 7 (unbuffered) reaches a pH close to 8. After 48 hours, LB starting pH 7 (unbuffered) reaches a pH between 9 and 10; this pH range is maintained after 72 hours. In these conditions, dormancy would correlate with a drop in CFU, indicating a subpopulation of cells have reached a nonculturable state. LB pH 7 buffered with 100 mM HEPES was used as a control to maintain neutral pH over 72 hours, making it less likely for cells to enter dormancy. For cells grown in the buffered condition, we would expect a smaller drop in CFUs, and overall higher CFU counts compared to the unbuffered conditions. We expected the Δ*toxR* mutant to show a larger drop in CFU compared to WT as it should be entering a dormant, nonculturable state at a faster

rate since there is no functional ToxR to be proteolyzed. We also expected the Δ*relA* mutant to show a smaller drop in CFU compared to WT, as it should be producing less (p)ppGpp, hence entering a nonculturable state at a slower rate.

As shown in Figure 2, the largest drop in CFUs occurs between 24 and 48 hours in LB pH 7 (unbuffered) and LB pH 9.3 (unbuffered). CFU counts leveled off between 48 and 72 hours. The fact that CFU counts leveled off around 10^7 instead of approaching zero reinforces the idea that VBNC formation occurs in a subpopulation of cells as a bet-hedging strategy. If all the cells formed a VBNC state, then no cells would be ready to proliferate if a favorable condition were encountered. On the contrary, if all cells remain culturable in favorable conditions and none form a VBNC state, then no cells would be ready to survive any unexpected stressors. Regarding differences between strains, the Δ*toxR* mutant experienced a greater drop in CFU compared to WT in LB (unbuffered) and LB pH 9.3 at 48 hours, which was expected (Fig. 2A and 2B). The Δ*relA* mutant exhibited no significant difference in CFUs compared to WT, which went against the expectation that the Δ*relA* mutant would exhibit a slower drop in CFUs (Fig. 2A and 2B). While the Δ*toxR*Δ*relA* mutant seems to fall between Δ*toxR* and Δ*relA* in CFUs at 48 hours in LB (unbuffered) and LB pH 9.3, the differences are insignificant (Fig. 2A and 2B). No significant differences were seen between strains when grown in LB HEPES, and all CFU counts in LB HEPES remained relatively high (~10^9) over 72 hours (Fig. 2C).

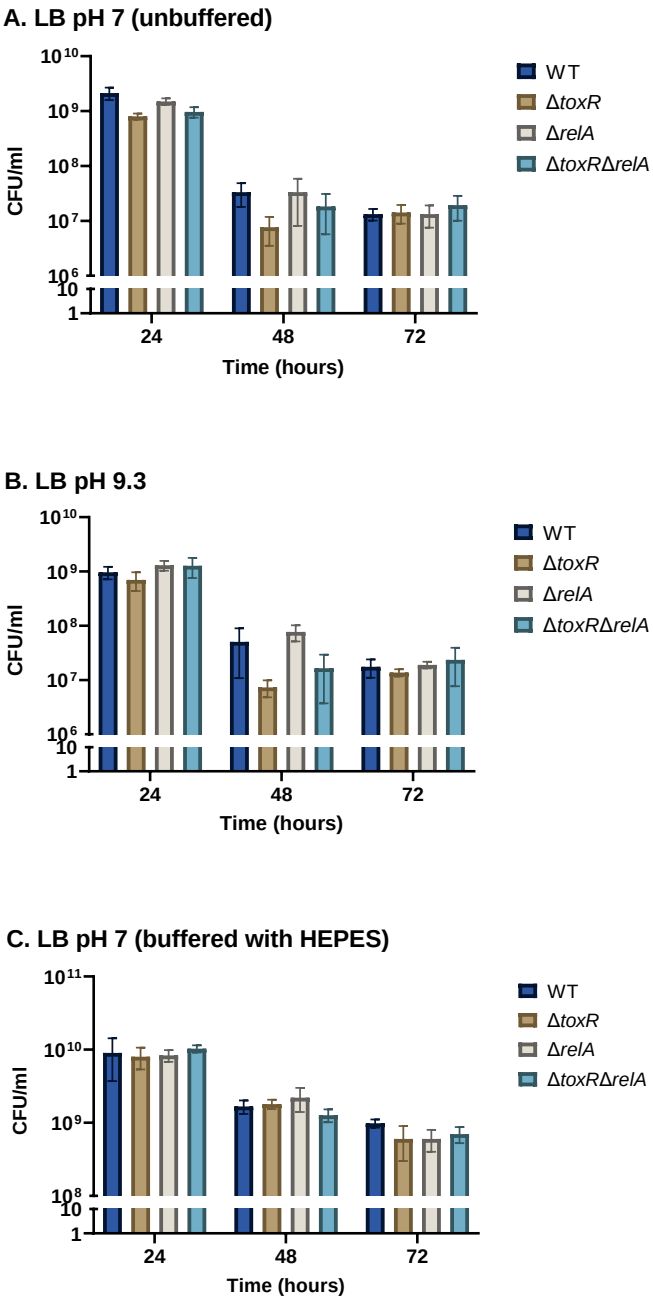

Figure 2: Culturability of *toxR* and *relA* mutants after 24, 48, and 72 hours. (A) CFU/ml of C6706 WT, Δ*toxR*, Δ*relA*, and Δ*toxR*Δ*relA* grown for 24, 48, and 72 hours in LB starting pH 7 (unbuffered). **(B)** CFU/ml of C6706 WT, Δ*toxR*, Δ*relA*, and Δ*toxR*Δ*relA* grown for 24, 48, and 72 hours in LB starting pH 9.3 (unbuffered). **(C)** CFU/ml of C6706 WT, Δ*toxR*, Δ*relA*, and Δ*toxR*Δ*relA* grown for 24, 48, and 72 hours in LB pH 7 buffered with 100 mM HEPES. The bars represent the mean of three independent experiments and the error bars indicate the standard deviation.

