CHARACTERIZATION OF THE *VIBRIO CHOLERAE* RND EFFLUX SYSTEMS

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The resistance-nodulation-division (RND) family efflux systems are ubiquitous transporters that have been extensively studied due to their ability to efflux a broad range of xenobiotic substrates. There is increasing evidence to suggest that the RND efflux systems have physiological roles beyond xenobiotic resistance to include pathogenesis and cellular stress responses. The *Vibrio cholerae* genome encoded six RND efflux systems: VexAB, VexCD, VexEF, VexGH, VexIJK, and VexLM. This work sought to characterize the role of these systems in antimicrobial resistance, pathogenesis, and environmental stress.

The *V. cholerae* RND efflux systems were characterized according to their substrate specificity and contribution to antimicrobial resistance. The VexAB, VexCD, VexGH, and VexIJK RND efflux systems were shown to contribute to resistance against multiple antibiotics, bile salts, and other detergents. In addition to functioning in antimicrobial resistance, the RND efflux systems were found to function in the extrusion of metabolic intermediates. In the absence of RND efflux, the accumulation of potentially toxic metabolites induced the expression of *vexRAB, vexGH,* and the Cpx membrane stress response system. Subsequent studies showed that independent activation of the Cpx system also resulted in the induction of *vexRAB* and *vexGH* expression, which indicated that the Cpx system and the *vexRAB* and *vexGH* RND efflux systems were reciprocally regulated. Collectively these results suggested a model whereby the

vexRAB and *vexGH* RND efflux systems function to reduce cellular stress resulting from endogenous metabolites, xenobiotics, and factors that induce a membrane stress response.

The VexAB RND system was also found to be positively regulated by VexR, a TetR-family regulator that was encoded within the same operon (i.e. *vexRAB*). VexR activated *vexRAB* expression in response to VexAB efflux substrates and in the absence of RND efflux activity. The latter phenotype suggested that the VexR modulated *vexRAB* expression in response to endogenous cues. Overall the data presented in this thesis supported the conclusion that the *V. cholerae* RND efflux systems functioned in responses that were essential for environmental adaptation including intestinal colonization, xenobiotic resistance, metabolic imbalance, and membrane stress.

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“Not only so but we also rejoice in our trials, because we know that trials produce perseverance; perseverance, character; and character, hope.”

1.0 INTRODUCTION

1.1 CHOLERA

Vibrio cholerae is the causative agent of the diarrheic disease cholera (1). The burden of cholera has largely been abolished in developed countries due to the formation of public sanitation works (2, 3). However, cholera remains a global health concern as it is now endemic in many regions of the world, affecting underdeveloped communities and refugee camps which often lack access to clean drinking water, proper sanitation, and medical care (1, 3, 4). In many ways, those most affected by the morbidity of cholera are the children who contract cholera during key developmental stages (5). The loss of fluids and electrolytes characteristic of the disease are not only an immediate threat to their life and health, but can cause lasting developmental retardation which may follow the child into adulthood (6). Cholera is estimated to annually afflict ~5 million people; as such it remains a significant public health problem (7).

Cholera-like diseases have been long recorded throughout history (8). The first in modern history originated in India in 1817, but it was not until 1883 that bacteria were demonstrated to be the cause of cholera (9). Between 1817 and 1923 there were six recorded cholera pandemics (8, 10). The seventh and ongoing pandemic began in 1961, spreading from Indonesia to India and the Middle East. In the 1970s, it moved to Africa and reached South America in the 1990s (11-14).

1.2 *VIBRIO CHOLERAE*

V. cholerae is a Gram-negative, motile, facultative anaerobic bacteria that is the causative agent of the disease cholera. More than 200 serogroups of *V. cholerae* have been isolated from the environment. However, only two serogroups have been associated with epidemic cholera. The O1 serogroup has been shown to be responsible for the 5th, 6th, and 7th pandemics (8). In 1992 a new serogroup (O139) was reported as the cause of a cholera outbreak in Bangladesh and India leading some to refer to the event as the 8th cholera pandemic (14-17). Despite the emergence of the O139 serogroup, the O1 serogroup still remains the dominant cause of present day cholera outbreaks. Although the incidents are rare, non-O1/non-O139 strains have been isolated from human infections, but were not associated with epidemic disease (18-21).

The O1 serogroup can further be divided into two biotypes: classical and El Tor. These biotypes are determined by physiological differences such as resistance to polymyxin B, hemolysin activity, the number of genes encoding cholera toxin (CT), and the presence of mannose-sensitive hemagglutinin (MSHA) (22). The classical biotype was associated with the 5th and 6th pandemics, while the 7th is caused by the El Tor biotype (8). The El Tor biotype is associated with a milder form of cholera and appears to have an advantage in survival in the aquatic environment (23). The O139 serogroup is believed to have evolved from the O1 serogroup El Tor biotype via a seroconversion event (24, 25). The El Tor strains are predominant in regions where the O1 serogroup is found.

1.2.1 *V. cholerae* in the aquatic environment

V. cholerae is a facultative human pathogen and a natural inhabitant of aquatic ecosystems (26-29). *V. cholerae* can attach to the surfaces of marine species such as plants, copepods, crustaceans, and insects (27, 30). The O1 serogroup has been shown to bind to the chitin of these marine organisms (31) and form biofilms which contributes to acid tolerance following human ingestion of *V. cholerae* contaminated food or water (32). One key difference between the El Tor and classical biotypes is the production of the MSHA (mannose sensitive hemagglutinin) type IV pili. The MSHA type IV pili is produced in the El Tor O1 and O139 strains and is involved in the adherence to chitin of zooplankton (33). In contrast, the classical strains do not assemble the MSHA pili (33). This likely contributes to the increased ability of the El Tor strains to survive in the aquatic environment (23).

The association of *V. cholerae* with zooplankton is assumed to provide protection against environmental stresses, largely due to formation of biofilms on these surfaces. Reports have shown that the El Tor O1 and O139 strains form biofilms on abiotic surfaces in a manner that is dependent on the expression of the *Vibrio* polysaccharide (VPS) exopolysaccharide (34-37). In addition to VPS exopolysaccharide, the El Tor O1 serogroup requires MSHA and flagellar motility to form normal biofilm (35, 36). The O139 serogroup does not require either MSHA or flagellar motility (35, 36). This has been attributed to a high level of expression of the VPS polysaccharide in the O139 serogroup. While the expression of VPS polysaccharide is found to contribute to environmental survival, its expression was found to inhibit colonization in the infant mouse model, suggesting that it decreases virulence (36).

1.2.2 Transmission and infection

Humans acquire *V. cholerae* through ingestion of contaminated food or water. In areas where *V. cholerae* is endemic, poor sanitation is the primary cause of cholera outbreaks (1). After ingestion, the bacteria must survive and pass through the gastric acid barrier of the stomach. The infectious dose of *V. cholerae* reported in human volunteer studies is fairly high (10^6-10^{11} colony forming units) (38), likely due to the sensitivity of *V. cholerae* to acidic conditions (39). After passage through the stomach, the bacteria penetrate the mucus lining of the intestinal epithelium, where they adhere to and colonize the intestinal epithelia (40). The primary site of *V. cholerae* colonization is the small intestine. During colonization, *V. cholerae* produces cholera toxin (CT) causing the diarrheic symptoms of cholera (discussed below) thereby disseminating the bacteria back into the environment (40).

Human-shed *V. cholerae* was found to have a greatly increased infectivity compared to in vitro grown strains (41). This was found to be transitory as human-shed samples, which were further cultured in vitro, no longer display a competitive advantage in vivo. Therefore, passage through the host was found to give *V. cholerae* a hyper-infectious phenotype that is believed to contribute to the epidemic spread of cholera.

In the transition from the aquatic environment to its colonization niche in the small intestine, *V. cholerae* is exposed to several environmental changes such as temperature, acidity, and osmolarity. Additionally, the bacteria encounter innate host defenses such as bile salts, organic acids, complement secreted by intestinal epithelial cells, and defensins produced by Paneth cells (42, 43). *V. cholerae* has developed mechanisms in order to survive and overcome these host defenses, including limiting the uptake and accumulation of antimicrobial compounds. This is accomplished by modulating the outer membrane permeability in conjunction with the

extrusion of toxic compounds via active efflux transporter (as discussed below) (9, 44-47). Further contributing to the ability of *V. cholerae* to colonize under these hostile conditions is the ability to produce virulence factors, likely in response to these transitional signals (1).

1.2.3 Major virulence factors

The disease cholera is characterized by a profuse watery diarrhea, referred to as rice water stool. At the apex of infection, an adult can lose >20 L of liquid per day (48). Due to this drastic loss of fluids, untreated cholera can lead to dehydration, hypotensive shock, and death within 12h of the first symptoms (38). Without intervention cholera has a 60% mortality rate which can be lowered to ~1.5% with oral or intravenous rehydration (23). The massive loss of fluid is largely due to the action of the CT (49). CT is an AB-type enterotoxin that is secreted across the bacterial outer membrane into the extracellular environment (50). It is a hetero-oligomer composed of a single active A subunit in association with a ring formed by five B subunits (50-55). The CT-B pentamer binds to the GM_1 ganglioside receptor on the surface of epithelial cells (56-58). The CT-A subunit is enters into the host cytosol, where it is activated by the reduction of a disulfide bond releasing CT-A1 subunit (59-61). The active CT-A1 subunit then ADP-ribosylates the G-protein Gsα, a GTP-binding regulatory protein associated with adenylate cyclase, which leads to the constitutive production of cyclic AMP (cAMP) (62-65). cAMP increases chloride and bicarbonate secretion while simultaneously inhibiting sodium chloride absorption (49, 66). Through osmotic processes, this results in passive water loss from the cell into the lumen, resulting in the massive diarrhea that is characteristic of the disease (63, 64).

The toxin co-regulated pilus (TCP) is the other major virulence factor in *V. cholerae*. The TCP is a type IV pilus that is required for intestinal colonization in both humans and animal

models (67, 68). Although TCP does not appear to directly bind to the intestinal epithelium, it does cause aggregation of *V. cholerae* and induces microcolony formation within the intestine (69). Additionally, the TCP acts as a receptor for the CTXϕ bacteriophage which carries the genes that encode for CT (*ctxAB*) (70). TCP is a polymer composed of the TcpA pilin subunits (71). The genes involved in TCP biosynthesis are encoded in the *tcpA-F* operon. The TCP operon is encoded on the *Vibrio* Pathogenicity Island (VPI) which encodes additional accessory colonization factors (*acfA-D*) (72, 73).

1.3 THE TOXR REGULON

1.3.1 ToxT

The *V. cholerae* virulence factors CT and TCP are regulated under a complex hierarchical regulatory system called the ToxR regulon (Figure 1). The ToxR regulon includes three major transcriptional activators: ToxT, ToxR, and TcpP (as reviewed in (23)). Within this hierarchy, the primary activator of *ctxAB* and *tcpA-F* is ToxT, a member of the AraC/XylS family of transcriptional activators. The C-terminal domain of ToxT encodes a conserved AraC DNA binding domain, while the N-terminal domain appears to encode dimerization and substrate binding domains (74, 75). The N-terminal domain of AraC proteins are thought to be involved in effector binding and/or multimerization (74, 75). A number of small effector molecules have been shown to negatively affect ToxT activity including virstatin, capsaicin, bile, and fatty acids. It is thought that these effector molecules function to inhibit ToxT through a common mechanism of binding to the N-terminal domain (76-83).

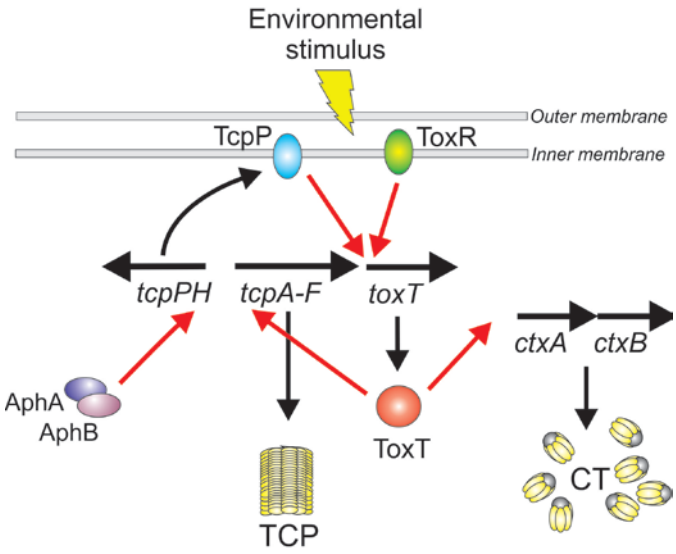

Figure 1. The *V. cholerae* ToxR regulon.

The ToxR regulon is a hierarchical regulatory system that controls *V. cholerae* virulence factor production in response to unknown environmental stimuli. Induction of the ToxR regulon begins with AphA and AphB binding to the *tcpPH* promoter to activate TcpP production. TcpP then binds with ToxR at the *toxT* promoter to activate ToxT production. ToxT then directly activates the expression of the *tcpA-F* operon and the *ctxAB* genes which result in the production of the toxin co-regulated pilus (TCP) and cholera toxin (CT), respectively.

ToxT activates the expression of *ctxAB* and *tcpA* by binding to a degenerate 13bp DNA sequence located in their respective promoters called the Toxbox. A Toxbox sequence is found upstream of all known ToxT activated genes (84). While the configuration of the Toxboxes differ at the various promoters, it is always located upstream of, but not overlapping, the -35 promoter element (85). The histone-like protein H-NS is able to counteract the activation of *ctxAB* and *tcpA-F* transcription by binding to this same DNA region and repressing transcription

(86-88). H-NS is further able to reduce the expression of virulence genes by binding to the *toxT* promoter and repressing *toxT* transcription (87).

1.3.2 ToxRS and TcpPH

The expression of *toxT* is activated by the interaction of two membrane bound transcriptional activators: ToxR and TcpP (Figure 1). ToxR is a membrane localized DNA binding protein. ToxR is composed of a cytoplasmic DNA binding/transcriptional activation domain, a transmembrane domain, and a periplasmic signaling domain (89-92). Wild type ToxR activity requires ToxS, another protein that is localized to the inner membrane. ToxS is hypothesized to interact with ToxR through its periplasmic domain. The exact role of ToxS is unclear, but it likely influences stability and/or enhances dimerization of ToxR, thereby serving as a positive effector of ToxR function (93-95).

Similar to ToxR, TcpP is also a membrane bound transcriptional activator that has a cytoplasmic localized DNA binding/transcriptional activation domain, a transmembrane domain, and a periplasmic domain. Furthermore, TcpP also requires a membrane bound effector protein, TcpH. TcpP and TcpH are thought to interact through their periplasmic domains (96, 97). The genes for TcpP and TcpH are encoded in a single operon whose transcription is responsive to environmental signals, such as temperature and pH (98). TcpP is regulated post-translationally through a regulated proteolytic event that requires at least two proteases that degrade TcpP (99, 100). Interaction with TcpH prevents proteolytic degradation as demonstrated by the rapid degradation of TcpP in cells lacking TcpH (96). TcpP has been shown to be proteolyzed in wild type cells grown under virulence repressing conditions (99). The transcriptional and post-translational regulation of TcpP suggested that control of its activity is important to the cell (23).

ToxR and TcpP function together to activate *toxT* expression in response to unknown environmental stimuli. Under virulence inducing conditions ToxR and TcpP bind directly to the *toxT* promoter (101). ToxR is reported to have a higher affinity for the *toxT* promoter than TcpP (101). The overexpression of TcpP can compensate for loss of ToxR, but ToxR overexpression cannot compensate for the loss of TcpP (101-104). This suggested that ToxR provides *toxT* promoter recognition, while TcpP is more directly responsible for transcriptional activation. The current hypothesis is that ToxR binds the *toxT* promoter and interacts with TcpP, which then stimulates expression from the *toxT* promoter (23).

ToxR also regulates the production of two outer membrane proteins, OmpU and OmpT, independent of TcpP (45, 105, 106). ToxR is required for the expression of OmpU, which contributes to antimicrobial resistance and functions as an adhesin (107). In contrast, ToxR represses the expression of *ompT* as observed by the induction of *ompT* expression in cells lacking *toxR*. Expression of *toxT* is associated with increased susceptibility to bile salts due to its large diameter pore. The regulation of these proteins by ToxR has been shown to be important for intestinal colonization and antimicrobial resistance (9, 106).

1.3.3 AphA and AphB

While *toxRS* appears to be constitutively active, the expression of *tcpPH* is regulated by two transcriptional activators in a manner similar to ToxR/TcpP activation of *toxT* (Figure 1). AphA and AphB are transcriptional activators that are encoded by unlinked genes. AphA belongs to a family of regulators with homology to the PadR repressor (108), while AphB is a LysR-type regulator (109). Analogous to ToxR, AphA binds the promoter of *tcpPH*, but requires interaction with AphB to induce transcription (109, 110). AphB binds downstream of AphA and interacts

with AphA, which is thought to stabilize AphB and result in activation of *tcpPH* (109, 110). One of the differences between the El Tor and classical biotypes of *V. cholerae* is due to a point mutation in the *tcpPH* promoters, which affects the ability of AphB to bind to *tcpPH* (111). This variation is attributed for the differential regulation of *toxT* between the El Tor and classical strains of *V. cholerae* (109, 112).

1.3.4 LeuO

Recent work in our laboratory identified a new component to the ToxR regulon (113). LeuO is a LysR-family regulator that was found to reduce virulence factor production when induced by cyclo(Phe-Pro). Cyclo(Phe-Pro) is a cyclic dipeptide produced by *V. cholerae* that has been reported to repress virulence factor production (114). Overexpression of *leuO* has been shown to repress *aphA* expression leading to reduced CT and TCP production. Interestingly, induction of *leuO* expression by cyclo(Phe-Pro) was shown to be dependent on ToxR. This suggested a novel role for ToxR in environmental sensing of cyclo(Phe-Pro). Thus providing the first example to show that ToxR can function to repress virulence factor production in response to environmental signals (113).

1.4 ACTIVE EFFLUX TRANSPORTERS

In the course of transitioning from the aquatic environment to its colonization niche in the human small intestine, *V. cholerae* must adapt to dramatic environmental changes. The gastrointestinal tract contains a number of barriers to colonization by *V. cholerae* including the acid barrier of the

stomach, the production of bile acids in the intestine, competition from the microbiome, antimicrobial products produced by the microbiome, and products of the innate immune system (115). Resistance to the diffusible antimicrobial compounds is accomplished by reducing the rate of influx combined with the expression of active efflux systems (9, 44-47, 116). As discussed above, *V. cholerae* regulates the rate of diffusion across its outer membrane by modulating the production of the OmpT and OmpU porins (44, 45, 107). Though outer membrane permeability is important in antimicrobial resistance, high level resistance is dependent upon energy-dependent efflux provided by RND family of transporters (as discussed below) (117-119).

1.4.1 RND-family efflux systems

There are five families of efflux systems that have been defined in Gram negative bacteria. The five families are differentiated from each other based on sequence similarity, the number of pump components, the number of transmembrane-spanning regions in the pump proteins, and the energy source used for substrate translocation (120). The five families include: the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic-compound extrusion (MATE) family, the small multidrug resistance (SMR) family, and the resistance nodulation division (RND) family (120, 121). Most bacteria encode multiple efflux systems belonging to several different families. Further, many bacteria encode multiple efflux pumps from each individual family. For example, *V. cholerae* encodes six RND efflux systems whereas *P. aeruginosa* encodes 12 (122, 123).

The RND family of efflux systems have been a focus in antimicrobial research for more than a decade due to their due to their association with multiple antibiotic resistance in Gram negative pathogens (as discussed below). There are a number of examples of RND efflux

systems exhibiting extremely broad substrate specificity. This includes the *V. cholerae* VexAB-TolC (119), *Escherichia coli* AcrAB-TolC (124), the *Neisseria gonorrhoeae* MtrCDE, and *P. aeruginosa* MexAB-OprM systems (125); all of which have been shown to efflux chemically diverse antimicrobial compounds including dyes, detergents, antibiotics, and antimicrobial peptides (126).

1.4.1.1 Structure of the RND efflux systems

RND efflux systems are tripartite transporters consisting of an outer membrane pore protein (OMP) that is homologous to *E. coli tolC*, a periplasmic membrane fusion protein (MFP), and an integral cytoplasmic membrane RND-family pump protein (127, 128). The energy for transport is provided by the proton motive force and the RND pumps function as proton antiporters. These three components function together to form a channel for the export of substrates from within the cell envelope to the external environment.

The crystal structure for all three components of the RND efflux systems have been solved and used to model the structure of the tripartite transport complex (Figure 2) (127). The first structure determined was for TolC from *E.coli* (shown in red in Figure 2). In *Enterobacteriaceae* (and *Vibrio*), TolC is a promiscuous protein that also functions as the OMP of other transport systems including the MFS and ABC transporters (129). TolC was found to assemble into a tightly woven trimer that formed a single 12-stranded beta-barrel that traversed the outer membrane with a long alpha-helical bundle extending into the periplasmic space (130, 131). The second component of the RND efflux systems that was crystalized was the *E. coli* AcrB RND pump protein (shown in blue in Figure 2). AcrB formed a homotrimer with a three-fold rotational symmetry (128, 132-136). The periplasmic portion of AcrB was found to have similar dimensions to the tip of the periplasmic localized TolC alpha-helical bundle and thereby

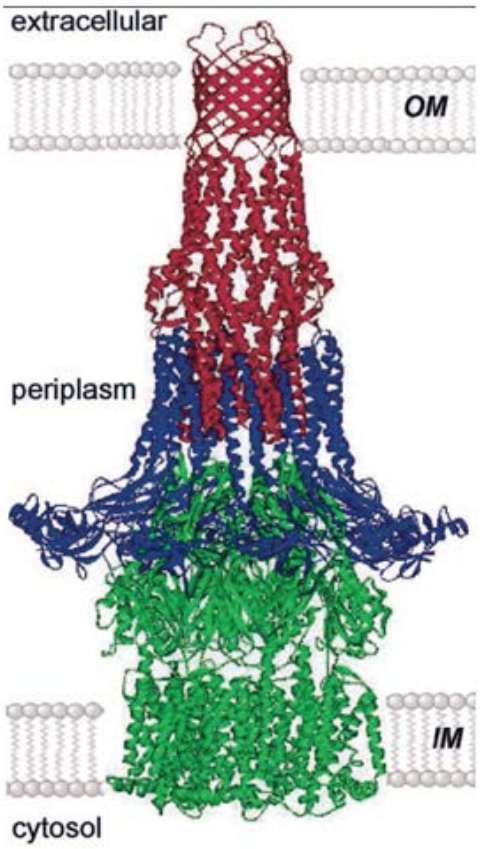

Figure 2. Structural model for tripartite RND efflux systems.

The tripartite RND efflux system was generated from the crystal structure of *E. coli* TolC (red), *E. coli* AcrB (green), and *P. aeruginosa* MexA (blue). A trimer of the TolC outermembrane protein in an open state and is shown forming minimal contacts with the AcrB pump which exists as a trimer in the cytoplasmic membrane. Surrounding the interface of the two is a ring of nine molecules of the MexA membrane fusion protein, which is a homolog of AcrA, the natural partner of AcrB/TolC. The figure was adapted from Eswaran et al., 2004, full reference in text(127).

was predicted to encode the TolC-binding domain (132). The final component to be solved was two different membrane fusion proteins (shown in green in Figure 2); MexA from *P. aeruginosa* and AcrA from *E. coli* (137, 138). Both proteins were found to be structurally conserved and formed elongated structures that had a tendency to be packed side-by-side. Based on this observation they were hypothesized to wrap around and stabilize the interaction between TolC to the RND pump protein. The MFP is proposed to first bind to the pump protein, after which the TolC is recruited and binds to the complex to form the tripartite transporter (139).

Substrates are thought to be captured by the RND pump protein either from the cytosol or from the phospholipid bilayer and then transported through TolC into the extracellular space (127, 132). The periplasmic domain of the RND pump determines the substrate specificity of the RND efflux system (140-144). In *E. coli*, chimeric studies with AcrB and AcrD RND pump proteins revealed that an exchange of the periplasmic domains caused coinciding changes in substrate specificity (145, 146). Genetic and biochemical studies with AcrB revealed that the substrate binding pocket was rich in aromatic residues which interacted with the various substrates of AcrB (133, 135). The crystal structure of this binding pocket has been solved with several substrates bound, including bile, which confirmed the location of the substrate binding pocket (141, 147).

1.4.1.2 Function of the RND efflux systems in antimicrobial resistance

The RND efflux systems have been shown to be important for antimicrobial resistance in a multitude of bacterial genera (reviewed in (129)). Many bacterial genera (e.g. the *Enterobacteriaceae*, *Vibrionaceae* and *Pseudomonads*) encode multiple different RND efflux systems that contribute to antimicrobial resistance. For example the *P. aeruginosa* MexAB-OprM, MexXY-OprM, MexCD-OprJ, and MexEF-OprN RND efflux systems have all been

shown to efflux a range of different antibiotics and antimicrobial compounds (148, 149). Further, mutational upregulation of these systems have been correlated with clinically significant multiple drug resistance in patient isolates and the evolution of multiple drug resistant strains (150-152).

The RND efflux systems also play an important role in antimicrobial resistance in the *Enterobacteriaceae*. There have been a number of studies showing that the AcrAB-TolC system was upregulated in multiple drug resistant clinical isolates (153-155). AcrAB-TolC is the principal RND efflux system in *E. coli* that contributes to resistance against antibiotics, acriflavin, ethidium bromide, SDS, triton X-100, bile salts, and short chain fatty acids (156-159). Although AcrAB-TolC has been shown to be overexpressed in multidrug resistant clinical isolates, it alone is not sufficient to cause clinically relevant increases in drug resistance. Rather, it is thought that AcrAB-TolC functions in conjunction with secondary mutations or the acquisition of other drug resistance traits. For example, high level ciprofloxacin resistance in *E. coli* has been associated with increased expression of AcrAB-TolC in conjunction with a mutation in topoisomerase which is one of the ciprofloxacin target genes (153-155, 160).

The AcrAB-TolC RND efflux system in *Salmonella enterica* serovar Typhimurium functions in a similar manner as the *E. coli* AcrAB-TolC. Substrates of this efflux system include antibiotics, acriflavin, ethidium bromide, SDS, triton X-100, cetrimide, and bile salts (161-166). In both human and veterinary isolates, along with laboratory mutants, the overexpression of AcrAB-TolC is associated with multidrug resistance (162, 164, 165).

The overexpression of the RND efflux systems has been associated with multidrug resistance in several species of bacteria. It is interesting to note that overexpression of the RND efflux systems in laboratory mutants is often detrimental to the cell. This suggests that there is selective pressure to maintain the overexpression phenotype in the clinical setting that is absent

in the laboratory. Further, the fact that overexpression of the RND efflux systems is detrimental to growth provides an explanation for why the expression of these systems are tightly regulated (as discussed below).

1.4.1.3 Function of the RND efflux systems in other aspects of bacterial biology

The contribution of RND efflux systems to antibiotic resistance is an easily scored phenotype with clinical relevance. Thus it has been the primary focus of those studying these systems. However, phylogenetic analysis has shown that the RND efflux systems have evolved independent of antibiotic selection (167, 168). Although the native role of the RND efflux systems in bacterial physiology is unclear, recent studies have suggested a function for the RND efflux systems independent of their role in antimicrobial resistance (reviewed in (169). This is exemplified by reports implicating RND efflux systems in diverse phenotypes such as biofilm formation (170, 171), iron acquisition (172), plant-bacteria interactions (173), lipid transport (174, 175), bacterial virulence (123, 129, 176), extrusion of toxic metal effectors (177), and the removal of metabolic byproducts from within the cell (178).

1.4.1.4 Role of the RND efflux systems in virulence

The RND efflux systems have been found to be required for virulence in several bacterial species, such that loss of RND-mediated efflux caused in vivo attenuation (123, 129, 163, 179-187). In *V. cholerae* and in *Salmonella*, the loss of RND-mediated efflux reduced pathogenicity (123, 188, 189). The loss of RND efflux in *Salmonella* reduced the expression of virulence genes encoded on the Salmonella Pathogenicity Islands (188, 189). Similarly, the loss of the *V. cholerae* RND efflux systems caused a decrease in virulence factor production through repression of the ToxR regulon (discussed below). In *Campylobacter* the RND efflux systems

have been shown to influence virulence indirectly via effects on motility (190, 191). The RND efflux systems have also been linked to pathogenesis due to their ability to efflux quorum sensing molecules in *Burkholderia* (192) and *Pseudomonas* (129, 193, 194). In addition to the linkage of the RND efflux system to the expression of virulence genes, there are numerous examples of the RND efflux systems contributing to virulence by providing resistance to antimicrobial compounds that are present in the host (124, 147, 187, 195-197).

1.4.1.5 Metabolic waste disposal

Studies of metabolic mutants in *E. coli* revealed upregulation of the *acrAB-tolC* RND efflux system (198). This suggested that AcrAB-TolC RND efflux system may have an innate role in removal of excess and potentially toxic metabolic intermediates that accumulated as a result of the disruption of the metabolic pathways. Subsequent studies have shown that loss of TolC induced the expression of *acrAB* and the Cpx and BaeSR stress response systems in *E. coli* (199, 200). These collective results led to the hypothesis that the native function of the RND efflux systems was to remove endogenous metabolites from within the cell that would otherwise prove stressful to the cell. Further support for this hypothesis was provided by Ruiz and Levy who showed that mutation of certain metabolic genes induced *acrAB* expression and that the addition of the metabolic intermediates to culture media induced *acrAB* expression in wild type cells (178).

1.4.1.6 *V. cholerae* RND efflux systems

The *V. cholerae* genome encoded six RND efflux systems (VexAB, VexCD, VexEF, VexGH, VexIJK, and VexLM) (122, 123). Each RND efflux system was separately encoded in an operon structure containing the RND efflux pump protein and at least one associated MFP whose gene

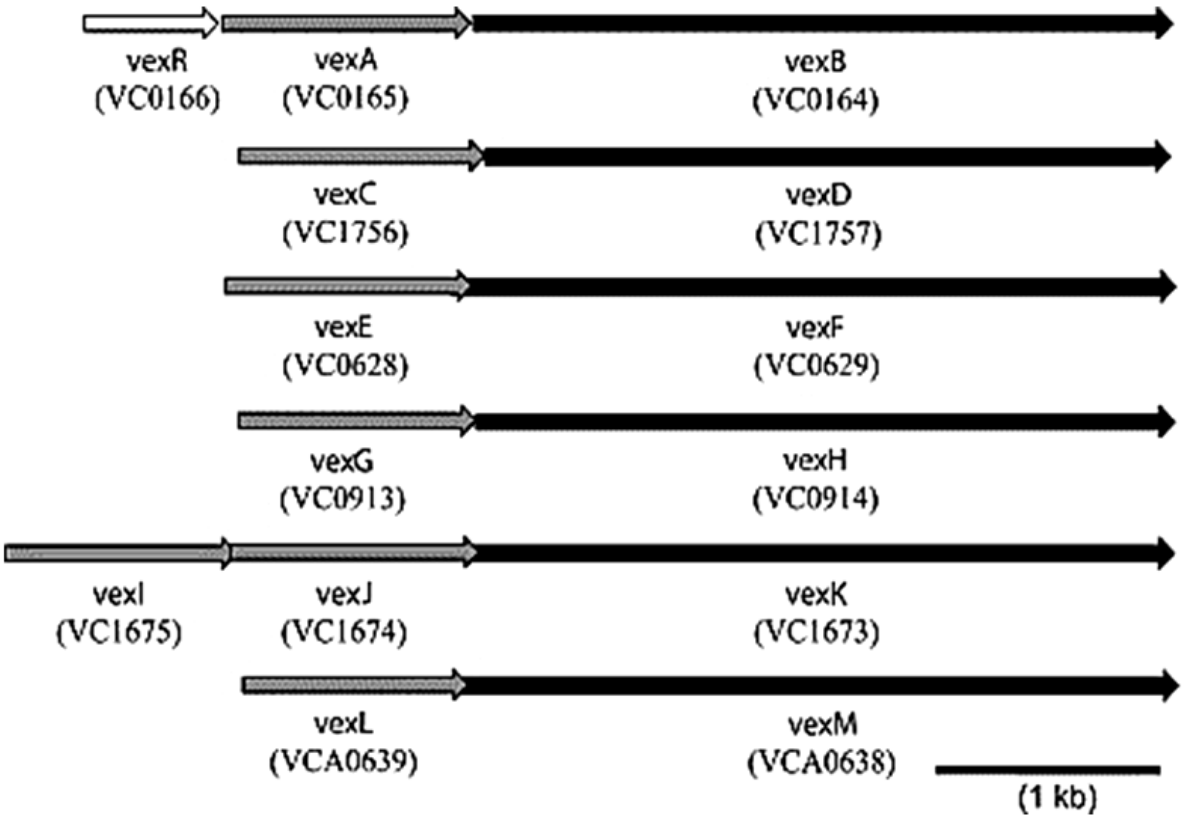

Figure 3. *V. cholerae* gene arrangement of the RND efflux systems.

