

Characterization of the links between RND family multidrug efflux systems and adaptive responses in *Vibrio cholerae*

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The Gram-negative bacterium *Vibrio cholerae* is the causative agent of the diarrheal disease cholera. *V. cholerae* is a natural inhabitant of marine ecosystems and causes infection following the consumption of *V. cholerae* contaminated food or water. Therefore, the lifestyle of pathogenic *V. cholerae* requires rapid transitions between aquatic ecosystems and colonization of the human gastrointestinal tract. These transitions are marked by swift and dramatic changes in environmental conditions including fluctuations in temperature, pH, salinity, oxygen tension, and the presence of antimicrobial compounds. For successful transitions between these disparate environments, *V. cholerae* must be able to sense and adapt to environmental fluctuations, this is achieved through the use of adaptive responses such as the induction of antimicrobial resistance mechanisms. Multidrug efflux pumps belonging to the RND superfamily contribute to adaptive resistance to a broad range of antimicrobial compounds including antibiotics, immune effectors, and detergents. Interestingly, RND-mediated efflux has also been linked to bacterial adaptive responses outside of antimicrobial resistance, suggesting RND efflux pumps perform native functions in bacterial physiology. Consistent with this, work from our laboratory has linked *V. cholerae* RND-mediated efflux to virulence factor production, metabolism, and stress responses. Yet, how RND-mediated efflux contributes to these adaptive responses remains mostly unknown. In the work presented in this dissertation we sought to explore the mechanistic links between *V. cholerae* RND efflux pumps and adaptive responses, and to exploit these links to identify novel

signals and regulatory networks that participate in adaptive responses important for the *V. cholerae* life cycle. We found that the RND efflux pumps participate in iron acquisition, maintenance of membrane integrity, and metabolism through the secretion of vibriobactin. Further, we linked RND efflux to virulence factor production through the regulation of *ompR* expression. We defined the role of OmpR in *V. cholerae* biology, and found that it responds to components of bile and environmental pH, and contributes to the alkaline pH response. Collectively, this work illustrates some of the native function of *V. cholerae* RND efflux pumps, and indicates that they contribute to bacterial adaptive responses through the modulation of the intracellular concentration of signaling molecules.

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I dedicate this dissertation to Covid19, during the throws of which I completed this thesis, and to the countless number of bacteria that gave their lives for the work here in.

1.0 Introduction

1.1 Cholera

Cholera is an acute diarrheal disease caused by the bacterium *Vibrio cholerae*. The disease cholera is characterized by a rapid onset of profuse watery diarrhea, that when left untreated quickly leads to dehydration and death. Cholera is an ancient disease, with reports of cholera-like disease dating back to antiquity, and is thought to have originally emerged from the Ganges river delta in India. Cholera infections are classified as either endemic or epidemic. In areas where *V. cholerae* is endemic and have poor water sanitation there are seasonal occurrences of cholera, that do not require external introduction of pathogenic *V. cholerae*. Epidemic cholera, by contrast, is the result of the introduction of exogenous pathogenic *V. cholerae*, typically from an endemic location, into an immunologically-naive population resulting in a cholera disease outbreak. Following introduction into a population epidemic *V. cholerae* is capable of establishing in the new environment to become endemic (1). *V. cholerae* also causes pandemic cholera outbreaks. Seven cholera pandemics have been recorded, the first one began in 1817 and the most recent, ongoing, 7th pandemic began in 1961 (2). An estimated 1.3 billion people are at risk for cholera in endemic countries, and although cases are underreported, the burden of cholera in endemic countries is estimated at 2.86 million cases and 95,000 deaths annually. Today, the greatest burden of cholera cases and deaths occur in Africa (3).

1.2 *Vibrio cholerae*

V. cholerae is a Gram-negative, motile, facultative, comma shaped bacillus bacterium belonging to the Vibrionaceae family. *V. cholerae* was first identified by Filippo Pacini in 1854 from post mortem cholera patients, and was first isolated in pure culture from cholera patients in Egypt by Robert Koch in 1883 (4). *V. cholerae* is classified into serogroups based on the O antigen of the lipopolysaccharide (LPS) portion of its outer membrane. While there have been over 200 different serogroups of *V. cholerae* identified, only two have been associated with pandemic disease, serogroups O1 and O139, with serogroup O139 only recently emerging in 1992 (2, 5).

1.2.1 *V. cholerae* in the aquatic environment

Members of the Vibrionaceae family are aquatic organisms and natural inhabitants of marine ecosystems (6, 7). Although *Vibrio* species make up only a small fraction of the total bacterial species present in the marine environment at any time, usually under 0.1%, they are capable of swift blooms that rapidly increase their relative concentration to other marine organisms. Blooms can result in *V. cholerae* reaching over 1% of total marine microbe population. *V. cholerae* is an opportunistic heterotroph and takes advantage of shifts in environmental factors to facilitate this swift population expansion. Changes in conditions such as the introduction of excess iron, plankton blooms, decreased salinity, and increased temperature are all correlated with the increase in the environmental *V. cholerae* population (8-10).

Environmental *V. cholerae* is often found associated with the chitinous surface of planktonic zooplankton (11, 12). *V. cholerae* colonizes chitinous surfaces through the use of the attachment factors N-acetylglucosamine binding protein A (GbpA) and the mannose-sensitive

hemagglutinin pilus (MSHA) (13, 14). The chitin-associated *V. cholerae* cells then develop into surface-attached biofilms (15). During zooplankton colonization *V. cholerae* produces several chitinase enzymes that degrade chitin oligosaccharides in order to utilize them as a carbon and nitrogen source (11, 15, 16). Consistent with this, zooplankton blooms are often followed by *V. cholerae* blooms (17). Colonization of zooplankton is an environment that is extremely important to the persistence of *V. cholerae*, as it is a niche that not only supplies *V. cholerae* with a source of nutrients from chitin, in the generally nutrient-poor aquatic ecosystem, but chitin-associated *V. cholerae* biofilms are more resistant than planktonic cells to environmental assaults such as changes in temperature and pH, and predation by protists (18, 19).

Colonization of chitinous surfaces in the marine environment is also an important niche for the evolution of *V. cholerae*. Chitin colonization induces both type VI secretion and natural competence (20, 21). Together, these two phenotypes allow for robust horizontal gene transfer (HGT) by liberating genetic material from neighboring bacterial cells, and incorporation of exogenous DNA into the *V. cholerae* genome. Chitin-induced HGT facilitates the transfer of large portions of DNA, allowing *V. cholerae* to rapidly accumulate multiple adaptive traits, and can even enable serogroup conversion (22, 23). Collectively, the colonization of chitinous surfaces within the marine environment allows *V. cholerae* to persist in harsh nutrient-poor aquatic ecosystems, and facilitates the continued evolution of the bacterium.

1.2.2 *V. cholerae* infection and transmission

Pathogenic strains of *V. cholerae* evolve from nonpathogenic environmental strains through the acquisition of virulence genes via HGT (24, 25). Gene transfer events have marked the evolution of environmental strains into human pathogens, including the acquisition of two

pathogenicity islands referred to as *Vibrio* Seventh Pandemic island-1 (VSP-1) and *Vibrio* Seventh Pandemic island-2 (VSP-2) in the currently circulating El Tor pandemic strains (26). The *V. cholerae* pathogenicity islands encode virulence genes whose products are required for disease development, and regulatory proteins that control virulence gene expression *in vivo*. (27-30). Pathogenic *V. cholerae* strains present in the marine environment are transmitted to humans through the consumption of *V. cholerae* contaminated food or water. Biofilm and zooplankton-associated pathogenic *V. cholerae* cells are more infectious than planktonic cells (31-34). Following ingestion, *V. cholerae* cells survive transit through the acid barrier of the stomach through the induction of acid tolerance response (ATR) genes. These genes include proton antiporters such as *clcA* and *nhaA*, and decarboxylases such as *cadA* (35-38). Collectively ATR gene products work to decrease the intracellular proton concentration to prevent acidification of the cytosol during transit through the acidic environment of the stomach. Following passage through the stomach *V. cholerae* enters the more alkaline environment of the small intestine where it then represses ATR genes to maintain cytosolic pH homeostasis (35). The initial location of *V. cholerae* host colonization is the distal small intestine, where *V. cholerae* preferentially colonizes intestinal crypts, a process dependent on attachment factors GbpA and the toxin coregulated pilus (TCP) (13, 39). Following initial colonization *V. cholerae* replicates to form microcolonies on the epithelial surface. *In vivo* microcolonies are typically clonal, indicating that very few *V. cholerae* cells are involved in the initial colonization of the host epithelium (40, 41). *V. cholerae* is an exclusively extracellular pathogen, and remains in the lumen of the host small intestine, associated with intestinal enterocytes, for the duration of infection. It is in this environment that *V. cholerae* induces the expression of virulence factors, which ultimately lead to the development of the enterotoxin-mediated secretory diarrhea that is the hallmark of cholera disease.

Epidemic transmission of *V. cholerae* occurs through the fecal-oral route due to the contamination of food and drinking water with pathogenic *V. cholerae* cells that are shed in the diarrheal purge from cholera patients. In preparation for host exit, infecting *V. cholerae* cells repress virulence gene expression and induce the expression of genes important for transmission and survival in the aquatic environment. This includes the expression of genes that allow *V. cholerae* to detach from the host epithelium and be shed within the diarrheal purge (42-45). These genes also contribute to the development of a hyperinfectious phenotype in human-shed *V. cholerae*, which increases their transmission capabilities, and contributes to epidemic spread. The hyperinfectious phenotype correlates with biofilm production, which has been shown to contribute to increased transmission in animal infection models (32, 46). Collectively, these phenotypes drive the *V. cholerae* infectious cycle by facilitating both transmission and dissemination.

1.2.3 *V. cholerae* virulence factors

V. cholerae virulence factors are the gene products that collectively allow for *V. cholerae* to colonize and survive within the host. *V. cholerae* colonization of the human gastrointestinal tract provides access to a nutrient-rich environment that enables the robust and rapid expansion of *V. cholerae* cells before disseminating back into the marine environment. Therefore, the acquisition and *in vivo* induction of virulence genes is an important adaptation that facilitates the expansion and persistence of *V. cholerae* in the environment.

V. cholerae encodes numerous genes that have been implicated as virulence factors, including the RTX (repeat in toxin), Zot (zonula occludens toxin), and Ace (accessory cholera enterotoxin) toxins, a type VI secretion system, and the GbpA attachment protein (13, 47-50). The two most important *V. cholerae* virulence factors are cholera toxin (CT) and the toxin coregulated

pilus (TCP), as they are both required for the development of cholera. CT is encoded for in the *V. cholerae* chromosome by the *ctxAB* operon. These genes are acquired by *V. cholerae* through a HGT event via infection with a lysogenic phage, the CTX phage (29). Structurally CT is an AB₅ enterotoxin consisting of a catalytically active A subunit surrounded by a pentamer of 5 B subunits (52). During *V. cholerae* colonization of the host small intestine expression of *ctxAB* is induced, and CT is secreted from the bacterial cell into the external environment through the use of the type II secretion system (53). Once in the extracellular environment the B subunits of CT bind to GM₁ gangliosides on the surface of intestinal cells, this binding induces host cellular uptake of CT through caveolae-mediated endocytosis, and CT enters into the host enterocyte in a lipid raft (54-56), and translocates to the host cell endoplasmic reticulum (ER). Within the ER the CT A1 chain is unfolded and liberated from the rest of CT by cellular protein disulfide isomerase (PDI) (57). Unfolded A1 is retrotranslocated from the ER into the host cell cytoplasm, where it refolds to its native conformation and enzymatically induces the constitutive activation of adenylyl cyclase through the ADP-ribosylation of G_sα subunit protein (58). This leads to increased intracellular cAMP concentrations, which signals the release of chloride ions and water from the cell via the cystic fibrosis transmembrane regulator (CFTR) (59, 60). The mass movement of fluid from intestinal cells into the intestinal lumen results in a profuse secretory diarrhea, the hallmark symptom of the disease cholera (61).

The genes that encode for TCP are cotranscribed with *ctxAB*. TCP is a Type IV pilus that is required for *V. cholerae* colonization of the host (62). The genes encoding for TCP are also acquired through HGT, and exist in the TCP gene cluster within the *V. cholerae* pathogenicity island VSP-1. The major pilin subunit protein of TCP, TcpA, is encoded by the *tcpA* gene and is used as a marker for TCP production during *in vitro* studies (63-65). TCP pili between adjacent

cells facilitate cell aggregation, and allow for microcolony formation, an important stage in *V. cholerae* infection (39). Interestingly, TCP is the receptor for the CTX phage, and therefore plays an important role not only in the virulence of *V. cholerae*, but also in the evolution of pathogenic *V. cholerae* strains (29).

1.2.4 The ToxR regulon

The ToxR regulon is a hierarchical regulatory cascade that controls production of both CT and TCP in *V. cholerae*. The ToxR regulon is named after the ToxR transcriptional regulator that was first discovered for its role in control of CT production (66, 67). ToxR is a membrane-bound master transcriptional regulator that is a component of the ancestral *V. cholerae* genome; it is found in all *Vibrios* including both environmental and pathogenic strains of *V. cholerae* (68). Although ToxR plays a central role in the expression of horizontally acquired *V. cholerae* virulence genes, its regulon also includes other ancestral genes, including *ompU* and *ompT*, the two primary *V. cholerae* outer membrane porin genes (69, 70).

Following host entry, the induction of the ToxR regulon occurs in a hierarchical and temporal fashion beginning with the expression of two cytoplasmic regulators *aphA* and *aphB* (Fig. 1). AphA and AphB are transcription factors which together induce the expression of the membrane-bound regulator *tcpP* (71, 72). TcpP, along with ToxR bind to and induce the expression of the *toxT* promoter, ToxT is a cytoplasmic transcription factor, and the direct transcriptional activator of genes that encode for CT and TCP production (Fig. 1) (73). Induction of the ToxR regulon, and thus CT and TCP production, is controlled in response to a range of environmental cues that include components of bile, temperature, pH, oxygen, bicarbonate,

peptides, and small molecules (74-78). The cumulative effect of all of these signals modulates the production of *V. cholerae* virulence factors in response to changes in environmental conditions.

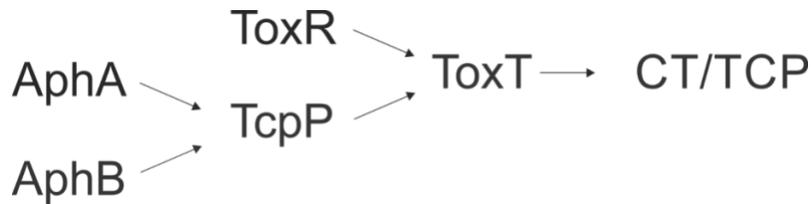


Figure 1 The ToxR regulon

Induction of the ToxR regulon takes place in a temporal and hierarchical manner and begins with cytoplasmic transcription factors AphA and AphB binding to and inducing the expression of the *tcpP* promoter. TcpP is a membrane bound transcriptional regulator that along with ToxR, another membrane bound regulator, induce the expression of *toxT*. ToxT is a cytoplasmic transcription factor that directly binds to, and induces the expression of the genes that encode for CT and TCP.

1.3 Bacterial adaptive responses

During their life-cycle, many pathogenic bacteria must rapidly transition between disparate environmental niches, each niche presenting with specific barriers to colonization. Colonization barriers include both physical and chemical stressors that threaten the viability of bacterial cells such as nutrient limitation, temperature, pH, osmolarity, antimicrobial compounds, reactive oxygen and nitrogen species, host immune effectors, and predation by other cells. To successfully colonize, survive, and replicate in spite of these barriers bacteria must be able to sense and swiftly adapt to the abrupt changes in environmental stressors. This is achieved through the induction of an array of adaptive responses. Bacterial adaptive responses are the collective inducible systems that alter the bacterial transcriptome and proteome to allow bacteria to resist physical and chemical stressors, repair cellular damage done from stressors, and acquire required nutrients in order to

successfully colonize and persist within an individual niche. Adaptive responses are broad and include antimicrobial resistance mechanisms, metabolic adaptations, stress responses, motility, nutrient acquisition pathways, biofilm production, virulence factor production, chemotaxis and others (79-81).

Adaptive responses are induced in response to activating signals which can be both exogenously and endogenously derived. Bacteria constantly survey their environment for the presence of adaptive response inducing cues. Due to the complex life cycle of pathogenic *V. cholerae* requiring successful transition between both the marine environment and host colonization, *V. cholerae* relies on adaptive responses for its pathogenic success. As *V. cholerae* passes through different regions of the host gastrointestinal tract it induces genes to adapt to changing environmental conditions. For example, upon entrance into the host gastrointestinal tract *V. cholerae* must pass through the acid barrier of the stomach. To withstand this harsh environment, the acid tolerance response genes are induced in response to the low pH in the stomach (36-38, 82). After leaving the stomach *V. cholerae* enters the host small intestine. In order to colonize and acquire nutrients within the small intestine niche-specific signals such as anaerobiosis, bile, and the presence of bicarbonate signal the induction of virulence factor production that facilitate colonization and disease development (75, 76, 83). The presence of antimicrobial bile salts within the small intestine also signals the induction of active efflux and a reduction of membrane permeability to aid in resistance to these toxic compounds (70, 84).

1.3.1 Two-component regulatory system structure and function

Extracellular signals that induce bacterial adaptive responses are transduced into the cell predominately by two-component regulatory systems (TCS). TCS are bacterial sensing systems

that monitor the extracellular environment for specific inducing signals, and in response regulate adaptive responses through transcriptional modulation. The prototypical TCS consists of a membrane-bound histidine kinase (HK) sensor protein coupled with a cognate cytoplasmic response regulator (RR). Signaling via TCS is achieved through a phosphorelay system. Specific environmental cues stimulate the activation of HKs through an extracytoplasmic sensing domain. Activation of the HK induces its autophosphorylation of a conserved histidine residue. The HK sensor then transfers that phosphate group to a conserved aspartate residue on its cognate RR. RRs are generally transcription factors and become active upon phosphorylation, but can also function by other mechanisms (85). Activated RRs control the differential expression of target genes to respond to the activating input signal. Collectively, activation of TCS via environmental cues results in changes in gene products that allow the bacteria to rapidly adapt to shifts in their environment (85, 86). Bacteria encode an array of TCS that sense and respond to a diverse range of both chemical and physical environmental cues. *V. cholerae* encodes for 43 HKs and 52 punitive RRs (87). Many of the adaptive responses induced by TCS aid pathogenic bacteria in disease development through the induction of a variety of antimicrobial resistant strategies including active efflux, and the induction of virulence factor production (88, 89).

1.3.1.1 The Cpx membrane stress response

The Cpx membrane stress response is a TCS that is conserved in Gram-negative bacteria. The Cpx system senses cell wall perturbations, and in response mediates an adaptive response that mitigates the damage. The system consists of the membrane bound sensor HK CpxA and cytoplasmic RR CpxR, along with an additional component, CpxP. CpxP is a periplasmic protein that regulates the activity of CpxA via modulation of the CpxA phosphorylation status (90). Activation of the Cpx system is induced through the CpxA-mediated sensing of membrane stress,

including misfolded extracytoplasmic proteins, periplasmic proteins with aberrant disulfide bonds, elevated pH, high salt concentrations, ROS, iron limitation, and others (91-95). Upon sensing membrane stress CpxA becomes activated and autophosphorylates, then transfers its phosphate group to CpxR. Phosphorylated CpxR functions as an active transcription factor. The CpxR regulon includes genes whose products collectively work to counteract extracytoplasmic stress, such as the induction of the extracytoplasmic protease DegP, and the Dsb system, which controls proper disulfide bond formation in extracytoplasmic proteins (92). Activated CpxR also functions as a repressor and controls the downregulation of nonessential envelope proteins (96). In *V. cholerae* the Cpx system has been linked to adaptive responses including iron acquisition, regulation of membrane permeability, drug efflux, and virulence factor production (94, 97-99).

1.3.1.2 The EnvZ/OmpR two-component system

The EnvZ/OmpR is a ubiquitous Gram-negative TCS that consists of the sensor HK EnvZ and RR OmpR. The EnvZ/OmpR system was first discovered in *E. coli* where it differentially regulates the expression of the major outer membrane porins *ompF* and *ompC* in response to environmental osmolarity (100-102). Additional work has shown that the EnvZ/OmpR system is also activated in a number of Gram-negative bacteria by low environmental pH (103, 104). EnvZ controls the phosphorylation status of OmpR through both kinase and phosphatase activity (105). Interestingly, OmpR has been found to function as an active transcription factor in both the phosphorylated and dephosphorylated states, and the relative concentration of phosphorylated and dephosphorylated OmpR within a cell determines the OmpR-mediated transcriptional outcome (106, 107). The EnvZ/OmpR TCS has been linked to regulation of bacterial adaptive responses including virulence, motility, and stress responses (103, 104, 108-110). The function of the EnvZ/OmpR system in *V. cholerae* remains poorly understood; however, recent work has shown

that *V. cholerae ompR* is induced by membrane intercalating agents, including bile components, and alkaline pH. This work indicated that OmpR also functions in defense against environmental alkaline pH and in regulation of virulence factor production through direct repression of *aphB* (108).

1.4 Mechanisms of antimicrobial resistance

A primary barrier to bacterial colonization of a niche is the presence of antimicrobial compounds. Antimicrobial resistance confers a selective advantage, as resistant bacterial populations outcompete their susceptible counterparts in the presence of antimicrobial compounds (111). Resistance mechanisms are considered either intrinsic or acquired traits. Intrinsic mechanisms are typically chromosomally encoded within the core genome of the bacteria, while acquired mechanisms are gathered by a bacterium through mutation or horizontal gene transfer (112). In the presence of antimicrobials, resistant bacterial strains have an advantage over their susceptible counterparts. Therefore, once resistant strains develop they rapidly become the dominate genotype (113). Mechanisms of antimicrobial resistance fall into three large categories: the reduction of the intracellular concentration of the antimicrobial compound, modification of the antimicrobial target, and inactivation of the antimicrobial compound (114).

The reduction of the intracellular concentration of an antimicrobial is achieved through a reduction in membrane permeability in order to reduce diffusion into the cell, and the induction of active efflux to export the compound from the cell. During host colonization *V. cholerae* resists the toxic effects of the antimicrobial components of bile through the mechanisms that reduce the intracellular bile concentration. In the presence of bile *V. cholerae* reduces outer membrane

permeability through ToxR-mediated altered expression of outer-membrane porins *ompU* and *ompT*. OmpT has a larger pore opening compared to OmpU, so repression of *ompT* and induction of *ompU* results in retarded diffusion of certain antimicrobials across the outer membrane (60). In response to bile *V. cholerae* also induces the expression of active efflux pumps to extrude bile from the cell (70, 84). These adaptations work synergistically to reduce the cellular uptake of bile and therefore confer bile resistance.

Antimicrobial compounds exert their toxic effects through interaction with components of bacterial cells. Therefore, resistance to antimicrobials can develop through modification of these bacterial cellular targets to reduce interaction with the antimicrobial. Such is the case for cationic antimicrobial peptide (CAMP) resistance in *V. cholerae*. During infection cells of the human innate immune response secrete CAMPs which bind to and disrupt the outer membrane of Gram-negative bacteria. In the presence the CAMPs *V. cholerae* modifies the lipid A portion of LPS with glycine and diglycine residues. This modification decreases the negative charge on LPS and reduces CAMP interactions with the bacterial outer membrane, and thus confers CAMP resistance (115).

Many antimicrobial compounds induce their bacterially toxic activity through a reactive moiety within their chemical structure, such as β -lactam antibiotics. The β -lactam ring is the reactive moiety found in all β -lactam antibiotics. β -lactamase enzymes that are secreted by bacterial cells hydrolyze this ring, and thus confer resistance to β -lactams. These enzymes are one of the most well studied antibiotic resistance mechanisms in bacteria. *V. cholerae*, like many Gram-negative bacteria, in response to β -lactam antibiotics produces and secretes β -lactamase enzymes in order to cleave the β -lactam rings, thereby conferring resistance (116).

1.4.1 Multi-drug efflux pumps

Multidrug efflux pumps are proteins imbedded into cell membranes that function in extrusion of toxic compounds from cells. They are found in diverse lifeforms, including humans, where they function in resistance to cancer drugs (117). In bacteria multidrug efflux pumps play a profound role in antimicrobial resistance. Many multi-drug efflux systems have the capability of effluxing a wide range of chemically disparate substrates originating both from the environment surrounding the bacteria and internally from products of bacterial metabolism. The efflux substrates of multidrug efflux pumps include not only antibiotics but also heavy metals, detergents, dyes, cell metabolic intermediates, and others (118-122). Efflux pumps are placed into one of 6 families; RND (resistance-nodulation-division), MFS (major facilitator superfamily), MATE (multidrug and toxic compound extrusion), SMR (small multidrug resistance), ABC (ATP-binding cassette), and the recently discovered PACE (proteobacterial antimicrobial compound efflux) (123-125). The action of multidrug pumps require the utilization of energy, as they are active transporters that shuttle substrates from the cell against the concentration gradient; some systems use the proton motive force for energy while others use chemical gradients or ATP (118).

Many efflux pumps are chromosomally encoded and are ancient genetic elements that predate the use of modern antibiotics to treat bacterial infections (126). These factors, coupled with the constitutive basal expression of efflux pumps makes them the primary contributor to bacterial intrinsic drug resistance (127). In the presence of substrates efflux pump expression is often induced in order to increase the removal of the substrate from the cell. Therefore, efflux pumps play a paramount role in both intrinsic and induced antimicrobial resistance (84, 118).

1.4.1.1 RND family efflux pumps and their role in antimicrobial resistance

RND family efflux pumps were first discovered in the early 1990's in *E. coli* and *Pseudomonas aeruginosa* due to their contribution to antibiotic resistance, and have since been recognized as being ubiquitous in Gram-negative bacteria (128, 129). The genes for RND efflux pumps are generally chromosomally encoded, however, recent reports have indicated that they can be plasmid-borne, and transferred via HGT (130-133). RND pumps are tripartite protein complexes consisting of an inner membrane bound transport protein, a periplasmic adapter protein, and a conserved outer membrane pore protein, which is typically homologous to the *E. coli* TolC outer membrane pore (134). Collectively the pump complex spans both membranes of the Gram-negative cell wall, as the periplasmic adapter protein links the inner membrane pump and outer membrane pore protein with no physical interaction between the two membrane proteins (135). Efflux is achieved following substrate capture from either the periplasmic or cytoplasmic compartment. The inner membrane pump protein utilizes energy supplied by the proton motive force to shuttle substrates through the outer membrane pore protein, releasing the substrate to the external environment (136, 137). Efflux pump substrate specificity is determined in part by residues in the periplasmic portal of the RND pump protein that exist at the interface with the cytoplasmic membrane (138, 139). Individual Gram-negative species typically encode multiple RND pumps. RND efflux has a profound effect on bacterial antimicrobial resistance as RND pumps are capable of effluxing a wide range of structurally and chemically diverse antimicrobial compounds including antibiotics, detergents, antimicrobial peptides, and dyes (140, 141). Reports have indicated that there is redundant substrate specificity for antibiotics between specific RND pumps in an individual species, which suggests functions outside of antibiotic resistance have

selected for their maintenance (84, 142, 143). The selective pressures that maintain redundant efflux mechanisms within bacterial genomes remains poorly understood.

1.4.1.2 The function of RND efflux pumps in adaptive responses

There is a growing body of literature that indicates that RND efflux pumps play a role in bacterial adaptive responses outside of their function in antimicrobial resistance, however how RND efflux pumps contribute to many of these responses is poorly understood. RND efflux has been linked to iron acquisition (94, 144), biofilm production, (145-147) membrane homeostasis, (94, 97) virulence, (148-150) and other responses. As such, RND efflux substrates include endogenously-produced compounds, such as siderophores, small molecules, and products of bacterial metabolism (94, 121, 144, 151). Many RND efflux substrates are effector molecules whose intracellular presence is sensed by the bacteria to regulate adaptive responses. Therefore, by modulating the intracellular concentrations of signaling molecules, RND efflux pumps play a central role in the expression of adaptive responses. We hypothesize that a native physiological function of RND efflux pumps is to modulate the intracellular concentration of metabolic intermediates and by products, and that impaired efflux results in the activation of adaptive responses due to the intracellular accumulation of these metabolic products. Exploration of the mechanistic links between RND efflux and bacterial adaptive responses provides an opportunity to explore the native functions and the evolutionary pressures that have selected for the development and maintenance of multiple redundant RND systems in individual bacterial genomes.

1.4.1.3 RND efflux pumps in *V. cholerae*

V. cholerae encodes six RND family multidrug efflux pumps: VexAB, VexCD, VexEF, VexGH, VexIJK, and VexLM (42, 152). *V. cholerae* RND efflux has been linked to resistance to a range of antimicrobial compounds including antibiotics, bile, antimicrobial peptides, and detergents. Like in many bacteria, the *V. cholerae* RND pumps exhibit redundant antimicrobial substrate specificity but VexAB plays the most prominent role in antimicrobial resistance. (84, 143, 149, 150). VexAB is induced during both human and animal model infections, indicating that RND efflux is likely important in the host during *V. cholerae* infection (42, 46, 153). Like in many Gram-negative bacteria, the *V. cholerae* RND efflux pumps are linked to bacterial adaptive responses in addition to their role in antimicrobial resistance, including virulence factor production, metabolism, iron acquisition, and stress responses. The loss of *V. cholerae* RND efflux results in reduced virulence factor production and an inability to colonize the infant mouse small intestine, along with induction of active efflux, activation of stress responses including both the Cpx and EnvZ/OmpR systems, as well as robust metabolic transcriptome changes (84, 94, 97, 108, 121, 150). These findings, coupled with the fact that the *V. cholerae* RND efflux pumps are capable of effluxing bacterial-derived signaling compounds, collectively indicate that the *V. cholerae* RND efflux effects adaptive responses through modulation of the intracellular concentration of signaling molecules (78, 84, 94, 121).

1.5 Goals of Dissertation

RND multidrug efflux pumps have been linked to a wide range of adaptive responses in Gram-negative bacteria including virulence, stress responses, iron acquisition, and biofilm

production (154). How RND-mediated efflux functions in these diverse phenotypes remains largely unknown. Work in our laboratory has linked RND-mediated efflux to virulence factor production and stress responses in the Gram-negative human pathogen *Vibrio cholerae* (84, 150). These observations lead to the hypothesis that RND efflux pumps participate in the regulation of *V. cholerae* adaptive responses by modulation of the intracellular concentration of specific signaling molecules. The work presented in the following chapters of this dissertation sought to identify the mechanistic links between *V. cholerae* RND efflux and bacterial adaptive responses.

The goal of the work in chapter 2 aimed to elucidate the mechanistic link between *V. cholerae* RND efflux and activation of the Cpx membrane stress response. Previous work indicated that the loss of RND efflux results in the constitutive induction of the Cpx system in *V. cholerae* (84, 99). This observation indicated that the *V. cholerae* RND efflux pumps functioned in maintaining the Cpx system in an inactive state, but the mechanistic link between RND efflux and the Cpx system remained unknown. A transposon screen for Cpx-suppressor mutants in a Δ RND strain discovered that Δ RND strains with mutations in genes within the biosynthetic pathway for the production of the siderophore vibriobactin suppressed the Cpx system. Based on this, we hypothesized that the RND pumps efflux vibriobactin. Exploration of this hypothesis led to the finding that the VexGH RND pump is primarily responsible for the export of vibriobactin, and a loss of this function results in the induction of the Cpx system through the intracellular accumulation of vibriobactin. Accumulation of vibriobactin induces the Cpx system via chelation of iron from iron-binding membrane proteins such as succinate dehydrogenase of the electron transport chain. In sum, this work illustrates one of the native functions for the *V. cholerae* RND efflux pump VexGH, and linked RND efflux to iron acquisition, maintenance of membrane homeostasis, and central metabolism. These findings shed light on some of the evolutionary

selective pressures that have maintained previously thought redundant efflux pumps in the *V. cholerae* genome.

The goal of the work presented in chapter 3 was to characterize the relationship between *V. cholerae* RND efflux and activation of the EnvZ/OmpR two-component system. Recent work in our laboratory found that loss of RND-mediated efflux resulted in broad changes in the *V. cholerae* transcriptome. Differentially expressed genes in a Δ RND strain cultured in virulence factor inducing conditions had an enrichment for response regulators from two-component systems, including an induction of *ompR*, compared to WT (121). This finding, coupled with previous work from our laboratory indicating that a Δ RND *V. cholerae* strain had attenuated virulence factor production (150) lead us to explore if *ompR* overexpression in a Δ RND strain contributed to the repression of virulence in that strain. This resulted in the finding that OmpR functioned as a repressor of *V. cholerae* virulence factor production via direct repression of *aphB*, and the overexpression of *ompR* in a Δ RND strain cultured in AKI medium contributed to virulence repression. Additional work discovered that the RND-dependent *ompR* inducing signal appeared to be membrane intercalating components of bile within AKI medium, and removal of these compounds through depletion of AKI medium with a C18 column repressed *ompR* expression, and restored virulence factor production in the Δ RND strain to WT levels. Collectively, this work uncovered a regulatory link between *V. cholerae* RND efflux and stress response activation, as well as virulence factor production. Further, it identified that OmpR's functions and inducing cues in *V. cholerae* are divergent from those in other Gram-negative organisms.