Viability and morphology of *toxR* and *relA* mutants under TPI conditions

V. cholerae cells exhibit an altered morphology when in dormant states like the VBNC state [4, 10]. Although *V. cholerae* is rod-shaped when culturable, it adopts a round, coccoid shape upon entering VBNC. To confirm that the TPI conditions are inducing a state of dormancy, and to ensure the previously observed drop in CFU is due to formation of a nonculturable state instead of strictly cell death, cells were stained with the LIVE/DEAD BacLight Bacterial Viability Kit and visualized via microscopy to determine their viability and morphology. Green and elongated cells represent viable cells that are still culturable. Green and round cells are viable but nonculturable. Dead or injured cells appear red. As shown in Figure 3, approximately 90% of cells remain viable at 24 hours in all conditions. After 48 hours in LB (unbuffered) and LB pH 9.3, approximately 50% of cells remain viable. If the drop in CFUs shown in Figure 2 was due to cell death instead of cells entering a dormant, nonculturable state, we would expect at least 90% of cells to appear red after 48 hours. For cells grown in LB buffered with HEPES, there was still a drop in viability between 24 and 48 hours, but approximately 70% of cells remained viable at 48 hours (Fig. 3C). No significant differences in viability were seen between strains.

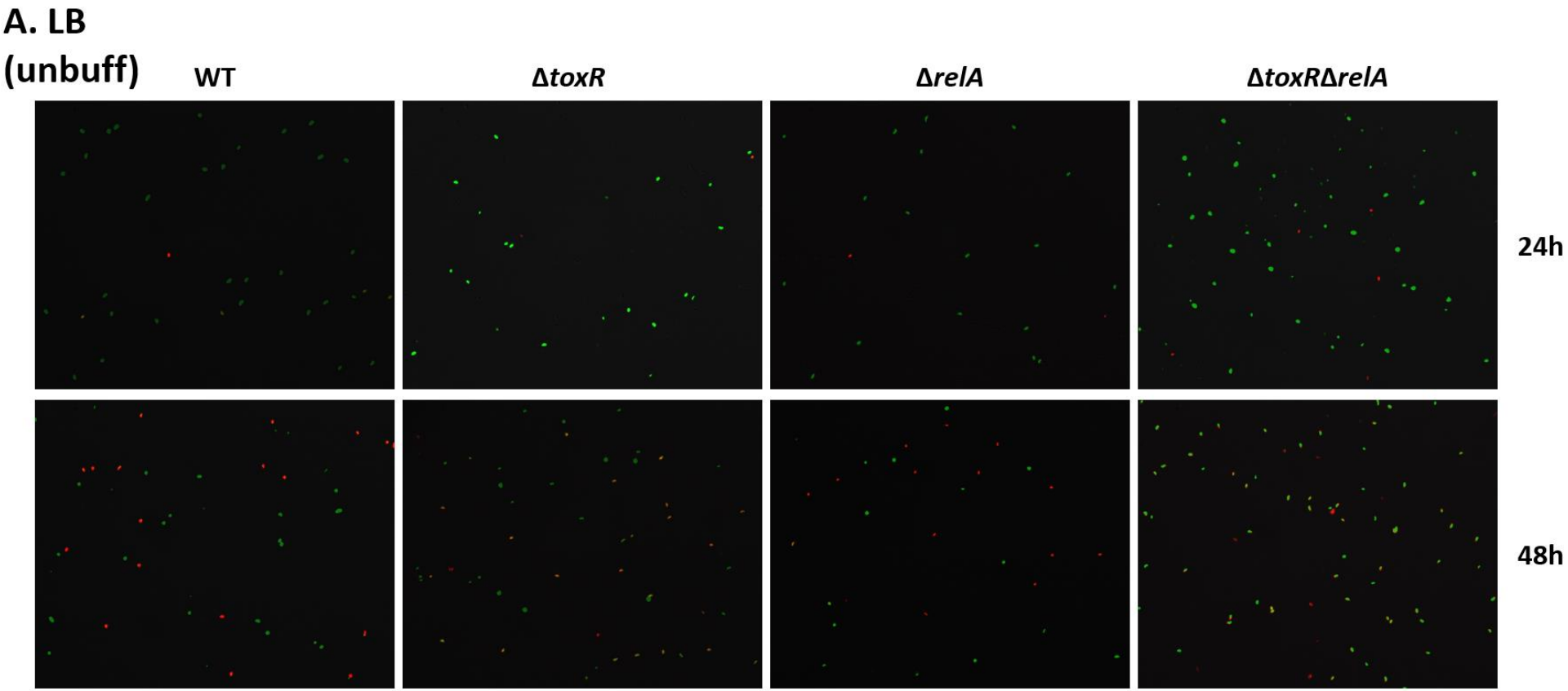

Figure 3A: Viability and morphology of *V. cholerae* mutants after 24 and 48 hours in LB pH 7 (unbuffered). Fluorescent images of C6706 WT, Δ*toxR*, Δ*relA*, and Δ*toxR*Δ*relA* grown for 24 and 48 hours in LB starting pH 7 (unbuffered). The cells were observed after treatment with Live/Dead viability staining. Viable and culturable cells appear green and elongated; viable but dormant cells appear green and round; dead cells appear red.

