COORDINATION OF *VIBRIO CHOLERAE* EARLY COLONIZATION PHENOTYPES

IN RESPONSE TO HOST INTESTINAL FACTORS

Amanda J Hay

A DISSERTATION

in

Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2016

Supervisor of Dissertation

_____
Jun (Jay) Zhu, Ph.D
Associate Professor of Microbiology

Graduate Group Chairperson

_____
Daniel Kessler, Ph. D
Professor of Cell and Developmental Biology

Dissertation Committee

Sunny Shin, Ph. D	Assistant Professor of Microbiology
Mark Goulian, Ph. D	Professor of Biology
Igor Brodsky, Ph. D	Assistant Professor of Microbiology
Mechthild Pohlschroder, Ph. D	Professor of Biology
Fevzi Daldal, PhD.	Professor of Biology

COORDINATION OF *VIBRIO CHOLERAE* EARLY COLONIZATION PHENOTYPES IN

RESPONSE TO HOST INTESTINAL FACTORS

dedicated to my parents
for their unwavering love and support

ABSTRACT

COORDINATION OF *VIBRIO CHOLERAE* EARLY COLONIZATION PHENOTYPES

IN RESPONSE TO HOST INTESTINAL FACTORS

Amanda Hay

Jun Zhu

Vibrio cholerae causes human infection through ingestion of contaminated food and water, leading to the diarrheal disease, cholera. In aquatic environments this bacterium displays an expression profile that is distinct from that observed during infection. It can also form matrix-encased aggregates known as biofilms, typically on chitinous surfaces, which can be important for transmission and infectivity. Upon entry into the host, a tightly regulated circuit coordinates induction of two major virulence factors: cholera toxin (CT) and a toxin co-regulated pilus (TCP). This study finds that bile components present in the host intestine can affect both of these processes. Certain bile salts, including taurocholate (TC), serve as host signals to activate *V. cholerae* virulence through inducing the activity of a transmembrane virulence regulator TcpP. In this study we show that Ca^{2+}, an abundant metal ion in the gut, enhances bile salt-dependent virulence activation. Induction of TCP by murine intestinal contents is counteracted when Ca^{2+} is depleted by the high affinity calcium chelator EGTA, suggesting that calcium present in the gut is a relevant signal for *V. cholerae* virulence induction in vivo. We further show that in conjunction with TC, Ca^{2+} affects dimerization and membrane diffusion of TcpP as analyzed by bacterial two-hybrid and fluorescence recovery following photobleaching assays. When applied to mature biofilms, TC induces an increase in number of detached cells with a concomitant decrease in biofilm mass. Inhibition of protein synthesis did not alter rates of detachment, suggesting that *V. cholerae* undergoes a passive dispersal.

Scanning electron microscopy micrographs of biofilms exposed to taurocholate revealed an altered, perhaps degraded, appearance of the biofilm matrix and cell-free media contains a higher amount of free polysaccharide with TC, suggesting an abiotic degradation of biofilm matrix by taurocholate. Furthermore*, V. cholerae* induces virulence in response to taurocholate only after exiting from the biofilm. Our data suggests a model in which *V. cholerae* ingested as a biofilm has co-opted the host-derived bile salt signal to sequentially detach from the biofilm and go on to activate virulence, which is further promoted by the synergistic effects of physiological levels of Ca^{2+}.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

CHAPTER 1: INTRODUCTION

***Vibrio cholerae* and cholera**

The diarrheal disease cholera is an important and ongoing health risk. The World Health Organization estimates that there are 3-5 million cases annually, with the majority of these being unreported[1]. The disease affects countries in which it is endemic, but also occurs in outbreaks, particularly following natural and humanitarian crises. The rapid spread and disease onset can quickly overburden health systems. This has been highlighted the case of Haiti. Following a devastating earthquake in 2010, an outbreak of cholera sickened 300,000 in the first ten months and has been an ongoing source of disease in subsequent years[2]. The bacteria responsible for cholera can cause disease following ingestion of contaminated food or water, and is transmitted via the fecal-oral route[3]. After an incubation period of as little as 18 hours, cholera patients experience vomiting and diarrhea, resulting in rapid dehydration and death if rehydration is not provided[3]. If a cholera patient resolves the disease, they may be temporarily protected, but re-infection is possible within five years. Various cholera vaccines are available, but do not meet global demand due to sub-optimal efficacy, insufficient production, or difficulty of administration in resource-poor settings[4].

The disease cholera likely dates back to pre-history; seven pandemic strains of the causative *V. cholerae* bacteria have been responsible for disease in the past two hundred years[5]. The seventh and current pandemic is dominated by the El Tor biotype, which may cause a more severe form of disease than past strains. As such, *V. cholerae* is sometimes considered a re-emerging disease[5]. Pathology during cholera is mediated

predominantly by cholera toxin (CT), which is sufficient to promote the hallmark "rice water stool" associated with the disease[3]. CT is produced by *V. cholerae* and then delivered to intestinal epithelial cells by a type 3 secretion system. CT is an oligomeric enterotoxin that consists of a single enzymatic Subunit A encased by a ring of five Subunit B proteins. Subunit B facilitates binding to epithelial cells. After the toxin is endocytosed, Subunit A goes on to initiate cellular processes resulting in host efflux of chloride and subsequent secretion of other salts and water[6]. The resulting diarrhea can lead to a fluid loss of up to several liters per day[3]. The second major virulence determinant is the toxin co-regulated pilus (TCP), which is essential for colonization in animal models and human volunteer studies[7]. This type IV pilus is important for bacteria-bacteria interactions and microcolony formation in the small intestine, although its full repertoire of functions is still under active investigation[8–10].

***Vibrio cholerae* regulation of virulence**

To achieve the diarrheal disease cholera, *V. cholerae* coordinates regulation of major virulence factors CT and TCP, depicted in Fig. 1.1[11]. The genes encoding proteins that make up the toxin (*ctxAB*) and pilus (*tcpA-F*) are directly activated by the cytosolic transcription factor, ToxT[12]. Two integral membrane regulators contribute to induction of ToxT. These regulators, ToxR and TcpP, in conjunction with helper proteins ToxS and TcpH, respectively, bind upstream of the *toxT* coding sequence[13]. An additional level of regulation occurs at the point of TcpP induction. AphA, AphB, and OhrR transcription factors work together to activate TcpP in response to environmental stimuli including low oxygen tension[14–17]. AphA also links quorum sensing and virulence regulation in *V. cholerae*[18,19].

V. cholerae can persist in brackish environments, and there are many strains of *V. cholerae* that are not considered pathogenic. Typically, only those strains possessing the two major virulence determinants are considered to cause endemic disease[20]. The genes encoding CT, *ctxAB*, are encoded from a lysogenic bacteriophage, CTXΦ, which uses TCP as a receptor and exists as an integrated prophage[21]. Genes encoding TCP structural proteins, ToxT, TcpPH, and accessory colonization factors (ACFs) are found on a pathogenicity island that was likely acquired through horizontal gene transfer[22]. Another major regulator, ToxR, is encoded on the ancestral genome, and as such plays a role in regulation of additional non-virulence related processes[23–25]. Various virulence associated genes and proteins comprise a tightly controlled regulatory cascade that can sense and respond to environmental conditions and signals[11].

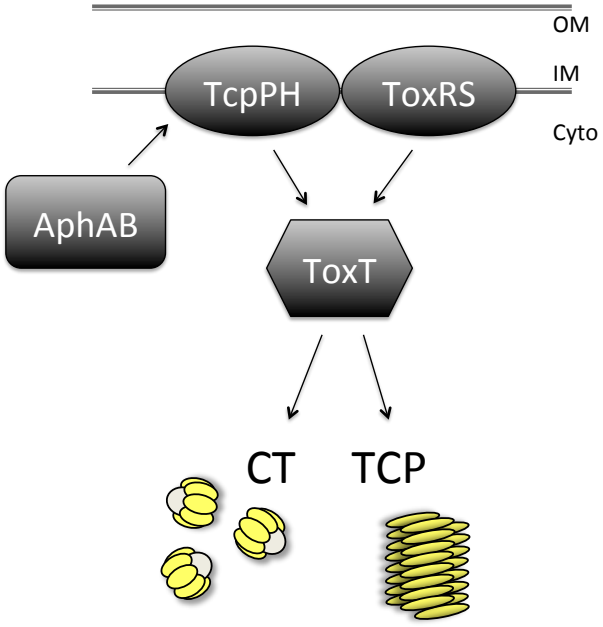


Figure 1.1 Schematic of virulence regulation in *V. cholerae*. AphA and AphB promote expression of TcpPH, which acts coordinately with ToxRS to induce ToxT and activate major virulence factors, cholera toxin (CT) and the toxin co-regulated pilus (TCP)

Composition and production of bile in the human gut

Following ingestion, *V. cholerae* will experience changing conditions as they will transit to the small intestines, which is the main site of colonization. Oxygen tension decreases and passage through the stomach exposes the bacteria to highly acidic conditions. In the intestines, the bacteria encounter various compounds such as bile and

bicarbonate, as well as an array of other immune defenses including mucin, antimicrobial peptides, and antibodies[26–28] At this interface, the intestinal epithelium is covered in a thick mucous layer overlaid by a second mucous layer that decreases in density as it extend toward the lumen[27]. Bile exists at high concentrations in the lumen, where its antimicrobial activity helps provide a barrier against bacterial outgrowth[29]. To successfully colonize the intestinal tract, *V. cholerae* and other enteric bacteria must adapt to the stress of bile.

As stomach contents are released the small intestine, bile is also deposited to aid

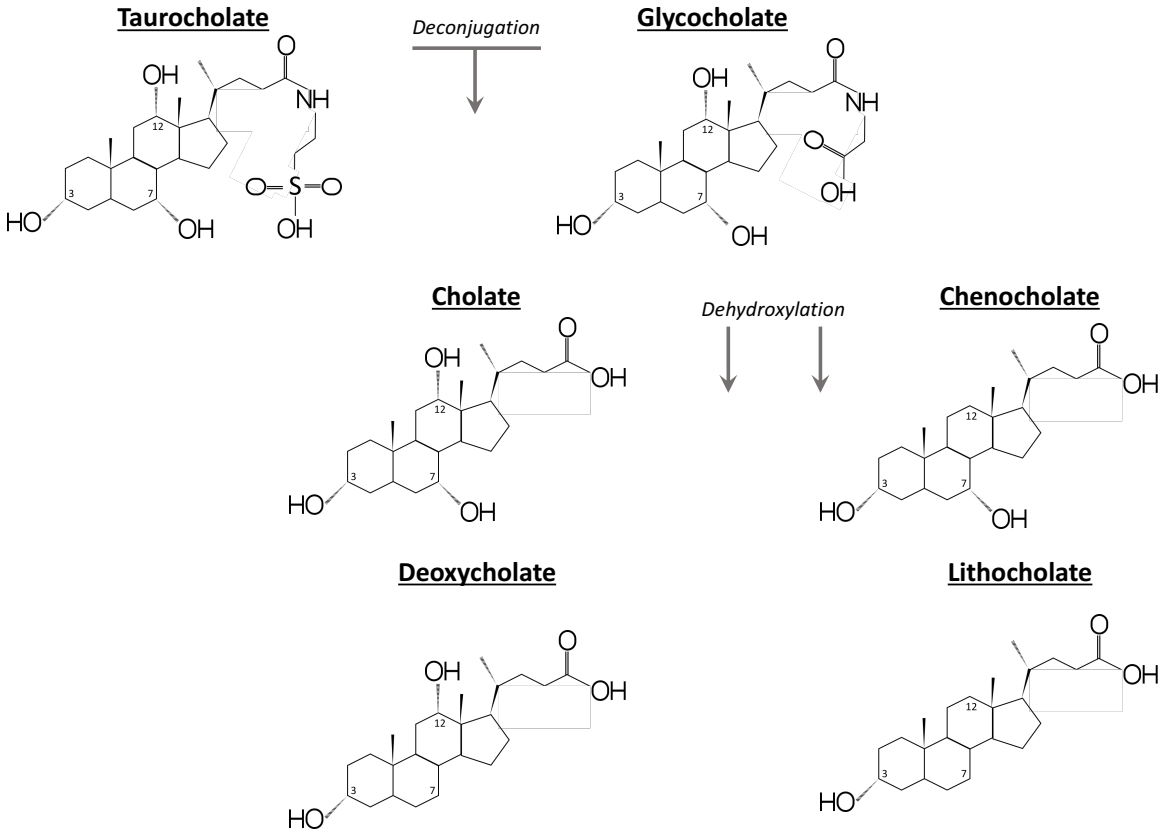


Figure 1.2 Structure of select bile salts. Primary bile salts cholate (cholic acid) and chenodeoxycholate (chenodeoxycholic acid) are synthesized in the liver, differing only by hydroxylation status at the 12C position. Before being stored in the gallbladder for use in digestion, primary bile salts are conjugated to a taurine or glycine group with an amide bond. Cholate conjugated to glycine forms glychocholate (glychocholic acid) and with taurine form taurocholic acid. Deconjutation by gut bacteria can return bile acids to cholate and chenodeoycholate. Once deonjugated, dehydroxylation by gut bacteria can occur at the 7C position. Dehydroxylation of cholate and chenodeoxycholate result in deoxycholate and lithocholate, respectively.

in digestion. Bile is a complex mixture composed of bile acids, bilirubin, cholesterol, phospholipids, fatty acids (both saturated and unsaturated) and ions such as Ca^{2+}, Na^{+} and Cl^{-} [30]. Bile salts in particular exert toxic effects on many bacteria, and are an important host signal for *V. cholerae*. A mixture of bile salts is synthesized in the liver from cholesterol precursors, with different bile having the potential to differentially affect bacterial physiology. The two main bile salts synthesized are cholic acid and chenocholic acid, and are termed primary bile acids (Fig. 1.2). They share a common sterol core, differentiated by the presence of a hydroxyl group at the C12 position. Secondary bile acids lack the C7 hydroxyl group (Fig. 1.2). Any of these bile acids may be conjugated with glycine or taurine amino groups. Before being released from the liver for storage in the gallbladder, primary bile acids are conjugated with a taurine or glycine group, to form taurocholate or glychocholate, respectively [31]. Following a meal, bile is released into the duodenum from the gallbladder where it is stored. Bicarbonate is also released, resulting in an increase of pH after deposition of acidic stomach juices[32].

***Vibrio cholerae* responses to bile**

Bile salts have antimicrobial properties that can affect the physiology of *V. cholerae* and other enteric bacteria[33]. Several responses to bile have been described for *V. cholerae* that contribute to survival and pathogenesis. Gram negative bacteria such as *V. cholerae* display some inherent resistance to bile compared to gram positive bacteria. This is due in part to the semi-permeable outer membrane that limits bile entry into gram negative cells[34]. Gram negative bacteria also efficiently pump bile salts and other toxic compounds out of the cell by use of the resistance–nodulation–division (RND) family of transporters. These three-member pumps traverse the inner membrane, periplasm, and outer membrane. In conjunction with TolC, an outer membrane

embedded pore complex, substrates are extruded from the cytoplasm into the environment[35]. The first and most well studied member, AcrAB, was discovered in E. coli[36]. AcrAB-TolC in *E. coli* can export a wide variety of substrates, and as such is considered a multidrug efflux pump[37]. This transporter family is generally known for its wide substrate specificity, but certain members do have higher affinity for specific compounds. For example, *V. cholerae* possesses RND pumps that when deleted, render bacteria broadly sensitive noxious compounds. However, *V. cholerae* mutants lacking the efflux pump VexCD have increased sensitivity to deoxycholate, but not to other compounds tested, suggesting substrate specificity to bile salt for this particular pump[38]. Efflux pumps such as AcrAB are constitutively expressed in many bacteria, but bile and other insults can either increase their expression or promote induction of axillary pumps with higher substrate specificity for bile[39]. *V. cholerae* efflux pumps VexAB and VexCD are both induced by 0.2% bile and VexCD (also named BreAB) was shown specifically to be induced by regulator BreR in response to deoxycholate[38,40,41]. Both efflux pumps were also induced in rabbit ileal loop model, indicating that these genes may play a role during infection *in vivo* [42]. AcrAB homologues are typically encoded as an operon, with TolC being encoded elsewhere on the chromosome, and often pairing with multiple pumps[35,43]. In *V. cholerae*, multiple RND family pumps are thought to pair with the same TolC, as deletion of this outer membrane protein renders the bacteria acutely sensitive to bile salts, while mutation of other putative TolC homologues does not[44].

When exposed to bile, many gram negative bacteria will attempt to exclude bile from entering the cell. To do this, many alter the expression of outer membrane porins that bile salts may pass through. Porins are comprised of beta barrel proteins that make up aqueous-filled channels. They are ubiquitous among gram negative bacteria and allow passive diffusion of molecules with little substrate specificity[45]. For *V. cholerae*,

OmpT is one of the primary porins, expressed under normal growth conditions[46]. OmpT is a “large” diameter porin in that it can accommodate diffusion of bile salt-sized substrates. Upon bile exposure, *V. cholerae* will instead express a “small” diameter porin, OmpU, which is thought to limit diffusion of bile salts and larger molecules. ToxR directly regulates expression of these two porins in the presence of bile by repressing the “large” diameter OmpT and activating the “small” OmpU porin[47]. When *ompT* and *ompU* promoters were swapped, resulting in ToxR-dependent bile activation of OmpT, these mutant cells displayed decreased bile resistance and greatly diminished colonization[48]. Further exploration of these two porins showed that wild type cells expressing the bile-induced OmpU had decreased outer membrane permeability and were better able to restrict anionic flux, supporting their proposed roles in bile resistance [47,49].

Conclusions

The work included in this dissertation adds to our knowledge of how *V. cholerae* responds to bile and its component parts. In Chapter 2, the response of *V. cholerae* grown in biofilms to conditions similar to those encountered *in vivo* is explored. Because *V. cholerae* biofilms likely represent a natural route of infection, we tested the responses of these biofilms to individual bile salts that would be encountered in the intestine. We found that taurine-containing bile salts such as TC can promote dispersal from mature biofilms, likely through physical disruption of the biofilm matrix components. We also found that virulence induction by the same bile salt, TC, occurred only following egress from biofilms. In further attempt to characterize the response of *V. cholerae* to intestinal conditions, we sought intestinal factors that contribute to induction of virulence associated genes. Building on previous identification of TC as a host promoted virulence

activating factor (VAF), Chapter 3 characterizes the contribution of calcium to this bile salt activation of major virulence determinant TcpA. We found that calcium acts in conjunction with TC to promote regulator activity and downstream activation of virulence genes. Calcium and TC affect TcpP-TcpP interaction and movement within the membrane, suggesting that the signals affecting bacterial induction of virulence may be more complex than previously appreciated. Taken together, we have proposed a model in which *V. cholerae* ingested as either a biofilm or as planktonic cells will respond to host signals to coordinate biofilm egress as well as timing and magnitude of virulence factor induction (See Figure 4.2). A better understanding of these early steps of infection may be informative for optimizing treatment and prevention strategies to combat the burden of cholera.

CHAPTER 2: HOST INTESTINAL SIGNAL-PROMOTED BIOFILM DISPERSAL INDUCES *VIBRIO CHOLERAE* COLONIZATION

Introduction

In its natural aquatic environment, *V. cholerae* is often associated with chintinous surfaces in a matrix-encased biofilm[50,51]. *V. cholerae* may be more infectious when ingested in this natural biofilm state, as a lower infectious dose is necessary to cause disease with biofilms compared to free-living planktonic cells. Additionally, incidence of cholera can be greatly reduced by simple filtration of contaminated water [52], suggesting that selective removal of bacterial biofilms is sufficient to reduce infection. Evidence also suggests that biofilm-like aggregates from rice water stool persist in the environment[53]. Efficacy of biofilm-initiated infection has been attributed to a concentrated infectious dose as well as protection during gastric passage[54]. However, even when biofilms are disrupted prior to infection, biofilm-derived *V. cholerae* cells out-compete their free-living planktonic counterparts[55]. This observation highlights the importance of understanding the early steps of infection in a more natural, biofilm route, especially taking into consideration the physiologic state of those biofilm-resident cells during this process.

