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Salvador Almagro-Moreno
Stefan Pukatzki *Editors*

# Vibrio spp. Infections

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Salvador Almagro-Moreno •
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Editors

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*Editors*
Salvador Almagro-Moreno
Burnett School of Biomedical Sciences
University of Central Florida
Orlando, FL, USA

Stefan Pukatzki
Department of Biology
The City College of New York
New York, NY, USA

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# Preface

In this book, we cover some of the novel advances in the diverse field of *Vibrio* research. The intention of the selected chapters is to provide a wide range of topics including some novel areas of research that can capture the breadth of the multidimensional nature of *Vibrio* infections: from molecular to epidemiological. For instance, in Chap. 2, Christopher Waters and his colleagues review new insights into *V. cholerae* biofilms ranging from molecular biophysics to microbial ecology. Specifically, they highlight recent developments into *V. cholerae* biofilms structure, their ecological role in environmental survival and infection, the regulatory systems that control them, and biomechanical insights into the nature of *V. cholerae* biofilms.

Francis Santoriello and Stefan Pukatzki discuss the Vibrio type VI secretion system (T6SS) in Chap. 3. They describe the structure of the T6SS in different *Vibrio* species and outline how the use of different T6SS effector immunity proteins controls kin selection. They summarize the genetic loci that encode the structural elements that make up the *Vibrio* T6SSs and how these gene clusters are regulated. Finally, they provide insights on T6SS-based competitive dynamics, the role of T6SS genetic exchange in those competitive dynamics, and roles for the *Vibrio* T6SS in virulence.

In Chap. 4, Sandra Sanchez and Wei-Leung Ng discuss motility control as a possible link between quorum sensing (QS) and surface attachment in *Vibrio* species. QS regulates a variety of behaviors that are important for the life cycle of many bacterial species including virulence factor production, biofilm formation, or metabolic homeostasis. Therefore, without QS, many species of bacteria cannot survive in their natural environments. In their chapter, they summarize several QS systems in different *Vibrio* species and discuss some emerging features that suggest that QS is intimately connected to motility control. They speculate that the connection between motility and QS is critical for *Vibrio* species to detect solid surfaces for surface attachment.

In Chap. 5, Karl Klose and Cameron Lloyd discuss the structure and regulation of the *Vibrio* flagellum and its role in the virulence of pathogenic species. They discuss the novel insights into the structure of this nanomachine that have recently been enabled by cryoelectron tomography. They also highlight recent genetic studies that have increased our understanding of flagellar synthesis specifically at the bacterial cell pole, temporal regulation of flagellar genes, and how it enables directional motility through run–reverse–flick cycles.

The ever-expanding list of environmental reservoirs of pathogenic *Vibrio* spp. keeps increasing. In Chap. 6, Diane McDougald and her colleagues discuss the critical role of these reservoirs in disease. As natural inhabitants of aquatic environments, *Vibrio* species have complex interactions with the other dwellers of their native ecosystems that drive the evolution of traits contributing to their survival. These traits also contribute to their ability to invade or colonize animal and human hosts. In their chapter, they summarize relationships of *Vibrio* spp. with other organisms in the aquatic environment and discuss how these interactions could potentially impact colonization of animal and human hosts.

The emergence of choleragenic *V. cholerae* remains a major mystery as only one group, the pandemic group, is capable of causing cholera in humans. In Chap. 7, Salvador Almagro-Moreno and his colleagues examine the emergence of pathogenic *V. cholerae* and cholera pandemic dynamics. The authors discuss the diverse molecular mechanisms associated with the evolution of pandemic *V. cholerae*, including the well-known mobile genetic elements that encode the critical virulence factors, and highlight novel discoveries that are shedding light on the constraints behind the unique distribution of pandemic clones. Finally, they provide an overview of the cholera pandemics from an evolutionary perspective.

In Chap. 8, Cecilia Silva-Valenzuela and Andrew Camilli examine the role of bacteriophages in the evolution of pathogenic *Vibrios* and discuss lessons for phage therapy. Bacteriophages were discovered over a century ago and have played a major role as a model system for the establishment of molecular biology. Despite their relative simplicity, new aspects of phage biology are consistently being discovered, including mechanisms for battling defenses put up by their *Vibrio* hosts. The authors discuss these mechanisms and contend that a deeper understanding of the arms race between *Vibrio* and their phages will be important for the rational design of phage-based prophylaxis and therapies to prevent against these bacterial infections.

