Characterization of the Cpx Response in *Vibrio cholerae*

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Microbiology and Biotechnology

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Abstract

The gram negative bacterial cell envelope is composed of the outer membrane, the periplasm and the inner membrane. These compartments are exposed directly to changes in the environment that are sensed and adapted to through different signaling transduction pathways. This often occurs through two-component signal transduction systems (TCS), which are broadly distributed among different bacterial species. The Cpx pathway is a TCS that employs the sensor histidine kinase CpxA and the response regulator CpxR, and regulates crucial adaptations to envelope stress response that affects many functions, including antibiotic resistance, across bacterial species. This system has also been implicated in the regulation of a number of envelope localized virulence determinants across bacterial species. The first goal of this thesis was to characterize the Cpx regulon members in the human pathogen *Vibrio cholerae* when the Cpx pathway is activated. For this purpose I characterized the transcriptional profile of the pandemic *V. cholerae* El Tor strain C6706 upon overexpression of *cpxR,* and the inducing cues that lead to the activation of the Cpx pathway. My data shows that the Cpx regulon of *V. cholerae* is enriched for genes encoding membrane localized and transport proteins, including a large number of genes known or predicted to be iron-regulated. The *V. cholerae* Cpx regulon included three strongly Cpx-regulated, putative ferric reductases that are likely directly regulated by CpxR. I present evidence that the function of these ferric reductases is likely tied to the up-regulation of iron-related genes by the Cpx response. Activation of the Cpx pathway also led to the expression of TolC, the major outer membrane pore, and components of two resistance-nodulation-division (RND) efflux systems in *V. cholerae.* I found that iron chelation, toxic compounds, or deletion of specific RND efflux components lead to Cpx pathway activation. Further, mutations that eliminated the Cpx response or members of its regulon resulted in growth phenotypes in the

presence of these inducers that, together with Cpx pathway activation, are partially suppressed by iron. Cumulatively, these results suggest that a major function of the Cpx response in *V. cholerae* is to mediate adaptation to envelope perturbations caused by toxic compounds and the depletion of iron. A second goal of this thesis was to characterize the effect of Cpx pathway activation on *V. cholerae* virulence factors. I found that activation of the Cpx pathway leads to a decrease in expression of the major virulence factors in this organism, cholera toxin (CT), and the toxin-coregulated pilus (TCP). The Cpx response controls virulence factor expression by repressing expression of the ToxT and TcpP regulators. I showed that the effect of the Cpx response on *ctxB* and *tcpA* expression is mostly abrogated in a cyclic adenosine monophosphate (cAMP) receptor protein (CRP) mutant, although expression of the *crp* gene is unaltered. These observations indicate that CRP function is affected by Cpx response activation. Altogether, the data presented here suggest a model whereby the Cpx response reduces production of CT and TCP by controlling the expression and function of regulators that function on the ToxR regulon in *V. cholerae*.

Preface

Some of the research conducted for this thesis forms part of a research collaboration, led by Professor T. L. Raivio at the University of Alberta, with Professor S. Pukatzki being the lead collaborator at the Department of Medical Microbiology & Immunology, University of Alberta. The *ex-vivo* animal model experiments referred to in Chapter 4 were performed by members of Professor S. Pukatzki's research group. The high throughput killing screen referred to in the Appendix was design and performed by myself and D. Unterweger, a PhD student in Professor S. Pukatzki research group.

Most of the content of Chapter 2 of this thesis has been published as N. Acosta, S. Pukatzki, and T.L. Raivio, "The *Vibrio cholerae* Cpx envelope stress response senses and mediates adaptation to low iron," Journal of Bacteriology, vol. 197, issue 2, doi:10.1128/JB.01957-14. I was responsible for the data collection and analysis as well as the manuscript composition. S. Pukatzki contributed to manuscript edits. T.L. Raivio was the supervisory author and was involved with concept formation and manuscript composition and edits.

Acknowledgments

I would like to thank my supervisor Dr. Tracy Raivio for, first, giving me the opportunity of joining her lab, and also for all her support and feedback during my PhD studies. It was a pleasure to work with you not only for all your knowledge, experience and coaching, but also for your incredible kindness. I learnt many things from you; you gave me the tools to become a scientist and the trust to develop my projects. Thank you so much for all your advice and being part of this important step in my life.

Thanks to my committee members, Dr. Stefan Pukatzki, for the great support in all aspects on the projects that we worked together, you were an important part in my academic development; it was a real pleasure to work in collaboration with you, I learnt valuable things from you and your lab members; and Dr. Christine Szymanski, for all your time, valuable and helpful comments, advices, and feedback that contributed a lot in this thesis. Thanks also to my thesis examiners, Dr. Mario Feldman and Dr. Victor J. DiRita, for accepting being part of my committee and for your time and effort.

Thank you to all my lab members, past and present. Stef, Roxana, Shannon, Julia, Stefan, Suey, Junshu, Randi, Margarita, and Alex- I really appreciate all your help and advices that contributed to this thesis. I learnt many things from each of you such as your kindness, collaboration and most importantly have fun doing science. It was a pleasure to work with this wonderful team. Also thanks to all Pukatzki's lab members, for your collaboration in many steps of this thesis, especially to Daniel who was a great person to work with.

Mom you have always been my engine, my motivation to go a step further; I really appreciate and thanks all your effort and support through my entire life. Thanks to you I am the person who

I am. Natalia, Melissa and Juan Sebastian you are not only my siblings but also my friends, I know I can always count on you.

Last but not least, David I thank you for being always with me, for supporting me and teaching me how to be a better person during any time no matter how difficult it is. We came here to reach this important academic step in our life but along this process we began a big journey full of joy and love. I can't wait to see what our next adventure is, but something that I know for sure is that no matter what, it will always be special and full of love.

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Appendix A

Appendix B

List of Nomenclature and Abbreviations

ABC: ATP-binding cassette

AMP: Ampicillin

A/E: Attaching and effacing

AMK: Amikacin

BAP: Bacterial alkaline phosphatase

BFP: Bundle forming pili

CAM: Chloramphenicol

CAMPs: Cationic antimicrobial peptides

cAMP: Cyclic adenosine monophosphate

CDPs: Cyclic dipeptides

CI: Clinical isolates

CPS: Counts per second

CRP: cAMP receptor protein

CT: Cholera toxin

CU: Chaperon usher

DAVID: Database for annotation, visualization, and integrated discovery

Dcytb: Duodenal cytochrome b_{561}

DIAMIDE: N,N-dimethylamide

DTT: Dithiothreitol

DMEM: Dulbecco's modified Eagle's medium

ELISA: Enzyme linked immunosorbent assays

HK: Histidine kinases

IM: Inner membrane

IMPs: IM proteins

IPTG: Isopropyl-β-D-thiogalactopyranoside

KAN: Kanamycin

LB: Luria-Bertani

LPS: Lipopolysaccharides

MDH: Malate dehydrogenase

MF: Membrane fusion

MFS: Major facilitator superfamily

MTR: Multiple transferable resistance

OM: Outer membrane

ONPG: O-Nitrophenyl β-D-galactopyranoside

OMPs: OM proteins

PAI: Pathogenicity island

PBPs: Penicillin binding proteins

PBPs: Periplasmic binding proteins

PMF: Proton motive force

POTRA: Polypeptide transport-associated

PPIases: Peptidyl-prolyl cis/trans isomerases

PWM: Position weight matrix

RND: Resistance-nodulation-cell division

RR: Response regulator

SM: Streptomycin

SMF: Sodium motive force

SPC: Spectinomycin

sRNA: small RNA

T1SS: Type I secretion system

T2SS: Type II secretion system

T3SS: Type III secretion system

T4SS: Type IV secretion system

T4P: type IV pili

T5SS: Type V secretion system

T6SS: Type VI secretion system

TCS: Two component system

TCP: Toxin co-regulated pilus

1 Chapter 1: General Introduction

1.1 Gram-Negative Bacterial Cell Envelope

Gram-negative bacteria possess a cell envelope that allows it to communicate, interact and protect itself from the environment. Additionally, this compartment is involved in functions such as selective passage of nutrients and waste products, motility, and cell integrity and shape (1). There are three principal components in the cell envelope; the outer membrane (OM), the periplasm, and the cytoplasmic or inner membrane (IM) (Figure 1-1).

1.1.1 *Outer membrane (OM)*

The OM is a selective barrier that allows protection from harmful or toxic components that are present in the environment (e.g. antibiotics). It is an asymmetrical bilayer composed of phospholipids (inner leaflet) and glycolipids (outer leaflet), that latter of which is composed mainly of lipopolysaccharide (LPS) (2) (Figure 1-1). The LPS is composed of lipid A, a core oligosaccharide and an O-antigen polysaccharide which varies in length (3). In general, the composition of the OM is enriched in saturated fatty acids and phosphatidylethanolamine (4-6). In addition to lipids, two types of proteins are found in the OM: Integral OM proteins (OMPs) and OM lipoproteins (7). The OMPs consist of antiparallel amphipathic β-strands that fold as β-barrel proteins (8). OMPs are synthesized in the cytoplasm with an N-terminal signal sequence and are then translocated across the IM by the Sec system (8). Once this OMP are translocated into the periplasm in an unfolded state, they interact with periplasmic proteins, principally with SurA which prevents the aggregation or misfolding of the OMP in the periplasm (9). In addition, there are other periplasmic proteins involved in the proper assembly of OMP such as Skp, FkpA and two periplasmic peptidyl-prolyl cis/trans isomerases (PPIases), PpiA and PpiD (10). The OMPs are then inserted into the OM by the Bam complex. This complex is highly conserved during evolution as it is conserved across all Gram-negative species, and it also has orthologs in

the mitochondria and chloroplasts of eukaryotes (11-13). The Bam complex is composed of the five β-barrel assembly machine components (i.e. BamA, -B, -C, -D, -E) in which BamA and BamD are essential for OMP insertion function (14, 15). In the current model, unfolded OMP bind to the polypeptide transport-associated (POTRA) domains of BamA which then trigger β-structure formation (9).

On the other hand, OM lipoproteins are anchored in the membrane through an N-terminal N-acyl-diacylglycerylcysteine and are transported to the OM by the Lol system (16). In this process, the acylation and folding take place after OM lipoprotein translocation by the Sec system (17). Lipidation occurs at the outer leaflet of the IM, where the Lgt enzyme transfers a diacylglyceryl moiety to the +1 cysteine of the conserved lipobox motif found at the N-terminal lipoproteins (18). Then the Lol system, which is composed by an ABC transporter in the IM (i.e. LolC, LolD and LolE), a periplasmic chaperone (i.e. LolA) and an OM receptor (i.e. LolB), is involved in the transport of OM lipoproteins to the OM depending on the absence of a few amino acid residues, which directly follows the lipidated residue (19).

1.1.2 *Periplasm*

The periplasm is an aqueous cellular compartment that occupies around 10% of the cell volume (20) and it is more viscous that the cytoplasm (21). It is composed of soluble proteins and the peptidoglycan layer (Figure 1-1). In addition, other periplasmic proteins are required for different roles such as transport and chemotaxis, cell envelope biogenesis and protein folding and degradation processes, which are required for cell envelope homeostasis (described in Section 1.3 and 1.4).

Because the periplasm is an oxidizing compartment, some of the soluble proteins are involved in catalyzing the formation of disulfide bonds (22). In this process the DsbA enzyme is involved, which is a periplasmic thiol-oxidoreductase that interacts with the cysteine residues of the substrate proteins and catalyze the introduction of disulfide bonds (23). Once DsbA is reduced it is reoxidized by an IMP, DsbB, which then transfers the electrons from the reduction to the membrane associated quinones (24). In addition, DsbC and DsbD proteins are involved in the rearrangement of incorrect disulfide bonds that are catalyzed by DsbA, through the recognition of misoxidase or misfolded proteins by DsbC, which is a disulfide isomerase, that is maintained in its reduced state by DsbD (25, 26).

Formation and transfer of disulfide bonds of proteins in the periplasm is a key component for bacterial virulence, since disulfide bond formation is required for many virulence factors in pathogens (27). For example, secreted toxins such as members of the AB5 toxin family (e.g. cholera toxin (CT) and the *Escherichia coli* heat labile toxin (Etx)) require disulfide bonds for the two subunits of the toxic fragment (i.e. A subunit) and the pentameric fragments (i.e. B subunit) for its proper folding (28). Furthermore, other examples in which the thiol-disulfide oxidoreductases activity of DsbA is important for virulence are for the proper formation of the *E. coli* heat-stable toxin (ST), the P Pili in uropathogenic *E. coli* (UPEC) and type 4 pili (e.g. Bundle-forming pili (BFP) and toxin-coregulated pili (TCP)) in enteropathogenic *E. coli* (EPEC) and *Vibrio cholerae* among others (27).

Finally, the peptidoglycan layer in the periplasm serves as an extracytoplasmic cytoskeleton that is required for bacterial cell shape and protection against osmotic lysis (29). The peptidoglycan is composed of glycan chains, which are repeating units of the disaccharide N-acetyl glucosamine-N-acetyl muramic acid, crosslinked by oligopeptides and by a β-1,4

glycosidic bond (30). Biosynthetic and hydrolyses enzymes required for peptidoglycan synthesis and re-modeling during cell division are localized in the periplasm. Some of those hydrolyses are involved in the cleavage of glycoside and amide bonds, such as carboxypeptidases and endopeptidases that trim peptides present in the peptidoglycan, amidases that remove peptides from the glycan chain, and transglycosylases that cleave the glycan chains(31). Additionally, synthases are required in the periplasm, such as transpeptidases (e.g. penicillin binding protein (PBP) 2 and PBP3) that are anchored in the IM and are involved in the attachment of the peptides of a nascent peptidoglycan chain with an existing chain (32). In addition there are some proteins that are associated with the peptidoglycan layer such as Braun's lipoprotein (Lpp), a small protein (58 amino acids), that is involved in anchoring the peptidoglycan layer to the OM (33).

1.1.3 *Inner membrane (IM)*

Different functions and cellular process are performed at the IM, such as respiration, metabolite exchange, lipid biosynthesis, protein secretion, transport, ATP generation and signal transduction (e.g. two-component system, described in Section 1.2) (1). The IM is a bilayer composed of phospholipids and proteins. Although, the composition of lipids varies between bacterial species, in *E. coli* and *V. cholerae* phosphatidylethanolamine comprises 70-80%, followed by phosphatidylglycerol (15-20%) and cardiolipin (≤ 5%) (34-36). As in the OM, there are two types of proteins in the IM: lipoproteins and integral IM proteins (IMPs) (Figure 1-1). IM lipoproteins are anchored in the inner membrane depending on their sorting signal, the residue immediately after the lipid-modified Cys residue, through an N-terminal N-acyl-diacylglycerylcysteine to the periplasmic leaflet (16). IMPs mainly contain α-helical bundles with α-helical membrane-spanning regions which require translocases and insertases for insertion into the inner membrane (37).

1.1.4 *Gram-negative surfaces structures*

Bacteria produce proteinaceous like organelles on their cell surface. These cell surface structures, such as pili, flagella and injectisomes, play important roles in adhesion, motility, secretion, uptake, infection and pathogenicity (38). For example, Gram-negative bacteria use structures such as pili in order to recognize and attach to their host cell, which recognize specific receptors in the cell, and bind to them through adhesins (39). One example of pili is the type IV pili (T4P) which is composed of thousands of assembled pilins subunits (40) and it is involved in adhesion to the cell surface, twitching motility, DNA uptake and cell signaling, among others (40, 41). For example, the TCP of *V. cholerae* is a T4P required for small intestine colonization (42).

Two general mechanisms have been described for pilus biogenesis assembly, in which the first one utilizes ATPases to power pilus assembly (43). In the second mechanism, the chaperon usher (CU) pathway, the assembly of the pili takes place at the OM; where a chaperone facilitates the folding of pilus subunits, which are targeted to a pore forming protein, the usher, which assembles the subunits into a pilus which is then secreted (44).

The flagellum is a long filament which is responsible for cell motility through the rotation of flagellar filaments in both counter-clockwise (CCW) and clockwise (CW) directions (45). The flagellum is a large macromolecular complex composed of several proteins, around 30 different ones (46) which constitute the three main structures in the flagellum: the basal body (or reversible motor), the filament (or helical screw) and the hook (or universal joint), that connects the basal body and the filament (47). The basal body is localized in the IM, whereas the hook and the filament extend outwards the cell surface (48). In addition, the reversible motor could be driven by either proton or sodium motive force (PMF or SMF, respectively) (49). For example, *V.*

cholerae, which is a highly motile bacterium, possesses a single polar flagellum, which is driven by SMF (50). Finally, the transcription of the flagellar regulon is organized by a transcriptional hierarchy known as flagellar gene hierarchy, where there is a temporally regulation of the genes involved in flagellar assembly (47, 51).

Gram-negative bacteria have developed the ability to deliver effector proteins into the host cytosol or extracellular milieu through specialized injection systems. Six secretion systems have been described in Gram-negative bacteria (52). The Type I secretion systems (T1SSs) are tripartite systems which facilitate the transport of proteins across the cell envelope. T1SSs are composed of an ATP-binding cassette (ABC) transporter, an adaptor protein and an outer membrane pore (53). The Type II secretion systems (T2SSs) use a two-step mechanism for translocation, where the effector protein is first translocated through the IM by the Sec or the Tat pathways and then it is translocated by the T2SS through the outer membrane (54). For example, *V. cholerae* uses the T2SS to secrete the CT in a folded state from the periplasm across the outer membrane (55). The type III secretion system (T3SS) is a complex system which needs more than 20 proteins for it assembling (56). The T3SS is important in the virulence of pathogenic bacteria (e.g. *Yersinia* spp., *Shigella* spp., *Salmonella* spp., *Pseudomonas* spp., and in *E. coli*); and it has been demonstrated that the lack of this system usually leads to render the ability of bacteria to cause infection (57). For example, in *V. cholerae* the presence of a T3SS has been demonstrated to be essential for the pathogenesis of non-pandemic *V. cholerae* strains (i.e. AM-19226 strain) (58).

The type IV secretion system (T4SSs) secretes a wide range of substrates, such as single proteins, protein-protein complexes and protein-DNA complexes, directly into host cells by a one-step or a two-step mechanism (59). The type V secretion systems (T5SSs) use the Sec

pathway to translocate either the precursor protein (for the autotransporter system) or the translocator and effector proteins (for the two-partner secretion systems) across the IM (60). Once they are located in the periplasm the translocator domain of the precursor protein is inserted in the OM and then allows the surface localization of the passenger domain. In the two-partner secretion system, the translocator protein allows the secretion of the effector protein across the OM (61). Finally, the T6SS is encoded by many Gram-negative bacteria and it is found as a gene clusters that consists of approximately 15–25 conserved genes. This is likely to be the most common of the large specialized secretion systems in bacteria (see description on Appendix B) (62).

1.2 Two-Component Signal Transduction Systems (TCS)

Two component systems (TCS) are the most prevalent signaling pathway in bacteria, and they are involved in the response to many different inducing cues, mainly related to stress response and the environment (63). The TCSs have been studied in bacteria since they were first characterized in genetic screens in *E. coli* mutants, which could not survive under nitrogen starvation, and cell envelope stress conditions (64). TCSs are generally composed of a sensor kinase and a response regulator (65).

1.2.1 *Sensor histidine kinases*

Sensor histidine kinases (HK) are localized in the inner membrane of Gram-negative bacteria and have a periplasmic domain, which is thought to be the receiver of the signal that is then transduced across the membrane to trigger a change in the cell in relation to a stimulus (Figure 1-2). Upon the recognition of an input signal by the sensor kinase, it autophosphorylates itself, using ATP as donor, at a conserved histidine residue. After that, the sensor kinase becomes a

phosphodonor that transfers a phosphoryl group to its cognate response regulator (RR) (Figure 1-2) (66). When there are no inducing cues the histidine kinase can sometimes function as a phosphatase to dephosphorylate its cognate response regulator (66). In addition to the phosphatase activity of histidine kinases, there are other mechanisms that modulate response regulator activity. For example, small inhibitory proteins or peptides, such as IraP, which has been discovered in *E. coli* and regulates the response regulator RssB (67). RssB controls RpoS, an alternative σ factor in stationary phase in this bacterium (68). Other inhibitory proteins are CpxP, which is part of the Cpx two component system (described in Section 1.4) and modulates the autokinase activity of the sensor kinase CpxA in *E. coli* (69, 70), and SdA, which inhibits the sensor kinase KinA/B in *Bacillus subtilus*, that is involved in sporulation (71).

1.2.2 *Response regulators*

Once the response regulator is phosphorylated at its conserved aspartate phosphorylation site by the sensor kinase, it induces a conformational change in the C-terminal transcriptional regulatory domain that drives its homodimerization with other response regulator proteins (65, 72). Such change leads to the activation of its DNA binding domain that allows the control of gene expression, either by activating or repressing transcription (Figure 1-2). The DNA-binding domains are categorized based on sequence similarity with the three major classes of response regulators (i.e. OmpR-like, NarL-like and NtrC-like) (66). Additionally, the phosphorylation of the response regulator can not only lead to changes in gene expression by participating in DNA binding and transcriptional control, but sometimes also affects other processes such as enzymatic activities, binding to RNA and enabling protein-protein interactions (73).

1.2.3 *Phosphotransfer specificity*

Many of the response regulators that have been described are only active when phosphorylated, thus ensuring specificity in response to an inducing signal sensed by the cognate sensor kinase as well as in the response towards the stimulus (74). The specificity of the phosphoryl group transfer to the response regulator is determined by the molecular recognition between the histidine kinase and the response regulator (75, 76). For example, Skerker and collaborators (77) described how the specificity of TCSs relies on the intrinsic ability of a histidine kinase to recognize its cognate response regulator by analysing different chimeras of the *E. coli* sensor kinase EnvZ. That study showed that a specific cluster of amino acids in the histidine sensor kinase is an important determinant of its substrate specificity (77). Additionally, it has been shown that other mechanisms may be involved in the specificity of recognition. A study of two histidine kinases, EnvZ and CpxA (described in Section 1.4), and their cognate response regulators, OmpR and CpxR (described in Section 1.4), respectively, showed that in the absence of EnvZ, CpxA can phosphorylate OmpR or in the absence of CpxA, EnvZ phosphorylates CpxR. However, this does not happen when both histidine kinases are present in *E. coli* (78). These results suggest that the abundance of a response regulator under a given condition can prevent cross talk between two component pathways (66).

1.3 Cell Envelope Stress Responses

Bacteria encounter different changes in their environment such as changes in temperature, osmolarity, and pH, which may cause the disruption of cell envelope homeostasis. Cell envelope stress responses are transcriptional responses that can sense several stressors (e.g. heat, ethanol, oxidative stress and chemical treatment), that may impair different components of the cell

envelope. During this process signal transduction systems are necessary in order to transduce the information from the cell envelope to the cytoplasm for gene expression regulation (79). Five envelope stress responses have been identified in *E. coli*: the σ^E, Bae (Bacterial Adaptive Response), Rcs (Regulator of Capsular Synthesis), Psp (Phage -Shock Protein) and Cpx (Conjugative Pilus Expression) responses. In general, the Cpx, σ^E, Rcs and Psp pathways play an important role in the maintenance of the cell envelope and regulation of virulence factor production (79).

1.3.1 *The σ^E pathway*

To begin the process of transcription initiation in response to different stimuli, multisubunit polymerases require specific σ factor(s) in addition to the RNA polymerase machinery (80). The alternative sigma factor σ^E (or RpoE) is the transcriptional regulator of the σ^E pathway, which is essential in some organisms for colonization (81). RpoE was initially discovered as a supplementary heat shock sigma factor required for the transcription of σ^{32} (or RpoH) in response to conditions that impair cell envelope homeostasis, specifically growth at high temperature (82). Many genes have been described to be part of the σ^E response which play an important role in the signal transduction cascade that senses misfolded or mislocalized proteins in the periplasm (83). These include genes that encode protein folding or degradation factors, thiol:disulfide oxido-reductases, and other genes with unknown function (81). Furthermore, RpoE activity modulates the expression of outer membrane proteins (OMPs) (e.g. OmpX, OmpT, OmpF and OmpC) (84). Compounds that generate misfolded proteins (e.g. ethanol, DTT (dithiothreitol)) activate RpoE, which autoregulates its own expression (85). Additionally, mutations in genes that encode proteins involved in the response to misfolded proteins in the cell envelope (e.g. *dsb*, *degP*, *surA* and *fkpA*) also increase RpoE activity (86).

Under non inducing conditions the σ^E pathway is repressed by the inhibition of RpoE by RseA, a membrane bound anti-sigma factor. Crystallization of the cytoplasmic domain of RseA in complex with RpoE showed that the RseA-RpoE interaction inhibits the association of RpoE with core RNA polymerase (87). In addition, the RpoE:RseA interaction is affected by the periplasmic protein RseB, which modulates the stability of the RpoE-RseA complex (88). The RseB:RseA:RpoE complex prevents RseA degradation by the DegP and DegS proteases (89). When bacteria encounter cell envelope stressors (e.g. mislocalized OMPs, shift in temperature and accumulation of LPS), the interaction between RpoE-RseA is disrupted first by the release of RseB from RseA due to its interaction with LPS (83, 90, 91). Subsequently, the protease DegS binds to mislocalized OMPs through its PDZ domains, specific protein-protein interaction motifs, activating the DegS catalytic activity which results in the cleavage of RseA (92). Next, RseP, a cytoplasmic membrane metalloprotease, and the cytoplasmic heat-shock proteases ClpXP and Lon finish the degradation RseA (93-95). This process allows the release of the RpoE factor and consequently its binding to the RNA polymerase holoenzyme which then induces the expression of RpoE dependent genes encoding chaperones, proteases, and others (81). RpoE also reduces the expression of several OMPs through the regulation of inhibitory small RNAs (sRNAs) (96).

The σ^E pathway is conserved in many Gram-negative bacteria and it has been demonstrated to be involved with virulence (86). For example, in *Salmonella enterica* serovar Typhimurium, *rpoE* mutants showed decrease in proliferation and survival in macrophages and increased susceptibility towards reactive oxygen species and antimicrobial peptides (97, 98). In addition, in *S. enterica* serovar Typhi deletion of *rpoE* resulted in a defect in motility, demonstrating that RpoE promoted the expression of flagellar genes. This is thought to occur through the regulation of the expression of *fliA* in this organism (99). In *Pseudomonas aeruginosa* AlgU (*rpoE*

orthologue) is involved in the transcription of the alginate biosynthetic operon, a capsular polysaccharide that is important in biofilm formation and disease causation (100). Deletion of *algU* in this pathogen generated defects in the formation of biofilms (101). In *Vibrio parahaemolyticus* deletion of *rpoE* increased sensitivity to polymyxin B, ethanol, and high-temperature stresses and generated defects in colonization (102). Furthermore, in both *Mycobacterium tuberculosis* and *Burkholderia pseudomallei* the mutation of *rpoE* reduced survival in macrophages (103, 104).

1.3.2 *Bae stress response*

The Bae response is regulated by a bacterial two-component regulatory system, which includes the sensor histidine kinase, BaeS, and the response regulator BaeR (105). The Bae response was first identified in a study that characterized the molecular mechanism leading to the control of *spy* expression in response to envelope stress (105). Spy (for spheroplast protein Y) is a periplasmic protein whose expression is induced in response to spheroplast formation (106) and it is also part of the Cpx regulon in *E. coli* (69). Recently, it has been shown that Spy is a novel ATP-independent periplasmic chaperone involved in preventing thermal aggregation of malate dehydrogenase (MDH) (107). Under inducing conditions BaeS senses environmental changes and transduces the information via phosphorylation of BaeR (108). Once BaeR is phosphorylated, it acts as a dimer and regulates the expression of its own operon (i.e. *baeS* and *baeR* genes), the *spy* gene, and genes involved in drug resistance and metal resistance. For example, the Bae system regulates the expression of the *mdtABC* operon, which encodes a resistance-nodulation- division (RND)-type efflux system, *mdtD* which belongs to the major facilitator superfamily (MFS) for metal transport and *acrD*, which encodes a component of a RND-type efflux system (105, 109, 110). The BaeR binding site 5'-

TTTTTCTCCATNATTGGC-3' is found at the promoter region of the *mdtABCD-baeSR* operon, *acrD* and *spy* genes (111). Some of the inducing cues that have been described for this stress response are induction of spheroplasting, overexpression of the pilin subunit PapG in the absence of its chaperone, exposure to compounds such as indole, tannin, flavonoid, sodium tungstate, and high levels of zinc or copper (105, 108, 112). Leblanc and collaborators (108) proposed that some of the described inducing cues that trigger the Bae response system (e.g. flavonoids and metals) may be involved in a process that generates oxidative stress and membrane damage, that consequently leads to the induction of the Bae response (108).

Studies have shown that overexpression of the response regulator BaeR leads to increased resistance to β-lactam antibiotics, novobiocin and deoxycholate (109, 113, 114). Additionally, deletion of *baeR* impairs adaptation to subinhibitory concentrations of indole (105). It has also been shown that mutation in the TolC-dependent efflux pumps in *E. coli* increases the activation of the Bae system, suggesting that BaeS may sense a metabolite that is effluxed via the AcrD and/or Mdt efflux pumps (115). Finally, previously it was shown that some of the regulon members of the Bae system are part of the transcriptome that changes in response to zinc (112). This suggests a possible relationship between the Bae system and zinc homeostasis in *E .coli* (116). In support of this idea, it was shown that the Bae response is involved with zinc detoxification (116). In summary, the Bae response is involved with the regulation of genes that are required for getting rid of specific envelope-damaging MdtABC substrates in order to maintain envelope homeostasis (108).

1.3.3 *Rcs stress response*

The Rcs response was first identified in a study that characterized genes involved in capsular polysaccharide biosynthesis (117). In that study it was found that *rcsA* and *rcsB* (for regulator of

capsular synthesis) are positive regulators of capsule synthesis and *rcsC* is a negative regulator of this process (117). Later studies showed that the mechanism by which the Rcs pathway modulates cell envelope homeostasis is by regulating the expression of genes involved in monitoring and maintaining cell surface components (86).

The Rcs stress response is regulated by a bacterial two-component regulatory system, which includes the sensor histidine kinase RcsC and the response regulator RcsB (118). In addition, RcsD, an inner membrane localized phosphorelay protein is required for the phosphorylation and dephosphorylation of the response regulator (119). In this phosphorylation process the signal travels from the histidine domain (H1) in RcsC to its aspartate domain (D1), then from there to the conserved histidine domain (H2) in RcsD. Finally, phosphate is transferred from the H2 domain in RcsD to the aspartate domain (D2) in RcsB (120). Recently a new domain has been identified in the N- terminal region of the phosphotransferase domain of RcsD named RcsD-ABL (for α/β/loop domain) (121). This RcsD-ABL domain stabilizes the interaction between RcsD and the effector domain of the response regulator RcsB, allowing the transference of the phosphoryl group to RcsB, which leads to gene expression regulation by RcsB (121).

The auxiliary protein RcsA forms a heterodimer with RcsB to induce the transcription of genes that possess a core recognition DNA binding site known as the RcsAB box (122, 123). Expression of such genes is low under normal laboratory conditions due to the stability of the RcsA protein which is modulated by the proteases ClpYQ and Lon (124, 125). Both RcsA and RcsB possess a LuxR type helix-turn-helix DNA binding domain that positively regulates the expression of genes involved in capsular synthesis as well as *rcsA* itself (117, 126). However, the expression of capsular synthesis genes is not detected when *rcsB* is mutated regardless of the levels of RcsA in the cell (120). In addition, the Rcs response also utilizes RcsF, another

auxiliary protein, which is important for signal transduction (127, 128). RcsF is an outer membrane lipoprotein that may sense some surface inducing cues, such as changes in LPS, that are then transmitted to RcsC (129). Interestingly, it has recently showed that RcsF is a surface-exposed lipoprotein localized by threading it through OMPs, which suggests that RcsF could sense surface related cues directly (130). The Rcs pathway has been not only described in *E. coli*, but also in *Klebsiella pneumoniae*, which is naturally mucoid (131-133). In addition in *Salmonella typhi* and *S. typhimurium* it has been shown that other auxiliary regulators (i.e. RmpA, TviA and PhoP) can substitute for the function of RcsA (134-136).

RcsB can also regulate, as a homodimer, the expression of a sRNA, RprA, in the absence of RcsA (137) and this regulation is independent of the RcsAB DNA binding site. Instead of being located 50-100 nucleotides upstream of the promoter region, the RcsB recognition site is immediately upstream of the -35 region of the RNA polymerase binding site (138, 139). The Rcs response regulates the expression of the stationary phase sigma factor RpoS at the translational level by inducing the expression of RprA and by repressing the transcription of *lrhA*, which encodes a repressor of RprA transcription (140, 141). In addition, studies have shown that RcsB also regulates the promoters of *osmC*, which encodes an osmotically inducible periplasmic protein, (138) and *ftsZ* which encodes a cell division protein responsible for septum formation (142, 143) in an RcsA-independent fashion.

In addition to the regulation of capsular genes, several studies have shown other targets of the Rcs response. This response negatively regulates the expression of the master operon for flagella synthesis (i.e. *flhlDC* operon) in *E. coli* (144) and genes involved in flagellar subunit synthesis and swarming in *S. typhi* and *Proteus mirabilis* respectively (135, 145, 146). Andresen and collaborators (147) showed that the Rcs response is involved in the regulation of motility and

plant cell wall degrading enzymes in the plant pathogen, *Pectobacterium carotovorum* subsp. *carotovorum* (147). Additionally, RcsB and RcsA regulate the expression of genes involved in curli expression (i.e. *csgDEFG* and *csgBA* operons) in *E. coli* (148). Curli are highly aggregated irregular surface structures that are involved in cell to cell contact among bacteria to facilitate colonization and the formation of biofilms (149).The negative regulation of flagella and curli suggests that the Rcs response may be involved in biofilm formation (86, 120). In support of this hypothesis, a recent publication showed that a mutation in *rcsB* in *Edwardsiella tarda* leads to a decrease in biofilm formation (150).

Recent studies have shown that the Rcs response is involved in cell envelope maintenance by regulating cell shape. Ranjit and Young (151) recently showed that the Rcs response is required to recreate a normal morphology *de novo* when lysozyme-induced spheroplasts are formed in *E. coli*. Further, Laubacher and collaborators (152) found that the Rcs response is important for the response to peptidoglycan stress such as in the presence of β-lactam antibiotics (152). It has also been shown that the presence of antimicrobial peptides not only activates the Rcs response, but also the σ^E and Cpx envelope stress responses (153). Additionally, the presence of human serum in extraintestinal *E. coli* (ExPEC) up-regulated genes that are regulated by the Rcs response, mediating serum resistance through induction of colanic acid production (154). Altogether, these findings suggest that the Rcs pathway plays an important role in response to stressors (e.g. serum and antimicrobial peptides) that may threaten the integrity of the cell wall structure.

1.3.4 *Phage-shock protein (Psp) stress response*

The Psp stress response was first discovered in a study of filamentous phage f1 infection of *E. coli* (155). In that study it was found that upon infection of the phage f1 there was an accumulation of an *E. coli* protein of 25 kDa as a result of the expression of the phage gene IV,

which encodes an integral membrane protein. This 25 kDa protein was later named phage shock protein A (PspA) (155). Brissette and collaborators (155) also found that the expression of *pspA* was induced by extracytoplasmic stressors such as ethanol and hyperosmotic shock (155). Additionally, *pspA* expression is induced due to the accumulation of mislocalized proteins (e.g. the OMP LamB) and or expression of precursors of the outer membrane protein PhoE (156, 157). Later it was determined that the *pspA* gene is encoded in the *pspABCDE* operon in *E. coli* (158). This operon is controlled by the σ^{54} factor and it is positively and negatively regulated at the transcriptional level (159). For example, *pspA* encodes a negative regulator of the *psp* operon, while *pspB* and *pspC* encode positive regulators of this operon (159). PspF is a bacterial enhancer-binding protein (bEBP) involved in the positive regulation of the σ^{54} factor and it is encoded upstream of the *psp* operon in the opposite orientation (160). As a bEBP protein, PspF uses ATP hydrolysis to isomerize the initial transcriptionally inactive σ^{54}-promoter DNA complex to an open complex (161). However, the precise roles of PspD, PspE and PspG in either Psp activation, transduction, regulation or response remain unknown (162).

The model of regulation of the Psp response suggests that protein-protein interactions are the main mechanism involved in signal transduction (86). Under non inducing conditions PspA interacts with PspF in the cytoplasm. This interaction inhibits PspF activity as an enhancer-binding protein activator of the σ^{54} factor, which is required for the transcription of the *pspABCD* operon and *pspG* (161). Under inducing conditions, such as mislocalization of a pore-forming outer membrane secretin protein to the inner membrane, PspA is relocalized to the inner membrane, which then forms a complex with PspB and PspC, which are membrane-bound proteins. The formation of this complex allows the interaction of PspF with the σ^{54} factor and therefore the transcription of the *psp* operon. The relocation of PspA to the cytoplasmic face of

the inner membrane is believed to also assist in maintaining the proton motive force (PMF), which is thought to be disrupted by Psp inducing signals (163). Additional roles for PspB and PspC have been proposed. For example, it has been suggested that both PspB and PspC prevent secretins from causing defects in cytoplasmic membrane permeability, although the mechanism remains undefined (164).

Several studies based on the inducing conditions or phenotypes associated with the deletion of *pspA* suggest the Psp response is involved with correcting a dissipation of PMF and problems associated with the mislocalization of secretin proteins (165-167). However, it is still unclear whether the Psp response is responsible for sensing direct changes in PMF or sensing downstream effects due to PMF dissipation (86). Additionally, this response has been implicated in long term stationary phase survival at high pH (i.e. pH 9) (168). Several studies have described an association between the Psp response and bacterial virulence in different organisms. For example, in *Yersinia enterocolitica* this stress response is important for virulence because a mutation in *pspC* leads to a decrease in virulence in a mouse model of infection. This may be related to the observation that mislocalization of the YscC secretin for the T3SS virulence determinant, in a *psp* mutant caused cell death due to changes in the inner membrane permeability (164, 169, 170). It is hypothesized that expression of YscC and the T3SS during infection leads to some inner membrane mislocalization of YscC, which is lethal unless an intact Psp response is present to adapt to this stressor (164). On the other hand, in *S. enterica* serovar Typhimurium it was found that a *pspA* deletion mutant is attenuated in mice because in this pathogen PspA is required for maintaining PMF-dependent metal import in macrophages (171). Similarly, studies in *Shigella flexneri* have shown that the *psp* operon is induced during

macrophage infection (172). In general, it appears that the Psp stress response is closely related to the maintenance of the inner membrane integrity, specifically PMF, when it is impaired.

1.3.5 *Cpx envelope stress response*

The Cpx pathway is an extracytoplasmic stress response that helps to maintain the integrity of the bacterial cell envelope by detecting and responding to damage to the cell envelope (173). The following section will review this system in detail.

1.4 The Cpx Envelope Stress Response

The Cpx envelope stress response was first discovered in a genetic screen for mutants that were impaired in the conjugative transfer of the F-plasmid in *E.coli* (174). More recent studies showed that the activation of the Cpx pathway leads to a reduction in levels of the regulatory protein TraJ, which is the activator of the *tra* genes involved in conjugal pilus production (175). The mechanism of this regulation is at the post-transcriptional level through the Cpx-dependent up-regulation of a protease complex that degrades TraJ (176, 177). In addition, other studies showed the first phenotypes associated with the *cpxRA* locus in which the Cpx signalling proteins are encoded. For example it was described that mutations in the *cpx* locus cause defects in the synthesis of acetohydroxyacid synthase I, an enzyme involved in the synthesis of some amino acids (i.e. isoleucine and valine), and growth defects when cells were grown in the presence of succinate (178, 179). Later studies showed that the *cpx* locus is involved in altering the protein composition of the outer and inner membranes (180).

1.4.1 *Cpx signal transduction*

The Cpx system is regulated by a bacterial two-component regulatory system, which includes the sensor histidine kinase CpxA (181), and the response regulator CpxR, an OmpR-like class of

response regulator (182, 183) (Figure 1-3). CpxA is an inner membrane sensor kinase that senses inducing cues in its periplasmic domain, then autophosphorylates and transfers the phosphate group to CpxR at a conserved aspartate residue (183). Recently, the crystal structure of the cytoplasmic region of CpxA was determined and used for mechanical modeling in order to understand how signals lead to the activation of the autokinase state in CpxA (184).

CpxR phosphorylation leads to the alteration in transcription of multiple genes by the direct binding of CpxR to DNA (Figure 1-3) (183, 185). Additionally, the modulation of CpxR phosphorylation is controlled by the presence of auxiliary proteins. For example, the periplasmic protein CpxP modulates the phosphorylation of CpxR by reducing the CpxA autokinase activity (Figure 1-3) (69, 70). Raivio and collaborators (186) showed that overexpression of CpxP inhibits Cpx pathway activity and the authors suggested that this inhibition is due to CpxP and CpxA interaction based on data that showed that when the periplasmic domain of CpxA is mutated there is no longer inhibition by CpxP (186). This hypothesis is supported by an *in vitro* study that reconstituted CpxAR signalling and showed that the presence of CpxP decreases the rate of CpxA autophosphorylation (70). The crystal structure of CpxP suggests that a positively charged concave surface may interact with negatively charged regions of the CpxA periplasmic domain (187, 188). Although CpxP is capable of inhibiting CpxA signalling activities, it is not required for the detection of some of the described Cpx inducing cues (e.g. NlpE overexpression, alkaline pH, PapE and PapG overexpression) (185, 186). Rather, it appears that removal of CpxP inhibition sensitizes the CpxA sensor kinase to activation. There is evidence suggesting that CpxP is important for augmenting the response of CpxA to misfolded proteins. Specifically, it has been shown that CpxP is required for the degradation of some misfolded protein inducers (189). Since the periplasmic protease DegP is also required for this process, it has been proposed

that CpxP may target misfolded proteins to the DegP protease, where it is degraded, together with the misfolded protein, thus relieving CpxP-mediated inhibition (189). In spite of this work, other studies indicate that CpxP is present in the presence of some inducing cues, but inactive (185). The mechanism by which it is inactivated remains obscure. Additionally, it has been proposed that CpxP may be involved with metal ion sensing due to the structural homology of CpxP and other periplasmic metal-binding proteins (e.g. CnrX and ZraP) (188), although the role of metal binding is currently unknown.

An additional signalling protein upstream of CpxA is the outer membrane lipoprotein NlpE. NlpE was first described in a screen for multicopy suppressors of the toxicity of an envelope-localized fusion protein (190) and it has subsequently been shown to be required for the activation of the Cpx pathway in response to adhesion to a hydrophobic surface (191). NlpE is not, however, required to sense other described Cpx inducing cues (185). Miyadai and collaborators (192) showed that anchoring of NlpE to the outer membrane is essential for Cpx induction (192). It has been proposed that NlpE could interact with the sensor kinase CpxA in the inner membrane (193), however the molecular mechanism by which NlpE stimulates CpxA upon overexpression or in response to adhesion remains unknown.

Cpx signal transduction can also be modulated downstream of CpxA. De Wulf and collaborators (194) reported that in *E. coli* K-12 strain MC4100 the expression of the auto-regulated *cpxRA* operon is activated at stationary phase (194). Furthermore, in an epistasis analysis it was shown that the expression of *cpxR* can be activated in a CpxA-independent manner in response to excess glucose or pyruvate in the cell and this regulation is related to growth and central metabolism through Pta-AckA (phosphotransacetylase-acetate kinase A) (195). In addition, it was shown that small cellular phosphodonors such as acetyl phosphate can

phosphorylate CpxR (183, 195, 196). Thus, some growth-related signals may lead to Cpx response induction in a manner that does not involve CpxA sensing of envelope cues.

Finally, the Cpx pathway affects other signalling systems through the regulation of connector proteins (i.e. MzrA and CacA). It has been shown that expression of *mzrA*, which encodes an inner membrane protein, is regulated by the Cpx pathway (197) and that regulation affects the activity of the osmo-regulated EnvZ-OmpR system (198), which is another two-component signal transduction pair required for the regulation of porin genes (199). Additionally, another study reported that the CacA (for Cpx activating connector-like factor A) protein activates the Cpx response in a CpxA and CpxR dependent manner, however the mechanism of CacA-mediated Cpx activation is not fully elucidated (200). Moreover, CacA expression is regulated by a specialized sigma factor, RpoS, which is required under conditions of nutrient deprivation or stress at stationary phase. The regulation of CacA by RpoS generates a link between the Cpx pathway and the general stress response of *E. coli* (200).

1.4.2 *Cpx inducing cues*

The *E. coli* Cpx system senses misfolded proteins in the bacterial cell envelope and mediates adaptation to envelope stress through a variety of mechanisms (201), therefore many of the known inducing cues of this system are related to generation of misfolded IM and/or periplasmic proteins (173). The effect of Cpx response induction is the regulation of the expression of genes encoding protein folding and degrading factors in order to maintain envelope homeostasis. For example, some of the inducing cues that trigger this pathway are aggregated UPEC P pilus subunits (i.e. PapE and PapG) and subunits of the EPEC BFP (Figure 1-3) (202-204). However, it is not clear whether other known inducing cues stimulate the Cpx response through the generation of misfolded proteins. These include: alkaline pH, over-expression of the lipoprotein

NlpE (see above), changes in osmolarity, attachment to hydrophobic abiotic surfaces and changes in membrane lipid composition (190, 191, 205-208).

Additionally, it has been reported that Cpx activity is induced by specific changes in inner membrane proteins. For example when *ftsH*, an inner membrane-bound protease, is mutated, the Cpx pathway activates the expression of another inner membrane protease *htpX* (209). In addition, it was reported that the absence of two membrane proteins (i.e. YqjA and YghB), that belong to the DedA membrane protein family, activates the Cpx pathway and other stress responses (i.e. Psp, Bae, and Rcs) in *E. coli*, suggesting their role in envelope homeostasis (210). Together with the previously described Cpx inducing cues, these data suggest that the positive regulation of IM proteases by the Cpx pathway is required to maintain quality control of IM proteins and that disruptions in this process lead to induction of CpxA (173).

Finally, abnormalities in cell wall peptidoglycan structure also induce the Cpx response. Evans and collaborators (211) showed that changes in peptidoglycan structure such as mutations in genes that encode penicillin binding proteins (PBPs), which are important for synthesizing and modifying the cell wall, not only activated the Cpx response but also the Rcs response in a CpxR-dependent manner (211). In that study it was suggested that envelope stress responses may be activated because of the role of PBPs peptidoglycan modification associated cell growth or the accommodation of macromolecular structures such as flagella. In the absence of such PBPs, the activation of the Cpx (and Rcs) response is required in order to down-regulate the expression of those structures (211).

1.4.3 *Physiological role of the Cpx response*

1.4.3.1 Envelope maintenance

Some of the Cpx regulon members in *E. coli* which are involved in the alleviation of protein misfolding stress are well characterized (212, 213). Thus, many Cpx regulon members encode envelope-localized protein folding and degrading factors. For example, the Cpx pathway regulates the expression of the periplasmic protein DegP (214) that can act as a serine protease or chaperone (215). Also the Cpx pathway controls the expression of *dsbA* (196, 216). DsbA is a soluble periplasmic protein that catalyses disulfide bond formation (217) and it has been shown to play an important role in the biogenesis of bacterial virulence factors (e.g. toxins, fimbriae and some components of the T3SS) (described on Section 1.1.2) (218). Expression of *ppiD* is also regulated by the Cpx pathway (196). PpiD assists in periplasmic protein folding and was discovered as a suppressor of the novobiocin sensitive phenotype conferred by mutation of the gene encoding the chaperone SurA (219). In addition, the Cpx response regulates the expression of the periplasmic chaperone, Spy (69). Although there is structural similarity between Spy and CpxP (107, 188, 220) , the inhibitory effect of CpxP on Cpx pathway activity is not mimicked by Spy (69).

1.4.3.2 Motility

The *tsr* and *motABcheAW* operons, related to chemotaxis and motility, were identified as members of the Cpx regulon by searching the *E. coli* genome for the presence of the CpxR consensus binding sequence (i.e. 5'-GTAAA(N)5GTAA-3') (194). The Cpx regulation of motility was confirmed by determining swarming phenotypes of different Cpx mutant strains (i.e. *cpxR* and *cpxA* mutants) as well as electrophoretic mobility shift analysis to measure CpxR binding to the *motABcheAW* promoter region (194). Moreover, De Wulf and collaborators (221),

using a matrix for the CpxR recognition motif, identified not only the *tsr* and *motABcheAW* operons, but also other genes involved in chemotaxis and motility (e.g. *aer, fliM, cheR, cheB, cheY, cheZ*) (221). In addition, MacRitchie and collaborators (222) showed that the Cpx regulated periplasmic protease DegP is required in the Cpx-mediated inhibition of motility (222). Further studies found that activation of the Cpx response down-regulates the expression of *flhC*, which is encoded in the class 1 flagellar operon required for the regulation of flagellar synthesis (223). Additionally, a study found that CpxR is involved in the regulation of flagellar genes in a RpoS dependent manner, where the stationary sigma factor up-regulates *cpxR* expression at exponential phase of growth, which leads to the repression of some flagellar biosynthesis genes (i.e. *fliA*, *flgM*, and *tar*) by CpxR (224). These results together suggest that the Cpx pathway may modify motility by modifying flagellar rotation, chemotactic responses and expression of flagella regulators.

1.4.3.3 Pathogenesis

Some studies suggest that the Cpx pathway has an effect on genes required for pathogenesis by regulating the expression of virulence-linked extracellular appendages. For example, in EPEC, the full activation of the Cpx pathway inhibits the expression of envelope localized virulence factors: The BFP (225), which is one of the major adhesion factors in EPEC (226), and the secretion of some T3SS translocator and effector proteins (i.e. EspA, EspB, EspD, and the translocated bacterial receptor Tir) (227). MacRitchie and collaborators (222) found that the Cpx pathway mediates post-transcriptional regulation of the T3SS and motility through the regulation of the protease/chaperone DegP (222). Similarly, the full activation of the Cpx response inhibits the expression of the P pilus in UPEC (228). Most likely, the function of the Cpx-mediated down-regulation of envelope appendages is to reduce unessential envelope protein traffic under

envelope stress conditions (86). Accordingly, a study found that constitutive activation of the Cpx response in EPEC attenuates virulence in the animal model *Galleria mellonella* (229).

Conversely, it has been reported that inactivation of the Cpx response diminishes the expression of virulence determinants, for example in EPEC this leads to a decrease in the expression of BFP (225). It has been shown that the Cpx pathway is required for the proper assembly of envelope localized appendages (e.g. T3SS and BFP) in EPEC through the regulation of folding factors such as DsbA, DegP and CpxP (222, 225). Additionally, it has been reported that the Cpx pathway is necessary for the expression of the UPEC P pilus in a similar fashion (230). In agreement with this, the Cpx pathway is activated when P pilus subunits become misfolded in the periplasm in the absence of their periplasmic chaperone, PapD (204). Further studies showed that inactivation of the Cpx response in UPEC by the deletion of the *cpxRA* operon reduced its ability to colonize the murine bladder and decreased virulence towards zebrafish embryos (231). Finally, a recent study showed that mutation of the response regulator *cpxR* leads to a decrease in colonization of *E. coli* commensal strain MP1 in a mouse model (232).

1.4.3.4 Biofilm formation

There is evidence that the Cpx pathway regulates the early steps of biofilm formation. For example, a screen for mutations that diminish biofilm formation in an adherent *E. coli* MC4100 strain, revealed that when *cpxA* was mutated there was a reduction in biofilm formation by affecting adherence to a solid surface (i.e. polystyrene) (233). The involvement of the Cpx pathway in biofilm formation in clinical isolates was evaluated as well by mutating the *cpxA* gene in two *E. coli* clinical isolates from the percutaneous trans-hepatic catheter and urine of patients suffering from cholecystisis as well as from infections related to urethral catheters (233). In these

two clinical isolates mutation of *cpxA* also reduced their ability to adhere to polystyrene (233). This study suggest that activation of the Cpx response, as a result of increasing levels of phosphorylated CpxR in the *cpxA* mutant, reduces biofilm formation (233).

The effects of the Cpx response on biofilm formation may arise from its regulation of the adhesive curli appendages. Recently, it was found that the Cpx pathway represses curli expression during envelope stress through the regulation of both the *csgBA* and the *csgDEFG* operons, which encode proteins that play roles in the secretion and assembly of CsgA curli subunits on the cell surface as well as transcriptional activation of both operons (206, 234, 235). Additionally CpxR also regulates CsgD-regulated genes, such as *adrA* (involved in biofilm matrix polysaccharide cellulose production), due to the negative regulation of the transcriptional activator CsgD (236).All these findings suggest the role of the Cpx pathway in biofilm formation, through the negative regulation of curli expression in *E. coli* (237).

1.4.3.5 Antimicrobial resistance

Several studies have suggested that the Cpx response is involved in antibiotic resistance. A study in *S. enterica* serovar Typhimurium showed that *nlpE* overexpression may contribute to cationic antimicrobial peptide (CAMPs) resistance, through the induction of the *amiA* and *amiC* genes in a CpxR dependent manner (238). Both genes encode a N-acetylmuramyl-L-alanine amidase involved in the cleavage of peptidoglycan during cell division (239). It is speculated that AmiA and AmiC could modulate the integrity of the peptidoglycan layer in response to CAMPs to confer resistance (238). In agreement with these results, our laboratory found that activation of the Cpx response regulates the expression of other proteins (i.e. *ycbB, ygaU, mltB and dacC*) involved in cell wall metabolism (223). Later a study demonstrated that those CpxR-regulated cell wall metabolism proteins are involved in antimicrobial resistance (240).

Studies in *E. coli* K-12 strains have shown that overexpression of the response regulator CpxR leads to increased levels of AcrD and MdtABC RND efflux pumps (114). Moreover, it has been reported that CpxR in conjunction with another response regulator, BaeR (described in Section 1.3.2), directly regulates the expression of *acrD* and *mdtABC* gene clusters (113). The regulation of *mdtABC* by the Cpx and Bae response has also been observed in other organism such as the plant pathogen *Erwinia amylovora* (241). Similarly, another study showed that overexpression of *nlpE* affects multidrug resistance in *E. coli,* mainly by the Cpx-dependent regulation of AcrD and MdtABC (242). Raivio and collaborators (223) showed that the Cpx response up-regulates efflux pumps expression and it down-regulates genes involved on electron transport chain (e.g. genes encoding NADH dehydrogenase, succinate dehydrogenase and cytochrome oxidase), which therefore could decrease PMF and increase antibiotic resistance to aminoglycosides and hydroxyurea (223). Studies in enterohaemorrhagic *E. coli* (EHEC) O157:H7 showed that the Cpx response controls the resistance to fosfomycin antibiotic through the downregulation of two transporters, GlpT and UhpT, involved in cotransport of fosfomycin (243). Finally, Mahoney and Silhavy (244) showed that the Cpx response confers resistance to amikacin, which could lead to the incorporation of mistranslated proteins into the IM that promotes changes in the homeostasis of this compartment (244). This study is in accordance with the previous observation that Cpx response up-regulates the expression of YccA, an IMP, which prevents jammed secretion machineries from being degraded by FtsH-mediated proteolysis (245).

Recently it was shown that a deletion in the *mtrC* gene*,* which encodes a multiple transferable resistance (MTR) efflux transporter in *H. ducreyi*, activates the Cpx response (246). It was suggested that this activation may contribute to the resistance towards cathelicidin LL-37, a human antimicrobial peptide, which may accumulate in the *mtrC* mutant and therefore impairs

the cell envelope integrity (246). Similarly, other studies found that another human antimicrobial peptide and CAMP, ApoEdpL-W and cationic polyethylenimine, trigger the Cpx response (153, 247). However the molecular mechanism of how these antimicrobial compounds induce the Cpx response is still unknown.

1.4.3.6 Novel Cpx pathway roles

Raivio and collaborators (223) used a microarray analysis to identify the Cpx regulon members of two different *E. coli* strains (i.e. MC4100 and EPEC) when the Cpx pathway was activated by transient NlpE overexpression in two different media conditions (223). As described on Section 1.4.1. NlpE overexpression induces the activation of the Cpx response in a CpxA dependent manner and it may requires the interaction between CpxA and NlpE (190, 191, 193). In this *E. coli* microarray study it was observed an enrichment of proteins that are inner membrane associated (223), which is in accordance with previous studies suggesting the importance of the Cpx response, which not only is involved in envelope stress responses associated to the OM, but also on the maintenance of the homeostasis in the IM compartment (209, 245, 248).

Raivio and collaborators (223) determined a set of core genes that were found to be Cpx-regulated, independently of strain background (i.e. *E. coli* MC4100 and EPEC) or media (i.e. LB and Dulbecco's modified Eagle's medium (DMEM)). In this set of genes most of them encoded proteins that are envelope localized, which reinforce the importance of Cpx stress response in the cell envelope. In that set of core genes there was an enrichment of genes characterized as "y" genes with unknown function, genes involved in cell wall modification enzymes (i.e. *dacC*, *slt*, *ycbB* and *ygaU*), genes involved in protein folding in the envelope (i.e. *cpxP*, *spy*, *yccA*, and *dsbA*), genes involved in motility and biofilm formation (i.e. *ydeH* and *flhC*), genes involved with

transport (e.g. *ompF*, *efeU*, *mglB* and *nhaB*), and genes involve in gene regulation (e.g. *mzrA* and *ydeH*) (223).

Additionally, it was found that the Cpx response regulon is involved with different novel cellular functions for this stress response depending on the media or strain analyzed. For example, genes involved in iron transport and siderophore synthesis were specifically down-regulated in EPEC in DMEM media (223). Clusters of genes (i.e. *nuo*, *sdh* and *cyo*) related to electron transport, TCA cycle and oxidative phosphorylation were enriched among down-regulated genes in almost all conditions tested (223). Interestingly, it was found that the down-regulation of those genes involved in respiration partially contributes to conferring antibiotic resistance most likely by limiting entry of drugs into the cell through the reduction of PMF (249). Finally, it was described novel sRNAs (i.e. CyaR, MicF, OmrA, OmrB, RprA, and RybB) that are part of the Cpx regulon in *E. coli* (223). A recent study demonstrated their role in the Cpx response and how it is the mechanism of regulation of those sRNA by the response regulator CpxR (250). It is interesting that some of these new roles of the Cpx response are not only regulated in *E. coli*, but also in other organisms (see sections 1.4.4 and 5.2.1), which suggests an evolutionary conservation for some functions of the Cpx regulation.

1.4.4 *The Cpx pathway in other bacterial species*

The Cpx pathway has also been studied in other bacterial species and it has been shown that some roles of the Cpx pathway in the regulation of virulence factors are shared between bacterial species (203, 251-253). For example, studies in *Shigella* spp., the causative agent of bacillary dysentery in humans, have shown that the Cpx pathway regulates the expression of the master activators for *Shigella* virulence (i.e. VirF and InvE) (205, 251). Nakayama and Watanabe (205) demonstrated that there is pH dependent regulation of *virF* expression that may depend on the

levels of CpxR phosphorylated (205). Furthermore a putative CpxR binding site in the promoter region of *virF* was found, and it was shown that deletion of *cpxR* in *S. sonnei* diminishes expression of *virF,* suggesting a role of the Cpx response in the positive regulation of VirF (254). Finally, Mitobe and collaborators (251) found that the Cpx response regulates the expression of the T3SS through the regulation of its activator, InvE*,* at the posttranscriptional level.

Similarly, the Cpx pathway regulates invasion genes in *S. enterica* serovar Typhimurium in a pH dependent manner, by regulating its major transcriptional activator, HilA (255). It was shown that this Cpx regulation of HilA has an effect on *sipC* expression, which encodes an effector molecule of the T3SS that is required for invasion (255). Other Cpx-related phenotypes in *S. typhimurium* have been described. For example, Humphreys and colleagues (256) established that the ability to invade human macrophages and epithelial cells and infect mice is decreased when the Cpx pathway is activated in *S. typhimurium* (256). Similar to the regulation of virulence factors in EPEC, full activation of the Cpx response decreases adhesion due to a negative regulation of curli expression (256).

Moreover, the Cpx response plays a role in the regulation of the T3SS and the adhesins of *Y. pseudotuberculosis* (252, 257). For example, when the Cpx pathway is activated in this organism there is a decrease of T3SS secreted effectors due to the Cpx regulation of genes involved in early steps during T3SS biogenesis (i.e. *yscJ*, *yscF*, and *lcrV*) (252). Congruent with this result it was reported that deletion of *cpxA* impairs the ability of *Y. pseudotuberculosis* to bind to and become internalized within mammalian cells. This phenotype was related to the role of the Cpx response in regulation of the major adhesins in *Y. pseudotuberculosis* (i.e. invasin, Ail and pH6 antigen) at the transcriptional level (257). Overall, the Cpx pathway plays an important role in bacterial pathogenesis in *Y. pseudotuberculosis*.

Finally, studies in *H. ducreyi* showed that the Cpx response down-regulates the expression of the lspB/lspA2 operon, which encodes proteins that compose a two partner secretion system required for virulence (Labandeira-Rey et al., 2009). Further the characterization of the Cpx regulon in this organism showed that it regulates the expression of genes not involved in the cell envelope stress response but instead genes involved with a number of virulence determinants (258, 259). Accordingly, Spinola and colleagues (2010) showed that activation of the Cpx response by deletion of *cpxA* in *H. ducreyi* exhibited a decrease in the formation of papules and pustules in human volunteers at the site of inoculation compared with the wild type strain (260).

Finally, in *V. cholerae*, the *cpxRA* operon organization is similar to *E. coli,* however at an amino acid level the degree of identity of the periplasmic domain of CpxA protein is low (i.e. 20.7% identity), which suggested differences between the inducing signal for this pathway between *E. coli* and *V. cholerae.* Using a *cpxP-lacZ* reporter it was previously shown that there is not basal activity of the Cpx pathway in *V. cholerae,* unlike *E. coli*. Moreover, the known inducing cues that trigger the Cpx pathway in *E. coli* (e.g. overexpression of NlpE and alterations in osmolarity) are not relevant in *V. cholerae* (261). These results suggest that *V. cholerae* Cpx pathway may sense different inducing cues, which is in agreement with the percentage of identity of the sensor domain of CpxA protein between *V. cholerae* and *E. coli*. When it was tested if any *cpx* mutants (i.e. *cpxR, cpxA* and *cpxA**) have an effect on a suckling mouse model colonization, none of the mutants had any effect on intestinal colonization, suggesting that the Cpx pathway may not have any role in colonization (261).

Slamti and Waldor (261) showed that the activation of the Cpx pathway in *V. cholerae* is required to stress related to high salinity (261). For example, when different concentrations of sodium chloride (i.e. 170, 250 and 500 mM) were added to basal medium of yeast extract and

tryptone, there was an increase on the signal of the *cpxP-lacZ* reporter, which suggests that the Cpx pathway may be involved in sensing the salinity that *V. cholerae* could encounter in estuarine environments. Finally, in this study it was determined which genes could trigger the activation of the *V. cholerae* Cpx pathway using a transposon mutagenesis screening. The results highlighted that most of the genes are related to the cell wall: 50% are localized in the inner membrane, 4.5% are periplasmic and 13.6% are localized in the outer membrane. Mainly, genes involved in periplasmic disulfide bond isomerization (e.g. *dsbC* and *dsbD*) were found for being important in *V. cholerae* Cpx pathway activation. These results indicated that this pathway may sense misfolded envelope proteins which contain aberrant disulfide bonds (261).

1.5 *Vibrio cholerae*

V. cholerae is a Gram-negative bacterium which is the etiological agent of cholera. Cholera is a diarrhoeal water-borne disease that is endemic in geographic areas where there is poor sanitation and limited access to potable water. Recently, an epidemiological study of countries (endemic or non-endemic) with a recent history of cholera reported that about 1.4 billion people are at risk for cholera in endemic countries and an estimated 87 000 cholera cases occur in non-endemic countries (262). Several million cases of cholera occur each year and over a hundred thousand deaths (262). Children aged 1 to 5 years are more susceptible to cholera in endemic areas, such as India and Bangladesh (263). Cholera disease starts upon ingestion of water contaminated with *V. cholerae,* which can reach and pass the gastric acid barrier in the stomach and then colonize the epithelium of the small intestine (264). Once it is established in the small intestine it produces one of the major virulence factors, the cholera toxin (CT), which is

responsible for the disruption of ion transport by intestinal epithelial cells leading to the generation of watery diarrhea which is the hallmark of the cholera disease (264).

Seven different pandemics of cholera have been described since 1817 (265). The ongoing seventh pandemic started in 1961 in Indonesia and it was spread in the southeast Asian archipelago (266). Subsequently cholera incidence decreased slightly but it re-appeared when cholera outbreaks were reported in the 1990s in Africa and for the first time in Latin America, where Peru was the location of the first cholera epidemic in this region (267). Recent cholera outbreaks in Haiti, Cameroon and Zimbabwe (268-270) suggest the increase in the incidence of cholera not only in endemic areas but as well in the western hemisphere (271). Currently, more than 200 *V. cholerae* serogroups have been identified, in which the O1 serogroup has been associated with the pandemic spread of cholera (272). Furthermore, the O1 serogroup is classified into two biotypes: classical and El Tor, the latest is the causative agent of the seventh ongoing pandemic of cholera (264). Finally, it has been suggested that outbreaks caused by the *V. cholerae* O139 serogroup could be the initiation of the eighth pandemic of cholera (273).

The biotype classification of *V. cholerae* classical and El Tor strains is based on biochemical differences, in which it is included the susceptibility to polymyxin B antimicrobial peptide, chicken cell erythrocytes agglutination, hemolysis of sheep erythrocytes, and the production of acetylmethylcarbinol (264). Additionally, it has been described that there are differences between *V. cholerae* classical and El Tor strains that not only relay on biochemical differences but as well differences on gene expression of the master virulence regulator ToxT that therefore affect the production of the virulence factors CT and TCP (274). Previously, it was shown that there is a single base-pair difference at positions -65 and -66 of the classical and El Tor *tcpPH* promoters, respectively, which is responsible for the differential regulation of virulence gene expression in

classical and El Tor biotypes (275). Furthermore, El Tor strains produce a T4 pili, the mannose-sensitive hemagglutinin (MSHA), that is not expressed by the majority of *V. cholerae* classical strains and may play a role in the environment for association with environmental reservoirs for *V. cholerae* (276).

Although *V. cholerae* is a human pathogen, it is a marine bacterium that can be found as normal aquatic flora in estuarine and brackish waters (277, 278). Once *V. cholerae* cells are shed from the host, they could be either ingested by another human soon after shedding or settle in the environment as a reservoir (279). In the environment, *V. cholerae* could be found in association with zooplankton and phytoplankton, such as copepods and cyanobacteria respectively (280-283) or in association with abiotic surfaces through the formation of biofilms (284, 285). Generally, *V. cholerae* Non-O1 serogroup strains are much more commonly isolated from these environments than O1 serogroups, especially in areas outside of epidemic zones (264). Finally, during the life cycle of *V. cholerae*, the aqueous environment could be a potential reservoir for potential human infection where the cycle could start again (279).

1.5.1 *V. cholerae virulence gene regulation*

The ability of *V. cholerae* to cause cholera disease mostly depends on two major virulence determinants: CT and the TCP. CT is responsible of the watery diarrhea characteristic of this disease and TCP is involved in the formation of microcolonies and attachment in the human intestinal epithelia (286, 287). Gene regulation of both virulence factors mostly rely in a regulatory cascade known as the ToxR regulon (288-290). In this regulatory cascade ToxT, which is part of the AraC/XylS protein family (291), is the primary response regulator involved in the activation of the *ctxAB* genes, which encodes the CT, and the *tcp* operon where the TCP is encoded (292). The expression of ToxT is under control of two transmembrane regulators, ToxR

and TcpP, which bind to the *toxT* promoter region from positions -54 to -32 for TcpP binding, and from -104 to -68 for ToxR binding, having different affinities for those recognition sites (293). In addition, ToxR and TcpP require an inner membrane localized protein, ToxS and TcpH respectively, which have been suggested to be important for the stability and function of their activity (292, 294-297).

The *toxRS* operon is considered to be constitutively active (298, 299). On the other hand, expression of the *tcpPH* operon is under control of additional accessory activators, AphA and AphB. The binding of AphA in the promoter of *tcpPH* is from positions -101 and -71 and requires the binding of AphB in order to activate transcription of *tcpP*. The binding site for AphB is from -78 and -43, which is upstream of the AphA binding site (300, 301). Furthermore, the cAMP receptor protein (CRP) also regulates the expression of CT and TCP in both classical and El Tor biotype strains (302). CRP represses the *tcpPH* transcription by interfering with AphA/B mediated activation due to the CRP binding site at the *tcpPH* promoter region, which overlaps with the AphA and AphB binding (300).