Like most bacteria, *V. cholerae* can grow as a biofilm, or community of bacteria encased in a matrix. Entrance into a biofilm state of growth is a is a multistep developmental process bacteria can initiate when under stressful conditions such as nutrient limitation[50,51,56]. When *V. cholerae* encounters bile or other biofilm-inducing conditions it expresses a set of Vibrio Polysaccharide (VPS) synthetic genes (*vpsA*-*Q*) that result in production of an exopolysaccharide to encases the bacterial aggregate[57]. In addition to this major biofilm matrix component, *V. cholerae* also produces several

proteins that stabilize the matrix[58]. This induction is regulated by the major regulators VpsT and VpsR[59,60], which in turn are regulated by quorum sensing[54,61]. This pathway can be exogenously activated by signals such as starvation and bile[62].

Once they have entered a biofilm state of development, bacteria may employ methods to actively exit from a biofilm. Strategies may include egress by enhanced motility or repressed adherence, and targeting of matrix components for degradation by bacterially produced enzymes or small molecules[63]. Often, these systems involve complex genetic regulatory mechanisms that cue dispersal in response to internal and external cues such as quorum sensing and nutrient availability, respectively. For example, *Escherichia coli* K-12 dispersion is controlled by the global regulator, CsrA, which modulates carbon utilization and can drive the bacteria into a biofilm-repressive state[64]. For *Staphylococcus aureus*, glucose limitation promotes expression of the *arg* system, the activation of which leads to extracellular protease production and ultimately dispersion[65]. The opportunistic pathogen Pseudomonas aeruginossa can affect it own dispersal through production of surfactants that non-specifically disrupt cell-cell and cell-biofilm interactions, as well as a more explosive seeding dispersal in which hollowed out biofilm structures burst open to release highly motile planktonic cells[66,67]. *P. aeruginosa* detached planktonic cells utilize a distinct regulatory profile compared to biofilm or free-living planktonic cells, displaying enhanced virulence induction[67], once again highlighting the notion that biofilm resident or previously biofilm resident cells are physiologically distinct from their free-living planktonic counterparts. In this study, we find that *V. cholerae* biofilm dispersal may impact infection, and attempt to characterize this bile-salt promoted exit from biofilms.

Results

The bile salt taurocholate (TC) decreases biofilm formation. To better understand the interplay between *V. cholerae* biofilms and the host signal bile salts, we incubated *V. cholerae* in biofilm-inducing conditions in the presence of various bile salts. As previously reported, *V. cholerae* formed thicker biofilms in the presence of the bile salts deoxycholate (DC) and cholate (CC) (Fig. 2.1 A)[62]. Surprisingly, biofilm formation was reduced in the presence of taurocholate (TC) (Fig. 2.1 A). Biofilm formation was not significantly altered when grown in the presence of another conjugated bile salt, glychocholate (GC) (Fig. 2.1 A). In the presence of DC and CC, *V. cholerae* biofilm formation is enhanced via increased production of VPS[62]. Therefore, we measured *vpsA* expression during biofilm formation in the presence of other bile salts. At 18 hours, *vpsA* expression was increased when grown in the presence of DC, but unaltered in the presence of TC (Fig. 2.1 B), suggesting that decreased VPS production was not the cause of lower

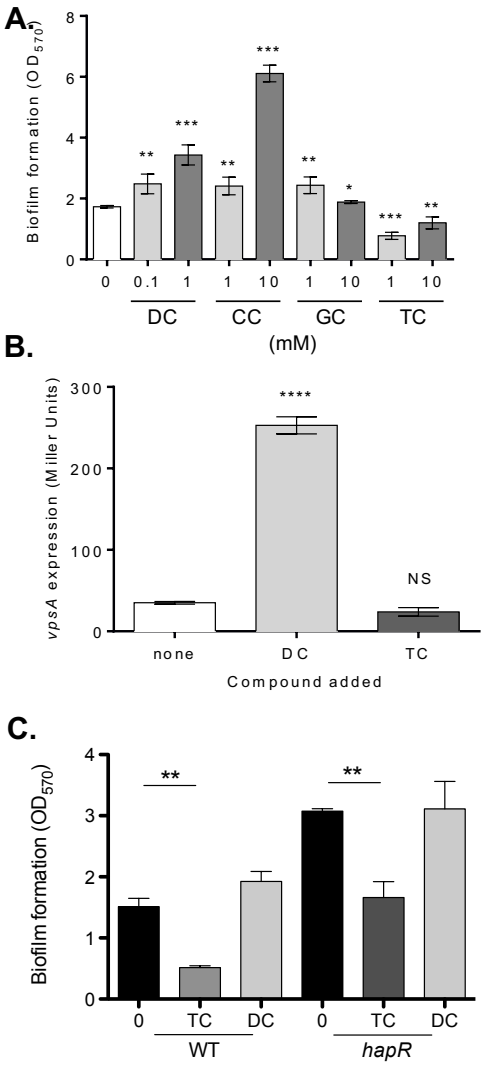


Figure 2.1 Effect of bile salts on biofilm formation. A. *V. cholerae* was inoculated into glass tubes and allowed to form biofilms for 24 hours at 22ºC. Biofilms of cultures grown in the presence of deoxycholate (DC), cholate (CC), glychocholate (GC) or taurocholate (TC) were quantified with crystal violet staining and data are presented in OD_{570} values. **B.** Expression of *vpsA* during formation of WT *V. cholerae* biofilms in the presence of 1 mM DC and TC for 18 hrs, as measured by the β-galactosidase assay. **C.** Biofilm formation of WT and Δ*hapR* mutant strains in the presence of TC or DC as quantified by CV staining. Data are means and s.d. of three independent experiments. Statistical significance reported as NS: no significance; *: P< 0.05; **: P < 0.005; **: P< 0.0005; ****: P< 0.0001.

biofilm formation in the presence of TC. Quorum sensing control over biofilm formation is executed through HapR, therefore Δ*hapR* mutant *V. cholerae* grow thicker biofilms than WT[54]. When grown in the presence of TC, Δ*hapR* mutants also show decreased biofilm formation (Fig. 2.1 C), suggesting that TC does not affect quorum sensing-regulation of biofilm formation.

The bile salt taurocholate (TC) promotes detachment of mature biofilms *in vitro*.

We next considered whether cells were less capable of residing in the biofilm, perhaps detaching at a more rapid rate than attaching or growing. To test this possibility, we performed detachment assays to determine whether bile salts affect the rate at which cells exit the biofilm. After 24 hours of growth, *V. cholerae* forms robust biofilms that

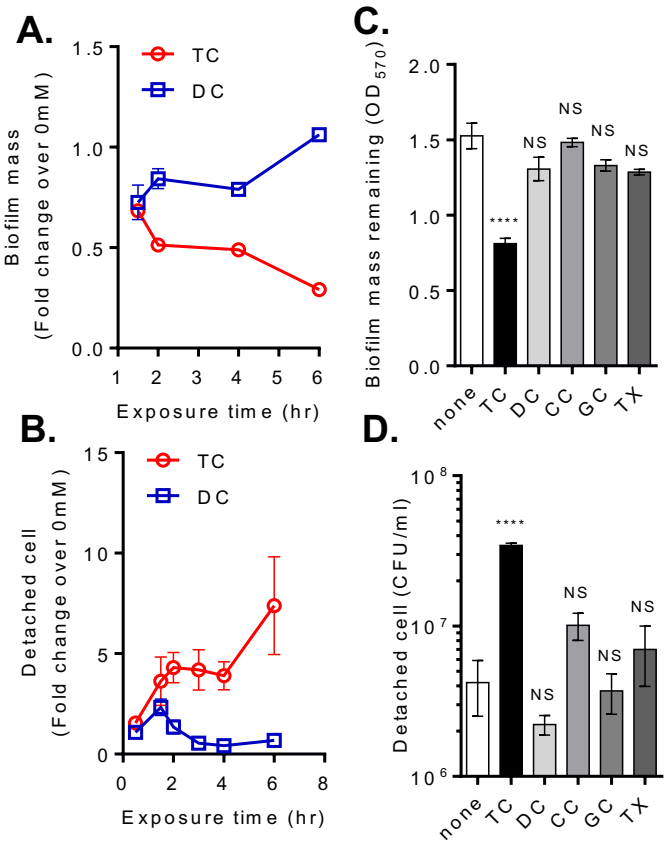


Figure 2.2 Effect of taurocholate (TC) on detachment of mature *V. cholerae* biofilms. **A**. Remaining biofilm mass after detachment for indicated time in the presence of 1mM taurocholate (TC) or 1mM deoxycholate (DC), presented as fold change of OD_{570} over no bile salts added for the same incubation period. **B.** Biofilm-derived planktonic cells when incubated in the presence of 1mM TC or 1mM DC presented as fold change of CFU/mL over no bile salts added for the same incubation period. **C, and D.** Remaining biofilm mass (OD_{570}) and detached cells (CFU/mL), respectively, following 2 hour exposure to TC, DC, cholate (CC), glychocholate (GC), and 0.1% Triton X (TX) All bile salts are at 1 mM final concentration. Data are means and s.d. of three independent experiments. Statistical significance reported as NS: no significance; ****: P< 0.0001.

display a characteristic pillar and tunnel architecture[56,68]. After washing away free-living planktonic cells, *V. cholerae* biofilms were incubated with fresh media supplemented with various bile salts. When biofilms were exposed to physiologic levels of TC[28], we found that there was a decrease in biofilm mass compared to biofilms incubated either with DC, or without bile salts (Fig. 2.2 A). Over this same time, we also observed an increase in the number of biofilm-derived planktonic cells in the samples incubated with TC (Fig. 2.2 B). This enhanced dispersal was not observed with CC, which shares structure with TC, but lacks a conjugated taurine group. Nor was it observed with another conjugated bile salt, GC, or with the detergent Triton X100 (TX) (Fig. 2.2 C, D) indicating the specificity of the bile salt taurocholate to promote detachment.

Taurocholate alters *V. cholerae* biofilm matrix structure. To further understand the nature of TC-enhanced dispersal, *V. cholerae* biofilms were imaged using scanning electron microscopy following incubation with or without TC

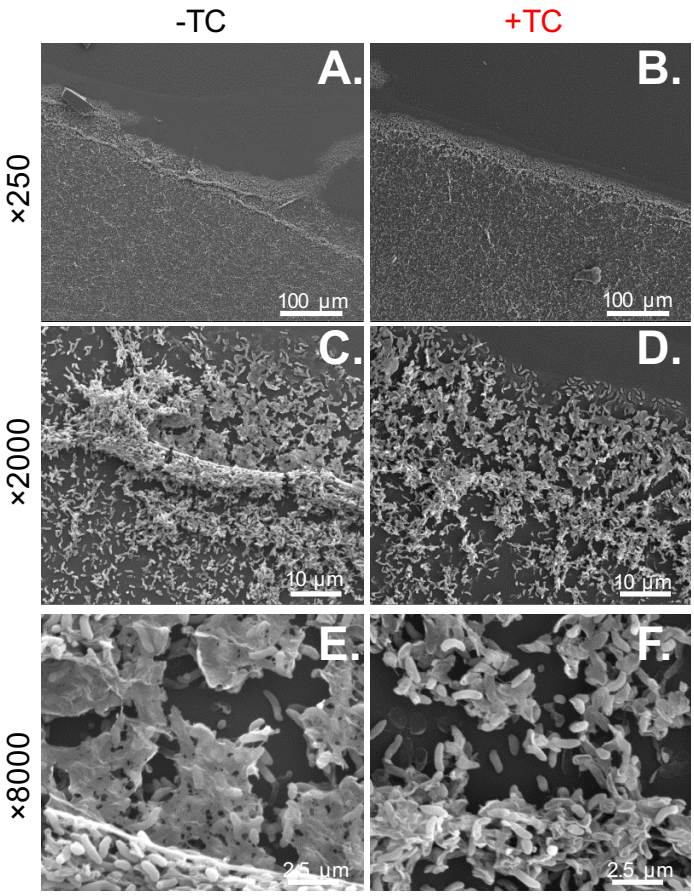


Figure 2.3. Visualization of TC-treated biofilms Representative images of *V. cholerae* biofilms imaged using scanning electron microscopy after 24 hours of growth on a 55x55 mm glass coverslip followed by 1 hour exposure to 1mM TC (**B**, **C**, **F**) or no TC (**A**, **C**, **E**). Images at 250x (**A**, **B**) are zoomed in to 2000x (**C**, **D**) and 8000x magnification (**E**, **F**).

for one hour (Fig. 2.3). At 2000x and 8000x magnification, individual cells within the biofilm matrix are clearly visible, as well as a sheet like-substance (Fig. 2C-F). Based on comparison to other previously published SEM images of biofilms, the sheets seen in the images are likely biofilm matrix[69–71]. Visual analysis suggests that TC-exposed biofilms have an altered appearance compared to unexposed biofilms in that there is less visible matrix and cells are less densely clustered. This initial assessment supports our hypothesis that TC enhances dispersal of biofilms. We thus considered that if the biofilm matrix was disturbed and there were fewer biofilm-resident cells, one of these phenomenon might proceed and cause the other.

***V. cholerae* TC-induced biofilm dispersal is passive.** To understand how taurocholate affects *V. cholerae* biofilm matrix, we first tested whether *V. cholerae* actively promotes its own dispersal from the biofilm in response to bile salts, possibly disrupting the biofilm matrix during this process. To examine whether TC enhances detachment of *V. cholerae* biofilms through altered production of VPS, we found that *vpsA* expression was not significantly altered in detached cells or biofilms exposed to TC or DC as compared to unexposed cells (Fig. 2.4 A). We then tested whether TC promotes detachment through quorum sensing pathways involving HapR[54]. However, the Δ*hapR* mutant strain displayed TC-enhanced detachment at levels similar to wild type (Fig. 2.4 B,C). To test whether *V. cholerae* induces other proteins to promote its own detachment in response to TC, we repeated detachment experiments in the presence of the protein synthesis inhibitor, chloramphenicol. At low levels, chloramphenicol can inhibit growth without causing cell death. Because biofilms may display enhanced resistance to antibiotics, we confirmed that chloramphenicol is able to inhibit protein synthesis in biofilm-resident cells[68]. When biofilms of wild-type *V. cholerae*

harboring an arabinose-inducible green fluorescent protein (GFP) construct (*pBAD-gfp*) were treated with arabinose and chloramphenicol for 2 hours, fluorescence induction was blocked compared to treatment with arabinose only (Fig 2.4 E). We then found that *V. cholerae* detachment was not significantly altered by the addition of chloramphenicol in the presence or absence of TC (Fig 2.4 F), suggesting that dispersal is not dependent on *V. cholerae* proteins induced in response to TC. Taken together, these data suggest that *V. cholerae* undergoes a passive dispersal.

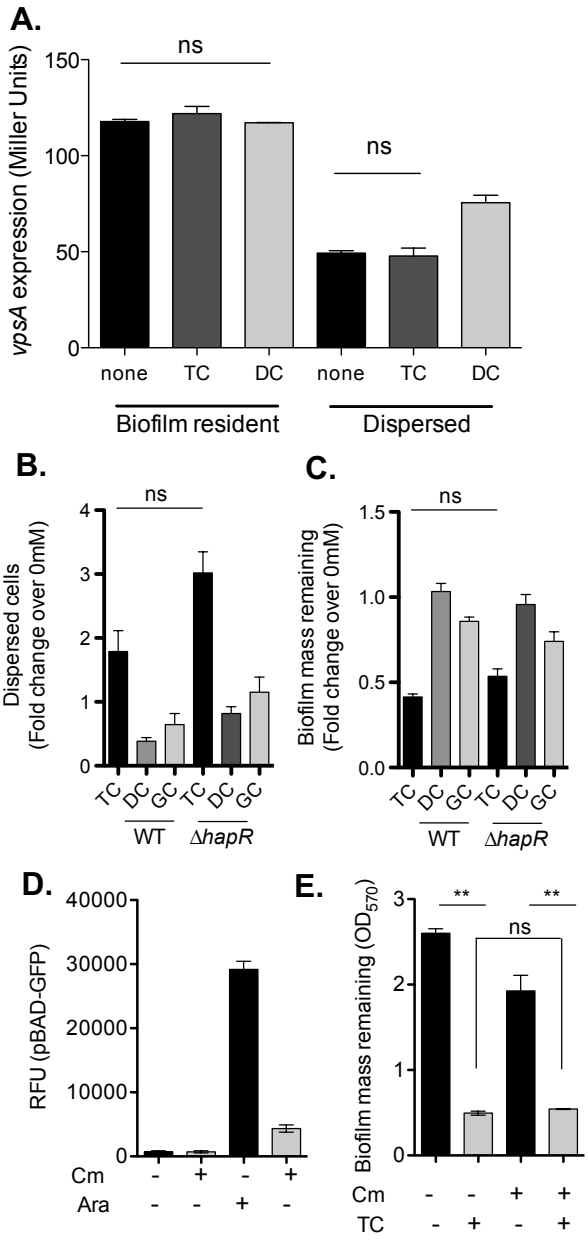


Figure 2.4. *V. cholerae* responses during bile salt detachment A. Level of expression of *vpsA* in biofilm-resident and detached cells, as measured with the β-galactosidase assay. Cells were collected following two hours of detachment. TC and DC were supplemented to final concentrations 1 mM. **B**. Following growth for 24 hours in biofilm inducing conditions wild type *V. cholerae* biofilms were incubated with the protein-synthesis inhibitor chloramphenicol (Cm) and TC for two hours. Biofilm remaining was quantified with CV staining. **C.** Control for efficacy of Cm to block protein translation in which pBAD-GFP harboring biofilms were assessed for synthesis of GFP and normalized by OD_{600}. **D.** Biofilm-derived planktonic cells when incubated in the presence of 1mM TC, DC or GC **E.** Detachment of WT and Δ*hapR* mutant strains in the presence of 1mM TC, DC, or GC in which remaining biofilm mass is quantified by CV staining. As Δ*hapR* strains form thicker biofilms, data are presented as fold change over the no supplement condition. Data are means and s.d. of three independent experiments. Statistical significance reported as NS: no significance; **: $P < 0.005$.