The goal of the work in chapter 4 focused on further defining the function of OmpR in *V. cholerae* biology beyond modulation of virulence. The work presented in chapter 3 found that the

function of OmpR in *V. cholerae* was divergent from other Gram-negative bacteria, as *V. cholerae* *ompR* did not respond to changes in media osmolarity, and did not control production of the *V. cholerae* major outer membrane porins OmpU and OmpT. Instead, *V. cholerae* OmpR functioned as a virulence repressor and was induced by membrane intercalating compounds (108). These observations indicated that the *V. cholerae* OmpR homologue was the subject of divergent evolution, and regulated novel genes, and was controlled by novel inducing cues. To investigate the undetermined function of OmpR in *V. cholerae* we defined the *V. cholerae* OmpR regulon. To do this we determined the transcriptome of $\Delta ompR$ cells overexpressing *ompR* through the use of RNA-sequencing (RNA-seq). This resulted in the finding that OmpR represses both components of the ToxR regulon and genes involved in the *V. cholerae* acid tolerance response (ATR) including *clcA*, *cadC*, and *cadBA*. This finding, coupled with previous reports that OmpR responds to changes in environmental pH in other Gram-negative species lead to the hypothesis that the *V. cholerae* OmpR homologue functioned in response to changes in media pH. Exploration of this hypothesis lead to the finding that *ompR* is induced by alkaline pH, and in response represses both virulence and *cadBA* expression through repression of *aphB*. Further analysis found that OmpR was required for fitness in alkaline conditions, as an $\Delta ompR$ mutant strain had reduced growth at high pH, compared to WT. Together, these findings indicate that the *V. cholerae* OmpR homologue has evolved to serve novel functions in virulence factor repression and alkaline pH response. These functions are specifically suited to the lifestyle of *V. cholerae*, which meets alkaline pH both in the rice-water stool of cholera patients, and in the marine environment.

2.0 The *Vibrio cholerae* VexGH RND Efflux System Maintains Cellular Homeostasis by Effluxing Vibriobactin

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2.1 Project summary

Resistance-nodulation-division (RND) superfamily efflux systems have been widely studied for their role in antibiotic resistance, but their native biological functions remain poorly understood. We previously showed that loss of RND-mediated efflux in *Vibrio cholerae* resulted in activation of the Cpx two-component regulatory system, which mediates adaptation to stress resulting from misfolded membrane proteins. Here, we investigated the mechanism linking RND-mediated efflux to the Cpx response. We performed transposon mutagenesis screening of RND-deficient *V. cholerae* to identify Cpx suppressors. Suppressor mutations mapped to genes involved in the biosynthesis of the catechol siderophore vibriobactin. We subsequently demonstrated that vibriobactin secretion is impaired in mutants lacking the VexGH RND efflux system and that

impaired vibriobactin secretion is responsible for Cpx system activation, suggesting that VexGH secretes vibriobactin. This conclusion was bolstered by results showing that *vexGH* expression is induced by iron limitation and that *vexH*-deficient cells exhibit reduced fitness during growth under iron-limiting conditions. Our results support a model where VexGH contributes to cellular homeostasis by effluxing vibriobactin. In the absence of *vexGH*, retained vibriobactin appears to chelate iron from iron-rich components of the respiratory chain, with the deferrated proteins functioning to activate the Cpx response. Our collective results demonstrate that a native function of the *V. cholerae* VexGH RND efflux system is in vibriobactin secretion and that vibriobactin efflux is critical for maintenance of cellular homeostasis.

2.2 Introduction

Vibrio cholerae is a Gram-negative bacterium and the causative agent of the life-threatening diarrheal disease cholera. *V. cholerae* is a native inhabitant of aquatic environments from which humans acquire cholera through the ingestion of *V. cholerae*-contaminated food or water (155, 156). Following ingestion, *V. cholerae* colonizes the small intestine, where it produces a variety of virulence factors that result in the production of a severe secretory diarrhea that is the hallmark of the disease cholera (156)

The ability of *V. cholerae* to colonize and replicate in the human gastrointestinal (GI) tract is dependent upon its ability to adapt to its environment. This includes overcoming colonization barriers provided by the presence of toxic antimicrobial compounds such as bile salts, fatty acids, and products of the innate immune system. Many of these compounds also serve as environmental cues that activate the expression of adaptive responses in *V. cholerae* that facilitate survival and

replication in the GI tract. One component of these adaptive responses is enhancement of antimicrobial resistance. This is accomplished by a multifactorial response that includes the expression of active efflux systems, reduced outer membrane permeability, and the expression of stress response systems that mitigate cellular damage resulting from exposure to toxic molecules (123).

Active efflux systems belonging to the resistance-nodulation-division (RND) superfamily are critical for intrinsic and induced antimicrobial resistance in Gram-negative bacteria, including *V. cholerae* (123). The RND efflux systems are ubiquitous tripartite transporters that exhibit a broad substrate specificity that includes antibiotics, detergents, antimicrobial peptides, and dyes (140). For this reason, RND transporters play a critical role in the evolution of multiple-antibiotic-resistant bacteria. However, the native function of the RND efflux systems in most bacteria is poorly understood. Numerous studies across multiple genera have linked RND transporters to the expression of diverse phenotypes, suggesting that their function in bacterial biology extends beyond their well-established role in antimicrobial resistance (154). However, the mechanisms by which RND transporters contribute to most of these phenotypes are not known.

The *V. cholerae* genome encodes six RND efflux systems. In addition to mediating resistance to antimicrobial compounds, the *V. cholerae* RND efflux systems are also required for virulence gene expression and colonization of the infant mouse (143, 149, 150). We recently showed that two *V. cholerae* RND multidrug efflux systems (i.e., *vexAB* and *vexGH*) and the Cpx envelope stress response were reciprocally regulated (97). The Cpx system is a two-component system that regulates adaptive responses to perturbations that generate misfolded envelope proteins (99). Environmental stimuli that activate the *V. cholerae* Cpx response include high salinity, iron stress, proteins containing aberrant disulfide bonds, and loss of RND-mediated efflux (95, 97, 99).

We found that mutation of *vexAB* and *vexGH* resulted in constitutive activation of the Cpx system and that activation of the Cpx system resulted in the upregulation of *vexAB* and *vexGH*. While the molecular mechanism involved in the reciprocal regulation of the RND transporters and the Cpx system is unknown, the genetic linkage between the Cpx response and the expression of these two broad-spectrum RND transporters indicates that *V. cholerae* employs a multifaceted strategy to alleviate extracytoplasmic stress by activating efflux to remove deleterious molecules from the cell while mitigating cellular damage via the Cpx response.

In this study, we sought to define the molecular mechanisms linking RND-mediated efflux to the activation of the *V. cholerae* Cpx system. To identify genes involved in this process, we performed a transposon mutagenesis screen to identify suppressors of the Cpx system in a RND-deficient *V. cholerae* mutant. The results of this screen identified several suppressors that mapped to genes involved in biosynthesis of the catechol siderophore vibriobactin. Subsequent analyses showed that vibriobactin secretion was impaired in RND-deficient *V. cholerae* and that the inability of the RND mutants to secrete vibriobactin resulted in activation of the Cpx system. These findings supported the novel conclusion that the RND efflux systems function in vibriobactin secretion. We further found that *vexGH* expression was regulated by iron and that VexGH directly contributed to vibriobactin secretion, suggesting that a native function of VexGH is to efflux vibriobactin. The intracellular accumulation of vibriobactin in mutants lacking *vexGH* appeared to be directly responsible for the activation of the Cpx system. Further, this vibriobactin-dependent activation of the Cpx system was dependent on aerobic respiration and succinate dehydrogenase; suggesting that retained vibriobactin directly impairs the function of iron-rich components of the respiratory chain. The inability to efficiently secrete vibriobactin in RND-deficient cells led to attenuated growth under iron-limiting conditions. Our collective studies demonstrate a

physiological function of a *V. cholerae* RND efflux transporter in iron acquisition and the maintenance of cellular homeostasis.

Table 1 Strains, plasmids, and oligonucleotides used in chapter 2

Strain:	Genotype:	Source:
<i>E. coli</i>		
EC100Dpir+	<i>F. mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) Φ 80 <i>dlacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1 endA1 araD139</i> Δ (<i>ara, leu</i>)7697 <i>galU galK</i> λ - <i>rpsL</i> (<i>Strr</i>) <i>nupG pir-116</i>	Epicentre
SM10 λ pir	<i>thi-1 thr leu tonA lacY supE recA::RP4-2-4-Tc::Mu Km_r</i> (λ . <i>pirR6K</i>)	(157)
<i>V. cholerae</i>		
JB58	01 El Tor strain N16961 Δ <i>lacZ</i> Sm _r	Lab collection (150)
JB485	JB58 Δ <i>vexB</i> Δ <i>vexD</i> Δ <i>vexF</i> Δ <i>vexH</i> Δ <i>vexK</i> Δ <i>vexM</i>	This work
XBV247	JB58 Δ <i>vibF</i>	This work
XBV251	JB485 Δ <i>vibF</i>	This work
XBV310	JB58 Δ <i>vibC</i>	This work
XBV307	JB485 Δ <i>vibC</i>	(158)
JB150	JB58 Δ <i>tolC</i>	(150)
JB116	JB58 Δ <i>vexH</i>	(99)
LS Δ R	JB58 Δ <i>cpxR</i>	This work
DK56	JB58 Δ <i>vibF</i> <i>cpxP-lacZ</i>	This work
DK53	JB485 Δ <i>vibF</i> <i>cpxP-lacZ</i>	(97)
DT1460	JB485 <i>cpxP-lacZ</i>	(97)
DT1458	JB58 <i>cpxP-lacZ</i>	(97)
Plasmids:	Description:	
pTL61T	<i>lacZ</i> transcriptional reporter, Amp _r	(159)
pXB229	pTL61T containing the <i>vexGH</i> promoter	(97)
pDK4	pTL61T containing the <i>irgA</i> promoter	This work
pDK7	pTL61T containing the <i>hutA</i> promoter	This work
pWM91	Allelic exchange vector, Amp _r	(43)
pWM91- Δ <i>vibF</i>	Used deletion of <i>vibF</i>	This work
pWM91- <i>vibC</i>	Used for deletion of <i>vibC</i>	This work
pBAD18	Arabinose inducible expression vector, Amp _r	(160)
pBAD18- <i>vibF</i>	<i>vibF</i> expression vector	This work
pNJ17	TnAraOut mariner transposon mutagenesis plasmid	(161)
Oligonucleotides:	Sequence (5'-3'):	
PVC0475-F-XhoI	AACTCGAGAGACGTTCCATATTTGGACCG	
P-VC0475-R-BamHI	GGGGATCCAAACATTAAGCCTAGTGTCACAG	
P-VCA0576-R-BamHI	TTGGATCCGATCGCGCTAGAGAGCAGAG	
P-VCA0576-F-XhoI	AACTCGAGCCGCCAACTGTGATGAGAGTC	
<i>vibF</i> -F1-SmaI	TACCCGGGACCAACTTAAACAGCAGCCTCGC	
<i>vibF</i> -F2	GCGGCTTATTGGGAAGATGAAGCTTGTCGCTCTCCGTCCATG	
<i>vibF</i> -R1-SpeI	CCACTAGTCATTCTTTCATCTCCAGATATAC	
<i>vibF</i> -R2	AGCTTCATCTTCCCAATAAGCCGCTTGCATTGCTGTCATTTTC	
VibC-F1-BamHI	GCGGGATCCCCAACCGAAATTGCGCAGC	
VibC-F2	GGTCGGATACACCTCAACGATGTGCTCACCGCTTAAGGAG	
VibC-R-SacI	GCGAGCTCCAGAGTAGTGCTAAAGGCGGCACCAAC	
VibC-R2	GCACATCGTTGAGGTGTATCCGACCACTTCACGCTTCATGACC	
VC2209-pBAD-F	ATGAATTCTCTTTAAAGATGAAAGAAA	
VC2209-pBAD-R	ATGTGACCTACCCCATGGACGGAGA	
VC2209-pBAD-F2	GTTTGGCTTCTGCACTTTTCGGGG	
VC2209-pBAD-R2	TGGGAGACGCAGCATCAATAGG	

2.3 Materials and methods used in chapter 2

2.3.1 Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 1. *E. coli* strain EC100D λ pir⁺ was used for cloning, and *E. coli* strain SM10 λ pir was used to conjugate plasmids into *V. cholerae*. *V. cholerae* O1 El Tor strain N16961 Δ lacZ was used as the WT control in all experiments. Bacteria were routinely grown in LB broth or on LB agar at 37°C. Modified T-medium was prepared as previously described (162). Anaerobic conditions were achieved with the BD GasPak EZ Pouch System. Antibiotics were used at the following concentrations: streptomycin, 100 μ g/ml; carbenicillin, 100 μ g/ml; kanamycin, 50 μ g/ml.

2.3.2 Plasmid and mutant construction

The plasmids and oligonucleotides used in this chapter are listed in Table 1. Transcriptional reporters for *irgA* (VC0475), *vibF* (VC2209) and *hutA* (VC0576) were constructed by cloning the promoter region of each respective gene in front of the *lacZ* gene in pTL61T. Briefly, N16961 genomic DNA was used as a template for PCR with primers P-VC0475 and F-XhoI/P-VC0475-R-BamHI. The resulting amplicon was digested with the XhoI and BamHI restriction endonucleases before being ligated into similarly digested pTL61T to generate pDK4. pDK6 (*vibF-lacZ*) and pDK7 (*hutA-lacZ*) were constructed similarly using the promoter specific PCR primers listed in Table 1. The *vibF* (VC2209) deletion construct was constructed as follows. Primers *vibF*-F1 and *vibF*-R2 and primers *vibF*-F2 and *vibF*-R1 were used in separate PCRs with N16961 genomic DNA. The resulting ~1.5-kb amplicons were collected and used as the template

for second-round PCR amplification with the flanking *vibF*-F1 and *vibF*-R1 PCR primers. The resulting ~3-kb amplicon was then digested with the SpeI and SmaI restriction endonucleases before being ligated into similarly digested pWM91 to generate pWM91- Δ *vibF*. The *vibC* (VC0773) deletion construct was constructed in a similar manner with primers *vibC*-F1 and *vibC*-R2 and primers *vibC*-F2 and *vibC*-R1. Unmarked in-frame deletion of *vibC* and *vibF* in each respective strain was constructed by allelic exchange with pWM91- Δ *vibC* and pWM91- Δ *vibF* as previously described (150). The *vibF* gene was cloned into pBAD18 in a two-step cloning procedure. N16961 genomic DNA was used as a PCR template for two concurrent PCRs with primers VC2209-pBAD-F1 and VC2209-pBAD-R2 and primers VC2209-pBAD-F2 and VC2209-pBAD-R1 to produce a 2,957-bp fragment and a 4,366-bp fragment, respectively. The 2,957-bp amplicon was digested with the EcoRI and KpnI restriction endonucleases before being ligated into the similarly digested pBAD18 vector. The resulting plasmid was then digested with the KpnI and Sall restriction endonucleases and ligated with the similarly digested 4,366-bp replicon to produce pBAD18-*vibF*.

2.3.3 Identification of Cpx suppressors

A mariner transposon library was generated in JB485::*cpxP-lacZ* with pNJ17 as previously described (161). The transposon library was then plated onto LB-X-Gal plates and incubated at 37°C. The following day, white colonies were selected and the transposon insertion sites in the white colonies were identified by DNA sequencing of arbitrary PCR products as previously described (161).

2.3.4 Growth analysis

Growth curves were generated in microtiter plates as follows. The strains indicated were grown overnight in T-medium with FeSO₄ supplementation. The overnight cultures were then washed once in 1 volume of phosphate-buffered saline (PBS) before being diluted 1:100 in fresh T-medium plus or minus FeSO₄. Two-hundred-microliter volumes of the diluted cultures were then placed in triplicate wells of a 96-well microtiter plate. The microtiter plates were then incubated at 37°C with constant shaking, and the OD at 600 nm (OD₆₀₀) was measured every 30 min. The OD at each time point was averaged and plotted against time to generate the growth curves reported.

2.3.5 Analysis of Cpx expression on agar plates

A chromosomal *cpxP-lacZ* reporter was used to assess the activation state of the Cpx system as previously described (97). Briefly, overnight LB broth cultures of the test strains were inoculated into fresh LB broth and incubated with shaking for 1 h, and then the cultures were normalized to an OD₆₀₀ of 0.1. The cells were then collected by centrifugation and resuspended in 1 volume of PBS. The cultures were then diluted 1:1,000 in PBS, and 2 µl of the diluted culture was spotted onto the surfaces of LB agar plates containing 160 µg/ml X-Gal and other additives as indicated. The inoculated plates were incubated overnight at 37°C before being photographed.

2.3.6 Transcriptional reporter assays

V. cholerae strains containing the reporter plasmids indicated were collected from the surfaces of LB agar plates and resuspended in LB broth to an OD₆₀₀ of 0.6. The cultures were used to inoculate 5 ml of fresh LB broth (1:100), which was incubated at 37°C with shaking for 3 h, and then culture aliquots were collected in triplicate and the β-galactosidase activity was quantified (time zero). The remaining cultures were then treated by addition of the carrier and dipyriddy (130 μM), FeSO₄ (130 μM), or both before being incubated at 37°C with shaking for an additional hour, when aliquots were taken in triplicate for the β-galactosidase assay. The experiments were performed independently at least three times, and β-galactosidase production was calculated and displayed in Miller units.

2.3.7 CAS assays

CAS assays were performed as previously described (163). Briefly, the test strains were cultured in T-medium without FeSO₄ supplementation at 37°C with shaking for 18 h. Cleared culture supernatants were then generated by centrifugation before triplicate 100-μl aliquots of the supernatant were collected from each strain. The amount of siderophore in the supernatants was then assessed by mixing 100 μl of modified CAS assay solution with 100 μl of culture supernatant in a 96-well microtiter plate. The solutions were then allowed to equilibrate for 3 h, and then the absorbance at 630 nm was measured with a Biotek Synergy 4 microplate reader. Total siderophore production was assessed by using 18 h cultures of the strains indicated that had been subjected to five freeze-thaw cycles. The resulting cell lysate were then used in the CAS assay as described above.

2.3.8 Cross-feeding growth stimulation assays

Cross-feeding growth stimulation bioassays were performed as previously described (164). Briefly, the growth indicator plates were prepared by inoculating 10 μ l of an overnight saturated culture of the *V. cholerae* $\Delta vibC$ mutant indicator strain into 20 ml of $\sim 50^{\circ}\text{C}$ LB agar containing 270 μM dipyriddy. The inoculated agar was then poured into 100-mm petri plates and allowed to solidify. The use of 270 μM dipyriddy in the LB agar was empirically determined to inhibit the growth of the $\Delta vibC$ mutant indicator strain. The solidified agar was then inoculated with the test strains by spotting 5- μ l aliquots of fresh overnight broth cultures in triplicate onto the surfaces of the indicator plates. After drying, the plates were incubated at 37°C for 24 h, and then the diameter of the zone of growth of the $\Delta vibC$ mutant indicator strain was measured.

2.4 Results

2.4.1 Identification of Cpx suppressors in RND-negative *V. cholerae*

We recently found that mutation of the *V. cholerae* RND family transporters resulted in constitutive expression of the Cpx system (97, 99). To elucidate the mechanism behind this phenotype, we performed a transposon mutagenesis screen to identify suppressors of the Cpx system in RND-negative strain JB485. We generated a transposon library in JB485 bearing a chromosomal *cpxP-lacZ* reporter and screened $\sim 10,000$ mutants on lysogeny broth (LB) 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) agar plates for white colonies; *cpxP* is positively regulated by CpxR and serves as a reporter of the activation state of the Cpx system (95,

97, 99). The transposon screen resulted in the identification of six transposon mutants that contained insertions in four independent genes.

Three of the six transposon insertions mapped to vibriobactin biosynthesis genes *vibF* (two hits) and *vibD* (1 hit). Vibriobactin is a catechol siderophore utilized for iron acquisition and is the only known siderophore produced by *V. cholerae* (165). Vibriobactin is produced by a sequential biosynthetic pathway that consists of the products of the *vibABCDEFGH* genes (Fig. 2). Strains containing mutations in any of the *vib* genes do not produce vibriobactin (166). Thus, the finding that *vibD* and *vibF* insertions suppressed the Cpx system suggested that vibriobactin contributed to induction of the Cpx system in JB485. Two hits mapped to *sdhA*, which encodes a subunit of succinate dehydrogenase (i.e., complex II). Succinate dehydrogenase is an iron-sulfur protein that catalyzes the oxidation of succinate to fumarate in the electron transport chain (ETC) and the Krebs cycle (167). One insertion mapped to *epsK*, which encodes a component of the type II secretion system. Interestingly, all four of the suppressor genes are regulated in response to iron (168, 169), suggesting that RND efflux-dependent activation of the Cpx system might be linked to vibriobactin production and/or iron homeostasis.

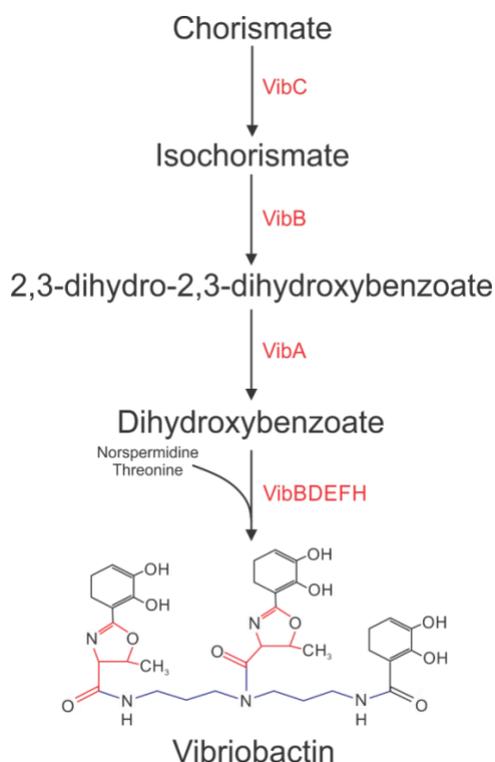


Figure 2 The vibriobactin biosynthesis pathway

The vibriobactin biosynthesis pathway. Vibriobactin is composed of three molecules of dihydroxybenzoate (shown in black) attached to a norsperimidine backbone (blue). One dihydroxybenzoate molecule is attached directly to the norsperimidine backbone, the other two DHB molecules are attached through cyclized threonine linkers (red). This figure was adapted from: Payne SM, Mey AR, Wyckoff EE. 2016. *Vibrio* iron transport: evolutionary adaptation to life in multiple environments. *Microbiol Mol Biol Rev* 80:69–90. doi:10.1128/MMBR.00046-15.

2.4.2 Vibriobactin is responsible for Cpx activation in strains lacking RND-mediated efflux

The above described screening implicated vibriobactin production in the activation of the Cpx system in JB485. To confirm this, we constructed an in-frame *vibF* deletions in wild-type (WT) strain JB58 and mutant strain JB485. We then introduced the chromosomal *cpxP-lacZ* reporter into each of the respective strains before assessing the activation status of the Cpx system on LB X-Gal agar plates. The results showed that JB58 and its isogenic Δ *vibF* mutant produced

white colonies on LB X-Gal agar (Fig. 3A), indicating that the Cpx system was inactive. RND-negative strain JB485 produced blue colonies on LB X-Gal agar, confirming constitutive Cpx activation as previously reported (97). In contrast, the JB485 Δ *vibF* mutant produced white colonies on the same agar, confirming the results of our transposon screening. As a control, we examined the test strains on LB X-Gal agar containing CuCl₂, a documented inducer of the Cpx system (99). All of the test strains produced blue colonies in the presence of CuCl₂, confirming that the Cpx system was functional in each strain.

We further verified that *vibF* was responsible for Cpx induction in JB485 by performing *vibF* complementation studies. We cloned *vibF* under control of the arabinose-regulated promoter in pBAD18 and transformed the resulting plasmid (i.e., pBAD18-*vibF*) and the empty-vector control into the WT and JB485 Δ *vibF* strains bearing a chromosomal *cpxP-lacZ* reporter. We then examined the activation state of the Cpx system in the presence or absence of arabinose on LB X-Gal plates. The results revealed that all of the strains produced white colonies in the absence of arabinose (Fig. 3B). Growth of the strains in the presence of arabinose demonstrated that ectopic *vibF* expression in JB485 Δ *vibF* activated *cpxP-lacZ* expression, as indicated by the production of blue colonies. This confirmed that *vibF* is required for Cpx activation in the absence of RND-mediated efflux. These results supported the conclusion that induction of the Cpx system in cells lacking RND-mediated efflux is dependent on vibriobactin production.

Vibriobactin biosynthesis is negatively regulated by the master iron regulator Fur. On the basis of this fact, we hypothesized that if induction of the Cpx system in JB485 is a result of vibriobactin production, then addition of iron to the growth medium would repress vibriobactin production and suppress the Cpx system. To test this, we cultured the WT, JB485, and isogenic Δ *vibF* mutant strains bearing the *cpxP-lacZ* reporter on LB X-Gal plates containing 500 μ M

FeSO₄. The results revealed that FeSO₄ suppressed *cpxP-lacZ* expression in JB485, confirming our hypothesis (Fig. 3A). Manganese can also complex with Fur to repress Fur-regulated genes (170, 171). We therefore repeated these experiments with LB X-Gal plates containing 500 μM MnCl₂. The results revealed that manganese also suppressed the Cpx system in JB485 (Fig. 3A). Together, these results were consistent with the notion that vibriobactin production was responsible for activation of the Cpx system in *V. cholerae* cells that lacked RND-mediated efflux.

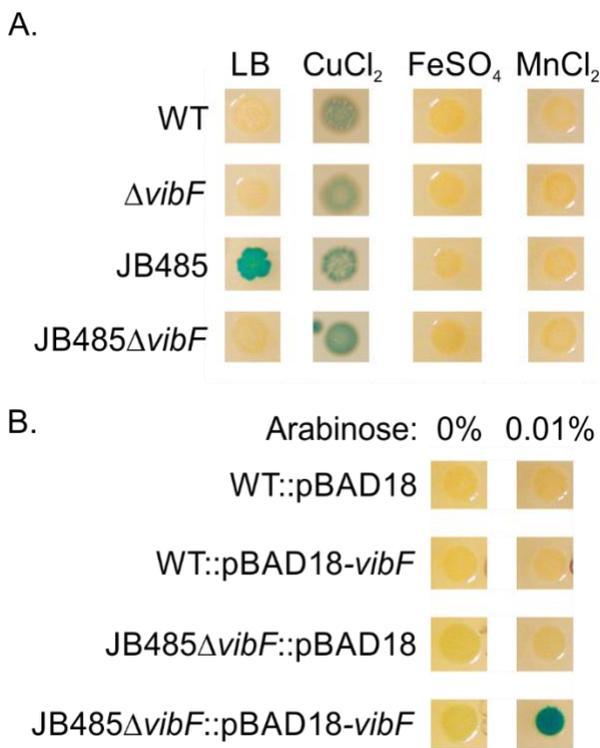


Figure 3 Expression of the Cpx system in RND efflux deficient *V. cholerae* is dependent upon vibriobactin production.

Strains harboring a chromosomal *cpxP-lacZ* reporter were inoculated onto the surface of LB X-gal agar plates and incubated overnight at 37°C before being photographed. (A) Effect of FeSO₄, CuCl₂, and MnCl₂ on the *cpxP-lacZ* expression in the indicated *V. cholerae* strains. All three compounds were added to the LB agar at a final concentration of 500 μM. (B) Effect of ectopic *vibF* expression on *cpxP-lacZ* expression in WT and JB485Δ*vibF*. WT and JB485Δ*vibF* harboring the indicated expression plasmids following growth on LB X-agar plates containing 0% or 0.01% arabinose.

2.4.3 JB485 culture supernatants contain reduced amounts of siderophore

The above-described results indicated that vibriobactin production is required for activation of the Cpx system in RND efflux-deficient *V. cholerae*. Vibriobactin is produced in the cytoplasm before being secreted by an unknown mechanism. On the basis of this, we hypothesized that the *V. cholerae* RND efflux systems functioned in vibriobactin secretion and that activation of the Cpx system in JB485 might have resulted from intracellular vibriobactin accumulation due to the absence of RND-mediated efflux. If this was true, we posited that JB485 culture supernatants should contain reduced amounts of iron-chelating compounds than the WT. To test this, we quantified siderophore secretion in the WT and JB485 strains by using the chrome azurol S (CAS) assay. We used a *vibC* mutant as a negative control in these studies because VibC catalyzes the first step in vibriobactin biosynthesis (Fig 2). This circumvents potential problems associated with downstream *vib* mutations (e.g., *vibF*) that may accumulate biosynthetic intermediates that could influence the CAS assay. The CAS assay is based on the formation of a chromogenic complex made up of CAS (163), hexadecyltrimethylammonium bromide, and Fe³⁺ that can be quantified spectrophotometrically. The presence of strong iron chelators like siderophores titrates Fe³⁺ from the dye complex, resulting in decreased absorbance at 630 nm. Thus, the absorbance is inversely proportional to the amount of iron-chelating agents present in the culture supernatant (163).

We cultured the WT, JB485, and Δ *vibC* mutant strains in minimal T-medium without iron supplementation to equivalent optical densities (OD's), growth conditions demonstrated to induce *V. cholerae* siderophore production (162). Cell-free supernatants from the resulting cultures were then assessed with the CAS assay. The results showed a higher absorbance ratio in JB485 than in the WT, indicating that the JB485 supernatants contained lower amounts of siderophore than the WT supernatant (Fig. 4A). In contrast, a *vibC* mutant showed the greatest increase in the

absorbance ratio, which was consistent with its defect in vibriobactin production. Analysis of whole-cell lysates showed that there was no difference in total siderophore production between the WT and JB485, while siderophore production in the $\Delta vibC$ mutant was decreased (Fig. 4B). Together, these data demonstrated that siderophore secretion was impaired in JB485 and suggested that the RND transporters functioned in siderophore secretion. However, the fact that the *vibC* supernatants contained lower amounts of siderophore than the RND-deficient cells suggests that other mechanisms must also contribute to siderophore export.

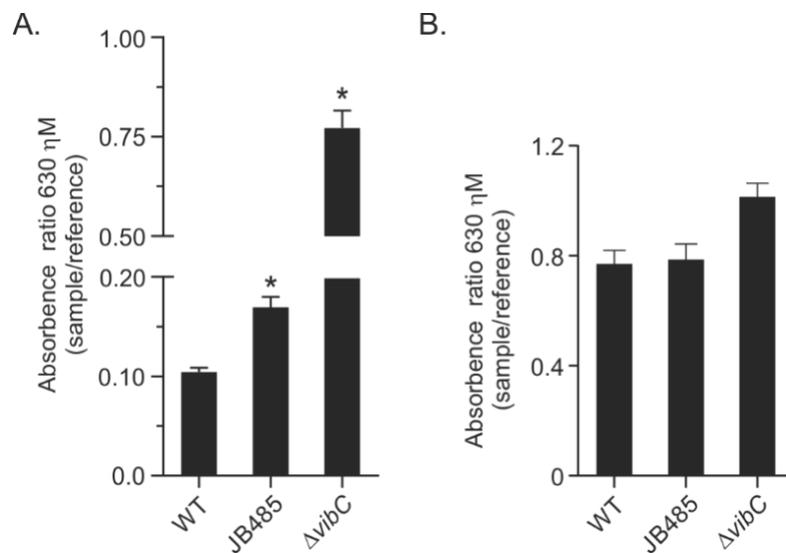


Figure 4 Culture supernatants from RND efflux deficient *V. cholerae* contain reduced concentrations of siderophore.

The CAS assay was used to quantify siderophore production in (A) cell free culture supernatants and (B) whole cell lysates of the indicated strains following growth to saturation in T-medium without iron supplementation. The data presented is the average +/- SD of three independent * = P<0.0001, relative to WT determined by Dunnett's multiple-comparison test.

2.4.4 Vibriobactin secretion is impaired in JB485

The CAS assay is nonspecific and does not discriminate between specific siderophores (i.e., vibriobactin) and other iron-binding compounds. We therefore performed growth stimulation cross-feeding assays to determine if the secreted iron-binding compound observed as described above was vibriobactin (164, 172). The cross-feeding assays assess the ability of a vibriobactin-producing test strain to stimulate the growth of a vibriobactin-negative indicator strain in iron-limiting agar. The indicator strain in our assays was JB58 $\Delta vibC$. The $\Delta vibC$ mutant cannot produce vibriobactin but retains the ability to use exogenously supplied vibriobactin (172).

The results of the cross-feeding assays showed that the WT strongly stimulated the growth of the indicator strain, whereas an isogenic $\Delta vibC$ mutant failed to stimulate growth (Fig. 5). This indicated that trans complementation of the $\Delta vibC$ mutant indicator strain was dependent upon vibriobactin production by the test strains. JB485 showed a lesser ability to stimulate the growth of the indicator strain than the WT (Fig. 5). This finding was consistent with the CAS assay and further supported the conclusion that JB485 was impaired in vibriobactin secretion. Deletion of *vibC* in JB485 completely blocked its ability to stimulate the growth of the $\Delta vibC$ mutant indicator strain. This suggested that the growth-stimulatory compound secreted by JB485 was vibriobactin. These results, combined with the CAS assay results, strongly suggest that vibriobactin secretion is impaired in JB485 and support the novel conclusion the *V. cholerae* RND transporters function in vibriobactin secretion.

