# Vibrio Cholerae:

# Epidemiology of Cholera, Virulence, Ecology, Biofilms and Vibrio Phages

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
Bachelors in Biotechnology

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August 2021

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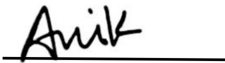

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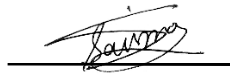

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# Approval

The thesis titled "Vibrio Cholerae: Epidemiology of Cholera, Virulence, Ecology, Biofilms and Vibrio Phages" submitted by Anik Dibya Islam (ID:18136066) and Syeda Saima Sajida (ID: 17336014) of Spring and Summer semester 2017, respectively has been accepted as satisfactory in partial fulfillment of requirement for the degree of Bachelors in Biotechnology.

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## Ethics Statement:

No living organism was used or harmed in this study.

# Abstract

Every year there are a million cases of acute diarrheal disease around the world that is caused by the strain, Vibrio Cholerae due to poor sanitation and hygiene practices. It mostly affects children and can be deadly if left untreated. It has come to light that V. Cholerae can change from planktonic to biofilm state and the biofilm state provokes the infection and disease transmission leading to an outbreak. This review will highlight all the essential aspects of Vibrio Cholerae's lifestyle and classification specifying the biotypes, summarize the latest knowledge on the epidemiology, transmission of the disease, virulence factors of V. Cholerae and its regulation involved in enhancing the pathogenicity and its ecological persistence in the aquatic and human intestine environment. Furthermore, it discusses the three stages of biofilm development – surface attachment, microcolony formation and dispersal, involvement of genes and regulators in regulating the biofilm formation and the process of intestinal colonization caused by the bacteria. Lastly, significant emphasis has been given on the possible ways to prevent biofilm formation and its infection; especially on the recent findings related to Vibrio Phages in controlling V. Cholerae.

**KEYWORDS:**

Vibrio cholerae, Epidemiology, Ecology, virulence, biofilms, vibrio phages, intestinal colonization, bile, quorum sensing, matrix protein, Vibrio polysaccharides, phage therapy, signaling molecule, ToxR Regulation, CT, TCP.

## Acknowledgement

We are grateful to Almighty Allah for bestowing upon us His blessings which have enabled us to come this far in our education in sound health and allowed us to finish our thesis successfully amid the COVID-19 pandemic.

We convey our heartfelt gratitude to our supervisor, Iftekhar Bin Naser, Ph.D. (Associate Professor and Coordinator of Biotechnology Program, Department of Mathematics and Natural Sciences, Brac University), for his continuous support and guidance throughout our work and undergrad journey. This thesis would not have been a success without his valuable advice and feedback.

Lastly, we would like to thank our parents for always supporting us in our academic endeavors.

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## List of Acronyms

CT - Cholera Toxin

TCP - Toxin-Coregulated Pilus

c-di-GMP - Cyclic Diguanosine Monophosphate

cAMP - Cyclic Adenosine Monophosphate

LPS - Lipopolysaccharide

EPS - Exopolysaccharide

HAP - Haemagglutinin/protease

ORF - Open Reading Frame

SI - Small intestine

Fla- Flagella

Che - Chemotaxis

VPS - Vibrio polysaccharide

MSHA - mannose-sensitive haemagglutinin type IV pilus

HGT -Horizontal Gene Transfer

DRCF - Dual role colonization factors

VBNC - Viable but nonculturable

CVEC - Conditionally, viable environmental cells

AEA - Alternator electron acceptor

TMAO - trimethylamine N-oxide

AMM - Alpha-methylmannoside

VcBMC - V. cholerae biofilm matrix clusters

T2SS - type VI secretion system

HNS - Histone-like nucleoid structuring protein

QS - Quorum Sensing

AI - Anti-inducer

CRP - cAMP Regulatory Protein

Fis - Factors for inversion stimulation

IHF - Integration hosting factor

RITARD - removable intestinal tie-adult rabbit diarrhea

# Chapter 1: Introduction

Vibrio cholerae is a Gram-negative, motile, curved rod-shaped bacterial pathogen of the Vibrionaceae family (Reidl & Klose, 2002; Silva & Benitez, 2016a) that resides in an aquatic reservoir and infects humans. Vibrio cholerae of serogroup O1 and O139 is the known cause of intestinal life treating cholera affects third world populations in impoverished countries with inadequate sanitation and yearly causing an estimated 2.9 million cases and 95000 (Merrell et al., 2000a; vanden Broeck et al., 2007a). Cholera is an acute diarrheal illness caused by pathogenic strains of Vibrio cholerae, which colonizes the small intestine for 12–72 hours after intake. Cholera causes stomach pains, vomiting, diarrhea, and fluid losses of up to 1 liter per hour, leading to significant fluid loss and metabolic acidosis, which may cause hypotonic shock, circulatory collapse, and death within 12 hours after the initial symptoms (Teschler et al., 2015a).This is why globally, the second most common cause of death among children under five years is diarrheal cholera (Nelson et al., 2009a). Symptomless carriers of cholera generally cause epidemic outbreaks, especially in vulnerable populations like small children, the elderly, or travelers (vanden Broeck et al., 2007b). Cholera is prevalent in impoverished nations like southern Asia, Africa, and Latin America, lacking clean water supply and proper sanitation (Bueno et al., 2020a), and is associated with seasonal epidemics that coincide with climate changes and other external factors (Faruque, Albert, et al., 1998a; Silva & Benitez, 2016a). V. cholerae enters its human host via contaminated water and food, passes through the stomach acid barrier, colonizes the small intestine, manufactures cholera toxin, and eventually causes watery diarrhea (Merrell et al., 2000b). Rice watery diarrhea usually contains 1010-1012 vibrios per liter. Symptomatic individuals may shed vibrios before illness (Cash et al., 2015a) and for 1–2 weeks after illness, while asymptomatic individuals usually had 103 vibrios per gram of feces for one day (Mosley et al., 1968). Thus, the number of symptomatic individuals affects the amount of V. cholerae shed for transmission. Then the pathogen spreads rapidly through the feces–oral pathway, exploiting the brief hyper-infective stage to infect other individuals.

Classical and El Tor biotypes of V. cholerae O1 vary in severity of clinical symptoms and expression and regulation of main virulence factors. Humans have had seven cholera pandemics. The seventh and current pandemic is characterized by the recurrent appearance of serogroup O139,

which originates from the El Tor biotype and shows a novel lipopolysaccharide (LPS) and a capsule. Toxigenic V. cholerae produces a variety of pathogenic components that work in concert to cause cholera pathogenesis. In addition to the synthesis of CT, which causes severe diarrhea, V. cholerae has genes for a colonization factor called toxin-coregulated pilus (TCP) and a regulatory protein called ToxR, which controls the expression of both CT and TCP. These are all vital virulence features for V. Cholerae (Faruque, Albert, et al., 1998b). Moreover, because it spends most of its life cycle outside of the human host in the aquatic environment, Vibrio cholerae has been researched extensively as a model organism for studying biofilm development in environmental infections. Vibrio's capacity to form biofilms (matrix-enclosed, surface-associated communities) is dependent on certain structural genes (flagella, pili, and exopolysaccharide synthesis) and regulatory pathways, according to recent research (two-component regulators, quorum sensing, and c-di-GMP signaling) (F. H. Yildiz & Visick, 2009). Biofilm formation is critical to the V. cholerae life cycle. Thus, scientists have focused their attention on the molecular processes that underlie it, as well as the signals that activate biofilm construction or dispersion (Teschler et al., 2015a). Bacteriophages, viruses that infect and kill bacteria, are gaining popularity as antibiotic replacements (Letchumanan et al., 2016a). The efficacy of phage treatment against bacterial infectious illnesses has also been shown in Western nations using animal models since 1980. There may be an efficient technique to manage pathogenic bacteria without disrupting the normal microflora by using Bacteriophage lytic enzymes, which destroy the species in which they were created (Bhowmick et al., 2009; Bvsc&ah, 2015).

This review focuses on all the essential aspects of Vibrio Cholerae's lifestyle. It summarizes current scientific knowledge on the epidemiology, genetics, and ecological persistence of toxigenic V. cholerae, specifying its biotypes, outbreaks, and epidemiological potential, highlighting main virulence factors in the aquatic ecosystem and human intestine. Lastly, significant emphasis has been given on Vibrio Cholerae Biofilms and the recent findings related to Vibrio Phages in controlling V. Cholerae.

# Chapter 2: Vibrio Cholerae

## 2.1 Taxonomy

The genus Vibrio was used to classify a broad range of gram-negative, rod-shaped bacteria possessing polar flagella. However, by the mid-1960s, specific taxonomic criteria for the genus Vibrio had been established. The International Subcommittee on Taxonomy of Vibrios proposed a transitional classification that eliminated the bulk of species formerly known classified as Vibrio. Taxonomic investigations on related species, on the other hand, have shown a tight connection between the three genera Vibrio, Aeromonas, and Plesiomonas. It is feasible to distinguish individuals of the genus Vibrio from related genera based on biochemical features (Faruque, Albert, et al., 1998b).

KINGDOM: Bacteria, PHYLUM: Proteobacteria, CLASS: Gammaproteobacteria, ORDER: Vibrionales, FAMILY: Vibrionaceae, GENUS: Vibrio, SPECIES: cholerae, Gram-negative, curved rod-shaped bacterium (Faruque, Albert, et al., 1998b).

## 2.2 Biotypes

Serogroups of V. cholerae strains are characterized by the structure of their cell surface lipopolysaccharides. Only the O1 and O139 serotypes of V. cholerae have been linked to Cholera illness by generating cholera toxin (CT) and causing cholera pandemics out of the approximately 200 known serogroups of V. cholerae (Faruque & Nair, 2002; Reidl & Klose, 2002) .The O1 strain differs in the severity of clinical symptoms and the expression and regulation of key virulence factors (Silva & Benitez, 2016b). The O1 serotype is divided into classical and El Tor biotypes distinguished by phenotypic characteristics (biochemical properties) such as sensitivity to polymyxin B and susceptibility to phage infection (Conner et al., 2016; Faruque, Albert, et al., 1998a) .Infections caused by non-O1 and -O139 serogroups or non-toxigenic O1 strains, on the other hand, are uncommon and seem to have little clinical significance (Reidl & Klose, 2002).

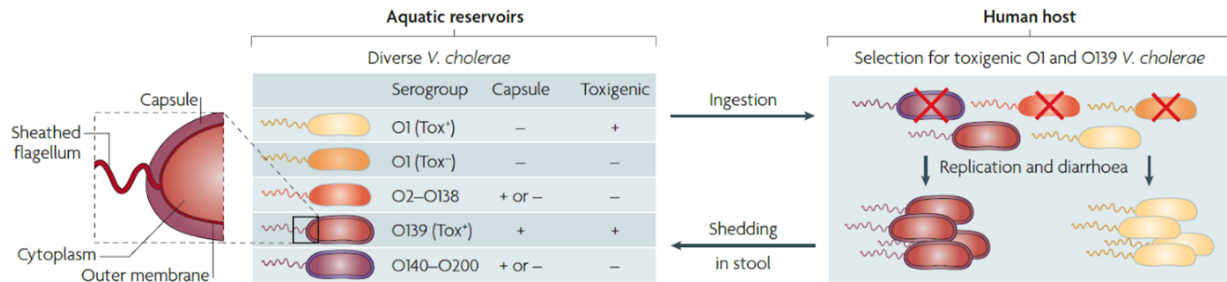


*Figure 1 Phylogenetic relationship of Vibrio cholerae strains (Nelson et al., 2009b).*

### 2.2.1 The genetic difference between biotypes

The El Tor (and O139) and classical biotypes generated different cholera toxin (CT). The El Tor and Classical CT A-subunits have the same amino acid sequence, while the B-subunits contain biotype-specific amino acid changes at positions 18 and 47. Tyr-18 and Ile-47 were deemed El Tor (and O139) biotype markers, whereas His-18 and Thr-47 were considered Classical (Sánchez & Holmgren, 2011).

### 2.2.2 Evolution of biotypes

Out of the seven pandemics, the classical biotype produced the first six and has gone extinct by now (Faruque & Mekalanos, 2012**).** The El Tor biotype replaced the classical biotype as the primary epidemic strain in 1961, causing the longest seventh pandemic today (Charles & Ryan, 2011; Conner et al., 2016; Faruque & Mekalanos, 2012). Although the O139 serogroup was responsible for catastrophic outbreaks in the 1990s, the El Tor strain remains the most common worldwide (Nelson et al., 2009b**).**This serogroup changing happened many times in cholera-endemic areas during the past decade, indicating that acquired immunity plays a role in serogroup emergence. It also implies that fast evolution and genomic rearrangement of O1 and O139 strains lead to cholera persistence and reemergence (Conner et al., 2016).

The El Tor strains are thought to be more environmentally friendly than the classical strains, whereas the traditional classical strains are thought to cause more severe cholera. The conservation of a predominantly El Tor genomic backbone having numerous genomic islands matching the classical strain confirms that El Tor and classic strains are genetic hybrids. El Tor variations are linked with enhanced ecological resilience, pathogenicity, disease severity, and dispersion globally

(Conner et al., 2016). Moreover, the El Tor biotype has a competitive growth advantage over classical biotype strains in vitro and in vivo cholera infection models, partly explaining the classical biotype's displacement due to more efficient adaptation in the environment (Faruque & Mekalanos, 2012). Additionally, the El Tor biotype may be more suited to aquatic reservoirs than the conventional biotype, increasing the incidence of asymptomatic infections (Charles & Ryan, 2011).

A multigene mutation in the O antigen-coding region of a progenitor O1 El Tor strain gave rise to the O139 serogroup in 1992 (Nelson et al., 2009b) **.**Comparative analysis suggests V. cholerae O139 is closely related to V. cholerae O1 and likely originated from an El Tor strain. This happened by possible genetic changes in the serotype-specific gene cluster (Faruque, Albert, et al., 1998a). Despite having a polysaccharide capsule and a modified lipopolysaccharide, O139 strains are comparable to El Tor O1 strains in terms of viral DNA sequences and multi-locus enzyme electrophoresis (Faruque, Albert, et al., 1998a; Tacket et al., 1998).

## 2.2.3 'Hybrid' El Tor strains

Over the last decade, El Tor variants with morphological and genetic features of classical biotype V. cholerae have been identified and referred to as 'Atypical', 'altered,' 'hybrid, and 'Matlab' El Tor strains. They include the Matlab variations from Bangladesh, the Mozambique variants, the new Haitian variants, and many more unusual El Tor variants from across the globe. Since then, these atypical El Tor variants exhibiting features (like expressed cholera toxin) of both the classical and El Tor strains have been isolated from several countries in Asia and Africa (Conner et al., 2016; Nelson et al., 2009b). These new atypical strains have entirely replaced the prototypic El Tor strains. They express a different cholera toxin than normal El Tor bacteria in more significant quantities temporally, leading to increased case fatality rates and disease severity during cholera (Charles & Ryan, 2011)**.**

# Chapter 3: Epidemiology

Cholera's seasonal pattern of occurrence in regions of endemic infection and explosive outbreaks that start in several locations at once indicate that environmental variables may have had a role in the epidemic's onset (Faruque, Albert, et al., 1998a).

There are five hallmarks of the epidemiology of cholera which includes (i) a significant level of pooling of cases by area and season, (ii) increased rate of infection in 1 to 5 years of aged patients in endemic areas, (iii) constant change in the antibiotic resistance patterns from one year to another, (iv) clonal diversity of epidemic strains, (v) protection against the disease through enhanced sanitation and hygiene practices and preexisting immunity (Faruque, Albert, et al., 1998a).

Cholera has caused seven pandemics since 1817, having a devastating impact on populations worldwide (Peterson & Gellings, 2018). The classical biotype of O1 serogroup of V. Cholerae had afflicted the previous six pandemics (Watnick & Kolter, n.d.). However, the seventh pandemic was marked by the prevalence of the O1 serogroup of the El Tor biotype, which has gained a signaling system based on cyclic GMP–AMP (cGAMP) (Yoon & Waters, 2019). There is also a recurring emergence of serogroup O139, which originated from the El Tor biotype and showed a new lipopolysaccharide (LPS) and capsule (Charles & Ryan, 2011; Silva & Benitez, 2016a). V. cholerae O1 El Tor adapted to survive in estuarine and freshwater aquatic reservoirs because of biofilm-associated form (Naser et al., 2017); (Watnick & Kolter, n.d.). Therefore it causes long-term cholera outbreaks and endemic disease. Moreover, this biotype is more resistant to antibiotics and is linked to an increased case fatality rate (Charles & Ryan, 2011).

Cholera epidemics occur twice a year in Bangladesh and India's Ganges Delta area (Shamim Hasan Zahid et al., 2008). Right after the monsoon, the most significant number of cholera cases are observed from September to December. In the spring, between March and May, to some extent, a lower rise in cholera cases is seen (Berk et al., 2012). Throughout the last two centuries, significant outbreaks of the disease have taken place (Conner et al., 2016). The recent epidemics in which 100 000 cases were recorded in Zimbabwe in 2008, resulting in at least 4000 fatalities, at least 300 000 individuals were infected, and about 5000 were dead in Haiti in 2010 (reported as of 17 April 2011) (Charles & Ryan, 2011).

Currently, cholera is endemic in southern Asia, Africa, and Latin America due to poverty and inadequate sanitation (Faruque, Balakrish Nair, et al., 2004; Faruque, Naser, et al., 2005; Peterson & Gellings, 2018; Silva & Benitez, 2016a). Seasonal outbreaks occur extensively in these areas (Silva & Benitez, 2016a) and based on environmental variables such as rainfall, salinity, temperature, and plankton blooms, the timing and intensity of such an outbreak fluctuates (Conner et al., 2016). In cholera-endemic nations, over 1.4 billion people are at risk of contracting the disease. Approximately 2.8 million cholera cases (uncertainty range: 1.4-4.3) and about 91 000 deaths occur in the endemic nations (uncertainty range: 28 000 to 142 000), whereas an estimated 87 000 cases and 2500 deaths are seen in the non-endemic countries (Ali et al., 2015).

# Chapter 4: Transmission

In densely populated regions carrying hyper infectious toxigenic strains, people contribute to cholera transmission via inadequate sanitation and contaminated water/food (Reidl & Klose, 2002). Oral consumption of V. cholerae-contaminated food or drink usually initiates infection. Next, the bacteria must survive the stomach's acid barrier and enter the mucus-coating covering the intestinal epithelia. Because V. cholerae cells are acid sensitive and are exposed to low pH in the gastrointestinal compartment (Cash et al., 2015b), the infectious dosage in human volunteers is high (106 to 1011 CFU). The surviving bacteria infiltrate intestinal epithelial cells, generating CT and cholera symptoms (Holmgren & Svennerholm, 1977).
The small intestine is the leading colonization site for V. cholerae. Bacterial cells are susceptible to differences in temperature, acidity, and osmolarity as they move from the aquatic environment to the human body. They must also survive in the gastrointestinal environment, including growth inhibitors like bile salts and organic acids and innate immune components like complement produced by intestinal epithelial cells (Holmgren & Svennerholm, 1977) and defensive barrier produced by Paneth cells (Mallow et al., 1996).