Finally, quorum sensing is involved in the regulation of virulence factors through the regulation of *aphA* expression. For instance, HapR, a LuxR homolog quorum sensing regulator in *V. cholerae* (303), represses *aphA* transcription (304). Specifically, at low cell density the quorum sensing system 1, which is composed by the CAI-1 autoinducer and the sensor kinase CqsS, and the quorum sensing system 2, which is composed of the LuxPQ two component system (TCS) (305), become phosphorylated and then transfer the phosphorus to LuxU and subsequently to LuxO, which leads the transcription of four small regulatory RNAs (sRNAs), Qrr1-4 (306). Then these sRNA in combination with the RNA-binding protein Hfq are in involved in the post-transcriptional regulation of *hapR* transcripts (306, 307).

1.5.2 *V. cholerae iron transport*

Iron is a central regulatory element as it is an important nutrient for many bacterial species. It is required for different cellular process such as energy generation and electron transport (308). Despite the abundance of iron in nature, it is difficult to acquire by many microorganisms due to the fact that Fe^{3+} has low solubility (10^{-18}M) at a physiological pH of 7. Besides, in humans, upon iron absorption in the gut, iron is bound to proteins such as transferrin in the serum, lactoferrin in secretory fluids and in granules of polymorphonuclear leukocytes; in addition iron is stored within ferritin in cells (309). In contrast, under anaerobic conditions Fe^{2+} is soluble and it is readily available for uptake by microorganisms (Figure 1-4) (308). Bacteria use different methods to acquire iron from the environment or from the host in order to solve iron-supply problems (Figure 1-4), such as iron ligands called siderophores (described in Section 1.5.2.1) and transporters of heme (described in Section 1.5.2.2).

1.5.2.1 Siderophore transport

One of the methods that is widely distributed and used among bacteria in order to acquire iron is the production of siderophore-mediated iron transport systems. Siderophores are low molecular weight iron ligands that bind to highly specific receptor proteins located in the OM that then transport the iron bound siderophore complex across the outer membrane via energy dependent systems (Figure 1-4) (310, 311). Once the siderophore complex is localized in the periplasm, it is recognized and bounded by specific periplasmic binding proteins (PBPs). Then, this siderophore complex-PBP is translocated into the cytoplasm across the IM by ATP-binding cassette (ABC) transporters (311). *V. cholerae* only synthesizes a catechol siderophore called vibriobactin which is encoded in two gene clusters (312), where ViuA is its OM receptor (313). It has been suggested that the gene downstream of *viuA*, *viuB,* is required for vibriobactin utilization by

removing the iron form the iron-vibriobactin complex (314). However, *V. cholerae* can utilize siderophores that are produced by other bacterial species. For example, the *V. cholerae* genome encodes proteins (i.e. IrgA and VctA) with sequence similarity to enterobactin OM receptors such as CirA and FepA from *E. coli* (315). *V. cholerae* can also utilize other siderophores such as agrobactin and fluvibactin which have similar structures to vibriobactin (312). Furthermore, *V. cholerae* possesses two periplasmic binding protein-dependent ABC transport systems, ViuPDGC and VctPDG, which are required for transport of vibriobactin and enterobactin respectively (315, 316). Recently, it has been shown that the ABC transporter VctPDGC can promote iron acquisition in the absence of siderophores (317).

1.5.2.2 Heme transport

Similarly to iron-siderophore uptake, heme is taken up via energized OM receptors and transported to the periplasm (Figure 1-4), where it is translocated to the cytoplasm through the IM via ABC-dependent periplasmic permeases (318). *V. cholerae* can use both heme and hemoglobin as a source of iron. It encodes different heme receptors such as HutA, HutR and HasR (319). Although, the fate of heme and hemoglobin is not well known once inside the cell, the HutZ protein has been demonstrated to be involved in promoting heme utilization as an iron source in *V. cholerae* (312). Recently, the crystal structure of HutZ has been solved revealing that it shares homology with the protein HugZ, a heme oxygenase from *Helicobacter pylori*. A mismatch in one amino acid residue required for heme oxygenase activity could explain the deficiency of HutZ in heme oxygenase activity (320).

1.5.2.3 TonB transduction systems

The energy that is required for the active transport of iron-siderophore complexes and heme across the OM is transduced via a complex of proteins called the TonB energy-transduction

system (321) (Figure 1-4). The TonB system is composed of the TonB, ExbB, and ExbD proteins, which are located in the inner membrane and found in cells at a ratio of 1:7:2 (322). It couples the PMF created by the electrochemical potential of the inner membrane in order to open the channels in the TonB-dependent outer membrane transporter (TBDTs) proteins (323-325).

V. cholerae possess two TonB systems, TonB1 and TonB2. Both are encoded in different locations in the *V. cholerae* genome. The *tonB1* system is co-transcribed with the genes required for transport of heme across the IM, *hutWXZ,* (326) and the *tonB2* system genes are located in an operon with unknown function (312). There is both specificity and redundancy with respect to the OM receptors that the two TonB systems energize (327). For example, the TonB1 system functions in vibriobactin transport, whereas TonB2 is required for enterobactin and heme transport. However, both the TonB1 and TonB2 systems function with the heme receptors HutA and HutB (319, 328). Furthermore, there are structural differences between both the TonB1 and TonB2 proteins. TonB1 is longer and thought to span more of the periplasm than TonB2. This may reflect the ability of TonB1 more efficiently transport heme in high osmotic conditions such as marine environments (328). Finally, a mutation of the *tonB* system in *V. cholerae* leads to a reduced ability to compete with the wild type strain during animal models of infection, suggesting the importance of these systems in virulence (328).

1.6 Objectives

Bacteria have developed different signalling pathways, such as that controlled by CpxA and CpxR, in order to transduce information from the cell envelope to the cytoplasm, when they encounter different inducing cues in the environment. The role of the Cpx pathway in maintaining protein folding homeostasis in the cell envelope among different bacterial species is

well understood, especially in the model organism *E. coli*. However, the Cpx regulon members and inducing cues that trigger this stress response in *V. cholerae* are poorly understood. In addition, the effect of activation of the Cpx response in relation to virulence gene expression in *V. cholerae* is unknown.

The general objective of this thesis is to characterize the Cpx response in *V. cholerae*. I defined three specific objectives to address the role of the Cpx pathway in the *V. cholerae* El Tor strain C6706:

1. To characterize the Cpx regulon members in *V. cholerae* when the Cpx pathway is activated and to compare the level of conservation with the regulon members in other bacterial species.

2. To determine the novel inducing cues of the Cpx pathway in *V. cholerae* and their role in the regulation of gene expression by the Cpx pathway.

3. To characterize the effect of Cpx pathway activation on *V. cholerae* virulence attributes, specifically in the expression of envelope localized virulence factors, and to determine whether these effects are at transcriptional or posttranscriptional levels.

1.7 Figures

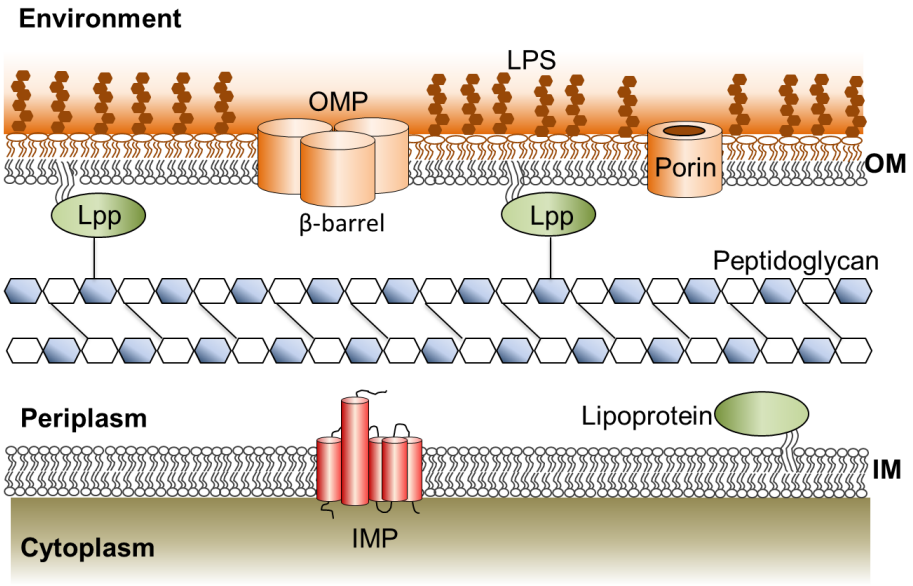

Figure 1 - 1 The Gram-negative bacteria cell envelope.

The cell envelope is composed of three compartments: the outer membrane (OM), the periplasm, and the cytoplasmic or inner membrane (IM). The OM is an asymmetrical bilayer composed of phospholipids (inner leaflet) and lipopolysaccharides (LPS) (outer leaflet). Two types of proteins can be found in the OM: OM proteins (OMP), which are trimeric β-barrel porins, and lipoproteins such as Lpp or Braun's lipoprotein. The periplasm is an aqueous cellular compartment and it is composed of soluble proteins and the peptidoglycan layer. The inner membrane is a bilayer composed of phospholipids and two types of proteins: integral IM proteins (IMP), which span the IM with hydrophobic α-helices, and lipoproteins. Figure adapted from (7).

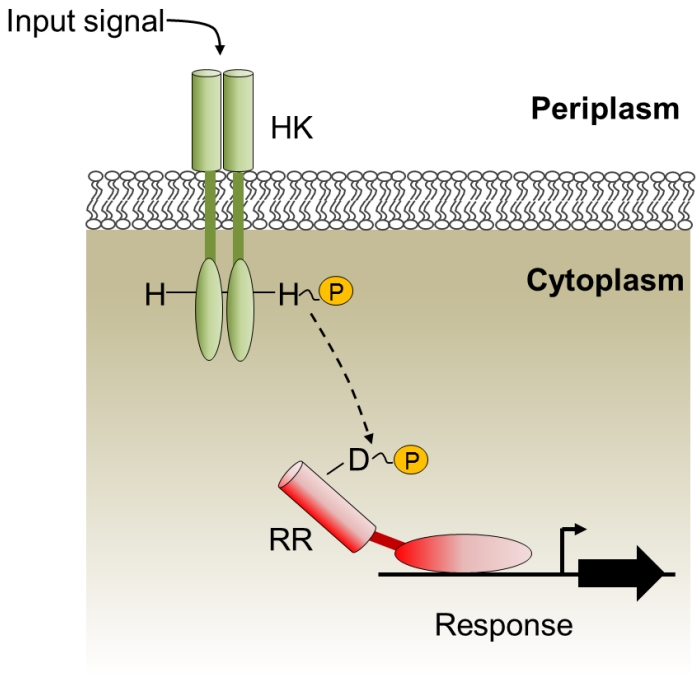

Figure 1 - 2 Two component signal transduction system.

Two component systems are composed of a sensor histidine kinase (HK) and a response regulator (RR). The HK is generally localized in the inner membrane of Gram-negative bacteria and has a periplasmic domain, which is thought to be the receiver of the input signal. Upon the recognition of an input signal, HK autophosphorylates itself at a conserved histidine (H) residue using ATP. The HK then phosphorylates its cognate response regulator (RR) at a conserved aspartate phosphorylation site (D). This induces a conformational change in the C-terminal transcriptional regulatory domain of the RR that leads to the activation of its DNA binding domain and the control of gene expression. Figure adapted from (329).

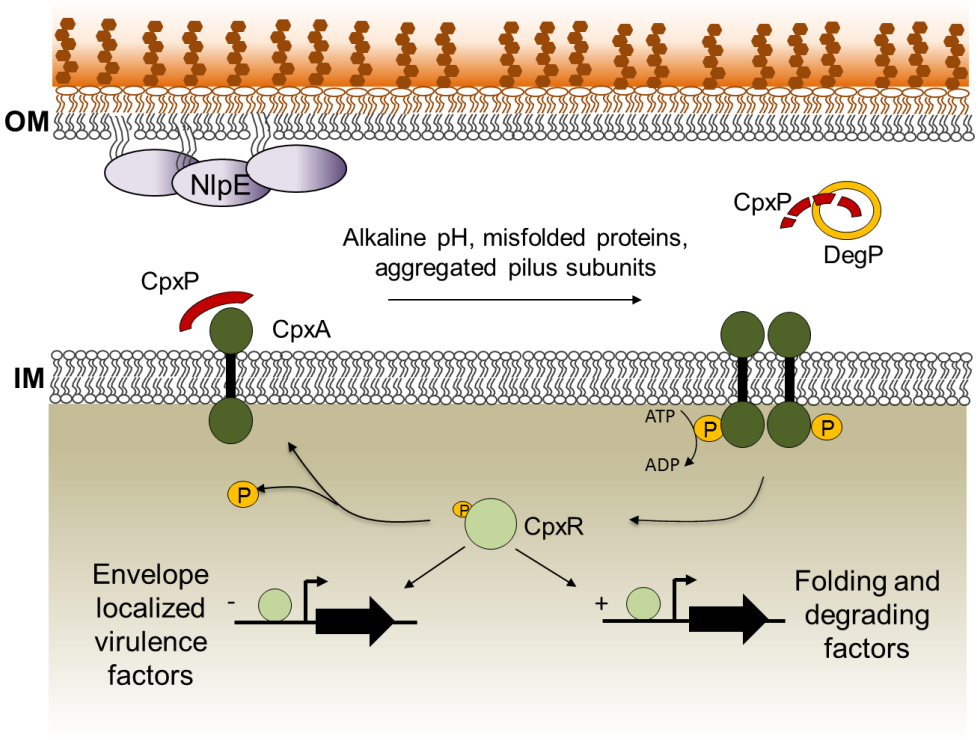

Figure 1 - 3 The Cpx envelope stress response in *E. coli*.

Under inducing conditions such as alkaline pH, the presence of misfolded proteins, aggregation of pilus subunits, and overexpression of the lipoprotein NlpE, the sensor kinase, CpxA, senses the inducing cues in the periplasm. Then it autophosphorylates and transfers the phosphate group to the response regulator, CpxR. Then CpxR mediates changes in gene expression, either by activating protein folding and degrading factors or repressing envelope localized protein complexes, including virulence factors. Under non inducing conditions CpxA acts as a phosphatase to maintain CpxR in an unphosphorylated state. (OM) outer membrane, (IM) inner membrane. Figure adapted from (86).

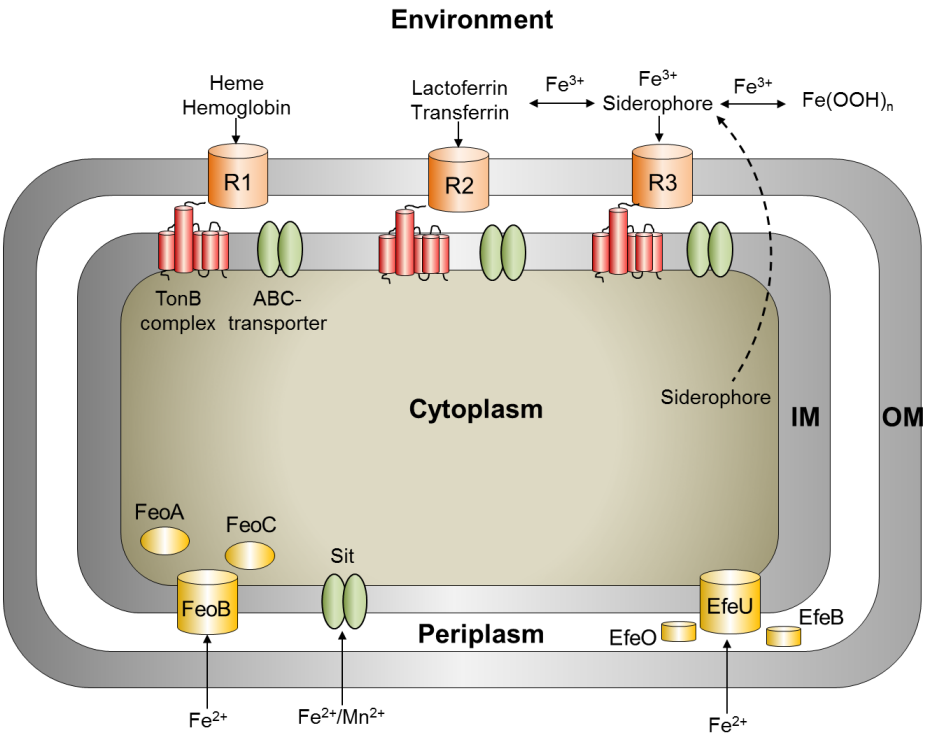

Figure 1 - 4 Fe^{2+} and Fe^{3+} transport systems in Gram-negative bacteria.

Under anaerobic conditions Fe^{2+} is soluble and can diffuse across the outer membrane (OM) to the periplasm where it is transported by various systems (e.g. Feo, Sit and Efe systems) across the inner membrane (IM). Heme is taken up into the cytoplasm through specific receptors (R1). Transferrin and lactoferrin release Fe^{3+}at the cell surface which is taken up into the cytoplasm through specific receptors (R2). Fe^{3+} transport requires its ligation with siderophores. Siderophores are synthesized in the cytoplasm and released by specific export systems. Once the iron-siderophore complex is formed, it binds to a specific receptor protein (R3) located in the OM and is transported across the IM into the cytoplasm via an energy dependent system, the TonB complex, and ATP-binding cassette (ABC) transporters. Figure adapted from (325).

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2 Chapter 2 Characterization of the Cpx regulon and determination of the novel inducing cues of the Cpx pathway in *Vibrio cholerae*.

2.1 Introduction

Perturbations of the bacterial cell envelope can induce cell envelope stress responses. During this process signal transduction pathways, such as two-component systems (TCS), are necessary for transducing the information from the cell envelope to the cytoplasm for gene expression regulation (79). TCS are the most prevalent signaling pathways in bacteria, and they are involved in the response to different inducing cues, mainly related to stress response and environmental changes (63). The Cpx envelope stress response is an example of a TCS, which is composed of the sensor histidine kinase CpxA, and the response regulator CpxR (330). This system senses envelope stress and regulates the expression of genes involved in maintaining cell envelope homeostasis to increase bacterial survival under adverse conditions (253).

CpxA is an inner membrane (IM) protein that autophosphorylates upon detecting an inducing cue and then becomes a phosphodonor and transfers a phosphoryl group to a conserved aspartate of its response regulator, CpxR (183, 330). CpxR phosphorylation leads to the alteration in transcription of multiple genes by the direct binding of CpxR to DNA (183, 185). In addition, CpxR phosphorylation is modulated indirectly by a periplasmic protein, CpxP, which reduces CpxA activity (185, 186) through a possible direct interaction with the periplasmic sensing domain of CpxA (69, 70, 187, 188).

The Cpx system in *Escherichia coli* senses misfolded proteins in the bacterial cell envelope and regulates protein folding and degrading factors, such as DegP, DsbA and PpiD, involved in the alleviation of the envelope stress (196, 213, 214, 216). For example, some of the inducing cues of this pathway are aggregated Uropathogenic *E. coli* P pilus subunits and subunits of the Enteropathogenic *E. coli* bundle forming pilus (BFP) (203, 204). Likewise, there are other

inducing cues that can trigger the Cpx pathway but may not be related to misfolded proteins such as overexpression of the outer membrane (OM) lipoprotein NlpE, changes in membrane composition, alkaline pH, and attachment to hydrophobic abiotic surfaces (190, 191, 208, 331).

The Cpx pathway has been studied in other bacterial species and, although in all cases it has been shown to regulate envelope-associated functions, species-specific adaptations are also apparent. For example, in *Haemophilus ducreyi,* the Cpx response appears not to be as tightly linked to protein misfolding, but rather was shown to control the expression of many genes, predominantly in a negative fashion, including several major virulence determinants (258, 259). Similarly, in *V. cholerae* El Tor N16961, recent evidence indicates that the Cpx pathway may be involved in sensing and mediating adaptation to high salinity, in addition to misfolded envelope proteins that contain aberrant disulfide bonds (261). A recent microarray study reported that, in *V. cholerae* El Tor N16961 strain, a major function of the Cpx response is to control efflux pump expression (332) as has been shown in *E. coli* (213) and *Klebsiella pneumonia* (333).

V. cholerae is a facultative pathogen which has human and environmental stages during its life cycle (334). It is the causative agent of cholera, a major cause of morbidity and mortality in countries lacking suitable water sanitizing practices (335, 336). Cholera is transmitted to humans by the ingestion of contaminated water or food. More than 200 serogroups of *V. cholerae* have been described: O1 serogroup strains of *V. cholerae* have been associated with the pandemic spread of cholera (272), whereas non-O1 strains can cause diarrhea but do not cause epidemics (337). Serogroups O1 and O139 are known to produce the main virulence factors in this bacterium: the toxin co-regulated pilus (TCP) and cholera toxin (CT) (287). CT is an ADP-ribosylating enterotoxin (338) that leads to the watery diarrhea associated with *V. cholerae* (279).

During colonization of the human gastrointestinal tract (GI), *V. cholerae* encounters stressful conditions, such as changes in pH, CO_2, osmolarity, and the presence of antimicrobial compounds, such as bile, in the intestinal lumen (339). In order to protect itself from bile, *V. cholerae* has several resistance mechanisms. One of these includes induction of expression of genes involved in efflux, such as *vceB*, *tolC*, *acrA*, *vexB*, *vexD* and *vexH* (340-345). Another mechanism is the modulation of OM permeability, by regulating the differential expression of *ompU* and *ompT* in a ToxR-dependent manner (346, 347). It has been shown that OmpU is important for resistance to organic acid stress, a condition that *V. cholerae* could encounter either during the course of colonization of the small intestine or in the environment (348).

In addition to stresses related to antimicrobial compounds, *V. cholerae* encounters others stresses such as iron limitation either in the human host or in the environment. Iron is an essential nutrient, being important in biological processes such as energy metabolism, DNA replication and oxygen transport (349, 350). In the human host, iron is sequestered by the presence of iron binding proteins such as transferrin or lactoferrin, a host defense mechanism that prevents pathogen growth (351). While iron is abundant in the aerobic environment, it exists as an insoluble ferric form that decreases its bioavailability (349); to overcome both of these iron limitation conditions, *V. cholerae* has multiple iron transport systems (e.g. siderophores, receptors for iron binding proteins, TonB1 and TonB2 systems) that may allow acquisition of iron in each of these two environments (312).

In the current study, we characterized the genes that are regulated by CpxR in *V. cholerae* El Tor C6706 strain under virulence-inducing conditions likely to simulate those encountered during an infection (352). Unlike a recently reported transcriptome study performed under laboratory growth conditions in LB on *V. cholerae* El Tor N16961 strain (332), we found that the Cpx

pathway regulates the expression of genes with diverse function in the cell. Notably, in addition to the reported alteration in the expression of genes involved in resistance-nodulation-cell division (RND) efflux pumps (332), we found that activation of the Cpx pathway leads to changes in the expression of genes involved in iron acquisition under AKI conditions, previously shown to maximize virulence factor production in *V. cholerae* (352). Based on the identity of the Cpx-regulated genes reported here, we identified new Cpx inducing cues in *V. cholerae* C6706 related to iron and efflux and showed that the Cpx response, and members of its regulon, is important for survival in the presence of these inducers.

2.2 Materials and Methods

2.2.1 *Growth conditions*

Cultures were routinely grown and maintained in Luria–Bertani (LB) broth with the appropriate antibiotics at 37°C and stored at -80°C in LB broth containing 10% glycerol. AKI conditions were used for *V. cholerae* El Tor C6706 in vitro virulence-inducing conditions (352). Briefly, overnight cultures grown in LB broth were inoculated into AKI medium (1.5% Bacto peptone, 0.4% Difco yeast extract and 0.5% NaCl) at a 1:10,000 dilution, followed by 6 hours of static growth at 37°C, and then transferred to shaking growth conditions (125-m flask) at 37°C. Antibiotics (all from Sigma) were used at the following concentrations in selective media: ampicillin (Amp), 100 μg/ml; kanamycin (kan), 50 μg/ml; streptomycin (Sm), 100 μg/ml and spectinomycin (Spc), 25 μg/ml. L-arabinose (Sigma) was added to growth media to a concentration of 0.1% for CpxR induction experiments. O-Nitrophenyl β-D-galactopyranoside (ONPG) (Sigma) was added to growth media to a concentration of 10 mg/ml for β-galactosidase assays.

2.2.2 *Bacterial strains and plasmids*

A spontaneous streptomycin-resistant variant of *V. cholerae* El Tor C6706 strain was used as the parental strain to create all the *V. cholerae* strains used in this study. All bacterial strains and plasmids used in this study are listed in Table 2-1. *V. cholerae* El Tor C6706 mutants used in this study originated from a transposon insertion library (353). In order to facilitate the analysis of gene expression using luminescent reporter genes carried on a plasmid encoding Kan^R, the Kan^R resistance cassette, the promoterless *lacZ* and the *gfp* transcriptional reporter were excised from the insertion mutation of some mutant strains. To remove the kanamycin cassette from the transposon insertion mutation of the mutants used (i.e. VC2692, VC2436, VC0914 and VC0164), the pBR-flp plasmid was transformed onto *V. cholerae* El Tor C6706 mutants and Flp-mediated excision of the kanamycin-resistance cassette was performed as previously described by Silva and Blokesch (354). Briefly, transformed mutants with the pBR-flp plasmid (Table 2-1) were grown on LB agar plates with ampicillin at 37°C for 24 hours. Then, antibiotic sensitivity was tested by re-streaking the clones on both LB agar plates with ampicillin and plates with kanamycin. Kanamycin sensitive clones were isolated and cured of plasmid pBR-flp by overnight growth in LB broth without any antibiotic at 30°C. Serial dilutions of the overnight culture were plated on LB agar plates without any antibiotic, and then ampicillin-sensitivity of single colonies was tested by streaking the clones on LB agar plates with or without ampicillin. To confirm the right Flp-mediated excision event in the transposon mutants, which left behind a 192 in-frame fragment with a FLAG epitope, PCR using primers flanking the mutated genes (Table 2-2) was performed. These flipped-out insertion mutations will be denoted in this paper as *cpxR*, *tolC*, *vexH* and *vexB* mutants. To confirm that the transposon insertion mutants carrying the Kan^R cassette exhibited the same phenotypes as the isogenic mutants in which the Kan^R cassette had been excised, we tested the bile sensitivity phenotypes of the *tolC::Kan, tolC, vexB::Kan,* and

vexB mutants after we flipped out the kanamycin cassette. All pairs of mutants, before and after the Flp-mediated excision, exhibited indistinguishable bile sensitivity relative to each other (data not shown).

To generate the *cpxR* overexpression vector (pCpxR) and (pCpxREc), the *cpxR* gene of *V. cholerae* El Tor C6706 and *Escherichia coli* were cloned in pBAD24 (355). The *cpxR* gene was amplified using primers cpxRF-cpxRR and ECCpxRF- ECCpxRR for *V. cholerae* and *E. coli* respectively (Table 2-2). The PCR products were then purified, using a QIAquick PCR purification kit (Qiagen), following the manufacturer's instructions. Then, the PCR products were cloned into the XmaI and PstI sites of the pBAD24 plasmid, using standard techniques. Inserts were confirmed by sequencing using primers flanking the multiple cloning site of pBAD24 (pBADF, and pBADR, Table 2) (355).

To construct the *cpxP–lux* reporter plasmid, the promoter region of *cpxP* was amplified by PCR using primers luxFcpxP and luxRcpxP (Table 2-2) and cloned between the EcoRI and BamHI sites of the pJW15 vector (227). The resulting plasmid was designated pN3.

2.2.3 *β-galactosidase assay*

To assess if there is any activation of the Cpx pathway in *E. coli* MC4100 by heterologous expression of the *V. cholerae* CpxR homologous, we quantified the expression of *cpxP*-lacZ and *cpxR*-lacZ transcriptional fusions using a β-galactosidase assay as previously described (223). Briefly, subcultures (1:50) of the *E. coli* strains carrying either pBAD24, the vector control, or the pCpxR plasmid were grown by quintuplicate in LB broth at 37°C with aeration for 6 hours. For Cpx induction, cultures were grown for 2 hours and then induced with 0.1% of L-arabinose and returned to growth for additional 4 hours. After the 6 hours of subculture, cells were

resuspended in 2 ml of 1×Z-buffer. An aliquot of the suspension was transferred to a 96-well microtiter plate and the OD_{600} was read. Subsequently, cells were lysed by adding chloroform and 0.1% SDS and were left standing for 10 minutes at room temperature. Then 50 µl of the lysed cell mixture was diluted in 1×Z-buffer (150 µl) in 96-well microtiter plates. To each well 50 µl of 10 mg/ml ONPG was added and the absorbance at 420 nm was read every 30 seconds for 20 minutes and Miller units were calculated.

2.2.4 *V. cholerae expression array*

For analysis of the *V. cholerae* El Tor C6706 CpxR regulon, the effect of *cpxR* overexpression in *V. cholerae* El Tor C6706 was evaluated using two independent RNA preparations for each of the non-induced (no arabinose) and induced (0.1% arabinose) conditions. Total RNA was extracted from cultures in AKI condition (as explained above) at 37°C (352). After 6 hours of static growth at 37°C, and transfer to shaking growth conditions, we added 0.1% of arabinose to induce the overproduction of *cpxR* and then after 25 minutes of growth 1 ml of culture was harvested and resuspended in 1 ml of TRIzol reagent (Ambion). RNA was extracted as recommended by the manufacturer (Ambion). RNA samples were quantified using the NanoDrop-1000 (Thermo Scientific). To assess the quality of the RNA, a sample of the isolated RNAs was run on the Agilent 2100 BioAnalyzer using the Agilent Prokaryote Total RNA Nano Kit (Agilent Technologies, Inc.), following the manufacturer's instructions. Then, one microgram of total RNA was subjected to a DNase treatment according to the manufacturer's instructions (Invitrogen). The resulting RNA was reverse transcribed using SuperScript III reverse transcriptase (Invitrogen), according to the manufacturer's instructions. Each cDNA sample was labeled with Cy3 dye using the NimbleGen Dual-Color DNA Labeling Kit (Roche), according to the manufacturer's protocols. For each condition, the hybridization experiment was performed

using a Roche NimbleGen *V. cholerae* El Tor N16961 Gene expression array (Roche). To ensure that there was no cross hybridization between samples in our experiments, each cDNA sample was labeled with a tracking control. Following hybridization, the arrays were washed, dried, and then scanned using a NimbleGen MS200 Microarray Scanner (Roche). Microarray data were analyzed using Partek Genomics Suite software (Partek). Principal component analysis (PCA) was performed to determine the clustering of biological and technical replicates. Genes showing a ≥ 2 or ≤ -2 fold differences in expression between the non-induced and induced condition, with a $P < 0.05$ were classified as CpxR regulated genes. To determine the functional category of the differential gene expression profile, genes were clustered according to their functional category established using the J. Craig Venter Institute (formerly TIGR) database for *V. cholerae* El Tor N16961 (http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?org=gvc).

For analysis of gene expression of wild type *V. cholerae* El Tor C6706 and the *cpxR* mutant (EC16554) in the presence of iron chelator, total RNA was extracted from bacterial strains grown for 3 hours as spots on yeast extract plus tryptone agar plates with and without 2,2'-Bipyridyl (100 μM) using two independent RNA preparations for each of the conditions. Total RNA was extracted and reverse transcribed as described above.

2.2.5 *Quantitative RT-PCR (qRT-PCR)*

We used qRT-PCR to validate the effect of *cpxR* overproduction on gene expression and the changes in gene expression for *V. cholerae* El Tor C6706 and the *cpxR* mutant (EC16554) in the presence of 2,2'-Bipyridyl (100 μM). For each target gene, specific primers (Table 2-3) were designed to amplify nucleotide fragments of ≈100 bp. qRT-PCR were performed using a 7500 Fast Real-Time PCR System (Applied Biosystems). The amplification reactions were carried out with equal amounts of cDNA (100 pg/μl) as initial template, and each reaction contained 225

nmol of primers (Table 2-3) and 5 µl of master mix as previously described (213). *gyrA* was used as an endogenous control since it did not show variation in gene expression under the conditions tested by microarray analysis.

2.2.6 *Luminescence assay on agar plates*

The luminescence activity produced by the *cpxP-lux* reporter gene (pN3) or the vector control (pJW15) in wild type *V. cholerae* El Tor C6706 and *cpxR*, *tolC*, *vexH* and *vexB* mutants was measured on LB agar plates in the absence and presence of different inducing cues. Bacterial strains were grown as lawns on LB agar plates with the appropriate antibiotics at 37°C. Bacterial cells were pelleted from overnight LB cultures carrying the appropriate antibiotics in volumes normalized according to the absorbance measured at 600 nm and spotted in triplicate onto pre-warmed LB agar or LB agar plates supplemented with NaCl (100 mM), $MgCl_2$ (100 mM), $CuSO_4$ (125 µM), diazenedicarboxylic acid bis (N,N-dimethylamide, diamide) (125 µM) or yeast extract plus tryptone agar plates supplemented with 2,2'-Bipyridyl (100 µM). In some experiments, $FeSO_4$ (80 µM) was added alone or in combination with other chemicals as indicated. Plates were incubated at 37°C overnight and luminescence was visualized using the ChemiDoc MP system (BIORAD). Assays were performed at least three times and one representative experiment is shown.

Quantification of the luminescence activity produced by either the *cpxP-lux* reporter gene (pN3) or the vector control (pJW15) was determined by individually resuspending each spot on 1 ml of LB broth, from which 200 µl were transferred to a 96-well microtiter plate and the counts per second (CPS) and OD_{600} were read. Measurements were done using a Wallac 1420 multilabel plate reader (Perkin-Elmer).

2.2.7 *Susceptibility assays*

For susceptibility assays in M9 minimal medium single colonies of strains to be tested were inoculated into 5 ml of LB broth plus antibiotics in triplicate and grown at 37°C with aeration overnight. For complementation assays, exogenous CpxR was induced from the pCpxR vector with a 0.01% volume of L-arabinose in the overnight cultures. The next day, a 1:50 dilution was made in 1 ml of M9 medium plus 0.2% succinate for each biological replicate, with the appropriate antibiotics, and supplemented with 80 μM 2,2'-Bipyridyl plus 80 μM $FeSO_4$. Subcultures were grown at 37°C in a 96-well microtiter plate in triplicate with shaking, and the OD_{600} was measured using a Wallac 1420 multilabel plate reader (Perkin-Elmer) every hour over a period of 11 hours.

For susceptibility assays in AKI conditions single colonies of strains to be tested were inoculated into 5 ml of LB broth plus antibiotics in triplicate and grown at 37°C with aeration overnight. For complementation assays, exogenous CpxR was induced from the pCpxR vector with a 0.001% volume per culture of L-arabinose in the overnight cultures. The next day, a 1:10,000 dilution was made in 10 ml of AKI medium, with the appropriate antibiotics in the presence of 0.5 mM diamide plus 80 μM $FeSO_4$, and grown at 37°C without shaking. After 5 hours of growth, 200 μl of culture was transferred to a 96-well microtiter plate in triplicate, and grown at 37°C with shaking. OD_{600} was measured using a Wallac 1420 multilabel plate reader (Perkin-Elmer) over a period of 6 h.

2.3 Results

2.3.1 *Virtual footprint analysis*

A recent microarray study reported that 25 genes were Cpx-regulated in the O1 *V. cholerae* El Tor N16961 (332). We set out to characterize the Cpx regulon in *V. cholerae* El Tor strain C6706, as it has a functional quorum sensing system and there is some evidence of a connection between the Cpx response and quorum sensing, since a quorum-sensing inducer regulates *cpxA* expression in *Photorhabdus luminescens* (356). Further, the Cpx response has been linked to the regulation of virulence genes in many pathogens (for a recent review, see reference Vogt and Raivio (173)), and quorum sensing plays a role in this process in *V. cholerae* (303).

As the *E. coli* and *V. cholerae* CpxR proteins are very similar (60.3% identity) (261), and the DNA binding domain is almost 100% conserved, we used the previously described *E. coli* CpxR binding site (5'-G(T/C)AA(A/C)(N)5(G/C)(T/A)(A/C/T)(A/C)(A/G/C)-3') (221) to search for Cpx regulon members in *V. cholerae* El Tor N16961, using the Virtual Footprint tool (http://prodoric.tu-bs.de/vfp/)(357). We scanned the *V. cholerae* El Tor N16961 genome as it is closely related to *V. cholerae* El Tor strain C6706, as found by phylogenetic analysis of the whole genome (358). Furthermore, based on a genome-wide single nucleotide polymorphisms (SNP) analysis, it was determined that 123 El Tor strains, which include N16961 and C6706 strains, differ by only ~50–250 SNPs across the entire genome (359). A total of 52 genes were found to have a putative CpxR binding site in their promoter region (Table 2-4). Of these, 34 genes are located on the large chromosome, and 18 are located on the small chromosome of *V. cholerae* (Table 2-4). Interestingly, two of those genes (i.e. VCA0537 and VCA0538) contain each one two putative CpxR binding sites in their promoter region (Table 2-4). Cluster analysis of these genes indicated they were mostly classified as hypothetical proteins (data not shown). As

expected, *cpxR* and *cpxP*, which are genes found to be Cpx-regulated in all organisms that possess them, contain the binding sites with the highest position weight matrix (PWM) scores (Table 2-4) and therefore the most similarity to the *E. coli* CpxR binding site. This is not surprising; given that heterologous expression of the *V. cholerae* CpxR homologue can complement an *E. coli* MC4100Δ*cpxR* strain carrying a *cpxR-lacZ* or *cpxP-lacZ* transcriptional reporter gene, restoring their expression (Figure 2-1). Further, the transcriptional regulatory domain (HTH) of CpxR in *V. cholerae* and *E. coli* is well conserved.

2.3.2 *V. cholerae CpxR regulon*

Microarray analyses in *H. ducreyi* and *E. coli* has shown that Cpx response induction leads to changes in expression of hundreds of genes, some that are directly regulated by CpxR, as well as many (most) that are not (223, 258). Thus, to determine if the above genes are bonified *V. cholerae* Cpx regulon members and identify additional candidates that may be indirectly regulated by CpxR, we examined the transcriptional profile of the pandemic *V. cholerae* El Tor C6706 using a microarray, based on the nucleotide sequence of the annotated *V. cholerae* El Tor strain N16961 (see above). Since activation of the Cpx response leads to inhibition of virulence determinant expression in many pathogens (225, 227, 252, 360), we performed our experiments in AKI media, using conditions previously shown to maximize virulence factor production (352). A total of 174 genes were differentially regulated (i.e. at least a two-fold increase or decrease in expression) upon transient overexpression of *cpxR*. Of these, 133 genes were up-regulated, and 41 were down-regulated (Table 2-5). Comparison of the Virtual Footprint results with our *V. cholerae* El Tor C6706 microarray data revealed that 16 genes containing a putative CpxR binding site were Cpx-regulated under these conditions (Figure 2-2, 13 were positively affected while 3 were negatively affected). These genes were amongst those found to be most strongly

CpxR-regulated (Figure 2-2, Table 2-5), which validates our approach and may suggest their direct regulation by CpxR. The remaining genes presumably contain weaker binding sites that were not detected by Virtual Footprint under the settings we used and/or are regulated indirectly.

To further examine the function of the Cpx response in *V. cholerae* C6706, we classified the 174 genes by gene clustering based on the J. Craig Venter Institute (formerly TIGR) database (http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?org=gvc). Overexpression of *cpxR* in *V. cholerae* alters the expression of genes involved in diverse cellular functions (Figure 2-3). Among these, the largest category was hypothetical proteins (39.7%). There was enrichment for genes encoding membrane-localized proteins, which mainly belonged to the group encoding transport and binding proteins (16.7%), energy metabolism (7.5%), protein fate (4%), and cell envelope (2.3%) (Figure 2-3). A noticeably large number of the up-regulated genes predicted to have a role in transport and binding of substrates were previously shown, or predicted, to be involved with iron acquisition, usage, or storage (Table 2-6). Similarly, as reported while this paper was being prepared for submission, genes encoding proteins involved in multidrug resistance (332) were enriched for, as were cation/proton antiporter systems (Table 2-5). Gene products involved in regulatory function were also regulated by the Cpx pathway. For example, these included a predicted two-component regulatory system (VC1638, VC1639) and a putative TetR family transcription factor (VC0166) (Table 2-5).

The strongest CpxR down-regulated genes (i.e. VC2468-VC2470) are clustered together on the large chromosome of *V. cholerae*. They encode two hypothetical proteins and an L-aspartate oxidase, and they are located downstream of the *rpoE* gene, which was previously shown to be part of the CpxR regulon in *E. coli* (213, 223). Altogether, our data suggest that the *V. cholerae* Cpx response controls genes involved in diverse functions mainly involved with the membrane

and transport. Recent *E. coli* and *H. ducreyi* Cpx microarray studies similarly identified Cpx regulon members as being enriched in membrane-localized proteins and transport functions, suggesting that the Cpx response plays a conserved role in mediating adaptation at the inner membrane (223, 258).

2.3.3 *Induction of the Cpx response changes the expression of virulence regulators*

Since the Cpx response negatively regulates virulence in other pathogens (173), we examined changes in gene expression upon overproduction of CpxR in AKI conditions, when virulence factors are produced. In our study, the transcripts of the genes encoding cholera toxin and the TCP were not detectable. This is likely because we performed our analysis at an early time point (25 minutes after transfer to shaking conditions in AKI media) that precedes the accumulation of high levels of these virulence determinants (16 hours after transfer to shaking conditions in AKI media) (352). In spite of this, the transcript levels of genes encoding products involved in virulence factor regulation were altered. The expression of the *toxRS* operon and *phoB*, both of which encode regulators of cholera toxin and TCP expression in *V. cholerae* (290, 292, 361), was changed upon induction of CpxR expression. (Table 2-5, 4.4, 4.0, and 2.2 fold elevation, respectively). Although we did not find a putative CpxR binding site in the promoter region of *toxR,* it was found for *phoB* (Figure 2-2). To our knowledge, this is the first study to report that an envelope stress response system regulates the expression of the *toxR* and *toxS* genes. These data suggest that prolonged activation of the Cpx response in *V. cholerae* strain C6706 may lead to downstream effects on virulence factor production (Refers to Chapter 4).

2.3.4 *The V. cholerae C6706 Cpx regulon is enriched for iron-related genes*

Our microarray analysis indicated that among the differentially expressed genes, 36 (20.7%) (Table 2-6) are involved with iron metabolism and acquisition in *V. cholerae*, based on the gene

ontology classification that is part of the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (362, 363). These included genes that encode components of the TonB1 energy-transduction system (VCA0910-12), genes involved in heme utilization, such as those encoding the HutBCD ABC transport system (VCA0913-VCA0915), the *hutWXZ operon* (VCA0907-VCA0909) (364), and the heme receptor protein HutA (VCA0576), genes for vibriobactin siderophore biosynthesis (VC0771) and transport (VCA0227, VCA0229, VCA0230), and genes for ferrichrome transport (VC0200–VC0201). Interestingly, a previous study showed that a transposon insertion in the *hutA* gene activates the Cpx pathway in *V. cholerae* (261). To confirm the observed changes in gene expression in the microarray for some of these genes, qRT-PCR was performed and the relative quantification was in accordance with the microarray data (Figure 2-4A, data not shown).

The most strongly CpxR regulated gene related to iron was VCA0151, which was up-regulated 179.36 fold when *cpxR* was overexpressed (Table 2-6). This change in gene expression was confirmed by qRT-PCR, showing a 147 fold change upon *cpxR* overexpression (Figure 2-4A). VCA0151 possesses a putative CpxR binding site (Figure 2-2) and is predicted to encode a putative ferric reductase (inner membrane localized). Ferric reductases are key enzymes for the iron assimilatory pathway that function by reducing chelated Fe(III) either before or after transport into the bacterial cytoplasm (365). Thus, in addition to the up-regulation of iron acquisition genes (see above, Table 3), the Cpx pathway also appears to regulate genes required for the reduction and intracellular incorporation of iron.

The Cpx response in *V. cholerae* El Tor C6706 also regulates the expression of genes that encode proteins involved in energy metabolism and oxidative phosphorylation, which utilize iron-containing cofactors. For example, CpxR strongly up-regulates the expression of two

predicted cytochrome b_{561} orthologs in *V. cholerae*, VCA0249 and VCA0538. Both showed more than a 38-fold change upon activation of the Cpx pathway (Table 2-6) that was confirmed by qRT-PCR (Figure 2-4A). The VCA0249 and VCA0538 genes have one and two putative CpxR binding sites, respectively, within their upstream regulatory region (Figure 2-2, Table 2-4) and their expression depends on iron availability in *V. cholerae* (366). Cumulatively, our data suggest that reallocation of iron within the cell may be related to either or both the Cpx inducing signal and the subsequent adaptation conferred by the *V. cholerae* Cpx response.

2.3.5 *The V. cholerae C6706 Cpx pathway increases expression of RND genes*

Six RND-efflux pump encoding loci have been annotated in the *V. cholerae* genome (367). As was recently reported in *V. cholerae* El Tor N16961 (332), we discovered that activation of the Cpx pathway in strain C6706 also leads to increased expression of two of these (i.e. VexAB and VexGH). A RND efflux system is composed of three components: the RND pump protein, a membrane fusion (MF) protein and an OM pore protein (368). *vexA* (VC0165) encodes a MF protein and its expression changes 3.69-fold (Table 2-5). *vexB* (VC0164) encodes a RND pump protein, whose expression upon Cpx activation changes 8.15-fold (Table 2-5). Additionally, the expression of the *vexR* (VC0166) gene, that is located upstream of the *vexAB* operon, changes 19-fold compared to the non-inducing condition (Table 2-5). VexR is a putative TetR family transcriptional regulator that may control the expression of the *vexAB* operon (343). Interestingly, we found a putative CpxR-binding site 57 bp upstream of the transcription start site of the *vexR* gene (Figure 2-2). Taken together, these results suggest the Cpx pathway may regulate expression of the VexAB RND efflux system directly.

Activation of the Cpx pathway also strongly upregulated the expression of the *vexGH* operon. VexG (VC0913) encodes a putative MF protein and VexH (VC0914) encodes a putative RND

pump protein and their expression was changed 22.41 and 28.24-fold respectively (Table 2-5). The observed changes in gene expression of both the *vexAB* and *vexGH* operons were verified by qRT-PCR (Figure 2-4B). This data is in accordance with a previous study (332) that also found that the CpxR positively regulates the expression of *vexAB* and *vexGH* operons (332). Bina and collaborators (343) previously reported that it is likely that all the RND efflux systems described in *V. cholerae* utilize the OM protein TolC. In our microarray the expression of *tolC* (VC2436) increased by 2.3-fold (Table 2-5) when *cpxR* was overexpressed. This change was confirmed by qRT-PCR (Figure 2-4B). Furthermore, upon *cpxR* overexpression in *V. cholerae* El Tor C6706, we found an increase in intensity of a band in OM preparations that we identified as TolC by mass spectrometry (data not shown). This result confirms our microarray and qRT-PCR data and suggests that up-regulated efflux is an important component of the Cpx response.

2.3.6 *The V. cholerae C6706 Cpx response is induced by iron limitation and inhibition of efflux pumps*

Previously, Slamti and Waldor (261) reported that the activation of the Cpx pathway in *V. cholerae* is required to mediate cellular responses towards changes in salinity and oxidative stress (261). For example, they found an increase in the signal of a *cpxP-lacZ* reporter when different concentrations of sodium chloride and $CuSO_4$ were added to a basal medium of yeast extract and tryptone (261). To test if the previously described inducing cues (i.e. chloride ions and $CuSO_4$) of the Cpx pathway in *V. cholerae* El Tor strain N16961 also activate the Cpx pathway in the C6706 strain background, we constructed a *cpxP-lux* transcriptional reporter using the previously described reporter plasmid pJW15 (227). We then measured the luminescence activity of wild-type strain C6706 carrying either the vector control, pJW15, or the *cpxP-lux* transcriptional reporter grown on agar plates supplemented with 100 mM NaCl, 100 mM $MgCl_2$ or 125 μM

$CuSO_4$. As expected, we found an increase in luminescence of the *cpxP-lux* reporter, but not the pJW15 vector control, for those inducing cues (Figure 2-5). In contrast to *E. coli,* alkaline pH did not activate the Cpx response in *V. cholerae* under the conditions examined here (data not shown).

Slamti and Waldor (261) also previously showed that mutations impacting periplasmic disulfide bond isomerization induce Cpx signaling. Consistent with this observation, we found that diamide, an oxidizing agent that reacts with low molecular weight thiols and promotes protein disulfide cross-linking (369), activates the Cpx pathway. Luminescence of *V. cholerae* El Tor C6706 carrying the *cpxP*-lux reporter was elevated compared to the vector control in the presence of 125 μM diamide (Figure 2-5E). There was no increase in *cpxP-lux* reporter activity upon exposure to H_2O_2 (data not shown), suggesting that the activation of the Cpx pathway in *V. cholerae* C6706 by diamide is not due to a general oxidative stress.

We found in our microarray data that genes that are induced by iron limitation are also up-regulated upon *cpxR* overexpression. To determine whether iron limitation in the cell is a signal for the activation of the Cpx pathway, we examined the activation of transcription of the *cpxP-lux* reporter in the presence of an iron chelator 2,2'-Bipyridyl. The presence of 100 μM 2,2'-Bipyridyl strongly activated the Cpx pathway, as the signal from the *cpxP–lux* reporter was elevated in comparison with the vector control (Figure 2-5F). For reasons that we do not yet understand, Bipyridyl acted as an inducing cue only on LB plates lacking NaCl. Changes in activity of the *cpxP-lux* reporter were confirmed to be CpxR-dependent for the above mentioned inducing cues (i.e. sodium chloride, $CuSO_4$, diamide and 2,2'-Bipyridyl) by measuring luminescence of the same reporter gene in a *cpxR* mutant (Figure 2-6).

To confirm the observed changes in the activation of transcription of the *cpxP-lux* reporter in response to the above stimuli, luminescence activity of each individual spot was quantified and corrected for cell density (OD_{600}) (Figure 2-7A-E). These data confirmed that $MgCl_2$, $CuSO_4$, diamide and 2,2-Bipyridyl all activate expression of the *cpxP-lux* reporter gene, in a CpxR-dependent fashion (Figure 2-6 and2-7). Conversely, NaCl, which appeared on plates to induce the Cpx response in some sections of spotted lawns of bacteria (Figure 2-5), actually appears to inhibit the Cpx response when luminescence of the entire spotted lawn is taken into account (Figure 2-7). These data suggest that the previously reported induction of the Cpx response by NaCl is a complex phenomenon that may depend on other, context-dependent signals.

To determine whether the activation of the Cpx pathway by the above mentioned inducing cues leads to changes in gene expression of some of the genes that we found to be CpxR-regulated, we extracted RNA from wild-type strain C6706 and the *cpxR* mutant (EC16554) grown on agar plates in the presence and absence of 2,2'-Bipyridyl. As expected, there was an increase in the expression of *cpxP* upon the presence of the iron chelator in the wild-type strain C6706, which was largely obliterated in the *cpxR* mutant (EC16554) (Figure 2-8A). The expression of *cpxP* appears to also be negatively regulated by CpxR, since basal levels of transcription were elevated in the absence of *cpxR* (Figure 2-8A). This is novel, since CpxR-dependent expression of *cpxP* has not previously been observed. Similar results were obtained when we analyzed the expression of additional genes identified as Cpx regulon members in the microarray data, such as *cpxA* and *vexH* (Figure 2-8B and C). CpxR seems to be required for basal expression of some Cpx regulon genes we identified, since *vexH* was expressed at lower levels in the absence of CpxR (Figure 2-8C). Additional regulators may be involved in the iron regulation of some Cpx regulated genes, since expression of *vexH* was still elevated in the *cpxR*

mutant by 2,2-Bipyridyl, although not to the same extent as in the wild-type strain (Figure 2-8C). These results support our microarray and luminescence data and suggest that iron chelation is a CpxR-dependent activating signal.

Since we also found that genes encoding efflux pumps and the TolC porin are strongly induced by the Cpx response, we speculated that these efflux pumps may rid the cell of a compound(s) that is an inducer of the Cpx pathway. To determine whether the Cpx response is activated when the two Cpx-regulated RND efflux pumps (i.e. VexB and VexH) and TolC are absent, we measured the luminescence activity of wild-type C6706 and *tolC, vexB* and *vexH* mutants carrying either the vector control, pJW15, or the *cpxP-lux* reporter grown on LB agar plates. As was recently reported by Taylor et al. (332) and Slamti et al. (261) for *V. cholerae* El Tor strain N16961, we found a dramatic increase in Cpx pathway activity when these efflux pump components were absent from the cell. The luminescence of the *cpxP-lux* reporter compared to the vector control was greatly elevated in the *tolC, vexB* and *vexH* mutants compared to wild-type C6706 (Figure 2-5G-I). These results were in accordance with the quantification of the luminescence activity (Figure 2-7F). Cumulatively, these observations suggest that the Cpx response in *V. cholerae* C6706 is induced by depletion of iron, aberrant disulfide bond formation, and defects in RND-pump mediated efflux.

2.3.7 *The V. cholerae C6706 Cpx response is required for adaptation to low iron and aberrant disulfide bond formation*

Induction of the Cpx response by the above mentioned stressors, iron limitation and aberrant disulfide bond formation, suggests that in *V. cholerae* C6706 the Cpx pathway is important for adaptation to them. To investigate whether Cpx pathway activation is necessary for survival when iron is limiting, we examined growth of wild-type C6706 and the *cpxR* (EC16554) mutant

in the presence of 2,2'-Bipyridyl. We observed no difference in the growth of the wild-type C6706 strain compared to the *cpxR* (EC16554) mutant in the presence of 2,2'-Bipyridyl in AKI conditions or on LB agar plates (data not shown). Since we found that the Cpx response regulates respiratory enzymes (Table 2-5), we wondered if iron depletion might induce the Cpx pathway because it affects iron-containing respiratory enzymes. Accordingly, we measured the ability of wild-type C6706 and the *cpxR* (EC16554) mutant to grow in the absence and presence of 2,2,'-Bipyridyl under growth conditions where the cells are forced to respire, namely in M9 minimal medium supplemented with succinate. Under these conditions, the *cpxR* (EC16554) mutant showed a defect in late exponential growth compared to the wild-type strain in the presence of 80 μM 2,2'-Bipyridyl (Figure 2-9 A and B). Complementation of the *cpxR* (EC16554) mutant with exogenous CpxR supplied by arabinose induction from the P_{BAD} promoter on pCpxR rescued this phenotype (data not shown). Our results suggest that the Cpx pathway in *V. cholerae* may be important for adaptation to conditions under which respiration is necessary and when iron is limiting.

To similarly test whether the Cpx response may be important for survival under conditions that impact disulfide bond formation, we examined the ability of wild-type C6706 and the *cpxR* (EC16554) mutant to grow in the presence of 0.5 mM diamide. The *cpxR* (EC16554) mutant showed a strong growth defect in AKI medium supplemented with 0.5 mM diamide compared to the wild-type strain (Figure 2-9 C and D). Complementation of the *cpxR* (EC16554) mutant with exogenous CpxR supplied by arabinose induction from the P_{BAD} promoter on pCpxR rescued this growth defect (data not shown).

2.3.8 *Iron can suppress Cpx response induction and Cpx dependent growth phenotypes*

Since our transcriptional data clearly links the Cpx response to adaptation to iron limitation, and iron chelation is a Cpx inducing cue, we wondered if Cpx sensed stresses might be related to low iron. To investigate this, we analyzed the effect of iron supplementation on the expression of a *cpxP-lux* reporter gene in the presence of *V. cholerae* Cpx response inducers. Addition of 80 μM $FeSO_4$ to 2,2'-Bipyridyl agar plates decreased the activity of the *cpxP-lux* reporter (Figure 2-10A). Moreover, addition of 80 μM $FeSO_4$ to agar plates containing the inducers diamide, $CuSO_4$ and $MgCl_2$ decreased the activity of the *cpxP-lux* reporter in a similar fashion (Figure 2-10B-D). Quantification of these stimuli with the presence or absence of iron was in accordance with the luminescence activity images (data not shown).

It has been hypothesized that the expression of the RND efflux pump VexH may be regulated in response to the iron status of the cell, most likely during the iron limitation conditions that *V. cholerae* encounters during late infection (344, 370, 371). To test whether the induction of the Cpx response by the inactivation of the VexH efflux pump is also related to iron, we examined the luminescence of a *cpxP-lux* reporter gene in wild-type *V. cholerae* C6706 and the *vexH* mutant grown on LB agar plates in the presence and absence of iron. As with the other Cpx inducing cues examined, supplementation of the *vexH* mutant with exogenous $FeSO_4$ decreased activation of *cpxP-lux* expression (Figure 2-10E, compare top and bottom panels, Figure 2-7F). Similarly, addition of exogenous $FeSO_4$ decreased activation of *cpxP-lux* expression in the *tolC* and *vexB* mutants (Figure 2-10 F and G, and Figure 2-7F). Our results suggest that iron is an important modulator of Cpx pathway signaling in *V. cholerae* El Tor C6706 in the presence of many, seemingly diverse, inducers.

To determine whether iron supplementation would also rescue the observed growth defect of the *cpxR* (EC16554) mutant in the presence of inducers, we added $FeSO_4$ back to cultures of wild-type C6706 and the *cpxR* (EC16554) mutant grown in the presence 80 μM 2,2'-Bipyridyl or diamide. The addition of 80 μM $FeSO_4$ improved the growth defect of the *cpxR* (EC16554) mutant grown in minimal succinate media in the presence of 2,2'-Bipyridyl, relative to the wild type strain (Figure 2-9 A and B). Consistent with the effects of exogenous iron on Cpx signalling, we found that 80 μM $FeSO_4$ also rescued the growth of the *cpxR* (EC16554) mutant in the presence of diamide under AKI conditions (Figure 2-9 C and D).

2.4 Discussion

We set out to study the Cpx regulon in *V. cholerae* El Tor strain C6706 in order to examine if this stress response performs conserved functions in the distantly related enteric diarrheal pathogens *V. cholerae* and *E. coli.* Since the Cpx response negatively regulates virulence in numerous other bacteria (173), we reasoned that strain C6706 was a better candidate than strain N16961 in which to begin our study of the *V. cholerae* Cpx response, as the quorum sensing circuitry, which has been shown to impact virulence determinant expression, is intact in this strain (303). Although this study was performed at time points prior to the accumulation of cholera toxin and the TCP, we found that expression of two important regulators of virulence is altered under these conditions. Both the *toxRS* and *phoB* loci exhibited elevated expression when CpxR was over-expressed (Table 2-5). Thus, downstream effects of the Cpx response on virulence factor was determined (Please refers to Chapter 4).

Curiously, this finding varies from a recent Cpx microarray study (332). These authors investigated the Cpx regulon in *V. cholerae* O1 El Tor N16961 strain by examining gene

expression changes that occurred in the presence of a *cpxA24* allele (183) that leads to constitutive activation of the Cpx response. Surprisingly, they reported changes in expression of only 25 genes, while the limited induction of CpxR over-expression used in this study lead to significant, two-fold changes in the expression of 174 genes (Table 2-5). At this point, we do not know the reason for this discrepancy, since more genes, not less, would be expected to exhibit changes in expression upon constitutive activation of the Cpx response. Two possibilities are strain differences and growth conditions. Taylor et al. (332) performed their work in *V. cholerae* El Tor strain N16961, which is known to contain a natural frame-shift mutation in the gene encoding the major quorum sensing regulator HapR (303). Perhaps this, and/or other mutations in this strain impact the Cpx regulon. Similarly, our study was performed in AKI medium under virulence factor inducing conditions, while Taylor et al. (332) examined the Cpx regulon in LB. As would be expected, growth conditions impact the genes that are changed upon Cpx pathway induction (223). Perhaps more genes subject to Cpx regulation are expressed during growth in AKI media as compared to LB. Regardless of these differences, both studies uncovered the Cpx regulation of common efflux pump components, suggesting that this Cpx-mediated adaptation is important under a variety of environmental conditions.

We found that the *V. cholerae* C6706 Cpx envelope stress response shares common features with that of other Gram-negative bacteria, like the regulation of inner membrane associated functions including transport, respiration, and efflux. We also discovered that the Cpx response is intimately connected to the regulation of genes involved in cellular iron homeostasis. To our knowledge, this is the first demonstration of a role for the Cpx envelope stress response in the regulation of iron acquisition. Mey and collaborators (366) reported the characterization of the ferric uptake regulator protein (Fur) and iron regulon in *V. cholerae* O395. A comparative

analysis (Table 2-6) with our data showed a high degree of overlap (see above). Fur regulates the level of intracellular iron by modifying expression of genes involved in iron uptake, storage, and metabolism (372). When the level of intracellular ferrous iron is raised, Fur blocks the transcription of iron uptake genes and positively regulates genes involved in iron storage, iron metabolism and antioxidant defense, mainly through the repression of the small RNA (sRNA) RyhB (373). As a consequence the cell can regulate the amount of iron acquisition to enable cellular functions and prevent damage caused by hydroxyl radicals (373). Fur was not found as part of the CpxR regulon under the *in vitro* conditions tested here, which implies that another regulator or regulatory mechanism may be involved in the regulation of iron acquisition in *V. cholerae* El Tor C6706 by the Cpx pathway. Although some genes predicted to be involved with iron homeostasis (e.g. VCA0151, VCA0249, VCA0538) contain upstream CpxR consensus binding sites, most others do not (Tables 2-5 and 2-6). How are these genes regulated by the Cpx response? One possibility could be the Cpx-regulated putative VC1638/VC1639 TCS. Comparative analysis of VC1638 showed it has similarity with the response regulator FeuP in *V. nigripulchritudo*, a homologue of which is involved with iron acquisition in *Rhizobium leguminosarum* (374). Another possibility could be through the Cpx regulation of the sRNA regulator *ryhB*. Further analyses to dissect the mechanism(s) of Cpx regulation of iron acquisition genes are required to understand the role of the Cpx pathway and iron.

The *V. cholerae* Cpx pathway not only regulates expression of well-characterized iron acquisition genes such as those encoding siderophores, multicomponent receptors and energy dependent transport systems (Table 2-6), but it also strongly regulates expression of three uncharacterized genes, VCA0151, VCA0249, and VCA0538, the products of which are predicted to encode an oxidoreductase and two cytochrome b_{561} ferric reductases (366). Induction of the

Cpx response by overexpression of *cpxR* also leads to changes in expression of genes encoding proteins predicted to contain iron cofactors, as well as VC2690, an iron-efflux transporter, *bfd* (VC0364), a bacterioferritin-associated ferredoxin and *bfr* (VC0365), a bacterioferritin (Table 2-5). The regulation of these genes by the Cpx response, and their involvement with iron, is apparently physiologically important, since mutation of *cpxR* leads to growth phenotypes under iron limiting and/or in the presence of Cpx-sensed envelope stress cues (Figure 2-9). Further, these phenotypes, as well as the induction of the Cpx response, can be relieved with exogenously added iron (Figures 2-9 and 2-10). Cumulatively, these results suggest a close connection between the Cpx envelope stress response and the control of iron homeostasis through its ability to control expression of iron acquisition functions, iron-containing respiratory enzymes, and factors involved in iron-related detoxification and storage.

In addition to confirming previously characterized *V. cholerae* Cpx inducing signals, we identified unique activating cues. Consistent with the enrichment for genes involved with iron homeostasis in our microarray, we found that 2,2'-Bipyridyl, a compound that chelates iron, activates the *V. cholerae* C6706 Cpx pathway (Figures 2-5 and 2-10) Additionally, we demonstrated that diamide and the mutation of certain efflux components leads to activation of the *V. cholerae* Cpx response (Figures 2-5 and 2-10). Previously, Slamti and Waldor (261) showed that mutations and conditions leading to aberrant disulfide bond formation in secreted proteins serve to activate the Cpx response in *V. cholerae* N16961. Our finding that diamide, an oxidizing agent that reacts with low molecular weight thiols and promotes protein disulfide cross-linking (369), is also a Cpx inducing cue is in line with this observation. Slamti and Waldor (261), together with Taylor et al. (332), showed that mutation of *tolC, vexB,* or *vexH* caused activation of the Cpx response in *V. cholerae* El Tor strain N16961. We demonstrate a similar

phenomenon here in *V. cholerae* El Tor strain C6706 (Figure 2-5). These data support the hypothesis put forth here and by others that Cpx induction in these mutants is due to the accumulation of a specific metabolite that can only be handled by these efflux pumps. Toxic, Cpx-inducing metabolites likely also induce the Cpx response in other organisms, since it has been demonstrated in *E. coli* that the Cpx pathway is activated in a *tolC* mutant (115) and in *Sinorhizobium meliloti*, a symbiotic partner of the leguminous plant *Medicago sativa*, the absence of a functional TolC leads an increased susceptibility to antimicrobial agents and activation of the Cpx response (375).

Surprisingly, we found that Cpx induction by diverse cues could be reversed in all cases by the addition of exogenous iron (Figure 2-10). The simplest explanation for this observation is that iron chelation is a common component of the Cpx inducing signal. Disruptions in disulfide bond formation in the periplasm (expected in the presence of diamide, a *dsbD* mutation, or $CuSO_4$ (261)) lead to defects in the ligation of iron-containing heme B to apocytochrome c (376-378). *V. cholerae* is predicted to have 14 c-type cytochromes in the cell envelope (379). Further, the connection of VexH expression to cellular iron status (344, 370) suggests that VexH may be required for the detoxification of bio-products of iron chelation in *V. cholerae* El Tor C6706. These findings are all consistent with the generation of a Cpx-inducing signal caused by iron chelation. Given the link between periplasmic disulfide bond equilibrium and maturation of iron containing respiratory proteins, together with the observed regulation of electron transport chain components in *V. cholerae* and *E. coli* (223), one idea is that Cpx sensed envelope stresses lead to iron chelation that result in elevated levels of, and/or defective, apo-co-factors in the envelope that are toxic (Figure 2-11). This model is supported by the recent finding that the periplasmic accumulation of the siderophore enterobactin in *E. coli* efflux pump mutants is toxic, and these

conditions also lead to activation of the Cpx response, amongst others (380). In that study it was shown that accumulation of non-iron loaded enterobactin in the periplasm when *tolC* is deleted, leads to growth defect and morphological changes as that enterobactin may chelate iron from iron-dependent reactions involved in cell division or peptidoglycan synthesis (380). The ensuing regulation of iron acquisition functions, respiration complexes, and efflux pump components could then simultaneously restore co-factor levels, correct respiration defects, and rid the cell of toxic molecules related to respiratory protein/co-factor damage upon iron chelation. Alternatively, the effect of iron chelation and supplementation on Cpx signaling and the growth of *cpx* mutants may be unrelated to events in the envelope, and instead reflect a signal that is generated in the cytoplasm. Future studies are directed towards distinguishing these possibilities and elucidating the connection between the Cpx envelope stress response and the iron status of the cell.

2.5 Tables and Figures

Table 2 - 1 Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Source or reference
Strains		
C6706	*V. cholerae* El Tor C6706 strain; (Sm^R) streptomycin-resistant spontaneous	Dr. J. Mekalanos, Harvard Medical School
TR70	MC4100 λRS88 [*cpxP-lacZ*] *cpxR1::spc*	(69)
TR237	MC4100 λRS88 [*cpxR-lacZ*] *cpxR1::spc*	(186)
NA9	TR70 carrying pBAD24 plasmid; (Spc^R Amp^R)	This study
NA10	TR70 carrying pCpxR plasmid; (Spc^R Amp^R)	This study
NA41	TR70 carrying pCpxR Ec plasmid; (Spc^R Amp^R)	This study
NA220	TR237 carrying pBAD24 plasmid; (Spc^R Amp^R)	This study
NA223	TR237 carrying pCpxR plasmid; (Spc^R Amp^R)	This study
NA38	TR237 carrying pCpxR Ec plasmid; (Spc^R Amp^R)	This study
NA45	C6706 carrying pBAD24 plasmid; (Sm^R Amp^R)	This study
NA44	C6706 carrying pCpxR plasmid; (Sm^R Amp^R)	This study
NA69	C6706 carrying pJW15 plasmid; (Sm^R kan^R)	This study
NA58	C6706 carrying pN3 plasmid; (Sm^R kan^R)	This study
EC16554	Derivative of C6706 strain carrying TnFGL3 insertion in the *cpxR* gene (VC2692) (Sm^R kan^R)	(353)
EC23411	Derivative of C6706 strain carrying TnFGL3 insertion in the *vexB* gene (VC0164) (Sm^R kan^R)	(353)
EC16862	Derivative of C6706 strain carrying TnFGL3 insertion in the *vexH* gene (VC0914) (Sm^R kan^R)	(353)
EC2859	Derivative of C6706 strain carrying TnFGL3 insertion in the *tolC* gene (VC2436) (Sm^R kan^R)	(353)
NA46	EC16554 carrying pCpxR plasmid; (Sm^R Amp^R kan^R)	This study
NA142	*cpxR* mutant, EC16554 marker-less deletion mutant (Sm^R kan^S)	This study
NA288	*vexH* mutant, EC16862 marker-less deletion	This study

	mutant (Sm^R kan^S)	
NA290	*vexB* mutant, EC23411 marker-less deletion mutant (Sm^R kan^S)	This study
NA357	*tolC* mutant, EC2859 marker-less deletion mutant (Sm^R kan^S)	This study
NA261	NA142 carrying pJW15 plasmid; (Sm^R kan^R)	This study
NA263	NA142 carrying pN3 plasmid; (Sm^R kan^R)	This study
NA299	NA288 carrying pJW15 plasmid; (Sm^R kan^R)	This study
NA300	NA288 carrying pN3 plasmid; (Sm^R kan^R)	This study
NA301	NA290 carrying pJW15 plasmid; (Sm^R kan^R)	This study
NA302	NA290 carrying pN3 plasmid; (Sm^R kan^R)	This study
NA366	NA357 carrying pJW15 plasmid; (Sm^R kan^R)	This study
NA368	NA357 carrying pN3 plasmid; (Sm^R kan^R)	This study
Plasmids		
pBAD24	pBAD vector, pBR322 ori, *araC*,(Amp^R)	(355)
pCpxR	pBAD24 carrying *cpxR* of *Vibrio cholerae* C6706 (Amp^R)	This study
pCpxREc	pBAD24 carrying *cpxR* of *Escherichia coli* (Amp^R)	This study
pJW15	pNLP10 with p15 *ori* reporter vector (Kan^R)	(227)
pN3	*cpxP* promoter cloned into *luxCDABE* reporter vector pJW15 (Kan^R)	This study
pBR-flp	FLP^+, λ $cI857^+$, λ p_R from pCP20 integrated into EcoRV site of pBR322 (Amp^R)	Dr. Melanie Blokesch, Global Health Institute, Switzerland

Table 2 - 2 Primers used in this study.