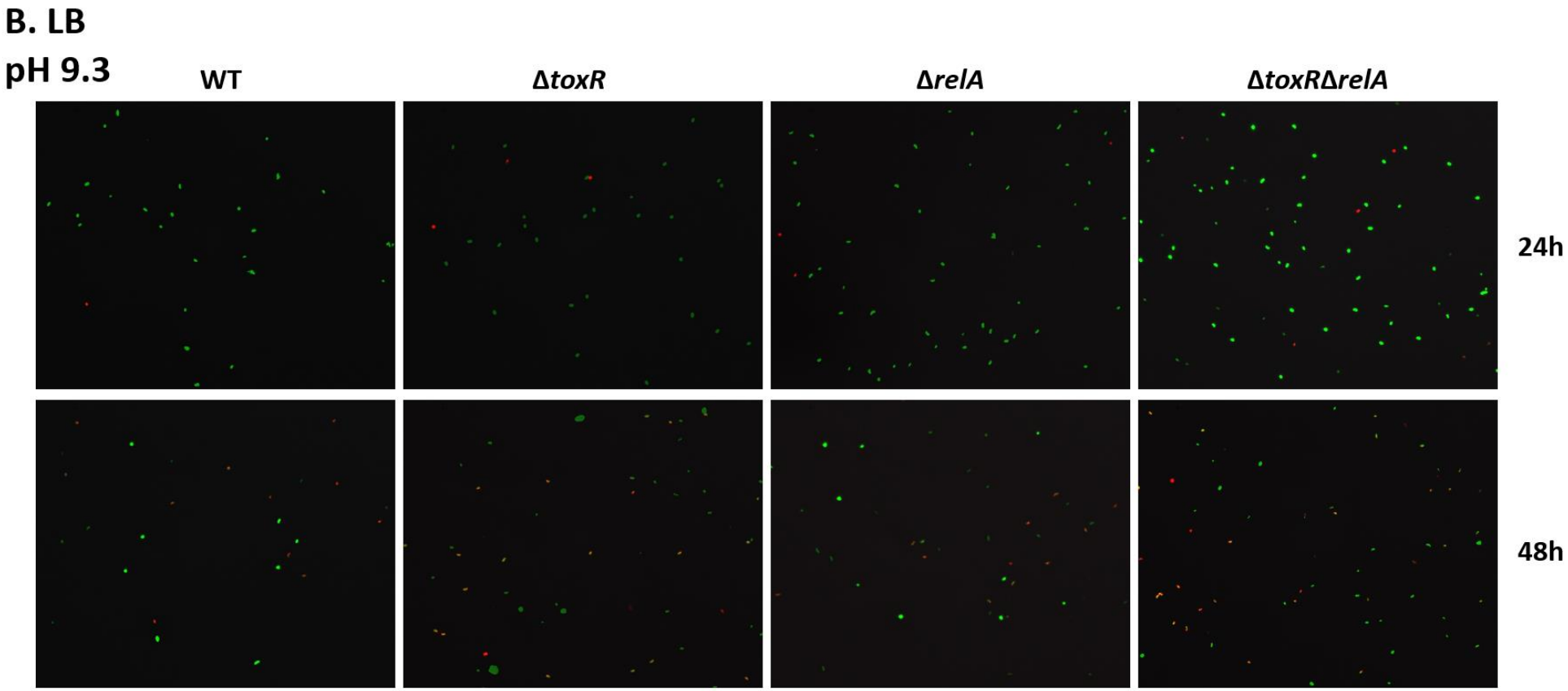

Figure 3B: Viability and morphology of *V. cholerae* mutants after 24 and 48 hours in LB pH 9.3 (unbuffered). Fluorescent images of C6706 WT, Δ*toxR*, Δ*relA*, and Δ*toxR*Δ*relA* grown for 24 and 48 hours in LB starting pH 9.3 (unbuffered). The cells were observed after treatment with Live/Dead viability staining. Viable and culturable cells appear green and elongated; viable but dormant cells appear green and round; dead cells appear red.