The expression of the *vexGH* RND efflux system has been reported to be under the influence of both the Cpx system and Fur (97, 173). We found that activation of the Cpx system resulted in *vexGH* upregulation via CpxR, while *vexGH* mutation resulted in activation of the Cpx system (97). A separate study showed that the *vexGH* promoter contains a Fur box and is regulated

in response to iron availability (95). On the basis of these findings and our above-described data, we hypothesized that VexGH is directly involved in vibriobactin secretion. To test this, we examined a $\Delta vexH$ mutant in the cross-feeding bioassay. The results showed that the $\Delta vexH$ mutant phenocopied JB485 for growth stimulation of the indicator strain, confirming that VexGH functions in vibriobactin secretion (Fig. 5).

There was a large difference between the levels of growth stimulation observed in the RND mutant and the $\Delta vibC$ mutant (Fig. 5). This indicated that vibriobactin can be secreted by additional mechanisms besides RND-mediated efflux. To determine if other active efflux systems contribute to vibriobactin export, we examined a $\Delta tolC$ mutant. TolC serves as the outer membrane pore protein for many different transport systems, including the RND family, major facilitator family, and ATP binding cassette transporters (174). The expression of *tolC* is also influenced by CpxR (95, 97). The $\Delta tolC$ mutant was slightly less able to stimulate indicator strain growth than the RND mutant (Fig. 5). This suggests that other active transport systems likely contribute to vibriobactin secretion. However, the fact that *tolC* deletion did not completely abrogate growth stimulation indicates that vibriobactin can escape from the cell in the absence of TolC-dependent active efflux. It is unknown how this occurs, but it is possible that vibriobactin could escape through porin channels. The fact that the *ompT* porin is regulated by Fur supports this idea (175). Taken together, the cross-feeding results suggested that the RND efflux systems, and VexGH in particular, function in vibriobactin secretion.

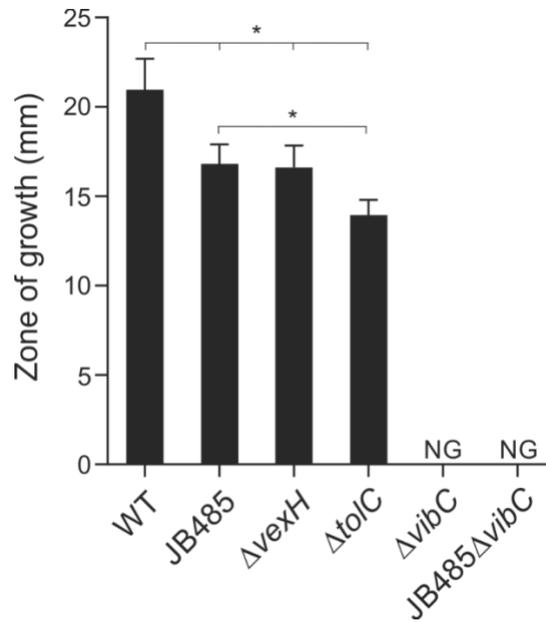


Figure 5 Vibriobactin secretion is impaired in *V. cholerae* lacking RND-mediated efflux.

The indicated *V. cholerae* strains were examined using the cross-feeding growth stimulation assay. The producer strains were seeded onto the surface of solidified LB agar plates containing 270 μ M dipyriddy and the Δ vibC indicator strain before the plates were incubated at 37°C. The diameter of the zone of stimulated growth of the Δ vibC indicator strain was then measured after 24 hours. The data is the average \pm SD of three independent experiments performed in triplicate. * = $P < 0.05$ determined by a t test. Abbreviations: NG, no growth.

2.4.5 *vexGH* regulation by iron is independent of the Cpx system

The above-described results indicated that the RND transporters are involved in iron acquisition. The expression of many RND efflux systems are regulated in response to environmental cues and/or by their respective efflux substrates (84, 97). Thus, we hypothesized that if any of the RND transporters are involved in iron acquisition then their expression would be regulated in response to iron. We tested this by assaying for iron-dependent changes in the expression of each of the six *V. cholerae* RND systems following growth in LB broth plus or minus the iron-chelating chemical dipyriddy. The results revealed that dipyriddy

increased *vexGH* expression (Fig. 6) but did not significantly affect the expression of the other five RND systems (data not shown). The addition of an equimolar amount of FeSO₄ to the dipyriddy cultures returned *vexGH* expression to a level that was equivalent to that of cells grown in the absence of dipyriddy (Fig. 6), confirming that increased *vexGH* expression was a result of iron limitation and not due to a nonspecific effect of dipyriddy. These results indicated that *vexGH* is regulated in response to iron, a conclusion that is consistent with a report linking *vexGH* to the Fur regulon (173).

We previously reported that *vexGH* was positively regulated by the Cpx system (97), while others reported that the Cpx system was activated in response to iron limitation during growth in the presence of dipyriddy on LB agar lacking NaCl (95). This suggested the possibility that the Cpx system was responsible for *vexGH* activation under the iron-limiting conditions described above. To address this possibility, we repeated the above-described experiments with a $\Delta cpxR$ mutant. The $\Delta cpxR$ mutant mirrored the WT results (Fig. 6). The fact that *vexGH* was induced to similar levels under iron-limited conditions in both the WT and the $\Delta cpxR$ mutant indicated that the iron-dependent regulation of *vexGH* transcription occurred by a CpxR-independent mechanism. We presume that the iron-dependent regulation occurred by a Fur-dependent process as previously reported (173), but additional work is required to confirm this.

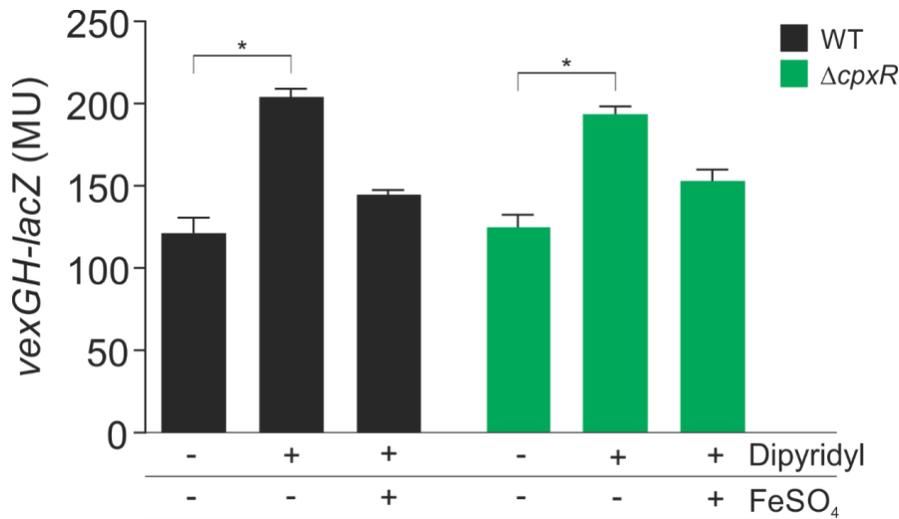


Figure 6 Iron dependent regulation of *vexGH* is independent of the Cpx system.

WT and an isogenic $\Delta cpxR$ mutant were cultured in LB broth in the presence or absence of 130 μM dipyridyl or 130 μM dipyridyl plus 130 μM FeSO_4 . After 3 hr growth at 37°C with shaking, triplicate aliquots were assayed for β -galactosidase activity. The presented data is the average \pm SD of three independent experiments. * = $P < 0.0001$ determined by Dunnett's multiple-comparison test.

2.4.6 RND-deficient mutants are not iron stressed

We hypothesized that the defect in vibriobactin secretion in JB485 may result in a reduced ability to obtain iron. In *V. cholerae*, as in many bacteria, the control of iron homeostasis is mediated by the ferric uptake regulator (Fur), which regulates the expression of iron acquisition genes in response to iron availability (169, 173, 176). Fur functions primarily as a repressor. Under iron-replete conditions Fur binds Fe_{2+} , which enables binding to conserved DNA sequences (i.e., Fur box) in the promoters of target genes to repress their expression. Under iron-depleted conditions, the Fe_{2+} binding equilibrium is shifted and iron is released from Fur, resulting in the derepression of target genes (177, 178). On the basis of this, we postulated that if the RND-negative mutant was iron stressed, then the expression of Fur-regulated genes would increase

relative to that in the WT. We therefore compared the expression levels of four Fur-regulated genes (*irgA*, *hutA*, *tonB*, and *vibF*) in the WT and JB485 strains (169, 173). The results revealed no significant difference in *irgA*, *tonB*, or *vibF* expression between the WT and JB485 (Fig. 7A). In contrast, *hutA* expression was higher in JB485 than in the WT. We previously showed that *hutA* was one of the most highly upregulated genes in the *V. cholerae* Cpx regulon (97); suggesting that *hutA* expression, like *vexGH* expression, is regulated by both Fur and the Cpx system. It is noteworthy that CpxR has also been linked to the expression of some iron acquisition genes in *V. cholerae* (95). To determine if increased *hutA* expression in JB485 was due to an iron acquisition defect or due to the Cpx system, we compared *hutA* expression in the WT, a $\Delta cpxR$ mutant, JB485, and JB485 $\Delta cpxR$ during growth in LB broth. The results showed that deletion of *cpxR* in the WT did not affect basal-level *hutA* expression but that *cpxR* deletion in JB485 reduced *hutA* expression to WT levels (Fig. 7B). This confirmed that the increased *hutA* expression in JB485 was due to CpxR and not due to an iron acquisition defect. From these results, we concluded that loss of RND-mediated efflux did not result in an iron acquisition defect under the conditions tested.

The above-described conclusion was further validated by comparing the growth of the WT, $\Delta vibF$ mutant, JB485, and JB485 $\Delta vibF$ strains in iron-limited T-medium. The results revealed that the WT and the $\Delta vibF$ mutant exhibited equivalent growth in iron-limited T-medium (Fig. 7C). Since *vibF* is essential for vibriobactin production, these results demonstrate that vibriobactin is dispensable for *V. cholerae* growth in iron-limited T-medium. In contrast JB485 exhibited impaired growth in iron-limited T-medium, whereas an isogenic JB485 $\Delta vibF$ mutant exhibited WT growth kinetics (Fig. 7C); suggesting that the inability to efflux vibriobactin was deleterious to growth under iron-limiting conditions. Control experiments in iron-sufficient T-medium

confirmed that JB485 did not exhibit a nonspecific growth defect in T-medium (Fig. 7D). Collectively, these results suggested that RND-mediated efflux is dispensable for growth under iron-sufficient conditions but enhances fitness during growth under iron-limiting conditions. The decreased fitness of the RND-negative mutant under iron-limiting conditions was likely due to the combined effects of increased vibriobactin production and a defect in vibriobactin efflux.

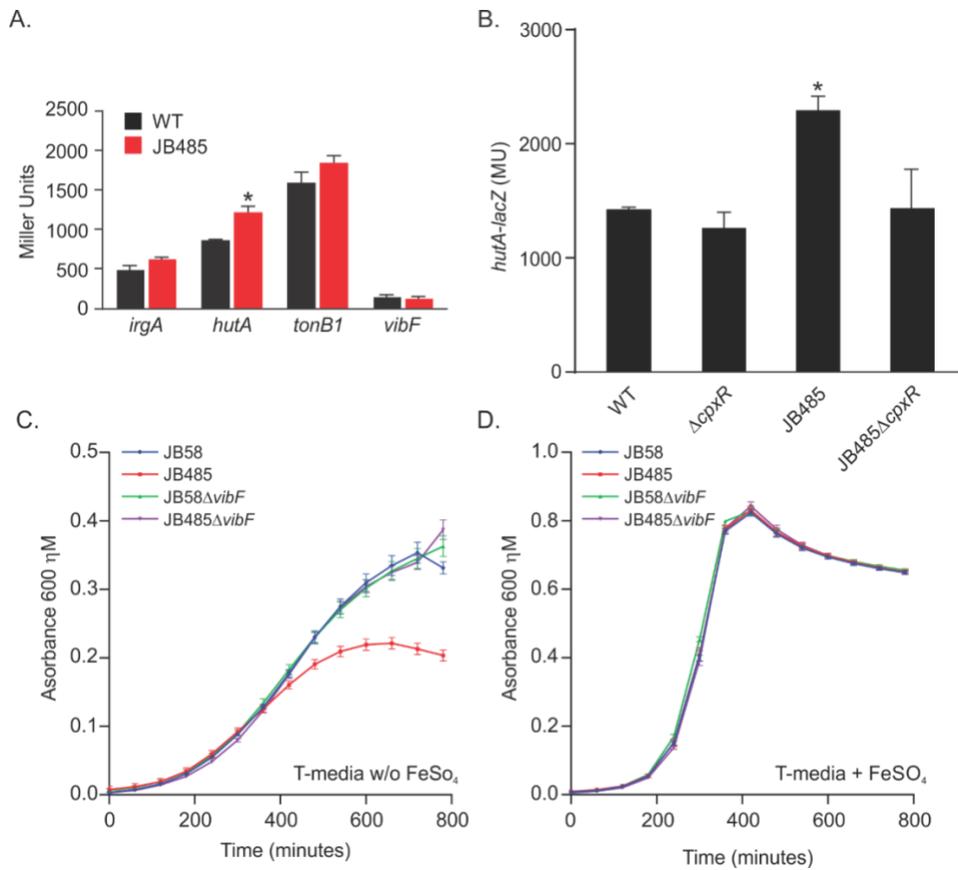


Figure 7 Loss of RND-mediated efflux does not affect the expression of Fur regulated genes.

(A) WT strain JB58 or the RND negative mutant JB485 bearing transcriptional reporters for the indicated Fur-regulated genes were cultured in LB broth to middle logarithmic phase when the expression of the reporters was assessed by the β -galactosidase assay. * = $P < 0.05$ relative to WT determined by *t* test. (B) Iron-dependent upregulation of *hutA* is independent of CpxR. The indicated *V. cholerae* strains bearing a *hutA-lacZ* transcriptional reporter were cultured as described in panel A. * = $P < 0.05$ relative to WT determined by *t* test. (C) RND deficient *V. cholerae* has impaired fitness in iron-deplete media. Overnight T-medium cultures of indicated strains were diluted 1:100 in fresh T-medium without (C), or with (D), FeSO₄ supplementation and cultured at 37°C with shaking in a microtiter plate reader. Cell growth was recorded every 30 minutes as the optical density at 600 η M. The presented data is the average \pm SEM of three independent experiments.

2.4.7 Oxidative stress activates the Cpx response in *V. cholerae*

Our collective data suggest that an inability to secrete vibriobactin results in activation of the Cpx system. However, the mechanism by which retained vibriobactin activated the Cpx system was unclear. The fact that we identified *sdhA* as a Cpx suppressor in JB485 suggested that aerobic respiration is required to generate the Cpx-activating signal. SdhA is part of complex II (i.e., succinate dehydrogenase) of the ETC (167, 179). Reactive oxygen species (ROS) are produced by the ETC as a byproduct of aerobic respiration. ROS can oxidize proteins and result in the formation of aberrant disulfide bonds and misfolded proteins, which can serve as activating signals for the Cpx system (99). If aerobic respiration is required for activation of the Cpx system in JB485, then growth under anaerobic conditions should suppress Cpx expression in JB485. We tested this by culturing JB485 *cpxP-lacZ* on LB X-Gal agar under anaerobic conditions. The results confirmed our hypothesis and showed that anaerobic growth completely suppressed the *cpxP-lacZ* reporter in JB485 (Fig. 8).

We next tested if ROS alone could induce the Cpx response. This was done by culturing the *cpxP-lacZ* reporter strains on agar plates containing paraquat. Paraquat is an oxidative-stress-inducing agent that catalyzes superoxide formation (180). Growth on paraquat activated the Cpx system in the WT, $\Delta vibF$ mutant, JB485, and JB485 $\Delta vibF$ strains under aerobic conditions but not under anaerobic growth conditions (Fig. 8). These findings confirmed that oxidative stress generated by paraquat was sufficient to activate the *V. cholerae* Cpx system. We performed similar experiments with hydrogen peroxide. However, hydrogen peroxide did not activate the Cpx system, which was consistent with a previous report (95). These divergent results may stem from the fact that paraquat was reported to catalyze superoxide production in *V. cholerae*, whereas

hydrogen peroxide abrogated superoxide production (181). From these results, we concluded that ROS can activate the Cpx response in *V. cholerae*.

The fact that Cpx suppressors in JB485 mapped to respiratory complex II suggested that complex II may be directly responsible for generating the Cpx-activating signal. We therefore tested if chemical inhibition of complex II in the WT or JB485 affects Cpx activation. To do this, we inoculated the WT, $\Delta vibF$ mutant, JB485, and JB485 $\Delta vibF$ *cpxP-lacZ* reporter strains onto LB X-Gal agar containing thenoyltrifluoroacetone (TTFA). TTFA binds to the quinone binding site on complex II, preventing ubiquinone binding and thus electron transport (182). Growth of the WT and the $\Delta vibF$ mutant in the presence of TTFA did not affect the activation state of the Cpx system (Fig. 8), suggesting that inhibition of complex II activity was not sufficient to activate the Cpx system in the WT or to suppress Cpx activation in JB485. In contrast, TTFA activated the Cpx system in a *vibF*-independent manner in JB485. This was evidenced by the fact that JB485 produced dark blue colonies and JB485 $\Delta vibF$ produced diffuse light blue colonies on the TTFA plates (Fig. 8). We cannot explain the RND efflux-dependent effects of TTFA on the Cpx system. It is possible that TTFA is a substrate for the RND transporters. This would result in increased TTFA uptake in JB485, which could serve to activate the Cpx system through an ROS-specific pathway, as electrons are unable to be efficiently passed from complex II. Alternatively, the absence of RND-mediated efflux may result in pleiotropic effects on the Cpx system in JB485 that are compounded by TTFA.

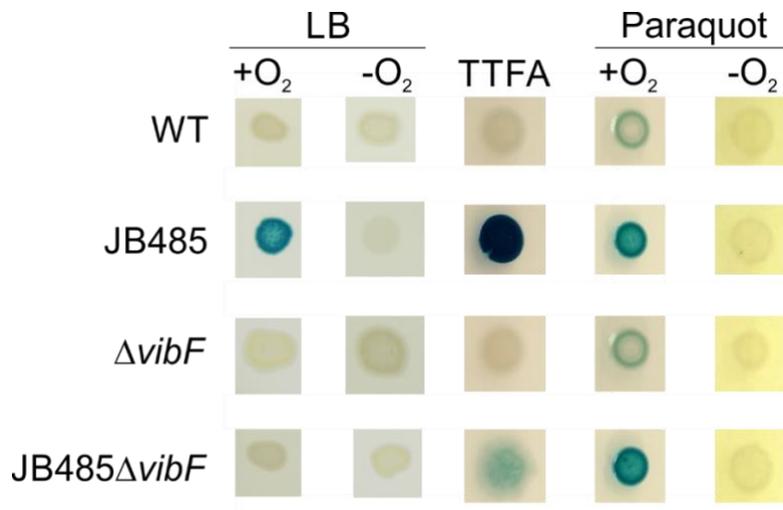


Figure 8 Reactive oxygen species activate the *V. cholerae* Cpx system.

The indicated *V. cholerae* strains bearing a chromosomal *cpxP-lacZ* reporter were inoculated onto the surface of LB X-gal agar plates, or plates containing 2.5 μ M of the complex II inhibitor 2-Thenoyltrifluoroacetone (TTFA), or 500 μ M of the superoxide producing drug paraquat. The plates were incubated overnight at 37°C in the presence or absence of oxygen before being photographed. Anaerobic conditions were achieved using a BD GasPak EZ Pouch Systems.

2.5 Discussion

In this chapter, we have expanded on the function of the *V. cholerae* RND efflux systems by demonstrating that the VexGH RND transporter contributes to vibriobactin export. As discussed below, our results provide the first evidence to link RND-mediated efflux to iron acquisition and the maintenance of cellular homeostasis in *V. cholerae* and provide insight into the selective pressures for the maintenance of what was previously thought to be a redundant RND transporter.

Our results demonstrate that the VexGH RND efflux system functions in vibriobactin export. This conclusion was supported by several lines of evidence, including the fact that there were reduced amounts of vibriobactin present in culture supernatants of mutants lacking *vexH* (Fig. 4) and that vibriobactin export was impaired in strains lacking RND-mediated transport (Fig. 5). Iron is a cofactor for many biological processes and is therefore an essential nutrient for nearly all life forms (183). Vibriobactin is produced by *V. cholerae* as a mechanism to acquire environmental iron (165). Vibriobactin is synthesized in the cytoplasm before being secreted. In many Gram-negative bacteria, siderophore secretion appears to occur by a two-step process where the siderophore is first translocated into the periplasm before being secreted into the external environment. Once outside the cell, siderophores bind to ferric iron before being taken back up into the cell via specific transporters, delivering the iron payload to the cytoplasm for use in metabolism. Although the processes of vibriobactin biosynthesis and uptake are well understood (184, 185) the mechanism by which vibriobactin is secreted into the environment is unknown. Our results strongly suggest that VexGH contributes to this process.

The finding that a Δ *vexGH* mutant phenocopied an RND null mutant for vibriobactin secretion (Fig. 5) suggested that the VexGH RND system may be the primary RND transporter involved in vibriobactin secretion. This conclusion is bolstered by the finding that *vexGH* is coregulated with the vibriobactin biosynthetic genes in response to iron (173). However, we cannot completely exclude the possibility that other RND transporters contribute to vibriobactin secretion that may have been missed because of the sensitivity of the assay. It is also possible that the mutation of individual RND transporters affects expression of redundant transporters, which could mask the phenotype of specific RND mutants in vibriobactin export. In addition to being regulated by Fur, *vexGH* is also positively regulated by the Cpx system in response to cell envelope

perturbations (95, 97). This suggests that the function of VexGH extends beyond vibriobactin secretion to protecting the cell from deleterious effects of toxic environmental compounds. The latter function is consistent with previous studies showing that VexGH is a multidrug efflux system that provides resistance to bile acids, nonionic detergents, ampicillin, and novobiocin (149).

All six *V. cholerae* RND transporters were found to be important for virulence factor production (150). The expression of *vexAB* was upregulated in human- and animal-shed *V. cholerae*, while that of *vexGH* and *vexIJK* was induced *in vivo* in human volunteers (150, 153, 186). These collective studies highlight the importance of the RND transporters in pathogenesis, but the contribution of each individual system to pathogenesis was unclear. We previously showed that four of the *V. cholerae* RND transporters had overlapping substrate specificity for bile, an important barrier to colonization (149, 150). VexB, VexD, VexH, and VexK functioned in bile salt resistance, with VexB and VexD being major contributors relative to VexH and VexK, which provided modest but equivalent contributions to bile salt resistance (149). Interestingly, while the antimicrobial susceptibility profile of mutants lacking the VexBDH or VexBDK efflux pumps were similar, the former strain was nearly 4 log units more attenuated during single-strain colonization of the infant mouse intestine than the latter strain (149). This suggested that VexH contributed much more to intestinal colonization than VexK. The mechanism behind this is unclear, but on the basis of our results showing that VexG functions in vibriobactin secretion and that vibriobactin retention is detrimental to growth under iron-limited conditions, we suggest that the inability of the Δ *vexH* mutant to secrete vibriobactin likely contributed to its *in vivo* attenuation. It is also possible that the inability of the Δ *vexH* mutant to secrete vibriobactin attenuated colonization because of impaired iron acquisition. The fact that vibriobactin-negative mutants are not attenuated for infant mouse colonization argues against this possibility (187).

We previously showed that RND-mediated efflux maintained the *V. cholerae* Cpx system in a suppressed state during growth under standard laboratory conditions (97). Although the mechanism linking RND efflux to the Cpx system was unknown, we and others proposed that Cpx activation in mutants with impaired RND transport likely resulted from the intracellular accumulation of toxic metabolites that were normally removed from the cell by the RND transporters (95, 97). Our results here demonstrate that the RND-dependent toxic metabolite was vibriobactin. The inability of the RND mutant cells to secrete vibriobactin was detrimental, as evidenced by the activation of the Cpx system and the vibriobactin-dependent decreased fitness of an RND-deficient mutant during growth under iron-limited conditions. *V. cholerae* is likely to encounter iron limitation in both the host and aquatic ecosystems, which makes these results particularly relevant to its biology (188-190).

V. cholerae, like many Gram-negative bacteria, encodes multiple RND efflux systems with overlapping substrate specificities. Yet the selective pressures maintaining these redundant systems in the *V. cholerae* genome are unclear. The fact that vibriobactin retention was detrimental to the cell could provide the selective pressure for the maintenance of *vexGH* in members of the family *Vibrionaceae*. The detrimental effects of vibriobactin retention could also explain our finding that RND-independent mechanisms also contributed to vibriobactin export, including active transporters and porins. It is interesting to speculate that the effects of siderophore retention defined here may apply to other bacteria where efflux has been linked to Cpx activation and siderophore export (144, 191-194). The conservation of similar findings between distantly related genera suggests that our results extend beyond *V. cholerae*.

Although we were unable to identify the precise mechanism by which vibriobactin activated the Cpx system in the Δ *vexGH* mutant, the fact that complex II mutations and anaerobic

growth suppressed the Cpx system suggested that the activating signal likely involved a toxic byproduct of aerobic respiration, most likely ROS, which can attack protein disulfide bonds, resulting in aberrant bond formation, a known Cpx-inducing signal. Our results showing that the Cpx system was induced upon exposure to oxidizing agents (e.g., CuCl₂ and paraquat) support this idea. On the basis of these observations, we propose a model (Fig. 9) whereby, in the absence of RND-mediated efflux, iron-free vibriobactin accumulates within the cell and chelates iron from the iron-rich components of the ETC. The removal of iron from ETC components then generates the Cpx-inducing cue in one of two ways. The chelation of iron from the Fe-S centers in complex II (or other ETC components) could directly result in the formation of abnormal disulfide bonds in the deferrated proteins. Alternatively, the chelation of iron from ETC components could result in increased ROS production, which could then catalyze the formation of nonnative disulfide bonds in periplasmic proteins (195). We note that these two scenarios are not mutually exclusive and it is possible that combinations of both signals may contribute to Cpx activation. This model also explains the observation that iron addition suppressed the Cpx system in RND-deficient cells. Iron addition could suppress the Cpx response by several mechanisms, including repression of vibriobactin production (via Fur), inhibition of iron chelation from ETC components, and/or replacement of iron in deferrated ETC components. This model is consistent with recent reports showing that dipyriddy activated the Cpx response in *V. cholerae* (95) and can be extended to other organisms where efflux has been associated with activation of the Cpx response and/or the induction of stress responses (144, 191-194, 196).

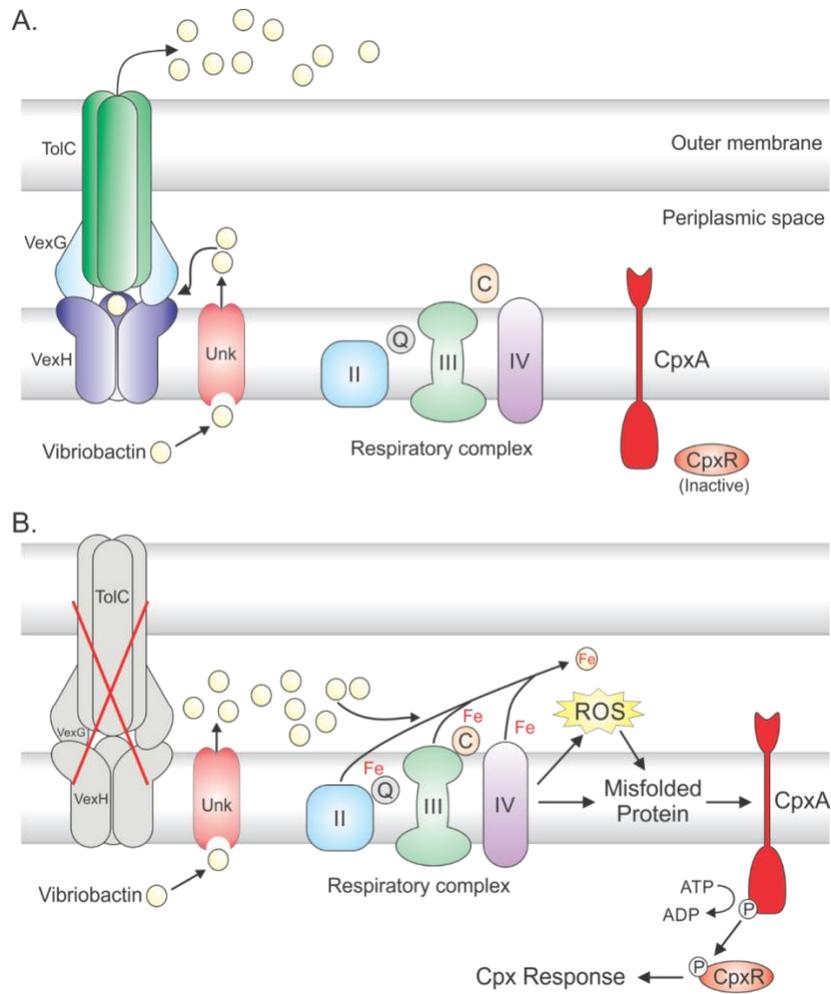


Figure 9 Putative model for activation of the Cpx system in a $\Delta vexGH$ mutant.

(A) In WT cells vibriobactin is produced and exported into the periplasmic space by an unknown transporter. Periplasmic vibriobactin is then secreted to the external environment by the VexGH-TolC RND efflux system and the CpxRA system is inactive. (B) In the absence of *vexGH*, iron-free vibriobactin accumulates in the periplasmic space and chelates iron from the iron-rich membrane bound components of the respiratory chain. The chelation of iron from the respiratory components leads to altered protein structures and/or increased ROS production; both of which can activate the Cpx system. Abbreviations: Unk, unknown vibriobactin transporter; ROS, reactive oxygen species; II, complex II; III, complex III; IV, complex IV, C, cytochrome C; P, phosphate; Fe, iron.

3.0 *Vibrio cholerae* OmpR Represses the ToxR Regulon in Response to Membrane Intercalating Agents That Are Prevalent in the Human Gastrointestinal Tract

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3.1 Project summary

Multidrug efflux systems belonging to the resistance-nodulation-division (RND) superfamily are ubiquitous in Gram-negative bacteria. RND efflux systems are often associated with multiple antimicrobial resistance and also contribute to the expression of diverse bacterial phenotypes including virulence, as documented in the intestinal pathogen *Vibrio cholerae*, the causative agent of the severe diarrheal disease cholera. Transcriptomic studies with RND efflux-negative *V. cholerae* suggested that RND-mediated efflux was required for homeostasis, as loss of RND efflux resulted in the activation of transcriptional regulators, including multiple environmental sensing systems. In this report, we investigated six RND efflux-responsive regulatory genes for contributions to *V. cholerae* virulence factor production. Our data showed

that the *V. cholerae* gene VC2714, encoding a homolog of *Escherichia coli* OmpR, was a virulence repressor. The expression of *ompR* was elevated in an RND-null mutant, and *ompR* deletion partially restored virulence factor production in the RND-negative background. Virulence inhibitory activity in the RND-negative background resulted from OmpR repression of the key ToxR regulon virulence activator *aphB*, and *ompR* overexpression in wild-type cells also repressed virulence through *aphB*. We further show that *ompR* expression was not altered by changes in osmolarity but instead was induced by membrane-intercalating agents that are prevalent in the host gastrointestinal tract and which are substrates of the *V. cholerae* RND efflux systems. Our collective results indicate that *V. cholerae ompR* is an *aphB* repressor and regulates the expression of the ToxR virulence regulon in response to novel environmental cues.

3.2 Introduction

The Gram-negative bacterium *Vibrio cholerae* is the causative agent of the life-threatening diarrheal disease cholera. *V. cholerae* is an aquatic organism that infects humans following the consumption of *V. cholerae*-contaminated food or water. After ingestion, *V. cholerae* colonizes the epithelium of the small intestine to cause disease by a process that is dependent upon virulence factor production. The two most important *V. cholerae* virulence factors are the toxin-coregulated pilus (TCP), which mediates intestinal colonization, and cholera toxin (CT), an enterotoxin that is responsible for the secretory diarrhea that is the hallmark of the disease cholera. CT and TCP production is under the control of a hierarchical regulatory system known as the ToxR regulon (39, 197). Activation of the ToxR regulon begins with the expression of two cytoplasmic transcriptional regulators, *aphA* and *aphB* (71, 72). AphA and AphB function synergistically to

activate *tcpP* expression. TcpP then binds along with ToxR to the *toxT* promoter to activate *toxT* expression. ToxT directly activates the expression of the genes that encode CT and TCP production (197).

The expression of adaptive responses is important for the success of *V. cholerae* as a pathogen. This includes tight regulation of the ToxR regulon, which is known to limit virulence factor production to the host gastrointestinal tract. Thus, the ToxR regulon has evolved to respond to specific environmental signals within the host (198). Other genes which are important for survival and persistence in aquatic ecosystems must be repressed during host entry for successful colonization (35, 82, 199). Late in infection, in preparation for host exit, *V. cholerae* downregulates virulence genes while activating genes required for dissemination and transmission (42-44, 77). Although the genetic mechanisms involved in ToxR regulon activation have been extensively studied, less is known about how environmental signals influence ToxR regulon expression *in vivo*.