Primer name	Oligonucleotide sequence 5' to 3' (restriction sites underlined)
cpxRF	TTCCCGGGATGGCACATATCCTTTTGATC
cpxRR	CTCTGCAGTTACTCCTCCTGAACCATTAAG
ECCpxRF	TTCCCGGGATGAATAAAATCCTGTTAGTTGATG
ECCpxRR	CTCTGCAGTCATGAAGCAGAAACCATCAG
pBADF	CTGTTTCTCCATACCCGTT
pBADR	CTCATCCGCCAAAACAG
luxFcpxP	TTGAATTCGCAAAATCGCACGAATGC
luxRcpxP	TTGGATCCCTGTTTCCAGATCCCACGTT
P7cpxR	GGAGGAGATAACCGAGAAATCC
P8cpxR	CTGTGCCGCGTTCTAATTTC
VC2436F	ATGAAAAAACTGCTTCCATTA
VC2436R	TTACTTCTTCGCGACTTTTAG
VC0914F	ATGTGGTTGTCAGATGTATCGG
VC0914R	TTAGTGTTGAGCGCGGCCTTTC
VC0164F	ATGCGCTTTACTGATGTTTTTATAAA
VC0164R	TTACAGCTGACGATGAGCCG

Table 2 - 3 qRT-PCR primers used in this study.

Gene	Primer sequence 5' to 3'	
	Forward	**Reverse**
VC2691	GGGATCTGGAAACAGCTTGA	CACGCATCTCTTTGAGTTGG
VC2692	ATCGGGTGATCGGCTTAGA	ACTCGCGATCGCTAAAAGG
VC2693	CGAAGTGCAGCCACTGAGTA	CAGCTTCAAATTGCGCATC
VCA0151	AGAGCACACCCACCAGTACA	GCACAAATCCGGATCAATTT
VCA0249	CAAAGAAGGGGCGATTCC	TAAACCATGAACCGCTTTCG
VCA0538	TTCATCTCCGAAAATTGAAGG	AACAAACAATCCAACGTACATCA
VCA0270	ACCAGTGGTGCGGGATATAG	GAAATGAGGCGCATCTCG
VCA0910	CAAGGCATCACAAGTCAACC	AACTTGTGCGCTCACTAAAGC
VCA0911	GTAAGCGTCCGGTGCTGTAT	TTCGCGCAATGACTTATCAA
VCA0912	AGTGGATAAGCGTCCGGTAG	TTTGACTAATTGTTGAACCTCAGC
VCA0913	ACAACACGCTCAAAAGCTGA	TTAGCTTGCAACGCATTGAT
VCA0576	TGCCTCAGACATTGAACAGC	GGGGTATATTTGAATAGGCCTTC
VC2436	GCGCTAGGCACACTAAGCTC	TTGGTTATAAATCTCTGCCAGGT
VC0166	GTCTTTGCAAGAACGCTTCC	CAAGAGCCAGCCAACGTC
VC0165	AGGCTCCATCTCGGCAAT	CCGCTTGTACTTGGATCAGG
VC0164	TTGGTATTGCGTCAATGGTG	TGGTTACTGTGGCTTGGCTA
VC0913	CGTGCAGTTTGAAGGTTCG	ATTTAAGGTTTCTTGATTGATACGC
VC0914	GCTCAAAACGTGGGCTGA	CCCGTCATAAACGGTGACTT

Table 2 - 4 Virtual Footprint analysis of the CpxR binding site in the *V. cholerae* genome.

ORF	Description of the gene product	PWM Score[a]	Strand	ATG distance
VC2691	Periplasmic protein CpxP	13.80	+	98
VC2692	Transcriptional regulator CpxR	13.80	+	75
VC1226	Thiopurine methyltransferase	13.71	+	91
VC0493	Hypothetical protein	13.69	-	5
VCA0549	PhnA protein	13.67	+	3
VCA0550	Hypothetical protein	13.67	+	166
VCA0538	Cytochrome b561, putative	13.65	+	74
VCA0537	Hypothetical protein	13.65	+	298
VC0166	TetR family transcriptional regulator	13.63	+	57
VC0167	ATP-dependent DNA helicase Rep	13.63	+	74
VCA0151	Oxidoreductase, putative	13.63	-	50
VCA0784	Trans-2-enoyl-CoA reductase	13.63	-	38
VC0988	Proton/peptide symporter family protein	13.62	+	102
VC0989	Hypothetical protein	13.62	+	145
VCA0249	Cytochrome b561, putative	13.55	+	74
VCA0195	Hypothetical protein	13.51	-	66
VC0586	Carbonic anhydrase, putative	13.22	-	83
VC0587	Sulfate permease family protein	13.22	-	50
VC1131	Membrane protein, putative	13.14	-	15
VC1865	Hypothetical protein	13.12	-	4
VC1785	Transcriptional regulator	13.11	+	16
VC1786	DNA repair protein RadC, putative	13.11	+	83
VCA0537	Hypothetical protein	13.01	+	101
VCA0270	D-alanyl-D-alanine carboxypeptidase	13.01	+	97
VCA0538	Cytochrome b561, putative	13.01	+	271
VC0941	Serine hydroxymethyltransferase	12.95	+	188
VC0940	Hypothetical protein	12.95	+	81
VC1758	Phage family integrase	12.92	+	80
VCA0536	Hypothetical protein	12.90	+	337
VCA0779	Hypothetical protein	12.89	-	3
VC2187	Flagellin FlaC	12.85	+	307
VC1590	Acetolactate synthase	12.81	+	13
VC2303	Hypothetical protein	12.79	-	11
VC2304	Hypothetical protein	12.79	-	43
VC2534	Magnesium transporter	12.70	+	95
VC0786	D-amino acid dehydrogenase small subunit	12.67	-	27
VC0787	LysR family transcriptional regulator	12.67	-	59
VC0185	Transposase, putative	12.64	-	30

VCA1065	Hypothetical protein	12.63	-	28
VC0718	Recombination associated protein	12.61	-	96
VC0719	DNA-binding response regulator PhoB	12.61	-	126
VCA1085	Hypothetical protein	12.59	+	221
VC1993	2,4-dienoyl-CoA reductase	12.53	-	101
VC1994	protease IV	12.53	-	114
VCA0685	iron(III) ABC transporter, periplasmic iron-compound-binding protein	12.34	-	92
VC1369	Hypothetical protein	12.31	+	134
VCA1032	Hypothetical protein	12.31	-	210
VC0684	Peptidyl-prolyl cis-trans isomerase, FKBP-type	12.26	+	132
VC2703	Potassium/proton antiporter	12.25	+	326
VC0545	Alanyl-tRNA synthetase	12.25	-	42
VC2704	Hypothetical protein	12.25	+	38
VCA0924	Hypothetical protein	12.25	-	15
VCA0925	Dihydroorotase	12.25	-	338
VCA0786	Hypothetical protein	12.21	-	63

a. The putative CpxR binding sites were predicted using the program Virtual Footprint (http://prodoric.tu-bs.de/vfp/) (357). Position Weight Matrices (PWM) represent the similarity to a degenerate DNA pattern derived from the *E. coli* CpxR binding site (221).

Table 2 - 5 Individual *V. cholerae* El Tor C6706 genes with statistically significant differential expression when *cpxR* was overexpressed.

ORF	Description of the gene product	Fold change[a]
Up-regulated		
VCA0151	Oxidoreductase, putative	179.36
VCA0139	Hypothetical protein	94.34
VC2691	Periplasmic repressor CpxP	77.84
VC2692	Transcriptional regulator CpxR	50.00
VCA0538	Cytochrome b561, putative	46.62
VCA0249	Cytochrome b561, putative	38.00
VC0914	Multidrug resistance protein, putative	28.24
VC0915	Hypothetical protein	24.81
VC0913	Hypothetical protein	22.41
VCA0152	Putative monovalent cation/H+ antiporter subunit G	21.10
VCA0154	Hypothetical protein	19.97
VC0166	TetR family transcriptional regulator	19.00
VC1225	Hypothetical protein	18.43
VC0587	Sulfate permease family protein	18.36
VCA0782	ABC transporter, ATP-binding protein	17.97
VCA0153	Putative monovalent cation/H+ antiporter subunit F	16.61
VCA0781	Hypothetical protein	14.92
VC1326	Hypothetical protein	12.71
VCA0990	DEAD-box ATP dependent DNA helicase	12.62
VCA0539	Hypothetical protein	12.02
VC1327	Galactose/methyl galactoside transporter ATP-binding protein	11.99
VC0916	Phosphotyrosine protein phosphatase	11.06
VCA0155	NADH dehydrogenase, putative	10.63
VC1328	Beta-methylgalactoside transporter inner membrane component	10.39
VC1224	Hypothetical protein	9.87
VC1596	Galactose-1-phosphate uridylyltransferase	9.81
VCA0780	UDP-glucose 6-dehydrogenase	8.98
VC1594	Aldose 1-epimerase	8.69
VC0164	Multidrug resistance protein, putative	8.15
VC1325	Galactoside ABC transporter, periplasmic D-galactose/D-glucose-binding protein	7.96
VCA0783	Arylesterase	7.78
VCA0270	D-alanyl-D-alanine carboxypeptidase	7.36
VCA0198	Site-specific DNA-methyltransferase, putative	7.28
VC1595	Galactokinase	6.41
VC0494	Hypothetical protein	6.15
VC1324	Hypothetical protein	5.71

VC1187	Hypothetical proteins	5.67
VCA0271	Hypothetical protein	5.60
VCA0914	Hemin ABC transporter, permease protein, putative	5.33
VCA0912	TonB system transport protein ExbD1	5.25
VCA0913	Hemin ABC transporter, periplasmic hemin-binding protein HutB	5.22
VCA0911	TonB system transport protein ExbB1	5.02
VCA0733	Hypothetical protein	4.69
VCA0092	Hypothetical protein	4.48
VC0984	Cholera toxin transcriptional activator	4.44
VC0917	UDP-N-acetylglucosamine 2-epimerase	4.43
VCA0910	TonB1 protein	4.38
VC0174	Hypothetical protein	4.37
VC0963	VisC protein, putative	4.36
VC0364	Bacterioferritin-associated ferredoxin	4.21
VC2617	Arginine/ornithine succinyltransferase, putative	4.20
VC2690	Ferrous iron efflux protein F	4.04
VC0983	Regulatory protein ToxS	4.02
VC2360	Endonuclease IV	4.01
VC0982	Selenoprotein W-related protein	3.92
VCA0989	Hypothetical protein	3.88
VCA0909	Coproporphyrinogen III oxidase	3.80
VC2337	LacI family transcription regulator	3.72
VC0919	Serine acetyltransferase-related protein	3.71
VC0165	Hypothetical protein	3.69
VC0768	Bifunctional GMP synthase/glutamine amidotransferase protein	3.69
VCA0614	Formate-tetrahydrofolate ligase	3.63
VCA0103	Sulfate permease family protein	3.60
VCA0732	Hypothetical protein	3.35
VCA0714	Diacylglycerol kinase	3.34
VCA0908	HutX protein	3.24
VC1637	Hypothetical protein	3.17
VC1797	Hypothetical protein	3.14
VCA0907	Heme-binding protein HutZ	3.13
VCA0576	Heme transport protein HutA	3.11
VC1639	Sensor histidine kinase	3.10
VCA0550	Hypothetical protein	3.10
VC2618	Acetylornithine aminotransferase	3.08
VC2312	Murein transglycosylase A	3.05
VC1638	DNA-binding response regulator	2.97
VC0746	RNA methyltransferase	2.96
VC1503	Hypothetical protein	2.94

VCA0643	Conserved hypothetical protein	2.93
VC0740	Hypothetical protein	2.86
VC0365	Bacterioferritin, bfr	2.84
VCA0976	Hypothetical protein	2.77
VC0200	Iron(III) compound receptor	2.74
VC2616	Succinylglutamic semialdehyde dehydrogenase	2.73
VC1412	Hypothetical protein	2.71
VC0743	Preprotein translocase subunit SecD	2.70
VC1640	50S ribosomal protein L25	2.70
VC1798	Eha protein	2.67
VC0871	Hypothetical protein	2.66
VC1571	Quinol oxidase, subunit I, qxtA	2.64
VC1688	Hypothetical protein	2.63
VCA0230	Iron(III) ABC transporter, ATP-binding protein	2.61
VC1188	Malate dehydrogenase	2.60
VC1742	Hypothetical protein	2.60
VC1812	Hypothetical protein	2.59
VCA0217	GGDEF family protein	2.59
VCA0560	GGDEF family protein	2.55
VC0201	Iron(III) ABC transporter, ATP-binding protein	2.54
VC0493	Hypothetical protein	2.54
VCA0229	Vibriobactin and enterobactin ABC transporter, permease protein, vctG	2.53
VC0739	S-adenosylmethionine:tRNA ribosyltransferase-isomerase	2.51
VCA0845	Hypothetical protein	2.49
VC0873	Hypothetical protein	2.48
VC2146	Conserved hypothetical protein	2.46
VCA0939	Sensory box/GGDEF family protein	2.45
VC2313	Hypothetical protein	2.44
VC1413	Methyl-accepting chemotaxis protein	2.44
VC0922	Hypothetical protein	2.44
VC0863	Hypothetical protein	2.43
VC1323	Hypothetical protein	2.43
VCA0227	Vibriobactin and enterobactin ABC transporter, periplasmic vibriobactin/enterobactin-binding protein, vctP	2.42
VC0742	Preprotein translocase subunit YajC	2.40
VC1653	Sensory box sensor histidine kinase/response regulator VieS	2.39
VC1975	2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase/2-oxoglutarate decarboxylase, menD	2.38
VC2226	Phosphoribosylaminoimidazole synthetase	2.35
VC2436	Outer membrane channel protein	2.31
VC1958	Hypothetical protein	2.29
VC2734	General secretion pathway protein C	2.24

VC0163	Conserved hypothetical protein	2.24
VC1976	Menaquinone-specific isochorismate synthase	2.23
VCA0977	ABC transporter, ATP-binding protein	2.20
VC0202	Iron(III) ABC transporter, periplasmic iron-compound-binding protein	2.19
VC0719	DNA-binding response regulator PhoB	2.18
VCA0644	NADH oxidase, putative	2.17
VC0872	Hypothetical protein	2.14
VCA0915	Hemin importer ATP-binding subunit	2.13
VCA0216	Hypothetical protein	2.12
VC1306	Hypothetical protein	2.11
VC0167	ATP-dependent DNA helicase Rep	2.11
VC0744	Preprotein translocase subunit SecF	2.07
VCA0659	Protein F-related protein	2.06
VC0771	Vibriobactin-specific isochorismatase	2.05
VCA0864	Methyl-accepting chemotaxis protein	2.01
VCA0537	Hypothetical protein	2.01
Down-regulated		
VC1649	Trypsin, putative	-2.00
VC1082	Response regulator	-2.01
VCA0219	Haemolysin, hlyA	-2.01
VC1520	ABC transporter, ATP-binding protein	-2.02
VC1132	ATP phosphoribosyltransferase	-2.03
VC1410	Multidrug resistance protein VceA	-2.04
VC1081	Response regulator	-2.06
VCA0615	Peptide methionine sulfoxide reductase	-2.07
VCA0137	Sn-glycerol-3-phosphate transporter	-2.11
VCA0108	Conserved hypothetical protein	-2.15
VC0634	Transcription elongation factor GreA	-2.16
VCA0691	Acetoacetyl-CoA reductase	-2.17
VC1938	Hypothetical protein	-2.23
VCA0981	Hypothetical protein	-2.24
VCA0546	Hypothetical protein	-2.25
VCA0712	Pyrazinamidase/nicotinamidase	-2.26
VC1409	Multidrug resistance protein, putative	-2.26
VC0996	Hypothetical protein	-2.28
VC0566	Protease DO	-2.30
VC1578	Hypothetical protein	-2.30
VC0545	Alanyl-tRNA synthetase	-2.35
VC1941	Hypothetical protein	-2.37
VCA0950	Hypothetical protein	-2.39
VCA0645	Hypothetical protein	-2.40
VCA1063	Ornithine decarboxylase	-2.41

VCA0046	Hypothetical protein	-2.45
VCA0209	Hypothetical protein	-2.49
VCA1064	Hypothetical protein	-2.50
VCA0784	Trans-2-enoyl-CoA reductase	-2.53
VC1678	Phage shock protein A	-2.57
VCA0223	Protease	-2.76
VCA0689	Conserved hypothetical protein	-3.01
VC2006	Chemotaxis protein CheV	-3.06
VC0546	Hypothetical protein	-3.29
VCA0842	Hypothetical protein	-3.29
VCA0136	Glycerophosphodiester phosphodiesterase	-3.39
VC2470	Hypothetical protein	-3.56
VCA0843	Glyceraldehyde-3-phosphate dehydrogenase	-4.25
VCA0549	PhnA protein	-8.60
VC2469	L-aspartate oxidase	-9.06
VC2468	Hypothetical protein	-16.14

a. Genes showing a ≥ 2 or ≤ -2 fold differences in expression between the non-induced and induced condition ($P < 0.05$). Numbers represent average fold change observed from two biological replicates with two technical replicates. Positive and negative stands for genes whose expression was up-regulated or down-regulated, respectively.

Table 2 - 6 Genes implicated in iron transport or function in *V. cholerae* El Tor C6706 that are CpxR regulated.

ORF	Description of the gene product	Fold change[a]	Mey *et al.* (366)	
			fur/ WT[b]	Low / high iron[c]
VCA0151	Oxidoreductase, putative	179.36		
VCA0538	Cytochrome b561, putative	46.62	2.1	1.3
VCA0249	Cytochrome b561, putative	38.00		
VCA0152	Putative monovalent cation/H+ antiporter subunit G	21.10		
VCA0154	Hypothetical protein	19.97		
VCA0153	Putative monovalent cation/H+ antiporter subunit F	16.61		
VCA0155	NADH dehydrogenase, putative	10.63		
VCA0914	Hemin ABC transporter, permease protein, putative	5.33	10.4	ND
VCA0912	TonB system transport protein ExbD1	5.25	13	23.9
VCA0913	Hemin ABC transporter, periplasmic hemin-binding protein HutB	5.22		
VCA0911	TonB system transport protein ExbB1	5.02	18.7	32.1
VCA0910	TonB1 protein	4.38	17.1	50
VC0364	Bacterioferritin-associated ferredoxin	4.21	3.7	3.2
VC2690	Ferrous iron efflux protein F	4.04		
VCA0909	Coproporphyrinogen III oxidase	3.80	20	52.1
VCA0908	HutX protein	3.24	ND	51.3
VCA0907	Heme-binding protein HutZ	3.13	8.2	ND
VCA0576	Heme transport protein HutA	3.11	20.4	37.3
VC0365	Bacterioferritin, bfr	2.84	3.7	3.2
VCA0976	Hypothetical protein	2.77	19.6	53.4
VC0200	Iron(III) compound receptor	2.74	5.4	10.2
VC1688	Hypothetical protein	2.63	2.6	2.9
VCA0230	Iron(III) ABC transporter, ATP-binding protein	2.61	3.7	7.9
VCA0217	GGDEF family protein	2.59	1.2	3.2
VC0201	Iron(III) ABC transporter, ATP-binding protein	2.54	3.4	5.8
VCA0229	Vibriobactin and enterobactin ABC transporter, permease protein, vctG	2.53	2.3	4.1
VC1323	Hypothetical protein	2.43		
VCA0227	Ferric vibriobactin enterobactin transport system substrate-binding protein	2.42	4.8	8.9

VCA0977	ABC transporter, ATP-binding protein	2.20	12.7	20.5
VC0202	Iron(III) ABC transporter, periplasmic iron-compound-binding protein	2.19		
VCA0915	Hemin importer ATP-binding subunit	2.13	2.7	5.8
VCA0216	Hypothetical protein	2.12	3.7	7.2
VC0771	Vibriobactin-specific isochorismatase	2.05	11.7	10.1
VCA0537	Hypothetical protein	2.01		
VCA0219	Haemolysin, hlyA	-2.01		
VCA0784	Trans-2-enoyl-CoA reductase	-2.53	0.95	0.5

a. Genes related or involved with iron based on gene ontology classification that is part of DAVID program (362) showing a ≥ 2 or ≤ -2 fold difference in expression between the non-induced and induced condition ($P < 0.05$). Numbers represent average fold change observed from two biological replicates with two technical replicates. Positive and negative stands for genes whose expression was up-regulated or down-regulated, respectively.

b. Mean expression ratio of *fur* mutant relative to wild-type parental strain *V. cholerae* O395 from previously published gene expression data (366). ND: not determined.

c. Mean expression ratio of wild-type strain *V. cholerae* O395 grown in the absence relative to the presence of iron supplementation from previously published gene expression data (366). ND: not determined.

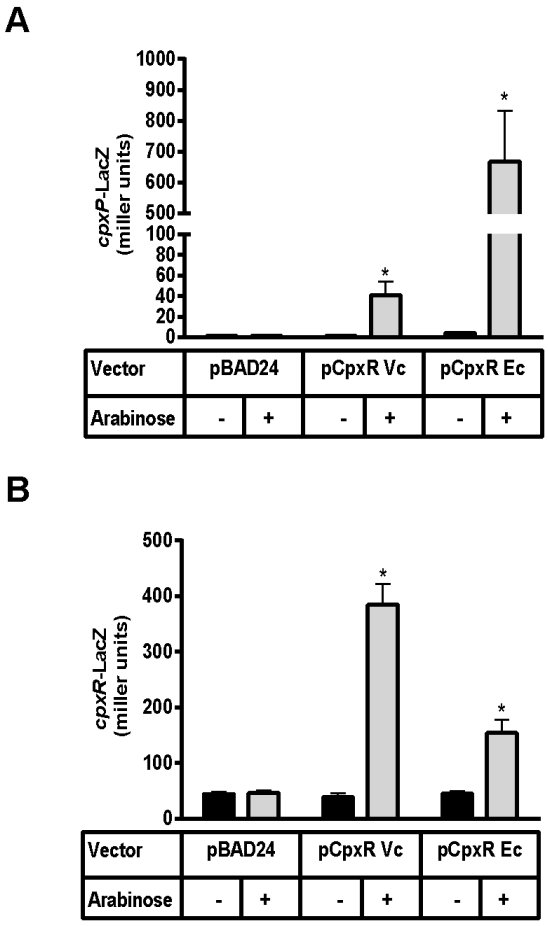

Figure 2 - 1 Heterologous expression of CpxR of *V. cholerae* in *E. coli* MC4100 Δ*cpxR*.

Strains carrying single-copy transcriptional A) *cpxP- lacZ* and B) *cpxR-lacZ* reporters were subcultured into LB broth and grown with shaking at 37°C for 6 hours. For CpxR induction from the pCpxR Vc and pCpxR Ec plasmids, after 2 hours of growth cultures were induced with 0.1% of arabinose and then grown for an additional 4 hours. Cells were lysed and the β-galactosidase levels were measured. Vc: *Vibrio cholerae*, Ec: *Escherichia coli.*

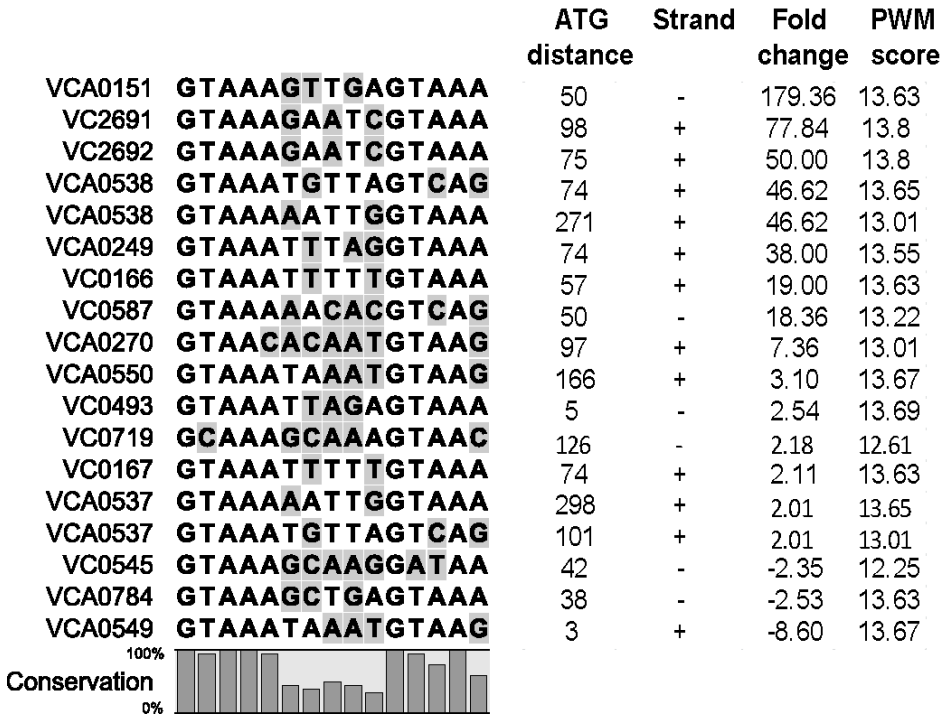

Figure 2 - 2 CpxR binding site in some *V. cholerae* El Tor C6706 CpxR-regulated genes.

Alignment of promoter regions of 16 CpxR-regulated genes found in our microarray that contain a putative CpxR binding site. The presence and the strand on which the putative CpxR binding site was found were predicted using the program Virtual Footprint (357). Fold change determined in our microarray data when *cpxR* was overexpressed (Table 2-5). Grey highlighted residues were not conserved. Position Weight Matrices (PWM) represent the similarity to a degenerated DNA pattern i.e. *E. coli* CpxR binding site (221).

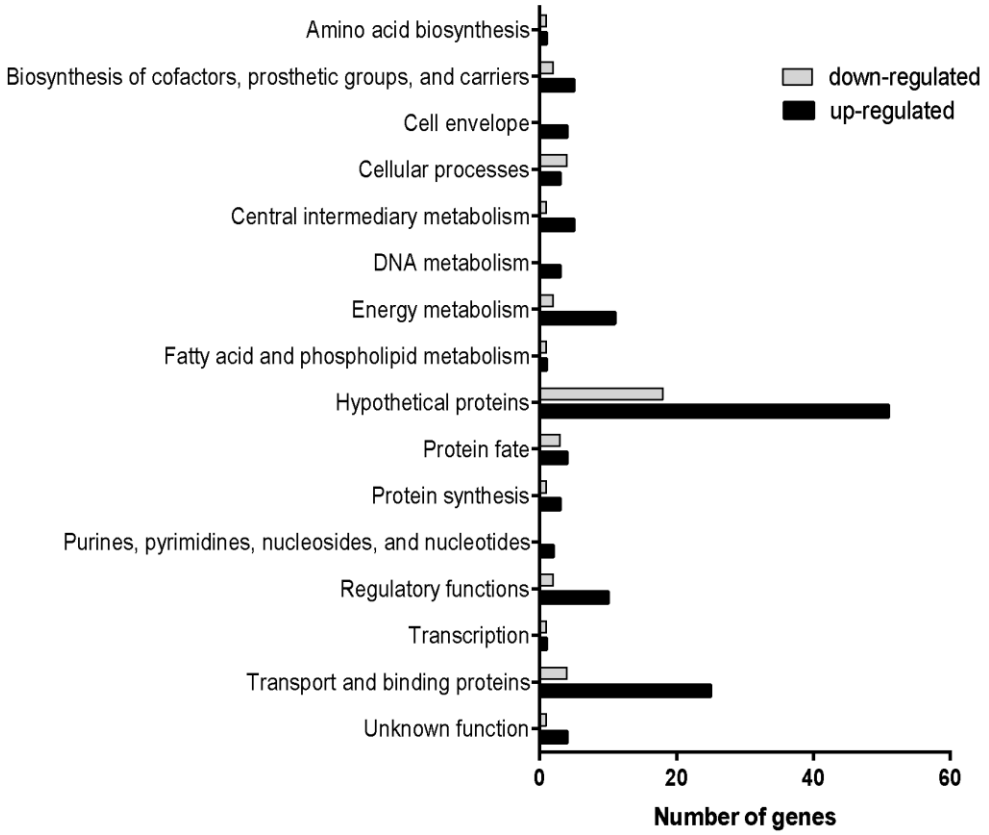

Figure 2 - 3 Functional clustering of *V. cholerae* CpxR regulated genes.

Functional categories were determined based on TIGR data of the 174 genes showing ≥ 2 or ≤ -2 fold differences in expression when *cpxR* was overexpressed.

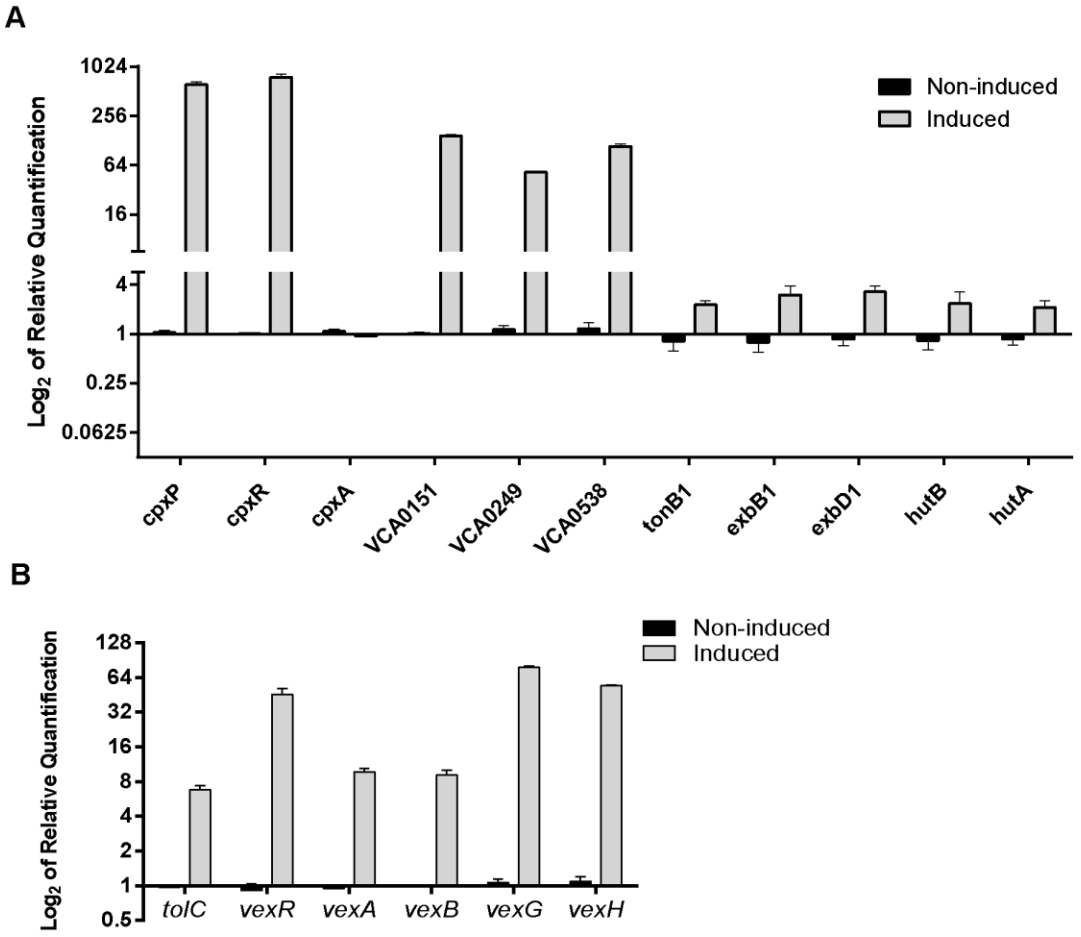

Figure 2 - 4 Transcriptional analyses of some genes that were found in the microarray either up-regulated or down-regulated.

RNA was isolated from cultures of *V. cholerae* El Tor C6706, carrying the overexpression plasmid pCpxR, in the absence (non-induced) (black bars) or presence of 0.1% arabinose (induced) (grey bars) and converted to cDNA. The cDNA was subjected to qRT-PCR analysis of the A) Cpx operon genes and genes related to iron and B) RND efflux encoded genes. Transcript levels were normalized to *gyrA* (endogenous control) for each gene in each condition and the relative quantification was determined. Experiments are representative of two biological replicates, each performed in triplicate. Error bars indicate the standard deviation (SD).

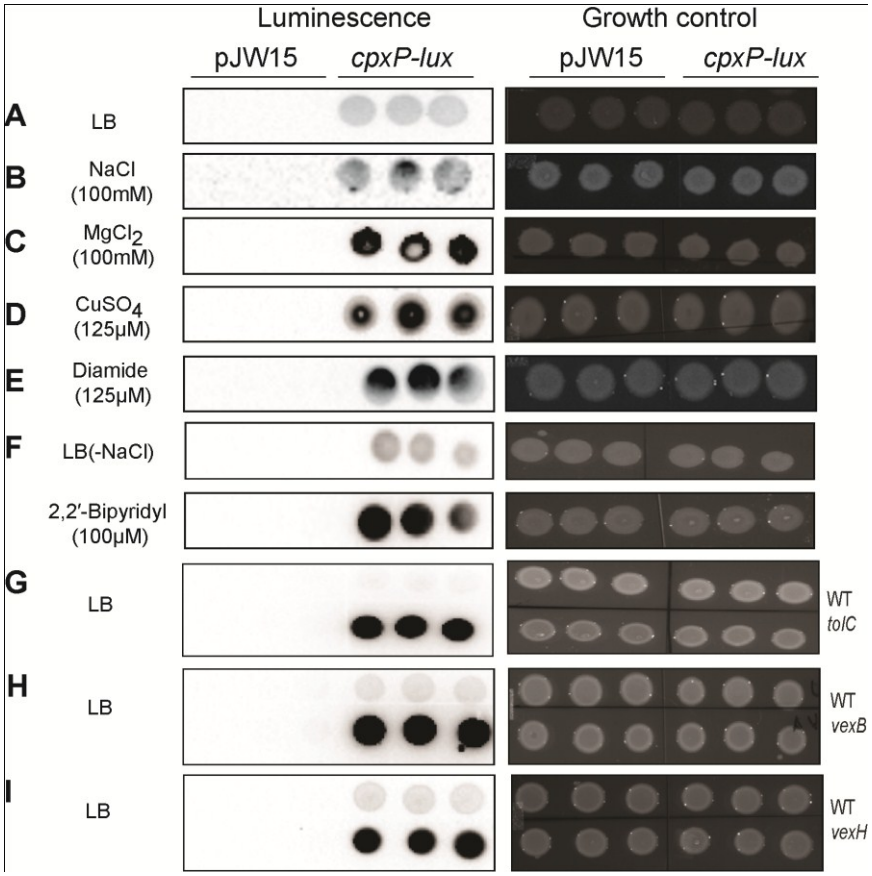

Figure 2 - 5 Induction of the *V. cholerae* Cpx pathway in response to various stimuli.

Luminescence activity of wild-type *V. cholerae* El Tor C6706, *tolC, vexB* and *vexH* mutants harboring either the pJW15 (vector control) or the *cpxP*-lux reporter plasmid (pN3). Luminescence was determined by measuring the chemiluminescence of the strains growing in LB plates and LB plates supplemented with NaCl (100 mM), $MgCl_2$ (100 mM), $CuSO_4$ (125 μM), diamide (125 μM) and yeast extract plus tryptone agar plates supplemented with 2,2'-Bipyridyl (100 μM). Each strain was spotted by triplicate and one representative luminescence image is shown.

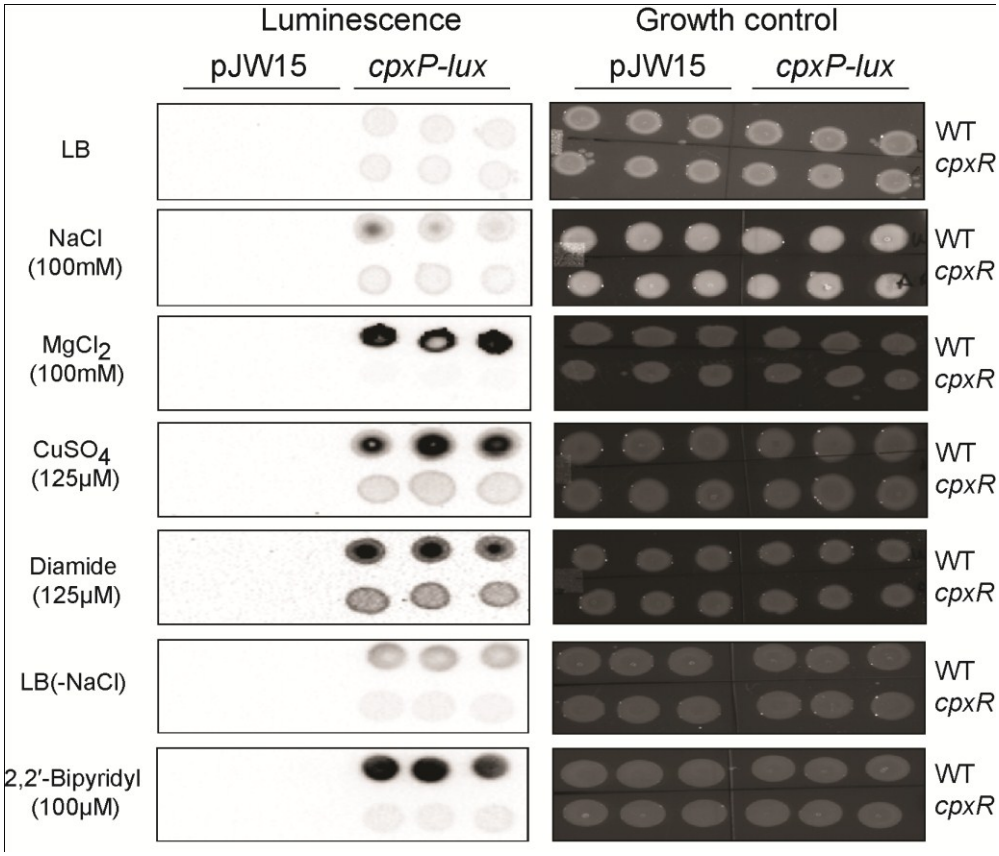

Figure 2 - 6 Induction of the *V. cholerae* Cpx pathway in response to various stimuli is CpxR dependent.

Luminescence activity of *V. cholerae* El Tor C6706 or the *cpxR* mutant harboring either the pJW15 (vector control) or the *cpxP*-lux reporter plasmid (pN3). Luminescence was determined by measuring the chemiluminescence of the strains growing in LB plates and LB plates supplemented with NaCl (100 mM), $MgCl_2$ (100 mM), $CuSO_4$ (125 μM), diamide (125 μM) and yeast extract plus tryptone agar plates supplemented with 2,2'-Bipyridyl (100 μM). Each strain was spotted by triplicate and one representative luminescence image is shown.

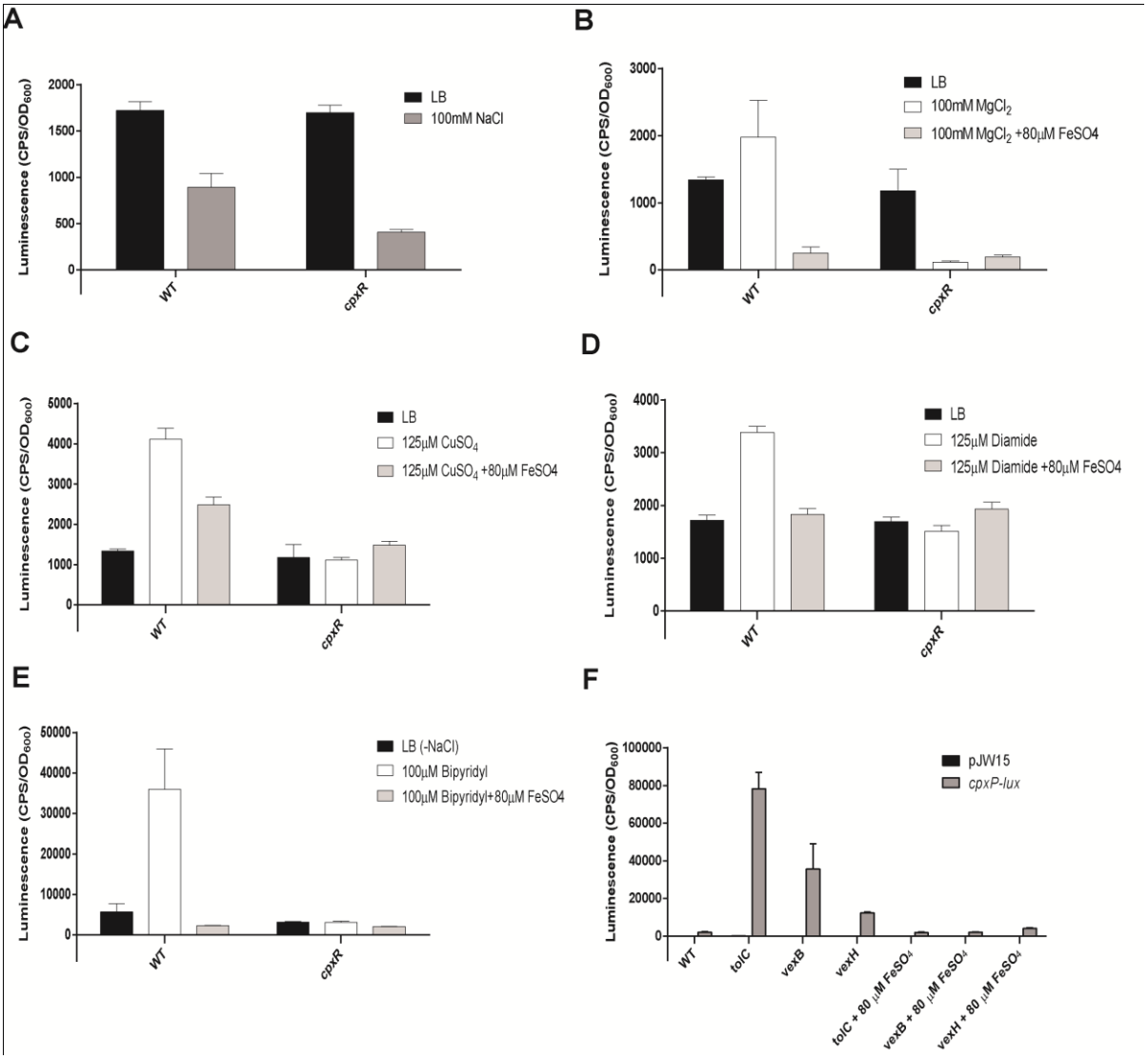

Figure 2 - 7 Quantification of the induction of the *V. cholerae* Cpx pathway in response to various stimuli.

Luminescence quantification of wild-type *V. cholerae* El Tor C6706, *cpxR*, *tolC, vexB* and *vexH* mutants harboring either the pJW15 (vector control) or the *cpxP*-lux reporter plasmid (pN3) under different conditions (refers to Figure 2-6 and Figure 2-10) were measured and reported as count per second (CPS) corrected for cell density (OD_{600}). Error bars indicate the standard deviation (SD).

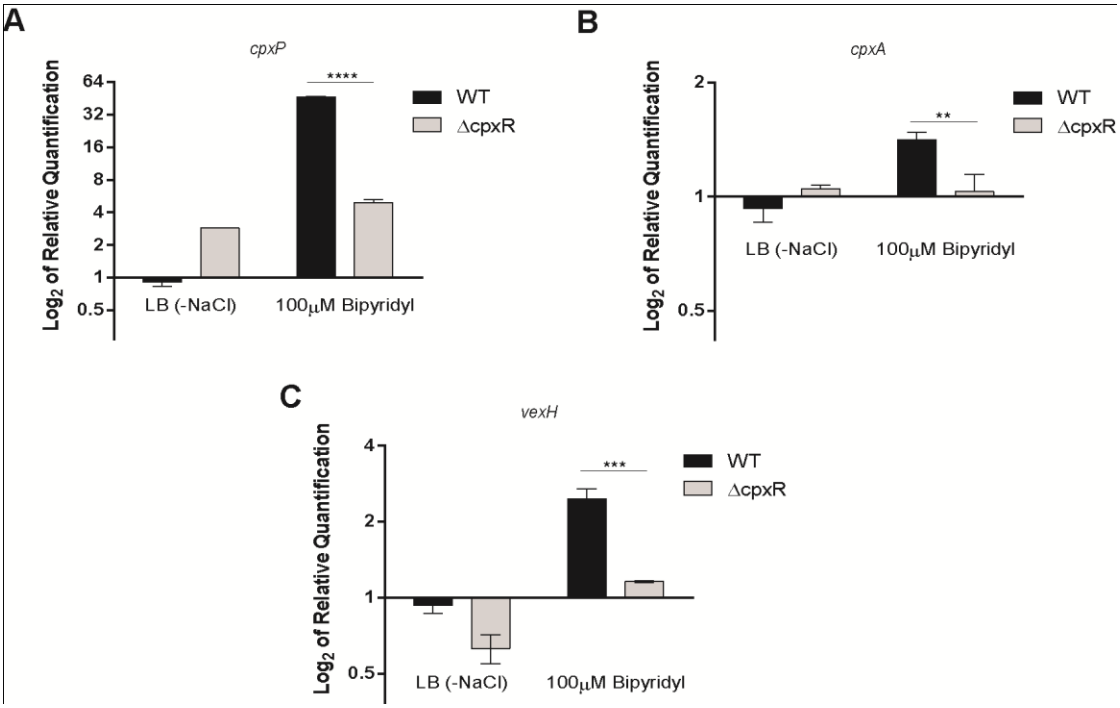

Figure 2 - 8 CpxR-regulated genes in response to iron chelation.

RNA was isolated from bacterial spots on agar plates of *V. cholerae* El Tor C6706 (black bars) and the *cpxR* mutant (EC16554) (grey bars), in the absence or presence of 2,2'-Bipyridyl (100 μM) and converted to cDNA. The cDNA was subjected to qRT-PCR analysis of *cpxP* (A), *cpxA* (B) and *vexH* (C). Transcript levels were normalized to *gyrA* (endogenous control) for each gene in each condition and the relative quantification was determined. Experiments are representative of two biological replicates, each performed in triplicate. Error bars indicate the standard deviation (SD).

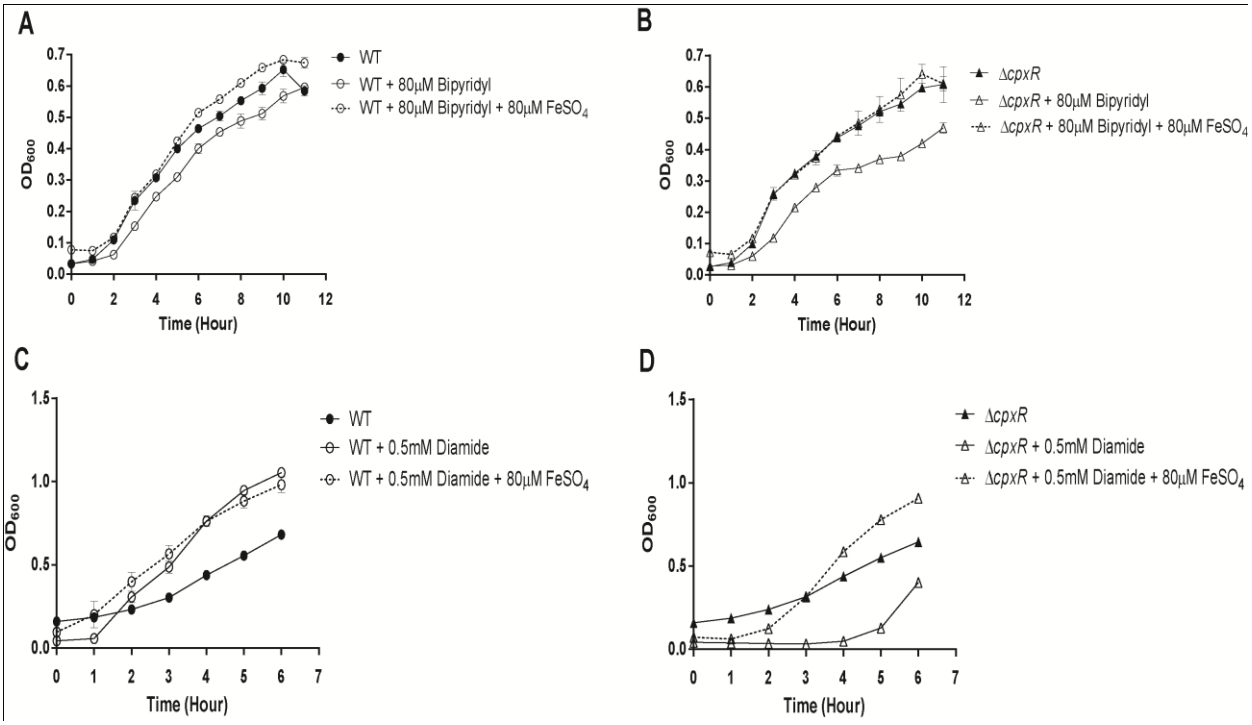

Figure 2 - 9 The Cpx pathway is involved in adaptation to low iron and aberrant disulfide bond formation.

A and B) Overnight cultures of wild-type C6706 and *cpxR* (EC16554) mutant were subcultured in M9 minimum medium supplemented with 0.2% succinate in the presence or absence of 80 μM 2,2'-Bipyridyl -/+ 80 μM $FeSO_4$ and allowed to grow at 37°C with shaking, while the OD_{600} was monitored over the course of 11 h. C and D) Overnight cultures of wild-type C6706 and *cpxR* (EC16554) mutant were subcultured in AKI medium in the presence or absence of 0.5 mM diamide -/+ 80 μM $FeSO_4$, and allowed to grow at 37°C without shaking for 5 h. Then cells (200 μl) were transferred into a 96-well microtiter plate in triplicate and the cultures were allowed to grow at 37°C with shaking, while the OD_{600} was monitored over the course of 6 h. Error bars indicate the standard deviation (SD) (some of the error bars are smaller than the symbols).

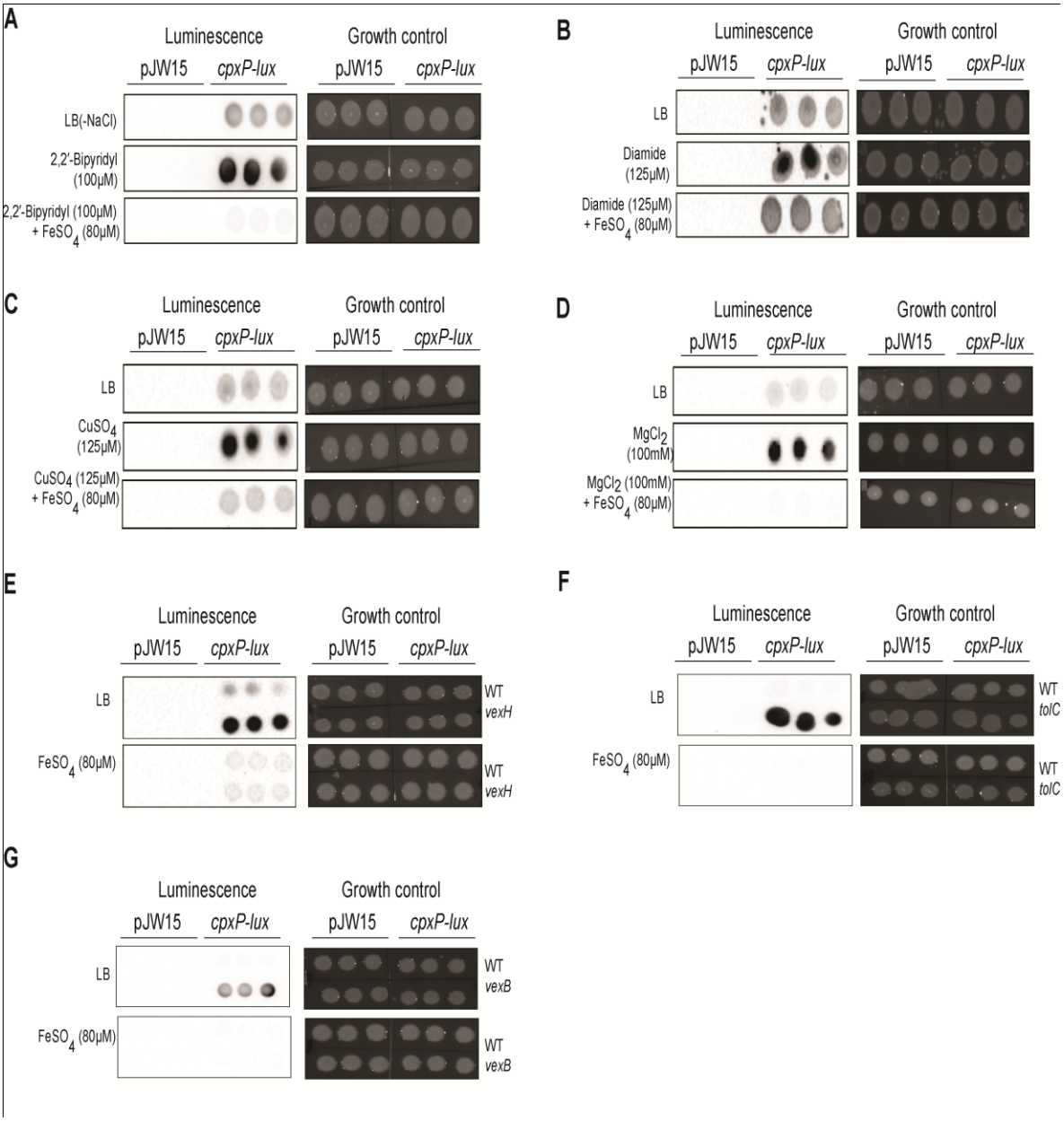

Figure 2 - 10 Iron is an important modulator for the Cpx pathway signaling.

Luminescence activity of wild-type *V. cholerae* El Tor C6706, *vexH, tolC* and *vexB* mutant harboring either the pJW15 (vector control) or the *cpxP*-lux reporter plasmid (pN3). Luminescence was determined by measuring the chemiluminescence of the strains growing in LB plates and LB plates supplemented with 2,2'-Bipyridyl (100 μM) -/+ 80 μM $FeSO_4$, diamide (125 μM) -/+ 80 μM $FeSO_4$, $CuSO_4$ (125 μM) -/+ 80 μM $FeSO_4$ and $MgCl_2$ (100 mM) -/+ 80 μM $FeSO_4$. Each strain was spotted by triplicate and one representative luminescence image is shown.

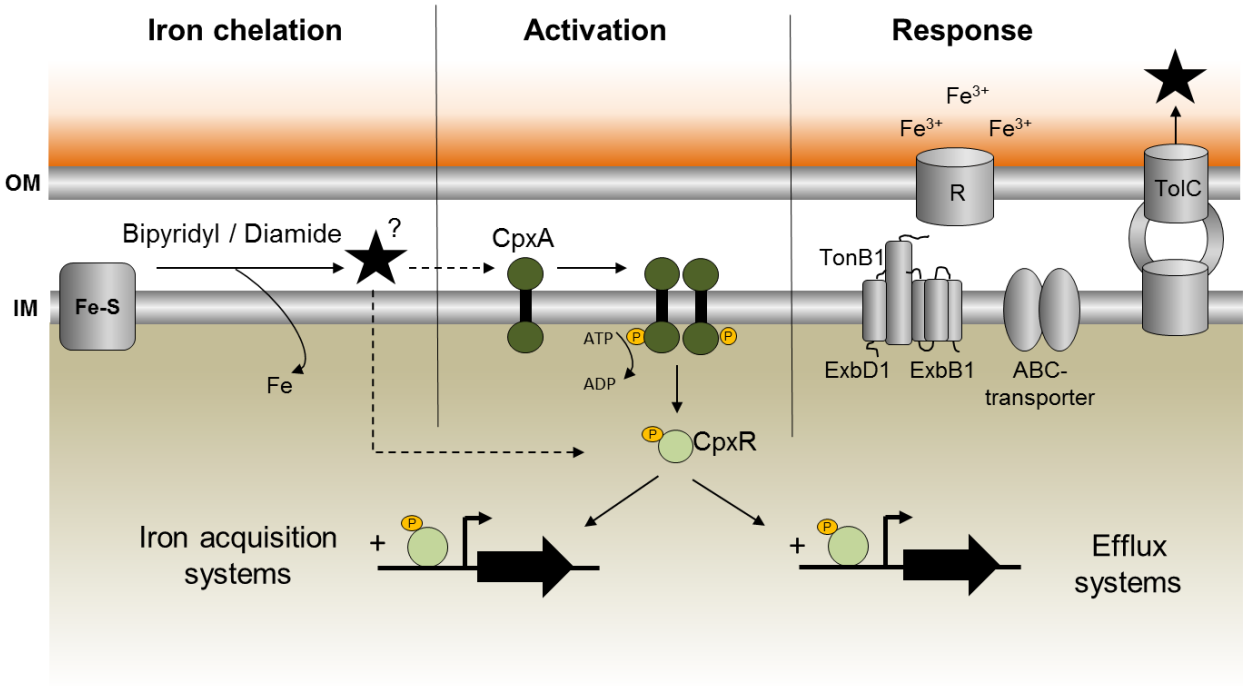

Figure 2 - 11 The *V. cholerae* El Tor C6706 Cpx envelope stress response senses and mediates adaptation to low iron.

Changes in iron homeostasis in the inner membrane (IM) caused by iron chelation (e.g. addition of bipyridyl) induce the Cpx pathway in *V. cholerae*. Unknown signal (e.g. toxic compound represented by a star) could be sensed directly or indirectly by the sensor kinase CpxA and then it phosphorylates the response regulator CpxR. Then CpxR activates the expression of genes involved in iron acquisition in order to maintain iron homeostasis. Additionally, resistant nodulation efflux systems are CpxR-dependently activated to pump out unknown toxic compounds that are generated during disruption of iron homeostasis in the IM. (OM) outer membrane.

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3 Chapter 3: Insights of the role of CpxR-regulated putative ferric reductases in *Vibrio cholerae* El Tor C6706

3.1 Introduction

Although iron is the second most abundant mineral in the earth, it is not readily available for usage in the environment because it is mostly found in the ferric iron (Fe^{3+}) form, which is water insoluble and metabolically inactive (381). Iron became an essential element for both Bacteria and Archaea in the early oxygen-free stages of the earth's evolution, where many chemical reactions that involved iron occurred (382). The introduction of an oxygen environment on earth resulted in a challenge for the assimilation of ferrous iron (Fe^{2+}) (383). To overcome this challenge ferric reductases emerged (384). Bacterial ferric reductases catalyze the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) which can then be transported across the inner or cytoplasmic membrane by specific iron transporters. This process of reduction of Fe^{3+} for the purpose of intracellular incorporation is named assimilatory iron reduction and it is found in prokaryotes and eukaryotes that live in aerobic and neutral environments (365). When ferric reductases act as terminal reductases in an electron transport chain, this process is known as dissimilatory iron reduction and it is mostly found in ferric iron-respiring microbes (365).

The ferric reductase domain (FRD) superfamily is widely distributed in many organisms (385), and assimilatory ferric reductases are found in all living organisms with exception of a group of lactic acid bacteria (365). Ferric iron reduction occurs as a result of the transfer of electrons from cytosolic NADPH or NADH to generate the reduced ferrous iron form. This mechanism involves a flavin cofactor (e.g. FMN and FAD) in prokaryotes and a cytochrome b-type in yeast (365). In bacteria, ferric reductases can be classified in three groups based on their cellular localization: periplasmic and cytoplasmic reductases; extracellular ferric reductases; and membrane-bound ferric reductases. Periplasmic and cytoplasmic ferric reductases possess broad substrate specificity towards complexes with ferric iron (Fe^{3+}) (386). Similarly, extracellular

reductases also exhibit broad substrate specificity but they require a flavin as cofactor. It has been suggested that extracellular ferric reductases are required for pathogens (e.g. *E. coli, Pseudomonas aeruginosa* and *S. typhimurium*) to acquire iron from the host (387). Finally, membrane-bound ferric reductases are suggested to be part of membrane-bound electron transport chains that use different donors (e.g. NADH, glycerol-3 phosphate and L-lactate) for the reduction of Fe^{3+} (365).

Three putative membrane-bound ferric reductases have been identified in *Vibrio cholerae*, VCA0151, VCA0249 and VCA0538, whose function is still unknown. Both VCA0249 and VCA0538 are regulated by the iron-dependent Ferric uptake regulator (Fur) through the regulation of the small RNA (sRNA) *ryhB* or direct binding to their promoter regions (366, 388). Fur is an important regulator of genes related to iron depending on the intracellular concentration of this ion (372). For example, under conditions when iron concentration is elevated in the cell, Fur is active and represses the transcription initiation of genes involved in iron uptake and positively regulates the expression of genes encoding iron storage (373). This positive regulation is mediated through the negative regulation of RyhB expression by Fur, where RyhB in conjunction with the RNA chaperone Hfq blocks the synthesis of iron storage genes among others (389). On the other hand, when the levels of iron decreases, Fur becomes inactive and then the expression of genes encoding iron uptake and the RyhB is de-repressed (373).

Recently, we found that activation of the Cpx response by transient overexpression of CpxR under virulence inducing conditions not only activates the expression of genes involved in iron acquisition but also positively up-regulates the expression of the VCA0151, VCA0249 and VCA0538 ferric reductases (described in Section 2.3.4). Similarly, Taylor and collaborators (332) found that constitutive activation of the Cpx response regulates the expression of those ferric

reductases when grown in LB broth (332). The role of these ferric reductases is mediating adaptation to Cpx-sensed envelope stress is unknown.

In this study on *V. cholerae* El Tor strain C6706, we characterized the putative role that the ferric reductases VCA0151, VCA0249 and VCA0538 have upon activation of the Cpx response. Additionally, we examined the basis for the regulation of these ferric reductases by CpxR, which was found to be independent of the growth media. We found that the Cpx pathway induces the expression of VCA0151, VCA0249 and VCA0538 in both virulence-inducing AKI medium, as well as in LB broth. Furthermore, we observed that under iron limitation conditions the Cpx response contributes to the regulation of the VCA0538 gene. Although we previously found that transcription of *fur* was not significantly altered when the Cpx response was activated, we determined that Fur is not required for the up-regulation of VCA0151 and VCA0249 by the Cpx response. Finally, we found that when CpxR is overexpressed in the VCA0151, VCA0249 and VCA0538 mutants, the previously observed up-regulation of genes involved in iron acquisition was diminished. These data suggest that the VCA0151, VCA0249, and VCA0538 ferric reductases play an important role in the Cpx-dependent up-regulation of the iron regulon.

3.2 Materials and Methods

3.2.1 *Bacterial strains, plasmids and growth conditions*

All bacterial strains and plasmids used in this study are listed in Table 3-1. *V. cholerae* El Tor strain C6706 was used as the parental strain to create all the *V. cholerae* strains used in this study. VC2692 (EC16554), VC2106 (EC16469), VCA0151 (EC11645), VCA0249 (EC9670) and VCA0538 (EC16172) mutants were obtained from a transposon insertion library in *V. cholerae* El Tor strain C6706 (353). Cultures were routinely grown and maintained in Luria-Bertani (LB)

broth with the appropriate antibiotics at 37°C with shaking and stored at -80°C in LB broth containing 10% glycerol. Antibiotics (all from Sigma) were used at the following concentrations in selective media: streptomycin (Sm), 100 µg/ml; ampicillin (Amp), 100 µg/ml and kanamycin (kan), 50 µg/ml. L-arabinose (Sigma) was added to growth media at a concentration of 0.1% for overexpression of CpxR.

3.2.2 *RNA extraction and expression arrays*

To analyse gene expression of wild type *V. cholerae* El Tor strain C6706 upon activation of the Cpx response (i.e. overexpression of CpxR) in LB broth, two independent RNA preparations for each of the conditions (i.e. non-induced and induced) were extracted from subcultures growing in LB broth at 37°C with aeration until the OD_{600} was 0.3. Then cultures were induced with 0.1% arabinose (final concentration) and after 25 minutes of growth 1 ml of culture was harvested and resuspended in 1 m of TRIzol reagent (Ambion). Total RNA was extracted and reverse transcribed as previously described (390).

For time-course experiments examining RNA samples after transient CpxR overexpression in *V. cholerae* El Tor strain C6706, two independent RNA preparations for each of the non-induced (no arabinose) and induced (0.1% arabinose) conditions at different time points (i.e. 5, 10 and 25 minutes) were examined. Total RNA was extracted from *V. cholerae* El Tor strain C6706 that was subcultured in LB broth at 37°C with aeration. When cultures reached an $OD_{600} \approx 0.3$, 0.1% (final concentration) arabinose was added to induce the overproduction of *cpxR* and after 5, 10 and 25 minutes of growth 1 ml of culture was harvested and resuspended in 1 ml of TRIzol reagent (Ambion). Total RNA was extracted and reverse transcribed as previously described (390).

To analyze gene expression of wild type *V. cholerae* El Tor strain C6706 and the *cpxR* mutant (EC16554) in the presence of 2,2'-Bipyridyl (100 μM), total RNA was extracted and reverse transcribed as previously described (390), using two independent RNA preparations for each of the conditions.

To determine the changes of gene expression in VC2106 (EC16469), VCA0151 (EC11645), VCA0249 (EC9670) and VCA0538 (EC16172) mutants compared to wild- type strain C6706 upon activation of the Cpx pathway, two independent RNA preparations for each of the non-induced (no arabinose) and induced (0.1% arabinose) conditions was extracted from cultures grown in AKI conditions at 37°C as previously described (390). Total RNA was extracted and reverse transcribed as previously described (390). To compare the previously described CpxR regulon members (described in Section 2.3.2) in wild type C6706 strain to that of the VCA0151 (EC11645), VCA0249 (EC9670) and VCA0538 (EC16172) mutants, we activated the Cpx response by over-expression of CpxR and used microarray analysis as previously described (390). For each condition, the hybridization experiment was performed using a Roche NimbleGen *V. cholerae* El Tor N16961 Gene expression array (Roche). We used fold cut-offs of ≥ 2 or ≤ -2 to identify differences in gene expression between wild-type strain C6706 and the VCA0151 (EC11645), VCA0249 (EC9670) and VCA0538 (EC16172) mutants.

3.2.3 *Quantitative RT-PCR (qRT-PCR)*

qRT-PCR analysis of the RNA extractions performed in this study were performed using a 7500 Fast Real-Time PCR System (Applied Biosystems). The amplification reactions were carried out as previously described (390). *gyrA* was used as an endogenous control. The sequences of all primers used for qRT-PCR are given in Table 3-2.

3.2.4 *Growth curves*

For growth curves in LB broth medium single colonies of the strains to be tested were inoculated into 5 ml of LB broth plus antibiotics in triplicate and grown at 37°C with aeration overnight. The next day, a 1:100 dilution was made in 1 ml of LB broth medium, with the appropriate antibiotics. Subcultures were grown at 37°C in a 24-well microtiter plate in triplicate with shaking, and the OD_{600} was measured using a Wallac 1420 multilabel plate reader (Perkin-Elmer) every hour over a period of 6 hours.

