Effect of temperature and heavy metals on biofilm formation of vibrio cholerae

By

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A thesis submitted to the Department of Biotechnology in partial fulfilment of the requirements for the degree of M.Sc. in Biotechnology.

Department of Biotechnology

BRAC University

Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material that has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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Approval

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of Fall, 2023 has been accepted as satisfactory in partial fulfilment of the requirement for the degree of M.Sc. in Biotechnology on 5/01/2026

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Abstract

Vibrio cholerae, is a waterborne pathogen capable of persisting in aquatic environments through its ability to form biofilms, which allow survival, transmission, and increased resistance. Environmental temperature fluctuation and heavy metals play critical roles in influencing biofilm development. This review discusses current research showing that temperature and heavy metals are two of the most important abiotic factors influencing *V. cholerae* biofilm development, architecture, and importance to public health.

Temperature is one of the key regulators that dictate the planktonic–biofilm transition. Generally low to moderate temperatures (15–25 °C) promote *V. cholerae* biofilm, and this correlates with an increase in intracellular c-di-GMP, activation of the regulatory network, activation of cold-adaptation pathways, and increased expression of adhesive proteins, e.g. MSHA pili and GbpA. These responses support *V. cholerae* attachment and survival in seawater during seasonal transitional periods. The exposure to heavy metals such as Cd, Cu, Pb, Zn, Ni, Hg, and As imposes oxidative stress, destabilization of the cellular membrane, and inhibition of multiple enzymes. *Vibrio cholerae* can manage its level of toxicity by regulating it through a collection of metal responsive regulators. In addition, toxic metal stress frequently selects for enhanced EPS production, thicker biofilms, and co-selection of metal tolerance, biofilm proficiency, and potentially antimicrobial resistance. The review suggests that temperature acts as a primary regulatory factor, while metal toxicity would function solely as a selective pressure for regulating biofilm architecture. The survival strategy of bacteria may interact synergistically in cholera endemic estuaries where there are both seasonal temperature increases, along with increased industrial discharge, VBNC transitions, and outbreak timing.

Notable knowledge gaps include limited experiments on temperature and heavy metals affecting *V. cholerae* biofilm formation and persistence in aquatic ecosystems. Therefore, it needs to resolve molecular cross-talk between metal stress signalling, quorum sensing, and temperature-controlled c-di-GMP pathways. Integrating metal monitoring for cholera, WASH interventions, and the need for implementation of One Health frameworks as methods for reducing the persistence of the environmental reservoir and the potential for outbreaks.

Keywords: *Vibrio cholerae*, biofilm formation, temperature variation, heavy metals, environmental stressors, ecosystems, bacterial persistence, environmental adaptation, public health risk.

Dedication

This piece of work is dedicated to my husband, whose constant support and patience and encouragement made this work possible.

Acknowledgement

I would like to take this opportunity to thank my supervisor, Associate Professor and Chairperson Iftekhar Bin Naser.

Thank you, Sir, for providing guidance and support at every step of this journey.

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List of Acronyms and Abbreviations

AMR – Antimicrobial resistance

AMP – Adenosine monophosphate

ATP – Adenosine triphosphate

Bap1 – Biofilm-associated protein 1

C6706 – Vibrio cholerae El Tor strain C6706

Cd – Cadmium

c-di-GMP – Cyclic diguanylate monophosphate

Cr – Chromium

CT – Cholera toxin

Cu – Copper

DGC – Diguanylate cyclase

DNA – Deoxyribonucleic acid

eDNA – Extracellular DNA

El Tor – El Tor biotype of *V. cholerae*

EPS – Extracellular polymeric substances

Fe – Iron

GbpA – N-acetylglucosamine-binding protein A

GMP – Guanosine monophosphate

HapR – HapR (quorum-sensing master regulator in V. cholerae)

Hg – Mercury

LPS – Lipopolysaccharide

MDR – Multidrug Resistance

Mn – Manganese

MSHA: Mannose-sensitive hemagglutinin (adhesin/pilus system)

mshA – mshA gene (MSHA pilus component)

Ni – Nickel

O1 – V. cholerae serogroup O1

O139 – V. cholerae serogroup O139

Pb – Lead

PDE – Phosphodiesterase

P-type ATPase – P-type ATPase (membrane ion transporter family)

QS: Quorum sensing

RbmA: Rugosity and biofilm structure modulator A

RbmC: Rugosity and biofilm structure modulator C

ROS: Reactive oxygen species

SST: Sea surface temperature

UV: Ultraviolet

V.: *Vibrio*

V. cholerae: Vibrio cholerae

VBNC: Viable but non-culturable

Chapter 1

Introduction

1.1 Background and Significance

A microscopic single-celled organism, bacteria, are found worldwide in millions. They prefer to live in a different environment due to their cellular structure and metabolic processes. Some are responsible for causing diseases, such as *Vibrio cholerae*. This curved-shaped gram-negative bacterium is responsible for causing enteric disease like diarrhoea. *Vibrio cholerae* can persist in an aquatic environment on biotic or abiotic surfaces by forming biofilm. Formation of biofilms enhances the survival ability of the bacteria, and those cells serve as reservoirs for infection. That is why understanding the factors that influence biofilm formation in seasonal variation can help interpret cholera outbreaks.

Among those numerous factors, the most crucial factor is temperature. Since *V. cholerae* is mesophilic and tends to grow between 20°C and 45°C. The bacterium tends to live in warm environments (~ 37°C) inside the human body. It also shifts their living temperature into (4-30)°C depending on the season and geographical changes when it is outside the body (Colwell et al., 2003). However, shifting the temperature to cooler in the aquatic environment initiates them to form Biofilm. In the lower temperatures, the survival strategy of *V.cholerae* produces cold shock proteins to adapt to the environmental changes. Nearly 5 million cases of cholera infection are annually reported, and the number of deaths is 0.1million (Conner et al., 2016). Because of its ability to produce toxins and infect the human gut, annual outbreaks also occur in different parts of the world, notably in Asia, Africa, and the Americas (Alam et al., 2006).

Outbreak of *Vibrio cholerae* depends on various environmental factors, including metals, salinity, and plankton (Huq et al., 2005). Heavy metals such as: cadmium (Cd), copper (Cu), lead (Pb), zinc (Zn), mercury (Hg), and arsenic (As) influence a significant number of microbial colonies and biofilm formation. Environmental conditions, temperature changes, and exposure to heavy metals affect the formation of biofilm. Fluctuation of the temperature influences the gene expression, and cyclic-di-GMP signalling is also responsible for the alteration of the growth of the *Vibrio cholerae* and biofilm formation. Biofilms are cell aggregates or surface-attached bacterial communities enclosed in an extracellular matrix. In the aquatic environment, *V. cholerae* is highly pathogenic as it forms biofilms on the surfaces of plankton, which greatly increases its pathogenic ability for survival (Broza et al., 2008).

Additionally, biofilm-like aggregates of *V. cholerae* have been found in the surface waters of Bangladesh in a partially dormant “conditionally viable” state (Faruque et al., 2006). The

combined effect of temperature fluctuations and heavy metal stress can be more alarming. Countries (Bangladesh, India) where cholera occurs frequently due to seasonal changes might face the extreme scenario. Rising temperature and climate change, with a high population where industrial toxic materials are used, can modify the ecological balance of *V. Cholerae.* The developed survival strategies may also initiate biofilm formation. Despite the importance of those two significant factors, the scientific study has rarely focused on the two combined factors. This review focuses on the findings from recent research to highlight how these abiotic factors (temperature and heavy metals) regulate the biofilm formation of Vibrio cholerae.

1.2 Temperature as a key Environmental factor

Temperature is one of the most significant factors of *V. cholerae* survival and behavior. Environmental studies show correlations between sea–surface temperature (SST), plankton blooms, and cholera incidence. Studies reveal that lower temperatures (15–25 °C) enhance biofilm formation, while higher temperatures (≥37 °C) suppress biofilm formation and promote motility and virulence traits.

Mechanistically, temperature influences:

- c-di-GMP signaling(increasing at low temperatures)
- Expression of biofilm regulators (VpsR, VpsT)
- Cold-shock proteins (CspV)
- Temperature-dependent translation factors (BipA)
- Adhesin production (MSHA pili, GbpA)

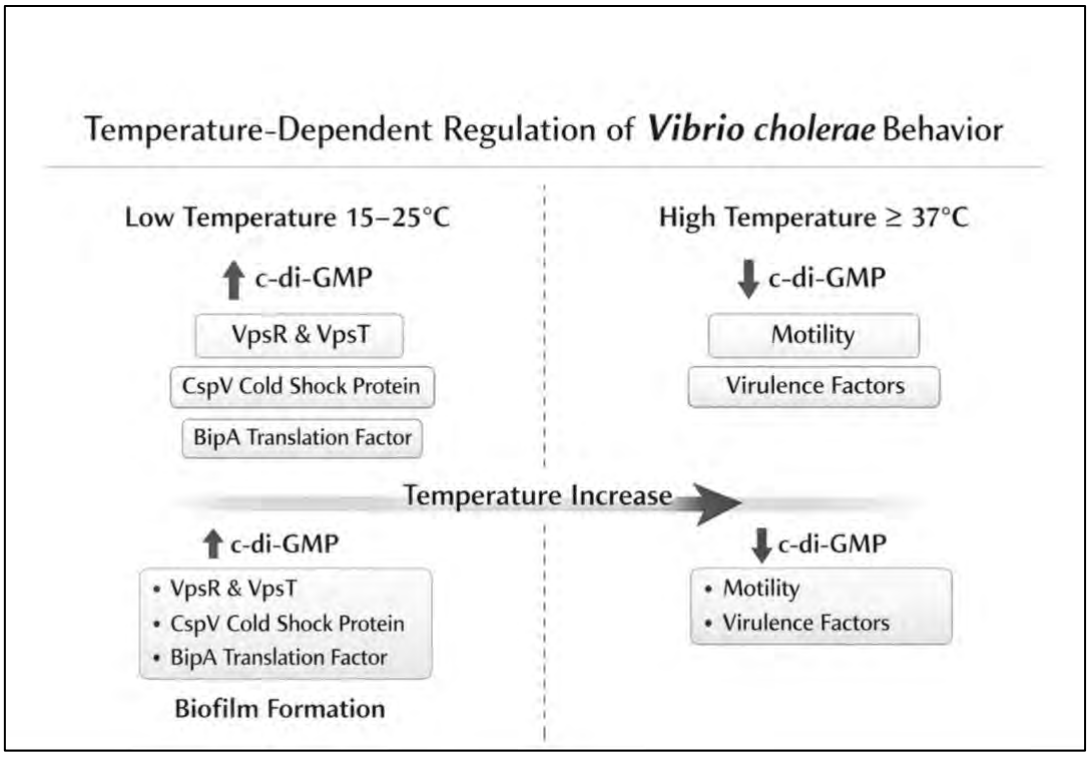

Figure 1: Temperature regulation of bacterial biofilm formation

Chapter 2

2.1 Taxonomy

Vibrio cholerae is a Gram-negative, motile, curved rod-shaped bacterial pathogen of the Vibrionaceae family that resides in an aquatic reservoir and infects humans (Finkelstein, 1996). It is classified within the phylum Gammaproteobacteria, order Vibrionales and genus Vibrio. Taxonomic investigations on related species have shown a tight connection between the three genera Vibrio, Aeromonas, and Plesiomonas.

The International Subcommittee on Taxonomy of Vibrios proposed a transitional classification that eliminated the bulk of species formerly classified as *Vibrio*. The distinguishing methods of differentiate individuals of the genus Vibrio from related genera based on biochemical features.