Schematic of the genes of the *Vibrio cholerae* RND efflux systems. The gene for each RND efflux pump (black arrows) is encoded in an operon with at least one associated membrane fusion protein (gray arrows). Only the *vexAB* operon has an associated TetR regulator encoded by *vexR* (open arrow). Listed below each gene is the ORF number and gene name. The loci are shown to scale. Figure adapted from Bina et al., 2008, full citation in the text (123).

was located upstream of the pump gene (as depicted in Figure 3) (123). All six RND efflux systems appear to share the same TolC OMP which was encoded by itself on the chromosome (123). The loss of RND-mediated efflux, whether by mutation or by use of chemical inhibitors, caused a significant increase in antimicrobial susceptibility, down-regulated the ToxR regulon, diminished CT and TCP production, and attenuated colonization. Consistent with the role of the RND efflux systems in antimicrobial resistance, and pathogenesis, studies found that *vexAB*, *vexGH*, and *vexIJK* were upregulated during human and animal colonization (41, 201, 202). Three of the RND efflux systems (VexAB, VexCD, and VexIJK) were shown to contribute to the antimicrobial resistance and intestinal colonization phenotypes.

The VexAB RND efflux system is functionally analogous to the *E. coli* AcrAB system. VexAB exhibited a high basal level of activity and provided *V. cholerae* with its intrinsic antimicrobial resistance (119, 203, 204). The VexAB RND efflux system mediated resistance to bile salts, non-ionic detergents, and a variety of antibiotics (e.g. ampicillin, erythromycin, novobiocin, and polymyxin B). In contrast, the VexCD and VexIJK RND efflux systems were functionally redundant with VexAB and appeared to only efflux bile acids and detergents, respectively (119, 123). A mutant lacking the *vexBDK* genes was found to be attenuated during murine colonization. However, *vexBDK* produced wild type levels of CT and TCP, suggesting the contribution of one or more of the remaining RND efflux system (VexEF, VexGH, and VexLM) to virulence factor production. Furthermore, the mechanism(s) by which the RND efflux systems influence virulence gene expression were unknown.

1.5 REGULATION OF RND EFFLUX SYSTEMS

Bacterial adaptation to environmental cues involves a range of transcriptional responses that are triggered and controlled by regulatory proteins. Often these regulatory proteins are able to respond to environmental or cellular cues by modulating transcription of target genes. Most environmental responsive microbial transcriptional regulators can be classified as one-component or two-component regulatory systems (205). In one-component systems, the regulator is a two-domain protein with a signal receiving domain and a DNA-binding domain which transduces the signal to a transcriptional response. In two-component systems the sensing and signaling domain are encoded on separate proteins. In this case the environmental stimulus is detected by the sensor protein which then activates, by phosphorylation, the DNA-binding response protein to effect the expression of target genes.

1.5.1 TetR family regulators

The TetR family of transcriptional regulators is one of the largest groups of one-component regulatory systems and is widely distributed among bacteria. Members of this family control the expression of genes whose products are involved in diverse physiological processes including antibiotic resistance, metabolism, stress responses, biofilm formation, quorum sensing, and pathogenicity (as reviewed in (206)). TetR proteins are defined by a highly conserved N-terminal DNA binding domain and a highly variant C-terminal domain that interacts with small-molecule ligands (206). The ligand binding domain is capable of binding to effector molecules which modulate the interaction of the DNA binding domain with its target sequences. In the case of RND efflux systems, the activity of TetR regulators is often modulated by binding substrates of

the linked RND efflux system. In many cases, TetR proteins appear to be capable of binding a diverse set of ligands that correspond to the multiple substrates of linked RND efflux systems (206).

Ahn et al. predicted regulatory targets of TetR-family proteins by genome context and concluded that a gene neighboring a *tetR* regulator was highly probable as the target of a TetR if an intergenic region of <200bp separated the genes (207). The probability that a certain gene was the target of TetR was lowered as the intergenic region increased. The vast majority of characterized TetR family regulators act as transcriptional repressors and in most cases also autoregulate their own expression (206, 208). The majority of studied TetR regulators have been reported to regulate the expression of antimicrobial efflux pumps, including the *E. coli* AcrR which regulates the expression of *acrAB* RND efflux system.

The TetR family of regulators was named after TetR, a regulator of the tetracycline TetA efflux pump in *E. coli* (206). In the Tet regulatory system, *tetR* is encoded upstream and divergent from *tetA* (209). The intergenic region encodes two palindromic operator sequences that are bound by a TetR homodimer thereby repressing transcription from both promoters. Tetracycline, the substrate of TetA, activates expression of *tetA* by binding to the TetR dimer, changes its conformation thereby lowering its affinity for DNA (209). Then, TetA exports tetracycline from within the cell which renders the cell resistant to tetracycline.

1.5.1.1 TetR family proteins in *V. cholerae*

One of the *V. cholerae* RND efflux systems has previously been reported to be regulated by a regulator belong to the TetR family. Cerda-Maira et al. reported that the expression of the *V. cholerae* VexCD RND efflux system was regulated by BreR (VC1746), a TetR-family protein

that was encoded by an unlinked gene (210). BreR was determined to negatively regulate the expression of both itself and of *vexCD*. The expression of both *vexCD* and *breR* were found to be induced by the presence of the bile salts cholate, deoxycholate, and chenodeoxycholate; all of which were shown to be substrates of *vexCD* (210). They further showed that deoxycholate interfered with BreR DNA binding and allowed expression of both *breR* and *vexCD*.

Among the six *V. cholerae* RND efflux systems only the *vexAB* system encoded a linked TetR-family regulatory gene, which has been named *vexR* (Figure 3). The *vexR* gene was encoded as the first gene in a three gene operon that included *vexA* and *vexB*; a genetic organization that is distinct from most RND efflux systems where the TetR regulator is encoded in the opposite orientation from the RND efflux system (206). The function of *vexR* in the expression of the *vexRAB* operon has not been reported.

1.5.2 Two-component regulatory systems

Two-component signal transduction systems allow bacteria to respond to environment signals through the combined efforts of a sensing component which usually encodes a histidine kinase activity and a cytosolic response regulator (211-214). The sensing component is a protein embedded in the membrane that responds to specific environmental signals. Once stimulated, the protein auto-phosphorylates a histidine residue and transfers this phosphate onto a conserved aspartate residue in the cytoplasmic response regulator, thereby activating it (213, 214). The cytoplasmic response regulator is a transcription factor whose activity is altered upon phosphorylation. Once phosphorylated, the response regulator then effects the expression of its target genes, thereby modulating a cellular response to the environmental signal (213, 214). In

the absence of the inducing signal the sensing component (the histidine kinase) can also function as a phosphatase which maintains the regulatory system in the off state (213, 214).

1.5.2.1 Cpx two-component regulatory system

The Cpx two-component system is widely distributed among Gammaproteobacteria including the *Enterobacteriaceae* and *Vibrionaceae*. This system has been well characterized in *E. coli* where it has been shown to alleviate membrane stress caused by misfolded proteins (254, 255). In this regulatory system (Figure 4A) (211), CpxA functions as a membrane associated sensor histidine kinase. Upon stimulation, CpxA autophosphorylates itself and then transfers the phosphate to a conserved aspartate residue on the cytoplasmic CpxR response regulator (reviewed in (254, 255)). Phosphorylated CpxR (CpxR~P) then modulates the expression of its target genes by binding to a consensus binding sequence located in their promoter regions (215). In the absence of stimuli, CpxA acts as a phosphatase on CpxR and renders it inactive.

CpxR~P also regulates its own expression (i.e. the *cpxRA* operon) and the divergently transcribed *cpxP* (Figure 4) (211, 216). CpxP is a periplasmic protein that appears to repress CpxR activation by interacting with the periplasmic domain of CpxA and inhibiting its kinase activity. CpxP may also exhibit chaperone activity (254, 255). *CpxA** mutants lead to constitutive activation of the Cpx system and have been useful in analyses of Cpx regulons. The *cpxA** mutation inactivates CpxA phosphatase activity, resulting in the accumulation of activated CpxR (i.e. CpxR~P) (254, 255). The dual activation of both the *cpxRA* and *cpxP* is hypothesized to function in both amplifying the cellular stress signal and in restoring the cell to basal levels upon loss of stimulation (211).

1.5.2.2 *E. coli* Cpx

The *E.coli* Cpx system has been proposed to function in alleviating stress resulting from cell envelope perturbations that are generally associated with cell envelope stress (254, 255). Consistent with this idea, the majority of stimuli that activate the Cpx system have been predicted to result in the production of misfolded or damaged cell envelope proteins (reviewed in (254, 255)). Activators of the Cpx system in *E. coli* include alkaline pH, copper, changes in inner membrane lipid composition, overproduction of the outermembrane lipoprotein NlpE, accumulating misfolded variants of the maltose binding protein, and elevated osmolarity. However, the effects of misfolded proteins on Cpx induction are not universal as there are examples of misfolded proteins that fail to activate the Cpx system (254, 255).

Once activated, the Cpx system modulates the expression of genes whose products function in alleviating cellular stress. In *E. coli* CpxR~P activates genes encoding the extracytoplasmic protease DegP, chaperones such as the peptidyl prolyl cis/trans isomerase PpiA, and the disulfide oxidoreductase DsbA (254, 255). Together, these proteins may be able to respond to misfolded proteins either by repairing or eliminating the damaged envelope protein.

1.5.2.3 Cpx functions beyond responding to misfolded proteins

While the Cpx system has been shown to function in alleviating cellular stress due to misfolded or damaged proteins, there is mounting evidence that the function of the Cpx system extends beyond maintaining the cell envelope (217). In *E. coli*, cell swarming was reduced by the CpxR~P mediated repression of *motB* and *aerR* (218, 219); and activation of the Cpx system was reported to reduce growth in biofilms (220). Furthermore, there are several reports indicating that the Cpx system is important for pathogenicity in *E. coli* and in related pathogens.

In enteropathogenic *E. coli*, the disruption of CpxR was associated with a reduction of type IV bundle forming pili production and of adherence to eukaryotic cells (221). The Cpx system in uropathogenic *E. coli* regulates the expression of factors involved in the assembly of pili and a *cpxR* null mutant in uropathogenic *E. coli* produced fewer and shorter pili than wild type (221). In *S. enterica* serovar Typhimurium, the mutation of *cpxA* caused attenuation in mice (222). Furthermore, the Cpx pathway of *Shigella sonnei* responds to changes in extracellular pH by regulating the expression of the type III secretion system (223). Together these indicated that the activation of Cpx pathway functions in pathogenicity through the genes which it regulates.

1.5.2.4 *V. cholerae* Cpx system

In *V. cholerae*, the *cpx* locus is organized in a manner similar to *E. coli*: *cpxRA* is encoded in an apparent operon with *cpxP* being encoded immediately upstream and divergently from *cpxRA* (211). The intergenic region between the *V. cholerae cpxRA* and *cpxP* encodes a CpxR conserved binding site, which in *E. coli* mediated CpxR activation of target genes (211, 219). While the genomic arrangement of the Cpx system in *V. cholerae* is similar to *E. coli*, the predicted amino acid sequences vary between these two systems (Figure 4B) (211). The CpxR proteins had the highest identity being 60%, while the CpxP proteins were only 21.6% identical. CpxA was 43.6% identical between the two genera, but there was a much high level of divergence in the periplasmic sensing domain of the protein. The CpxA periplasmic domain, which is thought to interact with CpxP, was found to be 20.7% identical. The cytosolic domain, which interacts with CpxR was found to be 54.3% identical. The variance in amino acid sequences found in CpxP and the CpxA periplasmic domain suggested that the Cpx systems in these two species respond to different stimuli (211).

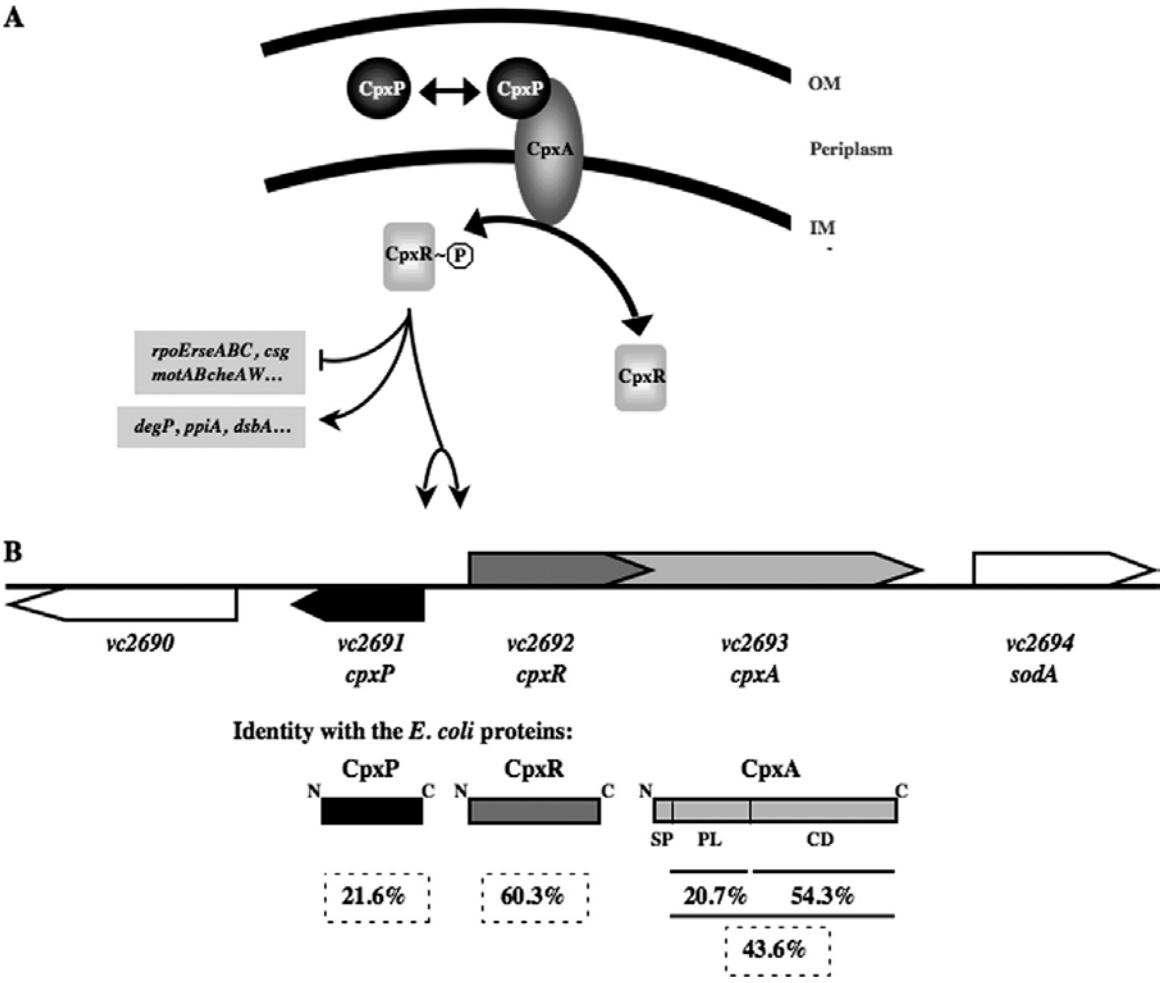

Figure 4. Cpx regulon.

(A) Schematic of the *E. coli* Cpx pathway. OM, outer membrane; IM, inner membrane. (B) Organization of the *V. cholerae cpxRA* operon. The predicted sequence of *V. cholerae* and *E. coli* Cpx proteins were aligned using DNA strider. SP, signal peptide; PL, periplasmic loop; CD, cytoplasmic domain." Figure adapted from Slamti et al., full citation in the text (204).

Although a number of Cpx-inducing stimuli have been described in *E. coli*, studies suggested that these stimuli are not conserved in *V. cholerae* (211). For example, the *E. coli* Cpx system is activated by increased osmolarity, but not by increased salinity (211, 224). In contrast, the *V. cholerae* Cpx system functions in an opposite manner; it is not responsive to osmolarity, but is activated by increased salinity (211). Additionally, the *E. coli* Cpx system is active under standard laboratory growth conditions, whereas the *V. cholerae* Cpx system is inactive. The differences in physiological roles of the Cpx system in *E. coli* and *V. cholerae* may be related to the distinct environmental niches these organisms occupy and appear to be reflected in amino acid sequence variability in the signaling domain of CpxA (211, 225).

While the physiological roles of the *V. cholerae* and *E. coli* Cpx systems appear to differ, deletion of *tolC* activated the Cpx system in both organisms (200, 211). In *E. coli*, the activation of the Cpx system was linked to loss of TolC-dependent efflux (200). TolC functions as the outer-membrane pore component of several *V. cholerae* transport systems including RND family transporters (226). Thus, we speculated that the *tolC*-dependent activation of the *V. cholerae* Cpx system resulted from the loss of RND efflux activity.

1.6 GOALS OF DISSERTATION

The RND family efflux systems are well known for their ability to efflux a broad range of antimicrobial substrates. However, there is increasing evidence to suggest that the RND efflux systems have physiological roles beyond xenobiotic resistance to include pathogenesis and cellular stress responses. The *Vibrio cholerae* genome encodes six RND efflux systems: VexAB,

VexCD, VexEF, VexGH, VexIJK, and VexLM. This work sought to characterize the role of these systems in antimicrobial resistance, pathogenesis, and environmental stress.

In the first chapter of these studies, the goal was to elucidate the roles of the VexEF, VexGH, and VexLM RND efflux systems in regards to RND-mediated antimicrobial resistance, murine colonization, and virulence factor production. Loss of RND efflux activity in the cell caused a significant increase in antimicrobial susceptibility, reduced virulence factor production, downregulation of the ToxR regulon, and attenuated murine colonization (123). Three of the RND efflux systems (VexAB, VexCD, and VexIJK) were found to partially contribute to these phenotypes (123). This latter finding suggested additional RND efflux systems may also contribute to the observed phenotypes. Therefore, a genetic approach was used to create a panel of RND efflux pump mutants which was used to determine the function of the remaining three RND efflux systems (i.e. VexEF, VexGH and VexLM) in these phenotypes. The results of these studies showed that VexGH contributed to RND-mediated antimicrobial resistance and murine colonization in a manner that was redundant with the previously characterized RND efflux systems (176). VexEF and VexLM were not found to contribute to antimicrobial resistance against any tested substrates nor were they found to contribute to murine colonization. VexAB, VexCD, VexGH, and VexIJK were determined to be major contributors to virulence factor production (176). Although virulence factor production was reduced in the Δ*vexBDHK* mutant compared to wild type, it was still significantly higher than the RND deficient mutant suggesting the contribution of VexEF and/or VexLM.

The second chapter of this work focused on the characterization of the relationship between the RND efflux systems to the Cpx stress response system in *V. cholerae*. Initial characterization of the *V. cholerae* Cpx system by Slamti and Waldor found that the *cpx* locus

was organized in a similar manner to *E. coli* (211). However, the environmental cues which induced the *E. coli* Cpx system did not appear to be conserved in *V. cholerae.* This suggested that the Cpx system fulfilled a different physiological role in *V. cholerae*. While the physiological roles of the *V. cholerae* and *E. coli* Cpx systems appeared to differ, deletion of *tolC* activated the Cpx system in both organisms (200, 211). TolC functions as the outer-membrane pore component of several *V. cholerae* transport systems including RND family transporters (227). Thus, we speculated that the *tolC*-dependent activation of the *V. cholerae* Cpx system resulted from the loss of RND efflux activity. Therefore, we explored the linkage between the RND efflux systems and the Cpx system. CpxR was found to function as a positive regulator of the VexAB and VexGH RND efflux systems (211). Conversely, mutation of *vexRAB* or *vexGH* resulted in the activation of the Cpx system, which suggested that the VexAB and VexGH RND efflux systems functioned in the regulation of the Cpx system. Activation of the Cpx system did not affect virulence factor production in *V. cholerae*. The induction of the Cpx system upon loss of RND-mediated efflux suggested that the accumulation of innate RND efflux substrates caused cellular stress. The overall results showed that the Cpx system and the RND efflux systems were reciprocally regulated and that the RND efflux systems likely function to alleviate cellular stress due to the accumulation of potentially deleterious endogenous compounds.

The final chapter of this work focused on the regulation of the *vexAB* RND efflux system by VexR. The expression of RND efflux systems is often regulated by a linked TetR family transcriptional regulator (208). The VexAB system was encoded in a three gene operon that included an uncharacterized upstream TetR family regulator (i.e. *vexR*). Based on this, we hypothesized that VexR was a regulator of the VexAB RND efflux system. The results of this

work revealed that VexR functioned as a positive regulator of the *vexRAB* operon, a finding that is in contrast to most RND-associated TetR regulators which function as repressors. Furthermore, *vexRAB* was found to be upregulated, in a *vexR*-dependent manner, in response to the efflux status of the cell. This suggested that *V.cholerae* was able to sense the loss of RND efflux activity. We hypothesized that this sensing was the result of the intracellular accumulation of an endogenous compound that was likely a substrate of the RND efflux systems. We therefore tested whether metabolic intermediates could function as inducers of the RND efflux systems by quantifying *vexRAB* expression in a panel of metabolic mutants. The results of these experiments showed that *vexRAB* was upregulated in several metabolic mutants, including tryptophan biosynthetic mutants. Furthermore, indole, an intermediate in tryptophan biosynthesis, was found to induce *vexRAB* expression and be a substrate of VexAB. The collective results indicated that VexR positively regulated *vexRAB* in response to VexAB efflux substrates and that the VexAB RND system plays a central role in modulating the intracellular level of potentially toxic metabolites.

2.0 *VIBRIO CHOLERAE VEXH* ENCODES A MULTIPLE DRUG EFFLUX PUMP THAT CONTRIBUTES TO THE PRODUCTION OF CHOLERA TOXIN AND THE TOXIN CO-REGULATED PILUS

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2.1 INTRODUCTION

Vibrio cholerae is a Gram negative, motile, facultative anaerobic bacterium, and the causative agent of cholera, a severe diarrheal disease, which untreated can rapidly lead to dehydration, hypotensive shock, and death. *V. cholerae* is a common inhabitant of aquatic environments where it can survive and persist in association with aquatic plants and animals. Humans acquire cholera by ingesting *V. cholerae* contaminated food or water (1). Upon ingestion, *V. cholerae* colonizes the small intestine where a complex regulatory cascade is induced, resulting in the production of several important virulence factors including cholera toxin (CT) and the toxin co-regulated pilus (TCP) (67, 228). CT is an AB-type enterotoxin that is responsible for the secretory diarrhea that is characteristic of cholera (229). The TCP is a type IV bundle forming pilus that is essential for intestinal colonization of both humans and laboratory animals (67, 68, 230, 231). CT and TCP production are tightly controlled by a hierarchical regulatory system called the ToxR regulon (232, 233). In response to unknown stimuli, ToxR and TcpP, two membrane associated transcriptional regulators, activate transcription of *toxT* (101, 228, 234, 235). ToxT, an AraC-family transcriptional regulator, directly activates the expression of the *ctxAB* and the *tcpA-F* operons which encode for the production of CT and the TCP, plus a number of accessory virulence genes (89, 236, 237).

In order to colonize and survive in the host, *V. cholerae* must protect itself from the toxic effects of antimicrobial compounds that are present in the gastrointestinal tract (GI). *V. cholerae* does this by limiting the uptake and intracellular accumulation of toxic antimicrobial molecules

that are present in the GI tract. This is accomplished by modulating the outer membrane permeability (e.g. through the production of porin proteins and cell envelope modifications) in conjunction with efflux of the antimicrobial molecules via active efflux transporters (9, 44-47). There are five different active efflux systems described in bacteria: the ATP-binding cassette superfamily (ABC), the small multidrug resistance family (SMR), the multi antimicrobial extrusion protein family (MATE), the major facilitator superfamily (MFS), and the resistance-nodulation-cell division superfamily (RND) (120). The RND family is particularly interesting because of its broad substrate specificity and its association with multidrug resistance in many Gram negative pathogens. Individual RND efflux systems, including the *V. cholerae* VexAB-TolC (119), *Escherichia coli* AcrAB-TolC (124), and *Pseudomonas aeruginosa* MexAB-OprM systems (125), have been shown to efflux chemically diverse antimicrobial compounds including: dyes, detergents, antibiotics, and antimicrobial peptides (126).

RND efflux systems are tripartite transporters that function as proton-substrate antiporters (238, 239). RND efflux systems are composed of an outer membrane pore protein (OMP) that is homologous to *E. coli tolC*, a periplasmic membrane fusion protein (MFP), and an integral cytoplasmic membrane pump protein belonging to the RND superfamily of transporters (127, 131, 132, 137, 238). These three components function to form a channel for the extrusion of substrates from within the cell envelope to the external environment. Most Gram negative pathogens encode multiple RND efflux systems; *V. cholerae* encodes six. In *V. cholerae*, each RND system is separately encoded in an operon structure wherein the RND efflux pump protein has at least one associated MFP whose gene is located upstream of the pump gene. It appears that all six RND efflux systems share the same TolC OMP which is encoded separately on the chromosome (123). Previous work in our laboratory showed that three of the RND efflux pumps

(VexB, VexD, and VexK) were required for antimicrobial resistance in vitro. The VexB RND efflux pump exhibited a very broad substrate specificity and contributed resistance to bile acids, detergents, and several antibiotics. In contrast, the VexD and VexK RND pumps appeared to only efflux bile acids and detergents, respectively (119, 123).

Recently, our laboratory reported that the *V. cholerae* RND efflux systems were not only important for antimicrobial resistance and intestinal colonization, but were also important for CT and TCP production (123). A mutant that lacked all six RND efflux pumps (i.e. ΔRND) was attenuated for CT and TCP production and was hypersensitive to antibiotics. Although the VexB, VexD, and VexK efflux pumps contributed to in vitro antimicrobial resistance, a mutant lacking the *vexBDK* genes produced WT levels of CT and TcpA. This finding suggested that one or more of the three remaining RND pumps (VexF, VexH, and VexM) must function in virulence factor production. In this study we have further characterized these three RND efflux pumps. Using a genetic approach to generate mutant strains with the RND efflux pumps deleted in various permutations, we found that the VexH RND efflux pump contributed to antimicrobial resistance, CT and TCP production, and successful colonization of the infant mouse small intestine. VexF and VexM did not appear to function in antimicrobial resistance in vitro, but were required for high level production of CT and TCP.

Table 1. Bacterial strains, plasmids, and oligonucleotides

Strain	Genotype	Strain #	Source
Vibrio cholerae			
N16961-Sm	Spontaneous streptomycin-resistant 01 El Tor strain N16961 Δ*lacZ*	JB58	(119)
Δ*vexH*	N16961-Sm Δ*vexH*	JB116	(123)
Δ*vexDH*	N16961-Sm Δ*vexD* Δ*vexH*	JB186	(123)
Δ*vexDHM*	N16961-Sm Δ*vexD* Δ*vexH* Δ*vexM*	JB386	(123)
Δ*vexDF*	N16961-Sm Δ*vexD* Δ*vexF*	JB435	(123)
Δ*vexDFHM*	N16961-Sm Δ*vexD* Δ*vexF* Δ*vexH* Δ*vexM*	JB459	(123)
Δ*vexDFHKM*	N16961-Sm Δ*vexD* Δ*vexF* Δ*vexH* Δ*vexK* Δ*vexM*	JB464	(123)
Δ*RND*	N16961-Sm Δ*vexB* Δ*vexD* Δ*vexF* Δ*vexH* Δ*vexK* Δ*vexM*	JB485	(123)
Δ*vexB*	N16961-Sm Δ*vexB*	JB495	(119)
Δ*vexK*	N16961-Sm Δ*vexK*	JB528	(123)
Δ*vexBK*	N16961-Sm Δ*vexB* Δ*vexK*	JB531	(123)
Δ*vexD*	N16961-Sm Δ*vexD*	JB692	(119)
Δ*vexBD*	N16961-Sm Δ*vexB* Δ*vexD*	JB694	(119)
Δ*vexBDHK*	N16961-Sm Δ*vexB* Δ*vexD* Δ*vexH* Δ*vexK*	DT12	This study
Δ*vexBHK*	N16961-Sm Δ*vexB* Δ*vexH* Δ*vexK*	DT23	This study
Δ*vexBDH*	N16961-Sm Δ*vexB* Δ*vexD* Δ*vexH*	DT30	This study
Δ*vexBH*	N16961-Sm Δ*vexB* Δ*vexH*	DT60	This study
Δ*vexHK*	N16961-Sm Δ*vexH* Δ*vexK*	DT64	This study
Δ*vexDK*	N16961-Sm Δ*vexD* Δ*vexK*	DT70	This study
Δ*vexDHK*	N16961-Sm Δ*vexD* Δ*vexH* Δ*vexK*	DT76	This study
Escherichia coli			
EC100D*pir+*	*F⁻ mcrA* Δ*(mrr-hsdRMS-mcrBC) Φ80dlacZ*Δ*M15* Δ*lacX74 recA1 endA1 araD139* Δ*(ara, leu)7697 galU galK λ⁻ rpsL (Str^R) nupG pir+*		Epicentre
SM10λ*pir*	*thi-1 thr leu tonA lacY supE recA::*RP4-2-4-Tc::Mu Km^r (λ *pir*R6K)		(240)
Plasmids			
pWM91	Suicide plasmid vector used for allelic exchange		(241)
pM132	pWM91::ΔVC0914		(123)
pM133	pWM91::ΔVC1673		(123)

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2.2 RESULTS

2.2.1 Function of VexH in Antimicrobial Resistance

Deletion of *vexH* alone did not affect *V. cholerae* susceptibility to any of the tested antimicrobial compounds (Table 2). This is consistent with the reported functional redundancy among the *V. cholerae* RND efflux pumps (123). Deletion of *vexH* in a Δ*vexB* background resulted in increased sensitivity to Triton X-100, ampicillin and novobiocin suggesting that this detergent and these antibiotics were substrates for the VexH RND efflux pump. This finding was corroborated by the corresponding increase in susceptibility to ampicillin and novobiocin in the Δ*vexBDKH* strain relative to the parental Δ*vexBDK* strain (Table 2). Novobiocin was also found to be a substrate for the VexB and VexK RND efflux systems as evidenced by the increased susceptibility observed for these mutants.

VexB and VexD were previously shown to efflux bile salts (119). Therefore *vexH* was deleted in the Δ*vexBD* background in order to test if *vexH* contributed to bile salt resistance. The resulting Δ*vexBDH* mutant exhibited an increase in susceptibility to cholate and deoxycholate (Table 2). A similar increase in bile salt susceptibility was observed following the introduction of the *vexH* deletion into a Δ*vexBDK* background. These results support the conclusion that bile salts were substrates for the VexH RND efflux pump. The observation that the cholate and deoxycholate susceptibility results were identical for the Δ*vexBDHK* strain and the ΔRND strain supported the conclusion that the VexB, VexD, VexH, and VexK RND efflux pumps were responsible for *V. cholerae* resistance to bile acids in vitro.

Table 2. Antimicrobial susceptibility of *V. cholerae* RND mutants

	Mean length of mutant growth relative to WT (s.d.)[1]							
	Cholate		**Deoxycholate**		**Triton X-100**	**Novobiocin**	**Ampicillin**	
Strain	**5%**	**0.05%**	**3%**	**0.01%**	**0.01%**	**0.6μg/mL**	**10μg/mL**	**2μg/mL**
N16961-Sm	100(0)	100(0)	100(0)	100(0)	100(0)	100(0)	100(0)	100(0)
Δ*vexB*	100(0)	100(0)	100(0)	100(0)	34.4(±3.1)^{A}	66.7(±25.5)^{A}	22.9(±7.8)^{A}	100(0)
Δ*vexH*	100(0)	100(0)	100(0)	100(0)	100(0)	75.7(±29.4)	100(0)	100(0)
Δ*vexBD*	NG	58.9(±14.1)^{A}	NG	73.9(±5.5)^{A}	35.6(±1.6)^{A}	58.9(±13.5)^{A}	40.0(±14.6)^{A}	100(0)
Δ*vexBH*	100(0)	100(0)	100(0)	100(0)	24.0(±4.3)^{B}	19.3(±2.7)^{B}	NG	20.6(±3.9)^{B}
Δ*vexBK*	100(0)	100(0)	100(0)	100(0)	23.9(±4.8)^{B}	23.2(±10.5)^{B}	22.0(±10.2)^{A}	100(0)
Δ*vexBDH*	NG	31.1(±1.6)^{C,D}	NG	47.2(±0.1)^{C,D}	26.4(±8.2)	19.3(±2.6)	NG	20.7(±1.7)^{B}
Δ*vexBDK*	NG	38.9(±1.6)^{C,D}	NG	47.2(±0.1)^{C,D}	23.1(±4.8)	24.6(±9.5)	23.8(±10.4)^{A}	100(0)
Δ*vexBHK*	100(0)	100(0)	100(0)	100(0)	24.3(±4.6)	8.2(±8.9)	NG	22.0(±1.1)^{B}
Δ*vexDHK*	34.4(±1.6)^{A}	100(0)	100(0)	100(0)	100(0)	62.2(±31.8)	100(0)	100(0)
Δ*vexBDHK*	NG	25.9(±3.32)	NG	33.3(±8.9)	23.6(±3.3)	12.8(±5.4)	NG	19.2(±2.3)^{B}
Δ*RND*	NG	25.9(±3.32)	NG	34.8(±6.8)	23.8(±5.9)	10.2(±8.8)	NG	23.0(±2.9)^{B}

Antimicrobial susceptibility was determined using antibiotic and detergent gradient agar plates. [1]The length of the mutant bacterial growth across the 90x90 mm gradient plate normalized to 100 mm. Data represents the average of three of more experiments with the standard deviation in parenthesis. Unpaired t-test was used to determine significance. $^{A}p<0.001$ compared to N16961-Sm; $^{B}p<0.05$ compared to Δ*vexB*; $^{C}p<0.05$ compared to Δ*vexBD*; $^{D}p<0.05$ compared to Δ*vexBDHK*. NG: no bacterial growth. doi:10.1371/journal.pone.0038208.t001

2.2.2 The VexF and VexM Pumps do not Function in Antimicrobial Resistance in vitro

The Δ*vexBDHK* mutant had the same antimicrobial susceptibility profile as the ΔRND mutant for all of the tested antimicrobial compounds, including cholate, deoxycholate, Triton X-100, SDS, erythromycin, Polymyxin B, novobiocin, ampicillin, and penicillin (Table 2 and data not shown). This suggested that neither VexF nor VexM functioned in antimicrobial resistance in vitro.