Growth curves in AKI conditions in the presence of 0.5 mM diamide were carried out as previously described (390). Briefly, single colonies of strains to be tested were inoculated into LB broth plus antibiotics in triplicate and grown at 37°C with aeration overnight. Subcultures were made in AKI medium, with the appropriate antibiotics in the presence of 0.5 mM diamide without and with 80 μM $FeSO_4$, and grown at 37°C statically. After 5 hours of growth, cultures were transferred to a 96-well microtiter plate in triplicate, and grown at 37°C with shaking. OD_{600} was measured using a Wallac 1420 multilabel plate reader (Perkin-Elmer) over a period of 6 h.

3.3 Results

3.3.1 *VCA0151, VCA0249, and VCA0538 genes encode putative ferric reductases*

We previously found that the most up-regulated genes upon Cpx activation encode putative ferric reductases, which suggests that the Cpx response regulates genes required for the reduction and intracellular incorporation of iron. We found that activation of the Cpx response up-regulates the expression of VCA0151, VCA0249, and VCA0538 (Chapter 2: Tables 2-5 and 2-6) (390). To assess the function of these putative ferric reductases, we performed an *in silico* characterization of their predicted cellular localization using the bioinformatics tool PSORTb v3.0.2

(http://www.psort.org/psortb/index.html) (391), and searched for motifs found in the PFAM database (http://pfam.xfam.org/) (392). This analysis showed that the VCA0151 gene is located on the small chromosome of *V. cholerae* (367). VCA0151 is predicted to be a transmembrane protein with 450 amino acids that encodes a putative ferric reductase with 3 domains: ferric reductase like transmembrane component, which is found in flavocytochromes capable of moving electrons across the plasma membrane, oxidoreductase FAD-binding domain and oxidoreductase NAD-binding domain (Figure 3-1A). The VCA0249 gene is also located on the small chromosome of *V. cholerae* (367).VCA0249 is predicted to be a transmembrane protein with 177 amino acids that encodes a putative cytochrome $b_{561}$with 2 domains: prokaryotic cytochrome b_{561}, which is predicted to be transmembrane-localized, and predicted membrane protein (DUF2231) domain (Figure 3-1B). Similarly, the VCA0538 gene is also located in the small chromosome of *V. cholerae* (367) and encodes a 181 amino acid transmembrane protein with 2 domains: prokaryotic cytochrome b_{561} and predicted membrane protein (DUF962) domains (Figure 3-1C). Because of their putative role as ferric reductases and their regulation by the Cpx response we named VCA0151, VCA0249, and VCA0538 as FcpR1, FcpR2 and FcpR3, respectively, for Ferric-reductase CpxR Regulated protein.

3.3.2 *The Cpx pathway regulates ferric reductases in a media independent manner*

We previously confirmed the Cpx regulation of *fcpR1, fcpR2, and fcpR3* when cells are growing under virulence inducing condition (i.e. AKI conditions) (described in Section 2.3.4) (390). To test if this Cpx regulation was media-dependent, we assessed changes in gene expression of *fcpR1*, *fcpR2* and *fcpR3* when the Cpx response is activated by CpxR over-expression in LB broth. As was observed under AKI conditions (Figure 2-4A), in LB we found that activation of the Cpx response strongly up-regulated *fcpR1*, *fcpR2* and *fcpR3* (Figure 3-2).

There was a 41-fold change in *fcpR1* transcript levels upon *cpxR* overexpression (Figure 3-2). Similarly, *fcpR2* and *fcpR3* showed 24- and 44-fold changes in transcript levels, respectively, upon activation of the Cpx pathway. Although the ratio of induction was lower compared to the AKI experiments (*fcpR*1: 179.36, *fcpR2*: 38 and *fcpR*3: 46.62 fold), these data suggest that regulation of *fcpR1*, *fcpR2* and *fcpR3* by the Cpx response is independent of the growth media.

We previously noted that there are putative CpxR binding sites in the promoter regions of *fcpR1*, *fcpR2* and *fcpR3* (Chapter 2: Figure 2-2), which suggests direct regulation by the response regulator CpxR. To determine if these putative ferric reductases are directly regulated by CpxR, we performed time-course experiments examining RNA samples after transient CpxR overexpression. As expected, the expression of *cpxP*, a known CpxR regulon member, was induced within the first 5 minutes after induction (Figure 3-3). Similarly, we found that the expression of *fcpR1*, *fcpR2* and *fcpR3* by CpxR is altered within 5 minutes of CpxR overexpression (Figure 3-3). The fast kinetics of *fcpR1*, *fcpR2* and *fcpR3* expression is suggestive of a direct regulation by CpxR. Notably, *fcpR3* transcription was induced to noticeably higher levels than *cpxP* at 5 minutes post-induction (16-fold higher, Figure 3-3C).

3.3.3 *fcpR1, fcpR2, and fcpR3 are differentially affected by iron limitation in a partially CpxR dependent manner.*

We previously showed that the Cpx response is induced by iron chelation when *V. cholerae* El Tor strain C6706 is grown in the absence of exogenously added NaCl (390). Further, we showed that expression of *fcpR3* was induced by iron limitation, in a CpxR dependent manner (390). Since *in silico* analyses indicated that FcpR1, FcpR2, and FcpR3 are ferric reductases, we wished to determine if they were also iron regulated, and if so, whether this regulation required CpxR. We therefore isolated RNA from cultures of wild-type and *cpxR* mutant *V. cholerae* El Tor strain

C6706 grown in the absence and presence of the iron chelator 2,2'-Bipyridyl, and used qRT-PCR to quantify the levels of the *fcpR1, fcpR2,* and *fcpR3* transcripts .

The transcription of all three ferric reductases was diminished in the absence of CpxR (Figure 3-4), as expected based on their positive regulation by this transcription factor (Figures 3-2, 3-3). Interestingly, however, transcription of *fcpR1, fcpR2,* and *fcpR3* was differentially affected by iron chelation (Figure 3-4). The presence of 2,2'-Bipyridyl in LB plates did not affect *fcpR1* expression in the wild-type C6706 strain and had only a small, stimulator effect in the *cpxR* mutant (Figure 3-4A). On the other hand, as previously reported (388), there was a decrease in *fcpR2* expression under iron limitation conditions that still occurred in the *cpxR* mutant, although to a lesser degree (Figure 3-4B). Finally, as we previously showed (390), there was an increase in expression of *fcpR3* in the presence of 2,2'-Bipyridyl, and this regulation was partially CpxR-dependent, because *fcpR3* expression was still elevated in the *cpxR* mutant by 2,2-Bipyridyl, although not to the same extent as in the wild-type strain (Figure 3-4C). These results support our hypothesis that Cpx response is required for the regulation of the putative ferric reductases FcpR1, FcpR2, and FcpR3 in *V. cholerae*. Our experiments additionally show that iron limitation has differential effects on the expression of each of these ferric reductases; having no impact on *fcpR1*, inhibiting *fcpR2*, and stimulating *fcpR3* expression (Figure 3-4).

3.3.4 *CpxR acts independently of Fur to regulate cpxP, fcpR1, fcpR2, and fcpR3 transcription*

Our results indicated that *fcpR2* and *fcpR3* transcription are negatively and positively affected by iron limitation, respectively. Further, this regulation still occurs in a *cpxR* mutant, albeit to a lower extent (Figure 3-4B, C). The major iron regulatory protein in many organisms is the Fur repressor. Fur is a DNA binding protein that, in the presence of iron, binds to the promoters of genes encoding iron uptake and utilization proteins to inhibit their transcription (373).

Additionally, Fur also acts in an iron sparing manner through its control of a sRNA RyhB (389). When Fur-mediated repression of *ryhB* transcription is relieved by iron limitation, RyhB can act at the post-transcriptional level to inhibit the production of iron-containing proteins (393). It has previously been shown that RyhB inhibits FcpR2 expression, while Fur inhibits *fcpR3* transcription (366, 388). Since we previously showed that low iron can affect Cpx-dependent gene expression, we wished to determine if RyhB or Fur could be involved with the effect of the Cpx response on FcpR2 and FcpR3 production.

To do this, we first asked whether the Cpx response had any impact on RyhB expression. We isolated RNA from wild-type and *cpxR* mutant strains grown in the absence and presence of the iron chelator 2,2'-Bipyridyl and used qRT-PCR to measure *ryhB* transcript levels. As previously observed in another *V. cholerae* El Tor strain, N16961(388, 394), we found that the expression of the sRNA *ryhB* increased upon iron limitation. Levels of *ryhB* RNA were elevated 11 fold in the presence of 2,2'-Bipyridyl in LB plates in the wild-type El Tor strain C6706 (Figure 3-4D). We observed that the mutation of *cpxR* led to a small decrease in expression of *ryhB* (i.e. 0.71 fold change) (Figure 3-4D), suggesting a weak positive regulation of this sRNA by the Cpx pathway. However, under iron limitation conditions, in the *cpxR* mutant the expression of *ryhB* was still enhanced 14.5 fold, indicating that CpxR is not involved in the stimulation of *ryhB* transcription in response to low iron (Figure 3-4D). Based on these results, we conclude that expression of *fcpR2* is likely independently regulated by RyhB and CpxR. RyhB acts to diminish expression of FcpR2 in low iron (Figure 3-4B), and the positive regulation of *fcpR2* transcription by CpxR is independent of this regulation.

Although in our microarray, in which CpxR was overexpressed, Fur was not found as part of the CpxR regulon in *V. cholerae*, we also sought to determine whether the regulation of the

putative ferric reductases by the Cpx response involved Fur. We determined if in a *fur* mutant the expression of *fcpR1*, *fcpR2* and *fcpR3* changed with respect to the wild-type C6706 strain upon overexpression of CpxR. As expected, our positive control for the activation of the Cpx response, *cpxP*, exhibited increased expression (i.e. 517 fold change) upon activation of the Cpx response (Figure 3-5A). Interestingly, the expression of *cpxP* appears to be positively regulated by Fur, since basal levels of transcription were decreased (i.e. 0.09 fold) in the absence of *fur* (Figure 3-5A). In spite of this, the expression of *cpxP* increased upon activation of the Cpx pathway in the *fur* mutant (170 fold, Figure 3-5A), however not to the same extent as observed in the wild type C6706 strain (517 fold, Figure 3-5A). Since the effect of Fur and CpxR on *cpxP* expression is additive (Figure 3-5A), we conclude that these transcription factors act independently to control *cpxP* transcription. This is a novel finding, since Fur-dependent expression of *cpxP* has not previously been observed.

As previously observed in *V. cholerae* El Tor strain N16961 (388), deletion of *fur* resulted in diminished *fcpR2* transcription (Figure 3-5C), most likely due to elevated levels of RyhB (see above). Conversely, in the absence of Fur, expression of *fcpR3* was elevated about 2 fold (Figure 3-5D), also as previously seen in *V. cholerae* El Tor strain N16961 (366). Although, there was a significant difference in the expression of *fcpR1* and *fcpR2* between the wild type C6706 strain and the *fur* mutant when the Cpx pathway was activated (Figure 3-5B and C), overexpression of CpxR still strongly induces the expression of *fcpR1* and *fcpR2*, regardless of the *fur* mutation, suggesting that the CpxR-mediated regulation works independently of Fur. Similarly, there was not a significant difference in *fcpR3* in gene expression between wild-type C6706 strain and the *fur* mutant upon overexpression of CpxR, suggesting that the CpxR mediated regulation of that ferric reductases is Fur-independent (Figure 3-5D).

3.3.5 *CpxR regulated ferric reductases are required for adaptation to aberrant disulfide bond formation*

To characterize the function of FcpR1, FcpR2, and FcpR3 under standard laboratory conditions, we determined whether the absence of those genes has any impairment in growth under different conditions. We first determined if deletion of *fcpR1*, *fcpR2* and *fcpR3* is detrimental for growing in LB broth. We observed no difference in the growth of the wild-type C6706 strain compared to the *fcpR1* and *fcpR3* mutants in LB medium (Figure 3-6 A and C). However, there was a growth defect for the *fcpR2* mutant compared with the wild type C6706 strain (Figure 3-6B). These data suggest that FcpR2 may be important for growth in the absence of stress.

We previously found that the Cpx pathway is required for adaptation to low iron under conditions where the cells are forced to respire (described in Section 2.3.7) (390). Since FcpR2 and FcpR3 were found to be iron regulated (366, 388) (described in Section 2.3.4), we wondered whether these putative ferric reductases were involved in this phenotype. We compared the ability of the *fcpR1*, *fcpR2* and *fcpR3* mutants with respect to the wild-type C6706 strain to grow in the absence and presence of the iron chelator, 2,2'-Bipyridyl, under growth conditions where the cells are forced to respire. Although a *cpxR* mutant exhibits a growth defect under these conditions ((390), Chapter 2: Figure 2-9), we found no statistical difference between the growth rates of the wild-type C6706 strain and the *fcpR1, fcpR2,* or *fcpR3* ferric reductase mutants (data not shown). Our results suggest that the individual Cpx regulation of *fcpR1, fcpR2,* and *fcpR3* is not important in the ability of the Cpx pathway to mediate adaptation to iron limitation when cells are forced to respire. At this time, we cannot rule out that FcpR1, FcpR2, and/or FcpR3

play redundant roles in this phenotype. Future studies should involve the creation of these mutant strains to test this hypothesis.

We and others previously found that the Cpx response is required for adaptation to aberrant disulfide bond formation (261, 390) (described in Section 2.3.7). To test if the strongly Cpx-regulated ferric reductases, FcpR1, FcpR2 and FcpR3, are required for this adaptation, we monitored the growth of the wild-type C6706 strain and *fcpR1*, *fcpR2* and *fcpR3* mutants in the presence of diamide, an oxidizing agent that reacts with low molecular weight thiols and promotes protein disulfide cross-linking (369). There was a significant decrease in survival for all mutants compared with wild-type C6706 when treated with diamide under AKI conditions (Figure 3-6,D-F). These results indicate that some of the CpxR-regulated genes involved, or predicted to be involved, in energy metabolism and iron are required to survive in the presence of the Cpx inducing cue generated by diamide. We previously showed that iron supplementation could restore the ability of a *cpxR* mutant to grow in the presence of diamide-induced stress ((390), Chapter 2: Figure 2-9). Interestingly, iron supplementation increased only the survival of a strain carrying an *fcpR2* mutation when grown in the presence of diamide (Figure 3-6E). These data imply that the stress reducing functions of FcpR1, FcpR2, and FcpR3 may be somewhat unique.

3.3.6 *cpxP and cpxRA expression are differentially regulated and influenced by deletion of fcpR1, fcpR2, or fcpR3*

The fact that *fcpR1, fcpR2,* and *fcpR3* are amongst the genes that are most strongly up-regulated upon Cpx response activation suggests that they play an important role in adaptation to envelope stress in *V. cholerae.* We therefore wondered if mutation of *fcpR1, fcpR2,* and/or *fcpR3* would generate a Cpx-sensed envelope stress. To determine if this was true, we first used qRT-

PCR to measure transcription of the Cpx-regulated gene *cpxP* in wild-type C6706 and its isogenic mutants lacking the ferric reductases in the absence and presence of CpxR over-expression. Since the *cpxRA* operon is known to be auto-activated in other enteric bacteria (186), we also measured expression of these genes under the same conditions.

Surprisingly, the results of this experiment revealed that *cpxP* and *cpxA* (and possibly *cpxR)* expression are differentially regulated in *V. cholerae* El Tor strain C6706, which is novel compared to other enteric bacteria (186). This was manifest in three ways. First in a wild-type strain C6706, activation of the Cpx response by CpxR over-expression induced *cpxP* transcription, but not that of *cpxA* (Figure 3-7, compare A and B). Transcription of *cpxP* was induced approximately 512 fold by CpxR overexpression (Figure 3-7, compare A and B). High levels of *cpxR* transcript were also detected, however we could not make any conclusions concerning *cpxRA* autoregulation based on this result, since we induced the Cpx response by over-expressing *cpxR* from an episomal copy encoded on a plasmid (Figure 3-7, compare A and B). Surprisingly, no increase in *cpxA* transcription was detected when *cpxR* was over-expressed (Figure 3-7, compare A and B).

The second observation that suggests that *cpxP* expression is uncoupled from that of the *cpxRA* operon is that deletion of the putative ferric reductases FcpR1, FcpR2, or FcpR3 had differential effects on transcription of *cpxP* and *cpxA*. We found that expression of *cpxP* was 0.065 fold that of the wild-type strain C6706 in the *fcpR1* mutant (Figure 3-7A). Similarly deletion of *fcpR2* and *fcpR3* also decreased the expression of *cpxP* to 0.215 and 0.62 fold that of C6706 respectively (Figure 3-7A). In contrast, there was not a significant change in expression of *cpxR* when *fcpR1* or *fcpR2* were deleted (Figure 3-7A) and deletion of *fcpR3* lead to 7 and 2 fold increases in expression of the *cpxR* and *cpxA* genes respectively (Figure 3-7A).

A third observation indicating that *cpxP* and *cpxA* expression are distinctly regulated derives from the fact that Cpx response activation in the *fcpR1-3* mutants exerted differing effects on *cpxP* and *cpxA* message levels (Figure 3-7, compare A and B). Despite the down-regulation of *cpxP* transcription in the ferric reductase mutants (Figure 3-7A), activation of the Cpx response by CpxR over-expression still resulted in robust activation of *cpxP* transcription, although this activation was dampened to a level about 10-fold less than that observed in a wild-type C6706 strain (Figure 3-7, compare A and B). In contrast, while CpxR over-expression failed to stimulate *cpxA* transcription in the wild-type C6706 strain, *cpxA* transcript levels were approximately 20 fold higher in the *fcpR1-3* mutants (Figure 3-7, compare A and B).

Together, these results demonstrate that the *cpxP* and *cpxRA* loci are differentially regulated in *V. cholerae* El Tor strain C6706, suggesting that a unique regulatory circuitry has evolved in *V. cholerae* to control the Cpx response, or alternatively that this circuitry has not yet been uncovered in other similar organisms. The data indicate either that Cpx signalling is down-regulated in the ferric reductase mutants and CpxR over-expression can only partially overcome this effect, or that a separate signalling pathway contributes to down-regulation of *cpxP* expression in the *fcpR1, fcpR2,* and *fcpR3* mutants. We favour the first explanation, since we have shown that all other Cpx-regulated genes analyzed are also down-regulated, even in the presence of CpxR over-expression, when either *fcpR1, fcpR2,* or *fcpR3* is deleted (see below).

3.3.7 *Transcriptomic analysis of the Cpx regulon in the absence of putative ferric reductases FcpR1, FcpR2, or FcpR3*

The Cpx response is activated by iron depletion ((390), Chapter 2: Figures 2-5,10) and strongly up-regulates three putative ferric reductases ((390), Figures 3-2, 3-3, 3-5), as well as many genes involved in iron uptake and metabolism. Further, deletion of any of the *fcpR1, fcpR2,*

or *fcpR3* genes affects *cpxP* expression and alters Cpx signalling (Figure 3-7). We therefore wondered if Cpx-dependent alterations in iron metabolism as a result of the very strong increase in expression of the putative FcpR1-3 ferric reductases might be responsible for some of the gene expression changes observed upon Cpx pathway activation. To investigate this question, we used microarray analysis of RNA isolated from cultures grown in the absence and presence of CpxR overexpression to characterize the Cpx regulon in each of the *fcpR1, fcpR2,* and *fcpR3* mutants and compared those regulons to that described for the wild-type C6706 strain ((390), Chapter 2: Table 2-5). We found that the Cpx-dependent activation of most of the iron-regulated genes we identified as part of the Cpx regulon was diminished or absent in each of the *fcpR1, fcpR2,* and *fcpR3* mutants (Table 3-3, Figure 3-8).

To confirm the observed changes in gene expression for some of the iron regulated genes, we performed qRT-PCR analysis. Under non-inducing conditions in the *fcpR1* and *fcpR3* mutants there was no change in the basal expression of *tonB*, *hutB* and *exbB1* genes relative to the wild-type strain (Figure 3-7C). However, deletion of *fcpR2* decreased the expression of *tonB*, *hutB* and *exbB1* 0.09, 0.03 and 0.225 fold, respectively (Figure 3-7C). Consistent with our microarray analysis, under inducing conditions (i.e. transient overexpression of CpxR), we found that the previously observed activation of those genes in a Cpx dependent manner was abolished in the ferric reductases mutants (Figure 3-7D). In some cases, CpxR over-expression even resulted in a dramatic inhibition of expression of the *tonB, hutB,* and *exbB1* genes (Figure 3-7D, *fcpR2* mutant). These data strongly suggest that the strong, Cpx-dependent up-regulation of the putative FcpR1, FcpR2, and FcpR3 ferric reductases leads to alterations in cellular iron homeostasis that impact Cpx-dependent signalling and/or alter other regulatory pathways in the cell that are

sensitive to iron. Further analyses will be required to understand the mechanism(s) involved in this regulation in *V. cholerae*.

3.4 Discussion

In this study we investigated the role of three putative ferric reductases in relation to Cpx dependent signalling and adaptation. Previously, we demonstrated that overexpression of CpxR in *V. cholerae* El Tor strain C6706 results in very strong activation of the *fcpR1*, *fcpR2* and *fcpR3* transcripts under conditions that are known to induce virulence expression in this organism (390). *In silico* characterization of FcpR1 showed that this protein contains domains that are also found in the ferric reductase protein family: a heme-containing 6 transmembrane ferric reductase domain and two C-terminal cytoplasmic FAD-binding and NADPH-binding domains (395). Interestingly, those domains are also shared with NADPH oxidases that share homology with ferric reductases (396).

FcpR2 and FcpR3 are putative cytochrome b_{561} orthologs in *V. cholerae* and their expression depends on the iron regulator Fur (366, 388). Cytochrome b_{561} proteins are integral membrane proteins which contain two heme molecules, and are reducible by ascorbate (397). These proteins are widely distributed among phylogenetically distant species (398), but they are most studied in higher organisms (399). The bacterial cytochrome b_{561} domains differ from those of eukaryotes in that a different architecture is involved in the coordination of the two heme groups (400). However, despite the architectural differences among cytochrome b_{561} proteins, their expression is all regulated by iron limitation. For example, the duodenal cytochrome b_{561} (Dcytb) in mice is a homolog of cytochrome b_{561} and its expression depends on iron status and hypoxia (401). In that study, it was described that Dcytb is a ferric reductase required for the transport of

ferrous iron into intestinal duodenal mucosal cells (401). Interestingly, the same iron limitation conditions are also expected to be encountered by *V. cholerae* during colonization in the human host (402), and expression of *fcpR2* and *fcpR3* depends on iron availability in this organism (366, 388).

In the current study, we found that the increased expression of the FcpR1-3 CpxR regulated ferric reductases is independent of the growth media. Transcription levels of *fcpR1*, *fcpR2* and *fcpR3* were found to be highly induced when the Cpx pathway was activated under standard laboratory condition (i.e. LB broth) and we previously demonstrated their Cpx-dependent up-regulation in virulence inducing AKI conditions ((390), Chapter 2: Figures 2-4A, Tables 2- 5,6). This suggests that the CpxR regulation of these putative ferric reductases is not only required under *in vitro* virulence inducing conditions in *V. cholerae* El Tor strain C6706 (i.e. AKI conditions). Similarly, Taylor and collaborators (332) also reported that the Cpx response regulates the expression of *fcpR1*, *fcpR2* and *fcpR3* when cells are grown in LB broth (332), however the induction was not as strong as what we observed (Figure 3-2). These differences could be related to the methodology that was used in order to induce the Cpx pathway and/or strain differences between the studies.

We previously found that *fcpR1*, *fcpR2* and *fcpR3* contain a putative CpxR binding site in their promoter regions (where *fcpR3* possess two) (390). We investigated the mechanism by which CpxR regulates the expression of these ferric reductases. We found that their fast kinetics of expression after transient CpxR overexpression at different time points were similar to those observed for *cpxP*, a gene that is very strongly positively regulated by CpxR. Interestingly, the expression of *fcpR3* is higher in comparison with that observed for *cpxP*. A study in *E.coli* suggested that genes that are known to be directly regulated by CpxR change their expression

within 30 minutes (225). Further, genes directly regulated by CpxR contain a strong consensus binding site that is located within 100 bp of the translational start site (213), as do *fcpR1-3.* These results strongly support the conclusion that CpxR directly binds to the promoter regions of *fcpR1*, *fcpR2* and *fcpR3* to regulate their expression. A future study to confirm the direct regulation of *fcpR1*, *fcpR2* and *fcpR3* by CpxR could be the construction of transcriptional fusions of the promoter region in which the CpxR binding site is mutated of *fcpR1*, *fcpR2* and *fcpR3*, with the light-producing *luxCDABE* operon encoded on previously described reporter plasmid pJW15 (227), and determine luminescence activity in the wild type and mutated version of the promoter region when the Cpx pathway is activated.

While we currently have no evidence for the enzymatic activities of FcpR1-3 as ferric reductases, our data demonstrate that these proteins impact cellular iron homeostasis. We found that the CpxR-mediated regulation of some of these ferric reductases is required to survive envelope stresses, including the generation of aberrant disulfide bonds by diamide (Figure 3-6). Further, the survival defect could be corrected by the addition of exogenous iron (Figure 3-6E). This suggests that a link between the requirement of these ferric reductases for adaptation to envelope stress and iron. Further, our analysis of the Cpx regulon in the *fcpR1-3* mutants demonstrated that activation of iron-regulated genes by CpxR over-expression no longer occurs when any of these putative ferric reductases are deleted (Table 3-3, Figure 3-8). These data strongly suggest that deletion of either *fcpR1, fcpR2,* or *fcpR3* leads to changes in iron status that may impact Cpx signalling or alter other iron-sensitive regulatory pathways in the cell. Together, these data support a role for the putative FcpR1-3 iron reductases in regulating iron homeostasis in *V. cholerae.* A future study in order to determine the putative ferric reductase activity of FcpR1, FcpR2, and FcpR3 could be assessed using a colorimetric assay in which ferric iron

reduction is quantified using a colorimetric Fe^{2+} indicator (e.g. bathophenanthroline disulfonic acid (BPDS) or 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine) and a source of electrons (e.g. NADPH or NADP) (403).

It is of interest that activation of the Cpx response not only up-regulates the expression of *fcpR3* (VCA0538), but also the downstream gene VCA0539 (Chapter 2: Table 2-5). Blast analysis of VCA0539 showed that it has 55% of identity with the YceI protein in *E. coli*. YceI is predicted to be a periplasmic protein whose expression is modulated at high pH (404) and at increased concentration of NaCl regardless of the oxygen availability (405). Interestingly, our research group previously reported the *yceI* is part of the CpxR regulon in *E. coli* K-12 strain MC4100 and enteropathogenic *E. coli* (EPEC) strain E2348/69 (223). In addition, *yceI* is encoded in a predicted operon with *yceJ,* which also encodes a putative inner membrane cytochrome b_{561} protein with a c-terminal domain located in the cytoplasm (406). These results suggest that VCA0538 and VCA0539 in *V. cholerae* and YceI and YceJ in *E. coli* may play a conserved role in the adaptation to envelope stress conferred by the Cpx response. Further analyses are required to elucidate their mechanism in that process.

It is interesting that Fur positively regulates the expression of *cpxP* (Figure 3-5A). This, and the fact that the Cpx response also regulates many iron-related genes in *V. cholerae* El Tor C6706 (390) suggest a tight connection between the Cpx envelope stress response and iron homeostasis. Fur positively regulates the expression of genes mainly through the negative regulation of the sRNA RyhB (407). Microarray studies in *V. cholerae* performed in the absence of RyhB did not identify *cpxP* as part of the RyhB regulon (388, 394). However, as previously reported (261), there is no basal expression of *cpxP* in *V. cholerae* in the absence of stress, which could imply that in those studies there were no measurable *cpxP* transcripts, therefore impeding the detection

of any effect of mutation of *ryhB* on *cpxP* expression. Additionally, studies that assessed the direct and indirect targets of the transcriptional regulator Fur by microarray coupled ChIP/microarray analysis have shown that *cpxP* does not possess a putative Fur binding site (366, 370). This suggests that RyhB may regulate the expression of *cpxP* under conditions where both transcripts are expressed, i.e. in the presence of cell envelope stress and iron limitation. Similarly, connections have been reported between the Cpx pathway and small regulatory RNAs in other organisms (250).

We performed transcriptomic analysis to better understand the role of the CpxR regulated ferric reductases upon activation of the Cpx response. We found that the absence of the putative FcpR1-3 ferric reductases lead to a decrease in expression of *cpxP*, suggesting that they are either involved in unidentified regulatory events that control *cpxP* expression and/or that they affect Cpx-dependent adaptation in *V. cholerae,* and therefore alter Cpx signaling. In addition, we found that deletion of *fcpR3* induced the expression of both *cpxR* and *cpxA*. Again, this could suggest that Cpx signaling events are altered because deletion of *fcpR3* changes levels of Cpx inducing cues, or it might indicate that additional, unidentified regulatory pathways are influenced in the *fcpR3* mutant that impact expression of *cpxRA.* Although we cannot currently distinguish among these possibilities, these data indicate the importance of FcpR1-3 in the control of expression and/or activity of the CpxP, R, and A signal transduction proteins in *V. cholerae*.

It has been shown in *E. coli* that the Cpx response is autoregulated, where once CpxR is phosphorylated it binds upstream of the *cpxRA* operon to induce further expression of the CpxA and CpxR signaling proteins during envelope stress (186). In addition, CpxR could be phosphorylated by a small molecular weight phosphodonor such as acetyl-phosphate (183, 196). However, we found that activation of the Cpx response does not lead to changes in expression of

cpxA transcripts in the wild-type C6706 strain, but we observed that in the *fcpR1*, *fcpR2* and *fcpR3* mutants, *cpxA* expression increases more than 20 fold. These data suggest that deletion of the *fcpR1-3* genes affects the expression of *cpxA* in a CpxR independent manner. In support of this conclusion, we found a putative transcriptional start site in the coding region of the *cpxR* locus. Finally, we reported that activation of the Cpx response in the ferric reductase mutants (i.e. *fcpR1*, *fcpR2* and *fcpR3*) affects the previously observed CpxR-regulation of genes related to iron. Finally, this study suggests that at least some of the CpxR iron regulated genes (i.e. *fcpR1*, *fcpR2* and *fcpR3*) can affect Cpx signaling and Cpx adaptation (e.g. to aberrant disulfide bond formation) in *V. cholerae* El Tor strain C6706.

3.5 Tables and Figures

Table 3 - 1 Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Source or reference
Strains		
C6706	*V. cholerae* El Tor C6706 strain; (Sm^R) streptomycin-resistant spontaneous	Dr. J. Mekalanos, Harvard Medical School
NA44	C6706 carrying pCpxR plasmid; (Sm^R Amp^R)	(390)
EC16554	Derivative of C6706 strain carrying TnFGL3 insertion in the VC2692 (Sm^R kan^R)	(353)
EC16469	Derivative of C6706 strain carrying TnFGL3 insertion in the VC2106 gene (Sm^R kan^R)	(353)
NA377	EC16469 carrying pCpxR plasmid; (Sm^R kan^R Amp^R)	This study
EC11645	Derivative of C6706 strain carrying TnFGL3 insertion in the VCA0151 gene (kan^R)	(353)
EC9670	Derivative of C6706 strain carrying TnFGL3 insertion in the VCA0249 gene (kan^R)	(353)
EC16172	Derivative of C6706 strain carrying TnFGL3 insertion in the VCA0538 gene (kan^R)	(353)
NA255	EC11645 carrying pCpxR plasmid; (Sm^R kan^R Amp^R)	This study
NA257	EC9670 carrying pCpxR plasmid; (Sm^R kan^R Amp^R)	This study
NA259	EC16172 carrying pCpxR plasmid; (Sm^R kan^R Amp^R)	This study
Plasmids		
pCpxR	pBAD24 carrying *cpxR* of *Vibrio cholerae* C6706 (Amp^R)	This study

Table 3 - 2 qRT-PCR primers used in this study.

Gene	Primer sequence 5' to 3'	
	Forward	**Reverse**
cpxP	GGGATCTGGAAACAGCTTGA	CACGCATCTCTTTGAGTTGG
fcpR1	AGAGCACACCCACCAGTACA	GCACAAATCCGGATCAATTT
fcpR2	CAAAGAAGGGGCGATTCC	TAAACCATGAACCGCTTTCG
fcpR3	TTCATCTCCGAAAATTGAAGG	AACAAACAATCCAACGTACATCA
ryhB	TTGACACGACATTGCTCACA	CGAGGTCAAAGCCAATTTTT
cpxR	ATCGGGTGATCGGCTTAGA	ACTCGCGATCGCTAAAAGG
cpxA	CGAAGTGCAGCCACTGAGTA	CAGCTTCAAATTGCGCATC
tonB1	CAAGGCATCACAAGTCAACC	AACTTGTGCGCTCACTAAAGC
hutB	ACAACACGCTCAAAAGCTGA	TTAGCTTGCAACGCATTGAT
exbB1	GTAAGCGTCCGGTGCTGTAT	TTCGCGCAATGACTTATCAA

Table 3 - 3 Comparative analysis of expression profiling of Cpx regulated genes in wild type *V. cholerae* El Tor C6706, *fcpR1*, *fcpR2* and *fcpR3* mutants when the Cpx pathway is activated.

ORF	Description of the gene product	Fold Change[a]			
		WT[b]	*fcpR1*	*fcpR2*	*fcR3*
VCA0151	Oxidoreductase, putative	179.36	-22.30	-4.62	
VCA0139	Hypothetical protein	94.34			
VC2691	Periplasmic repressor CpxP	77.84		-3.43	
VC2692	Transcriptional regulator CpxR	50			
VCA0538	Cytochrome b561, putative	46.62			
VCA0249	Cytochrome b561, putative	38		-11.94	
VC0914	Multidrug resistance protein, putative	28.24			
VC0915	Hypothetical protein	24.81			
VC0913	Hypothetical protein	22.41			
VCA0152	Putative monovalent cation/H+ antiporter subunit G	21.1	-13.64	-3.36	
VCA0154	Hypothetical protein	19.97	-8.28	-3.04	
VC0166	TetR family transcriptional regulator	19			
VC1225	Hypothetical protein	18.43			
VC0587	Sulfate permease family protein	18.36	-5.62	-13.35	
VCA0782	ABC transporter, ATP-binding protein	17.97			
VCA0153	Putative monovalent cation/H+ antiporter subunit F	16.61	-12.18	-2.89	
VCA0781	Hypothetical protein	14.92			
VC1326	Hypothetical protein	12.71			
VCA0990	DEAD-box ATP dependent DNA helicase	12.62			
VCA0539	Hypothetical protein	12.02			-4.20
VC1327	Galactose/methyl galactoside transporter ATP-binding protein	11.99			
VC0916	Phosphotyrosine protein phosphatase	11.06	2.31	3.02	
VCA0155	NADH dehydrogenase, putative	10.63	-8.91	-3.42	
VC1328	Beta-methylgalactoside transporter inner membrane component	10.39			
VC1224	Hypothetical protein	9.87			
VC1596	Galactose-1-phosphate uridylyltransferase	9.81		-2.35	
VCA0780	UDP-glucose 6-dehydrogenase	8.98			
VC1594	Aldose 1-epimerase	8.69			
VC0164	Multidrug resistance protein, putative	8.15			
VC1325	Galactoside ABC transporter, periplasmic D-galactose/D-glucose-binding protein	7.96			
VCA0783	Arylesterase	7.78			
VCA0270	D-alanyl-D-alanine carboxypeptidase	7.36			
VCA0198	Site-specific DNA-methyltransferase, putative	7.28			
VC1595	Galactokinase	6.41			
VC0494	Hypothetical protein	6.15	-4.21	-6.59	-2.90

VC1324	Hypothetical protein	5.71	-3.52	-5.00	-2.93
VC1187	Hypothetical proteins	5.67			
VCA0271	Hypothetical protein	5.6			
VCA0914	Hemin ABC transporter, permease protein, putative	5.33		-3.47	
VCA0912	TonB system transport protein ExbD1	5.25	-2.17	-9.21	
VCA0913	Hemin ABC transporter, periplasmic hemin-binding protein HutB	5.22	-3.48	-13.55	-3.96
VCA0911	TonB system transport protein ExbB1	5.02	-2.10	-11.90	
VCA0733	Hypothetical protein	4.69	-4.30	-5.97	-4.35
VCA0092	Hypothetical protein	4.48		-2.86	
VC0984	Cholera toxin transcriptional activator	4.44			
VC0917	UDP-N-acetylglucosamine 2-epimerase	4.43	6.27	17.18	
VCA0910	TonB1 protein	4.38	-6.10	-85.02	-2.28
VC0174	Hypothetical protein	4.37			
VC0963	VisC protein, putative	4.36			
VC0364	Bacterioferritin-associated ferredoxin	4.21		-4.29	
VC2617	Arginine/ornithine succinyltransferase, putative	4.2	-2.02		-2.22
VC2690	Ferrous iron efflux protein F	4.04			
VC0983	Regulatory protein ToxS	4.02			
VC2360	Endonuclease IV	4.01	-2.45		
VC0982	Selenoprotein W-related protein	3.92			
VCA0989	Hypothetical protein	3.88	2.12	2.51	2.46
VCA0909	Coproporphyrinogen III oxidase	3.8		-12.69	
VC2337	LacI family transcription regulator	3.72			
VC0919	Serine acetyltransferase-related protein	3.71	3.85	11.73	
VC0165	Hypothetical protein	3.69			
VC0768	Bifunctional GMP synthase/glutamine amidotransferase protein	3.69	2.00		
VCA0614	Formate--tetrahydrofolate ligase	3.63		2.13	
VCA0103	Sulfate permease family protein	3.6			
VCA0732	Hypothetical protein	3.35	-8.33	-49.44	-4.44
VCA0714	Diacylglycerol kinase	3.34			2.51
VCA0908	HutX protein	3.24	-3.83	-24.74	-3.98
VC1637	Hypothetical protein	3.17	-2.14	-12.26	
VC1797	Hypothetical protein	3.14	-2.54	-3.56	-2.41
VCA0907	Heme-binding protein HutZ	3.13		-4.56	
VCA0576	Heme transport protein HutA	3.11	-4.95	-17.94	-6.28
VC1639	Sensor histidine kinase	3.1		-6.78	-2.04
VCA0550	Hypothetical protein	3.1		2.47	
VC2618	Acetylornithine aminotransferase	3.08			
VC2312	Murein transglycosylase A	3.05		-3.93	-2.20
VC1638	DNA-binding response regulator	2.97	-2.18	-8.42	

VC0746	RNA methyltransferase	2.96	2.18	2.08	
VC1503	Hypothetical protein	2.94	-2.18	-2.53	
VCA0643	Conserved hypothetical protein	2.93	-9.18	-20.74	
VC0740	Hypothetical protein	2.86	-3.66	-2.15	-2.61
VC0365	Bacterioferritin, bfr	2.84		-6.68	
VCA0976	Hypothetical protein	2.77		-3.67	
VC0200	Iron(III) compound receptor	2.74		-5.27	
VC2616	Succinylglutamic semialdehyde dehydrogenase	2.73			
VC1412	Hypothetical protein	2.71			
VC0743	Preprotein translocase subunit SecD	2.7	2.91	5.11	
VC1640	50S ribosomal protein L25	2.7			
VC1798	Eha protein	2.67	-2.26	-4.02	-2.07
VC0871	Hypothetical protein	2.66	-4.34	-14.58	-3.25
VC1571	Quinol oxidase, subunit I, qxtA	2.64	-4.38	-7.63	
VC1688	Hypothetical protein	2.63	-2.02	-4.11	
VCA0230	Iron(III) ABC transporter, ATP-binding protein	2.61			
VC1188	Malate dehydrogenase	2.6			
VC1742	Hypothetical protein	2.6			
VC1812	Hypothetical protein	2.59	-8.55	-17.59	-7.62
VCA0217	GGDEF family protein	2.59	-2.27	-4.86	
VCA0560	GGDEF family protein	2.55		-2.01	
VC0201	Iron(III) ABC transporter, ATP-binding protein	2.54		-2.56	
VC0493	Hypothetical protein	2.54	-3.78	-3.80	-4.19
VCA0229	Vibriobactin and enterobactin ABC transporter, permease protein, vctG	2.53			
VC0739	S-adenosylmethionine:tRNA ribosyltransferase-isomerase	2.51			
VCA0845	Hypothetical protein	2.49	-3.77	-9.55	
VC0873	Hypothetical protein	2.48	-2.32	-6.42	
VC2146	Conserved hypothetical protein	2.46			
VCA0939	Sensory box/GGDEF family protein	2.45		-2.49	
VC2313	Hypothetical protein	2.44	-2.58	-6.29	-4.12
VC1413	Methyl-accepting chemotaxis protein	2.44			
VC0922	Hypothetical protein	2.44	2.25	6.36	-2.53
VC0863	Hypothetical protein	2.43		4.39	
VC1323	Hypothetical protein	2.43	-2.17	-2.50	
VCA0227	Vibriobactin and enterobactin ABC transporter, periplasmic vibriobactin/enterobactin-binding protein, vctP	2.42	-2.44	-3.55	-2.63
VC0742	Preprotein translocase subunit YajC	2.4			
VC1653	Sensory box sensor histidine kinase/response regulator VieS	2.39			
VC1975	2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase/2-oxoglutarate decarboxylase,	2.38		3.38	

	menD				
VC2226	Phosphoribosylaminoimidazole synthetase	2.35	2.32	3.27	
VC2436	Outer membrane channel protein	2.31			
VC1958	Hypothetical protein	2.29			
VC2734	General secretion pathway protein C	2.24	2.23	3.58	
VC0163	Conserved hypothetical protein	2.24	-2.78	-2.44	
VC1976	Menaquinone-specific isochorismate synthase	2.23	3.22	6.05	
VCA0977	ABC transporter, ATP-binding protein	2.2	-2.48	-5.91	-2.90
VC0202	Iron(III) ABC transporter, periplasmic iron-compound-binding protein	2.19			
VC0719	DNA-binding response regulator PhoB	2.18			
VCA0644	NADH oxidase, putative	2.17	-3.43	-6.05	
VC0872	Hypothetical protein	2.14	-2.79	-10.20	-2.05
VCA0915	Hemin importer ATP-binding subunit	2.13			
VCA0216	Hypothetical protein	2.12	-3.37	-7.25	
VC1306	Hypothetical protein	2.11			
VC0167	ATP-dependent DNA helicase Rep	2.11		-2.01	
VC0744	Preprotein translocase subunit SecF	2.07		2.02	
VCA0659	Protein F-related protein	2.06	2.44		-2.30
VC0771	Vibriobactin-specific isochorismatase	2.05	-2.45	-11.95	
VCA0864	Methyl-accepting chemotaxis protein	2.01			-2.02
VCA0537	Hypothetical protein	2.01			-2.22
VC1649	Trypsin, putative	-2		-2.36	
VC1082	Response regulator	-2.01	-2.49	-2.43	
VCA0219	Haemolysin, hlyA	-2.01			
VC1520	ABC transporter, ATP-binding protein	-2.02		2.43	
VC1132	ATP phosphoribosyltransferase	-2.03			2.47
VC1410	Multidrug resistance protein VceA	-2.04			
VC1081	Response regulator	-2.06	-2.91	-3.06	
VCA0615	Peptide methionine sulfoxide reductase	-2.07			
VCA0137	Sn-glycerol-3-phosphate transporter	-2.11			
VCA0108	Conserved hypothetical protein	-2.15	2.02	2.41	
VC0634	Transcription elongation factor GreA	-2.16		-2.18	
VCA0691	Acetoacetyl-CoA reductase	-2.17		-9.17	-2.35
VC1938	Hypothetical protein	-2.23		-2.12	
VCA0981	Hypothetical protein	-2.24	-4.08	-8.71	-2.32
VCA0546	Hypothetical protein	-2.25		-5.33	2.55
VCA0712	Pyrazinamidase/nicotinamidase	-2.26	2.00	2.62	
VC1409	Multidrug resistance protein, putative	-2.26	2.09		3.51
VC0996	Hypothetical protein	-2.28			
VC0566	Protease DO	-2.3		2.15	2.12

VC1578	Hypothetical protein	-2.3	2.01	2.46	-3.11
VC0545	Alanyl-tRNA synthetase	-2.35			
VC1941	Hypothetical protein	-2.37			
VCA0950	Hypothetical protein	-2.39		2.52	2.13
VCA0645	Hypothetical protein	-2.4		-4.43	
VCA1063	Ornithine decarboxylase	-2.41			
VCA0046	Hypothetical protein	-2.45			
VCA0209	Hypothetical protein	-2.49	4.26	3.83	
VCA1064	Hypothetical protein	-2.5	-2.16	-3.25	
VCA0784	Trans-2-enoyl-CoA reductase	-2.53			
VC1678	Phage shock protein A	-2.57	-3.64	-3.77	
VCA0223	Protease	-2.76		-2.18	
VCA0689	Conserved hypothetical protein	-3.01		-11.00	-3.02
VC2006	Chemotaxis protein CheV	-3.06			
VC0546	Hypothetical protein	-3.29	-3.12	-2.78	-4.31
VCA0842	Hypothetical protein	-3.29	2.19	6.31	-2.04
VCA0136	Glycerophosphodiester phosphodiesterase	-3.39			
VC2470	Hypothetical protein	-3.56			
VCA0843	Glyceraldehyde-3-phosphate dehydrogenase	-4.25		8.69	
VCA0549	PhnA protein	-8.6	3.05	7.32	3.59
VC2469	L-aspartate oxidase	-9.06			2.03
VC2468	Hypothetical protein	-16.14		-5.01	2.43

a. Genes showing a ≥ 2 or ≤ -2 fold difference in expression between the non-induced and induced condition ($P < 0.05$). Numbers represent average fold change observed from two biological replicates with two technical replicates. Positive and negative stands for genes whose expression was up-regulated or down-regulated, respectively.

b. Genes previously characterized ((390), Chapter 2: Table 2-5) as part of the CpxR regulon in *V. cholerae* El Tor strain C6706.

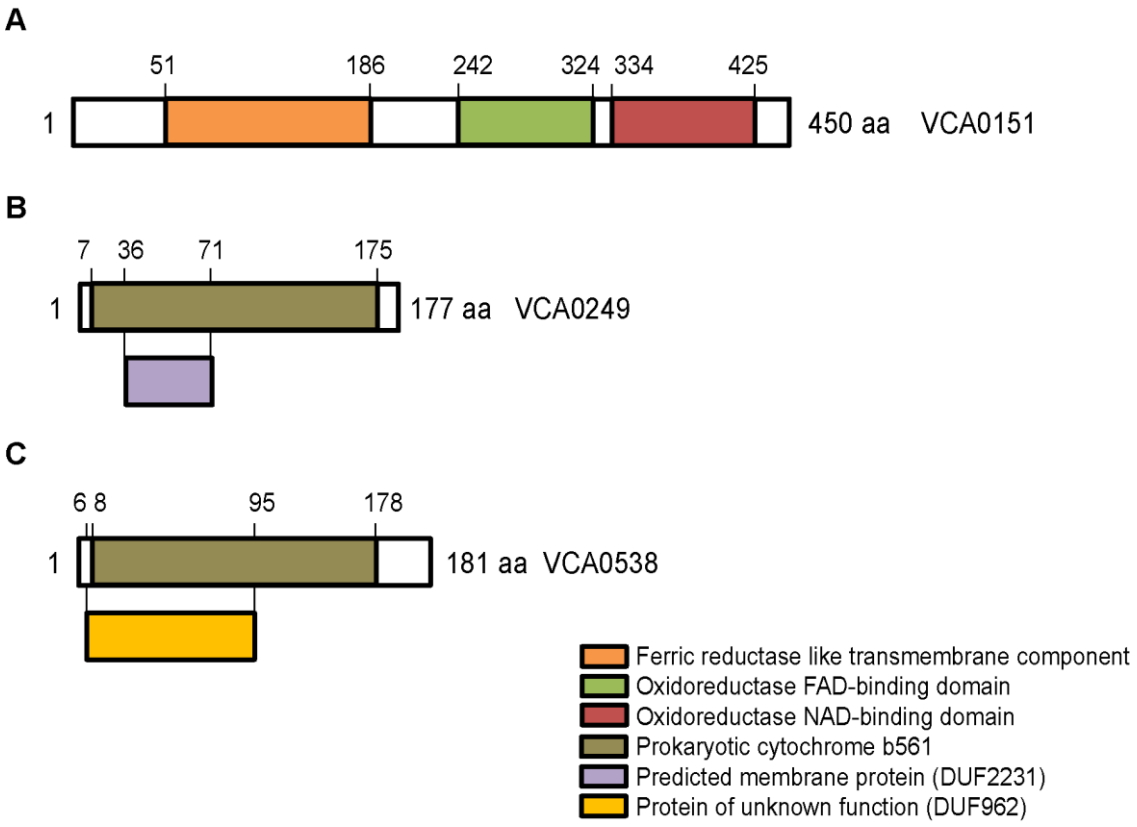

Figure 3 - 1 *In silico* characterization of Cpx-regulated ferric reductases: VCA0151, VCA0249, and VCA0538 domains.

Characterization of motifs found in VCA0151 (A), VCA0249 (B), and VCA0538 (C) proteins based on the PFAM database (http://pfam.xfam.org/) (392). aa: amino acids; DUF: domain of unknown function; FAD: flavin adenine dinucleotide; NAD: nicotinamide adenine dinucleotide.

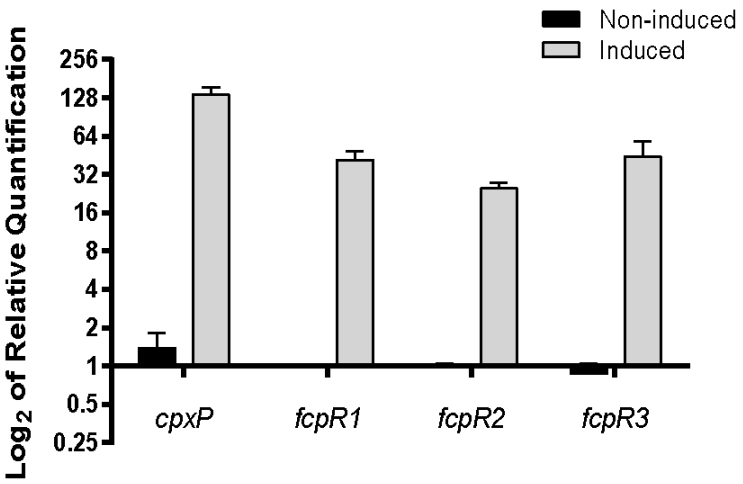

Figure 3 - 2 The Cpx response regulates expression of the *fcpR1, fcpR2,* and *fcpR3* genes that encode putative ferric reductases in a media independent manner.

RNA was isolated from cultures of *V. cholerae* El Tor strain C6706 carrying the overexpression plasmid pCpxR, in the absence (non-induced) (black bars) or presence of 0.1% arabinose (induced) (grey bars) to induce CpxR overexpression. RNA was extracted after the subcultures that grown in LB broth reached an OD_{600} of 0.3 and were induced by 25 minutes with arabinose. RNA was converted to cDNA. The cDNA was subjected to qRT-PCR analysis. Transcript levels were normalized to *gyrA* (endogenous control) for each gene in each condition and the relative quantification was determined. Experiments are representative of two biological replicates, each performed in triplicate. Error bars indicate the standard deviation (SD).

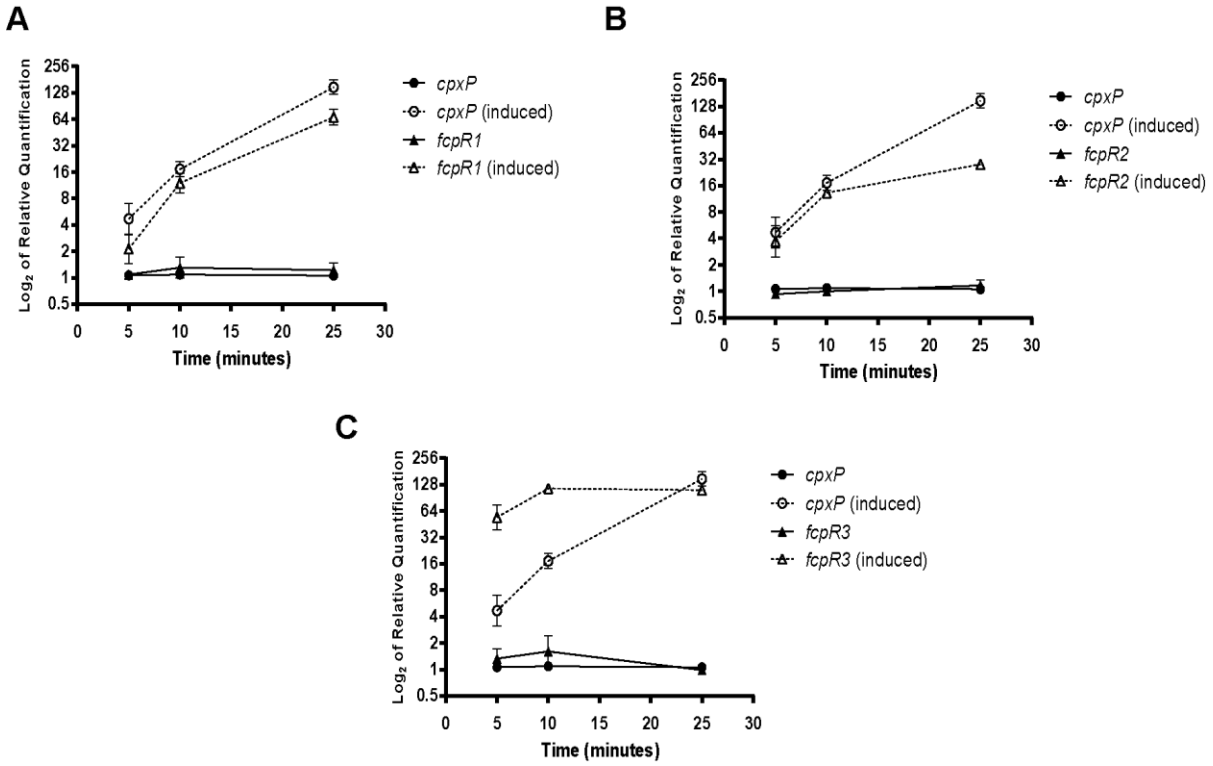

Figure 3 - 3 Kinetics of *fcpR1, fcpR2, and fcpR3* transcript accumulation after overexpression of CpxR.

RNA was isolated after 5, 10 and 25 minutes of induction of CpxR overexpression from cultures of *V. cholerae* El Tor strain C6706 carrying the overexpression plasmid pCpxR and growing in LB broth. RNA was converted to cDNA and it was subjected to qRT-PCR analysis. Continuous lines indicate transcript levels in cultures grown in the absence of induction, while discontinuous lines indicate transcript levels in cultures grown in the presence of 0.1% arabinose (induced).

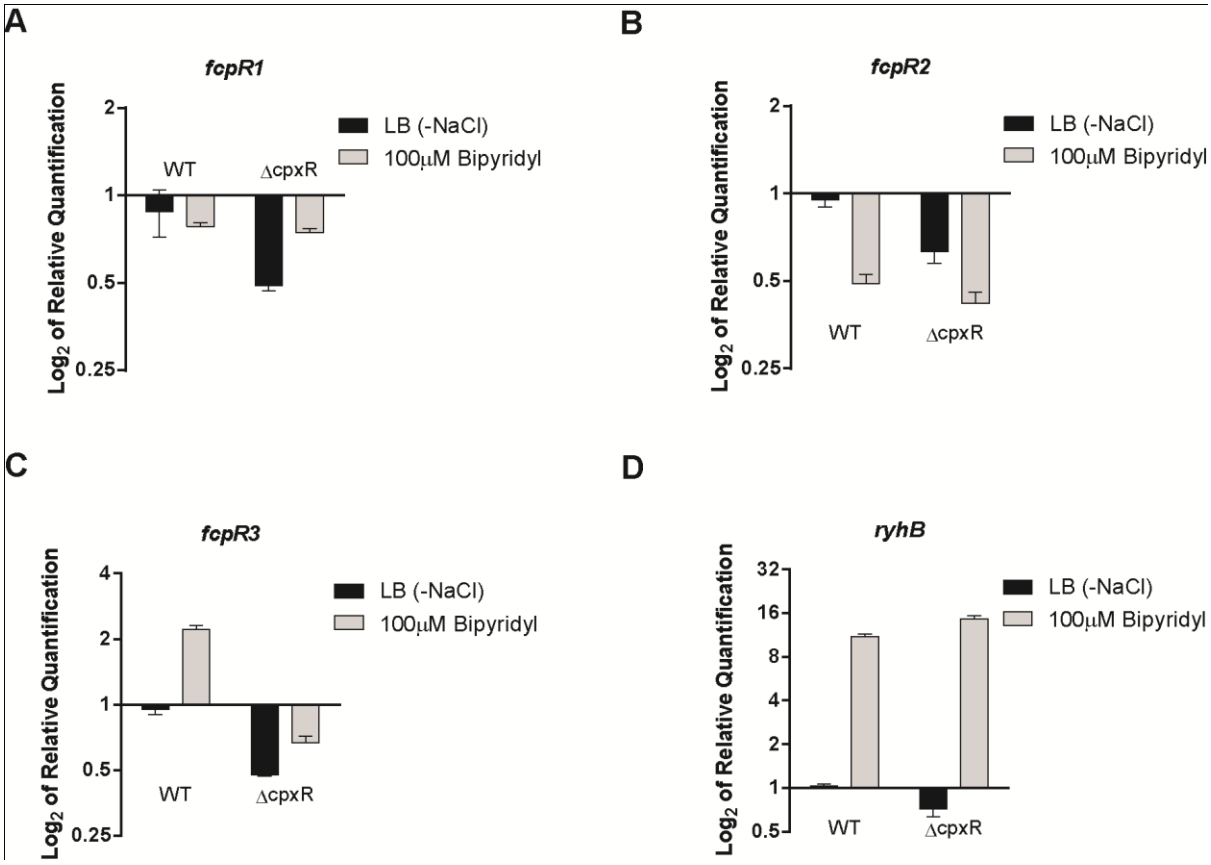

Figure 3 - 4 *fcpR1*, *fcpR2*, and *fcpR3* expression are differentially affected by iron limitation in a partially CpxR dependent manner.

RNA was isolated from bacterial spots on agar plates of *V. cholerae* El Tor C6706 and the *cpxR* mutant (EC16554), in the absence (black bars) or presence of 2,2'-Bipyridyl (100 μM) (gray bars) and converted to cDNA. The cDNA was subjected to qRT-PCR analysis of *fcpR1* (A), *fcpR2* (B), *fcpR3* (C) and *ryhB* (D). Transcript levels were normalized to *gyrA* (endogenous control) for each gene in each condition and the relative quantification was determined. Experiments are representative of two biological replicates, each performed in triplicate. Error bars indicate the standard deviation (SD).

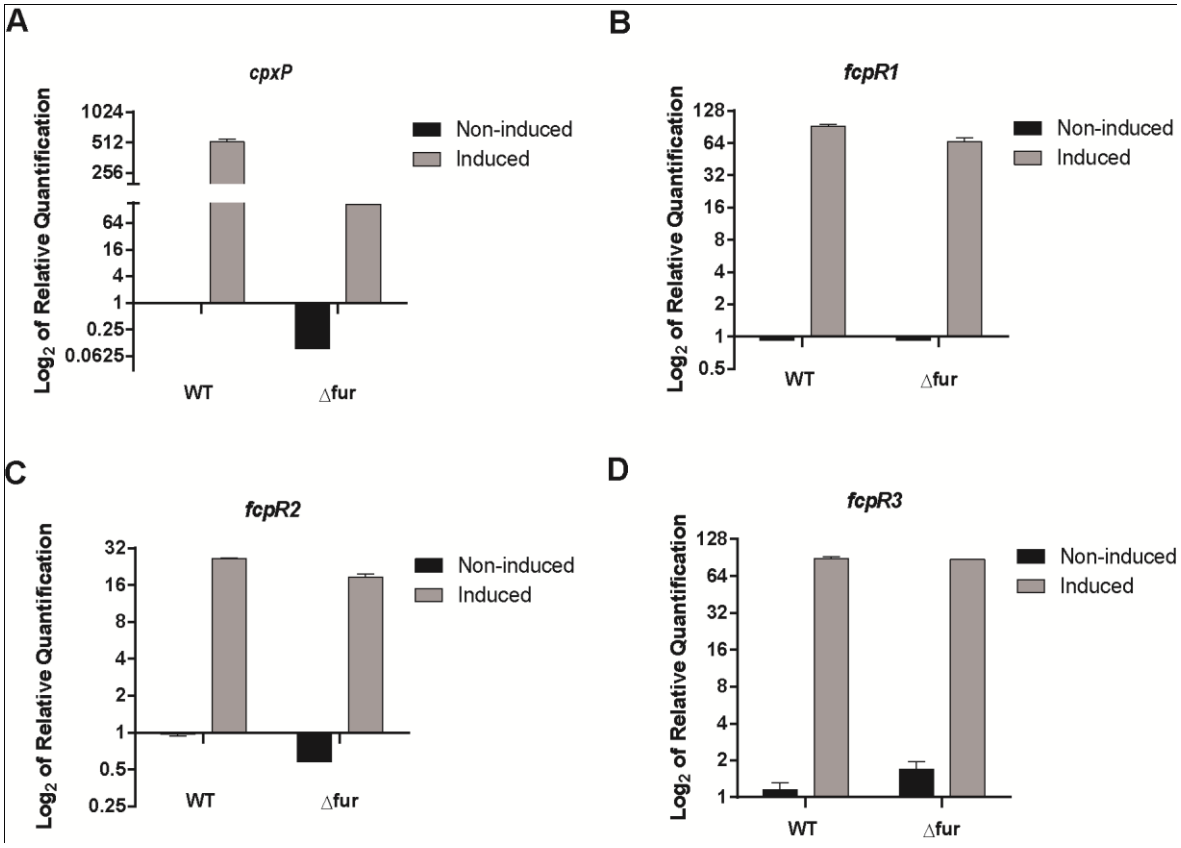

Figure 3 - 5 Transcriptional analyses of ferric reductases in wild type C6706 strain and *fur* mutant upon Cpx activation.

RNA was isolated from cultures of wild type *V. cholerae* El Tor C6706 and *fur* mutant, carrying the overexpression plasmid pCpxR, in the absence (non-induced) (black bars) or presence of 0.1% arabinose (induced) (grey bars) and converted to cDNA. The cDNA was subjected to qRT-PCR analysis of *cpxP* (A), *fcpR1* (B), *fcpR2* (C) and *fcpR3* (D). Transcript levels were normalized to *gyrA* (endogenous control) for each gene in each condition and the relative quantification was determined. Experiments are representative of two biological replicates, each performed in triplicate. Error bars indicate the standard deviation (SD).

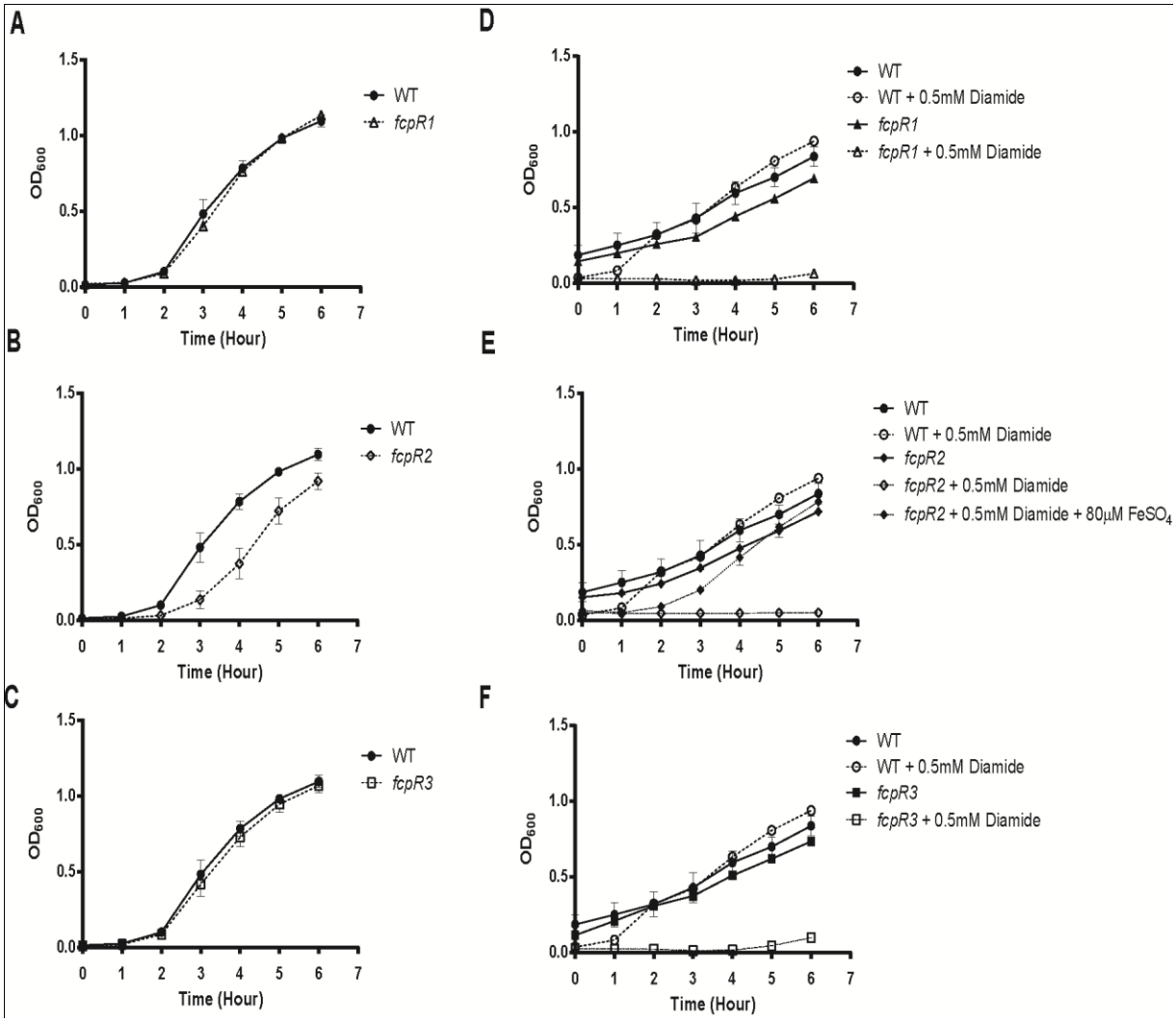

Figure 3 - 6 CpxR regulated ferric reductases are required for adaptation to aberrant disulfide bond formation.

(A-C) Overnight cultures of wild-type C6706 and *fcpR1* (EC11645), *fcpR2* (EC9670), and *fcpR3* (EC16172) mutants were subcultured in LB broth and the OD_{600} was monitored over the course of 6 h. (D-F) Overnight cultures of wild-type C6706 and *fcpR1* (EC11645), *fcpR2* (EC9670), and *fcpR3* (EC16172) mutants were subcultured in AKI medium in the presence or absence of 0.5 mM diamide and 0.5 mM diamide -/+ 80 μM $FeSO_4$, and allowed to grow at 37°C without shaking for 5 h. Then cells (200 μl) were transferred into a 96-well microtiter plate in triplicate and the cultures were allowed to grow at 37°C with shaking, while the OD_{600} was monitored over the course of 6 h. Error bars indicate the standard deviation (SD) (some of the error bars are smaller than the symbols).