2.2 Biotypes

Serogroups of *V. cholerae* strains are characterised by the structure of their cell surface lipopolysaccharides. Only the V. cholerae serotype O1 and serotype O139 infections in humans are associated with an epidemic form of cholera; these two infections cause cholera due to cholera toxin (CT). The cholera pandemics associated with the approximately 200 existing serogroups are due to the cholera toxin produced by the O1 and O139 serotypes (Kanungo et al., 2022). The O1 strain differs in the severity of clinical symptoms and the expression and regulation of key virulence factors (Mandal & Mandal, 2014). 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 (Samanta et al., 2015). However, few infections from strains classified as toxigenic strains. The majority of these cases are not expected to contribute to any significant medical problems.

2.3 Evolution of biotypes

Seven pandemics have been reported, each caused by the classical biotype until it was eliminated from existence. (Safa et al., 2008). In 1961, the El Tor biotype took over as the dominant epidemic strain and became responsible for the longest ongoing seventh pandemic (Safa et al., 2008).

Although serogroup O139 caused large deaths during the major epidemics of the 1990s, the El Tor strain is still responsible for most cases worldwide (Parvin et al., 2021). 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.

It is believed that the El Tor types of cholera are less harmful to the environment than the classical type. The preservation of a primarily El Tor genomic structure with multiple genomic islands homologous to the classical strain supports the conclusion that El Tor and classical type cholera strains are genetic hybrids. El Tor variants have been correlated with increased adaptability to environmental change; increased capacity for causing disease; increased severity of disease; and inter-region migration on a global basis.

2.4 Morphological and Physiological Characteristics

V. Cholerae is a facultative anaerobe and can survive in high pH. Nearly found all over the world as it can grow in approximately 4-42°C. However, the optimum temperature of growth is 30 - 37°C. The bacterium contains flagella for motility, which also helps in chemotaxis to uptake nutrients such as chitin, detritus and planktonic organisms.

V. cholerae can withstand the salinity fluctuation, high osmotic pressure and nutrient limitation, which allows it to be well-suited for survival in dynamic aquatic environments. The organism transitions between smooth, culturable forms and rugose variants, which display enhanced exopolysaccharide production and biofilm capacity.

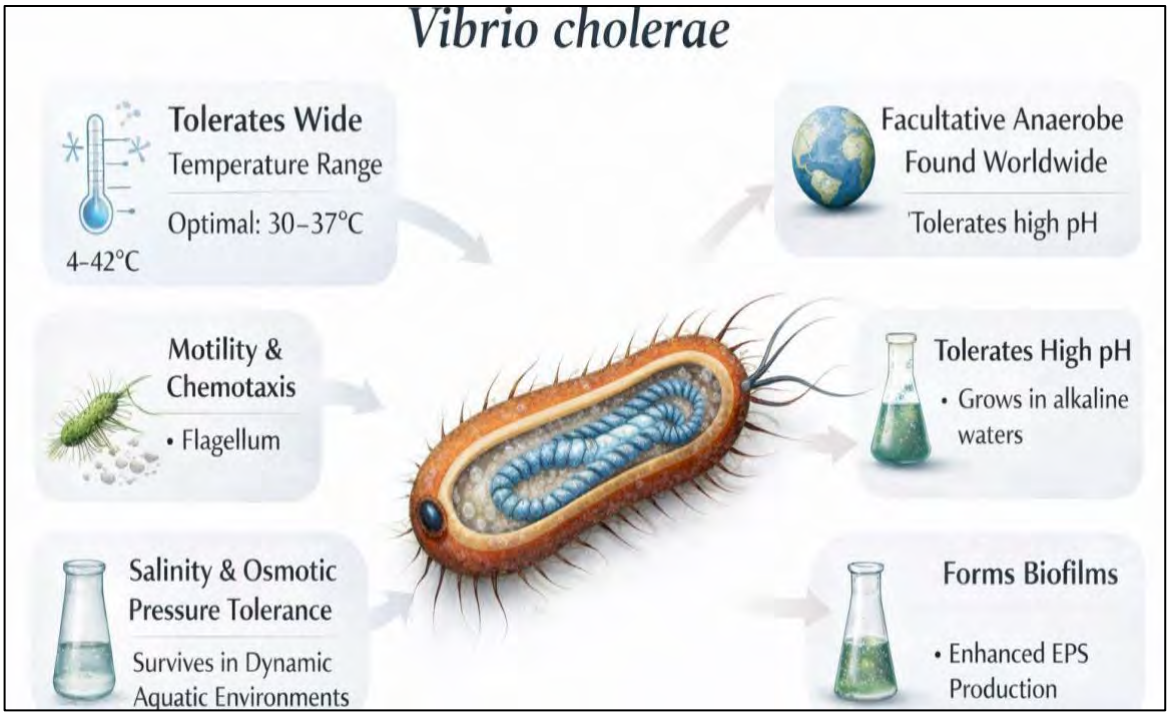

Figure 2: Characteristic of *vibrio cholerae*

2.5 Environmental Reservoirs and persistence as Biofilm

The species inhabits marine, estuarine, and freshwater environments and has strong associations with zooplankton, phytoplankton, shellfish, algae, and chitinous surfaces (Erken et al., 2015). Adhesions such as MSHA pili and GbpA help *Vibrio* spp. to attach the zooplankton surfaces. During infection on the human host, *Vibrio* colonises in the small intestine (~37°C), where biofilm formations are suppressed. However, upon release into the aquatic environment, those bacteria undergo environmental stresses which trigger the formation of biofilm because of their survival nature (Zhao et al., 2023). Changes in temperature and heavy-metal presence act as environmental signals that trigger significant changes in gene expression. Biofilms of *V. cholerae* made of microcolonies, extracellular polymeric substances (EPS), matrix proteins (RbmA, RbmC, Bap1), cell–cell adhesins, and structural polysaccharides VPS1 and VPS2 (Giglio et al., 2013). Biofilm gives protection against environmental stresses such as: lack of nutrition, oxidative damage and toxic compound presence. (Mahto et al., 2022). During inter-epidemic periods, bacteria undergo a lot of shock; these critical cycles are maintained by the biofilm. Temperature has a high impact on

bacterial motility, and at low temperatures (15 - 25°C), biomass and matrix production started to increase. C-di-GMP signalling, and biofilm matrix gene expression also have an effect on coordination with the temperature increase. Metals such as Cd^{2+}, Pb^{2+}, and Cu^{2+} have been reported to induce biofilm formation as a protective stress response in vibrios and other aquatic bacteria (Rather et al., 2021). Vibrio cholerae facilitates large-scale transmission following climate or pollution changes as it changes its nature due to environmental changes.

Chapter 3

3.1 *Vibrio cholerae* Biofilms

Biofilm is a survival feature of *Vibrio cholerae*, which helps it persist in aquatic environments, surface-sensitising pathways in an extracellular matrix that gives protection in a harsh environment, converting (Teschler et al., 2015).

Across bacterial species, biofilm develops in a multistage process:

(i) initial attachment

(ii) microcolony formation and maturation into a three-dimensional community.

(iii) dispersal of cells to seed new niches (Pombo et al., 2022).

Planktonic cells adhere by a single flagellum with surfaces. Those attachments activate surface-sensing pathways and adhesion expression (e.g., MSHA pili, GbpA, biofilm-specific adhesins) converts this reversible attachment into irreversible anchoring (Teschler et al., 2015b). Extracellular polymeric substances (EPS), Vibrio polysaccharides (VPS) and matrix proteins are secreted by adhesive cells to form microcolonies. The cell's coordination drove them to more matrix production and form two-dimensional surface-associated clusters to three-dimensional, tower-like structures (Yan et al., 2016).

Endogenous signals trigger the activation or deactivation of matrix production. In *V. cholerae*, dispersal is mediated by enzymatic degradation of VPS, modulation of c-di-GMP levels, and expression of motility-associated genes, allowing cells to escape from mature biofilms and colonize new surfaces (Bridges et al., 2020). In *V. cholerae*, this canonical progression is conserved but exhibits species-specific molecular underpinnings and architectural features (Teschler et al., 2015c).

3.2 Structure of biofilm:

V. cholerae forms highly organized biofilms with reproducible architecture. Early-stage biofilms begin as monolayers of rod-shaped cells aligned parallel to the surface. As divisions proceed, vertical growth induces a 2D to 3D transition, yielding three-dimensional clusters characterized by anisotropic mechanical stresses and directional cell arrangements (Yan et al., 2016b). Matrix

envelopes encasing these clusters, composed of VPS and matrix proteins (RbmA, RbmC, Bap1). Pellicles or surface films also have matrix-mediated surface tension effects (Teschler et al., 2015d). Pellicles formed at fluid interfaces exhibit complex morphogenesis, including wrinkling and fractal patterns driven by mechanical instabilities in the matrix, coordinated by specific matrix proteins and quorum-sensing regulators (Qin & Bassler, 2022).

These physical properties influence not only resilience but also dispersal, as cracks and fractures can release multicellular clumps that remain infectious. The extracellular matrix of *V. cholerae* biofilms is a composite of polysaccharides, proteins, lipids, and extracellular DNA. The primary polysaccharide scaffold of Vibrio cholerae biofilms has two specified gene clusters, vps-1 and vps-2, which are required for the maturation of biofilm. This structure creates a gel-like network that surrounds cells, maintaining biofilm integrity and providing protection against environmental stress. Mutations in vps genes can drastically reduce the amount of biofilm production, which can demolish the persistence of infection-causing ability (Teschler et al., 2015e). RbmA, RbmC, and Bap1 are the three dedicated matrix proteins that ensure the persistence of V. cholerae biofilms (Giglio et al., 2013b).

RbmA is a periplasmic protein that localizes primarily at cell–cell interfaces and promotes cell clustering of microcolonies. Deletion of rbmA results in loosely packed clusters and altered biofilm morphology (Smith et al., 2015). RbmC is a large, secreted protein that forms flexible envelopes around cell clusters. RbmC binds VPS surfaces, contributing to adhesion to biotic interfaces. Loss of RbmC compromises the encapsulation of clusters and impairs adhesion to certain host-derived surfaces (Huang et al., 2023). Bap1 is enriched at the biofilm–substrate and plays a significant role in maintaining pellicle integrity (Kaus et al., 2019). Bap1 gives mechanical strength to pellicles and assists in attachment at fluid interfaces under stress (Hollenbeck et al., 2014). These proteins form a hierarchical system where VPS provides a scaffold; RbmA holds cells together; RbmC and Bap1 attach clusters to surfaces, and control pellicle morphology (Teschler et al., 2015f).