As a result, V. cholerae has evolved to survive, invade, and express virulence factors like CT in hostile environments. After V. cholerae secretes the CT, cholera symptoms appear. Massive watery diarrhoea causes hypotensive shock and fatality within 12 hours of the initial symptoms (Reidl & Klose, 2002).This watery diarrhea releases hyper infectious vibrio cholerae back to the environment. Recent research in Bangladesh suggests that 15–30% of a cholera patient's nearby contacted persons get cholera shortly after the index case (J. B. Harris et al., 2008; Weil et al., 2009). V. cholerae that pass through the human infection is 'hyper infectious,' relative to in vitro produced organisms, and this state remains for hours in aquatic settings (Charles & Ryan, 2011; Merrell et al., 2002) . Researchers have implied different mathematical models, indicating hyperreflectivity is necessary for cholera outbreaks. Many reasons have been linked to this hyper infectious condition, including V. cholerae shed in feces, which is transiently motile but chemotactically deficient, enhanced expression of TCP, a colonization component required for human pathogenicity (Alam et al., 2005) , and acid-adapted V. cholerae (Angelichio et al., 2004). This hyper infectivity helps cholerae to attain an epidemic form through asymptomatic patients.

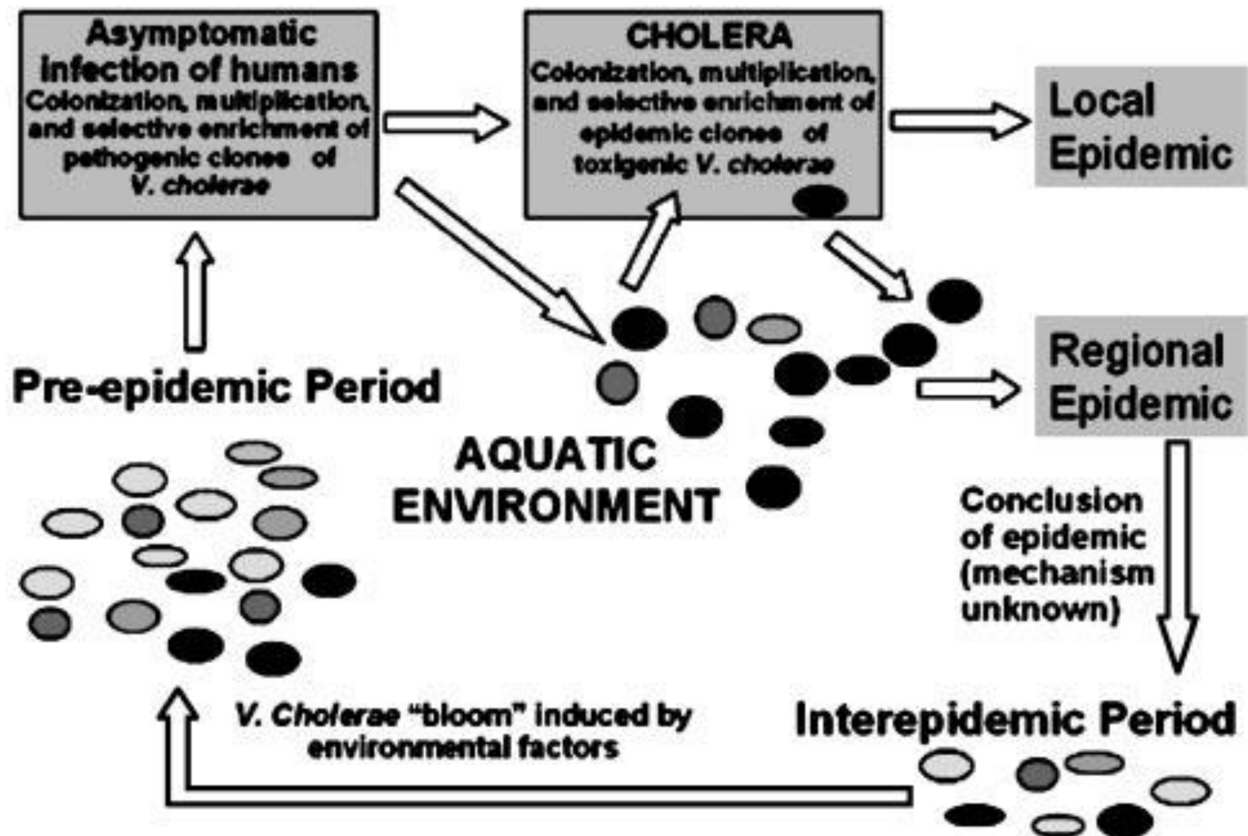


*Figure 2: Human asymptomatic infection with virulent V. cholerae strains just before a seasonal cholera outbreak occurred in an endemic region.*

Also, cholera stools include a combination of two morphologically different cell populations: clumped biofilm-like cells and single planktonic cells. Biofilm-like cells are more infectious than planktonic cells as they have increased virulence gene expression, involved in intestinal colonization (Faruque et al., 2006; Nielsen et al., 2010), explained in the latter part of this review paper.

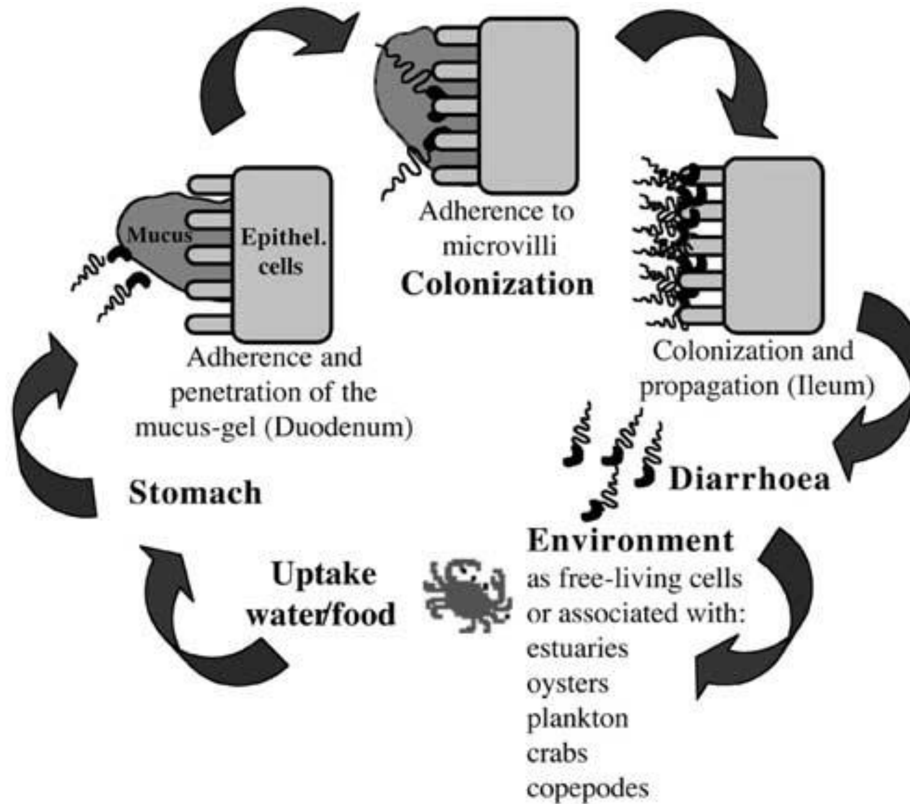


*Figure 3: The V. cholerae transmission cycle. The transmission cycle of the cholera-causing bacterium V. cholerae is shown here in a simplified form (Reidl & Klose, 2002).*

The seasonal transmission of V. cholerae from the aquatic environment to humans is linked to certain endemic situations. Severe weather changes (A. M. Harris et al., 1998), zooplankton blooms (Constantin De Magny et al., 2008; Reidl & Klose, 2002; Reyburn et al., 2011); variations in water temperature (Constantin De Magny et al., 2008; Reyburn et al., 2011); function of lytic bacteriophage (Faruque, Naser, et al., 2005) can be used to predict the likelihood of an outbreak. In recent years, there has been increasing evidence that lytic bacteriophages and bacterial biofilms may build up and terminate a cholera outbreak and thus contribute to human transmission (Cash et al., 2015b).

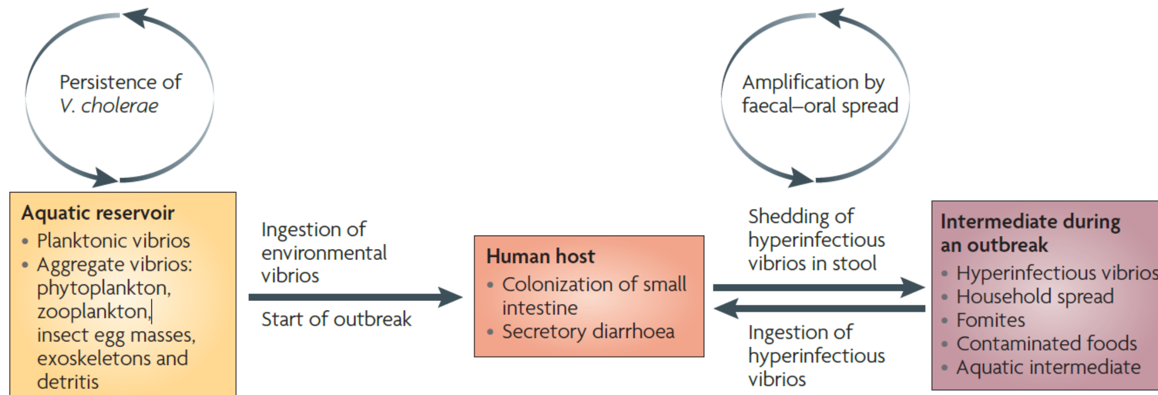


*Figure 4: Vibrio cholerae life cycle and transmission from the host perspective: In aquatic settings, toxic Vibrio cholerae strains coexist with non-toxic strains due to biofilm development on biological surfaces and chitin as a carbon and nitrogen source. Toxigenic strains inhabit the small intestine, proliferate, produce cholera toxin, and are excreted back into the environment by the host in secretory diarrhea. The stool-shed pathogens are temporarily hyper infectious, allowing them to spread the epidemic to new hosts. The role of hyper infectivity in fast cholera epidemic propagation is unknown. V. cholerae remains hyper infectious for at least 5 hours after passage from patients into the aquatic environment, indicating that hyper infectivity plays a role in transmission in regions of high crowding, where another person is likely to come into touch with the organism in a short period (Nelson et al., 2009b).Toxigenic V. cholerae replicates rapidly in the human intestine, resulting in stool shedding. This reseeds the aquatic environment, causes fecal-oral contamination, and adds to the explosive character of cholera epidemics (Boyd et al., 2021).*

# Chapter 5: Virulence

## 5.1 Pathogenesis

Vibrio cholerae colonizes the small bowel via TCP and interacts with intestinal epithelial receptors. Once adherent, the bacteria secrete toxin and hemagglutinin/protease (HA/protease), which nicks the CT-A subunit at Arg192, resulting in two distinct subunits linked by a single disulfide bond. This post-translational alteration is critical for successful toxin and increased cAMP generation, resulting in enormous electrolyte and water secretion into the intestinal lumen, with bacterial excretion. The patient's feces resemble rice water and may exceed 10 l each day (vanden Broeck et al., 2007a).

## 5.2 Virulence Factors

Molecular research showed that all cholera-causing bacteria contain a collection of virulence genes required for human disease. This includes genes for cholera toxin (CT), toxin coregulated pilus (TCP), and ToxR, which co-regulates the production of CT and TCP (Faruque & Nair, 2002).

To cause cholera disease successfully, Vibrio Cholerae first has to reach and colonize the small intestine epithelium and then manufacture a protein enterotoxin that hinders the ion transport process by intestinal epithelial cells, ultimately causing massive fluid efflux characteristic of cholera illness (Thelin & Taylor, 1996). Thus, the Vibrio cholera pathogenesis is considered a complicated process in which the pathogen is aided by several factors (Jonson et al., 1990). Molecular research showed that all cholera-causing bacteria contain a collection of virulence genes required for human disease (Faruque & Nair, 2002; Jonson et al., 1990). The V. cholerae genome comprises two chromosomes, of which chromosome one harbor all virulence genes (vanden Broeck et al., 2007a). **Toxin co-regulated pilus (TCP)** and **cholera toxin (CT)** are the main virulence determinants of V. cholerae required for pathogenesis in humans and animal models (Moorthy & Watnick, 2005). Cholera toxin (CT) is an AB5 family ADP-ribosyltransferase responsible for the disease's characteristic profuse rice-watery diarrhea, and toxin-coregulated pilus (TCP) is a type IV pilus that mediates adhesion and microcolony formation and is required for intestinal colonization in neonate mice and humans (Faruque & Nair, 2002). By colonizing with

TCP and secreting CT, V. cholerae cells are expelled in large quantities during diarrhea, allowing for long-term survival and selection advantage over nonpathogenic strains. The primary virulence genes of V. cholerae seem to be grouped in at least two cluster regions (CTX element & TCP pathogenicity island) identified on the chromosome (Everiss et al., 1994; Novais et al., 1999; Pearson et al., 1993; Trucksis et al., 1993).

The genes encoding the CT subunits are ctxA and ctxB genes, that make up an operon found in the prophage (an integrated phage genome in the host bacterial chromosome) of the lysogenic filamentous bacteriophage CTXF (Faruque & Mekalanos, 2012; *Phage Transfer: A New Player Turns Up in Cholera Infection*, n.d.) . This gene cluster is known as the **CTX genetic element** (Pearson et al., 1993; Waldor & Mekalanos, n.d.). Moreover, transforming novel toxigenic strains from nontoxigenic progenitors may be linked with CTXF propagation (Faruque, Asadulghani, et al., 1998). CTXF is a unique filamentous phage since it may integrate into the V. cholerae chromosome(s) or reproduce as a plasmid (Sánchez & Holmgren, 2011). CTX can integrate onto the V. cholerae chromosome at a particular attachment site known as attRS, producing persistent lysogens (Waldor et al., n.d.; Waldor & Mekalanos, n.d.). A CTX genome contains two parts: the 4.6-kb core region encodes CT, and the 2.5-kb RS2 region encodes CTX genome's control, replication, and integration activities (Waldor et al., n.d.). CTXF, like other bacteriophages, needs a receptor on V. cholerae to attach and proliferate. This receptor has been identified as the toxin coregulated pilus (TCP). Furthermore, the genes needed to make colonization factor TCP form a vast cluster known as the **V. cholerae pathogenicity island or TCP island** (306. Genetic Organization and Sequence of the Promoter-Distal Region of the Tcp Gene Cluster of Vibrio Cholerae, n.d.; Faruque et al., 2004; Faruque & Nair, 2002; Goldberg et al., 1990; Waldor & Mekalanos, n.d.), which is located separately from CTX genetic element (Sánchez & Holmgren, 2011). The TCP pathogenicity island and CTX genetic element structures indicate horizontal gene cluster transfer as a potential method for generating new V. cholerae pathogenic clones. Transformation of epidemic and pandemic cholera seems to be multistep processes that need TCP pathogenicity island. Because TCP Island has a receptor that transforms harmless non-toxigenic strains to toxigenic form through a process known as phage conversion, when infected by CTX phage (Faruque & Mekalanos, 2012; Karaolis et al., 1998). CTX utilizes the TCP as a receptor to infect V. cholerae cells. Thus, TCP expression by the bacteria is required for CTX susceptibility

(Faruque & Nair, 2002).It has been shown that toxigenic V. cholerae strains may also generate extracellular CTX particles (Faruque, Asadulghani, et al., 1998; Waldor & Mekalanos, n.d.).

The TCP pathogenicity island and the CTX element may have enabled some V. cholerae strains to adapt to the human intestinal environment (Faruque, Albert, et al., 1998a). The CTX genetic element confers a survival advantage to V. cholerae, hence to the bacteriophage, leading to an intestine enrichment of toxigenic V. cholerae. So CTXF improves the evolutionary fitness of its host and hence its own nucleic acids. With increasing host immunity to particular toxigenic clones of V. cholerae, new toxigenic clones develop and naturally replace older clones. In this way, the formation of novel toxigenic V. cholerae strains and their selective enrichment during cholera outbreaks is critical for the survival and evolution of V. cholerae and the genetic elements that transmit virulence genes.

TCP colonization of the small intestine is thought to be an essential component of V. cholerae infection strategy. Although the tcpA gene encodes the major subunit of TCP formation and function of the pilus assembly requires the protein products of several other genes located on the chromosome adjacent to the tcpA gene, these constitute the tcp gene cluster. At least 15 open reading frames are found in the tcp cluster. The entire region of nearly 40-kb flanked by the att-like sequences, including the TCP/ACF gene clusters, the integrase, and transposase genes, appears to constitute a pathogenicity island (Faruque & Nair, 2002). TCP must be expressed early in infection for the pathogen to colonize the small intestine and cause diarrhea by expressing CT. Additionally, the genes for regulatory protein ToxR, which co-regulates CT and TCP expression, are necessary. The **ToxR regulon** regulates cholera toxin, and TCP gene expression also contains ToxR, TcpP, and ToxT. Expression of ToxT requires TcpP and ToxR. TcpP and ToxR activities need TcpH and ToxS. ToxR may also start cholera toxin gene transcription independently of TcpP and ToxT (Raskin et al., 2020). Finally, **integrons** are discovered in the V. cholerae genome, which are gene expression elements that acquire open reading frames (ORFs) and convert them to functional genes. This allows bacteria to entrap genes from other microbes, allowing infectious genes to cluster and propagate genes for other metabolic activities. Molecular epidemiological monitoring of cholera in endemic regions has shown temporal variations in the characteristics of toxigenic V. cholerae and the development of new epidemic variants which frequently replace preexisting variants (Faruque, Albert, et al., 1998a; Faruque et al., 1994, 1997; Waldor et al., n.d.).

### 5.2.1 Cholera Toxin

In 1884, Koch suggested that the symptoms produced by Vibrio cholerae might involve a "poison". After De's seminal 1959 discovery of a diarrheagenic exo-enterotoxin in Vibrio cholerae (classical biotype) cell-free culture filtrates, much has been learned about cholera toxin (CT), perhaps the most well-known of all microbial toxins. Finkelstein and LoSpalluto isolated the toxin in 1969 and found it to be an 84 kDa protein. Initially, the toxin was believed to be made up of just one component that could aggregate into varied sizes and toxicity.

CT belongs to the superfamily of AB toxins (CT), which is encoded within the genome of the filamentous bacteriophage CTX and thus, it is horizontally transferred (Nesper et al., 2002). It is an oligomeric protein composed of two kinds of subunits:  a heterodimeric A-subunit (CT-A, Mr ~27,400, one single "heavy" toxicactive subunit) and a homopentameric B-subunit (CT-B, Mr ~58,000, oligomer consisting of many identical "light" subunits responsible for receptor binding) (vanden Broeck et al., 2007a). Meanwhile, the cell membrane receptor for CT was discovered as GM1, perhaps the first chemically-defined biologic receptor. The five identical B monomers (Mr ~11,600) are organized in a ring-like structure with single binding sites for the plasma membrane receptor of epithelial cells, monosialoganglioside GM1 (Sánchez & Holmgren, 2011; vanden Broeck et al., 2007a).