Polysaccharide content is released during taurocholate and taurodeoxycholate treatment of biofilms. We considered the possibility of abiotic degradation, either directly or indirectly resulting from TC exposure, and sought to find a possible mechanism for such degradation. Calcium and other divalent cations stabilize the exopolysaccharide, and their removal can cause collapse of the biofilm [72]. Because TC can bind to and chelate Ca^{2+} we questioned whether removal of stabilizing calcium by TC can lead to biofilm matrix disruption and therefore release of cells. We repeated experiments to quantify biofilm detachment in the presence of TC, Ca^{2+}, or both, yet supplemental calcium did not affect TC-enhanced detachment (Fig. 2.6). We next investigated the main component of this matrix, VPS, for disruption by TC. Using the

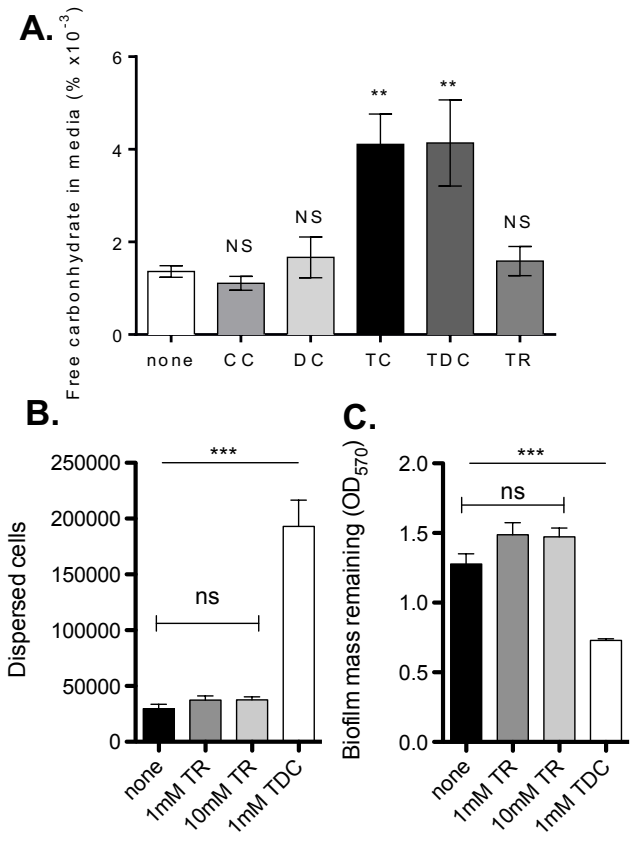


Figure 2.5. Carbohydrate content released from *V. cholerae* biofilms during incubation with bile salts and taurine-containing compounds A. To reduce growth and background from rich media, biofilms were incubated in M9 minimal media with 0.2% glycerol for 2 hours. Media was filter sterilized before carbohydrate content was estimated by phenol-sulfuric acid method and % carbohydrate values were calculated by fitting to a standard glucose curve. For bile salts and taurine (TR), final concentration is 1 mM. **B and C.** Detached cells and remaining biofilm mass when mature biofilms were incubated in the presence of 1mM taurodeoxycholate (TDC), 1mM TR or 10 mM TR for two hours. Data are means and s.d. of three independent experiments. Data are means and s.d. of three independent experiments. Statistical significance reported as NS: no significance; **: $P < 0.005$; ***: $P < 0.0005$.

phenol-sulfuric acid method, we estimated the polysaccharide content released during detachment. Cell-free filtered supernatants from biofilms exposed to TC contain a greater amount of carbohydrates than those exposed to DC or unexposed (Fig. 2.6 A). Due to the specificity of the detachment phenomenon to TC, we looked more closely at the taurine side group of this bile salt. Taurine alone is insufficient to enhance dispersal. (Fig. 2.5 B,C). We repeated experiments with another bile acid taurodeoxycholate (TDC), which is also conjugated to a taurine group. When incubated with TDC, *V. cholerae* displayed an enhanced rate of detachment, similar to TC (Fig. 2.5 B,C). Likewise, TDC-exposed biofilms have a greater polysaccharide content released from them compared to unexposed, while no difference is seen for those biofilms exposed to taurine alone (Fig. 2.5 A).

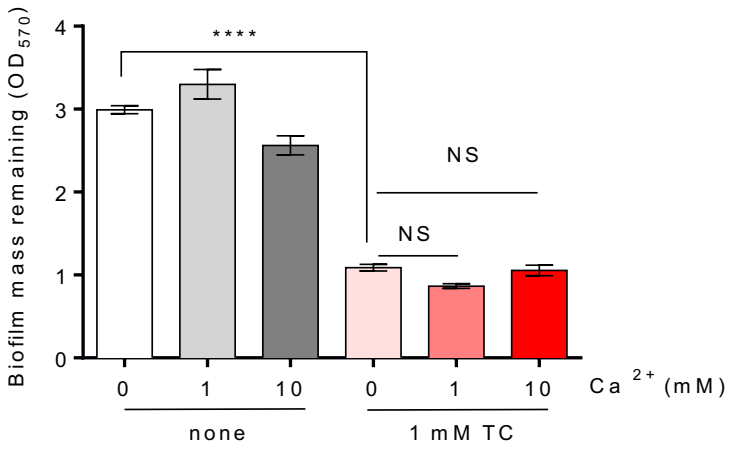


Figure 2.6. The effects of Ca^{2+} on *V. cholerae* mature biofilm dispersal. *V. cholerae* was inoculated into glass tubes and allowed to form biofilms for 24 hours at 22ºC. Biofilms were washed and incubated with media containing 1mM TC, and $CaCl_2$ at the indicated concentration. After two hours, remaining biofilm mass was quantified by crystal violet staning and reported as OD_{570}. Data are means and s.d. of three independent experiments. Statistical significance reported as NS: no significance; ****: P < 0.0001.

Detachment precedes induction of virulence program. It is likely that biofilms or biofilm-like particles, not just cells in a planktonic state, serve as transmission agents for human infection by *V. cholerae*[53]. Presumably, cells must exit from these biofilms before they can swim to sites of infection at the intestinal epithelium, as highlighted by the importance of motility during infection[73]. Because TC may be the cue for this *in vivo* detachment, and is also a host-derived virulence inducer[54,74], we investigated the

relationship between biofilm dispersal and virulence induction upon exposure to taurocholate. We tested whether detachment and expression of the virulence program were related by measuring detachment in a Δ*toxT* mutant strain of *V. cholerae*, in which major virulence gene expression is abolished[75]. Virulence gene expression is not required for detachment, as the detachment rate of the Δ*toxT* mutant biofilms did not differ significantly from wild type when exposed to TC (Fig. 2.7 A). To test whether dispersal affects virulence induction, we tested the response to virulence inducing conditions by cells that had either detached from or remained resident in a biofilm. We first used a luminescence reporter to test overall population level expression of the major virulence determinant TcpA. Fig. 2.7 B shows that *tcpA* expression was low in non-inducing conditions for biofilm resident cells and for detached cells, as was expected. Following incubation with inducer TC, TcpA was robustly induced in cells that had detached from biofilms, but low expression persisted in in those cells remaining biofilm-resident (Fig. 2.7 B). We then used a *tcpA-gfp* reporter paired with flow cytometry to assess individual cell levels of virulence gene expression (Fig. 2.7 C). To track live cells, this strain also harbors a constitutive *ptet-mCherry* construct. As seen in Fig. 2.6 C (i. and iii), *tcpA* expression is uniformly low in non-inducing conditions (top right quadrant), with very few outliers. In TC-exposed samples, a majority of the detached cell population induces *tcpA* at least an order of magnitude above background levels (Fig. 2.6 C iv). In agreement with population level experiments, TC-exposed biofilm resident cells do not induce *tcpA* (Fig. 2.6 C ii). Interestingly, lack of induction in this population was also uniform; that is, robust *tcpA* induction was observed in very few of the biofilm resident cells. To ensure that there was not a sub-population of TcpA- expressing cells at the liquid-biofilm interface, we used fluorescence microscopy to visually analyze biofilms of

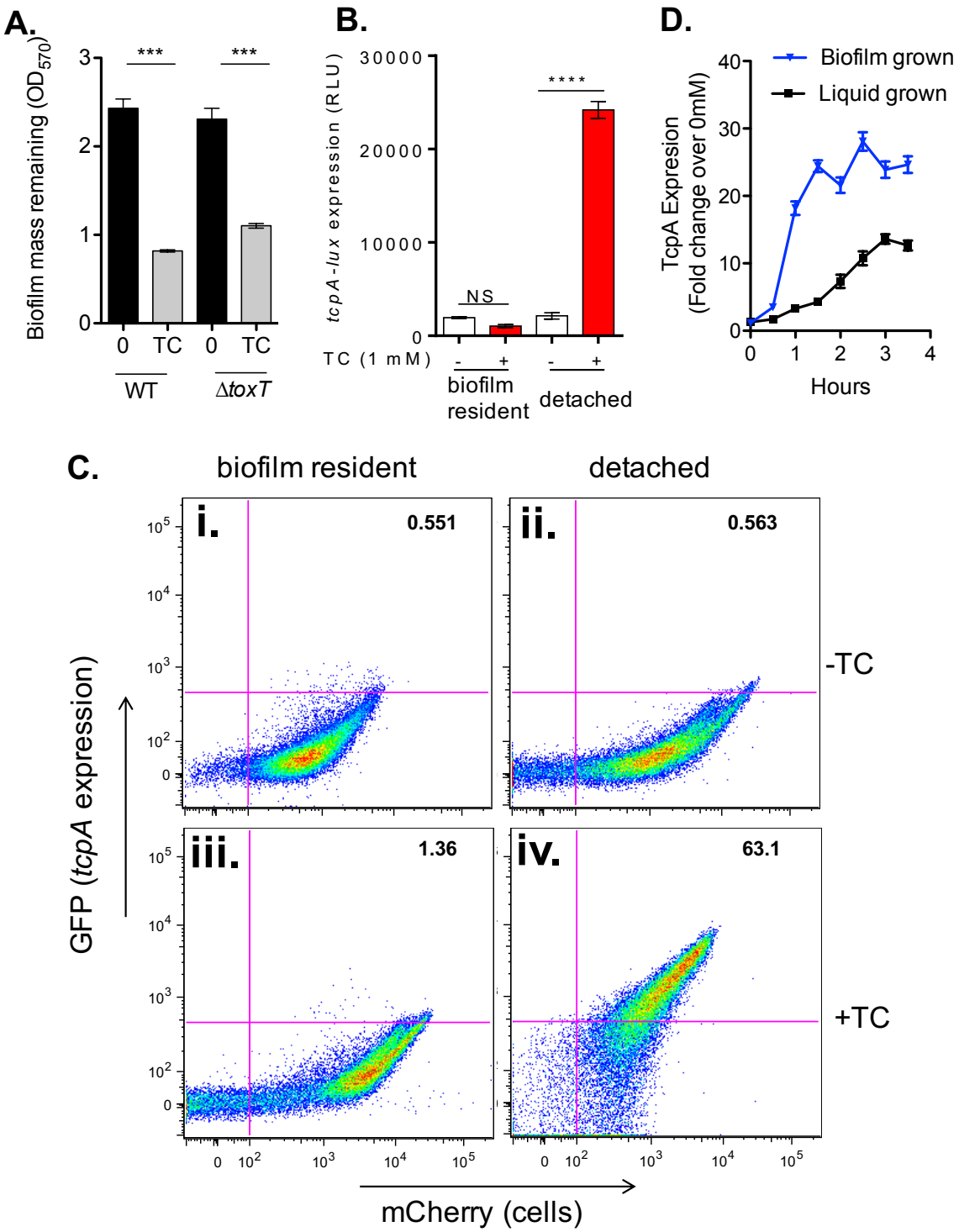


Figure 2.7. Contribution of biofilm dispersal to colonization and virulence A. Remaining biofilm mass (OD_{570}) following 2 hour exposure to TC for WT and Δ*toxT* mutant *V. cholerae*. Expression of major virulence factor *tcpA* in biofilm resident cells and detached cells as shown by **B**. *tcpA* promoter-luciferase reporter fusion (P_{tcpA}-*lux*). Data are means and s.d. of three independent experiments. Statistical significance reported as ****: P< 0.0001. **C**. Flow cytometry plot of *V. cholerae* harboring a plasmid with constitutive mCherry (P_{tet}-*mCherry*) and P_{tcpA}-*gfp*, in which mCherry indicates cells and GFP indicates *tcpA* expression. Average % of GFP cells from three independent experiments are listed in each graph. **D.** TC-promoted induction of TcpA as measured by *ptcpA-lux*. Cells were grown in either liquid cultures or biofilms that were manually dispersed. Cells were washed and added to fresh media containing TC. Data reported as fold change over 0mM. Statistical significance reported as NS: no significance; ***: P < 0.0005; ****: P < 0.0001.

the same strain harboring both *ptcpA-gfp* and *ptet-mCherry* plasmids. When incubated with TC, biofilm resident cells throughout the structure were found to express mCherry. Cells on the surface of the biofilm did not appear to have different levels of GFP than those internal to the biofilm (data not shown). These results suggest that although biofilm resident cells are exposed to TC, they are unable to fully promote expression of their virulence program until they have exited the biofilm environment. It is unclear whether detachment enhances the expression of virulence factors, or merely enables it. However, manually disrupted biofilms are able to rapidly induce *tcpA* in when exposed to TC, indicating that these cells are responsive to TC once removed from the biofilm environment (Fig. 2.7 D). The sequential nature of these evens highlights the potential importance of detachment during natural infection with *V. cholerae* biofilm particles.

Discussion

Using the suckling mouse model of infection, the field has learned a great deal about the physiologic and regulatory changes that *V. cholerae* undergoes as it transitions from aquatic resident to enteric pathogen. However, a majority of these studies have been conducted with planktonic, free swimming bacteria. Less is known about the changes that biofilm-resident *V. cholerae* undergo during this transition and while traversing through the gut. Because only certain bile salts enhance detachment of *V. choelrae* biofilms, it is clear that the mechanism of action is not simply detergent or surfactant in nature. The specificity of the observed enhanced detachment phenotype to taurine-containing bile acids hints at biochemistry at play that is unique to this class of molecules. Taurine contains a sulfonic acid, which is relatively rare among naturally occurring compounds. Sulfuric acid has long been used to degrade cellulose for industrial purposes, with recent studies describing similar properties for other sulfonic

acid containing compounds[76,77]. Work from the Wiredu group showed that out of an array of sulfonic acid containing compounds, those with hydrophobic R core groups were the most efficient at catalyzing hydrolysis of cellulose[76]. Because the amphipathic taurocholate molecule orients at interfaces, with the sterol group along the interface, this hydrophobic side group may be important to enable the acidic group to come in close enough proximity with the carbohydrate, and perhaps to position it properly[78]. These observations could explain why taurine is insufficient to promote detachment without a hydrophobic R group, and why only TC and TDC can promote detachment. Further work is needed to confirm or rule out this possibility, but we speculate that TC and TDC enhance *V. cholerae* detachment through directly promoting hydrolysis of the VPS component of the biofilm matrix. The role of a small molecule in disruption of biofilm matrix is not unprecedented, as *P. aeurginosa* produces rhamnolipid, a surfactant that when highly expressed can disrupt cell-matrix interactions[79].

For many bacteria, dispersal is a means of accessing new niches during different stages of their life cycle or in response to various cues[63]. *P. aeruginosa* active dispersal is orchestrated and achieved through an explosive release of bacteria from a hollowed out shell in the biofilm. Chua. *et. al*. found that not only do these dispersed cells have a distinct gene expression signature compared to both planktonic and biofilm cells, but that dispersion can specifically induce a hyperinfectious state that primes the detached bacteria for further infection[67]. Other studies have highlighted the importance of hyperinfectivity gained during host passage and biofilm residence for *V. cholerae*[80]. For example, *V. cholerae* strains that produce excess VPS exhibited lower stochastic shedding of bacteria from biofilms, likely because cells were more deeply embedded in the matrix. These trapped cells were deficient in their ability to establish colonies in new locations[81]. Our findings suggest that the converse occurs as well: reduction of biofilm

matrix, perhaps due to TC-mediated degradation, frees cells and enables them to induce new genes and colonize locations inaccessible to biofilm aggregates. Thus, we propose a model (See Figure 4.2) in which ingested biofilms encounter bile salts in the proximal small intestine that degrade the biofilm matrix, perhaps directly through hydrolysis. As they are freed, individual cells are able to induce a virulence program in response to TC, and go on to their sites of infection.

Chapter 2 was adapted from publication Hay and Zhu, (2014) [82].

CHAPTER 3: CALCIUM ENHANCES BILE SALT-DEPENDENT VIRULENCE ACTIVATION IN *VIBRIO CHOLERAE*

Introduction

A number of environmental conditions such as oxygen concentration and bicarbonate have been shown to influence *V. cholerae* expression of virulence genes[15,32]. Our group recently completed a study to identify additional host compounds that induce virulence *in vivo*[74]. To accomplish this, *V. cholerae* was applied to segments of murine intestine and was found to activate a *ptcpA-lux* virulence reporter. Murine intestinal extracts were then purified and retention of virulence inducing activity was confirmed before extracts were separated by 2D thin layer chromatography (TLC). To identify specific activating compounds, the TLC plate was covered with *ptcpA-lux* reporter *V. cholerae* embedded in a layer of soft agar. Two luminescent spots were found, indicating TcpA activation by two discrete compounds. Subsequent fractionation and analysis demonstrated that the first Virulence Activating Factor (VAF1) is the bile salt taurocholate[74]. In attempt to identify VAF2, intestinal extracts were fractionated by several methods and tested for inducing activity. Two observations were then made: VAF2 is only able to induce TcpA in in the presence of VAF1, and VAF2 is a heat stable, inorganic, ionic compound. As mammalian gastrointestinal tracts contain complex environments, we tested whether known ions found in bile affect virulence gene expression. We first examined calcium, and found that it did in fact enhance bile salt dependent virulence activation. Calcium is part of the biliary secretion released into the proximal intestine following a meal and is abundant in intestine[83]. The importance of calcium signaling and regulation has been well defined in eukaryotic cells[84,85]. More recently, bacterial responses to and regulation of calcium homeostasis have been

investigated. Calcium-regulated processes in bacteria include cell division, biofilm formation, and pathogenesis[86–88]. In the gut, calcium is present as free Ca^{2+}, but can also be bound to bicarbonate and to bile salts[83,89].

When bile is released into the proximal small intestine, several of its component parts may affect virulence in *V. cholerae*. Bile salts such as taurocholate and glycocholate stimulate virulence factor production, while fatty acids inhibit ToxT activity by directly binding to the protein[74,90,91]. We found that calcium enhances bile salt-induced virulence gene expression through modulating TcpP protein movement and activity. Our study adds to the growing body of work suggesting that calcium signaling is as relevant to bacterial physiology as eukaryotic, and further sheds light on the ways in which pathogens have co-opted host-resident signals for efficient colonization.

Results

Calcium enhances bile salt-induced virulence. To examine the effect of calcium on *V. cholerae* virulence gene expression, we measured the expression of *tcpA*, which encodes a major virulence determinant[10], in the presence calcium and known activator, taurocholate (TC). We found that calcium alone did not induce *tcpA* (Fig. 3.1 A). When calcium was supplied with TC, we observed an increase in TcpA expression greater than that of TC without added calcium (Fig. 3.1 A). Other ions including K^+ and Mg^{2+} had no effect on virulence expression with or without TC (Fig. 3.1 A), suggesting that this enhanced virulence induction is not due to a general salt or ion effect, but is specific to calcium. We were unable to draw conclusion of the effect of another divalent cation, Ba^{2+}, because this ion had a negative effect on growth at relatively low concentrations (data not shown). We then tested whether calcium-enhanced virulence is specific for

TC. We have previously shown that the bile salts TC and glychocholate (GC), but not deoxycholate (DC), promote virulence induction[74]. We found that calcium promoted *tcpA* induction only for known inducers TC and GC in a dose-dependent manner (Fig. 3.1 B). These data suggest that calcium acts together with the bile salts to promote activation of virulence for *V. cholerae*.