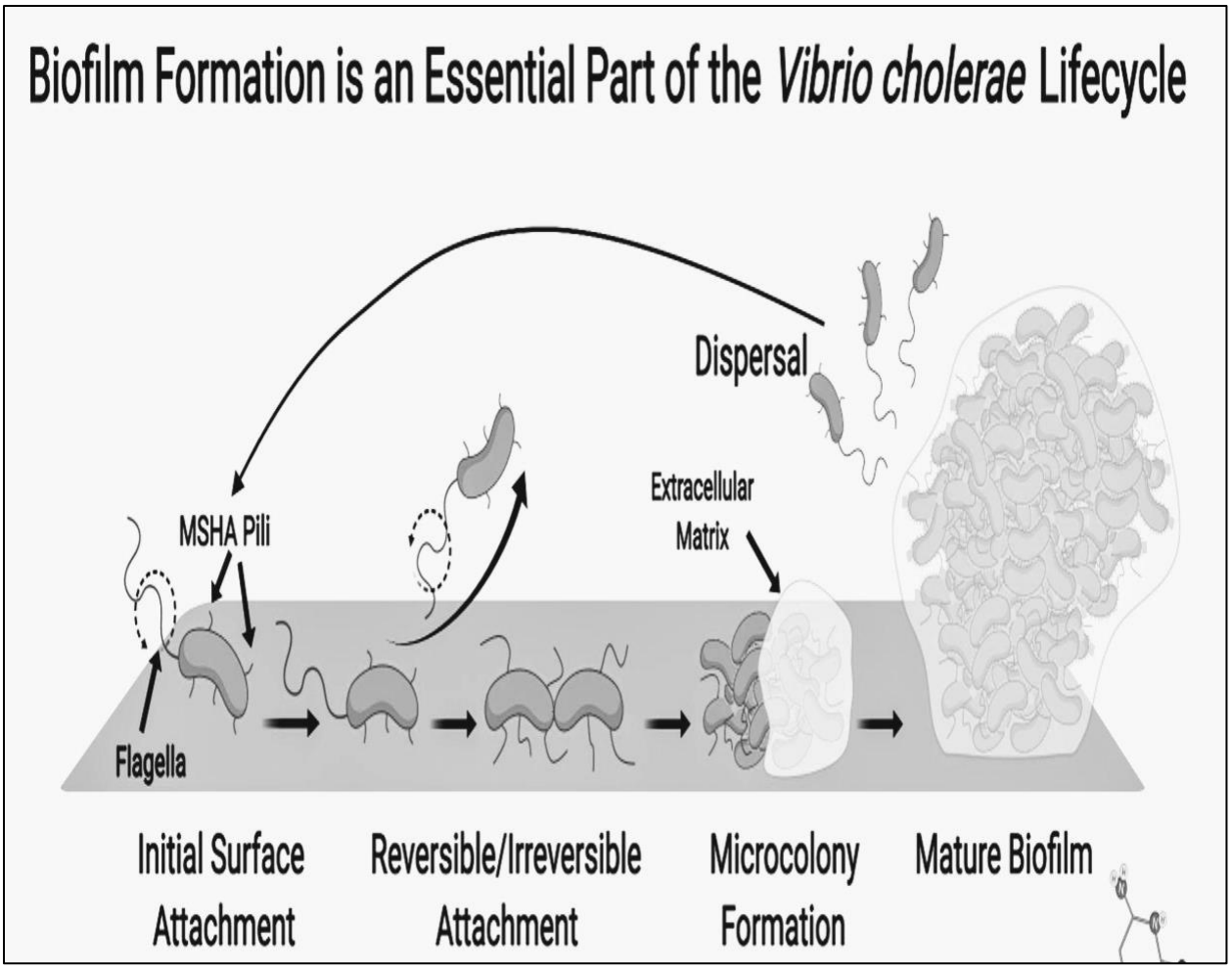

Figure 3: Vibrio cholerae Biofilm formation

Formation of a V. cholerae biofilm followed by some key points;

1. Surface sensing

When something touches a surface it changes the way the flagella around. The flagella are like tails that help things move. So when physical contact happens with a surface the flagellar rotation is altered. This means the flagella do not spin around in the way as they did before the physical contact with the surface. The change in rotation is because of the physical contact with the surface. The surface sensing is connected to the pathways that make c-di-GMP. This means that when the surface sensing happens it affects how the c-di-GMP is made. The surface sensing and the c-di-GMP synthesis pathways are linked together.

2. Initial attachment

MSHA type IV pili: Mediate adhesion to abiotic surfaces, Bind chitinous substrates. GbpA, which is also known as GlcNAc-binding protein A does an important job. It binds to two things: chitin and mucin. GbpA is really good at sticking to these things, that is why it's called GlcNAc-binding protein A. GbpA plays a role in binding to chitin and mucin. Biofilm specific adhesins like Bap1 and RbmC are really important. They help biofilms stick to surfaces. Bap1 and RbmC make sure the biofilms do not get washed away. This is because Bap1 and RbmC stabilize the attachment of biofilms to the surface. So basically Bap1 and RbmC are like the glue that holds biofilms in place.

3. Early biofilm establishment

Pili sticks out from the surface of the cell. They work as the main things that hold the cell in place. The pili extends outward from the cell surface. They function as the primary anchors for the cell. Cells start to make things in a pattern. The cells begin secretion in a matrix. This is how the cells work together to make the matrix secretion happen. The cells are very important for the matrix secretion to start.

4. Stabilization and microcolony formation

The production of VPS which's short for Vibrio polysaccharide, is started when it is needed. VPS production is triggered when certain things happen. This means that the VPS production process begins. The creation of VPS or Vibrio polysaccharide is underway. Cells transition from 2-D flat layers to 3-D structures

5. Biofilm maturation

Enhanced matrix accumulation. Formation of RbmC–Bap1 envelope clusters (Bap1 and RbmC, Maintain pellicle localization)

6. Genetic dependence of attachment

In the environment, mshA mutants show reduced biofilm biomass and impaired pilus-mediated adhesion.

7. Environmental stress response

Hostile conditions trigger pathway switching nutrient depletion, oxidative stress, osmolarity changes.

8. Biofilm dispersal

Cells come loose from the biofilm. This is when the cells that are part of the biofilm start to detach. The mature biofilm is like a group of cells that stick together and when the cells detach from the mature biofilm they are no longer a part of this group. The cells that detach from the biofilm can then move on and do other things.

Cells that are dispersed exhibit characteristics. The dispersed cells show some things that're interesting to see. Dispersed cells do things that are unique to them and increased colonization efficiency

9. Transmission and re-entry

Biofilm-derived cells often shed in diarrheal stools during outbreaks and re-enter aquatic environments or new human hosts.

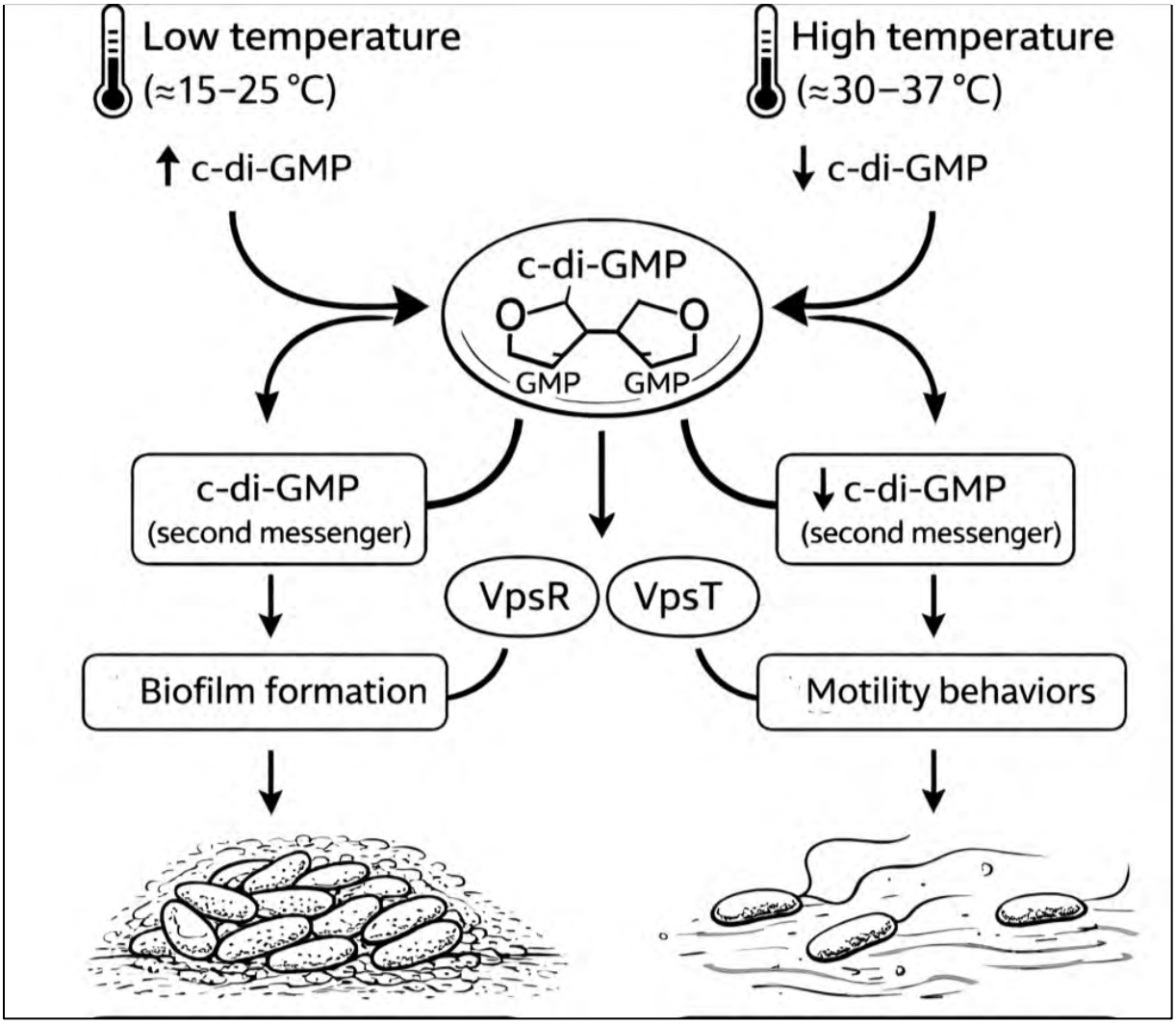

Figure 4: Temperature-dependent regulation of c-di-GMP signaling and biofilm formation in *Vibrio cholerae*

3.3 Functional roles of biofilms in environmental survival and pathogenesis

Biofilm gives protection against stress tolerance such as desiccation, UV radiation, oxidative stress, antimicrobials, protozoan predation, and heavy-metal toxicity (Teschler et al., 2015b). The matrix acts as a barrier so that it can protect the bacterial cells from the harsh environment specially in an environment where the contamination and toxic materials are heavily available. Biofilms create microenvironments that concentrate extracellular enzymes and metabolic by-products.

When the environment condition fluctuates the depth of the biofilm and its metabolic pathway are also affected.

V. cholerae cells are known as hyperinfectious which require lower infectious dose than planktonic cells (Tamayo et al., 2010b).

These biofilm clumps dispersed from the human body which indicates it was not affected by the gastric acid in the stomach (Silva & Benitez, 2016). Recent studies also prove that these biofilm cells act aggressively to protect bacterial cells in the human body by killing immune cells and ensuring pathogenesis (Vidakovic et al., 2023).

Chapter 4

4.1 Regulatory Signaling pathway

Vibrio cholerae's ability to form biofilms is influenced by the interplay of diverse environmental signals— temperature, nutrient levels, osmotic pressure, and heavy metal exposure— that together form a hierarchical system of regulation. A complex series of cascades of signal transduction, the primary mechanism that governs how quickly or slowly *Vibrio* spp. transition from planktonic to sessile states, involves the levels of cyclic di-GMP, the actions of the transcription factors VpsR and VpsT, the influences of quorum sensing (Conner et al., 2017) and also the environmental regulators BipA and CspV (Liu et al., 2024).

All these interacting pathways coordinate the formation of biofilms by promoting attachment to surfaces, the synthesis of a matrix, and eventually the dispersal of the organism into the aquatic environment, thereby creating a consistent synchrony between biofilm growth and the environment in which it is formed (Teschler et al., 2015). This chapter describes how each of these regulatory systems operates at the molecular level and describes how the signalling patterns in *V. cholerae* are influenced by changes in temperature and by heavy metal toxicity.

4.2 c-di-GMP Signalling as the Central Regulatory Switch

In many bacterial systems, c-di-GMP is the most important intracellular messenger for biofilm assembly. It has become a canonical system to understand this signalling molecule within the bacterium *V. cholerae*. When c-di-GMP accumulates to high levels inside a cell, it activates the transcription of the biofilm matrix genes, represses motile genes, and promotes the physiological shift towards sessile behaviour. Conversely, at low levels, it promotes motility and virulence of the organism (Teschler et al., 2015). The intracellular concentrations of c-di-GMP are maintained by the competing activities of two different bacterial systems: DGCs that convert GTP to c-di-GMP and PDEs that degrade c-di-GMP. In *V. cholerae* there are also more than 40 proteins encoded in the genome. The temperature-responsive dynamics of DGCs underscore the centrality of c-di-GMP signalling in coordinating biofilm formation with seasonal and microenvironmental thermal conditions.