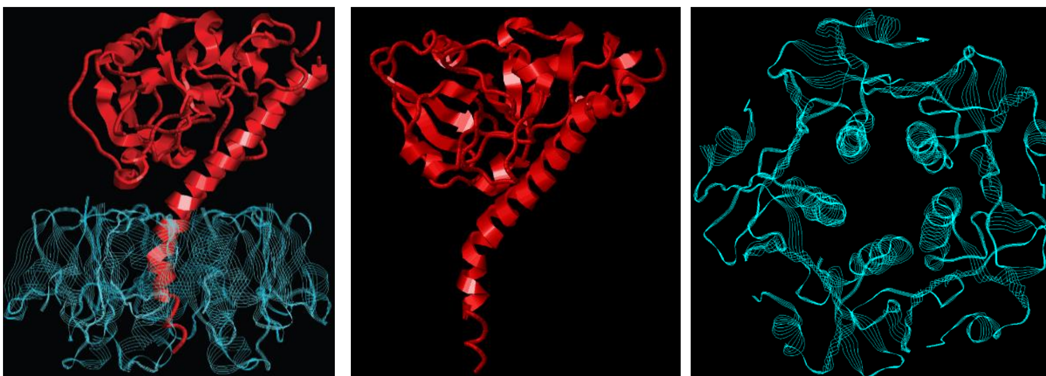

*Figure 5: Crystallographic structure of cholera toxin (a), its A (b), and B-subunits (c). (Sánchez & Holmgren, 2011).*

Assembled CT contains CTA component contained in CTB pentamer, responsible for toxin binding to cells. CTA is post-translationally changed by a V. cholerae protease, resulting in two polypeptide chains, CTA1 and CTA2 (CT-A1 Mr ~22,000 and CT-A2 Mr ~5,400), connected by a disulfide bond (vanden Broeck et al., 2007a). CTA1 has the toxic action (enzymatic ADP-ribosylating) of CTA, whereas CTA2 inserts CTA into the CTB pentamer. In the CT-B pentamer, the CT-A2 polypeptide binds the CT-A1 and CT-B subunits together. This polypeptide, CT-A1, is a catalytic mono-ADP ribosyltransferase. It is involved in ADP-ribosylation of the Gs-subunit of a stimulatory GTP-binding regulatory protein (AC). The CTB pentamer is kept together by 130 hydrogen bonds and 20 salt bridges. These numerous polar connections, along with subunit packing through hydrophobic contacts, may explain pentameric CTB's remarkable resilience against proteases, bile components, and other intestinal stimuli. Pentamer-pentamer interactions may increase stability(Sánchez & Holmgren, 2011)**.**

## 5.2.2 Toxin-coregulated pilus (TCP)

TCP is a major intestinal colonization factor encoded in the vibrio pathogenicity island (Karaolis et al., 1998). The significance of toxin-co-regulated pilus (TCP) in classical-biotype colonization has been demonstrated time and time again (Herrington et al., n.d.; Sharma et al., n.d.; Sun et al., 1990, 1991; Taylor et al., 1987). This clinical research shows that TCP expression is critical for V. cholerae O139 to colonize the gut, produce diarrhea, and activate immunological responses. Volunteers with a tcpA deletion defect had no diarrhea and significantly reduced colonization and immunological responses. TCP is 8 nm diameter, 1-4 mm long, thin, flexible pilus consisting of 1000 homopolymers of the TcpA subunit that self-associate (in an interwoven manner) to hold cells together in microcolonies (Li et al., 2008). The acquisition of the vibrio pathogenicity island is a critical event in the evolution of epidemic strains of V. cholerae since TCP also serves as a receptor for CTX (Li et al., 2008; Waldor & Mekalanos, n.d.).

### 5.2.3 Motility

Microbes rely on chemotaxis and motility to reach their niche destinations, locate nutrients, and detect signals from other bacteria or the host (Pauer et al., 2021). The alternative RNA polymerase subunits 54 and 28 and the 54-dependent transcriptional activators FlrA and FlrC are required for motility expression. The flagellar regulatory hierarchy controls multiple virulence genes. The ctx, tcp, and acf genes were elevated in flagellar regulatory mutants. Motility is believed to contribute to Vibrio cholerae virulence, although its function in pathogenesis is unknown. Recent research suggests that virulence factor production and motility traits are inversely linked. The authors suggest that when V. cholerae colonizes the intestinal cell surface, virulence factor expression increases while motility is downregulated. Upregulation of CT, TCP, extracellular protein secretion, HlyA, thermolabile hemolysin, GbpA, and the T6SS by downregulation of flagellar production would be expected to extend epithelial cell surface colonization and pathogenesis (Syed et al., 2009) .In non-laboratory settings, specific nonmotile mutants express greater amounts of CT and TCP than wild-type strains (Faruque, Balakrish Nair, et al., 2004).

# Chapter 6: Regulation of Virulence

Multiple mechanisms regulate virulence-associated genes in V. cholerae and the expression of many essential virulence genes is tightly controlled, allowing several genes to react similarly to changing environmental circumstances (Dirita et al., 1991). Extensive study of the molecular basis of cholera pathogenesis has revealed that a unique regulatory system with a cascade of regulatory factors coordinates the expression of the essential virulence factors, CT and TCP. The regulatory system comprises of three transcriptional activators, two inside the VPI (**ToxT and TcpP**) and one within the ancestral Vibrio chromosome (**ToxR**) (Karaolis et al., 1998; Kovach et al., 1996).

**The master regulator, ToxR**, is a 32-kDa transmembrane protein that is itself controlled. With increased CT expression, the ToxR protein binds to a tandemly repeated 7-bp DNA region upstream of the ctxAB structural gene (Dirita et al., 1991; Miller et al., 1987; Miller & Mekalanos, 1984). ToxR controls the expression of ctxAB as well as at least 17 other genes in the ToxR regulon, including the TCP colonization factor (Taylor et al., 1987), the accessory colonization factor (Petersont & Mekalanos, 1988), the OMPs OmpT and OmpU (Millert & Mekalanos, 1988), and three other lipoproteins (Parsot et al., 1991). Thus, ToxR is the primary regulator of CT and other key virulence factors in V. cholerae. ToxR expression is controlled by environmental variables (Parsot et al., 1991). The ToxR regulon contains **ToxT-dependent and ToxT-independent branches** in V. cholerae. It regulates virulence genes, indicating that the organism has evolved a system for sampling and reacting to its environment. The ToxR protein may serve as a 'scaffold protein that enables TcpP to attach close to the RNA polymerase binding site (Krukonis et al., 2000). ToxR and tcpR transcription are both stimulated by the same set of signals (Murley et al., 2000). This virulence-regulatory cascade seems to be started by **TcpP** expression, which is controlled by two additional activators, **AphA** and **AphB** (Kovacikova & Skorupski, 1999; Skorupski & Taylor, n.d.). These two activators connect to the tcpP promoter and synergistically stimulate transcription (Kovacikova & Skorupski, n.d.). The inducing environmental circumstances presumably affect the activity of AphA and AphB, whose genes are situated in the ancestral Vibrio chromosome (Kovacikova & Skorupski, 1999). AphA and AphB mutants lack tcpP and toxT transcription, intestinal colonization, and CT expression, indicating these genes are essential for virulence (Kovacikova & Skorupski, n.d.).

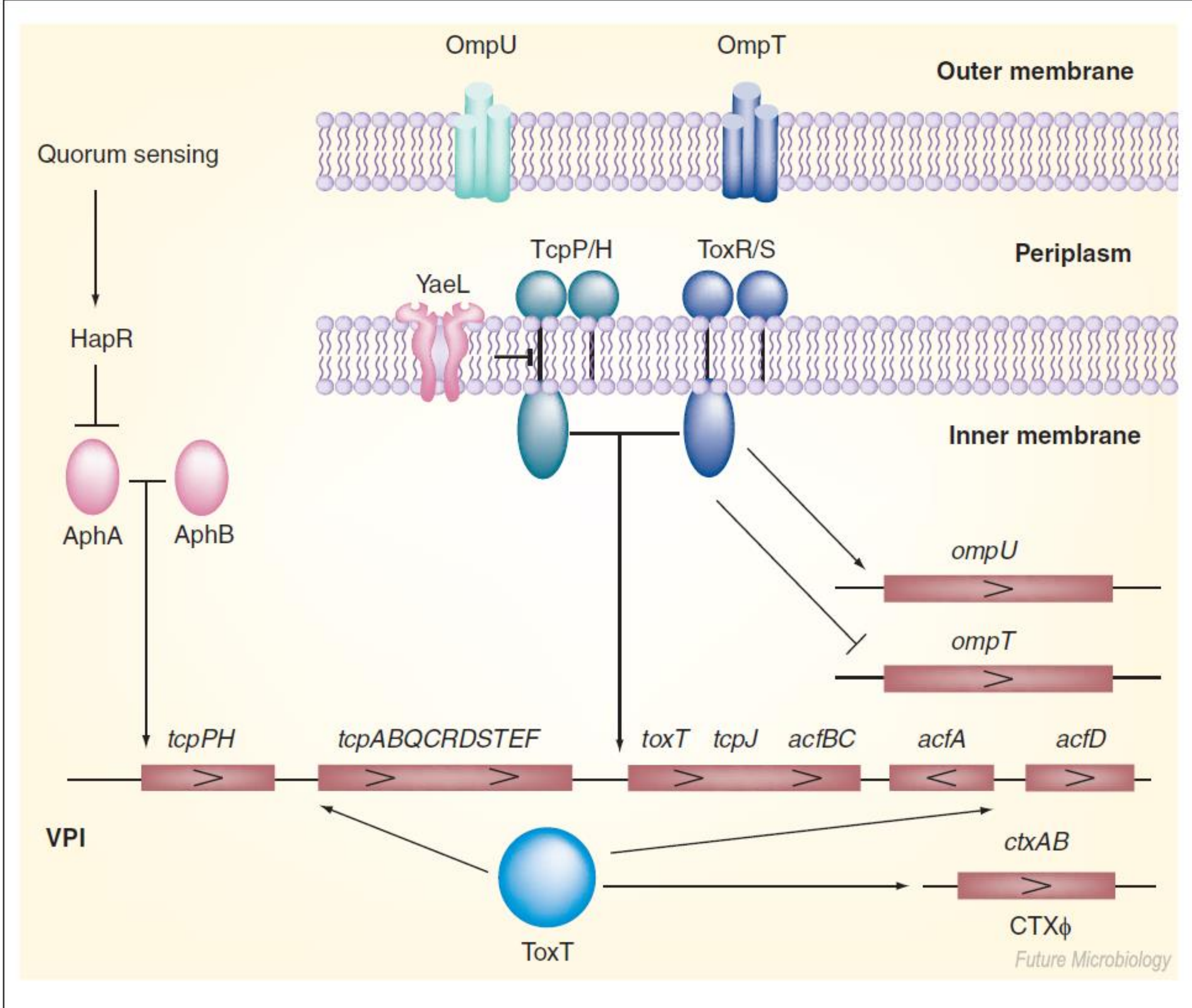


*Figure 6: ToxR Regulon Adapted from (Childers & Klose, 2007)*

**ToxT** directly activates the ctx and tcp gene clusters and other genes (e.g., acf, aldA, tagA) (Childers & Klose, 2007; Dirita et al., 1991). Strains lacking ToxT are avirulent as they can't produce CT or TCP (Dirita et al., 1991). The transmembrane transcriptional activators ToxR and TcpP regulate toxT gene transcription (Ha¨se & Mekalanos, 1998; Higgins, 1994). ToxT is transcribed in the intestine and is controlled by ToxR and TcpP (Childers & Klose, 2007). While ToxT seems to be produced in a transcriptionally active state, bile and temperature (environmental signals) may suppress ToxT transcription. This finding led to the hypothesis that the virulence

cascade occurs in two separate stages in the gut environment: toxT is transcribed within the lumen of the intestine in the presence of bile but ToxT protein stays inactive until the bacteria breach the mucus lining and reach the cell surface, where decreased bile concentrations allow for ToxT-dependent production of CT and TCP. Ctx and tcp transcription in the gut differs from transcription in the laboratory, according to Camilli and colleagues. Also, unlike in the laboratory, ctx transcription required TCP expression in the gut, indicating that bacteria must colonize the intestinal cell surface to receive a signal (reduced bile concentrations) (Reidl & Klose, 2002).

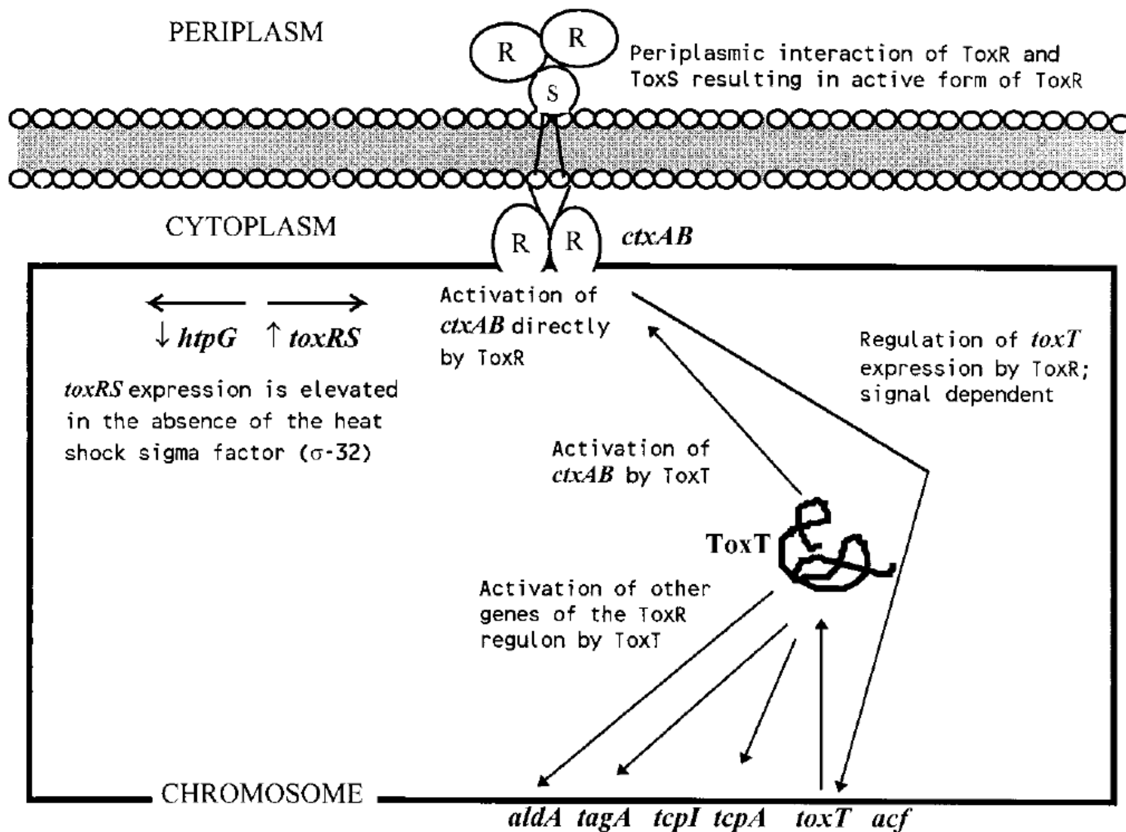


*Figure 7 Model for the ToxR/ToxT regulatory cascade of V.cholerae Adapted from (Faruque, Albert, et al., 1998a)*

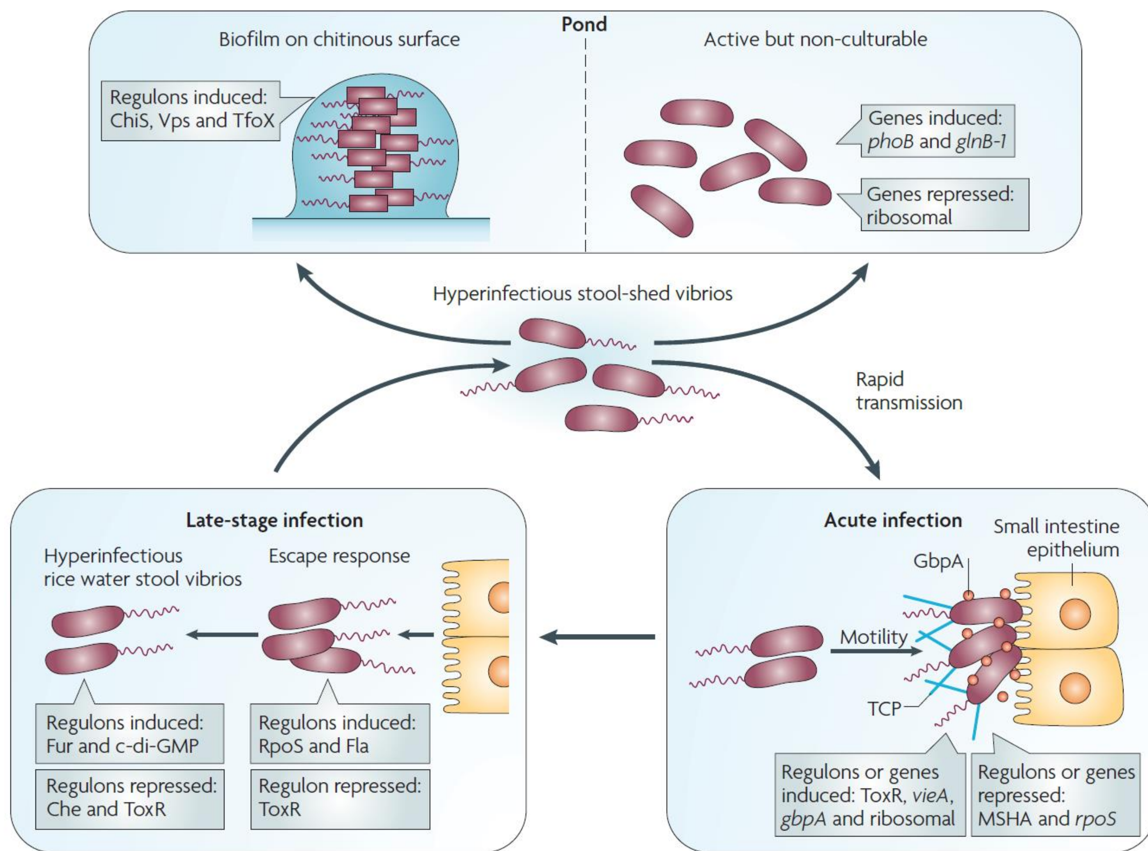


*Figure 8: Illustration of Vibrio cholerae gene expression patterns at various life stages: To colonize the small intestinal epithelium, Vibrio cholerae utilizes motility, mucinases and other factors to breach the mucus gel. Induction of vieA encodes a phosphodiesterase that hydrolyzes the second messenger cyclic di-GMP, and ToxR-regulated genes, including CT and TCP. Several genes are also inhibited, including the chitin-binding MSHA pilus and the stress sigma factor RpoS. In late infection stage V. cholerae detachs from the epithelium and prepares for transfer to another host (household spread) or entrance into the aquatic environment. Activation of genes for c-di-GMP production, nutrition scavenging mechanisms (such as the Fur regulon), and motility (Fla) is accompanied by suppression of genes for chemotaxis (Che) and the ToxR regulon. These result in a hyper infective 'motile but non-chemotactic' condition. If another host does not quickly consume bacteria in feces, they either establish themselves in the aquatic environment by consuming chitin or decay into an 'active but non-culturable' condition. Upon exposure to chitin, V. cholerae activates genes involved in chitin adhesion and catabolism (the ChiS regulon) and genes involved in genetic competence (the TfoX regulon). The Vps regulon encodes extracellular polysaccharides, which are required for biofilm development. As V. cholerae tries to adapt to nutrient-poor circumstances, significant changes in gene expression occur. These include phosphate and nitrogen starvation genes (phoB and glnB-1) and translation machinery genes (Nelson et al., 2009b).*

# Chapter 7: Ecology

## 7.1 Environment

V. cholerae, including serogroup O1 and O139 pandemic strains, have been proven to live naturally in aquatic environments, making them facultative human pathogens. They attach to plants, filamentous green algae, copepods (zooplankton), crustaceans, and insects in the marine environment.

