LEUO REGULATION OF ADAPTIVE RESPONSES IN *VIBRIO CHOLERAE*

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LeuO is a LysR-type transcriptional regulator that is conserved among many gram negative enteric pathogens. LeuO is a bifunctional regulator that is capable of both repression and activation of gene targets. The function of LeuO has been characterized in several members of the *Enterobacteriaceae* where it has been found to be a global regulator of diverse phenotypes, often by functioning as an H-NS antagonist. In this work we sought to characterize LeuO in *Vibrio cholerae*. RNA sequencing was used to identify LeuO regulated genes, many of which appeared to be involved in environmental adaptation. Interestingly, our results suggested that LeuO did not function as an H-NS antagonist in *V. cholerae*, but appeared to function redundantly with H-NS at many H-NS regulated promoters. In subsequent experiments, genetic approaches were used to confirm the RNA sequencing studies and to define the function of LeuO in *V. cholerae* adaptive responses. We found that LeuO was part of a complex regulatory cascade in which the one-component virulence regulator ToxR directly activated *leuO* expression in response to environmental cues. LeuO then went on to directly regulate genes that contributed to bile resistance, acid tolerance, and cationic antimicrobial peptide resistance. Our collective results suggest a model whereby LeuO contributes to temporal and spatial regulation of adaptive responses during *V. cholerae* passage through the host gastrointestinal tract.

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1.0 INTRODUCTION

1.1 CHOLERA

Cholera is an acute diarrheal disease caused by the gram negative bacterium *Vibrio cholerae* (Reviewed in (1, 2)). There are a reported 1.4-4.3 million cholera cases every year which result in an estimated 28,000-142,000 deaths worldwide (3). Although an acute disease, cholera outbreaks are a major public health risk and can have widespread socioeconomic impacts. Fear of trade-sanctions and travel restrictions in locations with cholera often results in a lack of cholera reporting and in extreme instances a delay in the response of global disease response networks. Cholera is endemic in many regions of the world, particularly in developing regions. Cholera outbreaks are commonly associated with the displacement of large populations, for example following natural disasters, political turmoil, and war.

1.1.1 Epidemiology

There are more than 200 serogroups of *V. cholerae* based on the O-antigen of their lipopolysaccharide. The majority of *V. cholerae* serogroups can cause mild cases of diarrhea but are not capable of causing cholera epidemics. Only two *V. cholerae* serogroups have been documented to cause outbreaks: those exhibiting the O1 and O139 serogroup. The O139 serogroup was first identified in Bangladesh in 1992 and subsequently spread to South America

(4). However, disease caused by the O139 serogroup has largely disappeared from South America and is now confined to South-East Asia. The O1 serogroup is responsible for the majority of cholera outbreaks and is further divided into two biotypes: the El Tor and classical biotypes. Historically, there have been seven recorded cholera pandemics (5). The first six pandemics were caused by classical strains between 1817 and 1923. The seventh pandemic, which is currently ongoing, began in 1961 and was caused by the El Tor strain. The disease cholera is endemic in around 50 countries most of which are in Asia and Africa. These countries have been linked to as the starting point of localized epidemic outbreaks as well as the pandemic spread of *V. cholerae* (6). Cholera epidemics are often attributed to a combination of poor sanitations, overcrowding, lowered immunity, and environmental factors such as seasonality or natural disasters.

1.1.2 Transmission

Cholera is typically found and spread in underdeveloped countries and areas with inadequate water treatment, poor sanitation, and improper hygiene. Cholera is transmitted through the ingestion of *V. cholerae* contaminated food or water. An infectious dose is roughly estimated to be between 10^5-10^8 colony forming units (7, 8). People infected with *V. cholerae* can become symptomatic or asymptomatic carriers. Approximately 4 out of 5 people infected with *V. cholerae* become asymptomatic carriers, where they do not develop disease symptoms, but the bacteria is present in their feces for 1-10 days after infection. People who are symptomatic shed the bacteria from 2-14 days after infection. Once disseminated back into the environment, human shed *V. cholerae* are thought to exist in a short-lived hyperinfectious state which contributes to transmission to a subsequent host (9).

1.1.3 Symptoms

Following the ingestion of *V. cholerae* contaminated food or water, the onset of cholera symptoms can take between 2 hours and 5 days. The duration between ingestion and symptom development is dependent upon the initial inoculum (10). Among people who develop symptomatic cholera, 80% exhibit mild to moderate diarrheal symptoms. The remainder of symptomatic people develops typical severe disease symptoms which include a profuse watery diarrhea, vomiting, and muscle cramps. Depending upon the stage of disease, some patients will exhibit signs of dehydration. Cholera-related diarrhea, often referred to as rice-water stool, is rapid and immense with fluid losses of up to 1 liter per hour. This rapid loss of body fluids leads to dehydration and hypovolemic shock. Without treatment, death can occur within hours.

1.1.4 Treatment and Prevention

Cholera is a self-limiting disease that can be successfully treated with oral rehydration therapy. Oral rehydration therapy is based on the use of oral rehydration salts to replace fluid and electrolytes that are lost in the secretory diarrhea. Intravenous fluids are also administered to people exhibiting symptoms of severe dehydration. In some severe cases, patients are also administered antibiotics. Antibiotics have been shown to diminish the duration of diarrhea, reduce the volume of rehydration fluids needed, reduce bacterial shedding, and to reduce symptoms of the disease. Mass administration of antibiotics in epidemic and endemic settings is not recommended, as it has no effect on the spread of cholera and contributes to increasing antimicrobial resistance.

The main approach for the prevention of cholera is the provision of proper sanitation. However, as evidenced by the endemic nature of cholera in many parts of Asia, building sanitation infrastructure in developing countries has proven to be difficult. Only recently have preventative strategies using vaccines begun to be employed. Currently there are two WHO pre-qualified oral cholera vaccines: Dukoral and Shanchol. Both vaccines are killed whole cell vaccines that contain O1 *V. cholerae.* Shanchol also contains O139, while Dukoral contains recombinant cholera toxin B subunit. Both vaccines have a two-dose regimen and require cold storage. Protection through vaccination has been shown to be effective but short lived with reduced efficacy in young children. The efficacy of these vaccines in epidemic outbreaks is currently being assessed in Haiti. Currently there are no countries that require cholera vaccination.

1.2 VIBRIO CHOLERAE

V. cholerae is a gram negative facultative human pathogen. In order to successfully cause the disease cholera, *V. cholerae* must enter into the host, overcome host defenses, colonize host tissue, release cholera toxin, and then disseminate back into the aquatic environment.

1.2.1 *V. cholerae* in the environment

The natural reservoir for *V. cholerae* is the aquatic environment; *V. cholerae* can be found in estuaries, brackish waters, and oceans in many areas of the world. *V. cholerae* can be isolated from aquatic reservoirs as individual free-living cells (i.e. planktonic cells), or in biofilms

associated with aquatic organism or abiotic surfaces. The population dynamics of *V. cholerae* in the aquatic ecosystem is influenced by a multitude of environmental factors (Reviewed in (11)). Algae blooms have been correlated with increased prevalence of *V. cholerae* in the environment, presumably due to the increase in nutrient availability. Seasonal rainfall has been shown to affect *V. cholerae* distribution in the environment. It is even proposed that global warming has impacted water temperature and acidification which has influenced the prevalence of *V. cholerae* in aquatic reservoirs. The ever changing aquatic ecosystem requires *V. cholerae* be able to adapt to a multitude of environmental conditions.

1.2.2 Host defenses

V. cholerae enters the human host through ingestion of *V. cholerae* contaminated food or water. Passage of *V. cholerae* through the gastrointestinal tract exposes the cells to a number of host defenses (Reviewed in (12)). *V. cholerae* must first survive passage through the gastric acid barrier of the stomach. Surviving bacteria then enter the lumen of the small intestine where they are exposed to high concentrations of bile acids and other organic acids plus products of the resident flora. *V. cholerae* then must migrate through the mucus layer, where they encounter products of the innate immune system including antimicrobial peptides and antibodies. *V. cholerae* has evolved a number of responses to mitigate the effects of these host defenses. Common mechanisms with which bacteria can combat these stressors include: altering their outer membrane, the efflux of harmful substrates, enzymes that chemically degrade or inactivate antimicrobials, and mutations in DNA that change gene products which are targets of the antimicrobials (13).

1.2.3 *V. cholerae* virulence factors

Once *V. cholerae* has entered the small intestine it traverses the mucous layer and migrates towards the epithelial surface where it produces attachment factors that facilitate colonization and replication (12). Early during *V. cholerae* infection, virulence factors are produced which contribute to colonization and disease development. Two of the major virulence factors produced by *V. cholerae* is cholera toxin (CT) and the toxin-coregulated pilus (TCP). CT is an AB-type enterotoxin consisting of a single A subunit and five B subunits (Reviewed in (14, 15)). The B subunit binds to GM_1 gangliosides that are located on the surface of epithelial cells to deliver the A subunit. The A subunit then enters into the host cytoplasm before being activated by the reduction of a disulfide bond which releases the active A1 subunit. The A1 subunit then ADP-ribosylates Gsα, a GTP-binding regulatory protein that is associated with adenylate cyclase. ADP-ribosylation of Gsα leads to constitutive cyclic AMP production (cAMP). High concentrations of cAMP then increase chloride and bicarbonate secretion while inhibiting sodium chloride uptake which then results in osmotic water loss into the intestinal lumen. The massive water secretion into the lumen produces the hallmark secretory diarrhea that is characteristic of the disease cholera. The TCP is a Type IV pilus that has been shown to be essential for intestinal colonization (16). While the TCP is not required for adherence to enterocytes, it has been shown to be required for microcolony formation *in vivo* (17). In addition to CT and TCP there are a number of accessory toxins and accessory colonization factors that contribute to *V. cholerae* pathogenesis.

While *V. cholerae* virulence factors have been shown to be induced upon host entry, recent studies have shown that virulence factors are repressed late in infection prior to dissemination in the diarrheal purge (18, 19). Late in infection *V. cholerae* also induces the

expression of other genes which are largely unknown that contribute to dissemination and transmission (20). The regulatory mechanisms controlling this phenotype shift late in infection have not been defined.

1.3 THE TOXR REGULON

V. cholerae major virulence factors, CT and TCP, are under the control of a hierarchical regulatory cascade called the ToxR regulon (Reviewed in (21)). Production of virulence factors require the proper conditions, as such, many of the proteins in this cascade are influenced by environmental stimuli (22). Inside the intestines, fine-tuned regulation and spatial-temporal regulation of virulence factors is critical. There are three primary transcriptional regulators in the ToxR regulon: ToxT, TcpP, and ToxR. The ToxR regulon has been further subdivided into ToxT-dependent and ToxT-independent branches. The ToxT-dependent branch is responsible for producing CT and TCP (Fig. 1) while the ToxT-independent regulates the production of outer membrane porins and the downregulation of the ToxR regulon in response to environmental cues (Fig. 2).

1.3.1 The ToxT-dependent branch

The virulence factors CT and TCP are under direct control of ToxT. ToxT is an AraC-like transcriptional activator that regulates expression of the genes *ctxAB* and *tcpA-F* operons which encode CT and TCP, respectively (23). ToxT activity at its target promoters has also been shown

to be influence by environmental factors including bicarbonate and fatty acids. The expression of *toxT* is coordinately regulated by ToxRS and TcpPH (24).

ToxR is a transmembrane transcriptional regulator and a one-component signal transducing protein (25). ToxR has an amino-terminal cytoplasmic DNA-binding domain and a carboxy-terminal periplasmic domain. The periplasmic domain of ToxR is able to sense environmental signals and alter the DNA-binding activity of the cytosolic DNA binding domain. ToxR directly interacts with ToxS, another transmembrane localized protein. ToxS has been shown to stabilize ToxR and is necessary for optimal ToxR transcriptional activity (26, 27). ToxR activity at its target promoters has been shown to be influence by a variety of environmental stimuli including peptides and bile salts (25, 27-29).

TcpP is a ToxR-family transmembrane transcriptional regulator (30). TcpP directly interacts with TcpH, another transmembrane localized protein. TcpH has been shown to be responsible for protecting TcpP from proteolytic degradation (31). In the absence of TcpH, TcpP is rapidly degraded which may play a role in downregulation of the ToxR regulon late in infection. TcpP activity, like ToxR, is also thought to be influenced by environmental stimuli including bile salts (32). Expression of *tcpPH* is coordinated regulated by AphA and AphB; two unlinked cytoplasmic regulatory proteins (33, 34). AphA is a member of the winged-helix family of transcription factors while AphB is a LysR-type transcriptional regulator. The expression of *aphA* and activity of AphB are also influence by environmental stimuli. The expression of *aphA* is under control of the *V. cholerae* quorum sensing systems while AphB activity is influenced by pH and oxygen tension (35, 36).

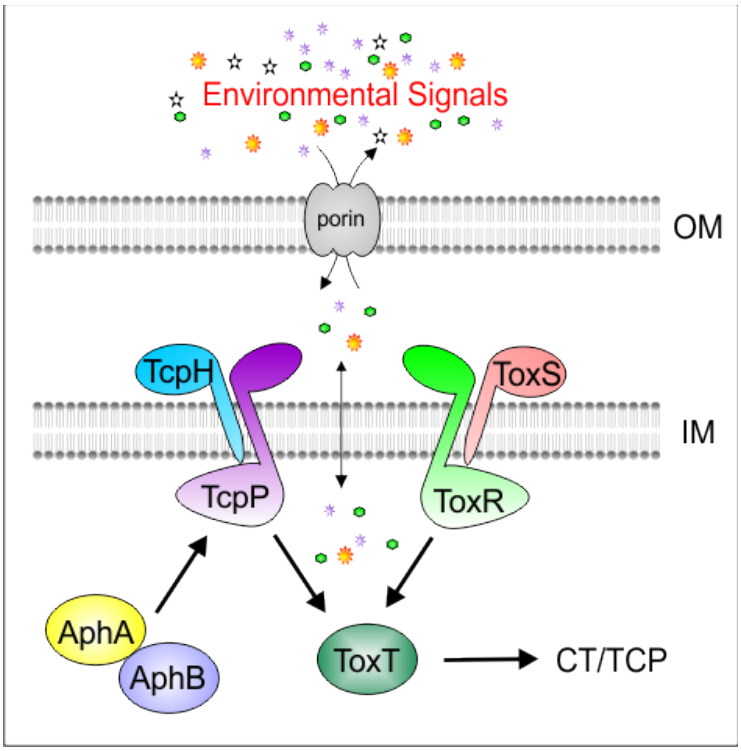

Figure 1. The ToxT-dependent branch of the ToxR regulon.

The ToxT-dependent branch of the ToxR regulon is a cascade that controls production of cholera toxin (CT) and the toxin coregulated pilus (TCP). Porins are located in the outer membrane (OM) while the transmembrane proteins ToxR, ToxS, TcpP, and TcpH are located in the inner membrane (IM). AphA, AphB, and ToxT are all cytoplasmic transcriptional regulators.

1.3.2 The ToxT-independent branch

V. cholerae produces two major outer membrane proteins: OmpU and OmpT. OmpU and OmpT are general diffusion porins which are responsible for allowing the diffusion of nutrients, metabolites, and signaling molecules into and out of the cell (37). The genes encoding for OmpU and OmpT are reciprocally regulated by ToxR which binds to conserved direct repeat elements that are located in the *ompU* and *ompT* promoters (38, 39). ToxR positively regulates *ompU* and negatively regulates *ompT*. OmpU is preferentially produced during growth in rich media or in

minimal media containing certain amino acids, bile, or mucin (40). In nutrient poor environments, or in a *toxR* mutant strain, the porin profile is reversed and OmpT becomes predominant while OmpU is no longer produced. Consistent with activation of *ompU* expression by bile salts, the production of OmpU is associated with bile salt resistance while the production of OmpT is associated with bile salt susceptibility; a phenotype that is related to the pore properties of each respective porin (41). OmpU and OmpT production has been found to be regulated in response to different environmental stimuli including bile, osmolality, organic acids, cyclic dipeptides, and amino acids (28, 29, 40, 42).

LeuO is a new member of the ToxT-independent branch of the ToxR regulon in *V. cholerae*. Recent studies in our laboratory showed ToxR activated *leuO* expression in response to an endogenously produced cyclic dipeptide cyclo(Phe-Pro) (43). Overexpression of *leuO* was then linked to downregulation of the ToxR regulon and attenuated production of CT and the TCP. These studies also showed that cyclo(Phe-Pro) signaling was dependent on the ToxR periplasmic domain; a finding that supported the conclusion that the ToxR periplasmic domain functioned in environmental sensing. The fact that cyclo(Phe-Pro) accumulated in culture supernatants at high cell density (29), such as occurs late in infection, suggested a model whereby ToxR may mediate the expression of genes late in infection in response to accumulated cell metabolites. Collectively the results of this study provided the first evidence that ToxR could function as a virulence repressor and suggested *leuO* functioned downstream of ToxR to modulate *V. cholerae* environmental adaptation in response to extracellular cues.

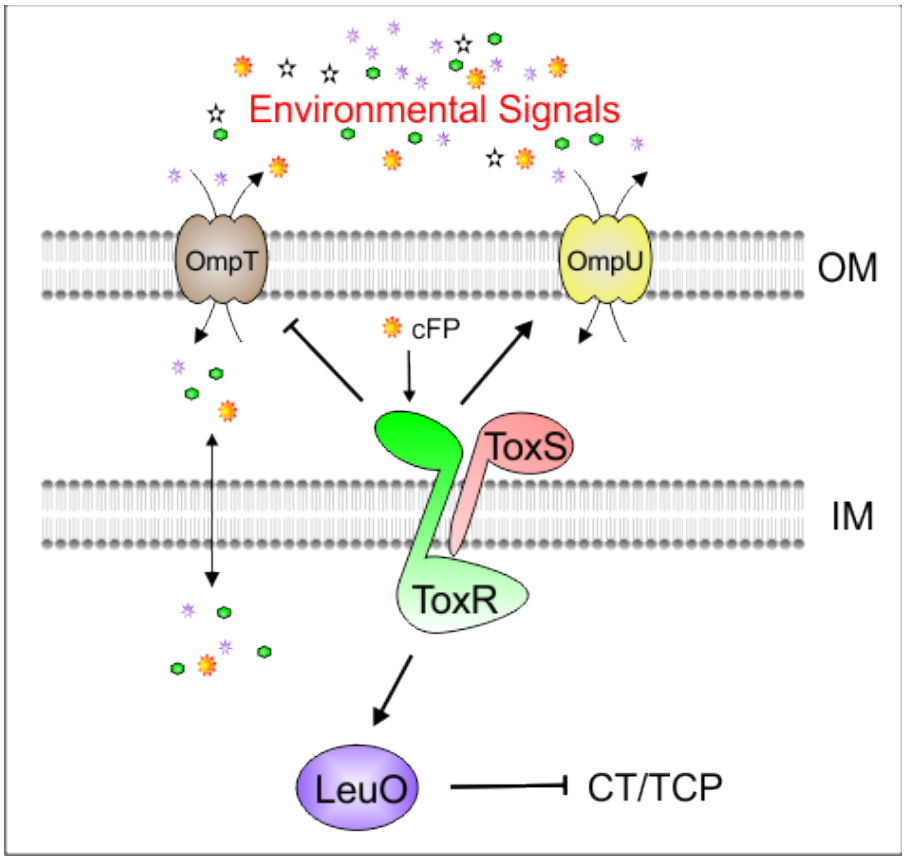

Figure 2. The ToxT-independent branch of the ToxR regulon.

The ToxT-independent branch of the ToxR regulon involves ToxR regulation of genes not essential for the production of cholera toxin (CT) or the toxin coregulated pilus (TCP). OmpU and OmpT are porins located in the outer membrane (OM) and are reciprocally regulated by ToxR. ToxR regulates LeuO, a cytoplasmic transcriptional regulator that represses CT and TCP production. The small molecule cyclo(Phe-Pro) (cFP) is one of the environmental signals that is directly sensed by ToxR and influences this regulatory cascade.

1.4 LEUO

LeuO is a LysR-type transcriptional regulator that is produced in many gram negative bacteria. LeuO is been best studied in the *Enterobacteriaceae*, where LeuO has been shown to be maximally expressed at high cell density and at stationary phase (44). LeuO was first

characterized in *Salmonella enterica* as being part of a promoter relay mechanism that functioned to regulate the expression of leucine biosynthesis genes (45, 46). Subsequent studies in the *Enterobacteriaceae* have suggested that LeuO is a global regulator that regulates the expression of diverse genes (47, 48). LeuO is also a bifunctional regulator, capable of regulating targets through repression or activation.

1.4.1 LysR-type transcriptional regulators

The LysR-type transcriptional regulators (LTTRs) are the largest family of bacterial genetic regulators (Reviewed in (49)). LTTRs have an N-terminal DNA binding domain with a winged helix-turn-helix motif, a long linker domain, and a C-terminal effector-binding domain. Although the DNA-binding domain is well conserved amongst LTTRs, the consensus binding sequence is not well defined and contains an AT-rich region with imperfect dyad symmetry. There are 38 LTTRs annotated in the *V. cholerae* genome, this large number can give insight as to why there is little conservation in binding sequences amongst LTTRs. With theoretically many LTTRs produced in one cell at any given time, specificity for target genes requires that each protein have its own unique characteristics. The multimeric state of LTTRs also contributes to specificity with the majority of LTTRs being found to function as tetramers. This conformation implies that LTTRs are able to regulate transcription through bending of the DNA. Dimerization is often achieved through the linker domain. The effector-binding domain is divided into two subdomains with a co-inducer binding cleft between them. Co-inducer binding is a common way in which substrates are used in feedback loops to regulate LTTR protein activity. Co-inducers have been found to also increase the LTTRs affinity for DNA or alter the degree of DNA-

bending. There are often homologs of an LTTR protein amongst bacteria that are structurally and functionally similar but regulate diverse cellular processes based on the respective organisms.

1.4.2 Roles of LeuO in the *Enterobacteriaceae*

LeuO function is well characterized in a number of *Enterobacteriaceae*. In both *Escherichia coli* and *S. enterica* serovar Typhimurium, LeuO has been shown to function as an antagonist of H-NS, the histone-like nucleoid structuring protein (47, 48). H-NS is a small chromatin-associated global transcriptional silencer found in *Enterobacteriaceae* (50). Approximately one half of the LeuO gene targets were shown to overlap with H-NS targets; however, H-NS is not required for LeuO binding and LeuO does not displace H-NS from the DNA.

LeuO has been shown to regulate a multitude of different phenotypes in the *Enterobacteriaceae*. In *E. coli* LeuO has been shown to regulate citrate fermentation, fimbriae production, and biofilm production (48). LeuO was also shown to be upregulated in response to low phosphate, branched amino acid starvation, and in response to the stringent response alarmone guanosine pentaphosphate (ppGpp) (44, 51, 52). In *S. enterica* LeuO has been shown to regulate virulence genes including OmpS1 and OmpS2 porins, and genes in the Salmonella pathogenicity island I (53-55).

LeuO also appears to be a global regulator in the family *Vibrionaceae* where it has been linked to diverse phenotypes. For example, in *V. parahaemolyticus*, LeuO has been shown to regulate the expression of a type III secretion system and cell swarming (56, 57). In *V. vulnificus*, LeuO has been shown to regulate cell wall degradation and in *V. cholerae* LeuO has been linked to biofilm production and virulence factor production (43, 58, 59).

1.5 GOALS OF DISSERTATION

ToxR is a global regulator that has been shown to regulate the expression of its target genes in response to a variety of environmental stimuli; a phenotype that is exemplified by the function of ToxR in activating virulence gene expression following host entry. Studies from our laboratory recently showed that ToxR could also repress virulence gene expression at high cell density via activation of *leuO* expression. This observation suggested the possibility that ToxR may function to regulate the *V. cholerae* host exit program or to regulate *V. cholerae* adaptive responses to the dynamic environments found in the host gastrointestinal tract. Since LeuO functioned downstream of ToxR, and has been shown to regulate diverse genes in other bacteria, we hypothesized that LeuO plays an important role in regulating the expression of adaptive responses in *V. cholerae*. This work sought to characterize the function of LeuO in *V. cholerae*.

The goal of Chapter 2 was to investigate the relation between LeuO and bile resistance. I found that *leuO* transcription increased in response to bile and bile salts, but not in response to other detergents. The bile-dependent increase in *leuO* expression was contingent on ToxR which was found to bind directly to the *leuO* promoter. I further showed that the periplasmic domain of ToxR was required for basal *leuO* expression and for the bile-dependent induction of both *leuO* and *ompU* transcription. *V. cholerae* mutants that did not express *leuO* exhibited increased bile susceptibility suggesting that LeuO contributes to bile resistance. My collective results demonstrated that ToxR activated *leuO* expression in response to bile and that LeuO was a component of the ToxR-dependent responses that contribute to bile resistance. In this work I showed that the function of ToxR in bile resistance extended beyond porin regulation to include *leuO*.

The goal of Chapter 3 was to investigate the relationship between LeuO and acid tolerance. *V. cholerae* is a neutrophilic enteric pathogen that is extremely sensitive to acid. As *V. cholerae* passages through the host gastrointestinal tract it is exposed to a variety of environmental stresses including low pH and volatile fatty acids. Exposure to acidic environments induces expression of the *V. cholerae* acid tolerance response. A key component of the acid tolerance response is the *cad* system, which is encoded for by *cadC* and the *cadBA* operon. CadB is a lysine/cadaverine antiporter and CadA is a lysine decarboxylase which function together to counter low intracellular and extracellular pH. CadC is a membrane associated transcription factor that activates *cadBA* expression in response to acidic conditions. I investigated the role of the LysR-type transcriptional regulator LeuO in the *V. cholerae* acid tolerance response. Transcriptional reporter assays revealed that *leuO* expression repressed *cadC* transcription, indicating that LeuO was a *cadC* repressor. Consistent with this, *leuO* expression was inversely linked to lysine decarboxylase production and *leuO* overexpression resulted in increased sensitivity to organic acids. Overexpression of *leuO* in a *cadA* mutant potentiated killing by organic acids suggesting that the function of *leuO* in the acid tolerance response extended beyond its regulation of the *cad* system. Collectively, these studies have identified a new physiological role for LeuO in *V. cholerae* acid tolerance.

The goal of Chapter 4 was to investigate the relationship between LeuO and resistance to cationic antimicrobial peptides (CAMPs). Antimicrobial susceptibility screens revealed that *leuO* deletion resulted in increased resistance to polymyxin B. Polymyxin B is cationic peptide-like antibiotic. The polymyxin B mechanism of action, like other CAMPs, is related to its function in disruption of cell membranes. The initial binding of CAMPs to gram negative bacteria is mediated by electrostatic interactions between the positively charged CAMP and the negatively

charged lipopolysaccharide (LPS). As such bacteria have evolved mechanisms to resist the bactericidal effects of cationic antimicrobial peptides by altering the charge of their LPS via chemical modification. In *V. cholerae* the genes encoded by the *almEFG* operon have been shown to be required for CAMP resistance. The AlmEFG proteins function to modify *V. cholerae* lipid A with glycine and diglycine. This modification changes the charge of the lipopolysaccharide, making it more electropositive and thus reducing the electrostatic interactions with CAMPs. The *almEFG* operon is positively regulated by the CarRS two-component system. CarR is the response regulator and CarS the environmental sensor with downstream targets being regulated in response to polymyxin B and bile. I investigated whether LeuO affected CAMP resistance through regulating the expression of *carRS*. I showed through transcriptional reporter assays and gel shift assays that LeuO was a direct repressor of *carRS*. *V. cholerae* mutants that did not express *leuO* exhibited increased resistance to polymyxin B, and conversely overexpression of *leuO* made cells more susceptible to polymyxin B. My collective results demonstrated that LeuO contributed to cell surface remodeling and polymyxin B resistance through the regulation of *carRS*.