Diguanylate Cyclase	Temperature Response	Functional Role	Key References
CdgA	Strong induction at low temperatures	Activates matrix gene expression via VpsR/T	Townsley & Yildiz, 2015
CdgH	Induction at 15–22 °C	Controls VPS operon expression	Krasteva et al., 2010
CdgK	Low-temperature responsive	Enhances c-di-GMP accumulation	(Townsley & Yildiz, 2015)
CdgL	Upregulated in cold shock	Supports VPS synthesis	(Townsley & Yildiz, 2015)
CdgM	Induced at 15 °C	Contributes to thick biofilm morphology	Townsley & Yildiz, 2015
VpvC	Major regulator at moderate temperatures	Stabilizes VpsT activation	Krasteva et al., 2010

Table 1: Functional roles and temperature responses of Diguanylate cyclase.

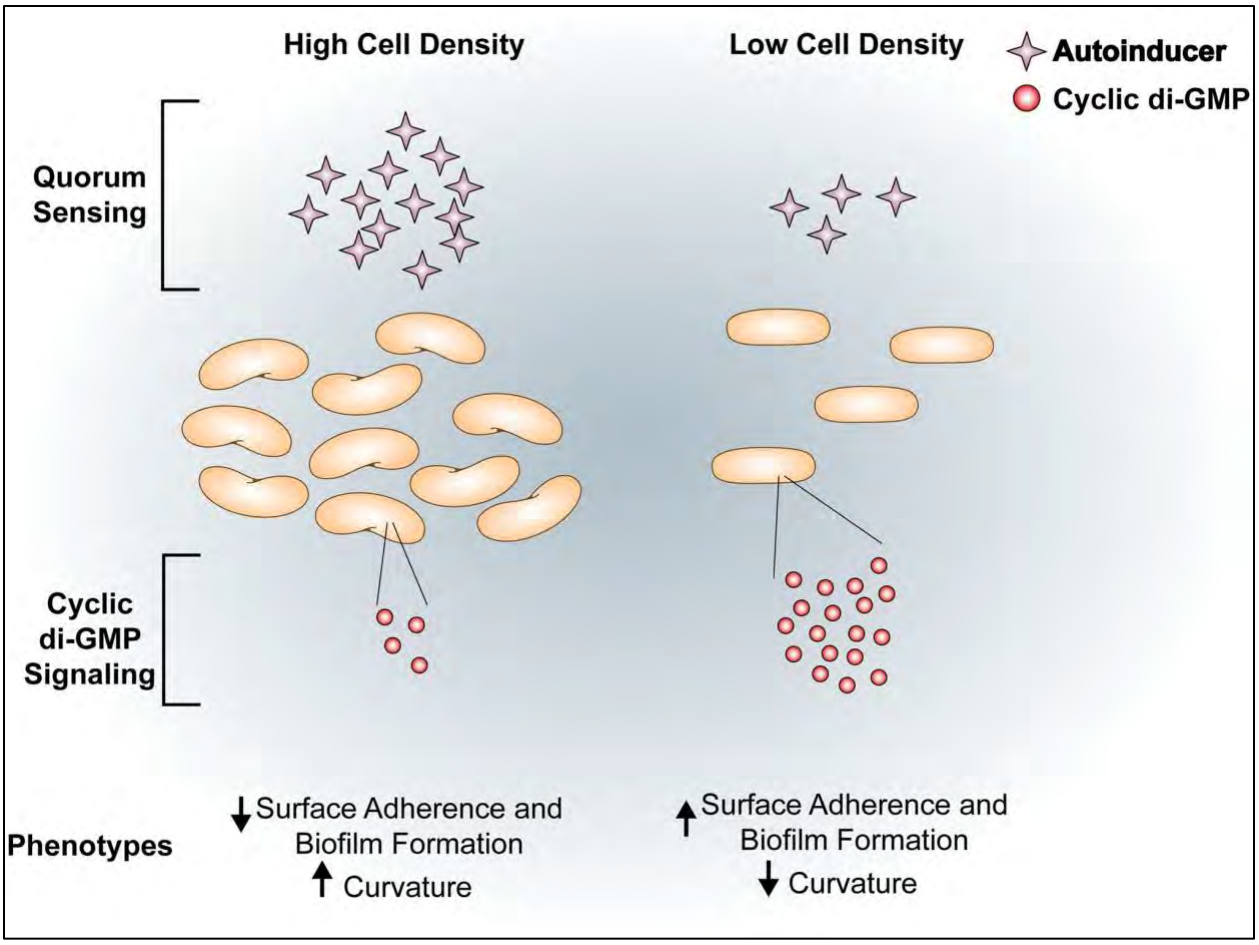

Figure 5: Cyclic di-GMP inhibits flagellar motility by regulating motor (Adapted from Fernandez et al., 2020)

When *V. cholerae* cells are at low density, they produce very few quorum-sensing autoinducers. This allows for a net increase in the intracellular levels of c-di-GMP as a result of increased diguanylyl cyclase (DGC) activity. The level of c-di-GMP also serves as a second messenger for numerous processes occurring within the cell. The presence of c-di-GMP allows for cell adhesion to surfaces and biofilm development, while preventing motility of the bacterium due to repression of flagellar motility and activation of surface-associated behaviors.

At higher levels of c-di-GMP, the expression of the curvature determinant, CrvA, is repressed. As a result, cells produced by *V. cholerae* are straighter. Reducing the curvature of the cells facilitates better packing of cells and forming more stable structures in biofilms; thus, enhancing the formation of sessile communities. Simultaneously, c-di-GMP inhibits flagellar motor function and further inhibits motility and reinforces attachment to surfaces. Conversely, once the *V. cholerae*

population reaches a high density and the cell's production of quorum-sensing autoinducers is increased, the intracellular c-di-GMP levels are decreased. Because there is an absence of c-di-GMP, the repression of CrvA is relieved allowing for normal curvature of *V. cholerae*, which increases the bacteria's swimming capabilities. Therefore, in high densities, flagellar motility is encouraged while surface-associated behavior and biofilm development are discouraged.

4.3 Integration of Multiple Regulatory Pathways

The regulation of biofilm formation by *V. cholerae* is not a single pathway, but instead an interconnected signalling network made up of multiple signals acting to affect the formation of organised biofilms. This network is influenced by temperature, heavy metal toxicity, quorum sensing, and the amount of c-di-GMP produced during growth. At lower temperatures, DGCs and CspV are activated while HapR is inhibited, allowing to produce structured biofilms to occur. Heavy metals contribute to the formation of biofilms by increasing the quantity of EPS produced and activating the stress response pathways of the bacteria. At higher temperatures, the bacteria will have increased motility, decreased matrix production, and activation of virulence pathways. The combination of these signals will determine if V. cholerae creates a dense, environmentally stable biofilm or will progress toward a host-associated life cycle.

Chapter 5

5.1 The temperature effect on the growth of V. cholerae

Temperature is a crucial impact factor for bacterial growth. Some kinds of bacteria allow themselves to form biofilm as a defence mechanism or survival strategy, which allows the bacteria to continue growing in a harsh environment. Biofilm is an EPS, that allows bacteria to withstand threats like antibiotics, desiccation, and immune responses. Temperature is necessary for the growth of *Vibrio cholerae*; it also mediates the transition between environmental reservoirs and the human host.

Vibrio cholerae are found in a diverse aquatic habitat where they are subjected to fluctuating temperatures ranging from 12–30°C and the human gut (~37 °C). Biofilms enhance survival under environmental stress and facilitate transmission (Conner et al., 2016). Temperature affects growth rate, motility, gene regulation, and importantly, biofilm formation (Lutz et al., 2013).

Studies showed that rising temperature of sea and river are also interconnected with the *V. cholerae* infection (De Magny et al., 2008). Conducted research on ecological changes runs a series of molecular studies which resulted in low-to-moderate temperatures nearly (15–25 °C) enhancing biofilm formation, whereas 37 °C tends to favour a more motile, less biofilm-associated state. C-di-GMP signalling, cold-responsive transcription (CspV), temperature-dependent translational control (BipA), and adhesins (MSHA pili and GbpA) mediate colonization indicates the changes of the temperature into biofilm.

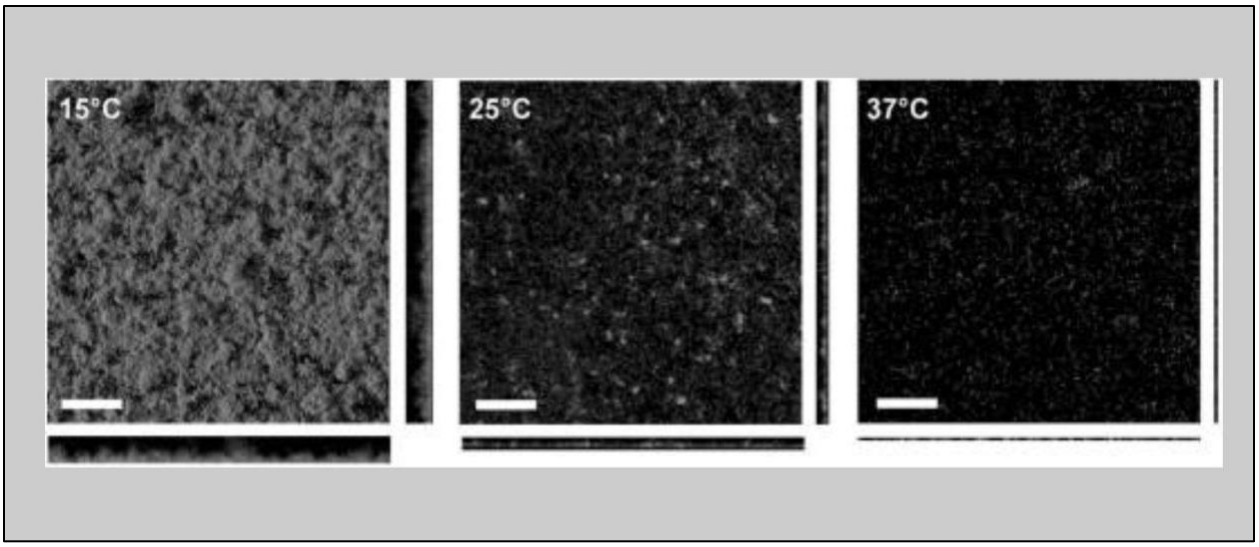

Figure 6: Biofilm structures of wild-type *V. cholerae* formed at 3 different temperatures (Adapted from Townsley & Yildiz, 2015)

A study on *V. cholerae* El Tor C6706 where it is found that at low temperature (15 °C and 25 °C) biofilm formation is induced compared with 37 °C. Biofilms formed at 15 °C were thicker and more structured than those at 37 °C. It is also confirmed that low-temperature growth enhances both biofilm biomass and c-di-GMP levels, reinforcing that this is a reproducible phenotype not limited to a single assay condition. When *V. cholerae* cultures were grown at 15 °C instead of 37 °C, intracellular c-di-GMP increased markedly, and this increase coincided with higher vps promoter activity and enhanced biofilm biomass. If a rapid temperature shifts downward 37 °C to 15 °C then it raises c-di-GMP within about an hour and shows changes of the growth rate (Townsley & Yildiz, 2015c). In another study where the work on cold-response proteins (CspV) and BipA showed biofilm formation in mutants at nearly (22–25)°C (L. Townsley et al., 2016). These findings highlight the *V. cholerae* c-di-GMP signalling, which note that low growth temperature is a potent inducer of c-di-GMP accumulation and biofilm formation.