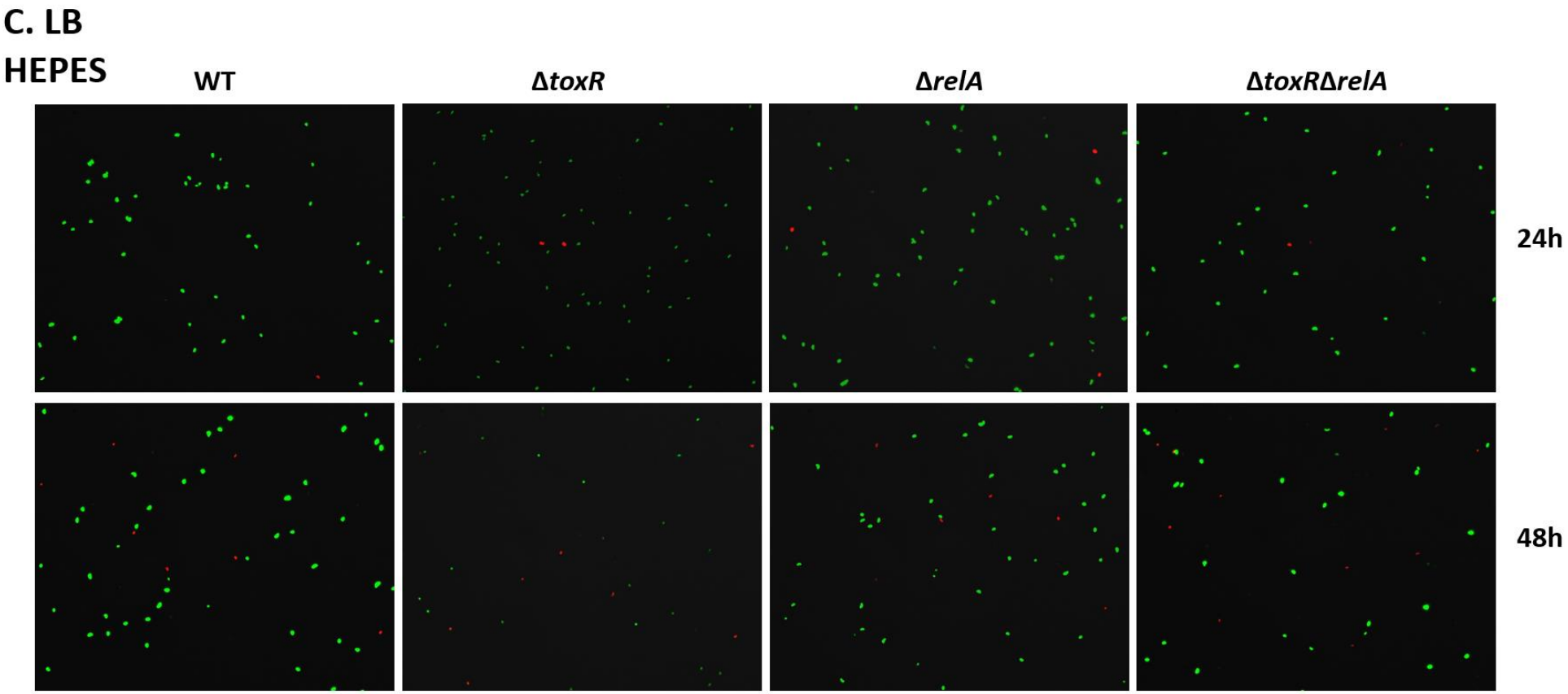

Figure 3C: Viability and morphology of *V. cholerae* mutants after 24 and 48 hours in LB pH 7 (buffered). Fluorescent images of C6706 WT, Δ*toxR*, Δ*relA*, and Δ*toxR*Δ*relA* grown for 24 and 48 hours in LB pH 7 buffered with 100 mM HEPES. The cells were observed after treatment with Live/Dead viability staining. Viable and culturable cells appear green and elongated; viable but dormant cells appear green and round; dead cells appear red.

Effect of RelA on ToxR stability under TPI conditions

During late stationary phase, when nutrients are limited and the media alkalinizes, proteolysis of the virulence regulator ToxR is known to facilitate entry of *V. cholerae* into a dormant state [33]. As mentioned previously, we hypothesized that the stringent response regulator RelA feeds into the proteolytic cascade of ToxR, as RelA produces the alarmone (p)ppGpp; (p)ppGpp activates transcription of RpoE, which activates transcription of proteases involved in the proteolysis of ToxR (Figure 1). In a Δ*relA* mutant, we would expect lower levels of (p)ppGpp, therefore less ToxR proteolysis, leading to increased ToxR stability compared to WT.

To investigate the effect of RelA on ToxR stability, C6706 WT and Δ*relA* strains were grown in LB starting pH 7 (unbuffered) and LB pH 7 (buffered with HEPES). Protein extracts were obtained at 24 and 48 hours for analysis with a ToxR immunoblot. As shown in Figure 4, ToxR levels decrease significantly between 24 and 48 hours in WT and Δ*relA* in LB (unbuffered), while ToxR levels remain relatively stable between 24 and 48 hours in LB (buffered), which is expected. Regarding the relationship between RelA and ToxR proteolysis, Figure 4A shows that Δ*relA* exhibits a similar decrease in ToxR stability as the WT. While there appears to be a slight difference in ToxR stability between C6706 WT and Δ*relA* (54% vs. 44%), the difference is minimal. Therefore, RelA alone is not sufficient to control ToxR proteolysis in *V. cholerae*. Further study should be done to identify the role of other stringent response regulators (such as SpoT) in ToxR proteolysis.