One of the goals of my thesis was to test the hypothesis that LeuO was a global regulator in *V. cholerae.* To test this, I utilized RNAseq to define the LeuO transcriptome (Appendix B). The results of this experiment confirmed that LeuO was a global regulator in *V. cholerae*. I found that LeuO affected the expression of 113 genes with 80 being repressed and 33 being activated by LeuO. The RNAseq analysis showed that in addition to regulating genes involved in acid tolerance, CAMP resistance, biofilm and pathogenesis, LeuO also regulated genes involved in cell metabolism and a number of stress responses.

2.0 *VIBRIO CHOLERAE LEUO* TRANSCRIPTION IS POSITIVELY REGULATED BY TOXR AND CONTRIBUTES TO BILE RESISTANCE

Work described in this section was published in the Journal of Bacteriology (J Bacteriol. 2015 Nov 15;197(22):3499-510. doi: 10.1128/JB.00419-15) by authors Vanessa M. Ante, X. Renee Bina, Mondraya F. Howard, Sameera Sayeed, Dawn L. Taylor and James E. Bina

2.1 INTRODUCTION

Vibrio cholerae is a gram-negative bacterial pathogen and the causal agent of the severe diarrheal disease cholera. *V. cholerae* exists naturally in aquatic reservoirs and is capable of colonizing the human small intestine. The transition of *V. cholerae* from the aquatic ecosystem to growth in the human gastrointestinal tract is mediated by transcriptional responses that are required for colonization and disease development. Many of the genes that contribute to intestinal colonization are under control of the membrane associated regulatory protein ToxR; which functions as one of the primary regulators in the ToxR regulon (Reviewed in (21)). The ToxR regulon is divided into two branches, a ToxT-dependent branch which controls the expression of virulence factors, and a ToxT-independent branch which reciprocally regulates the production of outer membrane porins OmpU and OmpT. The ToxT-dependent branch of the ToxR regulon is a hierarchical regulatory cascade that regulates the expression of genes encoding for the production of cholera toxin (CT) and the toxin co-regulated pilus (TCP) in response to environmental cues in the host.

ToxR is a membrane associated regulatory protein that belongs to the winged-helix family of transcriptional regulators (60, 61). ToxR is a one-component signal transducing protein that is composed of a periplasmic signaling domain and a cytoplasmic DNA binding domain that are linked by a single transmembrane spanning domain (25). *toxR* is encoded in an operon along with *toxS* which is located downstream of *toxR*. ToxS is an inner membrane protein, which is thought to interact with ToxR to facilitate its transcriptional activity (26, 27). The ToxR periplasmic signaling domain is thought to sense and transduce environmental stimuli to affect the activity of the cytoplasmic DNA binding domain at its target genes. ToxR has been shown to

respond to a variety of environmental stimuli including acidity, nutrient availability, salinity, small molecules, and bile (25, 28, 29, 42).

It has been shown that ToxR plays an essential role in modulating adaptive responses that contribute to bile resistance. Bile is produced by the liver and secreted at high concentrations into the small intestine to aid in the digestion of lipids. Bile is composed primarily of bile salts, but also contains significant amounts of phospholipids, cholesterol, protein and bilirubin. While bile is important in digestion, bile also provides a barrier against intestinal colonization by restricting bacterial growth in the small intestine; presumably through its detergent-like effects on bacterial cell membranes (62). As such, enteric pathogens have evolved methods to overcome this barrier. This includes the modulation of outer membrane porin proteins to decrease the rate of diffusion of toxic molecules across the outer membrane and by the expression of active efflux systems which remove bile salts from within the cell envelope (63-67). In *V. cholerae,* resistance to the antimicrobial effects of bile is due to the combined action of multiple factors including active efflux and ToxR regulated genes. This is evident by the observation that *toxR* mutant strains exhibit greatly increased susceptibility to bile and bile salts (68).

The elevated susceptibility of *toxR* mutant strains to bile salts has been linked to the expression of the *ompU* and *ompT* porins (68). OmpU and OmpT are general diffusion porins located in the outer membrane. Porins are responsible for allowing the diffusion of nutrients, metabolites, and signaling molecules into and out of the cell (37). OmpU and OmpT have been found to be regulated in response to environmental stimuli including bile, osmolarity, organic acids, cyclic dipeptides, and amino acids (25, 28, 29, 42). The genes encoding for OmpU and OmpT are reciprocally regulated by ToxR; which binds to conserved direct repeat elements that are located in the *ompU* and *ompT* promoters (38, 39). ToxR positively regulates *ompU* and

negatively regulates *ompT*. OmpU is preferentially produced during growth in rich media or in minimal media containing certain amino acids, bile, or mucin (40). In nutrient-poor environments, or in a *toxR* mutant strain, the porin profile is reversed and OmpT becomes predominant while OmpU is no longer produced. Consistent with activation of *ompU* expression by bile salts, the production of OmpU is associated with bile salt resistance while the production of OmpT is associated with bile salt susceptibility (41). This phenotype is presumably related to the fact that OmpU, in contrast to OmpT, is an anion selective porin which restricts passage of negatively charged compounds (41, 69).

Previous studies in our laboratory suggested that in *V. cholerae* ToxR activated *leuO* expression in response to cFP (43). Increased *leuO* transcription was linked to down regulation of the ToxR regulon and attenuated CT and TCP production. These results suggested that *leuO* functioned downstream of ToxR to modulate gene expression in response to environmental cues. LeuO is a LysR-family transcription factor that was first identified as a regulator of leucine biosynthetic genes in *Salmonella typhimurium* (45). Subsequent studies have shown LeuO to be a global regulator of diverse and unrelated phenotypes in the *Enterobacteriaceae*. For example, in *Salmonella enterica* LeuO has been shown to regulate outer membrane proteins, virulence genes, transport genes, biofilm production, and quorum sensing (70). Likewise, LeuO has been shown to be involved in the regulation of genes involved in carbohydrate utilization, phage resistance, acid shock, temperature adaptation and biofilm production in *Escherichia coli* (48). LeuO has also been associated with virulence gene regulation in *Yersina enterocolitica* (71). The function of LeuO as a global regulator appears to be conserved in the *Vibrionaceae* where LeuO has been shown to contribute to biofilm production, cell wall degradation, and virulence gene regulation (43, 57-59).

Since bile acids are an important environmental cue during *V. cholerae* pathogenesis, and since ToxR regulates the expression of many of its target genes in response to bile salts, we tested the hypothesis that *leuO* expression was also modulated in response to bile via ToxR. The results of our experiments showed that *leuO* expression was activated upon exposure to bile salts by a mechanism that was dependent on ToxR. Multiple approaches were used to show that ToxR acted directly at the *leuO* promoter and that the ToxR periplasmic domain was required for basal *leuO* expression and the bile-dependent induction of both *leuO* and *ompU* expression, respectively. Mutants that failed to express *leuO* exhibited reduced survival upon exposure to lethal concentrations of bile, indicating that LeuO contributed to bile resistance. Collectively our results indicated that ToxR activated *leuO* and *ompU* expression in response to bile salts by a mechanism that was dependent on the ToxR periplasmic signaling domain and that LeuO contributed to the ToxR-mediated bile resistance.

Table 1. Strains, plasmids and oligonucleotides used in Chapter 2.

Strain, plasmid or oligonucleotide	Relevant characteristics	Source
Strains		
Escherichia coli		
EC100λpir	*supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 (λpirR6K)*	Epicenter
SM10λpir	*thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu Kmr (λpirR6K)*	Lab collection
Vibrio cholerae		
JB58	O1 El Tor strain N16961 Δ*lacZ*, Sm^R	Lab collection
XBV222	JB58Δ*leuO*	(43)
DT733	JB58Δ*toxRS*	(43)
SS4	JB58 $toxR^{\Delta ppd}$	This study
Plasmids		
pCM10	*luxCDABE* reporter plasmid, Km^R	(72)
pJB906	pCM10 containing the *leuO* promoter	(43)
pTL61T	*lacZ* transcriptional reporter plasmid, Cb^R	(73)
pXB266	pTL61T containing the *leuO* promoter region with two ToxR binding sites	
pVA258	pTL61T containing the *leuO* promoter region with one ToxR binding site	This study
pVA261	pTL61T containing the *leuO* promoter region with no ToxR binding sites	This study
pXB233	pTL61T containing the *vexRAB* promoter	(74)
pXB228	pTL61T containing the *vexEF* promoter	(75)
pXB229	pTL61T containing the *vexGH* promoter	(75)
pXB230	pTL61T containing the *vexIJK* promoter	(75)
pXB231	pTL61T containing the *vexCD* promoter	(75)
pXB232	pTL61T containing the *vexLM* promoter	(75)
pBAD18	Arabinose regulated expression plasmid, Cb^R	(76)
pBAD33	Arabinose regulated expression plasmid, Cm^R	(76)
pXB298	pBAD18Km expressing *leuO*	(43)
pVA94	pBAD18 expressing *leuO*	This study
pXB289	pBAD18 expressing *toxRS*	(43)
pXB286	pBAD18 expressing $toxR^{\Delta ppd}S$	(43)
pXB302	pBAD33 expressing *toxRS*	This study
pDT1391	pBAD33 expressing $toxR^{\Delta ppd}S$	This study
pWM91	Suicide plasmid vector used for allelic exchange	(77)
pWM91::Δ$toxR^{ppd}$	pWM91 containing a fragment of *toxR* harboring a deletion of the periplasmic domain	This study
Oligonucleotides	Sequence (5' to 3')	
P_{leuO1}-F	CGCCCGGGAAATGCATTTTTATAGATTTTT	
P_{leuO2}-F	CGCCCGGGAATCGTATTGATTATTAAGGCT	
P_{leuO}-R	GGGGATCCGCGTCTTTTTTATCTAACATTTGCATGCCT	
$toxR^{\Delta ppd}$-F1	GGGAGCTCGGTCCTCAAAAGAGATAT	
$toxR^{\Delta ppd}$-F2	CTGCTCACTAACTAGGATCTTGCTAT	
$toxR^{\Delta ppd}$-R1	AACCCGGGCATGCCGCTCAGTCAGG	
$toxR^{\Delta ppd}$-R2	AGCAAGATCCTAGTTAGTGAGCAGTA	
5'BIO	GCGGGAGTCGGCAGCG	
leuO-F-EMSA	GCGGGAGTCGGCAGCGGTTAAAACATTTTTGACGTGAATATTAGTG	
leuO-R-EMSA	GCGGGAGTCGGCAGCGCGTCACTAGCGATAAATATGCATAAATC	
ompU-F-EMSA	GCGGGAGTCGGCAGCGCAATTAGATTGCGTGCATTT	
ompU-R-EMSA	GCGGGAGTCGGCAGCGTTTTTTTTACTCCCAAAGTTC	
vexR-F-EMSA	GCGGGAGTCGGCAGCGTGCAAAACAGGGGGTATTAG	
vexR-R-EMSA	GCGGGAGTCGGCAGCGGCCGTACACTATTTCAGACA	
leuO-qPCR-F	GACCACTTCGCCACAAATCACCA	
leuO-qPCR-R	CGTTGGATGGCGGAAAATGCG	
ompU-qPCR-F	ACACCGTATAGGCTGTCATTG	
ompU-qPCR-R	GTGCTGAAGCTCGCCTATCTC	
gyrA-qPCR-F	CAATGCCGGTACACTGGTACG	
gyrA-qPCR-R	AAGTACGGATCAGGGTCACG	

doi: 10.1128/JB.00419-15.

2.2 RESULTS

2.2.1 Bile salts induce expression of *leuO*.

In response to bile salts, ToxR has been shown to activate *ompU* and *ctxAB* expression while repressing *ompT* expression (68, 78). Given that ToxR activates the expression of at least some of its target genes in response to bile salts, we hypothesized that ToxR may also regulate *leuO* expression in response to bile salts. To test this hypothesis we introduced a *leuO-lacZ* reporter plasmid pXB266 into WT strain JB58. The resultant strain was then cultured in the presence of bile or the bile salt deoxycholate to middle logarithmic growth phase when *leuO* expression was quantified by the β-galactosidase assay. The results showed a ~5-fold increase in *leuO* expression in the presence of bile and a ~6-fold increase in *leuO* expression in the presence of deoxycholate compared to the LB broth control (Fig. 3A). These results confirmed the hypothesis that *leuO* expression was upregulated in the presence of bile or deoxycholate.

Bile salts and other components of bile exhibit detergent-like properties that can affect the permeability barrier of the outer membrane and compromise the integrity of the cytoplasmic membrane (62). This alluded to the possibility that *leuO* was upregulated as a result of the deleterious effects from the detergent properties of bile/deoxycholate on the cell envelope. If this was true, we hypothesized that exposure of *V. cholerae* to other classes of membrane active detergents should also result in *leuO* upregulation. We therefore tested the effects of two anionic detergents (SDS and Sarcosyl) and one nonionic detergent (Triton X-100) on *leuO-lacZ* expression. The results showed that exposure of JB58(pXB266) to these three detergents did not significantly affect *leuO* expression (Fig. 3A). Taken together, these results suggested that *leuO*

induction was specific for bile and bile salts and was probably not a result of the detergent-like properties of bile or deoxycholate.

The finding that bile salts induced *leuO* expression prompted us to investigate if cFP and bile salts functioned synergistically to regulate *leuO* expression. To test this we quantified *leuO* expression in WT strain JB58 containing pJB906 (*leuO-lux*) during growth in LB broth containing DOC, cFP, or DOC and cFP. As expected, the addition of DOC to the media activated *leuO* expression (Fig. 3B). However, the addition of cFP to the media did not significantly increase *leuO* expression compared to the DMSO control. cFP did activate *leuO* expression at high (non-physiological) concentrations of cFP (data not shown). As cFP was shown to activate *leuO* expression under AKI growth conditions, this result suggests that cFP activity is dependent on the growth conditions (43). The addition of both DOC and cFP to the growth media resulted in a small, but reproducible decrease in *leuO* expression compared to cells grown in media containing DOC and DMSO. Although the presence of DOC and cFP appeared to decrease *leuO* expression, the differences were not statistically significant. Based on these results we concluded that cFP and DOC do not work synergistically to increase *leuO* expression during growth under standard laboratory conditions. It remains to be determined how cFP and DOC affect *leuO* expression *in vivo*.

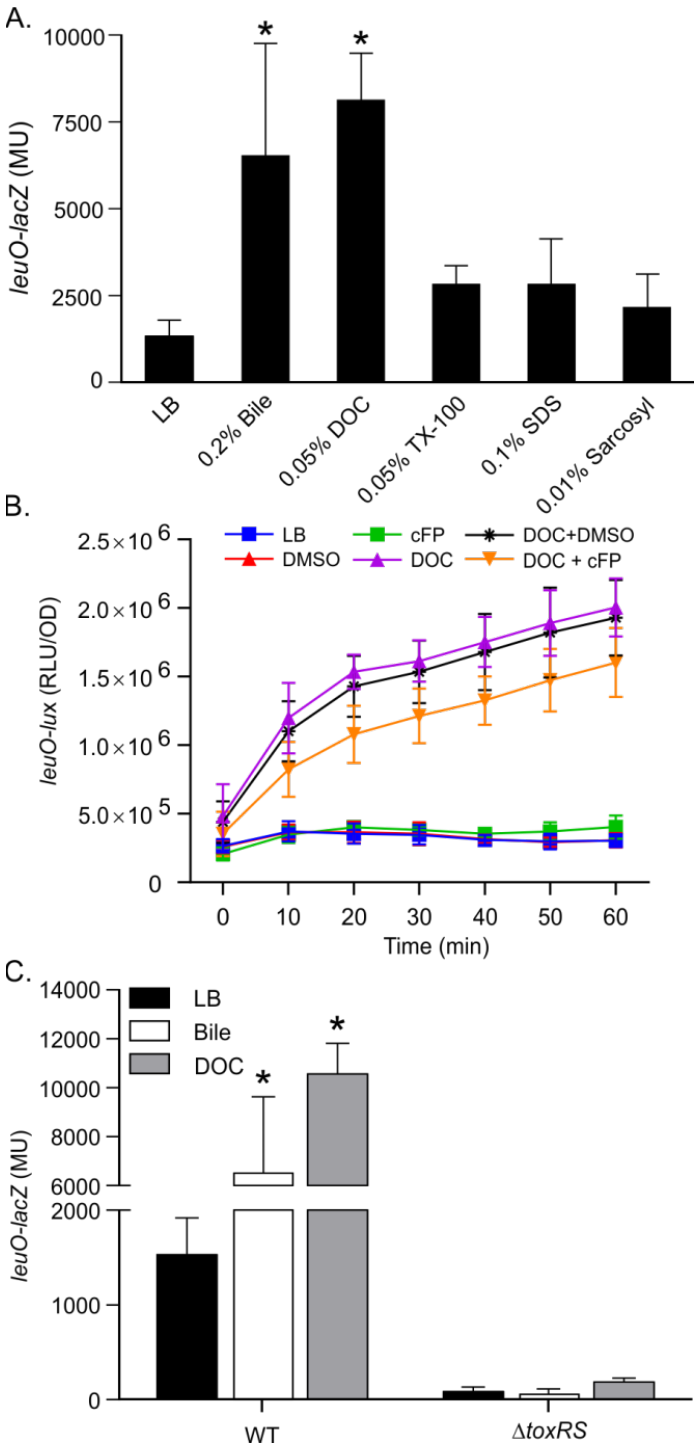

Figure 3. Induction of *leuO* transcription by bile and bile salts requires ToxR.

Induction of *leuO* transcription by bile and bile salts requires ToxR. (A) WT strain JB58 carrying the *leuO-lacZ* reporter plasmid pXB266 was grown in LB broth or LB broth supplemented with bile or the indicated detergents. Culture aliquots were collected at middle logarithmic phase and assayed for *leuO-lacZ* expression by the β-

galactosidase assay. (B) The expression of *leuO-lux* in WT strain JB58::pJB906 (*leuO-lux)* during growth in LB broth containing the indicated compounds. DOC was used at 0.0125%, cFP at 1 mM and DMSO at 0.1%. (C) WT strain JB58 and Δ*toxRS* strain DT733 carrying pXB266 (*leuO-lacZ)* were grown in LB broth or LB broth plus 0.2% bile or 0.1% deoxycholate. Culture aliquots were collected at middle logarithmic phase and assayed for *leuO-lacZ* expression by the β-galactosidase assay. The presented data are the mean +/- SD of three independent experiments. Statistical significance in panel A was determined using One-Way ANOVA with Dunnett's post-test comparing each mean with the LB control; *=P<0.01. Statistical significance in panel B was determined using Two-Way ANOVA with Tukey's post-test; *=<0.0001. Abbreviations: DOC, deoxycholate; TX-100, Triton X-100; SDS, sodium dodecyl sulfate; MU, Miller Units; DMSO, dimethyl sulfoxide; RLU, Relative Light Units. doi: 10.1128/JB.00419-15.

2.2.2 Upregulation of *leuO* by bile is dependent on ToxR.

ToxR regulates OmpU and OmpT production in response to bile salts. This suggested the possibility that ToxR may be responsible for the bile-dependent upregualtion of *leuO* in response to bile and bile salts. To test this we compared *leuO-lacZ* expression in JB58(pXB266) and an isogenic Δ*toxRS* mutant DT733(pXB266) that had been cultured in the presence and absence of bile or deoxycholate as described above. The results showed that *leuO* expression in JB58 was increased on exposure to deoxycholate and bile relative to growth in LB alone (Fig. 3C) as shown above. In contrast, *leuO* expression in the Δ*toxRS* mutant grown in LB broth decreased by ~15-fold relative to JB58. This indicates that ToxR is a positive regulator of *leuO*, confirming previous findings (43). Deletion of *toxRS* also abolished the bile- and deoxycholate-dependent upregulation of *leuO* (Fig. 3C). Together these data indicated that ToxR was required for basal *leuO* expression and for the increased *leuO* expression in response to bile and bile salts. Further, given that none of the other tested detergents affected *leuO* expression, we speculate that the

ToxR-dependent upregulation of *leuO* was specific for bile salts, and was not due to other components of bile or due to a general membrane stress response.

2.2.3 ToxR acts directly on the *leuO* promoter.

Previous studies showed that ToxR binds to direct repeat elements that represent a ToxR consensus binding sequence in the *toxT*, *ompU*, *ompT*, and *ctxAB* promoters to regulate their transcription (25, 38, 39, 79, 80). The gene encoding LeuO (VC2485) is encoded downstream from VC2486 in an apparent two-gene operon. Sequence analysis of the *leuO* promoter revealed the presence of two putative ToxR consensus binding sequences, suggesting that ToxR may directly regulate *leuO* expression (43). The distal ToxR binding site (i.e. site A in Fig. 4A) is located from -126 to -112 relative to the start codon for VC2486 while the proximal ToxR binding site (i.e. site B in Fig. 4A) is encoded on the complementary strand from -104 to -90 relative to the start codon for VC2486. To determine if both ToxR binding sites were required for *leuO* expression, derivatives of the *leuO* promoter lacking one or both ToxR consensus sequences were transcriptionally fused to the *lacZ* gene in pTL61T. All together we generated three *leuO-lacZ* reporters: pXB266 (WT *leuO* promoter), pVA258 (deletion of the distal ToxR binding site), and pVA261 (no ToxR binding sites) (Fig. 4A).

We introduced these three *leuO-lacZ* reporter plasmids into WT strain JB58 and the Δ*toxRS* mutant strain DT733. The resulting strains were then grown to middle logarithmic phase when *leuO-lacZ* expression was quantified using β-galactosidase assays (Fig. 4B). Consistent with above data, the results of these tests showed high levels of β-galactosidase production in JB58 containing the native *leuO* promoter (i.e. pXB266) and very little β-galactosidase production in the Δ*toxRS* mutant containing the same promoter reporter. In contrast, very little β-

galactosidase was produced in JB58 or the Δ*toxRS* mutant containing the *leuO* reporters lacking one (pVA258) or both (pVA261) ToxR consensus binding sites. These results confirmed that ToxR was required for *leuO* expression and suggested that sites A and B were both required for basal *leuO* expression in *V. cholerae.*

We next tested to see if the addition of bile could bypass the requirement for both ToxR binding sites in the *leuO* promoter to induce *leuO* expression. We therefore cultured WT strain JB58 bearing the three *leuO-lacZ* reporter plasmids (Fig. 4A) in the presence and absence of bile and quantified *leuO* expression. The results showed that only the full-length *leuO* promoter (i.e. pXB266) supported the activation of *leuO* expression in the presence of bile (Fig. 4C). Very little β-galactosidase was produced in JB58 containing the *leuO* reporters lacking one (pVA258) or both (pVA261) ToxR consensus binding sites in the presence or absence of bile. These results indicated that both ToxR binding sites are required for the bile-dependent induction of *leuO* expression.

The presence of the ToxR binding sequences in the *leuO* promoter suggested that ToxR acts directly on the *leuO* promoter. If this were true, we hypothesized that ToxR expression in a heterologous host would result in activation of the *leuO* promoter. To test this we expressed *toxRS* from the pBAD33 arabinose inducible promoter in an *E. coli* host that contained each of the three *leuO-lacZ* reporters described above (i.e. pXB266, pVA258 or pVA261). We cultured the recombinant strains to middle log phase in the presence of arabinose (to induce *toxRS* expression) before quantifying *leuO-lacZ* expression using β-galactosidase assays. The results showed that expression of *toxRS* from pBAD33::*toxRS* resulted in high and equal expression from the native *leuO* promoter (pXB266) and the promoter lacking the distal ToxR binding site (pVA258) (Fig. 4D). β-galactosidase production was greatly diminished in the strain bearing the

leuO promoter that lacked both ToxR consensus sequences (pVA261). The ~18-fold increase in *leuO-lacZ* expression in the *E. coli* pBAD33::*toxRS* cultures bearing pXB266 and pVA258 relative to the strain bearing pVA261 suggests that ToxR directly binds to the ToxR consensus binding sites in the *leuO* promoter to facilitate activation of *leuO* transcription in *E. coli*. These results also imply that in *E. coli*, in contrast to *V. cholerae*, ToxR can bind to the proximal ToxR consensus site in pVA258 to activate transcription.

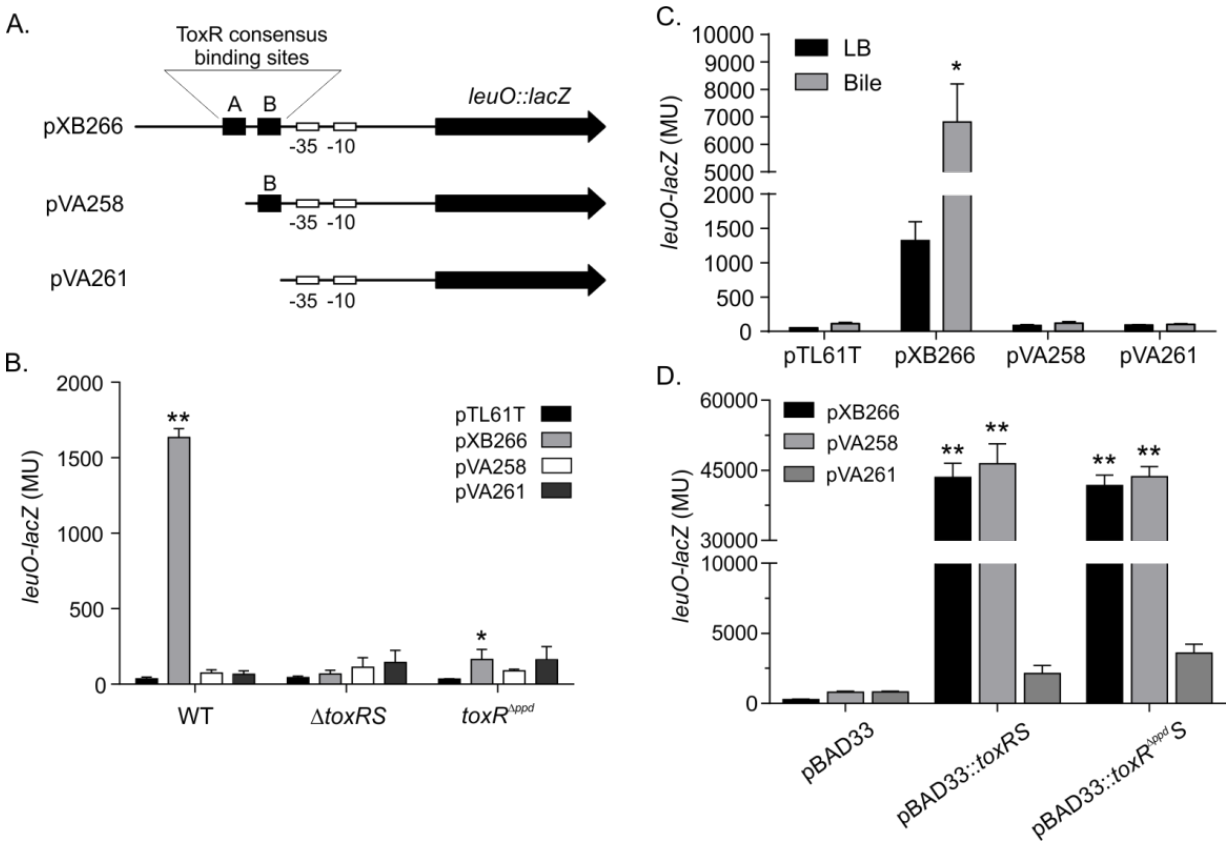

Figure 4. ToxR consensus binding sequences are required for ToxR activation of *leuO* transcription.