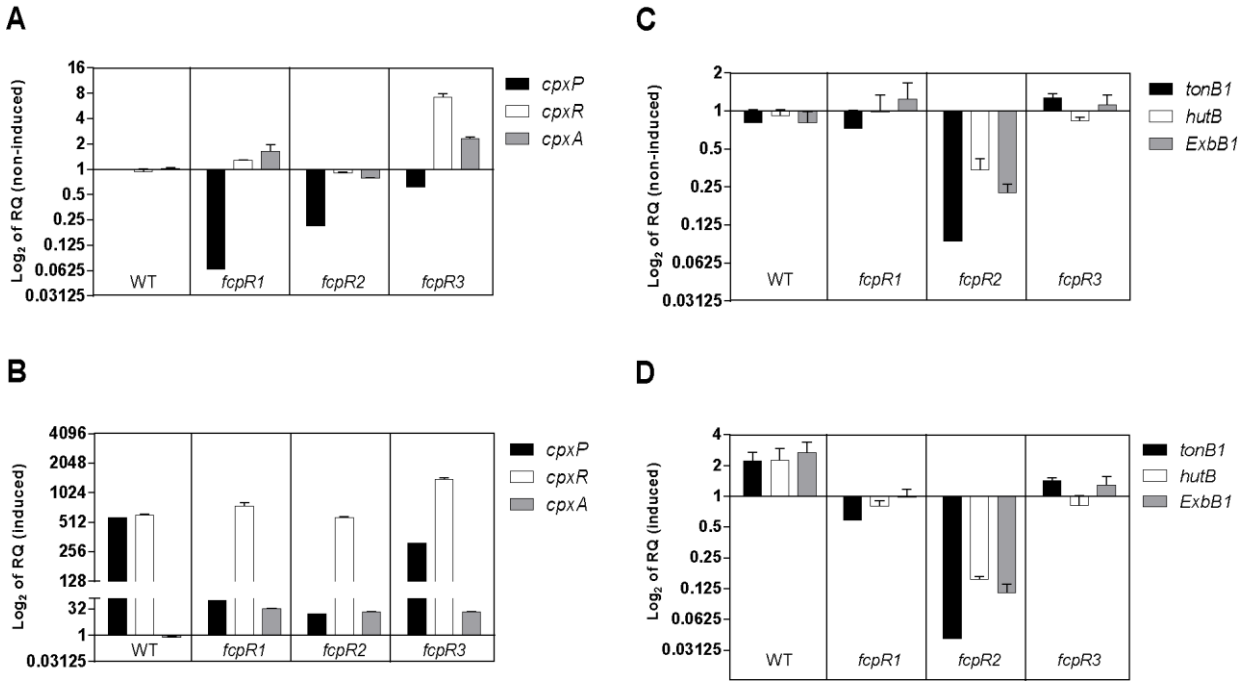

Figure 3 - 7 Transcriptomic analysis of the Cpx regulon in the absence of putative ferric reductases FcpR1, FcpR2, or FcpR3.

RNA was isolated from cultures of wild type *V. cholerae* El Tor C6706 and *fcpR1*, *fcpR2* and *fcpR3* mutants, carrying the overexpression plasmid pCpxR, in the absence (non-induced) (A and C) or presence of 0.1% arabinose (induced) (B and D) and converted to cDNA. The cDNA was subjected to qRT-PCR analysis. Transcript levels were normalized to *gyrA* (endogenous control) for each gene in each condition and the relative quantification was determined. Experiments are representative of two biological replicates, each performed in triplicate. Error bars indicate the standard deviation (SD).

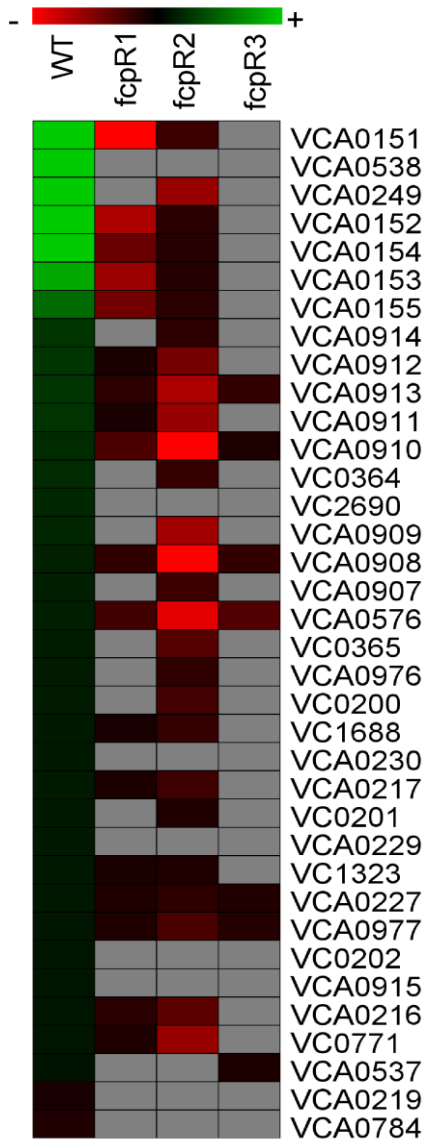

Figure 3 - 8 Comparative analysis of expression profiling of genes implicated in iron transport or function in wild type *V. cholerae* El Tor C6706, *fcpR1*, *fcpR2* and *fcpR3* mutants when the Cpx pathway is activated.

Relative quantification value (RQ) was obtained for the genes related or involved with iron based on gene ontology classification that is part of DAVID program (362) showing a ≥ 2 or ≤ -2 fold difference in expression when the Cpx pathway was activated ($P < 0.05$). Numbers represent average RQ observed from two biological replicates with two technical replicates. Positive and negative stands for genes whose expression was up-regulated or down-regulated, respectively. Gray represents no significant change, positive (+, green) and negative (-, red) stands for genes whose expression was up-regulated or down-regulated, respectively.

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4 Chapter 4: The Cpx system regulates virulence gene expression in *Vibrio cholerae*

4.1 Introduction

V. cholerae, a curved Gram-negative bacterium, is the causative agent of the waterborne disease cholera. More than 200 *V. cholerae* serogroups have been identified, of which the O1 serogroup has been associated with the pandemic spread of cholera (272). Furthermore, the O1 serogroup is classified into two biotypes: Classical and El Tor; the latter is responsible for the seventh ongoing cholera pandemic (264). Recent cholera outbreaks in Haiti, Cameroon and Zimbabwe (268-270) suggest the increase in the incidence of cholera in the western hemisphere (271).

V. cholerae colonizes the human small intestine, where it produces its main virulence factors, toxin co-regulated pilus (TCP) and cholera toxin (CT). TCP, a type IV pilus, enables small intestine colonization and the establishment of microcolonies (42, 287, 408). CT is produced and secreted in a folded state from the periplasm across the outer membrane by the type II secretion system (T2SS) (55). This AB5-type ribosylating enterotoxin (338) leads to the secretion of Cl^- and water from intestinal epithelial cells which is the hallmark of the watery diarrhea associated with *V. cholerae* (279, 409). Virulence gene expression in *V. cholerae* is controlled by a regulatory cascade known as the ToxR regulon (286, 289, 371, 410). Two membrane-bound DNA binding proteins, TcpP and ToxR, are critical for coordinated expression of the master virulence regulator, ToxT, in response to different environmental stimuli (i.e. temperature and pH) (293, 297, 411-413). ToxT directly activates the expression of the genes responsible for the biosynthesis of CT and TCP (410, 414). In addition, *tcpPH* promoter activation is positively regulated by two cytoplasmic regulators, AphA and AphB; and negatively regulated by cAMP receptor protein (CRP) and by quorum sensing via HapR, a LuxR homolog that represses *aphA* transcription (300-302, 304, 415, 416). Finally, an additional regulator, type VI secretion system

regulator A (TsrA), modulates the expression of CT, TCP, and another envelope localized virulence factor, the type VI secretion system (T6SS) in *V. cholerae* (417). The T6SS plays an important role in cytotoxicity towards amoebae and mammalian macrophages (418) as well as inter and intra-bacterial interactions by conferring toxicity towards other bacteria (419, 420).

Bacterial pathogens utilize envelope-localized signal transduction systems to sense and modulate expression of genes in response to environmental signals. The Cpx envelope stress response is controlled by a two-component regulatory system (TCS), which is composed of the sensor histidine kinase CpxA, and the cytoplasmic response regulator CpxR (330). CpxA is an inner membrane (IM) protein that autophosphorylates upon detecting an inducing cue via its periplasmic sensing domain and then becomes a phosphodonor to its response regulator, CpxR, at a conserved aspartate phosphorylation site (183, 330). CpxR phosphorylation leads to up-regulation and down-regulation of multiple genes by direct binding of CpxR to DNA (183). Additionally, the Cpx pathway regulates and is regulated by a periplasmic protein, CpxP, which reduces CpxA autokinase activity (69, 70). The Cpx system senses bacterial cell envelope stress such as misfolded proteins and regulates the expression of diverse genes involved in maintaining cell envelope homeostasis (253).

In many Gram-negative pathogens, the Cpx pathway regulates virulence gene expression (for a recent review, see (253)). For example, the Cpx pathway regulates the expression of a number of virulence determinants in enteropathogenic *Escherichia coli* (EPEC) (type III secretion system (T3SS), bundle-forming pili (BFP) and motility), uropathogenic *E. coli* (UPEC) (P pilus), *Yersinia pseudotuberculosis* (T3SS), *Legionella pneumophila* (Type IV secretion system (T4SS), *Shigella sonnei* (virulence regulators and T3SS), *Salmonella enterica* serovar Typhimurium (invasion factors), *Xenorhabdus nematophila* (*nilA*, *nilB*, and *nilC*) and in *Haemophilus ducreyi*

(*flp-tad* and *lspB-lspA2* operons, *dsrA*, *ncaA*, and *hgbA*) (205, 222, 225, 227, 228, 251, 252, 254-256, 259, 360, 421).

Studies on the human pathogen *V. cholerae* have shown that the activation of the Cpx pathway in *V. cholerae* N16961 is required to mediate cellular responses to perturbations in the cell envelope, and also to stresses related to salinity (261). Recently, we found that activation of the Cpx response in the *V. cholerae* El Tor strain C6706, which is closely related to *the V. cholerae* N16961 strain (358), leads to changes in the expression of genes involved in iron acquisition under virulence inducing conditions (390). Additionally, we and others found that the Cpx pathway regulates genes involved in antimicrobial resistance (332, 390). Finally, our microarray analysis also suggested that the Cpx pathway positively regulates the expression of the *toxR* gene (390), which encodes an important virulence factor regulator in *V. cholerae* (288).

In the current study, we characterized the effect of the activation of the Cpx response on different envelope localized virulence factors in the *V. cholerae* El Tor strain C6706. We confirmed that the Cpx pathway positively regulates the expression of the transcriptional regulator ToxR. Furthermore, we found that the Cpx pathway regulates the expression of one of the major outer membrane (OM) porins in *V. cholerae*, OmpT, in a ToxR-independent manner. Our results indicate that activation of the Cpx pathway negatively regulates the expression of virulence determinants such as CT and TCP at the transcriptional level. We show that activation of the Cpx pathway leads to down-regulation of *tcpP* and *toxT* expression, primarily through changes in CRP-mediated gene regulation. Overall, our work suggests that the Cpx response in *V. cholerae* El Tor C6706 negatively regulates CT and TCP expression through the down-regulation of the *tcpPH* promoter and via altered function of the catabolite activator protein.

4.2 Materials and Methods

4.2.1 *Growth conditions*

Bacteria were grown in Luria-Bertani (LB) broth with the appropriate antibiotics at 37°C with aeration unless otherwise noted and stored at -80°C in LB broth containing 10% glycerol. For *V. cholerae in vitro* virulence induction, AKI conditions were used as previously described (352). Briefly, overnight cultures grown in LB broth were inoculated into AKI medium at a 1:10.000 dilution. After 6 h of static growth at 37°C, the culture was transferred to a 125-ml flask and shaken (225 rpm) at 37°C for 16 h. Antibiotics (all from Sigma) were used at the following concentrations in selective media: ampicillin (Amp), 100 μg/ml; kanamycin (Kan), 50 μg/ml and streptomycin (Sm), 100 μg/ml. L-arabinose (Sigma) was added to growth media to a concentration of 0.1% for CpxR induction experiments.

4.2.2 *Bacterial strains and plasmids*

All strains and plasmids used in this study are listed in Table 4-1. All primers used in this study are listed in Table 4-2. A streptomycin-resistant variant of *V. cholerae* El Tor C6706 strain was used as the parental strain to create all the *V. cholerae* strains used in this study. In-frame deletion of *toxR* and *phoB* were performed as described by Metcalf et al. (423). Briefly, in-frame deletion mutants were constructed by overlap extension PCR method. The first PCR reactions were performed using the primers F1 and R1, and F3 and R3 (Table 4-2), which flank the upstream region of *toxR* and *phoB* genes respectively; and F2 and R2 and F4 and R4 (Table 4-2), which flank the downstream region of the *toxR* and *phoB* genes respectively. The second PCR reactions were performed using as a template the PCR products F1-R1 and F2-R2 for *toxR* knockout construct and F3-R3 and F4-R4 for *phoB* knockout construct. DNA amplified was cut with BamHI and NotI and band purified, using the GeneJET Gel Extraction Kit (Thermo

Scientific), following the manufacturer's instructions. These DNA fragments were cloned into the BamHI and NotI restriction sites of the suicide vector pWM91 (424) and transformed into *E. coli* strain DH5α-λpir. Then, *E. coli* strain SM10λpir was used as the donor to mobilize the plasmid into *V. cholerae* El Tor C6706 via conjugation for the selection of recombinants carrying either the *toxR* or *phoB* deletion mutations. *V. cholerae* El Tor C6706 *ompU*, *ompT, ompR* and *cpxR* mutants used in this study were originated from a transposon insertion library (353).

To construct *toxR–lux, ctxA-lux*, *tcpA-lux*, *tcpP-lux* and *toxT-lux* reporter plasmids, the promoter region of *toxR, ctxA, tcpA, tcpP* and *toxT* were amplified by PCR using primers listed in Table 4-2 and cloned between the EcoRI and BamHI sites of the pJW15 vector (227). The resulting plasmids were designated pN4, pN5, pN7, pN9 and pN10.

4.2.3 *RNA analyses*

We used qRT-PCR to validate the effect of *cpxR* overproduction on *toxR* and *toxS* gene expression as previously described (390). For analysis of the Cpx regulation of virulence factors, wild-type *V. cholerae* El Tor C6706, the *cpxR* mutant, and C6706 strain carrying the overexpression plasmid pCpxR were evaluated using two independent RNA preparations. Total RNA was extracted from cultures in AKI condition (see section 4.2.1). After the static growth at 37°C, cultures were transferred to shaking growth conditions, 0.1% of arabinose was added to induce the overproduction of *cpxR*. Then when cultures reached an OD_{600} ~0.8, 1 ml of culture was harvested and resuspended in 1 ml of TRIzol reagent (Ambion) and total RNA was extracted as previously described (390). For each target gene, specific primers (Table 4-2) were designed to amplify nucleotide fragments of ≈100 bp. qRT-PCR was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems) as previously described (390).

4.2.4 *Luminescence assay*

The luminescence activity produced by the vector control (pJW15) and the *toxR–lux, ctxA-lux*, *tcpA-lux*, *tcpP-lux* and *toxT-lux* reporter plasmids in wild-type *V. cholerae* El Tor C6706 carrying pCpxR were performed under AKI medium (352). Briefly, overnight cultures grown in LB were subcultured into AKI condition (see section 4.2.1). After the 6 h of static growth, 198 µl of culture were transferred to a 96-well microtiter plate and induced with 0.1% of arabinose and returned to 37°C with agitation. The OD_{600} and the luminescence counts per second (CPS) were read every hour for up to 4 h post-induction. For time course luminescence assay analysis, followed the 6 h of static growth at 37°C, cultures were transferred to shaking growth conditions (125-ml flask) and induced with 0.1% of arabinose to overexpress of *cpxR*. Every 2 h 200 µl of sample were collected, the OD_{600} and the CPS were read for a period of 16 h post-induction. Measurements were done using a Wallac 1420 multilabel plate reader (Perkin-Elmer).

4.2.5 *Detection of outer membrane profile*

Outer membrane (OM) samples were collected by subculturing *V. cholerae* strains in AKI conditions as described in section 4.2.1. After transference and induction with 0.1% of arabinose of the strains carrying the pCpxR or pBAD24 plasmids, cells were harvested in volumes normalized by OD_{600} when cultures reached an OD_{600} ~0.8. OM samples were collected and extracted as previously described (425). OM preparations were electrophoresed on an SDS–10% PAGE, followed by staining with Coomassie blue for visualization.

4.2.6 *Western blot analysis*

Expression of CT, TCP and T2SS expression were measured by Western blot against the Ctx-B subunit, TcpA, EpsL and EpsG respectively. Briefly, whole-cell lysates and supernatant were collected by subculturing *V. cholerae* strains in AKI conditions as described above. The strains

carrying the pCpxR or pBAD24 plasmids were induced right after the transference to flask with 0.1% of arabinose to induce the overproduction of *cpxR,* and then samples were collected after 16 h of incubation at 37°C. For time course Western blot experiments, every 2 h 1 ml of equivalent of sample was collected for a period of 16 h post-induction. The expression of the T6SS was measured by Western blot against the Hcp protein. Briefly, whole-cell lysates and supernatant were collected by subculturing *V. cholerae* O37 serogroup strain V52 1:100 in LB broth for 1.5 h at 37°C before being induced with 0.1% arabinose, followed by an additional incubation at 37°C until they reached an OD_{600} ~0.65 when samples were collected. For all Western blot analysis, supernatants were filtered through 0.22 μm low protein-binding polyvinylidine fluoride (PVDF) syringe filters (Millipore) and concentrated with 20% trichloroacetic acid (TCA). Whole-cell lysates and supernatant were electrophoresed on SDS–PAGE (10 or 12%) gels and transferred to nitrocellulose membranes as previously described (186). The blots were incubated with a 1:5000 dilution of anti-CtxB, a 1:100000 dilution of anti-TcpA, a 1:10000 dilution of anti-CpxR, a 1:100000 dilution of anti-EpsG, a 1:20000 dilution of anti-EpsL, a 1:500 dilution of anti-Hcp (426) and a 1:25,000 dilution of anti-rabbit immunoglobulin G-alkaline phosphatase conjugates (Sigma). Blots were developed as previously described (225).

4.2.7 *GM1-ELISA*

CT production was determined by a GM1-based enzyme linked immunosorbent assays (ELISA) as described previously by (427), using *V. cholerae* El Tor C6706 strains carrying either pBAD24 or pCpxR culture supernatants under AKI conditions (see Section 4.2.1). Briefly, overnight cultures grown in LB were subcultured into AKI condition (see section 4.2.1). After the 6 h of static growth at 37°C, the culture was transferred to a 125-ml flask and shaken (225 rpm) at 37°C for 16 h. after this time the supernatant was collected and 200 μl of it were added

and serial dilutions (1:3) were done immediately with 2 mg/ml of BSA buffer in a 96-well microtiter plate coated with GM1 gangloside. Then, the 96-well microtiter plate was incubated by 30 minutes at 37°C and three consecutives wash steps with PBS buffer were performed. GM1-ELISA was performed using a dilution 1:2000 of anti-CtxB and a dilution 1:2000 of anti-rabbit immunoglobulin G-alkaline phosphatase conjugates (Santa Cruz Biotechnology). A CT standard curve was generated to estimate the amount of CT in the supernatant samples. The color intensity was measured at OD_{405} nm in a BioRad xMark Microplate Spectrophotometer (BioRad).

4.2.8 *Bacterial killing assay*

To assess the effect of Cpx activation on the expression of T6SS components, the susceptibility of *Escherichia coli* MG1655R to T6SS-mediated killing by the predator strain *V. cholerae* O37 serogroup strain V52 was assessed as described previously (419). Briefly, predator and prey strains were grown as lawns on LB plates plus selective antibiotics and resuspended in LB broth. Prey and predator were mixed in a 1:10 ratio and spotted onto pre-dried LB agar plates with the absence or presence of 0.1% arabinose to induce the Cpx pathway. After an incubation of 4 h at 37˚C, each spot was harvested, serially diluted, and spotted onto a LB plates plus selective antibiotics and were incubated overnight at 37°C. Surviving prey (CFU/ml) were enumerated.

4.2.9 *Motility analysis*

Two microliters of overnight cultures of *V. cholerae* El Tor C6706 and *V. cholerae* O37 serogroup strain V52, which carry either pBAD24 or pCpxR plasmids, were inoculated onto 0.3% LB agar plates with the absence or presence of 0.1% arabinose to induce the overproduction of CpxR. The diameter of the swim zones was recorded after 16 h of growing with the appropriate antibiotics. All the inoculations were made in triplicate.

4.2.10 *Expression of cpxP-lux reporter in vivo*

For studying the activation of the Cpx pathway in a mouse model, the experiments were performed by Daniel Unterweger, Ben Kostiuk and Verena Bachmann (PhD student, MSc Student and former Postdoctoral Fellow respectively, in Dr. Stefan Pukatzki's lab, Department of Medical Microbiology & Immunology; University of Alberta). Briefly, for *in vivo* expression of *cpxP* during mice colonization, colonies of *V. cholerae* El Tor C6706, carrying either the vector control, pJW15, or the *cpxP-lux* reporter plasmid, were inoculated into 5 ml of LB broth and grown at 37°C with aeration overnight. The *tcpA-lux* reporter was used as a positive control for luminescence activity. The next day, $1x10^7$ CFU/ml were inoculated perorally to 5-day-old infant mice. Luminescence imaging of the mice was performed at 18 to 20 hours when the gastrointestinal tract of the mice were collected and kept in PBS for imaging using an IVIS Spectrum Imager System (Perkin Elmer). The heat bar indicates the luminescent intensity scale.

4.3 Results

4.3.1 *The Cpx response positively regulates the toxRS operon*

We previously demonstrated that activation of the Cpx pathway in *V. cholerae* El Tor strain C6706 leads to an increase in expression of the *toxS* (VC0983) and *toxR* (VC0984) genes (390). ToxR is a transcriptional regulator located in the inner membrane that contains an N-terminal domain with strong homology to the OmpR/PhoB protein family (428). ToxR activity is enhanced by the presence of the transmembrane protein, ToxS (292). To verify the observed regulation of *toxS* and *toxR* transcription by the Cpx pathway, we performed a qRT-PCR analysis and observed an increase in expression of both genes when the Cpx response was activated by means of CpxR overexpression (Figure 4-1A). For this experiment and all those in which the Cpx

pathway was activated, we used a previously constructed CpxR overexpression vector (pCpxR) in *V. cholerae* (390). We have shown that this method of Cpx pathway activation recapitulates induction of the Cpx response using envelope stress signals (390).

To determine if the positive regulation of ToxR by CpxR occurs at the transcriptional level, we constructed transcriptional fusions of the promoter region of *toxR* with the light-producing *luxCDABE* operon encoded on previously described reporter plasmid pJW15 (227). We used this reporter to measure expression of *toxR* when the Cpx pathway is activated. A *cpxP-lux* reporter was utilized as a positive control for the induction of the Cpx pathway using the same background strain and conditions. The *cpxP-lux* reporter was induced strongly by Cpx response activation, producing increased luminescence within the first hour after stimulation of CpxR over-expression from an arabinose-inducible promoter on the pBAD24 plasmid (Figure 4-1B). As previously reported (274, 429), the activity of the *toxR-lux* reporter was mostly constant throughout growth under AKI conditions (Figure 4-1B). When CpxR expression was induced, luminescence produced by the *toxR-lux* reporter increased steadily over 2 h to a level approximately twice that observed under non-induced conditions (Figure 4-1B). After 2 h, *toxR-lux* transcription remained at high levels throughout the experiment (Figure 4-1B). Although *toxR* expression is generally considered to be constitutive (299), our data suggests that CpxR, an envelope stress transcriptional regulator, positively regulates the expression of the *toxRS* operon. The delay in induction of *toxR* expression relative to that of the strongly regulated *cpxP-lux* reporter gene upon CpxR over-expression suggests that this effect is indirect (Figure 4-1B).

4.3.2 *The Cpx pathway regulates OmpT expression*

ToxR regulates the expression of two OM proteins in *V. cholerae*, OmpU and OmpT, by directly activating the *ompU* promoter and repressing the expression of the *ompT* promoter,

which is important for bile resistance (347, 430-432). In *E. coli*, the Cpx pathway regulates the expression of OmpC and OmpF, the major porin constituents of the OM (433). To test whether the activation of the Cpx pathway similarly regulates the expression of OmpU and OmpT in *V. cholerae* El Tor strain C6706, we examined the porin content in the OM in the absence and presence of CpxR overexpression. Wild-type C6706 carrying either the vector control (pBAD24) or the pCpxR plasmid were grown in AKI conditions in the presence of arabinose to induce CpxR expression. When OM preparations were analyzed by SDS-PAGE, OmpT levels were reduced when *cpxR* was overexpressed by addition of the inducer arabinose, compared to the non-inducing condition or in the vector control (Figure 4-2A). The absence of this band in an *ompT* transposon insertion mutant confirmed that the protein diminished by CpxR overexpression was OmpT (Figure 4-2A, compare lanes 4 and 6). No change in the level of the OmpU porin was observed upon CpxR over-expression (Figure 4-2A, compare lanes 3 and 4).

ToxR inhibits *ompT* expression and the levels of OmpT in the OM are elevated in cells lacking ToxR (431, 432). To determine if the negative regulation of OmpT by CpxR is ToxR-dependent, we examined the porin content in the OM from the wild-type C6706 strain and the *toxR* isogenic mutant under Cpx activating conditions (i.e. overexpression of CpxR) under AKI conditions. As expected, since ToxR is an *ompU* activator and *ompT* repressor (430, 431), in the *toxR* mutant there was a decrease in OmpU levels and an increase in OmpT levels in the OM (Figure 4-2B, lanes 5 to 8). However, when the Cpx pathway was induced in the *toxR* mutant by the addition of arabinose to stimulate CpxR overexpression, OmpT levels were still diminished (Figure 4-2B, compare lanes 4 and 8), but as in the *toxR* mutant background there is more OmpT levels, it suggest that the negative regulation of OmpT by CpxR could be both direct and indirect through the possible regulation of ToxR.

In *E. coli,* the Cpx response controls porin expression by up-regulating a small inner membrane protein called MzrA that stimulates the EnvZ-OmpR two-component system (198, 434). The response regulator OmpR in turn inversely regulates the expression of the two major OMPs in *E. coli*, up-regulating OmpC production and inhibiting expression of OmpF (435). To assess if the OmpR (VC2714) homologue in *V. cholerae* El Tor C6706 was involved in the Cpx regulation of OmpT, we compared the porin content in OMs isolated from wild-type C6706 and an *ompR* transposon insertion mutant under Cpx activating conditions. OmpT levels changed in the *ompR* transposon insertion mutant upon CpxR over-expression in an identical manner to that observed in the wild-type C6706 strain, which suggests that the negative regulation of OmpT by CpxR is also OmpR-independent (Figure 4-3, lanes 3 and 4 to 7 and 8).

4.3.3 *The V. cholerae Cpx response regulates the expression of CT and TCP*

Since CpxR overexpression resulted in increased levels of *toxR* transcription, a major virulence factor regulatory protein in *V. cholerae,* we sought to determine whether the Cpx system is involved in the regulation of virulence factors as shown previously in other Gram-negative pathogens (253). We determined the effect of the activation of the Cpx pathway on the expression of cholera toxin and TCP, the two major virulence factors in *V. cholerae* El Tor strain C6706, in the presence of the pCpxR over-expression plasmid or the pBAD24 vector control and grown under AKI conditions (352). Expression of CT was measured by western blot using antibodies directed against the Ctx-B subunit. As shown in Figure 4-4A, secretion of cholera toxin was abolished upon the over-expression of CpxR in *V. cholerae* El Tor C6706 compared with the vector and uninduced controls (compare lanes 3, 4, and 5 with lane 6). To confirm the reduction of expression of cholera toxin in the supernatant upon activation of the Cpx pathway, levels of cholera toxin in the culture medium of cells grown in the presence and absence of

arabinose were also assessed using an ELISA. In agreement with the western blots (Figure 4-4A), the results showed that the Cpx pathway has a large effect on cholera toxin synthesis (Figure 4-4B).

In *V. cholerae*, the type II secretion system (T2SS) is required for extracellular secretion of several proteins including cholera toxin (436, 437). To test if the observed reduction of cholera toxin in the supernatant when the Cpx pathway was activated was due to a downregulation of the T2SS by the Cpx pathway, we measured the expression of EpsL and EpsG, both components of the T2SS in *V. cholerae* (438, 439). We determined that the activation of the Cpx pathway does not have any effect on the expression of the T2SS. No differences in the expression of EpsL and EpsG were observed in samples collected from the CpxR over-expression strain as compared to the vector control strain (Figure 4-5).

To analyze TCP protein levels upon activation of the Cpx pathway, we examined the expression of TCP by measuring the expression of TcpA, the major subunit of TCP, in whole-cell lysates from cells grown under AKI conditions, which is favorable for TCP expression in *V. cholerae* (352). TcpA expression was reduced in wild-type C6706 carrying the pCpxR vector in the presence of arabinose compared to the uninduced sample and the vector control (Figure 4-4A, lanes 3, 4, and 5 to lane 6), showing a similar trend to that seen with the expression of Ctx-B (Figure 4-4A). These results suggest that the Cpx pathway negatively regulates expression of CT and the TCP in *V. cholerae* El Tor strain C6706.

To investigate if the Cpx pathway also plays a role in regulating the expression of other *V. cholerae* envelope localized virulence factors, we determined if overexpression of CpxR had an effect on T6SS in *V. cholerae* O37 serogroup strain V52, which constitutively expresses an active

T6SS (418). We also examined motility in *V. cholerae* El Tor strains C6706 and V52. Using western blotting and a T6SS-mediated bacterial killing assay as previously described (419), we found that CpxR overexpression did not alter the synthesis and secretion of the hemolysin-coregulated protein (Hcp), the major T6SS component (Figure 4-6A), or T6SS-mediated virulence of *V. cholerae* V52 towards *E. coli* MG1655 (Figure 4-6B). These results suggest that the Cpx envelope stress response is not involved in regulating the biogenesis of the T6SS in *V. cholerae* V52, which is consistent with a previous study that showed that the elaboration and assembly of the T6SS is a dynamic process that occurs in the bacterial cytosol (440). Furthermore, in contrast to *E. coli* (222), activation of the Cpx pathway did not impact motility in the *V. cholerae* strains El Tor C6706 or V52 (Figure 4-7).

4.3.4 *Transcription of both ctxA and tcpA is inhibited by the Cpx pathway*

To determine if the negative regulation of CT and the TCP by CpxR occurred at the transcriptional level, we constructed *ctxA-lux* and *tcpA-lux* transcriptional reporter genes using the previously described reporter plasmid pJW15 (227). We used these reporters to measure *ctxA* and *tcpA* transcription when the Cpx pathway was activated by over-expressing CpxR under conditions shown to maximize CT production (352). As previously reported (429), expression of *ctxA* was detected after the AKI medium cultures were switched from static to shaking growth conditions (aerobic) and increased linearly over 6 h of growth (Figure 4-8A). Consistent with these results, cholera toxin was detectable in the supernatants of cultures grown in AKI medium 6 h after the cultures were switched to aerating conditions, and increased over 16 h of growth (Figure 4-8B). Interestingly, *ctxA* expression was dramatically diminished when the Cpx pathway was activated, where the luminescence resulting from the *ctxA-lux* reporter decreased over time (Figure 4-8A) and there was no detectable cholera toxin in the supernatant (Figure 4-8B).

Similarly, *tcpA-lux* expression was significantly lower in *V. cholerae* El Tor C6706 carrying the pCpxR overexpression plasmid in the presence of arabinose (Figure 4-8C). Consistent with this finding, we observed a decrease in TcpA protein levels in the cell pellets of cultures when the Cpx pathway was activated (Figure 4-8D). These findings suggest that the Cpx-mediated negative regulation of CT and TCP occurs at the transcriptional level.

4.3.5 *The Cpx pathway down-regulates transcription of multiple regulators of virulence*

It is probable that the negative regulation of CT and TCP by the Cpx pathway is indirect, because a consensus CpxR binding site is not found in the promoter regions of the *ctx* or *tcp* genes by the Virtual Footprint tool (http://www.prodoric.de/vfp/) (357). Thus, we examined known regulators of CT and TCP in order to determine whether the Cpx pathway inhibits virulence factor production indirectly. Cholera toxin and TCP expression are controlled by a hierarchical regulatory system known as the ToxR regulon. ToxR in conjunction with another inner membrane regulator, TcpP, positively controls the expression of the master virulence regulator ToxT (for a review, see (288)). We were unable to assess whether the Cpx-mediated CT and TCP down-regulation we observed occurs through ToxR, since those virulence factors were not detectable in a *toxR* mutant (data not shown).

To determine if CpxR affected the direct regulator of CT and TCP expression, ToxT, in *V. cholerae* El Tor strain C6706, we constructed a *toxT-lux* luminescent reporter gene as previously described (227). Interestingly, *toxT* expression was repressed upon activation of the Cpx pathway. The luminescence of the *toxT-lux* reporter decreased over time after the addition of the inducer arabinose to stimulate CpxR over-production (Figure 4-9A). Because *toxT* regulation is dependent on the expression of upstream regulators in the ToxR virulence cascade (289, 290), we also measured the expression of *tcpP* upon CpxR over-expression in the same fashion. The

expression of the transcriptional regulator TcpP was also repressed when the Cpx pathway was activated under AKI conditions, in a similar fashion to *toxT* (Figure 4-9B). Thus, our data suggest that the Cpx response inhibits *toxT* transcription through its negative effect on transcription from the *tcpPH* promoter.

It has been shown that the transcriptional regulator PhoB influences virulence factors by directly inhibiting the expression of *tcpPH* (361). Previously, we found that activation of the Cpx pathway in *V. cholerae* El Tor strain C6706 leads to an increase in expression of *phoB* transcript levels (390). However, activation of the Cpx response in a *phoB* null background still led to decreased production of Ctx-B and TCP, suggesting that the Cpx pathway regulates expression of CT and TCP in a PhoB independent manner (Figure 4-10).

The *tcpPH* operon is positively regulated by direct binding of the LysR type transcription factor AphB, in conjunction with the AphA protein, to its promoter region (301, 415, 416). To determine if Cpx regulation of *aphA* and/or *aphB* transcription might be involved in the negative effect of the Cpx response on *tcpPH,* we used qRT-PCR to measure the expression of these genes when the Cpx response was activated by CpxR overexpression (Figure 4-11A). We observed that, while expression of the accessory regulator AphA was unaffected by Cpx pathway activation, there was a 2-fold decrease in the expression of *aphB* when *cpxR* was overexpressed (Figure 4-11A). These data suggest that the Cpx response affects the expression of regulators upstream of the ToxR virulence cascade.

In both classical and El Tor *V. cholerae* strains it has been demonstrated that CRP has a negative effect on expression of CT and TCP, and this appears to be due to its ability to repress expression of the *tcpPH* operon (300, 302). Accordingly, we sought to determine if CRP could

be involved in the inhibition of CT and TCP production by the Cpx response. To do this, we examined CT and TCP production in a *crp* mutant when the Cpx pathway was activated (Figure 4-12). As previously reported (441), in the absence of CRP, levels of both CT and TCP were elevated (Figure 4-12). While activation of the Cpx response by CpxR over-expression in the absence of CRP resulted in a small decrease in TCP and CT levels, the magnitude of this change was obviously smaller than that seen in a wild-type C6706 strain background (Figure 4-12, compare lanes 3 and 4 to 7 and 8). These data suggest that Cpx-mediated inhibition of CT and TCP production is, in large part, dependent on CRP.

4.3.6 *Negative regulation of virulence by CpxR over-expression is not a pleiotropic phenotype*

All of our studies examining Cpx regulation involved overexpression of the regulator CpxR. Although we have previously published that this condition accurately recapitulates activation of the Cpx response by envelope stress signals sensed via CpxA (390), we wanted to confirm that the negative effect of the Cpx response on virulence factor production was not an artificial effect of CpxR overexpression. Accordingly, we examined the transcript levels of virulence related genes in the wild-type C6706 strain and the *cpxR* mutant under AKI virulence-inducing conditions. Consistent with all of our previous observations, there was an increase in the expression of *ctxB*, *tcpA* and *tcpP* in the *cpxR* mutant, and a decrease in the *toxR* transcript level in the same background strain (Figure 4-11B). This data supports our finding that the Cpx response negatively regulates virulence, through the ToxT regulatory cascade, even in the absence of ectopic overexpression of CpxR.

4.3.7 *Activation of the Cpx pathway in vivo model*

For studying the activation of the Cpx pathway in a mouse model, expression of the *cpxP-lux* reporter was assessed during mice colonization. Preliminary data showed that the *cpxP-lux*

reporter is expressed in mice as luminescence activity was observed in comparison with the negative control mouse that was inoculated with the vector control (i.e. pJW15) (Figure 4-13A,B). As expected, it was observed that the mouse inoculated with the positive control, *tcpA-lux*, showed high luminescence activity (Figure 4-13C). These data suggest that at least *ex vivo*, the *V. cholerae* Cpx response is activated in a mouse model.

4.4 Discussion

Several studies have proposed that the Cpx pathway plays an important role in bacterial pathogenesis by regulating, positively and negatively, envelope-localized virulence factors (253). Based on these studies, we hypothesized that the Cpx pathway may also regulates envelope-localized virulence factors in *V. cholerae*. In support of this hypothesis, we recently reported that that the Cpx response regulates the expression of the *toxRS* operon (390). Expression of the *toxRS* operon has been considered to be constitutively active in LB broth or under virulence-inducing conditions, although supplementation of minimum medium with a mixture of asparagine, arginine, glutamic acid, and serine (NRES) amino acids increases the amount of ToxR (298, 299, 442). However, we confirm here via qRT-PCR and luminescent reporter gene assays that activation of the Cpx response leads to up-regulated transcription from the *toxRS* promoter (Figure 4-1). The mechanism by which the Cpx response regulates the expression of the *toxRS* operon remains unclear; however, this regulation appears to be independent of the growth conditions (it occurs in both LB and AKI conditions) (data not shown). In addition, we think that this regulation is indirect since there was a delay in the induction of *toxR* expression when the Cpx response is activated, and there is not a putative CpxR binding site in the promoter region of *toxR*.

Previous studies in *E. coli* have reported that the Cpx pathway regulates the expression of OM proteins (433) and some of its regulon members are required for their proper assembly (443). Since we found that CpxR positively regulates the expression of *toxR*, which regulates the expression of the two major OM porins in *V. cholerae* (430-432), we assessed whether the activation of the Cpx pathway also impacted OmpU and OmpT levels. We found that activation of the Cpx pathway leads to a decrease in the expression of the OmpT porin. A decrease in OmpT protein levels was detected in OM preparations when CpxR was over-expressed (Figure 4-2). Similarly, a recent microarray study showed that activation of the Cpx response in the *V. cholerae* El Tor strain N16961, which is closely related to the *V. cholerae* El Tor strain C6706 used in this study (358), also resulted in downregulation of *ompT* expression (332). Although ToxR negatively regulates the production of OmpT (431, 432) and we found that the Cpx pathway activates the expression of the *toxRS* operon, the negative regulation of *ompT* expression by the Cpx pathway appears to be ToxR-independent (Figure 4-2B). Interestingly, the expression of OmpT is also regulated by CRP (431, 444), whose activity was also found to be regulated by the Cpx response in this study (see below). Thus, the possibility remains that the Cpx response turns off OmpT production via an effect on CRP rather than through direct regulation, since we did not find a putative CpxR binding site in the promoter region of *ompT*. Thus, regulation of OMP expression appears to be conserved between relatively diverse enteric pathogens, although it occurs via different regulatory circuitries. We previously showed that the Cpx pathway also regulates the expression of another important OMP, TolC (390). Together, these observations suggest an important role for the Cpx response in the trafficking of toxic components across the OM in *V. cholerae* El Tor strain C6706.

Our analyses showed that the activation of the Cpx response lead to a decrease in the expression of some of the major virulence factors in *V. cholerae*, CT and TCP (Figure 4-4). Most likely, this negative regulation is at the transcriptional level since we found a decrease in the expression of transcriptional reporters for both the *ctxA* and *tcpA* genes when the Cpx response was activated (Figure 4-8). This observation is in apparent contradiction to our finding that when *cpxR* is overexpressed in *V. cholerae* El Tor strain C6706 it up-regulates the expression of the virulence regulator ToxR (390), an activator of *ctxA* and *tcpA* expression (288, 410). In this regard, a recent study showed for the first time that ToxR can negatively regulate the production of CT and TCP in response to cyclic dipeptides (CDPs) (445). Bacterial production of cyclo (Phe-Pro) leads to ToxR-mediated activation of the LysR transcription factor LeuO, which in turn represses CT production (445). At this point we cannot say whether Cpx-mediated activation of ToxR production is involved in the inhibition of CT and TCP via CDPs, or some other mechanism. A future goal of this work will be to examine this question by determining if ToxR-dependent regulation of *leuO* expression is altered when the Cpx response is induced. Interestingly, CDPs have been shown to accumulate in a growth-dependent manner in *V. cholerae*, specifically at stationary phase (446), where the Cpx response is also enhanced in *E. coli* (194).

CT, TCP and other virulence-associated genes are controlled by a regulatory network known as the ToxR regulon (289, 290), in which the primary direct transcriptional regulator of virulence gene expression is the ToxT protein (for a review, see (299)). We found that activation of the Cpx response led to a decrease on the expression of *toxT* (Figure 4-9). This suggests that the negative regulation of CT and TCP by the Cpx response may be indirectly due to a decrease in *toxT* expression. In support of this model, we found that CpxR negatively regulates the transcription of

additional genes in the virulence regulatory hierarchy, upstream of *toxT,* including the transmembrane response regulator TcpP (Figure 4-9). We suspect that this is also indirect, since we were not able to identify a potential CpxR binding site in the *tcpPH* promoter region.

The *tcpPH* locus is controlled by environmental signals (i.e. temperature and pH) and the transcriptional regulators AphA, AphB, and CRP (297, 300, 302, 415, 416). AphA and AphB interact and bind directly upstream of the *tcpPH* operon to activate transcription (301, 415, 416). CRP binds to DNA sequences that overlap the AphA and AphB binding site and is thought to repress *tcpPH* transcription, perhaps partly by interfering with AphA/B mediated activation (300). It has been proposed that CRP mediated repression of CT and TCP expression, which occurs in both classical and El Tor biotype strains of *V. cholerae* (302), may be mediated by its negative effect on AphA and AphB activity (300). We found here that the expression of CT and TCP is not affected in the *crp* mutant upon activation of the Cpx response, suggesting that the negative regulation of *tcpPH* expression by the Cpx response is mainly modulated by the regulation of CRP.

At present, we cannot say exactly how the Cpx response regulates CRP. In our previous microarray study (390), *crp* was not found to be part of the Cpx regulon, and we confirmed here that overexpression of CpxR has no effect on *crp* transcript levels (Figure 4-11A). These data indicate that the Cpx response stimulates CRP activity post-transcriptionally, but we do not know how this occurs. One possibility is that a Cpx-regulated sRNA affects translation or stability of the CRP encoding mRNA. In this regard, we have shown that the Cpx response controls several known sRNAs in *E. coli* (250); however, it is not known whether this is true in *V. cholerae,* and no sRNAs have been described to date that regulate CRP expression. An alternative explanation could be that the Cpx response alters the expression of sugar transporters that in turn affect levels

of cAMP and CRP function. In support of this model, it has been demonstrated that mutation of *cpxR* in *E. coli* leads to changes in the expression of the CRP-regulated sRNA gene *cyaR* in a manner that is dependent on the Cpx-regulated glucose permease PtsG (250). We previously found that activation of the Cpx response impacted sugar transporters in *V. cholerae* as well, including the genes involved in galactose uptake (VC1325, VC1327, VC1328) and utilization (VC1594, VC1595, VC1596) (390). Based on these data, a plausible model is that the Cpx response negatively regulates CT and TCP expression indirectly by changing the activity of CRP in *V. cholerae* El Tor strain C6706, which in turn results in alterations in expression of activator proteins found at the top of the virulence regulatory hierarchy.

In contrast to our results, Taylor and collaborators (332) recently reported that activation of the Cpx response had no impact on the expression of virulence factors (i.e. CT and TCP) in *V. cholerae* O1 El Tor strain N16961. At this point, we do not know the reason for this discrepancy, but it could be related to strain differences. For example, it is known that in *V. cholerae* O1 El Tor strain N16961 there is a frameshift mutation in the *hapR* gene (303), therefore the quorum sensing circuitry involved in the regulation of virulence factors is not intact in this strain. We also observed differences in the members of the Cpx regulon identified by Taylor and colleagues (332) compared to our study (390), further supporting the supposition that important Cpx-related regulatory network differences may exist between *V. cholerae* El Tor strains C6706 and N16961.

The impact of the Cpx response on virulence gene regulation raises the question of when the Cpx pathway may be important in the life cycle of *V. cholerae*. It was previously reported that there is an absence of intestinal growth defects of *cpx* mutants (i.e. *cpxR*, *cpxA*, *cpxP* and *cpxA**) using the suckling mouse model of *V. cholerae* (261). In spite of this, *cpxP* expression is induced in the small intestine of the mouse (447) and some Cpx regulon members (390) are enriched in

transcriptome data sets (Figure 4-14) described for *V. cholerae* isolated from human volunteers, human stool and vomitus samples, and from animal models (i.e. rabbit intestinal loop model, mice and crab-shell) (285, 371, 447-452). In addition, several studies have suggested that expression of virulence factors (e.g. CT and TCP) is reduced during late infection (371, 445, 449-452). We speculate that the Cpx pathway may play a role in the adaptation of *V. cholerae* during late infection. This may be required for the negative regulation of virulence factors during the transition between the host and the environment. Possibly the limitation of nutrients such as iron during late infection (371, 447, 449) triggers the activation of the Cpx response in *V. cholerae* (390).

Collectively, the findings presented in our study support the conclusion that the Cpx pathway in *V. cholerae* El Tor C6706 is involved in virulence gene regulation. In general, it is likely that the activation of the Cpx response downregulates envelope appendages in order to decrease unessential envelope protein traffic under envelope stress conditions (86). In pathogens, this may be an adaptive function associated with exit from the host. Taken together, our findings suggest that CpxR negatively regulates the two major virulence determinants of *V. cholerae,* CT and TCP, from the top of the ToxR regulatory cascade, in large part through regulating the activity of CRP which impacts the expression of *tcpPH* and the downstream regulator *toxT* in *V. cholerae* El Tor C6706.

4.5 Tables and Figures

Table 4 - 1 Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Source or reference
Strains		
C6706	*V. cholerae* El Tor biotype strain C6706; (Sm^R) streptomycin-resistant	Dr. J. Mekalanos (Harvard Medical School)
V52	*V. cholerae* O37 seregroup strain, Δ*hapA*, Δ*rtxA*, Δ*hlyA*; (Sm^R)	(418)
V52Δ*vasK*	V52 mutant lacking *vasK* gene, carrying pBAD24 plasmid; (Sm^R Amp^R)	(418)
NA24	V52 carrying pBAD24 plasmid; (Sm^R Amp^R)	This study
NA3	V52 carrying pCpxR plasmid; (Sm^R Amp^R)	This study
NA45	C6706 carrying pBAD24 plasmid; (Sm^R Amp^R)	(390)
NA44	C6706 carrying pCpxR plasmid; (Sm^R Amp^R)	(390)
NA335	C6706 mutant lacking *toxR* gene; (Sm^R)	This study
NA396	C6706 mutant lacking *phoB* gene; (Sm^R)	This study
NA111	NA44 carrying pJW15 plasmid; (Sm^R Amp^R kan^R)	This study
NA70	NA44 carrying pN3 plasmid; (Sm^R Amp^R kan^R)	This study
NA102	NA44 carrying pN4 plasmid; (Sm^R Amp^R kan^R)	This study
NA103	NA44 carrying pN5 plasmid; (Sm^R Amp^R kan^R)	This study
NA107	NA44 carrying pN7 plasmid; (Sm^R Amp^R kan^R)	This study
NA115	NA44 carrying pN9 plasmid; (Sm^R Amp^R kan^R)	This study
NA109	NA44 carrying pN10 plasmid; (Sm^R Amp^R kan^R)	This study
NA339	NA335 carrying pBAD24 plasmid; (Sm^R Amp^R)	This study
NA343	NA335 carrying pCpxR plasmid; (Sm^R Amp^R)	This study
NA399	NA396 carrying pBAD24 plasmid; (Sm^R Amp^R)	This study
NA401	NA396 carrying pCpxR plasmid; (Sm^R Amp^R)	This study
EC16554	Derivative of C6706 strain carrying TnFGL3 insertion in the *cpxR* gene (VC2692) (Sm^R kan^R)	(353)
EC18098	Derivative of strain C6706 carrying TnFGL3 insertion in the *ompU* gene (VC0633) (Sm^R kan^R)	(353)
EC4591	Derivative of strain C6706 carrying TnFGL3 insertion in the *ompT* gene (VC1854) (Sm^R kan^R)	(353)
EC10705	Derivative of strain C6706 carrying TnFGL3 insertion in the *ompR* gene (VC2714) (Sm^R kan^R)	(353)

EC14253	Derivative of strain C6706 carrying TnFGL3 insertion in the *crp* gene (VC2614) (Sm^R kan^R)	(353)
NA242	EC10705 carrying pCpxR plasmid; (Sm^R,$Kan^R Amp^R$)	This study
NA410	EC14253 carrying pBAD24 plasmid; (Sm^R,$Kan^R Amp^R$)	This study
NA409	EC14253 carrying pCpxR plasmid; (Sm^R,$Kan^R Amp^R$)	This study
Escherichia coli MG1655R	F- λ- ilvG- rfb-50 rph-1, carrying pBAD24 plasmid; (Rif^R Amp^R)	Dr. T.L. Raivio (University of Alberta)
Escherichia coli DH5α λpir	fhuA2 Δ(argF-lacZ)U169 phoA glnV44 ϕ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17	Dr. Daniele Provenzano (University of Texas at Brownsville)
Escherichia coli SM10λpir	KmR, thi-1, thr, leu, tonA, lacY, supE, recA::RP4-2-Tc::Mu, pir	Dr. J. Mekalanos (Harvard Medical School)
Plasmids		
pBAD24	pBAD vector, pBR322 ori, *araC*,(Amp^R)	(453)
pCpxR	pBAD24 carrying *cpxR* of *Vibrio cholerae* C6706 (Amp^R)	(390)
pJW15	pNLP10 with p15 *ori* reporter vector (Kan^R)	(227)
pN3	*cpxP* promoter cloned into *luxCDABE* reporter vector pJW15 (Kan^R)	(390)
pN4	*toxR* promoter cloned into *luxCDABE* reporter vector pJW15 (Kan^R)	This study
pN5	*ctxA* promoter cloned into *luxCDABE* reporter vector pJW15 (Kan^R)	This study
pN7	*tcpA* promoter cloned into *luxCDABE* reporter vector pJW15 (Kan^R)	This study
pN9	*tcpP* promoter cloned into *luxCDABE* reporter vector pJW15 (Kan^R)	This study
pN10	*toxT* promoter cloned into *luxCDABE* reporter vector pJW15 (Kan^R)	This study
pWM91	*oriR6K mobRP4 lacI* p*tac* tnp mini-Tn*10*Km; (Kan^R ,Amp^R)	(424)

Table 4 - 2 Primers used in this study.

Primer name	Oligonucleotide sequence 5' to 3' (restriction sites underlined)
F1	TTGGATCC CTTTACCTTCTTCACGCAGAT
F2	TTAGATGTTCGGATTAGGAAAAGTGTGTGAGTAGGATC
R1	GATCCTACTCACACACTTTTCCTAATCCGAACATCTAA
R2	CTGCGGCCGCCTTCAGCACTTTGGCTTCT
F3	CTGGATCCCAGGAAAACCGACATTTC
F4	AATTATGTCTAGAAGGATTTCAACCAAAGCCTAAGAGG
R3	CCTCTTAGGCTTTGGTTGAAATCCTTCTAGACATAATT
R4	TTGCGGCCGCAGAAACGCTCCGTTAAAC
luxFtoxR	TTGAATTCCCGTACCCGATTTAGCAA
luxRtoxR	TTGGATCCCGTTGCTGCCTAATCGAA
luxctxAF	TTGAATTCACGGCTTACACGACAATCCA
luxctxAR	TTGGATCCTGGCATAAGACCACCTGACT
luxtcpAF	TTGAATTCAGCCGCCTAGATAGTCTGTG
luxtcpAR	TTGGATCCATCAATCGCACGCTGAGCCA
luxtcpPF	TTGAATTCTCTTGTGCCTGCTGAGAACT
luxtcpPR	TTGGATCCTGGTGTACCAATCAGCCT
luxtoxTF	TTGAATTCTGGTGCAATGATCGCAGT
luxtoxTR	TTGGATCCAAGCTTTGCAATTCCACT
toxSF	CATCGCCATGGGTATTCTTC
toxSR	GTCACTCCCCCAATATAACCAG
toxRF	GATTAGGCAGCAACGAAAGC
toxRR	AATCACCTCGTTTGGACGTT
ctxBF	GCGATTGAAAGGATGAAGGA
ctxBR	ATCGCATGAGGCGTTTTATT
tcpAF	TTGGTCAGCCTTGGTAAGGT
tcpAR	CCCCATAGCTGTACCAGTGAA
tcpPF	TGAAAGTCTAACTCAGGCAATCAA
tcpPR	TTTCGATCAACGTCTTATGTTCA
aphAF	AACCGTGCGTGATGAGTTTA

aphAR	GGTAAGGTTCTGCCGATTGT
aphBF	GATGCTGCGTGAATTTCTTG
aphBR	TGAGCTCCAATCCGACAGTA
crpF	TCAGGTCAAATGGCTCGTC
crpR	ACGTCTAGGAACGCAAGGTC

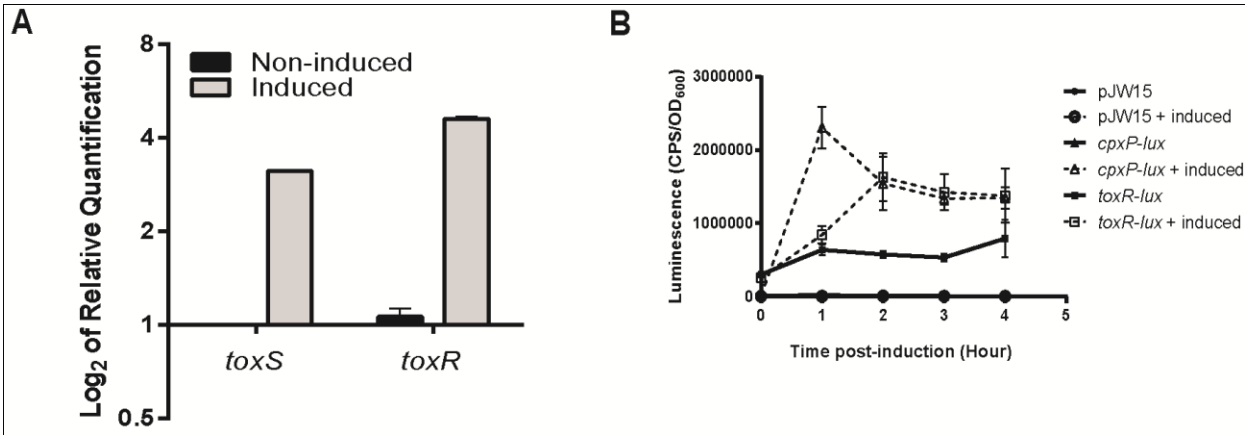

Figure 4 - 1 Activation of the Cpx pathway regulates *toxR*.

(A) qRT-PCR analysis of *toxS* and *toxR* transcript levels. RNA was isolated from cultures of *V. cholerae* C6706, carrying the overexpression plasmid pCpxR, in the absence (non-induced) (black bars) or presence of 0.1% arabinose (induced) (grey bars) and converted to cDNA. The cDNA was subjected to qRT-PCR analysis using the primers described on Table 4-2. (B) Luminescence activity of *V. cholerae* C6706, carrying the overexpression plasmid pCpxR, transformed with the vector control (pJW15), *cpxP-lux* or *toxR-lux* reporters. CpxR was overexpressed by adding 0.1% of arabinose (induced). Reporter gene expression was measured as counts per second (CPS) corrected for cell density (OD_{600}). Time 0 represents the time when cells were induced with 0.1% of arabinose and shifted to shaking conditions, after 6 h of statistic grow on AKI medium. The overall average and standard deviation resulting from two separate experiments performed in quintuplicate are shown.

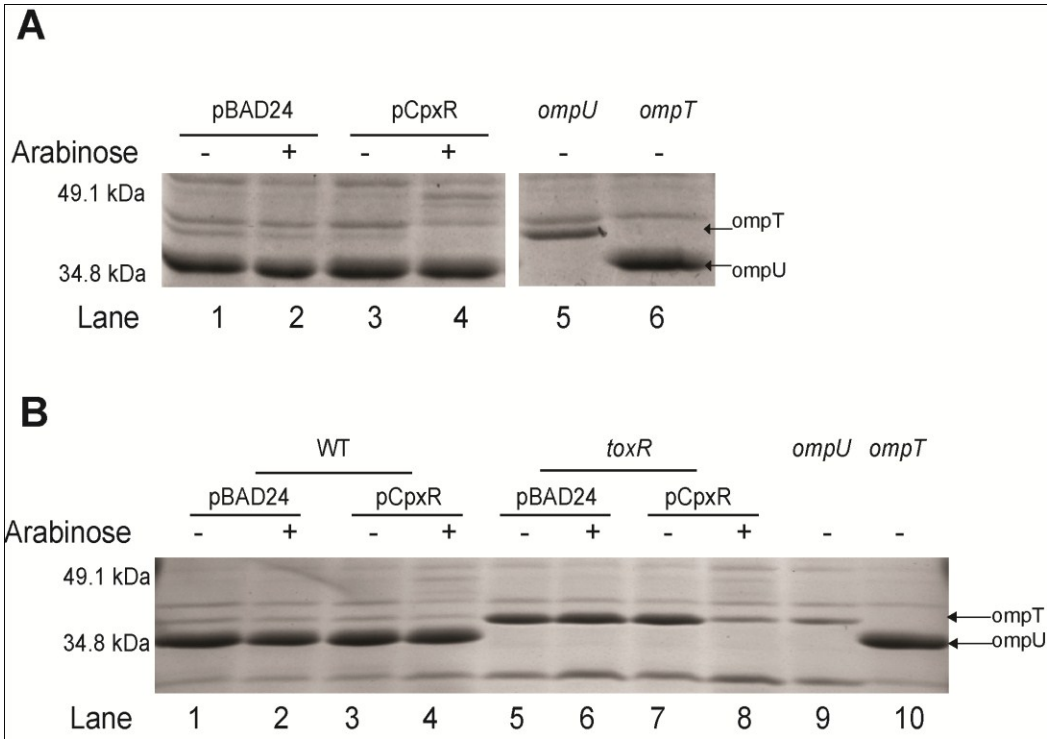

Figure 4 - 2 Activation of the Cpx pathway down-regulates OmpT expression.

(A) Outer membrane (OM) protein profiles of *V. cholerae* El Tor strain C6706 carrying either pBAD24 (lane 1 and 2) or pCpxR (lane 3 and 4), *ompU* (lane 5) and *ompT* (lane 6). (B) OM protein profiles of wild-type strain C6706 carrying either pBAD24 (lane 1 and 2) or pCpxR (lane 3 and 4); and a *toxR* mutant carrying either pBAD24 (lane 5 and 6) or pCpxR (lane 7 and 8), *ompU* (lane 9) and *ompT* (lane 10) mutant. All strains were grown in AKI conditions at 37°C. OM proteins were resolved by 10% SDS-PAGE followed by staining with Coomassie blue. The Cpx pathway was activated, by inducing CpxR over-expression with 0.1% of arabinose. Samples were collected from each strain at least three times; one representative SDS-PAGE is shown.

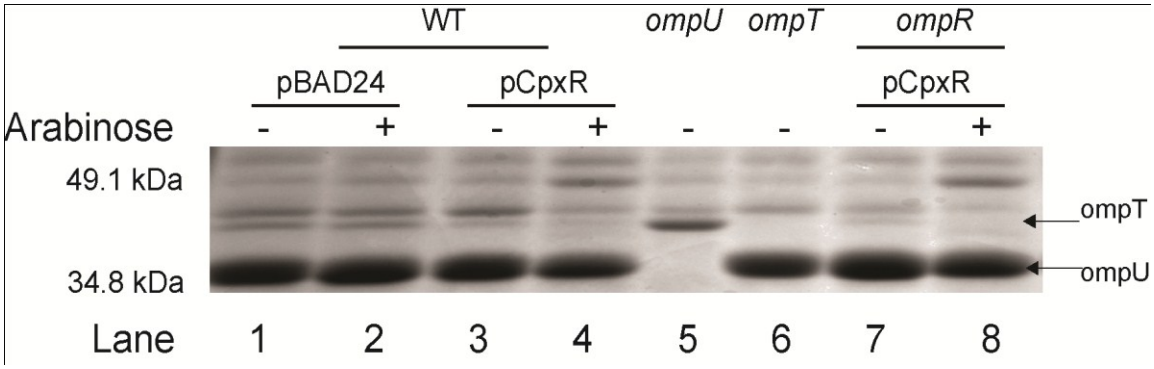

Figure 4 - 3 Cpx-mediated negative regulation of OmpT expression is OmpR-independent.

Outer membrane (OM) protein profiles of wild-type strain C6706 carrying either pBAD24 (lane 1 and 2) or pCpxR (lane 3 and 4), *ompU* (lane 5), *ompT* (lane 6) and *ompR* strain carrying pCpxR (lane 7 and 8) grown in AKI conditions at 37°C. OM proteins were resolved by 10% SDS-PAGE followed by staining with Coomassie blue. The Cpx pathway was activated by over-expressing CpxR from an arabinose inducible promoter in the presence of 0.1% of arabinose. Samples were collected from each strain at least three times; one representative SDS-PAGE is shown.

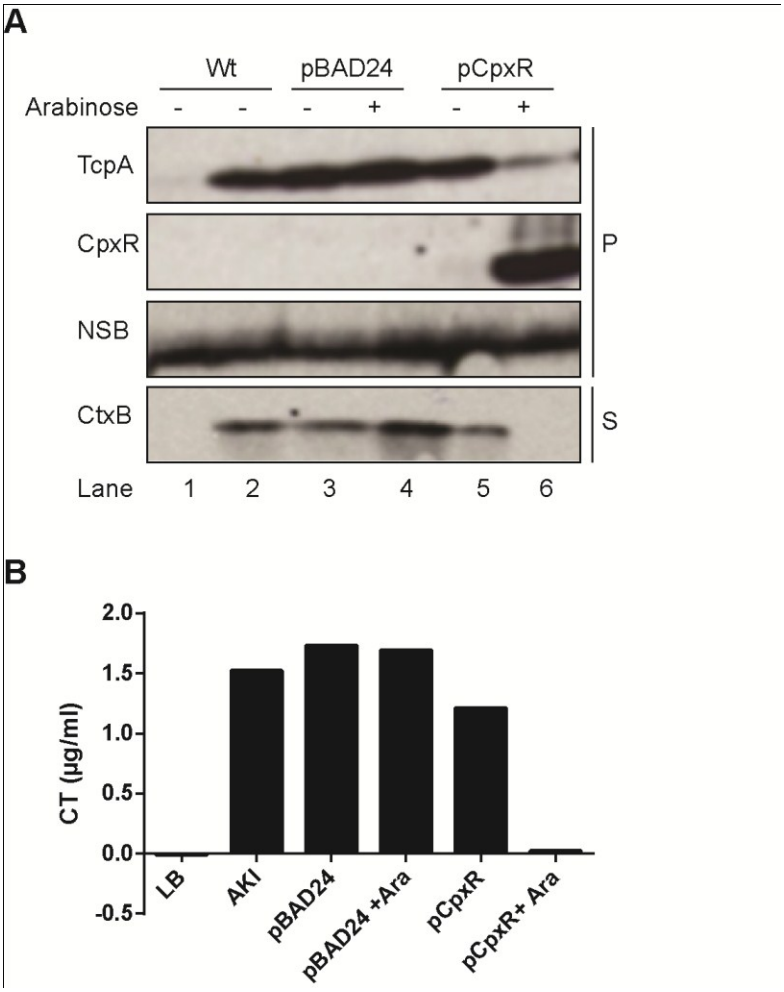

Figure 4 - 4 Cholera toxin and TCP are reduced when the Cpx response is activated.

Cell pellets (P) and supernatants (S) were collected from *V. cholerae* El Tor C6706 strains grown in LB (lane 1) or AKI medium (lanes 2 to 6). Subcultures were grown for 6 h statically at 37°C before the addition of 0.1% of arabinose to induce CpxR over-expression, followed by an additional 16 h of incubation at 37°C. Samples were collected from wild-type C6706 strain (lanes 1 and 2), C6706 carrying the vector control (pBAD24) (lanes 3 and 4) or the over-expression plasmid pCpxR (lanes 5 and 6) and analyzed by (A) Western blot against TcpA, CpxR and CtxB or (B) ELISA. Non-specific band (NSB).

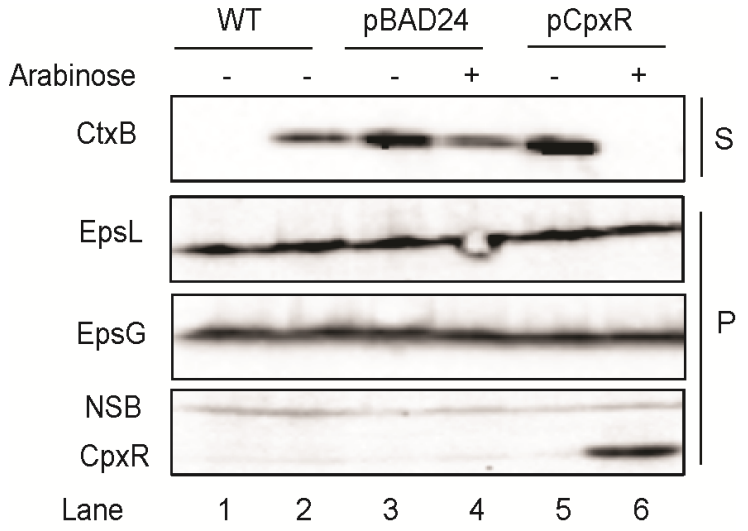

Figure 4 - 5 Activation of the Cpx pathway in *V. cholerae* does not affect the expression of the T2SS.

Cell pellets (P) and supernatants (S) were collected from *V. cholerae* C6706 grown in LB (lane 1) or AKI medium (lanes 2 to 6). Subcultures were grown for 6 h statically at 37°C before the addition of 0.1% arabinose to induce CpxR over-expression, followed by an additional 16 h of incubation at 37°C. Western analysis using antibodies directed against CtxB, EpsL, EpsG and CpxR from wild-type C6706 strain (lanes 1 and 2), C6706 carrying the vector control (pBAD24) (lanes 3 and 4) or the over-expression plasmid pCpxR (lanes 5 and 6). Non-specific band (NSB).

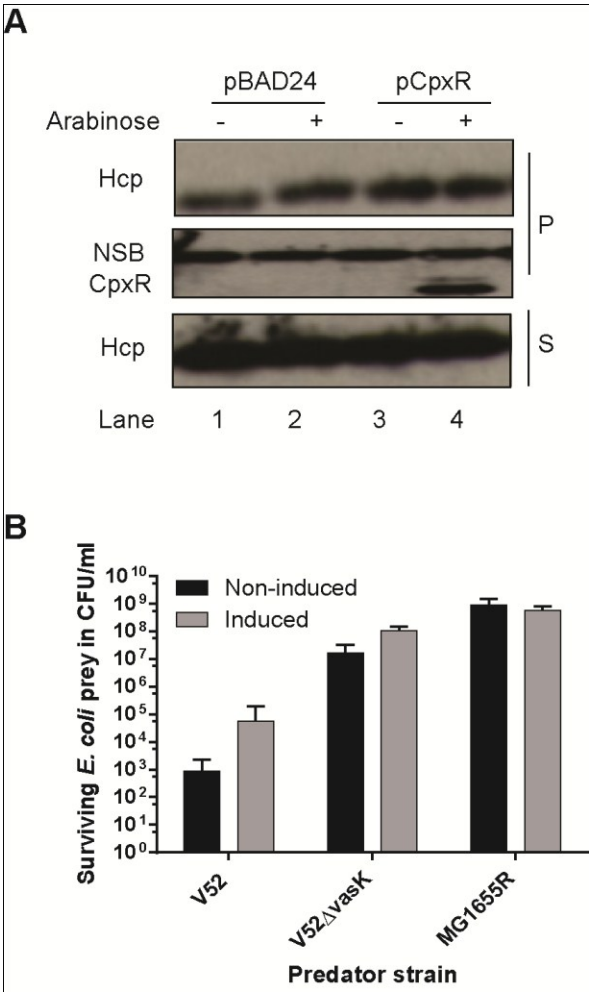

Figure 4 - 6 The Cpx response does not affect Hcp expression in *V. cholerae* V52.

(A) Cell pellets (P) and supernatants (S) were collected from *V. cholerae* V52 carrying the vector control (pBAD24, lanes 1 and 2) or pCpxR (lanes 3 and 4). Subcultures were grown in LB for 1.5 h at 37°C before the addition of 0.1% arabinose to induce CpxR over-expression. Western blot analysis was performed using antibodies directed against Hcp and CpxR. (B) Bacterial killing assay using *E. coli* MG1655R (pBAD24) as prey strain and *V. cholerae* strains V52 (pCpxR) and V52Δ*vasK* (pBAD24) as predator strains. *E. coli* MG1655R (pBAD24) was also included as predator strain for a negative control of T6SS-mediated killing. Surviving *E. coli* prey was calculated by counting the number of viable cells after 4 h of killing in the absence (non-induced) (black bars) or presence of 0.1% arabinose (induced) (grey bars). Non-specific bands (NSB).