2.2.3 Multiple RND Efflux Pumps Contribute to Virulence Factor Production

CT production in the *V. cholerae* ΔRND mutant was decreased by 70% relative to WT (Figure 5A) with a corresponding decrease in TCP production (Figure 5B). This is in agreement with previously reported work (123) and was used as a RND efflux-negative reference for analysis of CT and TCP production by the RND mutant strains generated in this study. CT and TcpA production in the Δ*vexBH*, Δ*vexHK*, Δ*vexDK*, Δ*vexBDH*, Δ*vexBDK*, Δ*vexBHK*, Δ*vexDHK*, Δ*vexDFHM*, and Δ*vexDFHKM* mutants (Figure 5A, 5B, and data not shown) was not statistically different from WT. In contrast, CT and TcpA production in the Δ*vexBDHK* mutant was reduced by 45% relative to WT (Figure 5A and 5B), suggesting that these four efflux pumps were required for virulence factor production. The observation that the presence of a functional copy of any one of these four RND efflux pumps resulted in a WT phenotype suggested that there is redundancy among these pumps for their function in CT and TCP production. The finding that the Δ*vexBDHK* mutant produced more CT and TcpA than the ΔRND mutant suggested that VexF and/or VexM also contributed to virulence factor production and support the conclusion

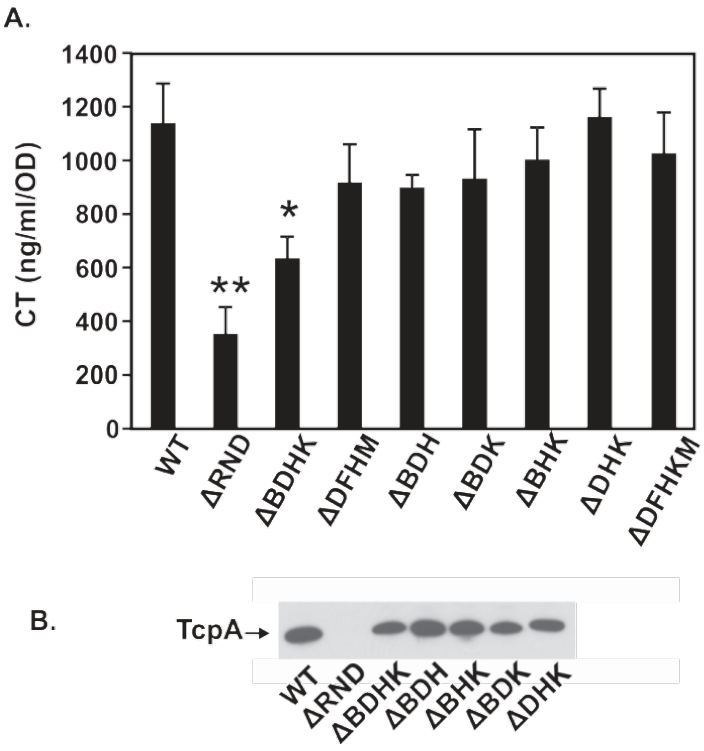

Figure 5. CT and TCP production by RND mutants.

CT and TCP production in the indicated strains was determined following growth under AKI conditions. CT (A) and TcpA (B) were detected by CT GM_1-ELISA and TcpA Western immunoblotting, respectively. Error bars represent the standard deviation of the mean from three or more experiments. Statistical analysis was performed by one-way ANOVA. *$p<0.05$ compared to wild type (WT); **$p<0.05$ compared to all tested strains.doi:10.1371/journal.pone.0038208.t001

that at least five of six RND efflux pumps are required for high-level production of CT and TcpA.

2.2.4 VexH Contributes to in vivo Colonization

The competitive index (CI) is a measure of fitness of a test strain relative to the WT strain for colonization of the infant mouse small intestine. Mutants that are able to compete equally with the WT strain exhibit a CI of ~1, whereas mutants that are outcompeted by the WT (i.e. attenuated mutants) will have a CI of <1. Analysis of the mutants constructed in this study showed that the Δ*vexBH*, Δ*vexHK*, Δ*vexDK*, Δ*vexDHK* and Δ*vexBHK* strains competed equally with the WT strain (Figure 6A). Similar results were previously reported for the Δ*vexBD* strain (123). In contrast, the Δv*exBDH* and Δ*vexBDHK* mutants were found to be severely attenuated and could not be recovered from the infant mice when inoculated at a 1:1 ratio (data not shown). The in vivo attenuation of these mutants did not emanate from an apparent growth defect as all of the mutants competed equally with WT during in vitro competitive growth assays and there were no differences in the growth kinetics of the strains in minimal media (data not shown). There was a possibility that the detection limit of the infant mouse colonization assay hindered our ability to recover severely attenuated mutant strains (e.g. Δ*vexBDH*, Δ*vexBDHK*, and ΔRND) in the intestinal homogenates. To compensate for this the challenge inoculum was biased for the mutant strains by 100-fold (i.e. 1:100 ratio of WT to mutant cells) which resulted in an ~2 log increase in the detection the limit. To validate that the biased input did not affect the CI, we tested the Δ*vexDHK* strain which competed equally with the WT strain at the 1:1 ratio. The results showed that the Δ*vexDHK* competed equally with the WT strain at the 1:100 input ratio,

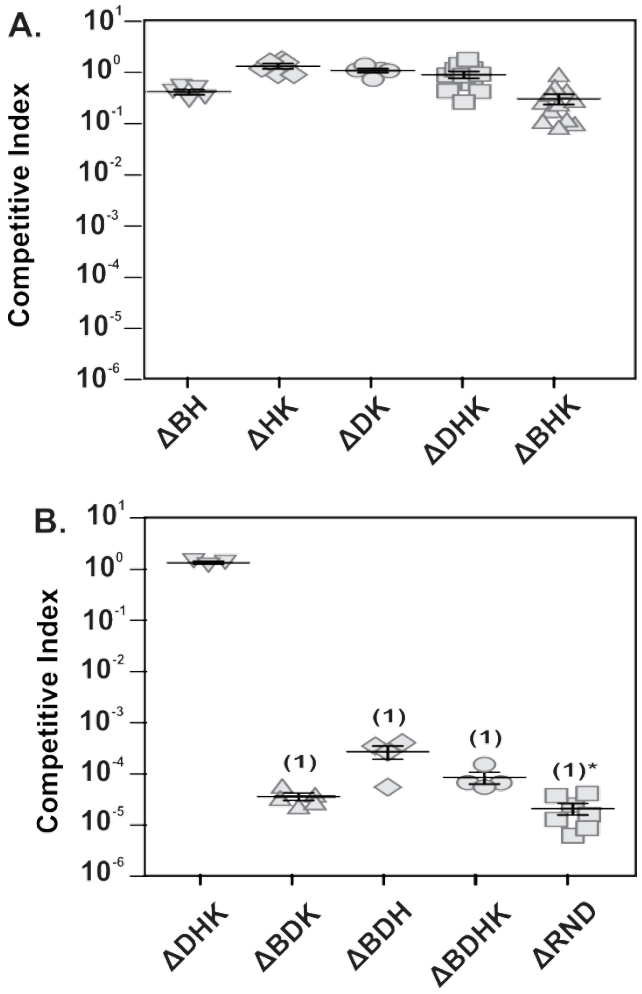

Figure 6. Infant mouse colonization assays with the RND efflux mutants.

Competition assays were performed using the infant mouse colonization assay as described in the Materials and Methods. Infant mice were challenged with a ~10^5 cfu inoculum containing a mixture of wild type and the indicated mutant at a ratio of 1:1 (A) of 1:100 (B). The competitive index was calculated as the ratio of mutant to wild type recovered from the small intestine, corrected for the ratio of mutant to wild type that was present in the inoculum. Each symbol represents one mouse. *The ΔRND mutant was not recovered from mice necessitating the calculation of a theoretical CI as described in the Materials and Methods. Mean and standard deviation are indicated by horizontal bars. Significance was determined using the Mann-Whitney U t-test. (1) $p<0.01$.

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confirming that the biased input did not affect the CI value (Figure 6B). The modified assay was then used to test the Δ*vexBDH*, Δ*vexBDK*, Δ*vexBDHK*, and ΔRND strains. The results of this analysis confirmed the severely attenuated phenotype of each strain (Figure 6B). However, the Δ*vexBDH* and Δ*vexBDHK* strains, which could not be recovered from infant mice when inoculated at the 1:1 ratio, were recovered in 30% of the challenged mice using the modified assay. Using data from the colonized mice, the Δ*vexBDH* and Δ*vexBDHK* strains had CI's that were reduced by 3.7 and 4.1 log units (Figure 6B). The ΔRND strain still could not be recovered from the mice which is consistent with this mutant having the greatest colonization defect with a >4.8 log reduction in its CI.

Despite the modifications to the colonization competition assay, the ability to quantify colonization differences between highly attenuated mutants (e.g. the Δ*vexBDH*, Δ*vexBDHK*, and ΔRND strains) was still limited. Therefore, we assessed the ability of these three highly attenuated strains to colonize the infant mouse small intestine in the absence of the WT competitor strain (Figure 7). Mice were challenged with the mutants at two inocula: ~10^6 cfu/mouse and ~10^8 cfu/mouse. The 10^6 cfu/mouse inoculum was equal to the mutant titer used in the modified competition assay while the 10^8 cfu/mouse inoculum was used to determine if increasing the challenge dose would facilitate colonization by the mutant strains.

The WT strain and Δ*vexBDK* mutant colonized 100% of the challenged infant mice when administered at 10^6 cfu/mouse. However, the Δ*vexBDK* mutant exhibited an apparent in vivo growth defect since the mutant replicated to a final in vivo titer was ~2 log units lower per mouse than was observed with the WT (Figure 7A). Inoculation of mice with 10^6 cfu of the Δ*vexBDH* mutant resulted in colonization of about 50% of the challenged mice. In the successfully colonized mice, the Δ*vexBDH* titers were at least 5-logs lower than was observed in mice

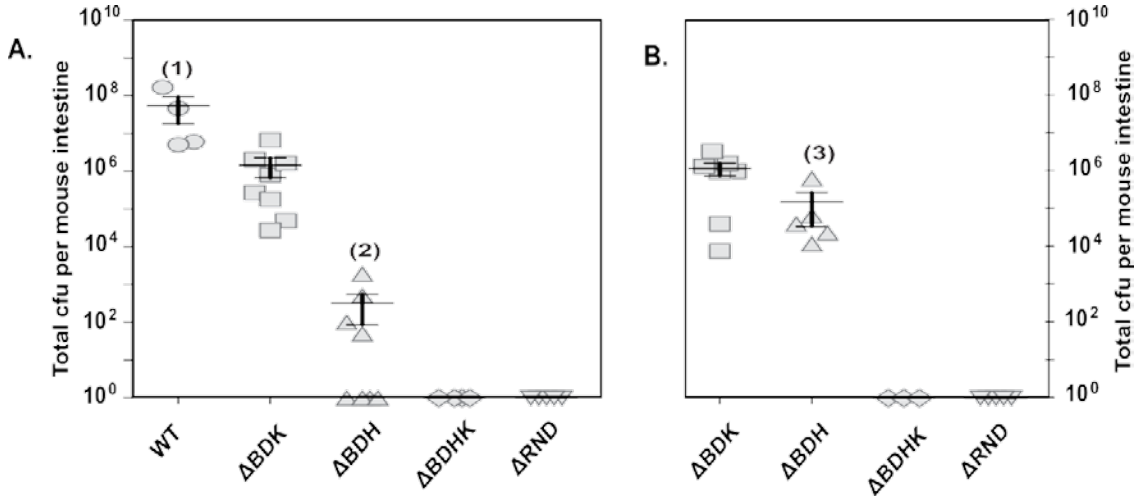

Figure 7. Colonization of the infant mouse small intestine by *V. cholerae* RND efflux mutants.

Infant mice were challenged with ~6x10^6 cfu (A) or ~8x10^7 cfu (B) of the indicated *V. cholerae* mutant. Bacterial loads in the small intestine were assessed after overnight incubation. Means and standard deviation are indicated by horizontal bars. The Mann Whitney U t-test was used to determine significance. (1) $p<0.05$ compared to all tested strains; (2) $p<0.05$ compared to Δ*vexBDK*; (3) $p<0.05$ compared to the ~6x10^6 cfu (A) Δ*vexBDH* challenge.

doi:10.1371/journal.pone.0038208.t001

challenged with the WT strain and 3-logs lower than the output observed in mice challenged with the Δ*vexBDK* mutant (Figure 7A). This indicates that the in vivo growth defect of the Δ*vexBDH* mutant was significantly greater than what was observed for the Δ*vexBDK* mutant. This is also consistent with the severe attenuation of this mutant in the colonization competition assays. When the challenge inoculum was increased to 10^8 cfu/mouse the Δ*vexBDH* mutant successfully colonized 100% of the challenged mice. In addition, the bacterial titer in each mouse also increased by 3-logs to a level that was equivalent to what was observed with the Δ*vexBDK* mutant (Figure 7B). The increase in the output titer was limited to the Δ*vexBDH*

mutant and was not observed when mice were challenged with 10^8 cfu of the Δ*vexBDK* mutant. The Δ*vexBDHK* and ΔRND mutants did not successfully colonize the intestinal tract at either inoculum level (Figure 7). This indicates that if either of these strains is able to colonize the infant mouse small intestine, the mutants were present at very low levels that were below our limits of detection. Since we were unable to distinguish an in vivo difference between these two strains, other approaches will be required to assess the function of VexF and VexM in vivo.

2.3 DISCUSSION

Deletion of *vexH* in the WT background did not result in an observable phenotype. There were two plausible explanations for this lack of phenotype: either VexH did not contribute to antimicrobial resistance, or its contribution was masked due to redundancy with one or more of the other five *V. cholerae* RND efflux pumps. The latter was proven true since the introduction of the *vexH* deletion into *V. cholerae* lacking the *vexBDK* RND efflux pumps resulted in increased susceptibility to a number of antimicrobial compounds (Table 2). This showed that VexH possessed a relatively broad substrate specificity that was second only to VexB (Table 2 and data not shown). VexH contributed to cholate, deoxycholate, Triton X-100, novobiocin, and ampicillin resistance, but not to penicillin or erythromycin resistance (which were VexB substrates). Redundant substrate specificity between VexH and VexB is consistent with the observation that VexH has the largest amino acid sequence identity in common with VexB among the *V. cholerae* RND efflux pumps (123). The *V. cholerae* Δ*vexBDHK* mutant exhibited the same antimicrobial susceptibility profile as the ΔRND strain (Table 2). This suggests that VexB, VexD, VexK, and VexH are the only RND efflux pumps that contribute to antimicrobial

resistance in vitro. Although these four RND efflux pumps were redundant for some substrates, they do not have equal activity. All four pumps contributed to bile acid resistance, yet the presence of VexB or VexD is sufficient to provide a WT level of resistance in the absence of VexH and/or VexK (Table 2) (119). Only in a Δ*vexBD* mutant background can the contribution of VexH and VexK be observed. Together this suggests that VexB and VexD are major contributors to bile acid resistance in vitro, while VexH and VexK have minor roles. This conclusion is likely only relevant to *V. cholerae* grown under the conditions used in our assays as there are reports to suggest that the expression of the RND efflux systems are responsive to environmental cues including those present in vivo in rabbit ileal loops and in humans (119, 202, 210, 242).

The antimicrobial susceptibility results also suggested that neither VexF nor VexM contributed to antimicrobial resistance in vitro. This was a surprising finding as *vexF* from a non-O1 *Vibro* was reported to produce a functional efflux system when expressed in *E. coli* in conjunction with *V. cholerae tolC* (243). This discrepancy may reflect strain or functional differences of VexF in a heterologous system (243). Alternatively, it is also possible that VexF or VexM are expressed under conditions or efflux substrates other than tested in this study (see below). The finding that *V. cholerae* produces redundant RND efflux pumps that function in bile acid and detergent resistance seems to be an important adaption to facilitate colonization of the small intestine. Redundant bile efflux pumps would provide an obvious benefit since intestinal bile is a natural host defense that microorganisms must overcome in order to colonize the small intestine (1). Consistent with bile salts being a major substrate of the RND efflux pumps, a number of studies have suggested that bile salts and other components of bile function to induce the expression of the RND efflux systems. In 2004, Chatterjee et. al. (244) reported that *V.*

cholerae grown in bile accumulated lower amounts of hydrophobic compounds than *V. cholerae* grown without bile, a phenotype they attributed to bile-dependent induction of active efflux. More recently, we and others have shown that transcription of the *vexAB* and *vexCD* RND efflux systems are upregulated in the presence of bile acids (123, 210). These results are consistent with the hypothesis that substrates of the individual RND efflux pumps function as effectors to upregulate the expression of the respective RND efflux system. While the chemical effectors that control the expression of *vexH* are unknown, a recent study has suggested that *vexH* expression may be responsive to the iron status of the cell (41, 245, 246). This finding, combined with the hypothesized iron limiting conditions *V. cholerae* may encounter late during infection (201), could explain the in vivo induction of VexH in humans during infection (242). If *vexH* transcription is up-regulated during in vivo colonization as a response to iron availability, then VexH could have a greater role in vivo than indicated by our in vitro analysis.

The function of the RND efflux systems in mediating resistance to host defenses is correlated with the ability of many bacterial pathogens to survive, invade, and colonize their hosts (129, 179, 181, 247). We therefore expected that *V. cholerae* RND efflux mutants with similar antimicrobial susceptibility profiles would behave similarly in vivo, but our results revealed this to be false. For example, the Δ*vexBDK* and Δ*vexBDH* mutants exhibited similar susceptibility profiles for bile salts and detergents, but the Δ*vexBDH* mutant was more attenuated in vivo than the Δ*vexBDK* mutant and less attenuated than the Δ*vexBDHK* and ΔRND mutants. Consistent with this the Δ*vexBDH* mutant required a two-log higher inoculum than did the Δ*vexBDK* mutant to efficiently colonize the small intestine (10^8 vs. 10^6 cfu/mouse, respectively; Figure 7). This is in contrast to WT which can efficiently colonize when administered at inoculums of 10^3–10^4 cfu/mouse (248). Even at the higher inoculums, neither mutant was able to

reach titers in the intestine equivalent to WT. It was noteworthy that administration of the Δ*vexBDH* mutant at 10^8 cfu/mouse resulted in a three-log increase in the bacterial outputs from the colonized mice relative to inoculation at 10^6 cfu/mouse. This phenomenon was not observed with the Δ*vexBDK* mutant (Figure 7B). Together this suggests that the in vivo roles of the RND efflux systems do not completely correlate with their contributions to in vitro antimicrobial susceptibility of WT *V. cholerae*. The fact that the Δ*vexBDH* mutant can survive to similar titers as the Δ*vexBDK* mutant when given at a high inoculum suggests that the Δ*vexBDH* mutant may be defective in colonization of the intestinal epithelium. The intestinal epithelium is covered by a thick mucus layer which provides a diffusion barrier against antimicrobial compounds that are present in the lumen (e.g. bile) (249). One implication of this is that the epithelial surface likely represents a more amenable environment for the survival of antimicrobial hyper-susceptible organisms like the Δ*vexBDK* and Δ*vexBDH* efflux mutants. Thus one possible explanation for the colonization difference observed between the Δ*vexBDK* and Δ*vexBDH* mutants is that they exhibit differential susceptibility to antimicrobial compounds that are present in the intestinal lumen.

This idea is supported by the observation that VexH has a broader substrate range than VexK (Table 2), which would make VexH more important during colonization than VexK. The finding that both VexH and VexK were induced during colonization of the human gut (242) corroborates the idea that these two RND efflux pumps are induced in vivo. Alternatively, it is possible that the colonization differences are due to unknown in vivo growth defects or differential effects on the in vivo induction of the ToxR regulon (see below). The CT and TCP bioassays showed that VexB, VexD, VexH, and VexK contributed to virulence factor production. However, the Δ*vexBDH*, Δ*vexBDK*, Δ*vexBHK*, and Δ*vexDHK* mutants were not

different from WT for CT and TCP production (Figure 5). This suggests that these four efflux systems were functionally redundant for CT and TCP production. Consistent with this result was the finding that VexB was able to complement for the loss of the five other RND efflux systems (123), which was evidenced by the observation that a *vexDFHKM* mutant (which is *vexB*+) was phenotypically identical to WT (Figure 6). The function of VexF and/or VexM in CT and TCP production was evident as the mutant that lacked *vexBDHK* was attenuated for CT and TCP production, while the mutant that lacked all six RND efflux systems (i.e. ΔRND) produced even less CT and TCP. This observation provides the evidence that VexF and/or VexM are required for WT CT and TCP production. This also indicates that neither VexF nor VexM are able to fully compensate for the loss of the other four RND efflux systems.

Although much is known about how RND efflux systems contribute to antimicrobial resistance, the mechanism of how they affect virulence factor production is not known. We previously showed that the *V. cholerae* RND efflux systems effect on virulence gene expression mapped to *tcpPH* transcription (123), but the connection between RND efflux systems and *tcpPH* transcription has not yet been determined. We hypothesize that the RND efflux systems function to modulate the intra- or extracellular concentration of a low molecular weight molecule that functions as a negative effector of *tcpPH* transcription. Efflux-dependent modulation of an effector molecule represents a mechanism that could be used to link efflux to gene expression. This process could be used to fine-tune the expression of virulence genes in response to the growth environment. For example the efflux of any given effector molecule, which would affect its cellular distribution, would be dependent upon the presence of competing efflux substrates in the bacterium's growth environment (e.g. components of bile in the GI tract). Consistent with this hypothesis, a number of potential low molecular weight effector compounds have been

described in the literature that affect virulence factor production including: fatty acids, bile acids, quorum sensing molecules, cyclic nucleotides, and cyclic peptides (77, 80, 114, 118, 250, 251). Significantly, all of these compounds have been reported to be effluxed in Gram negative bacteria (244, 252, 253) which suggests the possibility that effector efflux could be applicable to other bacterial pathogens where the RND efflux systems have also been reported to influence virulence factor production (179, 189). In addition to negatively affecting *tcpPH* transcription, efflux could also impact genes downstream of *tcpPH* in the ToxR regulon. For example, given the role of the RND efflux systems in bile resistance, it is possible that the loss of efflux could impact intracellular fatty acids pools and thus affect ToxT activity and virulence factor production (78, 254).

In summary, we have shown that VexH contributes to antimicrobial resistance and exhibits broad substrate specificity. VexH was found to be important for intestinal colonization and virulence factor production; phenotypes consistent with *vexH* being in vivo induced in humans (242). We have also shown that the *V. cholerae* RND efflux pumps have redundant functions, not only in antimicrobial resistance, but also in virulence factor production. Collectively these results support the conclusion that the RND efflux system contribute to *V. cholerae* pathogenesis in two ways. First, the RND efflux systems function to provide the bacterium with protection against antimicrobial compounds that are present in the host. Second, the RND efflux systems are required for efficient production of virulence factors.

3.0 RECIPROCAL REGULATION OF RND EFFLUX SYSTEMS AND THE CPX TWO-COMPONENT SYSTEM IN *VIBRIO CHOLERAE*.

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3.1 INTRODUCTION

V. cholerae is a facultative human pathogen that causes cholera, a severe acute diarrheal disease that is estimated to afflict 3-5 million people annually (255). People acquire cholera by ingestion of *V. cholerae* contaminated food or water (1). Once in the host environment, *V. cholerae* produces a variety of virulence factors that enable the pathogen to colonize the small intestine and to cause diarrhea. Two critical virulence factors co-regulated by the virulence activator ToxR are the Toxin Coregulated Pilus (TCP), a type IV pilus that is essential for colonization, and cholera toxin (CT), an enterotoxin that causes the secretory diarrhea that is the hallmark of cholera (67, 68, 228-231). In addition to the expression of TCP, intestinal colonization is also dependent upon *V. cholerae* overcoming host barriers in the human gastrointestinal tract. These barriers include antimicrobial compounds such as bile salts, fatty acids and components of the innate immune system. *V. cholerae* resistance to these factors is largely dependent upon the production of the Resistance-Nodulation-Division (RND) family of efflux systems (123, 176).

RND efflux systems are tripartite transporters that are ubiquitous among Gram-negative bacteria. Each RND efflux system is made up of three components: an outer membrane porin homologous to *E. coli tolC*; an integral cytoplasmic membrane pump protein belonging to the RND superfamily of transporters; and a periplasmic membrane fusion protein that links the outer membrane pore to the cytoplasmic membrane pump protein (127, 131, 137, 238, 239). These RND systems have garnered much attention in regards to xenobiotic resistance as a number of RND systems have been shown to efflux numerous chemically unrelated antimicrobial

compounds including dyes, detergents, antibiotics, and antimicrobial peptides (120, 126). As such, many of these broad-spectrum RND efflux systems are intricately linked to the development of multiple drug resistant organisms. Although antibiotic resistance provides an easily scored phenotype for many efflux systems, phylogenetic analysis indicates that the RND efflux systems evolved independently of antibiotic selection (167, 168). Thus the native role of the RND efflux systems in bacterial physiology remains unclear, but there is accumulating evidence to suggest that they influence bacterial physiology independent of their role in xenobiotic resistance. This is exemplified by reports implicating RND efflux systems in diverse phenotypes such as (reviewed in (169)) biofilm formation (170, 171), iron acquisition (172), plant-bacteria interactions (173), lipid transport (174, 175), bacterial virulence (123, 129, 176), extrusion of toxic metal effectors (177), and the removal of metabolic byproducts from within the cell (178).

V. cholerae encodes six RND efflux systems which are required for antimicrobial resistance, virulence factor production, and intestinal colonization (119, 123, 256). Functional characterization of the RND systems revealed that four of the six systems (i.e. VexAB, VexCD, VexGH, and VexIJK) mediate resistance to antimicrobial compounds in vitro (119, 123, 176). The VexAB RND efflux system exhibits a high basal level of activity and provides *V. cholerae* with its intrinsic antimicrobial resistance, a function analogous to *E. coli* AcrAB. The VexAB system is a multiple drug efflux system that mediates resistance to bile salts, non-ionic detergents, and a variety of antibiotics (e.g. ampicillin, erythromycin, novobiocin, and polymyxin B). The other three RND efflux systems are functionally redundant with VexAB. VexCD is a bile specific efflux system; VexGH mediates resistance to bile acids, non-ionic detergents, ampicillin and novobiocin; VexIJK effluxes bile acids, non-ionic detergents, and

novobiocin. The remaining two RND efflux systems, VexEF and VexLM, do not appear to influence resistance to antimicrobials. All six of the RND efflux systems contribute to virulence (123, 256) and strains lacking the RND efflux systems are severely attenuated in vivo (123, 256). Inactivation of the RND efflux systems results in down-regulation of the ToxR regulon, diminished CT and TCP production, and severe attenuation of growth in suckling mice (114, 123). The mechanism(s) by which the RND efflux systems influence virulence gene expression are unknown.

The Cpx two-component system is widely distributed among Gammaproteobacteria including the *Enterobacteriaceae* and *Vibrionaceae*. In this regulatory system, CpxA functions as a membrane associated sensor histidine kinase. Upon stimulation, CpxA autophosphorylates itself and then transfers the phosphate to a conserved aspartate residue on the cytoplasmic CpxR response regulator (reviewed in (257, 258)). Phosphorylated CpxR (CpxR~P) then modulates the expression of its target genes by binding to a consensus binding sequence located in their promoter regions. CpxR~P also regulates its own expression (i.e. the *cpxRA* operon) and the divergently transcribed *cpxP*. CpxP is a periplasmic protein associated with the *cpxRA* operon that appears to repress CpxR activation by interacting with CpxA and inhibiting its kinase activity, and may also exhibit chaperone activity (216). *CpxA** mutants lead to constitutive activation of the Cpx system and have been useful in analyses of Cpx regulons. The *cpxA** mutation inactivates CpxA phosphatase activity, resulting in the accumulation of activated CpxR (i.e. CpxR~P) (211, 215, 259). The Cpx system has been most extensively studied in *E. coli* where it has been shown to alleviate extracytoplasmic stress resulting from cell envelope perturbations that are generally associated with misfolded cell envelope proteins (260, 261). Consistent with this idea, the majority of stimuli that activate the Cpx system have been

predicted to result in the production of misfolded or damaged cell envelope proteins (reviewed in (257, 258)).

Although a number of Cpx inducing stimuli have been described in *E. coli*, studies suggest that these stimuli are not conserved in *V. cholerae* (211). For example, the *E. coli* Cpx system is activated by increased osmolarity, but not by increased salinity (211, 224). In contrast, the *V. cholerae* Cpx system functions in an opposite manner; it is not responsive to osmolarity, but is activated by increased salinity (211). Additionally, the *E. coli* Cpx system is active under standard laboratory growth conditions, whereas the *V. cholerae* Cpx system is inactive. The differences in physiological roles of the Cpx system in *E. coli* and *V. cholerae* may be related to the distinct environmental niches these organisms occupy and appear to be reflected in amino acid sequence variability in the signaling domain of CpxA (211, 225).

While the physiological roles of the *V. cholerae* and *E. coli* Cpx systems appear to differ, deletion of *tolC* activated the Cpx system in both organisms (200, 211). In *E. coli*, the activation of the Cpx system was linked to loss of TolC-dependent efflux (200). TolC functions as the outer-membrane pore component of several *V. cholerae* transport systems including RND family transporters (227). Thus, we speculated that the *tolC*-dependent activation of the *V. cholerae* Cpx system results from the loss of RND efflux activity. Here, we explored the linkage between RND efflux activity and the expression of the Cpx system. We show that CpxR functions as a positive regulator of the VexAB and VexGH RND efflux systems. Conversely, we found that mutation of *vexRAB* or *vexGH* resulted in the activation of the Cpx system, suggesting that the VexAB and VexGH RND efflux systems function in the regulation of the Cpx system. While the *V. cholerae* VexAB and VexGH RND efflux systems and the Cpx system were reciprocally regulated, the defect in virulence factor production in *V. cholerae* RND efflux mutants was independent of the

Cpx system. Together, our findings revealed a genetic linkage between the *V. cholerae* Cpx system and RND-mediated efflux and suggested that the *V. cholerae* Cpx system is activated in response to the accumulation of RND efflux substrates.

Table 3. Strains, plasmids and primers used in this study.