*V. vulnificus* continues being an underestimated yet lethal zoonotic pathogen. In Chap. 9, Carmen Amaro and Hector Carmona-Salido provide a comprehensive review of numerous aspects of the biology, epidemiology, and virulence mechanisms of this poorly understood pathogen. They emphasize the widespread role of horizontal gene transfer in *V. vulnificus*, specifically virulence plasmids, and draw parallels from aquaculture farms to human health. By placing current findings in the context of climate change, they contend that fish farms act as evolutionary drivers that accelerate species evolution and the emergence of new virulent groups. They suggest that on-farm control measures should be adopted both to protect animals from vibriosis and as a public health measure to prevent the emergence of new zoonotic groups.

Over the past few decades, the importance of specific nutrients and micronutrients in the environmental survival, host colonization, and pathogenesis of *V. cholerae* has become increasingly clear. For instance, *V. cholerae* has evolved ingenious mechanisms that allow the bacterium to colonize and establish a niche in the intestine of human hosts, where it competes with commensals and other pathogenic bacteria for available

nutrients. In Chap. 10, Fidelma Boyd and her colleagues discuss the carbon and energy sources utilized by *V. cholerae* and the current knowledge on the role of nutrition and intestinal colonization dynamics of the bacterium. They also examine how nutritional signals affect virulence gene regulation and how interactions with intestinal commensal species can affect intestinal colonization.

In Chap. 11, Jyl Matson and Jay Akolkar examine the role of stress responses in pathogenic *Vibrios* in host and environmental survival. Pathogenic *Vibrios* are regularly exposed to numerous different stress-inducing agents and conditions in the aquatic environment and when colonizing a human host. Naturally, they have developed a variety of mechanisms to survive in the presence of these stressors. The authors discuss what is known about important stress responses in pathogenic *Vibrio* species and their critical role in bacterial survival.

Ronnie Gavilan and Jaime Martinez-Urtaza provide a thorough review in Chap. 12 on *V. parahaemolyticus* epidemiology and pathogenesis, highlighting novel insights of this emergent foodborne pathogen. They address the microbiological and genetic detection of *V. parahaemolyticus*, the main virulence factors, and the epidemiology of genotypes involved in foodborne outbreaks globally. Interestingly, the epidemiological dynamics of *V. parahaemolyticus* infections remain obscure as the disease is characterized by the abrupt appearance of outbreaks in areas where the bacterium had not been previously detected. They discuss the recent studies that show the link between the appearance of epidemic outbreaks of *Vibrio* and environmental factors such as oceanic transport of warm waters and how recent genomic advances allow us to infer possible biogeographical patterns of *V. parahaemolyticus*.

During periods that are not conducive for growth or when facing stressful conditions, *Vibrios* enter a dormant state called viable but non-culturable (VBNC). In Chap. 13, Sariqa Hagley analyzes the role of VBNC in *Vibrio* survival and pathogenesis and the molecular mechanisms regulating this complex phenomenon. She emphasizes some of the novel findings that make "studying the VBNC state now more exciting than ever" and its significance in the epidemiology of these pathogens and its critical role in food safety.

One of the best studied aspects of pathogenic *Vibrios* are the virulence cascades that lead to the production of virulence factors and, ultimately, clinical outcomes. In Chap. 14, Jon Kull and Charles Midgett examine the regulation of *Vibrio* virulence gene networks from a structural and biochemical perspective. The authors discuss the recent research into the numerous proteins that contribute to regulating virulence in *Vibrio* spp. such as quorum sensing regulator HapR, the transcription factors AphA and AphB, or the virulence regulators ToxR and ToxT. The authors highlight how insights gained from these studies are already illuminating the basic molecular mechanisms by which the virulence cascade of pathogenic *Vibrios* unfolds and contend that understanding how protein interactions contribute to the host–pathogen communications will enable the development of new antivirulence compounds that can effectively target these pathogens.

The critical role of environmental reservoirs in the distribution of pathogenic *Vibrios* and how they can potentially drive outbreaks are beginning to be understood. In Chap. 15, Brandon Ogbunugafor and Andrea Ayala explore the increasingly appreciated contribution of birds in the spread of pathogenic *Vibrios* and its epidemiological consequences. To date, eleven of the twelve pathogenic *Vibrio* species have been isolated from aquatic and ground-foraging bird species. The authors discuss the implications that these findings have for public health, as well as the One Health paradigm. They contend that as pathogenic *Vibrios* become more abundant throughout the world as a result of warming estuaries and oceans, susceptible avian species should be continually monitored as potential reservoirs for these pathogens.