To confirm that calcium-enhanced virulence gene expression is relevant in the gut environment, we tested virulence activation of intestinal extracts that were treated with the high affinity calcium chelator EGTA. *V. cholerae* incubated with intestinal extracts expressed high levels of *tcpA* (Fig. 3.1 C). When cultures were incubated with

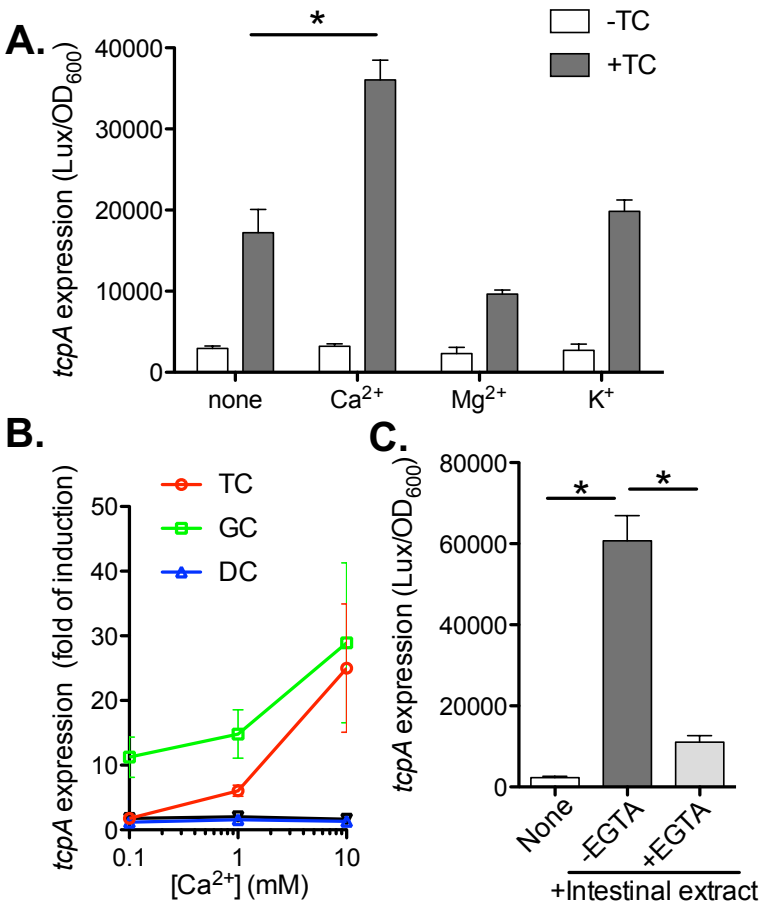


Figure 3.1 Contribution of Ca^{2+} to virulence gene expression in *V. cholerae* A. *tcpA* expression in the presence of taurocholate (TC) and different metal ions. *V. cholerae* containing P_{tcpA}-*luxCDABE* plasmids were grown in LB containing 10mM ion without or with 0.1 mM TC and grown microaerobically until mid-log (OD600 ~0.2). Luminescence was measured and normalized for growth against the OD600. **B.** Bile salt effects. *tcpA* expression was measured for cultures grown in 0.1 mM TC, 0.1 mM glycocholate (GC), and 0.1 mM deoxycholate (DC) in the presence of indicated calcium. **C.** Intestinal extracts. *tcpA* expression was measured without and with intestinal extract or intestinal extract incubated with EGTA. Intestinal extracts were incubated for 12 hours with or without EGTA and added to cultures at a final EGTA concentration of 0.25mM. The data shown are from three independent experiments. *: Student t-test, $P < 0.05$.

EGTA-treated intestinal extracts, virulence expression declined drastically (Fig. 3.1 C). This decrease of virulence induction was not due to the EGTA effects on bacterial growth as the amount of EGTA added in the intestinal extracts did not affect *V. cholerae* growth (data not shown). Together these results imply the importance of calcium for *V. cholerae* virulence expression *in vivo.*

Ca^{2+} promotes TcpP induction of ToxT. The regulatory genes and proteins that culminate to achieve virulence in *V. cholerae* are well-described and act in a highly regulated process [11] (Fig 1.1). To understand how calcium can promote virulence expression in *V. cholerae*, we examined if calcium affects the expression of the known virulence regulators. When incubated with calcium alone or calcium with TC, expression of *aphB*, *aphA*, *tcpP*, and *toxR* were unaffected, as measured by a promoter-luciferase transcriptional

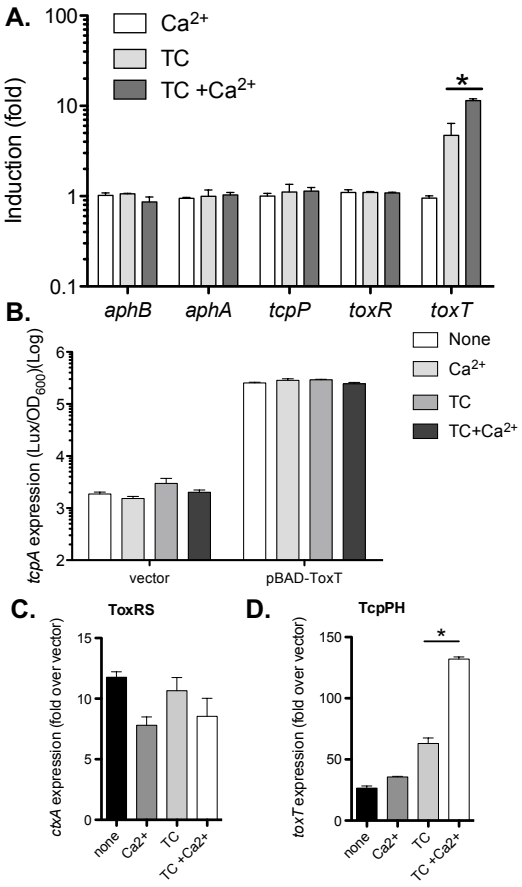


Figure 3.2 Effect of Ca^{2+} on virulence regulatory genes and proteins. A. Expression of *toxT, toxR, aphB, aphA, and tcpP* genes. Wild type containing indicated promoter-*lux* reporters were grown at 37°C microaerobically until mid-log phase and luminescence was measured. When indicated, 10 mM $CaCl_2$ and 0.1 mM TC was included in the medium. **B.** *tcpA* expression in *ΔtoxT mutant V. cholerae* containing P_{tcpA}-*luxCDABE* and pBAD-*toxT* plasmids. Cultures were grown in LB containing 0.05% arabinose, 0.1 mM TC, 10mM $CaCl_2$ or both until mid-log (OD600 ~0.2). Luminescence was measured and normalized for growth against the OD_{600}. **C.** and **D.** ToxR and TcpP in *E. coli* **C.** Induction of *ctxA* by ToxR (pBAD-*toxRS*) in *E. coli* when exposed to TC, $CaCl_2$, or both together. **D.** Induction of *toxT* by TcpP (pBAD-*tcpPH*) in *E. coli* when exposed to TC, $CaCl_2$, or both together. The data shown are from three independent experiments. *: Student t-test, $P < 0.05$.

fusion reporters (Fig. 3.2 A). Calcium alone also did not alter the expression of *toxT* (Fig. 3.2 A), encoding the master virulence regulator[12]. Addition of TC induced *toxT*, consistent with the previous report[74]. Importantly, addition of both calcium and TC further enhanced the expression of *toxT* (Fig. 3.2A), suggesting that calcium-promoted virulence induction acts upstream of ToxT. As *toxT* is regulated by TcpP and ToxR, it is possible that calcium affects the activity of either or both TcpP and ToxR. Alternatively, calcium may post-translationally act on the ToxT protein. To test these hypotheses, we first examined whether calcium affects ToxT activity independent of expression. When we used a Δ*toxT* mutant strain with a ToxT expression vector (pBAD-*toxT*) to control for the level of ToxT protein, we found that calcium, TC, or both did not affect *tcpA* induction by ToxT (Fig. 3.2 B), indicating that calcium does not affect ToxT activity. We then tested

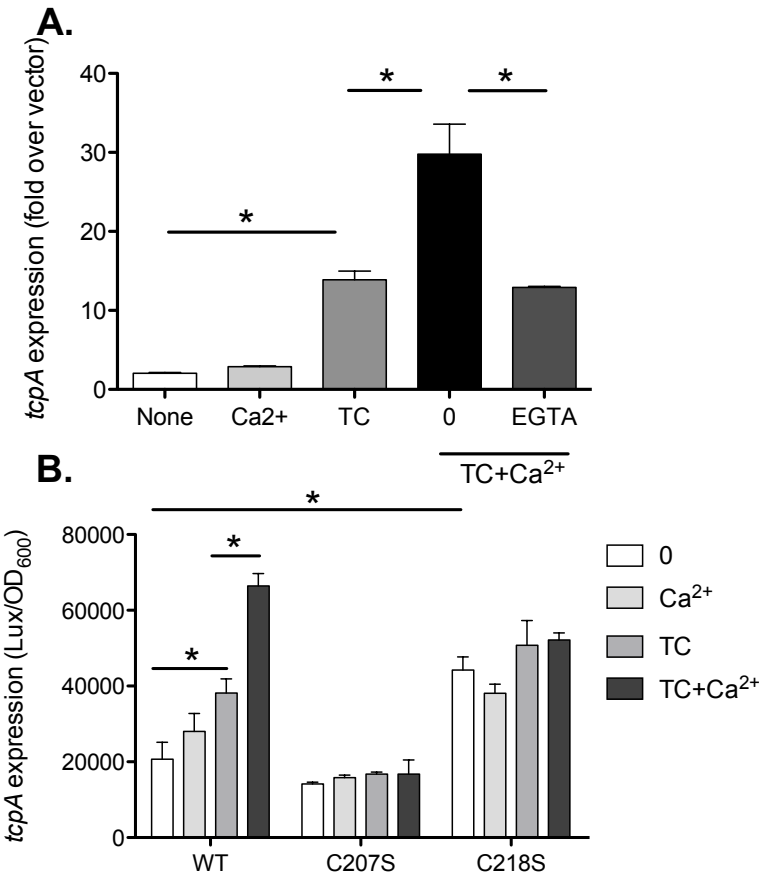


Figure 3.3. Calcium effects on TcpP induction of virulence. A. Effect of Ca^{2+} on TcpP-mediated *tcpA* expression. Expression of *tcpA* with controlled levels of TcpP *(ΔtcpPH; ΔtoxR* with pBAD-*tcpPH*) when exposed to 0.1mM TC, 10mM $CaCl_2$, or both together in the presence or absence of the calcium-specific chelator, EGTA **B.** Activation of ToxT by TcpP and its cysteine mutant derivatives. Expression of *toxT* by wild type TcpP and mutants with $TcpP^{C207S}$ (null activity) or $TcpP^{218S}$ (constitutive activity) were tested in the presence of 0.1 mM TC, 10 mM $CaCl_2$, or both together. Cells were grown at 37°C microaerobically until mid-log phase and luminescence was measured. The data shown are from three independent experiments. *: Student t-test, $P < 0.05$.

whether calcium affects ToxR or TcpP activity in *E. coli*. We found that calcium alone, TC, and TC+Ca^{2+} did not alter ToxR-induced *ctxA* expression (Fig. 3.2C), whereas addition of calcium and TC enhanced TcpP-dependent *toxT* expression in *E. coli* (Fig. 3.2 D). These data suggest that calcium may modulate TcpP activity to enhance bile salt-activated virulence gene expression.

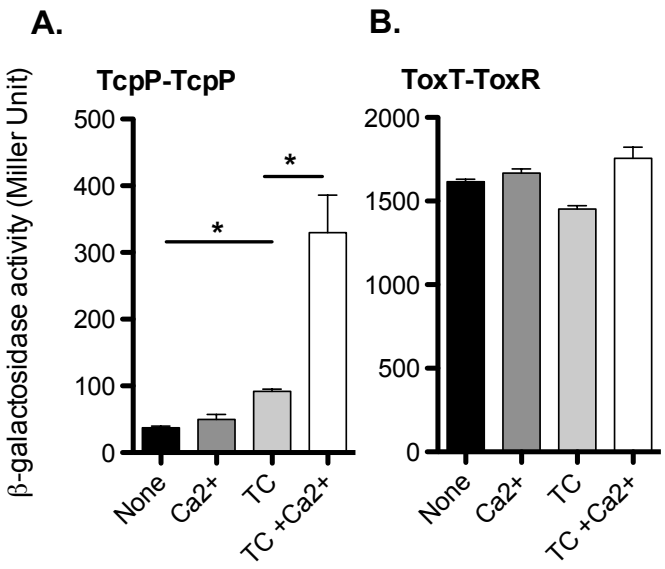


Figure 3.4. Effect of Ca^{2+} and TC on TcpP-TcpP interaction. *E. coli* cells harboring the adenylate cyclase two-hybrid for TcpP or ToxR were grown microaerobically until mid-log phase and then assessed for β-galactosidease activity, reported in Miller Units. **A.** TcpP interaction in the presence of 1 mM TC, 10 mM $CaCl_2$, or both and **B.** ToxR interaction in the presence of 1mM TC, 10mM $CaCl_2$, or both together. The data shown are from three independent experiments. *: Student t-test, $P < 0.05$.

Ca^{2+} promotes TC-dependent TcpP interaction and activity. To confirm that calcium affects the activity of TcpP to induce virulence, we constitutively expressed TcpPH in a Δ*tcpPH*/Δ*toxR* mutant strain of *V. cholerae* and measured *tcpA* expression with calcium and/or EGTA. Calcium alone did not have an effect on *tcpA*, however, the same concentration of calcium did enhance *tcpA* expression in the presence of TC (Fig 3.3 A). To verify that this affect was due to calcium, we added different amounts of EGTA in the medium. We found that EGTA could eliminate the enhanced virulence expression (Fig. 3.3 A). Taken together, these data suggest that calcium affects virulence at the level of TcpP in *V. cholerae*. To further dissect the possible role of calcium on TcpP activity, we examined TcpP cysteine mutants. Previously we discovered that two cysteine residues in the periplasmic domain of TcpP are critical for TcpP activity[74]. TcpP homodimerization

is required for optimal activation of TcpA. To test whether calcium may affect the activity of TcpP cysteine mutants, we compared calcium's effect on induction of *tcpA* by $TcpP^{WT}$, $TcpP^{C207S}$, and $TcpP^{218S}$. We found that unlike that of wild type, calcium displayed little effects on the activity of $TcpP^{C207S}$ (null activity), and $TcpP^{218S}$ (constitutive activity) (Fig. 3.3 B). As these two cysteine residues are involved in TcpP-TcpP intermolecular disulfide bond formation[74], these data suggest that calcium may modulate TcpP interaction. To further investigate how calcium affects TcpP activity, we examined whether calcium alters TcpP interaction using a bacterial two-hybrid system[92]. We found that while calcium alone had little effect, in the presence of TC, calcium greatly enhanced TcpP-TcpP interaction (Fig. 3.4 A). To ensure that this calcium effect is not a general on any membrane-bound protein, we measured ToxR-ToxR interaction as a control. We found that ToxR-ToxR interaction was unaffected by calcium under all conditions tested (Fig. 3.4 B). These data suggest that calcium may act to enhance virulence activation in the presence of TC by increasing Tcp-TcpP interaction, which may lead to increased induction of downstream virulence factors.

Calcium affects TcpP membrane diffusion. We next sought to elucidate the mechanism by which calcium and TC affect TcpP activity. TcpP is membrane-bound regulatory protein and the transmembrane domain of this protein is required for virulence activation[74,93]. Bile salts are known to be detergents and to interact with membranes[94–96]. We thus considered the possibility that calcium may affect the interactions between bile salts, TcpP proteins, and/or the bacterial inner membrane. In attempt to quantify these relationships, we performed fluorescence recovery after photobleaching (FRAP) experiments[97]. We expressed GFP-TcpP in *V. cholerae* and monitored fluorescence recovery on a confocal microscope following bleaching of cell

portions with an argon laser. To calculate recovery, an equal area of the bleached (A_1) and unbleached (A_2) portions of the cell were measured and recovery rate was calculated[98]. Representative analysis can be seen in Fig. 3.5. Fluorescent TcpP cells grown in the presence of both TC and Ca^{2+} had a decreased rate of recovery compared to controls (Fig. 3.6 A). Those grown with calcium or TC alone did not have significantly

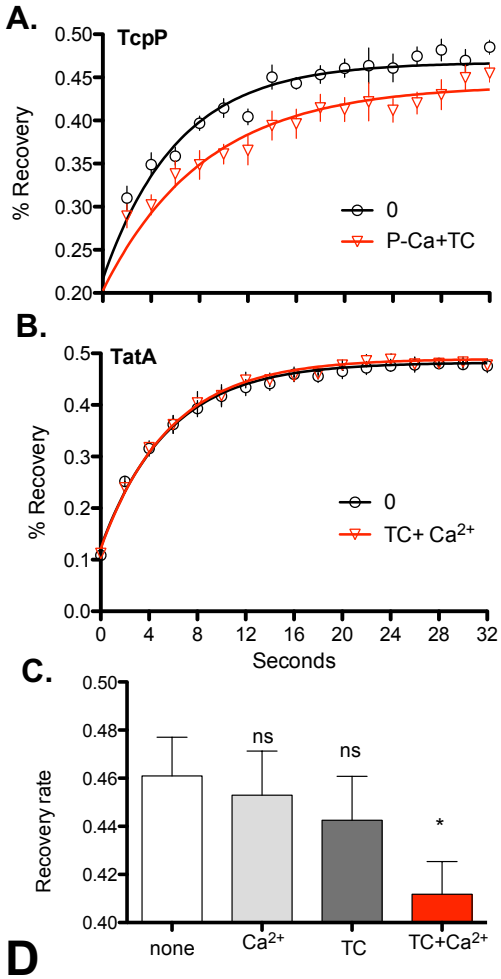
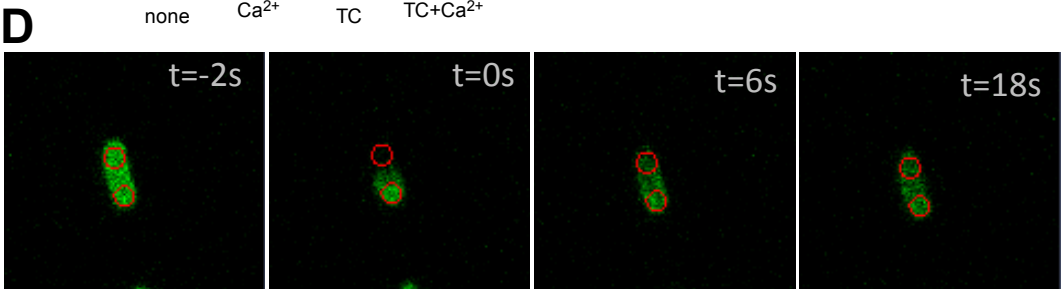


Figure 3.5 Fluorescence recovery following photobleaching (FRAP) of fluorescently labeled membrane proteins and analysis. Cells were grown to mid-log phase in the presence of arabinose with 1 mM TC and 10 mM $CaCl_2$ where indicated. Following near complete photobleaching with an argon laser, fluorescence intensity was recorded within two equally sized circular regions, one in the middle of the bleached compartment $A_1(t)$ and the other in the middle of the unbleached compartment for a given time $A_1(t)$. The fluorescence recovery data were normalized to the total remaining fluorescence with recovery rate reported as $\text{Recovery}(t) = 100 * (\frac{A_1(t)}{A_1(t)+A_2(t)})$ and fitted to an exponential association curve. **A.** GFP-TcpP Recovery. **B.** TatA-GFP recovery. **C.** TcpP in the presence of TC, Ca^{2+}, or both. **D.** Representative timecourse images of fluorescence recovery of fluorescently labeled TcpP. Cells expressing fluorescently labeled TcpP (pBAD-*gfp-tcpPH*) were grown to mid-log phase in the presence of arabinose before microscopy. Images were captured on ZEN 2012 software at 2s intervals preceding and immediately following bleaching. For this representative cell, $A_1(t)$ corresponds to the upper circle in the bleached compartment and $A_2(t)$ corresponds to the lower circle in the unbleached compartment.



different recovery from control (Fig. 3.6C). FRAP is typically used as a proxy to measure membrane fluidity by measuring fluorescent proteins within the membrane[99]. We considered the possibility that membrane fluidity was decreased in the presence of TC and Ca^{2+} together for *V. cholerae*. To further explore this possibility, we performed FRAP on *V. cholerae* cells harboring fluorescently labeled TatA. TatA is an inner membrane protein with homologues in several bacterial species that is commonly used for FRAP due to the high tolerance for this protein in the inner membrane[97,100]. Fluorescent TatA showed no difference in recovery between untreated cells and those grown in the presence both TC and Ca^{2+} (Fig. 3.6B). These data imply that TC and Ca^{2+} may not affect overall membrane fluidity, but may instead affect membrane movement of TcpP. Ongoing work is underway to better understand the nature of membrane alteration and mechanism by which TcpP dimerization is affected by bile salts and calcium.