Non-O1 and non-O139 strains are more often isolated from rivers and coastal regions than O1 and O139 strains, and most environment O1 strains are non-toxigenic, which is intriguing. V. cholerae O1 has been observed in marine creatures. The presence of MSHA type IV pili, uncharacterized chitin-binding proteins, and secreted chitinase enzyme suggests that V. cholerae may be closely attached with chitin structures in the environment (e.g., zooplankton such as copepods). Thus, as a result, these strains develop acid tolerance. Non-o1 and non-o139 V. cholerae strains can be recovered from chironomid egg masses (Chironomus sp., Diptera). These egg masses were discovered in waste stabilization ponds and provide a rich nutritional niche for V. cholerae strains (Reidl & Klose, 2002)

## 7.2 Evolution of Vibrio pathogenicity traits from the virulence gene pool of the aquatic environment

While Vibrio cholerae is a human disease, aquatic environments are home to various Vibrio species, including pathogenic and nonpathogenic, with varying virulence gene loads. Presumably, virulence-associated genes are dispersed with lesser virulence potential than epidemic strains (Faruque, Albert, et al., 1998a). Moreover, the intimate connection of V. cholerae with surface water and the people interacting with water suggests the significance of water ecology in Vibrio cholera's life cycle (Faruque & Nair, 2002). Recent research shows that virulence genes or their homologs are distributed across environmental V. cholerae isolates of various serogroups, indicating an environmental reservoir of virulence genes (Chakraborty et al., 2000; Mukhopadhyay et al., 2001; Rivera et al., 2001). Although the precise environmental involvement of virulence-associated variables and the selection pressures for V. cholerae-carrying virulence genes or their

homologs are unknown, the possibility of new epidemic strains arising from environmental progenitors seems plausible. In a study, structural virulence genes ctxAB, tcpA, toxR, and toxT genes were found in environmental isolates of V. cholerae from Calcutta. PCR confirmed the existence of these virulence genes or homologs in environmental V. cholerae serotypes and ribotypes. The tcpA gene of an ecological strain had 97.7% identity to the tcpA gene of V. cholerae O1. TcpA-positive strains have the toxin coregulated pilus (TCP), as shown by autoagglutination and electron microscopy. The ctxAB strains generated CT as indicated by GM1 ELISA and passage in rabbit ileal loops. This research proved the existence of virulence genes in different environmental strains of V. cholerae, thus revealing fresh insights into the ecology. It is unclear if the virulence genes found in ambient strains of V. cholerae are leftovers from failed lateral gene transfers or whether they serve a purpose for these environmental strains (Chakraborty et al., 2000). Other than pandemic gene clusters, current research has identified several virulence alleles in environmental V. cholerae strains, including tcpA, tcpF, and toxT alleles, and the CTX prophage repressor rstR alleles in vibrios of diverse non epidemic serogroups. Environment-specific virulence gene alleles developed in response to selection forces that varied across environment and host. This phenomenon is not surprising given the pathogen's aquatic home; therefore, clinical and environmental alleles of distinct genes appear plausible. Also, clinical strains of V. cholerae may have acquired virulence genes or alleles from an ecological pool. While environmental strains may acquire virulence genes and become human pathogens, they are unlikely to achieve pandemic potential by acquiring TCP and CT genes alone. On the other hand, another research discovered a TCP (non-O1 non-O139) strain that shares ribotypes with many toxigenic O1 strains. Thus, recent research indicates that pathogen evolution may be more complex than previously believed (Walther & Ewald, 2004). The virulence-associated gene clusters identified in certain environmental V. cholerae strains exhibited significant genetic variability. Some of these genes may contribute to fitness in nature, and inter-strain gene exchange may be meaningful in the evolution of this species. These environmental genes may have influenced the virulence genes carried by clinical vibrios. A crucial combination of different genes may be necessary for the origination of a potential pathogenic strain (Mukhopadhyay et al., 2001).

It is clear from the existing data on cholera epidemiology, bacterial reservoirs, bacteriophage-mediated lysogenic conversion, genetic evolution, virulence gene transfer and V. cholerae survival and enrichment, that the ecosystem for V. cholerae includes many components. These are the bacteria, the aquatic environment, CTXF, other unknown genetic elements involved in virulence gene transfer, and the host population's intestinal environment (Faruque, Albert, et al., 1998a). It has long been assumed that intricate **interactions between bacteria and their hosts** determine how microorganisms overcome host defenses (Brown et al., 2006; Day et al., 2007). Additionally, increasing data suggest that **horizontal gene transfer (HGT)** often occurs through microbial interactions in the environment. The significant pathogenic genes in V. cholerae are clustered in several regions of the V. cholerae chromosome, and the structure of these pathogenic gene clusters indicates that these are capable of being propagated horizontally (Karaolis et al., 1998; Kovach et al., 1996; Pearson et al., 1993; Waldor & Mekalanos, n.d.). Major virulence genes in V. cholerae seem to have recently been acquired via phages or unknown horizontal gene transfer events (Lin et al., 1999). The development of a strain with epidemic potential requires microevolution of individual genes and a critical mix of various horizontally acquired gene clusters (Faruque, Chowdhury, et al., 2004). Horizontal gene transfer (HGT) has been linked to the pathogenicity and ecological fitness of vibrios. For example, researchers have shown that V. cholerae O139 epidemic strains evolved from the O1 El Tor strain through homologous recombination (Blokesch & Schoolnik, 2007) V. cholerae acquired the genes for toxin coregulated pilus (TCP) production from a filamentous phage genome via HGT occurring in the aquatic environment (Davis & Waldor, 2003). Toxin-encoding filamentous bacteriophage CTXF uses TCP as its receptor. Virulence traits may be transferred from pathogenic bacteria to harmless bacteria, which serve as natural reservoirs for those genes in the environment through this horizontal gene transfer process. For example, V. mimicus has been implicated in developing novel toxigenic V. cholerae isolates (Fidelma Boyd et al., 2000). Other environmental Vibrio species (e.g., V. alginolyticus and non-O1/O139 V. cholerae) have been discovered in the Mediterranean Sea and globally to possess V. cholerae virulence-related and virulence-regulatory genes (Baffone et al., 2006; Xie et al., 2005). Even though serogroup transition from non-O1 to O1 via horizontal gene transfer is possible, the results do not support this occurring very frequently in the environment. For example, the development of V. cholerae O139 from an O1 El Tor strain is the only universally acknowledged serogroup transition event (Faruque, Chowdhury, et al., 2004).

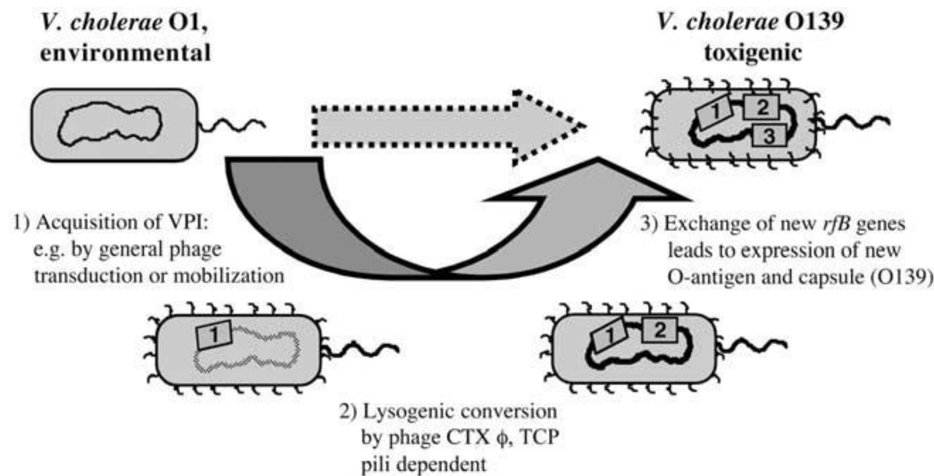


*Figure 9: Possible horizontal gene transfer events are important for V. cholerae virulence are shown, which led to the acquisition of essential virulence factor-encoding genes (Reidl & Klose, 2002).*

Recent research shows that V. cholerae has a flexible mechanism for acquiring genes from different species. Site-specific recombination between the circularized cassette and the recipient **integron** transfers an ORF or gene cassette into an integron. This is a unique class of integrons that can grab ORFs and turn them into functional genes. Given that V. cholerae has an unusual integron system, integrons may be involved in acquiring harmful genes and genes for other biological activities (Faruque, Albert, et al., 1998a).

Emergent human activities may also promote the development of virulence characteristics in the environment (Lebarbenchon et al., 2008). For example, using significant quantities of **antibiotics**, particularly non-biodegradable chemicals used in human treatment, guarantees that these drugs stay in the aquatic environment for extended durations, exerting selection pressure for a long time. Consequently, antibiotic-resistant bacteria have emerged in aquatic environments (Cabello, 2006).

Virulent environmental strains are enriched in the **intestinal environment of a mammalian host**. The research on Horizontal gene transfer also supports the hypothesis that environmental V. cholerae strains may adapt to the intestinal environment by developing virulence genes (Faruque, Albert, et al., 1998a). It is hypothesized that, in addition to seasonal variables causing a bloom of diverse V. cholerae in the environment, epidemics may be preceded by a steady enrichment of pathogenic strains either in the gut of an aquatic animal or, more likely, in humans who drink

surface water (Faruque, Chowdhury, et al., 2004). **Human colonization** produces a hyper infectious bacterial condition that persists after dispersion, perhaps contributing to the cholera epidemic spread (Merrell et al., 2002).

The V. cholerae **connection with chitin** was recently responsible for inducing the competence state in the bacterium, that is, the capability to acquire exogenous genetic material during growth on chitin via transformation. A recent independent study's findings support the idea that the same 53 kDa surface membrane protein (p53) that binds chitin particles and copepods also binds to intestinal epithelial cells in culture. Due to their characteristics, these compounds are called 'dual role colonization factors (DRCFs)'. DRCFs offer evidence that certain pathogen virulence factors utilized during infection may originate from their function in their natural habitat. This indicates that virulence is an adaptive mechanism arising in the environmental reservoir and such traits connect V. cholerae's human and aquatic lives (Pruzzo et al., 2008).

# Chapter 8: Environmental, intestinal stress responses and persistence strategy

V. cholerae has adapted to inhabit nutrient-rich human small intestine and aquatic habitats. Vibrios must resist harsh conditions such as nutritional restriction, UV exposure, high temperatures, oxidative stress, bacteriophage predation, and protozoan grazing in the aquatic environment. Climate fluctuation like temperature, salinity, the concentration of organic matter, and the presence of plankton affects Vibrio abundance in water. Plankton blooms may give the required infectious dosage for clinical cholera. Trophic regulation, including protozoan predation and bacteriophage lysis, has been demonstrated to limit V. cholerae growth in coastal waters (Vezzulli et al., 2008). Gastrointestinal Vibrios are subjected to low pH, bile acids, increased osmolarity, iron limitation, and antimicrobial peptides. Both conditions inhibit bacterial growth and multiplication in unique ways. However, the human small intestine offers more nutrients than aquatic settings. It has been shown that cholera patients may excrete 107–109 virulent Vibrios per mL of rice-watery stool. However, V. cholerae must overcome several harsh circumstances to achieve large titers in the stomach (Conner et al., 2016; Silva & Benitez, 2016a).

To survive harsh conditions in both human and aquatic hosts, V. cholera has similar survival mechanisms. V. cholerae forms biofilms on abiotic and biotic surfaces, actives general stress responses, enters a metabolically quiescent state, acquires and stores resources, and initiates defensive responses to particular physiological and biological threats (Conner et al., 2016). Biofilms protect V. cholerae against protozoan grazing in the environment, whereas planktonic equivalents are killed. The idea of a vibrio O1 or O139 aquatic reservoir indicates that the vibrios not only survive but are an essential part of the ecosystem. The capacity of V. cholerae O1 planktonic bacterial cells to interact with zooplankton, phytoplankton, and algae has been shown in microcosms enabling vibrios protection from the severe environmental conditions, acquire nutrition from the host, and therefore survive longer (Faruque, Albert, et al., 1998a).

The capacity to grow specific bacterial pathogens on the conventional medium is lost when transferred to aquatic settings from the host or laboratory. Despite their inability to be grown, these cells are capable of fundamental metabolic activities such as protein synthesis, respiration, and membrane integrity. In endemic regions, cholera outbreaks follow a seasonal pattern. In

interepidemic periods, toxigenic V. cholerae may exist in an unexplained ecological association with aquatic organisms, perhaps in the VNC form, until the next epidemic season, when environmental factors trigger the dormant bacteria to multiply and cause cholera outbreaks. Usually, stress circumstances compel vibrios to change to a viable but nonculturable (VNC) form that may induce infection and return to the culturable form when the conditions are preferable (Faruque, Albert, et al., 1998a). Microarray research revealed dramatic transcriptional alterations when bacteria reached the VNC stage. Enzymes for phosphate and nitrogen fixation were upregulated, whereas protein synthesis and energy metabolism genes were downregulated, keeping with low amounts of carbon sources, phosphate, and nitrogen fixation observed in aquatic settings (Nelson et al., 2009b).

With the changing oxygen concentrations, V. cholerae develops a range of four respiratory oxygen reductases: three bd-type oxygen reductases that directly take electrons from the ubiquinol pool and one cbb3-type haem-copper oxygen reductase. Although these enzymes have a high affinity for oxygen, nothing is known about their biochemistry in V. cholerae. For example, V. cholerae may develop without oxygen by respiring organic and inorganic alternate electron acceptors (AEA), such as fumarate and trimethylamine N-oxide (TMAO). In the absence of oxygen and an AEA, V. cholerae may ferment sucrose, dextrin, maltose, glucose, mannitol, sorbitol, lactose, and starch. The strain of V. cholerae ferments differently. El Tor N16961 may create 2,3-butanediol as a fermentative neutral end product, preventing medium acidification. However, since the traditional biotype O395 cannot generate 2,3-butanediol, its viability during glucose mixed fermentation is impaired (Bueno et al., 2020b).

# Chapter 9: Vibrio cholerae biofilms

In biofilms, cells cling to a surface, or one other as free-floating aggregates contained inside a matrix (L. Yang et al., 2012). So, bacteria (single or multiple species), a self-produced extracellular protective matrix which is made of extracellular polymeric substances (EPSs) and other substances (such as polysaccharides, proteins, nucleic acids, extracellular DNA, lipids, cell components, and sticky substances) together create a bacterial biofilm. EPS has a definitive role in providing structural rigidity, cohesiveness, coordinating other physical properties, and providing a distinct architecture to the biofilm (Silva & Benitez, 2016a; L. Yang et al., 2012). Bacterial biofilms provide many **advantages** to the bacterial communities, including antibiotic resistance, protection from immune defense cells, and better survival chances against acid, osmotic and oxidative stresses (L. Yang et al., 2012) .Studies on genetic and microscopic analysis of gram-negative organisms conclude that creating mature, three-dimensional bacterial biofilms is a developmental process with consecutive, discrete stages. The planktonic stage, the monolayer stage, and the biofilm stage are the main stages of this process (Moorthy & Watnick, n.d., 2005).

The formation of V.cholerae biofilms occurs in aquatic and intestinal environments, and this bacteria is found mainly in the water bodies in both planktonic and biofilm states throughout the year (Teschler et al., 2015b). A community of Vibrios develops biofilm by attaching to biotic surfaces (human intestinal mucosa) or abiotic surfaces (chitinous exoskeleton of crustaceans) where the cells aggregate and get enclosed by extracellular matrix (Conner et al., 2016; Silva & Benitez, 2016a). This biofilm protects V.cholerae from nutrient constraints or attacks by bacteriophages and protozoans. V.cholerae biofilms have a primary role in disease transmission because they contain more significant doses of bacteria and hyper infective cells. The biofilms are protected during transit via the stomach's gastric acid barrier, enabling more germs to reach the small intestinal colonization site. Also, they defend against pH, bile acids, chlorine, and predation inside the human body (Davey & O'toole, 2000).

## 9.1 Stages of Vibrio Cholera biofilm formation

There are multiple stages of formation of V.cholerae biofilm - the bacteria inspects its suitable surface and then attaches onto it, then forming microcolonies that eventually give rise to an organized, three-dimensional structure (Teschler et al., 2015b).

### 9.1.1 Surface Attachment

Both the human gut and the aquatic environment need surface adhesion for colonization; hence surface attachment is the first step of biofilm formation. Before producing a biofilm, V. cholerae goes through the planktonic and monolayer phases where the attachment to the abiotic or biotic surface is mediated by two exterior structures: the flagellum and pili (Mewborn et al., 2017; Moorthy & Watnick, n.d., 2005; Watnick & Kolter, n.d.). Motile V. cholerae of serogroup O1 and O139 assess solid surfaces (Biswas et al., 2020) before attaching to the favored surface, which depends on the various pili that are expressed by the bacteria (Mewborn et al., 2017). For example, the mannose-sensitive hemagglutinin (MSHA) helps V. cholerae attach to borosilicate and the exoskeleton of planktonic crustaceans; the N-acetylglucosamine binding protein GbpA enables to adhere to chitin and intestinal mucin; the chitin-regulated pilus ChiRP increases attachment to chitinous surfaces. The toxin coregulated pilus (TCP) facilitates attachment to cultured intestinal cells, intestinal microvilli, and microcolony development, and V. cholerae colonization of the suckling mouse and human gut requires type IV pilus (Mewborn et al., 2017). During this stage, the flagellar genes are positively expressed (Moorthy & Watnick, 2005), and the motility of V.cholerae is propelled by a single polar flagellum powered by a Na+ motor. Hydrodynamic forces acted on the flagellum when the cells swept near surfaces and produced a torque on the cell body, deflecting cell swimming direction into curved clockwise pathways (Biswas et al., 2020; Conner et al., 2016; Zamorano-Sánchez et al., 2019). After a suitable surface has been selected, the Mannose-Sensitive Haemagglutinin type 4 surface pili (MSHA-pili) transiently adhere to the surface. Their binding is mechano-chemical in nature essential in nature V. cholerae's shift from planktonic to biofilm state (Biswas et al., 2020; Conner et al., 2016). A non-metabolizable counterpart of mannose - mannose or a-methyl mannoside (AMM) prevents MSHA from interacting with the surface (Moorthy & Watnick, n.d.). Surface attachment causes flagellar gene transcription to be repressed and inhibits the production and function of flagella, which causes cells to remain permanently attached stably to the surface in a monolayer (Moorthy & Watnick,

2005; Zamorano-Sánchez et al., 2019). Permanent attachments are differentiated from transitory attachments by their resistance to the action of AMM after they have been established. The flagellar mutant monolayer is likewise immune to AMM's effects. This backs with the theory that persistent attachment requires the absence of flagellar mobility (Moorthy & Watnick, 2005).