V. cholerae is exposed to disparate environments within both the aquatic ecosystem and the human gastrointestinal tract. *V. cholerae* survival and growth in these niches require rapid adaptation to environmental conditions. *V. cholerae* enters humans from aquatic ecosystems that are typically aerobic and alkaline. The bacterium must then pass through the gastric acid barrier of the stomach before entering the duodenum and migrating to the epithelial surface, where it colonizes the crypts of the small intestine. Successful transition between these dissimilar environments requires that *V. cholerae* modulates its transcriptional responses so that specific genes are only expressed during colonization of appropriate niches. In *V. cholerae*, like most bacteria, this is achieved by environmentally responsive regulatory systems that monitor the

extracellular environment by use of a range of membrane-bound sensors such as ToxR and two-component signal transduction systems (TCSs) (87).

TCSs are widespread phospho-relay systems that modulate gene expression in response to environmental cues. They consist of a membrane-bound histidine kinase sensor protein coupled with a cytosolic response regulator. In the presence of appropriate stimuli, the sensor autophosphorylates a conserved histidine residue before transferring the phosphate to a conserved aspartate residue on the response regulator, activating the response regulator. Activated response regulators function to modulate adaptive responses by effecting the expression of target genes. Response regulators are typically transcription factors but can also function by other mechanisms (85). The adaptive responses mediated by TCSs are broad and include virulence, motility, metabolism, and stress responses.

One of the better characterized TCSs is the EnvZ-OmpR system that is ubiquitous in Gram-negative bacteria (200). EnvZ is the membrane-associated sensor kinase, and OmpR the response regulator that functions as a transcription factor. EnvZ/OmpR was first discovered in *Escherichia coli* and was shown to regulate the expression of its two major outer membrane porin proteins (201), *ompC* and *ompF*, in response to environmental osmolarity (100-102). The function of OmpR as an osmoregulator has been extended to a number of other bacterial genera (109, 202, 203). OmpR has also been linked to other phenotypes in Gram-negative bacteria, including virulence (109, 202, 204-208) and acidic tolerance (103, 203, 209-212). The *V. cholerae* OmpR homolog, open reading frame (ORF) VC2714, has been little studied, and its role in *V. cholerae* biology is unknown.

The RND efflux systems are ubiquitous tripartite transporters in Gram-negative bacteria that play critical roles in antimicrobial resistance. Many RND efflux systems exhibit broad

substrate specificity and have the capacity to efflux multiple substrates that are both structurally and functionally unrelated (123, 140). The RND systems play critical roles in antimicrobial resistance by exporting toxic compounds from the cytosol and periplasm into the extracellular environment. Although RND efflux pumps have been widely studied for their role in multiple antibiotic resistance, they also impact many other physiological phenotypes in bacteria (148). This was recently documented in *V. cholerae*, where the RND systems were shown to be required for cell homeostasis (94, 97). The absence of RND efflux in *V. cholerae* resulted in downregulation of the ToxR regulon and altered expression of genes involved in metabolic and environmental adaptation (121, 150), including several TCSs. The results of these studies suggested that RND-mediated efflux modulated homeostasis by effluxing cell metabolites, which served as concentration-dependent environmental cues to initiate transcriptional responses via periplasmic sensing systems. This observation suggested the possibility that these TCSs may have contributed to the virulence attenuation observed in RND efflux-impaired *V. cholerae*.

In this work, we investigated six regulatory genes that were induced in the absence of RND-mediated efflux for their contribution to virulence factor production in *V. cholerae*. This revealed that VC2714, encoding a homolog of *E. coli* OmpR, functioned as a virulence repressor in *V. cholerae*. We documented that VC2714 repressed the expression of the key virulence regulator *aphB*. We further showed that *ompR* expression was regulated in response to detergent-like compounds, which are prevalent in the host gastrointestinal tract and are substrates of the RND transporters. Our collective results suggest that the *V. cholerae* EnvZ/OmpR TCS has evolved to regulate virulence in response to novel environmental stimuli.

Table 2 Strains, plasmids and oligonucleotides used in chapter 3

Strain:	Genotype:	Source:
<i>E. coli</i>		
EC100Dpir+	<i>F. mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) Φ 80 <i>dlacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1 endA1 araD139</i> Δ (<i>ara, leu</i>)7697 <i>galU galK</i> λ - <i>rpsL</i> (<i>Str</i> κ) <i>nupG pir+</i>	Epicentre
SM10 λ pir	<i>thi-1 thr leu tonA lacY supE recA::RP4-2-4-Tc::Mu Km_r</i> (λ . <i>pirR6K</i>)	(157)
<i>V. cholerae</i>		
JB58	01 El Tor strain N16961 Δ <i>lacZ</i> Sm _r	Lab collection
Δ RND	JB58 Δ <i>vexB</i> Δ <i>vexD</i> Δ <i>vexF</i> Δ <i>vexH</i> Δ <i>vexK</i> Δ <i>vexM</i>	(213)
DK243	JB58 Δ <i>ompR</i>	This Work
DK246	Δ RND Δ <i>ompR</i>	This Work
Plasmids:	Description:	
pBAD33	Arabinose regulated expression vector	(160)
pTB1	pBAD33 expressing VC0486	This Work
pTB3	pBAD33 expressing VC1320-VC1319	This Work
pTB5	pBAD33 expressing VC1081	This Work
pTB7	pBAD33 expressing VC1638	This Work
pTB9	pBAD33 expressing VC1825	This Work
pTB11	pBAD33 expressing VC2714 (<i>ompR</i>)	This Work
pDK14	pBAD33 expressing VC2714-D55E	This Work
pDK15	pBAD33 expressing VC2714-D55A	This Work
pTB15	pBAD33 expressing VC1320	This Work
pTL61T	Reporter plasmid for making transcriptional fusions to <i>lacZ</i> , Amp _r	(159)
pDK9	pTL61T containing the <i>ompR</i> promoter	This Work
pXB192	pTL61T containing the <i>toxT</i> promoter	(213)
pXB193	pTL61T containing the <i>ctxAB</i> promoter	(215)
pXB194	pTL61T containing the <i>tcpA</i> promoter	(215)
pXB201	pTL61T containing the <i>toxRS</i> promoter	(215)
pXB202	pTL61T containing the <i>aphA</i> promoter	(215)
pXB203	pTL61T containing the <i>aphB</i> promoter	(215)
pXB266	pTL61T containing the <i>leuO</i> promoter	(216)
pWM91	Suicide vector used for allelic exchange, Amp _r	(157)
pWM91- Δ <i>ompR</i>	Suicide vector used for deletion of <i>ompR</i>	This Work
Primers:	Sequence (5' – 3'):	
P-VC2714-F-XhoI	GGCTCGAGA AACTCGATTGAGTATGAGAAAGG	
P-VC2714-R-XbaI	AATCTAGACCATGATCCCACCTAACTGTTGTTC	
VC2714-F1-XhoI	TTCTCGAGTGCGGCTTTGCTGTGGCGAC	
VC2714-R1-BamHI	GCGGATCCCACCTTGGCTGCGATTGCTAAC	
VC2714-F2	ATGCGCGCTTGCCTTCTGATGGTAAAGCCGCAAC	
VC2714-R2	ACCATCAGGAACGCAAGCGCGCATCATCTACCAC	
VC2714-F-SacI	AAGAGCTCAACAGTTAGGTGGGATCATG	
VC2714-R-SmaI	TTCCCGGGCTAAAAGAAGTTAGTTGGCGGC	
<i>aphA</i> -F	GCAGAACCCTTACCGTCTGCAA	
<i>aphA</i> -R	GCGTAATAAGCGGCTTCGATT	
<i>aphB</i> -F	ATCGGTGAAGTGAAAGACATTTTGG	
<i>aphB</i> -R	GATGTTGATGCAACTCTTCAGCAT	
<i>ToxRS</i> -F	CGTCAAAACGGTTCCGAAACG	
<i>ToxRS</i> -R	CGCGAGCCATCTCTTCTCAA	
<i>tcpP</i> -F	TAGCCGGCATTACTCATGATCTAC	
<i>tcpP</i> -R	TTGTTATCCCCGGTAAACCTTGC	

Table 2 continued

Primers:	Sequence (5' – 3'):
<i>gyrA</i> -F	CAATGCCGGTACACTGGTACG
<i>gyrA</i> -R	AAGTACGGATCAGGGTCACG

3.3 Materials and methods used in chapter 3

3.3.1 Bacterial strains and culture conditions

The bacterial strains and plasmids used in this chapter are listed in Table 2. *E. coli* strains EC100Dpir and SM10λpir were used for cloning and plasmid conjugation, respectively. *V. cholerae* strain JB58 was used as the WT in all experiments. Bacterial strains were routinely grown at 37°C in lysogeny broth or on LB agar. AKI growth conditions were used to induce *V. cholerae* virulence gene expression as previously described (217). The modified T-medium was prepared as previously described (162). Antibiotics were used at the following concentrations: streptomycin (218), 100 µg/ml; carbenicillin (Cb), 100 µg/ml; and chloramphenicol (20 µg/ml for *E. coli* and 2 µg/ml for *V. cholerae*). The C18-conditioned AKI medium was prepared as follows. Sep-Pak C18 cartridges (Waters) were preconditioned with 10 ml of 100% methanol followed by 10 ml of sterile double-distilled water (ddH₂O) before 50 ml of AKI broth was passed through the cartridge and the flow-through fraction was collected and used as a conditioned AKI broth. Molecules that were retained on the C18 columns following the passage of LB or AKI broth medium were eluted from the column with 10 ml of 100% methanol. The eluates were concentrated by evaporation. The resulting residue was resuspended in a volume of LB broth that was identical to the volume of the extracted AKI broth and filter sterilized prior to use.

3.3.2 Plasmid and mutant construction

Oligonucleotides used in this chapter are listed in Table 2. Chromosomal DNA from the WT was used as the PCR template for cloning experiments. The *ompR-lacZ* reporter plasmid pDK9 was generated as follows. The *ompR* promoter region was amplified by PCR using the P-VC2714-F-XhoI and P-VC2714-R-XbaI oligonucleotide primers. The resulting amplicon was digested with XhoI and XbaI restriction endonucleases and ligated into a similarly digested pTL61T vector to generate the plasmid pDK9. The *ompR* expression vector pTB11 was created by amplifying *ompR* using the VC2714-F-SacI and VC2714-R-SmaI oligonucleotide primers. The resulting 766-bp fragment was digested with SacI and SmaI restriction endonucleases and ligated into similarly digested pBAD33 to generate pTB11. The other expression plasmids (pTB3, pTB5, pTB7, pTB9, and pTB15) were made in a similar manner. The OmpR aspartate 55 mutant expression plasmids were constructed as follows. Internal fragments (364 bp) of the *ompR* gene containing D55E and D55A mutations were custom synthesized by Integrated DNA Technologies. The resulting fragments were digested with PmlI and EcoRI before being ligated into similarly digested pTB11 to generate plasmids pDK14 and pDK15. The *ompR* (VC2714) deletion construct was constructed as follows. Primer pairs *ompR*-F1/*ompR*-R2 and *ompR*-F2/*ompR*-R1 were used in separate PCRs with N16961 genomic DNA. The two resulting amplicons (~1.5 kb each) were collected and used as the template for the second-round PCR amplification with the flanking *ompR*-F1 and *ompR*-R1 PCR primers. The resulting ~3-kb amplicon was then digested with the SpeI and SmaI restriction endonucleases before being ligated into similarly digested pWM91 vector to generate pWM91- Δ *ompR*. pWM91- Δ *ompR* was then used to delete *ompR* through allelic exchange as previously described (150). All plasmids were validated via DNA sequencing.

3.3.3 Transcriptional reporter assays

V. cholerae and *E. coli* strains containing the indicated *lacZ* reporters were cultured under AKI conditions, in LB broth, or in a modified T-medium. At the indicated times, aliquots were collected in triplicate, and β -galactosidase activity was quantified as previously described (219). The experiment quantifying *ompR* expression during growth under varying NaCl concentrations was performed as follows. WT strains harboring pDK9 were cultured under virulence factor-inducing conditions in AKI medium containing the indicated NaCl concentrations for 5 h. Culture aliquots were then collected in triplicate, and β -galactosidase production was assessed. The experiments quantifying gene expression responses to bile salts, deoxycholate, SDS, oxgall, indole, and ethanol were performed as follows. The indicated strains were grown in LB broth at 37°C with shaking for 4 h when the indicated compounds were added to the cultures. Thereafter, the cultures were then incubated with shaking for an additional hour before culture aliquots were collected in triplicate and β -galactosidase production was assessed. All of the transcriptional reporter experiments were performed independently at least three times.

3.3.4 Determination of CT and TcpA production

CT production was determined by ganglioside GM1 enzyme-linked immunosorbent CT assays as previously described by use of purified CT (Sigma) as a standard (150). The production of TcpA was determined by Western immunoblotting as previously described (77).

3.3.5 Growth curve experiments

Growth curves were generated in microtiter plates. Overnight cultures of WT, Δ RND, $\Delta ompR$, and Δ RND $\Delta ompR$ strains grown in LB broth were washed in phosphate-buffered saline (PBS) and then diluted 1:10,000 in fresh LB broth containing either 0.172 M NaCl or 0.5 M NaCl. Two hundred microliters of the diluted cultures were then aliquoted in triplicate wells of a 96-well microtiter plate. The microtiter plates were then incubated at 37°C with constant shaking, and the optical density at 600 nm (OD₆₀₀) was measured every 30 min using a BioTek Synergy microplate reader.

3.3.6 Quantitative real-time PCR

V. cholerae strains were grown under AKI conditions for 3.5 h when total RNA was isolated from the cultures using TRIzol (Invitrogen) per the manufacturer's directions. cDNA was generated from the purified RNA using the Maxima First Strand cDNA synthesis kit (Thermo). The expression levels of specific genes were quantified by amplifying 25 ng of cDNA with 0.3 μ M primers using the SYBR green PCR mix (Thermo) on a StepOnePlus real-time PCR System (Applied Biosystems). The relative expression levels of genes in the mutant and WT cultures were calculated using the $2^{-\Delta\Delta CT}$ method. The presented results are the means standard deviations from three biological replicates, with each biological replicate being generated from three technical replicates. DNA gyrase (*gyrA*) was used as the internal control.

3.4 Results

3.4.1 *V. cholerae* OmpR represses virulence factor production

The loss of RND-mediated efflux resulted in downregulation of the ToxR regulon and diminished CT and TCP production (150), suggesting that there are one or more factors linking efflux to virulence factor production. Transcriptional profiling of an RND-negative ($\Delta vexB \Delta vexD \Delta vexF \Delta vexH \Delta vexK \Delta vexM$) *V. cholerae* mutant during growth under AKI conditions (i.e., virulence-inducing conditions) showed that the expression of a number of regulatory genes, including several TCSs, was increased in the absence of RND efflux (121). We hypothesized that one or more of these regulatory genes may have contributed to RND efflux-dependent virulence repression. To test this, we expressed six regulators (i.e., VC0486, VC1320 and VC1319, VC1081, VC1638, VC1825, VC1320, and VC2714) from the arabinose-regulated promoter in pBAD33 in wild-type (WT) *V. cholerae* during growth under AKI conditions in the presence of 0.05% arabinose and quantified CT production (160). VC0486 encodes an uncharacterized DeoR family regulator. VC1320 (214) and VC1319 (160) encode the CarRS TCS that is involved in regulating lipopolysaccharide (LPS) remodeling and VPS production (220-222); *carR* (pTB15) and *carRS* (pTB3) were independently expressed. VC1081 encodes an uncharacterized response regulator. VC1638 was recently shown to regulate the expression of VCA0732 in response to polymyxin B (223). VC1825 is an AraC family regulator that regulates a phosphotransferase system (PTS) transporter (16). VC2714 encodes an uncharacterized response regulator. The results showed that only pTB11, expressing VC2714, repressed CT production (Fig. 10A). VC2714 encodes a homolog of the *E. coli* osmotic stress regulator OmpR with 92.1% amino acid sequence similarity and hereafter will be referred to as *ompR*.

To further verify that *V. cholerae* OmpR was a virulence repressor, we repeated the above experiment in WT *V. cholerae* harboring plasmid pTB11 during growth under AKI conditions in the presence of increasing arabinose concentrations and quantified CT and TcpA production. The results showed an arabinose-dependent inhibition of both CT and TcpA production (Fig. 10B). Based on these results, we conclude that OmpR functions as a virulence repressor in *V. cholerae*.

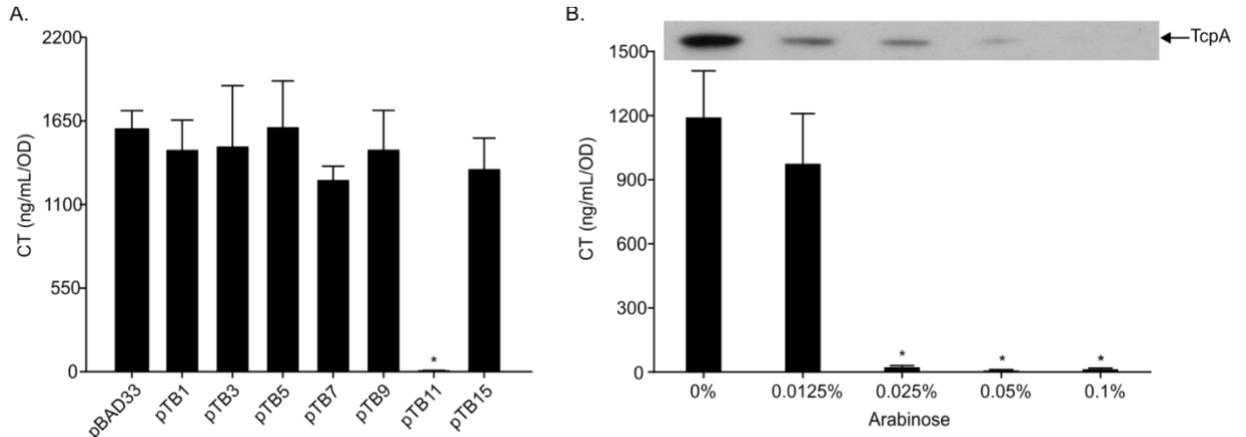


Figure 10 Overexpression of *ompR* represses virulence factor production

(A) WT *V. cholerae* harboring pBAD33 or the indicated expression plasmids were cultured under AKI conditions with 0.05% arabinose for 24h when culture supernatants were used for CT quantitation by GM1 ELISA. The pBAD33-based expression plasmids encode the following genes: pTB1 - VC0486; pTB3 - VC1320-VC1319; pTB5 - VC1081; pTB7 - VC1638; pTB9 - VC1825; pTB11 - VC2714; pTB15 - VC1320. The data represents the mean +/- SD of three independent experiments. * $P < 0.0001$ relative to pBAD33 determined by Dunnett's multiple comparisons test. (B) WT *V. cholerae* harboring pTB11 (pBAD33-*ompR*) was cultured under AKI conditions with indicated arabinose concentrations for 24h when culture supernatants were used for CT quantitation by GM1 ELISA and the cell pellets for TcpA immunoblotting, respectively. CT data represents the mean +/- SD of a minimum of three independent experiments. * $P < 0.0001$ relative to 0% determined by Dunnett's multiple comparisons test. The TcpA immunoblot is representative of a minimum of three independent experiments.

3.4.2 OmpR contributes to virulence repression in RND efflux-deficient *V. cholerae*

To verify that *ompR* was upregulated in RND-deficient *V. cholerae* as previously indicated in a transcriptomics data set (121), we introduced the *ompR-lacZ* transcriptional reporter plasmid pKD9 into WT and isogenic RND efflux-negative (Δ RND) *V. cholerae* strains and quantified *ompR* expression in both strains following growth in LB broth, in minimal T-medium, and under AKI conditions. The results showed significantly increased *ompR* expression in Δ RND relative to WT during growth under AKI conditions but no significant difference in LB broth or minimal T-medium (Fig. 11A). These findings confirm the previous study and suggest that the RND efflux-dependent induction of *ompR* is dependent on growth under AKI growth conditions.

We next tested if *ompR* contributed to the virulence repression observed in the RND-negative strain. To address this, we created *ompR* deletion strains in WT and RND-negative *V. cholerae* and quantified CT and TcpA production in WT, Δ RND, and their respective isogenic Δ *ompR* mutants. Confirming previous studies (150), the RND negative strain produced significantly reduced amounts of CT and TcpA relative to WT (Fig. 11B). Consistent with the finding that OmpR is a *V. cholerae* virulence repressor, deletion of *ompR* in WT resulted in a slight but not statistically significant increase in both CT and TcpA production (Fig. 11B). Deletion of *ompR* in the Δ RND background partially restored CT and TcpA production in the Δ RND Δ *ompR* strain relative to the parental Δ RND strain, but the magnitude of the increase did not reach WT levels (Fig. 11B). To determine if deletion of *ompR* was sufficient for the increased virulence factor production found between the Δ RND Δ *ompR* and Δ RND strains, *ompR* was complemented in the Δ RND Δ *ompR* strain by use of plasmid pTB11. Complementation of *ompR* in the Δ RND Δ *ompR* strain resulted in repression of both CT and TcpA production, indicating that deletion of *ompR* was in fact responsible for increased virulence factor production in the

Δ RND Δ ompR strain. Together, these data suggest that *ompR* contributes to virulence attenuation in the RND-negative background but that other factors are also involved in virulence repression.

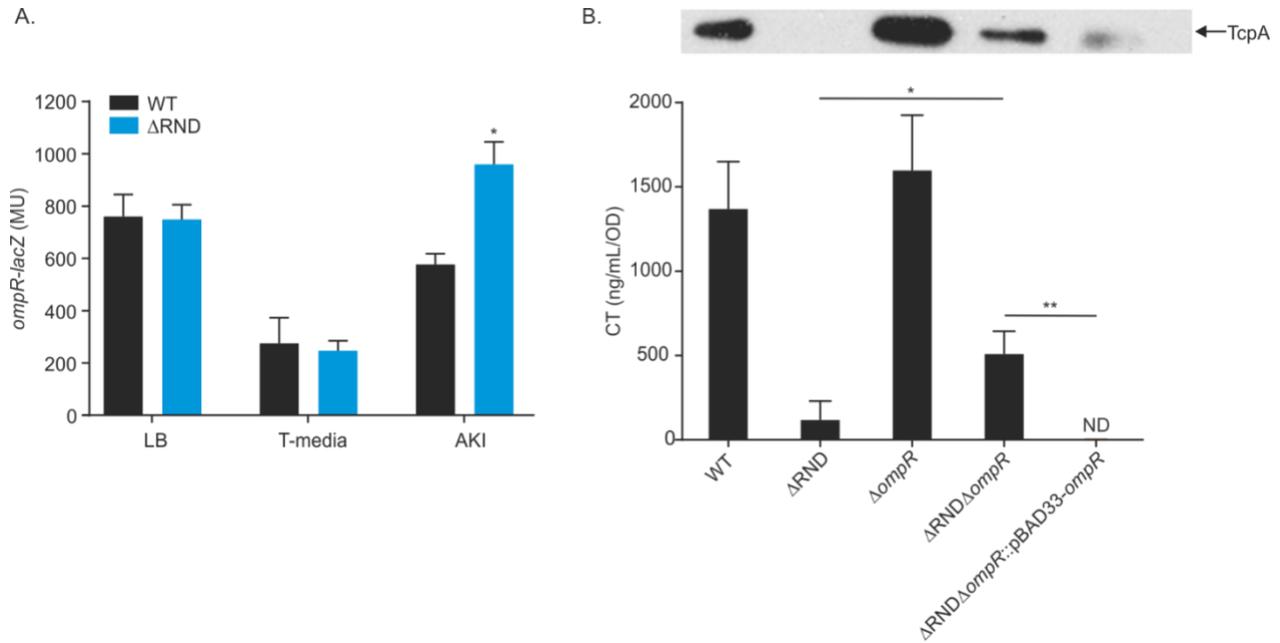


Figure 11 OmpR represses virulence factor production in RND efflux negative *V. cholerae*

(A) WT and Δ RND *V. cholerae* strains harboring an *ompR-lacZ* reporter plasmid were cultured under the indicated conditions for 5h when β -galactosidase activity was quantified. Data represents the mean \pm SD of three independent experiments performed in triplicate. * $P < 0.0001$ relative to WT determined by a t-test. (B) The indicated *V. cholerae* strains were cultured under AKI conditions with the Δ RND Δ ompR::pBAD33-ompR strain being cultured in the presence of 0.05% arabinose. At 24h culture samples were collected and CT and TcpA

production were assessed by GM1 ELISA and TcpA immunoblotting, respectively. The CT data represents the mean \pm SD of a minimum of three independent experiments. “ND” denotes non-detectable levels of CT. * $P < 0.05$, ** $P < 0.01$ relative to the parental strain, determined by Tukey's multiple comparisons test. TcpA immunoblot is representative of a minimum of three independent experiments.

3.4.3 *V. cholerae* OmpR represses *aphB* expression

The above results suggested that OmpR was a virulence repressor, but the mechanism by which it attenuated virulence factor production was unclear. As CT and TCP production is positively regulated by the ToxR regulon, we hypothesized that OmpR repressed components of the ToxR regulon. If this was true, then *ompR* deletion in Δ RND should increase the expression of the affected ToxR regulon genes relative to the parental strain Δ RND. We therefore compared ToxR regulon gene expression in Δ RND and its isogenic Δ *ompR* mutant during growth under AKI conditions. The results showed that *ompR* deletion in the RND-negative strain Δ RND did not significantly affect *aphA* expression (Fig. 12A) but did result in increased expression of *aphB* and the ToxR regulon genes downstream from *aphB* (i.e., *tcpP*, *toxT*, *ctxA*, and *tcpA*) (Fig. 12B through F). Δ RND and Δ RND Δ *ompR* had comparable levels of *toxR* expression, indicating that virulence repression by OmpR was not due to reduced *toxR* expression (Fig. 12G). As *aphB* is one of the most upstream regulators in the ToxR regulon, these results suggested that OmpR attenuated virulence factor production by repressing *aphB* in Δ RND.

To test if OmpR affected ToxR regulon expression in efflux-sufficient cells, we repeated the above experiments in the WT during growth under AKI conditions. The results showed that *ompR* deletion in the WT did not affect *aphA* expression but resulted in increased expression of *aphB* and its downstream target *tcpP* (Fig. 12I and J) but not the other ToxR regulon genes (Fig. 12H, J, K, L, M, and N). This is consistent with the observation that deletion of *ompR* did not significantly affect CT or TcpA production in the WT (Fig. 12B). Collectively, these results support the conclusion that OmpR is an *aphB* repressor and that OmpR regulation of *aphB* is relevant in WT cells during growth under AKI conditions.

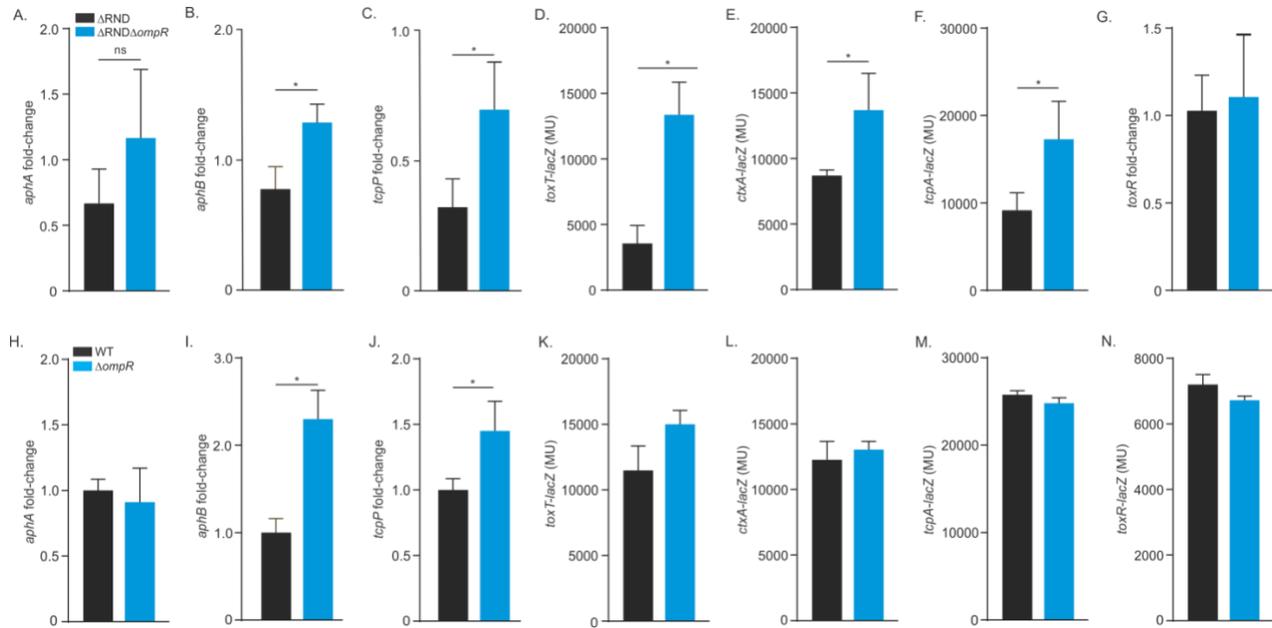


Figure 12 OmpR represses the ToxR regulon

V. cholerae strains were cultured under AKI conditions when gene expression was assessed using *lacZ* promoter reporters (panels D-F and K-N) or qRT-PCR (panels A-C, G-I, and J) as described in Section 3.3.6. Reporter gene expression in Δ RND and Δ RND Δ ompR *V. cholerae* strains. (H-N) Reporter gene expression in WT and Δ ompR *V. cholerae* strains. The results presented in panels A-C and H-J were generated at 3.5h post inoculation, the remaining assays were generated at 5h post inoculation. Data represents mean and SD of at least three independent experiments performed in triplicate. * P<0.05 relative to parental strain determined by a t-test.

3.4.4 Ectopic *ompR* expression represses *aphB* transcription in *E. coli*

To further confirm that OmpR can repress *aphB*, we tested if ectopic *ompR* expression altered *aphB* expression in *V. cholerae* and the heterologous host *E. coli*. In the first set of experiments, we expressed *ompR* from pTB11 in WT *V. cholerae* bearing *lacZ* transcriptional reporters for *aphA* and *aphB* during growth under AKI conditions in the presence of varying arabinose concentrations to induce *ompR* expression. The results showed a small arabinose dose-dependent increase in *aphA* expression (Fig. 13A); the biological significance of this finding is

unclear. By contrast, we observed an arabinose dose-dependent decrease in *aphB* expression (Fig. 13B), confirming that OmpR is an *aphB* repressor. Although OmpR may have a weak ability to induce *aphA* expression, its ability to repress *aphB* appears to be dominant, as the net consequence of *ompR* regulation of *aphA* and *aphB* is repression of *tcpP* (Fig. 12C and J).

In the second set of experiments, we expressed *V. cholerae ompR* from pTB11 in *E. coli* bearing *aphA-lacZ* or *aphB-lacZ* transcriptional reporters to address whether OmpR acted directly at the respective promoters. The *E. coli* strains were cultured to mid-log phase in the presence of varying arabinose concentrations when *aphA-lacZ* or *aphB-lacZ* expression was quantified. The results showed that arabinose addition had little effect on *aphA* expression (Fig. 13C). By contrast, there was an arabinose dose-dependent decrease in *aphB* expression (Fig. 13D), consistent with OmpR being an *aphB* repressor. These results suggest that OmpR may act directly at the *aphB* promoter; however, we cannot exclude the possibility that OmpR could be acting through an intermediate that is present in both *E. coli* and *V. cholerae*.

As OmpR has been shown to function as an active transcription factor in both the unphosphorylated and phosphorylated forms, we investigated if the phosphorylation of *V. cholerae* OmpR plays a role in its repression of *aphB*. To this end, OmpR point mutants mimicking constitutive phosphorylation (D55E) and constitutive dephosphorylation (D55A) of the conserved aspartate were cloned into pTB11 (225, 226). The mutant *ompR* constructs were then expressed in *E. coli*, and *aphA* and *aphB* expression was quantified as before. Consistent with the above findings, expression of the phosphomimic *ompR* mutants did not significantly affect *aphA* expression (Fig. 13E). By contrast, expression of the *ompRD55E* allele strongly repressed *aphB* expression, whereas expression of the *ompRD55A* allele only marginally repressed *aphB*, and to a far lesser extent than the *ompRD55E* allele (Fig. 13F). Taken together, these results indicate that

both phosphoforms of *V. cholerae* OmpR have the capacity to repress *aphB* transcription, but the phosphorylated form (i.e., *ompRD55E*) is predominately responsible for *aphB* repression. Collectively, these results support the conclusion that OmpR negatively regulates the ToxR regulon via directly repressing *aphB* transcription, and they suggest that the phosphorylated form of OmpR is primarily responsible for this repression.