(A) Schematic diagram of the *leuO* promoter in the indicated *leuO-lacZ* reporter plasmids. The location of the two putative ToxR consensus binding sites are indicated by the black boxes and denoted by the letters A (distal) and B (proximal). The putative -10 and -35 promoter elements are indicated by the white boxes. pXB266 contains the native *leuO* promoter which contains both ToxR consensus binding sites; pVA258 contains only the proximal ToxR binding site; and pVA261 lacks both ToxR binding sites but still maintains the -35 and -10 basal promoter elements.

(B) WT strain JB58, Δ*toxRS* strain DT733, and *toxR*$^{\Delta ppd}$ strain SS4 containing the indicated *leuO-lacZ* reporter plasmid were grown in LB broth to middle logarithmic phase when aliquots were collected and assayed for *leuO-lacZ* using the β-galactosidase assay. (C) WT strain JB58 containing the indicated *leuO-lacZ* reporter plasmids were grown in LB broth or LB broth plus 0.2% bile to middle logarithmic phase when aliquots were collected and assayed for *leuO-lacZ* expression using a β-galactosidase assay. (D) *E. coli* strains containing the indicated *toxRS* expression plasmid pBAD33::*toxRS*, pBAD33::*toxR*$^{\Delta ppd}$*S*, or pBAD33 and one of the *leuO-lacZ* reporter plasmids pXB266, pVA258 or pVA261 were grown in LB broth containing 0.08% arabinose to middle logarithmic phase when aliquots were collected and assayed for *leuO-lacZ* expression using the β-galactosidase assay. Statistical analysis for panel B was determined using Two-way ANOVA with Dunnett's post-test comparing the mean of each plasmid to the control pTL61T in the designated strain; Panel C statistical analysis was determined using Two-way ANOVA with Tukey's post-test comparing the overexpression plasmid to the pBAD33 control plasmid with the designated second plasmid; *=P<0.05, **=P<0.0001. doi: 10.1128/JB.00419-15.

2.2.4 Activation of *leuO* requires the ToxR periplasmic domain.

The periplasmic domain of ToxR was shown to be important for the cFP-dependent activation of *leuO* expression (43), but dispensable for basal *ompU* expression and virulence factor production (43, 60). We therefore examined whether the ToxR periplasmic domain contributed to *leuO* expression during growth in LB broth. To test this we generated a *V. cholerae* mutant (SS4) that produced a *toxR* allele in which we deleted the carboxy-terminal periplasmic domain (i.e. ToxR$^{\Delta ppd}$). This mutant allele is localized to the membrane and was previously shown to be functional (43, 60). We then introduced pXB266, pVA258 and pVA261 into the *toxR*$^{\Delta ppd}$ mutant SS4. The strains were then cultured as described above when *leuO-lacZ* expression was quantified. The results showed that deletion of the ToxR periplasmic domain largely abolished *leuO* expression from all three *leuO-lacZ* reporters (Fig. 4B). This indicated that the ToxR periplasmic domain was important for *leuO* expression.

We also tested whether the ToxR periplasmic domain was necessary for activation of *leuO* expression in *E. coli*. *E. coli* strains containing each of the *leuO-lacZ* reporter plasmids were transformed with plasmid pBAD33::*toxR*$^{\Delta ppd}$*S* (pXB286) and the resulting strains were cultured as described above before being assayed for *leuO-lacZ* expression. The results of these experiments mirrored the results obtained with *E. coli* expressing the WT *toxRS* allele. Overexpression of *toxR*$^{\Delta ppd}$*S* from pXB286 activated both, the WT *leuO* promoter in pXB266 and, the *leuO* promoter lacking the distal ToxR binding site in pVA258, to similar levels (Fig. 4D). Further, the magnitude of activation was similar to that observed with the strain expressing the WT *toxRS* allele. This suggested that *toxR*$^{\Delta ppd}$ allele produces a functional protein that is able to bind to the ToxR consensus sequences in the *leuO* promoter and to activate *leuO* transcription. This suggests that the inability of ToxR$^{\Delta ppd}$ to activate *leuO* expression in *V. cholerae* was not due to its inability to bind to DNA, but rather due to other factors that are dependent on the presence of the ToxR periplasmic domain.

2.2.5 ToxR can directly bind the *leuO* promoter.

To further support the conclusion that ToxR bound directly to the *leuO* promoter, gel electrophoretic mobility shift assays were performed with the *leuO* promoter. The EMSAs were performed with crude membrane fractions that were isolated from *V. cholerae* strain DT733 (Δ*toxRS*) containing pBAD18 or pBAD18 expressing *toxRS* or *toxR*$^{\Delta ppd}$*S*. As has been found with other gel shift assays using ToxR membrane fractions, the bound DNA probes do not enter the gel and are left in the wells of the gel, thus the binding of ToxR to the labelled probe should be assessed by the disappearance of free probe and not by the presence of a shifted band (61). The results showed that the ToxRS positive membrane fractions bound directly to the *leuO*

promoter which resulted in a decrease in the abundance of free *leuO* probe (Fig. 5A, lane 2). The decrease in free *leuO* probe was largely abolished in the presence of ToxRS negative membrane fractions (lane 7). Taken together these results indicated ToxR can directly bind to the *leuO* promoter.

To determine whether ToxR binding to the *leuO* promoter was specific, we performed binding competition assays by including a 10-fold excess of unlabeled competitor DNA in the binding reactions. If ToxR bound specifically to the *leuO* promoter fragment, adding in excess unlabeled *leuO* promoter would prevent a shift in the free probe. When excess *leuO* DNA was added in the assay, there was a reduced shift in the labeled *leuO* probe (Fig. 5A, lane 3). The *leuO* promoter was also competed with a known ToxR specific promoter, *ompU*, which competed for *leuO* binding and prevented the shift in the free probe (lane 4). The addition of a non-specific competitor encompassing the *vexR* promoter, which is not regulated by ToxR, did not alter the level of free *leuO* probe (lane 5) indicating that the observed shift was specific for the *leuO* promoter. These same binding conditions were also used for ToxRS negative membranes (Fig. 5A, lanes 6-10). The results showed that although there was some decrease in free probe between the no protein control and the ToxRS negative membrane control, the decrease was much less than observed with ToxRS positive membranes and was unaffected by the addition of any of the unlabeled competitor probes. These results confirmed the specificity of the ToxR positive membranes for the *leuO* promoter.

Gel shift assays were also performed to determine if deletion of the ToxR periplasmic domain altered ToxR binding to the *leuO* promoter. These experiments were performed as described above with membrane fractions from DT733 containing pBAD18 expressing *toxR*$^{\Delta ppd}$*S*. The results showed that the ToxR$^{\Delta ppd}$S membranes had comparable shifts as those of

the ToxRS membranes. The ToxR$^{\Delta ppd}$S membrane fractions shifted the *leuO* promoter (Fig. 5B, lane 2). The *leuO* shift was competed by the addition of 10-fold excess unlabeled *leuO* or *ompU* promoter probes, but not by the addition of the nonspecific *vexR* promoter probe (lanes 3-5). These same binding conditions were also used for ToxR$^{\Delta ppd}$S negative membranes (Fig. 5B, lanes 6-10). The results showed a decrease in free probe between the no protein control and the ToxR$^{\Delta ppd}$S negative membrane control, but the decrease was much less than observed with ToxR$^{\Delta ppd}$S positive membranes and was unaffected by the addition of any of the unlabeled competitor probes; confirming the specificity of the ToxR positive membranes for the *leuO* promoter. From these results we concluded that the ToxR periplasmic domain was not required for ToxR binding to the *leuO* promoter.

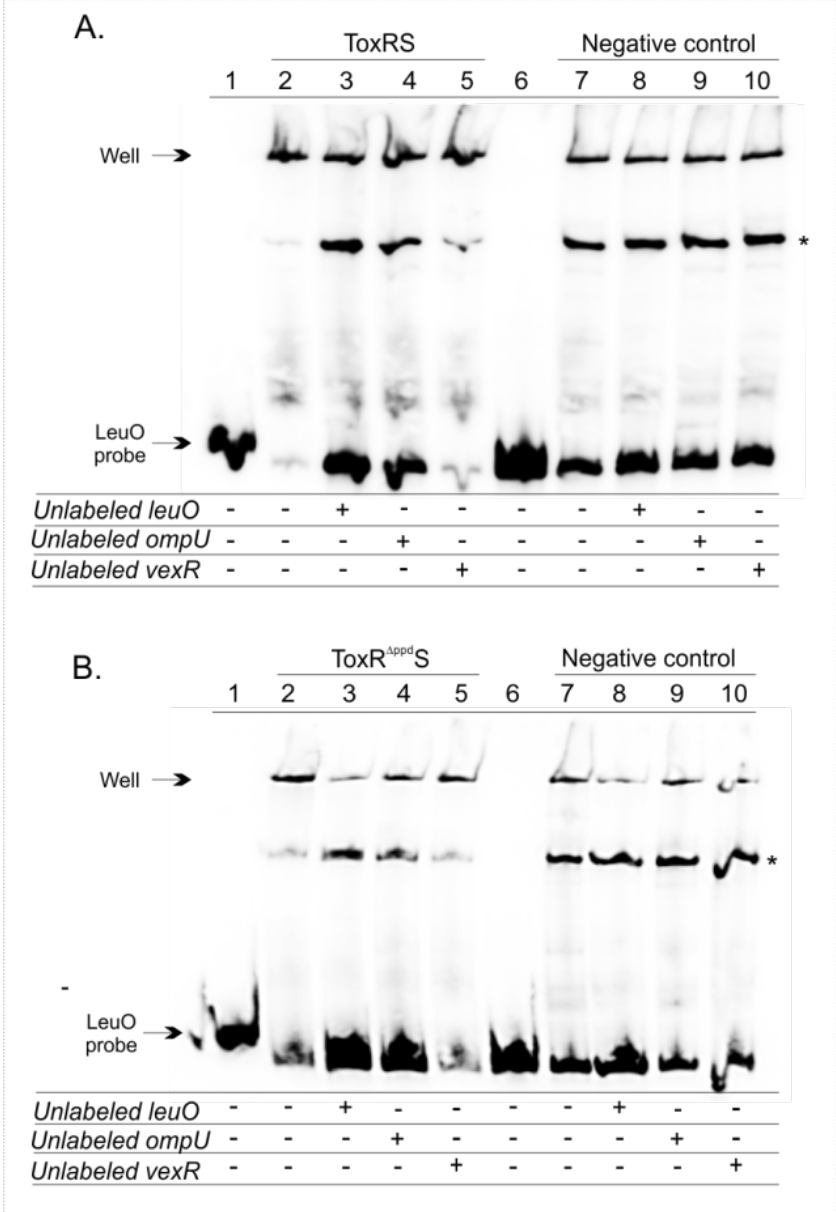

Figure 5. ToxR and ToxR$^{\Delta ppd}$ interact with the *leuO* promoter.

A biotinylated DNA fragment encompassing the *leuO* promoter was incubated with *V. cholerae* cytoplasmic membrane fractions isolated from strain DT733 (Δ*toxRS*) that overproduced ToxRS or ToxR$^{\Delta ppd}$S from pBAD18 described in the Materials and Methods. The negative control membranes are ToxRS negative and were isolated from DT733 containing the empty vector control (i.e. pBAD18). The binding reactions contained either no membranes (lane 1 and 6) or equal amounts of the indicated membranes (lanes 2-5 and 7-10). Unlabeled competitor DNA probes encompassing the *leuO*, *ompU* or *vexR* promoters were added as indicated to the binding reaction at a 10-fold excess relative to the biotinylated *leuO* DNA probe. Equal aliquots of binding reaction mixtures were then

electrophoresed on nondenaturing 5% acrylamide gels and the position of the *leuO* probe was visualized by chemiluminescence detection as described. (A) ToxR binding to the *leuO* promoter. The ToxR binding mixture contained 3 mg/ml of ToxRS positive membranes while control binding reactions contained an equivalent amount of ToxRS negative membranes. (B) ToxR$^{\Delta ppd}$ binding to the *leuO* promoter. The ToxR$^{\Delta ppd}$ binding mixture contained 1 mg/ml of ToxR$^{\Delta ppd}$S positive membranes while control binding reactions contained an equivalent amount of ToxRS negative membranes. The asterisks denote an unknown nonspecific mobility shift. doi: 10.1128/JB.00419-15.

2.2.6 The ToxR periplasmic domain is important for responding to bile.

The role of the ToxR periplasmic domain in environmental sensing is poorly understood. ToxR inversely regulates the *ompU* and *ompT* expression. OmpT is expressed during growth in minimal media while OmpU is expressed during growth in rich media. The addition of bile salts or the amino acids asparagine, arginine, glutamic acid, and serine (NRES) to minimal media results in ToxR-dependent porin switching that mimics growth in rich media (i.e. expression of *ompU* and repression of *ompT*) (77). The mechanism by which ToxR activates *ompU* expression in minimal media differs for bile salts and NRES (40). The addition of NRES to minimal media results in *toxR* upregulation which is sufficient to increase *ompU* expression. In contrast, bile salts activate *ompU* expression via a process that does not result in *toxR* upregulation and may involve transcriptional activation (40).

We took advantage of *ompT/ompU* switching system described above to test the contribution of the ToxR periplasmic domain on *leuO* and *ompU* expression in response to bile. We cultured WT strain JB58, Δ*toxRS* strain DT733 and the *toxR*$^{\Delta ppd}$ strain SS4 in T-minimal media or T-media containing bile or NRES and quantified *leuO* and *ompU* expression by qRT-PCR. The results showed that *leuO* expression was induced ~24-fold in response to bile and unaffected by the addition of NRES in the WT strain (Fig. 6A). The addition of NRES or bile to

the media did not affect *leuO* expression in the *toxRS* or *toxR*$^{\Delta ppd}$ mutants. By contrast to *leuO*, *ompU* expression increased in response to both NRES (13-fold) and bile (22-fold) relative to the control cultures (Fig. 6B). The expression of *ompU* was abolished under all conditions in the Δ*toxRS* mutant. The expression of *ompU* in the *toxR*$^{\Delta ppd}$ mutant following exposure to NRES increased to a level that was similar to that observed in WT, further confirming that the *toxR*$^{\Delta ppd}$ allele produced a functional protein in *V. cholerae*. The expression of *ompU* in the *toxR*$^{\Delta ppd}$ mutant following exposure to bile resulted in a much lower level of *ompU* induction than was observed in WT. Exposure to bile resulted in a ~21-fold increase in *ompU* expression in WT, but only a ~3-fold increase in the *toxR*$^{\Delta ppd}$ mutant (Fig. 6B). Taken together, these results provide additional evidence that the ToxR$^{\Delta ppd}$ protein is functional and that the periplasmic domain of ToxR is critical for the induction of *ompU* and *leuO* in response to bile. This suggests the possibility that bile/bile salts may affect ToxR activity by a process that requires the periplasmic domain.

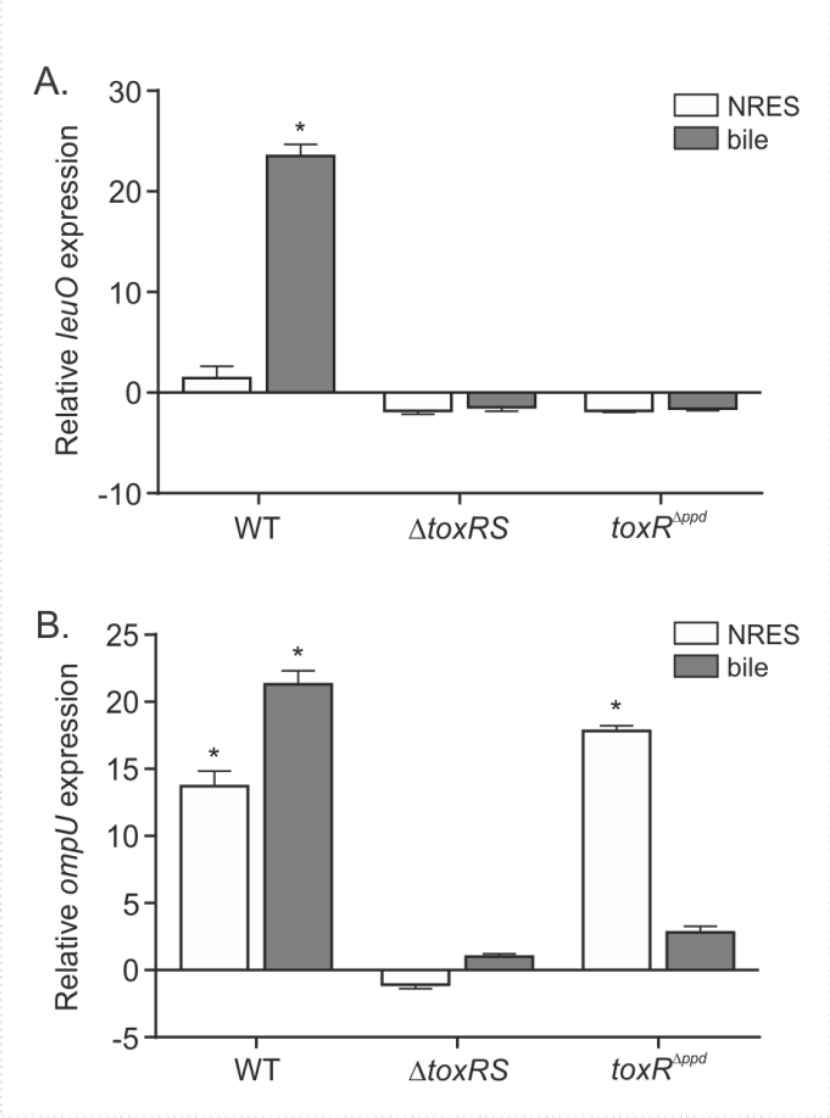

Figure 6. The ToxR periplasmic domain is required for *leuO* and *ompU* upregulation in response to bile.

qRT-PCR was used to determine the effect of the amino acids NRES and bile on (A) *leuO* and (B) *ompU* expression during growth of JB58 (WT), DT733 (Δ*toxRS*), and SS4 (*toxR*$^{\Delta ppd}$) in modified T-media. The strains were cultured to an OD_{600} ~0.3 before being transferred to media supplemented containing either 50 mM NRES or 0.2% bile. The cultures were then grown for an additional 15 minutes before aliquots were collected for RNA isolation as described in the Materials and Methods. The presented data is the mean +/- SD of three independent experiments. Statistical significance was determined using a one-sample t test comparing the sample mean to a hypothetical value of +/-1; *=P<0.01. doi: 10.1128/JB.00419-15.

2.2.7 LeuO contributes to *V. cholerae* bile resistance.

Since ToxR functions in bile resistance (28, 68), we hypothesized that LeuO may also contribute to *V. cholerae* bile resistance. To test this we performed bile killing assays. In these experiments we quantified the survival of WT *V. cholerae* (JB58) and isogenic *leuO, toxRS, toxR*$^{\Delta ppd}$ deletion mutants upon exposure to a lethal concentration of bile for one hour as described in the Materials and Methods (Appendix A). The results of these experiments showed a ~4-fold decrease in the recovery of the *leuO* mutant strain XBV222 relative to WT strain JB58 when exposed to 20% bile (Table 2). This suggested that the presence of *leuO* provided a survival advantage to *V. cholerae* in the presence of bile. The Δ*toxRS* mutant strain DT733 and the *toxR*$^{\Delta ppd}$ mutant SS4 were not recovered when exposed to 20% bile. This finding is consistent with the role of ToxR in bile resistance and is likely attributable to the combined dysregulation of *ompU, ompT* and *leuO* expression in the Δ*toxRS* mutant. We therefore performed the killing assays with the *toxR* mutant strains using 10% bile. When exposed to 10% bile, the Δ*toxRS* mutant strain exhibited a ~11-fold decrease in recovery relative to WT; suggesting that the Δ*toxRS* mutant was more sensitive to bile than the Δ*leuO* mutant. The *toxR*$^{\Delta ppd}$ mutant exhibited a ~7-fold decrease in recovery compared to WT. This indicated that the *toxR*$^{\Delta ppd}$ mutant exhibited greater susceptibility to bile than did a Δ*leuO* mutant. As ToxR$^{\Delta ppd}$ has previously been shown to be sufficient for *ompU* expression under standard laboratory conditions, these results suggest the possibility that the periplasmic domain is required for expression of other factors, in addition to *leuO*, that could contribute to bile resistance.

The above data suggested that LeuO contributed to *V. cholerae* survival in the presence of bile. If this was true, then overexpression of *leuO* in a Δ*toxRS* mutant should provide a survival advantage upon exposure to a lethal concentration of bile. We tested this by introducing

pBAD18-*leuO* or pBAD18 into the Δ*toxRS* mutant DT733. We chose a Δ*toxRS* mutant for these experiments to negate *leuO* and *ompU* expression. We cultured the *toxRS* deletion strain bearing the pBAD18-*leuO* plasmid pVA94 or the empty vector control (pBAD18) to log phase in the presence of 0.1% arabinose to induce *leuO* expression. Aliquots of the induced cultures were then exposed to lethal concentration of bile before being processed as described above. The results showed that *leuO* overexpression resulted in a ~5-fold increase in cell recovery relative to the empty vector control (Table 2). This indicated that LeuO contributed to *V. cholerae* survival in the presence of bile by a mechanism that is likely independent of *ompU*. Taken together the results of these experiments confirm the importance of ToxR in bile resistance and support the conclusion that ToxR-activation of *leuO* expression contributes to bile resistance.

Table 2. Bile killing assays.

	Fold change in recovery (SD)	
Strains:	10% bile	20% bile
Δ*leuO*/WT	1	-4.5 (2.1)[a]
Δ*toxRS*/WT	-11.3 (3.7)[a]	NR[b]
toxR$^{\Delta ppd}$/WT	-7.0 (1.4)[a]	NR
Δ*toxRS* pBAD18::*leuO*/Δ*toxRS* pBAD18	5.2 (1.4)[a]	NR

[a] $P < 0.01$; [b] NR = Not Recovered; SD = standard deviation

doi: 10.1128/JB.00419-15.

2.2.8 LeuO does not contribute to bile resistance through regulation of the RND efflux pumps.

Our laboratory has previously shown that the *V. cholerae* RND-family efflux systems are major contributors to *V. cholerae* bile resistance (63-67). We therefore tested whether the contribution of *leuO* to bile resistance was mediated by upregulation of any of the RND efflux systems. We quantified the expression of all six of the RND efflux pumps in WT strain JB58 and the Δ*leuO* mutant strain XBV222 in response to bile using *lacZ* promoter reporter fusions. The strains were cultured in the absence or presence of bile to middle logarithmic growth phase when expression of the individual RND efflux systems was quantified. The results showed that in the absence of bile there were no significant differences in expression of any of the RND efflux pumps in the Δ*leuO* mutant compared to the WT strain (Fig. 7). This indicated that LeuO did not contribute to the basal expression of any of the efflux pumps. When the same reporter strains were cultured in the presence of bile, as expected, *vexRAB* and *vexCD* expression were found to be significantly induced. However, there were no significant differences found in any of the RND efflux pumps expression between the WT and *leuO* deletion strain in response to bile (Fig. 7). This indicated that LeuO does not regulate the expression of the RND efflux systems and is not likely working through the RND efflux pumps to contribute to bile resistance.

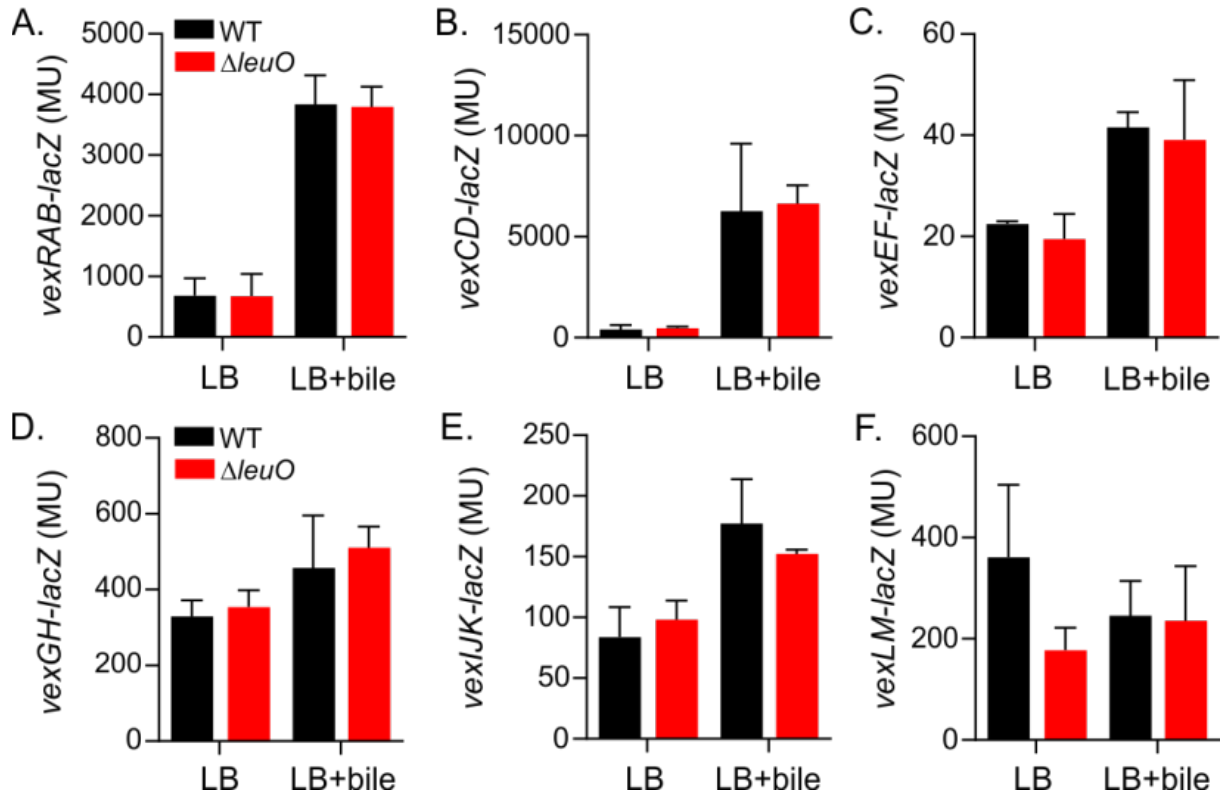

Figure 7. Effect of *leuO* on the expression of the *V. cholerae* RND efflux systems.