Discussion

In order to achieve efficient colonization and cause disease, *V. cholerae* must integrate many signals from the host environment to coordinate proper timing and level of expression of virulence genes. To add to our knowledge of the relevant virulence regulators and signals that activate them *in vitro*, we find in this study that physiologically relevant levels of calcium promote bile salt-dependent virulence activation[85,101]. This occurs through enhanced interaction of TcpP proteins concurrent with altered TcpP protein membrane diffusion. Our data suggest that TcpP may have more complex regulation than previously appreciated.

Detailed studies have shown that TcpP is the direct activator of ToxT and that ToxR facilitates TcpP binding at the *toxT* promoter[11]. Both ToxR and TcpP have a membrane spanning region, a periplasmic region, and a cytoplasmic region containing a DNA binding domain. ToxR cytoplasmic DNA-binding domain is sufficient to induce some proteins whose expression TcpP does not participate in[25]. However, membrane localization of ToxR is required for its role in promoting ToxT expression[93,102]. All three domains of TcpP are required for virulence activation, while the membrane-spanning and periplasmic regions are sufficient for TcpP dimerization[74]. Additionally, TcpP and ToxR interact at the membrane and at the *toxT* promoter, but this interaction is abolished when the TcpP membrane spanning region is swapped with the ToxR membrane region[93,102]. These findings suggest that the interaction of ToxR and TcpP at the membrane is important for proper virulence induction. Therefore, calcium and bile salts affecting TcpP membrane movement may play an important role in regulating TcpP activity. Increased production of TcpA in the presence of calcium was specific and not replicated by other similar metal ions such as Mg^{2+}. This finding, paired with the observation that calcium does not seem to act on a new pathway, but rather to enhance all known effects of bile salts such as TC, suggest that these molecules may act together as one bound molecule. The binding of Ca^{2+} to bile salts in the gut, particularly conjugated bile salts such as TC and GC, has long been appreciated. Bile acids are considered to be protective against gall stones resulting from calcium precipitation by buffering intraluminal Ca^{2+}[89]. Numerous biochemical studies have described the preferential binding of bile salts (BS) to Ca^{2+} at a 2:1 ratio, resulting in a $CaBS_2$ molecule with unique biochemical properties[89,103,104]. Thus, it is likely that *V. cholerae* and other intestinal pathogens encounter not just bile salts in the gut, but bile salts in various forms

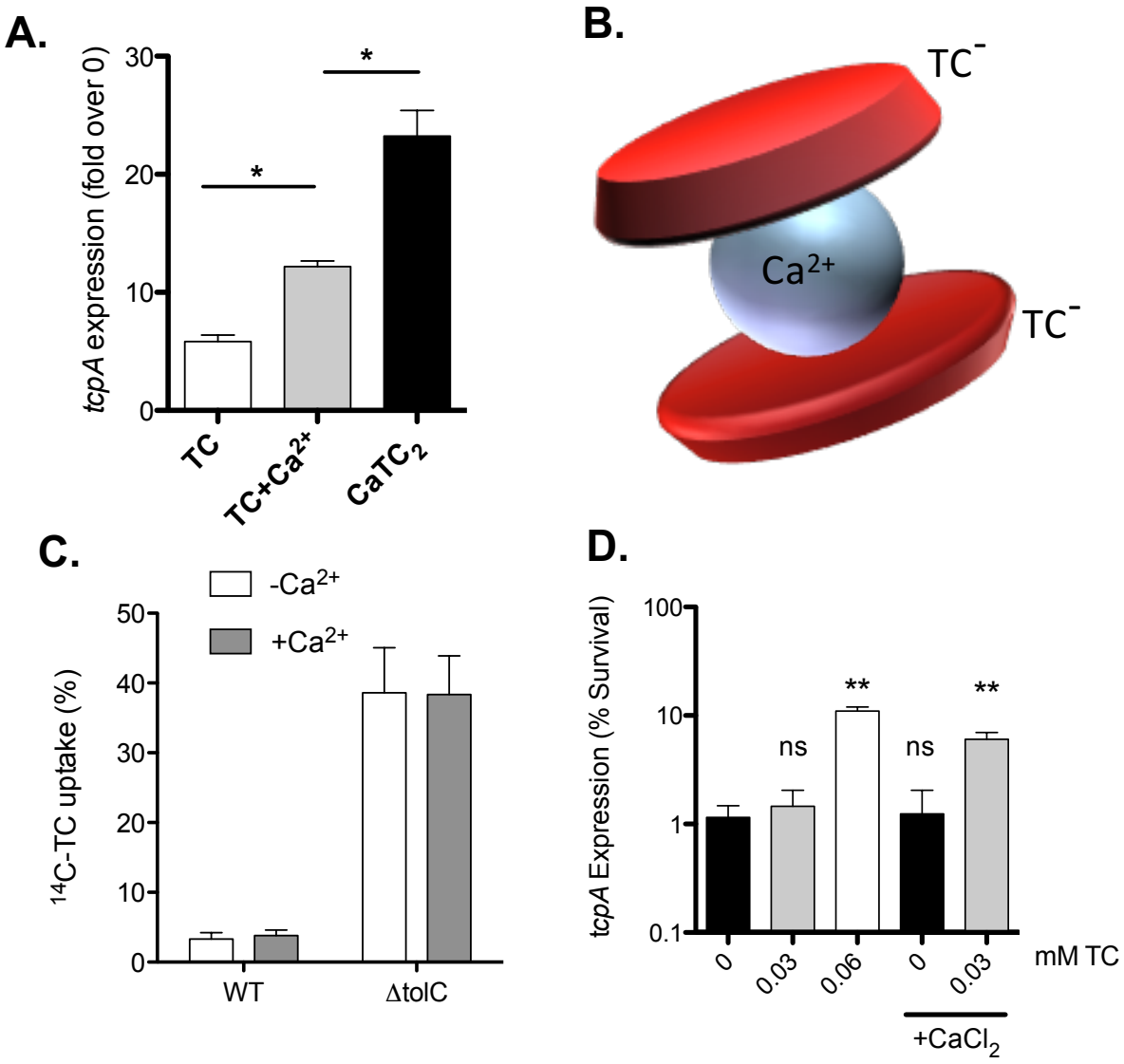


Figure 3.7. Effect of $CaTC_2$ on the expression of virulence gene *tcpA* A. Effect of $CaTC_2$ on the expression of virulence gene *tcpA*. *V. cholerae* induction of TcpA when incubated with synthesized $CaTC_2$ compared to TC, $CaCl_2$, or TC+Ca^{2+} *V. cholerae* containing P_{tcpA}-*luxCDABE* plasmids were grown in LB containing 0.1mM TC, 0.1mM TC with 10mM $CaCl_2$, or 0.1mM $CaTC_2$, Cultures were grown microaerobically until mid-log (OD600 ~0.2). Luminescence was measured normalized for growth against the OD600. B. Model of $CaTC_2$ binding based on quasi-elastic light-scattering [107] **C.** Accumulation of ^{14}C-taurocholate in *V. cholerae.* Cultures of WT or efflux deficient (Δ*tolC*) *V. cholerae* were grown at 37º to mid-log with or without 10mM $CaCl_2$ and exposed to 200µM TC and 20µM ^{14}C-taurocholate. Aliquots of cells were taken at indicated time points, pelleted, and count per minute (CPM) of C^{14} in cells was measured. TC uptake is reported as percentage CPM in cells compared to total CPM. **D.** *V. cholerae* cultures that were incubated with the indicated amount of TC, $CaCl_2$, or both. were treated +/- zeocin (25µg/ml) for 30 min at 37°C and plated on LB agar. Data reported as percentage of survival of treated cells compared to untreated controls. The data shown are from three independent experiments. *: Student t-test, $P < 0.05$.

of binding to calcium and other complexes. We synthesized $CaTC_2$ and found that it induced virulence significantly higher than TC or TC with additional calcium (Fig. 3.7 A). This suggests that $CaTC_2$ may be physiologically relevant *in vivo*. $CaTC_2$ has different properties than the ionic form of TC^-, which would likely predominate in the gut environment compared to the protonated form[105]. For example, ionized bile salts preferentially partition in the outer hemileaflet of the phospholipid bilayer. Calcium-bound bile salts, such as $CaTC_2$, instead partition between the two hemileaflets, residing among the hydrophobic lipid core[94,103]. This difference may be due to the way in which bile salts are hypothesized to bind to calcium ions. Bile salts are rigid amphipathic molecules with the sterol core. This core is a hydrophobic, with the hydroxyl groups orienting to one side, providing a hydrophilic face[78,106]. Using quasielastic light-scattering measurements, D'Archivio and colleagues found that a $CaBS_2$ molecule consist of a calcium ion fit in between to bile salt molecules, as depicted by the cartoon in Figure 3.7 B[107]. The same study also demonstrated that bile salts bind with a higher affinity to calcium than sodium, and that calcium likewise has a higher affinity for conjugated bile salts than unconjugated.

The difference in charge and placement of $CaTC_2$ compared to TC^- could inherently change the interaction between bile salt, membrane, and perhaps between TcpP protein(s). Further studies are required to fully elucidate the effect of these complex bile salt-based molecules on bacterial physiology and the role they play *in vivo*. We tested whether calcium, possibly in the $CaTC_2$ form, may enhance bacterial uptake of bile salts, thereby increasing TcpP activity. We incubated ^{14}C-labeled TC with wild type *V. cholerae* in the absence and in the presence of calcium and measured the amount of ^{14}C-TC in cells after 30'. We did not observe any difference of TC uptake between cultures with and without calcium (Fig. 3.7 C). To avoid the possible effect of

export of bile salts by efflux pumps, we performed the same uptake assay using Δ*tolC* mutants, which abolish efflux pump activity in *V. cholerae*[44]. Again, calcium did not affect TC uptake in Δ*tolC* mutant strains (Fig. 3.7 C). These data suggest that at least under the conditions tested, calcium does not affect bacterial bile salt uptake. To further explore how calcium may affect virulence activation in *V. cholerae* we tested the threshold of TC to induce virulence in the presence and absence of Ca^{2+}. Using a zeocin reporter strain (*ptcpA-sh ble*)[108] We found that the minimum concentration of TC to activate *tcpA* expression was decreased in the presence of Ca^{2+}(Fig 3.7 C). We hypothesize that TC is a more potent virulence activator when bound to Ca^{2+}. More in-depth biochemical studies are necessary to directly detect calcium-bile salt species in the context of infection.

The ideas that bacteria respond to bile salts by altering membranes, and therefore likely affecting membrane proteins, is in fact not new. In response to bile, *Bifidobacteira animalis* displays decreased membrane fluidity due to changes in lipid composition as well as an altered protein: phospholipid ratio[109]. The contribution of protein diffusion to pathogenesis has also been explored in the opportunistic fugal pathogen, C*andida albicans,* where mutants lacking the ability to modulate protein diffusion are defective for virulence[110]. In *V. cholerae,* the diffusion of TcpP within the inner membrane has also been investigated. Using single molecule tracking[111], the authors from this study concluded that the presence of ToxR and *toxT* promoter both affect diffusion of a single fluorescently labeled TcpP molecules. They also noted that a portion of TcpP proteins were immobile and suggested that this halting was due to TcpP binding at the *toxT* promoter. A mutant strain lacking the *toxT* promoter region had fewer immobile TcpP events[111]. In our experiments, TcpP mobility was shifted in conditions

that are both strongly virulence inducing and physiologically relevant[28,85,112]. This study reinforces the notion that pathogens are exquisitely adapted to the environments and niches that they colonize. Further research will better elucidate whether the calcium-bile salts signal is sensed by other enteric pathogens, and if there are other specific signals used that exist in the complex intestinal milieu.

Chapter 3 was adapted from Hay *et. al.* 2016, submitted

CHAPTER 4: DISCUSSION

Bile and the intestinal landscape

Bile is one of the major physical barrier that *V. cholerae* must cope with in order to successfully colonize the human host. We noted that exposure to different types of bile salts results in drastically different phenotypic outcomes for *V. cholerae*, even though each bile salts is present in the gut. A better understanding of the distribution of bile salts along the intestines helps to explain this phenomenon. Following release into the proximal small intestine, bile salts progress distally along the small and eventually large intestine. Bile salt concentration decreases as bile salts are reabsorbed, altered by bacteria, and incorporated into fecal matter[105]. Consequently, the bacterial load and community makeup of the microbiota changes along the intestine as depicted in Figure 4.1. Bile salts are thought to decrease in concentration from the lumen towards the intestinal epithelium due to the layers of mucous secreted by host cells. This decrease in bile salts into the mucosal layer is accompanied by an increase in bicarbonate towards the epithelium[113]. Similarly, bacteria are most concentrated in the lumen, and decrease in concentration further into the mucosal layers.

Bile composition also changes along the intestine as extensive modification of bile salts occur by commensal bacteria in both the small and large intestine. The most common modifications are deconjugation and dehydroxylation. Deconjugation is the removal of the amino side group and is performed by bile salt hydrolase enzymes by bacteria primarily in the small intestine. Numerous classes of both gram positive and negative bacteria have been found to have distinct bile salt hydrolases with variable affinity for different bile salts[114]. Primary bile salts that evade uptake in the small intestine and reach the large intestine will undergo dehydroxylation at the 7C position, resulting in

bacterial mediated formation of secondary bile salts. Dehydroxylation is performed by a smaller number of bacteria, typically *Clostridium sp*., but is an efficient process, converting nearly all primary bile salts to secondary[115–117] Thus, taurine and glycine conjugated bile salts are in highest abundance in the proximal small intestine, making up a large fraction of the bile acid milieu[118]. As bile acids are altered by the microbiome and recirculated, the ratio of primary and conjugated bile acids decreases as secondary, tertiary, and unconjugated bile acids increase (See Figures 4.1 and 1.2). Therefore, bacteria entering the small intestine will likely encounter high concentrations of conjugated bile salts, which concurs with the evidence that these molecules having strong pro-colonization effects on V*. cholerae* physiology.

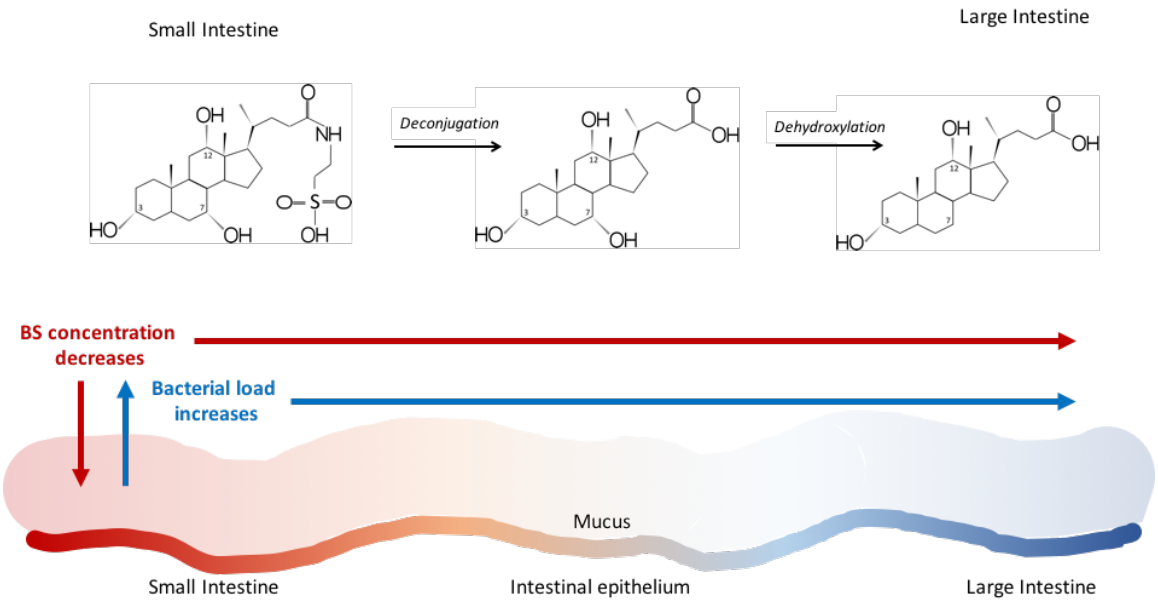


Figure 4.1. Bile and the intestinal landscape. Primary, conjugated bile salts are released into the proximal small intestine, where they are in high abundance and concentration. Modification of bile salts includes deconjugation in the small intestine and dehydroxylatoin in the large intestine, resulting in deconjugated and secondary bile salts, respectively. Moving distally, bile salt concentration decreases, and bacterial burden consequently increases. Bile concentration decreases into the mucus layer towards the epithelium, which also holds a lower concentration of bacteria.

Bile as a general virulence modulating signal

V. cholerae is not the only pathogen to integrate the bile signal into regulation of its virulence program. For example, bile positively regulates virulence for *Campylobacter jejuni,* a gram negative bacteria that is a major source of food poisoning. *C. jejuni* colonizes the distal ilium and colon, where it likely encounter bile salts modified by the microbiome. In response to deoxycholate, *C. jejuni* increases expression of major virulence factors including the Campylobacter invasion antigens (Cia), leading to more rapid invasion of epithelial cells.[119,120] Bile salts also positively modulate virulence for the enteric pathogen *Shigela flexnarii*. This response was specific the dehydrocylated bile salts such as deoxycholate and chenodeoxycholate, but not for other bile salts and detergents tested. Under these conditions, bile salts promote invasion and efficient utilization of type 3 secretion system, which contributes to pathogenicity[121,122]. Bile salts have the opposite effect on *Salmonella enterica* serovar *Typhimurium*, repressing virulence associated genes and phenotypes. In particular, bile decreased invasion of epithelial cells *in vitro* in cell culture and *ex vivo* in sections of mouse jejunal tissue[123]. This can be at least partially attributed to a bile-dependent decrease of type 3 secretion mediated by Salmonella Pathogenicity Island 1 (SPI1)[123,124]. Exposure to deoxycholate also led to decreased expression of genes on both Salmonella Pathogenicity Island 1 and 2[124,125]. This repression has been suggested to result in delay of virulence expression in the bile salt rich lumen until bacteria can access the epithelium where they can utilize invasion related proteins for inection[123].

V. cholerae has complex responses to bile, regarding regulation of virulence associated genes. Original studies suggested that bile inhibits virulence. In the Classical O395 biotype, which has high basal production of cholera toxin (CT), crude bile decreased toxin production[126]. Subsequently, in this same strain it was found that no

individual bile salt decreased CT production, leading to the discovery that fatty acids repress virulence through binding directly with regulator ToxT[90,91,127]. This same study suggested that CT could actually be induced directly by ToxR in the presence of cholate in the Classical strain[127]. In the El Tor biotype, secondary and unconjugated bile acids such as deoxycholate do not promote virulence, while the conjugated primary bile salts taurocholate and glychocholate do[74]. In fact, the former two bile salts stimulate growth as a biofilm, which is antithetical to virulence within the host[62]. Bile has also been shown to promote motility, which is required for efficient colonization [73,126]. Because bile and bile salts vary in concentration and composition at different portions of the intestine, different sets of compounds may represent unique spatio-temporal signals that can be integrated into gene expression for pathogens like *V. cholerae*. For example, the abundance of conjugated bile acids in the proximal small intestine may promote motility and expression of virulence determinants, to drive colonization. More distally, an abundance of secondary bile acids maybe be sensed as signal to activate aquatic and survival genes for the impending exit from the host.