### 9.1.2 Macro colony formation

After the first step of cell attachment, V cholerae progresses to the colony formation step where it produces an extracellular matrix in the three-dimensional direction which is composed of Vibrio polysaccharides (VPS), three matrix proteins (RbmA, Bap1, and RbmC), and a small number of nucleic acids (eDNA) (Berk et al., 2012; Biswas et al., 2020). Both the VPS and proteins play a crucial role in forming the biofilm matrix because it was seen that mutation in genes that encodes for VPS and protein inhibited the biofilm development. However, the nucleic acid or extracellular DNA is also an essential part of the extracellular matrix, although it is unclear how it interacts with the known VPS and proteins(Guttenplan & Kearns, 2013).

**VPS:** VPS is a polysaccharide generated soon after surface adhesion that creates a polymeric network of cells to form the three-dimensional biofilm (Berk et al., 2012; Conner et al., 2016). Two types of VPS were found in the biofilm: the major variant of the polysaccharide component of VPS has a repeating unit of [→4) -α-L-GulpNAcAGly3OAc-(1→4) -β-D-Glcp-(1→4) -α-D-Glcp-(1→4) -α-D-Galp-(1→]n, while the minor variant replaces the α-D-Glc with α-D GlcNAc (F. Yildiz et al., 2014).

The exact structure of VPS is unclear; however, it has been suggested that the sugar composition of VPS mainly consisted of glucose and galactose with some xylose, mannose, and N-acetyl glucosamine (Fong et al., 2006; Guttenplan & Kearns, 2013). On the large chromosome, there are 18 genes of VPS, which are divided into two vps clusters: vps-I-cluster and vps-II-cluster, with 12 genes and six genes, respectively (Fong et al., 2010; Guttenplan & Kearns, 2013; F. H. Yildiz & Schoolnik, 1999). These genes are grouped into six classes with various assumed functions: class I has VpsA and VpsB, which encodes for the nucleotide sugar precursors; class II has VpsD, VpsI, VpsK, and VpsL that encodes glycosyltransferases; class III has VpsE, VpsH, VpsN, and VpsO

genes that encode VPS polymerization and export proteins; class IV has VpsC and VpsG which encodes for acetyltransferases; class V has VpsU which encodes the phosphotyrosine-protein phosphatase; and class VI contains VpsF, VpsJ, VpsM, VpsP and VpsQ genes that encode the hypothetical proteins . The hypothetical proteins are essential for the biofilm development because without vpsF, vpsJ, or vpsM genes, the colony corrugation is lost, as is the capacity to produce pellicles, as well as biofilm and VPS synthesis, but when 15 of the 18 vps genes were deleted in-frame, the strains had less colony corrugation than the wild type (Fong et al., 2010).

The V. cholerae biofilm matrix cluster (VcBMC) comprises the vps-1, rbm, and vps-2 clusters, which encode numerous genes involved in VPS production and the main biofilm proteins RbmA, Bap1, and RbmC (Fong et al., 2006, 2010; Fong & Yildiz, 2007). An 8.3 kb rbm cluster with six genes encodes matrix proteins separates the vps-I and vps-II clusters (Fong et al., 2006; Fong & Yildiz, 2007; F. H. Yildiz & Schoolnik, 1999). Biofilm development requires VPS interaction with biofilm matrix proteins because it was seen that RbmA, RbmC, and Bap1 are not maintained at a solid-liquid interface in deletion mutants that cannot generate VPS, and RmbC is required for VPS inclusion throughout the biofilm (Berk et al., 2012). GalU and galE, two other genes involved in UDP-glucose and UDP-galactose synthesis, necessary for VPS synthesis, are also required for biofilm formation (Nesper et al., 2001).

**Matrix protein:** The extracellular matrix contains three proteins: RbmA, RbmC, and Bap1, which are mainly necessary for the microcolony, and mature biofilm formation are secreted by the type II secretion system (T2SS) during biofilm development and preserve spatial and temporal patterns (Conner et al., 2016; Zamorano-Sánchez et al., 2019). RbmA comprises two fibronectin type III (FnIII) folds, which are often seen in cell surface receptors and cell adhesion proteins, according to the crystal structure. A linker segment links the FnIII folds of two RbmA monomers, producing a bilobal structure with distinct surface characteristics (Giglio et al., 2013). RbmA accumulates on the cell surface after initial attachment and VPS synthesis, facilitating the cellular adhesion, architecture, and biofilm stability (Biswas et al., 2020; Conner et al., 2016). According to the structure predictions and phenotypic assessments, RbmA has been found to be a sugar-binding protein; however, the actual mechanism by which the protein acts is yet to be determined. RbmA might function as an agglutinin, hold cells together or anchor the biofilm to the carbohydrate-rich substrates, or function by binding to the carbohydrates in the biofilm's VPS matrix, resulting in

the densely packed and well-organized biofilm structure seen in the wild-type rugose form (Fong et al., 2006).Loss of RbmA can lead to the production of fragile biofilms that can easily be dissolved in detergents and reduce cells' organization into clusters (Berk et al., 2012; Fong et al., 2006).V. cholerae has two kinds of morphology: Smooth colony morphology and Rugose colony morphology. The cells are buried in a rugose exopolysaccharide in the rugose variant, giving it a wrinkled morphology. V.cholerae may convert to the rugose morphology because the rugose variant enhances the biofilm development. It is also resistant to harsh environments such as acid, UV light, chlorine, and complement-mediated serum bactericidal activity, thus providing an adaptive advantage and improved survivability for the bacteria (Rashid et al., 2003). Hence, it has been studied that for maintaining the rugose colonial morphology, RbmA is a necessity that also produces architecturally mature wild-type biofilm (Fong et al., 2006).

Next, there are two other proteins - Bap1 and RbmC, which are homologs of each other as both have FG-GAP and carbohydrate-binding domains, as well as three probable N-acetylglucosamine-binding sites, according to computational analysis, which has also shown that both the proteins function to maintain the colony rugosity and stabilize pellicle and biofilm structures (Fong & Yildiz, 2007). Both Bap1 and RbmC have four overlappings (VCBS) domains that may be involved in cell adhesion and have four and two FG-GAP domains, respectively, involved in ligand recognition and binding (Absalon et al., 2011; Duperthuy et al., 2013). RmbA secretion is followed by Bap1 secretion at the cell-attachment surface interface and on the substrate surrounding the cells. Bap1 is continually released into solution by the founder cell and other early components of the new biofilm, after which it accumulates on surrounding surfaces, according to its radially symmetrical distribution relative to the founder cell (Berk et al., 2012). Bap1 helps the biofilm proliferate at the water-air interface by maintaining pellicle strength and hydrophobicity (Biswas et al., 2020). The RbmC is then secreted at specific locations on the outer cell surface where RbmC and Bap1 generate flexible envelopes that may expand when cells divide as the biofilm grows. Constant production of biofilm components promotes the formation of a properly developed biofilm constituted of ordered clusters of cells, VPS, RbmA, Bap1, and RbmC (Biswas et al., 2020; Conner et al., 2016).

### 9.1.3 Dispersal

Dispersal is the last step of biofilm formation, and it seems to be quite active in many species, probably to enable the colonization of new habitats. Swarming/seeding dispersal, in which individual cells are released from a microcolony into the bulk fluid or surrounding substratum; clumping dispersal, in which aggregates of cells are shed as clumps or emboli; and surface dispersal, in which biofilm structures move across surfaces are the three distinct biofilm dispersal strategies (Hall-Stoodley et al., 2004).

Dns and Xds, two extracellular nucleases, have been linked to biofilm creation and dissemination via regulating eDNA, which is involved in nutrition supply and biofilm structure (Seper et al., 2011). Competent cells may pick up eDNA produced by cell lysis or active secretion during chitin development, where it might serve as a source of organic nutrients or be integrated into the genome. It might also stay in the biofilm matrix, which seems to have a critical structural role. Dns and Xds nucleases elimination enhanced biofilm development irrespective of VPS production, changed biofilm structure, and hampered biofilm detachment. On the flip side, data suggests that these nucleases degrade eDNA, reducing biofilm formation and perhaps facilitating diffusion. In vivo colonization was also shown to be impaired, suggesting that dispersion may be required for host colonization (Seper et al., 2011).

Also, the rbmB gene, which is a part of the VcBMC's rbm cluster, encodes a putative polysaccharide lyase that may be involved in VPS breakdown and cell separation (Fong & Yildiz, 2007). Despite the absence of experimental evidence for RbmB's enzymatic activity, strains missing RbmB generate more biofilms than bacteria encoding the protein (Fong & Yildiz, 2007). Lastly, bile is an active detergent that enhances the biofilm formation (Zhu & Mekalanos, 2003a) in the intestine; however, lately, it has been known that a component of bile salt called taurocholate plays a role in the degradation or dispersal of biofilms (Almagro-Moreno et al., 2015; Hay & Zhu, 2015).

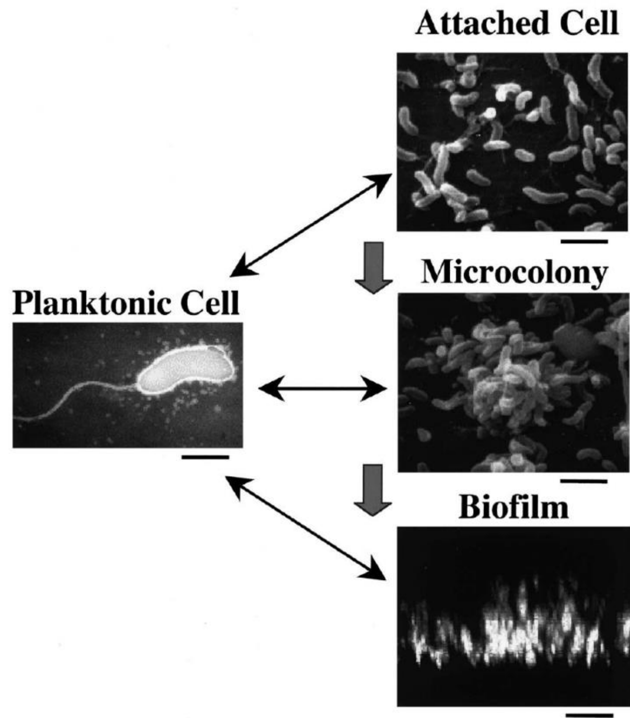


*Figure 10: Steps in Vibrio Cholerae biofilm formation (Faruque, Albert, et al., 1998a)*

# Chapter 10: Gene regulation of Vibrio Cholerae biofilm

The biofilm formation of V.cholera is regulated by an integrated network of positive regulators - VpsR and VpsT, Negative regulators - HapR, H-NS, CtyR, alternative sigma factor, signaling molecules - c-di-GMP, cAMP and (p)ppGpp, sRNA.

## 10.1 Positive regulators

There are three positive regulators of biofilm formation and VPS production; the VpsR, VpsT (Liu et al., 2015), and AphA. VpsR was first identified as one of the positive regulators, and it is known as the master regulator of biofilm formation and VPS production (Conner et al., 2016). It belongs to the response regulatory family called the two-component signal transduction systems (TCS). This VpsR regulator possesses three other components that are part of the signal transduction systems; the response regulator N-terminal domain, an ATP binding motif located at the central region of VpsR, which is crucial for alternative sigma 54-factor interaction, and C-terminal helix-turn-helix DNA-binding domain (F. H. Yildiz et al., 2001). VpsR activates the genes of vps (Haugo & Watnick, n.d.) by binding to the promoter region of vps to control the expression of the gene. This explains the importance and the impact of the VpsR in biofilm formation because it was seen that a disruption of the vpsR gene led to the inhibition of positive regulator VpsR and matrix protein which eventually terminated the biofilm formation (Conner et al., 2016). The expression of two other genes, vpsL, and vpsA, which are part of the vps cluster of EPSETr biosynthesis genes, is also upregulated by VpsR, promoting the synthesis of EPS and biofilm (F. H. Yildiz et al., 2001). Moreover, the VpsR also activates the genes of matrix protein, the eps genes that translate a section of Type 2 Secretion System which is necessary for matrix protein secretion and activates the genes that encode AphA, which is a significant virulence regulator that plays a part in the development of the biofilms and also somewhat in the pathogenesis of V.cholera (Conner et al., 2016). Asp59, a conserved aspartate residue in VpsR, appears to be essential for its function because the transition of this aspartate to alanine makes VpsR inactive, while the change to glutamate makes VpsR active, proving that phosphorylation does regulate the DNA binding of VpsR. (Lauriano et al., 2004a; Teschler et al., 2015b). By binding to the receptor proteins VpsR and VpsT, cyclic diguanylic acid (c-di-GMP) induce the biofilm exopolysaccharide and extracellular protein matrix synthesis at the transcriptional level (Conner et al., 2017; Mewborn et al., 2017). In most cases,

response regulators work in tandem with a sensor histidine kinase, but their control of the vps gene expression for rugose colonial morphology of V. cholera O1 El Tor is yet to be investigated (Casper-Lindley & Yildiz, 2004). Lastly, vpsR expression is positively controlled by VpsT. However, VpsR has a considerable effect on its own expression (Casper-Lindley & Yildiz, 2004) and it is negatively controlled by HapR. However, additional variables are likely to be involved, and further research is required to define its regulation completely (Beyhan et al., 2007).

The second positive response regulator of vps genes identified was VpsT. VpsT is also important in the rugose variant of V. cholerae. It is needed to develop a corrugated colonial morphology, biofilm development, and maximum vps gene expression (Casper-Lindley & Yildiz, 2004). Just like VpsR, the VpsT regulator positively controls the expression of vpsA and vpsL to induce the biofilm and EPS synthesis and bind to the tiny signaling molecule cyclic dimeric guanosine monophosphate (c-di-GMP) to upregulate the transcription of VcBMC genes (Conner et al., 2016). VpsT activates and controls vps genes' expression by binding to the promoter region of vps; however, a mutation in the vpsT reduces the vps gene and matrix protein gene expression that prevents biofilm formation VpsR (Casper-Lindley & Yildiz, 2004; M. Yang et al., 2010). The VpsR, AphA, and alternative sigma factor RpoS has upregulated the expression of vpsT, but the HapR directly downregulated the expression of vpsT (He et al., 2012; M. Yang et al., 2010).

Overall, both the VpsR and VpsT regulons overlap widely in terms of positively regulating each other's expression (Zamorano-Sánchez et al., 2015) and upregulating the transcription of vps genes and other biofilm-related genes. Still, the role of gene regulation played by VpsR in biofilm development is much more significant than VpsT. The binding site of VpsR and VpsT is located in the upstream regulatory region of vps-II cluster, but it has been discovered that the upstream regulatory region of vps-I, the rbmA gene, and the vpsT gene are also the binding sites for VpsR and VpsT (Conner et al., 2016).

## 10.2 Negative regulators

**HapR and Quorum Sensing:** The master biofilm repressor protein, HapR, negatively regulates the biofilm matrix formation in V.cholera (Beyhan et al., 2008). The biofilm matrix formation and

dispersal from biofilm depend on the repression and activation of *hapR,* respectively controlled via the quorum sensing (QS) pathway (Conner et al., 2016; Teschler et al., 2015b). HapR protein is a DNA-binding transcription factor that responds to cell density (Conner et al., 2016) by starting a gene-expression program that changes cells from a low-density individual state to a high-density one (Hammer & Bassler, 2009; Waters et al., 2008a).

The mechanism that enables cell-to-cell communication among bacteria to coordinate population activity by producing, secreting, and detecting chemical signal molecules called Autoinducers (AIs) is known as Quorum Sensing (QS) (Waters et al., 2008b). V. cholerae generates two autoinducers, namely, genus-specific autoinducer-1 (CAI-1) and a second signal termed autoinducer-2 (AI-2) with their chemical names (S)-3-hydroxytridecan-4-one and (2S,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate, respectively (Liang et al., 2008; Waters et al., 2008a). The bacteria employ parallel phosphorelay signaling networks to react to these signaling molecules' autoinducers (Waters et al., 2008c). The production and detection of the autoinducers in V. Cholerae are dependent on their respective signal receptors. Therefore, it is predicted that for quorum sensing in V. cholerae the system 1 is made up of an autoinducer (CAI-1) and its signal receptor or cognate sensor CqsS and the autoinducer (AI-2) and its cognate sensor LuxP/Q make up System 2 (Hammer & Bassler, 2003; Liang et al., 2007; Thompson et al., 2014). Along with the autoinducers and their related cognate sensors, the regulatory pathway regulating cell density-dependent metabolic responses also includes a signal transduction cascade that employs phosphorylation and dephosphorylation of transcriptional regulatory proteins, non-coding small RNAs, and RNA chaperons (Hoque et al., 2016).

When the cell density is low, the concentration of autoinducers CAI-1 and AI-2 is also low. Their signal receptors CqsS and LuxP/Q respectively act as kinases where the phosphate transfers from the signal receptors to LuxO, the response regulator via LuxU. The phospho-LuxO (LuxO-P) interacts with the alternative sigma factor 54 (RpoN) and activates the expression of the gene that encodes quorum-regulated small RNAs (sRNAs), Qrr1–4 (Hammer & Bassler, 2003; Waters et al., 2008a). Qrr sRNA interacts with the sRNA chaperone Hfq to inhibit the translation of hapR that encodes HapR, the master regulator of Quorum sensing (Conner et al., 2016; Sultan et al., 2010). As the expression of the hapR gene is repressed, the biofilm development is positively regulated, and also the production of virulence factor occurs due to the upregulation of aphA-

dependent virulence genes (Hammer & Bassler, 2003). On the other hand, at high cell density, the concentration of CAI-1 and AI-2 are high, LuxO dephosphorylates by the effect of signal receptors, CqsS and LuxP/Q, and the expression of Qrr1–4 sRNA are repressed (Conner et al., 2016). The translation of *hapR* occurs, and HapR protein is synthesized, which eventually reduces the biofilm formation by binding to the regulatory region of the vps-II operon and vpsT gene to down-regulate the gene expression that encodes the biofilm transcriptional activator, VpsT (Conner et al., 2016; Hammer & Bassler, 2009; Waters et al., 2008a) . Moreover, HapR prevents the virulence factor production by binding to aphA promoter to inhibit the transcription of aphA, but the protein enhances the Zn-metalloprotease, HA/protease (HapA) production by activating the expression of hapA gene that promotes the detachment and dispersal of V.cholerae (Hammer & Bassler, 2003; Mewborn et al., 2017; Silva et al., 2006; Thompson et al., 2014).