V. cholerae WT strain JB58 and Δ*leuO* mutant strain XBV222 carrying *lacZ* promoter fusion reporter plasmids for (A) *vexRAB-lacZ* (pXB233), (B) *vexCD-lacZ* (pXB231), (C) *vexEF-lacZ* (pXB228), (D) *vexGH-lacZ* (pXB229), (E) *vexIJK-lacZ* (pXB230) or (F) *vexLM-lacZ* (pXB232). All strains were cultured in LB broth in the presence or absence of 0.2% bile before being assayed for β-galactosidase activity as described in the Materials and Methods. The presented data is the mean +/- SD of three independent experiments. doi: 10.1128/JB.00419-15.

2.3 DISCUSSION

The ability of *V. cholerae* to respond to its environment is essential for its success as an enteric pathogen. This is critical upon entrance into the human host where *V. cholerae* must express genes that are indispensable for colonization of the small intestine. Colonization and growth in the small intestine requires the expression of virulence genes plus the expression of genes that

combat antimicrobial agents which are present in the intestine. ToxR plays a critical role in this regard by regulating the expression of genes that are required for host adaptation. ToxR is thought to transduce *in vivo* signals to effect the expression of its target genes, but the mechanism by which this occurs is poorly understood.

In this study, we observed upregulation of *leuO* expression in response to bile and the bile salt deoxycholate by a process that was dependent on ToxR (Fig. 3). Bile salts have detergent-like properties which make them bactericidal. Expression of *leuO* was not altered by other membrane-active detergents (e.g. SDS, Triton X-100 or sarcosyl), indicating that *leuO* induction was not the result of general membrane stress response, but instead was directly in response to bile and bile salts. The upregulation of *leuO* in response to bile suggested the possibility that *leuO* may function in bile resistance. Support for this conclusion was provided by the observation that *leuO* deletion resulted in increased bile susceptibility while *leuO* over expression resulted in increased bile resistance (Table 2). In light of recent studies showing *leuO* is expressed in the intestine using an infant mouse model (43), we speculate that the findings observed here may extend to the host. These results were also similar to what has been reported for OmpU, a porin that is associated with bile resistance in *V. cholerae* and whose expression is also activated by ToxR in response to bile and deoxycholate (68).

While the contribution of *leuO* to bile resistance is clear, the mechanism by which *leuO* impacts bile resistance was not resolved. The Δ*leuO* mutant strain exhibited a bile susceptibility phenotype that was intermediate relative to the Δ*toxRS* mutant (Table 2). This suggested that LeuO likely affected bile resistance by a mechanism that was distinct from *ompU/ompT* regulation. This conclusion was confirmed by the finding that *leuO* overexpression provided a survival advantage in a *toxRS* negative strain exposed to bile (Table 2). Bile resistance results

from the synergistic affects of reduced outer membrane permeability and active efflux (66). In *V. cholerae* the RND efflux systems are major contributors to bile resistance suggesting a potential mechanism by which LeuO could affect bile resistance. However, our results showed that there was no difference in the expression of any of the RND efflux systems in a *leuO* mutant grown in the presence or absence of bile (Fig. 7) suggesting that *leuO* affects bile resistance by a mechanism that is independent of the RND efflux systems. There are a number of other potential mechanisms by which LeuO could impact bile resistance including the expression of other transport systems, production of other porins, alterations in cell physiology, and alterations to the cell envelope.

The expression of *leuO* was previously found to be dependent on ToxR (43). Sequence analysis of the *leuO* promoter revealed the presence of two putative ToxR consensus binding sites, both of which were required for basal-level *leuO* expression in *V. cholerae* (Fig. 4B). This finding was reminiscent of what was observed for ToxR activity at the *ompU* promoter where the most distal ToxR binding site was needed for full *ompU* activation in *V. cholerae* (39). By contrast, ToxR activated expression from the *leuO* promoter lacking the distal ToxR binding site (i.e. pVA258) in *E. coli* (Fig. 4D). The lack of expression from the same mutant promoter in *V. cholerae* suggested that other factors affect *leuO* expression in *V. cholerae*. We do not know what these factors are, but there are a number of potential explanations for this result. It is possible that other DNA binding proteins interact with the *leuO* promoter and impede ToxR binding at the proximal site. It is also possible that ToxR may bind sequentially to the two ToxR binding sites in *V. cholerae*, having to bind to the distal site first which then facilitates binding at the proximal site. This tandem fashion of binding is similar to other DNA-binding domains of OmpR-family proteins which generally interact as dimers with direct repeat DNA sequences

(80). This idea is also supported by cooperative binding studies comparing ToxR oligomerization and regulation of target promoters containing multiple operator elements in *E. coli* and *V. cholerae* (81).

The periplasmic domain of ToxR has been implicated in responding to environmental signals, but how ToxR regulates its target genes in response to these signals is poorly understood. The finding that the ToxR periplasmic domain was required for the upregulation of both *leuO* and *ompU* in response to bile (Fig. 6) suggested the possibility that the periplasmic domain acts as a bile sensor which can affect the activity of the cytoplasmic DNA binding domain at its target promoters. The mechanism by which the periplasmic domain senses bile is unclear. There are a number of potential mechanisms by which bile could affect ToxR activity. Bile could facilitate ToxR interaction with ToxS. ToxS has been shown to contribute to ToxR stability and to enhance its activity at target genes (25-27). Alternatively, bile could potentially affect conformational changes in the ToxR periplasmic domain that affect DNA binding. Bile could also affect disulfide bond formation in the two cysteine residues located in the ToxR periplasmic domain. There is evidence that disulfide bond formation in the periplasmic domain of ToxR and ToxR-like proteins affect their activity. For example, disulfide bond formation in ToxR has been shown to contribute to *ompU* regulation in response to some growth conditions (82). ToxR and TcpP have also been shown to form homodimers and heterodimers (83, 84) and the bile salt taurocholate has been shown to induce intermolecular disulfide bond formation in the periplasmic domain of TcpP (32). Similarly, *E. coli* CadC has been shown to form disulfide bonds in response to pH which result in activation of *cadBA* transcription (85). Additional work will be required to differentiate between these potential mechanisms.

Previous studies showed that cFP activation of *leuO* resulted in the downregulation of the ToxR regulon (43). The data presented herein show that *leuO* expression is also activated by bile and bile salts. While the fatty acid components of bile have been linked to downregulation of the ToxR regulon (86), bile salts have been shown to either be neutral or to enhance virulence gene expression (32, 78). Thus, the role of LeuO in virulence gene regulation is a paradox. Although there are a number of potential explanations for the differential effects of LeuO on virulence, we suspect that bile salts and CDPs differentially affect the expression (or activity) of other proteins that contribute to virulence gene expression. For example, bile salts and cFP may differentially affect HNS or CRP; both of which have been shown to suppress the ToxR regulon and thus could contribute to the observed phenotype (87, 88). Studies to resolve the role of LeuO in virulence gene regulation are ongoing in our laboratory.

3.0 THE LYSR-TYPE REGULATOR LEUO REGULATES THE ACID TOLERANCE RESPONSE IN VIBRIO CHOLERAE

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by authors Vanessa M. Ante, X. Renee Bina, and James E. Bina

3.1 INTRODUCTION

Vibrio cholerae is a neutrophilic bacterium that is extremely sensitive to even mild acidic conditions (89). *V. cholerae* naturally persists in aquatic reservoirs with a neutral pH, variable nutrient availability, and ambient temperatures. *V. cholerae* is also an enteric pathogen that encounters a variety of environmental stresses while passing through the human gastrointestinal tract. Following ingestion *V. cholerae* encounters a dramatic change in pH from near neutral pH to a pH $\leq$ 2 in the human stomach. Passage of *V. cholerae* from the stomach into the small intestine further exposes the bacterium to an environment that contains a combination of inorganic acids and organic acids (90). Exposure of *V. cholerae* to acidic conditions results in the induction of an acid tolerance response. The acid tolerance response can be divided into two distinct branches: an inorganic acid tolerance response and an organic acid tolerance response (91).

The *V. cholerae* acid tolerance response encompasses diverse genes that function together to mitigate the effects of acid stress. This includes alterations in the outer membrane, the expression of genes that function in the regulation of K^+ and Na^+ homeostasis, and biofilm production (42, 92, 93). The acid tolerance response is likely an important factor for *V. cholerae* pathogenesis. For example, biofilm production has been shown to enhance acid tolerance which contributes to pathogenesis by providing protection from acid stress during passage through the gastric acid barrier of the stomach (93, 94). In addition, pre-activation of the *V. cholerae* acid tolerance response has been shown to impart a competitive advantage for colonization of the infant mouse intestine relative to unadapted cells (91). Taken together these results suggest that the acid tolerance response may play a crucial role in the both initial infection with *V. cholerae*

and the subsequent development of hyper-infectivity that has been observed in human and animal shed *V. cholerae* (9, 95, 96).

An important subset of genes that are induced in both the *V. cholerae* inorganic and organic acid tolerance response is the *cad* system. The contribution of the *cad* system to acid resistance is conserved among a number of enteric bacteria (97). The *cad* system includes three genes that are involved in maintaining the intracellular pH while also neutralizing the external pH. CadC is a ToxR-family transcriptional regulator that positively regulates the expression of the *cadBA* operon (98). CadA is a lysine decarboxylase that converts lysine to cadaverine while consuming a proton and producing carbon dioxide. CadB is a lysine-cadaverine antiporter that is localized to the cytoplasmic membrane. Tight regulation of the *cad* system is necessary as alterations in the intracellular pH is detrimental to the cell (99).

In *V. cholerae* it has been shown that AphB, a cytoplasmic DNA-binding protein, positively regulates the *cad* system in response to low pH or low oxygen by directly binding to the *cadC* promoter (36). Upregulation of the *cad* system contributes to the maintenance of the intracellular pH. The expression level of the *cad* system returns to a low constitutive level upon neutralization of the external environmental. The molecular mechanism by which *V. cholerae* downregulates the *cad* system are not known. In *Escherichia coli*, the *cad* system is repressed in two ways: the first is through feedback inhibition by cadaverine, the second is through the transcriptional regulator LeuO which functions by repressing *cadC* expression (100). In *V. cholerae* cadaverine does not repress the *cad* system (91), but it is unknown if LeuO influences *cadC* expression.

LeuO is a LysR-type transcriptional regulator that shares 50% identity and 75% similarity to *E. coli* LeuO. Our laboratory has shown that *V. cholerae leuO* is positively regulated by the

virulence regulator ToxR, often in response to environmental signals (43, 101). Expression of *leuO* is induced by the endogenously produced cyclic dipeptide cyclo(Phe-Pro). In response to cyclo(Phe-Pro) LeuO has was shown to repress the production of essential virulence factors by downregulating the ToxR regulon. Expression of *leuO* is also induced by bile salts and contributes to *V. cholerae* bile resistance (101). Preliminary transcriptomic profiling experiments performed in our laboratory indicated that the *cad* system was differentially regulated in a *V. cholerae leuO* mutant, suggesting that that LeuO may regulate the *cad* system. In the present study, we expanded upon this observation and tested the hypothesis that LeuO functioned as a regulator of the *V. cholerae cad* system. The results of our studies showed that LeuO was a repressor of *cadC* expression and directly bound to the *cadC* promoter. LeuO was also shown to regulate the production of CadA (lysine decarboxylase) and to contribute to *V. cholerae* survival after exposure to organic acid. LeuO overproduction in a *cadA* mutant also resulted in increased acid sensitivity suggesting that that the contribution of LeuO to acid tolerance extends beyond the *cad* system. Taken together, our studies have identified a new physiological role for LeuO and indicate LeuO is a component of the *V. cholerae* acid tolerance response.

Table 3. Strains, plasmids and oligonucleotides used in Chapter 3.

Strains	Characteristics	Source
E. coli		
EC100λpir	*supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 (λpirR6K)*	Epicenter
SM10λpir	*thi-1 thr leu tonA lacY supE recA::*RP4-2-Tc::Mu *kmR (λpirR6K)*	Lab collection
BW25113	*F⁻ Δ(araD-araB)567 lacZ4787Δ::rrnB-3 LAM⁻ rph-1 Δ(rhaD-rhaB)568 hsdR514*	(102)
ER2566	*fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mcr- 73::miniTn10--TetS)2 [dcm] R(zgb-210::Tn10--TetS) endA1 Δ(mcrCmrr)114::IS10*	New England BioLabs
V. cholerae		
JB58	*V. cholerae* O1 El Tor strain N16961 Δ*lacZ*, Sm^R	Lab collection
XBV222	JB58Δ*leuO*	(43)
XBV148	JB58Δ*aphB*	This study
JB804	*V. cholerae* O1 El Tor strain C6706 Sm^R	(103)
XBV144	JB804 Δ*lacZ*	This study
VA412	XBV144 Δ*leuO*	This study
EC20568	C6706 Tn::VC2485 (*leuO*)	(104)
EC17926	C6706 Tn::VC0278 (*cadA*)	(104)
Plasmids		
pTL61T	*lacZ* transcriptional reporter plasmid, Cb^R	(73)
pXB239	pTL61T containing the *cadC* promoter region	This study
pXB203	pTL61T containing the *aphB* promoter region	(105)
pBAD18	Arabinose regulated expression plasmid, Cb^R	(76)
pVA94	pBAD18 expressing *leuO*	(101)
pBAD18Km	Arabinose regulated expression plasmid, Km^R	(76)
pXB298	pBAD18Km expressing *leuO*	(43)
pBAD33	Arabinose regulated expression plasmid, Cm^R	(76)
pVA126	pBAD33 expressing *leuO*	This study
pWM91	Suicide plasmid vector used for allelic exchange	(106)
pDLT	pWM91 containing a fragment of *lacZ* harboring an internal deletion	(107)
pWM91Δ*leuO*	pWM91 containing a fragment of *leuO* harboring an internal deletion	(59)
pWM91Δ*aphB*	pWM91 containing a fragment of *aphB* harboring an internal deletion	This study
pMAL-c2	IPTG-inducible expression vector for fusion of proteins to MBP and cytoplasmic expression, Cb^R	New England BioLabs
pVA175	pMAL-c2 expressing *leuO*	This study
Oligonucleotides	**Sequence (5` to 3`)**	
P_{cadC}-*F*	TTCTCGAGTCGGGCTATCGACTGTACGATG	
P_{cadC}-*R*	GTTCTAGACACCACACACCGATGAAGAGCGAAATTATAA	
aphB-F1	TTGGATCCGCCCCACGATGGCTCGCG	
aphB-F2	CGACTGGTTGTCACAAAGATCACCAGCCGGAAAAAGTGCGCCTG	
aphB-R1	GCGAGCTCCAGTGGGCGATATGGGCG	
aphB-R2	GGTGATCTTTGTGACAACCAGTCGAAAGAGGTTTAGGTCATCTAG	
LeuO-F	CCCCCGGGTTAGATAAAAAAGACGCAATGAGTGCC	
LeuO-R	CCTCTAGATAGAAACGTAGAATGAACAAAGGATC	
cadC-EMSA-F1	GCGGGAGTCGGCAGCGGATGGTTAAACAACCTAAGTT	
cadC-EMSA-R1	GCGGGAGTCGGCAGCGGAGCGAAATTATAAGTGCAC	
cadC-EMSA-F2	GCGGGAGTCGGCAGCGAATTTCGCTCTTCATCGGTG	
cadC-EMSA-R2	GCGGGAGTCGGCAGCGCATAGAATAGCTCTTTGTATC	
5'BIO	5'-biotin-GCGGGAGTCGGCAGCG	

doi: 10.1099/mic.0.000194.

3.2 RESULTS

3.2.1 LeuO regulates *cadC* expression.

Our preliminary transcriptome studies suggested the possibility that LeuO may regulate the *V. cholerae cad* system. The *cad* system is regulated by AphB, which functions as an activator of *cadC*. Once CadC is produced, it directly activates the expression of the *cadBA* operon. Therefore we tested if LeuO affected the expression of either of these two regulatory genes in *V. cholerae*. We first investigated *cadC* transcription by quantifying *cadC* expression levels in WT strain JB58 and an isogenic Δ*leuO* strain XBV222 using the *cadC-lacZ* transcriptional reporter pXB239. The test strains were cultured under AKI virulence gene inducing conditions and *cadC-lacZ* expression was quantified using β-galactosidase assays. The results showed that *cadC* expression peaked at 5 hrs and declined thereafter (Fig. 8A). Growth of *V. cholerae* under AKI conditions results in the acidification of the culture media during static growth (i.e. the first four hours). The reduction in pH appears to correlate with the generation of organic acid byproducts from fermentation metabolism. After the initial 4 hrs of static growth, the cultures are shifted to aerobic growth which results in alkalization of the media (data not shown). Thus, *cadC* expression appeared to correlate with the changes in the pH of the growth medium during growth under AKI conditions. The expression of *cadC* in the Δ*leuO* mutant mirrored expression in the WT strain except that the expression level was elevated in the Δ*leuO* mutant compared to WT. The elevated *cadC* expression observed in the absence of *leuO* supported the hypothesis that LeuO was a regulator of the *cad* system and was acting as a repressor of *cadC*.

If LeuO was a *cadC* repressor, then we hypothesized that *leuO* overexpression would repress *cadC* transcription in *V. cholerae*. To test this hypothesis *V. cholerae* WT strain JB58

was transformed with the expression plasmid pVA126 (pBAD33-*leuO*) and the *cadC-lacZ* reporter plasmid pXB239. The resulting strain was cultured under AKI growth conditions in AKI broth alone or AKI broth containing arabinose to induce *leuO* expression. Expression of *cadC* was then quantified at 5 hrs post-inoculation. The results showed that the induction of *leuO* expression by the addition of 0.02% arabinose resulted in a ~60% reduction in *cadC* expression (Fig. 8B). This finding further supported the conclusion that LeuO was a *cadC* repressor in *V. cholerae*.

There are several potential mechanisms for LeuO to affect *cadC* expression. LeuO could act directly at *cadC* by binding to its promoter and inhibiting transcription. Alternatively, LeuO could repress *cadC* expression indirectly by repressing the expression of its upstream activator *aphB*. To differentiate between these two possibilities, we examined the effect of *leuO* overexpression on *aphB* transcription. We therefore repeated the above experiments using WT strain JB58 carrying pVA126 (pBAD33-*leuO*) and an *aphB-lacZ* transcriptional reporter plasmid (pXB203). The results showed that the induction of *leuO* expression by the addition of 0.02% arabinose did not alter *aphB* expression (Fig. 8B). This indicated *aphB* is not regulated by LeuO and that the effects of LeuO on *cadC* transcription were likely independent of *aphB*.

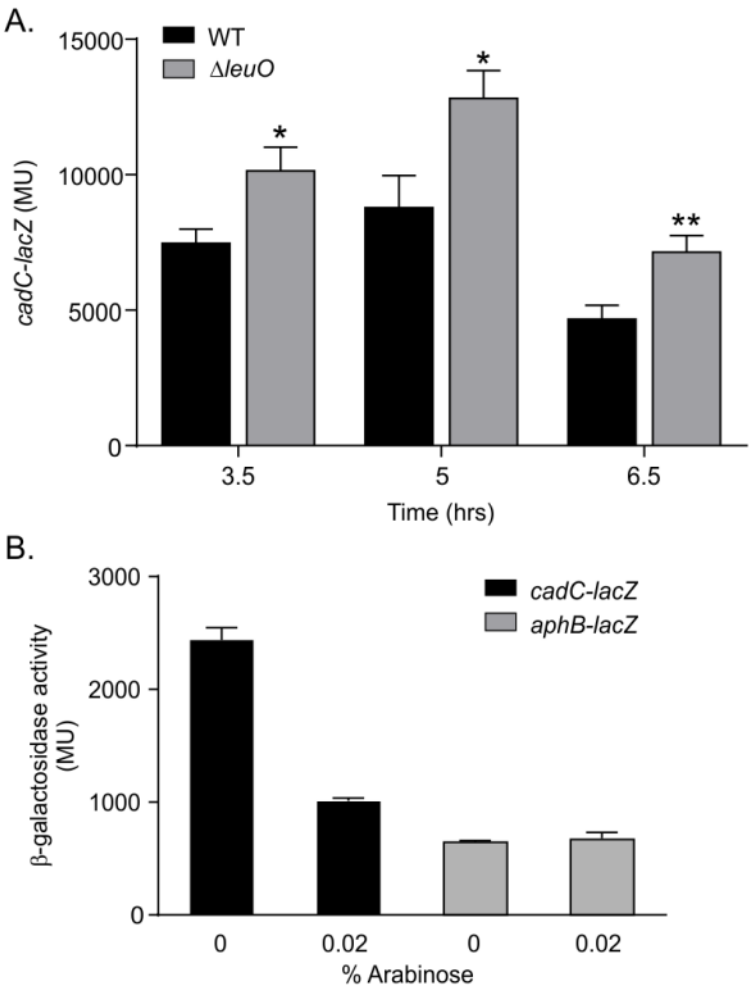

Figure 8. Effect of LeuO on *cadC* and *aphB* expression.

(A) WT *V. cholerae* strain JB58 and Δ*leuO* strain XBV222 carrying the *cadC-lacZ* reporter plasmid pXB239 were grown under AKI conditions. Culture aliquots were taken at the indicated times and assayed for β-galactosidase activity as described in the Materials and Methods. The presented data is the mean +/- SD of three independent biological replicates. (B) WT strain JB58 bearing pBAD33-*leuO* plasmid pVA126 and either the *cadC-lacZ* transcriptional reporter pXB239 (black bars) or the *aphB-lacZ* transcriptional reporter pXB203 (grey bars) were grown under AKI conditions in the presence or absence of 0.02% arabinose. Expression of the indicated reporter gene was assessed at 5 hrs by measuring β-galactosidase production. The presented data is the mean +/- SD of three technical replicates and is representative of two independent experiments. Statistical significance was determined using a t-test comparing the sample mean to the WT control mean; *=P<0.05, **=P<0.01. doi: 10.1099/mic.0.000194.

3.2.2 LeuO represses *cadC* expression by directly binding to its promoter.

The above results suggested that LeuO reduced *cadC* expression independently of *aphB*, but did not discriminate between LeuO affecting *cadC* expression directly or indirectly. To address this we examined whether *leuO* expression affected *cadC-lacZ* expression in a heterologous host. We introduced both the pBAD33-*leuO* expression plasmid pVA126 and the *cadC-lacZ* reporter plasmid pXB239 into *E. coli* and quantified *cadC-lacZ* expression following growth in LB broth for 5 hrs in the presence and absence of arabinose. The results showed a ~65% decrease in *cadC-lacZ* expression in LB broth containing 0.02% arabinose (Fig. 9A). This result indicated that genes unique to *V. cholerae* were not required for LeuO repression of *cadC* and suggested that LeuO may act directly at the *cadC* promoter. We note that these results do not exclude the possibility that LeuO could be acting indirectly through an intermediate gene present in *E. coli*.

To confirm further that LeuO was acting directly at the *cadC* promoter we performed gel shift assays. For these experiments, we purified LeuO as a translational fusion to the maltose binding protein (MBP) and generated two biotin labeled DNA probes from the *cadC* locus. The first DNA probe, named *cadC1*, contained the *cadC* promoter region from -79 to +1 relative to the *cadC* transcriptional start site as defined by Merrell *et al* (98)(Fig. 9B). This region of the *cadC* promoter also included the AphB binding site which was mapped to nucleotides -71 to -55 (36). The second DNA probe, called *cadC2*, was used as a negative control and contained nucleotides -8 to +77 relative to the *cadC* transcriptional start site. The results of the gel shift assays showed that LeuO-MBP bound to the *cadC1* DNA probe, but not to the *cadC2* DNA probe (Fig. 9B). Incubation of the *cadC1* DNA probe with MBP alone did not result in a shift, confirming that LeuO was responsible for the shift of the *cadC1* probe by the LeuO-MBP fusion

protein. Taken together these results confirmed that LeuO directly binds to a region in the *cadC* promoter that is present in the *cadC1* probe.

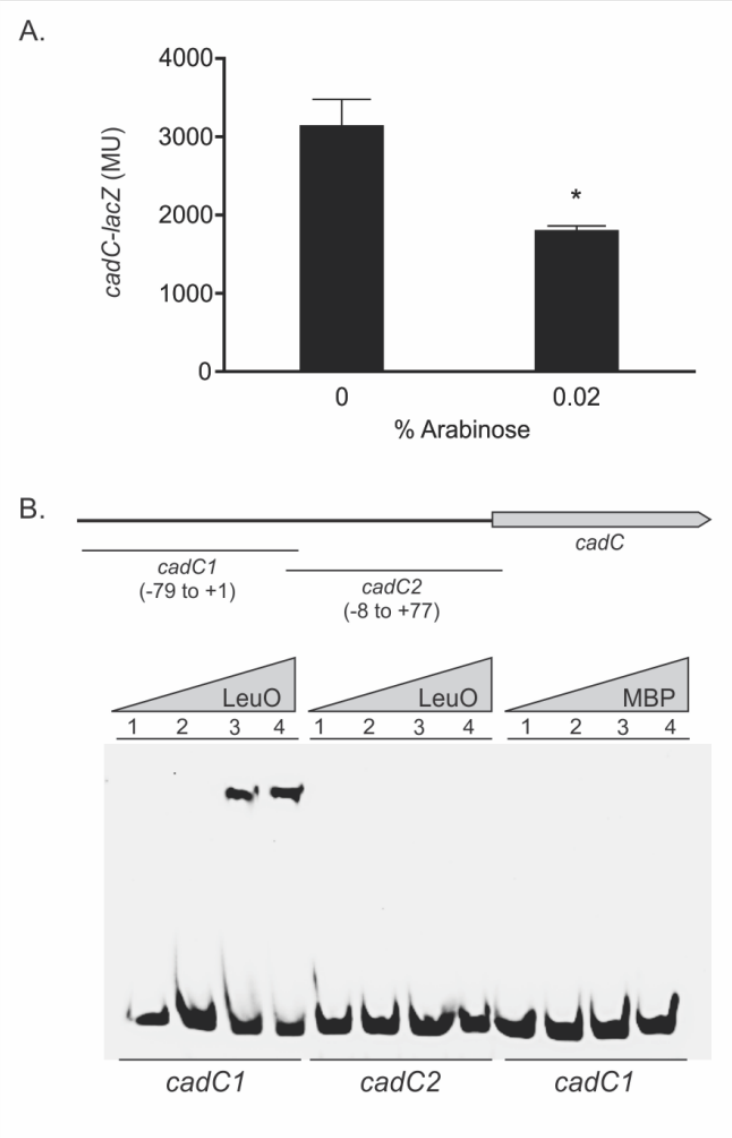

Figure 9. **Influence of LeuO on the *cadC* promoter.**

(A) *E. coli* containing the *cadC-lacZ* reporter plasmid pXB239 and the pBAD33-*leuO* plasmid pVA126 was grown in LB broth in the presence or absence of 0.02% arabinose for 5 hrs when β-galactosidase activity was determined. The presented data is the mean +/- SD of three independent experiments. Statistical significance was determined using a t-test comparing the mean of the induced strain to mean of 0% arabinose control; *=P<0.005. (B) Gel shift

assays were performed using purified LeuO-MBP or MBP and the two indicated DNA fragments from the *cadC* promoter. Nucleotide numbering listed for the *cadC1* and *cadC2* DNA fragments are relative to the *cadC* transcriptional start site. Biotin labeled *cadC1* or *cadC2* DNA fragments (1.5 nM) were incubated with either purified LeuO-MBP or MBP at 0 μM (lane 1), 10 μM (lane 2), 20 μM (lane 3), or 30 μM (lane 4) prior to electrophoresis. doi: 10.1099/mic.0.000194.