Heterogeneous effects of bile salts on bacterial phenotypes

While bile is a complex mixture, it is interesting to consider why exposure to different individual bile salts can have drastically different phenotypic outcomes within the same bacteria. One possible reason is that while the bile salts may be directly sensed by bacteria, they also signal indirectly through the cellular damage they cause. This is evidenced by the numerous bacteria that induce DNA repair and oxidative stress protection genes when exposed to certain bile salts[128–130]. It is also important to remember that bile salts are not equally toxic to cells, with deconjugated bile salts being the most toxic. To enter the cell cytoplasm of gram negative bacteria, bile acids must

pass through the outer membrane, which may occur through slow diffusion, or more likely through porins in the membrane[34]. For the plasma membrane of gram positive and gram negative bacteria, bile acids can insert into the outer hemileaflet and then “flip-flop” into the inner hemileaflet. More hydrophobic acids like deoxycholate “flip-flop” most quickly, with unconjugated primary acids like cholate doing so slowly. Conjugated bile acids showed little traversion to the inner hemileaflet in experiments using unilamellar vesicles[94,95] The implication is that a greater amount of deconjugated bile salts gain access to the plasma membrane and cytoplasm, where damage occurs via disruption of DNA, proteins, and membranes[131–134]. Additionally, based on pKa values, it is expected that deconjugated bile acids are more likely to be protonated and able to pass into membranes. Bile salts that are less toxic to cells, such as TC and GC, may therefore be interpreted as an entirely different signal. For example, conjugated bile salts such as TC and GC can initiate growth of spores for *Clostridium dificile*[135]. For *V. cholerae* these bile salts promote virulence induction and TC enhances biofilm egress[74,82]. However, the secondary bile acid deoxycholate has an opposite effect on these phenotypes. It damages *C. difficile* to the point of preventing infection[136]. For *V. cholerae* deoxycholate does not induce virulence, and promotes entry into a biofilm state. It is not surprising that within this landscape, pathogens like *V. cholerae* can fine-tune their responses to discrete conditions to find the optimal niche for survival and virulence induction.

Future Directions

Experiments that block protein synthesis demonstrated that V. cholerae does not likely egress from biofilms by inducing proteins in response to TC. Although we have suggested that *V. cholerae* undergoes a passive dispersal, there is still the possibility that a *V. cholerae* derived gene product may contribute to dispersal. For example, if an outer membrane or extracellular protein were produced during biofilm formation, it could be "activated" by TC to effect the biofilm matrix. A targeted screen for TC-insensitive

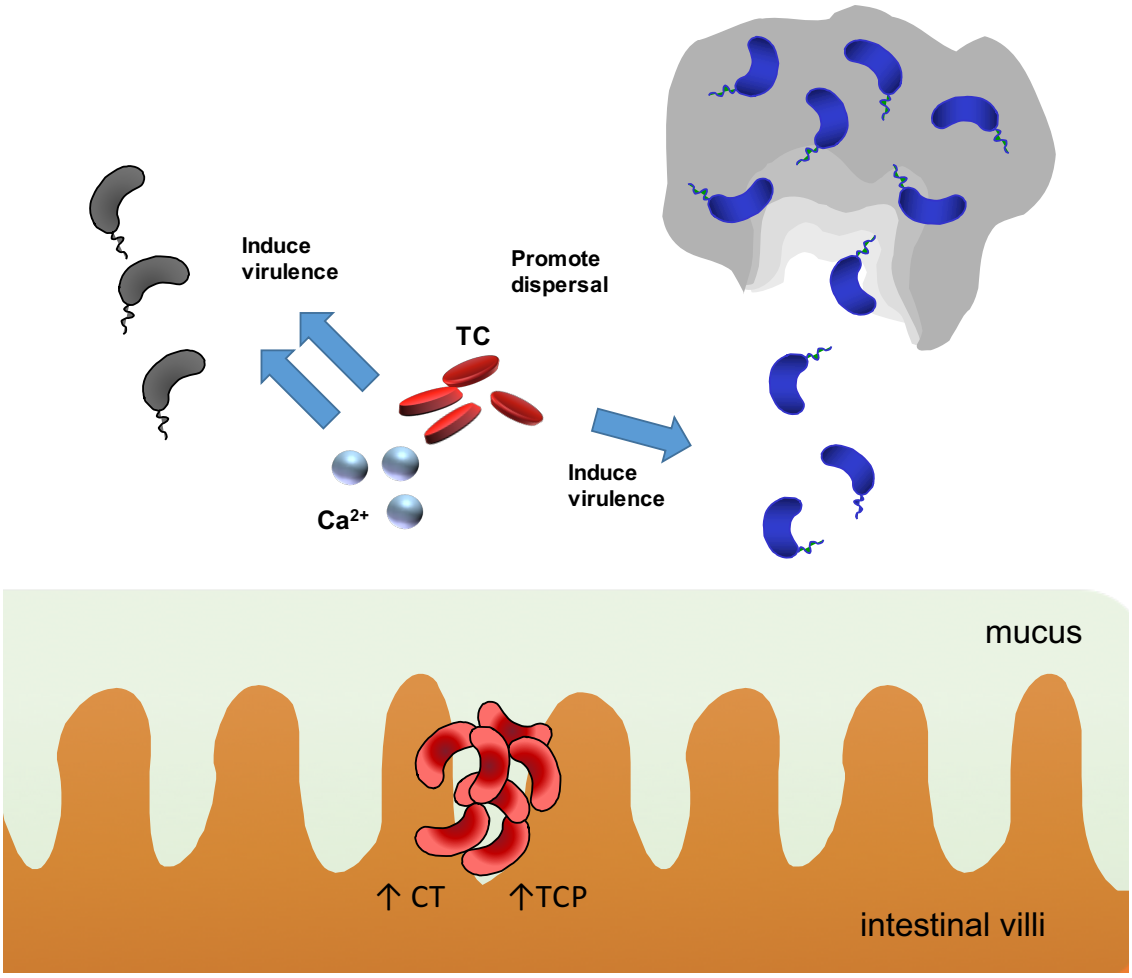


Figure 4.2. Working model: *V. cholerae* coordination of early colonization phenotypes. *V. cholerae* cells ingested as biofilm will reach the small intestine and be exposed to bile. The bile salt, taurocholate (TC), promotes dispersal from the biofilm. Dispersed cells or cells ingested in a free-living state are stimulated by the same TC signal to induce expression of virulence related products such as cholera toxin (CT) and the toxin co-regulated pilu (TCP). Calcium, or calcium bound to TC enhances this virulence activation by promoting virulence regulator activity.

mutants would be one way to address this hypothesis. A defined mutant library could be used to specifically test mutants for genes with characteristics such as extracellular and predicted enzyme activity[137]. Likewise, in an attempt to best replicate natural infection, it would be of interest to compare infectivity and *tcpA* expression for *V. cholerae* biofilms that are either host passaged or grown in high-salt, low nutrient conditions similar to those found in aquatic reservoirs.

Likewise, further studies are needed to better define the mechanism by which calcium and TC affect the interaction between TcpP and the membrane it is embedded in. Higher resolution studies of the movement of TcpP and its cysteine mutants can clarify whether dimerization is involved in diffusion rate. It would also be useful to confirm the presence and concentration of $CaTC_2$ *in vivo.* The signals that *V. cholerae* encounters during early infection may have large effects on its phenotype. This is particularly relevant as those with poor nutritional status are susceptible to enteric bacterial pathogens and likely have an altered set of signals and microbiota in the intestinal tract[138,139]dew. For example, undernourished patients typically have an altered or underdeveloped microbiome compared to healthy patients, as well as a distinct metabolomics profile[140,141] In further disruption of the homeostasis between host, bile, and microbiota, severe cholera effectively wipes out the patient's microbiome, with cholera representing the majority of bacteria present[142]. To build on the current study, further characterization of changes in bile composition during undernourishment and during disease may help be helpful in understanding susceptibility, disease course, and recovery for at-risk populations.

Conclusions

V. cholerae infection dynamics can differ based on the signals that bacteria encounter as well as the growth state of the bacteria. Growth in a biofilm enables cells to colonize the small intestine more quickly, and to a higher bacterial burden than planktonic grown cultures[55]. In this study, we have demonstrated that the host intestinal signal, taurocholate (TC), promotes detachment of *V. cholerae* from mature biofilms *in vitro*. Additionally, this step precedes the induction of virulence factors, also affected by bile salts. Conjugated bile salts are most concentrated where they empty into the proximal small intestine, and decrease in abundance in more distal portions of the gut[28]. Thus, *V. cholerae* will likely encounter high levels of TC upon entrance into the small intestine, as well as other bile components such as calcium. Careful studies investigating the timing of virulence factor production in a murine model suggest that there is an early TCP induction phase that occurs within two hours of inoculation, while cells are in the lumen, without which CT induction does not occur[143]. Our data is consistent with a model that can occur within this time frame. Cells ingested as a biofilm are met by TC, which promotes exit from the biofilm and potentiates virulence activation[82]. These detached cells, or planktonically grown ingested cells, respond to the same TC signal to induce virulence factors such as TcpA. Calcium, possibly bound to TC as $CaTC_2$ enhances this activation. Onset of symptoms in cholera is rapid, and death can occur within hours in the most severe cases[3]. It is important to understand the interactions between host and pathogen that occur during this critical incubation period. The culmination of this dissertation is to described novel ways in which *V. cholerae* integrates bile salts and other intestinal signals to coordinate early steps of infection.

CHAPTER 5: MATERIALS AND METHODS

Bacterial strains and growth conditions.

All *V. cholerae* strains used in this study were derived from E1 Tor C6706, and were propagated in LB media containing appropriate antibiotics at 37 °C[144]. In-frame deletions were constructed by cloning the regions flanking the target genes into suicide vector pWM91 containing a *sacB* counterselectable maker[145]. P_{vpsA}-*lacZ* transcriptional fusions were generated by cloning *vpsA* promoter into a plasmid containing a *lacZ* reporter[146]. Transcriptional *luxCDABE* reporters of promoter regions of *aphA*, *aphB*, *tcpP*, *toxR*, *toxT*, *ctxA* and *tcpA* have been described previously[15]. Plasmids for overexpressing virulence regulators were described previously[147]. For GFP-TcpP and TatA-GFP constructs, PCR-amplified fragments containing *gfp-tcpPH* or *tatA-gfp* coding sequences were cloned into pBAD24[148], and the resulting plasmids were introduced into *V. cholerae* by electroporation.

Biofilm formation assays

Assays to quantify biofilms were performed as previously described[61]. A 1:100 dilution of overnight-grown culture of *V. cholerae* strain was inoculated in LB broth into 10 × 75 mm borosilicate glass tubes and incubated for 22-24 h at 22°C. Subsequently, the tubes were rinsed three times with phosphate buffered saline (PBS) then filled with crystal violet stain. After 5 minutes, excess stain was rinsed off with de-ionized water. The biofilm-associated crystal violet was solubilized in dimethylsulphoxide (DMSO), and the OD_{570} of the resulting suspension was measured. All experiments were performed at least three independent times and samples were performed in triplicate.

Biofilm detachment assays

Supernatants from mature biofilms were aspirated. Biofilms on tubes were rinsed three times with PBS then fresh media pre-mixed with indicated compound was added gently as to not manually disrupt biofilm structure. After the indicated time, biofilm-derived planktonic cells were drawn from the supernatant, serially diluted, and plated on LB agar plates with appropriate antibiotics for enumeration. Remaining biofilms were quantified by crystal violet as described above, or biofilm-resident cells were collected after manual disruption by vortexing in the presence of PBS and glass beads.

Microscopy to examine biofilm structures

For scanning electron microscopy (SEM), a 1:100 dilution of overnight-grown culture of wild type *V. cholerae* was inoculated into 5mL of LB in 50-ml Falcon tubes containing 22-by-22-mm sterile glass coverslips. After 24 hours, coverslips were washed three times with PBS then placed into six-well plates with 2mL of fresh media pre-mixed with +/- 1mM taurocholate. Biofilms on coverslips for SEM were fixed in 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1M cacodylate buffer, pH 7.4, overnight at 4ºC. After several buffer washes the samples were post-fixed in 2.0% osmium tetroxide for 1 hour, washed again in buffer, and dehydrated in a graded ethanol series. Samples were treated with several changes of hexamethyldisilazane (HMDS) and then allowed to air dry prior to mounting and sputter coating with gold/palladium. SEM images were collected using a Philips XL20 scanning electron microscope.

Estimation of carbohydrate content during detachment

Supernatant from biofilm detachment assays were sterilized through 0.2 μM filters before carbohydrate content was estimated using a modified phenol-sulfuric acid method[149].

Briefly, 50 µL of each sample was added to a well of a 96-well microplate. To this, 150 µL of concentrated sulfuric acid was mixed in, followed by 30 µl of 5% phenol in water. Plates were covered and incubated for 10 minutes in a 90ºC water bath. Plates were cooled at room temperature, dried, and resulting OD_{490} was measured. A standard glucose curve generated during each independent experiment was used to convert sample OD_{490} values to percent sugar. To reduce background, M9 minimal media supplemented with 0.2% glycerol was used during detachment and biofilms were washed four times with PBS.

Beta-galactosidase assays during biofilm formation and detachment

Biofilms of *V. cholerae* Δ*lacZ* containing the *vpsA- lacZ* reporter plasmid were allowed to form and disrupted as described above. Biofilm- associated and detached cells were collected, washed, and resuspended in PBS to an OD600 ~ 0.2. Cultures were assayed for ß-galactosidase activity, which was normalized against the optical density at 600 nm and reported as Miller units as previously described[150].

Measurement of virulence gene expression during detachment

V. cholerae strains containing virulence promoter *luxCDABE* transcriptional fusions were used in liquid culture and detachment assays. Luminescence was measured using a Bio-Tek Synergy HT spectrophotometer and normalized for growth against OD_{600}. Luminescence expression is reported as light units/OD_{600}.

V. cholerae strains containing a constitutive P_{tet}-*mCherry* and inducible P_{tcpA}-*gfp* construct [15] were used for detachment assay. Immediately following detachment assay, data were collected on a LSR II flow cytometer (BD Biosciences) and post-collection data were analyzed using FlowJo version 9.7.5 (Treestar).

A TcpA reporter system was used (*lacZ::ptcpA-sh ble*) in which the zeocin- resistance gene sh ble[108] was fused to the promoter of tcpA and the resulting cassette was integrated into the lacZ locus of *V. cholerae.* Experiments were carried out on as described[147] on *V. cholerae* cells that were either biofilm resident or dispersed following incubation with +/-0.5mM TC. Cultures were treated +/- zeocin (25µg/ml) for 30 min at 37°C and plated on LB agar. Data reported as percentage of survival of treated cells compared to untreated controls.

Measurement of gene expression of virulence genes.

Overnight cultures of *E. coli* or *V. cholerae* strains containing promoter *luxCDABE* transcriptional fusions were subcultured at a dilution of 1:1000 in LB with indicated compounds and grown microaerobically until mid-log (OD_{600} ~0.2). Luminescence was measured using a Bio-Tek Synergy H1 spectrophotometer and normalized for growth against the OD600. Luminescence expression is reported as light units/OD_{600} unit.

A TcpA reporter system was used (*lacZ::ptcpA-sh ble*) in which the zeocin- resistance gene sh ble[108] was fused to the promoter of tcpA and the resulting cassette was integrated into the lacZ locus of *V. cholerae.* Experiments were carried out on as described[147] on *V. cholerae* cultures that were incubated with the indicated amount of TC, CaCl2, or boath. were treated +/- zeocin (25µg/ml) for 30 min at 37°C and plated on LB agar. Data reported as percentage of survival of treated cells compared to untreated controls.

Purification of intestinal extracts and calcium depletion.

All animal studies were carried out in accordance with the animal protocols that were approved by the Institutional Animal Care and Use Committee of University of

Pennsylvania. Intestinal extracts were purified as described[74]. Briefly, fragments of small intestines from 5-week-old CD-1 mice were cut open and flushed with double-distilled (ddH_2O). The intestinal flush was then autoclaved and extracted with phenol-chloroform and subsequently ethyl acetate. The aqueous phase was then precipitated with 70% (vol/vol) ethanol. The supernatant was dried using a rotary evaporator and resuspended with ddH_2O. EGTA (Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid) was incubated with intestinal extracts for 12 hours prior to use in experiments.

Preparation of calcium taurocholate ($CaTC_2$).

A solution of sodium taurocholate was acidified to ~pH 3 with 1M HCl then frozen and lyophilized to dryness. The resulting white powder was suspended in acetone and filtered through a pad of silica gel to remove sodium chloride byproducts. This material was evaporated to dryness with air giving taurocholic acid as a white film which was then dissolved in water. An aqueous solution of calcium hydroxide (0.5 eq, 1mg/mL solution) was added and the mixture was stirred for two days. This mixture was filtered through a pad of Celite to remove insoluble particulates, frozen, and lyophilized to dryness to give a white solid.

Bacterial two-hybrid system to determine TcpP-TcpP interaction.

Full-length *tcpP* or *toxR* fragments were cloned into pUT18C and pKT25 [92] vector as described previously[74,93]. Overnight cultures of *tcpP* and *toxR* reporters were subcultured at a dilution of 1:100 in LB medium containing 0.5 mM IPGT (isopropyl β-D-1-thiogalactopyranoside) with or without 1 mM taurocholate and/or 10 mM $CaCl_2$. TcpP-TcpP and ToxR-ToxR strains were then incubated without shaking at 30°C for 6 hrs.

Cultures were then assayed for β-galactosidase activity and the results were reported as Miller units as previously described[150].

Fluorescence recovery after photobleaching (FRAP) analysis.

Overnight cultures of *V. cholerae* strains containing either P_{BAD}-*tcpP-gfp* or P_{BAD}-*tatA-gfp* fusions were subcultured at a dilution of 1:1000 in LB with 0.1% or 0.05% arabinose, respectively, and grown aerobically to early log phase. Cultures were exposed to 10 mM $CaCl_2$ and/or 1 mM TC and grown microaerobically until mid-log (OD_{600} ~0.2). Cells were applied to microscope slides containing a soft agarose pad (1%) and visualized by fluorescence microscopy. Fluorescence microscopy was performed on a Zeiss LSM 710 confocal laser scanning microscope using a 63× oil immersion objective. ZEN 2012 software was used for acquisition of data. The 488-line of a 30 mW argon ion laser was used for both photobleaching and the subsequent fluorescence excitation/recording. For photobleaching, a power of 1.4 mW was applied onto selected regions within one of the compartments of the cell for 40 ms. Images were taken before photobleaching (t = −2 s), immediately after photobleaching (t = 0 s), and for thirty seconds at an interval of two seconds. Following photobleaching, the fluorescence within the compartment exposed was nearly fully depleted. FRAP recovery data was analyzed as described[98]. Briefly, recovery of fluorescence intensity was recorded within two equally sized circular regions, one in the middle of the bleached compartment $A_1(t)$ $F_1(t)$ and the other in the middle of the unbleached compartment $A_2(t)$. The fluorescence recovery data was normalized to the total remaining fluorescen$_2$ce for any given time point t. The recovery rate was thus:

$$\text{Recovery}(t) = 100 * \left(\frac{A_1(t)}{A_1(t)A(t)}\right)$$

C^{14}-labeled taurocholate uptake assays.