The first *Vibrio* genomes were sequenced 20 years ago revealing a functional and phylogenetic diversity previously unimagined as well as a genome structure indelibly shaped by horizontal gene transfer. Since then a plethora of genomes from pathogenic isolates has been added to the databases and an unprecedented degree of knowledge has been gleaned from them. In Chap. 16, Martinez-Urtaza and his colleagues highlight some of the major lessons that we have learned from *Vibrio* pathogen genomics in the past few decades. The initial glimpses into these organisms also revealed a genomic plasticity that allowed these bacteria to thrive in challenging and varied aquatic and marine environments, but critically also a suite of pathogenicity attributes. The authors outline how the advent of genomics and advances in bioinformatic and data analysis techniques provided a more cohesive understanding of how these pathogens have evolved and emerged from environmental sources, their evolutionary routes through time and space, and how they interact with other bacteria and the human host.

Orlando, FL — Salvador Almagro-Moreno
New York, NY — Stefan Pukatzki

# Contents

# About the Editors

**Salvador Almagro-Moreno** is Assistant Professor of Medicine at the University of Central Florida. He was the E.E. Just Postdoctoral Fellow at Dartmouth College in Dr. Ronald Taylor's lab where he studied molecular pathogenesis and emergence of pathogenic *Vibrios*. His research interests include elucidating the molecular adaptations developed by bacteria in order to colonize the human host and how environmental factors affect their pathogenic potential. His research program focuses on two model systems as distinct paradigms of pathogen emergence: *Vibrio cholerae*, the etiological agent of the severe diarrheal disease cholera, and *Vibrio vulnificus*, an emergent pathogen that causes deadly septicemia. His research approach strives to be holistic and multidisciplinary: "*From Bays to Bases*." It encompasses a mix of molecular biology, genomics, phylogenetics, pathogenesis, and ecology.

**Stefan Pukatzki** is the Sharon D. Cosloy Professor of Biology at the City College of New York. He did his postdoctoral studies at Harvard University in the laboratory of Dr. John Mekalanos where he co-discovered the type VI secretion system in bacteria using *Vibrio cholerae* as a model system. His laboratory explores why a minority of bacteria evolved mechanisms to harm the human host. This behavior is not common, because symbiotic strategies to co-exist with the host are abundantly available. His research on host–pathogen interactions allows us to observe an "arms race" of attack over evolutionary time, which generates concepts and approaches that can be applied to develop alternative therapies to treat antibiotic-resistant bacteria.

# *Vibrio* Infections and the Twenty-First Century

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Salvador Almagro-Moreno, Jaime Martinez-Urtaza, and Stefan Pukatzki

**Abstract**

The Vibrionaceae is a highly diverse family of aquatic bacteria. Some members of this ubiquitous group can cause a variety of diseases in humans ranging from cholera caused by *Vibrio cholerae*, severe septicemia caused by *Vibrio vulnificus*, to acute gastroenteritis by *Vibrio parahaemolyticus*. Planet Earth is experiencing unprecedented changes of planetary scale associated with climate change. These environmental perturbations paired with overpopulation and pollution are increasing the distribution of pathogenic Vibrios and exacerbating the risk of causing infections. In this chapter, we discuss various aspects of *Vibrio* infections within the context of the twenty-first century with a major emphasis on the aforementioned pathogenic species. Overall, we believe that the twenty-first century is posed to be both one full of challenges due to the rise of these pathogens, and also a catalyst for innovative and groundbreaking discoveries.

**Keywords**

*Vibrio* infections · Climate change · Cholera · Global warming · *Vibrio parahaemolyticus* · *Vibrio vulnificus*

## 1.1 *Vibrio* Infections

The Vibrionaceae encompasses a group of ubiquitous aquatic bacteria that inhabit freshwater, estuarine, and marine environments (Reen et al. 2006; Baker-Austin et al. 2018; Austin et al. 2020). Some members of this family can be pathogenic to humans and cause the majority of human infections caused by bacteria of aquatic origin (Baker-Austin et al. 2018). *V. cholerae* represents the best known and most widely studied pathogenic species within the Vibrionaceae. A phylogenetically confined group of *V. cholerae*, the Pandemic Group (PG), causes the severe diarrheal disease cholera in humans (Chun et al. 2009; Boucher 2016; Shapiro et al. 2016). Toxigenic strains of *V. cholerae* belong to two serogroups, O1 and O139, the latter being close to extinction (Clemens et al. 2017; Kanungo et al. 2022). The O1 group can be further subdivided into Classical and El Tor strains, with the former having caused the first six pandemics of cholera,

S. Almagro-Moreno (✉)
Burnett School of Biomedical Sciences, University of Central Florida, Orlando, FL, USA

National Center for Integrated Coastal Research, University of Central Florida, Orlando, FL, USA
e-mail: samoreno@ucf.edu