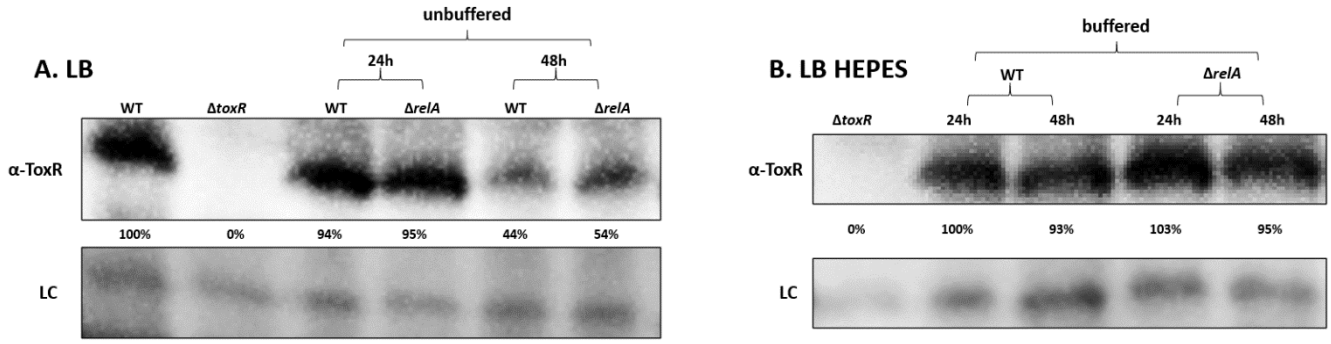

Figure 4: Effect of RelA on ToxR stability at nutrient limitation and alkaline pH. (A) ToxR immunoblot of protein extracts from C6706 WT and Δ*relA* grown in LB starting pH 7 (unbuffered) for 24 and 48 hours. **(B)** ToxR immunoblot of protein extracts from C6706 WT and Δ*relA* grown in LB pH 7 buffered with 100 mM HEPES for 24 and 48 hours. LC: loading control. Percentages represent intensity of bands relative to WT and LC.

DISCUSSION

Understanding the mechanisms by which bacteria adapt to their environment to enter dormant states is vital if we wish to eradicate chronic infections, antimicrobial resistance, or foodborne illness [18]. Under conditions not conducive to growth, such as nutrient-depleted marine environments or in the late stages of colonization of the human gut, *V. cholerae* can enter a VBNC state that increases its survival in stressful conditions [22]. In response to nutrient limitation and alkaline pH, *V. cholerae* enters a dormant state mediated by the proteolysis of the virulence regulator ToxR [33]. The proteolytic cascade of ToxR has been shown to involve the sigma-E envelope stress response, requiring the anti-sigma factor RseA and the sigma factor RpoE [33]. However, the mechanisms that initiate this cascade remain unknown. During late stationary phase, the stringent response is known to aid in bacterial survival and dormancy through production of the alarmone (p)ppGpp, primarily mediated by RelA [65, 67]. The ability of (p)ppGpp to activate transcription of RpoE suggested a potential relationship between the stringent response and ToxR proteolysis. This study focused on determining the role of the stringent response regulator RelA on dormancy and ToxR proteolysis in *V. cholerae* C6706.

To determine the role of RelA in the formation of a dormant, nonculturable state, CFU counts were performed, followed by fluorescent microscopy with LIVE/DEAD staining. As shown in Figure 2, there was no significant difference in culturability between Δ*relA* and WT strains. This is consistent with the results in Figure 3, as there are no major differences in viability or morphology between Δ*relA* and WT. To determine the role of RelA on ToxR stability, ToxR immunoblots were performed for WT and Δ*relA* strains grown in TPI conditions for 24 and 48 hours. As shown in Figure 4, RelA alone is not sufficient to control ToxR proteolysis in TPI

conditions. One possible explanation for these findings is the fact that there are multiple proteins responsible for the synthesis of (p)ppGpp, the second messenger of the stringent response [58]. While we tested RelA, known for being a widely conserved enzyme involved in the synthesis of (p)ppGpp, SpoT and RelV are two other enzymes capable of synthesizing (p)ppGpp in *V. cholerae* [59]. Although we would expect the absence of RelA to decrease intracellular levels of (p)ppGpp, it is possible that SpoT and/or RelV could compensate this difference. Based on our results, intracellular (p)ppGpp levels might be diminished, but they are unlikely to be abolished. To test this, intracellular (p)ppGpp levels could be measured in a Δ*relA* mutant under TPI conditions at various time points and compared to WT. Additionally, exogenous (p)ppGpp could be added to the cells to promote transcription of RpoE and potentially ToxR proteolysis; CFU counts and ToxR immunoblots could be used to determine if excess (p)ppGpp promotes ToxR proteolysis and dormancy.

Lastly, the roles of SpoT and RelV should be investigated to further elucidate the role of the stringent response in relation to ToxR proteolysis. SpoT is a bifunctional enzyme known to synthesize and hydrolyze (p)ppGpp [59]. It is possible that the synthetase activity of SpoT makes up for the absence of RelA. RelV, while not as widely conserved as RelA or SpoT, is also known to synthesize (p)ppGpp in *V. cholerae* [59]. RelV was discovered in *V. cholerae* when measuring intracellular concentrations of (p)ppGpp in a Δ*relA*Δ*spoT* mutant; under starvation conditions, sufficient intracellular concentrations of (p)ppGpp were still present, indicating there must be a third source besides RelA and SpoT [60]. Making Δ*spoT*, Δ*relV*, and a Δ*relV*Δ*spoT* mutants in a C6706Δ*relA* background could help define the relationship between the stringent response and ToxR proteolysis. This could be tested by growing the strains in TPI conditions and correlating

CFU counts, viability and morphology, ToxR immunoblots, gene expression, and intracellular (p)ppGpp levels.