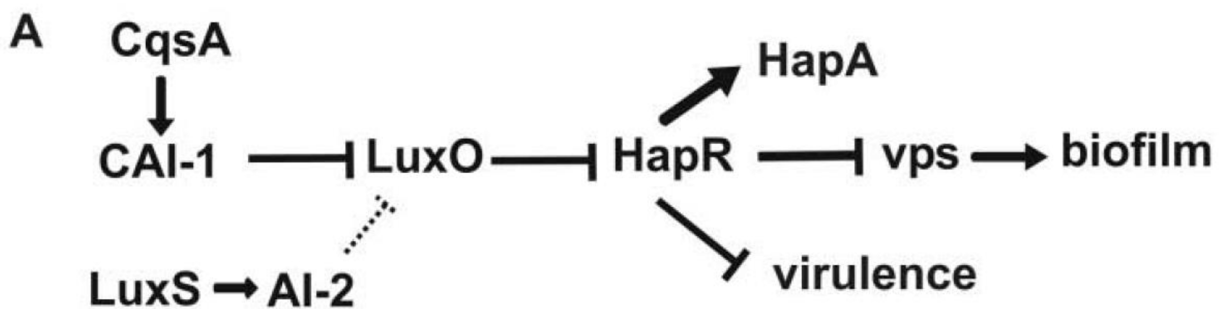


*Figure 11: Quorum Sensing pathway: Biofilm development in Vibrio cholerae is controlled by the quorum sensing-mediated regulation of biofilm formation. Positive impacts are shown by solid arrows, whereas negative effects are represented by solid T bars. Though AI-2 signaling does not alter biofilm formation, LuxO does react to AI-2 when looking at other phenotypes, as seen by the broken line (Zhu & Mekalanos, 2003b).*

There are other regulators which play a part in the regulation of HapR by Quorum sensing. VarS-VarA is a two-component system controlling quorum sensing that promotes post-transcriptional hapR expression through a route including the regulatory sRNAs CsrB, CsrC, and CsrD (Lenz et al., 2005; Liang et al., 2007; Tsou et al., 2011). These sRNAs CsrBCD regulate the global regulator CsrA's activity by binding, increasing the LuxO-P activity at low cell density (Liang et al., 2007). Therefore, Qrr1-4 is prevented from being activated by LuxO, resulting in reduced Qrr1-4 levels

and increased HapR synthesis (Lenz et al., 2005). Fis is a small nucleoid protein that promotes the breakdown of hapR mRNA and enhances the four qrr gene expression to reduce the level of HapR protein at low cell density and vice versa during high cell density (Lenz & Bassler, 2007; Liang et al., 2007). The cAMP receptor protein (CRP) is a global regulator best recognized for its role in carbon catabolite suppression, which occurs when a rapidly metabolizable carbon source (such as glucose) is present in the growth medium (Liang et al., 2007, 2008). Through its positive regulation of the CAI-I autoinducer synthase (CqsA) and negative regulation of Fis, CRP is responsible for increasing HapR synthesis (Liang et al., 2007). CRP is essential for the expression of CqsA, which leads to the formation of CAI-1, as per global gene expression profiling of a V. cholerae crp mutant (Liang et al., 2007). Accordingly, deletion of crp has been demonstrated to have a harmful influence on HapR and multiple HapR-regulated genes expression, and so reducing the HAP production, CT, EPS biosynthesis, udp gene expression (Liang et al., 2007), and biofilm development (Liang et al., 2008).

**H-NS (histone-like nucleoid structuring protein)**: It is a member of nucleoid-associated protein that consists of both factors for inversion stimulation (FIS) and the integration host factor (IHF) (Atlung & Ingmer, n.d.; Wang et al., 2015). It functions as a nucleoid organizer and transcriptional regulator where HNS negatively regulates the transcription or expression of vpsT, vpsL, and vpsA (164). A flexible linker connects an N-terminal oligomerization domain to a nucleic acid-binding domain in H-NS. H-NS inhibits gene expression by binding to promoters with high curvature AT-rich regions during transcription regulation (Ayala et al., 2015; Wang et al., 2015) .A result from Chip-seq had shown that both HNS and VpsT together regulate the expression of the important components of the biofilm matrix - the exopolysaccharides and protein components. Moreover, it was also found that there is an independent relationship between C-di-GMP pool and H-NS where an increase in the c-di-GMP pool due to environmentally induced fluctuation triggers a multi-locus H-NS anti-repression cascade that results in the liberation of vpsT from H-NS repression and allosteric activation of VpsT. Therefore, promoting the biofilm exopolysaccharide and protein matrix production has lowered the chances of H-NS binding to the downstream promoter regions of vps and rbm (Ayala, Wang, Benitez, et al., 2015; Wang et al., 2012). In contrast, a decrease in the c-di-GMP pool causes a decrease in vpsT expression, which is then reversed by H-NS displacing bound VpsT from promoters (Ayala, Wang, Silva, et al., 2015; Wang et al., 2012, 2015).

**CytR:** The V. cholerae CytR protein is another regulator discovered to repress the production of exopolysaccharides and biofilm growth, unlike the V. cholerae ΔcytR mutant (Haugo & Watnick, n.d.). The LacI repressor family comprises the E. coli CytR protein, and in response to low nucleoside concentrations, CytR in E. coli represses transcription of genes encoding proteins involved in nucleoside uptake and catabolism. A similar function was also detected in the CytR of V.cholera (Haugo & Watnick, n.d.). V. cholerae CytR negatively controls the transcription of the udp gene that encodes for uridine dephosphorylase and vps gene, which represses the biofilm development in both planktonic and biofilm-associated cells and also reduces the surface attachment by the planktonic cells (Haugo & Watnick, n.d.; Moorthy & Watnick, n.d.; Zhu & Mekalanos, 2003a).

## 10.3 Signaling molecule

Three small nucleotide signaling molecules are also responsible for controlling the biofilm development, namely, c-di-GMP, cyclic adenosine-monophosphate (cAMP), and guanosine 3′-diphosphate 5′-triphosphate and guanosine 3′,5′-bis (diphosphate) (p)ppGpp signaling.

**A second messenger signaling molecule called C-di-GMP:** is an essential molecule that helps regulate the shifting of V.cholera from planktonic or motile state to biofilm or sessile state (Tischler & Camilli, 2004). It happens when the cellular level of c-di-GMP is high, enabling the expression of the biofilm-related genes to produce the constituents like EPS and protein required for biofilm development. Moreover, high levels of c-di-GMP repress virulence factor expression and motility in V. cholerae, and low levels of c-di-GMP suppress biofilm formation and stimulate virulence factor expression and motility (Beyhan et al., 2006; Conner et al., 2017; Tischler & Camilli, 2005). The Diguanylate cyclases (DGCs) with GGDEF domains produce C-di-GMP from GTP, which is degraded to GMP by phosphodiesterases (PDEs) with EAL or HD-GYP domains (Conner et al., 2017; Tamayo et al., 2007). The genome of V.cholera encodes 31 proteins with a GGDEF domain, 12 proteins with an EAL domain, 9 proteins with a HD-GYP domain, and 10 proteins with a combined GGDEF- EAL domain (Tamayo et al., 2007). c-di-GMP is detected by PilZ, VpsT, and FlrA receptor proteins, as well as c-di-GMP riboswitches. (Conner et al., 2016, 2017).

Early in biofilm development, flagella and motility may be needed for finding and interacting with a suitable surface. Low levels of c-di-GMP seem to enhance motility, which is potentiated by PilZ-

containing proteins including PlzB and PlzC (Guttenplan & Kearns, 2013). It was also discovered from in-frame deletions in the genes encoding predicted DGCs and EAL domain-containing PDEs that there are four DGCs (CdgH, CdgK, CdgL, and CdgD) which prevents motility to activates biofilm formation (Guttenplan & Kearns, 2013) and two PDEs (CdgJ and RocS) which enhances motility in V.cholera. Hence, it was assumed that the activities of these proteins played an important role in the transition from planktonic to biofilm state of V.cholera. The concentration of c-di-GMP also affects the action of the vital regulator, FlrA. The binding of c-di-GMP to FlrA suppresses the expression of all flagellar genes as the concentration of c-di-GMP rises once the biofilm formation has started (Guttenplan & Kearns, 2013; Khan et al., 2020; Mewborn et al., 2017) . C-di-GMP also binds to VpsT and VpsR at high intracellular concentration, increasing the expression of Vps and rbm genes that encode for exopolysaccharide and protein matrix of biofilms, respectively (Mewborn et al., 2017).

QS may influence biofilm development in V. cholerae by altering intracellular c-di-GMP levels (Waters et al., 2008a). HapR expression in V. cholerae at high cell density inhibits biofilm formation in two ways. To begin, HapR modifies the expression of 14 genes that encode proteins with GGDEF and/or EAL domains that produce and destroy c-di-GMP molecules. When these genes are controlled by QS, intracellular levels of c-di-GMP are raised at low cell density and reduced at high cell density (Waters et al., 2008a). The biofilm transcriptional activator vpsT is induced by elevated c-di-GMP in the low-cell-density condition. Lastly, the same regulatory component in the biofilm transcriptional cascade, vpsT, is controlled by a congregation of HapR and c-di-GMP. HapR directly represses vpsT expression in addition to regulating it via regulation of c-di- GMP levels; hence, preventing biofilm formation at high cell density. (Beyhan et al., 2007; Waters et al., 2008a).

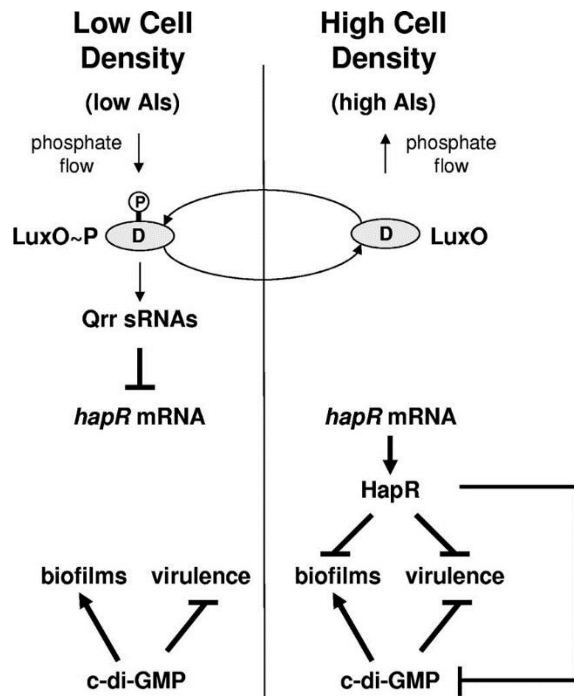


*Figure 12: QS and c-di-GMP interact to regulate gene expression in V. cholerae. To suppress translation of the master transcriptional regulator HapR, the response regulator LuxO (left) is phosphorylated. High AI concentrations (right side) dephosphorylate LuxO, halting qrr expression. Without Qrr sRNAs, HapR is generated. A biofilm-forming and virulence factor-expressing gene. c-di-GMP, like HapR, suppresses virulence factor expression but promotes biofilm formation. Here, HapR inhibits biofilm formation both directly (through vpsT) and indirectly (by c-di-GMP) (Waters et al., 2008a).*

Along with the regulation of biofilm and flagellum synthesis, C-di-GMP also controls the production of MSHA Pilus (Zamorano-Sánchez et al., 2019). High quantities of c-di-GMP increase transcription of msh, the operon encoding the MSHA pilus, vps, and other biofilm genes, while repressing transcription of flagellar genes, according to transcriptional profiling studies(Beyhan et al., 2006).

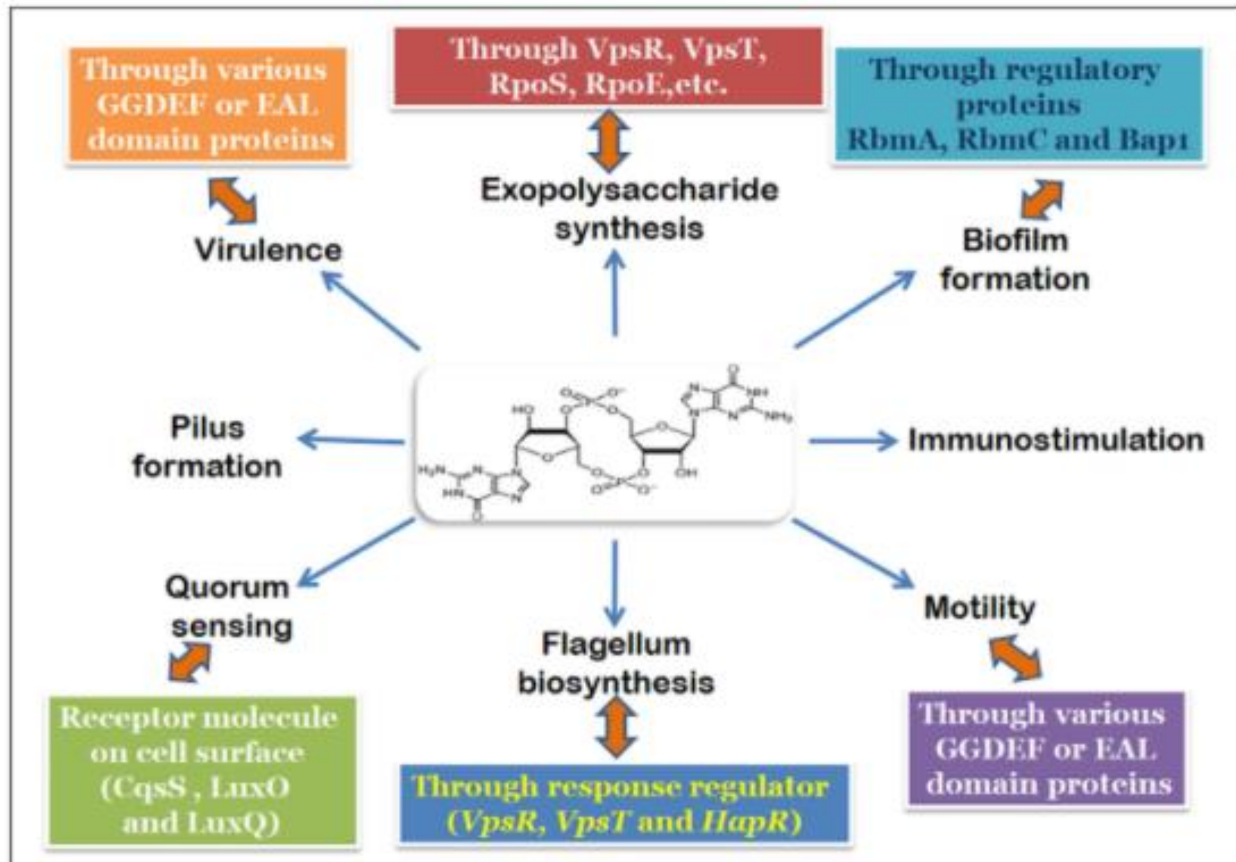


*Figure 13: The involvement of c-di-GMP in the regulatory pathway and constituents of biofilm development. Adapted from (Zamorano-Sánchez et al., 2019)*

**The second messenger cyclic adenosine-monophosphate cAMP**: functions as a repressor of V. cholerae biofilms development (Conner et al., 2016). When glucose is scarce, the adenylyl cyclase CyaA gets activated and produces high levels of cAMP, which binds to its cAMP receptor protein, CRP to form a cAMP-CRP complex (Liang et al., 2008). rbmA, rbmC, bap1, vpsR, and other vps genes encode for the essential biofilm components -protein matrix and exopolysaccharide are negatively regulated by the cAMP-CRP complex (Fong & Yildiz, 2008). It was found that cAMP-CRP also dysregulates a variety of DGC and PDE genes that govern c-di-GMP levels; for example, rocS, cdgA, cdgH, and cdgI (Fong & Yildiz, 2008). On the other side, HapR and the production of the QS autoinducer CAI-I are positively regulated by cAMP-CRP, allowing V. cholerae to detect changes in cell density and further increase the expression of HapR, which inhibits the formation of biofilms (Conner et al., 2016).

**(p)ppGpp:** lastly, biofilm formation is promoted by the stringent response triggered by nutritional stress and results in the synthesis of two molecules (p)ppGpp by RelA, SpoT, and RelV (Das et al., 2009; He et al., 2012; Raskin et al., 2007). Upregulation of (p)ppGpp has been demonstrated to promote the development of biofilms. All three (p)ppGpp synthases are required for vpsR transcription, while only RelA is required for vpsT transcription, suggesting that the synthases may potentially regulate biofilm genes directly (He et al., 2012). All in all, the c-di-GMP, cAMP, and (p)ppGpp pathways are essential for biofilm formation because they enable V. cholerae to rapidly react to different environmental inputs by altering internal levels of these tiny nucleotide signals.

## 10.4 sRNA

The role of sRNAs in regulating cellular activities is becoming more apparent. sRNA is known to regulate the levels of HapR in V.cholera; however, two more sRNAs have been found to influence biofilm development in V. cholerae. VrrA, whose expression is regulated by the sigma factor RpoE, inhibits the translation of the biofilm matrix protein RbmC by directly associating with its 5' end, thus limiting biofilm formation (Song et al., 2008, 2014). Biofilm production involves the sRNA RyhB, which is negatively controlled by iron and Fur, and it was seen that in low-iron media, a ryhB mutant has a deficiency in biofilm development, which may be corrected by adding more iron or succinate. Yet, the molecular mechanism through which RyhB regulates biofilm development remains unknown (Mey et al., 2005). Although the function of sRNAs in biofilm formation is unknown, these instances contribute to the complex regulatory network that regulates biofilm formation (Teschler et al., 2015b).

# Chapter 11: Intestinal colonization

In the first stage of intestinal colonization, humans may ingest Vibrio Cholerae in a variety of forms, including free-living, in a dormant condition known as viable but nonculturable (VBNC), in microcolonies, or a hyper infectious state known as CVEC (biofilm state) and V. cholerae may survive the high acidity of the human stomach if it is swallowed as part of a biofilm (Almagro-Moreno et al., 2015). Whether the biofilm is intact or scattered, the infectious dosage of V. cholerae obtained from biofilms is lower than the planktonic cells. It has been speculated that bacterial dispersion from the biofilms in the early phases of colonization may be facilitated by interaction with bile components after reaching the intestinal lumen. As the bacteria gets released in the lumen, it is protected against bile acids and other antimicrobial peptides by OmpU (Almagro-Moreno et al., 2015).

The motility and chemotaxis of V.cholerae are important aspects of early intestine colonization. V. cholerae of the wild type (chemotactic, motile) prefers to colonize the middle to the distal small intestine. Nonmotile or non-chemotactic mutants with clockwise flagellar rotation colonized the entire length of the small intestine. In contrast, motile but non-chemotactic mutants with clockwise flagellar rotation colonized just a portion of it (Butler & Camilli, 2004; Peterson & Gellings, 2018). The dispersed bacteria or planktonic cells swim to the intestinal mucosa and penetrate a highly viscous mucosal layer with mucin that acts as a barrier for the bacteria. In vivo colonization of V. cholerae is facilitated by N-acetyl-L-cysteine, a mucolytic agent (Millet et al., 2014) and haemagglutinin/protease (Hap), which is encoded by hapA, is a soluble mucinase produced by Vibrio cholerae, which can also assist in the breakdown of the mucin because a positive correlation between the ability of V. cholerae to pass through the mucus layer and the expression of hapA was observed in a column assay.

V. cholerae attaches to epithelial cells after penetrating the mucus layer and reaching the epithelium. V. cholerae seems to need to attach to epithelial cells to colonize the SI effectively. V. cholerae generates a variety of nonspecific adhesins that enables the bacterium first to identify whether it has found the suitable niche before completely attaching to that epithelial site. The flagellum (known to be involved in motility), Mam7, GbpA, OmpU, and FrhA are all adhesins that have been found in vivo and/or in vitro in V. cholerae (Almagro-Moreno et al., 2015).

In the last stage, V. cholerae develops microcolonies after adhering to the intestinal epithelium, mediated by TCP (Jude & Taylor, 2011), which is also an adhesion factor and promotes biofilm formation on chitin. According to recent research, microcolonies formed were clonal, but TCP stimulates the development of nonclonal aggregates in vitro via pilus–pilus interactions, which contradicts this observation. TCP may therefore have a role in microcolony formation in vivo via a process other than pilus–pilus contact and/or in collaboration with other variables (Silva & Benitez, 2016a).