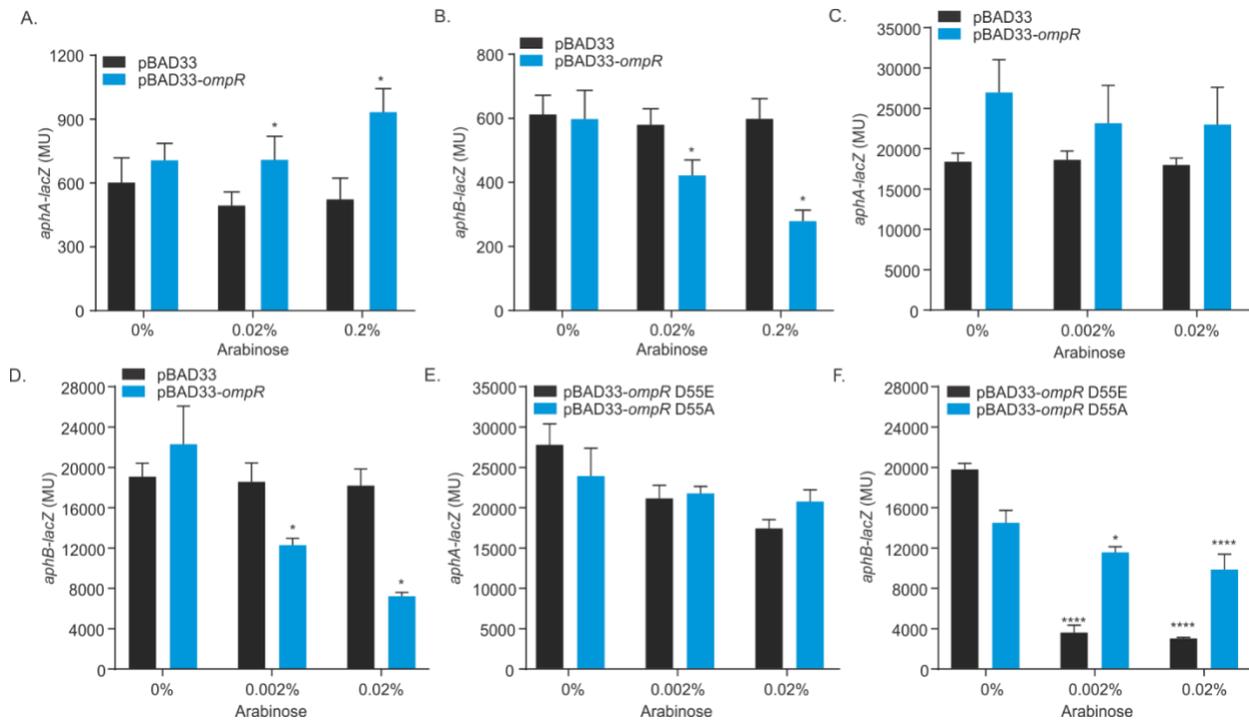


Figure 13. *V. cholerae* OmpR represses *aphB* expression

(A-B) WT *V. cholerae* harboring either pBAD33 or pBAD33-*ompR* (pTB11) with either *aphA-lacZ* (pXB202) or *aphB-lacZ* (pXB203) reporter plasmids were cultured under AKI conditions for 5h with the indicated arabinose concentrations when β -galactosidase activity was quantified. Data represents the mean +/- SD of three independent experiments performed in triplicate.

(C-D) *E. coli* strain EC100 harboring either pBAD33 or pBAD33-*ompR* (pTB11) with either *aphA-lacZ* (pXB202) or *aphB-lacZ* (pXB203) reporter plasmids were cultured in LB broth for 5h with the indicated arabinose concentrations when β -galactosidase activity was quantified. Data represents the mean +/- SD of three independent experiments performed in triplicate.

(E and F) *E. coli* strain EC100 harboring indicated pBAD33-*ompR* aspartate 55 mutant constructs (i.e. D55E or D55A) with either *aphA-lacZ* or *aphB-lacZ* reporter plasmids were cultured in LB broth with the indicated arabinose concentrations for 5h when β -galactosidase activity was quantified. Data represents the mean +/- SD of three independent experiments performed in triplicate.

* $P < .01$, **** $P < 0.001$ relative to 0% arabinose determined by Sidak's multiple comparisons test.

3.4.5 *V. cholerae ompR* is induced by bile salts and detergents

While the above data showed that OmpR functions as a virulence repressor through repression of *aphB*, we wished to address the environmental factors that modulate *ompR* expression in *V. cholerae*. OmpR has been extensively studied in *Enterobacteriaceae*, where it has been shown to function as an osmoregulator that mediates adaptive responses to osmotic stress (101, 204, 227). We therefore tested if *V. cholerae ompR* responded to changes in medium osmolarity by quantifying *ompR-lacZ* expression during growth under AKI conditions in standard AKI broth (86 mM NaCl), AKI broth with low NaCl (21.5 mM), and AKI with excess NaCl (250 mM). As shown in Fig. 14A, the NaCl concentration did not significantly affect *ompR* expression, suggesting that *ompR* was not regulated in response to osmolarity. Consistent with this, growth analysis showed that *ompR* was dispensable for growth in high-osmolarity broth up to 500 mM NaCl in both WT and Δ RND backgrounds (Fig. 14B and C). From these results, we conclude that *V. cholerae* OmpR is not regulated in response to medium osmolarity and therefore likely responds to different environmental stimuli than what is observed in *Enterobacteriaceae*.

The finding that *ompR* was induced in the absence of RND-mediated efflux (Fig. 14A) suggested that small molecules that accumulate intracellularly in the absence of RND efflux may play a role in *ompR* expression. Previous studies showed that a major function of the *V. cholerae* RND efflux systems was in resistance to hydrophobic and detergent-like molecules, including bile salts, fatty acids, and detergents (84, 150). We therefore tested if bile salts or detergents affected *ompR* expression as described above. The results showed that the addition of deoxycholate, bile salts, oxgall, and SDS to the growth media increased *ompR* expression (Fig. 14D). We also tested another small molecule, indole. Indole is a *V. cholerae* metabolite that is an RND efflux substrate and virulence repressor (78, 84). The data showed that indole did not affect *ompR* expression,

suggesting that altered *ompR* expression was specific for compounds with detergent-like properties. As detergents are associated with envelope stress due to their membrane-intercalating properties, we hypothesized that *ompR* may be induced in response to envelope stress. To test this, we quantified *ompR* expression following the induction of membrane stress by ethanol treatment (228). The results of these experiments showed that there was an ethanol dose-dependent increase in *ompR* expression (Fig. 14E). Taken together, these findings suggest that *V. cholerae ompR* is likely regulated in response to membrane perturbations resulting from exposure to membrane-intercalating agents.

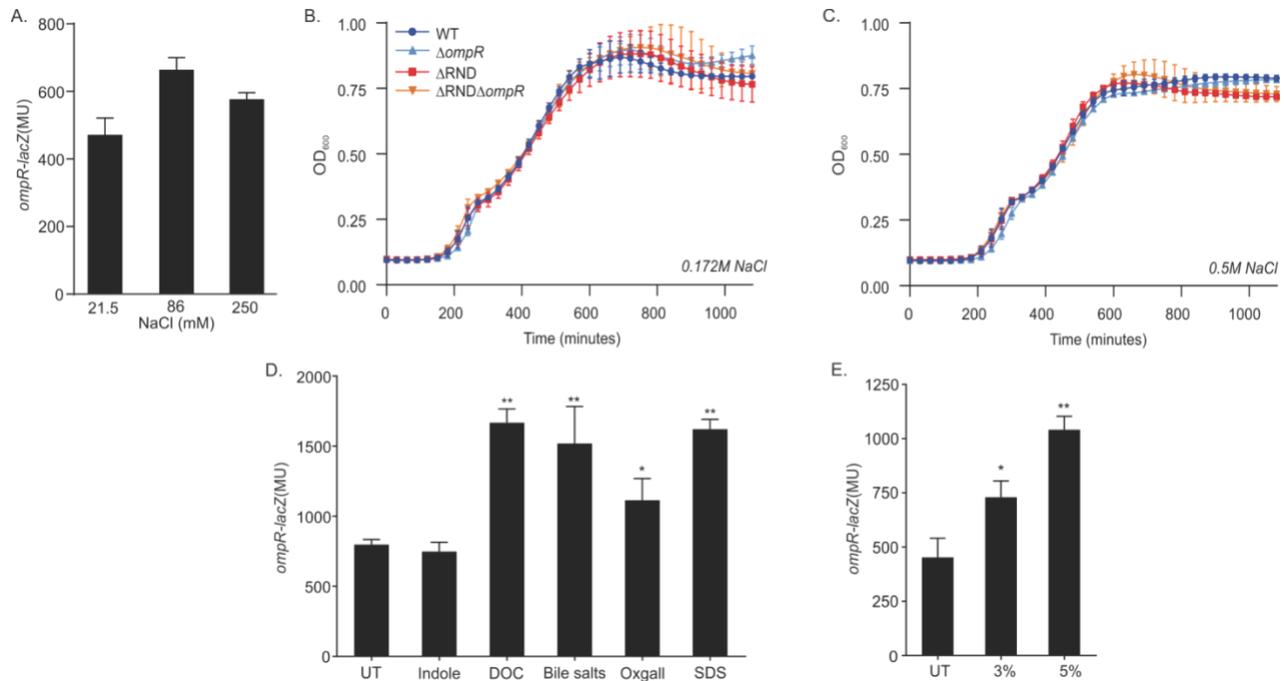


Figure 14 *V. cholerae ompR* does not respond to osmolarity but is induced by membrane intercalating agents

(A) WT *V. cholerae* harboring an *ompR-lacZ* reporter plasmid (pDK9) was cultured under AKI conditions with the indicated NaCl concentrations for 5h when β -galactosidase activity was quantified. (B-C) Growth analysis of *V. cholerae* $\Delta ompR$ mutants. Overnight LB cultures of *V. cholerae* WT, $\Delta ompR$, ΔRND , and $\Delta RND\Delta ompR$ were diluted 1:10,000 in either fresh (B) LB medium (0.172M NaCl), or (C) LB medium containing a total concentration of 0.5M NaCl and cultured at 37°C with constant shaking in a microtiter plate reader. Growth was recorded as the OD₆₀₀ every 30 minutes. Data indicates average of at least three independent experiments performed in triplicate. (D-E) WT *V. cholerae* harboring an *ompR-lacZ* reporter plasmid (pDK9) was cultured in LB broth for 4h when the indicated RND efflux substrates (D) or ethanol (E) were added to the culture medium. The cultures were then incubated with shaking for an additional hour when β -galactosidase activity was quantified. Data indicates the average \pm SD of three independent experiments performed in triplicate. “UT” denotes untreated samples, *P< .01, ** P<.001 relative to untreated determined by Dunnett's multiple comparisons test.

3.4.6 C18-depleted AKI medium nullifies *ompR* induction in an RND-negative *V. cholerae* strain

Based on the results above, we hypothesized that hydrophobic and/or nonpolar compounds present in the AKI medium were accumulating in the RND efflux-deficient strain Δ RND and activating *ompR* transcription. To test this, we generated a depleted AKI medium by passing AKI broth through a Sep Pak C18 cartridge to deplete nonpolar and hydrophobic compounds from the medium. We then quantified *ompR* expression in WT and RND-negative strains harboring pDK9 (*ompR-lacZ*) following growth under AKI conditions in AKI broth and in the C18-depleted AKI medium. The results showed increased *ompR* expression in Δ RND during growth in AKI broth as expected (Fig. 15). Growth of the WT in the C18-depleted AKI medium did not affect *ompR* expression when compared to expression in the standard AKI medium. However, growth of Δ RND in the C18-depleted AKI medium alleviated the increase in *ompR* transcription observed in the standard AKI medium. To determine if the hydrophobic compounds from the AKI medium that were retained on the C18 column were responsible for *ompR* induction in Δ RND, we eluted the retained compounds from the C18 cartridges used to extract AKI and LB broth. We then determined if the respective eluates contained *ompR*-inducing compounds by adding them to LB broth cultures of Δ RND and WT and quantifying *ompR-lacZ* expression. The results showed that the addition of the AKI medium C18 column eluate, but not LB C18 column eluate, activated *ompR* expression in Δ RND, while neither eluate had an effect on *ompR* expression in the WT (Fig. 15). Collectively, these data suggest that hydrophobic and/or nonpolar compounds present in the AKI medium are responsible for increased *ompR* expression in the RND-negative strain. The fact that the C18-depleted AKI medium did not affect *ompR* expression in the WT indicated that this phenotype was RND-dependent. Significantly, we also observed that the increase in *ompR*

expression in Δ RND was not dependent on growth under AKI conditions (i.e., static growth followed by shaken growth), as *ompR* expression was also enhanced in cultures grown in AKI broth under noninducing conditions (not shown). This observation, combined with the finding that *ompR* was not induced in Δ RND during growth in LB broth or T-medium (Fig. 11A), suggested that the *ompR*-inducing molecules were only present in the AKI medium. From these experiments, we conclude that hydrophobic and/or nonpolar compounds that are present in AKI medium, but not the LB medium, are responsible for *ompR* activation in Δ RND. Further, because this phenotype was RND efflux dependent, we infer that the inducing compounds are substrates for the *V. cholerae* RND efflux systems.

The observation that growth in C18-depleted AKI medium resulted in an RND efflux-dependent repression of *ompR* expression, coupled with the finding that OmpR is a virulence regulator, led us to explore if C18 depletion of the AKI medium also affected virulence factor production. To explore this, CT and TcpA production was quantified in WT and Δ RND strains cultured under AKI conditions in either standard AKI medium or a C18-depleted AKI medium. As previously illustrated, the Δ RND strain had reduced CT and TcpA production compared to WT when cultured in the standard AKI medium. Interestingly, growth of WT in the C18-depleted AKI medium resulted in a reduction in both CT and TcpA production (Fig. 15B). Inversely, growth of a Δ RND strain in the C18-depleted AKI medium resulted in an increase in both CT and TcpA production to levels relative to standard AKI-cultured WT. Collectively, these data suggest that the nonpolar and hydrophobic compounds that are extracted from the AKI medium by the Sep Pak C18 column play a role in both *ompR* expression and virulence factor production. The nature of these molecules will require further investigation.

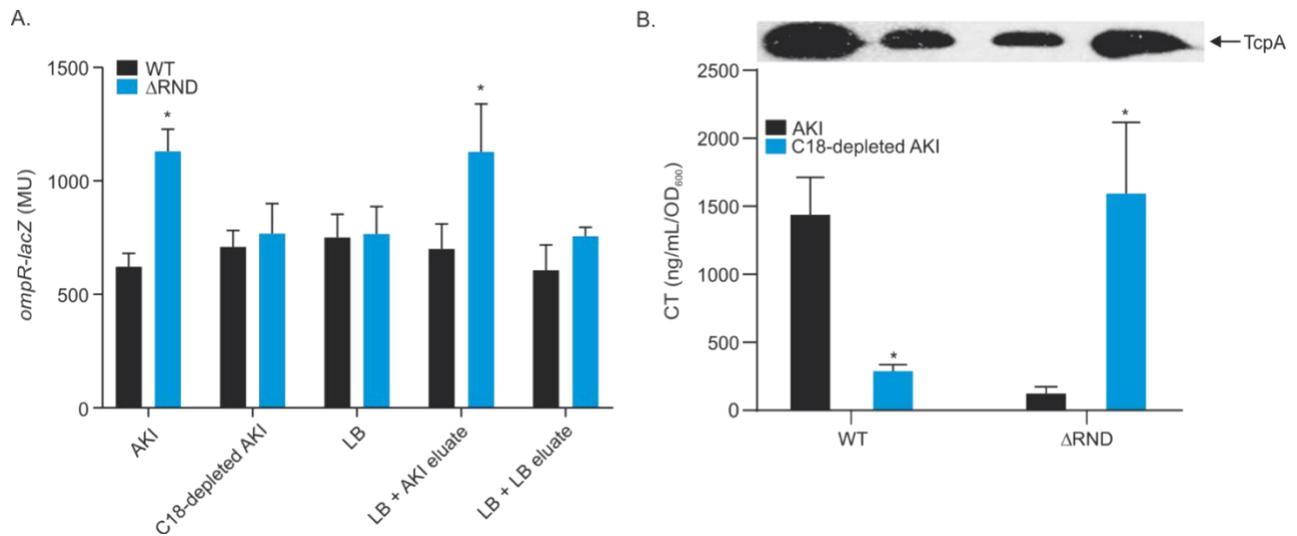


Figure 15 Conditioned AKI medium abolishes the RND efflux-dependent induction of *ompR* expression in *V.*

cholerae

(A) WT and Δ RND *V. cholerae* strains harboring an *ompR-lacZ* reporter plasmid (pDK9) were cultured in the indicated media for 5h when *ompR-lacZ* expression was quantified. * $P < 0.01$ relative to WT determined by a t-test.

(B) WT and Δ RND *V. cholerae* strains were cultured under AKI conditions in either AKI medium or C18-depleted AKI medium for 24h when CT and TcpA production were assessed by GM1 ELISA and TcpA immunoblotting, respectively. Data represents the mean +/- SD of three independent experiments. * $P < 0.01$ relative to AKI, determined by t-test. C18-depleted AKI medium and C18 column AKI medium eluates were prepared as described in section 3.3.

3.5 Discussion

V. cholerae is an inhabitant of the aquatic ecosystem that can colonize the human gastrointestinal tract to cause disease. The ability of *V. cholerae* to replicate in these two disparate ecosystems is dependent upon its ability to rapidly adapt to the changing environments it encounters. For example, upon host entry, *V. cholerae* must adjust to dramatic changes in temperature, pH, salinity, oxygen tension, and the presence of antimicrobial compounds. At the

same time, colonization of the intestinal tract requires the expression of niche-specific genes (e.g., virulence factors). Prior to exiting the host, *V. cholerae* must also regulate the expression of genes that are important for transmission and dissemination (42-44, 77). How all of these responses are integrated within the dynamic environment in the host is poorly understood. What is clear is that periplasmic sensing systems play a critical role in the process. This includes ToxR, which regulates host entry; the Cad system, which contributes to acid tolerance; the CarRS TCS, which mediates antimicrobial peptide resistance; OsrR, which regulates response to osmolality; and stress responses like the Cpx system that alleviate stress due to the presence of antimicrobial compounds in this host (37, 39, 220-222, 229, 230).

In this chapter, we interrogated the function of six regulatory genes on virulence factor production in *V. cholerae*. All of the tested regulatory genes were identified as being upregulated in an RND efflux-negative *V. cholerae* mutant (121). As RND-mediated efflux is required for virulence factor production, these induced regulatory genes represented potential efflux-dependent virulence repressors. We found that *ompR* contributed to repression of virulence factor production in the RND-null strain by repressing *aphB* expression. AphB is a key regulator in the ToxR virulence regulon (71). Previous studies have shown that AphB activity is modulated by low oxygen and acidic pH, but it was unknown whether expression of *aphB* was itself regulated (75). To our knowledge, OmpR is the first regulator shown to modulate *aphB* expression in *V. cholerae*. We further demonstrated that *ompR* was activated in response to membrane-intercalating compounds that are abundant in the host, suggesting that this regulatory circuit may be relevant *in vivo*.

Although the function of OmpR has been widely explored in *Enterobacteriaceae*, the function of the *V. cholerae* OmpR homolog has not been investigated previously. OmpR is known

as an osmoregulator in *Enterobacteriaceae*. Expression of *ompR* is induced at high salt concentrations, and OmpR regulates transcriptional responses to alleviate osmotic stress (100, 104, 231). Herein we report that *V. cholerae ompR* was not induced in response to osmolality and that *ompR* was dispensable for growth at high salt concentrations. These findings were consistent with two previous studies on *V. cholerae* responses to osmolarity (229, 232), neither of which identified *ompR* as one of the genes to respond to increased osmolarity. In the latter study, OsrR was identified as an osmoregulator which regulated motility and biofilm formation (229). We did not observe any effect of *ompR* on either of these two phenotypes (not shown), suggesting that OsrR and OmpR function independently. Taken together, these results suggest that *V. cholerae* OmpR has evolved to respond to different environmental stimuli and fulfill new functions.

Bacterial regulatory networks evolve in response to evolutionary pressures placed on individual species, as they inhabit specific niches (84, 233). TCSs have been suggested to evolve under such selective pressures to respond to novel stimuli and regulate diverse target genes to meet the needs of specific bacterial species (218). EnvZ/OmpR is an example of this. While EnvZ/OmpR is ubiquitous in *Gammaproteobacteria*, its function appears to have evolved divergently in several bacterial species (109, 205, 206, 212, 234). Our results suggest that this divergent evolution has also occurred in *V. cholerae*. We speculate that the lifestyle of *V. cholerae*, which involves growth in marine environments and the human host gastrointestinal tract, has selected for OmpR to respond to novel stimuli and to fulfill a novel physiological role in *V. cholerae*. Sequence comparison of the *V. cholerae ompR* locus to the *E. coli ompR* locus supports this hypothesis. While *V. cholerae* OmpR is 83% identical in amino acid sequence to its *E. coli* homolog, the *V. cholerae* EnvZ sensor kinase is only 47% identical to its *E. coli* counterpart.

OmpR functioned as a repressor of virulence factor production, and its expression was induced by the addition of membrane-intercalating compounds such as detergents and ethanol. Further, our OmpR phosphomimic studies suggested that it was the phosphorylated form of OmpR that was responsible for virulence repression. This likely explains the upregulation of *ompR* in the RND-negative background, as cells lacking RND-mediated efflux are hypersensitive to membrane-intercalating compounds due to the RND mutant's diminished ability to actively efflux these compounds from within the cell (84, 149, 150). We speculate that virulence repression in the RND-null mutant resulted from the intracellular accumulation of nonpolar and hydrophobic molecules that are present in AKI medium (e.g., fatty acids and detergent-like molecules). This hypothesis is supported by the finding that WT and RND-negative *V. cholerae* strains have comparable *ompR* expression when cultured in a C18 cartridge-depleted AKI medium. These molecules are likely the substrates of the RND transporters and thus accumulated in the RND-negative mutant, resulting in *ompR* induction and subsequent virulence repression. This is supported by the observations that OmpR contributes to virulence repression in an RND-negative strain and that growth of the RND-negative strain in the C18-extracted AKI medium attenuated *ompR* expression.

Bile salts are found at high concentrations in the lumen of the small intestine. The finding that *ompR* was activated in response to bile salts suggests the possibility that OmpR could contribute to spatial and temporal virulence regulation that has been observed *in vivo* (235). Following ingestion, *V. cholerae* enters the host small intestine, migrates to the intestinal epithelium, and activates virulence factor production while colonizing the intestinal epithelium. This suggests the possibility that high bile salt concentrations within the intestinal lumen may activate *ompR* to repress virulence factor production. Once *V. cholerae* traverses the mucus layer

to colonize the epithelial surface, where bile salt concentrations are reduced, OmpR-mediated virulence repression would be alleviated. This tight regulation of virulence factor production is paramount to the pathogenic success of *V. cholerae*. It is interesting to speculate that *V. cholerae* OmpR is one of multiple factors that converge on the ToxR regulon to ensure that it is only expressed in the appropriate *in vivo* niche. It is noteworthy that bile salts and fatty acids have pleiotropic effects on the ToxR regulon. Fatty acids have been shown to negatively affect ToxT activity (236). Bile salts have been implicated in intermolecular disulfide bond formation in TcpP (83). Bile acids, fatty acids, and other detergent-like compounds also signal through ToxR to repress *aphA* (121, 237). Thus, there seems to be a coordinated response by *V. cholerae* to these environmental cues to effect virulence factor production.

The induction of *V. cholerae ompR* in response to nonspecific membrane intercalating agents suggests that OmpR could also function as part of a generalized membrane stress response. Consistent with this, there is evidence that OmpR in *Enterobacteriaceae* may be a component of other stress response systems (238, 239). A conserved response to membrane stress in bacteria includes suppressing membrane protein production as a mechanism to alleviate envelope stress. Thus, OmpR-dependent virulence repression in *V. cholerae* could conceivably contribute to a membrane stress response because the ToxR regulon controls the expression of many membrane-bound and secreted proteins, including the two major outer membrane porins OmpU and OmpT (157). However, analysis of WT and $\Delta ompR$ whole-cell lysates by SDS-PAGE staining did not reveal any effect of OmpR on the production of OmpU and OmpT (not shown), which is consistent with the finding that OmpR did not affect *toxR* expression or protein production (Fig. 12 and data not shown). This contrasts what is observed in other bacterial species where OmpR regulates the expression of outer membrane porins (100-102, 203, 240).

4.0 *Vibrio cholerae* OmpR contributes to virulence repression and fitness at alkaline pH

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4.1 Project summary

Vibrio cholerae is a Gram-negative human pathogen and the causative agent of the life threatening disease cholera. *V. cholerae* is a natural inhabitant of the marine environment which enters humans through the consumption of contaminated food or water. The ability to transition between aquatic ecosystems and the human host is paramount to the pathogenic success of *V. cholerae*. The transition between these two disparate environments requires the expression of adaptive responses which are most often regulated by two-component regulatory systems such as the EnvZ/OmpR system, which responds to osmolarity and acidic pH in many Gram-negative bacteria. Previous work in our laboratory indicated that *V. cholerae* OmpR functioned as a virulence regulator through repression of the LysR-family transcriptional regulator *aphB*; however, the role of OmpR in *V. cholerae* biology outside of virulence regulation remained

unknown. In this chapter we sought to further investigate the function of OmpR in *V. cholerae* biology by defining the OmpR regulon through RNA-sequencing. This led to the discovery that *V. cholerae ompR* was induced at alkaline pH to repress genes involved in acid tolerance and virulence factor production. In addition, OmpR was required for *V. cholerae* fitness during growth in alkaline conditions. These findings indicate *V. cholerae* OmpR has evolved the ability to respond to novel signals during pathogenesis which may play a role in the regulation of adaptive responses to aid in the transition between the human gastrointestinal tract and the marine ecosystem.

4.2 Introduction

Vibrio cholerae is a Gram-negative facultative pathogen, and the causative agent of the disease cholera. *V. cholerae* is a native member of the marine microbiota. Pathogenic strains of *V. cholerae* enter the human host from the environment through the consumption of *V. cholerae* contaminated food or water. Following ingestion, *V. cholerae* traverses the gastrointestinal tract to ultimately colonize the surface of the distal small intestine (2). During this process *V. cholerae* activates the expression of virulence factors. The most important virulence factors for disease progression are the enterotoxin cholera toxin (CT), which is responsible for the secretory diarrhea that is the hallmark of the disease cholera, (241) and the toxin coregulated pilus (TCP), which facilitates intestinal colonization (39). Expression of CT and TCP are tightly controlled by a hierarchical regulatory cascade known as the ToxR regulon (197). Activation of this regulon begins with the expression of two cytoplasmic transcription factors, *aphA* and *aphB* (71, 72). Apha and AphB together activate the transcription of *tcpP*. TcpP then acts along with ToxR to activate

toxT expression. ToxT directly activates the expression of the genes that encode for the production of CT and TCP (197). Fine tuning of the ToxR regulon is in part mediated by factors affecting the production and activity of AphA and AphB and their activity (75, 77, 242).

The complex lifecycle of pathogenic *V. cholerae*, consisting of growth in aquatic ecosystems and the human gut, necessitates that *V. cholerae* rapidly adapt to sudden environmental changes, including changes in nutrient availability, osmolarity, temperature, and pH. There is an array of regulatory systems that control these adaptive responses during *V. cholerae* infection (43, 82, 199, 243). Early in infection *V. cholerae* genes involved in the acid tolerance response (ATR) are induced as the cells pass through the acidic environment of the stomach (36-38, 82). After exiting the stomach, *V. cholerae* adapts to the more alkali environment in the small intestine by repressing ATR genes, and a failure to do so results in an intestinal colonization defect (82). At the same time *V. cholerae* activates the expression of genes required for colonization and disease development (i.e. the ToxR regulon). During the late stages of infection, before exiting the host in the diarrheal purge, *V. cholerae* represses virulence genes while inducing genes required for dissemination and transmission (42-44, 77). The genetic mechanisms and environmental cues that contribute to adaptive responses that govern these late infection responses in *V. cholerae in vivo* remain largely unexplored.

Two-component regulatory systems (TCS) play a critical role in regulating adaptive responses to environmental cues. TCS are widespread in bacteria and transduce extracellular signals into transcriptional responses. The prototypical TCS consists of a membrane bound sensor histidine kinase (HK), and a cytoplasmic response regulator (RR) (7). The HK contains a signal sensing domain that monitors the extracellular environment for specific activating signals (7). Upon activation, the HK autophosphorylates, then transfers its phosphate group to a conserved

aspartate residue on its cognate RR. Phosphorylation of the RR's results in its activation which then functions to modulate transcriptional responses to the inducing cue (244). Most response regulators function as transcription factors but they can also effect adaptive responses by other mechanisms (85). One of the most well studied TCS is the EnvZ/OmpR system. EnvZ/OmpR is ubiquitous in Gram-negative bacteria and has been extensively studied for its role in regulating porin production in response to changes in environmental osmolarity and acidic pH (100, 101, 104, 200, 202). We recently reported that *V. cholerae* OmpR was a virulence repressor that regulated expression of the ToxR regulon by repression of *aphB* (242). Our work also showed that *V. cholerae ompR* did not respond to changes in osmolarity, the canonical *ompR* inducing signal in other Gram-negative bacteria. Instead, *V. cholerae ompR* was induced by membrane stress-inducing agents that are found within the host gut. Collectively, these findings suggested that OmpR evolved to respond to niche-specific environmental signals in *V. cholerae*.

In this chapter we further explored the function of OmpR in *V. cholerae*. Using transcriptional profiling and genetic approaches we documented that OmpR functions in the *V. cholerae* ATR by mechanisms that were mediated through its regulation of *aphB*. Further, we show that *ompR* was induced at alkaline pH and contributed to *V. cholerae* fitness at alkaline pH. Collectively, our results extend the function of *ompR* in *V. cholerae* biology to include both virulence factor production and adaptation to alkaline pH; two phenotypes that are relevant to human infection.

Table 3 Strains, plasmids, and oligonucleotides used in chapter 4

Strain:	Genotype:	Source:
<i>E. coli</i>		
EC100Dpir+	<i>F. mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) Φ 80 <i>dlacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1 endA1 araD139</i> Δ (<i>ara, leu</i>)7697 <i>galU galK</i> λ - <i>rpsL</i> (<i>Str_R</i>) <i>nupG pir+</i>	Epicentre
SM10 λ pir	<i>thi-1 thr leu tonA lacY supE recA::RP4-2-4-Tc::Mu Km_r</i> (λ . <i>pirR6K</i>)	(157)
<i>V. cholerae</i>		
JB58	01 El Tor strain N16961 Δ <i>lacZ</i> Sm _r	Lab collection (74)
JB485	JB58 Δ <i>vexB</i> Δ <i>vexD</i> Δ <i>vexF</i> Δ <i>vexH</i> Δ <i>vexK</i> Δ <i>vexM</i>	(242)
DK243	JB58 Δ <i>ompR</i>	(75)
XBV153	JB58 Δ <i>aphA</i>	(216)
XBV148	JB58 Δ <i>aphB</i>	
Plasmids:	Description:	
pTL61T	<i>lacZ</i> transcriptional reporter, Amp _r	(159)
pBAD33	Arabinose regulated expression vector	(160)
pWM91	Allelic exchange vector, Amp _r	(43)
pTB11	pBAD33 containing VC2714 (<i>ompR</i>)	(242)
pDK14	pBAD33 expressing VC2714-D55E	(242)
pDK15	pBAD33 expressing VC2714-D55A	(242)
pDK9	pTL61T containing the <i>ompR</i> promoter	(242)
pVA212	pTL61T containing the <i>cadB</i> promoter	(216)
pXB239	pTL61T containing the <i>cadC</i> promoter	(216)
pXB202	pTL61T containing the <i>aphA</i> promoter	(215)
pXB203	pTL61T containing the <i>aphB</i> promoter	(215)
pXB194	pTL61T containing the <i>tcpA</i> promoter	(121)
Oligonucleotides:	Sequence (5'-3'):	
P- <i>clcA</i> -F-xhaI	TTTCTCGAGCATGCGCAGGTTTCGTGGC	
P- <i>clcA</i> -R-xbaI	GGGTCTAGAGGCGTCTTATCTTTGGAG	
<i>gyrA</i> -F	CAATGCCGGTACACTGGTACG	
<i>gyrA</i> -R	AAGTACGGATCAGGGTCACG	
q- <i>ompW</i> .VCA0867-1	GGACGGAAAGTCGAATTAGC	
q- <i>ompW</i> .VCA0867-2	TCTGGTGGTGAGTTAGGTAG	
q- <i>ompV</i> .VC1318-1	CACCGTGGTAACCAAAGTTC	
q- <i>ompV</i> .VC1318-2	GCTGCCCAAACCTTACATTTCG	
q- <i>ompTa</i> .VC0972-1	TGACTGGCCATACTCTAACC	
q- <i>ompTa</i> .VC0972-2	CACGACCAGCAGAAATGTTG	
<i>OmpU</i> qPCR-1	ACACCGTATAGGCTGTCATTG	
<i>OmpU</i> qPCR-1	GTGCTGAAGCTCGCCTATCTC	
VC1854-RT1	CTGCGTCAGAACCCTTGTTG	
VC1854-RT2	AGCTGGGCTCGTGTATATGC	
qRT- <i>cadC</i> -F	CGTCCGCAGTCCAAAAAGG	
qRT- <i>cadC</i> -R	TCACTCAACACCAGCATCCG	
qRT- <i>cadB</i> -F	TCTGTTTGGTTGGACGCACT	
qRT- <i>cadB</i> -R	CCCACGAAAGACCACAGACA	
qRT- <i>cadA</i> -F	CGGGAATGAGCAAAGATGGC	
qRT- <i>cadA</i> -R	CCGATAGAGAACAGGAACAGCA	
qPCR- <i>clcA</i> -5	GAAGGTCCTACAGTACAA	

Table 3 continued

Oligonucleotides:	Sequence (5'-3'):
qPCR- <i>clcA</i> -3	CAGGGTATAGCGAAATTG
qRT- <i>aphB</i> -F	ATCGGTGAAGTGAAAGACATTTTGG
qRT- <i>aphB</i> -R	GATGTTGATGCAACTCTTCAGCAT

4.3 Materials and methods used in chapter 4

4.3.1 Bacterial strains and culture conditions

The bacterial strains and plasmids used in this chapter are listed in Table 3. *E. coli* strains EC100Dpir+ was used for cloning. *V. cholerae* strain JB58 was used as wild-type (WT) in all experiments. Bacterial strains were grown at 37°C in lysogeny broth (245) or on LB agar. AKI growth conditions were used to induce *V. cholerae* virulence gene expression as previously described (217). Modified T-medium was prepared as previously described (246). Antibiotics were used at the following concentrations: streptomycin (Sm) 100 µg/mL; carbenicillin (Cb), 100 µg/mL; chloramphenicol (Cm), 20 µg/ml for *E. coli* and 1 µg/ml for *V. cholerae*.