REFERENCES

1. Ayrapetyan M, Williams T, Oliver JD. (2018). Relationship between the viable but nonculturable state and antibiotic persister cells. *J Bacteriol 200*: e00249-18.
2. Bergkessel M, Basta DW, Newman DK. (2016). The physiology of growth arrest: uniting molecular and environmental microbiology. *Nat Rev Microbiol 14*: 549 –562.
3. Xu, H.S., Roberts, N., Singleton, F.L., Attwell, R.W., Grimes, D.J., and Colwell, R.R. (1982). Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. *Microb Ecol 8*: 313–323.
4. Colwell, R.R. (2000). Viable but nonculturable bacteria: a survival strategy. *J Infect Chemother 6*: 121–125.
5. Lothigius, Å., Sjöling, Å., Svennerholm, A.M., and Bölin, I. (2010). Survival and gene expression of enterotoxigenic *Escherichia coli* during long-term incubation in sea water and freshwater. *J Appl Microbiol 108*: 1441–1449.
6. Trevors, J.T., van Elsas, J.D., and Bej, A.K. (2013). The molecularly crowded cytoplasm of bacterial cells: dividing cells contrasted with viable but non-culturable (VBNC) bacterial cells. *Curr Issues Mol Biol 15*: 1–6.
7. Brenzinger, S., van der Aart, L.T., van Wezel, G.P., Lacroix, J.M., Glatter, T., and Briegel, A. (2019). Structural and proteomic changes in viable but non-culturable *Vibrio cholerae*. *Front Microbiol 10*: 793.
8. Zhong, J., and Zhao, X. (2019). Transcriptomic analysis of viable but non-culturable *Escherichia coli* O157:H7 formation induced by low temperature. *Microorganisms 7*: E634.
9. Lewis, K. (2010). Persister cells. Annu Rev Microbiol 64: 357-372.
10. Bakkeren, E., Diard, M. & Hardt, WD. (2020). Evolutionary causes and consequences of bacterial antibiotic persistence. *Nat Rev Microbiol 18*: 479–490.
11. Pasquaroli, S., Zandri, G., Vignaroli, C., Vuotto, C., Donelli, G., and Biavasco, F. (2013). Antibiotic pressure can induce the viable but non-culturable state in *Staphylococcus aureus* growing in biofilms. *J Antimicrob Chemother 68*: 1812–1817.
12. Somova, L.M., Andryukov, B.G., and Lyapun, I.N. (2019). Cell geteromorphism in the conditions of persistence of sapronoses causative agents in various environments. *AIMS Microbiol 5*: 147–157.
13. Ehrlich, G.D. (2002). Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media. *JAMA 287*: 1710–1715.
14. Anderson, M., Bollinger, D., Hagler, A., Hartwell, H., Rivers, B., Ward, K., and Steck, T.R. (2004). Viable but nonculturable bacteria are present in mouse and human urine specimens. *J Clin Microbiol 42*: 753–758.
15. Dinu, L.-D., and Bach, S. (2011). Induction of viable but nonculturable Escherichia coli O157:H7 in the phyllosphere of lettuce: a food safety risk factor. *Appl Environ Microbiol 77*: 8295–8302.
16. Zhao, X., Zhong, J., Wei, C., Lin, C.-W., and Ding, T. (2017). Current perspectives on viable but non-culturable state in foodborne pathogens. *Front Microbiol 8*: 580–580.
17. del Mar Lleò, M., Benedetti, D., Tafi, M.C., Signoretto, C., and Canepari, P. (2007). Inhibition of the resuscitation from the viable but non-culturable state in *Enterococcus faecalis*. *Environ Microbiol 9*: 2313–2320.