Overnight cultures of wild type and Δ*tolC* mutants[44] were subcultured 1:100 into LB medium with or without added $CaCl_2$ aerobically until mid-log (OD_{600}~0.2). Cells were concentrated 10-fold in uptake buffer (uptake buffer: 1mM $MgCl_2$, 10mM Tris-Cl (pH 7.5), 137 mM NaCl) with or without $CaCl_2$ and incubated with 200 μM taurocholate and 20 μM ^{14}C-taurocholate. Aliquots of cells were taken at indicated timepoints, pelleted, and count per minute (CPM) of ^{14}C in cells was measured on a Beckman scintillation counter. TC accumulation is reported as percentage of CPM in cells compared to total CPM.

BIBLIOGRAPHY

1. Ali M, Lopez AL, Ae You Y, et al. The Global Burden of Cholera. *Bull World Health Organ*. 2012;90(3):209-218. doi:10.2471/BLT.11.093427.
2. Cravioto A, Lanata CF, Lantagne DS, Nair GB. Final report of the independent panel of experts on the cholera outbreak in Haiti. *United Nations Public Heal Rep*. 2011:1-32.
3. Sack DA, Sack RB, Nair GB, Siddique A. Cholera. *Lancet*. 2004;363(9404):223-233. doi:10.1016/S0140-6736(03)15328-7.
4. Desai SN, Clemens JD. An overview of cholera vaccines and their public health implications. *Curr Opin Pediatr*. 2012;24:85-91. doi:10.1097/MOP.0b013e32834eb625.
5. Banerjee R, Das B, Balakrish Nair G, Basak S. Dynamics in genome evolution of Vibrio cholerae. *Infect Genet Evol*. 2014;23:32-41. doi:10.1016/j.meegid.2014.01.006.
6. Sánchez J, Holmgren J. Cholera toxin structure, gene regulation and pathophysiological and immunological aspects. *Cell Mol Life Sci*. 2008;65(9):1347-1360. doi:10.1007/s00018-008-7496-5.
7. Herrington DA, Hall RH, Losonsky G, Mekalanos JJ, Taylor RK, Levine MM. Toxin, Toxin-coregulated pili, and the toxR Regulon are Essential for Vibrio Cholerae Pathogeneis in Humans. *J Exp Med*. 1988;168(October):0-5.
8. Krebs SJ, Taylor RK. Protection and attachment of Vibrio cholerae mediated by the toxin-coregulated pilus in the infant mouse model. *J Bacteriol*. 2011;193(19):5260-5270. doi:10.1128/JB.00378-11.
9. Reguera G, Kolter R. Virulence and the Environment : a Novel Role for Vibrio cholerae Toxin-Coregulated Pili in Biofilm Formation on Chitin Virulence and the

Environment : a Novel Role for Vibrio cholerae Toxin-Coregulated Pili in Biofilm Formation on Chitin. 2005. doi:10.1128/JB.187.10.3551.

10. Taylor RK, Millert VL, Furlong DB, Mekalanost JJ. Use of phoA gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. *Proc Natl Acad Sci U S A*. 1987;84(May):2833-2837.
11. Matson JS, Withey JH, DiRita VJ. Regulatory networks controlling Vibrio cholerae virulence gene expression. *Infect Immun*. 2007;75(12):5542-5549. doi:10.1128/IAI.01094-07.
12. DiRita VJ, Parsot C, Jander G, Mekalanos JJ. Regulatory cascade controls virulence in Vibrio cholerae. *Proc Natl Acad Sci U S A*. 1991;88(12):5403-5407. doi:10.1073/pnas.88.12.5403.
13. Krukonis ES, DiRita VJ. DNA binding and ToxR responsiveness by the wing domain of TcpP, an activator of virulence gene expression in Vibrio cholerae. *Mol Cell*. 2003;12(1):157-165. doi:10.1016/S1097-2765(03)00222-3.
14. Kovacikova G, Lin W, Skorupski K. The LysR-type virulence activator AphB regulates the expression of genes in Vibrio cholerae in response to low pH and anaerobiosis. *J Bacteriol*. 2010;192(16):4181-4191. doi:10.1128/JB.00193-10.
15. Liu Z, Yang M, Peterfreund GL, et al. Vibrio cholerae anaerobic induction of virulence gene expression is controlled by thiol-based switches of virulence regulator AphB. *Proc Natl Acad Sci U S A*. 2011;108(2):810-815. doi:10.1073/pnas.1014640108.
16. Liu Z, Wang H, Zhou Z, et al. Differential Thiol-Based Switches Jump-Start Vibrio cholerae Pathogenesis. *Cell Rep*. 2016;14(2):347-354. doi:10.1016/j.celrep.2015.12.038.
17. Kovacikova G, Lin W, Skorupski K. The virulence activator AphA links quorum

sensing to pathogenesis and physiology in Vibrio cholerae by repressing the expression of a penicillin amidase gene on the small chromosome. *J Bacteriol*. 2003;185(16):4825-4836. doi:10.1128/JB.185.16.4825-4836.2003.

18. Lin W, Kovacikova G, Skorupski K. The quorum sensing regulator HapR downregulates the expression of the virulence gene transcription factor AphA in Vibrio cholerae by antagonizing Lrp- and VpsR-mediated activation. *Mol Microbiol*. 2007;64(4):953-967. doi:10.1111/j.1365-2958.2007.05693.x.
19. Zhu J, Miller MB, Vance RE, Dziejman M, Bassler BL, Mekalanos JJ. Quorum-sensing regulators control virulence gene expression in Vibrio cholerae. *Proc Natl Acad Sci U S A*. 2002;99(5):3129-3134. doi:10.1073/pnas.052694299.
20. Faruque SM, Albert MJ. Epidemiology , Genetics , and Ecology of Toxigenic Vibrio cholerae. 1998;62(4):1301-1314.
21. Faruque SM, Mekalanos JJ. Phage-bacterial interactions in the evolution of toxigenic Vibrio cholerae. *Virulence*. 2012;3(7):556-565. doi:10.4161/viru.22351.
22. Karaolis DK, Johnson J a, Bailey CC, Boedeker EC, Kaper JB, Reeves PR. A Vibrio cholerae pathogenicity island associated with epidemic and pandemic strains. *Proc Natl Acad Sci U S A*. 1998;95(6):3134-3139. doi:10.1073/pnas.95.6.3134.
23. Krukonis ES, Yu RR, DiRita VJ. The Vibrio cholerae ToxR/TcpP/ToxT virulence cascade: Distinct roles for two membrane-localized transcriptional activators on a single promoter. *Mol Microbiol*. 2000;38(1):67-84. doi:10.1046/j.1365-2958.2000.02111.x.
24. Provenzano D, Schuhmacher DA, Justin L, Klose KE, Barker JL. The Virulence Regulatory Protein ToxR Mediates Enhanced Bile Resistance in Vibrio cholerae and Other PathogenicVibrio Species The Virulence Regulatory Protein ToxR

Mediates Enhanced Bile Resistance in Vibrio cholerae and Other Pathogenic Vibrio Species. 2000. doi:10.1128/IAI.68.3.1491-1497.2000.Updated.

25. Crawford JA, Krukonis ES, DiRita VJ. Membrane localization of the toxR winged-helix domain is required for TcpP-mediated virulence gene ctivation in Vibrio cholerae. *Mol Microbiol*. 2003;47(5):1459-1473.

26. Perez-Lopez A, Behnsen J, Nuccio S-P, Raffatellu M. Mucosal immunity to pathogenic intestinal bacteria. *Nat Rev Immunol*. 2016;16(3):135-148. doi:10.1038/nri.2015.17.

27. Johansson ME V, Ambort D, Pelaseyed T, et al. Composition and functional role of the mucus layers in the intestine. *Cell Mol Life Sci*. 2011;68(22):3635-3641. doi:10.1007/s00018-011-0822-3.

28. Eastwood M a, Boyd GS. The distribution of bile salts along the small intestine of rats. *Biochim Biophys Acta*. 1967;137(2):393-396. http://www.ncbi.nlm.nih.gov/pubmed/6051569.

29. Hofmann AF, Eckmann L. How bile acids confer gut mucosal protection against bacteria. *Proc Natl Acad Sci U S A*. 2006;103(10):4333-4334.

30. Hofmann AF. The Gastrointestinal System. Salivary, Gastric, Pancreatic, and Hepatobiliary Secretion. In: *Handbook of Physiology*. 3rd ed. Bethesda, MD: Am. Physiol. Soc.; 1989:549-566.

31. Swann JR, Want EJ, Geier FM, et al. Systemic gut microbial modulation of bile acid metabolism in host tissue compartments. *Proc Natl Acad Sci U S A*. 2011;108 Suppl :4523-4530. doi:10.1073/pnas.1006734107.

32. Abuaita BH, Withey JH. Bicarbonate induces Vibrio cholerae virulence gene expression by enhancing ToxT activity. *Infect Immun*. 2009;77(9):4111-4120. doi:10.1128/IAI.00409-09.

33. Begley M, Gahan CGM, Hill C. The interaction between bacteria and bile. *FEMS Microbiol Rev*. 2005;29(4):625-651. doi:10.1016/j.femsre.2004.09.003.

34. Nikaido H. Molecular Basis of Bacterial Outer Membrane Permeability Revisited Molecular Basis of Bacterial Outer Membrane Permeability Revisited. *Microbiol Mol Biol Rev*. 2003;67(4). doi:10.1128/MMBR.67.4.593.

35. Fralick JOE a. Evidence that TolC is required for functioning of the Mar / AcrAB efflux pump of Escherichia Evidence that TolC Is Required for Functioning of the Mar / AcrAB Efflux Pump of Escherichia coli. *Am Soc Microbiol*. 1996;178(19):5803-5805.

36. Ma D, Cook DN, Alberti M, Pon NG, Nikaido H, Hearst JE. Molecular cloning and characterization of acrA and acrE genes of Escherichia coli. *J Bacteriol*. 1993;175(19):6299-6313. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=206727&tool=pmcentrez&rendertype=abstract.

37. Zgurskaya HI, Nikaido H. Bypassing the periplasm: reconstitution of the AcrAB multidrug efflux pump of Escherichia coli. *Proc Natl Acad Sci U S A*. 1999;96(13):7190-7195. doi:10.1073/pnas.96.13.7190.

38. Bina JE, Provenzano D, Wang C, Bina XR, Mekalanos JJ. Characterization of the Vibrio cholerae vexAB and vexCD efflux systems. *Arch Microbiol*. 2006;186(3):171-181. doi:10.1007/s00203-006-0133-5.

39. Chatterjee A, Chaudhuri S, Saha G, Gupta S, Chowdhury R. Effect of bile on the cell surface permeability barrier and efflux system of Vibrio cholerae. *J Bacteriol*. 2004;186(20):6809-6814. doi:10.1128/JB.186.20.6809-6814.2004.

40. Cerda-Maira F a, Ringelberg CS, Taylor RK. The bile response repressor BreR regulates expression of the Vibrio cholerae breAB efflux system operon. *J*

Bacteriol. 2008;190(22):7441-7452. doi:10.1128/JB.00584-08.

41. Cerda-Maira F a, Kovacikova G, Jude B a, Skorupski K, Taylor RK. Characterization of BreR interaction with the bile response promoters breAB and breR in Vibrio cholerae. *J Bacteriol*. 2013;195(2):307-317. doi:10.1128/JB.02008-12.

42. Xu Q, Dziejman M, Mekalanos JJ. Determination of the transcriptome of Vibrio cholerae during intraintestinal growth and midexponential phase in vitro. *Proc Natl Acad Sci U S A*. 2003;100(3):1286-1291. doi:10.1073/pnas.0337479100.

43. Baucheron S, Nishino K, Monchaux I, et al. Bile-mediated activation of the acrAB and tolC multidrug efflux genes occurs mainly through transcriptional derepression of ramA in Salmonella enterica serovar Typhimurium. *J Antimicrob Chemother*. 2014;69(9):2400-2406. doi:10.1093/jac/dku140.

44. Bina JE, Mekalanos JJ. Vibrio cholerae tolC Is Required for Bile Resistance and Colonization. *Infect Immun*. 2001;69(7):4681-4685. doi:10.1128/IAI.69.7.4681.

45. Rigel NW, Silhavy TJ. Making a beta-barrel: assembly of outer membrane proteins in Gram-negative bacteria. *Curr Opin Microbiol*. 2012;15(2):189-193. doi:10.1016/j.mib.2011.12.007.

46. Chakrabarti SR, Chaudhuri K, Sen K, Das J. Porins of Vibrio cholerae: purification and characterization of OmpU. *J Bacteriol*. 1996;178(2):524-530. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=177687&tool=pmcentrez&rendertype=abstract.

47. Wibbenmeyer JA, Provenzano D, Candice F, Klose KE, Delcour AH, Landry CF. Vibrio cholerae OmpU and OmpT Porins Are Differentially Affected by Bile Vibrio cholerae OmpU and OmpT Porins Are Differentially Affected by Bile. 2002;70(1). doi:10.1128/IAI.70.1.121.

48. Provenzano D, Klose KE. Altered expression of the ToxR-regulated porins OmpU and OmpT diminishes Vibrio cholerae bile resistance , virulence factor expression , and intestinal colonization. 2000;97(18):10220-10224.

49. Simonet VC, Baslé A, Klose KE, Delcour AH. The Vibrio cholerae porins OmpU and OmpT have distinct channel properties. *J Biol Chem*. 2003;278(19):17539-17545. doi:10.1074/jbc.M301202200.

50. Lutz C, Erken M, Noorian P, Sun S, McDougald D. Environmental reservoirs and mechanisms of persistence of Vibrio cholerae. *Front Microbiol*. 2013;4(December):375. doi:10.3389/fmicb.2013.00375.

51. Yildiz FH, Visick KL. Vibrio biofilms: so much the same yet so different. *Trends Microbiol*. 2009;17(3):109-118. doi:10.1016/j.tim.2008.12.004.

52. Colwell RR, Huq A, Islam MS, et al. Reduction of cholera in Bangladeshi villages by simple filtration. *Proc Natl Acad Sci U S A*. 2003;100(3):1051-1055. doi:10.1073/pnas.0237386100.

53. Nelson EJ, Chowdhury A, Harris JB, et al. Complexity of rice-water stool from patients with Vibrio cholerae plays a role in the transmission of infectious diarrhea. *Proc Natl Acad Sci U S A*. 2007;104(48):19091-19096. doi:10.1073/pnas.0706352104.

54. Zhu J, Mekalanos JJ. Quorum sensing-dependent biofilms enhance colonization in Vibrio cholerae. *Dev Cell*. 2003;5(4):647-656. http://www.ncbi.nlm.nih.gov/pubmed/14536065.

55. Tamayo R, Patimalla B, Camilli A. Growth in a biofilm induces a hyperinfectious phenotype in Vibrio cholerae. *Infect Immun*. 2010;78(8):3560-3569. doi:10.1128/IAI.00048-10.

56. Watnick PI, Kolter R. Steps in the development of a Vibrio cholerae El Tor biofilm.

Mol Microbiol. 1999;34(3):586-595. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2860543&tool=pmcentrez&rendertype=abstract.

57. Fong JCN, Syed K a, Klose KE, Yildiz FH. Role of Vibrio polysaccharide (vps) genes in VPS production, biofilm formation and Vibrio cholerae pathogenesis. *Microbiology*. 2010;156(Pt 9):2757-2769. doi:10.1099/mic.0.040196-0.

58. Absalon C, Van Dellen K, Watnick PI. A communal bacterial adhesin anchors biofilm and bystander cells to surfaces. *PLoS Pathog*. 2011;7(8):e1002210. doi:10.1371/journal.ppat.1002210.

59. Tor E, Casper-lindley C, Yildiz FH. VpsT Is a Transcriptional Regulator Required for Expression of vps Biosynthesis Genes and the Development of Rugose Colonial Morphology in Vibrio VpsT Is a Transcriptional Regulator Required for Expression of vps Biosynthesis Genes and the Development of . *J Bacteriol*. 2004;186(5):1574-1578. doi:10.1128/JB.186.5.1574.

60. Yildiz FH, Dolganov NA, Schoolnik GK. VpsR , a Member of the Response Regulators of the Two-Component Regulatory Systems , Is Required for Expression of vps Biosynthesis Genes and EPS ETr -Associated Phenotypes in Vibrio cholerae O1 El Tor. *J Bacteriol*. 2001;183(5):1716-1726. doi:10.1128/JB.183.5.1716.

61. Hammer BK, Bassler BL. Quorum sensing controls biofilm formation in Vibrio cholerae. *Mol Microbiol*. 2003;50(1):101-114. doi:10.1046/j.1365-2958.2003.03688.x.

62. Hung DT, Zhu J, Sturtevant D, Mekalanos JJ. Bile acids stimulate biofilm formation in Vibrio cholerae. *Mol Microbiol*. 2006;59(1):193-201. doi:10.1111/j.1365-2958.2005.04846.x.

63. Kaplan JB. Biofilm dispersal: mechanisms, clinical implications, and potential therapeutic uses. *J Dent Res*. 2010;89(3):205-218. doi:10.1177/0022034509359403.

64. Jackson DW, Suzuki K, Oakford L, Simecka JW, Hart ME, Romeo T. Biofilm Formation and Dispersal under the Influence of the Global Regulator CsrA of Escherichia coli Biofilm Formation and Dispersal under the Influence of the Global Regulator CsrA of Escherichia coli. 2002. doi:10.1128/JB.184.1.290.

65. Boles BR, Horswill AR. Agr-mediated dispersal of Staphylococcus aureus biofilms. *PLoS Pathog*. 2008;4(4):e1000052. doi:10.1371/journal.ppat.1000052.

66. Davey ME, Caiazza NC, Toole GAO. Rhamnolipid Surfactant Production Affects Biofilm Architecture in Pseudomonas Rhamnolipid Surfactant Production Affects Biofilm Architecture in Pseudomonas aeruginosa PAO1 †. 2003;185(3). doi:10.1128/JB.185.3.1027.

67. Chua SL, Liu Y, Yam JKH, et al. Dispersed cells represent a distinct stage in the transition from bacterial biofilm to planktonic lifestyles. *Nat Commun*. 2014;5:4462. doi:10.1038/ncomms5462.

68. Kierek-Pearson K, Karatan E. Biofilm development in bacteria. *Adv Appl Microbiol*. 2005;57(05):79-111. doi:10.1016/S0065-2164(05)57003-5.

69. Reguera G, Kolter R. Virulence and the environment: a novel role for Vibrio cholerae toxin-coregulated pili in biofilm formation on chitin. *J Bacteriol*. 2005;187(10):3551-3555. doi:10.1128/JB.187.10.3551-3555.2005.

70. Marks LR, Parameswaran GI, Hakansson AP. Pneumococcal interactions with epithelial cells are crucial for optimal biofilm formation and colonization in vitro and in vivo. *Infect Immun*. 2012;80(8):2744-2760. doi:10.1128/IAI.00488-12.

71. Bomchil N, Watnick P, Kolter R. Identification and Characterization of a Vibrio

cholerae Gene , mbaA , Involved in Maintenance of Biofilm Architecture Identification and Characterization of a Vibrio cholerae Gene , mbaA , Involved in Maintenance of Biofilm Architecture. 2003. doi:10.1128/JB.185.4.1384.

72. Kostakioti M, Hadjifrangiskou M, Hultgren SJ. Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era. *Cold Spring Harb Perspect Med*. 2013;3(4):a010306. doi:10.1101/cshperspect.a010306.