4.3.2 Growth curve experiments

Growth curves were generated in 96-well microtiter plates. Overnight LB cultures of WT and $\Delta ompR$ strains were diluted 1:10,000 in fresh LB broth containing either 0 or 35 mM NaOH; 0.05% L-arabinose and 1 µg/mL Cm was added to the media for the complementation studies. Two-hundred microliters of the diluted cultures were then aliquoted into triplicate wells of a 96-

well microtiter plate and the plates were incubated at 37°C with shaking in a Biotek ELX-808 microplate reader and growth was then monitored as the OD₆₃₀ every 30 min.

4.3.3 Transcriptional reporter assays

V. cholerae harboring the indicated *lacZ* reporters were cultured under AKI conditions or in LB broth. At the indicated times, aliquots were collected in triplicate and β -galactosidase activity was quantified as previously described (219). For experiments assessing transcriptional response to alkaline pH, strains harboring the indicated *lacZ* transcriptional reporter fusions were cultured under AKI conditions for 4h when they were treated with 20mM NaOH to raise the pH to ~8.7 or treated with water as the control. Note that pH 8.7 was used for all of the transcriptional reporter analyses as it did not affect the growth of any of the analyzed strains under the tested conditions. The cultures were then incubated with shaking for an additional hour before culture aliquots were collected in triplicate and β -galactosidase production was assessed. All of the transcriptional reporter experiments were performed independently at least three times.

4.3.4 RNA sequencing

A *V. cholerae* $\Delta ompR$ strain harboring either pBAD33-*ompR* (pTB11), or the empty vector pBAD33 were cultured under AKI conditions in the presence of 0.05% arabinose for 4.5 h when total RNA was isolated using TRIzol according to the manufacturer's directions (Invitrogen) and further purified using a RNeasy kit with in column DNase treatment (Qiagen). The resulting RNA was then processed and sequenced by the University of Pittsburgh Health Sciences Sequencing Core at Children's Hospital of Pittsburgh. The methods for RNA process, sequencing and RNA-

seq analysis have been described (78). Briefly, the resulting FASTQ files from three independent experiments were mapped to the N16961 reference genome using CLC Genomics Workbench (version 10.1, Qiagen) and the default mapping parameters. Sample normalization and the identification of differentially expressed genes was accomplished using the Differential Expression for RNA-Seq tool in CLC Genomics Workbench. Genes showing a ≥ 2.0 -fold difference in expression and a false discovery rate P-value of ≤ 0.05 were identified as differentially expressed genes. The raw RNA sequencing data files have been deposited at the National Center for Biotechnology Information Sequence Read Archive under accession number SRP109296.

4.3.5 Quantification of CT production

CT production was determined by GM1 enzyme-linked immunosorbent assays as previously described using purified CT (Sigma) as a standard (213).

4.3.6 Quantitative real time PCR

V. cholerae strains harboring either pBAD33-*ompR* or pBAD33 empty vector were grown under AKI conditions with 0.05% arabinose for 3 or 5h when total RNA was isolated from the cultures using Trizol (Invitrogen) per the manufacturer's directions. cDNA was generated from the purified RNA using the Maxima First Strand cDNA Synthesis Kit (Thermo). The expression level of specific genes was quantified by amplifying 25 ng of cDNA with 0.3 μ M primers using the SYBR green PCR mix (Thermo) on a StepOnePlus real-time PCR System (Applied Biosystems). The relative expression level of genes in the mutant and WT cultures was calculated using the $2^{-\Delta\Delta CT}$ method. The presented results are the means \pm standard deviation from three

biological replicates, with each biological replicate being generated from three technical replicates. DNA gyrase (*gyrA*) was used as the internal control.

4.4 Results

4.4.1 Defining the *V. cholerae* OmpR regulon

Recent work in our laboratory documented that *V. cholerae* OmpR did not appear to function in response to osmolarity, but instead was a virulence repressor that was induced in response to membrane intercalating agents (242). This suggested that the function of OmpR in *V. cholerae* diverges from what is observed in other *Gamaproteobacteria*. To further explore the impact of OmpR on *V. cholerae* biology we used RNA sequencing (RNA-seq) to define the *V. cholerae* OmpR regulon during growth under virulence inducing conditions (i.e. AKI conditions). In these experiments we cultured a *V. cholerae* $\Delta ompR$ mutant bearing either pBAD33 or pBAD33-*ompR* under virulence inducing conditions in AKI broth containing 0.05% arabinose inducer. Total RNA was then isolated at 4.5h and used for RNA-seq as previously described (78). The results from three independent experiments was then analyzed to identify differentially expressed genes that were defined as genes exhibiting a ≥ 2 -fold change in expression in the pBAD33-*ompR* samples, compared to the empty vector control (i.e. pBAD33), with $P \leq 0.05$. This analysis resulted in the identification of 191 differentially expressed genes; 68 genes that were upregulated and 124 which were repressed by *ompR* overexpression (Table 5). Most of the differentially regulated genes were annotated as belonging to four functional groups (Table 4):

pathogenesis (9.9%), transport and binding proteins (19.9%), metabolism (25.7%) and uncharacterized (31.4%).

The validity of our approach for identifying OmpR-regulated genes was confirmed by the finding that *ompR* overexpression resulted in the repression of pathogenesis genes including most of the genes involved in CT and TCP production; a finding that is consistent with the recent report that OmpR was a *V. cholerae* virulence repressor (242). Further support for our approach was provided by the observation that 35% of the differentially expressed genes due to *ompR* overexpression were also identified as being differentially expressed in a *V. cholerae* RND (Resistance-Nodulation-Division) efflux negative mutant (Table 5), where *ompR* expression was constitutively upregulated (121, 242). Interestingly, overexpression of *ompR* did not affect the expression of its canonical sensor *envZ*. As *envZ* is encoded in a bicistronic operon downstream of *ompR*, this suggested that OmpR may not autoregulate its own expression in *V. cholerae*, as it does in other bacterial species (212, 247). This finding was confirmed by transcriptional reporter data showing that *ompR* expression was similar in WT and its isogenic $\Delta ompR$ mutant strain during growth under virulence inducing conditions (Fig. 16).

The observation that about half (~46%) of the differentially regulated genes fell into the metabolism and transport and binding functional groups suggested that a major role for OmpR is in metabolic and environmental adaptation (Table 4 and Table 5). Significantly, most of the regulated metabolism genes were repressed by OmpR. This included repression of genes involved in central and intermediary metabolism and genes involved in the electron transport chain. Upregulated genes included a fructose phosphotransferase system, sulfate metabolism genes and anaerobic metabolism genes. This was similar to what is observed in other *Enterobacteriaceae* where OmpR reprograms the cell transcriptome in response to inducing stimuli (248, 249).

OmpR has been linked to osmoregulation in the *Enterobacteriaceae*. In *V. cholerae* osmoadaptation has been linked to the osmoregulator regulator OsrR and involves increased biofilm production, ectoine biosynthesis, and the import of compatible solutes through OmpW, OpuD, and PutP transporters (229, 250-252). In the RNA-seq dataset, OmpR repressed *osrR* (2.6-fold) and *ompW* (3.38-fold) but did not affect the expression of biofilm genes (i.e. *vps* genes) or ectoine biosynthesis genes (i.e. *ectABC*, *opuD*, and *putP*). These results, coupled with previous findings that *V. cholerae ompR* was dispensable for growth at high osmolarity, led us to conclude that *V. cholerae* OmpR does not function in osmoadaptation under the tested conditions.

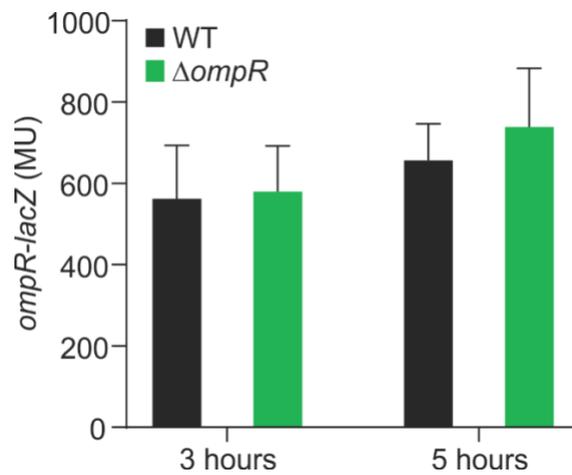


Figure 16 *V. cholerae* OmpR does not autoregulate its own expression

WT and $\Delta ompR$ *V. cholerae* harboring an *ompR-lacZ* reporter plasmid were cultured under virulence factor inducing conditions. Culture aliquots were collected at 3h and 5h to quantify *ompR-lacZ* expression as β -galactosidase activity. The data indicates the average \pm SD of a minimum of three independent experiments performed in triplicate. MU, Miller units.

Table 4 Functional classes of OmpR regulated genes in *V. cholerae*

Functional group	Upregulated genes	Downregulated genes	Total
Transcription	1	0	1
Transport and binding proteins	21	17	38
Biosynthesis of cofactors, prosthetic groups, and carriers	2	3	5
Cellular processes	1	2	3
Regulatory function	2	4	6
Metabolism	17	32	49
Cell envelope	0	5	5
Protein fate	0	1	1
Amino acid biosynthesis	0	3	3
DNA replication, recombination and repair	0	1	1
Pathogenesis	0	19	19
Conserved, hypothetical, and unknown	23	37	60
Total:	67	124	191

4.4.2 *V. cholerae* OmpR functions in OMP gene regulation

OmpR regulates the expression of outer membrane porin protein genes (253) in many Gram-negative bacteria (100, 101, 109, 202, 203). Consistent with this, the RNA-seq results suggested that *V. cholerae* *ompR* regulated the expression of three OMPs: *ompW* (VCA0867) and *ompV* (VC1318) were repressed by 3.38 and 2.02-fold respectively, and *chiP* (VC0972) was induced by 2-fold. We validated this finding by quantifying the effect of *ompR* overexpression on *ompW*, *ompV*, *chiP*, plus the two major *V. cholerae* OMPs *ompU* and *ompT*, which were not differentially regulated in the RNA-seq data. To do this, *V. cholerae* Δ *ompR* harboring either pBAD33 or pBAD33-*ompR* was cultured under AKI conditions for 5h in the presence of 0.05% arabinose, when gene expression was quantified by qRT-PCR (Fig. 17). The results showed that OmpR repressed *ompW* while activating *chiP*. The expression of *ompV* was also reduced, however, the results did not reach statistical significance. Significantly, OmpR did not have an effect on the

expression of the two major *V. cholerae* OMPs, *ompT* and *ompU*, consistent with our previous results (242). This contrasts finding in other *Enterobacteriaceae* where OmpR differentially regulates the production of their major OMPs, *ompC* and *ompF* (212, 231, 254). Collectively, these results validate the OmpR transcriptomic data, and indicate that *V. cholerae* OmpR retains conserved functions in the regulation of OMP genes, but not for the regulation of *ompT* and *ompU* which are under control of ToxR and other effectors (157, 175, 255, 256).

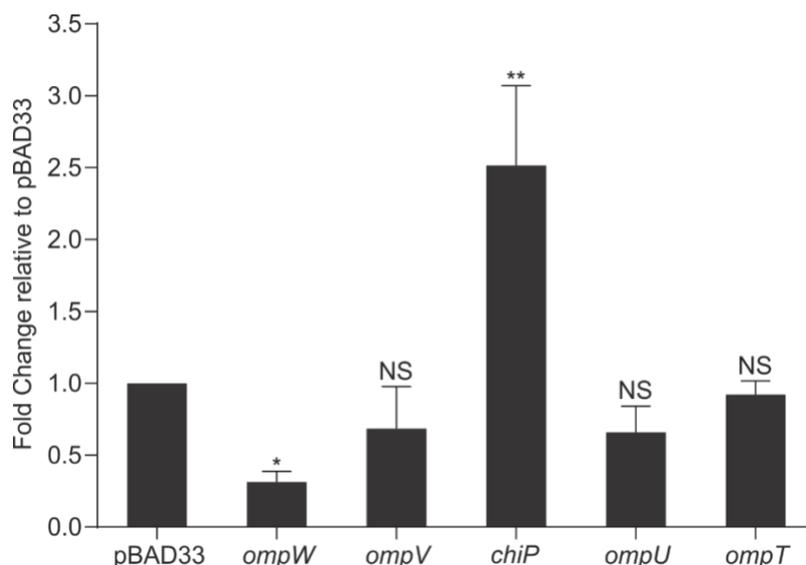


Figure 17 *V. cholerae* OmpR modulates porin gene expression

V. cholerae Δ *ompR* harboring either pBAD33 or pBAD33-*ompR* were cultured in AKI conditions with 0.05% arabinose for 5h when RNA was collected and used for qRT-PCR to quantify *ompW*, *ompV*, *chiP*, *ompU*, and *ompT* expression. The data indicates the average \pm SD of a minimum of three independent experiments performed in triplicate. * $P < 0.01$, ** $P < 0.0001$ relative to pBAD33 as determined using Dunnett's multiple comparisons test.

4.4.3 OmpR regulates acid tolerance genes and is induced by alkaline stress

OmpR has been studied for its role in activating ATR genes in response to low pH in a number of Gram-negative organisms (103, 203, 209-212, 257). Interestingly, the *V. cholerae ompR*

transcriptome indicated that OmpR repressed a number of ATR genes including *nhaP1*, *cadB*, *cadA*, and *clcA* (37, 38, 82, 258). The fact that *cadC* and *clcA* are positively regulated by AphB at low pH (75), and OmpR directly represses *aphB* transcription (242), led us to hypothesize that OmpR may function upstream of *aphB* to regulate genes involved in pH adaptation. To test this, we first sought to validate the RNA-seq results by quantifying the effect of *ompR* on the expression of the AphB-dependent genes *cadC*, *cadB*, *cadA* and *clcA* by qRT-PCR. Consistent with the RNA-seq data, the results showed that *ompR* overexpression repressed *cadB*, *cadA*, and *clcA* by ~2-fold (Fig. 18A). Expression of *cadC*, which encodes the direct regulator of the *cadBA* operon, was also reduced but to a lesser extent, mirroring the RNA-seq results.

The above results suggested that *V. cholerae* OmpR regulated ATR genes. We next tested if *ompR* was itself regulated in response to pH. We cultured WT harboring an *ompR-lacZ* reporter under AKI conditions for 4h when the culture media pH was altered through addition of either NaOH or HCl. We then quantified *ompR-lacZ* expression 60 min post exposure. The results showed that acidification of the media with HCl addition did not alter *ompR* expression (data not shown). By contrast, there was an alkaline pH-dependent induction of *ompR* expression, suggesting that *ompR* was regulated in response to alkaline pH (Fig. 18B). The fact that OmpR repressed ATR genes, whose expression is likely to be detrimental during alkaline stress, and was induced under alkaline pH suggested that OmpR may contribute to alkaline pH response in *V. cholerae*.

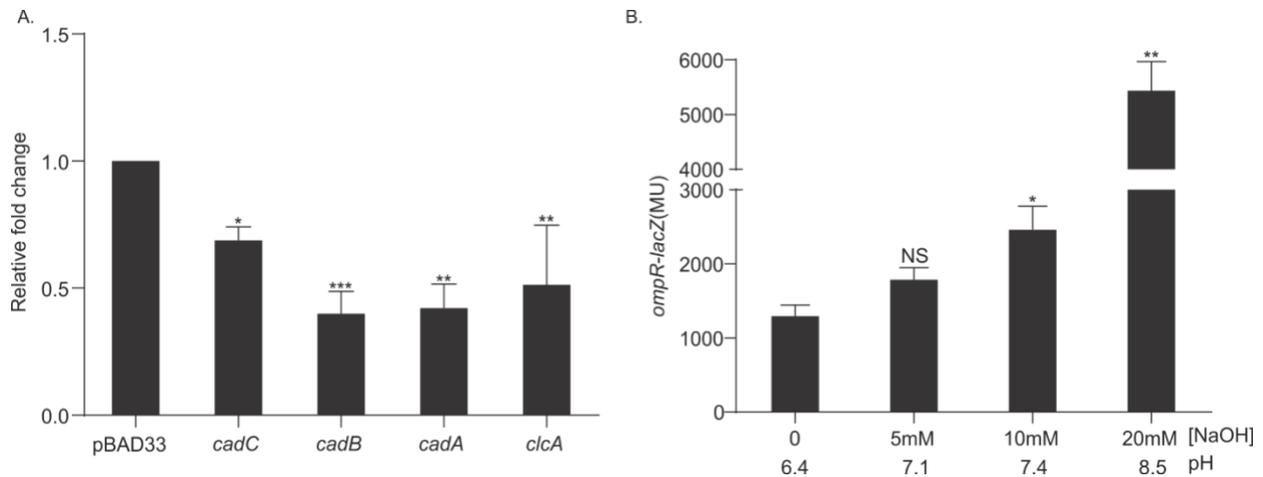


Figure 18 OmpR represses acid tolerance genes

(A) *V. cholerae* $\Delta ompR$ harboring either pBAD33 or pBAD33-*ompR* were cultured in AKI conditions with 0.05% arabinose. RNA was collected at 5h and used for qRT-PCR to quantify *cadC*, *cadB*, *cadA* and *clcA* expression. The fold change in gene expression in the pBAD33-*ompR* harboring strains are relative to the pBAD33 harboring strains. The data are the average +/- SD of a minimum of three independent experiments performed in triplicate. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$ relative to pBAD33 determined by Dunnett's multiple comparisons test. (B) WT *V. cholerae* harboring an *ompR-lacZ* reporter plasmid was cultured under AKI conditions for 4h before NaOH was added to the indicated concentrations; pH values of the media following NaOH addition at time of experiment are shown. β -galactosidase activity was then quantified 1h post treatment. The data indicates the average +/- SD of a minimum of three independent experiments performed in triplicate. * $P < 0.01$, ** $P < 0.0001$ relative to 0 determined by Dunnett's multiple comparisons test.

4.4.4 OmpR represses *aphB* at alkaline pH

The observation that *ompR* was induced by alkaline pH, combined with OmpR functioning as an *aphB* repressor (242), suggested that OmpR may regulate *aphB* expression during growth under alkaline pH. To test this, we cultured WT and $\Delta ompR$ strains bearing either an *aphA-lacZ* or *aphB-lacZ* reporter under AKI conditions for 4h before adding 20mM NaOH to raise the media pH to 8.7; the control for these experiments received an equivalent amount of water. The cultures

were incubated for an additional hour before quantifying *aphA* and *aphB* expression. The results showed that increased media pH had little effect on *aphA* expression in either strain (Fig. 19A). By contrast, raising the pH by NaOH addition decreased *aphB* expression in WT, but not in the $\Delta ompR$ mutant (Fig. 19B), indicating that OmpR repressed *aphB* transcription in response to alkaline pH.

To confirm that OmpR downregulated ATR genes at alkaline pH through *aphB*, we cultured WT, $\Delta ompR$, $\Delta aphA$, and $\Delta aphB$ strains harboring either *cadC-lacZ* or *cadB-lacZ* reporters under AKI conditions and quantified gene expression in response to alkaline pH, as described above. As expected, both *cadC* and *cadB* were repressed in WT at alkaline pH (Fig. 19C-D). The expression of *cadC* was also repressed at alkaline pH the $\Delta ompR$ mutant, suggesting that alkaline pH-dependent repression of *cadC* was OmpR-independent. By contrast, the alkaline pH-dependent repression of *cadB* was lost in the $\Delta ompR$ mutant, indicating that OmpR contributes to *cadB* repression at alkaline pH (Fig. 19D). Unexpectedly, the expression level of both *cadC* and *cadB* were repressed under standard AKI conditions in the *aphA* mutant relative to WT, and their expression further decreased to a level that was like WT at alkaline pH (Fig. 19C-D). This suggests that AphA may play a role in the basal expression of *cadC* and *cadB* at neutral pH, but that *aphA* likely does not contribute to their regulation at alkaline pH; additional studies will be required to assess the biological relevance of this observation. The expression of *cadC* and *cadB* were dramatically reduced in the *aphB* mutant (Fig. 19C-D) relative to WT during growth in standard AKI broth, confirming previous studies showing that AphB positively regulated the Cad system (75). Expression of *cadC* was not further repressed at alkaline pH in the *aphB* mutant (Fig. 19C), whereas *cadB* expression was repressed at alkaline pH in the *aphB* mutant (Fig. 19D). This suggests the presence of an AphB and OmpR-independent mechanism for *cadB* repression in

response to alkaline pH. From these results we concluded that the *V. cholerae* Cad system was repressed in response to alkaline pH and that OmpR contributed to *aphB* repression in response to alkaline pH. Our results further suggest that repression of the Cad system in response to alkaline pH is modulated by both AphB-dependent and independent mechanisms.

The expression of *cadBA* in *E. coli* and Salmonella is directly repressed by OmpR (107). The above findings indicated that *V. cholerae* OmpR represses *cadBA* expression in response to alkaline pH in an AphB-dependent manner. However, the above results did not exclude the possibility that OmpR also directly repressed the expression of *cadC* or *cadBA*. To address this possibility, we expressed *V. cholerae ompR* in *E. coli* bearing *cadC-lacZ* and *cadBA-lacZ* transcriptional reporters. The *E. coli* strains were cultured to mid-log phase in LB broth containing a range of arabinose concentrations, before *cadC* and *cadBA* expression were quantified (Fig. 19E-F). The results showed that overexpression of *V. cholerae ompR* did not affect the expression of either *cadC* or *cadBA* in *E. coli*, suggesting that OmpR indirectly regulated the Cad system in *V. cholerae*.

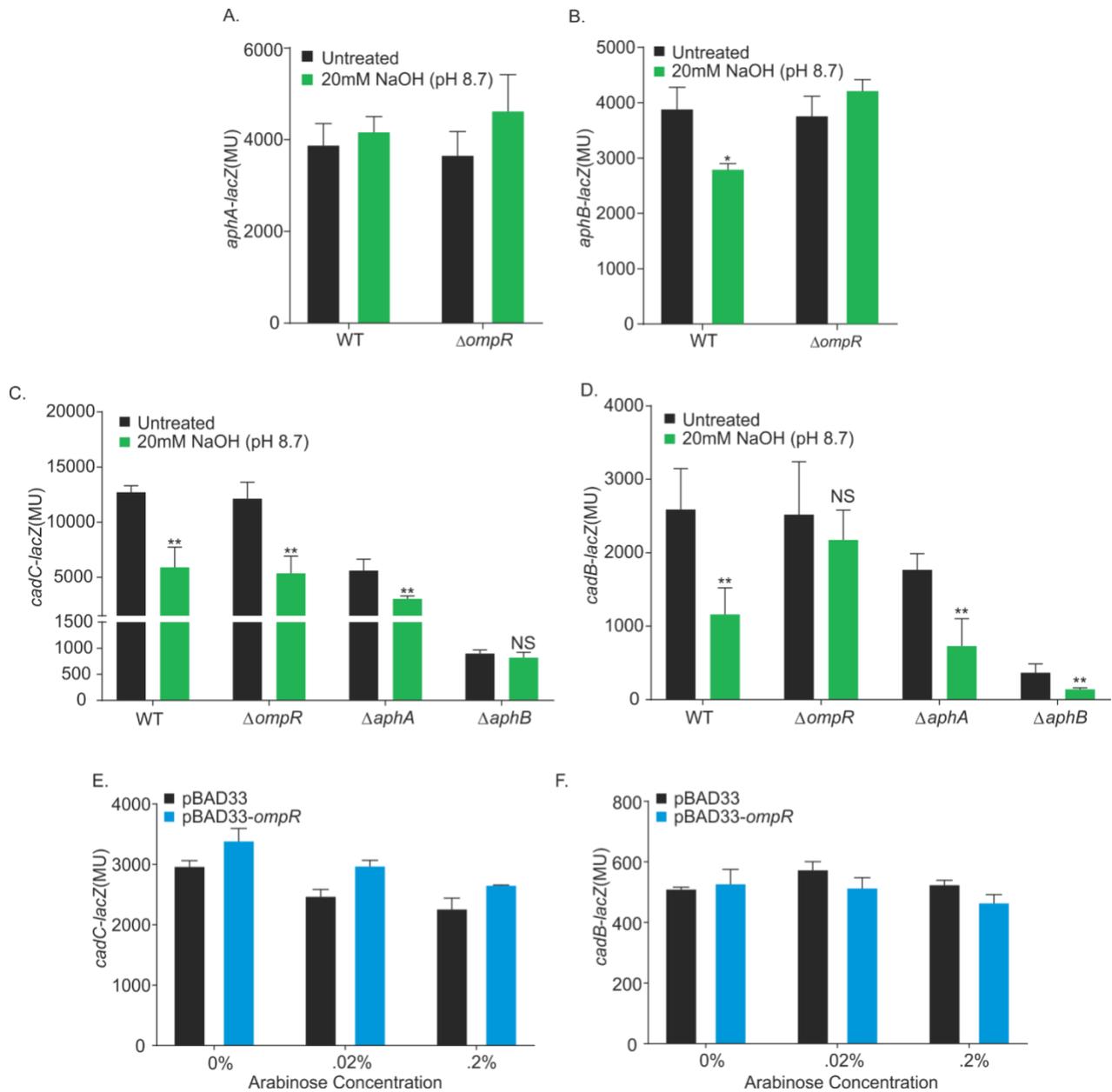


Figure 19 OmpR represses *aphB* and *cadB* expression at alkaline pH

The indicated *V. cholerae* strains harboring an (A.) *aphA-lacZ*, (B.) *aphB-lacZ*, (C.) *cadC-lacZ* or (D.) *cadB-lacZ* reporters were cultured under AKI conditions for 4h when 0 mM (black bars) or 20 mM NaOH (green bars) was added to the culture media; the addition of 20mM NaOH increased the media pH to 8.7. The cultures were then incubated for an additional hour when β -galactosidase activity was quantified. The data is the average \pm SD of at least three independent experiments with each experiment being performed in triplicate. * $P < 0.01$, ** $P < 0.001$ relative to 0mM control for each strain as determined by a Students t-test. (E. & F.) *E. coli* harboring either pBAD33

(black bars) or pBAD33-*ompR* (blue bars) plus a (E.) *cadC-lacZ* or (F.) *cadB-lacZ* transcriptional reporter plasmid were cultured in LB broth with the indicated arabinose concentrations for 5h when β -galactosidase activity was quantified. The data is the average \pm SD of three independent experiments with each experiment being performed in triplicate. Abbreviations: MU, Miller units.

4.4.5 Alkaline pH represses *V. cholerae* virulence factor production in an OmpR-dependent manner

Virulence factor production in *V. cholerae* is modulated in response to multiple environmental cues including pH (75, 259, 260). Expression of the ToxR regulon in El Tor strains is optimal in AKI broth at pH 7.4 (261). In classical *V. cholerae* strains elevated pH (~pH 8.5) has been used as virulence non-inducing conditions in multiple studies (157, 259, 260, 262, 263). As our results showed that *ompR* was induced at alkaline pH, and OmpR functioned as a virulence repressor through *aphB* repression (242), we hypothesized that OmpR may contribute to virulence repression at alkaline pH. To test this, we cultured WT and $\Delta ompR$ strains under AKI conditions in standard AKI broth at pH 7.4 (inducing conditions) and pH 8.7 (non-inducing conditions). We then quantified CT production at 6h and following overnight growth; 6h was assessed because AphB is thought to be most relevant during early induction of the ToxR regulon. When cultured in pH 7.4 AKI broth, the *ompR* mutant strain produced similar amounts of CT compared to WT at both time points tested, confirming the previous report (242) (Fig. 20A-B). By contrast, growth of WT in pH 8.7 AKI broth resulted in a pronounced reduction of CT production at both 6h and 24h. The alkaline-pH dependent virulence attenuation at 6h was OmpR-dependent, as CT production in the *ompR* mutant strain was not affected by high pH (Fig. 20A). At 24h, CT production was

highly attenuated in WT and $\Delta ompR$ at pH 8.7, but the extent of attenuation was less in the $\Delta ompR$ background.

CT and TCP production are coordinately regulated by the ToxR regulon. We therefore further explored the impact of alkaline pH on virulence by quantifying the expression of *tcpA* and *aphB* under the same conditions used above. The results showed that the expression of *tcpA*, which encodes the pilin subunit of the TCP, was repressed in an OmpR-dependent manner in response to alkaline pH (Fig. 20C). We also found that *aphB* expression was repressed in response to alkaline pH, by a mechanism that was partially dependent on OmpR (Fig. 20D). The fact that deletion of *ompR* did not fully restore *aphB* expression or CT production to WT levels (Fig. 20B) suggests that both OmpR-dependent and independent mechanisms contribute to virulence repression in response to alkaline pH. Based on these results we concluded that alkaline pH represses CT and TCP production. We further conclude that virulence repression is OmpR-dependent at early time points and mediated in part by OmpR-dependent repression of *aphB*. Our data suggest OmpR contributes to alkaline pH-induced CT repression at later time points, but that OmpR-independent mechanisms also exist.

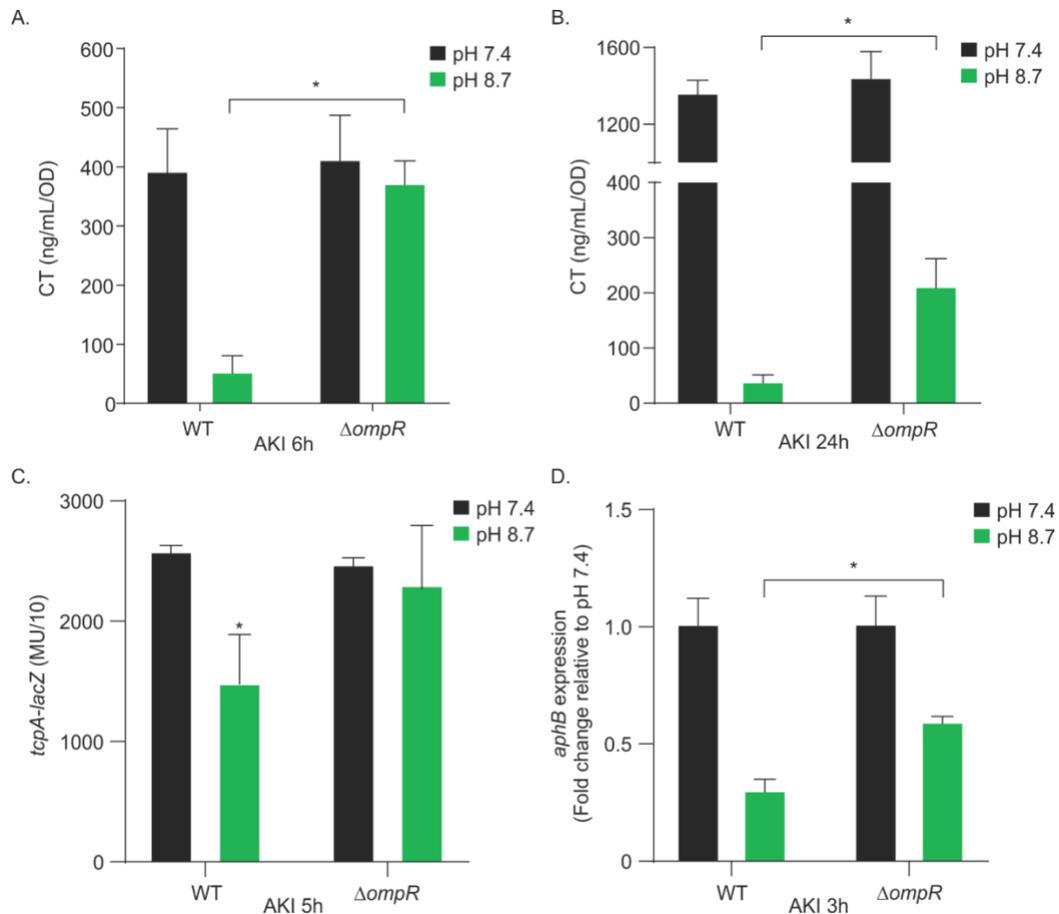


Figure 20 OmpR represses virulence factor production at alkaline pH

V. cholerae WT and $\Delta ompR$ were cultured under AKI conditions in standard AKI broth pH 7.4 or AKI broth pH 8.7. Culture aliquots were collected at (A.) 6h and (B.) 24h to quantify CT production by GM1 ELISA, or (C.) 5h and (C.) *tcpA-lacZ* and (D.) 3h when RNA was collected and used for qRT-PCR to quantify *aphB* expression. The data is the average \pm SD of three independent experiments. * $P < 0.001$ relative to WT, determined by a Students t-test. MU, Miller units.