Strains	Characteristics	Source
E. coli		
EC100Dpir	*supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 (λpirR6K)*	Epicentre
SM10λpir	*thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu Kmr (λpirR6K)*	Lab collection
BW25113	F-, DE(*araD-araB*)567, *lacZ4787*(del)::*rrnB*-3, LAM-, *rph*-1, DE(*rhaD-rhaB*)568, *hsdR514*	(262)
JW3883-1	F-, DE(*araD-araB*)567, *lacZ4787*(del)::*rrnB*-3, LAM-, *rph*-1, DE(*rhaD-rhaB*)568, *hsdR514, cpxR::Km*	(262)
V. cholerae		
JB58	*V. cholerae* O1 El Tor strain N16961 Δ*lacZ*, Sm^R	Lab collection
*cpxA**	JB58::*cpxA**	(211)
Δ*cpxR*	JB58Δ*cpxR*	(211)
JB485	JB58Δ*vexB* Δ*vexD* Δ*vexF* Δ*vexH* Δ*vexK* Δ*vexM*	(123)
DT1452	JB485Δ*cpxR*	This study
MKW589	Δ*cpxR lacZ::cpxP-lacZEc*	(211)
DT1458	JB58 *lacZ::cpxP-lacZEc*	This study
DT1572	JB58Δ*vexB lacZ::cpxP-lacZEc*	This study
DT1574	JB58Δ*vexD lacZ::cpxP-lacZEc*	This study
DT1578	JB58Δ*vexH lacZ::cpxP-lacZEc*	This study
DT1687	JB58Δ*vexK lacZ::cpxP-lacZEc*	This study
DT1576	JB58Δ*vexB* Δ*vexD lacZ::cpxP-lacZEc*	This study
DT1580	JB58Δ*vexB* Δ*vexH lacZ::cpxP-lacZEc*	This study
DT1689	JB58Δ*vexB* Δ*vexK lacZ::cpxP-lacZEc*	This study
DT1582	JB58Δ*vexD* Δ*vexH lacZ::cpxP-lacZEc*	This study
DT1584	JB58Δ*vexB* Δ*vexD* Δ*vexH lacZ::cpxP-lacZEc*	This study
DT1691	JB58Δ*vexB* Δ*vexD* Δ*vexK lacZ::cpxP-lacZEc*	This study
DT1478	JB58Δ*vexB* Δ*vexD* Δ*vexH* Δ*vexK lacZ::cpxP-lacZEc*	This study
DT1480	JB58Δ*vexB* Δ*vexD* Δ*vexF* Δ*vexH* Δ*vexK lacZ::cpxP-lacZEc*	This study
DT1482	JB58Δ*vexB* Δ*vexD* Δ*vexH* Δ*vexK* Δ*vexM lacZ::cpxP-lacZEc*	This study
DT1462	JB58Δ*vexB* Δ*vexF* Δ*vexH* Δ*vexK* Δ*vexM lacZ::cpxP-lacZEc*	This study
DT1476	JB58Δ*vexD* Δ*vexF* Δ*vexH* Δ*vexK* Δ*vexM lacZ::cpxP-lacZEc*	This study
DT1586	DT1452 *lacZ::cpxP-lacZEc*	This study
DT1460	JB485 *lacZ::cpxP-lacZEc*	This study
Plasmids		
pTL61T	*lacZ* transcriptional reporter plasmid, Cb^R, oriRK2	(263)
pXB228	pTL61T containing the *vexEF* promoter region	This study
pXB229	pTL61T containing the *vexGH* promoter region	This study
pXB230	pTL61T containing the *vexIJK* promoter region	This study
pXB231	pTL61T containing the *vexCD* promoter region	(123)
pXB232	pTL61T containing the *vexLM* promoter region	This study
pXB233	pTL61T containing the *vexRAB* promoter region	(123)
pXB265	pTL61T containing the *breR* promoter region	This study
pΔR	*cpxR*::Km allelic exchange vector	(211)
pJL1P'Z	Allelic exchange vector for placing *cpxP-lacZ* into the *V. cholerae* genome	(211)
pBAD33-*cpxR*	pBAD33 expressing *cpxR*	(211)
pBAD33	Arabinose regulated expression plasmid, Cm^R, p15A origin of replication	(264)

Table 3. (continued)

Strains	Characteristics	Source
PCR primers		
cpxR-F	GGTCAAGTGACGTATAGGGAGCG	
cpxR-R	GAGGTAGGGTCAATACCGCGAAC	
lacZ5	CTCTAGAAGCTTCTAGCTAGAGGG	
lacZ6	CCGCCACCTGACGTCTAAGAAACC	
P-166c-F-XhoI	TTCTCGAGGGGTCCGGAGACGTACT	
P-166c-R-XbaI	CGTCTAGAGGAGCTGTTTATCGCCG	
P-VC0628-F-XhoI	GGCTCGAGATATTTGATCGGCGGAGT	
P-VC0628-R-XbaI	GGCTCGAGATATTTGATCGGCGGAGT	
P-VC0914-F-XhoI	GCCTCGAGCACATCGCTCAAGTGCGC	
P-VC0914-R-XbaI	CGTCTAGATCTTTGGCCGATAGCACA	
P-VC1673-F-XhoI	GGCTCGAGACCGCAGCCTTGCTGGG	
P-VC1673-R-XbaI	AATCTAGACCCACCAGCAAAGTGGA	
P-VC1746-F-SmaI	AACCCGGGAATTCGGCTTTTTCTTTCCAAATCGGCAGTG	
P-VC1746-R-BamHI	AAGGATCCAATCAGCGCCAACCGTTTTTGCTCACTGAG	
P-VCA638-F-XhoI	GGCTCGAGGGGTTTGGTCGGCATCT	
P-VCA638-R-XbaI	CGTCTAGAGTGCGATACTCCAACTTA	

doi:10.1128/IAI.00025-14

3.2 RESULTS

3.2.1 Identification of CpxR regulated genes.

To gain a better understanding of the genetic basis of the *V. cholerae* Cpx response, we used microarrays to identify CpxR regulated genes. This was done by defining the effect of the *cpxA** mutation on the *V. cholerae* transcriptome. The *cpxA** mutation inactivates the phosphatase activity of CpxA, which results in accumulation of CpxR in its activated form (i.e. CpxR~P). Analysis of the microarray revealed that the levels of 25 transcripts were changed ≥2 fold in

Table 4. CpxR regulated genes

Gene category[a]	ORF #	Expression ratio (sd) *cpxA**	CpxR binding site?	Gene	Gene product
Cellular processes					
	vc2691	67.0 (16.8)	yes [b]	*cpxP*	periplasmic protein *cpxP*, putative
Cell envelope					
	vc1854	0.5 (0.1)	no	*ompT*	OmpT porin
Metabolism					
	vca0151	5.2 (2.3)	yes		oxidoreductase, putative
	vca0249	2.3 (0.3)	yes		cytochrome b561, putative
	vca0538	10.4 (3.3)	yes [c]		cytochrome b561
Regulatory functions					
	vc0166	3.2 (0.7)	yes (2x)	*vexR*	transcriptional regulator, TetR family
	vc2692	8.1 (1.7)	yes[b]	*cpxR*	transcriptional regulator CpxR
Transport and binding proteins					
	vc0042	0.5 (0.2)	yes	*trkH*	potassium uptake protein
	vc0164	2.4 (0.2)	yes[d]	*vexB*	RND efflux pump VexB
	vc0165	2.2 (0.3)	yes[d]	*vexA*	Membrane fusion protein VexA
	vc0913	14.8 (2.4)	yes	*vexG*	membrane fusion proteins VexG
	vc0914	12.4 (2.2)	yes[e]	*vexH*	RND efflux pump VexH
	vc2436	2.1 (0.3)	yes	*tolC*	outer membrane pore protein TolC
	vca0576	4.2 (2.2)	yes	*hutA*	heme transport protein HutA
	vca0603	2.6 (1.4)	no		ABC transporter substrate-binding protein
	vca0782	4.3 (1.9)	yes		ABC transporter, ATP-binding protein
Hypothetical & conserved hypothetical proteins					
	vc0191	2.0 (0.9)	yes		
	vc0806	0.5 (0.1)	no		
	vc0915	2.4 (1.1)	no		
	vc0938	5.5 (1.3)	no		
	vca0126	2.0 (0.5)	no		
	vca0139	51.1 (25.4)	yes (3x)		
	vca0162	3.0 (1.2)	no		
	vca0539	11.2 (2.0)	yes[c]		
	vca0781	3.1 (1.2)	yes[f]		

a. Gene categories were derived from Heidelberg et al. (122).
b. The CpxR consensus binding site was located in the intergenic region between the divergently transcribed *cpxP* and *cpxR* genes.
c. vca0538 and vca0539 are in an operon that contains 3 CpxR *c*onsensus binding sites located upstream of vca0538
d. vc0166, vc0165 and vc0164 are in an operon with two CpxR consensus binding site located upstream of vc0166
e. vc0913 and vc0914 are in an operon with a CpxR consensus binding site located upstream of vc0913
f. vca0782 and vca0781 are in an operon with a CpxR consensus binding site located upstream of vca0782
doi:10.1128/IAI.00025-14

response to the constitutive activation of CpxR; 22 genes were upregulated and three genes were downregulated (Table 4). We hypothesized that if CpxR directly regulated the expression of these genes, then their respective promoters would likely contain a CpxR consensus binding sequence. We therefore searched the putative promoter regions of the 25 genes for sequence similarity to the published CpxR consensus binding site ($GTAAN_6GTAA$) (265). Eighteen of the genes (72%) contained a putative CpxR consensus sequence in their respective promoters; all but one of these genes was upregulated by CpxR. An analysis of 25 randomly selected promoters identified two genes (8%) containing CpxR consensus binding sequences. Seven genes did not contain a CpxR binding site and were likely regulated in an indirect manner. The preferential location of CpxR consensus binding sites in the promoters of positivity regulated genes suggests that CpxR primarily functions as a transcriptional activator in *V. cholerae*.

Transcripts for *cpxP* and *cpxR*, which are known to be regulated by CpxR, were in the list of upregulated genes lending credence to our approach. CpxP is located next to *cpxR* and is expressed from a divergent promoter. A CpxR consensus binding sequence is located in the intergenic region separating these two genes, a location that is consistent with CpxR's known regulation of its own expression as well as the divergently transcribed *cpxP* (261, 266). In contrast to *E. coli*, the *V. cholerae* Cpx response did not appear to activate genes involved in alleviating membrane stress (e.g. *dsbA*, *degP*, and *fkpA*) resulting from misfolded membrane proteins (219, 265, 267); a finding that may reflect functional differences in the Cpx response in the *Vibrionaceae* versus the *Enterobacteriaceae*.

Notably, 11 of the 25 CpxR regulated genes were involved in maintaining the permeability barrier of the cell (Table 4). Five of the upregulated genes encoded for the production of two broad-spectrum RND efflux systems: VexAB, VexGH, and their cognate

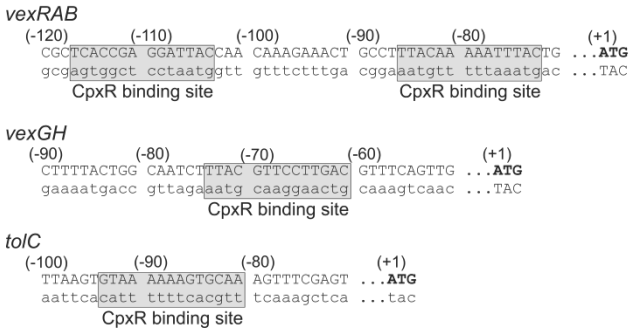

Figure 8. CpxR consensus binding sites in the *vexRAB*, *vexGH*, and *tolC* promoters.

Putative CpxR consensus binding sequences in the promoter regions of the *vexRAB*, *vexGH* and *tolC* genes are indicated by grey boxes. The start codon for each gene is marked by bold font. Numbering is relative to the start codon for each respective gene. doi:10.1128/IAI.00025-14

outermembrane pore component TolC. Furthermore, a putative CpxR consensus binding sequence was found in the promoter regions of *vexRAB*, *vexGH*, and *tolC*, suggesting that CpxR regulates their expression (Figure 8). One of the downregulated genes was *ompT*, which encodes a ToxR-regulated porin that possesses a large diameter pore. Repression of *ompT* is associated with decreased susceptibility to low molecular weight antimicrobial compounds such as bile salts (9, 268, 269).

3.2.2 CpxR is a positive regulator of the *V. cholerae* RND efflux systems.

The expression of *vexRAB* and *vexGH* RND efflux systems increased in the *cpxA** mutant (Table 4). This finding, combined with the presence of CpxR consensus binding

vexRAB and *vexGH* promoters (Figure 8), suggested that CpxR likely regulated the expression of these two RND efflux systems. To test this hypothesis and to validate the microarray results, we quantified *vexRAB* and *vexGH* expression in WT, in the *cpxA** mutant (which constitutively activates CpxR) and in a *cpxR* deletion mutant during growth in LB broth and during growth under AKI conditions. These growth conditions were selected as they represent conditions where the RND efflux systems have been shown to contribute to both antimicrobial resistance and virulence factor production (10). We measured reporter expression at two time points (2 and 4 h post inoculation for the LB cultures and 3.5 and 6.5 h post inoculation for the AKI cultures) to control for potential growth-dependent effects on expression.

With activation of CpxR, in the *cpxA** background, *vexRAB* expression was significantly increased relative to WT under AKI growth conditions. In contrast, the absence of CpxR, in the Δ*cpxR* background, did not influence *vexRAB* expression under either condition (Figures 9A and 9B). Thus, CpxR does not regulate the basal-level expression of *vexRAB*, but can enhance *vexRAB* expression under conditions where the Cpx system is activated. The expression level of *vexGH* was increased during growth in LB broth and under AKI conditions in the *cpxA** background (Figures 9C and 9D). However, the basal level of *vexGH* expression under AKI conditions appeared to be much lower than was observed in LB broth. In contrast to *vexRAB*, *cpxR* deletion resulted in a significant reduction in *vexGH* expression during growth in LB broth (Figure 9C), but not during growth under AKI conditions (Figure 9D), implying that CpxR positively effects the basal level expression of *vexGH* during growth in LB broth, but not under AKI conditions. Collectively these observations indicate that there are media-dependent differences in the activation of the Cpx system and that the Cpx system is likely inactive during growth under virulence gene inducing conditions. These findings validate the microarray results

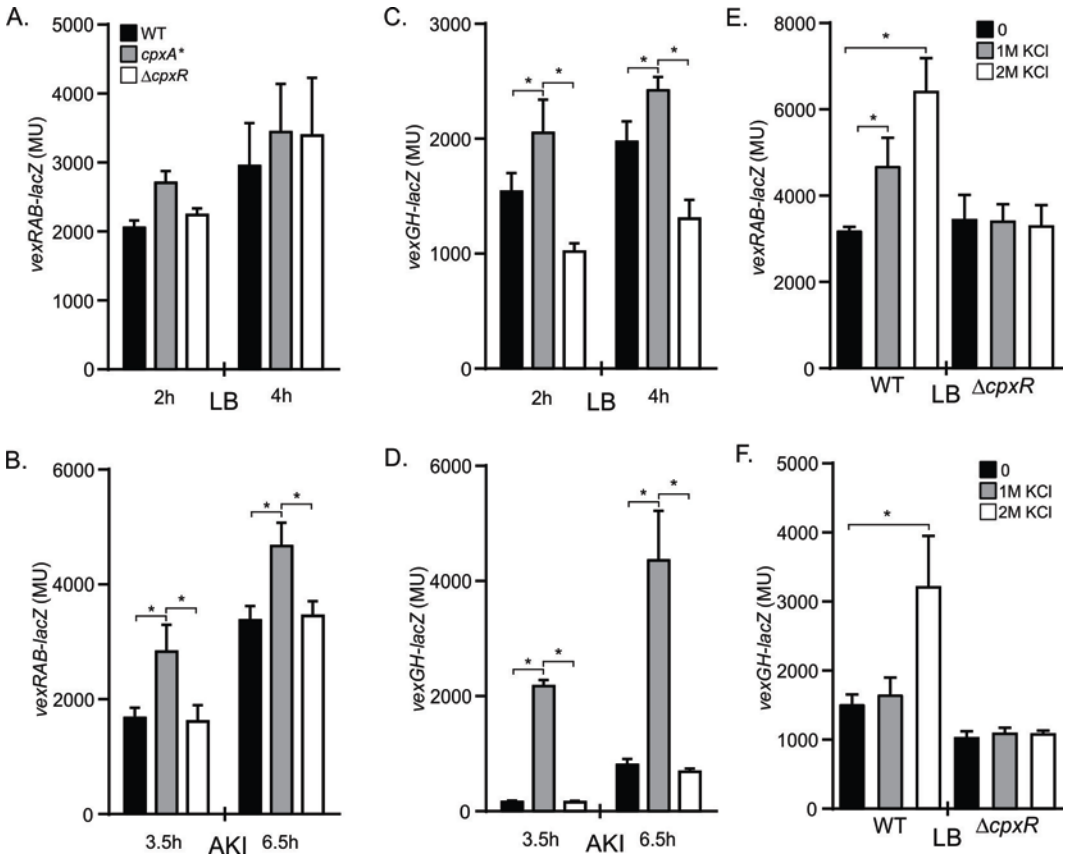

Figure 9. Expression of *vexRAB* and *vexGH* in *V. cholerae cpx* mutants.

The indicated *V. cholerae* strains bearing either a *vexRAB-lacZ* (A, B and E) or *vexGH-lacZ* (C, D and F) reporter were grown in LB broth (A and C), or under AKI conditions (B and D), or in LB broth containing KCl at indicated concentrations (E and F). Culture aliquots were taken at 2 h and 4 h (A and C), 3.5 and 6.5 h (B and D) or at 4 h (E and F) and assayed for β-galactosidase activity. The presented data is the mean +/- SD of three independent experiments. Statistical significance was determined by ANOVA with the Tukey-Kramer multiple comparisons test (A, B, C, & D). Panels E and F were compared to 0 KCl. * = P<0.001. doi:10.1128/IAI.00025-14

and support the conclusion that CpxR is a positive regulator of the *vexRAB* and *vexGH* RND efflux systems.

Since *vexRAB* and *vexGH* appeared to be positively regulated by CpxR, we predicted that their expression should increase with activation of the Cpx system. We therefore quantified *vexRAB* and *vexGH* expression in WT and a Δ*cpxR* mutant following growth in LB broth containing various concentrations of KCl, a known inducer of the *V. cholerae* Cpx system (211). The results showed a KCl concentration-dependent increase in *vexRAB* expression. Growth in 1M KCl resulted in a 1.5-fold increase in *vexRAB-lacZ* expression, while growth in 2M KCl caused a 2-fold increase in *vexRAB-lacZ* expression relative to growth in LB broth without KCl (Figure 9E). This phenotype was found to be dependent on CpxR as deletion of *cpxR* abolished the KCl-dependent induction of *vexRAB* (Figure 9E). The expression of *vexGH* was also induced by KCl as evidenced by a 2-fold increase in *vexGH-lacZ* expression during growth in 2M KCl relative to the LB control (Figure 9F). The induction of *vexGH* by KCl was also dependent on CpxR as deletion of *cpxR* abolished KCl-dependent increase in *vexGH* expression (Figure 9F). Together these results confirm that the data obtained with the *cpxA** mutant reflects activated CpxR and provides additional evidence to support the conclusion that the *vexRAB* and *vexGH* RND efflux systems are positively regulated by the Cpx system in a CpxR dependent manner.

3.2.3 Activation of the Cpx response enhances *V. cholerae* resistance to antimicrobial compounds.

Since both the *vexRAB* and *vexGH* RND efflux systems were upregulated in the *cpxA** mutant (Figure 9), we predicted that the *cpxA** mutant should exhibit enhanced resistance to antimicrobial compounds that are substrates for these two RND efflux systems (e.g. bile salts and

other detergent-like molecules). To test this hypothesis, we calculated the plating efficiency of CpxR activated *V. cholerae* on Thiosulfate-citrate-bile salts-sucrose (TCBS) agar. Growth of *V. cholerae* on TCBS agar is dependent upon the expression of the RND efflux systems which provide resistance to bile salts and other detergent-like compounds that are present in this medium (119, 123, 256, 270, 271). In these experiments we compared the plating efficiency of *cpxA** and Δ*cpxR* strains to WT. We also compared the plating efficiency of WT and the Δ*cpxR* mutant grown in LB broth +/- 2 M KCl. The results showed that there was a 3-fold increase in the recovery of the *cpxA** mutant relative to WT, while the recovery of the Δ*cpxR* mutant was not significantly different from WT (Figure 10). Activation of the Cpx system by growth in 2M KCl resulted in a 3-fold increase in recovery of the KCl grown cells relative to cells grown in LB without KCl. Growth of the Δ*cpxR* mutant in 2M KCl did not have a significant effect on its recovery which confirmed that this phenotype was dependent on *cpxR* (Figure 10). In conjunction with the previous data (Figure 9), these results provide further evidence to support the idea that activation of the Cpx system induces *vexRAB* and *vexGH* expression in a CpxR-dependent manner thereby enhancing antimicrobial resistance.

Next the effect of the Cpx system on *V. cholerae* antimicrobial susceptibility was investigated by examining the resistance of the WT, *cpxA** and Δ*cpxR* strains to antimicrobial agents using disk diffusion assays. The results showed that the *cpxA** mutant exhibited increased resistance to ampicillin, but not to other tested antimicrobial compounds (Table 5). Since VexAB and VexGH are the only two RND efflux systems that contribute to ampicillin resistance, this finding is consistent with the idea that both of these RND efflux systems are upregulated in the *cpxA** mutant. It is not surprising that differences in susceptibility were not observed for the other RND-dependent antimicrobial substrates as the contributions of VexAB and VexGH to

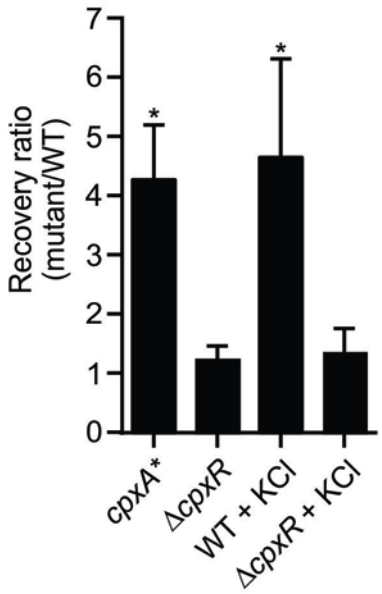

Figure 10. Effect of CpxR activation on the recovery of *V. cholerae* on TCBS agar.

V. cholerae WT, *cpxA** and Δ*cpxR* were cultured in LB broth or LB broth containing 2 M KCl before aliquots were diluted in PBS and plated in duplicate onto LB agar and TCBS agar plates for enumeration. The plates were incubated overnight at 37°C before the resulting colonies were counted. The recovery ratio for each mutant was calculated by the following equation: [the number of mutant colonies on TCBS/the number of mutant colonies on LB] divided by [the number of WT colonies on TCBS/the number of WT colonies on LB]. The results are the mean of three independent experiments +/- SD. Statistical significance relative to WT was determined by ANOVA with the Tukey-Kramer multiple comparisons test. * = $P < 0.001$. doi:10.1128/IAI.00025-14

Table 5. Susceptibility of *V. cholerae* strains to antimicrobial compounds by disk diffusion assays.

Strain	Zone of growth inhibition[a] (SD)							
	$CuCl_2$	SAR	DOC	TET	GENT	PXB	ERY	AMP
WT	25.3 (0.96)	17.7 (0.58)	8.5 (1.29)	26.5 (0.35)	18.8 (0.40)	0 (0)	24.0 (1.63)	10.0 (0.89)
Δ*cpxR*	25.5 (0.71)	15.0 (0)	8.0 (0)	28.3 (0.50)	18.0 (0)	0 (0)	23.5 (1.0)	9.4 (0.84)
*cpxA**	25.3 (0.50)	16.3 (0.50)	8.3 (0.96)	26.0 (0.82)	19.2 (0.75)	0 (0)	24.8 (2.75)	0 (0)[1]

[a]Zone of growth inhibition (measured in mm) to $CuCl_2$, sarcosyl (SAR), deoxycholate (DOC), tetracycline (TET), gentamicin (GENT), polymixin B (PXB), erythromycin (ERY), and ampicillin (AMP). Student t-test was used to determine if differences were significant compared to WT: [1]P<0.0001. doi:10.1128/IAI.00025-14

resistance to these substrates is masked due to redundancy among the six RND efflux systems (119, 123, 256). There was no significant difference in susceptibility to any of the compounds tested between the WT and the Δ*cpxR* mutant strain, suggesting that the tested xenobiotics did not function to activate the expression of the Cpx response. This was verified by plating *V. cholerae* containing a *cpxP-lacZ* reporter on LB agar containing subinhibitory amounts of these xenobiotics; no differences in expression of the reporter in the presence or absence of any of the compounds were noted (data not shown), confirming that the Cpx system was not activated in response to these antimicrobial compounds. Based on these observations, we conclude that the Cpx system is not required for *V. cholerae'*s intrinsic resistance to xenobiotic antimicrobial compounds, but its activation could enhance the pathogen's resistance to antimicrobials through increased expression of *vexRAB* and *vexGH*.

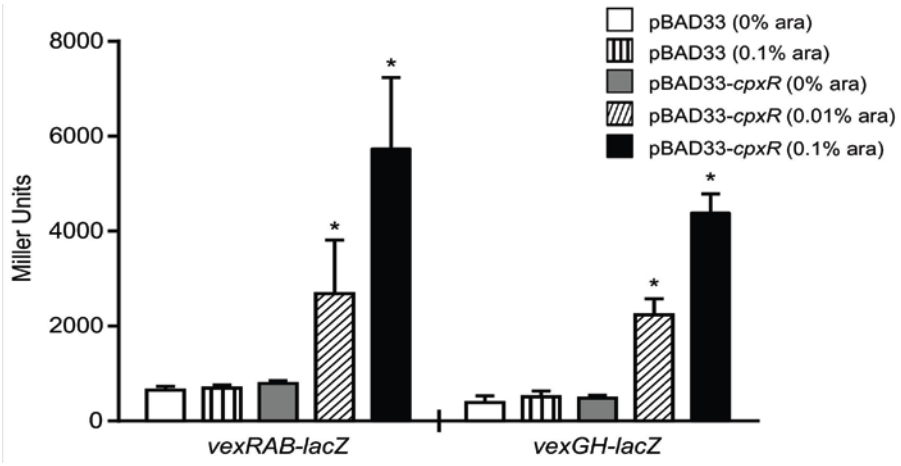

Figure 11. Ectopic expression of *cpxR* activates *vexRAB* and *vexGH* expression in *V. cholerae*.

V. cholerae containing the indicated RND efflux system reporters, and either pBAD33-*cpxR* or pBAD33, was grown in LB broth at 37°C in the presence or absence of arabinose as described in the methods. After 4 h of growth, triplicate aliquots were taken and assayed for β-galactosidase activity. The presented data is the mean +/- SD of three independent experiments. Statistical significance was determined by ANOVA with the Tukey-Kramer multiple comparisons test. * = P < 0.001. doi:10.1128/IAI.00025-14

3.2.4 Ectopic expression of *cpxR* activates *vexRAB* and *vexGH* expression.

The observation that the *vexRAB* and *vexGH* promoters contain CpxR consensus binding sequences (Figure 8), and were upregulated in a *cpxA** background (Figure 9), suggested that CpxR was a positive regulator of *vexRAB* and *vexGH*. To confirm this hypothesis, we expressed *cpxR* from the arabinose inducible promoter in pBAD33 in *V. cholerae* harboring either *vexRAB-lacZ* or *vexGH-lacZ* reporters. This resulted in a dramatic arabinose dose-dependent increase in both *vexRAB* and *vexGH* expression (Figure 11A). In contrast, *cpxR* expression did not affect the

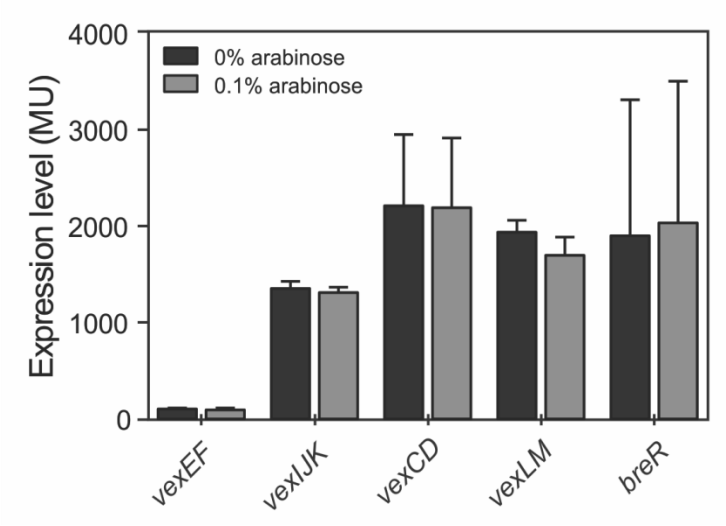

Figure 12. Effect of CpxR on the expression of the indicated RND efflux systems.

V. cholerae containing the indicated RND efflux system reporter plasmids and pBAD33-*cpxR* were grown in LB broth in the presence or absence of arabinose as described in the methods. Following 4 h growth, triplicate aliquots from each culture were collected and assayed for β-galactosidase activity. The results are the mean and SD from three independent experiments. doi:10.1128/IAI.00025-14

expression of any other RND efflux system (Figure 12); confirming the CpxR was specific for *vexRAB* and *vexGH*. These results confirmed our hypothesis that CpxR was a positive regulator of the *vexRAB* and *vexGH* RND efflux systems. Taken together, these observations are consistent with the hypothesis that CpxR functions as an activator at the *vexRAB* and *vexGH* promoters.

3.2.5 Mutation of *vexB* and *vexH* activate the Cpx system.

Our collective findings revealed that the VexAB and VexGH RND efflux systems are components of the Cpx response. This suggested the possibility that active efflux by the RND

efflux systems might function to suppress the Cpx response. To test this hypothesis, we used a chromosomal *cpxP-lacZ* reporter as an indicator of the activation state of the Cpx system; *cpxP* expression is regulated by CpxR (211). We compared *cpxP* expression in WT vs JB485, a strain lacking all six RND efflux systems (123). JB485 produced dark blue colonies on LB-X-gal plates whereas the WT and the JB485Δ*cpxR* mutant yielded white colonies (Figure 13A). Control cultures grown on agar plates containing 500μM $CuCl_2$ (a Cpx inducer) showed induction of *cpxP* in WT, but not the *cpxR* mutants validating that the *cpxP-lacZ* construct is faithfully reporting on the activation state of the Cpx system in each strain. Thus, the absence of RND efflux activity induces the Cpx system, a finding consistent with the idea that the RND efflux systems normally suppress the activity of the Cpx system in *V. cholerae*.

Previous studies have shown that four of the six RND systems (*vexB*, *vexD*, *vexH* and *vexK*) contribute to in vitro resistance of *V. cholerae* to antimicrobial compounds with both distinct and redundant roles (119, 123, 256). We therefore sought to determine which of the RND efflux systems contributes to Cpx suppression by examining *cpxP* expression in a panel of strains that included both single and multiple RND efflux mutants. For these experiments, the *cpxP-lacZ* reporter was introduced into the chromosome of each of the RND mutants. Expression of *cpxP* was then examined on LB-X-gal plates and LB-X-gal plates containing $CuCl_2$ (as a positive control). LB agar was used to screen for *cpxP* expression because previous studies have shown that the Cpx system was poorly expressed in LB broth (211). Among the four single RND efflux mutants (*vexB*, *vexD*, *vexH* and *vexK*) only the *vexB* mutant produced light blue colonies on LB-X-gal (Figure 13A); the *vexH* mutant produced colonies that appeared white to the naked eye but were discernable as faint blue in color under magnification, while the *vexD* and *vexK* mutant colonies were white in color. These observations suggest that *cpxP* expression is

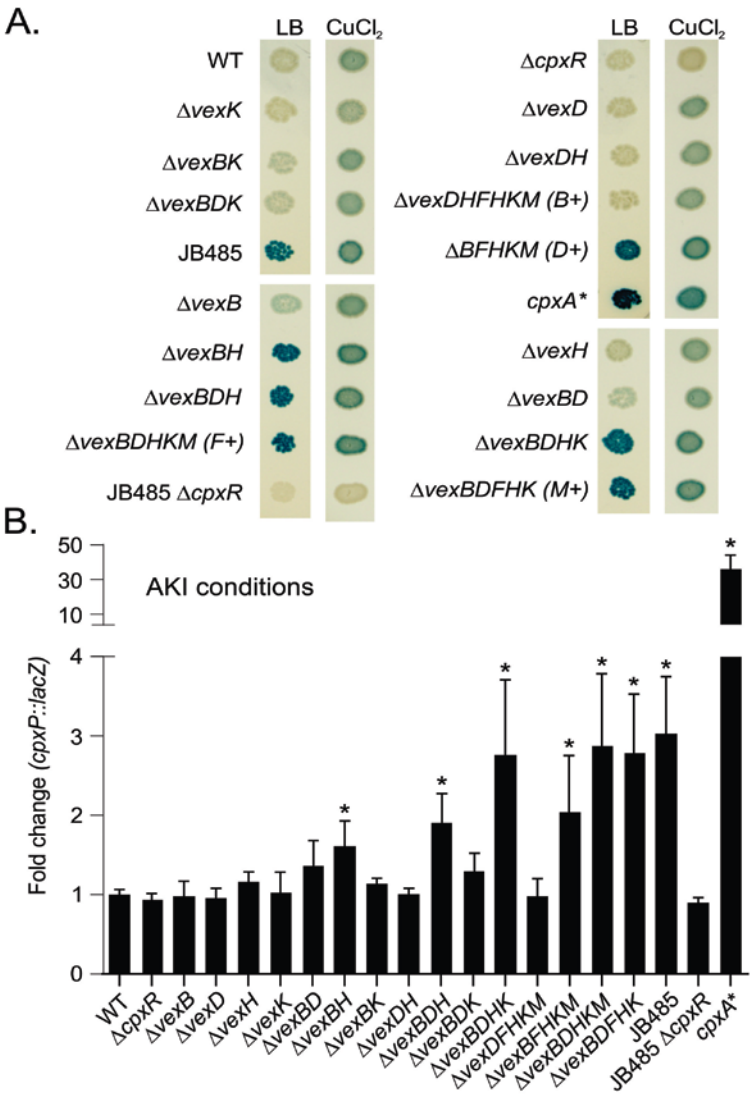

Figure 13. Induction of the Cpx system in *V. cholerae* RND efflux mutants.

Expression of a chromosomal *cpxP-lacZ* reporter in the indicated *V. cholerae* strains. (A) The indicated strains were inoculated onto the surface of LB-X-gal plates +/- 500 μM $CuCl_2$ before the plates were incubated overnight at 37°C and photographed. (B) The same *V. cholerae cpxP-lacZ* fusion strains were cultured under AKI conditions for 5 h when culture aliquots were collected and their β-galactosidase activity assayed. The fold change was calculated as the β-galactosidase activity (measured in Miller Units) in the mutant divided by the WT. The bars represent the mean +/- SD of three independent experiments. Statistical relevance as compared to WT was calculated using a one-way ANOVA with Dunnet's post-hoc test. * = P < 0.001. doi:10.1128/IAI.00025-14

activated in the absence of either *vexB* or *vexH,* (albeit to a very low level in the absence of *vexH*), but that the absence of either *vexD* or *vexK* does not induce *cpxP* expression. Furthermore, a *vexBDK* triple mutant produced colonies that were similar in color to the *vexB* single mutant, supporting the idea that *vexD* and *vexK* do not influence *cpxP* expression under these conditions. When the *vexB* and *vexH* deletions were combined, the resulting strain produced dark blue colonies similar in color to JB485 (Figure 13A), consistent with the hypothesis that these two RND efflux systems are functionally redundant for this phenotype. We hypothesized that if Cpx activation was a result of efflux activity provided by *vexGH* and/or *vexRAB,* then a strain lacking all five RND efflux systems except *vexRAB* should suppress expression of the Cpx system relative to JB485. Indeed, this was what we observed with the Δ*vexDFHKM(vexB+)* strain which produced faint blue colored colonies that were similar in color to the Δ*vexH* mutant (Figure 13A). We conclude that efflux activity provided by the VexAB and VexGH RND efflux systems maintains the Cpx system in a suppressed state during growth of *V. cholerae* on LB agar.