73. Butler SM, Camilli A. Going against the grain: Chemotaxis and infection in Vibrio cholerae. *Nat Rev Microbiol*. 2009;3(8):611-620. doi:10.1038/nrmicro1207.GOING.

74. Yang M, Liu Z, Hughes C, et al. Bile salt-induced intermolecular disulfide bond formation activates Vibrio cholerae virulence. *Proc Natl Acad Sci U S A*. January 2013:1-6. doi:10.1073/pnas.1218039110.

75. Higgins DE, Nazareno E, DiRita VJ. The virulence gene activator ToxT from Vibrio cholerae is a member of the AraC family of transcriptional activators. *J Bacteriol*. 1992;174(21):6974-6980.

76. Amarasekara AS, Wiredu B. Sulfonic Acid Group Functionalized Ionic Liquid Catalyzed Hydrolysis of Cellulose in Water : Structure Activity Relationships. 2014;2(3):102-107. doi:10.12691/rse-2-3-4.

77. Amarasekara AS, Wiredu B. Aryl sulfonic acid catalyzed hydrolysis of cellulose in water. *Appl Catal A Gen*. 2012;417-418:259-262. doi:10.1016/j.apcata.2011.12.048.

78. Hofmann a F, Small DM. Detergent properties of bile salts: correlation with physiological function. *Annu Rev Med*. 1967;18:333-376. doi:10.1146/annurev.me.18.020167.002001.

79. Boles BR, Thoendel M, Singh PK. Rhamnolipids mediate detachment of Pseudomonas aeruginosa from biofilms. *Mol Microbiol*. 2005;57(5):1210-1223. doi:10.1111/j.1365-2958.2005.04743.x.

80. Merrell DS, Butler SM, Qadri F, et al. Host-induced epidemic spread of the cholera bacterium. *Nature*. 2002;417(6889):642-645. doi:10.1038/nature00778.

81. Nadell CD, Bassler BL. A fitness trade-off between local competition and dispersal in Vibrio cholerae biofilms. *Proc Natl Acad Sci U S A*. 2011;108(34):14181-14185. doi:10.1073/pnas.1111147108.

82. Hay AJ, Zhu J. Host intestinal signal-promoted biofilm dispersal induces Vibrio cholerae colonization. *Infect Immun*. 2015;83(1):317-323. doi:10.1128/IAI.02617-14.

83. Hofmann a F, Mysels KJ. Bile acid solubility and precipitation in vitro and in vivo: the role of conjugation, pH, and Ca2+ ions. *J Lipid Res*. 1992;33(5):617-626. http://www.ncbi.nlm.nih.gov/pubmed/1619357.

84. Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol*. 2000;1(1):11-21. doi:10.1038/35036035.

85. Permyakov EA, Kretsinger RH. Cell signaling, beyond cytosolic calcium in eukaryotes. *J Inorg Biochem*. 2009;103(1):77-86. doi:10.1016/j.jinorgbio.2008.09.006.

86. Bilecen K, Yildiz FH. Identification of a calcium-controlled negative regulatory system affecting Vibrio cholerae biofilm formation. *Environ Microbiol*. 2009;11(8):2015-2029. doi:10.1111/j.1462-2920.2009.01923.x.

87. Domínguez DC, Guragain M, Patrauchan M. Calcium binding proteins and calcium signaling in prokaryotes. *Cell Calcium*. 2015;57(3):151-165. doi:10.1016/j.ceca.2014.12.006.

88. Sarkisova S, Patrauchan MA, Berglund D, Nivens DE, Franklin MJ. Calcium-Induced Virulence Factors Associated with the Extracellular Matrix of Mucoid Pseudomonas aeruginosa Biofilms Calcium-Induced Virulence Factors Associated with the Extracellular Matrix of Mucoid Pseudomonas aeruginosa Biofilms. *J Bacteriol*. 2005;187(13):4327. doi:10.1128/JB.187.13.4327.

89. Moore EW, Celic L. Interactions Between Ionized Calcium and Sodium Taurocholate : Bile Salts Are Important Buffers for Prevention of Calcium-Containing Gallstones. *Gastroenterology*. 1982;83(5):1079-1089. doi:10.1016/S0016-5085(82)80077-2.

90. Chatterjee A, Dutta PK, Chowdhury R. Effect of fatty acids and cholesterol present in bile on expression of virulence factors and motility of Vibrio cholerae.Chatterjee, A., Dutta, P. K., & Chowdhury, R. (2007). Effect of fatty acids and cholesterol present in bile on expression of virulence. *Infect Immun*. 2007;75(4):1946-1953. doi:10.1128/IAI.01435-06.

91. Plecha SC, Withey JH. The mechanism for inhibition of Vibrio cholerae ToxT activity by the unsaturated fatty acid components of bile. *J Bacteriol*. 2015;(March). doi:10.1128/JB.02409-14.

92. Karimova G, Pidoux J, Ullman A, Ladant D. A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proc Natl Acad Sci USA*. 1998;95(May):5752-5756.

93. Fan F, Liu Z, Jabeen N, Dillon Birdwell L, Zhu J, Kan B. Enhanced interaction of Vibrio cholerae virulence regulators TcpP and ToxR under oxygen-limiting conditions. *Infect Immun*. 2014;82(4):1676-1682. doi:10.1128/IAI.01377-13.

94. Donovan JM, Jackson AA. Transbilayer movement of fully ionized taurine-conjugated bile salts depends upon bile salt concentration, hydrophobicity, and

membrane cholesterol content. *Biochemistry*. 1997;36(38):11444-11451. doi:10.1021/bi9705927.

95. Kamp F, Hamilton J a, Westerhoff H V. Movement of fatty acids, fatty acid analogues, and bile acids across phospholipid bilayers. *Biochemistry*. 1993;32(41):11074-11086. doi:10.1021/bi00092a017.

96. Merritt ME, Donaldson JR. Effect of bile salts on the DNA and membrane integrity of enteric bacteria. *J Med Microbiol*. 2009;58(12):1533-1541. doi:10.1099/jmm.0.014092-0.

97. Van Den Wildenberg SMJL, Bollen YJM, Peterman EJG. How to quantify protein diffusion in the bacterial membrane. *Biopolymers*. 2011;95(5):312-321. doi:10.1002/bip.21585.

98. Skoog K, Soderstrom B, Widengren J, Von Heijne G, Daley DO. Sequential closure of the cytoplasm and then the periplasm during cell division in Escherichia coli. *J Bacteriol*. 2012;194(3):584-586. doi:10.1128/JB.06091-11.

99. Nenninger A, Mastroianni G, Robson A, et al. Independent mobility of proteins and lipids in the plasma membrane of Escherichia coli. *Mol Microbiol*. 2014;92(5):1142-1153. doi:10.1111/mmi.12619.

100. Ray N, Nenninger A, Mullineaux CW, Robinson C. Location and mobility of twin arginine translocase subunits in the Escherichia coli plasma membrane. *J Biol Chem*. 2005;280(18):17961-17968. doi:10.1074/jbc.M413521200.

101. Hofer AM, Brown EM. Extracellular calcium sensing and signalling. *Nat Rev Mol Cell Biol*. 2003;4(7):530-538. doi:10.1038/nrm1154.

102. Crawford JA, Krukonis ES, DiRita VJ. Membrane localization of the toxR winged-helix domain is required for TcpP-mediated virulence gene activation in Vibrio cholerae. *Mol Microbiol*. 2003;47:1459-1473.

103. Zimniak P, Little JM, Radominska A, Oelberg DG, Anwer MS, Lester R. Taurine-conjugated bile acids act as Ca2+ ionophores. *Biochemistry*. 1991;30(35):8598-8604.

104. Gu J-J, Hofmann AF, Ton-Nu H-T, Schteingart CD, Mysels KJ. Solubility of calcium salts of unconjugated and conjugated natural bile acids. *J Lipid Res*. 1992;33:635-646.

105. Hofmann AF. Bile Acids : The Good , the Bad , and the Ugly. 2013:24-29.

106. Maldonado-Valderrama J, Wilde P, Macierzanka A, Mackie A. The role of bile salts in digestion. *Adv Colloid Interface Sci*. 2011;165(1):36-46. doi:10.1016/j.cis.2010.12.002.

107. D'Archivio AA, Galantini L, Gavuzzo E, Giglio E, Mazza F. Calcium Ion Binding to Bile Salts. *Langmuir*. 1997;7463(10):3090-3095.

108. Bostock JM, Miller K, O'Neill AJ, Chopra I. Zeocin resistance suppresses mutation in hypermutable Escherichia coli. *Microbiology*. 2003;149(4):815-816. doi:10.1099/mic.0.C0111-0.

109. Ruiz L, Sanchez B, Ruas-Madiedo P, Reyes-Gavila, De Losn CG, Margolles A. Cell envelope changes in Bifidobacterium animalis ssp. lactis as a response to bile. *FEMS Microbiol Lett*. 2007;274(2):316-322. doi:10.1111/j.1574-6968.2007.00854.x.

110. Douglas LM, Konopka JB. Plasma membrane organization promotes virulence of the human fungal pathogen Candida albicans. *J Microbiol*. 2016;54(3):178-191. doi:10.1007/s12275-016-5621-y.

111. Haas BL, Matson JS, DiRita VJ, Biteen JS. Single-molecule tracking in live Vibrio cholerae reveals that ToxR recruits the membrane-bound virulence regulator TcpP to the toxT promoter. *Mol Microbiol*. 2015;96(1):4-13.

doi:10.1111/mmi.12834.

112. Brown EM, MacLeod RJ. Extracellular calcium sensing and extracellular calcium signaling. *Physiol Rev*. 2001;81(1):239-297. doi:10.1038/nrm1154\rnrm1154 [pii].

113. Flemstrom G, Isenberg JI. Gastroduodenal mucosal alkaline secretion and mucosal protection. *News Physiol Sci*. 2001;16(February):23-28. ISI:000169164000006\nd:\1Refman12\PDF\Ref_ID-5159.PDF.

114. Ridlon JM, Kang D-J, Hylemon PB. Bile salt biotransformations by human intestinal bacteria. *J Lipid Res*. 2006;47(2):241-259. doi:10.1194/jlr.R500013-JLR200.

115. Wells JE, Berr F, Thomas LA, Dowling RH, Hylemon PB. Isolation and characterization of cholic acid 7α-dehydroxylating fecal bacteria from cholesterol gallstone patients. *J Hepatol*. 2000;32(1):4-10. doi:10.1016/S0168-8278(00)80183-X.

116. Narushima S, Itoha K, Miyamoto Y, et al. Deoxycholic acid formation in gnotobiotic mice associated with human intestinal bacteria. *Lipids*. 2006;41(9):835-843.

117. Hirano S, Nakama R, Tamaki M, Oda H. Isolation and characterization of thirteen intestinal microorganisms capable of 7 Isolation and Characterization of Thirteen Intestinal Microorganisms Capable of 7a-Dehydroxylating Bile Acids. 1981;41(3):737-745.

118. Hofmann AF. The Continuing Importance of Bile Acids in Liver and Intestinal Disease. *Arch Intern Med*. 1999;159:2647-2658.

119. Malik-Kale P, Parker CT, Konkel ME. Culture of Campylobacter jejuni with sodium deoxycholate induces virulence gene expression. *J Bacteriol*. 2008;190(7):2286-2297. doi:10.1128/JB.01736-07.

120. Doig P, Yao R, Burr DH, Guerry P, Trust TJ. An environmentally regulated pilus-like appendage involved in Campylobacter pathogenesis. *Mol Microbiol*. 1996;20(4):885-894. doi:10.1111/j.1365-2958.1996.tb02526.x.

121. Olive AJ, Kenjale R, Espina M, Moore DS, Picking WL, Picking WD. Bile salts stimulate recruitment of IpaB to the Shigella flexneri surface, where it colocalizes with IpaD at the tip of the type III secretion needle. *Infect Immun*. 2007;75(5):2626-2629. doi:10.1128/IAI.01599-06.

122. Pope LM, Reed KE, Payne SM, Payne SM. Increased protein secretion and adherence to HeLa cells by Shigella spp . following growth in the presence of bile salts . These include : Increased Protein Secretion and Adherence to HeLa Cells by Shigella spp . following Growth in the Presence of Bile S. *Microbiology*. 1995;63(9):3642-3648.

123. Prouty AM, Gunn JS. Salmonella enterica Serovar Typhimurium Invasion Is Repressed in the Presence of Bile Salmonella enterica Serovar Typhimurium Invasion Is Repressed in the Presence of Bile. 2000;68(12):6763-6769. doi:10.1128/IAI.68.12.6763-6769.2000.Updated.

124. Prouty AM, Brodsky IE, Manos J, Belas R, Falkow S, Gunn JS. Transcriptional regulation of Salmonella enterica serovar Typhimurium genes by bile. *FEMS Immunol Med Microbiol*. 2004;41(2):177-185. doi:10.1016/j.femsim.2004.03.002.

125. Hernández SB, Cota I, Ducret A, Aussel L, Casadesús J. Adaptation and preadaptation of Salmonella enterica to Bile. *PLoS Genet*. 2012;8(1):e1002459. doi:10.1371/journal.pgen.1002459.

126. Gupta S, Chowdhury R. Bile affects production of virulence factors and motility of Vibrio cholerae. *Infect Immun*. 1997;65(3):1131-1134.

127. Hung DT, Mekalanos JJ. Bile acids induce cholera toxin expression in Vibrio

cholerae in a ToxT-independent manner. *Proc Natl Acad Sci U S A*. 2005;102(8):3028-3033. doi:10.1073/pnas.0409559102.

128. Prieto AI, Ramos-Morales F, Casadesús J. Repair of DNA damage induced by bile salts in Salmonella enterica. *Genetics*. 2006;174(2):575-584. doi:10.1534/genetics.106.060889.

129. Rodríguez-Beltrán J, Rodríguez-Rojas A, Guelfo JR, Couce A, Blázquez J. The escherichia coli sos gene dinf protects against oxidative stress and bile salts. *PLoS One*. 2012;7(4). doi:10.1371/journal.pone.0034791.

130. van der Veen S, Abee T. Contribution of Listeria monocytogenes RecA to acid and bile survival and invasion of human intestinal Caco-2 cells. *Int J Med Microbiol*. 2011;301(4):334-340. doi:10.1016/j.ijmm.2010.11.006.

131. Badie G, Heithoff DM, Sinsheimer RL, Mahan MJ. Altered levels of Salmonella DNA adenine methylase are associated with defects in gene expression, motility, flagellar synthesis, and bile resistance in the pathogenic strain 14028 but not in the laboratory strain LT2. *J Bacteriol*. 2007;189(5):1556-1564. doi:10.1128/JB.01580-06.

132. Cremers CM, Knoefler D, Vitvitsky V, Banerjee R, Jakob U. Bile salts act as effective protein-unfolding agents and instigators of disulfide stress in vivo. *Proc Natl Acad Sci U S A*. 2014;111(16):E1610-E1619. doi:10.1073/pnas.1401941111.

133. Nair PP, Davis KE, Shami S, Lagerholm S. The induction of SOS function in Escherichia coli K-12/PQ37 by 4- nitroquinoline oxide (4-NQO) and fecapentaenes-12 and -14 is bile salt sensitive: Implications for colon carcinogenesis. *Mutat Res - Fundam Mol Mech Mutagen*. 2000;447(2):179-185. doi:10.1016/S0027-5107(99)00205-5.

134. Sagawa H, Tazuma S, Kajiama G. Protection membrane against hydrophobic bile

salt-induced damage by liposomes and hydrophilic cell bile salts. *Am J Physiol*. 1993;264(5):835-839.

135. Francis MB, Allen C a, Shrestha R, Sorg J a. Bile acid recognition by the Clostridium difficile germinant receptor, CspC, is important for establishing infection. *PLoS Pathog*. 2013;9(5):e1003356. doi:10.1371/journal.ppat.1003356.

136. Buffie CG, Bucci V, Stein RR, et al. Precision microbiome reconstitution restores bile acid mediated resistance to Clostridium difficile. *Nature*. 2014;(2). doi:10.1038/nature13828.

137. Cameron DE, Urbach JM, Mekalanos JJ. A defined transposon mutant library and its use in identifying motility genes in Vibrio cholerae. *Proc Natl Acad Sci U S A*. 2008;105(25):8736-8741. doi:10.1073/pnas.0803281105.

138. Dewey KG, Mayers DR. Early child growth: How do nutrition and infection interact? *Matern Child Nutr*. 2011;7(SUPPL. 3):129-142. doi:10.1111/j.1740-8709.2011.00357.x.

139. Marcobal a, Kashyap PC, Nelson T a, et al. A metabolomic view of how the human gut microbiota impacts the host metabolome using humanized and gnotobiotic mice. *ISME J*. 2013;7(10):1933-1943. doi:10.1038/ismej.2013.89.

140. Subramanian S, Huq S, Yatsunenko T, et al. Persistent gut microbiota immaturity in malnourished Bangladeshi children. *Nature*. 2014;509(7505):417-421. doi:10.1038/nature13421.

141. Gordon JI, Dewey KG, Mills D a, Medzhitov RM. The human gut microbiota and undernutrition. *Sci Transl Med*. 2012;4(137):137ps12. doi:10.1126/scitranslmed.3004347.

142. Hsiao A, Ahmed a. MS, Subramanian S, et al. Members of the human gut microbiota involved in recovery from Vibrio cholerae infection. *Nature*. September

2014. doi:10.1038/nature13738.

143. Lee SH, Hava DL, Waldor MK, Camilli a. Regulation and temporal expression patterns of Vibrio cholerae virulence genes during infection. *Cell*. 1999;99(6):625-634. http://www.ncbi.nlm.nih.gov/pubmed/10612398.

144. Hsiao A, Liu Z, Joelsson A, Zhu J. Vibrio cholerae virulence regulator-coordinated evasion of host immunity. *Proc Natl Acad Sci U S A*. 2006;103(39):14542-14547. doi:10.1073/pnas.0604650103.

145. Metcalf WW, Jiang W, Daniels LL, Kim S-K, Haldimann A, Wanner BL. Conditionally Replicative and Conjugative Plasmids Carrying lacZa for Cloning , Mutagenesis , and Allele Replacement in Bacteria. *Plasmid*. 1996;13(35):1-13. doi:10.1006/plas.1996.0001.

146. Akakura R, Winans SC. Mutations in the occQ operator that decrease OccR-induced DNA bending do not cause constitutive promoter activity. *J Biol Chem*. 2002;277(18):15773-15780. doi:10.1074/jbc.M200109200.

147. Liu Z, Miyashiro T, Tsou A, Hsiao A, Goulian M, Zhu J. Mucosal penetration primes Vibrio cholerae for host colonization by repressing quorum sensing. *Proc Natl Acad Sci U S A*. 2008;105(28):9769-9774. doi:10.1073/pnas.0802241105.

148. Guzman LLM, Belin D, Carson MJ, Beckwith J, LUZ-MARIA GUZMAN MICHAEL J. CARSON, AND JON BECKWITH DB, LUZ-MARIA GUZMAN MICHAEL J. CARSON, AND JON BECKWITH DB. Tight Regulation, Modulation, and High-Level Expression by Vectors Containing the Arabinose PBAD Promoter. *J Bacteriol*. 1995;177(14):4121-4130. http://jb.asm.org/content/177/14/4121.abstract.

149. Masuko T, Minami A, Iwasaki N, Majima T, Nishimura S-I, Lee YC. Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. *Anal Biochem*.

2005;339(1):69-72. doi:10.1016/j.ab.2004.12.001.

150. Miller JH. *Experiments in Molecular Genetics*. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory; 1972.