Temperature	Biofilm Biomass	Regulatory Response	Key References

15 °C	highest; thick and structured	c-di-GMP, vps genes, CspV	(L. Townsley et al., 2016).
25 °C	High; stable biofilm	Moderate in matrix gene expression; adhesins	(Obana et al., 2020).
37 °C	Comparatively Lower; weak biofilm formation	c-di-GMP; motility; virulence	(Liberatore et al., 2025)

Table 2: Effects of Temperature on Vibrio cholerae Biofilm Biomass and Regulatory Response

Understanding how temperature and heavy metals jointly and independently regulate *V. cholerae* biofilm formation is essential for predicting persistence in natural habitats, anticipating seasonal cholera outbreaks, and designing environmental interventions.

5.2 Temperature-responsive pathways

The temperature effect is mediated primarily by diguanylate cyclases (DGCs) where around six diguanylate cyclases (DGC) CdgA, CdgH, CdgK, CdgL, CdgM and VpvC are required for low-temperature c-di-GMP elevation and biofilm induction and if these are lacking in strain then the c-di-GMP or biofilm formations are not get increased (Townsley & Yildiz, 2015c). This indicates that low temperature primarily acts by activating specific DGCs. These DSCs are large sensory proteins which respond to extracellular or membrane associated changes. However, several other studies also need to evaluate the exact temperature sensing mechanism of DGC (Almblad et al., 2021).

A number of large transcription factors VpsR and VpsT are activated by the c-di-GMP elevation. Binding protein VpsT is induced by the c-di-GMP. This incident also acts for DNA binding which activates the transcription biofilm genes (Fernandez et al., 2018). VpsR responds to c-di-GMP by

enabling it to integrate nutrient and messenger signals (Stauder et al., 2010b). In a low temperature, c-di-GMP increases which pushes VpsR or VpsT to form biofilm by VPS production and matrix synthesis. A cold-shock protein CspV expresses rapidly at a low temperature and influences the formation of biofilm. CspV mutants display impaired ability to form cold-induced biofilms and altered type VI secretion (Townsley & Yildiz, 2015c). In addition, *V. cholerae*'s association with zooplankton increases at a low temperature.

While c-di-GMP and CspV act in transcription, BipA exerts temperature-dependent control in translation. Studies explain BipA as a ribosome-associated GTPase. BipA is temperature-dependent, and it controls more than 200 proteins of the biofilm matrix and cell-surface structures (Del Peso Santos et al., 2021).

At temperatures below ~22 °C, BipA suppresses the production of biofilm-associated proteins; even matrix components appear as a smooth colony. When the temperature gets low, BipA normally acts to stop formation on cold-induced biofilm assembly. Proteomic analysis revealed that BipA's temperature-dependent regulation extends across multiple cellular processes and functions for transcriptional activation (Del Peso Santos et al., 2021).

These studies provided evidence that at low temperature biofilm gene transcription activates via c-di-GMP or VpsR or VpsT and CspV. However, BipA can decide how the signals will be translated. Cells initially get attached to the environmental surface and form biofilms. The adhesion process depends greatly on key adhesins: MSHA pili and GbpA (Stauder et al., 2010c). MSHA pili are type IV pili that mediate mannose-sensitive hemagglutination and adhesion to a variety of abiotic and biotic substrates (Lutz et al., 2013b). mshA and gbpA expression were significantly higher at 25 °C compared to 15°C, which indicates the bacterium can persist in the environment at warmer temperatures, especially in summer. If the mshA or gbpA genes are mutants, then the chitin attachment is reduced and decreased colonization of zooplankton surfaces (Stauder et al., 2010d). GbpA is a modular adhesin that binds chitin and interacts with the bacterial surface, and also acts as a key colonization factor in both environmental and host contexts. This represents environmental temperatures (~20–25 °C), typical of coastal waters where high mshA/gbpA expression facilitates colonization of chitinous zooplankton and other surfaces, seeding biofilms that act as long-term reservoirs.

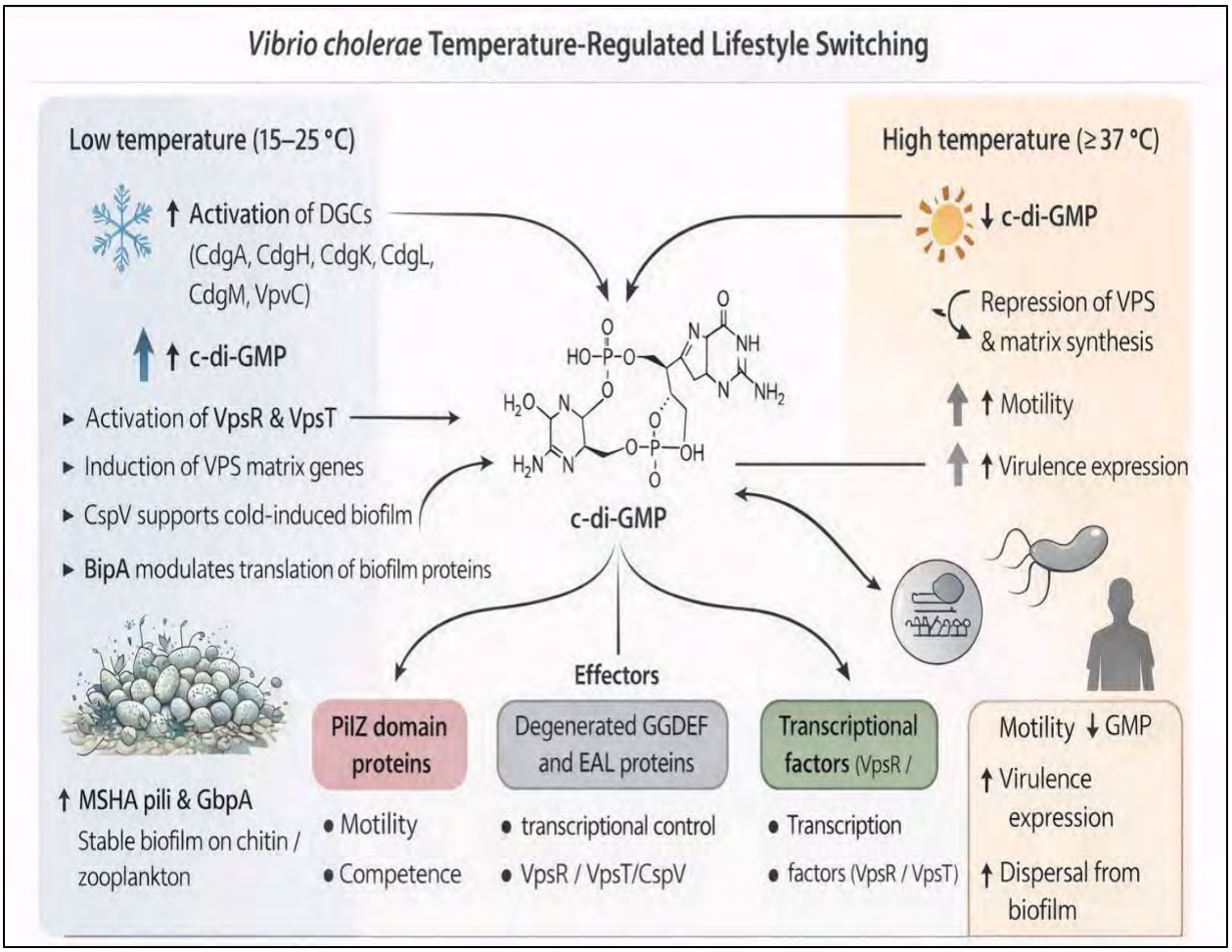

Figure 7: Temperature-dependent regulation of biofilm formation and virulence in Vibrio cholerae.

5.3 Temperature play as a critical factor

The range between low and moderate temperatures (approx. 15-25 °C) increases cyclic-di-GMP levels through specific diguanylate cyclases (DGCs), such as CdgA, CdgH, CdgK, CdgL, CdgM and VpvC. This temperature also results in increased activation of VPS (various protein secreting) and the expression of matrix genes driven by VpsR/VpsT. Additionally, low to moderate temperature regulates the expression of the cold-shock regulator CspV and biphasic translation of biofilm proteins regulated by BipA. Also, expression of the adhesin proteins MSHA and GbpA at this temperature supports colonization of both environmental surfaces and planktonic lifestyles

(White-Ziegler et al., 2008). Host temperature (37 °C) supports motility and virulence gene expression, reduces c-di-GMP levels and regulates the production of biofilm matrix components. Host temperatures also lead to planktonic or loosely adherent lifestyles suitable for colonization of the intestinal tract (Lupescu et al., 2025).

5.4 Effects of Temperature on VBNC State and Biofilm Persistence

Low temperatures strongly promote the VBNC state in *V. cholerae*, enabling survival during extreme stress without requiring continuous metabolic activity (S. Zhao et al., 2021).

Biofilms provide a stable microenvironment where cells can transition into VBNC more efficiently, aided by matrix insulation and reduced oxygen flux. VBNC cells embedded in biofilms have been shown to persist in aquatic systems for months, resuscitating when temperatures rise seasonally (Flemming et al., 2016). The temperature-dependent interplay between biofilms and VBNC behavior plays a major role in cholera seasonality, particularly in regions with distinct monsoon cycles.

Condition	**Temperature**	**VBNC Induction**	**Notes**	**Key References**
Artificial seawater	4 °C	Strong	Cells enter VBNC within 20–40 days	(Wu et al., 2016)
Nutrient-rich medium	4 °C	Weak	Nutrients delay VBNC entry	(Molloy, 2013)
22–37 °C seawater	Moderate–high	None	Cells die without VBNC transition	(Wu et al., 2016)

Biofilm-forming cultures	4–15 °C	High	EPS matrix enhances VBNC stability	(Molloy, 2013)

Table 3. Temperature-Dependent VBNC Induction and Biofilm Survival

5.5 Temperature effect on biofilm matrix and epidemiological consequences

The life cycle of the *Vibrio cholerae* greatly depends on the temperature variation. *Vibrio cholerae* faces many transitions throughout its lifestyle, which are mostly associated with warm temperatures, human gut conditions, and cooler aquatic reservoirs (4-25)°C. This shifting of temperature is not only associated with growth rate alteration but also with regulators. Regulatory networks affect gene expression, signalling molecules, and protein translation, which are all associated with biofilm formation. Multiple studies revealed that lowering the temperature significantly increases the architecture of biofilm biomass, also functioning as long-term environmental reservoirs, prolonged culturability and persistence. Under static growth conditions, the complexity of the biomass structure of *V. cholerae* biofilms is significantly greater at 15 °C and 25 °C compared to 37 °C. At 15 °C, biomass can be nearly 20-fold higher than at 37 °C. The maximum thickness of biomass recorded was almost double at 15 °C compared to 25 °C, whereas at 37 °C it showed 6.60 μm, which is lower than the other results (L. Townsley & Yildiz, 2015d). In low temperature, biofilm matrices provide barrier and protection from the environment, which makes them resistant (Lutz et al., 2013c). Conversely, at 37 °C, c-di-GMP levels are lower, and VpsR or VpsT activity is reduced, where colonization factors are tuned towards intestinal colonization rather than environmental persistence. Biofilms that are formed under warmer environments are typically thinner (Kim et al., 2020). This indicates that when the seasonal downshift in temperature occurs, biofilm formation is subsequently increased. Some microcosm study results show that V. Cholerae cells embedded in biofilms can be cultured for a longer period than planktonic cells (Alam et al., 2007). Since increasing temperature is associated with cholera outbreaks, a rise of 1 °C can double cholerae infection (Bekele et al., 2025). This indicates that cold temperatures act like an environmental cue to preserve biofilm.