We also examined whether the RND efflux systems influence the expression of the Cpx system during growth under virulence inducing conditions. For these experiments, each RND mutant was grown under AKI conditions before assaying for *cpxP* expression. There was no difference in *cpxP* expression among any of the single deletion mutants (Figure 13B). However, when a *vexB* mutant was combined with a *vexH* deletion, we observed an approximately 2-fold increase in *cpxP* expression. The *vexBK* and *vexDH* mutants did not appear to affect *cpxP* expression. This suggested that during growth under AKI conditions, *vexB* and *vexH* function in a redundant manner to limit activation of the Cpx system. There was an increase in *cpxP*

expression in the *vexBDHK* mutant relative to the *vexBDH* mutant, suggesting that the VexIJK RND efflux system contributed to the suppression of the Cpx system. The expression of *cpxP* in the *vexBDHKM(vexF+),* and *vexBDFHK(vexM+)* mutants was similar to the *vexBDHK* mutant. Expression of *cpxP* was similar to WT in the *vexDFHKM(vexB+)* mutant, confirming that the VexAB RND efflux system is sufficient to complement for the absence of the other five RND efflux systems. The increase in *cpxP* expression in JB485 relative to the *vexBFHKM(vexD+)* mutant suggests that the VexCD RND efflux system contributes to the suppression of the Cpx system during growth under AKI conditions. These findings are reminiscent of our previous observations regarding CT and TCP production in RND efflux deficient *V. cholerae*: where all six RND systems were required for high-level CT and TCP production (256). The concordance of these observations raises the intriguing possibility that the factor responsible for activation of the Cpx system during AKI growth may also contribute, by a Cpx-independent mechanism, to the virulence attenuation observed in JB485 (123).

3.2.6 CpxR contributes to *vexRAB* and *vexGH* expression in RND efflux negative *V. cholerae*.

The above data suggested that the Cpx system was activated by loss of the RND efflux systems (Figure 13). Based on this, and the finding that CpxR activated *vexRAB* and *vexGH* expression (Figures 9 and 11), we hypothesized that the expression of *vexRAB* and *vexGH* in JB485 would be upregulated in a CpxR-dependent manner. To test this we quantified *vexRAB* and *vexGH* expression in WT, JB485, and JB485 Δ*cpxR* during growth under AKI conditions. AKI conditions were selected as they showed the most dramatic effect on the expression of *vexRAB* and *vexGH* (Figure 9). The results showed a significant increase in the expression of both

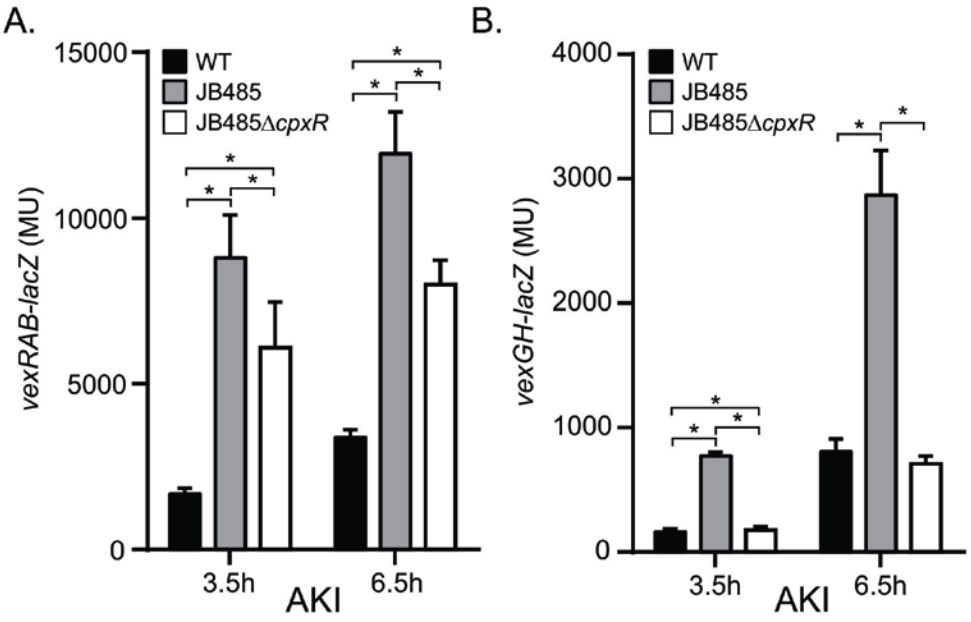

Figure 14. CpxR activates *vexRAB* and *vexGH* expression in the absence of RND efflux activity.

The indicated *V. cholerae* strains containing a *vexRAB* (A) or *vexGH* (B) reporter plasmid were grown under AKI growth conditions. Aliquots were taken at 3.5 and 6.5 hours and assayed for β-galactosidase activity. The presented data is the mean +/- SD of three independent experiments. Statistical significance was determined by ANOVA with the Tukey-Kramer multiple comparisons test. *=P<0.001. doi:10.1128/IAI.00025-14

vexRAB and *vexGH* in strain JB485 relative to WT at 3.5 and 6.5 hours (Figures 14A and 14B). The level of *vexRAB* expression in JB485 Δ*cpxR* was reduced relative to JB485, but was still greater than in WT (Figure 14A). This suggests that CpxR contributes to the induction of *vexRAB* in JB485, but that additional factors are also contributing to *vexRAB* upregulation. In contrast to *vexRAB*, the expression level of *vexGH* returned to WT levels in the JB485 Δ*cpxR* mutant, indicating that CpxR was responsible for the upregulation of *vexGH* in JB485 (Figure 14B). Together these results provide additional evidence supporting the conclusion that the loss of RND efflux activity results in CpxR activation and that CpxR functions as a positive regulator of *vexRAB* and *vexGH*.

3.2.7 The Cpx system does not affect CT or TCP production.

The RND efflux systems were shown to contribute to the production of both CT and TCP in *V. cholerae* (123). While still unknown, the mechanism by which the RND systems repressed CT and TCP production was linked to repression of the ToxR regulon (123). The upregulation of the Cpx system in the RND deficient mutant suggested the possibility that CpxR could function to repress CT TCP production was linked to repression of the ToxR regulon (123). The upregulation of the Cpx system in the RND deficient mutant suggested the possibility that CpxR could function to repress CT and TCP production. We investigated this hypothesis by quantifying CT and TCP production in WT, *cpxA**, Δ*cpxR*, JB485, and JB485Δ*cpxR* strains. If *cpxR* was responsible for attenuated CT and TCP production, then its deletion in the RND negative background should result in increased CT and TCP production. Likewise, CT and TCP production should be decreased in WT by the *cpxA** mutation due to constitutive activation of

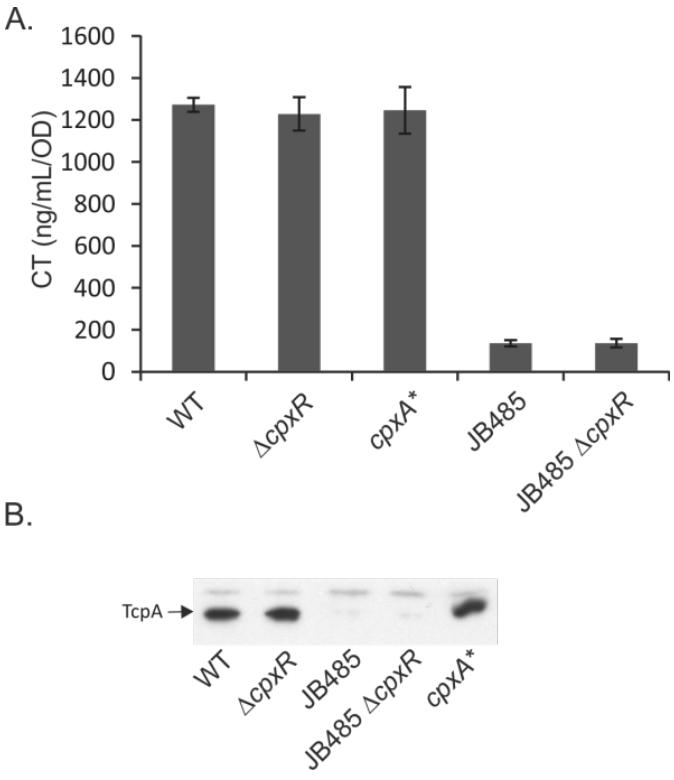

Figure 15. Quantification of CT and TcpA production in *V. cholerae* strains.

The indicated strains were grown under AKI growth conditions. Culture aliquots were collected following overnight growth and assayed for (A) CT production using a GM_1 ELISA and (B) TcpA production by Western blot using polyclonal antibody that was generated against TcpA. doi:10.1128/IAI.00025-14

the Cpx system. Our results showed that there was no significant difference in CT or TCP production between WT, *cpxA**, and Δ*cpxR* or between JB485 and JB485Δ*cpxR* (Figure 15). This indicated that the defect in virulence factor production in JB485 was not a result of the activation of the Cpx system. These results also suggested that the Cpx system does not function to regulate virulence factor production in *V. cholerae*; a result that is consistent with previous findings showing that the Cpx system was dispensable for *V. cholerae* colonization of the infant mouse small intestine (211).

3.3 DISCUSSION

Bacteria have evolved overlapping mechanisms to sense and respond to stress that can result from exposure to toxic molecules, adhesion to abiotic surfaces, or from misfolded proteins. The Cpx two-component system represents one such system that in *E. coli* mitigates envelope stress resulting from protein misfolding (258). *V. cholerae* also encodes a Cpx two-component system that shares a conserved genetic organization with the *E. coli* Cpx system (211). However, there are differences in the amino acid sequences of the sensor domains of the *V. cholerae* and *E. coli* CpxA and CpxP proteins and the *V. cholerae* Cpx system does not respond to stimuli that activate the *E. coli* Cpx system (211). Thus, the Cpx systems may play different roles in the physiology of these two related Gammaproteobacteria.

Our characterization of the *V. cholerae* CpxR transcriptome supports the idea that there are differences between the *V. cholerae* and *E. coli* Cpx regulons. One key difference we found is that genes involved in protein fate (e.g. *degP* and *dsbA)* were either not detected as being regulated or were found to be repressed by CpxR in *V. cholerae* (Table 4). This finding is similar

to *Haemophilus ducreyi*, where *degP* was also noted to be absent from the Cpx regulon (272). Together these observations suggest that the physiological role for Cpx systems may not be universally conserved among Gram-negative bacteria. Although *degP* and *dsbA* appear to be regulated independently of CpxR in *V. cholerae*, the activation of the Cpx system in *V. cholerae dsbC* and *dsbD* mutants (211), which likely results in misfolded cell envelope proteins, suggests that the Cpx system can respond to misfolded proteins. The mechanism by which this occurs remains to be determined, but could involve alternate sigma factor sigma (E) (*rpoE*) which has been shown to functionally overlap with the Cpx system in responding to extracytoplasmic stress in *E. coli* (219, 273).

The list of *V. cholerae* CpxR regulated genes included a number of genes that mediate uptake and efflux of low molecular weight antimicrobial compounds. This included the *ompT* porin (which was repressed) and the *vexRAB, vexGH, tolC* genes (which were upregulated). The latter loci encode for the production of two broad-spectrum RND efflux systems that contribute to *V. cholerae* resistance to multiple antimicrobial compounds and pathogenesis (119, 123, 176). Several lines of evidence suggest that the *vexRAB* and *vexGH* operons are regulated by CpxR: the promoters of both operons contain CpxR consensus binding sequences (Figure 8); both operons were upregulated in the *cpxA** mutant (Figure 9); ectopic *cpxR* expression activated their expression in *V. cholerae* (Figure 11); and KCl (a Cpx system activator) induced their expression in WT, but not in a Δ*cpxR* mutant (Figure 9). This conclusion is buttressed by the observation that the *cpxA** mutant exhibited increased resistance to ampicillin (an antibiotic substrate of the VexAB and VexGH RND systems) and exhibited a growth advantage on TCBS agar (Table 5 and Figure 10), and that induction of the Cpx system with 2M KCl provided a growth advantage for WT on TCBS, but not for a Δ*cpxR* mutant (Figure 10). While our data

strongly support the conclusion that CpxR is an activator of these two RND efflux systems, CpxR was not required for *V. cholerae* intrinsic resistance to antimicrobial compounds (Figure 10 and Table 5). This suggests that the Cpx system could contribute to xenobiotic resistance under Cpx-inducing conditions by further increasing the expression of *vexRAB* and *vexGH*; however, the Cpx system is not required for *V. cholerae* intrinsic resistance to xenobiotics.

Besides finding that the *V. cholerae* Cpx response promotes expression of RND efflux systems, we also found that inactivation of RND efflux stimulates the Cpx response. Mutation of *vexB* and/or *vexH* resulted in activation of the *V. cholerae* Cpx system. It is interesting to note that this phenotype was also observed with deletion of RND efflux systems in *H. ducreyi* and *Klebsiella pneumoniae* (274, 275) which suggests that the genetic linkage between the Cpx system and the efflux systems is not unique to *V. cholerae.* The mechanism(s) by which the RND efflux systems modulate the activity of the Cpx system are not known. However, since RND efflux systems function in small molecule export, we speculate that this phenotype is the result of the intracellular accumulation of an endogenously produced small molecule in the RND efflux mutants. Consistent with this hypothesis, recent studies suggest that a natural function of RND efflux systems may be to remove metabolic waste from within the cell (198). In *E. coli*, there is evidence to suggest that in the absence of RND-mediated efflux, metabolites accumulate in the cell and activate the expression of the MAR, Bae and Cpx stress response systems (178, 197-200, 276). These regulatory systems then activate the expression of the *acrAB*, *acrD* and *mdtABCD* RND efflux systems and other stress mitigating genes. Although *V. cholerae* appears to lack the MAR and Bae systems, we propose that a similar mechanism occurs in *V. cholerae.* We speculate that an as yet unidentified cellular metabolite accumulates in the absence of the RND efflux and activates the Cpx system. Our observation that different RND efflux pumps

were required to suppress the Cpx system during growth on LB agar versus growth under AKI conditions suggests that the Cpx system may respond to the accumulation of multiple different endogenous molecules. Determining the identity of such effector molecules is a key challenge for future studies.

We previously proposed that a small molecule accumulated in *V. cholerae* in the absence of RND-mediated efflux and repressed CT and TCP production during growth under AKI conditions (256). This proposal was supported by the fact that *V. cholerae* virulence factor production was dependent upon all six RND efflux systems and there was functional redundancy among the RND systems for this phenotype (114, 123, 256). The similarity of these results to the Cpx data, along with the inclusion of the ToxR-regulated *ompT* in the list of CpxR-responsive genes, suggest that attenuation of CT and TCP production in the RND deficient strain may be due to the activation of the Cpx system. Although the Cpx system has been linked to virulence in other pathogens (222, 277-281), our data indicate that the Cpx system does not affect *V. cholerae* virulence factor production. This was evidence by the fact that *cpxR* deletion, constitutive activation of the Cpx system (i.e. *cpxA**), or chemical activation of the Cpx system (i.e. with $CuCl_2$), did not affect CT or TCP production. This conclusion is further supported by a previous study which showed that the Cpx system was dispensable for *V. cholerae* intestinal colonization (211).

While our findings strongly suggest that the Cpx system does not exert a significant influence over the expression of the ToxR regulated virulence factors CT and TCP, we cannot exclude the possibility that the Cpx system plays a role in the regulation of other genes in vivo. Late in infection, *V. cholerae* is thought to encounter growth conditions conducive to the induction of the Cpx system including high cell density, nutrient limitation, and the likely

accumulation of metabolic waste/byproducts (41, 201). The finding that both the *vexAB* and *vexGH* RND efflux systems are upregulated late during infection in humans and animals (41, 201, 202, 242) are consistent with the hypothesis that Cpx system is also induced late in infection. Thus, it is tempting to speculate that the Cpx system may contribute to late gene expression during infection. Late induced genes contribute to important phenotypes. This includes genes that contribute to *V. cholerae* survival in the environment and genes that contribute to the hyper-infectious phenotype associated with human shed vibrios and thought to be a key factor responsible for the epidemic spread of cholera (41, 282).

4.0 VEXR IS A POSITIVE REGULATOR OF THE VEXAB RESISTANCE-NODULATION-DIVISION EFFLUX SYSTEM AND MEDIATES RELIEF FROM METABOLIC STRESS.

4.1 INTRODUCTION

Vibrio cholerae is a noninvasive Gram negative bacterial pathogen that causes the disease cholera. Cholera is a severe acute diarrheal disease that affects an estimated 3-5 million people per year (1). Untreated cholera can rapidly lead to dehydration, hypotensive shock, and death. Cholera is contracted by ingesting *V. cholerae* contaminated food or water (1). Following ingestion, *V. cholerae* colonizes the small intestine via a process that is dependent upon the induction of genes which are required for intestinal colonization and disease development. These in vivo expressed genes contribute to *V. cholerae* pathogenesis in diverse ways and range from traditional virulence factors (i.e. toxins and adhesins) to genes that facilitate *V. cholerae* survival in the gastrointestinal (GI) tract. *V. cholerae* persistence in the intestine is dependent upon its ability to overcome antibacterial barriers that are intrinsic to the GI tract, including the presence of high concentrations of toxic small molecules (such as bile acids and other detergent-like molecules), antimicrobial products generated by resident flora, and products of the innate immune system. In response to these toxic elements, *V. cholerae* activates genes which function to protect the cell by modulating its outer membrane (OM) permeability barrier and activating efflux transporters (123, 211, 283, 284). For example, in response to bile acids *V. cholerae* alters the porin composition of the OM to effectively reduce the rate of bile salt diffusion, and presumably the diffusion of other soluble toxic molecules, across the OM (284). In conjunction with reduced OM permeability, *V. cholerae* expresses RND-family transport systems that function to efflux bile salts, and multiple other antimicrobial compounds, from within the cell envelope to the external environment (123, 211). Together, the activated RND efflux systems and reduced OM permeability function synergistically to provide *V. cholerae* with high-level

resistance to lethal antimicrobial compounds present in the host. The importance of these responses in the pathobiology of this organism is highlighted by the fact that *V. cholerae* exhibits a greatly diminished ability to colonize the intestinal tract in the absence of these adaptive responses (123).

The RND efflux systems have been a focal point in bacterial antimicrobial resistance research due to the ability of individual RND systems to provide resistance to a broad range of chemically unrelated substrates that include antibiotics, detergents, dyes, and antimicrobial peptides. The RND efflux systems are found in most gram negative bacteria and function as proton-substrate antiporters (226, 238, 239, 265). Individual RND efflux systems are composed of three components: an outer membrane pore protein that is homologous to *Escherichia coli* TolC, an integral cytoplasmic membrane pump protein belonging to the RND superfamily, and a periplasmic membrane fusion protein that links the outer membrane pore protein to the RND pump protein (127, 131, 132, 137). Together these three components form a transport apparatus that spans the cell envelope and functions to efflux substrates from within the cell envelope into the external environment. Although the RND transport apparatus is responsible for the efflux of antimicrobials, phylogenetic analysis suggests that the RND efflux systems evolved independent of antibiotic selection (167, 168). Indeed, there is mounting evidence that the RND efflux systems are involved in diverse functions (reviewed in (169)) such as biofilm formation, iron acquisition, plant-bacteria interactions, lipid transport, bacterial virulence, divalent cation resistance, and the removal of metabolic byproducts from within the cell.

The *V. cholerae* genome encodes six RND efflux systems (123). Inhibition of the RND efflux systems renders *V. cholerae* hypersensitive to multiple antimicrobial compounds and attenuates the expression of virulence factors including cholera toxin (CT) and the toxin co-

regulated pilus (TCP) (119, 123, 176, 267, 285). Among the six RND efflux systems, the VexAB system was shown to be the primary system that contributes to intrinsic antimicrobial resistance in vitro (119, 123, 267). There have been several studies that suggest that the VexAB RND efflux system is important to *V. cholerae* pathogenesis. This includes finding that *vexAB* was induced in vivo in humans and animals, that *vexAB* expression was enhanced by the Cpx system, and that VexAB was required for high-level virulence factor production (35-37, 50). While there is ample evidence to suggest that VexAB is important in pathogenesis, the regulatory mechanisms controlling its expression are unknown.

In many bacteria regulation of the RND efflux systems is mediated by a linked TetR family regulator (208). The TetR family is a large family of regulatory proteins that function in diverse phenotypes including antibiotic resistance, metabolism, stress responses, and pathogenicity (206). TetR proteins contain two functional domains: a conserved N-terminal DNA-binding domain and a variant C-terminal ligand binding domain (206). The ligand binding domain is capable of binding to effector molecules that modulate the interaction of the DNA binding domain with its target sequences. In the case of RND efflux systems, the activity of their TetR regulators is often modulated by the binding of efflux substrates of the linked RND system. In many cases, TetR proteins appear to be capable of binding a diverse set of ligands that correspond to the multiple substrates of linked RND efflux systems (206). The vast majority of reported TetR family regulators function as repressors that bind the promoter region and repress transcription in the absence of bound ligands. In addition to regulating their specific target genes, many TetR regulators are also capable of regulating their own expression (206, 208).

In this work we tested the hypothesis that a TetR family protein regulated the expression of the *vexAB* efflux system. The *vexAB* locus encoded a linked gene, named *vexR*, which had

homology to TetR-family regulators. The uncharacterized *vexR* gene was the first gene in a three gene operon that included *vexA* and *vexB*; a genetic organization that was distinct from most RND efflux systems (206, 207). Our results confirmed that VexR functioned in the regulation of the *vexRAB* operon, but in a manner that was opposite to most TetR regulators associated with other RND efflux systems. Our results showed that VexR functioned as an activator of the *vexRAB* operon, whereas most RND-linked TetR regulators function as repressors. We further found that the *vexRAB* operon was upregulated, in a *vexR*-dependent manner, in response to the efflux status of the cell. This suggested that endogenous metabolic compounds may be native substrates for VexAB and can serve as activators of the *vexRAB* efflux operon. Consistent with this hypothesis, *vexRAB* was upregulated in several metabolic mutants, including tryptophan biosynthetic mutants. Further the indole, an intermediate in tryptophan biosynthesis, was found to induce *vexRAB* expression. Taken together our results suggested that VexR functions as a positive regulator of the *vexRAB* operon. We further posit that a native role of the VexAB RND efflux system is to remove excess cellular metabolites from within the cell that would otherwise accumulate to toxic levels.

Table 6. Strains, plasmids and oligonucleotides.

Strain:	Genotype:	Source:
Vibrio cholerae		
JB804	01 El Tor strain C6706, Sm^R	(231)
JB3	01 El Tor strain N16961, Sm^R	(119)
JB58	01 El Tor strain N16961 Δ*lacZ*, Sm^R	(119)
JB114	JB58 Δ*vexM*	(123)
JB116	JB58 Δ*vexH*	(123)
JB432	JB58 Δ*vexF*	(123)
JB464	JB58 Δ*vexD* Δ*vexF* Δ*vexH* Δ*vexK* Δ*vexM*	(123)
JB485	JB58 Δ*vexB* Δ*vexD* Δ*vexF* Δ*vexH* Δ*vexK* Δ*vexM*	(123)
JB495	JB58 Δ*vexB*	(119)
JB528	JB58 Δ*vexK*	(123)
JB692	JB58 Δ*vexD*	(119)
JB694	JB58 Δ*vexB* Δ*vexD*	(119)
JB718	JB58 Δ*vexR* Δ*vexD*	This study
XBV218	JB58 Δ*vexR*	This study
XBV220	JB58 Δ*vexR* Δ*vexB* Δ*vexD* Δ*vexF* Δ*vexH* Δ*vexK* Δ*vexM*	This study
Escherichia coli		
EC100D*pir+*	*F⁻ mcrA* Δ*(mrr-hsdRMS-mcrBC) Φ80dlacZ*Δ*M15* Δ*lacX74 recA1 endA1 araD139* Δ*(ara, leu)7697 galU galK λ⁻ rpsL (*Sm^R*) nupG pir+*	Epicentre
SM10λ*pir*	*thi-1 thr leu tonA lacY supE recA::*RP4-2-4-Tc::Mu Km^R (λ *pir*R6K)	(240)
ER2566	*F- glnV44(AS) galK2(Oc) rpsL704(strR) xylA5 mtl-1 argE3(Oc) thiE1 tfr-3 λ DE3 = λ sBamHIo* Δ*EcoRI-B int::(lacI::PlacUV5::T7 gene1) i21* Δ*nin5*	New England BioLabs
Plasmids:	**Description:**	
pBAD18	Expression plasmid, Cb^R, pBR322 origin of replication	(264)
pCM10	Vector for construction of *lux*CDABE transcriptional fusions, Km^R, ori101	(286)
pDT1076	pCM10 containing the *vexR* promoter region from N16961	This study
pDT1146	pMMB66EH::*vexR*	This study
pDT1777	pDT1076 with Cm-mark cassette inserted into the vector, Cm^R	This study
pJB703	pBAD18*::vexR*	This study
pMAL-c2	Expression plasmid for fusion of proteins to MBP and cytoplasmic expression, Cb^R, pBR322 origin of replication	New England BioLabs
pMMB66EH	Expression plasmid, Cb^R, oriV/T	(287)
pSC137	Vector for transposon mutagenesis of bacteria, Cm^R,oriR6K	
pSS35	pMAL-c2::*vexR*	This study
pTL61T	Vector for construction of *lacZ* transcriptional fusions, Cb^R, oriRK2	(9)
pWM91	Suicide plasmid vector used for allelic exchange, Cb^R, oriR6K/fl	(282)
pWM91:: Δ*vexR*	pWM91*::*Δ*vexR*	This study
pXB233	pTL61T containing the *vexR* promoter region from N16961	This study

Table 7. (continued)

Strain:	Genotype:	Source:
Oligonucleotides:	**DNA sequence (5' – 3'):**	
166b-F-XhoI	AACTCGAGGCAGAGAAATGTGATGT	
166b-R-XbaI	AATCTAGAGCCAAACAGCAGGATCG	
166c-F-XhoI	TTCTCGAGGGGTCCGGAGACGTACT	
166c-R-XbaI	CGTCTAGAGGAGCTGTTTATCGCCG	
Biotin	GCGGGAGTCGGCAGCG	
MCS4.VexA.R	CCGGATCCCATTCTGGTGCGAACTCCAAATTAGTGTTG	
VC0166-SacI-F	CTGAGCTCAAGGGTTCATATGCA	
VC0166-XbaI-R	TTTCTAGATTAGTGTTGAGTAATTGCA	
VC0166-F1	AAAGAGCTCATTTGCAGAGAAATGTG	
VC0166-F2	TTATCGCGGCGGGATGCAATTACTCA	
VC0166-R1	AATCTAGACACTTTTTCATTCTGGTG	
VC0166-R2	ATTGCATCCCGCCGCGATAAGGATTT	
VC0166-F-pMAL-SmaI	GGCCCGGGTTGCAGAGAAATGTGATGTCTGAAATAGTG	
VC0166-R-pMal-EcoR1	GGAATTCTTAGTGTTGAGTAATTGCATCC	
vexR-F1	GCGGGAGTCGGCAGCGATAATAATCCGCTCACCGAG	
vexR-R1	GCGGGAGTCGGCAGCGCCCCTGTTTTGCAATACACTTG	
vexR-F2	GCGGGAGTCGGCAGCGTGCAAAACAGGGGGTATTAG	
vexR-R2	GCGGGAGTCGGCAGCGGCCGTACACTATTTCAGACA	
XWL-BRL-F	CGCAGGGTTTTCCCAGTCACGAC	

4.2 RESULTS

4.2.1 Genetic organization of the *vexRAB* operon.

In many Gram negative bacteria the RND efflux systems encode a linked TetR family regulatory protein that functions as a repressor of the linked RND efflux system. The *V. cholerae* genome encodes six RND efflux systems; among these six, only the VexAB RND system contained a linked gene *vexR* (VC0166) which encoded a putative TetR-family regulator. The *vexR* gene was

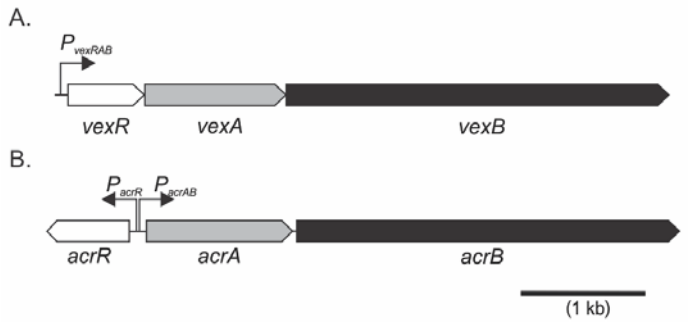

Figure 16. Genetic organization of RND efflux operons.

(A) Schematic of the *V. lerae vexRAB* operon. (B) Schematic of the *E. coli acrR-acrAB* locus. Genes encoding a TetR-family (white), membrane fusion family (grey), and RND-family (black) proteins are shown. Putative promoters for each respective operon are indicated by the thin black arrows.

present as the first gene in the *vexRAB* operon (Figure 16A). Relative to most other RND efflux systems, the genetic arrangement of *vexR* within the *vexRAB* operon is unusual. In most RND efflux systems that encode a linked TetR regulator, the regulatory gene is expressed from a divergently expressed promoter that is upstream of and overlaps with the RND efflux system promoter. This arrangement is demonstrated by the archetypical *acrAB* RND efflux system in *E. coli* where *acrR* is encoded upstream and divergently from the *acrAB* efflux system (Figure 16B). The AcrR protein functions to regulate the expression of the *acrAB* RND system in response to its antimicrobial substrates while also regulating its own expression (288).

4.2.2 Expression of *vexRAB* is induced in response to VexAB efflux substrates.

The role of the RND efflux pumps is to extrude their substrates out of the cell. As such, their expression is typically regulated by a feedback loop in response to the presence of their efflux

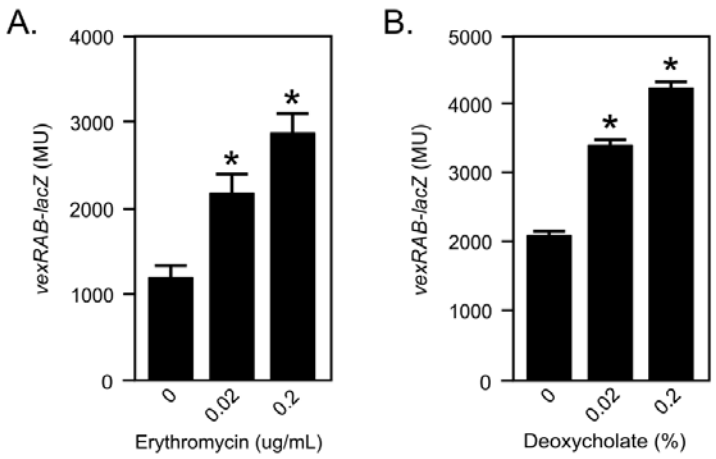

Figure 17. Effect of VexAB efflux substrates on *vexRAB* expression.

V. cholerae strain N16961 containing pXB233 (*vexRAB-lacZ)* was grown under AKI conditions for 5 h with the indicated concentrations of (A) erythromycin or (B) deoxycholate. Data is the mean ± SD of three independent experiments. Statistical significance was determined relative to the media control by one-way ANOVA with Dunnet's post-hoc test. *=P<0.05.

substrates. Since bile salts were shown to be major substrates of VexAB (119, 123), we hypothesized that *vexRAB* expression may be regulated in response to bile salts. To test this hypothesis we cultured *V. cholerae* under virulence gene inducing conditions in AKI broth containing sub-lethal concentrations of deoxycholate and measured *vexRAB* expression using a *vexRAB-lacZ* reporter. We also included erythromycin, an antibiotic that is a substrate of VexAB. The results of these experiments showed a concentration-dependent induction of *vexRAB* expression in response to both erythromycin (Figure 17A) and deoxycholate (Figure 17B). Deoxycholate at 0.2% resulted in a 2-fold increase in *vexRAB* expression while the presence of erythromycin at 0.2 μg/mL resulted in a nearly 3-fold increase in *vexRAB*

expression. Based on these results we concluded that the *vexRAB* operon was regulated in response to its efflux substrates.

4.2.3 VexR is required for expression of the *vexRAB* operon.

The finding that *vexRAB* expression was induced in response to VexAB efflux substrates indicated the possibility that the *vexRAB* system was regulated in a fashion that was similar to other RND efflux systems. Given that most TetR-family regulators function as repressors in the absence of their efflux substrates, we hypothesized that VexR would function to repress *vexRAB* expression. If this was true, then deletion of *vexR* should result in increased *vexRAB* expression. We therefore compared *vexRAB-lacZ* expression in WT and an isogenic Δ*vexR* mutant strain. The strains were grown in LB broth to an OD_{600} of 0.8 when *vexRAB-lacZ* expression was quantified. The results showed that *vexRAB* expression was significantly decreased in the Δ*vexR* strain relative to WT (Figure 18A). This finding was opposite to what was expected if VexR functioned as a repressor and suggested that VexR is a positive regulator of the *vexRAB* operon.