3.2.3 Lysine decarboxylase activity is influenced by LeuO.

CadC positively regulates the expression of *cadBA*, and thus the production of lysine decarboxylase (CadA), in response to low environmental pH (98). Based on this, we hypothesized that if LeuO repressed *cadC*, then *leuO* deletion of should result in increased *cadC* expression, and a corresponding increase in *cadBA* expression and lysine decarboxylase production. Likewise, *leuO* overexpression should result in decreased *cadC* expression and a corresponding decrease *cadBA* expression and lysine decarboxylase activity. To test this hypothesis we quantified lysine decarboxylase activity in *V. cholerae* strains lacking *leuO* or *aphB* and in a *V. cholerae leuO* negative mutant in which we ectopically expressed *leuO*. In contrast to *E. coli* (108), *V. cholerae* only encodes one lysine decarboxylase (i.e. CadA) which facilitates direct measurement of lysine decarboxylase production in *V. cholerae* cell lysates as a reporter for *cadA* expression (91).

We first quantified lysine decarboxylase production in WT strain JB58, Δ*leuO* strain XBV222, and Δ*aphB* strain XBV148. The results showed a 29% increase in lysine decarboxylase activity in the *leuO* mutant relative to WT (Fig. 10). Although this increase in lysine decarboxylase activity did not reach statistical significance (P=0.16), lysine decarboxylase activity was consistently elevated in the *leuO* mutant in multiple independent experiments. By contrast, deletion of *aphB* resulted in a 79% reduction in lysine decarboxylase activity. This was

expected, as AphB is a positive regulator of *cadC*. To provide further evidence that LeuO negatively regulated lysine decarboxylase production we quantified the effect of *leuO* overexpression from pBAD18Km-*leuO* (pXB298) on lysine decarboxylase production in a Δ*leuO* mutant (XBV222). The results showed that the addition of 0.02% arabinose to the growth media resulted in a 53% reduction in lysine decarboxylase activity (Fig. 10). The observation that *leuO* deletion appeared to increase lysine decarboxylase activity, while *leuO* overexpression decreased lysine decarboxylase activity, provided additional evidence to support the conclusion that LeuO was a negative regulator of the *cad* system in *V. cholerae*.

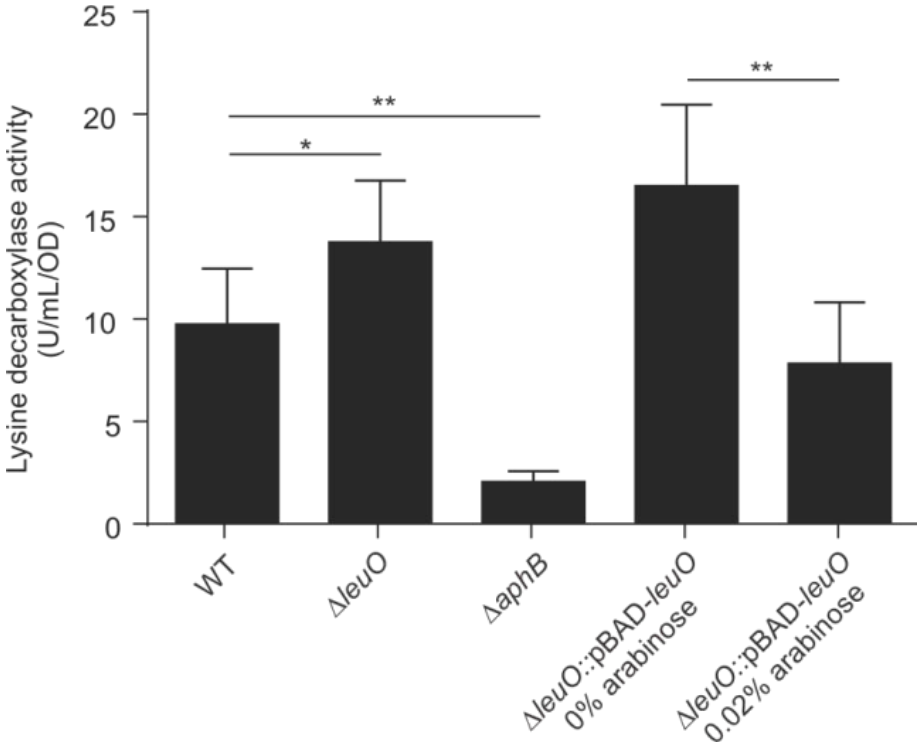

Figure 10. Impact of *leuO* on lysine decarboxylase production in *V. cholerae*.

The WT strain JB58, Δ*leuO* strain XBV222, Δ*aphB* strain XBV148, and Δ*leuO* strain carrying the pBAD18Km-*leuO* plasmid pXB298 were grown in AKI media under AKI conditions at 37°C for 4 hrs when lysine decarboxylase activity was quantified as described in the Materials and Methods. Strains containing the arabinose inducible pBAD18Km-*leuO* were grown in the presence or absence of 0.02% arabinose. Lysine decarboxylase specific

activity was defined as the amount of lysine converted to cadaverine per minutes divided by the optical density at 600 nm. The presented data is the mean +/- SD of three independent experiments. * P=0.16; **P>0.05. doi: 10.1099/mic.0.000194.

3.2.4 Effect of LeuO on *V. cholerae* survival following organic acidic challenge.

Studies have shown that the *cad* system contributes to an inducible acid tolerance phenotype whereby *V. cholerae* cells preadapted to mild acid conditions (i.e. pH 5.7) exhibit increased resistance to lethal acid challenge relative to unadapted cells (91). As our genetic and biochemical data suggested that LeuO repressed the *cad* system, we hypothesized that LeuO should also negatively affect *V. cholerae* acid tolerance (98). We tested this by challenging unadapted *V. cholerae* cells with varying concentrations of organic acids as described in the Materials and Methods (Appendix A). Since both *leuO* and the acid tolerance response were expressed *in vivo* (43, 92, 98), we chose to perform these assays using cells cultured under virulence gene inducing conditions (i.e. AKI conditions). We cultured WT, *leuO*, and *cadA* mutant strains for four hours under AKI conditions, which is the point where *cadC* expression was greatest (Fig. 8A), before exposing the cells to varying concentrations of organic acids that were present in the wells of microtiter plates. We then assessed cell viability at 15 and 30 minutes post organic acid challenge with the unadapted cells and 45 and 60 minutes post challenge with the adapted cells by replica plating culture aliquots from the microtiter plates onto LB agar plates.

The results for the unadapted cells revealed that there was no significant difference in the susceptibility of WT or the *leuO* mutant to organic acid challenge at either time point (Fig. 11A). This was an expected result given that LeuO appeared to be a *cadC* repressor. In contrast, the

cadA mutant exhibited an increase in susceptibility to the acid challenge as shown by decreased survival at 30 min relative to the WT control (Fig. 11A). This confirmed previous reports that *cadA* contributed to the *V. cholerae* acid tolerance response (91). In contrast to the unadapted cells, there was no apparent difference in organic acid susceptibility among any of the acid adapted mutant strains at either time point (Fig. 11B). This suggests that under virulence gene inducing conditions, other components of the acid tolerance response can compensate for the loss of *cadA*.

LeuO is a global regulator in the *Enterobacteriaceae*; a phenotype that appears to be conserved in the *Vibrionaceae*. This suggested the possibility the LeuO might affect the expression of other acid tolerance genes in addition to *cadC*. If this were true, *leuO* overexpression in *cadA* mutant should result in increased organic acid susceptibility. To test this, we repeated the acid killing assays using *leuO* and *cadA* mutants in which we ectopically expressed *leuO* (Fig. 11C and 11D). The results showed that *leuO* overexpression in the *leuO* mutant resulted in increased susceptibility of the unadapted cells to organic acid challenge (Fig. 11C). This finding confirmed that *leuO* expression enhanced *V. cholerae* susceptibility to organic acids and was consistent with the conclusion that LeuO repressed the *cad* system. Interestingly, ectopic expression of *leuO* in the *cadA* mutant also increased *V. cholerae* susceptibility to organic acid challenge (Fig. 11C). This finding indicated that the function of LeuO in organic acid tolerance extended beyond its regulation of the *cad* system.

We next tested whether LeuO affected the induction of an acid tolerance response phenotype. We therefore repeated the above experiments with AKI cultures that had been preadapted at pH 5.7 for one hour prior to organic acid challenge. The results showed increased organic acid resistance among the adapted cells relative to the unadapted cells with all of the

tested strains (Fig. 11). This was supported by comparison of cell viability between the 30 min unadapted cultures and the 45 min adapted cultures. Significantly, 60 min post challenge, there were no observable difference in survival between the WT, *leuO*, and *cadA* mutant strains (Fig. 11B) indicating that *V. cholerae* was able to mount an acid tolerance response in the absence of *leuO* and *cadA*. By contrast, when *leuO* was overexpressed in either the *leuO* or *cadA* mutants, the cells exhibited increased susceptibility to organic acid challenge relative to the empty vector control (Fig. 11D). This indicated that *leuO* overexpression negatively affected the ability of *V. cholerae* to mount an acid tolerance response. The fact that *leuO* overexpression in the *cadA* mutant resulted in increased acid susceptibility provided additional evidence to suggest that the function of *leuO* to acid tolerance extends beyond the *cad* system.

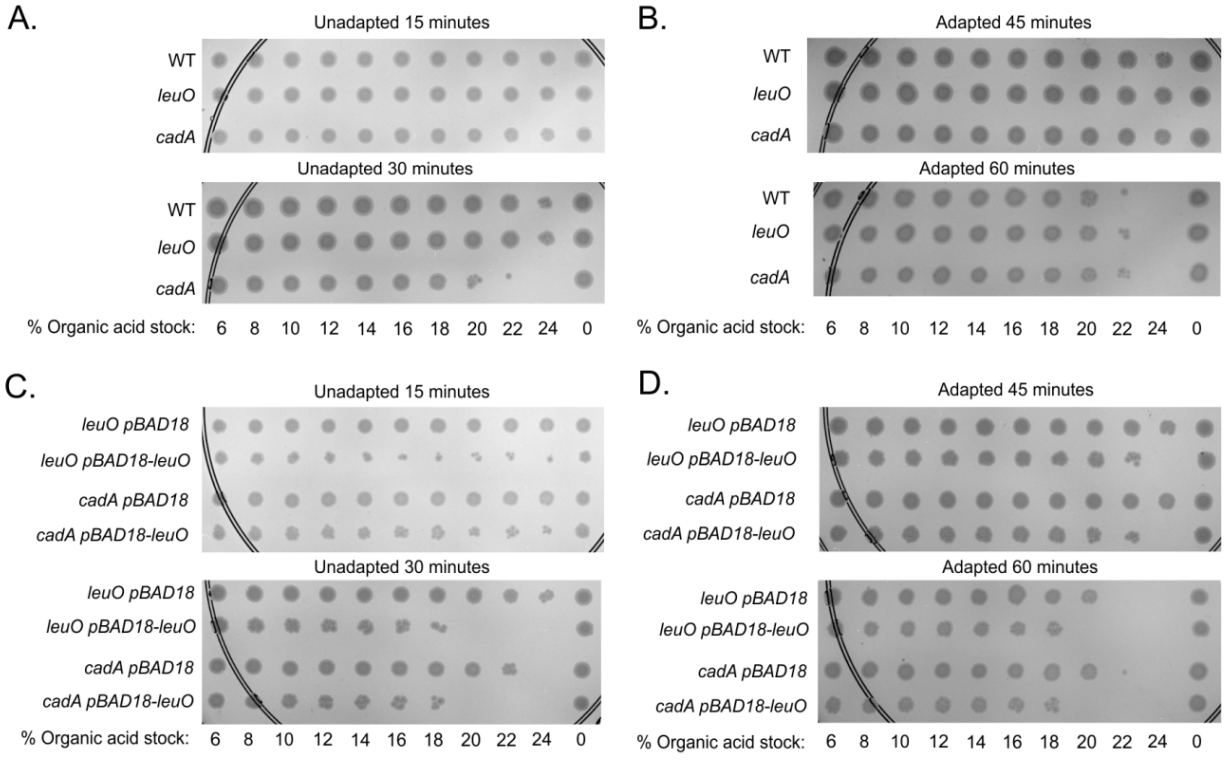

Figure 11. Effect of *leuO* on *V. cholerae* survival in organic acid.

(A) Survival of unadapted WT (XBV144), *leuO* (VA412) and *cadA* (EC17926) following organic acid challenge for 15 and 30 min. (B) Survival of adapted WT (XBV144), *leuO* (VA412) and *cadA* (EC17926) following organic acid challenge for 45 and 60 min. (C) Survival of unadapted *leuO* (EC20568) and *cadA* (EC17926) mutants containing pBAD18 or pBAD-*leuO* following organic acid challenge for 45 and 60 min. (D) Survival of adapted *leuO* (EC20568) and *cadA* (EC17926) mutants containing pBAD18 or pBAD-*leuO* following organic acid challenge for 45 and 60 min. All strains were cultured for four hours under AKI conditions before the organic acid challenge; 0.02% arabinose was added to the broth for strains containing pBAD18 or pBAD18-*leuO*. Unadapted cells (A and C) and adapted cells (B and D) were inoculated into microtiter plates containing the indicated final concentrations of the organic acid stock solution. The microtiter plates were then incubated at 37°C and cell viability was assessed over time by replica plating ~10 uL from each well of the microtiter plates onto the surface of an LB agar plate using a 96-well pin replicator. The agar plates were incubated overnight at 37°C before being photographed. The presented results are representative of at least three independent experiments. doi: 10.1099/mic.0.000194.

3.3 DISCUSSION

LeuO is a LysR-family regulator that has been shown to function downstream of ToxR in *V. cholerae* (43, 101). Several lines of evidence suggest that LeuO is a global regulator in the *Vibrionaceae* that functions in host adaptation and virulence. In *V. cholerae* LeuO has been shown to affect virulence factor production, biofilm production, and bile salt resistance (43, 59, 101). In *V. parahaemolyticus* LeuO has been shown to regulate the expression of type III secretion system, and serine protease production in *V. vulnificus* (57, 58). Taken together these results suggest that LeuO likely functions to regulate diverse genes involved in environmental adaptation in the *Vibrionaceae*.

In this work, we identified a new physiological function for LeuO in *V. cholerae* environmental adaptation. We found that LeuO regulated the expression of the *cad* system; a

finding that suggested that LeuO contributes to acid tolerance. The *V. cholerae cad* system is constitutively expressed at a low basal level, but is upregulated under conditions of low pH or low oxygen (36). Upregulation under these conditions is mediated by AphB binding to the *cadC* promoter. Once CadC is produced, it upregulates *cadBA* expression leading to the production of CadB (a lysine/cadaverine antiporter) and CadA (a lysine decarboxylase). CadA contributes to acid tolerance through its degradation of lysine to the polyamine cadaverine; a reaction that plays a key role in maintaining pH homeostasis within the cell.

While AphB positively regulates expression of the *cad* system, our results showed that LeuO negatively regulates the expression of the *cad* system. This conclusion was supported by the fact that *leuO* deletion increased *cadC* expression while *leuO* overexpression reduced *cadC* expression (Fig. 8). These results strongly suggested that LeuO was a *cadC* repressor. The negative effects of *leuO* on *cadC* transcription were further shown to affect the production of lysine decarboxylase production, the downstream target of CadC (Fig. 10). Taken together these results indicated that LeuO negatively regulates the expression of the *cad* system by repressing *cadC* transcription.

LeuO appeared to regulate the expression of the *cad* system by directly binding to the *cadC* promoter. This suggests the possibility that there may be interplay between AphB and LeuO in regulation of the *cad* system. Our results show that the expression of the *cad* system increased during static growth under AKI conditions before declining upon shift of the cultures to aerated growth (which is associated with alkalization of the media). Growth of El Tor strains under static AKI growth conditions results in low oxygen tension and low pH; conditions that have been correlated with AphB activation of *cadC* (36). By contrast, *leuO* expression appears to increase with cell density until it reaches its maximum level at late log phase (data not shown).

This suggests the possibility that LeuO may function to fine tune expression of the *cad* system by antagonizing AphB binding to the *cadC* promoter. The fact that both LeuO and AphB are LysR-family regulators, and that LysR-family regulators bind to T-N_{11}-A motifs (49), are consistent with this idea. Further, LeuO has been shown to regulate many of its target genes in the *Enterobacteriaceae* by functioning as an antagonist (48). Whether LeuO is functioning as an AphB antagonist in *V. cholerae* will require additional studies.

Overexpression of *leuO* in a *cadA* mutant increased *V. cholerae* susceptibility to organic acid in both adapted and unadapted cells (Fig. 11). This suggested that the contribution of LeuO to organic acid tolerance extended beyond the *cad* system. The mechanism by which this occurred is not known. The acid tolerance response in *V. cholerae* is complicated and involves diverse genes including the virulence regulator ToxR (42, 92). ToxR was shown to be required for the organic acid tolerance response through its regulation of the OmpU and OmpT porins. The fact that ToxR positively regulates *leuO* expression suggests that the role of ToxR in acid tolerance extends beyond porin regulation. In addition to *cadC*, AphB positively regulates other genes that contribute to acid tolerance (36, 109). While LeuO does not appear to affect production of OmpU or OmpT (101), it is possible that LeuO could affect the expression of other AphB-regulated genes that contribute to acid tolerance via a mechanism similar to what occurs with *cadC*. Alternatively, given that LeuO appears to be a global regulator, LeuO could affect acid tolerance through regulation of other unknown genes.

Although our data conclusively shows that LeuO represses *cadC* expression, the physiological relevance of LeuO repression of *cadC* and the acid tolerance response is not yet clear. Since *leuO* expression is induced by bile and LeuO contributes to bile salt resistance (101), one possibility is that downregulation of the acid tolerance response may contribute to bile

resistance. In *S. typhimurium* the acid tolerance response increased cell surface hydrophobicity (110); a phenotype that could result in increased susceptibility to detergent-like molecules like bile salts. If the *V. cholerae* acid tolerance response also resulted in increased cell surface hydrophobicity, *leuO* induction in response to bile salts may function to downregulate the acid tolerance response to decrease cell surface hydrophobicity and positively affect bile resistance. LeuO could also function in a feedback mechanism to modulate cadaverine production via *cadC* repression. Cadaverine is a polyamine that has two positive charges at neutral pH. Excess polyamines are growth inhibitory, which necessitates the regulation of their production (111). Cadaverine has also been found to reduce *V. cholerae* auto-agglutination, likely as a result of its positively charged amine groups electrostatically disrupting the pili interactions (112). Thus excess cadaverine could hinder intestinal colonization.

4.0 LEUO REGULATES THE CARRS TWO-COMPONENT SYSTEM AND IS A REPRESSOR OF POLYMYXIN B RESISTANCE IN VIBRIO CHOLERAE

Work described in this section was contributed

by Vanessa M. Ante,

X. Renee Bina, Mondraya F. Howard, and James E. Bina

4.1 INTRODUCTION

Vibrio cholerae is a gram negative human pathogen and the causative agent of the diarrheal disease cholera. People acquire cholera by ingestion of food or water that is contaminated with *V. cholerae*. Following *V. cholerae* ingestion, the organism colonizes enterocytes in the small intestine and replicates to high cell titers in the intestinal lumen before being disseminated from the host in a secretory diarrhea. Within the human gastrointestinal tract, *V. cholerae* is exposed to a variety of antimicrobial compounds including products of the innate immune system like cationic antimicrobial peptides (CAMPs). *V. cholerae* resistance to these antimicrobial compounds is dependent upon the induction of adaptive mechanisms that include the upregulation of antimicrobial efflux systems, modification of cell permeability through porin production, and biochemical modification of the cell surface.

CAMPs are short (~12-50 amino acids) amphipathic peptides that typically contain an excess of basic amino acids that result in a net positive charge (Reviewed in (113)). In gram negative bacteria, electrostatic interactions between the positively charged CAMPs and the negatively charged lipopolysaccharide (LPS) are thought to drive the initial interaction of CAMPs with the cell surface. Binding of CAMPs to the LPS results in outer membrane perturbation which facilitates CAMP uptake. Once across the outer membrane, CAMPs can disrupt the cytoplasmic membrane and/or inhibit critical cytoplasmic processes resulting in cell death. *V. cholerae* has evolved a number of mechanisms to resist the antimicrobial effects of CAMPs. This includes active efflux of CAMPs that have traversed the outer membrane via the VexAB-TolC RND-efflux pump (64) and the induction of an extracytoplasmic stress response (114). Moreover, LPS modification has been shown to be critical for *V. cholerae* CAMP resistance. Production of hexacylated lipid A via the MsbB acyltransferase confers polymyxin B

resistance in *V. cholerae* (115). Glycine and diglycine modification of the LPS by AlmEFG has also been shown to confer high level polymyxin B resistance in *V. cholerae* (116). AlmEFG functions to add glycine to hexacylated lipid A. Glycinylation of lipid A results in a net positive charge on lipid A which is thought to reduce the electrostatic interactions between CAMPs and LPS and lead to CAMP resistance.

CarRS was first identified in *V. cholerae* as a calcium-responsive negative regulator of biofilm production (117). Two subsequent studies have shown that CarRS also regulates cationic antimicrobial peptide resistance by positively regulating the expression of the *almEFG* operon (116, 118). Introduction of mutations into *carR* or the *almEFG* operon result in a ~100-fold decrease in *V. cholerae* resistance to the cationic-peptide like antibiotic polymyxin B. The molecular mechanisms controlling the expression of the *carRS* operon are unknown. Previous studies have shown that *carRS* expression is influenced by environmental cues (116). Growth of *V. cholerae* in the presence of polymyxin B resulted in upregulation of *carRS*, while growth in the presence of calcium or deoxycholate resulted in *carRS* repression.

We have recently found a number of *V. cholerae* phenotypes that are regulated by LeuO, a LysR-type transcriptional regulator. Expression of *leuO* is positively regulated by ToxR, often in response to environmental stimuli. The small molecule cyclo(Phe-Pro) has been shown to induce *leuO* expression leading to LeuO-dependent repression of virulence factor production (43). Expression of *leuO* was found to be induced by bile salts with LeuO contributing to bile resistance (101). LeuO has additionally been considered to play a role in the acid tolerance response through its regulation of the *cad* system (119). Previous studies have identified LeuO as a positive regulator of biofilm production through a yet unknown mechanism (59).

In this study, we show through transcriptional reporter and gel shift assays that LeuO is a direct repressor of *carRS*. We found that repression of *carRS* by bile salts was dependent on LeuO. *V. cholerae* mutants that did not express *leuO* exhibited increased resistance to polymyxin B, and conversely overexpression of *leuO* made cells more susceptible to polymyxin B. Our collective results demonstrate that LeuO contributes cell surface remodeling and polymyxin B resistance through the regulation of *carRS*.

Table 4. Strains, plasmids and oligonucleotides used in Chapter 4.

Strain, plasmid or oligonucleotide	Relevant characteristics	Source
Strains		
Escherichia coli		
EC100λpir	*supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 (λpirR6K)*	Epicenter
SM10λpir	*thi-1 thr leu tonA lacY supE recA::*RP4-2-Tc::Mu *kmR (λpirR6K)*	Lab collection
ER2566	*F⁻ glnV44(AS) galK2(Oc) rpsL704(strR) xylA5 mtl-1 argE3(Oc) thiE1 tfr-3* λ *DE3 =* λ *sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T gene1) i21 Δnin5*	New England BioLabs
Vibrio cholerae		
JB3	*V. cholerae* O1 El Tor strain N16961, Sm^R	Lab collection
JB58	*V. cholerae* O1 El Tor strain N16961, Sm^R Δ*lacZ*	Lab collection
XBV222	JB58Δ*leuO*	(43)
DT733	JB58Δ*toxRS*	(43)
JB461	JB3Δ*toxRS*	(18)
XBV302	JB58Δ*almE*	This study
Plasmids		
pTL61T	*lacZ* transcriptional reporter plasmid, Cb^R	(73)
pXB266	pTL61T containing the *leuO* promoter region	(43)
pVA289	pTL61T containing the *carRS* promoter region	This study
pMH53	pTL61T containing the *carRS* promoter region	This study
pBAD18Km	Arabinose regulated expression plasmid, Km^R	(76)
pXB269	pBAD18Km expressing VC2486 and *leuO*	(43)
pBAD33	Arabinose regulated expression plasmid, Cml^R	(76)
pVA126	pBAD33 expressing *leuO*	(119)
pXB302	pBAD33 expressing *toxRS*	(101)
pMAL-c2	IPTG-inducible expression vector for fusion of proteins to MBP and cytoplasmic expression, Cb^R	New England BioLabs
pVA175	pMAL-c2 expressing *leuO*	(119)
pWM91	Suicide plasmid vector used for allelic exchange, Cb^R	(106)
pWM91::Δ*almE*	pWM91::ΔVC1579	This study
Oligonucleotides	Sequence (5' to 3')	
P_{carRS}-F	AAACTCGAGAACACGCGGCGAGGAATTGAGTCAG	
P_{carRS}-R	CGGGGATCCGATAATGTAGAGACTGGGTTGG	
P_{carRS}-short-F	CGCTCGAGGTTTAATCACTGAGAGTGTAGCC	
P_{carRS}-short-R	GGGGATCCGTTGGTTAGACATGGGGACCTC	
almE-F1	CCCCCGGGCCACCAAGATACAAACTA	
almE-F2	TACAATTCTGCGGCGAGTCAGACATA	
almE-R1	ATGAGCTCGCTGCATCATGTCGGCTA	
almE-R2	TGTCTGACTCGCCGCAGAATTGTATG	
carRS-F-EMSA	GCGGGAGTCGGCAGCGGGCGAGGAATTGAGTCAGAAGCC	
carRS-R-EMSA	GCGGGAGTCGGCAGCGGAGACTGGGTTGGTTAGACATGGGG	
5'BIO	5`-biotin-GCGGGAGTCGGCAGCG	

4.2 RESULTS

4.2.1 LeuO regulates polymyxin B resistance in *V. cholerae.*

Phenotypic screening of a *V. cholerae leuO* deletion strain for alterations in antimicrobial resistance indicated that the Δ*leuO* mutant exhibited an increase in resistance to cationic antimicrobial peptides. To confirm this phenotype, we determined the minimum inhibitory concentration (MIC) of a *V. cholerae* Δ*leuO* mutant on polymyxin B gradient agar plates. The results of these experiments revealed that deletion of *leuO* resulted in a ~3-fold increase in the polymyxin B MIC relative to WT (Table 5). This was surprising as the El Tor biotype of *V. cholerae* is intrinsically resistant to high concentrations of polymyxin B. We previously have shown ToxR positively regulated *leuO* expression (101). Therefore we also determined the polymyxin B MIC for a Δ*toxRS* mutant. The results showed that in contrast to the Δ*leuO* mutant, the Δ*toxRS* mutant exhibited a >2-fold decrease in the polymyxin B MIC relative to WT (Table 5). This finding is consistent with the pleiotropic function of ToxR in polymyxin B resistance. ToxR is a global regulator that controls the expression more than 150 genes in *V. cholerae* (18) including the OmpU porin which has been linked to antimicrobial peptide resistance (120). The finding that mutation of *toxRS* resulted in a decrease in the polymyxin B MIC, while *leuO* mutation resulted in an increase in the polymyxin B MIC, suggests that porin dysregulation is dominant to *leuO* with respect to polymyxin B resistance. We also examined a Δ*almE* mutant. AlmE has been shown to be critical for the intrinsic polymyxin B resistance in *V. cholerae* (115). The Δ*almE* mutant exhibited a polymyxin B hypersensitive phenotype that was evidenced by a >100-fold decrease in its MIC. This finding was consistent with previous reports showing that the *almEFG* operon was required for cationic antimicrobial resistance.