Chapter 6

6.1 Heavy Metal - Induced biofilm formation in *V. Cholerae*

Heavy metals such as cadmium (Cd), lead (Pb), zinc (Zn), copper (Cu), nickel (Ni), chromium (Cr), manganese (Mn), mercury (Hg), and arsenic (As) are naturally present in many aquatic environments. However, if the level is marked too high by industrial effluents, mining, urban runoff, agricultural inputs, and sewage discharge, then it creates massive issues (Farias et al., 2024). Heavy metals are non-degradable elements that can persist for a long time, and often accumulate on the surface of the aquatic environment, which assists in forming biofilms. In many coastal regions where cholera is endemic, water bodies receive substantial heavy-metal loads, creating exposures for aquatic microbiota, including *Vibrio cholerae*. When these metals are present at lower levels, they might be helpful, but at higher concentrations they tend to disrupt the balance, causing cellular damage and DNA lesions. Biofilm formation is a survival strategy for the bacterium under stress; in the high metallic ion enriched environment, the extracellular polymeric matrix binds metal ions, reduces intracellular toxicity, and provides a structured refuge for bacterial persistence. Thus, heavy metals actively reshape the architecture, regulation, and ecological resilience of *V. cholerae* biofilms by reinforcing its extracellular matrix (Murphy et al., 2021).

Cadmium (Cd^{2+}) is one of the most potent inducers of biofilm formation among heavy metals in *V. cholerae*. This response involves oxidative stress activation, induction of antioxidant enzymes, and upregulation of EPS biosynthetic pathways (Cho et al., 2022). These regulatory responses are connected to increased VPS production, which suggests that matrix enhancement is not merely a protective physical barrier but a genetically programmed component of cadmium adaptation. This also supports naturally occurring Vibrio biofilms with increased resistance and ecological competitiveness (Lutz et al., 2013). Copper (Cu^{2+}) and zinc (Zn^{2+}) are essential trace elements but become toxic at high concentrations. Zinc disrupts protein folding and enzyme activity, activating ZntA efflux pathways (Bruins et al., 2000). Zinc produces similar effects by enhancing chitin exposure. In both cases, biofilm formation increases as part of a protective strategy.
Lead (Pb^{2+}) and arsenic (As^{3+}/As^{5+}) are prevalent contaminants in many *V. cholerae*–endemic regions, including Bangladesh and India (Bruins et al., 2000). Lead exposure has been shown to increase adhesion to surfaces and also creating a cross-linked matrix that becomes more resistant

to shear forces. This phenomenon explains why *V. cholerae* biofilms from lead-contaminated water sources often exhibit unusually high structural rigidity ((Harrison et al., 2009).

Heavy Metal	Biofilm Effect	Mechanism	Key References
Cadmium (Cd^{2+})	Strong induction of EPS; thicker biofilms	Oxidative stress, upward EPS, and metal sequestration	(Hussien et al., 2024)
Copper (Cu^{2+})	Moderate to higher level of induction	Efflux pump-activated periplasmic stress response	(Vergnes et al., 2022)
Lead (Pb^{2+})	Increase in rugosity and biofilm density	Metal-binding proteins get higher and larger amounts of matrix deposition	(Nong et al., 2019)
Zinc (Zn^{2+})	Variable; strain-dependent	Disrupts protein folding; triggers stress regulons	(Xia et al., 2021)

Table 4: Effects of Heavy Metals on *V. cholerae* Biofilm Formation

6.2 Mechanisms of heavy-metal tolerance in *V. cholerae*

Although detailed genetic mechanisms have not been proven, several lines of evidence have shown bacterial metal-tolerance strategies in *V. cholerae*. Genomic analyses of epidemic and environmental strains reveal multiple putative metal efflux systems (e.g., P-type ATPases, cation diffusion facilitators) often involved with antibiotic-resistance genes on chromosomes or mobile elements (Imran et al., 2018).

Proteomic studies under Cd^{2+}, Pb^{2+}, Zn^{2+}, and Ni^{2+} stress show upregulation of periplasmic chaperones, oxidoreductases, and putative metal-binding secreted proteins, which also affects enzymatic detoxification (Zhang et al., 2023). Heavy-metal exposure induces expression of

antioxidant defenses and DNA repair enzymes (Jasu & Ray, 2021). These mechanisms mirror those described in other Gram-negative bacteria and provide the baseline physiological capacity upon which biofilm-mediated tolerance can build. Direct mechanistic studies on heavy-metal–induced biofilm formation specifically in *V. cholerae* are still limited. Reviews on biofilm-mediated heavy-metal bioremediation consistently conclude that biofilms act as metal sponges, with the matrix providing abundant binding sites.

6.3 Evidence linking heavy metals to biofilm formation

Several recent studies on *Vibrio* spp. from aquaculture and shellfish directly examined relationships between heavy-metal contamination, resistance, and biofilm capacity (Lin et al., 2025) analyzed *Vibrio* spp. (including *V. parahaemolyticus* and *V. cholerae*) isolated from shellfish in metal-polluted coastal waters, where results showed that higher environmental concentrations of Cd and Cu were positively correlated with (i) multidrug resistance and (ii) strong biofilm-forming phenotypes.

This study indicates that metal-pollution can be considered as a pollutant for environments and can make *Vibrio* strains that are both resistant and proficient at biofilm formation (Lin et al., 2025b). Similar patterns were reported in studies conducted on fish and other aquatic shell creatures, where non-O1/O139 *V. cholerae* isolates with higher tolerance to Hg^{2+}, Cd^{2+}, and Pb^{2+} were mostly multidrug-resistant and carried virulence-associated genes. Although biofilm was not always quantified in that study, co-selection of heavy-metal tolerance, AMR, and biofilm traits in contaminated environments (Xu et al., 2019).

6.4 Structural Adaptations of Metal-Induced Biofilms

Exposure to heavy metals can significantly impact gene expression by gene alteration. Biofilms grown under environmental stress tend to be thicker, more wrinkled, and enriched in polysaccharides. Microscopical analysis revealed that vertical formation is also enhanced. This suggests an increase of microcolony density compared to biofilms grown in metal-free conditions.

These structural reinforcements work for multiple adaptive functions where metal sequestration in the outer EPS layers reduces cytoplasmic exposure.

Biofilm acts for supporting deeper layers that make a shield to protect from stress. This tendency of forming biofilms improves surface attachment and pellicle formation. Biofilms which are exposed with cadmium or copper stress often display vortex-like microcolonies (Chakraborty & Mondal, 2024). These reflect altered mechanical forces due to metal-binding cross-linking between EPS molecules and the structural effects which shows long-term persistence in polluted waters.

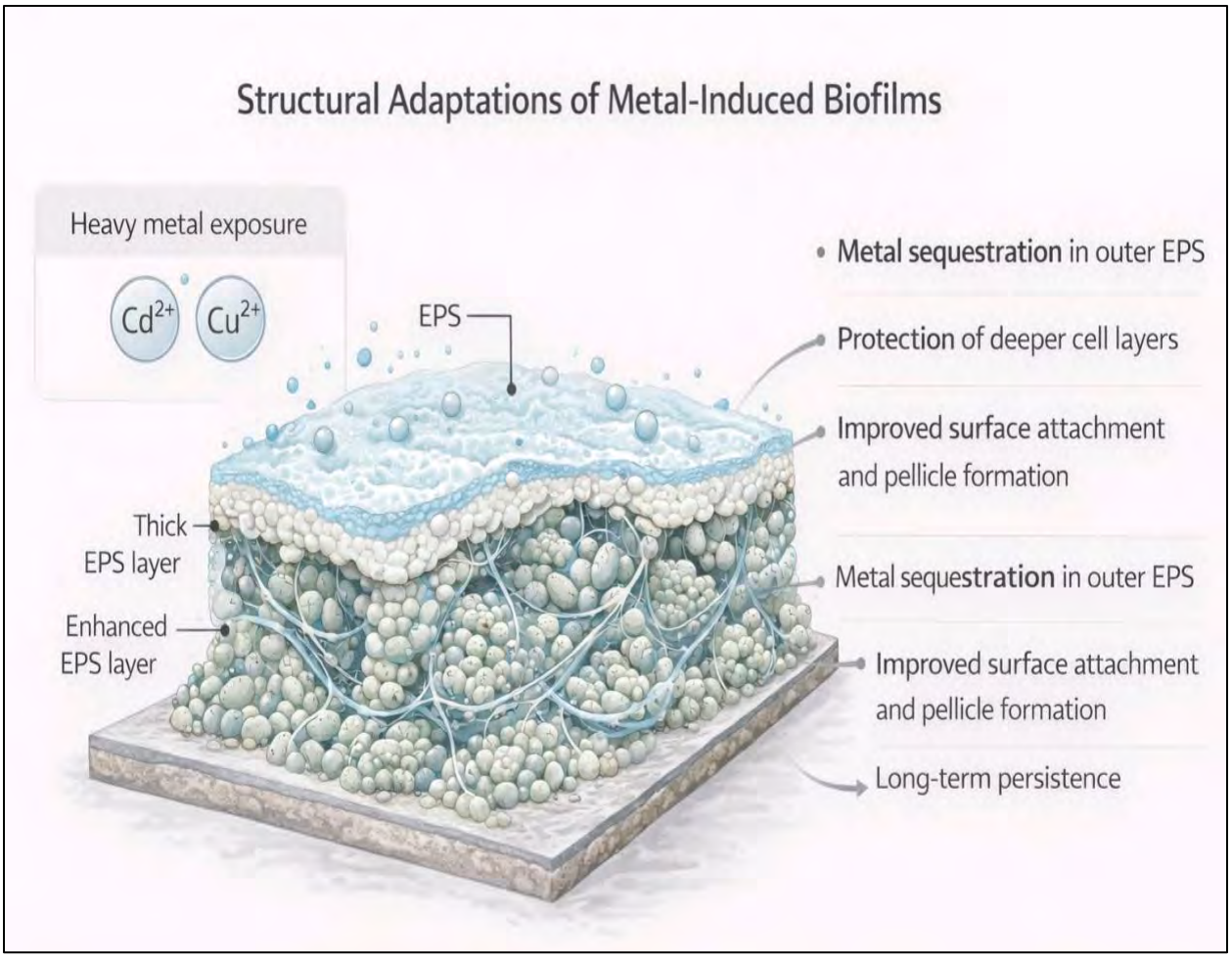

Figure 8: Structural Adaptations of Metal-Induced Biofilms

6.5 Ecological and Public Health Implications of Metal-Induced Biofilms

Heavy metals highly contaminate the water body and affect the aquatic animals and microbial community. Presence of heavy metals increases the abundance and environmental stability of *V. cholerae* biofilms. In regions like Bangladesh, where drinking water sources are contaminated with industrial effluents and other toxic components, metal-induced biofilms may act as persistent reservoirs for toxigenic strains (Chowdhury et al., 2025). As in cold season the bacteria mostly persist, biofilm stability increases the likelihood of VBNC transition, followed by resuscitation when temperatures increase. This ecological cycle aligns mostly with observed cholera in seasonal patterns (Lutz et al., 2013b).

In addition, metal-rich biofilms may facilitate horizontal gene transfer by prolonging cell proximity and protecting DNA from degradation (Michaelis & Grohmann, 2023). This process potentially accelerates the spread of virulence factors and disease which mostly becomes endemic in many parts of the world. This also increased the antibiotic resistance genes, and environmental survival traits.