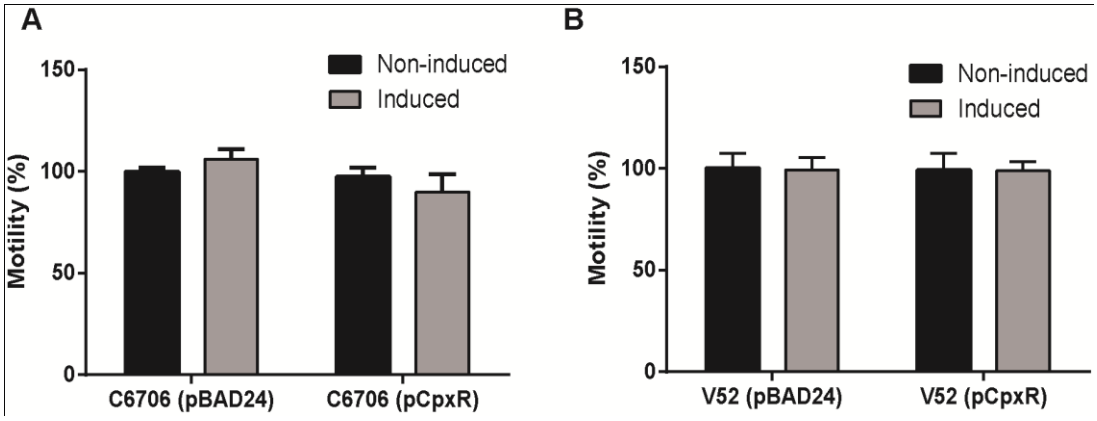

Figure 4 - 7 Motility of *V. cholerae* C6706 and V52 when the Cpx pathway is activated.

Overnight cultures for C6706 (A) and V52 (B) strains carrying either the vector control (pBAD24) or pCpxR were inoculated onto 0.3% LB agar plates (non-induced) or 0.3% LB agar plates containing 0.1% arabinose (induced). The growth diameter was recorded after 16 h. Each strain was inoculated by triplicate and the average and standard deviation are indicated.

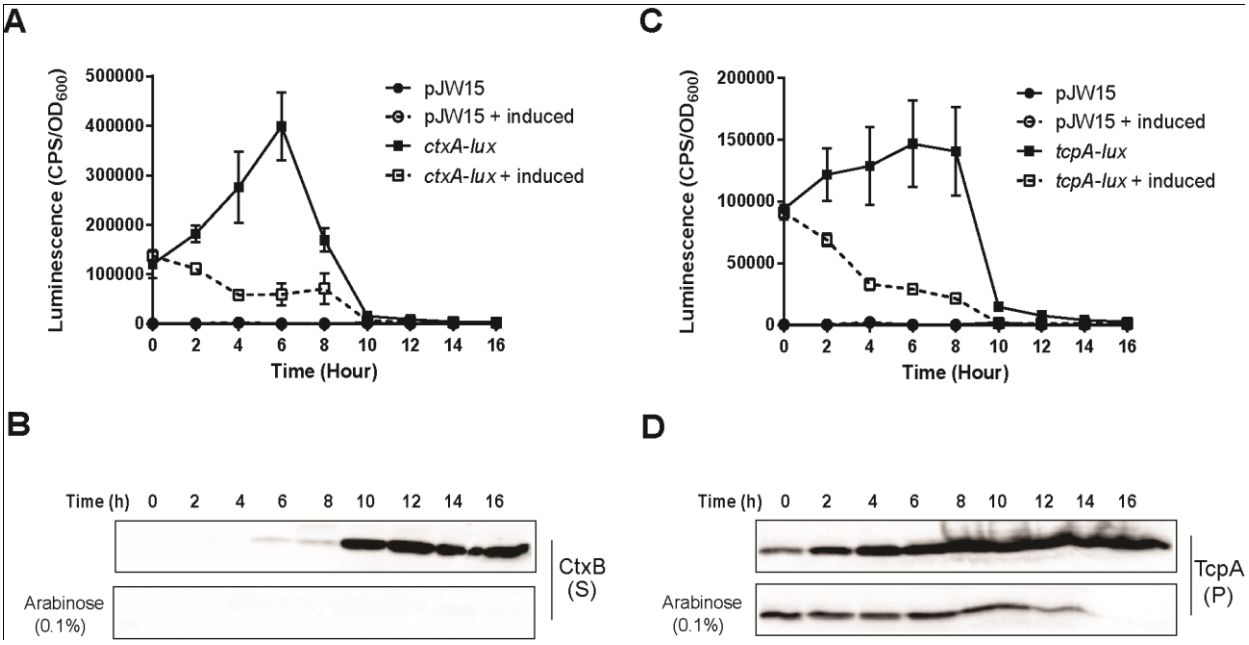

Figure 4 - 8 Activation of the Cpx pathway reduces the *ctxA* and *tcpA* expression at transcriptional level.

Luminescence activity of *V. cholerae* C6706 carrying the overexpression plasmid pCpxR, transformed with either the vector control (pJW15) or *ctxA-lux* (A) and *tcpA-lux* (C) reporters. Reporter gene expression was measured as counts per second (CPS) corrected for cell density (OD_{600}). The overall average and standard deviation resulting from two separate experiments performed in triplicate are shown. Pellet (P) and supernatants (S) samples were collected from *V. cholerae* C6706 carrying the overexpression plasmid pCpxR, grown under AKI conditions. Samples were analyzed by Western blot against CtxB (B) and TcpA (D). CpxR was overexpressed by adding 0.1% of arabinose (induced). Time 0 represents the time when cells were induced with 0.1% of arabinose and shifted to shaking conditions, after 6 h of statistic grow on AKI medium.

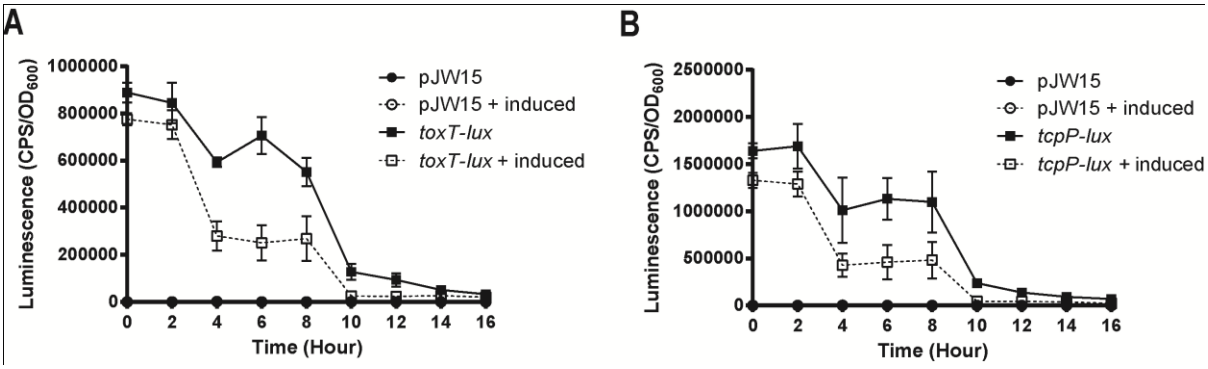

Figure 4 - 9 Cpx pathway regulates expression of major virulence factors regulators.

Luminescence activity of *V. cholerae* El Tor C6706, carrying the overexpression plasmid pCpxR, transformed with either the vector control (pJW15) or *toxT*-lux (A) and *tcpP*-lux (B), reporters. Reporter gene expression was measured as counts per second (CPS) corrected for cell density (OD_{600}). Time 0 represents the time when cells were induced with 0.1% of arabinose and shifted to shaking conditions, after 6 h of statistic grow on AKI medium. The overall average and standard deviation resulting from two separate experiments performed in triplicate are shown.

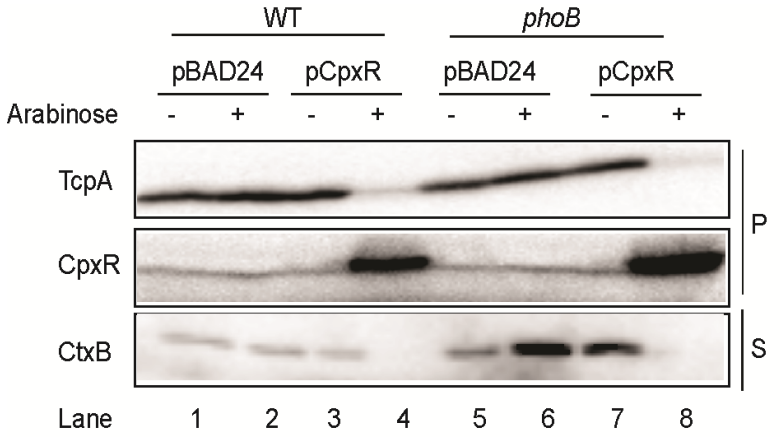

Figure 4 - 10 Cpx regulation of cholera toxin and TCP is PhoB independent.

Cell pellets (P) and supernatants (S) were collected from *V. cholerae* El Tor C6706 strain (lane 1 to 4) and *phoB* isogenic mutant (lane 5 to 8) grown in AKI medium. Subcultures were grown for 6 h statically at 37°C before the addition of 0.1% arabinose to induce CpxR over-expression, followed by an additional 16 h of incubation at 37°C. Samples were analyzed by Western blot against TcpA, CpxR and CtxB.

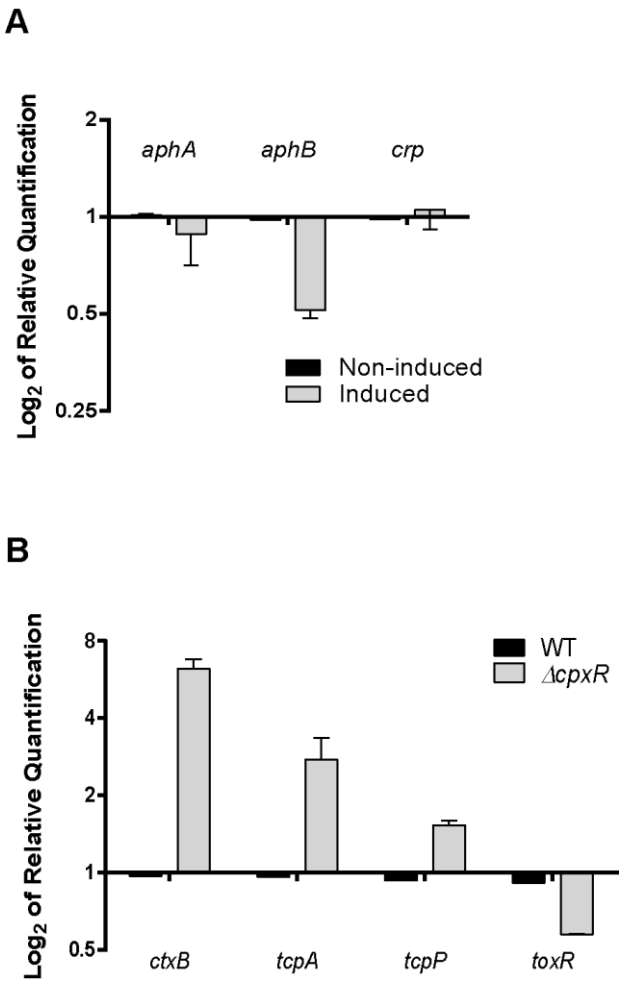

Figure 4 - 11 Cpx pathway regulates transcript levels of virulence factors and regulators.

qRT-PCR analysis of *aphA*, *aphB*, *crp, ctxB*, *tcpA, tcpP* and *toxR* transcript levels. RNA was isolated from cultures of (A) wild-type *V. cholerae* C6706 carrying the overexpression plasmid pCpxR, in the absence (non-induced) (black bars) or presence of 0.1% arabinose (induced) (grey bars) and of (B) *V. cholerae* C6706 strain (black bars) and the *cpxR* mutant (grey bars) and converted to cDNA. The cDNA was subjected to qRT-PCR analysis using the primers described on Table 4-2.

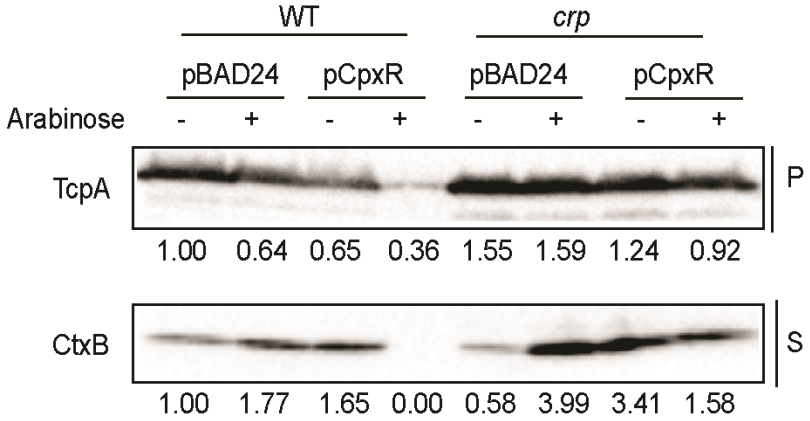

Figure 4 - 12 Cpx-mediated inhibition of CT and TCP production is diminished in the absence of cAMP receptor protein (CRP).

Cell pellets (P) and supernatants (S) were collected from wild-type *V. cholerae* C6706 and *crp* strains grown in AKI medium. Subcultures were grown for 6 h statically at 37°C before the addition of 0.1% of arabinose to induce CpxR over-expression, followed by an additional 16 h of incubation at 37°C. Samples were analyzed by Western blot using antibodies directed against TcpA and CtxB. The numbers below each lane indicate the intensity of either TcpA or CtxB band in each sample, relative to the wild-type strain.

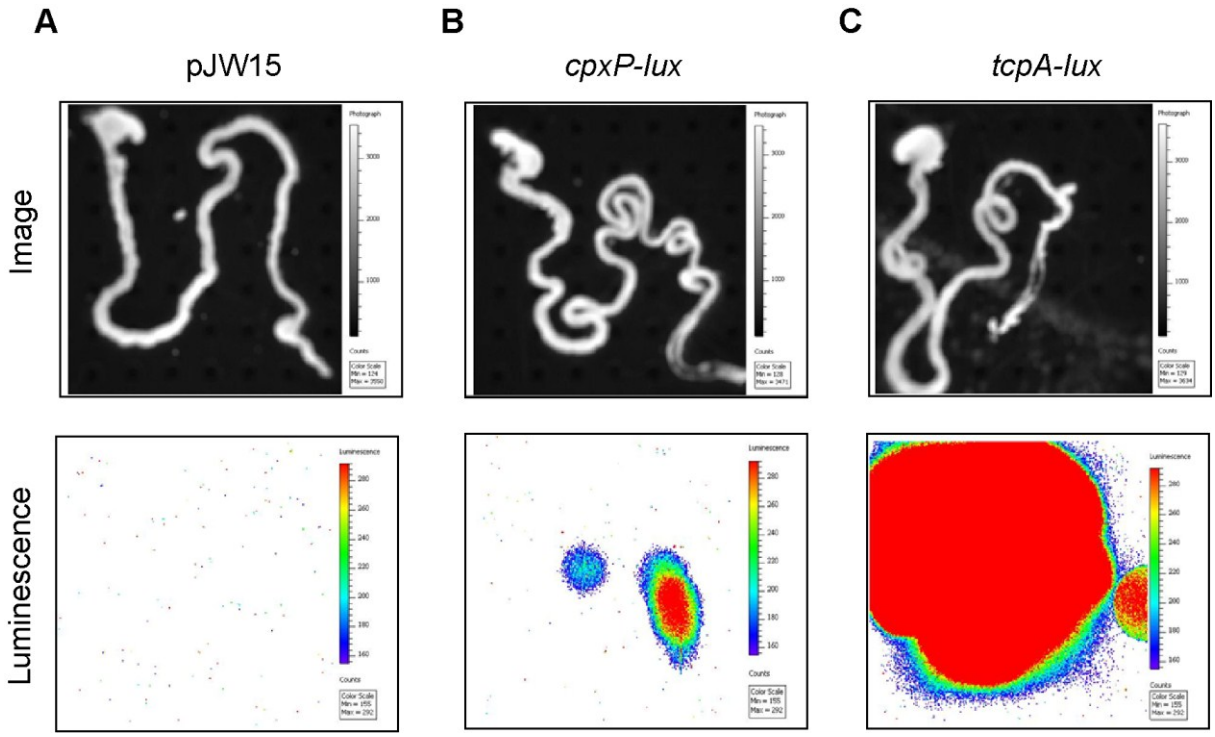

Figure 4 - 13 *Ex vivo* activation of the Cpx response in a mouse model.

Overnight culture of the *V. cholerae* El Tor C6706 strain carrying pJW15 (A), *cpxP-lux* (B) or *tcpA-lux* (C) was inoculated perorally to a 5-day-old infant mice. Luminescence imaging of the gastrointestinal tract extracted from the mice was performed at 18 hours with an IVIS Spectrum Imager System (Perkin Elmer). The heat bar indicates the luminescent intensity scale.

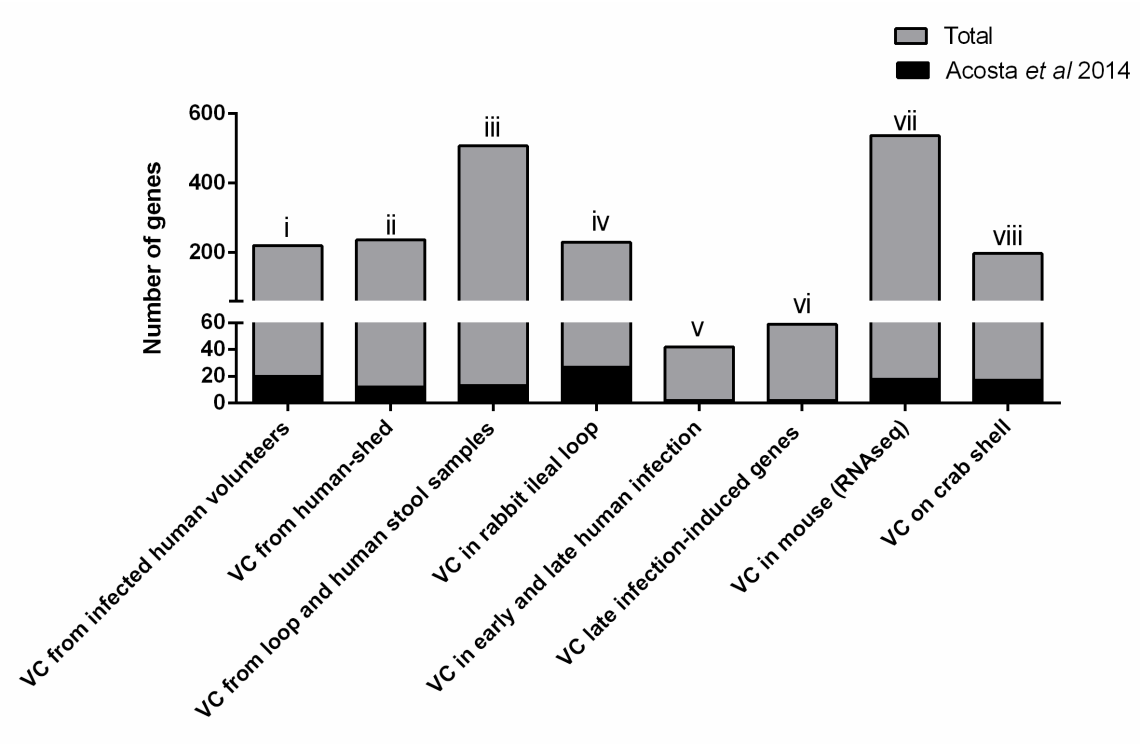

Figure 4 - 14 Comparative analysis between genes that changed expression when the Cpx pathway was activated and previous genomics research on *V. cholerae*.

Comparative analysis of the 174 genes that we found to be CpxR regulated (390) with genes that were either induced or repressed in samples from: i) human healthy volunteers infected with *V. cholerae* (448) ii) human cholera stool respect to stationary phase in vitro (449), iii) genes shared in intestinal loop model and human cholera stools (371), iv) rabbit intestinal loop model respect to exponential phase (450), v) stool or vomitus sample during early and late stages of human infection (452), vi) late infection-induced genes in mice (451), vii) mouse analyzed by RNAseq (447); and viii) in crab-shell (285). VC: *Vibrio cholerae*

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5 Chapter 5: General Discussion

5.1 Overview

The purpose of this thesis was to better understand the role of the Cpx envelope stress response in the human pathogen *V. cholerae*. More specifically, this work examined three main aspects: first, what are the Cpx regulon members in *V. cholerae*. Second, it investigated the inducing cues of this two component system, which may help to better understand the importance of the Cpx pathway during the life cycle of *V. cholerae*. Third, it determined the effect of Cpx pathway activation on *V. cholerae* virulence attributes, specifically elaboration of toxin-coregulated pili (TCP) and secretion of cholera toxin (CT), thus describing an additional level of regulation of these virulence factors in this organism.

5.2 *V. cholerae* Cpx regulon members

5.2.1 *Comparison of the Cpx regulon in V. cholerae to that of other organisms*

The Cpx pathway in *E. coli* is composed of the sensor kinase, CpxA, the response regulator, CpxR, and the regulatory periplasmic protein, CpxP, whose gene is divergently transcribed from the *cpxRA* operon (330, 331). Previous studies in *V. cholerae* El Tor N16961 have shown that, as in *E. coli,* the *cpx* loci are organized similarly and the *cpxR*, *cpxA* and *cpxP* genes are under the control of CpxR (261). Congruent with those studies, we found that when CpxR was over-expressed the most strongly induced gene was *cpxP* (VC2691), with a 77.84 fold change in expression. However, there was no change in expression of *cpxA* (VC2693), suggesting that expression of the *cpxRA* operon may not be autoregulated in *V. cholerae,* as it is in *E. coli.* Congruent with this, we found that deletion of the CpxR- regulated ferric reductases (i.e. *fcpR1-3*) resulted in differential expression levels of *cpxP and cpxRA*, which suggest that *cpxP* expression is uncoupled from that of the *cpxRA* operon. Additionally, it suggests that these ferric

reductases are either involved in unidentified regulatory mechanism that control *cpxP* expression and/or that they affect Cpx-dependent adaptation in *V. cholerae.*

Recently, our research group characterized the Cpx regulon in *E. coli* K-12 strain MC4100 and enteropathogenic *E. coli* (EPEC) strain E2348/69 (223). These results showed that the Cpx pathway regulates genes known or predicted to encode membrane-localized proteins (223). As previously shown in *E. coli* (223), over-expression of CpxR in *V. cholerae* lead to increases and decreases in the expression of genes with diverse functions in the cell (See Chapter 2). Similar to the *E. coli* microarray study, our microarray data showed enrichment for genes that encode proteins that are cell envelope localized, mainly in the inner membrane. We found that most of the genes encoding membrane localized proteins, including those that facilitate iron transport, were up-regulated in *V. cholerae* (See Chapter 2). In contrast with our results, EPEC genes involving iron transport and siderophore synthesis were down-regulated in DMEM media (223).

In *E. coli* a set of core genes were found to be Cpx-regulated, independently of strain background or media (223). These included genes involved in cell wall metabolism. In *V. cholerae* El Tor C6706 we found that some of the cell wall modifying enzymes, including *dacAII* (VCA0270) and *mltA* (VC2312), were also up-regulated in our microarray study (7.36 and 3.05-fold change respectively). Interestingly, there is a putative CpxR binding site in the promoter region of the *dacAII* gene, suggesting a possible direct regulation of *dacAII* by CpxR. A previous microarray study done with wild-type *H. ducreyi* 35000HP and the isogenic *cpxR* mutant, showed that, as in *V. cholerae,* the *dacA* homologue (HD2016) was up-regulated by the Cpx pathway (258). In addition, Evans and collaborators (211) proposed a model in which the Rcs and Cpx stress responses are required for activation of genes involved in the repair of cell wall damage in *E. coli* (211). These data suggest that as in *E. coli*, the Cpx pathway in *V. cholerae*

may be involved in the maintenance of envelope homeostasis by regulating cell wall modifying enzymes that can help to alleviate the effects of stressors in the cell envelope.

Additionally, we found that the activation of the Cpx pathway leads to ~3 fold increased expression of the *yajC*, *secD* and *secF* genes (VC0742-VC0744), which encode components of the Sec protein secretion pathway (454). Similarly, in *E. coli* the Cpx pathway positively regulates other components of the Sec pathway such as SecA and YccA (197, 213, 221). These results reinforce the hypothesis that the Cpx pathway plays an important role in relieving envelope stress by boosting protein secretion capacity to ensure that protein folding and degrading factors are secreted to the periplasm (253).

Finally, it has been reported that the Cpx response controls outer membrane (OM) porins and efflux systems expression in several organism (113, 114, 198, 241, 433). Similarly, we found that the Cpx response in *V. cholerae* regulates the expression of two RND efflux pumps (i.e. VexB and VexH), TolC and OmpT OM porins. In addition, mutants of those efflux pumps and TolC reciprocally activate the Cpx response in *V. cholerae* (332, 390). Moreover, it has been shown that the *tolC* mutation also leads to an increase on activation of the Cpx response in *E. coli* and *S. meliloti* (115, 375). All together, these data support the hypothesis where the Cpx pathway is required for maintenance of OM permeability and control of efflux pumps to extrude metabolites that are toxic for the cell.

5.2.2 *Transcriptional regulation of iron related genes*

In order to identify CpxR-regulated genes, we determined changes in the *V. cholerae* El Tor C6706 transcriptome after overexpression of the response regulator CpxR. Interestingly, we found that several genes that encode proteins required for iron acquisition were up-regulated by

the Cpx pathway. The most strongly Cpx up-regulated genes are predicted to be required for iron assimilation once ferric iron (Fe^{3+}) is internalized in the cell (Figure 5-1). We found that upon activation of the Cpx response there was an increase in expression of *fcpR1* (or VCA0151), which encodes a putative oxidoreductase and two putative cytochrome b_{561} orthologs, *fcpR2* (or VCA0249) and *fcpR3* (or VCA0538), which are predicted to be membrane-bound, diheme ferric reductases, that are required for reducing chelated ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) (365). Congruent with this putative role of *fcpR2* and *fcpR3* in iron assimilation, and our finding that the Cpx response is activated by iron limitation and involved in regulating iron uptake and storage genes, we determined that these genes are important for adaptation to some Cpx inducing conditions (See Chapter 3).

Both *fcpr2* and *fcpR3* are tightly regulated by iron availability in *V. cholerae*. As mentioned before, the expression of *fcpR3* is negatively regulated by Fur but independent of iron (366), while the expression of *fcpr2* is under control of the small RNA (sRNA) RyhB (388). Additionally, it was previously shown that the *fcpr2* gene is expressed in the intestines of infant rabbits, which is suggested to be an iron limited environment (447). However, whether these putative ferric reductases may play a role in iron assimilation in *V. cholerae* is unknown. An interesting possible future study would be to determine if *fcpr1*, *fcpr2* and *fcpr3* have ferric reductase activity. For example, ferric reductases are indispensable for growth on ferrioxamine B as the sole iron source (325), therefore this potential activity could be measured by assessing growth of wild-type *V. cholerae* El Tor C6706 strain and mutants carrying single, double, or triple mutations in the putative ferric reductase genes as previously described (455). In addition, diheme ferric reductases usually require heme for their stability and function (399, 456), thus the conserved histidines predicted to be involved in heme ligation in the putative ferric reductases

could be mutated, and the mutants could be tested in the above growth assay to confirm that heme binding is important (401, 457). These experiments will elucidate whether these ferric reductases may be involved in iron assimilation under envelope stress conditions. Congruent with this model it has been described that under normal conditions expression of cytochrome b_{561} is low in other organism such as *E. coli* (458), reinforcing the idea that they may be of particular importance during envelope stress.

Besides the up-regulation of those ferric reductases by the Cpx response, we also found that genes encoding proteins for iron acquisition, iron-containing respiratory enzymes, and factors involved in iron-related detoxification and storage were also positively controlled by CpxR. Although, it is unknown if this positive regulation by CpxR is direct or indirect, our transcriptomic analysis of the Cpx regulon in the absence of the putative ferric reductases FcpR1, FcpR2, or FcpR3, suggest that this regulation could be mediated by the role of these putative ferric reductases in regulating iron homeostasis and therefore it may impact the Cpx-dependent signalling or alter other iron-sensitive regulatory pathways in the cell.

Additionally, we found a putative CpxR binding site in the locus encoding the sRNA RyhB, which is iron-regulated by Fur in *V. cholerae* El Tor N16961 and O395 strains (388, 394). Preliminary data suggest that the Cpx pathway positively regulates the transcription of *ryhB*. Probably, the Cpx positive regulation of this sRNA could also explain the observed changes in our microarray data in the expression of genes that are related to iron and are Fur/RyhB regulated. For instance, it has been shown that RyhB also feedback-controls the expression of its own regulator, Fur (459), therefore activation of the Cpx response could decrease the levels of Fur. Moreover, RyhB not only act as a repressor, as it has been shown that it up-regulates mRNA translation of a protein involved in siderophore biosynthesis (460). However, these RyhB-Fur

circuitries of regulation have not been elucidated in *V. cholerae.* It is suggested, based on our data with *V. cholerae*, that at least for the regulation of the ferric reductases (i.e. FcpR2 and FcpR3), CpxR and Fur act independently to affect their transcription .

In addition, this possible regulation of RyhB by the Cpx response could also explain the decrease in expression of *cpxP* in the *fur* mutant (See Chapter 3). Further analyses are required to study the relationship between the Cpx response, RyhB expression, and the control of iron-related genes. One possible future study could determine the transcript levels of the CpxR regulon members upon activation of the Cpx response in both wild-type C6706 and an isogenic *ryhB* mutant. Interestingly, in a genetic screen for the detection of novel Fur-regulated genes in the causative agent of furunculosis in fish, *Aeromonas salmonicida* subsp. *salmonicida*, it was shown that *cpxR* could be a member of the Fur regulon in this organism (461). This data and ours suggest how in two distantly related pathogens which share similar environments (i.e. fresh and marine waters), the Cpx pathway may be associated with the regulation of iron-regulated and iron-storage/binding functions.

It was previously reported that perturbation to the cell envelope in *V. cholerae* by inactivating the type II secretion system (T2SS) leads to changes in iron homeostasis and oxidative stress, which is linked with the induction of extracytoplasmic stress responses, such as σ^E (462). However, in this study it was not assessed whether deletion of the entire T2SS operon would lead to an accumulation of cholera toxin in the periplasm and therefore activation of the extracytoplasmic stress responses (462). Sikora and collaborators (462) proposed that generalized cell envelope perturbations affect iron metabolism and oxidative stress in some gram-negative species, including *V. cholerae*. Here, we found that the Cpx pathway may be important for sensing conditions where iron is chelated or perhaps during envelope stress when iron

concentrations may change, rather than in response to nutritional limitation. Consequently the Cpx pathway positively regulates the expression of genes involved in iron acquisition in order to maintain cell envelope homeostasis (See Chapter 2, Figure 5-1). The fact that many of the genes that change in expression upon Cpx activation contain iron cofactors, together with the enrichment for genes involved in iron uptake upon CpxR over-expression, suggests that the Cpx response may be involved somehow in coordinating the expression of iron-containing respiratory enzymes with the levels of cellular iron in *V. cholerae* (Figure 5-1).

Recently, other studies have shown evidence of connections between the Cpx response and metal regulation in different organism. For example, CpxP has been linked to zinc homeostasis in *E. coli* (J. Wong and T. L. Raivio in preparation). Similarly, another study reported the transcriptional profile of *S.* Typhimurium upon exposure to either copper or zinc ions and found that, besides affecting the expression of genes involved in metal homeostasis, these metals also affect the expression of previously characterized CpxR regulon members and the σ^E envelope-stress response in *E. coli* (463). Interestingly, this study also found that TolC and the siderophore enterobactin are involved in the resistance to excess amounts of $CuSO_4$ (463). This is in line with the recent finding reported by Vega and Young (380), that periplasmic accumulation of the siderophore enterobactin in *E. coli* efflux pump mutants is toxic, which then leads to activation of different responses including the Cpx response (380). Additionally, it has been proposed that there may be an interplay between CpxR and SO2426, a gene in *Shewanella oneidensis* involved in chromate resistance, in response to metal stress (464). Congruently all these studies reinforce the proposed model in this thesis where we describe a new putative role for the Cpx pathway in sensing envelope stresses related to iron chelation that result in elevated levels of, and/or

defective apo-co-factors in the envelope that are toxic, which therefore require the RND efflux pumps and TolC for detoxification processes in *V. cholerae* (Figure 5-1).

5.3 *V. cholerae* Cpx inducing cues

Previously, it was reported that the inducing cues that trigger the Cpx pathway in *E. coli* are not relevant in *V. cholerae* (261), therefore, to characterize the inducing cues that activate the Cpx pathway in *V. cholerae*, we constructed a *cpxP-lux* transcriptional reporter. This fusion was designed to contain the upstream regulatory elements including the putative CpxR binding site for regulation of *cpxP*. As previously described (261), this study showed that $CuSO_4$ and conditions that lead to the formation of aberrant disulfide bonds, as shown in our study for the presence of diamide, activate the Cpx pathway.

It can be hypothesized that the main effect of diamide and $CuSO_4$ is interfering with proper disulfide bond formation in the periplasm. Previously, it was suggested that periplasmic proteins with aberrant disulfide bonds, as seen in *dsbD* mutants, may harm an undefined component of the cell envelope which then leads to the activation of the Cpx response (261). Here, we propose that the undefined components that may be harmed are cell envelope proteins that contain iron as a cofactor (e.g. energy metabolism proteins such as cytochromes that contain heme), therefore, in the presence of diamide or $CuSO_4$ there is a disruption of the center of iron-containing respiratory proteins, that then leads to changes in iron homeostasis in the cell envelope, which can be sensed by the Cpx pathway (See Chapter 2). For example, maturation of apocytochrome c requires the covalent ligation of a heme B cofactor to the two cysteines present in the Cys-Xaa-Xaa-Cys-His motif located in the periplasmic domain upon its translocation to the periplasm (376). This process requires the cytochrome c maturation (Ccm) system and DsbD, where DsbD promotes

the maturation of cytochrome c-type respiratory factors by transferring electrons through its direct interactions with CcmG (also known as DsbE) (376-378). Therefore, if DsbD is inactivated it could lead to the accumulation of apocytochrome c in the periplasm, which could be detrimental for the cell and induce the activation of the Cpx response, which ensures the proper regulation of iron acquisition functions to respiratory protein/co-factor when they are damaged upon stress.

In addition, other studies have shown that copper induces the Cpx response (197, 261, 465). Excess copper may induce the release of Fe^{2+} from Fe-S clusters (466), which could result in the induction of the Cpx pathway (Figure 5-1). Accordingly, we observed that the presence of exogenous iron suppresses both the induction of the Cpx response by diamide and $CuSO_4$ and the Cpx dependent growth phenotypes observed in a *cpxR* mutant. However, the mechanism of how CpxA may sense signals related to excess of copper or aberrant disulfide bond have not yet been determined. The crystal structure of CpxA has been determined in *V. parahaemolyticus*, which shares high sequence similarity with *V. cholerae* at the CpxA periplasmic domain (467). The possible ligands of this protein are still unknown, for instance whether *V. cholerae* CpxA can sense directly either iron or apocytochrome c, which lacks its iron cofactor.

Interestingly, this study and the Taylor *et al* (332) study found that the Cpx response in *V. cholerae* is induced by defects in RND-pump mediated efflux and the Cpx pathway leads to increased expression of these RND systems (i.e. VexAB and VexGH). Additionally, we showed that TolC, their common OM pore in *V. cholerae* (343), is also regulated by the Cpx response. We suggested that this activation may contribute to the resistance towards unknown molecule(s) which could be the product of the iron chelation process, that may accumulate in the *tolC, vexH and vexB* mutants and therefore impair cell envelope integrity (Figure 5-1). This model is in

accordance with previous studies reporting activation of the Cpx response in *tolC* mutants in different organism (115, 332, 407). Additionally, it seems that deletion of RND efflux systems in other organisms such as *Klebsiella pneumonia* and *H. ducreyi* (246, 333) also activates the Cpx response, suggesting that this role of the Cpx pathway is conserved among different bacterial species. Finally, another study reported that the BaeSR envelope stress response system, in *S. typhimurium,* is required to prevent the accumulation of toxic metabolites that may damage the membranes (468).

A current topic of study is the molecular mechanism of activation of the Cpx pathway and the nature of the unknown inducer(s) or metabolite(s) that might be accumulating and inducing the Cpx pathway in the *tolC* mutant. For this purpose, a transposon mutagenesis screen can be done in order to identify genes responsible for the induction phenotype seen in *tolC* mutants. In the present study we observed that only some of the inducing cues of the Cpx pathway in *E. coli,* among other bacterial species, are conserved with those in *V. cholerae* (e.g. mutation of *tolC*). This is expected as species-specific signalling events may be required due to the diversity of environments and niches among bacterial species.

In agreement with our microarray data, that showed that the Cpx regulon is enriched for genes required for iron acquisition, we found that 2,2'-Bipyridyl, a compound that chelates iron, activates the Cpx pathway. However, iron depletion only activated the *V. cholerae* Cpx response in low salt media, and it is not well understood why this is the case. Slamti and Waldor (261) suggested that the *V. cholerae* Cpx response may monitor salinity and mediate adaptation to upshifts in salinity, because in that study it was found that chloride ions activated the Cpx pathway (261). In addition, the authors proposed that Cl^- may alter the activity of one of the components of the Cpx pathway or alter indirectly an unknown factor(s) that affects Cpx signal

transduction (261). Fleischer and collaborators (70) also found that chloride salts impact CpxA signaling activities in an *in vitro* proteoliposome system (70). Similarly, another study showed that mutation of *cpxR* in *Cronobacter sakazakii* confers salt sensitivity, suggesting that the Cpx response along with other responses (i.e. sigma factors RpoN and RpoS) are important for resistance in hyperosmotic conditions (469). Cumulatively, all these studies suggest a close connection between the Cpx envelope stress response and changes in salinity, as previously reported (261), which may affect the signaling of other inducing cues (e.g. 2,2'-Bipyridyl) of the Cpx response in *V. cholerae*.

It seems that iron is an important modulator of Cpx pathway signaling in *V. cholerae* El Tor C6706 because exogenous iron decreases the activation of the Cpx response under iron limitation conditions or when efflux pump components are deleted. However, the mechanism is not well understood. Two models for how the Cpx response is suppressed by iron can be envisaged: the unknown molecule(s) that accumulates in the *tolC, vexH and vexB* mutants could chelate iron from important envelope proteins, such as respiratory chain components, therefore the presence of iron decreases the activation of the Cpx pathway; or, alternatively, iron inhibition may be working separately via a different pathway. However, it is still unknown where the inducing signal (i.e. iron chelation and deletion of efflux pumps) is sensed, for instance if the sensor kinase CpxA is in charge of sensing the unknown molecule(s) in the periplasm or if the signal is coming from the cytoplasm, which is then either transduced to the periplasm to regulate the activation of CpxR or there are other mechanisms for this activation. Further analysis such as the analysis of the effect of iron inhibition in *cpxA* mutants could be done to elucidate those possibilities.

Finally, iron uptake is an important virulence factor that facilitates colonization of the host (327) and *V. cholerae* has developed several iron acquisition strategies to survive in the human

host and in aquatic environments (325). Our data does not discard the hypothesis that the Cpx pathway may play an additional role in the regulation of genes required for iron acquisition in environments such as the human host, where there is an extremely low iron milieu (470). For example, bacteria need on average 10^{-7} to 10^{-5} M of iron for optimal growth (471), however, the concentration of free iron in the host is only about 10^{-24} M (472). In addition, colonic lumen iron absorption is considered a defence mechanism by restraining the growth and virulence of enteric pathogens (471).

5.4 Role of the Cpx response in *V. cholerae* pathogenesis

5.4.1 *Cpx response regulates toxRS and phoB expression*

In this study we found that the expression of the *toxR* changed upon induction of the Cpx pathway (See Chapter 2 and 4). ToxR is a transmembrane protein with a cytoplasmic localized DNA-binding/transcription activation domain (286). This domain is a member of the OmpR/PhoB family of transcriptional activators (428). In addition, ToxR protein function requires an inner membrane localized protein, ToxS, which has been suggested to be required for both stability and dimerization of ToxR (292, 294, 295) and was also found to be regulated by the Cpx response (See Chapter 2 and 4). However, comparison of the Cpx regulon members found in this study with those identified in a microarray study of wild type and *toxR* mutant strains in *V. cholerae* (371) showed that there is little overlap between the data sets. More studies are required to understand the role of activation of ToxR by the Cpx pathway, besides its role in virulence factors regulation (see below).

ToxR is a regulator of cholera toxin and TCP expression among other virulence determinants, such as the OmpT and OmpU in *V. cholerae* (289, 290, 347, 371, 430-432). Interestingly, as it

was previously reported (332), we found that OmpT is regulated by the Cpx response and in addition we found that this regulation is ToxR-independent, as activation of the Cpx response in a *toxR* mutant did not restore the decrease in OmpT protein levels compared to the wild-type C6706 strain (See Chapter 4). Because a putative CpxR binding site in the promoter of *ompT* was not found, we hypothesized that the regulation of OmpT expression by CpxR is indirect. One possible mechanism for this regulation may be related with the iron status upon Cpx activation that may lead to changes in Fur activity that then affect OmpT expression. This model could be supported with the fact that iron limitation is an inducing cue of the Cpx response ((390), See Chapter 2), and also because it was previously described that iron and Fur positively regulate *ompT* expression (473). Similarly to our results, the iron and Fur regulation of OmpT expression was also found to be independent from the modulation of ToxR production (473). This hypothesis may be tested by assessing the OMP profile upon activation of the Cpx pathway in the presence and absence of iron.

We also found that *phoB* expression changed upon induction of the Cpx pathway (See Chapters 2 and 4). A previous study showed that the response regulator PhoB, which is part of the two-component system PhoBR, negatively regulates the expression of virulence genes by repressing the *tcpPH* promoter (361). Since we reported that the Cpx pathway regulates the expression of *phoB* and it has a putative CpxR binding site in its promoter region spanning the positions -126 to -111 ((390), Chapter 2: Figure 2-2), we hypothesized that the Cpx pathway may inhibit CT and TCP production via *phoB*. However, a *phoB* deletion did not decrease the inhibitory effect of the Cpx response on virulence factor expression. Additional work will be required to determine the putative role of PhoB during Cpx-mediated adaptation. For example, recently a study showed that the expression of the Cpx and phosphate (Pho) responses in *E. coli*

are required for resistance to phosphorus starvation during gentamicin exposure (474). Future studies are required to determine if this role of phosphate regulation and Cpx response in *E. coli* is also conserved in *V. cholerae*.

5.4.2 *Cpx response regulates CT and TCP production*

The two major virulence factors involved in the outcome of cholera disease by *V. cholerae* are the TCP and the CT (288). The genes required for the expression of TCP and CT are located in two different pathogenicity islands within the large chromosome of *V. cholerae* and they were both acquired by horizontal gene transfer (475, 476). The TCP operon is encoded in the Vibrio Pathogenicity island (VPI) (477) and the *ctxAB* genes encoding the CT are located in a cluster of genes known as the CTX genetic element (475). These virulence determinants are tightly regulated by many transcriptional regulators at different levels (299).

The main direct virulence regulator of CT and TCP is ToxT (299). ToxT is an AraC/XylS protein family member (291), which binds at a conserved sequence known as the toxbox in the promoter region upstream of the -35 element of the *ctxAB* and *tcp* operons (414, 478). Additionally, this binding site is also shared with the histone-like protein H-NS, which strongly represses transcription of the *ctxAB* promoter and to a lesser extent the *tcpA* promoter, where ToxT competes with H-NS for binding to DNA and acts as an activator of transcription (479-481). Although bacterial two-hybrid analyses have suggested that ToxT can dimerize at its N-terminal domain, *in vitro* studies have suggested that ToxT binds to DNA as individual monomers (478, 482). In this study we found that the expression of *toxT* is affected upon activation of the Cpx response (See Chapter 4), which suggests that there is an additional level in the regulation of the virulence factors through ToxT. However, we did not find any putative CpxR binding site in the promoter region of *toxT* which suggests that this regulation is indirect.

Two transmembrane regulators are required to regulate the expression of ToxT: ToxR and TcpP. Although we found that the regulation of CT and TCP by the Cpx response is ToxR independent, based on our experimental set up we cannot rule out the possibility that ToxR could be part of the mechanism of this regulation (See Chapter 4). Recently, Bina and collaborators (445) proposed a mechanism for CT and TCP regulation in which cyclic dipeptides (CDPs), through ToxR, activate the expression of *leuO,* a global regulator that represses *aphA* expression (445). Additionally, in the same study it was suggested that other factors exist, in addition to ToxR, that likely contribute to *leuO* expression (445). Interestingly, Bina and collaborators (445) also found that the presence of CDPs regulate the expression of VexAB RND efflux system and its regulator VexR, which were also found to be Cpx regulated in this study (See Chapter 2), supporting the possibility that the Cpx response could be involved in the response to CDPs presence. CDPs play a role in virulence gene expression in *V. cholerae* as they act as effectors to regulate the expression of *tcpP* (446, 483). Therefore, future studies are required to determine if this regulation of *leuO* expression by ToxR, in the presence of CDPs, is CpxR dependent.

TcpP activity requires a membrane-bound effector protein, TcpH (296). It is thought that TcpH interacts with the TcpP periplasmic domain in order to prevent its degradation, which allows it to continue the regulation of virulence factors (296, 299). Expression of *tcpPH* operon is controlled by environmental signals such as temperature and pH (484). Interestingly, we found that the Cpx response also indirectly regulates the expression of *tcpP* at transcriptional level. This suggests that the Cpx pathway likely affects CT and TCP expression through downregulation of *tcpP* expression.

Additionally, TcpP expression is regulated by AphA, AphB and CRP (300-302, 415, 416). In this thesis we found that the negative regulation of virulence factors by the Cpx response was

mostly mediated by CRP (See Chapter 4). CRP is involved in carbon catabolite repression (485) and it has been shown that deletion of *crp* in *V. cholerae* affects multiple processes involved in pathogenesis, such as motility and intestinal colonization (441). In addition, CRP plays a role in the modulation of the HapR activation, a quorum sensing regulator and a LuxR homologue (441, 486). HapR is an important regulator of biofilm formation and rugosity in *V. cholerae*, where at high cell density it represses the expression of genes responsible for exopolysaccharide biosynthesis (*vps*) (487, 488). Moreover, HapR regulates the expression of the virulence factors, CT and TCP, by decreasing *aphA* transcription, which in turn indirectly represses *tcpPH* expression (304). Preliminary data suggests that activation of the Cpx response leads to a decrease in biofilm formation (data not shown), which fits with our finding that Cpx-dependent inhibition of virulence factor production is dependent on CRP as well. These results are in accordance with our working model (see below) which proposes that the Cpx response is involved in the regulation of virulence factors late in infection, where CT and TCP are known to be reduced (371, 445, 449-452), and the bacteria are also subject to iron limitation (332, 371, 447, 449).

In summary, in this thesis we found that the activation of the Cpx pathway regulates the expression of cholera toxin and TCP in *V. cholerae*, reinforcing the broad role of this pathway in the regulation of genes involved in virulence in different organism (253). Overexpression of CpxR inhibits the transcription from the *ctxA* and *tcpA* promoters, likely in an indirect manner. We propose that the Cpx pathway represses CT and TCP gene expression by (i) affecting the activity of CRP which directly regulates levels of *tcpP* and *tcpH* expression, (ii) negatively regulating expression of *aphB* and (iii) positively regulating the *toxR* transcription (Figure 5-2). For the latter, our data do not exclude the possibility that the Cpx response may interfere with

virulence gene regulation through the response regulator ToxR, as we could not determine if the negative regulation of virulence factors by the Cpx response is dependent on *toxR* null. Further studies are necessary to provide more evidence on this point.

Preliminary data suggest that the Cpx response is activated during colonization as seen in the mouse model experiments (See Chapter 4). Similarly, a recent study showed that inactivation of the Cpx response decreases colonization of *E. coli* in a mouse model (232).We speculate based on our transcriptional data (See Chapter 2) and the role of the Cpx pathway in negatively regulating virulence determinants (See Chapter 4), that this regulation will not be at early stages of infection because it would be detrimental for *V. cholerae* virulence, rather it would be upon exit or in the environment. In addition our microarray data suggest (See Chapter 2) that the Cpx pathway is important under conditions where bacteria have to respire which fits with our hypothesis that the Cpx response is important during exit of the host. For example, it was described that the Cpx pathway is active in cattle feces (489). Finally, the observation that the Cpx response regulates genes involved in iron acquisition as well as excretion of metabolic waste products (e.g. *tolC, vexH and vexB*) with previously characterized transcriptional analysis in *V. cholerae*, further suggests that the Cpx response may play a role during late infection where there is low iron limitation conditions and accumulation of metabolic waste (332, 371, 447, 449). Additionally, it has been suggested that *V. cholerae* also may encounter iron limitation during growth in association with crustaceous, such as crab, in the marine environment, as transcriptional data have shown that *V. cholerae* induces the expression of genes required for iron acquisition when it is growing in crab-shell, which is a source of chitin in the marine environment for this bacterium (285).

The Cpx pathway is not the only envelope stress response system that has been described to be important for the regulation of *V. cholerae* virulence factors. For example, the alternative sigma factor σ^E (also known as RpoE) has been shown to be important for intestinal survival in an infant mouse model, and for growth and survival in the presence of envelope stressors such as high temperature and ethanol (490). Additionally, through the regulation of VrrA, a sRNA, the RpoE factor also regulates the expression of OM proteins such as OmpA and OmpT in response to envelope stress (491, 492). Also, VrrA down-regulates TcpA levels (491) and modulates biofilm formation (493). These data reinforce the hypothesis that envelope stress response systems play important roles in envelope homeostasis that impact processes such as virulence and biofilm formation.

5.5 Concluding Remarks

Two component systems (TCS) are important signaling pathways in bacteria for responding to different changes in their environments. The Cpx response is a TCS involved in the regulation of expression of genes involved in maintaining cell envelope homeostasis. Initial studies have suggested that this stress response system was mainly involved in sensing signals derived from the generation of misfolded IM and/or periplasmic proteins. At the beginning of this project we wondered about the level of conservation of this role in *V. cholerae*, and if we could learn more about the Cpx response by characterizing the regulon in other species. This thesis suggest that the Cpx response is not only involved in the regulation of protein folding and degrading factors, but rather is also involved in mediating adaptation to novel inducing cues such as iron limitation or responding to the accumulation of toxic metabolites generated during cell growth.

In accordance with several studies that have proposed that the Cpx pathway plays an important role in bacterial pathogenesis by regulating virulence factors, we found that this role is also conserved in *V. cholerae*. This study increased our knowledge of the evolutionary importance of the Cpx response in virulence by elucidating the molecular mechanism(s) by which the Cpx pathway regulates gene expression in *V. cholerae*. Future studies should focus on the comparison of different Cpx regulon member and functions, which will help to elucidate conserved mechanisms of Cpx-regulated adaptation to envelope stress. Finally, knowledge of the conserved role of the Cpx response in the regulation of virulence determinants could be applied to the development of new antibacterial drugs to control different bacterial pathogens.

5.6 Figures

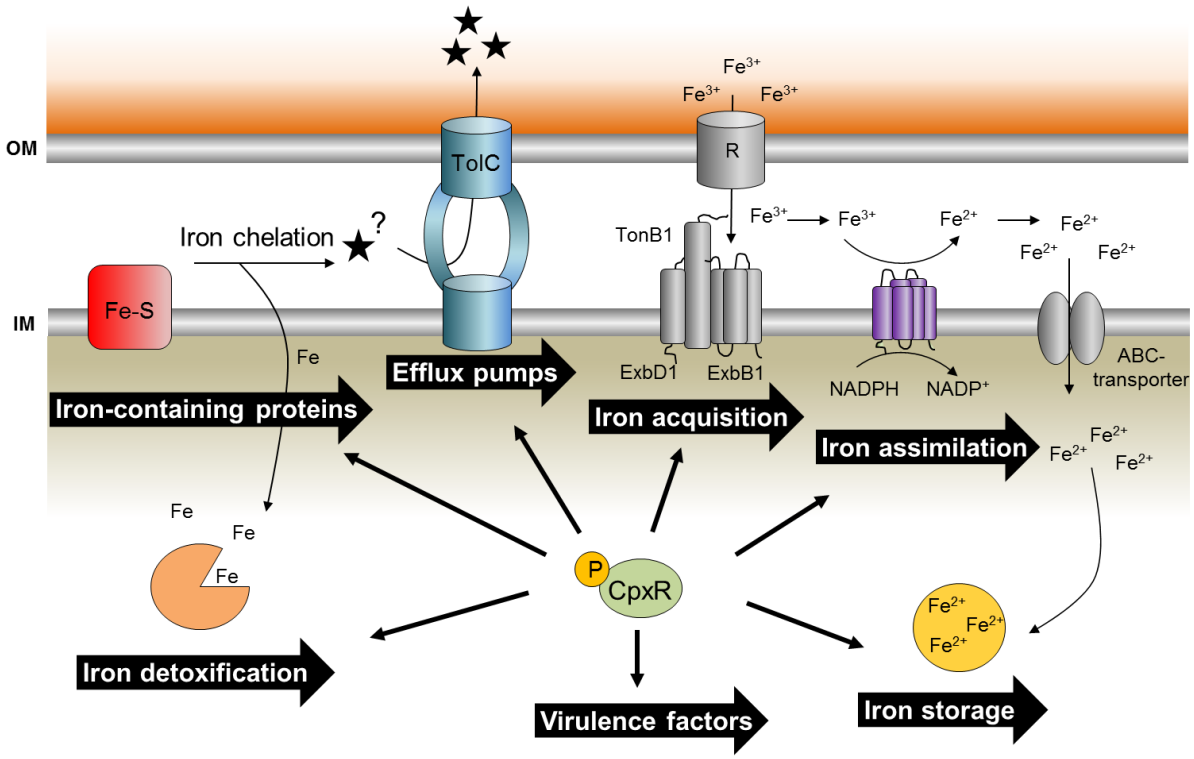

Figure 5 - 1 Summary of the activation of the Cpx response in *V. cholerae*.

Activation of the Cpx response in *V. cholerae* leads to changes in gene expression of genes involved in iron acquisition, including outer membrane (OM) receptors (R) and the TonB1-dependent acquisition system. The Cpx pathway also regulates expression of putative ferric reductases involved in iron assimilation (i.e. conversion of ferric (Fe^{3+}) to ferrous (Fe^{2+}) iron) using as a source of electrons cytosolic NADPH to generate the reduced ferrous iron. Then, specific ABC-transporters mediate entry of the iron to the cytoplasm compartment. At the same time activation of the Cpx response regulates the expression of iron-storage/binding encoding genes and detoxification proteins that may be involved in reduce the toxic effect of iron accumulation in the cytoplasm during the stress response. Finally, the Cpx pathway may play a role in responding to the accumulation of metabolic waste (i.e. represented by a star) that may be involved in iron chelation. (IM) inner membrane.

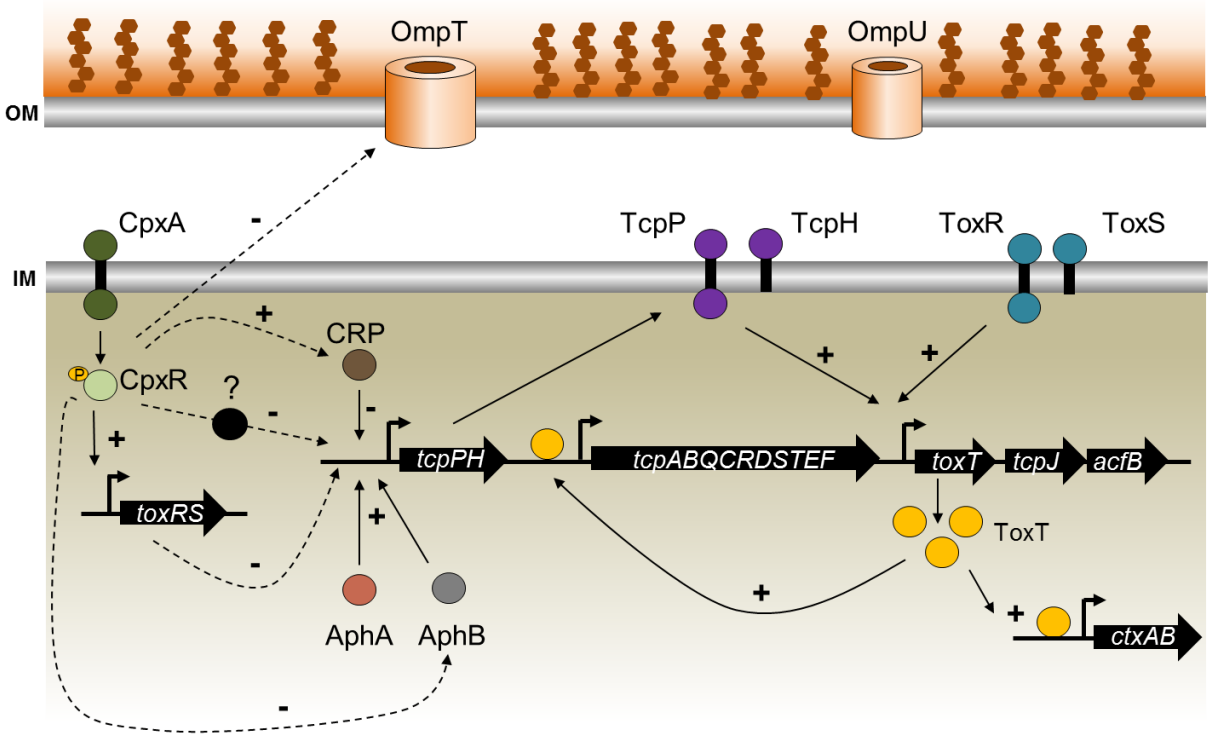

Figure 5 - 2 Regulatory network of the Cpx pathway and virulence factors regulation in *V. cholerae.*

Activation of the Cpx response leads to downregulation of some virulence factors, CT and TCP, in *V. cholerae* at transcriptional level. This negative regulation of CT and TCP by the Cpx response is mainly mediated by changing the activity of the negative regulator of the *tcpPH* promoter, CRP. Therefore, this leads to a reduction of the expression of *tcpP* and the downstream regulator of the ToxR regulatory cascade, ToxT. In addition, CpxR may regulate CT and TCP through the regulation of the virulence regulators ToxR and AphB. Finally, activation of the Cpx response mediated changes in the outer membrane (OM) composition by repressing the expression of the OM porin OmpT in a ToxR-independent manner. (IM) inner membrane.

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6 Appendix A: Characterization of the effect of Cpx pathway activation on the virulence attributes of enteropathogenic *Escherichia coli* (EPEC) clinical isolates

6.1 Introduction

Enteropathogenic *E. coli* (EPEC) is an important agent of the infantile diarrhea associated with *E. coli* pathotypes. It infects children worldwide having high prevalence in community and hospital settings (494). It is estimated that EPEC is the cause of 1.3 million deaths annually in children younger than 5 years (495). The hallmark of EPEC infections is the ability to produce attaching and effacing (A/E) lesions on the brush border surface of small intestinal enterocytes (Figure 6-1) (496). This phenotype is also produced by other *E. coli* pathotypes such as enterohaemorrhagic *E. coli* (EHEC) (497).

For the development of the attaching and effacing (A/E) lesions and intestinal colonization, EPEC first uses adhesins to attach to intestinal epithelial cells; some of them include the type IV bundle-forming pilus (BFP), a type III secretion system (T3SS), EspA filaments (arising from the T3SS), and intimin (498-500). Several studies have suggested that the BFP is one of the major adhesion factors in EPEC involved on the initial binding to host epithelial cells (Figure 6-1A) (226) and its operon is encoded in the *E. coli* adherence factor (EAF) plasmid, in which its transcriptional activator called plasmid encoded regulator A (PerA) is also encoded (501). The major structural component of the BFP is BfpA (Figure 6-1B), which is a bundlin protein (502). BfpB (Figure 6-1B) is a secretin protein which is the major component of the outer membrane subassembly of the BFP in EPEC (503).

After EPEC attaches to epithelial cells using the BFP, it leads to the formation of actin-rich pedestals by the translocation of virulence factors through the T3SS (Figure 6-1A) (504). The T3SS is encoded on a 35 kb pathogenicity island (PAI) known as the locus of enterocyte effacement (LEE) (505). The LEE also encodes secreted translocators (i.e. EspA, EspB and

EspD), effector proteins (i.e. EspF, EspG, EspH, Map and EspZ), and proteins required for intimate bacterial attachment (i.e. Intimin and Tir) to the host epithelial cells (497). For example, the secreted translocator protein, EspA (Figure 6-1B), is required for the formation of filamentous extensions to the T3SS needle complex (506, 507). In addition, the secreted translocators EspB and EspD are required for the formation of the translocation pore in the host cell membrane (Figure 6-1B), which allows the translocation of other effector proteins into the host cell cytosol (508).

As described in Section 1.4.3.3., several studies in different species suggest that the Cpx pathway regulates the expression of virulence-linked extracellular appendages at transcriptional and posttranscriptional levels (253). In wild-type EPEC strain E234869, the Cpx pathway inhibits the expression of BFP (225) and the secretion of some T3S translocator and effector proteins (i.e. EspA, EspB, EspD) (227). In addition, our research group recently found that the Cpx pathway mediates post-transcriptional regulation of the T3SS and motility (222). Although previous studies identified a relationship between the BFP or T3SS assembly and the Cpx signaling pathway in wild-type EPEC strain E2348/69 (203, 225), whether this relationship pertains to other EPEC clinical isolates (CI) is not known. We hypothesized that, as in the wild-type EPEC strain E2348/69, the Cpx pathway would also regulate the expression of BFP and the T3SS in EPEC clinical isolates in the same fashion. The main aim of this project was to evaluate the effect of the Cpx pathway in five EPEC clinical isolates on the elaboration of the BFP, T3SS and motility.

6.2 Materials and Methods

6.2.1 *Bacterial strains and plasmids*

All *E. coli* strains used in this study are listed in Table 6-1. The five EPEC clinical isolates (CI), previously characterized for their capacity to cause detachment of Lec2 cell monolayers, a techinique used for studying LA pattern and invasion of eukaryotic cells (509), were used for the characterization of the role of the Cpx pathway in EPEC clinical isolates. All strains were stored at -80ºC in LB broth containing 10% glycerol.

6.2.2 *Growth conditions*

For routine growth, EPEC strains were cultured in Luria-Bertani (LB) broth. For analysis of BFP profiles, EPEC strains were cultured in Dulbecco's modified Eagle's medium (DMEM/F12) (Gibco) containing 0.1 M Tris (pH 7.4) as previously described (203). Solid LB medium was prepared in the same manner as LB broth, except that bacto agar (Difco) was added to a final concentration of 1.5%. For motility assays, the final concentration of bacto agar was 0.3%. LB agar plates were supplemented with the appropriate antibiotics. Antibiotics (all from Sigma) were used at the following concentrations in selective media: chloramphenicol (Cam), 25 μg/ml; kanamycin (Kan), 50 μg/ml and amikacin (Amk), 3 μg/ml. For activation of the Cpx pathway the *nlpE* gene was overexpressed from the pCA-*nlpE* plasmid (510) with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma).

6.2.3 *Electrocompetent cells and transformation*

A single colony of each EPEC strain was used to inoculate 5 ml of LB broth with the appropriate antibiotic and grown at 37°C overnight with aeration. Then the culture was subcultured 1:50 into 50 ml of LB broth in a 125 ml Erlenmeyer flask and incubated at 37°C and

225 revolutions per minute (r.p.m). After the subcultures reached an OD_{600} of 0.5 to 0.6, they were dispensed into sterile 50 ml conical centrifuge tubes and centrifuged at 4000 rpm for 10 minutes at 22ºC. The supernatants were discarded, pellets were resuspended in 2 ml of LB broth, and 1 ml was transferred to a 1.5 ml microcentrifuge tube. Tubes were heated at 50°C for 30 minutes. After the heat shock treatment, tubes were chilled on ice for 2 minutes. Tubes were centrifuged at 13.200 r.p.m at 22ºC for 1 minute. Supernatants were discarded, and pellets were washed with 1 ml of 10% glycerol. Then tubes were centrifuged as above and pellets were washed with 500 µl of 10% glycerol. Then tubes were centrifuged as above and cells were washed with 200 µl of 10% glycerol. Tubes were centrifuged as above and supernatants were discarded, and pellets were resuspended in 1 ml of 10% glycerol and subsequently stored at -80ºC.

A 50 µl aliquot of electrocompetent cells was thawed on ice, and then 2.5 µl of purified plasmid was added and mixed. Cells were transferred to an electroporation cuvette (Molecular BioProducts) and after chilling on ice for 2 minutes, electroporation was carried out in a MicroPulser electroporator (Bio-Rad) set to 2.5 kV. Immediately after electroporation, 1 ml of LB broth was added to the cuvette and cells were transferred to a 1.5 ml microcentrifuge tube and incubated for 3 hours at 37°C. Transformants were plated on selective media and incubated at 37ºC overnight.

6.2.4 *BFP Western blot assay*

In order to assess the effect of activation of the Cpx pathway on BFP expression, whole-cell lysates were collected by subculturing the EPEC clinical isolates and reference strains in DMEM/F12 as previously described (203). Briefly, subcultures (1:50) were grown at 37°C with aeration until they reached an OD_{600} of 0.5. The strains harbouring the pCA-24N or pCA-*nlpE*

plasmids were induced after 1.5 hours of growing with 0.1 mM IPTG and then returned to 37°C. Whole-cell lysates were collected in a 1.5 ml microcentrifuge tube and centrifuged at 13.200 r.p.m for 2 minutes. Cell pellets were resuspended in 50 µl of loading buffer (dH_2O containing 6% SDS, 20% glycerol, 125 mM Tris-HCl pH 6.8, 10% β-mercaptoethanol, 0.2% bromophenol blue). Whole cell lysates were electrophoresed on an SDS–10% PAGE gels in a Mini-PROTEAN electrophoresis system (Bio-Rad) using running buffer (1 g SDS, 3.03 g Tris, 14.41 g glycine in 1 liter) and transferred to nitrocellulose membranes (Bio-Rad) as previously described (186). Non-specific reactions were blocked by incubation in a 10% skim milk solution for 3 hours. The blots were incubated with a 1:10.000 dilution of anti-BfpA or anti-BfpB (511, 512), a 1:10.000 dilution of anti- MBP-CpxR (186), or a 1:50.000 dilution of anti-bacterial alkaline phosphatase (BAP) and then with a 1:25.000 dilution of secondary anti-rabbit immunoglobulin G-alkaline phosphatase conjugates (Sigma). Blots were developed as previously described (225).

6.2.5 *T3SS secretion profile assay*

In order to assess the effect of activation of the Cpx pathway on the T3S of the T3 secreted proteins EspD, EspB and EspA, secretion profiles were obtained as previously described (513). Briefly, subcultures (1:50) of the EPEC strains were grown in DMEM media at 37°C with 5% CO_2 statically for 7 hours. At 2 hours post-subculturing the strains harbouring the pCA-24N or pCA-*nlpE* plasmids were induced with 0.1 mM IPTG. After the 7 hours of incubation, subcultures were collected in a 1.5 ml microcentrifuge tube and centrifuged at 13.200 r.p.m for 2 minutes. Supernatants were collected in another 1.5 ml microcentrifuge tube and centrifuged as above. 900 µl of the supernatants were chilled on ice for 5 minutes and secreted proteins were precipitated from the supernatant with 10% TCA for 30 minutes on ice. After precipitation, tubes were centrifuged at 13.200 r.p.m at 4°C for 10 minutes, supernatants were discarded, and

secreted proteins were washed with 1 ml of ice cold acetone for 30 minutes at -80°C. Then, tubes were centrifuged at 13.200 r.p.m at 4°C for 10 minutes, the supernatants were discarded and pellets were air dried for 1 hour. Pellets were resuspended in 10 µl of loading buffer. Secreted proteins were resolved by SDS-12% PAGE gels followed by staining with Coomassie brilliant blue (Bio-Rad).