Since *vexRAB* expression was induced in response to VexAB substrates, we examined whether the substrate-dependent induction of *vexRAB* was contingent on the presence of *vexR*. We chose to focus on deoxycholate as an inducer because it is a major substrate of VexAB and the most biologically relevant compound to *V. cholerae* pathogenesis. Growth of WT in the presence of 0.2% deoxycholate resulted in a ~5.5-fold increase in *vexRAB-lacZ* expression (Figure 18A); which was greater than the 2-fold increase that was observed under AKI growth conditions (Figure 17A). Deletion of *vexR* largely abolished the deoxycholate-dependent induction of *vexRAB* expression. This result further supported the conclusion that *vexR* is required for *vexRAB* expression. While VexR appears to be required for robust *vexRAB* expression, we observed that

the presence of deoxycholate still induced *vexRAB* expression by ~2-fold in the Δ*vexR* strain relative to growth in LB broth, although it was still greatly reduced compared to WT grown in LB broth (Figure 18A). This suggests that there may be other factors involved in regulating *vexRAB* expression. Taken together these findings strongly suggested that VexR functioned as a positive regulator of the *vexRAB* operon.

The above results were surprising since most TetR family regulators function as repressors. We therefore sought to further confirm that VexR was an activator of the *vexRAB* operon. If VexR was an activator, we hypothesized that recombinant *vexR* expression should activate expression of the *vexRAB* promoter in *E. coli*. We therefore co-transformed pDT1076 (*vexRAB-lux*) into *E. coli* with pBAD18 (empty vector control) or pBAD18::*vexR*. The resulting *E. coli* strains were then grown in LB broth plus or minus 0.2% arabinose (to induce *vexR* expression from the arabinose inducible promoter in pBAD18) and luminescence production was measured at zero, one and two hours. The results showed that the empty vector control (i.e. pBAD18) did not affect *vexRAB* expression (Figure 18B). This indicated that neither pBAD18, nor arabinose, affected the expression of the *vexRAB-lux* reporter. In contrast, there was a marked increase in luminescence production in the strain containing pBAD18::*vexR*. The presence of pBAD18::*vexR*, even in the absence of arabinose, was sufficient to induce *vexRAB* expression as evidenced by the increase in luminescence production at one and two hours (Figure 18B). This result indicated that the *vexR*AB promoter is very sensitive to VexR levels. The addition of arabinose to the AKI broth further increased *vexRAB* expression. This result suggested that VexR can activate expression of the *vexRAB* promoter in the absence of additional *V. cholerae* proteins. Based on these results, plus the results presented in Figure 18A, we concluded that VexR functioned as a positive regulator of the *vexRAB* operon.

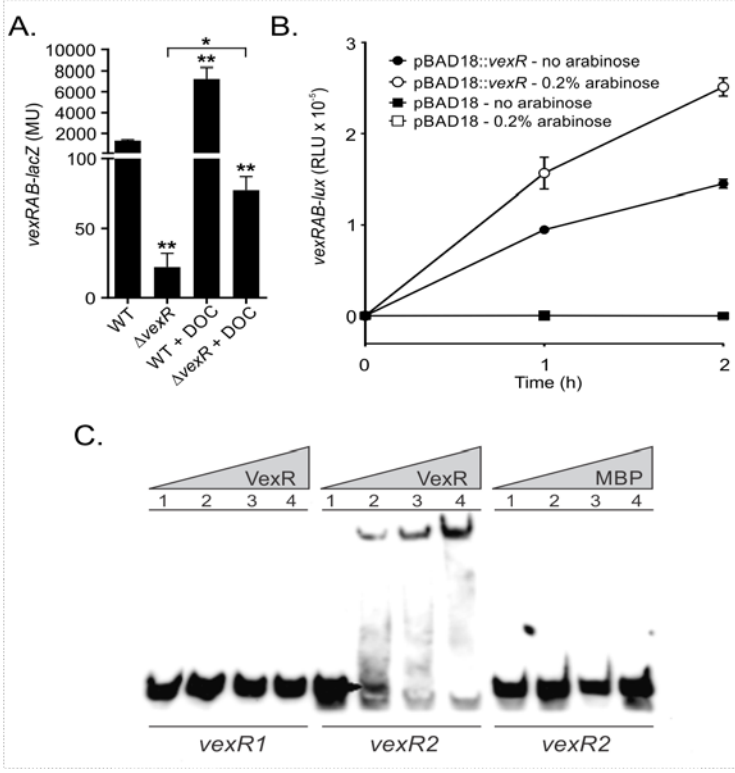

Figure 18. VexR is required for *vexRAB* expression.

(A) N16961 WT and Δ*vexR* containing pXB233 (*vexRAB-lacZ)* were grown in LB broth. At 3.5h deoxycholate (DOC) was added to a final concentration of 0.2% and the cultures were incubated for an additional 30 min before *vexRAB* expression was determined as described in the methods. Data is the mean of three independent experiments ± SD. Significance was determine by one-way ANOVA with Tukey-Kramer multiple comparison test. Unless otherwise indicated, asterisks are significance relative to WT (*p<0.05; **p<0.001). (B) *E. coli* containing pDT1076(*vexRAB-lux*) and either pJB703(pBAD18::*vexR*) or pBAD18 was grown in LB broth with or without 0.2% arabinose and bioluminescence was assayed at 0h,1h, and 2h. Error bars indicate the mean ± SD of three replicates. The results are representative of three independent experiments. (C) Gel mobility shift assay showing the binding of VexR to the *vexRAB* promoter. The promoter was split into two fragments (*vexR1*) -129 to -46 and (*vexR2*) -59 to +21 relative to the ATG start site. Biotin labeled DNA (2.5nM) from *vexR1* (lane 1-4) or *vexR2* (lane 5-12) fragments was incubated with either purified VexR-MBP or MBP as indicated at 0 nM (lane 1), 25 nM (lane 2), 100 nM (lane 3), or 250 nM (lane 4) prior to electrophoresis. Specific binding reaction, detection, and visualization are discussed in the Material and Methods.

4.2.4 VexR binds to the *vexRAB* promoter directly.

The above experiments suggested that VexR may act directly at the *vexRAB* promoter. To investigate whether this was true we performed gel mobility shift assays using VexR-MBP with the *vexRAB* promoter. The *vexRAB* promoter was split into two fragments that covered from -129 to -46 (*vexR1*) and -59 to +21 (*vexR2*) relative to the ATG start site (Figure 18C). The results of the gel shift assays show that purified VexR-MBP was able to shift the *vexR2* promoter fragment, but not the *vexR1* promoter fragment. Incubation of the *vexR2* fragment with MBP did not result in a shift confirming that the results were due to VexR (Figure 18C). These results validated that VexR directly binds to the *vexRAB* promoter and supports the conclusion that VexR directly regulates the expression of the *vexRAB* operon.

4.2.5 VexR contributes to antimicrobial resistance.

If VexR was a positive regulator of the *vexRAB* operon, we hypothesized that the deletion of *vexR* should increase *V. cholerae* susceptibility to antimicrobial compounds that are substrates of VexAB. To test this we determined the MIC of deoxycholate, erythromycin, and Triton X-100 for WT and the Δ*vexR* mutant. While deoxycholate, erythromycin, and Triton X-100 are all substrates of VexAB, other RND efflux systems possess redundant activity for efflux of deoxycholate and Triton X-100. The results showed a 2.7-fold decrease in the erythromycin MIC in the Δ*vexR* mutant (Table 7). In contrast, there was no change in the susceptibility of the Δ*vexR* mutant to deoxycholate or Triton X-100. These results contrast the resistance profile of a Δ*vexB* mutant. For example, the erythromycin MIC for the *vexR* mutant was 1.65 μg/mL compared to 0.07 μg/mL for the *vexB* mutant. Likewise, the Triton X-100 MIC for the *vexR* mutant was >3%

while the *vexB* mutant MIC was 0.0017%. This indicated that the *vexR* mutant exhibited a phenotype that was intermediate between WT and the Δ*vexB* strain. Since VexB is the only RND efflux pump that contributes to erythromycin resistance (119), these results indicate that *vexAB* is still expressed in the Δ*vexR* mutant, albeit at a lower level than in WT. This residual expression is consistent with the data above that deoxycholate still activated *vexRAB* expression, albeit to a low level, in the *vexR* deletion mutant (Figure 18A).

The fact that *vexR* deletion did not affect deoxycholate resistance was expected as previous studies have shown that the VexCD RND efflux system was redundant with VexAB for bile salt resistance (119, 267). Because of overlapping specificity for deoxycholate, mutation of both *vexB* and *vexD* are required to produce a bile salt hypersensitive phenotype. Therefore, to address whether *vexR* contributed to deoxycholate resistance we examined the effect of *vexR* deletion in a Δ*vexD* background. The results showed that deletion of *vexR* in a Δ*vexD* background reduced the deoxycholate MIC to 0.019%; a MIC that was 2.7-fold greater than the

Table 7. Antimicrobial susceptibility of *vexR* mutants.

		MIC (s.d.)[1]		
Strain	**Genotype**	**Ery (μg/mL)**	**Doc (%)**	**TX-100 (%)**
JB58	WT	4.40 (2.1)	>3 (0)	>3 (0)
XBV218	Δ*vexR*	1.65 (0.9)[2,3]	>3 (0)	>3 (0)
JB495	Δ*vexB*	0.07 (0.005)[2]	>3 (0)	0.0017 (0.0011)[2]
JB692	Δ*vexD*	ND	>3 (0)	ND
JB718	Δ*vexR*Δ*vexD*	ND	0.020 (0.001)[2,4]	ND
JB694	Δ*vexB*Δ*vexD*	ND	0.007 (0.003)[2]	ND

[(1)]Minimum Inhibitory Concentration (MIC) for erythromycin (Ery), deoxycholate (Doc), and Triton X-100 (TX-100) for the indicated N16961 strains with standard deviations in parenthesis. [(2)] P< 0.05 relative to WT. [(3)] P<0.05 relative to Δ*vexB*. [(4)] P<0.05 relative to Δ*vexBD*. ND=not determined.

deoxycholate MIC observed with the Δ*vexB*Δ*vexD* mutant (Table 7). The intermediate resistance phenotype of the Δ*vexR*Δ*vexD* mutant (relative to the Δ*vexB*Δ*vexD* mutant) was similar to the erythromycin results and further supported the conclusion that the *vexRAB* efflux system is expressed at a reduced level in the *vexR* mutant.

VexAB is the primary RND efflux system involved in Triton X-100 resistance (119). Consistent with this, a >1,700-fold increase in Triton X-100 susceptibility was observed in a Δ*vexB* mutant (Table 7). However, deletion of *vexR* did not affect Triton X-100 susceptibility (Table 7). This suggests that the low-level *vexAB* expression in the *vexR* mutant was sufficient to provide WT-level Triton X-100 resistance. Alternatively, it is possible that deletion of *vexR* resulted in the induction of other resistance traits that contributed to Triton X-100 resistance and thus compensated for the reduction in *vexRAB* expression in the *vexR* mutant.

The collective MIC data indicated that VexR was required for high-level resistance to erythromycin and deoxycholate, presumably by activating transcription of the *vexRAB* operon. When *vexR* was deleted, the contribution of VexAB to antimicrobial resistance was significantly reduced, but not to a level that was equal to a *vexB* mutant. This latter finding suggested that *vexAB* expression was not solely dependent on VexR.

4.2.6 Overexpression of *vexR* enhances resistance to deoxycholate.

We hypothesized that if *vexR* was required for *vexRAB* upregulation and antimicrobial resistance, then *vexR* overexpression should complement a Δ*vexR* mutant for growth in the presence of deoxycholate and erythromycin. We tested this hypothesis by determining the deoxycholate and erythromycin MIC for WT, Δ*vexR* and Δ*vexR*Δ*vexD* strains that expressed *vexR* from the

arabinose promoter in pBAD18. In these experiments the respective strains containing pBAD18 or pBAD18::*vexR* were grown on antimicrobial gradient agar plates that contained a range of different arabinose concentrations. However, we were unable to identify conditions where ectopic *vexR* expression complemented the tested mutants for deoxycholate or erythromycin resistance (data not shown). We considered that this result could have been an artifact of the pBAD18 expression system, and therefore we cloned *vexR* into the IPTG-inducible pMMB66EH expression vector and repeated the complementation experiments and obtained identical results. The reason that we were unable to transcomplement the *vexR* mutant is not clear. The inability to complement the *vexR* deletion likely did not result from the introduction of a secondary mutation during the construction of the *vexR* mutant since DNA sequencing confirmed the integrity of the *vexR* deletion construct (i.e. pWM91::Δ*vexR*) and the *vexRAB* locus in the *vexR* deletion strain. Also, the finding that the *vexR* mutant did not exhibit a defect in Triton X-100 resistance confirms that the *vexR* mutant produced a functional VexAB efflux system. Further, the Δ*vexR* and Δ*vexR*Δ*vexD* mutants were independently created from different parental strains, making it unlikely that the complementation defect was due to an unlinked spontaneous mutation. The lack of complementation was also not due to mutations introduced into the complementing plasmids as we also confirmed the DNA sequence of both complementing plasmids. This latter conclusion is further supported by the observation that the presence of pBAD18::*vexR* activated *vexRAB-lacZ* expression in *E. coli* (Figure 18B). Based on these results, we speculated that the *vexRAB* activation via *vexR* may be dependent upon specific physiological conditions or alternatively it is possible that activation could be dependent on DNA sequences that are internal to *vexR*.

Since the *vexR* deletion mutant could not be complemented by ectopic *vexR* expression, we sought another method to confirm that VexR contributed to antimicrobial resistance. We

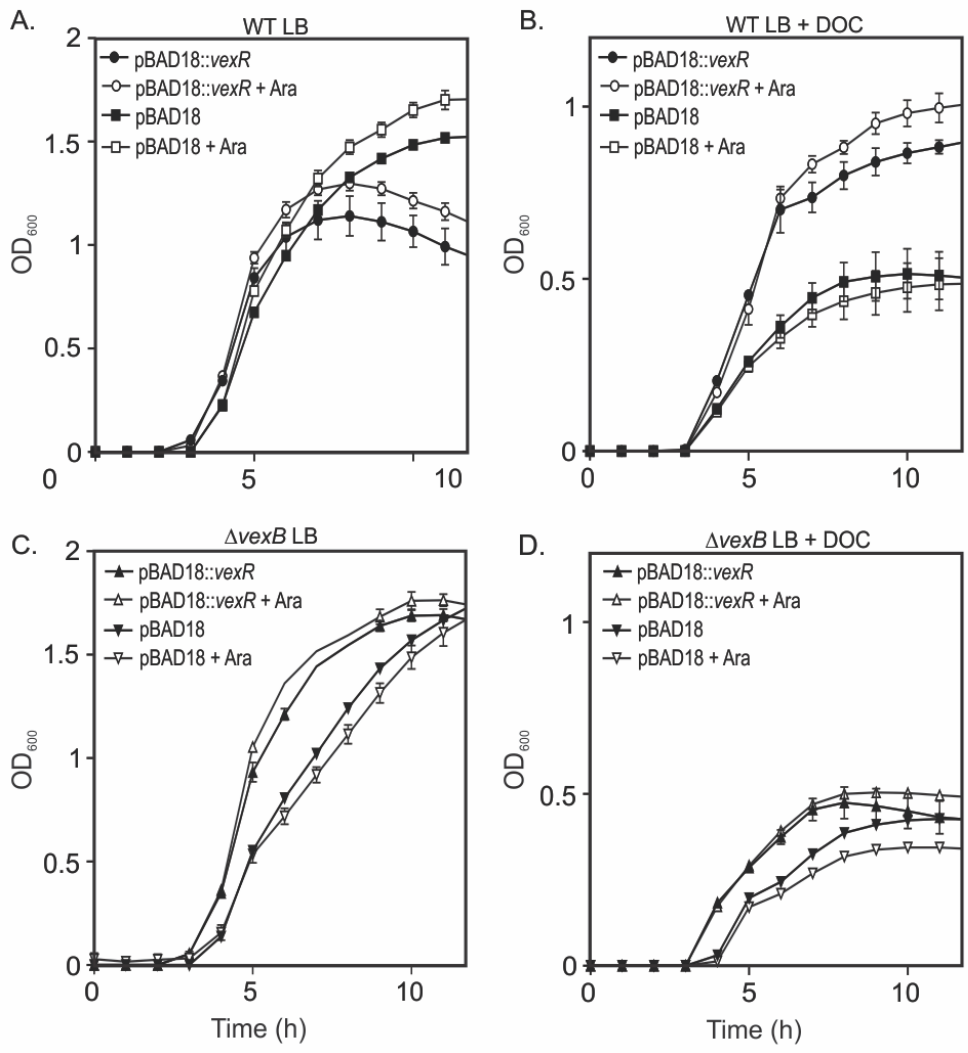

Figure 19. VexR contributes to *V. cholerae* survival under inhibitory antimicrobial conditions.

V. cholerae N16961 WT (A & B) and Δ*vexB* (C & D) containing pJB703(pBAD18::*vexR*) or pBAD18 were grown in triplicate wells of microtiter plates containing LB broth (A & C) or LB broth plus 0.015% deoxycholate (B & D). Expression of *vexR* was induced by adding 0.1% arabinose to the growth media as indicated. Cell growth was monitored as the change in the optical density at 600 nm and plotted versus time as the mean ±SEM. The results are representative of three independent experiments.

hypothesized that if *vexR* was indeed an activator of the *vexRAB* operon, then ectopic expression of *vexR* in WT should result in enhanced growth in the presence of sub-lethal concentrations of VexAB substrates. To test this hypothesis we performed growth curves for WT(pBAD18::*vexR*) and WT(pBAD18) in LB broth containing a sub-lethal concentration of deoxycholate. The results of this analysis showed that in the absence of deoxycholate, WT(pBAD18::*vexR)* replicated at a rate that was similar to WT(pBAD18) through six hours (Figure 19A). Thereafter WT(pBAD18) growth continued to increase whereas the strain bearing pBAD18::*vexR* plateaued and the cell density slightly decline through 12 hours. This indicated that overexpression of *vexR* during growth in LB broth was detrimental. We speculate that this is a result of increased *vexAB* expression which has been suggested to be growth inhibitory under non-selective conditions (208, 289). When the same strains were cultured in LB broth containing deoxycholate, the growth pattern reversed. In the presence of deoxycholate WT(pBAD18::*vexR*) exhibited a significant growth advantage over the empty vector control even in the absence of arabinose (Figure 19B). The addition of arabinose further enhanced growth of WT(pBAD18::*vexR)* relative to the same culture grown in the absence of arabinose (Figure 19C). The ectopic expression results were reminiscent of the findings observed for *vexR* activation of *vexRAB* in *E. coli* (Figure 18B) where *vexR* enhanced *vexRAB-lux* expression even in the absence of arabinose, and further enhanced *vexRAB-lux* expression with the addition of arabinose. These results provide additional support for the hypothesis that VexR is a positive activator of the *vexRAB* operon.

The above results suggested that ectopic *vexR* expression enhanced deoxycholate resistance, but did not allow us to discriminate if the resistance phenotype was mediated by VexAB or some other factor. If enhanced growth was due to VexR activation of VexAB

production, then deletion of *vexB* should alleviate the *vexR*-dependent growth enhancement in the presence of deoxycholate. We therefore repeated the above growth experiments in a *vexB* deletion strain. The results showed that the growth of the Δ*vexB*(pBAD18) control strain was slightly attenuated relative to Δ*vexB*(pBAD18::*vexR*) during growth in LB (Figure 19C). This suggests that *vexR* may regulate the expression of other genes, in addition to *vexAB,* that impart a growth advantage in LB broth. The growth of the Δ*vexB* strains was significantly inhibited in the presence of deoxycholate (Figure 19D). Unlike the WT strains, *vexR* expression did not significantly affect cell growth (Figure 19D). This confirmed that the enhanced growth observed with *vexR* overexpression in WT grown in the presence of deoxycholate resulted from *vexR*-dependent activation of the *vexRAB* operon (Figure 19B). Taken together the collective results strongly support the idea that the overexpression of *vexR* provided a growth advantage to *V. cholerae* in the presence of deoxycholate via activating production of the VexAB RND efflux system.

4.2.7 VexR contribution to CT production and murine colonization.

VexAB has been shown to contribute to virulence factor production and to murine colonization (123, 176). Our work here demonstrated that VexR was a positive regulator of *vexRAB* (Figure 18) and was induced by deoxycholate (Figure 17B). This suggested the possibility that bile acids could induce VexR production in vivo and thus affect virulence factor production and intestinal colonization. To test if *vexR* affected virulence, we quantified CT production in WT and Δ*vexR*. We also tested JB485 (which lacks all six RND efflux pumps) and its isogenic *vexR* deletion mutant for alterations in CT production. The rationale for testing JB485 is based on studies

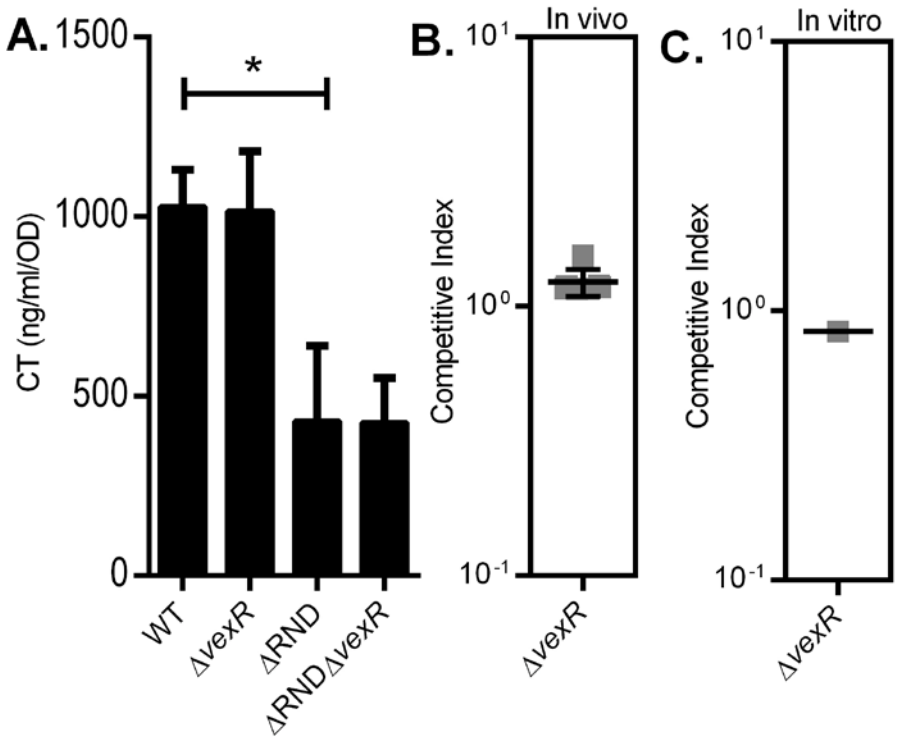

Figure 20. Effect of *vexR* on CT production and murine colonization.

(A) The indicated N16961 strains were grown under AKI growth conditions. Culture aliquots were collected following overnight growth and CT production was quantified using a GM1 ELISA. One-way ANOVA with Tukey-Kramer multiple comparisons was used to determine significance. *P=<0.01. (B & C) Competition assays to assess the role of *vexR* in intestinal colonization. (B) Infant mouse colonization competition assay. The competitive index was calculated as the ratio of Δ*vexR* to WT recovered from the small intestine, corrected for the ratio of Δ*vexR* to WT that was present in the inoculum. Each symbol represents one mouse. Mean and standard deviation are indicated by horizontal bars. (C) In vitro growth competition assay. The in vitro growth competitive index was calculated as described for the in vitro assay using cultures grown in LB broth.

showing that JB485 was attenuated for CT and TCP production and overexpressed *vexR* (123, 176); this suggested a possible link between VexR and virulence factor production (204). The results of the analysis showed that JB485 had a 65% reduction in CT production compared to WT, which corresponded to previous studies (123, 176). CT production in JB485Δ*vexR* was not significantly different from JB485 (Figure 20A). WT and Δ*vexR* were examined in the infant mouse colonization competition model to determine if *vexR* affected colonization. The results showed that the Δ*vexR* mutant competed equally with WT (Figure 20B). Taken together these results indicate that *vexR* does not significantly affect *V. cholerae* virulence factor production or intestinal colonization.

4.2.8 Deletion of the RND efflux systems induces *vexRAB* expression.

Recent studies have suggested that the RND systems have evolved to remove toxic metabolic byproducts from within the cell (178, 198, 199). If this was true, we hypothesized that the accumulation of metabolites in RND negative cells would activate the expression of *vexRAB*. The observation that *vexRAB* was upregulated in an efflux negative strain (JB485) was consistent with this hypothesis (204). To further expand upon this idea and to determine whether VexR functioned in response to the efflux status of the cell we introduced a *vexRAB-lacZ* reporter in WT, JB485 and JB485Δ*vexR*. The resulting strains were then cultured under AKI conditions for 5 h when *vexRAB* expression was assessed (Figure 19). The results showed a ~4.5-fold increase in *vexRAB* expression in JB485 confirming previous results (204). Deletion of *vexR* in JB485 reduced *vexRAB* expression to near background levels which suggested that the increase in *vexRAB* expression in JB485 was dependent upon *vexR* (Figure 19).

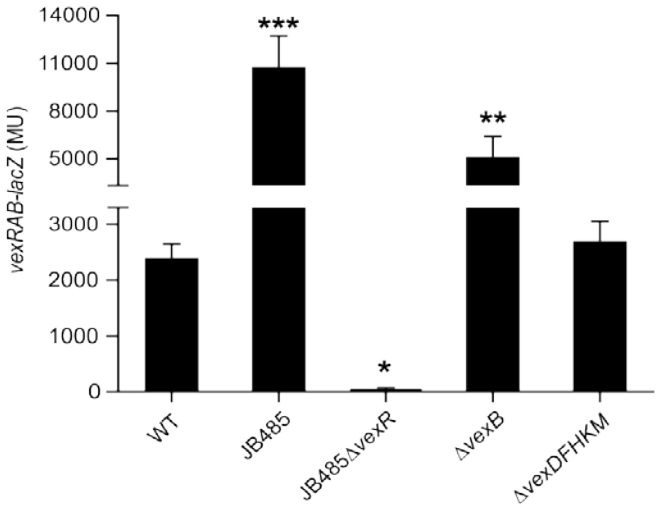

Figure 21. Expression of *vexRAB* in *V. cholerae* RND efflux mutants.

The indicated N16961 strains containing pXB233 (*vexRAB-lacZ*) were grown for 5 h under AKI conditions when *vexRAB* expression was quantified as described in the methods. Error bars indicate ± SD of three independent experiments. One-way ANOVA with Dunnet's post-hoc test was used to determine significant differences relative to WT. *=P<0.05; **=P<0.01; ***P=<0.0001.

We next tested whether increased *vexRAB* expression in JB485 was due to loss of *vexB* or a result of the deletion of all six RND efflux systems. We first quantified *vexRAB* expression in a Δ*vexB* mutant. The results showed that *vexRAB* expression increased in the *vexB* mutant (~3-fold), but to a level that was less than observed in JB485 (Figure 19). This suggested that at least one of the other RND systems can partially compensate for the loss of *vexB*. The expression of *vexRAB* in a Δ*vexDFHKM* (*vexB*+) was not significantly different from WT. This latter finding was consistent with previous work showing that *vexB*, due to its broad substrate specificity, was able to complement for the loss of the other five RND efflux systems (123, 176). Taken together these results indicate that *vexRAB* expression was responsive to the RND efflux status of the cell

and that deletion of *vexB* resulted in *vexRAB* expression being elevated, likely due to accumulation of one of its intracellular substrates. The higher level of *vexRAB* expression in JB485 relative to the *vexB* mutant indicates that one or more of the other RND efflux systems are able to efflux the inducting factor(s). Based on these results we concluded that *V. cholerae* compensates for reductions in RND efflux activity by upregulating *vexRAB* expression. Exactly how *V. cholerae* senses efflux activity was unclear, but we speculated this resulted from the intracellular accumulation of natural substrates of the RND efflux systems in the absence of RND efflux activity. Such compounds then presumably function as positive effectors of VexR.

4.2.9 The expression of *vexRAB* is altered in metabolic mutants.

We tested whether *V. cholerae* upregulated *vexRAB* expression in response to accumulation of metabolic byproducts. Our approach for these experiments was based on the assumption that the mutation of metabolic genes would disrupt biosynthetic pathways and result in the intracellular accumulation of chemical intermediates of the targeted biochemical pathway which would then activate *vexRAB* expression. To conduct these studies we obtained a number of metabolic mutants from a defined transposon mutant library that was constructed in *V. cholerae* El Tor strain C6706 (290). We selected mutants that targeted a number of different metabolic pathways including some that have been shown to affect *E. coli acrAB* expression (178). This included mutants that affected purine metabolism, cysteine metabolism, sulfur metabolism, vibriobactin biosynthesis, glycolysis, gluconeogenesis, pyruvate metabolism, amino acid metabolism, and the citric acid cycle (Table 8).

The metabolic mutants were transformed with pDT1777 (*vexRAB-lux*) and cultured under AKI conditions for five hours when *vexRAB* expression was quantified. While we were most

Table 8. *V. cholerae* C6706 mutants used in this study.

ORF # (gene)	Mutant ID[1]	NR well[1]
VC0027(*ilvA*)	EC8508	13-C5
VC0051(*purK*)	EC20412	29-B12
VC0052(*purE*)	EC1769	4-D2
VC0164(*vexB*)	EC23411	32-G3
VC0374(*pgi*)	EC24273	33-B8
VC0384(*cysJ*)	EC14462	21-E9
VC0385(*cysI*)	EC5082	9-D4
VC0386(*cysH*)	EC9587	14-C9
VC0537(*cysM*)	EC19978	34-C1
VC0767(*guaB*)	EC11960	18-E4
VC0774	EC4709	8-B6
VC0819(*aldA*-1)	EC11507	17-H6
VC0923	EC8862	14-B2
VC0968(*cysK*)	EC11848	18-B2
VC1061	EC10232	15-B8
VC1169(*trpA*)	EC5818	10-F1
VC1170(*trpB*)	EC24412	33-H10
VC1171(*trpC/F*)	EC12331	19-A2
VC1172(*trpD*)	EC11883	17-E12
VC1173(*trpG*)	EC11883	16-G12
VC1174(*trpE*)	EC11131	34-C5
VC1579	EC1872	4-G4
VC1732(*aroA*)	EC389	01-G8
VC1819(*aldA*-2)	EC14803	22-H2
VC2013(*ptsG*)	EC12803	19-D7
VC2092(*gltA*)	EC7541	11-A10
VC2209(*vibF*)	EC18511	27-D7
VC2348(*deoB*)	EC10553	16-A4
VC2362(*thrC*)	EC24541	33-G6
VC2363(*thrB*)	EC19558	28-H7
VC2364(*thrA*)	EC13310	20-B1
VC2558(*cysC*)	EC1335	03-G4
VC2559(*cysN*)	EC13560	20-H6
VC2560(*cysD*)	EC21282	30-C10
VC2649(*cysE*)	EC9914	15-C5
VCA0013(*malP*)	EC20144	29-G9
VCA0014(*malQ*)	EC2460	05-C2
VCA0765(*ybjU*)	EC4499	08-B3
VCA0886(*kbl*)	EC9834	15-H4
VCA0896(*zwf*)	EC3123	6-H3
VCA0987(*ppsA*)	EC14445	21-D11
VCA1046(*mtlD*)	EC21873	31-C6
1. The C6706 mutants were obtained from: Cameron DE, Urbach JM, Mekalanos JJ. 2008. A defined transposon mutant library and its use in identifying motility genes in *Vibrio cholerae*. PNAS 105:8736-8741.		

Table 9. Expression of *vexRAB* in C6706 metabolic mutants.