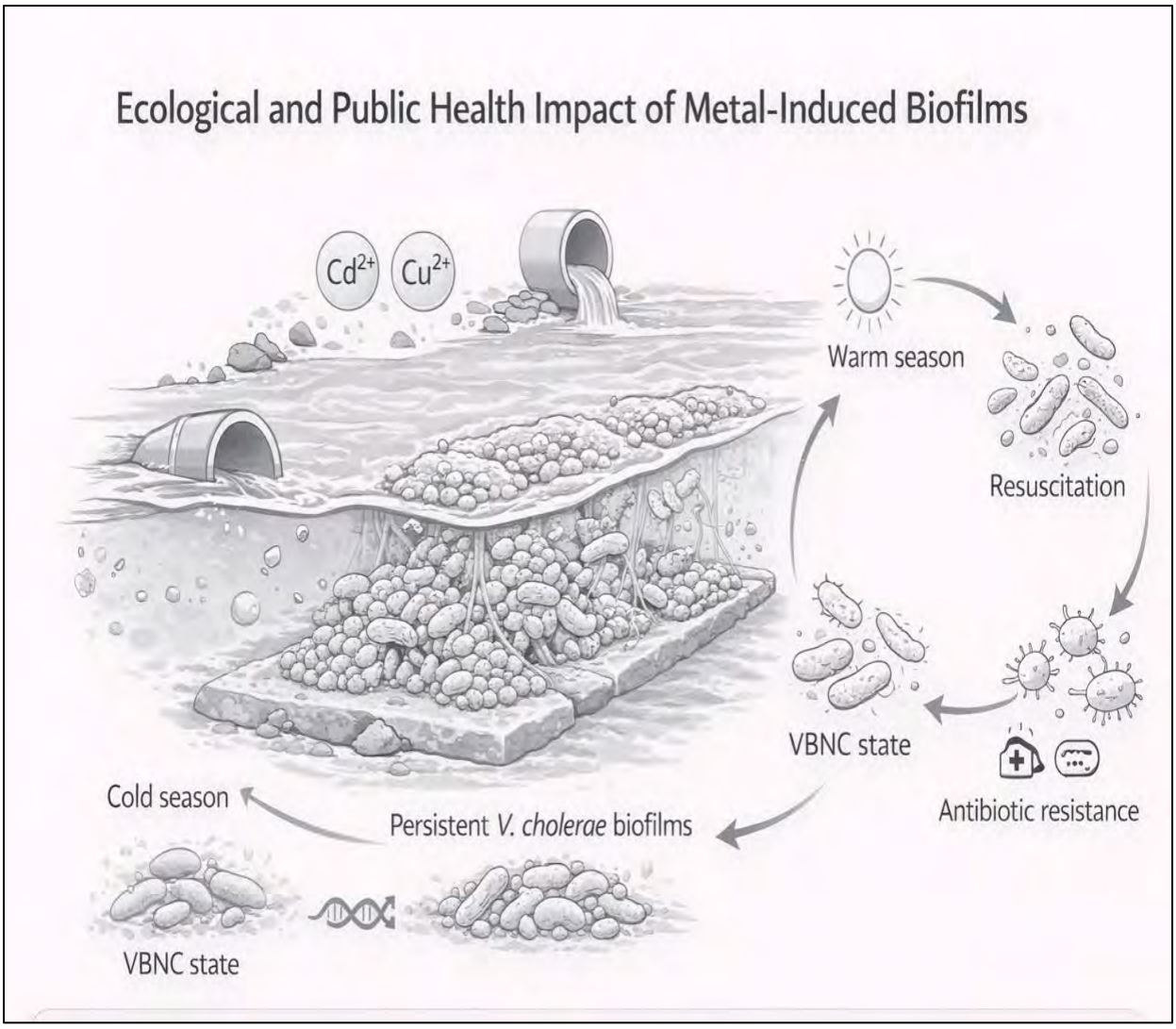

Figure 9: Ecological and Public Health Impact of Metal-Induced Biofilms

6.6 Heavy Metals, Quorum Sensing, and Community-Level Adaptation

Meta-analyses show co-occurrence of heavy-metal resistance, quorum sensing (QS), and biofilm genes in bacterial genomes. In *V. cholerae*, QS regulates biofilm formation, dispersal, and virulence.

Heavy-metal stress may alter QS signaling by affecting:

- Signal production
- Receptor activity

- Membrane integrity
- Cellular metabolism

Changes in QS can shift population behavior between planktonic and biofilm states. Chronic metal exposure may drive evolutionary coupling of QS, metal resistance, and biofilm pathways which results in metal-tolerant biofilm communities with enhanced environmental persistence.

Chapter 7

7.1 Interactions Between Temperature and Heavy Metals

Temperature can significantly impact the level of toxicity and environmental behavior of metals. Warmer temperatures may increase metal solubility and mobility, especially in regions like Bangladesh and India (Punia & Singh, 2025). At a lower temperature, the biofilm viscosity increases (Nedwell, 1999). Seasonal transitions in monsoon and dry season alter both temperature and metal concentration in endemic regions (Bhuyain et al., 2022). Therefore, biofilm responses to temperature and metals are not independent, and cross-modulation affects the VBNC state transitions and Outbreak seasonality (Lin et al., 2025c).

Heavy metals exert toxicity primarily through oxidative stress, protein misfolding, membrane destabilization, and disruption of enzyme activity. Reactive oxygen species (ROS) generated through metal redox cycling (particularly Cu^{2+} and Fe^{3+}) cause DNA damage, lipid peroxidation, and metabolic dysfunction (Pizzino et al., 2017). To counteract these effects, *V. cholerae* activates multiple stress-response pathways, including SoxR, OxyR, MerR-family regulators, and metal-specific efflux pumps (Watanabe et al., 2018). These responses have downstream effects on biofilm induction.

The EPS matrix acts as an ion-binding scaffold; its polysaccharides, extracellular DNA, and proteinaceous components chelate metal ions through negative charges and functional groups (Vandana et al., 2023). This reduces the bioavailable concentration of metals and protects cells deep within the biofilm. As a result, metal stress often increases EPS production by activating biosynthetic operons (vpsA-K) and matrix proteins such as RbmA, RbmC, and Bap1. These structural adaptations contribute to the increased thickness, density, and resistance properties of metal-induced biofilms.

Condition	Biofilm Behavior	Mechanistic Explanation	References
Low Temp (15–20 °C) + Metals	Synergistic increase in EPS and adhesion	metabolism but stress signalling (c-di-GMP, CspV)	(Conner et al., 2016)

Moderate Temp (25 °C) + Metals	Peak biofilm formation	Optimal enzyme activity + strong EPS response	(Harimawan et al., 2017)
High Temp (37 °C) + Metals	Reduced biofilm; higher toxicity	Higher membrane permeability; lowering matrix production	(Far, 2024)

Table 5: Combined Effects of Temperature and Heavy Metals on *V. cholerae* Biofilm Phenotypes

7.2 Combined stress in Vibrio: insights from multi-contaminant studies

Multi-contaminant studies provide insight into multi-contaminant stressors on *V. cholerae* biofilms but there have been few studies directly regarding temperature and heavy metals together acting on *V. cholerae*. Research on *Vibrio* spp. communities and other vibrios will provide indirect support for the multi-contaminant stressor interaction effects on *V. cholerae* biofilms. Joint exposure of heavy metals and petroleum hydrocarbons in biospecies associated with shellfish and Spoilage bacteria.

Studies reported the synergistic effects of industrial pollutants (heavy metals and petroleum hydrocarbons) on the species of vibrios, including *V. cholerae,* isolated from shellfish (Lin et al., 2025d). The researchers determined the concentrations of contaminants within shellfish tissues. Heavy Metals (Cd, Cu and Pb) and petroleum hydrocarbons both are frequently found as co-contaminants at higher concentrations.

The vibrios isolated from contaminated samples had a higher occurrence of antibiotic resistance and their bio-film forming abilities were greater than those from less contaminated samples (Mitsuwan et al., 2025). Analyses resulted in the conclusion that combined contamination was the most reasonable explanation for the differences in resistance versus bio-film formation patterns between the contaminated and non-contaminated populations of vibrios. The study results imply

that although temperature was not controlled for in this study, the collection of samples occurred over a period of time in a warm coastal environment, where the interaction of Temperature and contamination is a relevant factor in ecology; thus, the findings support the hypothesis that multiple contaminants have a cumulative impact on selecting for stronger vibrios in warmer water.

7.3 Cell-surface hydrophobicity and adhesins under combined stress

Results showed that elevated surface hydrophobicity and roughness due to heavy metal activity, heavy metals also promote the expression of MSHA pili on Vibrio cholerae/ Vibrio parahaemolyticus (Floyd et al., 2020). Adhesin production from environmental factors, such as temperature, affects initial attachment to particulate matter in estuarine regions. Removal of heavy metals from estuary environments may, thus, reduce density of microcolonies and biofilms (Von Hertwig et al., 2021).

Heavy metals increase hydrophobicity and surface roughness, thus facilitating enhanced non-specific adhesion of microorganisms (García et al., 2020). As a consequence, a combination of heavy metals and elevated temperature will create greater density of microcolonies and better stability of biofilms than either individual factor (Y. Lin et al., 2024).

7.4 VBNC state, biofilms, and dual stress

The ability of *V. cholerae* to exist in a VBNC state under some conditions allows biofilms to serve as a refuge for VBNC cells in order to later emerge from dormancy. Biofilms have also been shown to provide habitat for VBNC cells that are exposed to other environmental conditions that cause bacterial death (Lutz et al., 2013b). In addition to providing a refuge, biofilms may also facilitate bacteria in surviving long-term exposure to environmental stressors by providing a means to amass viable VBNC cells deep within the biofilm matrix as well as adequately retaining toxic metal concentrations within the biofilm structure while protecting them from detection by traditional culture techniques (Syed et al., 2021).

The ability of biofilms to create a habitat for VBNC cells indicates a strong need for additional research into the protective nature of biofilms for *V. cholerae* during dual stressful environments (Li et al., 2014). Regions that contain metals are being affected by environmental conditions associated with seasonal warming. Cholera-prone areas, such as South Asia, and portions of East Africa are characterized by areas known as "heavily used estuarine environments (Weng & Wang, 2019).

The main characteristics of these environments are:

- Discharges from monsoon-driven rivers carry metals, nutrients, and organic materials into these estuaries.
- Surface Water Temperature (SST) and river temperature rise seasonally during the summer months.
- The seasonal occurrence of plankton blooms creates an environment conducive to high population densities of *Vibrio cholerae* due to an abundance of surfaces for *V. cholerae* to attach (Racault et al., 2019).

Additionally, it has been suggested that in environments such as these, favorable thermal conditions associated with seasonal warming will result in an increase in the formation of biofilms on various substrates associated with (Xu et al., 2019b):

- Zooplankton
- Phytoplankton aggregates
- Sedimentary deposits
- Artificial substrates (nets, pipes, microplastics)

Heavy metal contamination will also favor the survival of metal-tolerant genotypes that are able to form biofilms by acting as a selective force. Aquaculture ponds and commercial coastal shellfish production systems are hot spots for the interaction of elevated sea temperatures and metal pollution.

- Warm shallow waters are conducive to rapid growth of *Vibrio* spp.

- Channeled nutrient flow from sediment and feed sources provides great sources of chronic exposure for Vibrios found on fish and shellfish products (Mohamad et al., 2019).

Surveys of *V. cholerae* isolates found in freshwater fish and marine shellfish indicate that isolates are frequently developing resistance to heavy metals and antibiotics and developing biofilm-forming phenotypes.

7.5 Implications for modeling, surveillance, and control

There are three main implications of modelling, monitoring, and controlling environmental reservoirs for co-stress effects of *vibrio cholerae*:

Impacts on reservoir persistence; impacts on the timing of outbreaks; and risk of the emergence of MDR-*V. cholerae* biofilm proficient clones. Heavy metals are rarely considered as factors in current models (Racault et al., 2019b).