J. Martinez-Urtaza
Department de Genetica I de Microbiologia, Facultat de Biociencies, Universitat Autonoma de Barcelona, Barcelona, Spain

S. Pukatzki
Department of Biology, The City College of New York, New York, NY, USA



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whereas El Tor being the source of the seventh and current pandemic (Balasubramanian et al. 2021). Other *V. cholerae* strains can cause sporadic disease in humans, known combined as non-O1 non-O139 strains. Even though infections by these strains are rare, they can cause severe gastrointestinal and extraintestinal infections (Deshayes et al. 2015). Cholera remains a major scourge in places with limited access to clean drinking water and poor sanitation infrastructure cholera outbreaks are increasing in frequency and intensity (Clemens et al. 2017; Kanungo et al. 2022). Nonetheless, as we discuss below, ambitious yet feasible frameworks are being proposed to eliminate the disease in the coming decades (Kanungo et al. 2022; Qadri et al. 2017; Editorial Lancet Gastroenterology Hepatology 2017; Francois 2020; Islam et al. 2022).

Infections by non-cholera Vibrios are commonly known as Vibriosis. The two most common and relevant ones are caused by *V. vulnificus* and *V. parahaemolyticus*. *V. vulnificus* is an emergent zoonotic pathogen that can cause a fulminant septicemia in susceptible hosts. The bacterium is typically contracted either through (a) the consumption of contaminated seafood, in particular oysters, resulting in gastroenteritis or primary septicemia or (b) exposure of wounds to sea water or products contaminated with the bacterium resulting in wound infections and secondary septicemia. *V. vulnificus* is the leading cause of seafood-associated deaths in the USA and is endemic to the Gulf and Southeastern coast (Phillips and Satchell 2017; López-Pérez et al. 2021). However, much uncertainty remains about the virulence of the organism (López-Pérez et al. 2019; Roig et al. 2018). For instance, recent genomic surveys determined that the known virulence factors of *V. vulnificus* strains are widespread within the species, with every strain analyzed encoding them (López-Pérez et al. 2019; Roig et al. 2018). Therefore, to date, the specific factors that allow only certain strains within the species to cause human disease remain to be elucidated. Furthermore, reliable markers that predict a high pathogenic potential of specific strains are still lacking, rendering this organism a unique threat to public health (Baker-Austin et al. 2018; Jones and Oliver 2009; Oliver 2012, 2015). *V. vulnificus* can also wreak havoc in aquaculture farms, a setting that allows the bacterium to quickly proliferate and be transmitted to humans. Besides the economic losses associated with this menace, novel hybrid strains can emerge in these settings as evidenced by a deadly Israeli outbreak in the 1990s (Paz et al. 2007; Amaro et al. 2015). Overall, efforts to understand and scrutinize the evolutionary and ecological trajectories of this pathogen are critical to prevent this zoonotic agent from expanding its narrow susceptible host range and habitat preference.

*V. parahaemolyticus* infections are associated with the consumption of raw or undercooked seafood and are characterized by a severe gastroenteritis that is distinct from cholera. Also, unlike *V. cholerae*, the bacterium cannot be transmitted from host to host or via the fecal-oral route. *V. parahaemolyticus* had been mostly restricted to Japan until the late 1960s, since then, infections associated with the bacterium started being reported worldwide turning this pathogen into a global public health concern (Baker-Austin et al. 2018; Letchumanan et al. 2014; Martinez-Urtaza and Baker-Austin 2020). In most cases, the disease resolves without the need for treatment, however, *V. parahaemolyticus* also can cause debilitating and dysenteric forms of gastroenteritis, necrotizing fasciitis, and septicemia in immunocompromised patients (Baker-Austin et al. 2018; Letchumanan et al. 2014; Martinez-Urtaza and Baker-Austin 2020; Zhang and Orth 2013). Since the date of its identification in 1953, *V. parahaemolyticus* infections have been reported in various parts of the world, causing outbreaks in Asia, Europe, and America. Pathogenic strains are mostly restricted to two serotypes, which are defined by somatic (O) and capsular (K) antigens (Baker-Austin et al. 2018; Letchumanan et al. 2014; Martinez-Urtaza and Baker-Austin 2020; Zhang and Orth 2013). Specifically, the O3:K6 (sequence type 3) and O4:K12 (sequence type 36) serotypes have been responsible for a large number of *V. parahaemolyticus* outbreaks and are associated with the pandemic expansion events of this