18. Gollan, B., Grabe, G., Michaux, C., Helaine, S. (2019). Bacterial persisters and infection: past, present, and progressing. *Annu Rev Mircobiol 73*: 359-385.
19. Oliver, J.D. (2010). Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiol Rev 34*: 415–425.
20. Li, L., Mendis, N., Trigui, H., Oliver, J.D., and Faucher, S.P. (2014). The importance of the viable but non-culturable state in human bacterial pathogens. *Front Microbiol 5*: 258–258.
21. Huq A, Small EB, West PA, Huq MI, Rahman R, et al. (1983). Ecological relationships between *Vibrio cholerae* and planktonic crustacean copepods. *Appl Environ Microbiol 45*: 275—283. PMID: 6337551
22. Almagro-Moreno S, Taylor RK (2013). Cholera: Environmental Reservoirs and Impact on Disease Transmission. *Microbiol Spectrum 1*(2):OH-0003-2012.
23. Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), Division of Foodborne, Waterborne, and Environmental Diseases (DFWED); https://www.cdc.gov/
24. Colwell, R.R., and Huq, A. (1994). Environmental reservoir of *Vibrio cholerae*. The causative agent of cholera. *Ann N Y Acad Sci 740*: 44–54.
25. Alam, M., Sultana, M., Nair, G.B., Siddique, A.K., Hasan, N.A., Sack, R.B., et al. (2007). Viable but nonculturable *Vibrio cholerae* O1 in biofilms in the aquatic environment and their role in cholera transmission. *Proc Natl Acad Sci U S A 104*: 17801–17806.
26. Lutz, C., Erken, M., Noorian, P., Sun, S., and McDougald, D. (2013). Environmental reservoirs and mechanisms of persistence of *Vibrio cholerae*. *Front Microbiol 4*: 375.
27. Silva-Valenzuela, C.A., Lazinski, D.W., Kahne, S.C., Nguyen, Y., Molina-Quiroz, R.C., and Camilli, A. (2017). Growth arrest and a persister state enable resistance to osmotic shock and facilitate dissemination of *Vibrio cholerae*. *ISME J 11*: 2718–2728.
28. Almagro-Moreno, S., Root, M.Z., and Taylor, R.K. (2015a). Role of ToxS in the proteolytic cascade of virulence regulator ToxR in *Vibrio cholerae*. *Mol Microbiol 98*: 963–976.
29. Darcan, C., Ozkanca, R., and Flint, K.P. (2003). Survival of nonspecific porin-deficient mutants of *Escherichia coli* in black sea water. *Lett Appl Microbiol 37*: 380–385.
30. Asakura, H., Kawamoto, K., Haishima, Y., Igimi, S., Yamamoto, S., and Makino, S.-i. (2008). Differential expression of the outer membrane protein W (OmpW) stress response in enterohemorrhagic *Escherichia coli* O157:H7 corresponds to the viable but non-culturable state. *Res Microbiol 159*: 709–717.
31. Kusumoto, A., Asakura, H., and Kawamoto, K. (2012). General stress sigma factor RpoS influences time required to enter the viable but non-culturable state in *Salmonella enterica*. *Microbiol Immunol 56*: 228–237.
32. Kusumoto, A., Miyashita, M., and Kawamoto, K. (2013). Deletion in the C-terminal domain of ClpX delayed entry of *Salmonella enterica* into a viable but non-culturable state. *Res Microbiol 164*: 335–341.
33. Almagro-Moreno, S., Kim, T.K., Skorupski, K., and Taylor, R. K. (2015b). Proteolysis of virulence regulator ToxR is associated with entry of *Vibrio cholerae* into a dormant state. *PLoS Genet 11*: e1005145–e1005145.
34. Bina J, Zhu J, Dziejman M, Faruque S, Calderwood S, et al. (2003). ToxR regulon of *Vibrio cholerae* and its expression in vibrios shed by cholera patients. *Proc Natl Acad Sci USA 100*: 2801–2806. PMID: 12601157

35. Miller VL, Taylor RK, Mekalanos JJ (1987). Cholera toxin transcriptional activator toxR is a transmembrane DNA binding protein. *Cell 48*: 271–279. PMID: 3802195
36. Carroll PA, Tashima KT, Rogers MB, DiRita VJ, Calderwood SB (1997). Phase variation in tcpH modulates expression of the ToxR regulon in *Vibrio cholerae*. *Mol Microbiol 25*: 1099– 111. PMID: 9350866
37. Häse CC, Mekalanos JJ (1998). TcpP protein is a positive regulator of virulence gene expression in *Vibrio cholerae*. *Proc Natl Acad Sci USA 95*: 730–734. PMID: 9435261
38. Krukonis ES, Yu RR, DiRita VJ (2000). The *Vibrio cholerae* ToxR/TcpP/ToxT virulence cascade: distinct roles for two membrane-localized transcriptional activators on a single promoter. *Mol Microbiol 38*: 67–84. PMID: 11029691
39. Matson JS, Withey JH, DiRita VJ (2007). Regulatory networks controlling *Vibrio cholerae* virulence gene expression. *Infect Immun 75*: 5542–5549. PMID: 17875629
40. DiRita VJ, Mekalanos JJ (1991). Periplasmic interaction between two membrane regulatory proteins, ToxR and ToxS, results in signal transduction and transcriptional activation. *Cell 64*: 29–37. PMID: 1898871
41. Taylor RK, Miller VL, Furlong DB, Mekalanos JJ (1987). Use of phoA gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. *Proc Natl Acad Sci USA 84*: 2833–2837. PMID: 2883655
42. Sanchez J, Holmgren J (2008). Cholera toxin structure, gene regulation and pathophysiological and immunological aspects. *Cell Mol Life Sci 65*: 1347–1360. doi: 10.1007/s00018-008-7496-5 PMID: 18278577
43. Miller VL, Mekalanos JJ (1988). A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires ToxR. *J Bacteriol 170*: 2575–2583. PMID: 2836362
44. Li CC, Merrell DS, Camilli A, Kaper JB (2002). ToxR interferes with CRP-dependent transcriptional activation of *ompT* in *Vibrio cholerae*. *Mol Microbiol 43*: 1577–1589. PMID: 11952906
45. Mey AR, Craig SA, Payne SM (2011). The effects of amino acid supplementation on porin expression and ToxR levels in *Vibrio cholerae*. *Infect Immun. 80*: 518–528. doi: 10.1128/IAI.05851-11 PMID: 22144480
46. Crawford, J.A., Kaper, J.B., and DiRita, V.J. (1998). Analysis of ToxR-dependent transcription activation of *ompU*, the gene encoding a major envelope protein in *Vibrio cholerae*.
Mol Microbiol 29: 235–246.
47. Provenzano, D., and Klose, K.E. (2000). Altered expression of the ToxR-regulated porins OmpU and OmpT diminishes *Vibrio cholerae* bile resistance, virulence factor expression, and intestinal colonization. *Proc Natl Acad Sci USA 97*: 10220–10224.
48. Mathur, J., and Waldor, M.K. (2004). The *Vibrio cholerae* ToxR-regulated porin OmpU confers resistance to antimicrobial peptides. *Infect Immun 72*: 3577–3583.
49. Mey, A.R., Craig, S.A., and Payne, S.M. (2012). Effects of amino acid supplementation on porin expression and ToxR levels in *Vibrio cholerae*. *Infect Immun 80*: 518–528.
50. Urban S (2009). Making the cut: central roles of intramembrane proteolysis in pathogenic microorganisms. *Nat Rev Microbiol 7*: 411–423. doi: 10.1038/nrmicro2130 PMID: 19421188
51. Heinrich J, Wiegert T (2009). Regulated intramembrane proteolysis in the control of extracytoplasmic function sigma factors. *Res Microbiol 160*: 696–703. doi: 10.1016/j.resmic.2009.08.019 PMID: 19778605