Modelling co-stress effects on environmental reservoirs, most mechanistic models developed to date for *V. cholerae* and the dynamics of *vibrio* spp. from an environmental viewpoint consider temperature & salinity as the two most important factors affecting their growth cycle. Heavy metals have been shown to be selected for more persistent biofilm forming strains, co-selected for the emergence of antimicrobial resistant strains and modify survival and VBNC behaviour.

To improve the accuracy of model predictions of reservoir persistence, review of the temporal dynamics of *V. cholerae* outbreaks; and the risk of the emergence of MDR *V. cholerae* biofilm proficient clones; energy utilising environmental reservoir co-stress contributors' heavy metals should be incorporated into future predictive models. This can be accomplished by developing future models to include metal concentrations directly in areas where metal discharge has been identified.

Monitoring of *V. cholerae* in the environment typically includes monitoring of four factors; temperature, salinity, the abundance of phytoplankton in the environment; and the numbers of *V. cholerae* present in water or plankton. Environmental monitoring programs operating in the coastal areas of polluted or industrialized areas should include the measurement of heavy metals. As well as biofilm sampling of metal-rich biofilm intensive niche environments where the greatest pressure will occur on the emergence of metal co-selected.

Chapter 8

8.1 Epidemology

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 (Hraib et al., 2023).

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 (Hu et al., 2016). The classical biotype of O1 serogroup of *V. Cholerae* had afflicted the previous six pandemics (Deen et al., 2019). 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) (Severin et al., 2018). There is also a recurring emergence of serogroup O139, which originated from the El Tor biotype and showed a new lipopolysaccharide (LPS) and capsule (Yu et al., 2012). *V. cholerae* O1 El Tor adapted to survive in estuarine and freshwater aquatic reservoirs because of biofilm-associated form. 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.
Cholera epidemics occur twice a year in Bangladesh and India's Ganges Delta area (Sack et al., 2021). 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 (Emch et al., 2008). Throughout the last two centuries, significant outbreaks of the disease have taken place. The recent epidemics in which 98,585 cases were recorded in Zimbabwe in 2008, resulting in at least 4000 fatalities (Cuneo et al., 2017). Currently, cholera is endemic in southern Asia, Africa, and Latin America due to poverty and inadequate sanitation (Bekele et al., 2025b). Approximately 2.8 million cholera cases and about 91 000 deaths occur in

the endemic nations, whereas an estimated 87 000 cases and 2500 deaths are seen in the non-endemic countries (*Oral Cholera Vaccine Stockpile for Cholera Emergency Response*, n.d.).

8.2 Biofilm-derived hyper infectious phenotype and transmission

The emergence of the hyper infectivity phenotype attributable to biofilm formation by Vibrio cholerae has significant population-level implications for cholera demonstrated that *V. cholerae* grown in a biofilm or recently excreted via rice-water stool has significantly increased infectivity compared to that observed from laboratory-grown, planktonic cultures when tested in animal models (Tamayo et al., 2010).

Key findings regarding the infectivity of biofilm-derived cells include:

Heavy Metals, Quorum Sensing, and Community-Level Adaptation

Aspect	Effect of Heavy Metals	Community-Level Outcome
Gene co-occurrence	Selection for metal-resistance traits	Coupling of QS and biofilm pathways
Quorum sensing signaling	Altered signal production and receptor activity	Shift between planktonic and biofilm states
Biofilm regulation	Stress-driven modulation of QS-controlled genes	Enhanced biofilm stability and persistence
Community adaptation	Chronic environmental pressure	Evolution of robust, metal-tolerant biofilm communities

Figure 10: Metal-contaminated waters promote persistent *V. Cholerae* biofilms and increased public health.

Studies found that natural routes of transmission of cholera may result from the consumption of biofilms or clumps derived from biofilms during an outbreak when bacterial loads in the environment and stools are high (Silva & Benitez, 2016). Similar research demonstrated that the hyper infectious phenotype of *V. cholerae* in a newly excreted state enhances the rate of cholera outbreak through associations between the bacteria and biofilm growth in aquatic environments, where *V. cholerae* is quickly ingested by the next host to maintain rapid cycling of transmission of the disease between person/environment and person (Nelson et al., 2009).

8.3 Implications for Cholera Epidemiology and Public Health

The factors of temperature fluctuation and metal pollution impact more than just environmental microbiology. They also affect how people become infected with cholera and suffer from cholera disease. There is a close correlation between seasonality of cholera outbreaks and environmental temperature changes; thus, warmer temperatures are associated with higher levels of cholera infection due to their support for the resuscitation of the cold-adapted VBNC population. Additionally, as a result of metal contamination in the aquatic environment, the establishment of *V. cholerae* reservoirs is more stable, and their ability to survive during inter-epidemic periods is greater.

The disinfecting techniques employed within the water treatment facilities used to disinfect bacteria-laden waters do not penetrate the biofilm formed on metals. As such, despite the treatment with chlorine, *V. cholerae* will continue to exist within the water. Additionally, due to the VBNC state of the organism, routine culturing techniques to detect V. cholerae within the outside environment cannot successfully identify V. cholerae reservoirs and provide an underestimating score regarding the cholera transmission risk within the polluted waters.

Research shows a strong correlation between the biofilm stage of a bacterium's development and the overall lifecycle of that species Nong et al. (2019b). Selected *V. cholerae* samples from various estuaries exhibited seasonal patterns related to biofilm production; thus, the ecological conditions in these estuarine environments provided an environment in which *V. cholerae* could establish its biofilm (Sultana et al., 2018). Local *V. cholerae* populations can persist through the seasonal variability in the environmental factors that affect the survival of this bacterium's biofilm life cycle.

Based on experimentation, we noted that biofilms may be growing due to changes in environmental conditions; biofilm growth appeared to enhance survival in dry and nutrient-poor conditions.

Public health responses must therefore account for the dual influences of temperature and pollution. Monitoring environmental conditions, reducing industrial discharge, and improving water treatment processes are critical components of cholera prevention strategies in affected regions.

8.4 Environmental reservoirs and spatial epidemiology

The environmental review documents emphasize the existence of a vast array of reservoirs for *Vibrio cholerae* throughout the environment. Soils phytoplankton (particularly copepods) are considered the most prominent natural *V. cholerae* reservoir; copepods can harbor between 10^4–10^6 cells of *Vibrio cholerae* on their exoskeleton(s) and within the biofilms (Islam et al., 2019).

Phytoplankton and detrital aggregates provide nutrient-rich habitats for *Vibrio cholerae* due to their ability to form biofilms on phytoplankton or marine snow (Racault et al., 2019c). Many species of fish and shellfish, such as hilsa and plowfish, possess biofilms of Vibrio cholerae on their gills, corals, and other tissues. The role of these organisms in facilitating *Vibrio cholerae* transmission has recently gained recognition. (Dey et al., 2022). The sediments, seaweeds, and manmade objects, such as: nets, microplastics, and pipes are the long-term locations of biofilms of *Vibrio cholerae,* and therefore can be reseeded to the water column regularly (Islam et al., 2019b).

Chapter 9

9.1 Knowledge gaps and future directions

Although the evidence is growing, there are still vast gaps in our understanding of the interactions between temperature and heavy metals on the virulence of *V. cholerae* biofilms:

- There are no direct dual-factorial studies available at this time.
- There have yet to be published studies that systematically examine the relationship between metal exposure concentrations and temperature changes while examining virulence through analysis of *V. cholerae* biofilm biomass, architecture and gene expression. Experimental studies using factorial designs (e.g., 15/25/30 °C × sub-inhibitory levels of Cd^{2+}, Zn^{2+}, Cu^{2+}), should be completed for quantifying synergism, additivity, and antagonistic effects.
- Molecular cross-talk pathways between the metals and temperature signaling pathways are very poorly defined. Current knowledge indicates that temperature signals through: c-di-GMP via DGC; CspV, BipA, and adhesins (Wang et al., 2023) metals signal through Fur; Stress responses to metals and altered Membrane and Secreted protein phenotypes (Fu et al., 2020).
- However, we do not know how these molecular signaling pathways are connected. For instance, whether metal stress can modulate the c-di-GMP signal, or alter QS or bipolar signal transduction pathways has not yet been studied.
- Natural Occurrences of Heavy Metals and Their Mixtures: Each of the specific heavy metals e.g. Fe, Cd and Cu have different chemical properties and will most likely act independently from one another to regulate biofilm formation and virulence. As metals are naturally occurring in the environment when in mixed-metal environments together with organic pollutants, more research is needed examining mixed-metal and organic pollutant scenarios on the biofilm formation and virulence on V. cholerae (G. Lin et al., 2025e).
- VBNC transition and resuscitation from stress. There is still a need to directly study the relationship between temperature-induced VBNC transitions and the effect of metals on biofilms. The use of long-term microcosm experiments along with real-world multi-stress scenarios of temperature, salinity, and metal presence would provide valuable information regarding determining the ratio between culturable, VBNC, and infectious bacterial cells.

- Public Health Connections. Although there are established relationships among pollution, Vibrio numbers, and AMR, there is currently a lack of direct epidemiological evidence linking the selection of biofilms based on heavy metals with the occurrence of cholera outbreaks. Therefore, integrative studies utilizing "One Health" approaches, including analyses of environmental metagenome data, Vibrio cholerae genomic information, pollution data, and clinical isolate data, are needed to complete this research loop.

Chapter 10

10.1 Conclusion

This review paper has presented a thorough review of the evidence to show how temperature and heavy metals are two major environmental factors that have affected the development and maintenance of *Vibrio cholerae* biofilms over time as well as the potential health consequences associated with exposure to these biofilms. It has also addressed the findings from the fields of molecular microbiology, environmental ecology, microbial physiology, and epidemiology, all of which show the significance of biofilms for *V. cholerae*, since biofilm formation serves as a primary means by which this organism adapts to its surroundings. Environmental temperature is an important regulatory factor in *V. cholerae* biofilm development as well as regulation of other physiological processes in these organisms. Specifically, exposure to low and moderate temperature ranges (15-25 °C) correlates with increased levels of c-di-GMP in the cells, activation of the VpsR/VpsT regulatory pathway, cold-responsive gene expression, and enhanced expression of surface adhesion proteins, these regulatory and physiological responses allow for the formation of dense and highly structured biofilms on both biotic and abiotic surfaces which are a means to survive over an extended period in an aquatic environment. The effects of heavy metals on biofilm behavior and the population structure of *V. cholerae* are extensive. Heavy metals such as cadmium, lead, zinc, nickel, and copper all have a direct impact on membrane characteristics. Biofilm-forming organisms can accommodate the responses within the biofilm structure, where the biofilm can provide protection against the adverse effects of toxic metal ions. Further, metal tolerance is often co-selected with the ability to produce strong biofilms and develop antimicrobial resistance, which suggests that these traits were selected to provide enhanced survival capability in the environment and to add to the difficulty of controlling these organisms through public health interventions. Through biofilm formation, *vibrio* spp. found in water bodies form a long-term habitat to provide support for further growth and development. To prevent future outbreaks, future strategies must also consider climate change impacts on environmental conditions and relate biofilm habitats to WASH as a primary tool for outbreak prevention. The results from this review provide evidence monitoring for heavy metals should be added to cholera surveillance systems in the municipalities of estuaries and urban waterways impacted by industrial discharges. The environmental and early warning systems that combine biofilm and plankton indicator use with remote sensing technology need to be increased. Finally, a One Health framework should be adopted.

Overall, this review indicates that high temperatures and toxic metals have a very strong correlation with each other and act as key driving forces behind the formation of biofilms in *Vibrio cholerae.* By working together to affect the expression of genetic regulatory pathways, modify the way the bacteria metabolize compounds, and facilitate ecological persistence through unique biofilm matrix architecture, these two key environmental elements impact the evolution of *Vibrio cholerae* and its subsequent epidemiological impact. Thus, biofilms also serve as the link between the bacteria's ability to survive within the environment and the development of human disease.

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