Mutated ORF (Gene Name):	Gene Product:	RLU/OD_{600} (±SD)*10^{-5}	Fold change[1]
C6706 (WT)	Wild type parental strain	16.8(3.8)	-----
VC0027(*ilvA*)	threonine dehydratase	26.9(6.7)***	1.6
VC0051(*purK*)	phosphoribosylaminoimidazole carboxylase ATPase subunit	21.0(2.7)	1.3
VC0052(*purE*)	phosphoribosylaminoimidazole carboxylase catalytic subunit	14.4(2.3)	0.9
VC0164(*vexB*)	RND multidrug efflux pump	35.2(6.6)***	2.1
VC0374(*pgi*)	glucose-6-phosphate isomerase	18.4(0.6)	1.1
VC0384(*cysJ*)	sulfite reductase	19.5(6.4)	1.2
VC0385(*cysI*)	sulfite reductase subunit beta	10.0(0.2)***	0.6
VC0386(*cysH*)	phosphoadenosine phosphosulfate reductase	17.4(2.0)	1.0
VC0537(*cysM*)	cysteine synthase B	12.7(5.1)	0.8
VC0767(*guaB*)	inosine 5'-monophosphate dehydrogenase	13.0(2.0)	0.8
VC0774	2,3-dihydroxybenzoate-2,3-dehydrogenase	10.4(1.7)***	0.6
VC0819(*aldA*-1)	aldehyde dehydrogenase	9.6(1.3)***	0.6
VC0923	serine acetyltransferase-related protein	9.8(0.9)***	0.6
VC0968(*cysK*)	cysteine synthase A	9.4(0.5)***	0.6
VC1061	cysteine synthase	5.6(0.8)***	0.3
VC1172(*trpD*)	anthranilate phosphoribosyltransferase	37.3(0.9)***	2.2
VC1579(*almE*)	enterobactin synthetase component F-related protein	34.6(1.3)***	2.1
VC1732(*aroA*)	3-phosphoshikimate 1-carboxyvinyltransferase	10.3(0.5)***	0.6
VC1819(*aldA*-2)	aldehyde dehydrogenase	3.3(1.3)***	0.2
VC2013(*ptsG*)	PTS system glucose-specific transporter subunits IIBC	12.0(0.7)*	0.7
VC2092(*gltA*)	citrate synthase	5.9(0.3)***	0.4
VC2209(*vibF*)	nonribosomal peptide synthetase VibF	24.6(1.8)***	1.5
VC2348(*deoB*)	phosphopentomutase	7.7(3.0)***	0. 5
VC2362(*thrC*)	threonine synthase	4.1(0.5)***	0.3
VC2363(*thrB*)	homoserine kinase	6.3(3.9)***	0.4
VC2364(*thrA*)	aspartokinase I/homoserine dehydrogenase	3.6(0.2)***	0.2
VC2558(*cysC*)	adenylylsulfate kinase	11.5(9.2)***	0.7
VC2559(*cysN*)	sulfate adenylate transferase, subunit 1	2.8(0.5)***	0.2
VC2560(*cysD*)	sulfate adenylyltransferase subunit 2	15.5(2.9)	0.9
VC2649(*cysE*)	serine acetyltransferase	10.1(0.1)**	0.6
VCA0013(*malP*)	maltodextrin phosphorylase	23.9(0.6)**	1.42
VCA0014(*malQ*)	4-alpha-glucanotransferase	20.5(1.7)	1.2
VCA0765(*ybjU*)	L-allo-threonine aldolase	3.7(0.3)***	0. 2
VCA0886(*kbl*)	2-amino-3-ketobutyrate coenzyme A ligase	5.3(4.0)***	0.3
VCA0896(*zwf*)	glucose-6-phosphate 1-dehydrogenase	11.7(0.6)	0.7
VCA0987(*ppsA*)	phosphoenolpyruvate synthase	8.1(4.0)***	0.5
VCA1046(*mtlD*)	mannitol-1-phosphate 5-dehydrogenase	36.4(0.7)***	2.2

Indicated C6706 strains bearing a *vexRAB*-lux reporter were grown in 96-well plates under AKI conditions for 5h before luminescence (RLU) and OD_{600} were measured. [1]Fold change=(mutant RLU/OD_{600})/(WT RLU/OD_{600}). Two-way ANOVA with Dunnet's post-hoc test to determine statistical significance relative to WT. *=P<0.01; **=P<0.001; ***=P<0.0001.

interested in the mutants that activated *vexRAB* expression, we noted several mutations reduced *vexRAB* expression. Based on our hypothesis that *vexRAB* expression is modulated by accumulation of metabolites, this would suggest that reduced *vexRAB* expression is due to loss of down-stream activators. However, we cannot exclude that the observed reduction of *vexRAB* expression was an artifact resulting from cellular byproducts inhibiting the luciferase activity of the reporter and not a true representation of the *vexRAB* promoter (291). The analysis identified three mutants (VC1172, VC1579, and VCA1046) which resulted in a >2-fold increase in *vexRAB* expression (Table 9). VC1172 encodes TrpD which functions in tryptophan biosynthesis; VC1579 encodes AlmE which is a lipid A modification enzyme (292); and VCA1046 encodes mannitol-1-phosphate 5-dehydrogenase and is involved in mannitol metabolism. These results confirmed that *vexRAB* expression was upregulated in response to interruption of at least three metabolic pathways. To further investigate this phenomenon we focused on the tryptophan biosynthesis due to the availability of mutants and two chemical intermediates in the tryptophan biosynthetic pathway.

4.2.10 Disruption of the tryptophan biosynthetic pathway affects *vexRAB* expression.

Mutation of *trpD* (VC1172) resulted in the strongest induction of *vexRAB* expression suggesting that metabolic intermediates of tryptophan biosynthesis may function as inducers of the *vexRAB* operon. To investigate this we examined *vexRAB* expression in six different tryptophan biosynthetic mutants (Figure 22A). The results showed that *vexRAB* expression increased 3-fold in the *trpB* mutant, and ~2-fold in the *trpA* and *trpD* mutants (Figure 22B). Mutation of *trpB* (VC1170) is predicted to result in indole accumulation while mutation of *trpA* (VC1169) and *trpD* (VC1172) would result in indole-3-glycerol phosphate and anthranilate accumulation,

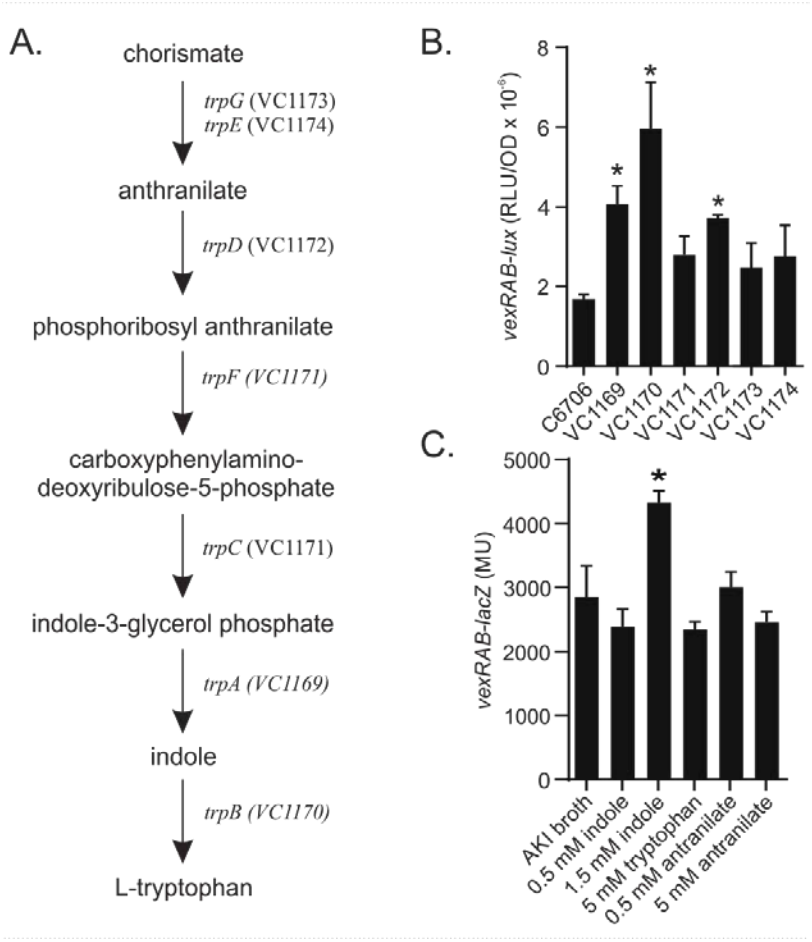

Figure 22. Indole activates *vexRAB* expression.

(A) Schematic of the *V. cholerae* tryptophan biosynthetic pathway. (B) The indicated C6706 strains bearing pDT1777 (*vexRAB-lux*) were grown in 96-well plates under AKI conditions for 5 h when luminescence (RLU) and OD_{600} were measured. Data is the mean +/- SEM of three independent assays. Two-way Anova with Dunnet's post-hoc test compared to WT was used to determine significance. *=P<0.01; **=P<0.0001. (C) *V. cholerae* N16961 containing pXB233 (*vexRAB-lacZ*) was grown with or without indole, L-tryptophan, or anthranillic acid at the indicated concentrations under AKI conditions for 5 h before *vexRAB* expression was determined as described in the methods. Bars indicate the mean +/- SD of three independent experiments. One-way ANOVA with Dunnet's post-hoc test was used to determine significant changes relative to growth in media alone. *=P<0.001.

respectively. This suggested that indole, indole-3-glycerol phosphate and anthranilate could be *vexRAB* inducers.

If intermediates from tryptophan biosynthesis were functioning as *vexRAB* inducers, we hypothesized that adding these compounds to the growth media would activate *vexRAB* expression. As anthranilate and indole were available from commercial sources, we tested if the addition of these compounds affected *vexRAB-lacZ* expression in WT. The results showed that indole at 1.5 mM increased *vexRAB* expression by ~1.5 fold (Figure 22C). This finding, combined with the observation that *vexRAB* was induced in the *trpB* mutant (Figure 22B), suggested that indole was a *vexRAB* activator. The addition of anthranilate or tryptophan did not have a significant effect on *vexRAB* expression (Figure 22C). The significance of this finding is unclear, but suggests that exogenous anthranilate does not induce *vexRAB* expression; we note that *trpD* mutation may have pleiotropic effects on other metabolic pathways which could have influenced *vexRAB* expression.

4.2.11 Indole is a substrate of the VexAB RND efflux system.

Indole is an intermediate in the biosynthesis of tryptophan. Indole is also produced by *V. cholerae* as a byproduct of tryptophan degradation and has been shown to function as a signaling molecule in biofilm formation (293, 294). At high concentrations indole is toxic, which suggested that it may be a substrate for the *V. cholerae* RND efflux systems. We therefore tested if the RND efflux systems contributed to indole resistance by comparing the growth of WT and JB485 on indole gradient agar plates. The results showed that the WT MIC was 2.1 mM while the JB485 MIC was 1.6 mM (Table 10). This suggested that the *V. cholerae* RND efflux systems contributed to indole resistance. In an effort to determine which RND efflux system contributed

Table 10. Minimum inhibitory concentration of indole for RND mutants.

Strain	Genotype	Indole MIC (s.d)[1]
JB58	WT	2.1 (0.1)
XBV218	Δ*vexR*	2.0 (0.2)
JB495	Δ*vexB*	1.8 (0.2)[2]
JB692	Δ*vexD*	2.0 (0.2)
JB432	Δ*vexF*	2.0 (0.1)
JB116	Δ*vexH*	2.1 (0.1)
JB528	Δ*vexK*	2.1 (0.1)
JB114	Δ*vexM*	2.0 (0.1)
JB485	Δ*vexBDFHKM*	1.6 (0.1)[3]

[(1)]Minimum Inhibitory Concentration (MIC) of Indole (mM) for the indicated N16961 strains. One-way ANOVA with Dunnet's post-hoc test was used to determine statistical difference relative to WT. [(2)] P< 0.05 relative to WT; [(3)] P<0.001 relative to WT.

to indole resistance we examined mutants lacking each of the six RND efflux systems pumps (i.e. *vexB, vexD, vexF, vexH, vexK, vexM*). Only the *vexB* mutant had a small but significant decrease in the indole MIC (1.8 mM), which was lower than the MIC exhibited by JB485 (Table 10). Although small, the change in the *vexB* MIC was consistent with the idea that indole is a substrate of the VexAB RND efflux system. The discrepancy between the *vexB* and JB485 MICs suggest that at least one of the other RND efflux systems function in indole resistance. All together this data indicates that indole is a substrate of the RND efflux systems and that indole functions as an inducer of the *vexRAB* operon.

4.3 DISCUSSION

VexR is a positive regulator of the *vexRAB* operon.

The VexAB RND efflux system is the only *V. cholerae* RND system that is associated with a linked TetR family regulator (Figure 16). In contrast to what is observed with most RND loci, VexR was encoded as the first gene in the *vexRAB* operon. Expression of *vexRAB* was largely dependent on VexR as evidenced by the finding that *vexR* deletion decreased *vexRAB* expression and abolished the substrate-dependent induction of *vexRAB* expression (Figure 18A). Deletion of *vexR* also resulted in increased susceptibility to antimicrobial compounds that were substrates of VexAB (Table 7). These results indicated that VexR functioned as a positive regulator of the *vexRAB* operon. This conclusion was confirmed in subsequent experiments showing that VexR bound directly to the *vexRAB* promoter (Figure 18C), that VexR was able to activate *vexRAB* expression in *E. coli* (Figure 18B), and that *vexR* overexpression in *V. cholerae* resulted in a *vexB*-dependent growth advantage in the presence of sub-lethal concentrations of deoxycholate (Figure 19).

The finding that VexR was a positive regulator was unexpected; to the best of our knowledge VexR is the first example of a RND-associated TetR-family regulator that functions as an activator. The vast majority of TetR-family regulators behave as transcriptional repressors. This finding highlights differences in the regulation of *vexRAB* relative to its orthologous *Enterobacteriaceae* system (i.e. *acrR-acrAB*). In *E. coli acrR* functions as a negative regulator of *acrAB* while global regulatory systems such as the Mar operon function to positively regulate *acrAB* expression in response to antimicrobial exposure (Figure 16) (206, 207). Although *V. cholerae* lacks the Mar operon, VexR appears to function in a similar role as the Mar operon by activating *vexRAB* expression in response to antimicrobial exposure. It is worth noting that the *vexRAB* and *vexGH* RND efflux systems are also positively regulated by the Cpx system in response to membrane stress (204). However, the Cpx system did not affect the expression of the

RND efflux systems in response to antimicrobial compounds that were substrates of the RND efflux systems (204). This suggests that *vexRAB* expression is under the influence of multiple regulatory systems that respond to distinct stimuli.

Metabolites activate the expression of the *vexRAB* operon.

The expression of *vexRAB* was highly elevated upon loss of RND-mediated efflux in the mutant JB485 (Figure 21). This upregulation was abolished when *vexR* was deleted (Figure 21), indicating that VexR positively regulates *vexRAB* expression in response to the efflux status of the cell. These data suggested that in the absence of RND-mediated efflux, a cellular product accumulates within the cell thereby inducing *vexRAB* expression. As we have demonstrated that *vexRAB* is induced by substrates of VexAB (Figure 17), this indicated that induction of *vexRAB* in JB485 was due to the accumulation of an endogenous substrate of the VexAB RND efflux system. Based on recent reports in *E. coli* (178, 198-200), we hypothesized that the accumulating compound would be a byproduct of cellular metabolism. The finding that *vexRAB* expression was upregulated in three of the 36 tested metabolic mutants provided evidence to support this hypothesis (Table 9). Altogether, this indicates the VexAB RND efflux system plays a role in removing excess metabolites from the cell. While *vexRAB* was induced in three of the metabolic mutants, it is also possible that other efflux systems may function in a similar or redundant role to remove metabolites from the cell. For example, the *E. coli acrEF*, *yfiK* and *aaeAB* efflux systems have been shown to be important for indole, cysteine-cystine, and p-hydroxybenzoate export, respectively (295-297). The fact that *vexRAB* expression in the Δ*vexB* was lower than what was observed in JB485 supports this conclusion that other RND efflux systems function in a similar manner (Figure 21).

The mutations that activated *vexRAB* expression disrupted three different metabolic pathways. This suggested that multiple metabolites can activate *vexRAB* expression (Table 9 and Figure 22B). The mutation of *trpB* resulted in the greatest increase in *vexRAB* expression. Since TrpB catalyzes the conversion of indole to tryptophan (Figure 22A), this suggested that indole was likely responsible for induction of *vexRAB* in the *trpB* mutant (Figure 22). Activation of *vexRAB* expression by exogenous indole supported this conclusion (Figure 22C). In addition, both JB485 and the Δ*vexB* mutant exhibited increased susceptibility to indole. This suggested that indole was a substrate for VexAB and likely additional RND efflux systems (Table 10). Based on the fact that indole was both an inducer and substrate of *vexRAB,* we conclude that the VexAB efflux system functions to remove indole from within the cell before it reaches concentrations that are detrimental to cell growth. Given the broad substrate specificity of the VexAB system, combined with the observation that *vexRAB* was induced in two other metabolic mutants; we propose that the function of VexAB in metabolic relief extends beyond indole.

Role of VexR in virulence factor production and colonization.

Inhibition of RND efflux activity was shown to attenuate virulence factor production in *V. cholerae* by an unknown mechanism (123), but was hypothesized to result from the accumulation of small molecules in the RND mutants that inhibited virulence factor production. Although our data shows that the RND-associated virulence defect is independent of *vexR*, we speculate that specific metabolites also function to negatively affect *V. cholerae* virulence factor production. Precedence for small molecule inhibition of virulence factor production includes virstatin, bile, and fatty acids (reviewed in (77)). Additional work will be required to identify the existence of endogenous molecules that modulate virulence factor production.

Conclusions.

Herein, we have shown that VexR functions as a positive regulator of the *vexRAB* operon. VexR appears to activate expression of the *vexRAB* operon in response to efflux substrates of the VexAB RND efflux system. The finding that VexR functions as a positive regulator was unusual. Although more than 20,000 distinct sequences in the genomic database putatively code for TetR family regulators, only a fraction have been characterized, the majority of which function as repressors (207, 298). To the best of our knowledge only ten members of the TetR family of regulators have been characterized as activators, none of which were associated with an RND efflux system (299-308).

We have established a novel role for VexAB in exporting cellular metabolites. We suggest that VexAB relieves cellular stress by preventing the accumulation of metabolic byproducts to toxic levels. This is supported by our data which indicates that indole was a substrate and inducer of the *V. cholerae vexRAB* RND efflux system. Our results further suggest that the substrates induce the production of VexAB via its cognate regulator (i.e. VexR). It is noteworthy that similar processes were documented in *E. coli* where metabolic mutants and metabolic byproducts were found to activate the *acrAB* RND system (178, 198-200). Therefore, the ability of the RND efflux systems to alleviate cellular stress due to excess metabolic intermediates appears to be an evolutionarily conserved mechanism.

5.0 CONCLUSION

The endemic and epidemic potential of the disease cholera has had a great historical impact and continues to be a public health concern in various communities throughout the world. The recurrent problem of cholera is largely due to the success of *V. cholerae* in overcoming challenges in both aquatic and in vivo environments in order to survive, colonize, and further disseminate. Essential to the survival of *V. cholerae* is its ability to adapt to rapid environmental changes by responding to environmental cues, to overcome antimicrobial challenge, and to produce virulence factors. In order to understand the mechanisms by which *V. cholerae* adapts and survives stringent environmental conditions, we focused on the RND family of multidrug transporters. The RND family of efflux systems is known for their ability to efflux a broad range of antimicrobial substrates and their contribution to multidrug resistance in several Gram-negative pathogens.

In this work, we sought to characterize the function of the RND efflux systems in *V. cholerae*. While the RND efflux systems are known for their ability to efflux a broad range of antimicrobial substrates, phylogenetic analysis suggests that they evolved independent of xenobiotic selection (167). This suggests that the RND efflux systems may have an innate role that is independent of antimicrobial challenge. We hypothesize that the innate function of the *V. cholerae* RND efflux systems is to efflux metabolic waste, which in the absence of RND-

mediate efflux accumulates, causes cellular stress which then affects virulence factor production. Our data herein supports this hypothesis.

The RND efflux systems in *V. cholerae* are redundant in contributing to antimicrobial resistance due to overlapping substrate specificities**.** Previously, the RND efflux systems were found to contribute to *V. cholerae* antimicrobial resistance against a broad range of substrates (123). Yet the contribution of individual pumps was difficult to elucidate as several RND efflux systems were able to compensate for the deletion of other pumps (as discussed in section 2.3). As such, a panel of RND mutants was created with various deletion permutations in order to distinguish the contribution of individual pumps to antimicrobial resistance. The functional redundancy of the *V. cholerae* RND efflux systems was not restricted to their involvement in antimicrobial resistance. Similar patterns of redundancy were observed for virulence factor production, murine colonization, and alleviating cellular stress (Sections 2.2.3, 2.2.4, and 3.2.5). Therefore, a similar approach was used to discern the roles of the RND efflux systems in contributing to each of these phenotypes**.**

It was interesting to note that the characterization of the RND efflux systems in regards to antimicrobial resistance provided insight into their roles in other functions. The VexAB RND efflux system was considered the major contributor to antimicrobial resistance as it had the broadest range of substrates and its presence could compensate for the deletion of all the other RND efflux systems. The supremacy of the VexAB RND efflux system was a phenotype that was echoed in other functions of the RND efflux systems. The presence of VexAB alone produced a wild type phenotype in regards to virulence factor production and murine colonization (Section 2.2.3 and (123)). Furthermore, VexAB was sufficient to alleviate cellular stress (Section 3.2.5); a phenotype that was likely due to the positive regulation of *vexAB* by

VexR and CpxR. The fact that the VexAB was able to compensate for loss of any or all of the other RND systems reinforces the idea that VexAB is the primary RND efflux system in *V. cholerae* and the other RND systems function in response to specific environmental stimuli. This is exemplified by finding that the *vexCD* system in induced only in the presence of bile ((210) and data not shown).

In this work, we have shown that VexR positively regulates the *vexRAB* operon; thereby contributing to VexAB mediated antimicrobial resistance (Section 4.2.3 and 4.2.5). Furthermore, we have shown that *vexRAB* is regulated in response to substrates of VexAB in a manner that is dependent on *vexR* (Section 4.2.2 and 4.2.8). Together this provides a likely mechanism by which the VexAB system can respond to antimicrobial compounds, contribute to virulence factor production, and compensate of the deletion of the other RND efflux systems. The accumulation of VexAB substrates would induce *vexRAB* expression. The production of VexR would be expected to further contribute to the activation of *vexRAB* expression, which would enhance the cell's response to the stimulus. The observation that the cell is highly sensitive to the production of VexR (Section 4.2.3 and 4.2.6) supports the hypothesis that VexR functions as a positive regulator to increase the production of the VexAB RND efflux system. The ability of VexR to function as a positive regulator is distinct compared to other members of the TetR family of regulators (as discussed in Section 4.3). This in conjunction with the observation that *vexAB* is the only *V. cholerae* RND efflux system with a linked regulator suggests that VexAB plays an important role in *V. cholerae* survival, as is supported by the diverse phenotypes to which VexAB contributes and the induction of *vexRAB* expression during human and animal in vivo colonization.

VexAB is similar to the *E. coli* AcrAB RND efflux system. Both RND efflux systems have a broad range of substrates and therefore are the major contributors to antimicrobial resistance in their respective species. However, the regulation of these two systems differs. Herein, we have presented evidence that the VexAB RND efflux system is positively regulated by both VexR and the Cpx system (Section 3.2 and 4.2). Contrarily, the AcrAB RND efflux system is repressed by its cognate TetR regulator (i.e. AcrR), but activated by the global regulatory system referred to as the MAR (multiple antibiotic resistance) regulon. However, in *V. cholerae* there is no known MAR regulon. Therefore we suggest that the *V. cholerae* VexR functions in a similar role to the *E. coli* MAR regulon. Together this would suggest the ability to elicit a rapid and robust response to accumulating substrates by induction of the RND efflux systems is a conserved mechanism in these organisms. This would be of obvious benefit to the ability of these species to survive in the presence of intestinal bile and other antimicrobial compounds. However, as both of these RND efflux systems are implicit in the extrusion of cellular metabolites, it may also indicate a common requirement for the disposal of metabolic waste.

As VexAB is a major RND efflux system in *V. cholerae*, it was used to determine which metabolic pathways produce endogenous substrates of the RND efflux systems. The expression of *vexRAB* was observed to be highly induced by mutants from three distinct metabolic pathways (Section 4.2.9). Mutants in the tryptophan biosynthetic pathway induced the expression of *vexRAB* due to the accumulation of indole, which was determined to be a substrate of the RND efflux systems (Sections 4.2.10 and 4.2.11). Together these results supported our hypothesis that the innate role of RND efflux systems is to extrude cellular byproducts. The accumulation of certain metabolites induced the expression of *vexRAB* in accordance with the observed feedback

mechanism of the RND efflux systems and their substrates (as discussed in Section 4.3). Furthermore, the deletion of all six RND efflux systems induced *vexRAB* expression higher than the deletion of *vexB* alone (Section 4.2.8). This suggested that the RND efflux systems have a redundant role in extruding cellular waste, such that one or more of the innate substrates is also a substrate for another RND efflux system. The loss of RND-mediate efflux causes cellular stress presumably due to the accumulation of the metabolic waste (as discussed below and in Section 3.3). Therefore, understanding which metabolites causes cellular stress and identifying the corresponding RND efflux systems that contribute to the expulsion of said metabolite could prove to useful to developing therapeutic targets.

It was interesting to note that the expression of *vexRAB* was induced by the disruption of three distinct metabolic pathways (Section 4.2.9). The sensitivity of *vexRAB* to structurally diverse metabolic substrates is in accordance with the ability of VexAB to provide resistance against a broad range of antimicrobial substrates (Section 2.2 and (119, 123)). Similar to *vexRAB*, the expression of the *acrAB* RND efflux system in *E. coli* was reported to be induced by mutants in four distinct metabolic pathways (309). These data indicate an evolutionary conserved mechanism of enteric bacteria to utilize the RND efflux systems in the extrusion of diverse metabolic byproducts.

The finding that the RND efflux systems have an innate role that is unrelated to xenobiotic resistance is further supported by the observation that loss of RND-mediated efflux causes cellular stress in the absence of antimicrobial challenge. Induction of the *V. cholerae* Cpx system activated the expression of both *vexRAB* and *vexGH* RND efflux systems, which suggested that the cell was dependent on active efflux to relieve stress. While it would be easy to assume that the antimicrobial resistance capability of the RND efflux systems is the target of the

Cpx stress response, our data did not support a link between antimicrobial challenge and induction of the Cpx system (as discussed in Section 3.3). This suggested that the role of the RND efflux systems in relieving cellular stress was not restricted to providing antimicrobial resistance, but rather that there was an innate role for the RND efflux systems in the absence of xenobiotic challenge. In support of this, our data clearly indicates that loss of RND-mediated efflux activates the Cpx system (Section 3.2.6). This was in agreement with previous reports that loss of TolC-mediated efflux induced the Cpx stress response system in both *V. cholerae* and *E. coli* (200, 211). As both of our tested conditions including rich media and no exogenous RND substrates, we concluded that the induction of the Cpx stress response was not due to a starvation stress response, nor to antimicrobial challenge, but rather due to the accumulation of an innate compound (as discussed in Section 3.3). In light of the ability of the *V. cholerae* RND efflux systems to extrude metabolic byproducts, this suggests that the loss of RND-mediated efflux allows an accumulation of metabolic waste that causes cellular stress and therefore induces the Cpx stress response. Furthermore, it suggests a role for the Cpx system to alleviate cellular stress from accumulating metabolites by inducing the *vexRAB* and *vexGH* RND efflux systems.

The RND efflux systems were determined to repress the Cpx system during growth in LB broth and under AKI conditions (Section 3.2.5). The VexAB and VexGH RND efflux systems were required for Cpx repression under both growth conditions, which corresponds to their regulation by the Cpx system and suggested a reciprocal regulation (as discussed in Section 3.3). The other RND efflux systems contributed to repression of the Cpx system during growth under AKI growth conditions but not in LB broth. These data implied that different metabolites accumulated under various growth conditions and require the different RND efflux systems for extrusion from the cell. It is possible that a different metabolite is accumulating under each

growth condition and requires a different set of RND efflux systems for extrusion from the cell. It is also possible that under both conditions there is a metabolite accumulating which is a substrate of VexAB and VexGH and that a different metabolite is accumulating under AKI conditions that require the function of the other RND efflux systems. While for the sake of reductionism only one or two substrates are suggested to be accumulating, we cannot exclude that there are multiple substrates accumulating under each growth condition that are effluxed by multiple pumps. All of these possibilities are supported by the ability of the *V. cholerae* RND efflux systems to efflux multiple substrates, to have overlapping substrate specificity, and to have a redundancy in function that allows some pumps to compensate for the loss of others.

The contribution of all six RND efflux systems to repression of the Cpx system during growth under AKI conditions had an interesting correlation with the ability of the RND efflux systems to contribute to virulence factor production. As the reduction in virulence factor production upon loss of RND-mediated efflux was attributed to the accumulation of a small molecule (as discussed in Section 2.3), this introduced the possibility that the accumulation of said small molecule coordinately induced the Cpx system. This also suggested the possibility that the Cpx system was responsible for repression of virulence factor production. However this hypothesis was proven to be incorrect as Δ*cpxR* and *cpxA** mutants were found to have no effect on CT or TcpA production compared to WT, nor did they significantly affect colonization (Section 3.2.7 and (211)).

The clinically relevant symptoms and epidemic potential of cholera is due to the ability of *V. cholerae* to colonize the intestine and elicit the secretory diarrhea that is characteristic of the disease. Therefore, understanding the regulation and action of the virulence factors TCP and CT is important to controlling morbidity and spread of this disease. The reduction of virulence factor

production upon loss of RND-mediated efflux has been a phenotype of interest. The RND efflux systems are not in themselves capable of binding to and regulating the expression of genes. Nor are they part of the cellular machinery required for assembly and/or extrusion of the TCP and CT. Together this suggested that the capability of the RND efflux systems to modulate virulence factor production was likely related to their innate ability to extrude molecular compounds (as discussed in Section 2.3). Our revelation that the innate role of the RND efflux systems is to expel metabolic waste suggests that the accumulating small molecules that affect virulence factor production are likely endogenous metabolites. Understanding the linkage between metabolism and virulence could potentially elucidate therapeutic approaches for *V. cholerae* and other gram negative pathogens where the RND efflux systems have been shown to be important for virulence.

The RND efflux systems that were major contributors to virulence factor production (VexAB, VexCD, VexGH, and VexIJK) also contributed to resistance against the bile salts cholate and deoxycholate (Sections 2.2.1 and 2.2.3). The overlapping substrate specificity indicates that the repression of virulence factors may be due the accumulation of a common substrate. While VexEF and/or VexLM also contributed to virulence factor production, antimicrobial susceptibility assays did not indicate redundant substrate specificity with VexAB, VexCD, VexGH, or VexIJK (Sections 2.2.1-2.2.3). Together, this suggests that there are multiple effector molecules extruded by the RND efflux systems that affect virulence factor production. However, we cannot exclude that it is a single un-identified molecule that is a substrate of all six RND efflux systems.

The metabolic intermediate indole was determined to be a substrate and inducer of the *vexRAB* operon (as discussed in Section 4.3). Previous work has shown that indole is a cellular

cue that can regulate bacterial gene expression in a number of bacteria. In *V. cholerae* indole has been shown to regulate genes involved in quorum sensing and biofilm formation (293). Therefore, we hypothesized that the accumulation of indole in an RND-deficient mutant caused reduced virulence factor production. Preliminary findings support this hypothesis; indole inhibited the production of CT and TcpA in a dose-dependent manner (Appendix C). The addition of 0.5 mM indole caused a reduction in virulence factor production that was similar the RND deficient mutant. As *V. cholerae* strains have been reported to produce up to 0.5mM of indole in culture supernatant (293, 310), this data indicates that biological levels of indole are sufficient to repress the production of CT and TCP. While further work is needed to determine the mechanism by which indole represses virulence factor production, we can conclude that loss of RND-mediated efflux can cause an accumulation of indole which is sufficient to repress production of CT and TCP.

V. cholerae modulates gene expression late during infection, downregulating virulence genes and upregulating genes involved in exiting the host and disseminating back into to the environment (41, 282). Passage through a host has been shown to induce a hyper-infectious phenotype which is suggested to be a large contributor to the epidemic spread of *V. cholerae* (41). Indole is a cellular cue that could be involved in the transition of *V. cholerae* from the host to the environment. Preliminary results indicate that indole can repress CT and TCP in a dose dependent manner (Appendix). As indole is cellular signal that is expected to increase with cell density, this is likely a signal during late infection that could repress virulence factor production. Coordinately, indole has been shown to induce biofilm in *V. cholerae* (293). Biofilm formation contributes to the hyper-infectious phenotype, protection of *V. cholerae* in the environment, and surviving that gastric barrier. Therefore, if true, the use of indole as a cellular signal is of crucial

importance to *V. cholerae* and suggests an innate role of the RND efflux systems during pathogenesis.

Conclusion

In this work, we have shown that the *V. cholerae* RND efflux systems contribute to *V. cholerae* survival and pathogenesis due to their ability to efflux a broad range of substrates. The diversity of substrates and the redundancy of these pumps were most easily categorized by their contribution to antimicrobial resistance. In addition to antimicrobial resistance, our work has established an intrinsic role of the RND efflux systems in extruding metabolic waste. In the absence of RND-mediated efflux, metabolic substrates accumulate and prove stressful to the cell and induce the Cpx system. In a wild type cell, the Cpx system would respond to the accumulation of metabolic waste by activating the *vexRAB* and *vexGH* RND efflux systems to reduce the intracellular levels of the stressful metabolites. Furthermore, the innate role of the RND efflux systems in expelling metabolic waste provides a mechanism by which the RND efflux systems repress virulence factor production. Upon loss of RND-mediated efflux, one or more cellular metabolites, such as indole, accumulates and represses the transcription of *ctxAB* and *tcpA*, likely through the ToxR regulon. Together these data indicate the *V. cholerae* RND efflux systems play an important role in *V. cholerae* survival and pathogenesis by regulating and responding to the accumulation of small molecules.