6.2.6 *Motility assay*

Two microliters of overnight cultures of EPEC strains were inoculated onto 0.3% LB agar plates. All the inoculations were made in triplicate. The diameter of the swim zones was recorded after 14 hours of growth in the presence of the appropriate antibiotics. Plates of strains carrying either the vector control pCA-24N or the overexpression plasmid pCA-*nlpE* were supplemented with 0.1 mM IPTG.

6.3 Results

6.3.1 *Overexpression of nlpE activates the Cpx pathway in EPEC clinical isolates*

Previously it was shown that overexpression of the lipoprotein NlpE in *E. coli* induces the Cpx pathway (190). To assess the effect of activation of the Cpx pathway on virulence determinants in the EPEC clinical isolates, we first transformed the pCA-24N and pCA-*nlpE* plasmids and determined if overexpression of *nlpE* in the EPEC clinical isolates also induces the Cpx response, by assessing changes in the expression of the response regulator CpxR. The *cpxRA* operon has been shown to be auto-regulated in *E. coli* K-12 strain MC4100 and also in EPEC type strain E2348/69 (186, 223), and so the levels of CpxR serve as a marker of Cpx response activation. Overexpression of *nlpE* lead to an increase in expression of CpxR in all five EPEC clinical isolates compared to the non-inducing condition or in the vector control (Figure 6-2). As a

control, the expression of CpxR was compared between the EPEC E2348/69 reference strain and the E2348/69 *cpxR::kan* mutant. The loss of the CpxR band in the E2348/69 *cpxR::kan* mutant confirmed that the protein increased in expression by NlpE overexpression was CpxR (Figure 6-2F) and this is indicative of the activation of the Cpx pathway in the EPEC clinical isolates.

6.3.2 *BFP is down regulated by the Cpx pathway in EPEC clinical isolates*

To assess if the Cpx envelope stress response system affects the expression of BFP in the five EPEC clinical isolates (CI) as previous shown in the EPEC E2348/69 reference strain (225), we first determined if in these clinical isolates there was an induction of BFP expression under virulence inducing conditions (i.e. DMEM/F12 medium). The expression of the BfpA and BfpB subunits of the BFP in the transformed EPEC clinical isolates was assessed by Western blot analysis. There were differences in the expression of BFP amongst the EPEC clinical isolates. For example, when EPEC clinical isolates CI2, CI3 and CI5 were grown in DMEM/F12 media, a virulence factor induction media, there was no induction of the expression of BfpA and BfpB (Figure 6-3, lanes 2, 3 and 5). The same phenotype was observed in the JPN15 strain, which is a modified version of strain E2348/69 which lacks the EAF plasmid that encodes the BFP operon (Figure 6-3). However, BfpA and BfpB were produced in the DMEM/F12 media in EPEC clinical isolates CI1 and CI4 (Figure 6-3, lanes 1 and 4), as was observed in the EPEC type strain (E2348/69) (Figure 6-3). To determine whether Cpx pathway activation would reduce BFP expression in EPEC clinical isolates CI1 and CI4 as is the case with the EPEC E2348/69 reference strain, we compared BFP synthesis in EPEC clinical isolates transformed with the vector control to those carrying the pCA-*nlpE* plasmid upon induction with IPTG. We found that when the Cpx system was activated, there was a reduction in the expression of BfpA and BfpB in

the EPEC clinical isolate CI1 (Figure 6-4A), but there was no effect of IPTG addition on BfpA and BfpB levels in the EPEC clinical isolate CI4 (Figure 6-4B).

6.3.3 *T3SS is down regulated by the Cpx pathway in some EPEC clinical isolates*

To assess whether the Cpx system was involved in the regulation of T3S in the EPEC clinical isolates as has been shown previously in the EPEC reference strain (E2348/69) (227), we determined the effect of overexpression of *nlpE* on the expression of T3SS translocators, such as the EPEC secreted proteins (Esp) (i.e. EspD, EspA and EspB). This was assessed by analysis of secreted proteins of bacterial cultures growing in DMEM medium. We found that the T3S substrates were only produced and secreted in the EPEC clinical isolates CI3, CI4 and CI5 in DMEM medium (Figure 6-5). Interestingly, as previously shown, we observed that overexpression of NlpE from pCA-*nlpE* drastically decreased the secretion of the EspD, EspA and EspB proteins compared to strains transformed with the empty vector (Figure 6-5, compare lanes 2 and 4) or in the absence of IPTG (Figure 6-5, compare lanes 3 and 4).

6.3.4 *Motility profile of EPEC clinical isolates when the Cpx pathway is activated.*

De Wulf and collaborators (194) showed that the Cpx pathway in *E. coli* regulates the expression of the *motABcheAW* operon and determined that mutations in Cpx related genes (i.e. *cpxR* and *cpxA*) have an effect on swimming phenotypes (194). In addition, our lab reported that the periplasmic protease DegP, which is Cpx regulated, is required for the Cpx-mediated inhibition of motility in *E. coli* (222). To examine whether the Cpx pathway was exerting the same effect on motility in the EPEC clinical isolates, we determined the effect of induction of the Cpx response on the swim zones of the five EPEC clinical isolates that were transformed with either the vector control or the pCA-NlpE plasmid in agar plates with and without IPTG.

Surprisingly, motility was not affected in any of the EPEC clinical isolates studied when the Cpx pathway was activated (Figure 6-6).

6.4 Conclusions and future directions

In this study, we showed that the Cpx pathway negatively controls the expression of BfpB in only one of the EPEC clinical isolates tested (i.e. CI1). This effect on envelope appendages is similar to what Vogt and collaborators (225) found in the E2348/69 reference strain, where the induction of the Cpx pathway inhibits transcription of the genes encoing the BFP during the activation of the Cpx response (225). Our studies showed that virulence factor production in DMEM medium may be regulated differently in some of the EPEC clinical isolates studied, since no BFP expression was detectable in three of the five CIs under conditions shown to up-regulate virulence factor production in EPEC strain E2348/69. However, the inhibitory effect of the Cpx response on expression of the BFP and the T3SS in EPEC is conserved. Our results provide further evidence in support of a conserved physiological role for the Cpx envelope stress response in the regulation of virulence-linked extracellular appendages in EPEC strains, a function that has also been shown in other pathogens such as *Vibrio cholerae*, *Yersinia pseudotuberculosis*, and *Legionella pneumophila*, among others (222, 225, 227, 252, 360, 421). Regulation of virulence factors by the Cpx response thus represents another conserved function for the Cpx response, in addition to it's well-established role in the regulation of cell envelope integrity (183). It is most likely that, under envelope stress conditions, the Cpx response is required to diminish protein traffic in the envelope (253). Finally, the data show that the effect of the Cpx response on motility in *E. coli* seems to be strain-specific.

It would be important that future studies determine if the Cpx inhibition of the BFP seen in EPEC clinical isolate 1 occurs at a transcriptional level, as it does in the EPEC E2348/69 reference strain (225). Also, it would be interesting to investigate if the inhibition of some of the T3SS translocators studied here (i.e. EspD, EspA and EspB) in three EPEC clinical isolates are regulated at a transcriptional level by CpxR, as in the EPEC E2348/69 reference strain (227). In addition, it would be interesting to increase our number of clinical samples in order to examine the role of the Cpx pathway in more EPEC clinical isolates.

6.5 Table and Figures

Table 6 - 1 Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Source or reference
Strains		
E2348/69	Prototypical EPEC O127:H6 strain	(514)
JPN15	Spontaneous pEAF-cured derivative of E2348/69	(515)
ALN88	E2348/69 *cpxR*::Kan; (Kan^R)	(203)
ALN195	E2348/69 *cpxA24*; (Amk^R)	(227)
EPEC CI1	EPEC clinical isolate 1, O111:H4	(509)
EPEC CI2	EPEC clinical isolate 2, O78:H11	(509)
EPEC CI3	EPEC clinical isolate 3, O142:H6	(509)
EPEC CI4	EPEC clinical isolate 4, O119:H6	(509)
EPEC CI5	EPEC clinical isolate 5, O142:H6	(509)
NA145	EPEC CI1 carrying pCA-24N plasmid; (Cam^R)	This study
NA150	EPEC CI2 carrying pCA-24N plasmid; (Cam^R)	This study
NA156	EPEC CI3 carrying pCA-24N plasmid; (Cam^R)	This study
NA162	EPEC CI4 carrying pCA-24N plasmid; (Cam^R)	This study
NA168	EPEC CI5 carrying pCA-24N plasmid; (Cam^R)	This study
NA174	EPEC CI1 carrying pCA-nlpE plasmid; (Cam^R)	This study
NA180	EPEC CI2 carrying pCA-nlpE plasmid; (Cam^R)	This study
NA186	EPEC CI3 carrying pCA-nlpE plasmid; (Cam^R)	This study
NA192	EPEC CI4 carrying pCA-nlpE plasmid; (Cam^R)	This study
NA198	EPEC CI5 carrying pCA-nlpE plasmid; (Cam^R)	This study
Plasmids		
pCA-24N	Expression vector with IPTG-inducible promoter; (Cam^R)	(510)
pCA-*nlpE*	pCA24N-based *nlpE* overexpression vector; (Cam^R)	(510)

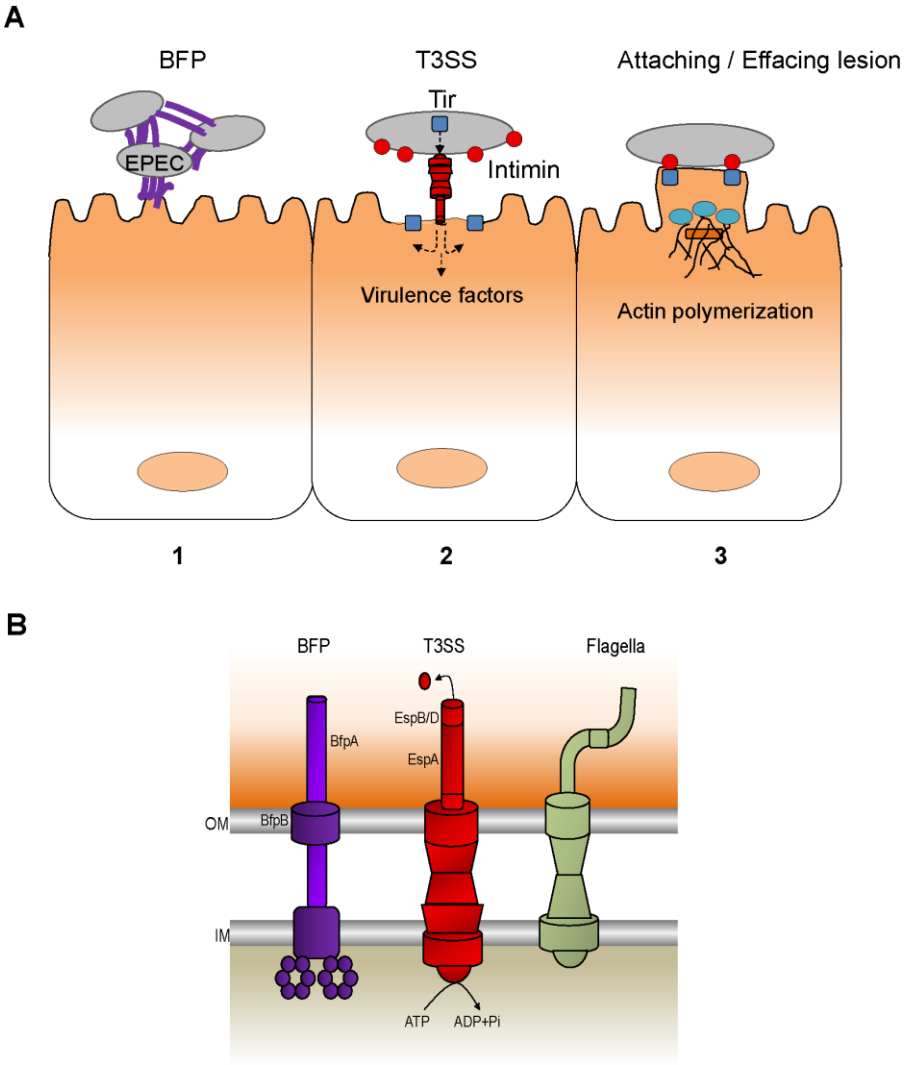

Figure 6 - 1 Enteropathogenic *Escherichia coli* (EPEC) adherence to epithelial cells and formation of the attaching and effacing (A/E) lesions.

(A) EPEC pathogenesis is thought to proceed by three steps, first EPEC attaches to the small bowel epithelial cells through the bundle-forming pilus (BFP). Second, EPEC secretes several virulence factors through a type III secretion system (T3SS), including the Tir receptor into host cells. Third, there is an interaction between Tir and intimin, an outer membrane protein in EPEC. This interaction allows the generation of a cytoskeletal rearrangement which results in the formation of a pedestal-like structure. (B) Major virulence-linked extracellular appendages in EPEC: BFP, T3SS and flagella. OM: outer membrane; PP: periplasmic space; IM: inner membrane. Figure adapted from (516).

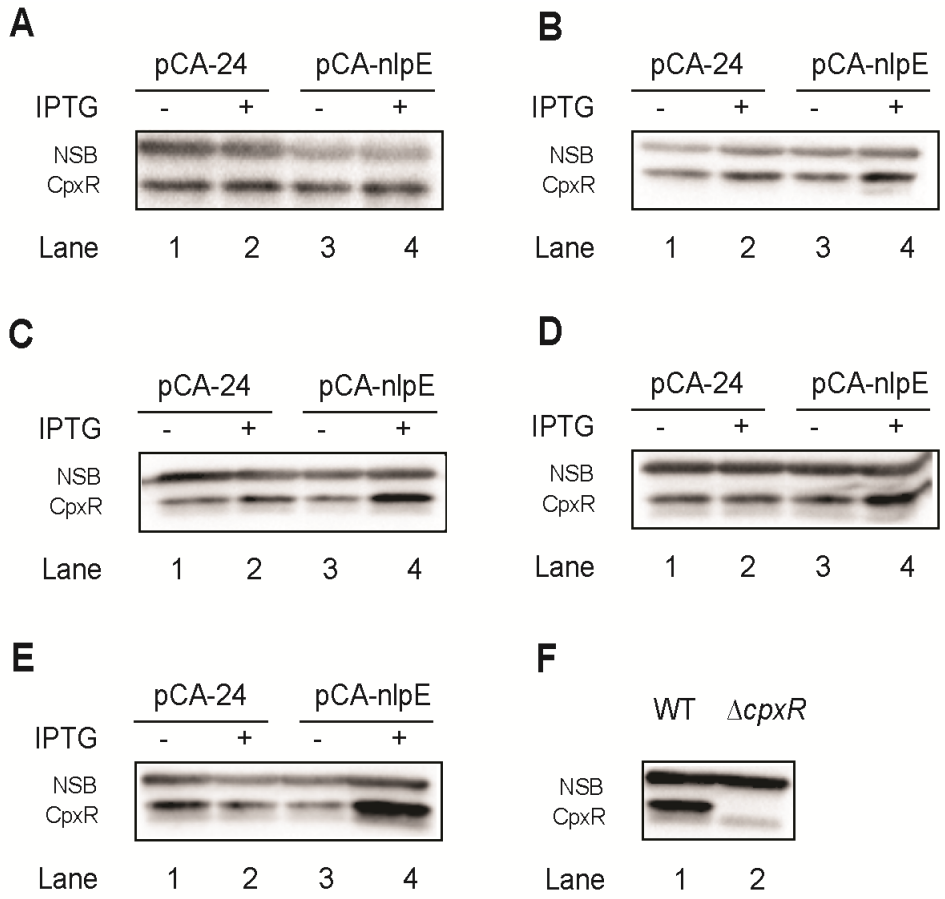

Figure 6 - 2 Overexpression of *nlpE* activates the Cpx pathway in EPEC clinical isolates.

Whole cell lysates of EPEC clinical isolates 1 (A), 2 (B), 3 (C), 4 (D), 5 (E), E2348/69 and E2348/69 *cpxR::Kan* (F) grown in DMEM/F12 at 37°C. Whole cell lysates were resolved by 10% SDS-PAGE followed by a Western blot against anti-CpxR. The Cpx pathway was activated by adding 0.1 mM IPTG. Samples were collected from each strain at least three times; one representative Western blot is shown. Non-specific bands (NSB).

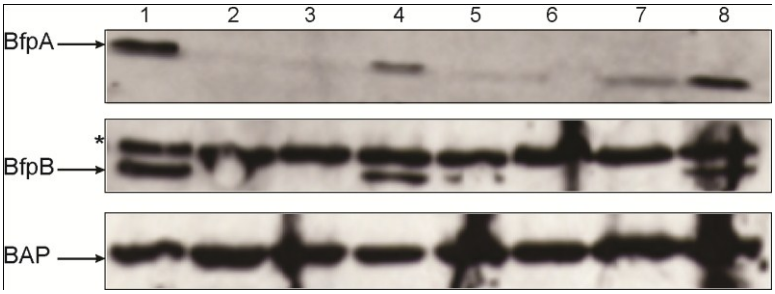

Figure 6 - 3 Expression of the BFP subunits, BfpA and BfpB, in EPEC clinical isolates.

Western blot analysis of BfpA, BfpB and BAP in EPEC clinical isolates (CI) CI1 (lane 1), CI2 (lane 2), CI3 (lane 3), CI4 (lane 4), CI5 (lane 5), JPN15 strain (lane 6), ALN195 strain (lane7) and E2348/69 EPEC strain (lane 8). Whole-cell lysates were collected by growing the EPEC clinical isolates in DMEM/F12 at 37°C. Samples were collected from each strain at least three times; one representative Western blot is shown. Asterisk denotes non-specific bands.

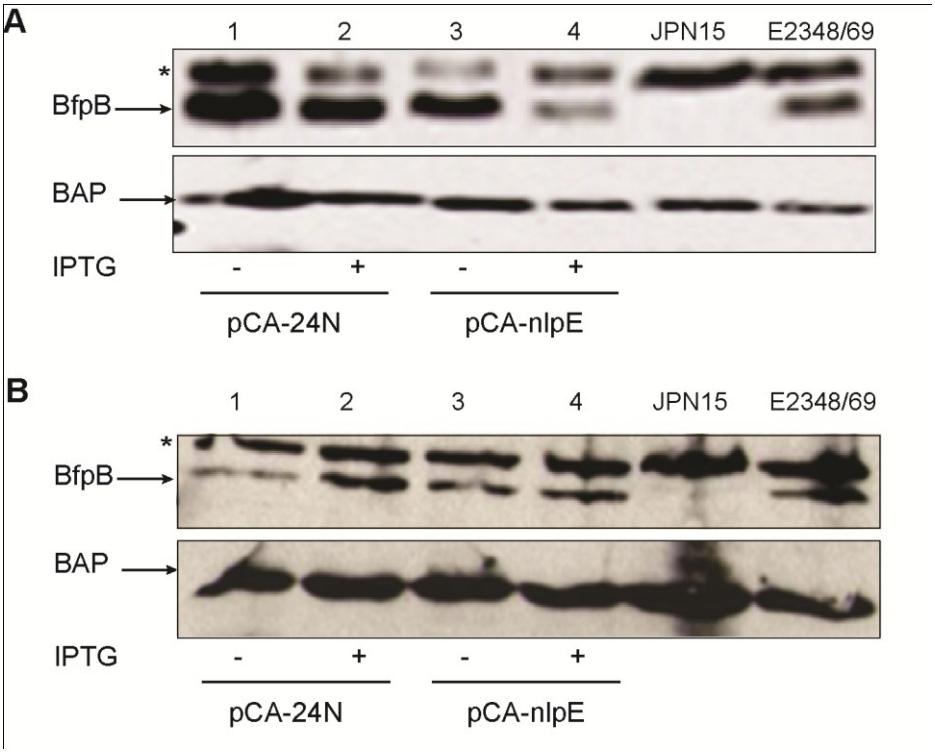

Figure 6 - 4 Effect on BFP expression when the Cpx pathway is activated in EPEC clinical isolates.

Western analysis of BfpB and BAP in EPEC clinical isolates (CI) 1 (A) and CI4 (B) were performed when the Cpx pathway was activated by overexpression of *nlpE* with 0.1 mM IPTG from the pCA-*nlpE* plasmid. JPN15 is a modified version of strain E2348/69 which lacks the EAF plasmid that encodes the BFP operon. Samples were collected from each strain at least three times; one representative Western blot is shown. Asterisks denote non-specific bands.

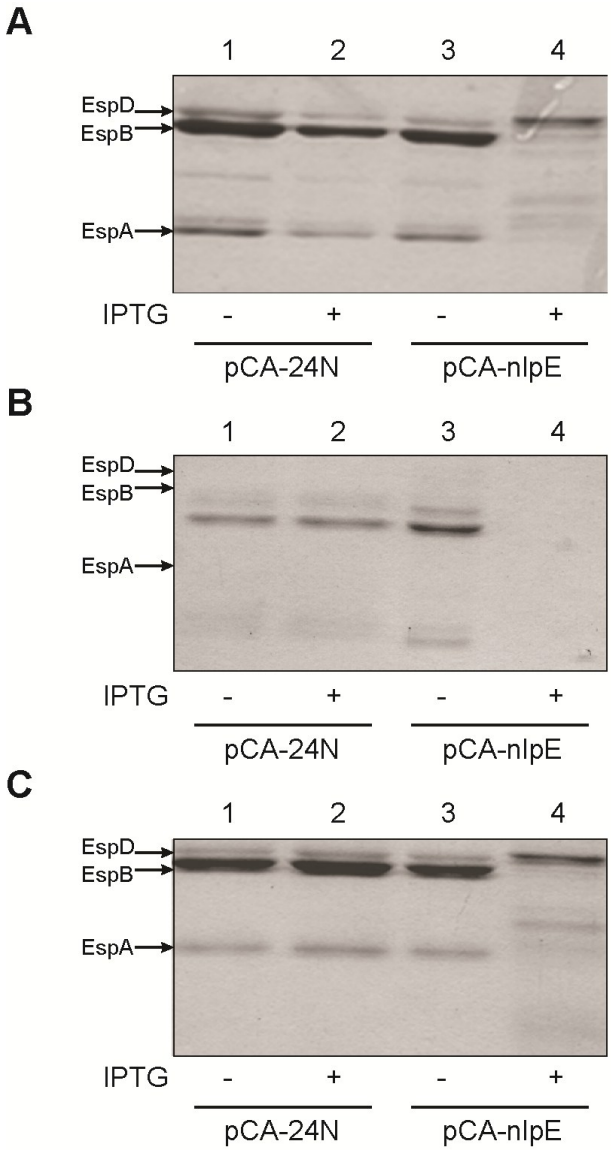

Figure 6 - 5 Activation of the Cpx pathway in EPEC clinical isolates diminishes the levels of EPEC T3 secreted proteins (Esp).

Secretion protein analysis of EspD, EspB and EspA was determined by growing the EPEC clinical isolates in DMEM at 37°C. Secreted proteins were precipitated from the supernatant with 10% TCA and were visualized by SDS-PAGE and Coomassie blue staining. For activation of the Cpx pathway 0.1 mM IPTG was added to the subcultures of transformed EPEC clinical isolates with either the vector control (pCA-24N) (lanes 1-2) or pCA-*nlpE* plasmid (lanes 3-4). Secretion profiles for EPEC clinical isolate CI3 (A); CI4 (B) and CI5 (C). Samples were collected from each strain at least three times; one representative SDS-PAGE gel is shown.

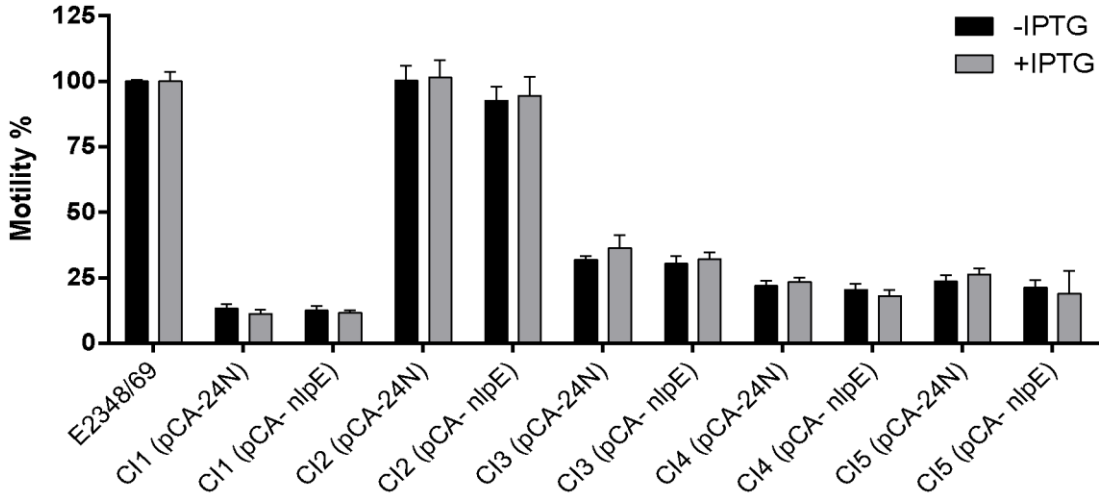

Figure 6 - 6 Motility profile of EPEC clinical isolates when the Cpx pathway is activated.

Overnight cultures for E2348/69 EPEC wild type and five EPEC clinical isolates were inoculated onto 0.3 % LB agar plates. The growth diameter was recorded after 14 hours for strains carrying the vector control pCA24N or the overexpression plasmid pCA-*nlpE*. All the inoculations were made in triplicate.

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7 Appendix B: Identification of the bacterial T6SS targets of *Vibrio cholerae* O37 serogroup strain V52

7.1 Introduction

The type VI secretion system (T6SS) in *Vibrio cholerae* O37 serogroup strain V52 is encoded in the virulence-associated secretion (VAS) gene cluster (418). Previously, Pukatzki and collaborators (418) showed that transposon insertions in the T6SS encoded genes (i.e. *vasK, vasA, vasF* and *vasH*) reduced the ability of amoeboid killing by *V. cholerea* V52. Additionaly, in that study two VAS encoded genes, *hcp-1* and *hcp-2,* were identified using microarray analysis of a *vasH* deletion mutant compared with wild-type V52. They encode hemolysin coregulated proteins (418). All the T6SS described so far have a common characteristic: the export of the Hcp protein outside of the cell through the T6SS (517). Overall, this secretion system is encoded by many Gram-negative bacteria and it is found as a gene cluster that consists of approximately 15–25 conserved genes (517). It is likely that the T6SS is the most common of the large specialized secretion systems in bacteria (62). In addition, there is evidence of an evolutionary relationship between some components of the T6SS and the tail proteins of bacteriophages T4 and λ (518).

The T6SS has been implicated in various processes. For example, it has been proposed that T6S is related to the resistance of *V. cholerae* V52 to *Dictyostelium* amoeba predation and contributes significantly to the cytotoxicity that it displays towards amoebae and mammalian macrophages (418); survival of *Burkholderia mallei* in macrophages (519); *Rhizobium leguminosarum* symbiosis of the nodulating pea and fixation of nitrogen (520); biofilm formation by enteroagregative *E. coli* (521); and modulation of quorum sensing in *V. anguillarum* (522); among others.

Although, it has been reported that the T6SS plays an important role in bacterial-host interactions (Figure 7-1), it has been proposed that the T6SS is mainly important for

interbacterial interactions by conferring toxicity towards other bacteria (Figure 7-1) (523). For example, MacIntyre and collaborators (419) showed that the T6SS of *V. cholerae* V52 has antimicrobial properties and that it may promote *V. cholerae* survival in the environment and/or during colonization of a host (419). However, the target(s) in prey cells of effectors that are secreted by the T6SS are unknown. The main aim of this project was to develop a high throughput killing platform in order to identify the *E. coli* gene product(s) that confer sensitivity to T6SS-dependent killing by *V. cholerae* V52. This project was developed and done in collaboration with Daniel Unterweger (a PhD student in Dr. Stefan Pukatzki's lab, Department of Medical Microbiology & Immunology; University of Alberta).

7.2 Materials and Methods

7.2.1 *Bacterial strains and growth conditions*

A total of 3985 *E. coli* K-12 BW25113 knockout mutants obtained from the Keio collection (524) were used as prey for the high throughput killing screen. *V. cholerae* V52 and the isogenic mutant *V. cholerae* V52 Δ*vasK* (418) were used as predator strains for the competition assays. Bacterial strains were routinely grown and maintained in Luria-Bertani (LB) broth with the appropriate antibiotics at 37°C with shaking at 225 r.p.m. and stored at -80°C in LB broth containing 10% glycerol. Antibiotics (all from Sigma) were used at the following concentrations in selective media: streptomycin (Sm), 100 µg/ml, and kanamycin (Kan), 50 µg/ml.

7.2.2 *High throughput killing screen*

To assess the susceptibility of the Keio mutants to resist T6SS-mediated killing, an *in vitro* growth competition assay was developed in collaboration with Daniel Unterweger (a PhD student in Dr. Stefan Pukatzki's lab, Department of Medical Microbiology & Immunology; University of

Alberta). *E. coli* K-12 BW25113 knockout mutants (kanamycin-resistant) were inoculated into 200 µl of LB broth in 96-well microtiter plates and grown at 37°C with aeration overnight. Overnight cultures of the predator strains (i.e. *V. cholerae* V52 and *V. cholerae* V52 Δ*vasK* mutant, as positive and negative controls, respectively, for T6SS-mediated killing) were inoculated into 5 ml of LB broth with the appropriate antibiotic. The next day, each, the predator and the prey strains were subcultured in LB broth in a 1:50 dilution, and were grown at 37°C with aeration until they reached an OD_{600} of 0.5. Prey and predator were mixed in a 1:1 ratio (200 µl of final volume) in a 96-well microtiter plate and 2 µl of the mixture were spotted onto a 96 well plate containing 150 µl of pre-dried LB agar. After an incubation of 4 hours at 37˚C, each well was washed with 50 µl of LB broth, supplemented with 50 µg/ml kan, and re-inoculated into 100 µl of LB broth supplemented with 50 µg/ml kan and allowed to grow at 37˚C with shaking. The OD_{600} was obtained after 1 (time 0) and 5 (time 1) hours of growth. The difference in OD_{600} (time 1- time 0) was determined and compared with the difference in the OD_{600} readings for the positive (i.e. *E. coli* K-12 BW25113 *spy::kan* mutant and *V. cholerae* V52) and negative controls (i.e. *E. coli* K-12 BW25113 *spy::kan* mutant and *V. cholerae* V52Δ*vasK*) for T6SS mediated killing assay.

7.2.3 *Standard bacterial killing assay*

To confirm positive hits obtained in the high throughput killing screen, the susceptibility of *E. coli* K-12 BW25113 knockout mutants to T6SS-mediated killing by the predator strain *V. cholerae* V52 was assessed as described previously (419). Briefly, predator and prey strains were grown as lawns on LB plates plus selective antibiotics and resuspended in LB broth. Prey and predator were mixed in a 1:10 ratio and spotted onto pre-dried LB agar plates. After an incubation of 4 hours at 37˚C, each spot was harvested, serially diluted, and spotted onto LB

plates plus selective antibiotics and were incubated overnight at 37°C. Surviving prey were enumerated as CFU/mL.

7.3 Results

To determine the bacterial T6SS targets of *V. cholerae* V52, a high throughput killing platform was developed and standardized in collaboration with Daniel Unterweger (a PhD student in Dr. Stefan Pukatzki's lab, Department of Medical Microbiology & Immunology; University of Alberta). A total of 3985 mutants of the *E. coli* Keio library (524) were screened for their susceptibility to T6SS-mediated killing by *V. cholerae* V52 (Table 7-1). For the Keio mutants that appeared to be resistant to T6SS killing in the high throughput killing screen, none were found to be resistant to T6SS-mediated killing when a standard killing assay (419) was performed (data not shown). One possible explanation for this phenomenon could be that in quite a few cases those mutants showed very low OD_{600} values at time 0, therefore after subtracting this value from that at time 1, it could be interpreted as a positive hit. A low OD_{600} reading at time 0 could due to a growth phenotype conferred by the particular mutation, or alternatively, perhaps the washing step was occasionally inefficient, such that less *E. coli* cells were washed off the spot after the 4 hour incubation. However, we suggest the inability to find possible targets could be attributed to the nature of T6SS mediated toxicity by *V. cholerae* V52, since it is now known that more than one effector is required for efficient killing (see below).

7.4 Conclusions and future directions

Our first goal for this project was to develop a high throughput killing screen to identify the bacterial target(s) in *E. coli* of *V. cholerae* T6SS-mediated killing. We successfully screened

3985 mutants from the Keio library (524). However, at the time that this project was finished the number of effector proteins known to be involved in conferring T6SS-mediated killing in *V. cholerae* V52 was unknown. Recently, Unterweger and collaborators (420) reported that various *V. cholerae* genomes encode three effector proteins that are required for the full T6SS-mediated intraspecies killing phenotype. In addition, for each effector protein, there is an immunity protein which is encoded immediately downstream from the effector that prevents the producing strain from being killed by it's own T6SS (420). For example, normally *V. cholerae* C6706 is resistant to killing by *V. cholerae* V52. However, when *V. cholerae* C6706 lacks one antitoxin, C6706 is killed in a T6SS-mediated manner (525). The toxin and antitoxin systems related to the T6SS-mediated killing were elucidated through bioinformatics and screening of a library of transposon mutants carrying mutations in the genes encoded on the genetic island containing the T6SS in *V. cholerae* (420, 526-528).

The *V. cholerae* T6SS effector proteins against prokaryotes are VCA0123 (VgrG3), VCA0020 (VasX) and VC1418 (TseL), and the individual toxins are associated with the VCA0124, VCA0021, VC1419 antitoxins respectively (420, 526-528). VgrG3 encodes a peptidoglycan degrading enzyme which may assist with the delivery of accessory T6SS toxins (526). VasX encodes a putative pore-forming toxin that when absent is unable to kill a mutant lacking the corresponding immunity gene (527). VasX was previously characterized as being required for the T6SS-mediated killing of *D. discoideum* (529). TseL is predicted to have a lipase domain that when mutated loses its antibacterial effect, suggesting that the lipase activity is required for the T6SS-mediated killing (527, 528). We hypothesize that because the T6SS-mediated bacterial killing is multi-factorial and the effectors target different cellular processes,

our screen could not identify the targets in the prey organism, since removal of one target would still leave other putative targets available for the toxic effects of distinct T6SS effectors.

In addition, studies have been reported in other bacterial species that implicating the T6SS in targeting toxins to other bacteria during competition between bacterial species, such as *Pseudomonas aeruginosa* (530). Hood and collaborators (531) characterized the T6SS in *P. aeruginosa*. In that study three substrates of the T6SS were identified, which they named proteins Tse1–3 (type six exported 1–3). These proteins are coregulated with the secretory system. One of these Tse proteins, Tse2, was found to be the toxin component of a toxin-immunity system that arrested the growth of some prokaryotic and eukaryotic cells when it was expressed intracellularly. This suggests that Tse2 might provide a fitness advantage to *P. aeruginosa*, especially in scenarios where that involve cell-cell interactions with other species, such as in chronic infections in patients with CF (531).

Burkholderia thailandensis is another example of how the T6SS confers advantages in fitness during competition with bacterial species. Schwarz and collaborators (532) characterized the five T6SS encoding gene clusters (T6SS-1, 2, 4, 5 and 6) in *B. thailandensis*. The T6SS-1 was required for *B. thailandensis* to persist in mixed biofilm assays (i.e. flow chambers) with *P.putida,* because mutations in some genes of the T6SS-1 (e.g. *clpV-1*) diminished its ability to persist and proliferate in the presence of *P. putida* within 24 hours. This phenotype was not due to defects in adhesion, because T6SS-1 mutant strains adhered equally well compared to the wild type strain and *P. putida*. On the other hand, T6SS-5 was required for virulence in mice because when mice were infected with T6SS-5 *B. thailandensis* mutants by aerosol-challenge and then monitored for ten days, they exhibited increased survival compared to those infected with the wild type strain (532).

Future studies are required to elucidate the *E. coli* gene product(s) that confer sensitivity to T6SS-dependent killing by *V. cholerae* V52. For this purpose our standardized high throughput killing platform could be used with *V. cholerae* V52 mutants that express only one of the three identified T6SS effector proteins (i.e. *V. cholerae* V52 ΔVCA0123 ΔVCA0020, ΔVCA0123 ΔVC1418 and Δ VCA0020 ΔVC1418) as predators and the *E. coli* knockout collection as prey. Another future direction in order to address this question could be conducting the screening when the effectors (i.e. VCA0123, VCA0020 and VCA0123) are overexpressed, instead of mutating the effector proteins, which may have a strong T6SS-mediated killing effect to the prey due to redundancy of the effector proteins alone. Identification of the possible(s) T6SS targets in the prey organism will aid in understanding the molecular mechanisms behind inter-species bacterial competition.

7.5 Table and Figure

Table 7 - 1 OD_{600} ΔT1-T0 of the *E.coli* Keio mutants after T6SS-mediated killing.

GenoBase ID	OD_{600} ΔT1-T0	GenoBase ID	OD_{600} ΔT1-T0	GenoBase ID	OD_{600} ΔT1-T0	GenoBase ID	OD_{600} ΔT1-T0	GenoBase ID	OD_{600} ΔT1-T0
thrA	0.08	rimJ	0.35	rfbA	0.08	ygjM	0.15	yjjB	-0.12
thrB	0.04	yceH	0.01	rfbD	0.05	ygjN	-0.03	yjjQ	0.02
thrC	-0.13	mviM	0.00	rfbB	-0.13	ygjQ	-0.08	bglJ	0.00
yaaX	0.07	flgN	0.16	galF	-0.09	ygjR	-0.89	fhuF	-0.19
yaaA	-0.01	flgM	-0.07	wcaM	0.38	ygjU	0.01	rsmC	-0.12
yaaJ	-0.03	flgM	-0.09	wcaL	0.03	ygjV	-0.04	holD	0.01
talB	-0.15	flgA	0.07	wcaK	-0.06	uxaA	-0.01	rimI	-0.11
mog	-0.15	flgB	0.05	wzxC	0.05	uxaC	-0.01	yjjG	0.02
yaaH	0.05	flgC	-0.13	wcaJ	0.00	exuT	0.26	osmY	0.03
yaaW	-0.03	flgD	-0.11	manB	-0.10	exuR	-0.02	yjjU	0.07
yaaI	0.02	flgE	-0.09	manC	0.00	yqjA	-0.05	yjjV	0.02
dnaK	0.01	flgF	-0.20	wcaI	0.13	yqjB	-0.21	yjjW	0.03
dnaJ	-0.01	flgG	-0.04	nudD	0.03	yqjD	-0.02	yjjI	0.04
nhaA	0.01	flgH	0.05	fcl	-0.05	yqjE	0.03	deoA	0.03
nhaR	0.22	flgI	-0.03	gmd	-0.05	yqjK	0.07	deoB	0.14
rpsT	-0.03	flgJ	-0.01	wcaF	-0.05	yqjF	-0.01	deoD	0.10
ileS	-0.02	flgK	-0.01	wcaE	-0.04	yqjG	-0.11	yjjJ	-0.27
fkpB	0.10	flgL	0.07	wcaD	0.06	yhaH	-0.05	lplA	0.08
rihC	0.32	rluC	-0.32	wcaC	0.01	yhaI	-0.05	smp	0.26
carA	0.00	yceF	0.04	wcaB	0.06	yhaJ	0.08	serB	0.08
carB	0.02	yceD	0.00	wcaA	0.02	yhaK	0.04	radA	0.01
yaaV	0.04	rpmF	-0.08	wzc	0.04	tdcD	-0.07	nadR	0.00
caiF	0.43	fabH	-0.01	wzb	-0.01	tdcC	-0.05	yjjK	0.06
caiD	0.49	fabF	-0.03	wza	0.00	tdcB	-0.01	slt	0.02
caiC	0.01	pabC	0.01	yegH	-0.06	tdcA	0.25	trpR	-0.07
caiB	-0.02	yceG	0.01	yegH	0.05	yhaB	0.01	gpmB	0.17
caiA	0.00	ycfH	-0.03	asmA	0.05	yhaC	-0.05	rob	-0.13
caiT	0.67	ptsG	0.06	dcd	0.03	garK	0.09	creA	0.02
fixA	0.01	fhuE	-0.02	udk	-0.01	garL	0.07	creB	0.06
fixB	0.56	ycfF	-0.06	yegE	-0.04	garP	0.00	creC	0.04
fixC	0.44	ycfL	0.06	yegE	0.02	garD	0.04	creD	-0.14
fixX	0.10	ycfM	0.00	alkA	-0.05	sohA	-0.01	arcA	0.04
yaaU	0.16	ycfN	-0.75	yegD	-0.11	yhaV	0.21	yjjY	-0.02
yabF	0.13	nagZ	-0.18	yegI	-0.04	agaZ	0.07	lasT	0.04
kefC	0.15	ycfP	-0.04	yegJ	-0.05	agaV	-0.04	thrL	0.01
apaH	0.21	ndh	0.07	yegK	-0.06	agaW	0.19	htgA	-0.04
apaG	-0.06	ycfJ	-0.03	yegL	-0.01	agaS	-0.07	htgA	-0.30
ksgA	0.03	ycfQ	0.04	yegM	-0.02	agaY	0.00	hokC	0.04
pdxA	0.05	ycfQ	-0.02	yegN	-0.33	agaB	0.10	yaaY	-0.02
surA	-0.25	ycfR	0.00	yegO	-0.02	agaC	0.01	caiE	-0.01
djlA	-0.10	JW1099	-0.04	yegO	-0.03	agaD	0.43	yabI	0.01
yabP	0.18	JW1099	0.02	yegB	0.01	agaI	0.13	leuB	-0.09
rluA	-0.09	mfd	-0.09	yegB	0.04	yraH	0.01	leuB	-0.06
hepA	-0.03	ycfT	0.07	baeS	0.11	yraI	0.10	yacG	0.08
polB	0.02	ycfX	0.05	baeR	0.00	yraJ	-0.05	hpt	0.05
araC	0.18	ycfX	0.06	yegP	0.03	yraK	-0.01	yadD	0.09
yabI	0.05	cobB	0.08	ogrK	-0.08	yraM	-0.02	ligT	-0.04
thiQ	-0.05	cobB	0.09	JW2068	0.00	yraN	0.09	ligT	-0.31
thiP	-0.02	ymfA	0.00	yegR	-0.07	yraO	0.33	eriC	0.03
tbpA	-0.02	potD	0.03	yegS	0.05	yraP	0.20	cdaR	0.03
yabN	0.17	potC	0.03	yegS	0.00	yraQ	0.04	yaeI	-0.03
setA	0.01	potB	-0.17	gatR	0.26	yhbO	-0.06	JW5015	-0.01
leuD	0.05	potA	0.59	gatD	-0.18	yhbP	0.25	yaeF	0.01
leuC	0.12	pepT	-0.05	gatC	0.02	yhbQ	0.36	yafD	0.02
leuA	0.03	ycfD	0.04	gatB	0.00	yhbS	0.39	mltD	-0.04
leuL	-0.16	phoQ	-0.09	gatZ	-0.04	yhbT	-0.06	yafV	-0.04
leuO	0.22	phoP	0.05	fbaB	0.02	yhbU	0.01	fadE	0.11

ilvI	0.08	trmU	-0.16	fbaB	-0.04	yhbW	-0.07	mbhA	0.03
ilvH	0.06	ymfB	0.00	yegT	0.00	mtr	0.09	yafX	-0.02
fruR	0.19	ymfB	-0.05	yegU	0.07	deaD	0.01	ykfF	0.05
mraZ	0.08	ymfC	0.08	yegV	0.05	nlpI	-0.01	JW5025	-0.08
mraZ	0.13	icd	-0.08	yegW	-0.09	JW3133	0.03	mmuP	-0.02
mraW	0.04	ymfD	0.14	yegX	0.01	rpsO	0.00	mmuP	-0.10
mutT	0.00	ymfE	0.01	thiD	0.00	truB	0.05	JW5028	0.03
yacG	0.04	lit	0.08	thiM	0.02	rbfA	0.03	JW5029	-0.01
yacF	-0.05	intE	0.33	yohL	0.08	argG	-0.19	JW5029	-0.01
coaE	0.07	ymfG	0.49	yohM	-0.09	secG	0.53	yagV	0.09
guaC	0.17	ymfH	0.61	yohN	-0.05	mrsA	0.06	ykgK	0.05
hofC	0.48	ymfI	-0.15	yehA	0.04	folP	-0.06	JW5032	-0.03
hofB	0.02	ymfJ	0.01	yehB	0.02	rrmJ	0.06	JW5032	-0.05
ppdD	-0.04	ymfL	0.01	yehC	-0.02	yhbY	-0.06	ykgL	0.06
nadC	0.01	ymfM	0.01	yehD	-0.14	greA	-0.03	JW5034	-0.01
ampD	0.12	ymfN	0.00	yehE	0.10	dacB	0.02	JW5034	-0.03
ampE	-0.08	ymfR	0.01	mrp	0.04	yhbE	0.19	rpmE2	-0.07
aroP	-0.16	ymfO	-0.03	yehI	-0.02	sfsB	-0.25	rpmE2	-0.12
pdhR	0.29	ymfP	0.10	yehK	-0.03	yrbA	-0.35	ykgA	0.04
aceE	0.05	ymfQ	-0.01	yehL	-0.02	yrbC	0.02	ykgB	-0.02
aceF	-0.16	ycfK	0.01	yehM	0.01	yrbD	-0.12	ykgI	0.09
lpdA	-0.01	ycfK	-0.05	yehP	-0.03	yrbE	0.00	ykgC	-0.06
yacH	0.04	tfaE	0.14	yehQ	0.02	yrbF	0.10	ykgC	-0.03
acnB	0.13	stfE	0.00	yehR	0.01	yrbG	0.06	ykgE	0.03
yacL	0.09	pinE	-0.09	yehS	0.12	yrbH	0.01	ykgG	0.05
speD	0.02	mcrA	0.05	yehT	0.04	yrbI	0.00	yahH	0.08
speE	0.00	JW5173	0.01	yehT	0.07	yhbG	0.14	yahM	0.05
yacC	0.00	elbA	0.05	yehU	0.06	rpoN	0.05	cynR	0.07
cueO	-0.02	ycgX	-0.03	mlrA	0.04	yhbH	0.15	mhpT	0.21
gcd	0.10	ycgE	-0.07	yehW	-0.02	ptsN	-0.11	JW5047	-0.10
yadG	-0.03	ycgF	-0.02	yehX	-0.02	yhbJ	-0.02	JW5049	-0.03
yadH	-0.03	ycgF	0.01	yehY	-0.55	ptsO	-0.05	JW5049	-0.01
yadI	0.00	ycgZ	0.07	yehZ	-0.02	yrbL	0.02	yaiU	0.07
yadE	0.02	ymgA	0.23	bglX	-0.19	mtgA	0.02	ampH	-0.01
panD	-0.08	ymgB	0.06	dld	0.02	elbB	-0.02	yaiZ	0.06
yadD	-0.01	ymgC	-0.08	yohC	0.00	arcB	-0.02	psiF	-0.05
panC	0.03	ycgG	-0.03	yohD	-0.09	yhcC	0.15	psiF	-0.03
panB	-0.11	ymgF	-0.03	yohF	-0.18	gltB	0.21	proY	-0.05
yadC	-0.03	ydeU	0.06	yohG	-0.03	gltD	0.14	yajI	0.02
yadK	0.05	ymgD	-0.02	yohG	-0.02	gltF	-0.05	thiJ	-0.01
yadL	0.04	JW5178	-0.02	yohI	-0.02	yhcA	0.10	yajQ	0.07
yadM	-0.03	JW5179	-0.01	yohJ	0.05	yhcD	0.23	yajR	0.09
htrE	0.11	JW1162	0.06	yohK	0.08	yhcE	0.02	bolA	0.04
ecpD	0.06	minC	0.04	cdd	0.08	yhcF	0.21	mdlB	-0.07
yadN	-0.07	ycgJ	0.02	sanA	0.04	yhcG	0.30	mdlB	-0.02
pcnB	0.03	ycgK	-0.01	yeiT	-0.05	yhcH	0.08	ylaB	0.06
yadB	-0.33	ycgL	0.00	yeiA	-0.05	nanE	0.01	ylaC	0.07
dksA	-0.49	ycgM	-0.03	mglC	-0.15	nanT	0.06	JW5064	0.02
sfsA	-0.14	ycgM	0.10	mglA	0.43	nanA	0.03	ybbM	0.09
hrpB	-0.02	ycgN	-0.02	mglB	-0.09	nanR	0.19	ybbN	0.05
mrcB	-0.04	hlyE	-0.03	galS	0.27	dcuD	0.04	JW5068	-0.05
fhuA	0.03	umuD	-0.02	yeiB	-0.10	sspB	0.05	JW5069	0.01
fhuC	-0.07	umuC	0.03	yeiG	0.04	sspA	-0.02	ybcJ	0.04
fhuD	0.00	nhaB	0.00	cirA	-0.09	yhcM	0.03	sfmH	0.00
fhuB	0.14	fadR	0.48	lysP	-0.11	degQ	-0.01	sfmF	-0.08
eriC	-0.11	ycgB	-0.02	yeiE	-0.01	mdh	-0.13	sfmF	-0.04
yadS	0.12	dadA	-0.08	yeiH	0.07	argR	-0.06	fimZ	0.10
btuF	0.05	dadX	-0.07	nfo	-0.04	yhcO	-0.01	JW5075	-0.05
mtn	-0.11	ldcA	-0.04	yeiI	-0.02	yhcP	-0.04	JW5075	-0.15
dgt	0.36	mltE	-0.09	yeiI	-0.01	yhcQ	0.08	ylcG	0.08
degP	-0.02	ycgR	-0.03	yeiJ	0.07	yhcS	0.02	nmpC	-0.02
cdaR	0.00	ymgE	-0.12	rihB	0.12	tldD	0.05	rzpD	0.02
yaeH	-0.02	ycgY	0.02	nsr	-0.01	yhdP	-0.02	rzoD	0.02
yaeI	0.00	treA	0.01	yeiM	0.06	cafA	0.04	ybcV	0.07
yaeI	-0.01	ycgS	-0.03	yeiC	-0.25	yhdE	0.00	cusS	0.09
glnD	-0.07	ycgT	-0.02	fruA	-0.13	yhdA	0.03	ybdF	0.06

hlpA	-0.04	ycgV	0.06	fruK	0.17	yhdH	-0.01	hokE	0.02
rnhB	0.28	ychF	-0.01	fruB	-0.24	yhdT	-0.01	fepA	0.04
ldcC	0.19	ychH	-0.02	setB	-0.13	panF	-0.03	citF	-0.06
yaeR	-0.02	ychM	0.02	yeiW	0.29	prmA	0.01	lipB	0.07
rof	0.00	sirB2	-0.01	yeiQ	-0.04	yhdG	-0.03	ybeB	0.03
yaeP	0.02	sirB1	0.01	yeiR	-0.04	fis	-0.09	ybeQ	-0.03
yaeQ	-0.02	chaA	0.01	yeiU	-0.08	yhdU	-0.12	gltI	-0.04
yaeJ	-0.09	chaB	0.07	spr	0.04	envR	0.65	gltI	-0.10
cutF	-0.03	chaC	0.01	rtn	-0.05	acrE	0.09	ybfG	-0.03
yaeF	-0.11	ychN	0.05	yejA	-0.05	acrF	-0.03	ybfG	-0.06
yaeB	0.07	ychN	0.05	yejB	-0.12	yhdV	0.03	kdpE	0.09
rcsF	0.14	ychO	0.07	yejE	-0.36	yhdW	-0.05	abrB	0.03
metQ	0.13	narL	0.05	yejF	-0.03	yhdZ	0.04	ybgO	0.05
metI	0.26	narX	0.21	rsuA	0.02	yrdB	0.02	ybgQ	0.05
metN	-0.01	narK	0.01	yejH	0.02	aroE	-0.08	tolB	-0.01
yaeD	-0.03	narG	0.02	rplY	0.32	smg	1.12	tolB	-0.05
dkgB	0.01	narH	0.08	yejK	-0.12	sun	0.20	JW5101	0.00
yafC	0.28	narJ	0.70	yejL	0.09	trkA	-0.09	ybhT	0.09
yafD	0.01	narI	0.11	JW2177	0.00	mscL	0.00	ybhJ	0.04
yafE	-0.04	tpr	-0.14	narP	0.07	yhdL	-0.12	ybhF	-0.01
gloB	0.12	purU	-0.34	ccmH	-0.03	zntR	0.22	ybhF	-0.15
yafS	0.04	ychJ	0.08	dsbE	-0.01	yhdN	0.04	ybiX	0.08
rnhA	0.11	ychJ	0.11	ccmF	-0.03	rpmJ	-0.37	ybiM	-0.01
dnaQ	0.05	ychK	0.01	ccmE	0.00	pioO	-0.08	ybiN	-0.03
yafT	0.06	hnr	0.02	ccmD	0.04	gspA	0.06	ybiO	0.00
yafU	-0.01	galU	0.68	ccmC	0.09	gspC	0.34	fsaA	0.11
yafV	0.02	hns	0.26	ccmB	0.09	gspD	-0.07	yliA	0.06
ivy	-0.01	tdk	-0.18	napC	-0.08	gspE	0.61	yliB	0.02
fadE	0.06	JW1227.5	-0.01	napH	0.21	hofF	-0.13	ybjG	-0.02
lpcA	-0.06	adhE	-0.09	napG	-0.18	hofG	0.11	ybjI	0.04
yafJ	0.00	ychE	-0.07	napA	0.20	hofH	0.72	ybjK	0.05
yafK	0.04	oppA	0.05	napD	0.04	gspJ	0.22	JW5115	-0.02
yafQ	0.09	oppB	0.04	napF	0.44	gspK	0.19	ybjT	0.02
dinJ	-0.04	oppC	0.01	eco	0.08	hofD	0.01	hcr	-0.04
yafL	0.02	oppD	0.02	mqo	0.14	bfr	-0.06	dmsA	-0.07
yafM	0.00	oppF	0.07	yojI	-0.17	bfd	0.02	ycaM	0.02
fhiA	0.02	yciU	-0.03	alkB	-0.01	chiA	0.10	ycaI	0.05
dinB	0.03	cls	0.13	ada	-0.06	tufA	0.04	ssuC	-0.04
yafN	0.27	kch	0.01	ompC	-0.02	yheL	-0.03	ycbQ	0.03
yafO	0.02	yciI	0.02	yojN	-0.15	yheM	-0.02	ycbV	-0.02
yafP	0.07	yciA	0.03	rcsB	-0.10	yheN	-0.05	ycbF	0.08
ykfJ	0.04	yciA	0.01	rcsC	-0.08	fkpA	0.88	ycbW	-0.01
prfH	-0.08	ispZ	-0.01	JW2207	-0.01	slyX	0.02	ycbX	0.07
pepD	-0.02	yciC	-0.04	atoS	-0.11	slyD	0.31	ymbA	-0.05
gpt	-0.02	ompW	-0.05	atoC	-0.02	yheV	0.07	yccS	-0.07
yafA	0.06	yciE	-0.02	atoD	-0.07	kefB	-0.13	mgsA	-0.07
crl	-0.14	yciF	-0.04	atoA	-0.04	yheR	0.12	yccU	0.01
phoE	-0.10	yciG	-0.03	atoE	0.06	yheS	0.01	yccX	-0.03
proB	0.02	trpA	-0.09	atoB	0.00	yheT	0.16	etp	0.04
proA	0.01	trpB	0.13	yfaP	-0.01	yheU	0.05	ymcD	0.02
ykfI	0.00	trpC	-0.25	yfaQ	-0.01	prkB	-0.03	cspH	-0.01
yafW	0.05	trpD	-0.01	yfaS	0.00	yhfA	0.02	torS	0.00
ykfH	-0.03	trpE	-0.01	yfaS	-0.02	crp	-0.10	ymdF	-0.04
ykfG	0.00	trpL	-0.02	yfaT	-0.02	argD	0.06	ycdG	0.09
yafX	-0.01	trpH	0.08	yfaA	-0.02	pabA	0.04	ycdH	0.00
ykfF	0.02	yciQ	0.02	ubiG	0.04	fic	0.30	ycdL	0.03
ykfB	-0.06	yciL	0.03	ubiG	-0.06	yhfG	-0.03	JW5140	-0.05
yafY	-0.02	btuR	-0.10	yfaL	0.05	ppiA	0.32	ycdN	-0.29
ypjK	0.01	yciK	-0.04	yfaL	-0.03	tsgA	0.00	ycdR	0.19
yafZ	-0.05	sohB	-0.05	yfaE	0.05	nirB	0.37	ycdT	0.02
ykfA	0.04	yciN	0.00	inaA	0.03	nirD	0.04	JW5145	0.02
perR	0.26	cysB	0.04	glpQ	-0.08	nirC	0.01	ycdZ	-0.02
insM	-0.02	acnA	-0.08	glpT	-0.01	cysG	-0.38	JW5148	-0.07
ykfC	0.06	pgpB	0.03	glpA	0.00	yhfL	0.08	JW5148	0.07
mmuM	0.06	yciS	-0.02	glpB	-0.09	yhfM	-0.13	ymdC	-0.03
afuC	0.11	yciM	-0.03	glpC	-0.14	yhfQ	0.03	yceK	0.01

afuB	-0.19	pyrF	0.11	JW2238.5	-0.01	yhfS	-0.06	yceP	0.00
JW0258	-0.03	yciH	-0.04	yfaU	0.03	yhfT	0.05	flgH	-0.01
yagB	-0.11	osmB	-0.09	yfaV	-0.17	php	0.03	yceF	-0.07
yagA	0.02	yciT	0.03	yfaW	0.01	yhfW	0.14	plsX	-0.06
yagE	-0.04	JW1277	-0.02	yfaW	0.12	yhfX	0.12	plsX	-0.22
yagF	-0.05	yciR	0.01	yfaX	0.03	yhfZ	0.30	ycfM	0.03
yagH	0.06	rnb	-0.24	cinA	-0.01	gph	0.01	ycfP	0.03
yagI	-0.06	yciW	-0.01	yfaZ	0.02	rpe	0.17	ycfQ	0.02
argF	0.04	ycjD	0.09	yfaO	0.01	dam	-0.04	ycfS	0.02
yagJ	-0.07	sapF	0.31	yfaO	-0.02	damX	0.30	JW5163	-0.05
yagK	-0.06	sapD	0.06	ais	0.07	aroB	-0.33	JW5163	-0.17
yagL	0.09	sapC	0.11	yfbE	0.04	hofQ	-0.02	ymfA	0.05
yagM	-0.18	sapB	0.03	yfbE	0.02	yrfB	-0.02	ycfC	-0.02
yagN	0.12	sapA	0.12	yfbF	-0.01	yrfC	0.01	ymfE	0.01
intF	-0.26	ymjA	-0.03	yfbF	-0.02	mrcA	0.08	JW5167	-0.01
yagP	0.06	ycjJ	-0.15	yfbG	-0.06	nudE	0.02	ymfI	0.05
yagQ	0.10	ycjL	0.09	yfbH	0.00	yrfG	0.03	JW5169	-0.04
yagR	-0.03	ycjC	0.01	yfbI	0.02	hslR	0.06	ymfP	0.00
yagR	-0.01	aldH	0.05	yfbI	-0.01	yhgE	0.08	ymfS	-0.04
yagS	0.09	ordL	0.08	yfbW	-0.01	pckA	0.14	stfE	0.07
yagS	0.07	goaG	-0.11	yfbJ	-0.01	envZ	0.03	JW5173	0.08
yagT	-0.03	pspF	0.31	pmrD	-0.02	ompR	0.04	ycgG	-0.03
yagU	0.01	pspA	0.30	menE	0.00	greB	-0.04	ydeU	0.06
yagU	0.00	pspB	0.01	menC	-0.13	yhgF	0.23	ymgD	0.04
ykgJ	-0.01	pspC	0.16	menB	0.01	feoA	-0.13	JW5178	0.04
yagV	-0.01	pspD	0.15	yfbB	-0.01	feoB	0.08	JW5179	-0.03
yagW	0.05	pspE	-0.03	yfbB	-0.13	yhgG	0.01	ycgN	-0.01
yagX	0.17	ycjM	0.05	menF	0.02	yhgA	0.02	hlyE	0.05
yagY	0.03	ycjN	0.00	elaB	0.48	bioH	0.04	dsbB	-0.07
yagZ	0.02	ycjO	0.13	elaA	0.53	yhgI	-0.12	dsbB	0.10
ykgK	0.04	ycjP	-0.06	elaC	-0.26	gntT	-0.02	JW5183	0.10
ykgL	-0.01	ycjQ	0.22	yfbK	0.08	malQ	0.04	ycgO	0.01
eaeH	-0.04	ycjR	0.01	yfbK	-0.10	malP	-0.02	ycgC	-0.03
ykgA	0.04	ycjS	0.04	yfbL	0.01	malT	0.22	ycgS	0.07
ykgB	0.07	ycjT	0.00	yfbL	-0.04	rtcB	0.03	ycgT	0.07
ykgI	-0.22	ycjU	-0.09	yfbN	0.11	rtcR	-0.07	dhaR	-0.10
ykgD	0.09	ycjV	-0.14	yfbO	0.32	glpR	0.19	ychM	-0.03
ykgE	-0.04	ompG	0.05	nuoN	0.03	glpG	-0.01	JW5191	-0.10
ykgF	0.02	ycjW	0.05	nuoM	-0.06	glpE	0.29	JW5194	0.03
ykgG	0.04	ycjX	-0.04	nuoL	-0.05	glpD	-0.24	tonB	-0.08
ykgH	0.09	ycjF	-0.03	nuoK	0.06	yzgL	0.05	yciO	0.00
betA	-0.22	tyrR	-0.07	nuoJ	0.07	glgP	0.38	yciQ	0.04
betB	-0.28	tpx	0.00	nuoI	0.06	glgA	0.09	yciX	0.07
betI	-0.07	ycjG	0.00	nuoH	0.05	glgC	-0.02	yciX	-0.04
betT	0.17	mpaA	-0.05	nuoG	0.03	glgX	-0.02	yciX	-0.31
yahA	0.06	JW1319.5	-0.01	nuoF	0.10	glgB	-0.13	yciW	0.03
yahB	0.35	ycjY	-0.03	nuoE	-0.06	yhgN	0.17	ycjK	-0.04
yahC	0.05	ycjZ	0.04	nuoB	-0.05	gntK	-0.19	ycjK	-0.11
yahD	-0.05	JW1321.5	0.02	lrhA	0.04	yhhW	0.02	ycjR	-0.02
yahE	0.03	mppA	-0.03	yfbQ	-0.01	yhhX	-0.03	JW5203	-0.02
yahF	0.01	ynaI	-0.11	yfbR	-0.02	yhhY	0.06	JW5203	-0.42
yahG	-0.05	ynaJ	0.03	yfbS	0.01	yhhZ	0.08	abgA	0.05
yahH	0.02	ydaA	0.08	yfbS	-0.23	yrhA	-0.11	ydaM	-0.02
yahI	0.15	fnr	-0.05	yfbT	0.08	yrhB	0.00	ydaQ	0.07
yahJ	-0.06	ogt	-0.01	yfbT	-0.02	ggt	0.04	lar	0.01
yahK	-0.16	abgT	0.01	yfbU	-0.06	yhhA	0.04	sieB	-0.10
yahL	0.03	abgB	-0.02	yfbV	0.13	ugpQ	0.01	sieB	-0.17
yahM	0.25	abgA	-0.03	ackA	0.03	ugpC	0.22	ydaG	-0.01
yahN	-0.04	abgR	0.04	pta	0.03	ugpE	0.42	ydaW	0.05
yahO	0.07	ydaL	0.02	yfcC	0.06	ugpA	-0.03	rzpR	-0.02
yahO	-0.03	ydaM	0.02	yfcC	-0.09	ugpB	-0.04	rzoR	-0.02
prpR	0.01	ydaM	0.02	yfcD	-0.02	livF	0.03	ydbJ	0.04
prpB	0.13	ydaN	0.03	yfcE	-0.02	livG	-0.01	ydbL	-0.02
prpC	0.21	ydaN	-0.02	yfcE	0.03	livM	-0.03	paaD	0.03
prpD	-0.04	JW1336.5	0.02	yfcF	0.10	livH	0.09	paaK	-0.07
prpE	0.04	dbpA	-0.04	yfcG	-0.01	livK	-0.04	paaK	-0.21

codB	0.07	ydaO	0.02	yfcG	-0.01	yhhK	0.03	ydbD	-0.16
codA	-0.14	intR	0.23	folX	0.02	livJ	-0.04	hrpA	0.04
cynR	-0.08	ydaQ	-0.14	yfcH	0.02	yhhF	0.05	cybB	0.06
cynT	0.05	ydaC	-0.02	yfcH	-0.12	yhhM	0.07	hokB	-0.03
cynS	0.09	recT	0.00	yfcI	-0.02	yhhN	0.02	hokB	-0.09
cynX	0.06	recE	0.44	hisP	0.00	zntA	0.02	ydcI	0.04
lacA	0.02	racC	-0.04	hisM	0.00	sirA	-0.12	JW5227	0.01
lacY	0.00	ydaE	-0.03	hisQ	-0.01	acpT	-0.12	ydcM	0.09
lacI	-0.63	kil	-0.04	hisJ	0.07	nikA	-0.02	ydcO	0.10
mhpR	0.22	ydaF	-0.05	argT	0.00	nikB	0.03	JW5230	-0.07
mhpA	0.02	ydaG	0.06	ubiX	0.04	nikC	0.24	JW5231	-0.05
mhpB	0.07	ydaS	-0.02	purF	0.13	nikD	0.06	JW5231	0.03
mhpC	-0.37	ydaT	-0.10	cvpA	0.04	nikE	-0.04	ydcX	0.07
mhpD	-0.01	ydaU	-0.12	dedD	-0.03	nikR	-0.09	yncA	0.01
mhpF	-0.24	ydaV	0.07	dedA	-0.06	yhhH	0.13	ansP	-0.02
mhpE	0.21	ydaW	0.01	truA	-0.30	yhhI	0.10	yncH	0.00
yaiL	0.10	rzpR	-0.07	usg	-0.01	yhiI	-0.04	JW5236	-0.03
yaiM	-0.31	trkG	0.09	pdxB	0.09	yhiJ	0.06	JW5236	-0.14
adhC	-0.19	ynaK	-0.02	div	-0.05	yhiL	0.06	JW5237	0.01
yaiN	0.00	ydaY	-0.03	yfcJ	-0.03	yhiN	-0.16	sfcA	0.00
yaiO	0.02	ynaA	0.03	yfcJ	-0.02	pitA	-0.24	bdm	0.06
JW0350	-0.02	JW1362	-0.16	yfcL	-0.05	uspB	0.13	yddS	0.10
yaiP	-0.02	JW1365	0.00	yfcM	0.09	uspA	0.11	yddV	0.05
yaiS	0.01	stfR	0.01	yfcA	0.10	yhiP	0.10	yddA	-0.05
tauA	0.04	tfaR	-0.09	aroC	0.00	prlC	0.01	ydeN	0.09
tauB	0.00	pinR	0.08	yfcB	0.03	yhiR	0.18	JW5244	-0.03
tauC	0.06	ynaE	0.09	yfcN	0.09	gor	-0.51	yneE	0.11
tauD	-0.22	ynaF	-0.04	yfcO	-0.07	arsR	-0.09	yneI	0.08
yaiT	-0.01	ompN	-0.03	yfcP	-0.01	arsB	0.02	marR	0.03
yaiU	-0.01	ydbK	0.04	yfcQ	-0.06	arsC	0.05	marA	0.01
yaiV	0.03	ydbJ	-0.05	yfcR	-0.02	yhiS	-0.19	eamA	-0.01
sbmA	-0.05	hslJ	-0.13	yfcS	-0.02	slp	0.00	JW5251	-0.07
yaiW	0.01	ldhA	0.02	yfcU	-0.02	yhiF	0.02	ydfO	0.05
yaiY	-0.07	ydbH	-0.06	yfcV	0.04	hdeA	0.00	gnsB	0.08
yaiZ	-0.04	ynbE	-0.07	sixA	0.04	hdeD	0.10	ynfN	-0.09
ddlA	0.07	feaR	0.08	yfcX	-0.02	yhiE	0.05	essQ	0.06
yaiB	0.02	feaB	0.16	yfcY	-0.10	yhiU	0.00	ydfU	0.07
phoA	0.06	tynA	-0.05	yfcZ	-0.07	yhiV	-0.10	JW5257	-0.13
yaiC	-0.02	maoC	0.22	fadL	0.00	gadW	0.05	ynfC	-0.03
proC	0.05	paaA	0.01	yfdF	0.06	gadX	0.01	ynfD	-0.04
yaiI	-0.02	paaB	0.01	vacJ	0.01	gadA	-0.03	ynfF	-0.11
aroL	0.05	paaC	0.02	yfdC	-0.03	yhjA	0.01	ynfH	-0.08
yaiA	0.02	paaD	-0.01	intS	-0.17	treF	-0.05	ynfI	-0.05
aroM	-0.04	paaE	0.22	yfdG	-0.02	yhjB	0.06	ynfJ	0.03
yaiE	0.02	paaF	0.02	yfdH	0.03	yhjC	0.02	ynfK	-0.03
ykiA	-0.01	paaG	-0.02	yfdH	0.01	yhjD	0.00	ydgJ	0.07
rdgC	0.03	paaH	-0.01	yfdI	0.08	yhjE	-0.03	JW5266	-0.05
yajF	0.02	paaI	0.05	yfdL	0.03	yhjG	0.11	slyA	0.15
yajF	0.27	paaJ	-0.01	xylU	-0.01	yhjH	-0.01	ydhL	0.19
araJ	0.02	paaX	0.05	yfdO	-0.03	dctA	-0.02	JW5269	0.17
sbcC	0.05	paaY	0.01	yfdP	-0.05	bcsZ	0.10	ydhO	0.15
sbcD	-0.16	ydbA	-0.03	yfdQ	-0.01	yhjR	0.02	ydhX	-0.05
phoB	0.07	ydbC	-0.06	yfdR	-0.04	yhjU	0.06	ydhV	0.14
phoR	0.43	ydbD	0.04	yfdS	-0.07	ldrD	0.95	sufB	0.17
brnQ	0.36	ynbA	-0.03	JW5386	-0.02	yhjV	0.59	ydiO	0.01
malZ	-0.51	ynbA	0.17	dsdC	0.23	dppF	0.03	ydiQ	-0.03
yajB	-0.03	ynbB	0.06	dsdX	0.01	dppD	0.04	JW5279	-0.05
queA	-0.01	ynbC	-0.02	dsdA	0.23	dppC	0.07	pfkB	0.19
tgt	-0.06	ynbD	-0.02	emrY	0.00	dppB	0.36	astD	-0.06
yajC	-0.21	acpD	0.03	emrK	0.00	dppA	-0.05	ydjY	0.16
yajD	0.01	hrpA	0.00	evgA	-0.04	yhjX	-0.06	ynjB	0.25
tsx	-0.04	ydcF	0.01	evgS	-0.06	tag	0.11	ynjC	0.18
yajI	-0.07	aldA	0.17	yfdE	-0.01	yiaC	0.78	ynjD	0.27
ybaD	0.08	gapC	-0.05	yfdV	0.00	yiaD	-0.03	ynjE	0.13
ybaD	0.01	cybB	0.07	yfdU	-0.08	yiaG	-0.14	ynjI	0.11
nusB	0.76	ydcA	-0.01	yfdW	0.08	cspA	-0.06	ydjH	0.02