The above data indicated that *leuO* negatively regulated polymyxin B resistance. If this was true, we hypothesized that *leuO* overexpression should result in increased *V. cholerae* susceptibility to polymyxin B. To test this, we compared polymyxin B susceptibility of WT and Δ*leuO V. cholerae* in which we overexpressed *leuO* from the arabinose regulated promoter in pBAD18Km. The results of these experiments showed that *leuO* overexpression in WT reduced the polymyxin B MIC by ~3-fold relative to the empty vector control (Table 5). Likewise, *leuO* overexpression in the Δ*leuO* mutant resulted in a >5-fold decrease in the polymyxin B MIC relative to the empty vector control. The fact that *leuO* overexpression resulted in increased polymyxin B susceptibility in both WT and the Δ*leuO* mutant supported the hypothesis that *leuO* negatively regulated polymyxin B resistance in *V. cholerae*.

Table 5. Polymyxin B susceptibility of *V. cholerae* strains.

Strain	MIC[1] (sd)
WT	114 (28.6)
Δ*leuO*	360 (2.1)[2]
Δ*toxRS*	50 (2.3)[2]
Δ*almE*	< 3 (0)[2]
WT (pBAD18Km)	118 (13.4)
WT (pBAD18Km-*leuO*)	40 (15.9)[3]
Δ*leuO* (pBAD18Km)	294 (45.6)
Δ*leuO* (pBAD18Km-*leuO*)	56 (5.6)[4]

[1.] Minimum inhibitory concentration (MIC) is in µg/mL with standard deviations in parenthesis.
[2.] $P \leq 0.01$ relative to WT
[3.] $P \leq 0.01$ relative to WT pBAD18Km
[4.] $P \leq 0.01$ relative to ΔleuO pBAD18Km

4.2.2 LeuO regulates expression of the *carRS* two-component regulatory system.

Preliminary RNA sequencing analysis comparing WT and a Δ*leuO* mutant identified *carRS* as a potential target for LeuO regulation. Given that a *carR* mutant has previously been shown to be highly susceptible to polymyxin B (116), this provided a potential mechanism for which LeuO regulated polymyxin B resistance. To validate the preliminary data, we introduced the *carRS-lacZ* plasmid pMH53 into WT strain JB58, Δ*leuO* strain XBV222, and Δ*toxRS* strain DT733. The resulting strains were then cultured in LB broth and assayed for β-galactosidase activity. The results showed that *carRS-lacZ* expression was increased ~40% in the Δ*leuO* mutant compared to WT (Fig. 12). In the Δ*toxRS* mutant, *carRS-lacZ* expression was comparable to the Δ*leuO* mutant. This was expected as ToxR is a positive regulator of *leuO* expression. This data would indicate that LeuO was a repressor of *carRS* expression and supported the hypothesis that LeuO regulated polymyxin B resistance through *carRS*.

Our lab has previously shown that *leuO* expression is induced in the presence of bile and the bile salt deoxycholate in a ToxR-dependent manner (101). It has also previously been found that *carRS* expression is repressed in the presence of bile and the bile salt deoxycholate (116). To determine if *carRS* repression in the presence of deoxycholate was dependent on LeuO, WT strain JB58, Δ*leuO* strain XBV222, and Δ*toxRS* strain DT733 carrying the *carRS-lacZ* plasmid were grown in LB broth containing 0.05% deoxycholate and assayed for β-galactosidase activity. Consistent with the previous published data, the results showed that *carRS-lacZ* expression was decreased >2-fold in the presence of deoxycholate compared to LB broth alone in the WT strain (Fig. 12). In contrast to this, the Δ*leuO* and Δ*toxRS* mutants displayed a ~2-fold increase in *carRS-lacZ* expression in the presence of deoxycholate compared to LB broth. Taken together

this data indicates that *carRS* expression is repressed by deoxycholate in a LeuO-dependent manner.

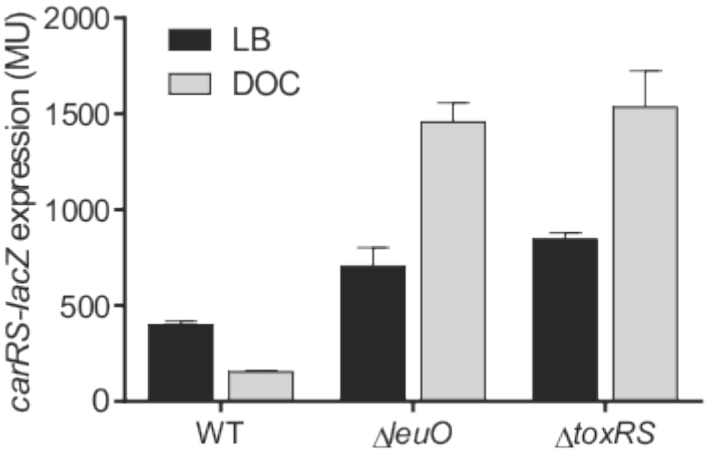

Figure 12. Expression of *carRS* is repressed by deoxycholate in a LeuO-dependent manner.

V. cholerae WT strain JB58, Δ*leuO* mutant strain XBV222, and Δ*toxRS* mutant strain DT733 carrying the *carRS-lacZ* reporter plasmid pMH53 were cultured in LB broth in the presence or absence of 0.05% deoxycholate (DOC) to stationary phase before being assayed for β-galactosidase activity as described in the Materials and Methods. The presented data is the mean +/- SD of two to three independent experiments.

4.2.3 LeuO acts directly on the *carRS* promoter.

The above reporter assays suggested that LeuO regulated *carRS* expression. However, it was unclear whether LeuO acted directly or indirectly at the *carRS* promoter. To address this, we tested the effect of *leuO* overexpression on transcription of the *carRS* promoter in a heterologous host. We introduced pBAD33 or pBAD33-*leuO* and the *carRS-lacZ* reporter plasmid pVA289 into *E. coli*. We then cultured the cells in the presence of varying concentrations of arabinose

before quantifying *carRS-lacZ* expression using a β-galactosidase assay. The results showed that the addition of arabinose to the cells containing the empty vector (pBAD33) did not affect *carRS-lacZ* expression (Fig. 13A). However, there was an arabinose dose-dependent reduction in *carRS-lacZ* expression in the cells containing pBAD33-*leuO*. This suggested that LeuO may directly repress *carRS* transcription. As a control, we also tested whether *toxRS* overexpression affected *carRS* expression. The results showed that the addition of arabinose to cells containing pBAD33-*toxRS* did not affect *carRS-lacZ* expression, thus confirming the specificity of *leuO* for the *carRS* promoter. These results suggested that LeuO acts directly at the *carRS* promoter; however, we noted the possibility that LeuO could be affecting *carRS* expression via an intermediate gene that is conserved in *E. coli* and *V. cholerae*.

To test whether LeuO functioned directly at the *carRS* promoter we performed electrophoretic mobility shift assays (EMSAs) using purified LeuO-MBP and the *carRS* promoter as previously described (119). Incubation of LeuO-MBP with a DNA probe covering the *carRS* promoter resulted in a mobility shift in the *carRS* probe starting at 2.5 μM (Fig. 13B lane 2). The *carRS* probe was further shifted at higher concentrations of LeuO-MBP (lanes 4 and 5), which may suggest LeuO oligomerization on the *carRS* promoter. The idea of LeuO forming oligomers is consistent with findings in *Salmonella enterica* serovar Typhi where LeuO has been shown to form tetramers (121). In contrast, incubation of the *carRS* probe with purified MBP did not result in a mobility shift, indicating that LeuO-MBP binding was due to LeuO and not due to non-specific binding by the MBP. Incubation of LeuO-MBP with a DNA probe derived from the *vexR* promoter did not result in a mobility shift indicating that LeuO binding was specific for the *carRS* promoter. The collective results of these experiments support the conclusion that LeuO directly binds to the *carRS* promoter to modulate its expression.

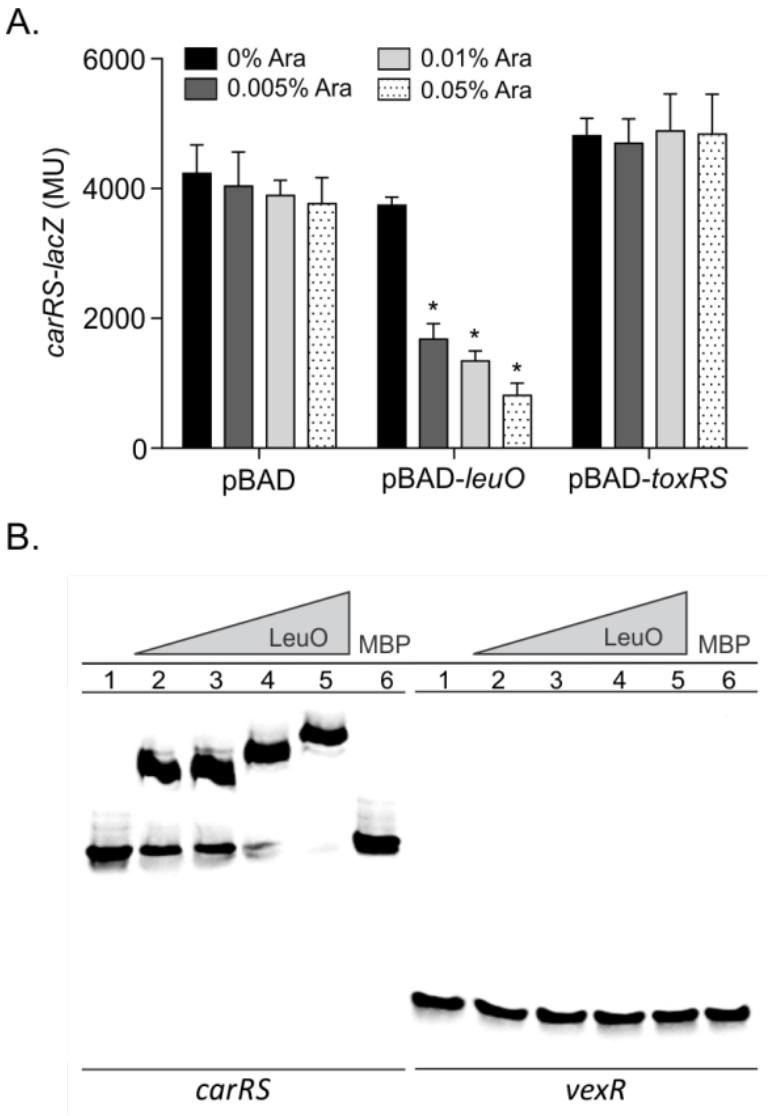

Figure 13. LeuO acts directly on the *carRS* promoter.

(A) *E. coli* carrying either pBAD33, the pBAD33-*leuO* plasmid pVA126, or the pBAD33-*toxRS* plasmid pXB302 and the *carRS-lacZ* plasmid pVA289 were grown in LB broth plus the indicated amount of arabinose to mid-logarithmic phase and assayed for β-galacatosidase activity as outlined in the Materials and Methods. The data presented is the average +/- standard deviation of three independent experiments. Statistical analysis was conducted using two-way ANOVA with Tukey's post-test *= $p<0.0001$. (B) Gel mobility shift assay. Biotin labeled DNA fragments *carRS* (1.5 nM) or *vexR* (3 nM) were incubated with LeuO-MBP at 0 μM in lane 1, 2.5 μM in lane 2, 5 μM in lane 3, 10 μM in lane 4, 25 μM in lane 5, or MBP at 25 μM in lane 6.

4.2.4 *V. cholerae* survival in polymyxin B.

The minimum inhibitory concentration data indicated that LeuO regulated polymyxin B susceptibility. To further investigate the role of LeuO in polymyxin B susceptibility we performed polymyxin B killing assays on WT, Δ*leuO*, Δ*almE* and Δ*toxRS V. cholerae* mutant strains. The cells were grown to middle logarithmic phase in LB broth before being incubated with a lethal dose of polymyxin B (500 µg/mL). We then assessed cell survival at 10 and 60 min by plating for viable cells. The results of the assay revealed that there was no significant difference between the rate of killing of the WT, Δ*leuO* and Δ*toxRS* mutants during the time course of this assay (Fig. 14A). The Δ*leuO* mutant did show a small increase in recovery in three of the four experiments relative to WT, but this difference did not meet the statistical significance threshold. By contrast, the Δ*almE* mutant was rapidly killed upon exposure to polymyxin B as evidenced by a ~3-log decrease in survival at 10 min and being unrecoverable at 60 min. These latter results are similar to previous reports and are consistent with the proposed role of *almE* in polymyxin B resistance (116).

In Fig. 12 we show that bile-dependent repression of *carRS* expression requires LeuO. Previous studies in our laboratory have shown that *leuO* transcription is upregulated upon exposure to bile salts and that LeuO contributed to bile salt resistance (101). This suggested the possibility that LeuO may contribute to polymyxin B and bile resistance in an inverse manner. To test whether bile salts affected *V. cholerae* survival upon exposure to lethal polymyxin B challenge, we repeated the above killing assays using *V. cholerae* cells that had been cultured in LB broth containing 0.05% deoxycholate. The WT results showed a ~1-log decrease in survival at 10 min and a >3-log decrease in survival at 60 min (Fig. 14B). By contrast, both the Δ*leuO* and Δ*toxRS* mutants did not exhibit a decrease in survival during the time course of the

experiment. The fact that the deoxycholate activates *leuO* expression and that the Δ*leuO* mutant was protected from the bactericidal activity of polymyxin B is consistent with the idea that LeuO is negatively regulating polymyxin B resistance in *V. cholerae*. Likewise, the Δ*toxRS* mutant was also protected and may be linked to its requirement for the upregulation of *leuO* expression by deoxycholate. Similar to cells grown in LB broth alone, the Δ*almE* mutant was rapidly killed by polymyxin B challenge confirming that *almE* is required for *V. cholerae* resistance to polymyxin B.

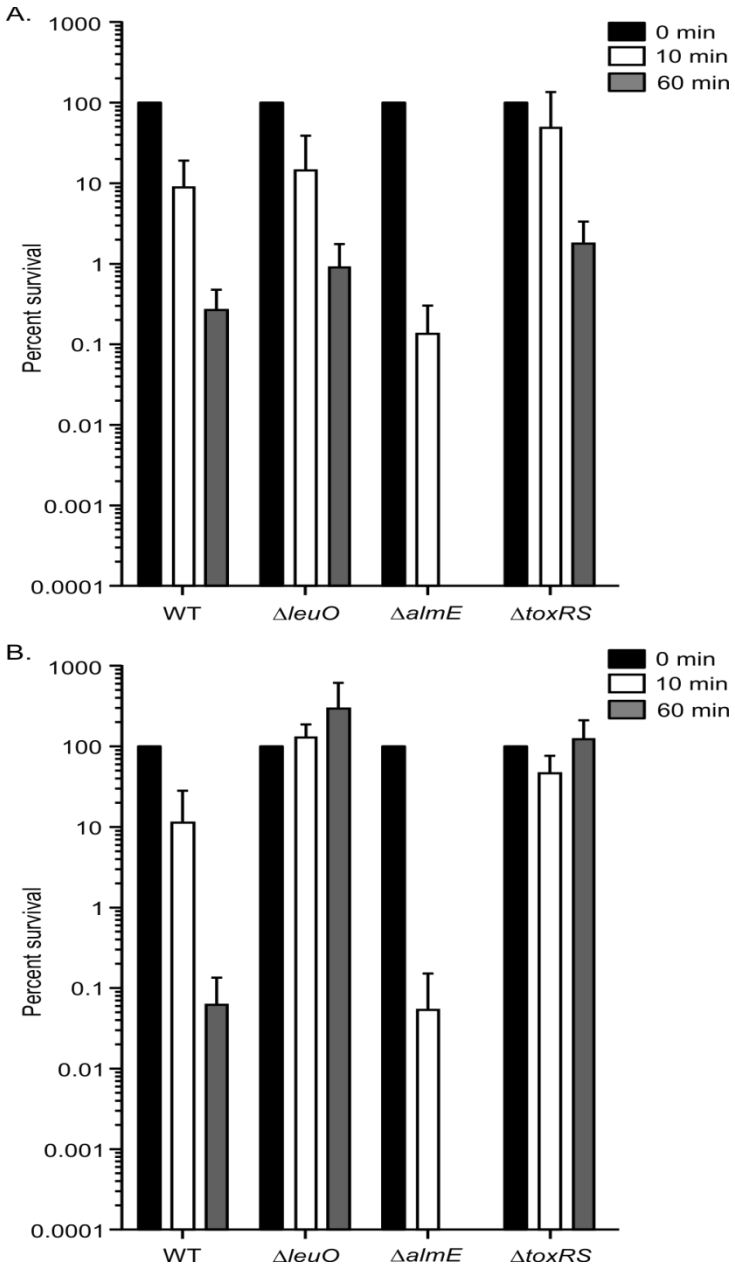

Figure 14. *V. cholerae* survival in polymyxin B.

V. cholerae WT strain JB58, Δ*leuO* strain XBV222, Δ*almE* strain XBV302, and Δ*toxRS* strain JB461 were grown in (A) LB broth or (B) LB broth containing 0.05% deoxycholate to middle logarithmic phase. Strains were then exposed to a lethal concentration of polymyxin B for 10 or 60 min before aliquots were plated onto LB agar to quantify the viable cell counts as described in the Materials and Methods. The percent survival for each strain was calculated as (CFUoutput/CFUinput) x 100. The data presented is the average +/- standard deviation of four independent experiments.

4.3 DISCUSSION

We have previously shown that *leuO* is regulated by ToxR, often in response to environmental signals. LeuO plays roles in resistance to environmental stressors, such as bile and acidity (101, 119). Given that LeuO regulates antimicrobial resistance, we tested if LeuO affected the minimum inhibitory concentration of polymyxin B. We found that deletion of *leuO* resulted in increased polymyxin B resistance compared to WT (Table 5). This observed difference in was further found to be the result of LeuO regulating LPS remodeling through *carRS*. In this study, we show that LeuO is a repressor of *carRS* expression. Through EMSAs we were able to determine that LeuO directly binds to the *carRS* promoter (Fig. 13). Through reporter assays we found that *carRS* is repressed by deoxycholate in a LeuO-dependent manner (Fig. 12).

During the course of infection *V. cholerae* is exposed to diverse environments depending on its location in the intestine. Cells that localize to the lumen are certainly exposed to high concentrations of bile salts. By contrast, cells that have traversed the mucosa to colonize the epithelial surface are somewhat protected from luminal bile salts due to the barrier properties of the mucous layer, but more likely to be exposed to high concentrations of cationic antimicrobial peptides that are produce by Paneth cells. We have previously shown that *leuO* expression was induced in response to bile salts and contributed to *V. cholerae* resistance to the antimicrobial effects of these anionic detergent-like molecules. These findings, combined with the data present in this report suggesting that LeuO indirectly regulates *almEFG*, suggest a model where LeuO differentially regulates resistance to cationic antimicrobial peptides and anionic bile salts in response to environmental cues.

To test this model we performed polymyxin B killing assays on *V. cholerae* following growth in the presence or absence of the bile acid deoxycholate. Deoxycholate has been

previously shown to induce the expression of *leuO* during growth in LB broth. We hypothesize that growth of *V. cholerae* in deoxycholate will result in *leuO* activation and decreased expression of *carRS* and its downstream target *almEFG*. Downregulation of *almEFG* will result in LPS that is more negatively charged and increased polymyxin B susceptibility. We found that pre-adapting cells in the bile salt deoxycholate did not affect subsequent susceptibility to polymyxin B in the WT strain (Fig. 14B). This would suggest that the initial decreased concentration of CarRS in the cell does not keep it from mounting a proper tolerance response to polymyxin B. This may suggest that the activator of *carRS* in response to polymyxin B is a able to displace LeuO from the *carRS* promoter. In contrast, Δ*leuO* and Δ*toxRS* mutants displayed significantly increased survival to polymyxin B when grown in the presence of deoxycholate. This was not observed in the Δ*almE* mutant, suggesting that this mechanism is dependent upon AlmE. The observed increase in polymyxin B resistance in the Δ*leuO* and Δ*toxRS* mutants may be attributed to an elevated concentration of CarRS in the cell prior to polymyxin B exposure.

5.0 CONCLUSIONS

The success of *V. cholerae* as a human pathogen is dependent upon its ability to rapidly sense and adapt to changes in its growth environment. *In vivo* this includes adaptation to the dynamic environments *V. cholerae* encounters during passage through the host gastrointestinal tract. In this work we show that LeuO functions downstream of ToxR to regulate a number of adaptive responses that may be important in pathogenesis including bile resistance, acid tolerance, and cationic antimicrobial peptide resistance.

5.1 THE LEUO REGULON

Transcriptome profiles of global regulators, like members of the LTTR family, can provide insight into their contributions to pathogenesis, metabolism, and cell division. The transcriptome profiles of LTTRs have been applied to vaccine development and for the treatment and diagnoses of bacterial infections (49). To better understand how LeuO regulates diverse cellular responses in *V. cholerae*, a goal of this proposal was to characterize the LeuO regulon in *V. cholerae*. To identify target genes of LeuO, RNA sequencing (RNAseq) analysis was used. LeuO was found to regulate the expression of 113 genes in the *V. cholerae* genome with the majority of the regulated genes being repressed (Appendix B). These genes were involved in diverse functions

including pathogenesis, metabolism, biofilm production, and stress responses. The gene targets identified will help to provide insight into the function of LeuO in future studies.

The considerable number of genes regulated by LeuO in *V. cholerae* was not surprising given that LeuO has been shown to be a global regulator in both *S. enterica* serovar Typhimurium and *E. coli*. LeuO has been characterized as functioning as an antagonist of the repressor H-NS in the *Enterobacteriaceae* (47, 48). Comparison of the LeuO regulon to the recently published *V. cholerae* H-NS regulon as identified by RNAseq data analysis (122), suggests that this is not conserved in *V. cholerae*. In contrast, it appears that LeuO functioned as a repressor at many H-NS suppressed genes including genes in the ToxR regulon. This suggests that LeuO may augment H-NS or alternatively be redundant for H-NS in *V. cholerae*.

One interesting observation from our RNAseq data is that a number of the genes identified as targets of LeuO in *V. cholerae* were horizontally acquired, such as the vibrio pathogenicity island and the superintegron that is unique to El Tor strains. Horizontally acquired genes are often maintained because they encode genes that enhance virulence or fitness and typically encode their own regulator. Additional LeuO targets include accessory colonization factors, the toxins MARTX and hemolysin, and a number of proteases involved in host escape and predator evasion. Taken together this indicates that LeuO regulation of pathogenesis extends far beyond the role it plays in CT and TCP production.

5.2 LEUO AND ADAPTIVE RESPONSES

Our results show that LeuO is a member of the ToxR regulon and that ToxR activates *leuO* expression in response to environmental cues. We have shown that the environmental cues can

be derived exogenously or endogenously (Section 2.2.2 and (43)). Exogenous molecules include components of bile, while endogenous molecules include cyclic peptides. Other endogenous molecules appear to be products of cell metabolism. This latter conclusion is based on the observation that deletion or inhibition of the *V. cholerae* RND efflux systems results in upregulation of *leuO* (unpublished); a phenotype that appears to be linked to the intracellular accumulation of molecules in the RND negative background that are normally removed from the cell by the RND efflux systems. Once *leuO* is activated by its effector molecules, LeuO then regulates genes that contribute to adaptive responses. In this work we have conclusively shown that *leuO* contributes to acid tolerance (Chapter 3), bile resistance (Chapter 2), and cationic antimicrobial peptide resistance (Chapter 4). However, based on our RNAseq data (Appendix B), we suspect that *leuO* may also regulate other genes late in infection, when the cells are at high cell density in the intestinal lumen, which contribute to dissemination and transmission phenotypes that have been observed in human shed *V. cholerae*. Based on our collect work with LeuO, we propose two models to explain the contributions of LeuO to *V. cholerae* pathogenesis.

In the first model we posit that LeuO is modulating adaptive responses based on spatial localization within the small intestine; whether the cell is localized to the lumen or the surface of the epithelium (Fig. 15). We hypothesize that following ingestion *V. cholerae* is exposed to organic acids and bile in the lumen of the intestine. These molecules are sensed by ToxR which results in activation of *leuO* expression (Section 2.2.2). LeuO then functions to activate genes that contribute to bile resistance while downregulating the *carRS* and *almEFG* genes. The downregulation of the latter genes in essence increases the net negative charge of the lipopolysaccharide (LPS). This increase in the negative charge results in electrostatic repulsion of anionic bile salts and makes the cell more resistant to the bactericidal effects of bile. By

contrast, we predict that *leuO* expression will be reduced in cells that have traversed the mucous layer, which has been shown to function as a diffusion barrier for bile salts (123). The down regulation of *leuO* results in derepression of *carRS* and increased *almEFG* expression (Section 4.2.2). The increase in *almEFG* expression will then result in glycine modification of LPS which will result in a net decrease in the negative charge of LPS. The decrease in LPS negative charge will then result in increased resistance to cationic antimicrobial peptides (CAMPs) that are predicted to be present at elevated concentrations at the epithelial surface. Future studies using a *leuO-gfp* reporter could be performed with a mucous expressing enterocyte cell line to test this model.

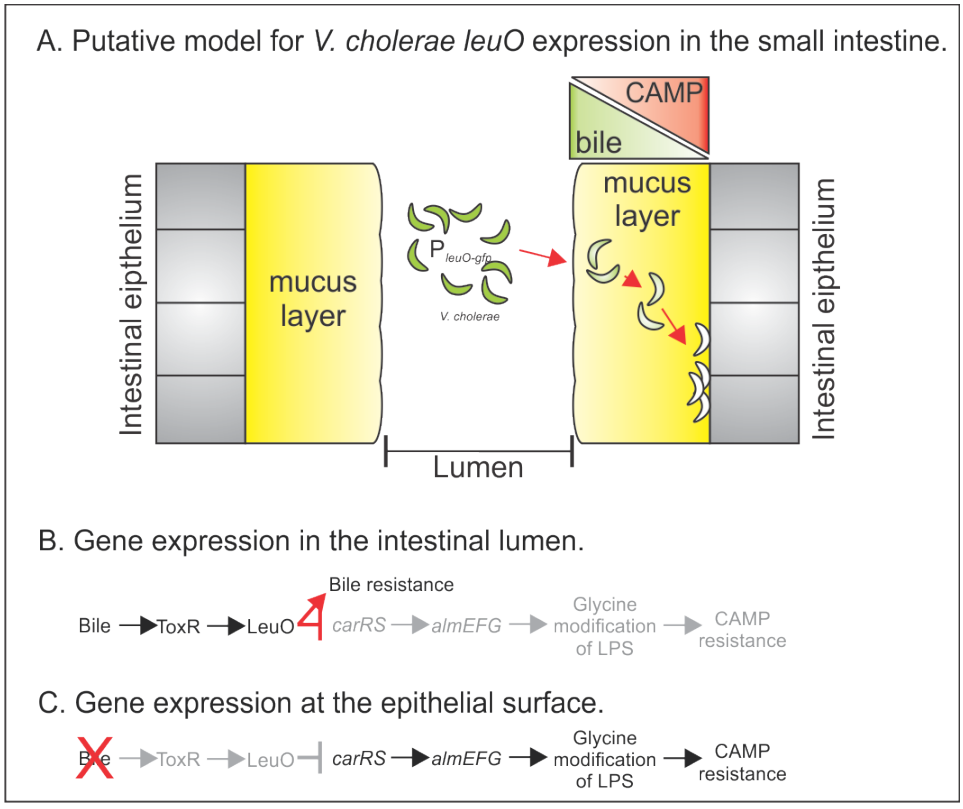

Figure 15. Putative model for *V. cholerae leuO* expression in the small intestine.