52. Alba BM, Gross CA (2004). Regulation of the *Escherichia coli* sigma-dependent envelope stress response. *Mol Microbiol 52*: 613–619. PMID: 15101969
53. Ades SE (2008). Regulation by destruction: design of the sigmaE envelope stress response. *Curr Opin Microbiol 11*: 535–540. doi: 10.1016/j.mib.2008.10.004 PMID: 18983936
54. Ades SE, Connolly LE, Alba BM, Gross CA (1999). The *Escherichia coli* sigma(E)-dependent extracytoplasmic stress response is controlled by the regulated proteolysis of an anti-sigma factor. *Genes Dev 13*: 2449–2461. PMID: 10500101
55. Kanehara K, Ito K, Akiyama Y (2002). YaeL (EcfE) activates the sigma(E) pathway of stress response through a site-2 cleavage of anti-sigma(E), RseA. *Genes Dev 16*: 2147–2155. PMID: 12183368
56. Alba BM, Leeds JA, Onufryk C, Lu CZ, Gross CA (2002). DegS and YaeL participate sequentially in the cleavage of RseA to activate the sigma(E)-dependent extracytoplasmic stress response. *Genes Dev 16*: 2156–2168. PMID: 12183369
57. Traxler MF, Summers SM, Nguyen HT, Zacharia VM, Hightower GA, Smith JT, Conway T. (2008). The global, ppGpp-mediated stringent response to amino acid starvation in *Escherichia coli*. *Mol Microbiol 68*: 1128 –1148.
58. Ronneau S, Hallez R. (2019). Make and break the alarmone: regulation of (p)ppGpp synthetase/hydrolase enzymes in bacteria. *FEMS Microbiology Reviews 43*: 389-400
59. Pal RR, Das B, Dasgupta S, Bhadra RK. (2011). Genetic components of stringent response in *Vibrio cholerae. Indian J Med Res 133*: 212-217.
60. Das B, Pal RR, Bag S, Bhadra RK. (2009). Stringent response in *Vibrio cholerae:* genetic analysis of *spoT* gene function and identification of a novel (p)ppGpp synthetase gene. *Mol Microbiol 72*: 380-98.
61. Amato SM, Brynildsen MP. (2014). Nutrient transitions are a source of persisters in *Escherichia coli* biofilms. *PLoS One 9*: e93110.
62. Mishra A, Taneja N, Sharma M. (2012). Viability kinetics, induction, resuscitation and quantitative real-time polymerase chain reaction analyses of viable but nonculturable *Vibrio cholerae* O1 in freshwater microcosm. *J Appl Microbiol 112*: 945–953.
63. Wan C, Zhang Q, Lee DJ, Wang Y, Li J. (2014). Long-term storage of aerobic granules in liquid media: viable but non-culturable status. *Bioresour Technol 166*: 464 – 470.
64. Boaretti M, Lleo MM, Bonato B, Signoretto C, Canepari P. (2003). Involvement of rpoS in the survival of *Escherichia coli* in the viable but non-culturable state. *Environ Microbiol 5*: 986–996.
65. Jayakumar, J.M., Balasubramanian, D., Reddi, G., and Almagro-Moreno, S. (2020). Synergistic role of abiotic factors driving viable but non-culturable *Vibrio cholerae*. *Environ Microbiol Rep 12*: 454-465.
66. Costanzo A, Ades SE. (2006). Growth phase-dependent regulation of the extracytoplasmic stress factor, sigmaE, by guanosine 3',5' -bispyrophosphate (ppGpp). *J Bacteriol 188*: 4627-34.
67. Haralalka, S., Nandi, S., and Bhadra, R.K. (2003). Mutation in the *relA* gene of *Vibrio cholerae* affects in vitro and in vivo expression of virulence factors. *J Bacteriol 185*: 4672–4682.