4.4.6 *V. cholerae* OmpR is required for fitness in alkaline pH

The observation that OmpR repressed ATR genes, and *ompR* expression was induced at alkaline pH, suggested that OmpR may contribute to fitness at alkaline pH. To test this, we determined the growth kinetics of WT, $\Delta ompR$, $\Delta aphA$, and $\Delta aphB$ strains in LB broth pH 7.4 and

LB broth pH 9.7. The results revealed that all strains had similar growth kinetics in LB broth pH 7.4 (Fig. 21A). By contrast, the $\Delta ompR$ mutant exhibited a pronounced growth defect relative to the other strains during growth at pH 9.7, as indicated by an increase in the lag phase (Fig. 21B). The growth attenuation of the $\Delta ompR$ mutant at pH 9.7 was complemented by ectopic *ompR* expression (*cf.* the black and green traces in Fig. 21D) whereas there was no difference in the growth of the two strains in LB pH 7.4 (Fig. 21C). This confirms that the observed alkaline pH-dependent phenotype was due to *ompR* mutation and not some other non-specific effect. We noted that the presence of pBAD33, independent of *ompR*, exhibited a non-specific negative effect on the growth of all strains at alkaline pH as shown by a decrease in the final culture density (Fig. 21D). The reasons for this are unknown. The $\Delta aphA$ and $\Delta aphB$ mutants exhibited little difference in growth kinetics relative to WT at both pH's tested, suggesting that their contribution to growth under the test conditions was minimal (Fig. 21B).

The above experiments confirmed that *V. cholerae ompR* contributed fitness during growth at alkaline pH. Previous studies in *E. coli* have shown that OmpR phosphorylation was not required for OmpR-dependent responses to pH (257). To determine if the phosphorylation status of OmpR played a role in *V. cholerae* response to alkaline pH, we repeated the above complementation experiments with plasmids expressing mutant alleles of *V. cholerae* OmpR that mimic phosphorylated OmpR (*ompR_{D55E}*) and dephosphorylated OmpR (*ompR_{D55A}*). Each of the mutant alleles were expressed in $\Delta ompR$ during growth in LB pH 7.4 and LB pH 9.7 as described above. During growth at pH 7.4 the *ompR_{D55E}* allele resulted in a longer lag phase relative to the *ompR_{D55A}* allele and the final density of both cultures (*ompR_{D55E}* and *ompR_{D55A}*) was decreased relative to WT (Fig. 21C). This suggests that the exclusive expression of either phosphomimic is detrimental for growth at neutral pH. The expression of all three *ompR* alleles (*i.e.* *ompR*, *ompR_{D55A}* and

ompR_{D55E}) complemented the $\Delta ompR$ mutant for growth at alkaline pH (Fig. 21D). This suggests that although OmpR is required for optimum fitness at alkaline pH, this phenotype is independent of the phosphorylation status of OmpR. Taken together the results suggest that *ompR* contributes to *V. cholerae* fitness during growth in alkaline conditions and that this process is independent of the OmpR phosphorylation status.

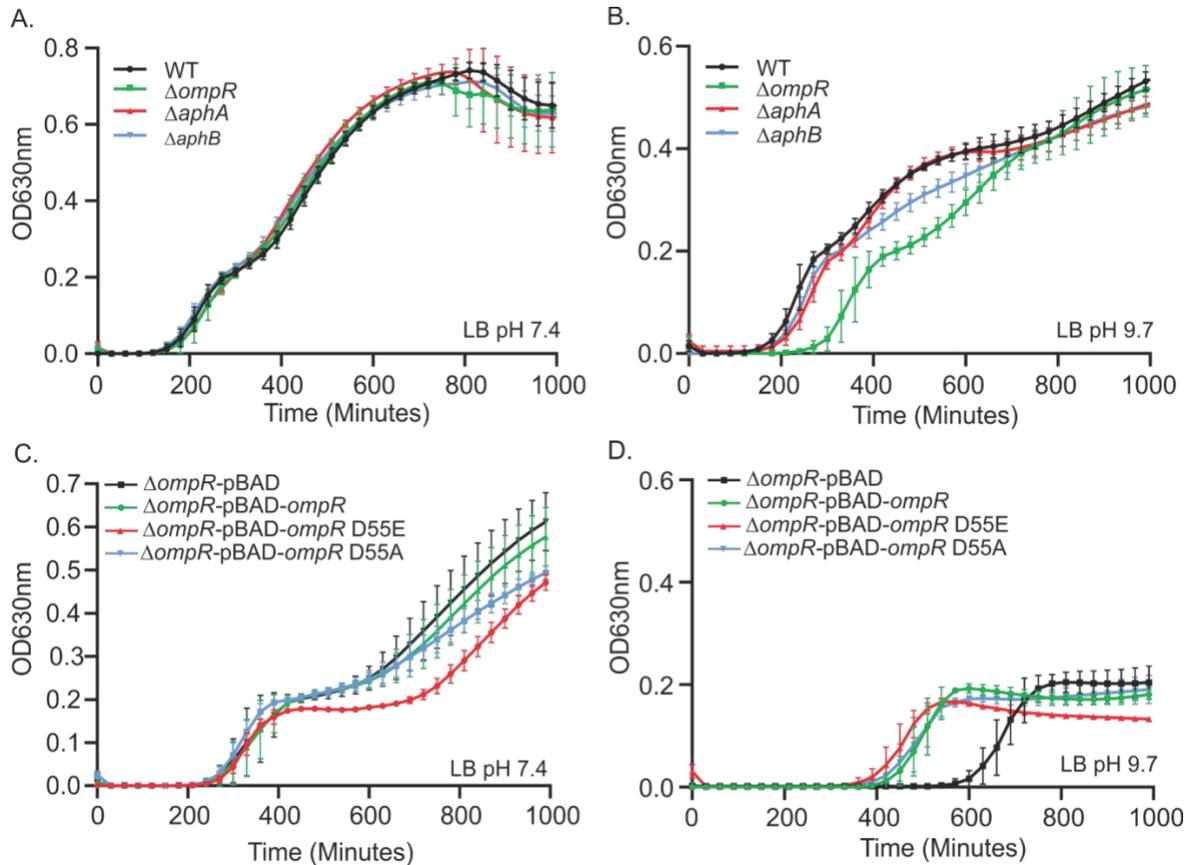


Figure 21 A *V. cholerae* $\Delta ompR$ is attenuated for growth at alkaline pH.

Overnight LB cultures of WT, $\Delta ompR$, $\Delta aphA$, and $\Delta aphB$ *V. cholerae* strains were diluted 1:10,000 in LB broth (A-C) or LB broth supplemented with 35 mM NaOH (pH 9.7) (B-D). Arabinose (0.05%) was included in the growth media for *V. cholerae* $\Delta ompR$ harboring pBAD33, pBAD33-*ompR*, pBAD33-*ompR_{D55E}*, or pBAD33-*ompR_{D55A}* (C-D). The cultures were distributed into the wells of a 96 well microtiter plate in triplicate. The microtiter plate was then incubated at 37°C with shaking in a plate reader and cell growth was monitored by reading the optical density at 630 nm every 30 minutes. The data is the average +/- SD of at least three independent experiments.

4.5 Discussion

The lifecycle of pathogenic *V. cholerae* relies on transitioning between marine ecosystems and the human gastrointestinal tract. The ability of *V. cholerae* to successfully cycle between these two disparate environments is dependent on the activation of TCSs, which sense and respond to environmental cues to coordinate the expression of adaptive responses. In this chapter we characterized the response of *V. cholerae* OmpR to alkaline pH. Based on our results we propose the following model where *V. cholerae* OmpR functions at alkaline pH to coordinately repress genes involved in virulence and acid tolerance via AphB (Fig. 22). At alkaline pH, OmpR directly represses *aphB* transcription resulting in downregulation of the ToxR virulence regulon to attenuate CT and TCP production. Repression of *aphB* also results in reduced expression of acid tolerance genes (e.g. *cadC*, *cadBA* and *clcA*) which likely contributes to *V. cholerae* fitness at alkaline pH. We speculate that this novel function of the *V. cholerae* EnvZ/OmpR TCS in regulating adaptive responses to alkaline pH may contribute to successful transition between the human gastrointestinal tract and marine ecosystems.

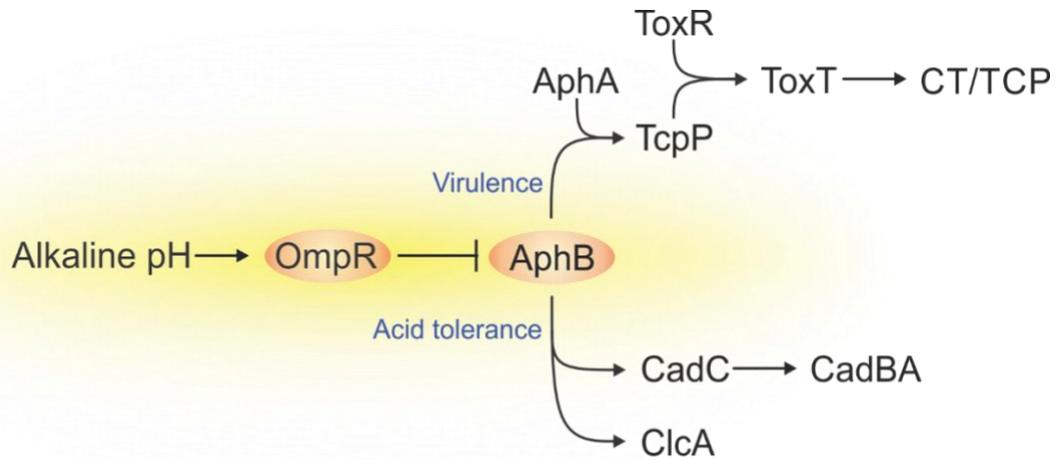


Figure 22 Model of *V. cholerae* OmpR in response to alkaline pH

Alkaline pH induces the expression of *V. cholerae ompR*, which then directly represses *aphB* transcription. Repression of *aphB* results in the downregulation of genes involved in both acid tolerance and virulence factor production, leading to enhanced fitness at alkaline pH and attenuated production of CT and TCP.

Bacterial regulatory networks evolve in response to selective pressures during growth in specific niches. This drives the evolution of regulatory networks to respond to novel signals, and to alter the constitution of their target regulons (233, 245). This divergent evolution results in conserved TCS systems fulfilling different physiological roles across bacterial species and genera, (218) as has been documented with the EnvZ/OmpR TCS. EnvZ/OmpR is conserved among *Gamaproteobacteria* bacteria, but multiple studies have shown divergence in OmpR-inducing signals and in the OmpR regulon among different species (109, 205, 206, 212, 234). This appears to also be the case in *V. cholerae*. The work presented here, and in a previous report (242), indicates that the function of EnvZ/OmpR in *V. cholerae* has diverged from other well-studied enteric pathogens. We speculate that the unique lifestyle of *V. cholerae* has supplied the selective pressures for evolution of *V. cholerae* EnvZ/OmpR.

Our collective results suggest that the EnvZ/OmpR TCS fulfils novel physiological roles

in *V. cholerae*. The observation that *ompR* was not induced by acidic pH, or media osmolarity, but was instead induced by alkaline pH suggested that OmpR likely functioned in adaptation to alkaline pH. Support for this hypothesis was provided by the observation that a *V. cholerae* Δ *ompR* mutant was attenuated for growth at alkaline pH. The discovery that OmpR repressed *V. cholerae* ATR genes (*nhaP1*, *cadBA*, and *clcA*) highlighted a potential mechanism for OmpR-dependent adaptation to alkaline pH. The *V. cholerae* ATR involves induction of AphB activity, and the increased expression of genes that are collectively predicted to prevent acidification of the cytoplasm (i.e. *cadBA*, *nhaP1*, and *clcA*) (75, 82, 258). The Cad system contributes to the ATR by preventing cytoplasmic acidification. CadA is a lysine decarboxylase that consumes a proton during the decarboxylation reaction to produce cadaverine which is then removed from the cell by the lysine-cadaverine antiporter CadB. ClcA and NhaP1 are H⁺/Cl⁻ and Na⁺/H⁺ antiporters, respectively, which are also predicted to facilitate survival at low pH by expelling protons from the cytoplasm. Conversely, the repression of these genes at high pH would block intracellular proton depletion and likely contribute to adaptive responses to counteract alkalization of the cytoplasm (82). It is interesting to note that this contrasts with the function of EnvZ/OmpR in the *Enterobacteriaceae*. In *E. coli* and Salmonella, *ompR* is induced by high osmolarity and acidic pH and reprograms the cell transcriptome to enhance survival at low pH. Recent studies have shown that during adaptation to low pH, *E. coli* and Salmonella acidifies their cytoplasm (>90 min) by a mechanism facilitated by OmpR-dependent repression of the Cad system (257, 264, 265).

Recent studies have shown that alkaline pH-dependent repression of *clcA* is critical for infection in the infant mouse infection model (82). In infant mice *clcA* is induced in the mouse stomach but repressed in the more alkaline intestine. Expression of *clcA* in alkaline environments is detrimental and failure to repress *clcA* in the intestine was associated with a 50-fold colonization

defect compared to WT (82). The expression of *clcA* in response to low pH is mediated by AphB (266), but the molecular mechanism involved in *clcA* repression in the intestine is unknown. Studies indicate that rice water stool from cholera patients is alkaline (pH 7.5-8.5), making it interesting to speculate that OmpR may contribute to *clcA* repression in the intestine (267), but additional work will be required to verify this. We note that there are conflicting reports regarding the contribution of *ompR* to intestinal colonization, suggesting that there may be strain differences among *V. cholerae* isolates. A recent study reported that *ompR* was required for colonization (268) while three other studies suggested that *ompR* was dispensable for colonization (87, 269, 270).

Previous studies have shown that alkaline pH represses *ctxAB* expression in classical biotypes of *V. cholerae* (71). Here we show this phenotype is conserved in El Tor biotypes and is in part due to OmpR-dependent repression of *aphB* at alkaline pH. Alkaline pH strongly repressed cholera toxin production at both 6h and 24h. Attenuated CT production at 6h during growth at high pH was abrogated by deletion of *ompR*. However, at 24h *ompR* deletion only partially restored CT production at alkaline pH. This indicates that OmpR is primarily responsible for alkaline pH-induced virulence repression at early time points during growth under AKI conditions and that other factors contribute to virulence repression at later time points. This is consistent with the ToxR regulon model where AphB, the target of OmpR repression, functions early during induction of the ToxR regulon. There are multiple OmpR-independent mechanisms that may also contribute to virulence repression in response to alkaline pH. For example, PepA, Crp and TcpI have all been reported to contribute to virulence regulation in response to alkaline pH in classical biotypes (73, 259, 260). In El Tor strains, ToxR proteolysis has been shown to occur in response to alkaline pH (271).

We speculate that alkaline induction of *ompR* reflects an evolutionally adaptation of *V.*

cholerae to its unique lifecycle. *Vibrio* spp. are native to alkaline marine environments (6, 7) and have evolved efficient mechanisms to grow at high osmolarity and alkaline pH. This adaptation serves *V. cholerae* well in the human gut where *V. cholerae* can rapidly multiply in the alkaline rice water stool before disseminating in the diarrheal purge to reenter aquatic ecosystems. The ability of *V. cholerae* to cycle between these two disparate environments requires adaptive transcriptional rewiring. Late in infection, in preparation to exit the host and enter the aquatic ecosystem, *V. cholerae* represses virulence genes and induces genes important in transmission and dissemination (42-44, 77). The signals and regulatory mechanisms that control this transcriptional shift are poorly understood. Both seawater and the rice water stool of cholera patients are markedly alkaline (267, 272), and the high pH in the marine environment is an important signal that has been shown to induce adaptation through transcriptional regulation (273). Therefore, we postulate that the alkaline pH-induced expression of *ompR* may represent a genetic mechanism that functions during the late stages of infection to repress virulence gene expression and adapt to alkaline pH in the lumen, thus preparing the bacteria for entrance back into the aquatic ecosystem (42-44, 77). This speculative model is supported by our results showing that alkaline-pH induction of *ompR* results in the transcriptional silencing of acid-tolerance and virulence genes, and that *ompR* contributes to fitness in alkaline pH, and activates the expression of the chitin-specific porin *chiP* (15, 274). Pathogenic *V. cholerae*'s transition between the host gastrointestinal tract and aquatic ecosystems may have selected for the divergent evolution of *V. cholerae* OmpR to respond to alkaline pH, an adaptation that appears to be specifically suited to the *V. cholerae* life cycle.

5.0 Conclusion

Cholera is an ancient disease that continues to be a significant health burden with an estimated 3 million global cases of cholera annually (3). The bacterium *Vibrio cholerae* is the causative agent of cholera. *V. cholerae* is a natural inhabitant of marine ecosystems and causes human infection following the consumption of *V. cholerae* contaminated food or water. Paramount to *V. cholerae*'s pathogenic success is its ability to transition between disparate niches in the marine environment and the human gastrointestinal tract. In moving between these two niches, *V. cholerae* is confronted with rapid fluctuations in environmental conditions including changes in nutrient availability, antimicrobial compounds, temperature, pH, oxygen and osmolarity. In order to survive these sudden environmental variations and successfully colonize new niches *V. cholerae* must be able to sense and swiftly adapt to environmental cues. These responses are mediated at the transcriptional level, and are collectively referred to as bacterial adaptive responses which reprogram the transcriptome by modulating the expression of niche-specific nutrient acquisition systems, antimicrobial resistance mechanisms, virulence genes, and stress response genes.

An adaptive response that is central to the *V. cholerae* life cycle is modulation of the expression of genes to enhance resistance to the toxic effects of environmental antimicrobial compounds. *V. cholerae*, like most Gram-negative bacteria, utilizes RND family multidrug efflux pumps for antimicrobial resistance. RND-mediated efflux confers resistance to a wide range of chemically and structurally dissimilar antimicrobial compounds including antibiotics, detergents, dyes, host immune effectors, and others (123, 140). In addition, a growing body of literature has linked RND efflux pumps to a broad range of bacterial phenotypes outside of their role in antimicrobial resistance including, but not limited to, metabolism, nutrient acquisition, stress

responses, biofilm production, and virulence factor production (94, 108, 145, 194, 275-279). Consistent with these findings, previous work in our laboratory has illustrated connections between *V. cholerae* RND-mediated efflux and virulence factor production, stress responses, and metabolism (84, 97, 121, 150). Yet, how RND efflux participates in these phenotypes remains poorly understood. The work presented in this dissertation sought to explore the links between *V. cholerae* RND-mediated efflux and virulence factor production and stress responses, and to exploit these links to uncover novel signals and genetic pathways that contribute to the expression of these responses. Through these studies we documented mechanistic links between *V. cholerae* RND-mediated efflux and iron acquisition, membrane homeostasis, metabolism, and virulence factor production. Collectively, the elucidation of these links and regulatory pathways has resulted in a better understanding of how RND-mediated efflux influences the expression of adaptive responses that *V. cholerae* utilizes during its lifecycle to achieve pathogenic success.

5.1 *V. cholerae* VexGH functions in regulation of membrane homeostasis and metabolism through vibriobactin secretion

Previous work in our laboratory indicated that the loss of RND efflux resulted in the constitutive activation of the Cpx membrane stress response, suggesting a link between RND efflux and the maintenance of membrane homeostasis (84). This led to the hypothesis that native efflux substrates that accumulated intracellularly in the absence of RND efflux were responsible for the induction of the Cpx system. To identify possible RND-dependent Cpx-inducing compounds we developed a transposon mutagenesis screen for Cpx suppressor mutants in a *V. cholerae* Δ RND mutant strain. This screen led to the discovery that mutation of genes involved

in the production of the catechol siderophore vibriobactin suppressed the Cpx system in the Δ RND background. Subsequent studies revealed that vibriobactin was a native substrate of the VexGH RND efflux pump, and that the absence of RND-mediated efflux resulted in the intracellular accumulation of vibriobactin and constitutive activation of the Cpx system (Section 2.4.2). Further analysis found that Cpx activation in the Δ RND strain was dependent on aerobic respiration, and that reactive oxygen species were capable of inducing the Cpx system. These findings collectively lead to a model for Cpx induction where the loss of RND-mediated efflux resulted in the intracellular accumulation of iron-free vibriobactin, which then chelated iron from iron-rich membrane-bound components of the electron transport chain. This chelation resulted in Cpx activation as a result of direct sensing of the deferrated proteins and/or the production of ROS from the deferrated proteins (Fig. 9). This work uncovered a previously unknown native physiological function of VexGH in effluxing vibriobactin, and linked *V. cholerae* RND efflux to iron acquisition, maintenance of membrane integrity, and metabolism.

RND efflux has been linked to the Cpx system in several Gram-negative bacteria. The loss of efflux has been shown to result in Cpx system activation in *V. cholerae*, *E. coli*, *Sinorhizobium meliloti*, and *Haemophilus ducreyi* (95, 97, 99, 192, 193, 280-282). However, the mechanism by which the loss of efflux results in membrane perturbations, the Cpx system inducing signal, remained poorly understood. The work presented in chapter 2 uncovered the mechanistic link between loss of RND efflux and induction of the Cpx system in *V. cholerae*; the intracellular accumulation of vibriobactin. These findings were consistent with a recent report in *E. coli* where loss of efflux resulted in activation of the Cpx system via the siderophore enterobactin (283). Taken together, these findings indicate a conserved function of RND-mediated efflux in both iron acquisition and repression of the Cpx system in these two species. We speculate that this phenotype

is likely conserved in most Gram-negative bacteria that produce both RND transporters and siderophores.

The finding that vibriobactin retention disrupted components of the ETC, and that Cpx system induction was dependent on aerobic respiration, indicates that the RND efflux pumps contribute to the maintenance of membrane integrity and may play a role in central metabolism. Consistent with this finding, work from our lab has shown that the *V. cholerae* RND efflux pumps export metabolic intermediates and that a loss of RND-mediated efflux resulted in broad transcriptional changes in metabolism-associated genes, compared to WT (84, 121). Collectively, these findings indicate that the *V. cholerae* RND efflux pumps function in cellular metabolism, likely through modulation of the intracellular concentration of metabolites. Similarly, RND efflux has been linked to metabolic pathways in multiple other Gram-negative species. Deletion of RND genes resulted in differential expression of many metabolic genes in *Salmonella* (275, 284), as well as global metabolomic alterations in *Acinetobacter baumannii* (285). Both the *E. coli* and *Pseudomonas aeruginosa* RND pumps extrude bacterial metabolites (151, 286, 287), and overexpression of RND efflux genes in *P. aeruginosa* resulted in compensatory alterations in metabolism gene expression (288, 289). The work presented in chapter 2 provide additional evidence that RND efflux pumps play a conserved role in bacterial metabolism in Gram-negative bacteria, likely through the modulation of the intracellular concentrations of bacterially derived metabolic compounds.

RND efflux pumps play a profound role in resistance to a wide range of antimicrobial compounds, however multidrug efflux pumps are ancestral genomic elements, and their presence in bacterial genomes predates the use of antibiotics to treat bacterial infections (290). This observation coupled with the fact that most Gram-negative bacteria encode multiple RND efflux

pumps with redundant substrate specificity suggests that the RND transporters have evolved to fulfill native physiological functions outside of their role in antibiotic resistance. These native functions have likely provided the selective pressures for the evolution and maintenance of multiple RND efflux pumps within individual bacterial genomes. The work presented in chapter 2 indicates that a native function of the *V. cholerae* VexGH RND efflux pump is to efflux vibriobactin. The loss of this native function impacts membrane integrity, metabolism, stress response activation, and fitness in iron-deplete conditions. Therefore, this function may represent one of the selective pressures that have maintained what was previously thought to be a redundant multiple-drug efflux pump within the *V. cholerae* genome. The fact that similar functions for RND efflux pumps have been identified in other Gram-negative species indicates that similar selective pressures may extend beyond *V. cholerae* into other bacterial genera and that our conclusions may be broadly applicable to all Gram-negative bacteria.

5.2 OmpR is a *V. cholerae* virulence repressor and contributes to virulence repression in a RND efflux-negative mutant

Previous work in our laboratory indicated that a Δ RND mutant strain of *V. cholerae* had repressed virulence factor production and was unable to colonize the infant mouse small intestine (150). This observation, coupled with recent findings that the transcriptome of the Δ RND strain was enriched for differentially expressed regulatory genes (121), lead to the hypothesis that the loss of RND efflux lead to the induction of regulatory genes that participated in virulence repression. To test this hypothesis we screened regulator genes that were induced in the Δ RND strain for a possible function in virulence factor production. This led to the finding the expression

of the response regulator *ompR* was increased in the Δ RND mutant when grown in AKI medium, and increased *ompR* expression contributed to virulence repression in the Δ RND strain. Interrogation of the function of OmpR in virulence factor production revealed that OmpR downregulated the ToxR regulon through direct repression of *aphB* (Section 3.4.4). These findings lead to the hypothesis that in the absence of RND efflux the intracellular accumulation of efflux substrates resulted in induction of *ompR*. We therefore set out to identify RND-dependent *ompR* inducing signals. We discovered that membrane intercalating components of bile, which are known RND efflux substrates, induced *ompR* expression in WT cells. These bile components, which are present, in AKI medium accumulated in Δ RND cells and induced *ompR*. Conversely, extraction of these compounds from AKI medium using a C18 column reduced *ompR* expression and alleviated OmpR-dependent virulence repression in the Δ RND background (Section 3.4.6). The findings that bile salts, a *V. cholerae* virulence modulatory signal (83), are present within the C18 fraction of AKI medium (Table 6) suggests that they are the membrane-intercalating bile components responsible for the RND-dependent *ompR* induction and virulence repression found in the Δ RND strain. However, more analysis is required to validate this hypothesis. In sum, these findings indicate that the *V. cholerae ompR* has evolved to be induced by bile, an important signaling cue that is present in the human small intestine, and that bile-induced *ompR* expression results in virulence gene repression via direct repression of the ToxR regulon.

The EnvZ/OmpR system is one of the most well studied two-component systems in bacteria. Canonically, *ompR* responds to changes in media osmolarity to regulate the expression of major outer-membrane porin proteins (100-102, 200). However, we found that the *V. cholerae ompR* homologue did not respond to changes in osmolarity, and was not required for growth at high NaCl concentrations (Section 3.4.5). Instead, *V. cholerae ompR* is induced by membrane

intercalating components of bile and repressed virulence gene expression. Although OmpR has been linked to the induction of virulence factors in other Gram-negative species (109, 204-208), to our knowledge, this is the first time that OmpR has been shown to function as a virulence repressor. Taken together, these findings suggest that *ompR* has evolved to respond to novel signals and perform novel regulatory tasks in *V. cholerae*.

Our work presented in section 3.4.6 that bile-induced *ompR* expression in a Δ RND mutant contributed to virulence repression uncovered a mechanistic link between *V. cholerae* RND efflux and virulence factor production. These findings indicate that the *V. cholerae* RND efflux pumps contribute to virulence gene expression via modulation of intracellular concentrations of known signaling molecules. RND efflux has been linked to the production of virulence factors in a range of Gram-negative bacteria in addition to *V. cholerae* (121, 148, 150, 154), but it is unknown how RND efflux impacts virulence gene expression in these other bacteria. We suggest that the findings presented here likely extend beyond *V. cholerae* to other bacterial genera, where RND efflux pumps may contribute to virulence regulation by similar mechanisms.

5.3 OmpR participates in the *V. cholerae* alkaline pH response

The work presented in chapter 3 documented that *V. cholerae* OmpR functions in repression of *aphB* to regulate virulence factor production. However, the functional role of OmpR in *V. cholerae* biology outside of virulence remained unknown. To explore the role of OmpR in *V. cholerae*, we sought to define the *V. cholerae* OmpR regulon through the use of RNA sequencing. This revealed that OmpR repressed components of the ToxR regulon, a finding that was consistent with the results from Chapter 3. OmpR was found to also repress genes that participate in the *V.*

cholerae acid tolerance response (ATR) including *clcA* and the Cad system (Section 4.4.3). This, coupled with numerous reports in other Gram-negative bacteria indicating that OmpR functions in response to acidic pH (103, 104, 203, 209-212, 227, 257) lead us to explore a possible role for *V. cholerae* OmpR in response to environmental pH. We found that *V. cholerae ompR* expression did not respond to acidic pH, but instead was strongly induced by alkaline pH; findings that were divergent from the function of OmpR in other Gram-negative bacteria. Alkaline pH induction of *ompR* resulted in the OmpR-dependent repression of *aphB*, *cadBA*, and virulence factor production. These findings indicated that *V. cholerae* OmpR has evolved to respond to alkaline pH to repress components of the acid tolerance response. Consistent with these findings OmpR was also required for fitness during growth under alkaline pH (Section 4.4.6).

Both OmpR and AphB are components of the *V. cholerae* ancestral genome, and all *Vibrios* including both pathogenic and non-pathogenic *V. cholerae* strains encode both regulators. This suggests that AphB and OmpR have native regulatory functions outside of regulation of virulence factor production in toxigenic strains of *V. cholerae*. Although both AphB and OmpR have been studied for decades, their native regulatory functions in *V. cholerae* remain poorly understood. The work presented in chapter 4 found that OmpR is required for optimal fitness in alkaline pH conditions, and in response to alkaline pH repressed the acid tolerance genes *cadBA* via regulation of *aphB* (Section 4.4.4). This is consistent with previous findings that AphB is a positive regulator of *cadBA*, and acidic pH induces *cadBA* expression via AphB (75). The finding that a $\Delta ompR$ mutant was attenuated under alkaline pH, while an $\Delta aphB$ mutant was not, indicates that the OmpR response to alkaline pH extends beyond its regulation of *aphB*. Our OmpR transcriptome data set (Table 5) represents a rich source of information that can be utilized to explore possible pathways that protect *V. cholerae* from alkaline pH induced stress. Collectively, these results suggest that

defense against alkaline pH likely represents a native function of *V. cholerae* OmpR, as alkaline pH is an environmental condition that *V. cholerae* faces both in the marine ecosystem and during human infection (267, 272).

Bacterial regulatory networks are extremely plastic and evolve in response to selective pressures placed on them by bacterial species moving to colonize new environmental niches. This results in conserved regulatory systems responding to novel signals, and regulating divergent genes in closely related bacterial species. The findings presented in chapters 3 and 4 indicate that the *V. cholerae ompR* has been the subject of divergent evolution. In gamaproteobacteria, the EnvZ/OmpR system canonically responds to environmental osmolarity and acidic pH (100-104, 200, 203, 209-212, 227, 257). Our results indicate that the *V. cholerae ompR* homologue does not respond to changes in osmolarity, or acidic pH (Sections 3.4.5 and 4.4.3). Instead, *V. cholerae ompR* is induced by membrane intercalating components of bile and alkaline pH (Sections 3.4.6 and 4.4.3), and in response regulates alkaline pH tolerance, and represses both virulence factor production and components of the ATR (Sections 3.4.6., 4.4.4, and 4.4.5). Therefore, these findings indicate that in *V. cholerae ompR* has evolved to respond to novel inducing cues and to control the expression of a regulon that is divergent from what is observed in other bacterial genera. We hypothesize that the unique lifestyle of pathogenic *V. cholerae* has supplied the selective pressure for this divergent evolution. As a native aquatic organism and enteric pathogen, bile and alkaline pH are two important environmental cues that pathogenic *V. cholerae* encounters during its infectious cycle. Bile serves as an important signal for enteric pathogens including *V. cholerae in vivo* (74, 237, 291). Alkaline pH is found both in the marine environment and during host infection, as sea water and rice water stool from cholera patients is markedly alkaline (267, 272). The alkaline pH of sea water serves as an environmental signal for marine bacteria (273).

Therefore, our findings that *V. cholerae ompR* responds to bile and alkaline pH to repress virulence and acid tolerance genes, while enhancing fitness at alkaline pH, are consistent with *ompR* having evolved novel functions specifically suited to the lifestyle of *V. cholerae*.