(A) *V. cholerae* containing a chromosomal *leuO-gfp* reporter are indicated by the comma shaped bacteria. Following *V. cholerae* ingestion, bile that is present in the intestinal lumen induces *leuO* expression which imparts a bile resistance phenotype. As *V. cholerae* migrates to the epithelial cell surface it traverses the mucus layer that acts as a

diffusion barrier to bile salts. *V. cholerae* penetrating the mucosa will thus be exposed to ever lessening concentrations of bile salts. This leads to a gradient of *leuO* expression based on the cells location in the mucus layer with the lowest expression being observed in *V. cholerae* cells localized to the epithelial surface. The repression of *leuO* expression at the epithelial surface results in deprepression of *carRS* and upregulation of *almEFG* which imparts resistance to cationic antimicrobial peptides (CAMP) that produce by cells localized to the epithelium and at maximal concentration near the epithelial surface. (B) *leuO* expression in *V. cholerae* localized to the intestinal lumen. Cells in the lumen are exposed to bile which induces *leuO* expression. LeuO then represses *carRS* while activating genes that contribute to bile resistance. (C) *leuO* expression in *V. cholerae* localized to the intestinal epithelium. The mucous layer acts as a bile diffusion barrier. This results in downregulation of *leuO* and upregulation of *carRS* and its target genes *almEFG*. The net result is glycinylation of LPS which imparts resistance to CAMP.

In addition to the spatial regulation based on location in the intestinal tract, we also speculate that LeuO is involved in regulation of genes late in the infection cycle. LeuO is a stationary phase protein and has been shown to be induced late in infection in the infant mouse model (43). Late in infection, the ToxR regulon is repressed before dissemination (18, 19), and we have shown that LeuO is a ToxR regulon repressor. Further, ToxR responds to cFP, a small molecule that accumulates in the growth media, which repressed virulence factor production in a *leuO*-dependent manner (43). Taken together, these observations suggest a second model for *leuO* during pathogenesis (Fig. 16). In this model we hypothesize that *leuO* functions to regulate genes late in infection that contribute to both *V. cholerae* dissemination into the aquatic ecosystem and transmission to a new host. We speculate that cell metabolites accumulate when *V. cholerae* is at high cell density. These metabolites signal through ToxR to activate *leuO* expression. LeuO then downregulates virulence factor production while activating the expression of genes that contribute to survival and persistence in the aquatic ecosystem (e.g. biofilm) and

genes that contribute to hyperinfectivity that is transiently observed in human shed *V. cholerae* (9, 20).

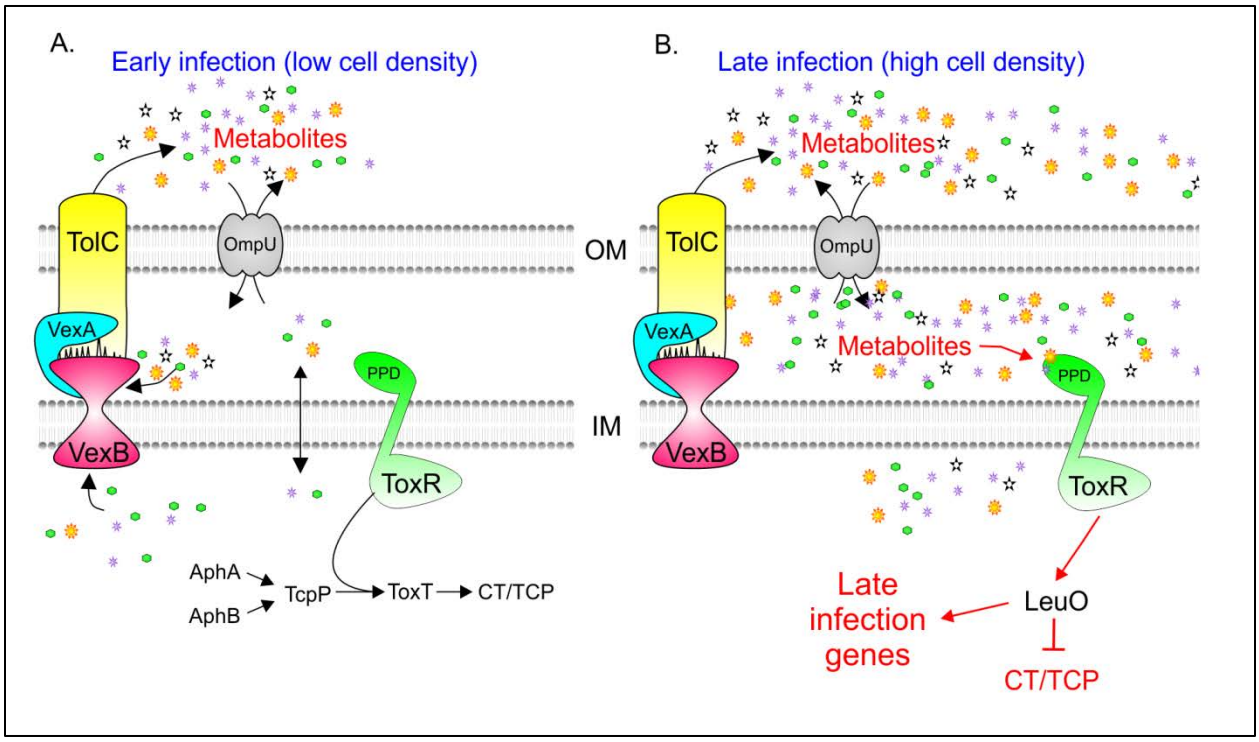

Figure 16. Putative model for the relationship between cell metabolites and *leuO* expression.

(A) At low cell density or early during infection the concentration of cell metabolites are low and ToxR functions with TcpP to activate the expression of the ToxR regulon which facilitates colonization and disease development. (B) Late in infection *V. cholerae* grows to high cell titers in the lumen. Nutrients become limiting and the concentration of cellular metabolites increases. The metabolites interact with the periplasmic sensing domain (PPD) of ToxR, which results in ToxR upregulating the expression of *leuO*. LeuO then represses virulence factor production while upregulating the expression of other genes that contribute to late infection phenotypes affecting dissemination and transmission.

APPENDIX A

MATERIALS AND METHODS

A.1 STRAINS, CHEMICALS, AND MEDIA

The bacterial strains used are listed in Tables 1, 3 & 4. *E. coli* strains EC100λ*pir* and SM10λ*pir* were used for cloning and plasmid mobilization, respectively. *E. coli* strains EC100λ*pir* or BW25113 were used for the two plasmid β-galactosidase reporter assays. *E. coli* strain ER2566 was used for purification of LeuO-MBP and MBP. The *V. cholerae* strains used in these studies were seventh pandemic O1 El Tor clinical isolates. *V. cholerae* strain JB58 (N16961Δ*lacZ* Sm^R) (124) or strain XBV144 (C6706 Δ*lacZ* Sm^R) were used as the wild-type (WT) control strains. Bacterial strains were grown at 37°C in Luria-Bertani (LB) broth or on LB agar. AKI growth conditions which are used to induce the ToxR regulon have been described previously (125). Modified T-media was prepared as previously described (40). Stock solutions of the detergents and bile (Difco Oxgall) were made in water and filter sterilized before use. An organic acid cocktail (1X) consisting of: 87 mM acetic acid, 25 mM butyric acid, and 37 mM propionic acid was used for the organic acid challenge assays. Acid adaptation media contained 0.1X organic acid cocktail in LB broth at pH 5.7. Bacterial stocks were maintained at -80°C in LB broth

containing 25% glycerol. Growth media was supplemented with carbenicillin (Cb) and streptomycin (Sm) at 100 μg/ml, kanamycin (Km) at 50 μg/ml, or chloramphenicol (Cm) at 1 μg/ml for *V. cholerae* or at 25 μg/ml for *E. coli* as required. Arabinose was added to growth media at the indicated concentrations to induce expression from the arabinose-regulated promoter in pBAD18, pBAD18Km and pBAD33.

A.2 PLASMID AND MUTANT CONSTRUCTION

Plasmids and oligonucleotides used are listed in Tables 1, 3 & 4. Genomic DNA from N16961Δ*lacZ* Sm^R was used as a PCR template for cloning. Plasmid reporters containing derivatives of the *leuO* promoter lacking one or both ToxR binding sites were constructed as follows. pVA258 (P_{leuO} lacking the distal ToxR binding site) was generated by PCR using the P_{leuO1}-F and P_{leuO}-R oligonucleotide primers. The resulting amplicon was digested with BamHI and XmaI restriction endonucleases and ligated into similarly digested pTL61T. pVA261 (P_{leuO} lacking both ToxR binding sites) was generated by PCR using the P_{leuO2}-F and P_{leuO}-R primers. The resulting PCR amplicon was digested with BamHI and XmaI restriction endonucleases and ligated into similarly digested pTL61T. The *cadC-lacZ* reporter plasmid pXB239 was constructed as follows. Briefly, the P_{cadC}-F/P_{cadC}-R PCR primer pair was used to amplify the *cadC* promoter region from the *V. cholerae* N16961 genome. The resulting PCR amplicon was then digested with XhoI and XbaI restriction endonucleases before being ligated into similarly digested pTL61T to generate pXB239. The β-galactosidase reporter construct pVA289 for *carRS-lacZ* was generated as follows. The PCR primers P_{carRS}-F/P_{carRS}-R were used to amplify the *carRS* promoter region from the *V. cholerae* N16961 genome. The resulting PCR amplicon

was digested with XhoI and BamHI restriction endonucleases before being ligated into similarly digested pTL61T to create pVA289. The β-galactosidase reporter construct pMH53 for *carRS-lacZ* was generated as follows. The PCR primers P_{carRS}-short-F/P_{carRS}-short-R were used to amplify the *carRS* promoter region from the *V. cholerae* N16961 genome. The resulting PCR amplicon was digested with XhoI and BamHI restriction endonucleases before being ligated into similarly digested pTL61T to create pMH53.

The *leuO* expression plasmid pVA94 (pBAD18::*leuO*) was constructed by moving *leuO* from pXB298 as a NheI and XbaI restriction fragment into the same sites in pBAD18. The *leuO* expression plasmid pVA126 (pBAD33::*leuO*) was constructed by removing the *leuO* fragment from pXB298 using XbaI and SspI restriction enzymes. The resulting ~1 kb *leuO* fragment was collected and ligated into pBAD33 digested with XbaI and SmaI. pXB302 (pBAD33::*toxRS*) was made by moving the *toxRS* genes from pXB289 as a SacI and SphI restriction fragment into the same sites in pBAD33. pDT1391 (pBAD33::$toxR^{\Delta ppd}S$) was made by moving the $toxR^{\Delta ppd}S$ genes from pXB286 as a SacI and SmaI restriction fragment into the same sites in pBAD33.

pWM91::Δ$toxR^{ppd}$, which contains a 94 amino acid C-terminal deletion of the ToxR periplasmic domain, was made by crossover PCR as previously described (126, 127). Briefly, primer pairs $toxR^{\Delta ppd}$-F1/$toxR^{\Delta ppd}$-R2 and $toxR^{\Delta ppd}$-F2/$toxR^{\Delta ppd}$-R1 were used in separate PCR reactions with N16961 genomic DNA. The resulting ~1 kb amplicons were collected and used as the template for second round PCR amplification with the flanking $toxR^{\Delta ppd}$-F1/$toxR^{\Delta ppd}$-R1 PCR primers. The resulting ~2 kb amplicon was then digested with SacI and SmaI restriction endonucleases before being ligated into similarly digested pWM91. The *aphB* deletion plasmid, pWM91::Δ*aphB*, was also constructed by PCR stitching. The *aphB*-F1/*aphB*-R2/ and *aphB*-F2/*aphB*-R1 PCR primer pairs were used to amplify ~1 kb regions flanking *aphB*. The resulting

PCR amplicons were used as the template for a second round of PCR using the *aphB*-F1 and *aphB*-R1 PCR primers. The resulting ~2 kb amplicon was digested with BamHI and SacI restriction endonucleases before being ligated into similarly digested pWM91 to generate pWM91::Δ*aphB*. The *almE* (VC1579) deletion plasmid pWM91::Δ*almE* was made by crossover PCR. Primer pairs *almE*-F1/*almE*-R2 and *almE*-F2/*almE*-R1 were used in separate PCR reactions with N16961 chromosomal DNA. The resulting ~1 kb amplicons were purified and used as templates in a second PCR amplification with the flanking *almE*-F1/*almE*-R1 PCR primers. The resulting ~2 kb amplicon was then digested with SacI and SmaI restriction endonucleases before being ligated into similarly digested pWM91 to generate pWM91::Δ*almE* .

Deletion of the ToxR periplasmic domain in *V. cholerae* strain SS4 was accomplished as follows. pWM91::Δ$toxR^{ppd}$ was conjugated into JB58 and plasmid cointegrants were selected for Sm and Cb resistance. Sm and Cb resistant cointegrants were then plated onto LB agar plates containing 5% sucrose and no NaCl. Sucrose resistant and Cb sensitive colonies were then screened by PCR using $toxR^{\Delta ppd}$-F1/$toxR^{\Delta ppd}$-R1 primers to confirm deletion of the ToxR periplasmic domain. Verification of $toxR^{\Delta ppd}$ in SS4 was accomplished by DNA sequencing of the *toxR* locus. Deletion of *V. cholerae aphB* (VC1049) was performed by allelic exchange as previously described (63). Briefly, *E. coli* SM10λ*pir* was used to conjugate plasmid pWM91::Δ*aphB* into *V. cholerae* JB58 and co-integrants were selected for Sm/Cb resistance. Several Sm/Cb resistant colonies were cultured on LB agar (without NaCl) containing 5% sucrose to select for resolution of the integrated plasmid. Sucrose resistant and Cb sensitive colonies were then screened by PCR using the *aphB*-F1/*aphB*-R1 PCR primers to confirm *aphB* deletion. To make a *V. cholerae almE* deletion mutant, pWM91::Δ*almE* was conjugated into JB58 and plasmid co-integrants were selected for resistance to Sm and Cb. Several Sm and Cb

resistant co-integrants were then plated onto LB agar (without NaCl) plates containing 5% sucrose. Sucrose resistant and Cb sensitive colonies were then screened by PCR using *almE*-F1/*almE*-R1 primers to confirm deletion of *almE*. This resulted in the identification of strain XBV302 which was further verified by DNA sequencing of the *almE* locus. Deletion of *lacZ* (VC2338) in JB804 was accomplished in an identical manner using pDLT to generate strain XBV144. Deletion of *leuO* (VC2485) in XBV144 was accomplished as previously described to generate strain VA412 (59). The C6706 transposon insertion mutants were graciously supplied by Dr. John Mekalanos (Harvard Medical School).

The LeuO-MBP purification plasmid pVA175 (pMAL-c2::*leuO*) was constructed by amplifying the *leuO* gene from N16961 using the LeuO-F/LeuO-R PCR primers. The resulting PCR amplicon was then digested with XbaI and SmaI restriction endonucleases and ligated to pMAL-c2 which had been restricted with XbaI and XmnI endonucleases to generate pVA175. This ligation resulted in a translational fusion of *leuO* to the C-terminus of *malE* (maltose binding protein). The DNA sequence of the protein purification construct was subsequently verified by sequencing.

A.3 REPORTER ASSAYS

β-galactosidase assays were performed as follows. *V. cholerae* strains carrying the *leuO-lacZ* reporter indicated in the figure legend were cultured overnight in LB broth at 37°C with shaking. The cultures were then diluted 1:100 into fresh LB broth and incubated at 37°C with shaking. Culture aliquots were collected in triplicate at mid-exponential phase (OD_{600} of ~0.5) to quantify β-galactosidase activity as previously described (128). *V. cholerae* strains harboring the *cadC*-

lacZ reporter plasmid pXB239 were grown under AKI conditions and culture aliquots were taken in triplicate at various times to quantify β-galactosidase activity. The effect of LeuO on *cadC* expression in *V. cholerae* consisted of growing strain JB58 containing the *cadC-lacZ* plasmid pXB239 and pBAD33-*leuO* plasmid pVA126 under AKI conditions in the presence or absence of 0.02% arabinose. Culture aliquots were collected in triplicate after 5 hrs to quantify *cadC-lacZ* expression. The effect of LeuO on *aphB* expression in *V. cholerae* was accomplished by growing strain JB58 containing the *aphB-lacZ* plasmid pXB203 and the pBAD33-*leuO* plasmid pVA126 under AKI conditions in the presence or absence of 0.02% arabinose. Culture aliquots were collected in triplicate after 5 hrs to quantify *aphB-lacZ* expression. *V. cholerae* strains carrying the *leuO-lacZ* reporter plasmid pXB266 or the *carRS-lacZ* reporter plasmid pMH53 were cultured overnight in LB broth at 37°C with shaking. The cultures were then diluted 100-fold into fresh LB broth containing 0.05% deoxycholate, 10 mM $CaCl_2$, or 5 μg/mL polymyxin B and incubated at 37°C with shaking. Culture aliquots were then collected in triplicate at mid-logarithmic phase (OD_{600} of ~0.5) or stationary phase (OD_{600} of ~1.0) to quantify β-galactosidase production. All of the reporter experiments were performed independently at least three times. Expression from the *lacZ* reporter was calculated and displayed as Miller Units (MU).

The *E. coli* two plasmid β-galactosidase reporter experiments were performed as follows. *E. coli* strain EC100λ*pir* bearing an expression plasmid (pBAD33, pXB302, or pDT1391) and a *lacZ* reporter plasmid (pXB266, pVA258, or pVA261) were cultured overnight in LB broth with shaking at 37°C. The overnight cultures were then diluted 1:100 into fresh LB broth plus or minus 0.08% arabinose and the cultures were incubated at 37°C with shaking. Culture aliquots were collected in triplicate at mid-exponential phase (OD_{600} of ~0.5) to quantify β-galactosidase activity as previously described (128). LeuO repression of *cadC* expression in *E. coli* was

accomplished as follows. Overnight cultures of *E. coli* strain BW25113 containing the *cadC-lacZ* plasmid pXB239 and the pBAD33-*leuO* plasmid pVA126 were diluted 1:100 in LB broth plus or minus 0.02% arabinose. The cultures were incubated at 37°C with shaking and aliquots were collected after 5 hrs to quantify *cadC-lacZ* expression using β-galactosidase activity. *E. coli* strain EC100λ*pir* containing an expression plasmid (pBAD33, pVA126, or pXB302) and the *carRS-lacZ* reporter plasmid pVA289 was cultured overnight in LB broth with shaking at 37°C. The overnight cultures were then diluted 100-fold into fresh LB broth containing 0%, 0.005%, 0.01%, or 0.05% arabinose and the cultures were incubated at 37°C with shaking. Culture aliquots were collected in triplicate at mid-exponential phase (OD_{600} of ~0.5) to quantify β-galactosidase activity. Expression from the reporter plasmids was calculated and displayed as Miller Units (MU).

The bioluminescence assays were performed as follows. *V. cholerae* strain JB58 containing pJB906 (*leuO-lux*) was cultured overnight in LB broth at 37°C with shaking. The overnight cultures were then diluted 1:100 into fresh LB broth and incubated at 37°C with shaking for two hours. Aliquots (100 µl) of the culture were then diluted into 100 µl of LB broth plus the indicated substrates (i.e. DMSO, DOC or cFP) and distributed into triplicate wells of a white 96-well microtiter plate with a clear bottom (Corning). In these experiments DOC was used at 0.0125%, cFP at 1 mM and DMSO at 0.1%. The plates were then incubated at 37°C and luminescence and the OD_{600} were measured at the indicated time points using a BioTek Synergy HT plate reader. The relative light units (RLU) for each sample were calculated by dividing the luminescence by the OD_{600}. The reported results are the average and standard deviation of three independent experiments.

A.4 PURIFICATION OF LEUO-MBP AND MBP

Proteins for the gel shift assays were purified as follows. *E. coli* ER2566 carrying plasmid pMAL-c2 or the pMAL-c2::*leuO* plasmid pVA175 were grown in LB broth overnight at 37°C with aeration. The cultures were then diluted 100-fold into LB broth containing Cb and incubated at 37°C with shaking to an OD_{600} ~0.5 when 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added and the cultures were incubated for an additional 2 hrs. The cells were then harvested by centrifugation and the pellet resuspended in column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA) plus 1 mM phenylmethylsulfonyl fluoride. The cells were then lysed with a M-11P Microfluidizer according to the manufacturer's instructions (Microfluidics). The resulting cell lysates were cleared of particulate matter by centrifugation at 15,000 x g for 20 min at 4°C. The clarified supernatant (i.e. LeuO-MBP or MBP) was then diluted 1:6 with column buffer and loaded onto a 0.8 x 7.0 cm chromatography column containing 1 ml of amylose resin (New England Biolabs). The column was equilibrated with 12 ml of column buffer before the clarified supernatant was run through. Bound proteins were eluted from the resin using elution buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 10 mM maltose). The purity of the eluted fusion proteins were analyzed by SDS–PAGE with Coomassie brilliant blue R-250 staining. Protein concentrations were determined using the Coomassie Plus (Bradford) Assay kit according to the manufacturer's instructions (Thermo Scientific).

A.5 MEMBRANE ISOLATION

V. cholerae DT733 (Δ*toxRS*) containing pXB289 (pBAD18::*toxRS*), pXB286 (pBAD18::*toxR*$^{\Delta ppd}$*S*), or pBAD18 was cultured in LB broth with shaking at 37°C to an OD_{600} of ~1.0 when expression from the arabinose promoter was induced by the addition of arabinose to 0.2%. The cultures were then incubated with shaking at 37°C for an additional hour when the cells were harvested by centrifugation. The cell pellet was resuspended in 10 mM Tris-HCl (pH 8.0) in 0.75 M sucrose. Spheroplasts were then induced by the addition of 150 μg/ml lysozyme followed by the addition of 2 volumes of buffer A (10 mM Tris-HCl, pH 8.0, 10 mM EDTA) (129, 130). The cells were then lysed by passage through a model M-11P Microfluidizier (Microfluidics). Particulate matter was removed from the cell lysate by centrifugation at 8,000 x g for 10 minutes at 4°C. The membrane containing supernatant was then subjected to centrifugation in a SW-28 rotor (Beckman) at 24,000 rpm at 4°C for 2 hours to pellet the membrane fraction. The membrane pellet was then suspended in 20% sucrose in buffer B (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and applied to a two-step 60% and 70% sucrose gradient. The sucrose gradients were then subjected to centrifugation in a SW-28 rotor at 23,000 rpm at 4°C for 18 hours. The inner and outer membrane fractions were then decanted from the sucrose gradients and diluted with two volumes of cold buffer B before being centrifuged in a Ti55 rotor (Beckman) at 47,000 rpm at 4°C for 1 hour. The resulting inner membrane pellets were resuspended in cold buffer B and frozen at -20°C until used. Protein concentrations were determined using the Pierce Coomassie Plus Protein Assay according to the manufacturer's directions.

A.6 ELECTROMOBILITY SHIFT ASSAY

The following is for the EMSA in Chapter 2 using membrane fractions. DNA probes for gel shift assays were generated by PCR using the primers listed in Table 1. The PCR primers for the EMSAs were engineered to include a 5' tail (5'GCGGGAGTCGGCAGCG 3') which facilitated biotinylation of the probes by a second PCR reaction using the 5'BIO PCR primer which hybridized to the 5' tail. The 5'BIO PCR primer was purchased from the manufacturer (IDT) with a 5' biotin label. The biotinylated probes were gel purified and quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific) before being used in the EMSAs. The DNA binding reactions were performed in a final volume 10 μl of binding buffer (10 mM Tris-HCl (pH 7.4), 5 mM NaCl, 50 mM KCl, 1 mM EDTA, 50 μg/ml BSA, 1.5 nM biotinylated probe and 10 μg/ml sheared salmon sperm DNA) containing the indicated amounts of the *V. cholerae* inner membranes. The binding reactions were incubated at 30°C for 30 minutes before being subjected to electrophoresis at 150 V for 1 hour on a non-denaturing 5% polyacrylamide TBE gel that had been pre-run with 5% thioglycolic acid for 1 hour at 150 V in 1x TBE buffer. The resolved gels were electroblotted to positively charged nylon membrane in 0.5x TBE buffer at 380 mAmps for 1 hour, before the nylon membrane was UV crosslinked at 120,000 microjoules using a Stratalinker 1800 Crosslinker (Strategene). The biotinylated probes were then detected using the Pierce Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific) and visualized using a FluorChem E imaging system (Protein Simple).

The following is for the EMSA in Chapter 3 using LeuO-MBP or MBP. The DNA fragments designated *cadC1* (the nucleotide sequence between -79 and +1 relative to the *cadC* transcriptional start site) and *cadC2* (the nucleotide sequence between -8 to +77 relative to the *cadC* transcriptional start site) were PCR amplified from the N16961 genome using the *cadC*-

EMSA-F1/*cadC*-EMSA-R1 and *cadC*-EMSA-F2/*cadC*-EMSA-R2 oligonucleotide primers, respectively (Table 3). The PCR fragments were then gel purified and 100 ng was used as a template for a second PCR reaction using the biotinylated 5'BIO oligonucleotide primer purchased from Integrated DNA Technologies (Coralville, IA). The resulting DNA fragments were end labeled with biotin. The biotin labeled probes (1.5 nM) were incubated with purified LeuO-MBP or MBP in amounts ranging from 0 to 30 μM in binding buffer containing 10 mM Tris (pH 7.4), 150 mM KCl, 0.1 mM dithiothreitol, 0.1 mM ethylenediaminetetraacetic acid (pH 8.0), and 200 μg/ml sheared salmon sperm. The binding reactions were incubated at room temperature for 20 min before being subjected to electrophoresis on a non-denaturing 5% TBE-PAGE in 0.25x TBE buffer at 200 V for 45 min. The DNA in the gel was transferred to a nylon membrane in 0.5x TBE buffer at 380 mAmps for 1 hr. The nylon membrane was then UV crosslinked at 120,000 microjoules using a Stratalinker 1800 (Stratagene). Biotin labeled DNA was detected using the Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific) and visualized using a Fluorchem E Digital Darkroom imager (Protein Simple).