pgpA	-0.08	JW1416.5	-0.02	yfdX	-0.02	hokA	0.04	ydjK	0.10
yajO	-0.17	trg	0.04	ypdI	0.02	glyS	0.08	yeaJ	0.02
xseB	0.35	ydcI	0.00	yfdY	-0.02	JW3532	0.03	yeaP	0.10
thiI	-0.14	ydcJ	-0.10	ddg	-0.03	yiaH	-0.05	yeaV	0.19
panE	0.01	ydcG	0.05	yfdZ	-0.02	yiaA	0.06	yeaW	0.62
yajQ	0.01	JW1421	-0.02	ypdA	0.02	xylB	-0.11	yoaB	0.16
yajR	0.15	ydcH	-0.06	ypdA	-0.02	xylA	0.19	yoaC	0.20
cyoE	0.00	rimL	-0.06	ypdB	-0.06	xylF	0.08	yebN	0.19
cyoD	0.19	ydcK	-0.07	ypdB	-0.22	xylG	-0.24	JW5298	0.03
cyoC	0.20	tehA	0.00	ypdC	0.10	xylH	-0.19	yebQ	0.09
cyoB	0.20	tehB	-0.02	ypdC	-0.05	xylR	-0.47	proQ	0.13
cyoA	0.13	ydcL	-0.10	ypdD	-0.07	bax	0.00	yebU	0.12
ampG	-0.36	JW1427.5	0.00	ypdE	-0.07	malS	0.42	yebV	0.02
yajG	-0.06	ydcM	0.12	ypdG	0.06	avtA	0.04	yebW	-0.03
bolA	0.45	ydcO	-0.10	ypdH	-0.25	ysaA	0.50	yebA	-0.01
tig	-0.09	ydcN	-0.02	yfeO	0.08	yiaJ	0.07	JW5305	-0.16
clpP	-0.02	ydcN	0.04	yfeO	-0.07	yiaK	0.03	yebB	0.12
clpX	0.00	ydcP	-0.06	ypeC	-0.04	yiaL	0.04	yecD	0.16
lon	0.04	yncJ	-0.03	mntH	-0.22	yiaM	0.06	yecN	0.19
hupB	-0.08	ydcQ	-0.10	nupC	-0.03	yiaO	0.02	yecM	0.18
ppiD	0.07	ydcR	0.05	yfeA	-0.04	lyx	0.49	yecT	0.16
ybaV	-0.19	ydcR	0.02	yfeA	0.08	sgbH	-0.09	otsA	0.11
ybaW	-0.01	ydcS	0.03	xapR	0.29	sgbE	-0.21	dcyD	-0.09
ybaW	-0.03	ydcS	0.00	xapB	0.05	yiaT	0.03	yedN	-0.06
ybaX	-0.01	ydcT	0.10	xapA	-0.03	yiaU	0.04	JW5315	-0.02
ybaE	0.03	ydcU	0.02	yfeN	-0.06	yiaV	0.02	yodD	0.08
cof	0.07	ydcU	-0.04	yfeR	0.02	yiaW	-0.03	yedS	0.08
ybaO	-0.08	ydcV	-0.02	yfeH	0.05	aldB	0.17	yedW	-0.01
mdlA	-0.06	ydcW	0.11	cysZ	-0.01	selB	0.35	yodB	0.08
glnK	-0.01	ydcX	0.01	cysK	-0.06	selA	0.17	yeeJ	0.18
amtB	0.03	ydcY	0.00	ptsH	-0.10	yibF	0.06	yeeL	0.06
tesB	-0.03	ydcZ	0.01	ptsI	0.09	rhsA	0.17	JW5326	0.02
ybaY	-0.08	yncB	-0.11	crr	1.01	yibA	-0.03	yeeP	-0.03
ybaZ	0.00	yncC	-0.04	pdxK	-0.03	yibG	0.10	dacD	0.17
ybaA	-0.27	yncD	-0.09	yfeS	-0.02	yibH	-0.13	nudD	0.08
ylaB	0.01	yncE	-0.02	cysM	0.02	yibI	0.06	yegH	0.12
ylaC	0.00	yncG	-0.02	cysA	0.19	yibI	0.04	yegM	0.08
maa	-0.09	yncH	0.04	cysW	0.04	mtlA	-0.03	yegP	0.00
hha	-0.04	rhsE	-0.05	cysU	-0.21	mtlD	0.31	gatR	0.14
ybaJ	-0.08	ydcD	-0.01	cysP	-0.27	mtlR	0.04	fbaB	0.11
acrB	0.01	JW5237	0.07	yfeT	-0.03	JW3576	-0.11	yegX	0.21
acrA	-0.01	ydcC	0.10	yfeT	0.04	yibL	0.19	yohN	0.15
acrR	0.01	ydcE	-0.03	yfeU	-0.02	lldP	-0.02	yehL	0.11
kefA	0.04	yddH	0.01	yfeV	-0.02	lldR	0.70	yehP	0.09
ybaM	0.03	yddH	0.05	yfeW	-0.02	lldD	0.05	yehR	0.05
priC	0.05	nhoA	0.00	yfeX	0.01	yibK	0.00	yehT	0.06
ybaN	0.02	yddE	0.05	ypeA	-0.05	cysE	-0.20	yehU	0.08
apt	-0.01	yddE	-0.01	ypeA	-0.12	secB	-0.09	JW5354	0.09
ybaB	0.04	narV	0.00	amiA	0.02	grxC	0.69	pbpG	0.08
recR	0.03	narW	-0.30	hemF	0.03	yibN	0.16	yohC	0.08
htpG	-0.05	narY	0.08	eutR	-0.01	gpmI	0.11	yeiW	0.06
aes	-0.06	narZ	0.04	eutK	-0.09	yibP	-0.01	yeiP	0.06
gsk	-0.20	narU	0.10	eutC	0.08	yibD	-0.08	yejO	0.08
ybaL	0.00	JW1465	-0.29	eutB	-0.04	tdh	0.01	yfaZ	-0.03
fsr	0.09	yddJ	-0.11	eutH	-0.27	kbl	-0.01	yfbE	0.11
ushA	0.13	yddK	-0.02	eutG	-0.02	rfaD	0.06	yfbJ	0.08
ybaK	-0.16	JW1468	-0.09	eutJ	0.00	rfaF	0.03	menD	0.11
ybaP	-0.03	yddG	-0.10	eutE	0.21	rfaC	-0.01	nuoC	-0.03
ybaQ	0.04	fdnG	-0.05	eutN	-0.31	rfaL	0.02	yfbT	0.08
copA	0.15	fdnH	0.04	eutM	-0.06	rfaZ	0.01	yfcE	0.17
ybaS	-0.02	fdnI	-0.01	eutD	0.06	rfaY	-0.02	yfcK	0.19
ybaT	0.07	yddM	0.01	eutP	0.01	rfaJ	-0.03	yfcM	0.04
cueR	0.05	adhP	0.02	eutS	0.09	rfaI	0.13	yfdI	0.05
ybbJ	-0.01	sfcA	-0.08	eutS	0.12	rfaB	0.02	tfaS	-0.05
ybbJ	0.12	rpsV	-0.20	ypfG	0.12	rfaS	0.07	yfdL	0.03
ybbK	-0.01	bdm	-0.04	ypfG	0.14	rfaP	-0.09	yfdN	0.01

ybbL	0.01	osmC	0.02	yffH	0.00	rfaG	0.03	JW5386	0.04
ybbM	-0.06	yddO	0.10	yffH	-0.04	rfaQ	-0.01	JW5387	0.11
ybbN	-0.04	yddP	0.05	aegA	0.03	mutM	-0.08	ypdA	0.05
ybbO	0.13	yddQ	0.01	narQ	-0.14	rpmG	-0.09	ypdH	0.16
tesA	-0.06	yddR	0.01	acrD	-0.17	pyrE	0.01	yfeA	0.12
ybbA	0.10	yddS	-0.12	yffB	-0.03	rph	0.08	JW5392	0.08
ybbP	-0.03	ddpX	0.08	JW2457	0.14	yicC	0.03	JW5393	0.04
rhsD	0.14	yddU	0.12	JW2457	-0.01	dinD	0.03	ucpA	0.02
ybbC	-0.03	yddV	-0.02	ypfH	0.08	yicG	0.22	yfeW	0.04
JW0488	0.01	yddW	-0.02	ypfH	0.08	yicF	-0.07	ypfH	-0.05
ybbD	0.09	xasA	0.03	ypfI	-0.01	rpoZ	-0.02	yfgE	0.02
ybbD	0.09	gadB	-0.14	ypfJ	0.02	trmH	-0.12	JW5398	0.05
JW5069	0.00	yddB	0.00	purC	0.02	recG	-0.05	yfgH	0.15
ybbB	0.06	ydeM	0.28	nlpB	0.01	gltS	-0.14	yfgJ	0.10
ybbS	0.03	ydeO	-0.02	gcvR	0.03	yicE	0.15	sseB	0.05
allA	0.08	ydeP	-0.03	bcp	0.04	yicH	-0.26	yphG	0.04
allR	0.37	ydeQ	-0.02	hyfA	0.04	yicI	0.08	yphH	0.05
gcl	0.04	ydeR	0.02	hyfB	-0.12	setC	-0.02	yfhK	0.08
hyi	-0.07	ydeS	-0.12	hyfC	0.05	yicL	0.25	yfhB	0.12
glxR	0.04	hipA	0.35	hyfD	0.01	nlpA	0.01	yfiP	0.03
ybbV	-0.02	hipB	-0.01	hyfE	-0.22	JW3636.1	-0.23	JW5410	0.09
ybbW	-0.03	ydeU	-0.01	hyfF	-0.13	yicP	0.03	JW5411	0.04
allB	0.02	ydeK	0.02	hyfG	-0.36	uhpT	-0.15	yfiL	0.04
ybbY	0.07	ydeV	-0.01	hyfH	0.12	uhpC	0.00	rimM	0.11
glxK	-0.03	ydeW	-0.03	hyfI	-0.10	uhpC	-0.04	yfjD	0.08
ylbA	-0.05	ego	0.05	hyfJ	-0.11	uhpB	-0.05	JW5417	0.12
allC	0.03	ydeY	0.00	focB	-0.22	uhpA	-0.03	yfjO	0.04
allD	0.12	ydeZ	0.03	perM	-0.12	ilvN	0.15	yfjP	-0.03
fdrA	-0.05	yneA	-0.05	yfgC	-0.02	ilvB	-0.02	ypjJ	0.05
ylbE	-0.02	yneB	0.02	yfgD	-0.07	ivbL	0.02	ypjA	0.06
ylbF	0.02	yneC	-0.02	uraA	-0.03	yidF	0.06	pinH	0.10
arcC	-0.07	tam	0.45	upp	0.04	yidG	-0.19	ypjC	0.09
purK	-0.09	yneE	-0.02	purM	-0.01	yidH	-0.05	ygaQ	0.04
purE	-0.02	uxaB	0.01	purN	-0.08	yidI	-0.10	JW5426	0.08
ppiB	-0.01	JW1515	0.02	ppk	0.01	yidJ	0.30	ygaT	0.08
ybcI	-0.02	JW1515	-0.05	ppx	0.14	yidK	-0.01	ygaY	0.08
sfmA	0.00	yneG	-0.05	yfgF	0.02	yidL	0.01	srlA	0.03
sfmC	-0.40	yneH	0.52	yfgF	0.01	JW3657	0.26	srlE	0.01
sfmD	-0.07	yneI	0.02	yfgH	-0.01	glvG	0.28	gutQ	0.08
fimZ	-0.06	yneJ	0.05	yfgH	0.03	glvB	0.02	ygaA	0.09
intD	0.11	yneK	-0.05	yfgI	0.03	glvC	0.17	ascG	-0.07
JW0526	0.10	sotB	0.23	guaA	0.03	yidP	0.04	JW5436	0.03
JW0527	-0.01	marC	-0.10	xseA	-0.09	yidE	0.04	rpoS	0.02
JW0530	0.01	marR	0.18	yfgJ	0.04	ibpB	-0.09	ygbF	-0.06
emrE	-0.27	marA	-0.12	yfgL	0.04	ibpA	-0.11	ygcI	0.05
ybcK	-0.02	marB	-0.05	yfgM	0.04	dgoK	-0.01	ygcQ	0.04
ybcL	-0.06	eamA	0.07	yfgA	0.07	yidA	0.00	ygcR	0.23
ybcM	0.00	eamA	-0.03	yfgA	0.03	yidB	-0.06	ygcU	0.06
ybcN	0.08	ydeE	0.03	yfgB	0.05	recF	-0.11	ygcW	-0.03
ninE	-0.02	ydeE	0.05	yfgB	0.09	trmE	0.02	ygcE	0.03
ybcO	0.01	ydeH	-0.03	ndk	0.00	tnaL	0.01	ygcG	0.06
rusA	-0.10	ydeI	-0.01	pbpC	0.00	tnaL	0.08	ygdI	0.04
ybcQ	0.07	ydeJ	0.06	yfhM	0.06	tnaA	0.02	amiC	0.07
JW0539.5	-0.01	dcp	-0.01	sseA	-0.04	yidY	-0.11	ygdB	0.07
essD	-0.06	ydfG	-0.11	sseB	-0.03	yidZ	-0.01	ppdB	-0.02
ybcS	-0.19	ydfH	-0.07	yfhJ	0.01	yieE	-0.19	JW5452	0.04
rzpD	-0.06	ydfZ	-0.06	fdx	0.07	yieF	0.09	yqeF	0.04
borD	-0.33	ydfI	-0.02	hscA	-0.08	yieG	-0.09	yqeH	-0.02
ybcV	0.47	ydfJ	-0.04	hscB	-0.82	yieH	-0.10	yqeJ	0.04
ybcW	0.03	pinQ	-0.01	yfhF	-0.01	yieI	0.10	ygeI	0.17
nohB	0.10	tfaQ	-0.02	nifU	0.05	yieJ	0.01	x	0.21
tfaD	0.03	tfaQ	0.00	iscS	0.00	yieC	0.06	ygeK	0.20
ybcY	0.01	stfQ	0.16	yfhP	-0.06	bglB	0.30	JW5459	0.07
ylcE	0.15	nohA	0.05	yfhQ	0.05	bglF	0.06	JW5460	0.15
appY	-0.12	gnsB	-0.02	yfhR	0.04	bglG	-0.04	ygeQ	0.21
ompT	-0.02	cspI	0.15	csiE	0.07	phoU	-0.01	xdhA	0.06

envY	-0.01	ydfP	-0.04	hcaT	0.04	pstB	-0.02	ygeW	0.02
ybcH	-0.04	ydfQ	0.06	hcaR	0.06	pstA	-0.05	guaD	0.07
nfrA	-0.08	ydfR	0.02	hcaR	0.16	pstC	0.18	ygfQ	0.00
nfrB	0.07	cspB	-0.03	hcaE	-0.03	pstS	-0.01	ygfS	0.04
cusS	-0.06	cspF	0.06	hcaF	-0.04	atpG	0.00	ygfT	0.01
cusR	0.07	ydfT	0.01	hcaC	0.02	atpA	-0.21	ygfU	0.04
cusC	-0.06	ydfU	0.07	hcaB	-0.13	atpH	-0.06	JW5471	0.04
cusF	-0.06	ydfU	0.03	hcaD	-0.09	atpF	-0.03	ygfB	0.04
cusA	0.05	rem	-0.07	yphA	-0.02	atpE	0.00	JW5474	0.09
pheP	-0.07	hokD	-0.07	yphB	0.15	atpB	-0.03	rpiA	0.10
ybdG	0.02	relE	-0.05	yphC	0.01	gidB	-0.05	ygfI	0.03
nfnB	0.15	relB	-0.10	yphD	-0.07	gidA	-0.02	yggP	0.07
ybdF	0.03	ydfV	0.01	yphE	0.10	mioC	-0.01	yggU	0.03
ybdJ	-0.01	flxA	0.00	yphF	0.07	asnC	-0.10	mltC	0.04
ybdK	0.10	ydfW	-0.01	yphF	-0.01	asnA	-0.01	yghE	0.00
hokE	-0.06	ydfX	0.04	yphG	0.10	yieN	-0.01	yghF	0.00
fepA	-0.04	dicC	0.06	yphG	-0.02	rbsD	-0.22	yghJ	0.08
fes	-0.10	ydfA	-0.01	yphH	0.01	rbsA	0.00	glcF	0.02
ybdZ	0.00	ydfC	0.01	glyA	0.01	rbsC	-0.10	glcE	0.02
entF	0.04	dicB	0.04	hmp	0.39	rbsB	0.09	yghO	0.10
fepE	-0.01	ydfD	0.08	glnB	0.02	rbsK	0.62	yghQ	0.07
fepC	0.00	ydfE	0.08	yfhA	-0.05	rbsR	0.21	yghS	0.00
fepG	-0.02	intQ	0.14	yfhG	0.00	yieO	0.01	yghU	0.05
fepD	-0.06	rspB	0.04	yfhK	-0.05	JW3736	0.28	JW5495	0.02
fepB	0.01	rspA	0.07	purL	0.19	yifE	-0.03	JW5496	0.10
entC	-0.03	ynfA	0.04	yfhD	0.04	yifB	-0.05	JW5497	0.13
entE	0.01	ynfB	0.05	yfhD	0.01	ilvL	-0.01	dkgA	0.05
entB	0.03	speG	0.01	yfhB	0.05	ilvL	-0.04	yqhG	0.00
entA	0.13	ynfC	0.02	yfhH	0.03	ilvG	0.02	ygiQ	0.09
ybdB	0.09	ynfE	0.16	yfhL	-0.09	ilvM	-0.01	tolC	0.13
ybdB	-0.01	ynfG	-0.06	pdxJ	-0.28	ilvE	0.00	ygiB	0.05
cstA	-0.04	mlc	-0.13	recO	0.00	ilvA	-0.02	yqiC	0.03
ybdD	0.06	ynfL	-0.03	lepA	0.07	ilvY	0.23	yqiG	0.05
ybdH	0.02	ynfM	0.02	rseC	-0.50	ilvC	0.05	yqiI	0.03
ybdL	0.03	asr	0.09	rseB	-0.12	ppiC	0.02	ygjO	-0.04
ybdM	-0.02	ydgD	0.04	rseA	-0.03	yifN	-0.06	ygjP	0.02
ybdN	-0.06	ydgE	0.00	nadB	0.05	rep	-0.07	ygjT	-0.08
ybdN	0.04	ydgF	0.24	yfiC	0.06	rhlB	-0.01	yqjC	0.00
ybdO	0.17	ydgG	-0.05	srmB	-0.03	rhoL	-0.04	yhaL	-0.03
dsbG	0.12	ydgG	0.06	yfiE	0.09	rhoL	0.14	yhaM	0.03
ahpC	-0.07	pntB	0.02	yfiK	0.08	rho	-0.30	yhaO	0.01
ahpF	0.07	pntA	0.08	yfiD	0.04	wecA	-0.07	tdcG	0.03
ybdQ	-0.22	ydgH	0.02	ung	0.02	wecB	0.09	tdcE	0.00
ybdR	-0.10	ydgH	-0.02	yfiF	0.00	wecC	0.06	tdcR	0.05
rnk	-0.10	ydgI	0.02	trxC	0.06	rffG	-0.09	garR	-0.03
rna	0.04	ydgB	-0.03	yfiP	0.06	rffH	0.00	yhbO	0.18
citT	-0.12	ydgC	0.03	yfiQ	0.03	rffC	-0.04	deaD	0.19
citG	-0.06	rstA	0.06	yfiM	0.01	rffA	-0.02	pnp	0.15
citX	-0.25	rstB	0.07	kgtP	-0.05	wzxE	0.00	yhbC	-0.01
citE	0.16	tus	0.06	clpB	0.15	wecG	0.06	yhbX	0.00
citD	-0.08	fumC	-0.04	JW2574	0.00	yifK	0.04	nanK	0.00
citC	0.06	fumA	-0.09	yfiH	0.07	aslB	0.00	yhcB	-0.01
dpiB	0.06	manA	-0.05	yfiH	0.13	aslA	-0.16	yhcN	-0.02
dpiA	0.09	ydgA	-0.01	rluD	0.03	hemY	0.05	yhcR	-0.05
dcuC	-0.05	uidC	0.63	yfiA	0.00	hemX	0.01	yhdP	0.16
crcA	-0.09	uidB	0.04	pheL	-0.06	cyaA	0.02	yhdJ	-0.01
cspE	-0.02	uidA	-0.01	pheA	-0.06	cyaY	0.09	zraP	0.03
crcB	-0.03	uidR	0.19	tyrA	-0.11	yzcX	-0.02	nfi	0.04
ybeM	-0.02	hdhA	-0.05	aroF	0.05	yifL	-0.15	thiG	-0.01
tatE	0.10	malI	-0.08	yfiL	0.06	yigA	-0.04	sthA	0.01
lipA	-0.03	malY	0.13	yfiR	0.05	xerC	-0.06	JW5552	0.03
ybeF	-0.04	add	0.45	yfiN	0.01	yigB	0.09	argB	-0.02
ybeD	-0.06	ydgJ	-0.09	yfiB	-0.02	uvrD	-0.23	JW5554	-0.01
dacA	-0.05	blr	0.04	ypjD	0.05	corA	-0.07	gldA	-0.06
rlpA	-0.05	ydgT	-0.01	yfjD	-0.11	yigF	0.13	cpxP	0.10
ybeA	0.07	ydgK	-0.01	recN	0.06	pldA	0.07	yiiM	-0.02

ybeB	0.01	rnfA	0.08	smpA	-0.39	JW3795	-0.14	yiiF	-0.01
cobC	0.02	rnfB	0.17	yfjF	0.04	pldB	0.02	yihS	0.01
ybeL	-0.03	rnfC	-0.03	yfjG	0.06	yigM	0.02	yihO	0.10
ybeQ	-0.07	rnfD	0.14	smpB	-0.05	metR	0.30	typA	0.08
ybeR	-0.05	rnfG	-0.03	intA	0.03	metE	0.01	JW5572	0.10
ybeS	0.00	rnfE	0.11	yfjH	0.07	udp	-0.04	yihF	0.07
ybeT	-0.06	nth	0.30	alpA	0.06	udp	-0.01	mobB	0.00
ybeU	0.04	ydgR	0.02	yfjI	0.07	rmuC	0.47	trkH	0.10
ybeV	0.00	gst	-0.37	yfjI	0.01	ubiE	-0.07	yigZ	0.18
hscC	-0.26	pdxY	-0.03	JW2606	0.00	tatA	0.00	tatD	-0.02
rihA	0.52	pdxH	0.03	yfjJ	0.09	tatC	-0.11	tatB	0.08
gltL	-0.06	ydhA	0.06	yfjK	0.08	fre	0.01	yigL	0.04
gltK	-0.04	ydhH	0.08	yfjL	-0.01	fadA	0.01	rhtB	0.06
gltJ	0.03	slyB	-0.04	yfjM	0.06	fadB	0.06	rhtC	0.03
JW0651	0.08	slyA	0.21	yfjN	0.05	pepQ	0.05	yigI	0.04
corC	0.08	ydhI	-0.06	yfjO	0.00	yigZ	-0.01	rarD	0.08
ybeY	0.02	ydhJ	-0.02	yfjP	0.09	mobA	0.06	yigG	0.11
ybeZ	0.01	ydhJ	0.10	yfjQ	0.03	yihD	-0.09	yigE	0.00
yleA	-0.06	ydhK	0.06	yfjR	-0.01	yihE	-0.03	dapF	0.12
ubiF	-0.02	sodC	0.33	JW2616	0.00	dsbA	0.15	wecF	0.03
asnB	-0.06	ydhF	-0.05	yfjS	0.09	yihG	-0.08	wzzE	0.01
nagD	-0.01	JW1640	0.07	yfjT	-0.31	polA	0.45	trxA	0.01
nagC	-0.10	ydhM	-0.04	yfjU	0.06	yihI	-0.05	gppA	0.01
nagA	-0.03	nemA	0.14	yfjV	-0.04	hemN	0.15	ilvD	0.04
nagB	-0.12	gloA	-0.01	yfjW	-0.01	glnG	-0.06	hdfR	0.03
nagE	0.24	rnt	0.07	JW2623.5	0.10	glnL	-0.01	yieP	0.00
ybfM	-0.09	lhr	0.01	yfjX	0.09	glnA	-0.06	kup	-0.05
ybfN	0.03	ydhD	-0.07	yfjY	0.02	yihL	-0.01	yieM	0.08
fur	-0.08	ydhD	0.12	yfjZ	0.00	yihM	-0.03	atpI	0.09
ybfJ	0.08	ydhO	0.04	ypjF	-0.01	yihN	-0.02	yieL	0.00
ybfE	-0.04	ydhO	-0.26	ypjA	-0.03	yshA	0.15	yieK	0.12
ybfF	-0.09	sodB	-0.02	ypjB	-0.03	yihP	0.09	tnaB	0.01
seqA	-0.29	ydhP	-0.06	ypjC	0.01	yihQ	0.00	yidX	0.05
pgm	-0.03	purR	-0.02	JW2631	0.02	yihR	-0.08	dgoR	0.07
ybfP	-0.06	ydhB	0.10	JW5426	-0.01	yihT	-0.03	dgoA	0.02
ybfG	0.03	ydhC	0.04	yqaD	-0.03	yihU	0.00	dgoT	0.05
potE	0.14	ydhC	-0.15	ygaT	0.05	rbn	-0.05	yidS	0.01
speF	-0.13	cfa	-0.04	ygaF	0.06	dtd	-0.19	yidR	-0.04
ybfK	-0.08	norM	-0.02	ygaF	0.04	yiiD	-0.01	yidR	0.01
ybfK	-0.01	norM	0.11	gabD	0.03	fdhE	0.56	yidQ	0.04
kdpE	0.05	ydhQ	-0.03	gabT	0.09	fdoI	0.09	emrD	0.01
kdpD	0.06	ydhR	0.03	gabP	-0.31	fdoH	0.02	JW5635	0.09
kdpC	0.11	ydhS	0.04	ygaE	0.00	fdoG	0.22	yicO	0.07
kdpB	0.01	ydhS	0.00	ygaU	0.12	fdhD	0.01	yicN	0.00
kdpA	0.04	ydhT	0.02	yqaE	0.05	yiiG	-0.02	yicM	-0.05
kdpF	0.17	ydhU	0.02	ygaV	0.03	frvR	0.23	JW5639	-0.03
ybfA	0.01	ydhW	0.00	ygaP	0.55	frvX	0.02	ttk	0.03
rhsC	0.15	ydhV	-0.02	stpA	0.19	frvA	0.01	radC	0.02
ybfB	0.06	ydhY	0.00	ygaW	0.06	rhaS	0.06	htrL	0.04
ybfB	0.01	ydhY	-0.01	ygaC	0.05	rhaR	-0.12	yibQ	0.02
ybfO	0.21	ydhZ	-0.03	ygaM	0.03	rhaT	0.17	yibP	0.08
ybfC	-0.05	pykF	-0.07	nrdH	0.36	sodA	-0.16	yiaY	0.02
JW0694	-0.05	lpp	-0.16	nrdI	0.06	cpxA	-0.01	JW5649	0.04
ybfL	-0.05	ynhG	-0.02	nrdE	-0.01	cpxR	0.00	sgbU	0.06
ybfD	0.07	sufE	0.00	nrdF	0.01	yiiP	0.10	yiaN	0.02
ybgA	-0.02	csdB	0.06	proV	-0.32	pfkA	0.17	yiaB	0.03
phrB	0.39	sufD	0.10	proW	-0.14	sbp	0.46	yiaF	0.03
ybgH	0.04	sufC	0.10	proX	0.04	cdh	-0.20	tkrA	0.03
ybgI	-0.03	sufB	-0.05	ygaY	0.02	tpiA	0.14	bisC	0.04
ybgJ	0.06	sufB	0.03	ygaZ	-0.02	yiiQ	-0.39	yhjY	0.00
ybgK	-0.10	sufA	0.09	ygaH	0.08	yiiR	0.42	yhjW	0.10
ybgL	-0.08	ydiH	-0.01	mprA	-0.16	yiiS	-0.09	yzpK	0.07
nei	-0.38	ydiI	-0.04	emrA	0.04	yiiT	0.11	JW5662	-0.01
abrB	-0.14	ydiJ	-0.18	emrB	0.02	fpr	-0.10	yhjT	0.03
ybgO	-0.02	ydiK	-0.03	luxS	-0.04	glpX	-0.02	yhjQ	0.05
ybgP	0.06	ydiM	-0.04	gshA	0.02	glpK	-0.07	bcsA	0.05

ybgQ	-0.05	ydiM	0.01	yqaA	-0.01	glpF	0.39	bcsC	0.04
ybgD	-0.02	ydiN	-0.07	yqaB	0.00	yiiU	-0.07	yhjK	0.03
gltA	0.03	ydiN	-0.02	yqaB	-0.31	menG	0.03	kdgK	0.09
sdhC	-0.13	ydiB	0.02	alaS	-0.02	menA	-0.01	hdeB	0.03
sdhD	-0.07	ydiB	-0.02	recX	0.03	cytR	-0.11	yhiD	-0.01
sdhA	-0.05	aroD	0.07	ygaD	0.02	priA	0.01	yhiQ	0.00
sdhB	-0.08	ydiF	0.01	mltB	0.00	rpmE	-0.11	yhiM	0.04
sucA	-0.09	ydiF	-0.02	srlB	-0.23	yiiX	-0.02	JW5675	0.04
sucB	-0.09	ydiO	0.01	srlD	-0.17	metJ	0.19	yhiH	0.04
sucC	-0.07	ydiP	-0.07	gutM	0.02	metB	-0.01	yhhJ	0.02
sucD	0.08	ydiP	0.02	srlR	0.16	metL	-0.21	rhsB	-0.06
farR	0.11	ydiQ	-0.01	gutQ	-0.04	metF	0.01	yhhT	0.03
hrsA	0.38	ydiR	-0.07	JW2678	-0.04	katG	0.19	yhhS	0.00
ybgG	-0.04	ydiS	-0.02	JW2679	-0.04	yijF	-0.04	dcrB	0.00
ybgG	0.00	ydiT	0.04	flrD	-0.07	fsaB	-0.06	yhhL	0.00
cydB	0.00	ydiD	0.02	flrR	0.01	frwC	0.02	yrhD	0.04
ybgT	-0.02	ydiD	0.15	hypF	0.30	frwB	0.02	gntR	0.01
ybgE	-0.08	ppsA	-0.07	hydN	-0.12	pflD	-0.04	gntU	0.09
ybgC	0.02	ydiA	-0.05	ascF	-0.01	pflC	0.15	rtcA	-0.01
ybgC	-0.04	aroH	0.06	ascB	0.06	frwD	0.00	yhgH	0.01
tolQ	0.00	ydiE	0.05	hycG	-0.12	yijO	-0.02	hslO	0.03
tolR	-0.44	ydiU	0.04	hycF	-0.24	yijP	-0.03	yrfD	0.02
tolA	-0.07	ydiV	0.06	hycE	-0.23	ppc	0.11	yrfA	0.03
pal	-0.01	nlpC	-0.02	hycD	0.01	argE	0.23	aroK	0.08
ybgF	0.06	btuD	-0.29	hycC	-0.04	argC	0.04	yhfY	-0.01
nadA	0.04	btuE	-0.57	hycB	-0.01	argH	0.30	yhfU	0.02
pnuC	0.06	btuC	-0.23	hycA	-0.03	oxyR	0.07	yhfR	0.06
zitB	-0.06	ihfA	0.17	hypA	-0.29	yijC	-0.06	yhfO	0.04
ybgS	-0.07	pheM	-0.27	hypB	0.06	yijD	-0.01	yhfN	0.04
aroG	-0.17	rpmI	-0.15	hypC	-0.04	trmA	-0.08	yhfK	0.05
galM	-0.06	arpB	0.06	hypD	0.02	btuB	0.56	yheO	0.02
galK	-0.27	ydiY	0.02	hypE	-0.01	coaA	-0.06	pshM	0.06
galT	-0.22	pfkB	0.17	fhlA	0.01	tufB	-0.06	gspL	0.08
galE	-0.71	ydiZ	0.04	ygbA	0.10	rplK	0.04	gspI	0.02
modF	-0.08	yniA	0.09	mutS	0.08	rplA	-0.06	gspD	0.05
modE	0.69	yniB	0.05	pphB	-0.02	htrC	-0.02	smf	0.01
ybhT	-0.07	yniC	-0.01	ygbI	-0.04	thiH	0.09	yrdD	0.02
modA	0.41	yniC	0.02	ygbJ	-0.03	thiS	-0.10	yrdA	-0.02
modB	0.39	ydjM	-0.01	ygbJ	-0.06	thiF	0.02	yjbF	0.03
modC	0.34	ydjN	-0.07	ygbK	-0.03	thiE	0.05	JW5712	0.02
ybhA	0.00	ydjO	-0.22	ygbL	0.05	thiC	0.03	ubiC	-0.05
ybhE	0.07	cedA	0.05	ygbM	0.04	rsd	0.02	zur	0.00
ybhD	-0.02	katE	0.11	ygbN	0.02	nudC	0.06	yjbN	-0.05
ybhH	0.07	ydjC	-0.04	rpoS	-0.10	hemE	-0.11	yjbO	0.09
ybhI	0.01	ydjC	0.00	pcm	-0.01	yjaG	0.08	yjbS	0.03
ybhJ	0.04	celF	-0.45	surE	-0.01	hupA	-0.15	yjcB	0.03
ybhC	-0.06	celD	-0.11	ygbO	0.01	yjaH	-0.17	JW5719	0.01
ybhB	0.05	celC	-0.10	ygbE	0.04	zraS	-0.02	JW5720	0.01
bioA	-0.10	celB	-0.08	cysC	-0.04	zraR	-0.03	yjcS	0.00
bioB	0.09	celA	-0.03	cysN	0.01	purD	0.01	phnQ	0.04
bioF	0.05	osmE	0.07	cysD	-0.02	purH	-0.02	phnQ	-0.02
bioC	0.17	cho	0.07	iap	0.03	yjaA	0.02	JW5728	-0.01
bioD	0.11	spy	-0.09	ygbF	0.03	yjaB	0.03	yjcZ	0.06
uvrB	0.12	astE	0.06	ygbT	0.07	metA	0.12	yjdB	0.03
ybhK	0.04	astB	0.06	ygcH	0.07	aceB	0.15	adiA	0.08
moaA	0.19	astA	0.04	JW2727	0.04	aceA	0.15	yjdO	0.02
moaB	0.04	argM	0.02	ygcJ	-0.03	aceK	-0.05	yjdC	0.01
moaC	0.37	xthA	0.10	ygcK	0.10	arpA	0.18	dsbD	0.10
moaD	0.11	ydjX	-0.02	ygcL	0.00	iclR	0.30	dcuA	0.11
moaE	-0.30	ydjZ	0.00	ygcB	0.05	metH	0.14	yjeI	0.07
ybhL	0.57	ynjA	-0.17	cysH	-0.12	yjbB	0.14	ecnA	0.01
ybhM	-0.01	ynjB	0.09	cysI	-0.06	pepE	0.01	sugE	0.03
ybhM	0.13	ynjE	-0.06	cysJ	0.07	yjbC	-0.09	yjeM	0.07
ybhN	-0.01	ynjF	0.06	ygcM	-0.01	yjbD	-0.18	rnr	0.06
ybhO	-0.33	ynjF	-0.03	ygcN	-0.01	lysC	0.21	yjfN	-0.01
ybhP	0.00	nudG	0.05	ygcO	0.01	pgi	0.14	yjfO	0.03

ybhP	0.03	ynjH	0.01	ygcP	-0.10	yjbE	0.00	sgaT	0.03
ybhQ	-0.07	gdhA	0.07	ygcQ	0.11	yjbG	-0.06	ytfB	-0.02
ybhR	-0.17	ynjI	0.04	ygcR	0.01	yjbH	0.27	fklB	-0.01
JW0776	0.01	topB	-0.02	ygcS	-0.12	psiE	0.32	ytfH	0.05
ybhS	-0.03	selD	0.21	ygcU	-0.01	xylE	0.08	ytfI	0.01
ybhG	-0.06	ydjA	0.05	ygcU	0.05	malG	0.22	ytfK	0.01
ybiH	0.15	ansA	-0.12	JW2743	0.05	malF	0.29	JW5751	0.08
rhlE	-0.10	pncA	-0.02	ygcW	0.05	malE	-0.10	ytfR	0.08
JW0782	0.00	pncA	-0.46	yqcE	0.00	malK	0.00	ytfT	0.06
ybiA	0.04	ydjE	-0.12	ygcE	0.03	lamB	0.02	yjfF	0.03
dinG	0.04	ydjF	0.15	ygcF	0.00	malM	-0.04	yjgF	-0.03
ybiB	-0.02	ydjG	-0.14	ygcG	0.03	malM	0.09	yjgK	0.01
ybiC	-0.15	ydjH	0.00	mazG	0.01	yjbI	-0.04	yjgL	0.01
ybiJ	-0.07	ydjH	0.01	chpA	0.06	dgkA	0.15	yjgM	0.02
ybiI	-0.13	ydjI	0.04	relA	-0.01	dinF	-0.08	yjgN	-0.01
ybiX	-0.01	ydjJ	-0.10	rumA	-0.03	yjbJ	-0.03	yjgB	-0.02
ybiL	-0.03	ydjL	0.38	barA	-0.02	yjbL	-0.05	JW5765	0.06
ybiN	-0.04	yeaC	0.05	gudD	0.43	yjbM	-0.04	JW5766	0.03
ybiO	0.00	msrB	0.03	gudX	-0.02	qor	-0.13	yjhB	0.02
glnQ	0.54	yeaD	-0.08	gudP	0.02	alr	0.23	yjhC	0.01
glnP	0.84	yeaE	-0.09	yqcA	-0.08	tyrB	0.02	yjhD	0.04
glnH	0.08	mipA	0.06	yqcB	0.02	aphA	-0.01	yjhU	0.02
dps	0.04	yeaG	-0.02	yqcC	0.01	JW4016	0.01	yjhH	0.10
ybiF	0.06	yeaH	-0.07	syd	0.06	yjbQ	-0.14	sgcX	0.09
ompX	0.01	yeaI	-0.02	yqcD	0.07	yjbR	-0.14	yjhT	-0.02
ybiP	-0.03	yeaI	0.04	yqcD	-0.06	uvrA	-0.03	yjhA	0.11
mntR	-0.02	yeaJ	0.05	ygdH	-0.11	yjcC	0.13	fimI	0.04
ybiR	-0.04	yeaJ	0.02	sdaC	0.18	soxS	0.23	JW5781	0.07
ybiS	0.02	yeaK	0.04	sdaB	-0.12	soxR	0.27	yjiD	0.06
ybiT	0.03	JW1777	-0.44	fucO	0.06	yjcD	0.12	yjiH	0.04
ybiU	-0.08	yeaL	0.07	fucA	0.01	yjcE	-0.07	kptA	0.04
ybiV	0.16	yeaM	-0.13	fucP	-0.05	yjcF	-0.07	kptA	-0.03
ybiW	-0.04	yeaN	0.14	fucI	0.10	yjcG	-0.13	yjiL	-0.01
ybiY	-0.12	yeaO	0.09	fucK	-0.03	yjcH	-0.05	yjiM	0.08
fsaA	-0.07	yoaF	0.03	fucU	0.00	acs	0.41	yjiT	0.05
moeB	-0.03	yeaQ	-0.05	fucR	0.27	nrfA	-0.02	mcrD	0.10
moeA	0.19	yoaG	0.01	ygdE	0.02	nrfB	0.05	mcrC	0.04
ybiK	0.06	yeaR	0.01	ygdD	0.07	nrfC	0.15	yjiA	0.01
yliC	-0.04	yeaR	-0.03	gcvA	0.07	nrfD	0.82	yjiY	0.08
yliD	0.01	yeaS	0.05	ygdI	-0.06	nrfE	0.27	yjjM	0.02
yliE	-0.11	yeaS	0.16	csdA	-0.03	nrfF	0.55	yjjN	-0.01
yliF	-0.14	yeaT	0.05	ygdK	-0.02	nrfG	0.31	mdoB	0.04
yliG	-0.08	yeaU	0.08	ygdL	-0.16	gltP	0.04	yjjA	0.04
yliH	0.08	yeaX	0.02	mltA	0.01	yjcO	-0.06	yjjP	0.08
yliI	0.03	rnd	0.10	argA	-0.03	fdhF	0.43	yjjZ	0.03
yliJ	-0.08	fadD	0.19	recD	-0.07	yjcP	-0.26	prfC	0.01
dacC	-0.04	yeaY	0.01	recB	0.02	yjcQ	0.25	JW5799	0.04
deoR	0.23	yoaA	-0.04	ptrA	0.04	yjcR	-0.12	nadR	0.18
ybjG	0.11	yoaB	0.03	recC	0.00	alsE	0.04	yjjX	0.04
cmr	0.00	yoaC	-0.25	ppdC	0.02	alsC	0.14	ybhR	0.12
ybjH	-0.10	yoaH	0.10	ygdB	-0.08	alsA	-0.03	hyfI	0.18
ybjI	-0.04	pabB	0.07	ppdB	0.01	alsB	0.18	leuB	-0.02
ybjJ	-0.03	yeaB	0.05	ppdA	0.10	rpiR	0.10	pcnB	0.03
ybjK	0.02	yeaB	-0.01	thyA	-0.12	rpiB	0.15	yadB	0.01
ybjL	0.02	sdaA	-0.04	thyA	-0.07	phnP	-0.07	fhiA	0.00
ybjM	0.17	yoaD	-0.04	ptsP	-0.07	phnO	-0.05	mbhA	0.05
grxA	0.12	yoaE	0.00	nudH	0.00	phnN	-0.05	ykfC	0.06
ybjC	-0.08	yoaE	0.18	nudH	-0.04	phnM	0.20	JW5814	0.03
nfsA	-0.01	manX	0.04	mutH	0.07	phnL	-0.01	tfaD	-0.03
rimK	-0.12	manY	0.02	ygdQ	-0.01	phnK	0.72	ybfE	-0.06
ybjN	0.00	manZ	-0.25	ygdQ	-0.05	phnJ	-0.07	ybhD	0.02
potF	0.25	yobD	0.00	ygdR	0.05	phnI	-0.17	potG	0.03
potG	-0.05	JW1810	0.04	tas	0.69	phnH	0.09	ybjS	0.00
potH	-0.01	rrmA	0.00	ygeD	-0.02	phnG	-0.01	ycfS	-0.01
potI	-0.07	cspC	0.01	ygeD	0.00	phnF	0.24	mltE	-0.04
ybjO	0.02	yobF	-0.02	aas	0.15	phnE	-0.21	abgT	0.04

ybjF	0.03	yebO	0.02	galR	0.28	phnD	-0.01	ydcH	-0.03
artJ	0.08	yobG	-0.35	lysA	-0.14	phnC	0.24	yddM	-0.03
artM	-0.02	kdgR	0.03	lysR	0.22	phnB	-0.05	yneF	0.01
artQ	-0.01	yebR	0.06	ygeA	0.03	phnA	0.15	asr	0.08
artI	0.06	yebS	0.15	araE	0.03	yjdA	-0.06	ydhL	0.03
artP	0.01	yebT	-0.03	kduD	-0.01	proP	0.02	ydiD	0.05
ybjP	-0.01	yebU	0.05	kduI	0.33	basS	-0.06	yebN	0.02
ybjQ	-0.01	yebV	0.00	yqeG	-0.41	basR	0.02	znuA	0.00
ybjQ	-0.07	yebW	0.01	yqeH	0.04	yjdE	-0.04	yedQ	0.00
ybjR	0.05	pphA	0.19	yqeI	0.04	adiY	0.25	yeeJ	-0.05
ybjS	-0.02	yebY	0.00	yqeJ	-0.02	melR	0.26	yeeY	0.02
ybjT	-0.02	yebZ	-0.02	yqeK	0.10	melA	-0.02	wzzB	0.01
ltaE	0.08	yobA	-0.08	ygeF	-0.04	melB	0.39	yegR	0.01
poxB	0.01	holE	0.20	ygeG	0.10	yjdF	-0.05	yohG	0.01
hcp	0.08	yobB	0.07	ygeH	0.13	fumB	-0.09	yejO	0.01
ybjE	0.06	yobB	-0.01	ygeI	-0.01	dcuB	0.28	elaD	0.00
aqpZ	0.00	exoX	0.04	x	0.00	dcuR	0.00	yfcB	-0.03
ybjD	-0.12	yebE	0.01	ygeK	-0.04	dcuS	0.07	yphC	-0.01
ybjX	0.07	yebF	-0.06	JW5460	0.04	yjdI	-0.06	ygaA	0.04
macA	0.07	yebG	-0.03	ygeP	0.07	yjdJ	-0.01	ygcI	0.00
macB	0.01	purT	0.06	ygeQ	0.03	yjdK	-0.07	ygcS	0.00
cspD	-0.12	eda	0.02	ygeR	-0.01	lysU	0.11	JW5846	0.01
yljA	0.08	edd	-0.04	xdhB	0.33	yjdL	0.21	prfB	-0.02
clpA	-0.02	zwf	0.07	xdhC	0.01	cadA	-0.05	yghO	0.00
aat	0.06	hexR	0.10	ygeV	0.04	cadB	-0.18	yqhC	-0.03
cydD	-0.09	pykA	0.15	ygeX	0.02	cadC	-0.05	yqjF	-0.07
trxB	-0.02	msbB	0.04	ygeY	0.06	cutA	-0.02	pnp	-0.03
lrp	0.23	yebA	0.35	ygeZ	-0.27	aspA	-0.05	yihO	-0.03
ycaJ	-0.01	znuA	-0.12	yqeA	-0.09	fxsA	0.58	ysgA	-0.04
dmsB	0.02	znuC	-0.43	yqeB	0.00	yjeH	0.01	yigL	-0.02
dmsC	-0.20	znuB	-0.07	ygfJ	0.02	groL	-0.06	recQ	0.00
ycaC	-0.02	ruvB	-0.02	ssnA	0.02	yjeJ	-0.08	trxA	-0.03
ycaD	-0.17	ruvA	0.02	ygfM	0.06	yjeK	-0.13	rbsD	-0.06
ycaM	0.27	yebB	0.04	xdhD	-0.07	efp	-0.07	yidX	-0.01
ycaN	-0.08	ruvC	0.02	ygfO	-0.03	ecnB	0.56	dgoT	-0.03
ycaK	-0.09	yebC	0.08	idi	0.02	blc	-0.06	yidR	-0.03
pflA	-0.04	nudB	0.02	lysS	0.11	ampC	-0.01	yicJ	0.00
pflB	-0.06	yecD	0.23	recJ	-0.08	frdD	0.00	bcsC	-0.04
focA	0.12	yecE	-0.04	dsbC	0.34	frdC	0.09	yhjK	-0.02
ycaO	-0.02	yecN	-0.01	xerD	0.09	frdB	0.13	yrhA	-0.03
ycaO	-0.01	yecO	0.32	fldB	0.06	frdA	0.14	aroK	-0.05
ycaP	0.06	yecP	0.29	ygfX	-0.08	poxA	-0.01	aidB	-0.02
serC	0.04	torZ	-0.29	ygfY	-0.05	yjeN	-0.35	yjfR	-0.04
aroA	0.03	torY	-0.01	ygfZ	-0.08	yjeO	-0.44	yjiK	-0.03
ycaL	0.16	cutC	-0.10	yqfA	-0.08	yjeP	-0.66	mcrB	-0.04
cmk	-0.09	yecM	-0.02	yqfB	-0.06	yjeQ	0.16	bglJ	-0.03
ihfB	0.22	yecT	0.13	bglA	-0.08	yjeS	-0.04	prfC	-0.05
ycaI	-0.02	flhE	0.00	ygfF	-0.02	amiB	0.17	ydhM	-0.03
ycaQ	-0.03	flhA	0.10	gcvP	-0.06	mutL	0.04	yfeH	-0.05
ycaR	0.08	flhB	-0.07	gcvH	0.00	miaA	-0.17	ypeB	-0.03
ycbJ	-0.10	cheZ	-0.11	gcvT	-0.09	hfq	-0.06	csiE	-0.02
ycbC	0.05	cheY	-0.08	visC	0.11	hflC	0.02	JW5710	-0.02
smtA	0.10	cheB	-0.06	ubiH	0.06	yjeT	0.16	JW5711	0.03
ycbB	0.06	cheR	-0.26	pepP	-0.45	purA	-0.01	JW5712	0.02
ycbK	0.03	tap	0.01	ygfE	0.06	yjeB	-0.05	JW5713	-0.05
ycbL	-0.03	tar	0.05	ygfE	-0.06	yjfH	-0.04	JW5714	0.00
aspC	0.04	cheW	0.44	ygfA	0.00	yjfI	0.46	JW5715	-0.05
ompF	-0.03	cheA	0.02	serA	0.02	yjfJ	0.34	JW5716	0.09
pncB	0.08	motB	0.02	rpiA	-0.02	yjfK	0.55	JW5717	0.03
pepN	-0.03	motA	0.05	yqfE	-0.01	yjfL	0.55	JW5718	0.03
ssuB	0.01	flhC	0.12	iciA	0.26	yjfM	0.35	JW5719	0.01
ssuD	-0.02	flhD	0.02	sbm	0.03	yjfC	0.20	JW5720	0.01
ssuA	0.05	yecG	0.04	argK	0.04	aidB	-0.07	JW5721	0.00
ssuE	0.00	otsA	-0.01	ygfG	0.11	yjfP	0.12	JW5725	0.04
ycbR	-0.08	otsB	-0.01	ygfH	-0.04	yjfQ	0.01	JW5726	-0.02
ycbS	-0.06	araH	-0.07	ygfI	-0.01	yjfR	-0.08	JW5728	-0.01

ycbT	-0.09	araG	0.07	yggE	-0.05	sgaB	-0.02	JW5729	0.06
ycbU	-0.11	araF	-0.15	yggA	-0.05	sgaA	-0.05	JW5730	0.03
ycbV	-0.09	ftnB	0.07	yggB	0.53	sgaH	0.13	JW5731	0.08
ycbF	0.32	ftnB	0.05	epd	0.03	sgaU	-0.01	JW5732	0.02
pyrD	0.01	yecJ	-0.06	yggC	0.03	sgaE	0.06	JW5733	0.01
ycbW	-0.12	yecR	0.04	yggD	-0.02	yjfY	0.08	JW5734	0.10
ycbX	-0.05	yecR	0.09	yggF	-0.04	rpsF	-0.11	JW5735	0.11
ycbY	0.05	ftnA	-0.36	cmtA	-0.06	priB	0.11	JW5736	0.07
uup	-0.02	yecH	0.07	cmtB	-0.02	rplI	-0.12	JW5737	0.01
pqiA	0.01	tyrP	0.08	tktA	-0.05	yjfZ	0.00	JW5738	0.03
pqiB	-0.09	yecA	-0.02	yggG	0.03	ytfA	-0.01	JW5739	0.07
ymbA	0.02	uvrC	-0.25	speB	0.02	cycA	0.37	JW5741	0.06
rmf	0.15	uvrY	0.12	speA	-0.05	ytfE	0.06	JW5742	-0.01
ycbZ	0.07	yecF	0.00	yqgB	-0.06	ytfF	0.41	JW5743	0.03
ycbG	-0.01	sdiA	0.19	yqgC	0.03	ytfG	-0.02	JW5744	0.03
ompA	0.01	yecC	0.02	galP	0.06	cpdB	0.03	JW5745	-0.02
sulA	0.02	yecS	0.01	sprT	0.02	cysQ	-0.02	JW5746	-0.01
yccR	-0.03	fliY	-0.06	endA	0.07	ytfJ	0.28	JW5747	0.05
yccS	0.10	fliZ	0.00	yggJ	-0.24	ytfL	-0.06	JW5748	0.01
yccF	0.04	fliA	-0.01	gshB	-0.01	msrA	-0.09	JW5749	0.01
helD	0.02	fliC	-0.08	yqgE	-0.03	ytfM	0.04	JW5751	0.08
yccT	0.01	fliD	-0.02	yggR	-0.14	ytfN	0.16	JW5752	0.08
yccU	0.00	fliS	0.07	yggS	0.05	ytfP	0.07	JW5753	0.06
yccV	-0.03	fliT	0.25	yggT	-0.07	yzfA	0.03	JW5754	0.03
yccW	-0.10	amyA	0.01	yggV	-0.06	chpB	0.36	JW5755	-0.03
yccX	-0.04	yedD	0.09	yggW	0.02	ytfQ	0.11	JW5756	0.01
yccK	0.04	yedE	0.12	yggM	-0.02	fbp	0.01	JW5757	0.01
yccA	-0.14	yedF	0.07	ansB	0.08	mpl	0.01	JW5758	0.02
hyaA	-0.07	yedK	-0.07	yggN	-0.02	yjgA	-0.02	JW5759	-0.01
hyaB	-0.03	yedL	0.04	yggL	0.02	pmbA	-0.07	JW5761	-0.02
hyaC	-0.49	yedN	0.05	yggH	-0.04	cybC	0.01	JW5763	0.04
hyaD	-0.01	yedN	0.08	mutY	-0.03	nrdG	-0.03	JW5764	0.09
hyaE	-0.11	yedM	0.38	yggX	0.00	nrdD	0.05	JW5765	0.06
hyaF	-0.10	fliE	-0.10	mltC	0.04	treC	0.32	JW5766	0.03
appC	-0.10	fliF	0.08	JW2931	-0.03	treB	0.14	JW5768	0.02
appB	0.05	fliG	0.03	nupG	-0.13	treR	0.28	JW5769	0.01
yccB	0.05	fliH	0.06	yqgA	-0.03	mgtA	0.18	JW5770	0.04
appA	0.01	fliI	0.04	yghD	-0.20	pyrI	0.39	JW5774	0.02
etk	-0.19	fliJ	0.02	yghG	-0.05	pyrB	-0.06	JW5775	0.10
etp	0.29	fliK	0.09	pppA	0.00	pyrL	0.30	JW5776	0.09
yccZ	-0.34	fliL	0.03	glcA	0.03	yjgH	0.03	JW5777	-0.02
ymcA	0.24	fliM	-0.05	glcB	-0.02	yjgI	0.02	JW5778	0.11
ymcB	-0.02	fliN	0.06	glcG	-0.01	yjgJ	0.03	JW5779	0.04
ymcC	-0.02	fliP	-0.08	glcD	-0.05	argI	-0.04	JW5781	0.07
ymcC	-0.14	fliQ	0.07	glcC	0.01	yjgD	-0.03	JW5782	0.06
ymcD	-0.20	fliR	0.06	yghR	-0.03	holC	0.00	JW5783	0.04
cspG	0.32	rcsA	0.02	yghT	-0.04	pepA	0.00	JW5784	0.04
sfa	-0.03	dsrB	-0.10	pitB	0.00	yjgR	0.03	JW5784	-0.03
gnsA	-0.03	yodD	0.08	gsp	-0.02	idnR	0.22	JW5785	-0.01
yccM	0.06	yedP	0.09	hybG	-0.01	idnT	0.37	JW5786	0.08
torS	0.03	JW1939	0.08	hybE	0.01	idnO	0.17	JW5787	0.05
torT	0.04	yodC	0.11	hybC	0.21	idnD	0.24	JW5788	0.10
torR	0.06	yedI	0.01	hybA	-0.01	idnK	0.14	JW5789	0.04
torC	-0.12	yedA	0.06	hyb0	0.02	intB	0.39	JW5790	0.01
torA	-0.15	vsr	0.10	yghW	0.04	yjgW	-0.10	JW5791	0.08
torD	-0.28	dcm	0.10	yghZ	-0.03	yjgX	0.05	JW5792	0.02
yccD	0.05	yedJ	0.03	yqhA	0.08	yjgZ	0.08	JW5793	-0.01
cbpA	0.03	yedR	0.07	yghA	-0.05	yjhE	0.04	JW5794	0.04
yccE	0.04	yedS	-0.01	exbD	0.39	JW4246	-0.02	JW5795	0.04
agp	0.04	hchA	-0.10	exbB	0.20	JW4246	0.01	JW5796	0.08
yccJ	0.04	yedV	0.00	metC	0.00	fecE	0.13	JW5797	0.03
wrbA	0.03	yedW	0.06	yghB	-0.04	fecD	0.12	JW5798	0.01
ymdF	0.03	yedX	-0.07	JW2977	0.16	fecC	-0.04	JW5799	0.04
ycdG	0.07	yedY	-0.05	yqhD	-0.15	fecB	0.16	JW5800	0.18
ycdH	-0.02	yedZ	-0.04	yqhH	0.08	fecA	0.01	JW5801	0.04
ycdH	0.21	yodA	-0.22	sufI	-0.02	fecR	0.04	JW5803	0.12

ycdI	0.05	yodB	0.00	parC	-0.05	fecI	-0.03	JW5805	0.18
ycdJ	0.06	yeeI	0.03	ygiS	0.19	yjhF	0.05	JW5807	-0.02
ycdK	0.07	JW1959	-0.03	ygiU	-0.03	yjhG	0.04	JW5808	0.03
ycdL	-0.03	yeeL	-0.04	ygiV	-0.05	yjhI	0.07	JW5809	0.01
ycdM	-0.03	shiA	-0.14	ygiW	0.08	sgcR	-0.01	JW5811	0.00
ycdC	-0.07	amn	0.05	qseB	0.05	sgcE	0.04	JW5812	0.05
putA	-0.13	yeeN	0.10	qseC	0.03	sgcA	0.02	JW5813	0.06
yzpU	0.02	yeeO	-0.08	ygiZ	0.01	sgcQ	-0.03	JW5814	0.03
ycdN	0.04	cbl	-0.05	mdaB	-0.06	sgcC	0.08	JW5815	-0.03
ycdO	0.07	nac	0.21	ygiN	-0.03	sgcB	0.07	JW5816	-0.06
ycdB	0.01	erfK	0.00	yqiA	-0.06	sgcX	0.03	JW5817	0.02
phoH	-0.18	cobT	0.11	icc	0.10	yjhP	0.04	JW5818	0.03
ycdP	0.00	cobS	0.08	yqiB	0.00	yjhQ	-0.17	JW5819	0.00
ycdQ	-0.02	cobU	-0.15	nudF	0.31	JW4269.5	0.09	JW5820	-0.01
ycdR	-0.01	JW5326	-0.03	tolC	-0.29	yjhR	0.01	JW5821	-0.04
ycdS	-0.02	JW1980	-0.14	ygiC	-0.08	yjhS	0.13	JW5822	0.04
ycdU	-0.05	flu	-0.02	ygiD	-0.09	fimB	-0.03	JW5823	-0.03
ycdW	-0.05	yeeR	-0.12	zupT	-0.02	fimE	-0.05	JW5824	-0.03
ycdX	0.03	yeeS	-0.05	ygiL	-0.01	fimA	0.08	JW5825	0.01
ycdY	-0.02	yeeT	-0.01	yqiG	0.07	fimC	0.03	JW5826	0.08
ycdZ	-0.02	yeeU	0.02	JW3017	0.06	fimD	0.02	JW5827	0.03
csgG	-0.05	yeeV	-0.03	glgS	0.04	fimF	-0.02	JW5828	0.05
csgF	-0.06	yeeW	0.03	yqiJ	-0.17	fimG	-0.07	JW5830	0.02
csgE	0.05	yeeX	0.04	yqiK	-0.04	fimH	0.10	JW5831	0.00
csgD	-0.01	yeeA	0.05	rfaE	-0.08	gntP	0.01	JW5832	0.00
csgB	0.05	gyrI	0.02	glnE	-0.07	uxuA	0.09	JW5833	-0.05
csgA	-0.19	sbcB	0.14	ygiF	-0.07	uxuB	0.26	JW5834	0.02
ymdB	0.00	yeeD	0.09	ygiM	-0.27	uxuR	0.21	JW5836	0.01
ymdB	-0.01	yeeE	0.04	upk	-0.03	yjiC	0.04	JW5837	0.01
mdoC	0.03	yeeY	-0.12	folB	0.06	yjiE	-0.03	JW5838	0.01
mdoG	0.21	yeeZ	0.26	ygiH	-0.03	iadA	0.01	JW5839	0.01
JW1036	0.01	hisL	0.01	ygiP	0.00	yjiG	0.03	JW5840	0.00
mdoH	0.02	hisG	0.06	ttdA	0.36	yjiJ	0.15	JW5841	-0.03
yceK	-0.04	hisD	-0.09	ttdB	0.31	yjiK	0.05	JW5842	-0.01
msyB	-0.10	hisC	-0.07	ygjE	-0.07	JW4296.5	0.04	JW5843	0.04
yceE	0.00	hisB	-0.02	rpsU	-0.04	yjiN	0.05	JW5844	0.00
htrB	0.05	hisA	-0.25	dnaG	-0.14	yjiO	0.01	JW5845	0.00
yceA	0.01	hisF	0.10	rpoD	0.01	yjiP	-0.22	JW5846	0.01
yceA	-0.05	hisI	-0.08	mug	-0.06	yjiR	0.12	JW5847	-0.02
yceI	-0.03	ugd	-0.05	yqjH	-0.02	yjiS	0.08	JW5848	0.00
yceJ	0.00	gnd	0.04	yqjI	-0.05	mcrB	0.05	JW5849	-0.03
yceO	-0.18	wbbL	0.05	aer	-0.03	yjiW	0.04	JW5850	-0.07
solA	0.04	wbbL	-0.05	ygjH	-0.18	hsdS	0.01	JW5851	-0.03
yceP	0.00	yefI	0.25	ebgR	0.17	hsdM	0.75	JW5852	-0.03
dinI	-0.05	wbbJ	-0.02	ebgA	0.08	hsdR	0.06	JW5853	-0.04
pyrC	-0.16	yefG	-0.23	ebgC	0.00	mrr	-0.11	JW5854	-0.02
yceB	-0.01	rfc	0.03	ygjI	0.07	yjiX	0.04	JW5855	0.00
grxB	0.47	glf	0.04	ygjJ	0.11	tsr	-0.07	JW5856	-0.03
yceL	-0.04	rfbX	0.04	ygjK	-0.02	yjjL	0.05	JW5857	-0.06
yceL	-0.03	rfbC	-0.14	fadH	0.06	dnaT	0.03	JW5858	-0.01
JW5859	-0.03	JW5863	-0.02	JW5868	-0.04	JW5872	-0.03	JW5877	-0.03
JW5860	-0.03	JW5864	-0.03	JW5869	-0.03	JW5873	-0.05	JW5878	-0.02
JW5861	0.00	JW5866	-0.05	JW5870	-0.03	JW5874	-0.03	JW5876	-0.05
JW5862	-0.04	JW5867	-0.02	JW5871	-0.04				

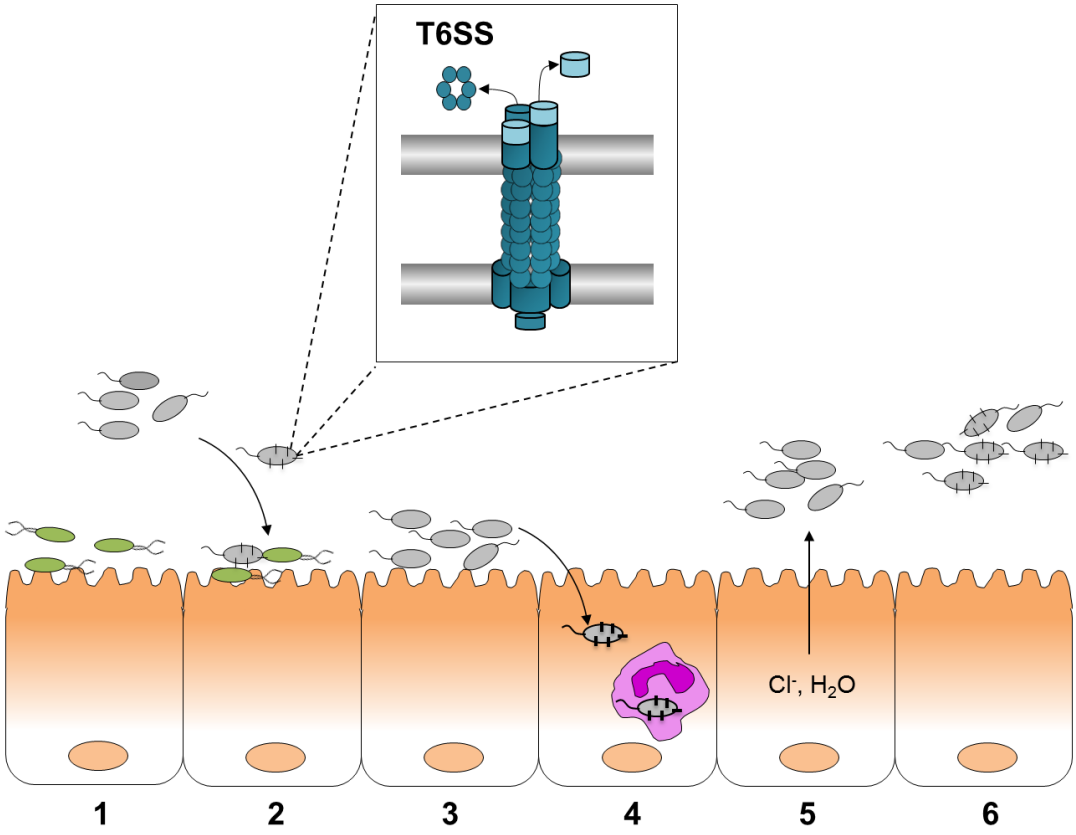

Figure 7 - 1 Model of the role of the T6SS of *V. cholerae*.

(1) The human small intestine could be colonized with commensal bacteria (green) or pathogens such as *V. cholerae* (grey). (2) Upon *V. cholerae* infection, it passes the gastric acid barrier and then reaches the small intestine, where *V. cholerae* can assembles the T6SS on its surface (black lines) in order to perform T6SS-mediated killing towards commensal bacteria. (3) Then *V. cholerae* colonizes the small intestine, (4) where it can use T6SS-mediated killing towards immune cells, allowing the infection to proceed. (5) *V. cholerae* produces and secretes the cholera toxin, which leads to the watery diarrhea associated with it and (6) then it leaves the human host. (7) *V. cholerae* can use the T6SS-mediated killing for intraspecific competition. Figure adapted from (523).

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