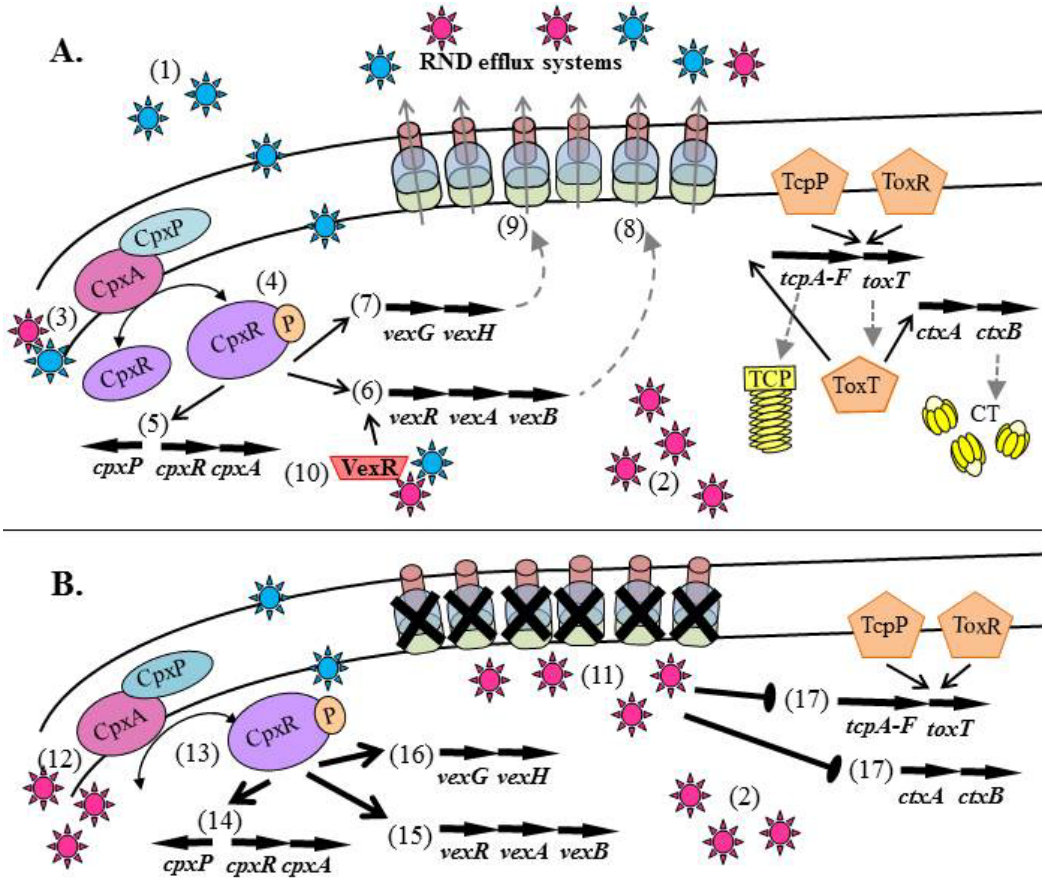

Figure 23. Model of the function of the RND efflux systems in *V. cholerae.*

The RND efflux systems extrude a broad range of substrates: (1) exogenous compounds such as antibiotics and bile salts; and (2) endogenous metabolic by-products such as indole. (A) In a WT cell, the accumulation of metabolic waste activates (3) the Cpx system. (4) Phosphorylated CpxR then induces expression of (5) *cpxRA*/*cpxP* and the (6) *vexRAB* and (7) *vexGH* RND efflux systems. This induction increases (8) VexAB and (9) VexGH production, thereby alleviating cellular stress. Furthermore, exogenous and endogenous substrates of VexAB induce the expression of (6) *vexRAB* in a (10) *vexR*-dependent manner, resulting in increased (8) VexAB production to extrude the compounds. (B) The loss of RND mediated efflux causes the accumulation of (11) metabolic substrates. This proves stressful to the cell and (12) induces the Cpx system, thereby constitutive activating (13) CpxR and causing the induction of (14) *cpxP*, (15) *vexRAB* and (16) *vexGH* expression. (11)The accumulation of one or more cellular metabolites represses (17) *ctxAB* and (18) *tcpA*, likely through the ToxR regulon.

APPENDIX A

MATERIALS AND METHODS

A.1 BACTERIAL STRAINS AND CULTURE CONDITIONS

The bacterial strains used in this study are listed in the Tables 1, 3, and 6 in each respective chapter. *Escherichia coli* EC100D*pir*+, SM10λ*pir,* and ER2566 were used for cloning, plasmid mobilization, and protein purification, respectively. *Vibrio cholerae* strains used in this study were derivatives of O1 El Tor strains N16961 and C6706 (122, 231, 290). *V. cholerae* strains JB3 (N16961-*lacZ*+ Sm^R), JB58 (N16961-Δ*lacZ* Sm^R), or JB804 (C6706-lacZ+ Sm^R) were used as the wild type (WT) control strain for all experiments as indicated. Bacterial strains were grown at 37°C in Luria-Bertani (LB) broth or on LB agar. *V. cholerae* was grown in AKI broth under AKI growth conditions for virulence inducing conditions (311). AKI growth conditions were as follows: a fresh saturated overnight LB broth culture of the indicated strain was inoculated 1:10,000 into 10 mL of AKI broth in a 18x175 mm test tube. The test tube was then incubated statically at 37°C for 4 h when the broth culture was transferred into a sterile 125 mL Erlenmeyer flask. The Erlenmeyer flask was then incubated with shaking overnight at 37°C

before CT and TCP production was assessed. Bacterial stocks were maintained at -80°C in LB broth containing 25% glycerol. Growth media was supplemented with carbenicillin (Cb) and streptomycin (Sm) at 100 μg/mL, kanamycin (Km) at 50 μg/mL, or chloramphenicol (Cm) at 1 μg/mL (for *V. cholerae*) or at 20 μg/mL (for *E.coli*) as required. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was added to LB agar plates at 40μg/mL unless otherwise indicated. Bacterial growth media was purchased from Difco (Lawrence, KS) and chemicals were purchased from Sigma-Aldrich (St Louis, MO).

A.2 PLASMID CONSTRUCTION

Plasmids and oligonucleotides used in this study are listed in the appropriate Tables associated with each chapter (Tables 1, 3, and 6). Enzymes for cloning experiments were purchased from New England Biolabs (Beverly, MA). β-galactosidase reporter constructs for *vexRAB*, *vexEF*, *vexGH*, *vexIJK*, and *vexLM* were constructed as follows. Briefly, the gene-specific PCR primer pairs (i.e. *166c-F-XhoI/166c-R-XbaI, P-VC0628-F-XhoI/R-XbaI, P-VC0914-F-XhoI/R-XbaI, P-VC1673-F-XhoI/R-XbaI,* and *P-VCA0638-F-XhoI/R-XbaI*) were used to amplify the promoter region for each operon from the *V. cholerae* N16961 genome. The resulting PCR amplicons were then digested with XhoI and XbaI restriction endonucleases before being ligated into similarly digested pTL61T to generate pXB233(*vexRAB-lacZ*), pXB228 (*vexEF-lacZ*), pXB229 (*vexGH-lacZ*), pXB230 (*vexIJK-lacZ*), and pXB232 (*vexLM-lacZ*). The *breR-lacZ* reporter (pXB265) was similarly created using the PCR primer pair *P-VC1746-F-SmaI/R-BamHI* and the indicated restriction endonucleases. The DNA sequence of the reporter constructs were subsequently verified by DNA sequencing. pDT1076 (*vexRAB-lux*) was generated by cloning the *vexRAB*

promoter from pXB233 into pCM10 as follows. The *vexRAB* promoter was PCR amplified from pXB233 using the BRL-F and166c-F-XhoI PCR primers. The resulting PCR amplicon was then made blunt ended before being restricted with BamHI. The resulting fragment was then cloned into pCM10 that had been linearized with EcoRI, made blunt-ended, before being restricted with BamHI. A Cm-marked version of pDT1076 was created by transposing the Cm-marked mariner transposon from pSC137 into pDT1076 to generate pDT1777. The reporter plasmids were verified by DNA sequencing

The *vexR* expression plasmids were constructed as follows. pJB703 was generated by amplifying *vexR* from N16961 using the VC0166F-SacI and VC0166-XbaI-R PCR primers. The resulting amplicon was restricted with SacI and XbaI endonucleases before being ligated with similarly digested pBAD18 generate. pDT1146 (pMMB66EH::*vexR*) was generated by amplifying the *vexR* gene from N16961 using the VC0166F-SacI and MCS4.VexA.R primers. The resulting amplicon was blunt ended, digested with BamHI, and then ligated with similarly treated pMMB66EH (kindly provided by Dr. Robert Shanks, University of Pittsburgh) to generate pDT1146. The sequence of *vexR* in both plasmids was confirmed by DNA sequencing. pSS35 was constructed by amplifying the *vexR* gene from N16961 using the VC0166-F-pMAL-SmaI and VC0166-R-pMal-EcoRI PCR primers. The resulting PCR amplicon was then digested with EcoRI and SmaI endonucleases to make and cloned into the same sites of pMAL-c2 (NEB). The DNA sequence of the protein purification construct was subsequently verified by sequencing.

The allelic exchange vector pWM91::Δ*vexR* was generated by crossover PCR as previously described (118, 119, 123, 312). Briefly, *vexR*-specific VC0166-F1-R2 and VC0166-F2-R1 PCR primer pairs (Table 6) were used in separate PCR reactions with N16961 genomic

DNA as a template. The resulting ~1kb PCR products were gel purified, pooled, and then used as the template for a second PCR reaction using the flanking F1-R1 PCR primers to generate the ~2 kb *vexR* deletion construct. The resulting ~2kb PCR amplicon was then purified, restricted with XbaI and SacI endonucleases before being ligated with similarly digested pWM91 to generate pWM91::Δ*vexR*. The resulting plasmid was then used to delete *vexR*. Briefly, pWM91::Δ*vexR* was conjugated into the *V. cholerae* and cointegrants were selected for Cb/Sm resistance. Several Cb/Sm resistant colonies were then streaked for single colonies onto LB agar (without NaCl) containing 5% sucrose to select for the resolution of the integrated plasmid. Several sucrose-resistant colonies were screened for Cb sensitivity to verify plasmid loss before the *vexR* deletion was confirmed by PCR using flanking primers.

A.3 MUTANT CONSTRUCTION

Unmarked in-frame deletions of the RND efflux pump protein gene in each respective strain was constructed by allelic exchange using genetic constructs and methods previously reported and as described above (118, 119, 123, 312). The Δ*vexBDHK*, Δ*vexBHK*, Δvex*BDH*, and Δ*vexBH* mutants were derived by deletion of *vexH* in strains JB740, JB531, JB694, and JB495, respectively. The Δ*vexHK*, Δ*vexDK*, and Δ*vexDHK* mutants were derived by deletion of *vexK* deletion in strains JB116, JB692, and JB186, respectively. The deletion of *vexR* is described above.

Deletion of *V. cholerae cpxR* was performed as previously described (211). Briefly, pΔR was conjugated into *V. cholerae* and co-integrants were selected for Sm/Km resistance. Several Sm/Km resistant colonies were streaked onto LB (without NaCl) containing 5% sucrose to select

for resolution of the integrated plasmid. Several resistant colonies were screened for Cb sensitivity to verify the loss of the plasmid, and for Km resistance to verify the presence of the Km cassette inserted in the *cpxR* gene. PCR using *cpxR*-F and *cpxR*-R primers was then used to confirm the presence of the *cpxR*::Km insert.

Introduction of the chromosomal *cpxP-lacZ* reporter into *V. cholerae* was performed as previously described (225). Briefly, the pJL1P'Z reporter construct was conjugated into *V. cholerae* strains and co-integrants were selected for Sm/Cb resistance. Several Sm/Cb resistant colonies were then streaked onto LB (without NaCl) containing 5% sucrose to select for resolution of the integrated plasmid. Sucrose resistant colonies were then screened for Cb sensitivity to verify plasmid loss before the *cpxP-lacZ* insertion was confirmed by PCR using the *lacZ*5 and *lacZ*6 primers. Construction of the *V. cholerae* RND efflux mutant strains was previously reported (119, 123, 256, 313).

A.4 TRANSCRIPTIONAL REPORTER ASSAYS

Strains were grown under the indicated growth conditions and culture aliquots were taken in triplicate at various time points post-inoculation to quantify β-galactosidase activity as previously described (9, 314). All reporter experiments were performed independently at least three. The *V. cholerae lacZ*::*cpxP-lacZ* reporter strains were cultured under AKI conditions as follows. Overnight cultures were diluted 1:10,000 into 73 mL of AKI broth in a 30 mm diameter glass cylinder. The cultures were then incubated statically at 37°C for 4 h when 10 mL aliquots were transferred into 125 mL Erlenmeyer flasks and grown at 37°C with shaking. Aliquots were then collected at the indicated times for the β-galactosidase assay. The two plasmid reporter

system utilized overnight cultures of *V. cholerae* containing pBAD33-*cpxR* or pBAD33 (negative control) and the indicated *lacZ* reporter (i.e. *vexRAB-lacZ* or *vexGH-lacZ*). The cultures were diluted 1:100 in LB broth plus or minus arabinose to achieve the indicated final concentration and the cultures were incubated with shaking at 37°C. Aliquots were then collected at the indicated times to measure gene expression using the β-galactosidase assay.

A.5 LUCIFERASE REPORTER ASSAYS

E. coli EC100D*pir+* containing pDT1041 and pDT1124 (or pBAD18) were grown overnight in LB broth before being diluted 1:200 into fresh LB broth with or without 0.2% arabinose. Aliquots (100μL) of the diluted cultures were then distributed in triplicate into the wells of white 96-well microtiter plates with clear bottoms and incubated with shaking at 37°C for the duration of the assay. Luminescence production and the OD_{600} at indicated time points were determined using a BioTek Synergy HT plate reader. The reported results are the average relative light units (RLU) for each test sample divided by the optical density. Two-Way ANOVA with Tukey's multiple comparison post-test was used to determine significance.

C6706 transposon mutants containing pDT1777 were grown overnight in LB broth before being diluted into LB or AKI broth. Strains grown in LB broth were diluted 1:200 from the overnight culture into fresh LB broth with Cm and grown under the same conditions as listed above. Strains grown under AKI conditions were diluted 1:10,000 into AKI broth with Cm. Aliquots (370μL) of the diluted cultures were then distributed in triplicate as described above. Plates were incubated statically at 37°C for 4h, at which point 215μL of culture was removed from each well for a final volume of 155μL/well. Plates were then grown with shaking at 37°C

for the remainder of the assay. Luminescence production and the OD_{600} at indicated time points were determined using a BioTek Synergy HT plate reader. The reported results are the average relative light units (RLU) for each test sample divided by the optical density. Two-way ANOVA with Dunnet's post-hoc test was used to determine significance relative to WT.

A.6 IDENTIFICATION OF CPXR BINDING SITES

Putative CpxR binding sites were identified in the promoter regions of the differentially regulated genes using CloneManager Professional Software (SciEd Software). The search was limited to 300 bp preceding the ATG start codon for each gene and used the published CpxR consensus binding site (i.e. $GTAAN_6GTAA$) with an allowance for limited mismatches without gap insertion (265). To estimate the frequency of CpxR binding sites in the *V. cholerae* genome, we randomly selected 25 genes (15 from the large chromosome and 10 from the small chromosome) using the random number generator function in Microsoft Excel. The promoters of these genes were then searched as described above for the presence of CpxR consensus binding site.

A.7 ANALYSIS OF *CPX* EXPRESSION ON AGAR PLATES

A chromosomal *cpxP-lacZ* reporter was used to examine the expression the Cpx system in a panel of various RND efflux mutants. Overnight cultures of the reporter strains were diluted 100-fold into LB broth and grown at 37°C with shaking for 1 h. The cultures were then normalized to

an OD_{600} of 0.1 and equal volumes of the cultures were spun down and the pellet resuspended in an equal volume of PBS. The resuspended cultures were then diluted 1000-fold before 2 μL was inoculated onto LB agar plates containing 160 μg/mL X-gal. As a positive control the same cultures were also inoculated onto LB agar plates containing 160 μg/mL X-gal and 500 μM $CuCl_2$. The inoculated plates were incubated overnight at 37°C before being photographed using a digital camera.

A.8 ANTIMICROBIAL SUSCEPTIBILITY TESTS

Antimicrobial susceptibility tests were performed using antibiotic and detergent gradient agar plates or by antibiotic disk diffusion. Antimicrobial susceptibility tests using gradient agar plates were performed as previously described (123, 269, 285, 315). Each 9 cm x 9 cm gradient plate was inoculated with six *V. cholerae* strains, including WT which served as an internal control, before being incubated at 37°C. The following day the length of bacterial growth along the antimicrobial gradient was recorded for each strain. Reported values represent the average from a minimum of three independent experiments. The minimum inhibitory concentration (MIC) of each strain was calculated by the percent growth across the plate multiplied by the antimicrobial concentration used in the plate. Statistical significance was calculated as indicated.

Antimicrobial susceptibility tests using disk diffusion were performed as previously reported (119) using the following antibiotic disks purchased from Becton Dickenson (Franklin Lakes, NJ, USA): ampicillin (10 μg/mL), tetracycline (10 μg/mL), gentamicin (10 μg/mL), erythromycin (15 μg/mL), and polymyxin B (300 μg/mL). Test compounds that were not

commercially available were prepared by spotting 20 μL of concentrated stock onto blank 6 mm disks from Becton Dickenson (i.e. 1M $CuCl_2$, 10% sarcosyl, and 10% deoxycholate). LB agar plates were inoculated with a lawn of the test strains using overnight cultures before the disks were aseptically placed on the surface of the agar plates. The plates were then inoculated overnight at 37°C before the zones of growth inhibition were measured in mm using a ruler. The student t-test was used to determine statistical significance in the zones of growth inhibition.

A.9 GROWTH ANALYSIS

Strains were grown overnight in LB broth and normalized to an OD_{600} of 1.0. The cultures were then diluted 1:20,000 into LB broth with or without arabinose and/or deoxycholate as indicated. Aliquots of 100μL of each strain were then distributed in triplicate into the wells of a flat bottom 96-well microtiter plate. The 96-well plate was then incubated in a BioTek ELx808 plate reader at 37°C with intermittent shaking and growth was monitored by measuring the absorbance at 600 nm every 20 min for 14h. The presented data is the average of triplicate samples for each time point.

A.10 PLATING EFFICIENCY

Overnight cultures of the tested *V. cholerae* strains were diluted 100-fold into LB broth or LB broth containing 2M KCl before being incubated at 37°C with shaking for 1 h. The cultures were then normalized to an OD_{600} of 0.25 when equal aliquots of each strain were spun down in a

microfuge and the resulting cell pellet resuspended in PBS. Serial dilutions of the cultures were then inoculated onto LB agar and Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) agar plates using an EDDY JET 2 Spiral Plater (IUL instruments). The agar plates were then incubated overnight at 37°C before the viable cells were enumerated using a Flash & Go automatic colony counter (IUL Instruments).

A.11 CT AND TCP QUANTIFICATION

CT and TCP production were assayed as previously described (123) from cultures grown under AKI growth conditions. CT production was determined by GM_1 enzyme-linked immunosorbent assay (ELISA) as previously described using purified CT (Sigma) as a standard. TCP production was quantified by Western immunoblotting using a polyclonal antibody that was directed towards TcpA, the pilin subunit of the TCP (285). The polyclonal antisera against CT and TcpA were kindly provided by Dr. John Mekalanos (Harvard Medical School, Boston, MA) and Dr. Jun Zhu (University of Pennsylvania). Immunoreactive proteins on the Western blots were visualized using the SuperSignal West Pico Chemiluminescent Detection Kit (Pierce Biotechnology).

A.12 GROWTH ANALYSIS IN THE INFANT MOUSE MODEL

The colonization phenotype of the RND efflux mutants were assessed using the infant mouse competition assay as previously described (123, 283, 285). Briefly, 5–7 day old mice were

separated from their mothers 2 h prior to inoculation. The infant mice were then anaesthetized with isoflurane (Aceto Pharm, NY) and inoculated by gavage using a 30 cm length of 0.011" x 0.024" polyethylene tubing that was attached via a 30.5 GA needle to a 1 cc syringe containing the inoculum. The inoculum consisted of a mixture of the wild type strain (*lacZ*+) and the RND mutant strain (*lacZ*-) at a 1:1 or 1:100 ratio (WT:mutant) and administered in a 50 mL volume that contained ~$2.5x10^4$ cfu of each strain; for the 1:100 inoculum the mutant titer was increased to ~$2.5x10^6$ cfu. An aliquot of the inoculum was also serially diluted and spread onto LB plates containing Sm and X-gal to verify the input ratio. Following inoculation the mice were kept in a humidified incubator at 30°C. The following day, the infected mice were sacrificed and the small intestine was removed from above the cecum and homogenized in 5 mL of sterile PBS. Serial dilutions of the homogenates were then spread onto LB agar plates containing Sm and X-gal. The agar plates were then incubated at 37°C overnight when the resulting bacterial colonies were quantified as WT (*lacZ*+) or mutant (*lacZ*-) based on colony color. A competitive index (CI) was calculated for each mutant strain as the ratio of the mutant to the WT in the input inoculum divided by the ratio of the mutant to WT in the output from the mouse intestinal homogenates. To determine the in vitro competitive index, an inoculum consisting of a 1:1 ratio of the test and control strain was inoculated into fresh LB or media and cultured with shaking overnight at 37°C before being serially diluted and spread onto LB plates containing Sm and X-gal to determine the output ratio. Standard bacteria growth assays in M9-glycerol minimal media were also performed to control for potential unknown metabolic differences that could affect growth of the mutants in vivo. A theoretical CI was calculated for mutant strains that could not be recovered from the mouse challenge experiments by using an artificial value of 1 recovered cfu for each strain.

The infant mouse colonization assay was performed identical to the competition assay with the exception that the inoculum consisted of a single strain of *V. cholerae* with the mice receiving either $2x10^6$ or $2x10^8$ cfu. Following overnight incubation, the intestinal homogenates were serially diluted before being spread onto LB-Sm agar plates to enumerate the bacterial loads in the small intestine of each mouse.

A.13 MICROARRAY ANALYSIS

RNA was isolated for the *cpxA** microarrays from WT and the *cpxA** mutant strain that were grown in LB broth at 37°C with shaking to an OD_{600} ~1.0. The subsequent steps of cDNA preparation and labeling, microarray hybridization, and data collection, were carried out as previously described (316). The experiment was repeated independently three times with two technical replicates for each individual experiment. The resulting data was processed as follows. The background subtracted spot intensities for each gene were subjected to global normalization before being averaged across all experimental samples. Genes exhibiting a change in expression of ≥2-fold with a coefficient of variation ≤ 0.6 across all experiments were defined as being differentially regulated. The microarray data has been deposited in NCBI's Gene Expression Omnibus and is available through accession number GSE55037.

A.14 PURIFICATION OF VEXR

VexR was purified as follows. *E. coli* ER2566 carrying pMAL-c2 or pSS35 were grown overnight at 37°C with aeration. The cultures were then diluted 100-fold into LB broth with Cb and incubated at 37°C with shaking until reaching an OD_{600} of ~0.5 when isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.3 mM and the cultures were incubated for an additional 2 h. The cells were then harvested by centrifugation, the supernatant removed, and the pellet resuspended in column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA) plus 1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were then lysed using a M-11P Microfluidizer processor according to the manufacturer's instructions (Microfluidics). The resulting lysates were cleared of particulate matter by centrifugation at 15,000 x g for 20 min at 4°C. The clarified supernatants (i.e. VexR-MBP or MBP) were diluted 1:6 with column buffer and loaded onto a 0.8 x 7 cm column containing 1 mL of amylose resin (New England Biolabs). The column was washed with 12 column volumes of column buffer before the bound proteins were eluted with elution buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 10 mM maltose). Protein concentrations were determined using the Coomassie Plus (Bradford) Assay kit according to manufacturer's instructions (Thermo Scientific).The purity of the eluted proteins were assessed by SDS–PAGE with Coomassie Brilliant Blue R-250 staining.

A.15 ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

DNA fragments designated vexR1 (the nucleotide sequence between -129 and -46 of the *vexR* promoter region) and vexR2 (-59 to +21 of the *vexR* promoter region) were amplified from the

N16961 genome using the primers *vexR*-F1/*vexR*-R1 and *vexR*-F2/*vexR*-R2, respectively. The fragments were then used as a template for a PCR amplified using the biotin primer resulting in biotin end labeled fragments. The biotin labeled probes (2.5 nM) were incubated with purified VexR-MBP or MBP in amounts ranging from 0 to 250 nM in 10 µl of binding buffer containing 10 mM Tris (pH 7.4), 150 mM KCl, 0.1 mM DTT, 0.1 mM EDTA (pH 8), and 200 µg/mL sheared salmon sperm DNA. The binding reactions were incubated at room temperature for 20 min before being subjected to electrophoresis on a nondenaturing 5% polyacrylamide gel in 0.25x TBE buffer at 200V for 45 min. The DNA in the gel was transferred to a nylon membrane in 0.5x TBE buffer at 380 mA for 1 h. The nylon membrane was then UV crosslinked at 120,000 microjoules using Stratalinker 1800. The biotin labeled DNA probes were then detected using the Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific) and documented using a FluorChem E imaging system (Protein Simple).

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APPENDIX B

ABBREVIATIONS

 # : number

% : percent

:: : insertion

~ : approximately

± : plus/minus

°C : degree centigrade

ANOVA : analysis of variance

β : beta

bp : base pair

Cb : carbenicillin

cc : cubic centimeter

cDNA : complementary DNA

cfu : colony forming units

CI : competitive index

cm : centimeter

Cm : chloramphenicol

cpxA* : cpxA mutant

CpxR~P : phosphorylated CpxR

CT : cholera toxin

$CuCl_2$: copper chloride

Δ : deletion

DNA : deoxyribonucleic acid

DTT : Dithiothreitol

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

***E. coli* :** *Escherichia coli*

EDTA : Ethylenediaminetetraacetic acid

ELISA : enzyme-linked immunosorbent assay

g(centrifuge) : gravitational acceleration

GA : gauge

GI : gastrointestinal

GM_1 : monosialotetrahexosyl ganglioside

h : hour

***H.drucreyi* :** *Haemophilus ducreyi*

HCl : hydrochloric acid

i.e. : id est; latin for “that is”

IPTG : Isopropyl β-D-1-thiogalactopyranoside

kb : kilo bases

KCl : potassium chloride

Km : kanamycin

lacZ : in reference to the b-galactosidase gene

LB : luria bertani

lux : in reference to the luciferase operon

M : molar

mA : milli Amps

MAR : multiple antibiotic resistance

MATE : multi antimicrobial extrusion protein family

MBP : maltose binding protein

MFP : membrane fusion protein

MFS : major facilitator superfamily

μg : micro gram

MIC : minimum inhibitory concentration

mL : milli liter

μL : micro liter

mm : milli meter

μm : micro meter

MU : Miller Units

NaCl : sodium chloride

NCBI : National Center for Biotechnology Information

ng : nano gram

NG : No bacterial growth

nm : nano meter

nM : nano Molar

number" : inch

OD : optical density

OD_{600} : optical density at 600 nano meters

OMP : outer membrane protein

P : P value

PAGE : Polyacrylamide gel electrophoresis

PBS : phosphate buffered saline

PCR : polymerase chain reaction

pH : measurement of acidity/basicity of an aqueous solution

PMSF : phenylmethylsulfonyl fluoride

RLU : relative light units

^{R} : resistance

RNA : ribonucleic acid

RND : Resistance-Nodulation-Cell Division family

s.d. : standard deviation

SDS : sodium dodecyl sulfate

Sm : streptomycin

SMR : small multidrug resistance family

TBE : Tris/Borate/EDTA

TCBS : Thiosulfate-citrate-bile salts-sucrose

TCP : Toxin co-regulated pilus

Tris : tris(hydroxymethyl)aminomethane

UV : ultra violet

V : volts

***V. cholerae* :** *Vibrio cholerae*

WT : wild type

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

APPENDIX C

PRELIMINARY DATA: INDOLE REPRESSES VIRULENCE FACTOR PRODUCTION

C.1 INTRODUCTION

The toxin co-regulated pilus (TCP) and cholera toxin (CT) are two virulence factors important for the colonization and pathogenicity of *V. cholerae*. They are encoded by the genes *tcpA-F* and *ctxAB*, respectively, whose expressions are coordinately regulated under a complex hierarchy called the ToxR regulon (as reviewed in Section 1.3). The production of these two virulence factors was significantly reduced upon the loss of RND-mediated efflux, whether by chemical inhibitors or genetic manipulation (Section 2.2.3 and (123, 285)). The reduction of CT and TCP production corresponded with the downregulation of the ToxR regulon.

The RND efflux systems are a family of multidrug transporters which have not been implicated in the direct binding and regulation of genes (as reviewed in Section 1.4). Nor are the *V. cholerae* RND efflux systems required for the assembly and/or extrusion of TCP and CT. Therefore this indicated that the RND efflux systems contributed to virulence factor production through their cognate ability of transporting small molecules (as discussed in Section 2.3). Indeed, as the expression and activity of the ToxR regulon has been shown to be modulated by

various small molecules (76-83, 113), it was suggested an endogenous substrate of the RND efflux systems accumulates in their absence and can repress *ctxAB* and *tcpA*.

V. cholerae has six RND efflux systems, all of which have been implicated in contributing to virulence factor production (Section 2.2.3). The four RND efflux systems which were major contributors to CT and TCP production (VexAB, VexCD, VexGH, and VexIJK) were also characterized as providing intrinsic antimicrobial resistance against common substrates (i.e. the bile salts cholate and deoxycholate) (Sections 2.2.1 and 2.2.3). Together, this suggested that the RND efflux systems have overlapping substrate specificities and that a common substrate of these accumulates upon loss of RND efflux to repress CT and TCP.

Characterization of the *V. cholerae* RND efflux systems has revealed an intrinsic role in extruding metabolic waste (as discussed in Section 4.3). The tryptophan biosynthetic intermediate indole was found to be a substrate of the RND efflux systems (Sections 4.2.9-4.2.11). Indole has also been reported to be a cellular cue which can regulate the expression of genes in various bacteria species, including *V. cholerae* (293, 317-319). Therefore, indole was a possible candidate for the inhibition of CT and TCP.

C.2 RESULTS

As indole was a substrate of the RND efflux systems, it was hypothesized that indole would accumulate upon the loss of RND-mediate efflux and repress the production of CT and TCP. In order to test this hypothesis, we assayed the ability of *V. cholerae* N16961 to produce virulence factors in the presence of various concentration of indole. The results showed a concentration

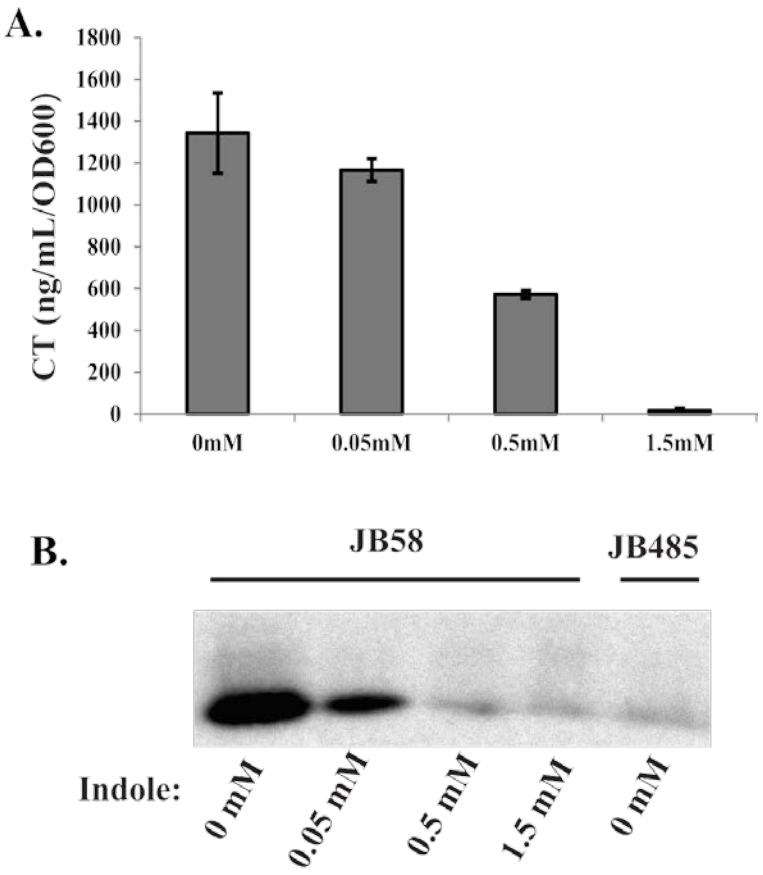

Figure 24. Indole repression of *V. cholerae* CT and TCP production.

V. cholerae N16961 strains were grown under AKI conditions with or without the indicated concentrations of indole. (A) The CT production of JB58 was detected by CT GM_1-ELISA. Error bars represent the standard deviation of the mean from three or more experiments. (B) TCP of JB58 and JB485 was detected by TcpA Western immunoblotting.

dependent inhibition of CT and TCP production in the presence of indole. The addition of 0.5 mM indole caused a 57.4% reduction in CT relative to WT, while 1.5 mM of indole reduced CT production 98.8% (Figure 24A). The production of TCP was similarly reduced in a dose dependent manner (Figure 24B). As *V. cholerae* strains have been reported to produce up to 0.6 mM of indole in culture supernatants (293, 310), this data indicates that biological levels of indole can repress the production of CT and TCP. The reduction of CT and TCP in WT treated with 0.5 mM indole is reminiscent of the RND deficient strain, which exhibited an ~70% reduction in CT/TCP compared to WT (Section 2.2.3 and (123)). These results support our hypothesis that the reduction of virulence factor production in the RND deficient strain is due to the accumulation of metabolic intermediates that are normally efflux by the RND efflux systems.

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