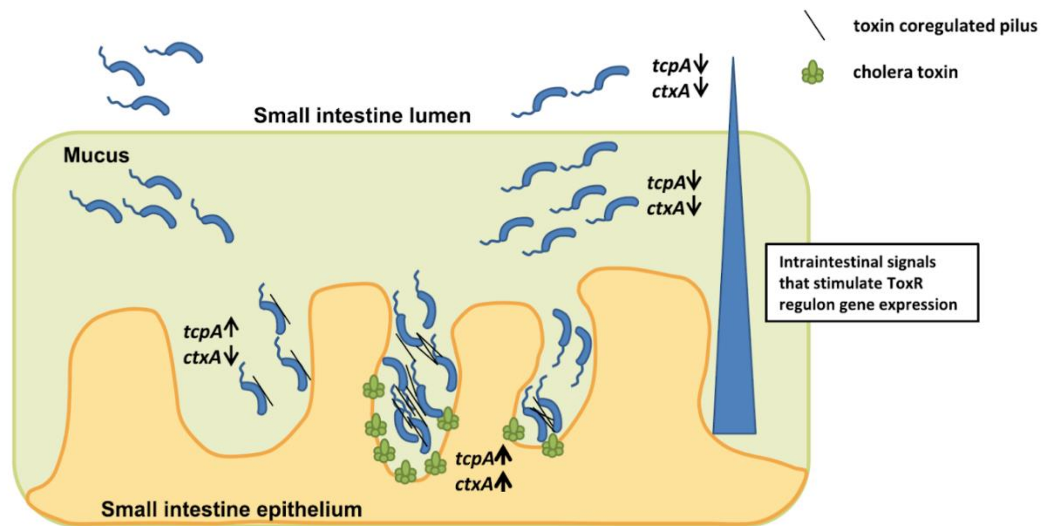


*Figure 14: Production of CT and TCP in small intestine (Peterson & Gellings, 2018)*

To cause the infection, vibrios express CT, which binds to the GM1 receptor in the apical membrane of intestinal epithelial cells. It has been indicated that inhibition of bacterial motility when bacteria adhere to the villi would increase CT expression, based on the negative relationship between motility and expression of virulence gene (Syed et al., 2009). Low bile and high bicarbonate levels enhance toxin delivery at this location, which is adjacent to the GM1 receptor. This spatiotemporal pattern of CT expression is in harmony with prior research showing that TCP expression leads to ctxAB transcription in vivo. Later, the bacteria negatively express the virulence factors and spread throughout the small intestine or return to the aquatic environment (Silva & Benitez, 2016a).

# Chapter 12: Possible ways to prevent Vibrio Cholerae infection and biofilms

## 12.1 Gut Microbiota

In recent years, researchers have begun to recognize the role of commensal gut microbes in enteric pathogen infections. New research combining genomics and machine intelligence links gut microorganisms to disease susceptibility. The generation of chemicals that modify V. cholerae intraspecies communication has been observed in studies of gut microorganisms that increase during cholera recovery. Inhabitant microbes of the gut disrupt V. cholerae biofilm production, a key element in colonization. Moreover, gut microbiota can suppress V. cholerae colonization by producing metabolites such as autoinducer signaling molecules, antimicrobial peptides, short-chain fatty acids, bile salts, and so on (Weil & Ryan, 2018). The gut microbiota's protective ability to withstand enteropathogenic infections is known as "colonization resistance." B. vulgatus can successfully reduce V. cholerae colonization and fluid buildup in the gut of a newborn mouse. The presence of B. vulgatus reduced V. cholerae colonization by 4.47-fold in the adult mouse gut (You et al., 2019).

## 12.2 Vps gene transcription deregulation scopes

The complicated regulation of vps gene transcription allows inhibiting the V. cholerae VPS-dependent biofilm. Many of the discovered regulators are linked to VPS production and biofilm development. V. cholerae, which uses a single polar flagellum to swim, must be immobilized to form biofilms. Deletion of flagellar synthesis genes increases vps gene transcription in numerous V. cholerae strains (Lauriano et al., 2004b; Watnick et al., n.d.). A mutation in the gene encoding the flagellar motor reduces vps transcription and biofilm formation. In contrast, treatment with phenamil, a selective inhibitor of the flagellar motor, reduces vps transcription and biofilm formation (Lauriano et al., 2004b).

CytR represses vps gene transcription (Haugo & Watnick, n.d.).CytR is a repressor of genes involved in nucleoside absorption and catabolism in Escherichia coli, and it is the same in V.

cholerae. Because sugar addition to the VPS exopolysaccharide needs phosphorylated nucleoside activation, nucleoside availability is a checkpoint in exopolysaccharide production and biofilm development (Kierek & Watnick, 2003; Moorthy & Watnick, n.d.).

Quorum sensing, a remarkable characteristic of bacterial communities, inhibits vps gene transcription at high cell densities. Autoinducers are a class of tiny chemicals that many organisms can make, secrete, and detect. Bacteria use autoinducer concentrations to determine cell density. It has been found that high quantities of a particular V. cholerae autoinducer inhibit vps gene transcription. This may regulate biofilm thickness by inhibiting biofilm association or allowing biofilm escape.

Recent study has focused on the involvement of cyclic diguanylate (c-di-GMP) in bacterial biofilm formation. The synthesis and degradation of c-di-GMP are tightly regulated in numerous organisms. GGDEF and EAL domains are involved in the production and breakdown of c-di-GMP. The genome of V. cholerae contains 31 GGDEF domain proteins, 12 EAL domain proteins, and 10 GGDEF/EAL domain proteins, indicating that this is an important method for gene transcription control. This is because at least one EAL protein has been found that represses VPS production but stimulates cholera toxin synthesis (Tischler & Camilli, 2005). Inhibiting c-di-GMP synthesis may increase cholera toxin production (F. H. Yildiz et al., 2001).

## 12.3 TCP gene transcription deregulation scopes

Tcp gene transcription regulation is as complicated as vps gene transcription regulation. Most tcp gene transcription alterations match ctx gene transcription alterations encoding cholera toxin (Haugo & Watnick, n.d.). Transcriptional activation of virulence genes tcp and ctx by ToxR and ToxS, two inner membrane-bound transcription factors. Hung et al. recently completed the first small-molecule screen for TCP and CTX expression inhibitors. This study used a V. cholerae strain with a tetracycline resistance gene chromosomally fused to the ctx promoter. This strain was grown in 384-well plates with 50,000 small molecules and tetracycline. Compounds that enhanced V. cholerae tetracycline sensitivity were studied as possible ctx gene transcription inhibitors. A total of 15 interesting compounds were discovered, with virstatin being the most researched. Virstatin

reduced TCP and CTX production by inhibiting ToxT. Oral virstatin inhibited V. cholerae colonization in the newborn mouse gut (Hung et al.,2005)

## 12.4 Scopes of Bacteriophage phage therapy

Learning about current research on bacteriophages can help us navigate a solution for vibrio cholera prevention and control. Bacteriophages (phages) are the most common and varied biological organisms on earth which are important drivers of bacterial diversity and community composition because they facilitate horizontal gene transfer (Hansen et al., 2019). It has a nucleic acid genome encased in a protein or lipoprotein coat or capsid, surrounded by lipid. It contains the information needed for its reproduction encoded in the phage nucleic acid. Moreover, phages are obligate intracellular parasites that require the host bacteria to reproduce (Bvsc&ah, 2015).

To reproduce intracellular phage, the virus particle adsorbs to the bacterial cell surface, injects its genome, and takes over the host metabolic machinery. The rupture of host cells and phage progeny completes the lytic cycle. Tail fibers help the lytic cycle's initial step, adsorption, by binding to phage receptors on the bacterial cell surface (Donlan, 2009). Phages can survive under harsh circumstances. Based on their survival tactics, phages have three lifestyles: lytic, lysogenic, and pseudolysogenic. A lytic phage enters a cell, grows, and bursts out, killing the bacteria. In a lysogenic life cycle, the phage does not multiply, but its genome does, and it is generally incorporated into the host genome or preserved as an extrachromosomal plasmid. Temperate phages can enter either the lytic or lysogenic cycle, while other phages are purely lytic. In a pseudo lysogenic life cycle, the phage neither lysogenizes nor lytically responds but stays inactive. When nutrition sources return, the phage can enter the lysogenic or lytic life cycle (Wei et al., 2011).

**Advantages:** Despite encouraging outcomes in some instances, antibiotic research has overtaken phage treatment. Antibiotic resistance in pathogenic microorganisms poses a severe danger to human health globally. Thus, phage treatment has regained popularity as an alternative therapeutic approach (Hansen et al., 2019; Letchumanan et al., 2016b). Humans have employed phage to treat infectious illnesses and to control biofilms. The traditional strategy for phage therapy has been to target pathogenic bacteria with obligate lytic phages, and the lytic capacity of phage mixtures has been used to decrease both mono species and dual-species biofilms. Some naturally generate

comparable depolymerases that allow breakdown or hydrolyze of polymers, thereby weakening the biofilm matrix's physical barriers and the capsular polysaccharides to get access to cell membranes and receptors. The fundamental purpose of many of these enzymes is to attack the cell wall and impair bacterial cells (Donlan, 2009; Hansen et al., 2019). Phages have already been used for biofilm treatment of Pseudomonas aeruginosa, E. coli, Staphylococcus aureus, and the results have been positive. Recent research in the USA looked into the usage of phages to treat biofilm disorders. While phage therapy could not wholly prevent biofilm development, it dramatically lowered biofilm biomass and cell density (Letchumanan et al., 2016b).

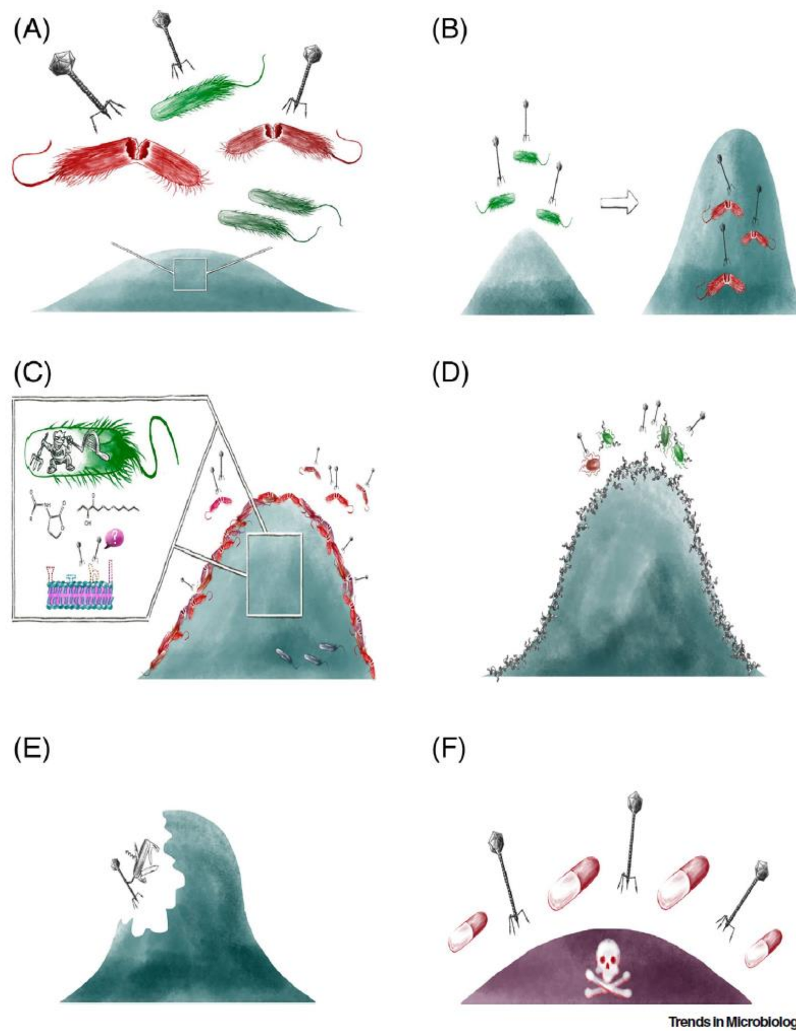


*Figure 15: Possible outcomes of phage-biofilm encounter. Adapted from (Hansen et al., 2019)*

Phages are effective against Gram-negative and Gram-positive bacteria, as well as multidrug-resistant diseases. There are several reasons why bacteriophages should be used to combat antibiotic resistance. A lytic phage can kill a bacterium, but antibiotics might not destroy it, allowing antibiotic resistance to evolve. Unlike antibiotics, phages are selective enough to minimize secondary infections and side effects and not change the gut microbiome. Unlike phages, antibiotics target all bacterial cells, creating secondary infections and allergies in the patient. Phages are eco-friendly and evolved through natural selection. Comparatively, developing new antibiotics takes years and requires extensive clinical studies before usage. (Letchumanan et al., 2016b).

**Disadvantages:** The disadvantages of phage should be considered, such as its limited host range, bacterial resistance to phage, and virulence genes encoded by a phage that can be incorporated into the host bacterial genome. The immune system might inactivate the phage; the phage preparations may also include endotoxin, which is dangerous. Though phage resistance is an emerging concern, the chances of this happening are 10-fold lower than antibiotics. Moreover, phage therapy can lead to the transferring of virulence genes to nonvirulent bacteria (Letchumanan et al., 2016b). Using phage mixes or specially designed phages may be an efficient way to get around these problems. Lytic bacteriophages may represent a novel anti-biofilm agent class. Concerns about antibiotic resistance have rekindled interest in phage treatment. Antimicrobial usage has grown in hospitals and other contexts. Antimicrobial resistance might be slowed by using phage to treat device-associated illnesses (Donlan, 2009). In the following section we discuss vibriophages which are the virus that target and eliminate vibrio cholerae specifically.

## 12.4.1 Vibrio phage that targets Vibrio Cholerae specifically

There are at least 200 bacteriophage species that infect V. cholerae, known as vibrio phages. Predatory phages target V. cholerae in aquatic and intestinal habitats, as observed initially a century ago (D’herelle & Malone, n.d.). Unlike lysogenic phages like CTX that encode the diarrhea-causing cholera toxin, lytic phages can change bacterial population dynamics by killing bacteria and releasing tens to hundreds of phage offspring.

The behavior of lytic phages that prey on V. cholerae was theorized in a 1927 paper linking cholera patient illness prognosis with co-occurrence of "strong or weak" phage (D'herelle & Malone, n.d.). After 75 years, phage predation was linked to naturally reducing cholera epidemic duration and intensity (Faruque, Naser, et al., 2005). In aquatic reservoirs, resistance and counter-resistance development may lead to cyclical patterns of rising V. cholerae populations, followed by phage amplification and bacterial decrease, followed by phage amplification and bacterial booms (Faruque, Naser, et al., 2005). Seasonal cholera outbreaks are said to be wiped off via phage predation (Bvsc&ah, 2015). A long-standing interest in cholera-associated phages has been sparked by these results and the use of phages to identify different forms of V. cholerae to combat cholera outbreak clonality and fresh appeal in harnessing phages to stop outbreaks of cholera (Yen et al., 2017). While cholera cases fluctuated seasonally, the presence of pathogenic V. cholerae strains in water samples often corresponded with the absence of detectable cholera phages. Interepidemic water samples had cholera phages but no live bacteria. The number of Vibrio phages capable of lysing a certain serogroup of V. cholerae was inversely correlated with the presence of a specific strain in most water samples. However, most water samples with V. cholerae O1 or O139 had no detectable phage that lysed the matching serogroup strain. These findings suggest that cholera phages can impact seasonality and even the formation of new pandemic serogroups or clones (Faruque, Naser, et al., 2005).

A study identified and sequenced 15 bacteriophages from stool samples of cholera patients over a 10-year surveillance period in Dhaka, Bangladesh. According to the findings, a single new bacteriophage type, ICP1, is present in all stool samples from cholera patients, but two other forms, ICP2 and ICP3, are temporary. ICP1 is a Myoviridae with a 126-kilobase genome and 230 open reading frames. Comparative sequencing analysis of ICP1 and related isolates show substantial genetic conservation. As the O1 antigen of lipopolysaccharide (LPS) functions as the ICP1 receptor, the prevalence of ICP1 in cholera patients suggests that ICP1 is well suited to predation of human-pathogenic V. cholerae O1 (Angermeyer et al., 2018; Seed et al., 2011).

Between 2001 and 2004, researchers in Dhaka, Bangladesh, explored the dynamics of V. cholerae-lytic phage interactions. These studies found an increase in cholera cases when lytic vibriophages in water decreased. Similarly, cholera epidemics tended to stop when virus concentrations in water surged. The environmental phage peak corresponded with increased phage excretion in cholera

patients' feces. Thus, late-stage phages reduce cholera transmissibility, finally causing the epidemic to collapse. Therefore, cholera phages in the environment and their multiplication in cholera patients significantly impact cholera epidemiology, primarily by eliminating the epidemic strain (Faruque & Mekalanos, 2012). Environmental phages may affect the epidemic incidence, cholera seasonality, and serogroup prevalence. The negative relationship between vibrio phage concentration and susceptible V. cholerae strains in water shows that outbreaks start during low phage concentration times (after floods and the monsoon season). Predictably, V. cholerae strains that were either phage-resistant (due to serogroup type) or lysogenic for widespread phages were immune to phage lysis. If lysogenic and nonlysogenic strains fought for the same environmental niche, the lysogenic bacterium would be provided with a ‘‘vibriocide" (i.e., phage) that might eradicate nonlysogenic competitors. Given the variations in lysogeny and susceptibility of clinical isolates to V. cholerae filamentous vibriophage, such territorialism might exist (Faruque, Naser, et al., 2005). Phages may also be necessary for pathogenic clone formation and territorialism among V. cholerae strains. This may have occurred through horizontal gene transfer and selection, as shown by the establishment and dominance of V. cholerae O139 in Bangladesh and India in 1992-93 (Shamim Hasan Zahid et al., 2008).