5.4 Model of the role of *V. cholerae* OmpR during host infection

We propose the following model of OmpR function during *V. cholerae* infection (Fig. 23). Following ingestion, *V. cholerae* enters the acidic environment of the stomach, and in response induces ATR genes. Exiting the stomach *V. cholerae* enters into the more alkaline environment of the small intestine and represses ATR genes (82). The high concentration of bile in the lumen of the small intestine induces *ompR* expression, which results in repression of ATR genes (e.g. *clcA*, and *cadBA*) and virulence genes, both through OmpR-dependent repression of *aphB*. *V. cholerae* traverses to the distal small intestine, its colonization niche (40, 41). To colonize the distal small intestine *V. cholerae* must penetrate the thick mucus layer that covers the epithelium (292). This mucus layer functions as a diffusion barrier for luminal bile, and thus the relative concentration of bile decreases proximal to the epithelium. As *V. cholerae* traverses the mucus the local concentration of bile decreases, resulting in reduced *ompR* expression. Decreased *ompR* transcription relieves repression on the ToxR regulon, and allows for virulence factor production. The production of virulence factors facilitates colonization of the epithelium, and results in the development of rice water stool, the hallmark symptom of *V. cholerae* infection. During the late stages of infection, in preparation for exit from the host into the alkaline aquatic ecosystem, *V. cholerae* represses virulence genes and induces genes required for dissemination and transmission (42, 44, 77, 243). The alkaline pH of rice water stool induces *ompR* expression, which results in

the repression of *aphB* and downstream virulence factors, while inducing genes required for fitness in an alkaline environment. Collectively, this model illustrates how the *V. cholerae* OmpR has evolved to meet the specific needs of *V. cholerae*, and functions in important aspects of host infection and likely environmental survival and persistence.

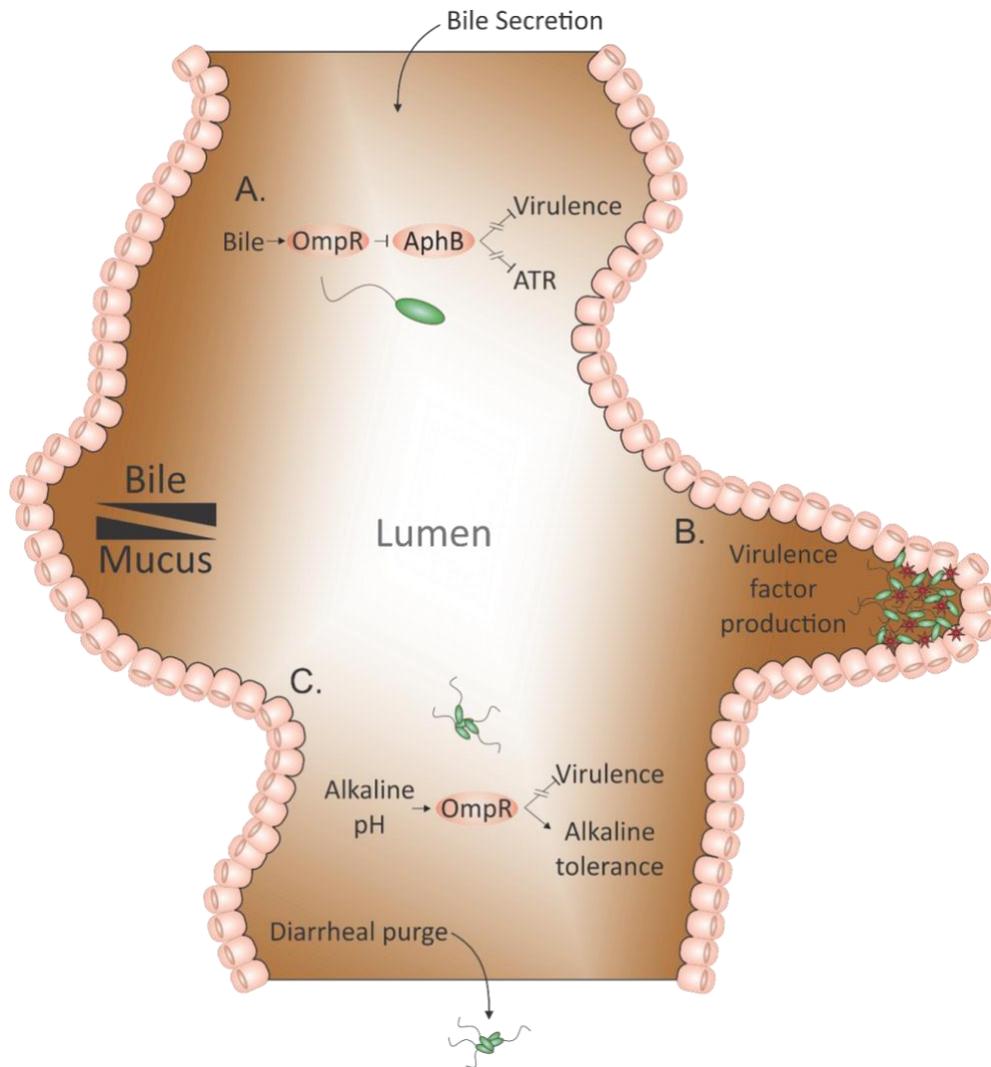


Figure 23 Model for the function of OmpR during *V. cholerae* infection

(A.) From the acidic stomach *V. cholerae* enters the lumen of the host small intestine where bile concentrations are high. Membrane intercalating agents within bile induce *ompR* expression, resulting in repression of virulence and acid tolerance genes. OmpR contributes to virulence repression as *V. cholerae* navigates to the distal small intestine where it colonizes enterocytes. (B.) Within the distal small intestine *V. cholerae* cells traverse the thick mucus layer that exists on top of intestinal cells. Bile concentrations are reduced across the mucus layer, resulting in decreased *ompR* expression, relieving OmpR-dependent repression of *aphB*, and virulence factor production is induced. (C.) During the late stages of infection, to prepare for host exit, the alkaline pH of cholera-induced rice water stool induces *ompR* expression, repressing virulence factor production and inducing alkaline pH tolerance to prepare for life within the alkaline marine environment.

5.5 Future directions

The findings presented in this dissertation have identified novel areas that warrant further investigation. Primarily, the identity of the compound(s) within the C18 fraction of AKI media that were responsible for the effects on virulence factor production found in chapter 3, and the genes within the OmpR regulon that contribute to the OmpR-dependent fitness at alkaline pH illustrated in chapter 4.

The extraction of AKI medium with a C18 column resulted in the repression of virulence factor production in WT cells, and restored virulence factor production in Δ RND cells (Fig. 15), the identity of the compound(s) present within AKI medium responsible for this differential phenotype remain unknown. Mass spectrometry analysis of C18-extracted AKI medium discovered that a number of bile salts are present within the C18 fraction of AKI medium (Table 6). Bile salts are an important virulence regulatory signal in a number of enteric pathogens including *V. cholerae* (83, 293), *Salmonella* (294), *Shigella* (295), and *E. coli* (296). Bile salts are efflux substrates of the *V. cholerae* RND efflux pumps (84, 150), therefore the growth of a Δ RND strain in the presence of bile salts results in their intracellular accumulation. Therefore, Δ RND cells cultured within AKI medium contain a higher concentration of bile salts, compared to efflux-proficient WT cells. Collectively, these observations indicate that an individual bile salt, or multiple bile salts in combination, are a possible dose-dependent *V. cholerae* virulence regulatory cue. The bile salts found within the C18 fraction of AKI medium, indicated in Table 6, thus represent attractive candidates for future studies to identify the exact species responsible for the observed dose-dependent virulence regulation.

Culturing a *V. cholerae* Δ RND strain in C18-depleted AKI medium resulted in reduced expression of *ompR*, and recovery of virulence factor production, both to WT levels (Fig. 15).

This, coupled with the finding that deletion of *ompR* only partially restored virulence factor production in a Δ RND *V. cholerae* strain (Fig 11) indicates that the virulence-modulatory compound(s) within the C18 fraction of AKI medium impact virulence through both OmpR-dependent and independent mechanisms. Therefore, analysis of any identified regulatory molecules within the C18 fraction of AKI medium will have to be assayed for their interaction with other virulence regulatory pathways in *V. cholerae*. This is supported by previous findings that the bile salt taurocholate modulates *V. cholerae* virulence through interaction with TcpP (83).

V. cholerae is highly alkaline tolerant and grows optimally at alkaline pH (297). For decades culture at high pH has been used as a means to enrich and isolate *V. cholerae* from fecal and environmental samples (51). Yet, the mechanisms that *V. cholerae* employs for such robust alkaline pH resistance remain largely unknown. The work presented in chapter 4 indicates that OmpR is an alkaline-responsive regulator, and is required for *V. cholerae* fitness at alkaline pH. To our knowledge OmpR is the first regulator identified to participate in *V. cholerae* alkaline fitness. In response to alkaline pH OmpR was found to repress ATR genes via repression of *aphB* (Fig. 19). However, the finding that a Δ *ompR* strain was attenuated at high pH, while a Δ *aphB* mutant was not (Fig. 21) indicates that OmpR contributes to the *V. cholerae* alkaline pH response in ways that are independent of its regulation of *aphB*. While OmpR-regulated genes that participate in the *V. cholerae* alkaline response remain unknown, our OmpR regulon data set (Table 5) represents a powerful tool for the identification of OmpR-dependent alkaline stress response genes.

Our OmpR regulon data set indicates that OmpR represses genes involved in central metabolism such as *sdh* genes, and induces expression of *cys* genes, whose gene products are involved in sulfate metabolism and the production of sulfur-containing amino acids. Previous

studies have indicated that central metabolism genes, including *sdh* genes, are repressed in *E. coli* and *Staphylococcus aureus* in response to alkaline pH (298, 299). Further, transcriptional profiling of *Shewanella oneidensis* and *E. coli* found that both species induce the expression of *cys* genes in response to high pH (253, 300). A possible explanation for this is that alkaline pH increases the cellular demand for sulfur containing amino acids to replace those damaged by alkaline stress, but this is yet to be studied. The finding that *V. cholerae* is an alkaline pH-responsive regulator that represses *sdh* and induces *cys* gene expression, coupled with the findings that and similar transcriptional responses have been identified in response to alkaline pH in other bacterial species collectively indicates possible conserved mechanisms in bacterial response to alkaline conditions in both Gram-positive and Gram-negative bacteria. Thus, these pathways are attractive candidates for interrogation to identify OmpR-dependent genes involved in alkaline pH fitness in *V. cholerae*. A defined transposon mutant library is available to us in the laboratory (301). Use of this library would allow for the high throughput screening of candidate pathways and genes identified from the OmpR regulon that are required for fitness at alkaline pH. Such studies would expand our knowledge of the mechanisms that *V. cholerae* employs for resistance to alkaline pH.

Bacterial antimicrobial resistance is rapidly emerging as a profound public health risk. The estimated annual medical expense due to patients infected with antimicrobial-resistant pathogens is as high as \$20 billion, along with an addition productivity loss of \$35 billion (302). This illustrates the extreme need for the development of novel treatment strategies to combat the development and spread of antimicrobial resistant pathogens. Due to the central role that drug efflux plays in antimicrobial resistance, drugs that target the activity of multidrug efflux pumps are an attractive treatment option. For decades such drugs, termed efflux pump inhibitors (EPIs), have been shown to be effective at blocking the action of multidrug efflux pumps and increasing

bacterial antibiotic susceptibility *in vitro* (303). However, no EPI's have been approved for clinical use (304, 305). The work presented in this dissertation highlights some of the consequences resulting from the loss of RND multidrug efflux. The loss of RND efflux in *V. cholerae* induced the Cpx and EnvZ/OmpR stress response systems, altered cellular metabolism, repressed virulence factor production, decreased membrane integrity, and reduced fitness in iron-deplete environments. This work illustrates that RND-mediated efflux is not only required for antimicrobial resistance, but also to maintain cell homeostasis and virulence factor production. Collectively, these findings indicate that successful drug-mediated inhibition of RND efflux could be an effective treatment method by impacting multiple important aspects of pathogen physiology, and thus gives additional validity to the development of drugs to selectively target bacterial multidrug efflux pumps.

The work presented in chapters 2 and 3 used multiple experimental screening approaches utilizing a RND efflux-null mutant strain of *V. cholerae* to uncover novel signals and regulatory pathways that participate in metabolism, iron acquisition, stress responses, and virulence factor production. These studies indicate that efflux-null mutant bacterial strains are a powerful tool that can be exploited to interrogate a wide range of bacterial adaptive responses. Consistent with findings that have linked multidrug efflux to a broad range of bacterial phenotypes in Gram-negative pathogens, similar approaches utilizing efflux-null mutants have been used to study efflux-linked phenotypes including metabolism, motility and virulence in *Salmonella* (275, 276), iron acquisition and fatty acid production in *E. coli* biofilm and virulence in *Acinetobacter baumannii* (278, 279), and quorum sensing in *Pseudomonas aeruginosa* (145). In sum, the work presented in this dissertation, in concert with this body of literature, lends validity to the use of

efflux-null mutants as a powerful and flexible model system to study efflux-linked phenotypes, and suggests that such approaches are broadly applicable in Gram-negative species.

5.6 Limitations

Using the data presented in chapter 2 we conclude that ROS produced during aerobic respiration resulted in the RND-dependent activation of the Cpx system through attack of periplasmic protein disulfide bonds (Section 2.5). We came to this conclusion as aberrant disulfide bond formation in periplasmic proteins was shown to be a *V. cholerae* Cpx-inducing signal (99). However, we cannot exclude the possibility that ROS induce the *V. cholerae* Cpx system through alternative pathways, such as ROS-induced oxidative damage outside of aberrant disulfide bond formation. The experiments performed could not discern between these possibilities, and it is conceivable that aerobic respiration and ROS may induce the Cpx system through multiple mechanisms simultaneously.

In chapter 2 we hypothesized that the intracellular accumulation of vibriobactin in the Δ RND strain results in constitutive Cpx activation through chelation of iron from iron-binding membrane proteins, however this hypothesis was not directly tested. Although we found that a Δ RND strain was not iron stressed (Fig. 7) the experiments performed could not determine if retained vibriobactin in the Δ RND strain in fact chelated intracellular iron. It is possible that vibriobactin accumulation results in Cpx activation through multiple pathways, or a different mechanism entirely. Further work will have to be performed to determine if retained vibriobactin does chelate iron from iron binding proteins. In addition, the secretion of vibriobactin in WT and Δ RND strains was not directly tested. Vibriobactin secretion was inferred using the cross-feeding

growth stimulation assay (Section 2.3.8, Fig. 5). To have full confidence in the presented model intra and extracellular vibriobactin would need to be directly measured in the strains used in Figure 5.

The work presented in chapters 3 and 4 uncovered functions for the TCS response regulator OmpR in *V. cholerae* that are divergent from its role in other Gram-negative species. However, there was no exploration of the HK that interacts with OmpR in *V. cholerae*. Canonically, EnvZ is OmpR's cognate HK, and *V. cholerae* encodes for an EnvZ homologue downstream of OmpR (VC2713). However, TCS response regulators, including OmpR, are known to occasionally interact with non-canonical HK's (306-308), therefore we cannot assume that OmpR's interacting partner in *V. cholerae* is EnvZ, and it is possible that OmpR's function is modulated by multiple HKs. Further studies will be required to determine the OmpR interacting partner(s) in *V. cholerae*.

We found in chapters 3 and 4 that *ompR* expression in *V. cholerae* is induced by novel signals, membrane intercalating compounds and alkaline pH, however the regulator(s) that function upstream of *ompR* that are responsible for *ompR* induction under the tested conditions were not explored. OmpR regulates its own expression in other Gram-negative species (103, 212), however, we found that *ompR* expression is not autoregulated in *V. cholerae* (Fig. 16). The expression of *ompR* in Salmonella is regulated by LtrR (224), while CRP regulates *ompR* expression in *E. coli* (309). The finding that CRP has no regulatory activity of *ompR* expression in *Yersinia pestis* (310) indicates that the control of *ompR* expression is divergent in related bacterial species. Recent ChIP-seq analysis of *V. cholerae* CRP-DNA binding did not indicate an interaction between *V. cholerae* CRP and the *ompR* promoter region, indicating that *ompR* expression may be regulated by other factors in *V. cholerae* (311).

Our conclusion that alkaline pH is a novel *ompR* inducing cue specific to *V. cholerae* (Section 4.5) is limited by published studies. It is possible that alkaline pH is an *ompR* inducing signal in other Gram-negative species, but has not been directly tested. Published transcriptomic data sets have not indicated differential *ompR* expression in response to alkaline pH in either in *E. coli* (298) or *Shewanella oneidensis* (253). These studies indicate that alkaline pH is not an *ompR* inducing signal in these species under the tested conditions, however, further work will need to be done to determine if alkaline pH induction of *ompR* is in fact specific to *V. cholerae*. Interestingly, a recent report indicated that the Salmonella *ompR* regulator *lrrR* is induced in response to alkaline pH. This finding suggests that *ompR* expression may be induced by alkaline pH in Salmonella as well as *V. cholerae* (312). However, to our knowledge this has not yet been directly tested.

The model for the *in vivo* function of *V. cholerae* OmpR in Figure 23 indicates that the two novel *V. cholerae ompR* inducing signals illustrated in chapters 3 and 4, membrane intercalating compounds and alkaline pH, are independent signals sensed by the cell. However, it is possible that these two signals impact cellular physiology in similar ways, which results in *ompR* induction through a conserved mechanism. In *E. coli* and Salmonella osmotic stress and acidic pH both result in *ompR* induction through the conserved mechanism of cytoplasmic acidification (257). This finding suggests that our findings indicating that bile and alkaline pH induce *V. cholerae ompR* expression may do so through a similar conserved mechanism.

Appendix A The *V. cholerae* OmpR regulon

Table 5 Genes differentially regulated during *ompR* overexpression as identified by RNAsequencing

Locus number	Gene name	Fold change (pBAD-ompR/pBAD)	FDR p-value
VC2698	<i>aspA</i>	-3.87	0
VC0280	<i>cadB</i>	-2.65	2.49E-11
VC2559	<i>cysN</i>	2.56	1.76E-03
VCA0747	<i>glpA</i>	3.11	1.62E-12
VCA0749	<i>glpC</i>	2.13	1.57E-07
VCA0657	<i>glpD</i>	2.68	9.11E-15
VC2092	<i>gltA</i>	-2.21	1.32E-07
VC1328	<i>mglC</i>	-2.87	9.69E-08
VC2714	<i>ompR</i>	98,433.39	0
VC1092	<i>oppB</i>	-3.29	0
VC1094	<i>oppD</i>	-2.07	1.03E-07
VCA1062	<i>potE</i>	-2.4	5.14E-05
VC2089	<i>sdhA</i>	-3.11	0
VC2088	<i>sdhB</i>	-2.04	5.00E-08
VC2091	<i>sdhC</i>	-3.69	0
VC0743	<i>secD_1</i>	-2.28	6.19E-09
VC2085	<i>sucC</i>	-2.06	1.06E-07
VCA0885	<i>tdh</i>	-2.88	8.84E-12
VC0824	<i>tpx</i>	-3.62	0
VC0036	VC0036	-2.26	3.27E-09
VC0112	VC0112	-2.8	1.67E-14
VC0168	VC0168	-2.29	5.09E-08
VC0200	VC0200	2.05	9.44E-07
VC0281	VC0281	-2.05	1.88E-05
VC0384	VC0384	2.36	3.03E-03
VC0385	VC0385	2.02	0.02
VC0389	VC0389	-2.46	3.42E-12
VC0391	VC0391	-2.73	3.42E-12
VC0430	VC0430	-2.43	1.27E-12
VC0483	VC0483	-2.1	2.38E-08
VC0538	VC0538	2.3	1.51E-03
VC0539	VC0539	2.31	3.04E-04
VC0540	VC0540	2.2	6.67E-04

Table 5 continued

Locus number	Gene name	Fold change (pBAD-ompR/pBAD)	FDR p-value
VC0573	VC0573	-2	1.70E-08
VC0586	VC0586	-2.05	1.68E-09
VC0606	VC0606	-2.79	1.67E-14
VC0607	VC0607	-2.35	6.67E-11
VC0687	VC0687	3.5	0
VC0700	VC0700	-3.03	0
VC0786	VC0786	-3.77	0
VC0794	VC0794	2.39	0.03
VC0795	VC0795	2.15	0.04
VC0796	VC0796	3.45	0.03
VC0819	VC0819	-2.23	2.16E-05
VC0820	VC0820	-2.79	3.40E-12
VC0821	VC0821	-2.23	3.70E-09
VC0826	VC0826	-2.14	1.25E-07
VC0827	VC0827	-2.22	1.66E-08
VC0828	VC0828	-2.65	5.16E-11
VC0829	VC0829	-2.64	6.03E-09
VC0830	VC0830	-2.93	7.02E-13
VC0831	VC0831	-3.12	1.52E-13
VC0832	VC0832	-3.24	3.28E-14
VC0833	VC0833	-3.1	1.10E-13
VC0834	VC0834	-3.17	0
VC0835	VC0835	-2.78	9.00E-11
VC0836	VC0836	-2.81	1.12E-12
VC0837	VC0837	-3.84	0
VC0841	VC0841	-2.17	8.87E-06
VC0844	VC0844	-3.03	5.36E-12
VC0845	VC0845	-2.96	1.44E-10
VC0873	VC0873	-2.06	2.97E-08
VC0884	VC0884	-2.2	7.87E-10
VC1075	VC1075	-2.37	1.36E-11
VC1091	VC1091	-2.87	0
VC1093	VC1093	-2.59	9.11E-15
VC1158	VC1158	-2.36	6.57E-13
VC1168	VC1168	-3.06	0
VC1264	VC1264	-2.09	1.78E-07
VC1272	VC1272	2.28	9.70E-13

Table 5 continued

Locus number	Gene name	Fold change (pBAD-ompR/pBAD)	FDR p-value
VC1301	VC1301	2.62	0
VC1318	VC1318	-2.03	2.03E-06
VC1325	VC1325	-5.32	6.11E-13
VC1327	VC1327	-3.83	2.07E-12
VC1329	VC1329	3.56	0
VC1422	VC1422	2.27	1.40E-09
VC1438	VC1438	-2.11	5.30E-11
VC1439	VC1439	-2.6	1.67E-14
VC1440	VC1440	-2.75	9.11E-15
VC1441	VC1441	-2.97	0
VC1442	VC1442	-2.92	0
VC1443	VC1443	-2.76	4.82E-14
VC1456	VC1456	-2.83	4.30E-10
VC1457	VC1457	-2.26	1.41E-05
VC1468	VC1468	2.24	4.45E-07
VC1492	VC1492	-2.28	2.33E-08
VC1510	VC1510	-2.17	2.31E-09
VC1511	VC1511	-2.07	2.74E-07
VC1513	VC1513	-2.05	8.58E-07
VC1514	VC1514	-2.05	1.47E-08
VC1560	VC1560	-2.47	3.56E-10
VC1581	VC1581	2.29	9.97E-08
VC1582	VC1582	2.21	4.53E-07
VC1589	VC1589	-4.27	2.01E-13
VC1590	VC1590	-5.38	3.32E-11
VC1591	VC1591	-7.04	8.88E-12
VC1592	VC1592	-2.49	3.15E-07
VC1655	VC1655	2.52	9.98E-05
VC1661	VC1661	-3.31	0
VC1669	VC1669	2.56	0
VC1687	VC1687	2.41	0
VC1692	VC1692	2.09	3.02E-06
VC1693	VC1693	2.32	3.46E-10
VC1694	VC1694	2.23	4.36E-04
VC1695	VC1695	-2.01	7.46E-07
VC1807	VC1807	6.77	0
VC1820	VC1820	27.39	0

Table 5 continued

Locus number	Gene name	Fold change (pBAD-ompR/pBAD)	FDR p-value
VC1821	VC1821	12.65	0
VC1822	VC1822	2.29	1.50E-10
VC1824	VC1824	3.06	0
VC1825	VC1825	5.52	0
VC1826	VC1826	17.66	0
VC1827	VC1827	14.78	0
VC1828	VC1828	9.28	0
VC1865	VC1865	2.23	6.67E-11
VC1870	VC1870	-2.19	2.59E-10
VC1905	VC1905	-2.76	9.02E-09
VC1953	VC1953	2.1	5.19E-06
VC1962	VC1962	-2.18	8.43E-09
VC1965	VC1965	-2.29	4.57E-10
VC1991	VC1991	2.01	3.45E-05
VC2031	VC2031	2.57	2.78E-12
VC2090	VC2090	-3.5	0
VC2100	VC2100	-2.01	2.36E-09
VC2337	VC2337	-2.36	1.47E-09
VC2366	VC2366	-2.18	2.23E-10
VC2367	VC2367	-2.21	1.71E-12
VC2469	VC2469	-2.86	0
VC2558	VC2558	2.19	5.29E-03
VC2560	VC2560	3.02	2.51E-04
VC2561	VC2561	3.61	5.50E-06
VC2569	VC2569	2.61	8.79E-08
VC2637	VC2637	-2.56	3.18E-09
VC2638	VC2638	-2.74	1.34E-08
VC2699	VC2699	-2.53	1.67E-14
VCA0029	VCA0029	-2.61	0
VCA0030	VCA0030	-2.67	1.32E-13
VCA0076	VCA0076	2.69	0
VCA0088	VCA0088	-2.43	0
VCA0094	VCA0094	4.15	0
VCA0095	VCA0095	3.9	2.95E-10
VCA0130	VCA0130	-2.66	4.89E-05
VCA0139	VCA0139	-2.31	4.16E-06
VCA0179	VCA0179	4.2	9.11E-15

Table 5 continued

Locus number	Gene name	Fold change (pBAD-ompR/pBAD)	FDR p-value
VCA0193	VCA0193	2.11	2.48E-09
VCA0219	VCA0219	-2.29	4.97E-10
VCA0229	VCA0229	2.14	7.43E-08
VCA0260	VCA0260	-2.29	8.87E-11
VCA0274	VCA0274	-2.4	5.92E-11
VCA0276	VCA0276	-3.07	1.02E-06
VCA0277	VCA0277	-3.1	1.29E-06
VCA0278	VCA0278	-2.36	3.01E-08
VCA0280	VCA0280	-2.09	3.32E-04
VCA0365	VCA0365	-126.3	0.03
VCA0378	VCA0378	3.09	0.04
VCA0412	VCA0412	2.5	0.02
VCA0454	VCA0454	3	1.07E-05
VCA0464	VCA0464	2.26	1.64E-04
VCA0526	VCA0526	-2.53	0
VCA0534	VCA0534	-2.03	2.09E-08
VCA0537	VCA0537	-2.27	9.81E-11
VCA0538	VCA0538	-2.04	6.79E-08
VCA0554	VCA0554	2.86	0
VCA0619	VCA0619	2.38	6.87E-10
VCA0628	VCA0628	-2.07	1.83E-05
VCA0630	VCA0630	-2.23	1.68E-09
VCA0631	VCA0631	-2.31	4.82E-12
VCA0632	VCA0632	-2.54	2.14E-12
VCA0678	VCA0678	-2.87	9.74E-07
VCA0679	VCA0679	-2.57	8.04E-08
VCA0732	VCA0732	-4.3	0
VCA0745	VCA0745	3.21	0
VCA0748	VCA0748	2.36	1.68E-09
VCA0798	VCA0798	2.04	2.64E-07
VCA0804	VCA0804	2.65	5.14E-05
VCA0845	VCA0845	-2.06	2.14E-05
VCA0862	VCA0862	2.67	2.33E-09
VCA0863	VCA0863	2.12	3.16E-06
VCA0867	VCA0867	-3.39	1.27E-07
VCA0886	VCA0886	-2.38	9.59E-07
VCA0909	VCA0909	2.35	8.87E-11

Table 5 continued

Locus number	Gene name	Fold change (pBAD-ompR/pBAD)	FDR p-value
VCA0924	VCA0924	-2.89	2.20E-13
VCA0935	VCA0935	3.29	5.69E-07
VCA0966	VCA0966	-2.02	2.09E-08
VCA0982	VCA0982	-2.21	0
VCA0983	VCA0983	-2.03	5.60E-07
VCA0994	VCA0994	-3.67	3.60E-12
VCA1012	VCA1012	2.6	0
VCA1013	VCA1013	2.69	0
VCA1063	VCA1063	-2.43	2.87E-05
VCA1079	VCA1079	-2.05	3.33E-08

Appendix B Identification of bile salts in the C18 fraction of AKI medium

Appendix B.1 Method for mass spectrometry analysis of bile salts present in the C18 fraction of AKI medium

Sep-Pak C18 cartridges (Waters) were preconditioned with 10 ml of 100% methanol followed by 10 ml of sterile double-distilled water (ddH₂O) before 50 ml of AKI broth was passed through the cartridge and the flow-through collected and used as C18 extracted AKI medium. Molecules that were retained on the C18 columns following the passage of AKI medium were eluted from the column with 10 ml (5X concentrated) of 100% methanol and used as C18 eluent. AKI broth medium, C18 extracted AKI medium, and C18 eluent were all submitted in triplicate for mass spectrometry analysis using bile salts internal standards. C18 eluent was calculated at 1X relative concentration.

Table 6 Bile salts concentrations measured in (analyte / ISTD) via mass spec analysis

Bile salt	AKI medium (SD)	C18 extracted AKI medium (SD)	C18 eluent (SD)
TβMCA	NF	NF	0.053 (0.011)
βMCA	17.253 (0.432)	NF	19.449 (1.496)
TUDCA	0.3570 (0.007)	NF	0.410 (0.036)
TCA	58.806 (1.503)	0.005 (0.004)	61.0378 (5.051)
GCA	53.100 (0.980)	NF	57.575 (3.538)
CA	45.922 (0.747)	NF	49.751 (4.224)
UDCA	0.123 (0.010)	NF	0.151 (0.016)
TDCDA	NF	NF	NF
TDCA	6.221 (0.106)	0.002 (0.0003)	6.955 (0.472)
GUDCA	0.028 (0.002)	NF	0.032 (0.002)
CDCA	0.516 (0.025)	NF	1.332 (0.153)
TLCA	0.1211 (0.005)	0.005 (0.0001)	0.179 (0.012)
GLCA	0.047 (0.003)	0.0008 (0.0004)	0.076 (0.006)
DCA	4.130 (0.061)	NF	4.925 (0.363)
GCDCA	2.598 (0.084)	NF	2.881 (0.173)
GDCA	10.547 (0.157)	NF	12.124 (0.799)
LCA	NF	NF	NF

TβMCA - Tauro-β-muricholic acid, βMCA - β-muricholic acid, TUDCA – Tauroursodeoxycholate, TCA – Taurocholate, GCA – Glycocholate, CA – Cholate, UDCA - Ursodeoxycholic acid, TDCDA – Taurochenodeoxycholate, TDCA – Taurodeoxycholate, GUDCA – Glycoursodeoxycholate, CDCA – Chenodeoxycholate, TLCA – Tauroolithocholate, GLCA – Glycolithocholate, DCA – Deoxycholate, GCDCA – Glycochenodeoxycholate, GDCA – Glycodeoxycholate, LCA - Lithocholic acid, NF – not found

Appendix C Abbreviations

β - beta

\pm - plus/minus

:: - gene insertion

~ - approximately

Δ - deletion

$^{\circ}\text{C}$ - degree centigrade

μg – micro gram

A - absorbance

ABC - ATP-binding cassette

Ace - accessory cholera enterotoxin

ANOVA - analysis of variance

ATR – Acid tolerance response

bp - base pair

CAMP - cationic antimicrobial peptide

CAS - chrome azurol S

Cb - carbenicillin

cDNA - complementary DNA

cf. – compare

CFTR - cystic fibrosis trans-membrane conductance regulator

ChIP-seq - chromatin immunoprecipitation-sequencing

Cm - chloramphenicol

CT – cholera toxin

CuCl₂ – copper chloride

ddH₂O - double-distilled water

DNA - deoxyribonucleic acid

DOC - deoxycholate

E. coli - *Escherichia coli*

e.g – for example

ELISA - enzyme-linked immunosorbent assay

EPI - efflux pump inhibitors

ER – endoplasmic reticulum

ETC – electron transport chain

FeSO₄ – iron sulfate

GbpA - N-acetylglucosamine binding protein

GI – gastrointestinal

GM1 - monosialotetrahexosyl ganglioside

h - hour

HCl - hydrochloric acid

HGT – Horizontal gene transfer

HK- Histidine kinase

i.e – for example

Kb - kilo bases

Km - kanamycin

lacZ - in reference to the β-galactosidase gene

LB - Luria Bertani

LPS – lipopolysaccharide

M - molar

MATE - multidrug and toxic compound extrusion

MFS - major facilitator superfamily

min – minutes

mL - milli liter

mm – millimeter

mM – millimolar

MnCl₂ - manganese chloride

MSHA - mannose-sensitive hemagglutinin

MU - miller units

NaCl – sodium chloride

NaOH – sodium hydroxide

OD - optical density

OD₆₀₀ - optical density at 600 nano meters

OMP - outer membrane protein

ORF – open reading frame

P - P value

PACE - proteobacterial antimicrobial compound efflux

PAGE - polyacrylamide gel electrophoresis

PBS - phosphate buffered saline

PCR - polymerase chain reaction

PDI - protein disulfide isomerase

pH - measurement of acidity/basicity of an aqueous solution

PTS - phosphotransferase system

R - resistance

RNA - ribonucleic acid

RNA-seq – RNA sequencing

RND - resistance-nodulation-division

RR – response regulator

SD - standard deviation

SDS - sodium dodecyl sulfate

SEM - standard error of the mean

Sm - streptomycin

SMR - small multidrug resistance

ssp – species

TCP – toxin coregulated pilus

TCS – two-component system

TTFA - thenoyltrifluoroacetone

V. cholerae: Vibrio cholerae

WT – wild-type

X-gal - 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

Zot - zonula occludens toxin

λ – lambda

μ L - micro liter

μM – micro molar

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