The following is for the EMSA in Chapter 4 using LeuO-MBP or MBP. The *vexR* DNA fragment was previously defined and consisted of the nucleotide sequence between -129 and -46 relative to the *vexR* translational start site (74). The DNA fragment containing the *carRS* promoter (the nucleotide sequence between -400 to +20 relative to the *carR* translational start site) was PCR amplified from the N16961 genome using *carRS*-F-EMSA and *carRS*-R-EMSA oligonucleotide primers (Table 4). The resulting PCR fragment was then gel purified and 100 ng was used as a template for a second PCR reaction using the biotinylated 5'BIO oligonucleotide primer purchased from IDT to produce the end-labelled DNA probe. The biotin labeled probes (*carRS* 1.5 nM, *vexR* 3 nM) were incubated with purified LeuO-MBP or MBP in amounts

ranging from 0 to 25 μM in binding buffer containing 10 mM Tris (pH 7.4), 150 mM KCl, 0.1 mM DTT, 0.1 mM EDTA (pH 8.0), and 200 μg/mL sheared salmon sperm. The 10 μl binding reactions were incubated at room temperature for 20 min before being subjected to electrophoresis on a non-denaturing 5% TBE-PAGE gel in 0.25x TBE buffer at 200V for 45 min. The gel was pre-run at 100V for 1 hr in 0.25x TBE prior to sample addition. The DNA in the gel was then transferred to a nylon membrane in 0.5x TBE buffer at 380 mAmps for 1 hr. The nylon membrane was then UV crosslinked at 120,000 microjoules using Stratalinker 1800 (Stratagene) before the biotin labeled DNA fragments were detected using Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific) and visualized using FluorChem E (Protein Simple).

A.7 LYSINE DECARBOXYLASE ASSAYS

Strains were grown in AKI media static at 37°C until the cultures reached an OD_{600} of ~0.1 (4 hrs). Strains containing pBAD18Km-*leuO* were grown in AKI media in the presence or absence of 0.02% arabinose. After 4 hrs, culture aliquots were collected and processed for the quantification of lysine decarboxylase activity as previously described with slight modification (131). Briefly, the cells were collected by centrifugation and normalized to an OD_{600}=1. The cell pellet was then washed with 1 ml of cold (4°C) Buffer A (1 M NaCl, 0.05 M Potassium Pi buffer pH 6.5) before being centrifuged and resuspended in 200 μl cold (4°C) Buffer B (20 mM Potassium Pi pH 5.8). 20 μl of $CHCl_3$ was then added to each sample followed by vortexing for 15 sec to disrupt the cell membrane. Quantification of lysine decarboxylase activity was then carried out in triplicate by combining 10 μl of the cell lysate with 110 μl prewarmed Buffer C (5

mM lysine, 0.1 mM pyridoxal 5`-phosphate, 16 mM Potassium Pi pH 5.8); a parallel mixture without lysine was also prepared to control of the level of endogenous polyamines, since these react in the assay as cadaverine. The enzymatic reaction was incubated at 37°C for 15 min before adding 120 µl of Stop Solution (1 M Na_2CO_3) and placing on ice. Lysine and cadaverine were then derivatized by adding 120 µl of 10 mM 2,4,6-Trinitrobenzene sulphonate to the mixture and incubating at 40°C for 4 min. After incubation, samples were chilled on ice. For phase separation, 1 ml toluene was added and thoroughly vortexed for 20 sec; *N*,*N*`-bistrinitrophenylcadaverine (TNP-cadaverine) is soluble in toluene and *N*,*N*`-bistrinitrophenyllysine (TNP-lysine) is toluene insoluble. Samples were then centrifuged at 2,000 rpm for 5 min to allow the phases to separate. The concentration of TNP-cadaverine was measured by removing the upper aqueous phase and reading the A_{340} in quartz cuvettes with a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific). Lysine decarboxylase activity was determined as the difference in A_{340} between the sample incubated with lysine and that incubated without. Specific activity, was calculated using the equation (A_{340}/(time x OD_{600})) x 1000, and is a measure of lysine converted to cadaverine per time in min per cell density.

A.8 BILE KILLING ASSAYS

Overnight cultures of the indicated strains were diluted 1:100 in LB broth plus or minus 0.1% arabinose (to induce expression from pBAD18) and incubated at 37°C with shaking for four hours. Culture aliquots were then collected and washed once with phosphate buffered saline (PBS) before being diluted in PBS to an OD_{600} of 0.5. Serial dilutions of each strain were then plated onto LB agar plates to enumerate the cell titer at time zero (CFU_{input}). Aliquots containing

~10^6 CFU of each strain were then added to PBS containing 10% bile (*toxR* mutant strains) or 20% bile (*toxR* positive strains); the bile concentrations were reduced for the *toxR* mutant strains due to their increased bile sensitivity. The cultures were then incubated statically at room temperature for 60 minutes when aliquots were collected, washed in PBS, and plated onto LB agar to quantify the viable cells (CFU_{output}). The recovery ratio of each strain was then calculated as the (CFU_{output}/CFU_{input}). The fold change in recovery was then determined by dividing the recovery ratio for the mutant strains by the recovery ratio for WT. Fold change in recovery for the *leuO* overexpressing strain was determined by dividing the recovery ratio for Δ*toxRS*::pBAD18-*leuO* by the recovery ratio for Δ*toxRS*::pBAD18. The presented data are the mean and SD of three independent experiments. Statistical significance was determined using Dunnett Multiple Comparisons Test relative to a recovery ratio of 1.0.

A.9 ORGANIC ACID CHALLENGE ASSAYS

The acid challenge assays were facilitated by obtaining mutant strains from an ordered *V. cholerae* C6706 transposon library (104). C6706 is highly conserved with N16961 differing by only 87 single nucleotide polymorphisms across the entire genome (132) and we have not observed differences in the LeuO regulon or acid tolerance between the two strains. Overnight cultures of each test strain were diluted 1:10,000 into 10 ml of AKI broth, in the presence or absence of 0.2% arabinose, in a test tube and incubated statically at 37°C for 4 hrs before the cultures were normalized to OD_{600}=0.1 before use. The analysis of unadapted cells was performed as follows. Aliquots (100 μl) of the respective normalized cultures were distributed into the wells of a 96-well microtiter plate that contained a linear range of the organic acid

cocktail in LB broth. For the acid adaptation analysis, the cells were resuspended in organic acid adaptation media at pH 5.7 and incubated for 1 hr at 37°C. The cells were then collected by centrifugation and resuspended in fresh LB broth from which 100 µl aliquots were distributed into the wells of a 96-well microtiter plate that contained a linear range of the organic acid cocktail in LB broth. The inoculated microtiter plates were then incubated at 37°C and ~10 µl aliquots from each well were replica plated at the indicated time points onto LB agar plates using a 96-pin replicator. The agar plates were then incubated at 37°C for 18 hrs when the agar plates were imaged using a Fluorchem E Digital Darkroom imager (Protein Simple).

A.10 ANTIMICROBIAL SUSCEPTIBILITY ASSAYS

Antimicrobial susceptibility assays were performed using gradient agar plates as previously described (64). The gradient agar plates were inoculated with saturated overnight cultures of the *V. cholerae* WT strain JB58, Δ*leuO* strain XBV222, Δ*almE* strain XBV302, and Δ*toxRS* strain JB461 and incubated overnight at 37°C. For strains bearing pBAD18Km or pBAD18Km-*leuO* 0.1% arabinose was included in the gradient agar plate. After overnight incubation, growth of each strain across the gradient was measured. The minimum inhibitory concentration (MIC) of each strain was calculated as the percent of growth across the plate multiplied by the antimicrobial concentration used in the plate. The presented results are the mean and standard deviation of three independent biological replicates. Statistical significance for the tested strains was determined using one sample student's t-tests.

A.11 POLYMYXIN B KILLING ASSAYS

Overnight cultures of the *V. cholerae* WT strain JB58, Δ*leuO* strain XBV222, Δ*almE* strain XBV302, and Δ*toxRS* strain JB461 were diluted 100-fold into fresh LB broth plus or minus 0.05% deoxycholate. The inoculated cultures were then incubated with aeration at 37°C until they reached an OD_{600} of ~0.5 (4 hrs). Culture aliquots were subsequently collected by centrifugation and pellets resuspended in phosphate buffered saline (PBS) to an OD_{600} of 0.1. Serial dilutions of each strain were then plated onto LB agar plates to enumerate the cell titer at time zero (CFU_{input}). The remaining cells were then collected by centrifugation and resuspended in LB broth containing 500 μg/mL polymyxin B and incubated at 37°C with aeration. Aliquots were then collected at 10 and 60 min, washed in PBS, and plated onto LB agar to quantify the viable cells (CFU_{output}). The percent survival of each strain was then calculated by dividing the number of cells recovered following 10 min or 60 min exposure to polymyxin B by the number of cells in the input at time zero (CFU_{output}/CFU_{input}) x 100. The reported data represents the average +/- standard deviation from four independent experiments.

A.12 RNA EXTRACTION AND QRT-PCR

RNA was extracted from *V. cholerae* cultures grown in modified T-media at 37°C with shaking to an OD_{600} of ~0.3 when NRES (50 mM) or bile (0.2%) was added to the cultures. The cultures were then incubated for an additional 15 minutes at 37°C with shaking before RNA was extracted using Trizol according to the manufacturer's instructions (Ambion). The resulting RNA was quantified using a Nanodrop spectrophotometer (Thermo) before being used to make

cDNA with the Maxima First Strand cDNA synthesis kit (Life Technologies) according to the manufacturer's instructions. The resulting cDNA was then used with gene specific primers (Table 1) and the SYBR Green PCR master mix (Thermo) to quantify gene expression using a StepOnePlus Real-Time PCR System (Applied Biosystems). The relative gene expression levels were then calculated by the $2^{(-\Delta\Delta CT)}$ method (133) using the A-subunit of DNA gyrase (*gyrA*) as an internal control. The presented results are the mean +/- standard deviation of three independent biological replicates, with each biological replicate being generated from at least two technical replicates.

APPENDIX B

RNASEQ

B.1 INTRODUCTION AND RESULTS

To better define the target genes that LeuO regulates, we preformed RNAseq analysis to determine the LeuO transcriptome. Total RNA was isolated from WT and Δ*leuO* mutant strains grown in LB broth containing bile. This condition was chosen as it would show a significant difference in LeuO production using physiologically relevant parameters. In the WT strain, bile will induce *leuO* expression ~6-fold and in the Δ*leuO* mutant no LeuO will be present. Given that both the WT and Δ*leuO* mutant were treated the same, bile-responsive genes will not be identified as changing expression unless regulated through LeuO. Analysis of the RNAseq transcriptome data indicated that 113 genes were regulated by LeuO: 33 genes upregulated, 80 genes downregulated (Table 6). The genes included in Table 6 were selected with the criteria of having a 2-fold change and p value <0.05. The fold change listed is the difference in WT compared to a Δ*leuO* mutant, therefore repression by LeuO denoted as a positive number and decimals indicate activation by LeuO.

Table 6. Selected genes regulated by *V. cholerae* LeuO as identified by RNAseq.

Fold change	P-value	Gene number	Gene name	Function	Putative Role
2.28	1.00E-04	VC0280	*cadB*	cadaverine/lysine antiporter	acid tolerance and metabolism
3.00	<1.00E-05	VC0281	*cadA*	lysine decarboxylase, inducible	acid tolerance and metabolism
2.66	<1.00E-05	VC0284		putative outer membrane iron receptor	iron transport
0.42	<1.00E-05	VC0481	*lysE*	Transport and binding proteins	metabolism
0.32	2.00E-04	VC0606	*glnB-1*	nitrogen regulatory protein PII	regulatory protein
2.56	0.0089	VC0612		cellobiose/cellodextrin-phosphorylase	chitin catabolism
3.12	0.0085	VC0613	*chb-1*	beta-N-acetylhexosaminidase	chitin catabolism
3.80	0.0037	VC0614		conserved hypothetical protein	chitin catabolism
2.35	0.0031	VC0615		endoglucanase-related protein	chitin catabolism
4.14	<1.00E-05	VC0616		peptide ABC transporter, ATP-binding protein	chitin catabolism
3.06	<1.00E-05	VC0617		peptide ABC transporter, ATP-binding protein	chitin catabolism
2.85	<1.00E-05	VC0618		peptide ABC transporter, permease protein	chitin catabolism
2.79	<1.00E-05	VC0619		peptide ABC transporter, permease protein	chitin catabolism
2.38	<1.00E-05	VC0620	*cbp*	peptide ABC transporter, periplasmic peptide-binding protein	chitin catabolism
0.18	<1.00E-05	VC0687	*cstA*	carbon starvation protein A	stringent response
2.49	<1.00E-05	VC0715	*frp*	nitroreductase A	metabolism
2.60	2.00E-04	VC0734	*aceB*	malate synthase A	metabolism
0.38	0.0189	VC0770		conserved hypothetical protein	
11.01	<1.00E-05	VC0796	*citC*	citrate (pro-3S)-lyase ligase	fermentation
18.13	<1.00E-05	VC0797	*citD*	citrate lyase, gamma subunit	fermentation
11.74	0.0492	VC0798	*citE*	citrate lyase, beta subunit	fermentation
12.26	0.0319	VC0799	*citF*	citrate lyase, alpha subunit	fermentation
10.66	0.0287	VC0800	*citX*	apo-citrate lyase phosphoribosyl-dephospho-CoA transferase	fermentation
0.45	0.0453	VC0802		hypothetical protein	
3.03	<1.00E-05	VC0829	*tcpB*	toxin co-regulated pilus biosynthesis protein B	pathogenesis
2.27	<1.00E-05	VC0831	*tcpC*	toxin co-regulated pilus biosynthesis outer membrane protein C	pathogenesis
1.99	0.0189	VC0833	*tcpD*	toxin co-regulated pilus biosynthesis protein D	pathogenesis
9.56	<1.00E-05	VC0838	*toxT*	TCP pilus virulence regulatory protein	pathogenesis
8.47	<1.00E-05	VC0839	*tcpJ*	leader peptidase	pathogenesis
9.92	<1.00E-05	VC0840	*acfB*	accessory colonization factor	pathogenesis
7.98	<1.00E-05	VC0841	*acfC*	accessory colonization factor	pathogenesis
5.14	<1.00E-05	VC0842		conserved hypothetical protein	pathogenesis
6.97	<1.00E-05	VC0843	*tagE-1*	TagE protein	pathogenesis
2.52	0.0245	VC0844	*acfA*	accessory colonization factor	pathogenesis
0.37	0.0489	VC0910	*treB*	PTS system, trehalose-specific IIBC components	trehalose metabolism
0.36	0.0317	VC0911	*treC*	trehalose-6-phosphate hydrolase	trehalose metabolism
2.34	0.0425	VC1294		hypothetical protein	

Fold change	P-value	Gene number	Gene name	Function	Putative Role
2.70	3.00E-04	VC1295		c-di-GMP phosphodiesterase	degrade c-di-GMP
2.42	<1.00E-05	VC1319	*carS*	sensor histidine kinase	biofilm and polymyxin B resistance
2.79	<1.00E-05	VC1320	*carR*	DNA-binding response regulator	biofilm and polymyxin B resistance
0.46	<1.00E-05	VC1321		hypothetical protein	
2.01	<1.00E-05	VC1444	*vpsV*	hypothetical protein	biofilm
2.16	<1.00E-05	VC1445	*vpsS*	sensor histidine kinase/response regulator	biofilm
3.40	2.00E-04	VC1449	*rtxH*	hypothetical protein	pathogenesis
2.59	<1.00E-05	VC1454	*rstA1*	RstA1 protein	pathogenesis
2.61	<1.00E-05	VC1463	*rstA2*	RstA2 protein	pathogenesis
2.70	0.012	VC1542	*ligA-2*	DNA ligase	DNA replication, recombination and repair
0.41	<1.00E-05	VC1560	*katG*	catalase/peroxidase	oxidative stress
2.21	<1.00E-05	VC1561		transcriptional regulator, LysR-family	regulatory protein
2.05	0.0245	VC1562		beta-lactamase-related protein/Zn-dependent hydrolase	
0.33	1.00E-04	VC1581		NADH dehydrogenase subunit L	metabolism
2.05	0.0187	VC1583	*sodC*	superoxide dismutase, Cu-Zn	oxidative stress
4.13	<1.00E-05	VC1585	*katB*	catalase	oxidative stress
8.63	<1.00E-05	VC1644		hypothetical protein	
5.69	<1.00E-05	VC1709		zinc protease	protease
2.55	0.0053	VC1947		transcriptional regulator, LysR-family	regulatory protein
2.44	<1.00E-05	VC2240		phenolic acid decarboxylase	metabolism
4.41	<1.00E-05	VC2370		sensory box/GGDEF family protein	synthesize c-di-GMP
0.39	<1.00E-05	VC2373	*gltB-1*	glutamate synthase, large subunit	biosynthesis of glutamate
0.42	<1.00E-05	VC2374	*gltD-1*	glutamate synthase, small subunit	biosynthesis of glutamate
4.69	<1.00E-05	VC2388		hypothetical protein	
3.99	0.0188	VC2621	*xds*	extracellular nuclease-related protein	extracellular nuclease
0.36	<1.00E-05	VCA0014	*malQ*	4-alpha-glucanotransferase	maltose biosynthesis
0.44	0.0116	VCA0023		hypothetical protein	
2.29	<1.00E-05	VCA0035		phosphatidylglycerophosphatase B	phospholipid degradation
4.41	<1.00E-05	VCA0044		hypothetical protein, predicted periplasmic protease	protease
2.23	6.00E-04	VCA0101		conserved hypothetical protein	
3.18	1.00E-04	VCA0139		hypothetical protein, putative acetyl CoA synthase homolog	
0.40	<1.00E-05	VCA0148		TagA-related protein	protease
2.80	<1.00E-05	VCA0160	*mtr*	tryptophan-specific transport protein	tryptophan metabolism
3.95	<1.00E-05	VCA0161	*tnaA*	tryptophanase	tryptophan metabolism
2.03	2.00E-04	VCA0163		conserved hypothetical protein	
2.89	0.0188	VCA0195		hypothetical protein	
2.87	0.0033	VCA0218	*tlh*	thermolabile hemolysin	pathogenesis
4.97	<1.00E-05	VCA0219	*hlyA*	haemolysin	pathogenesis

Fold change	P-value	Gene number	Gene name	Function	Putative Role
5.16	0.0032	VCA0223	*prtV*	protease	protease, predator protection
31.96	<1.00E-05	VCA0250	*amy1*	alpha-amylase	metabolism
2.15	0.0085	VCA0267	*emrD-3*	multidrug resistance protein D	multidrug resistance
13.59	<1.00E-05	VCA0344		hypothetical protein	
9.30	<1.00E-05	VCA0345		conserved hypothetical protein	
3.81	<1.00E-05	VCA0346		H-REV 107-related protein	
9.43	<1.00E-05	VCA0428		hypothetical protein	
5.03	<1.00E-05	VCA0431		hypothetical protein	
2.15	<1.00E-05	VCA0432		hypothetical protein	
0.35	<1.00E-05	VCA0447		haemagglutinin associated protein	pathogenesis
8.86	<1.00E-05	VCA0448		hypothetical protein	
0.34	<1.00E-05	VCA0536		conserved hypothetical protein	
0.22	<1.00E-05	VCA0556		hypothetical protein	
2.13	1.00E-04	VCA0565		sensor histidine kinase	regulatory protein
2.69	<1.00E-05	VCA0612	*mscL*	large-conductance mechanosensitive channel	osmotic shock protection
0.46	0.0089	VCA0641		conserved hypothetical protein	
3.79	0.0086	VCA0650		hypothetical protein	
0.49	0.0089	VCA0688	*phaC*	polyhydroxyalkanoic acid synthase	phospholipid metabolism
0.41	0.0111	VCA0690		acetyl-CoA acetyltransferase	phospholipid metabolism
0.26	0.0103	VCA0691		acetoacetyl-CoA reductase	phospholipid metabolism
2.18	<1.00E-05	VCA0721		hypothetical protein	
0.46	<1.00E-05	VCA0728		hypothetical protein	integron unique to El Tors
0.42	<1.00E-05	VCA0729		hypothetical protein	integron unique to El Tors
0.45	<1.00E-05	VCA0730		hypothetical protein	integron unique to El Tors
3.83	0.0031	VCA0748	*glpB*	anaerobic glycerol-3-phosphate dehydrogenase subunit B	glycerol metabolism
3.65	2.00E-04	VCA0749	*glpC*	anaerobic glycerol-3-phosphate dehydrogenase subunit C	glycerol metabolism
2.56	0.0016	VCA0834		hypothetical protein	
0.40	2.00E-04	VCA0860	*malS*	alpha-amylase	metabolism
2.49	<1.00E-05	VCA0862	*fadL-3*	long-chain fatty acid transport protein	transport
2.18	<1.00E-05	VCA0863	*volA*	lipase, putative	phospholipid degradation
0.38	3.00E-04	VCA0934		hypothetical protein	
0.28	<1.00E-05	VCA0943	*malG*	maltose ABC transporter, permease protein	maltose metabolism
0.38	2.00E-04	VCA0944	*malF*	maltose ABC transporter, permease protein	maltose metabolism
0.28	<1.00E-05	VCA0945	*malE*	maltose ABC transporter, periplasmic maltose binding protein	maltose metabolism
0.25	4.00E-04	VCA0952	*vpsT*	transcriptional regulator, LuxR-family	biofilm
0.21	<1.00E-05	VCA1028	*ompS*	maltoporin, cell envelope OMP	maltose metabolism
2.21	<1.00E-05	VCA1031		pseudo gene	chemotaxis

B.2 METHODS

B.2.1 RNA Isolation

V. cholerae strains used in this study are listed in Table 1. Overnight cultures of *V. cholerae* strains JB58 (WT) and XBV222 (Δ*leuO*) were diluted 100-fold into fresh LB broth containing 0.05% crude bile (oxgall). Cultures were incubated at 37°C with aeration to an OD_{600} of ~0.5 before RNA was extracted using Trizol according to the manufacturer's instructions (Ambion). The resulting RNA was treated with DNase and further purified on an RNeasy column according to the manufacturer's directions (Qiagen). RNA was quantified using a Nanodrop spectrophotometer (Thermo) before being sent for RNAseq experiments. Three individual biological replicates of each strain were used for RNA isolation and subsequently sent for RNAseq experiments.

B.2.2 Whole-transcriptome analysis with total RNA sequencing

Total RNA from each sample was assessed using Qubit 2.0 fluorometer and Agilent TapeStation 2200 for RNA quantity and quality. Total RNA libraries were generated using Illumina TruSeq Stranded Total RNA Sample Preparation Guide Rev. E. The first step involved the removal of ribosomal RNA (rRNA) using biotinylated, target-specific oligos combined with Ribo-Zero rRNA removal beads depleting samples of both cytoplasmic and mitochondrial rRNA, Gram-positive and Gram-negative bacterial RNA. Following purification, the RNA was fragmented using divalent cations under elevated temperature. The cleaved RNA fragments were copied into first strand cDNA using reverse transcriptase and random primers, followed by second strand

cDNA synthesis using DNA polymerase I and RNase H. These cDNA fragments then have the addition of a single 'A' base and subsequent ligation of the adapter. The products were purified and enriched with PCR to create the final cDNA library.

The cDNA libraries were validated using KAPA Biosystems primer premix kit with Illumina-compatible DNA primers and Qubit 2.0 fluorometer. Quality was examined using Agilent Tapestation 2200. The cDNA libraries were pooled at a final concentration 1.8 pM. Cluster generation and 75 bp paired read single-indexed sequencing was performed on Illumina NextSeq 500.

B.2.3 Analysis Methods

Sequencing analysis was done using Bacterial RNA-seq Analysis on Maverix Analytic Platform (Maverix Biomics, Inc, San Mateo, CA). Raw sequencing reads from Illumina sequencing platform that was converted into FASTQ file format were quality checked for potential sequencing issues and contaminants using FastQC. Adapter sequences, primers, Ns, and reads with quality score below 28 were trimmed using fastq-mcf of ea-utils and Trimmomatic. Reads with a remaining length of fewer than 20 bp after trimming were discarded. Pre-processed reads were mapped to the *Vibrio cholerae* genome (RefSeq Accession Number: NC_002505 and NC_002506) using EDGE-pro. Read counts for RefSeq genes generated by EDGE-pro were normalized across all samples and then used for differential expression analysis using DEseq. Significant differentially expressed genes were determined by adjusted P-value with a threshold of 0.05.

APPENDIX C

ABBREVIATIONS

% : percent

:: : insertion

~ : approximately

+/- : plus/minus

°C : degree centigrade

β : beta

Δ : deletion

A : absorbance

AKI : growth conditions which are used to induce the ToxR regulon

AMP : antimicrobial peptide

ANOVA : analysis of variance

bp : base pair

cAMP : cyclic AMP production

CAMP : cationic antimicrobial peptide

Cb : carbenicillin

cDNA : complementary DNA

cFP : cyclo(Phenylalanine-Proline)

CFU : colony forming units

Cm : chloramphenicol

CT : cholera toxin

DMSO : dimethyl sulfoxide

DNA : deoxyribonucleic acid

DOC : deoxycholate

DTT : Dithiothreitol

e.g. : exemplī grātiā ; latin for “for example”

E. coli : *Escherichia coli*

EDTA : Ethylenediaminetetraacetic acid

EMSA : Electromobility shift assay

g : gravitational acceleration

gfp : green fluorescence protein

GM1 : monosialotetrahexosyl ganglioside

hr : hour

H-NS : histone-like nucleoid structuring protein

i.e. : id est; latin for “that is”

IPTG : Isopropyl β-D-1-thiogalactopyranoside

kb : kilo bases

Km : kanamycin

lacZ : in reference to the β-galactosidase gene

LB : Luria-Bertani

LPS: lipopolysaccharide

LTTRs : LysR-type transcriptional regulators

lux : in reference to the luciferase operon

M : molar

mA : milli Amps

MBP : maltose binding protein

µg : micro gram

MIC : minimum inhibitory concentration

min : minutes

mL : milli liter

µL : micro liter

MU : Miller Units

NCBI : National Center for Biotechnology Information

ng : nano gram

nm : nano meter

nM : nano Molar

NR : not recovered

NRES : asparagine, arginine, glutamic acid, and serine

OD : optical density

OD_{600} : optical density at 600 nano meters

OMP : outer membrane protein

P : P value

PAGE : Polyacrylamide gel electrophoresis

PBS : phosphate buffered saline

PCR : polymerase chain reaction

pH : measurement of acidity/alkalinity of an aqueous solution

ppd : periplasmic domain

qRT-PCR : quantitative real time polymerase chain reaction

RLU : relative light units

R : resistance

RNA : ribonucleic acid

RNAseq : RNA sequencing

RND : Resistance-Nodulation-Cell Division family

rpm : revolutions per minute

S. enterica : *Salmonella enterica*

SD : standard deviation

SDS : sodium dodecyl sulfate

Sm : streptomycin

TBE : Tris/Borate/EDTA

TCP : Toxin co-regulated pilus

TX-100 : Triton X-100

U : units

V : volts

V. cholerae : *Vibrio cholerae*

WT : wild type

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