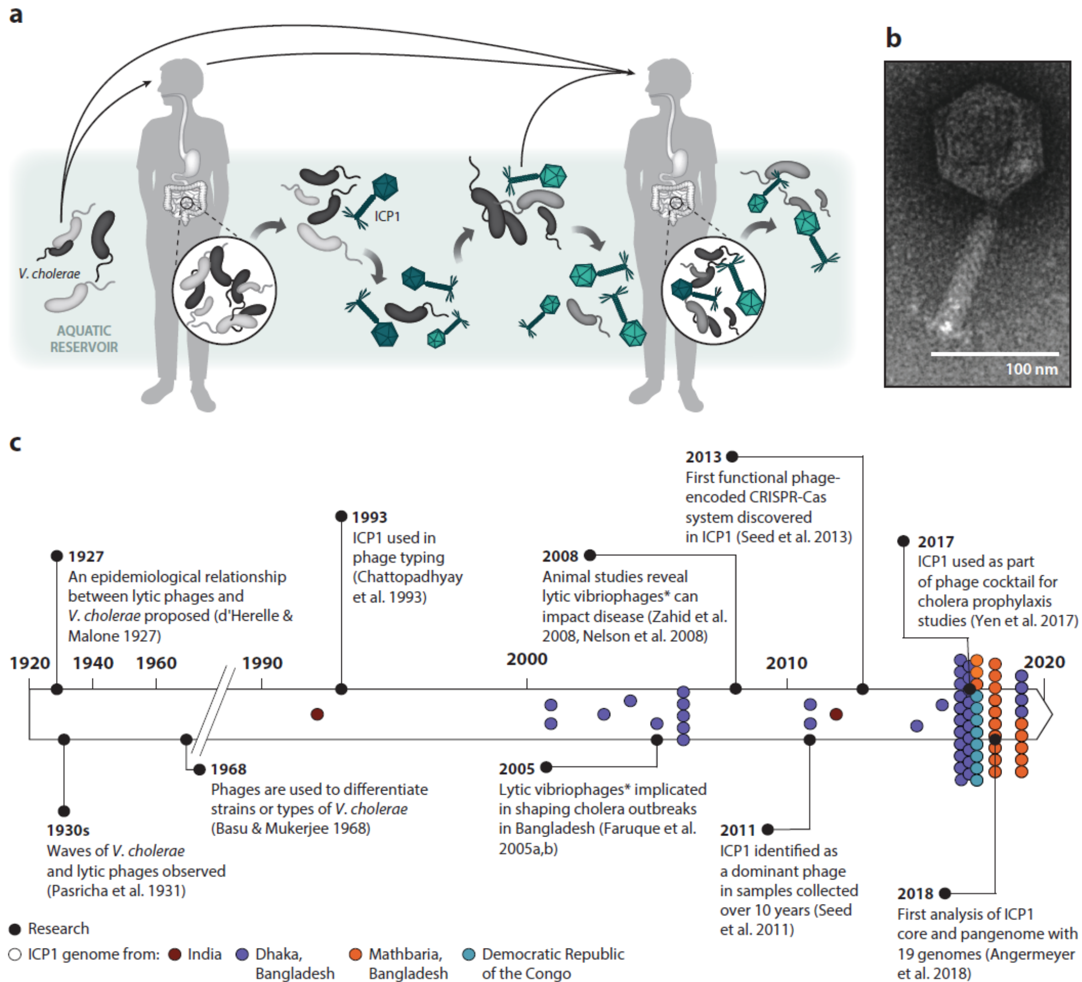


*Figure 16: ICP1 and epidemic Vibrio cholerae coexist in nature. (a) Toxigenic V. cholerae strains of the O1 serogroup invade the stomach, producing cholera. V. cholerae reproduces and shed in feces, reseeding the aquatic habitat. Predation by ICP1 (dark teal) on V. cholerae (light gray) causes selection of phage-resistant strains (black). Counter phage adaptations select novel ICP1 variations (light teal). Aquatic and intestine coevolution (appearance of dark gray bacteria) We don't know how phage predation affects illness outcome. (b) ICP1 electron micrograph (scale bar indicates 100 nm). This section highlights essential discoveries and sequenced isolates, color-coded by location of isolation, and the relevance of lytic phages in cholera epidemiology and ICP1 research. Asterisks denote ICP1 vibriophages utilized in this research (adapted from (Boyd et al., 2021))*

## 12.4.2 Vibrio phage cocktail for eradicating Vibrio Cholerae

As Jensen et al. predicted, if a rise in the environment's concentration of V. cholerae causes an epidemic, vibriophages would grow in density and promote a reduction in the outbreak's size (Jensen et al., 2006).

Water in cholera-endemic areas is known to have a wide range of phages that grow on V. cholerae and target distinct serogroups and strains. Phages generally influence the V. cholerae population through their predatory role. At the same time, the bacteria are known to withstand phage assault in numerous ways, including restriction-modification systems, mutations, receptor modulation, and biofilm development. Recent research examined the effect of providing three environmental phages on biofilm-associated V. cholerae in laboratory microcosms (4). In addition to degrading V. cholerae biofilm matrix, **one of the three phages could successfully kill planktonic** V. cholerae O1 and O139 cells. Thus, various phages may work together to control the incidence and spread of pathogenic V. cholerae in aquatic ecosystems. This is achieved by separating phages that destroy biofilms and phages that target planktonic V. cholerae cells to concurrently attack the extracellular polymeric biofilm matrix and bacterial organisms dispersed from biofilms. The findings may also help create effective phage-mediated water treatment methods to lower the danger of waterborne illnesses like cholera (4). Another study done for 5 years at the ICDDRB, Bangladesh, found that vibriophages in the host's feces reduce the infectious dosage of V. cholerae by tenfold (Nelson et al., 2007; Shamim Hasan Zahid et al., 2008).

Many nations have explored the efficiency of **five lytic vibrio phage cocktails** in treating Vibrio cholerae O1 biotype El Tor serotype O gawa MAK 757 (ATCC51352) infection in a rabbit model . Oral phage cocktail delivery following oral bacterial administration decreased bacterial shedding substantially (p 0.01). The rabbits seemed healthy and free of toxicity. It was determined that phage cocktail was more effective as a lytic agent than single phages. Oral delivery of an appropriate phage mixture might replace antibiotic therapy for cholera. In one investigation, an oral phage cocktail (ATCC-B1, B2, B3, B4, B5) was given to adult mice (Jaiswal et al., 2013). The study compared phage cocktails, antibiotics, and oral rehydration therapy for Vibrio cholerae infection. Vibrio phage B1, B2, B3, and B4 had roughly 40kb, whereas phage B5 had a genome size of

around 100kb. The marine phages could endure a pH range of 2–12 and a temperature range of 25–60C in vitro. The study found that compared to antibiotics and oral rehydration, the five-vibrio phage cocktail decreased the amount of Vibrio Cholerae cells in infected mice (Jaiswal et al., 2014). Previously, a phage cocktail was tested in a Vibrio cholerae 01 infected RITARD (removable intestinal tie-adult rabbit diarrhea) model. The phage combination provided considerable protection and prophylaxis against Vibrio cholerae infection (Bhowmick et al., 2009; Letchumanan et al., 2016b).

Newborn mouse and infant rabbit models of colonization and sickness were protected against V. cholerae infection when given an **oral cocktail of three virulent phages** (designated ICP1, ICP2, and ICP3) that had been identified from rice-water stool samples taken from cholera patients in Bangladesh (Reyes-Robles et al., 2018; Yen et al., 2017). To our knowledge, the efficiency of the ICP cocktail in killing V. cholerae cells is influenced by phage concentration in the gut during infection. The digestive tracts intricate design presumably hinders phage access to V. cholerae cells. The cocktail's composition and dosage might be improved in the future. The cocktail's phage ratio may be adjusted, and more phages could be introduced to kill V. cholerae. Also, while shorter prophylaxis duration resulted in 100% bacterial load reduction, optimizing the timing for each dosage is required before human usage. As a result of recurrent exposure to V. cholerae, numerous treatments may be required to entirely eliminate the illness (Yen et al., 2017).

### 12.4.3 Vibrio Phage-associated epidemiological model

In epidemic regions, cholera's incidence curve has a classic form, with a steady climb until a peak, then a quick decline. Even though cholera outbreaks like this often occur in endemic areas like Bangladesh, the cholera outbreaks are primarily self-limiting. Although environmental conditions have been linked to cholera outbreak, they do not sufficiently explain the epidemic's quick demise. For example, in a temperate climate, temperature and rainfall may not fluctuate much throughout an epidemic. The population developing sufficient immunity to halt the pandemic is also ruled out since outbreaks in endemic areas like Bangladesh return every year. During the pandemic, more cholera patients expelled both V. cholerae and the lytic phage JSF4, a vibrio phage that attacks V.

cholerae bacteria. JSF4 is one of the vibrio phages among many others that have been discovered in Bangladesh. As pandemic proceeds, the levels of V. cholerae and JSF4 phage in the environment increase. Finally, the environmental V. cholerae population collapses with the environmental phage plateau. The findings imply that the phage-induced death of the epidemic V. Cholera strain causes the ambient V. cholerae population to drop and the epidemic to end. Studies in the last several years have shown that an increase in the hyper infectious V. cholerae population drives cholera outbreaks and, moreover, a rise in virulent phages in sick persons and environmental reservoirs quenches outbreaks (Silva-Valenzuela & Camilli, 2019).

The results mentioned above can explain why cholera outbreaks are self-limiting in nature. When the balance of phage and bacteria favors the phage, the quantity of bacteria drops dramatically, causing the epidemic to collapse. Moreover, under normal settings, phage-resistant descendants of the epidemic strain do not rise quickly enough to maintain it. With the reemergence of the same strain during the next season's cholera season, it's clear that some virus cells survive the phage assault. Surviving phage assaults in the environment may be a crucial part of the cholera epidemic cycle. The metabolic state of V. cholerae cells and the optimal expression of phage receptors may impact phage infection susceptibility (Shamim Hasan Zahid et al., 2008).

The latest findings of the same study recommend revising a cholera epidemiological model. According to the newly revised model, an epidemic starts when human victims multiply V.cholerae strain by ingestion, intestinal colonization, and finally dissemination of hyper infectious V. cholera into the environment through stool. This produces an index case. As more cholera patients succumb to the disease, phages and the epidemic clone spread, increasing phage concentration in the environment, decreasing V. cholerae concentration. The domain cannot maintain a high load of live V. cholerae due to phage predation, and the infectious dosage rises due to the harmful impact of congesting phage with the inoculum. So, the balance swings quickly in favor of the phage, causing the epidemic to collapse. However, phage concentrations decrease over time due to physicochemical changes in the water or being swept away by floods or rain. As a result, V. cholerae strains with epidemic potential may re-grow, aided by a single clone of V. cholerae dominating the following epidemic cycle. If this idea is right, cholera phages like JSF4 might be utilized to stop epidemics in their tracks (Faruque, Johirul Islam, et al., 2005).

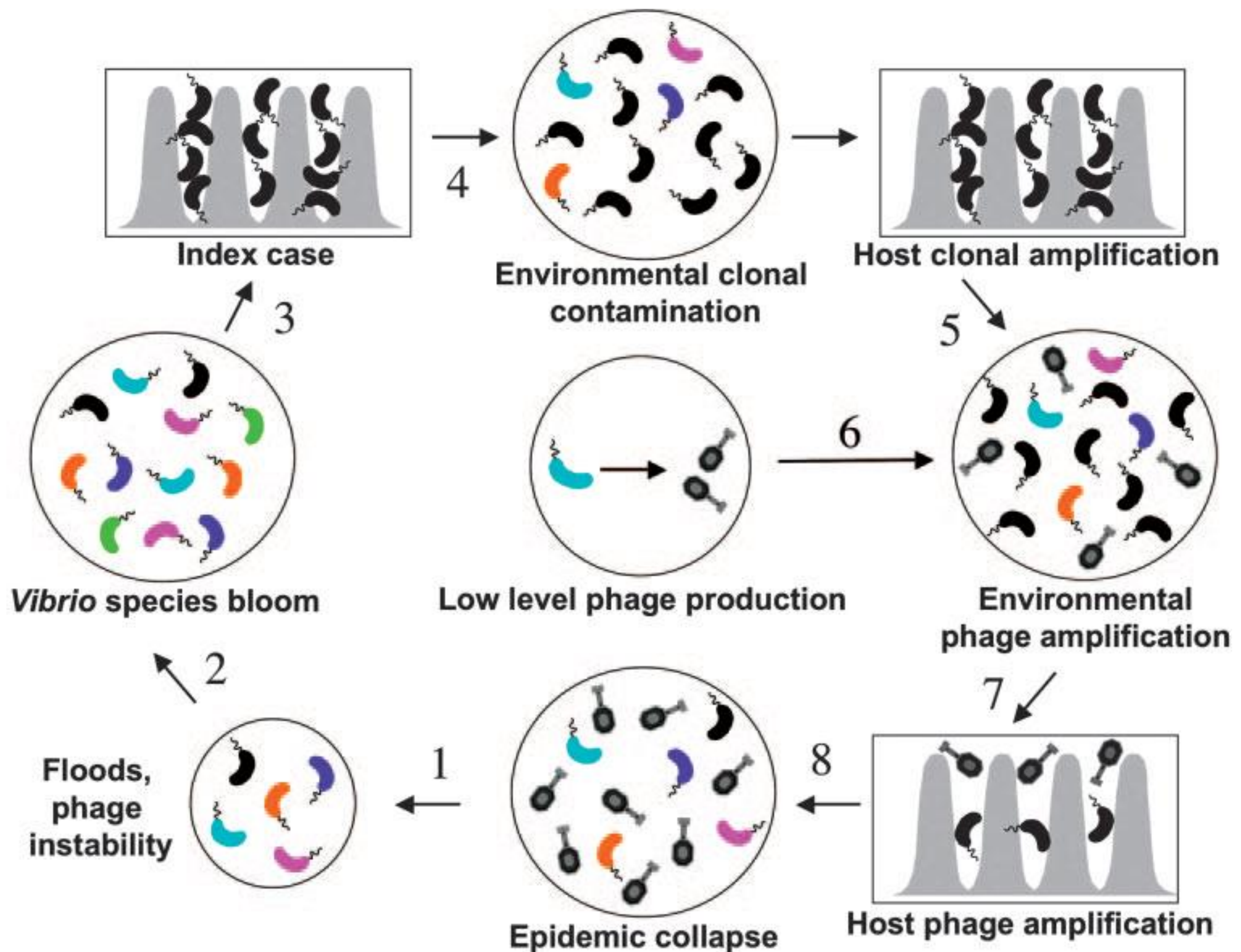


*Figure 17: Factors affecting seasonal cholera outbreaks. Environmental events are illustrated in circles, whereas human cholera fatalities are indicated in rectangles. Fluctuations or other physical-chemical variables that cause phage instability to reduce the number of viable aquatic lytic parasites. A bloom of non-pathogenic and pathogenic Vibrio species in local water sources. The setting in which the index case is infected by the pathogenic V. cholerae strain (black vibrios) is favorable. 4, The pathogenic clone's amplification seeds the environment. A pathogenic clone multiplies in the host and pollutes the environment, triggering an epidemic. Sixth, Phages formed by ambient Vibrio species (through lysogen induction or lytic growth) start to amplify on the pathogenic clone. The pathogenic clone and lytic phage are progressively consumed by Cholera sufferers, leading to phage proliferation in vivo. Eighth, phage shed by cholera sufferers infected the environment, amplifying phage on the infectious clone. The lowering concentration of the pathogenic clone in the environment and the increase in ambient phage concentration all contributed to a significant drop in the incidence of cholera transmission in the interepidemic period (Faruque, Johirul Islam, et al., 2005).*

## 12.4.4 How Vibrio cholera becomes resistant to vibriophages

To combat phage infection, bacteria employ a wide range of defense methods, including restriction-modification, abortive infection, cell surface receptor phase variation, phage-inducible

chromosomal islands, phage-inducible chromosomal islands, and bacteriophage exclusion systems and CRISPR-Cas systems (Reyes-Robles et al., 2018).

A mutation in the rfb gene cluster, which encodes the enzymes for LPS104 production, makes V. cholerae O1 resistant to.139 and many other vibrio phages. The vibrio phage cannot bind or infect bacteria without the LPS O antigen receptor. All studied rfb-null mutants of V. cholerae O1 are attenuated (Nelson et al., 2009b).

OMVs are nonreplicating spheres formed by bacteria. These structures are made up of lipids, proteins, and periplasmic components. OMVs have been found to help bacterial cell contacts and convey cargo proteins, toxins, and nucleic acids to nearby bacteria and host cells. Vaccination of adult female mice with V. cholerae OMVs protects their suckling pups from V. cholerae colonization. Others have investigated OMVs' ability to operate as decoys, which bacteria use to shield themselves from external assaults, including antibiotics, antimicrobial peptides, and bacteriophage infection. A study investigated whether V. cholerae OMVs can protect against the pathogenic phages ICP1, ICP2, and ICP3. While OMVs from two modern cholera pandemic strains can partially inhibit (neutralize) all three phages, this neutralization is receptor-dependent for two of the phages whose receptors have been identified, the presence of V. cholerae OMVs in aquatic reservoirs, biofilms, and the human intestinal tract is unknown. Also, no OMV mutants of V. cholerae have been discovered yet; therefore, the significance of OMVs in phage protection during infection or in aquatic microcosms remains unknown. Since V. cholerae produces OMVs, it is plausible to presume that OMV protection is proportional to OMV production rate and cumulative OMV concentration (Reyes-Robles et al., 2018).

Cholera outbreaks are self-limiting in nature since they diminish after a peak without active human intervention. However, how many bacteria survive to seed the environment for the next epidemic season is unknown. Thus, environmental or genetic alterations that increase bacterial resistance to phage predation may drive V. cholerae evolution. Toxin resistance may be exhibited by the metabolic condition of V. cholerae cells, insufficient production of phage receptors, or other phage-bacterium interactions mutations. These resistant descendants frequently lack the O1 antigen, which serves as the phage's receptor. Mutations in the cyaA or crp genes, which encode adenylate cyclase or cyclic AMP receptor protein, respectively, altered the susceptibility of V.

cholerae O1 strains to phages and the susceptibility linked with the bacteria's capacity to adsorb these phages. These findings imply that cAMP-CRP-mediated phage adsorption downregulation may help V. cholerae strains survive phage predation in the environment (Faruque & Mekalanos, 2012).

Another study discovered that quorum sensing, controlled by signal molecules called autoinducers, may protect V. cholerae against predatory phages. Virus cholerae mutants with inactivated AI synthase genes were more sensitive to phages than the original bacterium. Secondly, Phages typically use pathogenic V. cholerae receptors such cell surface lipopolysaccharide O side chain polysaccharides. So, bacteria mutants that have lost cell surface receptors essential for phage invasion may have a survival benefit. Thirdly, Exogenous autoinducers CAI-1 or AI-2 generated by recombinant strains harboring cloned AI synthase genes boosted V. cholerae survival and decreased phage titer in phage-bacteria mixed cultures. The effects of autoinducers appear to be mediated in part by quorum sensing-dependent haemagglutinin protease synthesis and in part by phage receptor downregulation. These findings may help create phage-mediated cholera control techniques (Hoque et al., 2016).

# Chapter 13: Conclusion

For many years, the molecular processes involved in V. cholerae biofilm development and their function in the ecological tenacity of the disease-causing bacteria have been widely investigated. The roles of extracellular matrix polysaccharides (VPS) and matrix proteins in generating a well-structured biofilm and the regulators involved in modulating biofilm gene expression have been recognized. However, there are more scopes of further investigation and discovery in the field of biofilm regulation. Additionally, more research is required to understand the dispersion process of V.cholerae from their biofilms (Teschler et al., 2015b). Moreover, extensive research is needed regarding the infection caused by the V.cholerae biofilms in humans. Since, with our insufficient knowledge, it has been suggested that biofilms created in the small intestine may be architecturally distinct from static biofilms formed in the LB medium (Silva & Benitez, 2016a). It has been indicated that V. cholerae may use environmental cues to synchronize gene expression and repression at various steps of the infectious cycle. Due to a lack of appropriate animal models, we are still understanding V. cholerae-host interactions. The predicted interaction between commensal bacteria and host signals to regulate V. cholerae gene expression is unknown after bacteria reach the small intestine during colonization. This creature's ability to perceive and respond to so many environmental cues require further study (Rothenbacher & Zhu, 2013).

Furthermore, it has been discovered that biofilm acts as a protective layer for the bacteria within it to protect against predation by protozoans or antimicrobial attacks, which still needs to be more explored; however, such protection makes the bacteria resistant to antibiotics, making it untreatable. Hence, With the rise of antibiotic-resistant bacteria, scientists have realized the necessity to investigate phages' medicinal potential. Understanding the molecular biology of phage–bacteria interactions help. Some studies have paved the way for bacteriophages to treat acute cholera infections instead of antibiotics. As multidrug resistance spreads and the manufacture of new chemical antibiotics slow, the area of bacteriophage treatment is quickly expanding. So that phage medications may best answer today's medical demands, the scientific community's discoveries must be combined with decades of phage treatment expertise in some areas of the globe.

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