pathogen (Baker-Austin et al. 2018; Letchumanan et al. 2014; Martinez-Urtaza and Baker-Austin 2020; Zhang and Orth 2013). The specific set of drivers that ultimately led to the expansion of these two serogroups remains unknown; however, molecular, and *in vivo* data indicate that these strains possess increased virulence capabilities compared with other serogroups (Martinez-Urtaza and Baker-Austin 2020; Zhang and Orth 2013). Strategies to reduce incidence of *V. parahaemolyticus* involve the identification and monitoring of the environmental abiotic conditions that significantly elevate its risk. Specifically, bivalve mollusks, such as oysters and mussels, can harbor large concentrations of this pathogen leading to increased risk of infection after ingestion (Martinez-Urtaza and Baker-Austin 2020). Therefore, monitoring virulent strains of *V. parahaemolyticus* in seafood products is a major health safety concern that must be prioritized to mitigate future outbreaks of this pathogen (Martinez-Urtaza and Baker-Austin 2020).

There are other *Vibrio* species that can be pathogenic to humans, however, their reduced incidence and severity are overshadowed by the three aforementioned pathogens. Those include *Vibrio fluvialis*, *Vibrio mimicus*, *Vibrio hollisae*, *Vibrio metschnikovii, Vibrio cincinnatiensis*, *Vibrio furnissii,* or more commonly *Vibrio alginolyticus*, which can cause gastroenteritis, wound, or ear infections (Baker-Austin et al. 2017, 2018). Nonetheless, the number of cases associated with some of these species such as *V. alginolyticus* or *V. fluvialis* continue increasing suggesting a potential source of concern over the coming years. Unfortunately, there are no global surveillance frameworks that systematically gather epidemiological data on pathogenic Vibrios, and very few countries have dedicated surveillance systems for them (Newton et al. 2012; Janda et al. 2015). Critically, it is imperative in order to prevent the unpredicted appearance of *Vibrio* outbreaks to prioritize the development of frameworks to assess the spread and distribution of these potential pathogens. Furthermore, monitoring is needed to reduce the impact that emergent strains or novel pathogenic species within the *Vibrio* group might have in human populations and aquaculture settings.

## 1.2 Vibrios and the Environment

In their natural environment, pathogenic Vibrios can be frequently found associated with other aquatic dwellers such as copepods and crustaceans (Huq et al. 1983; Tamplin et al. 1990; de Magny et al. 2011; Turner et al. 2014), arthropods and chironomid egg masses (Broza and Halpern 2001; Purdy and Watnick 2011), cyanobacteria (Epstein 1993; Greenfield et al. 2017; Reddi et al. 2018), shellfish (Phillips and Satchell 2017; Twedt et al. 1981; Hood et al. 1981; de Sousa et al. 2004), waterfowl (Halpern et al. 2008), or fish (Amaro et al. 2015; Senderovich et al. 2010; Novoslavskij et al. 2015; Messelhäusser et al. 2010). In addition, Vibrios generally face a wide range of abiotic and biotic stressors that pose a threat to their survivability such as nutrient limitation, pH changes, temperature, and salinity fluctuations, or protozoal grazing and phage predation (Almagro-Moreno and Taylor 2013; Lutz et al. 2013; Jayakumar et al. 2020). It appears that some of the mechanisms that allow these bacteria to colonize and persist in their natural environment provide preadaptations for virulence in the human host (Phillips and Satchell 2017; Zhang and Orth 2013; Broberg et al. 2011; Sakib et al. 2018; Cabanyero and Amaro 2020).

During adverse environmental conditions (e.g. antibiotic exposure, nutrient limitation) *Vibrio* cells enter a non-sporulating protective dormant state that enhances their survival and long-term persistence called viable but nonculturable (VBNC) (Almagro-Moreno and Taylor 2013; Lutz et al. 2013; Jayakumar et al. 2020). When external conditions become favourable (e.g. nutrient influx, reduction of antibiotics) dormant cells can recover from the

VBNC state, a phenomenon also known as awakening or resuscitation. VBNC cells pose a major public health risk, as these pathogens can be found in this state during interepidemic periods, furthermore, they are a difficult to detect and eradicate source of food and water contamination (Almagro-Moreno and Taylor 2013; Lutz et al. 2013).

The growth and overall distribution of pathogenic Vibrios is severely affected by external environmental conditions. *Vibrio* infections naturally have very marked seasonal distribution as their abundance is primarily driven by increased temperature, salinity, and rainfall events (Huq et al. 1984, 2013; Lobitz et al. 2000). During warm summer months, Vibrios populations experience drastic blooms, which increase the likelihood of susceptible individuals to become in contact with them and contract the diseases associated with their pathogenic species. Furthermore, extended periods of warm weather, driven by climate change, have provided suitable conditions for the proliferation of pathogenic *Vibrio* spp. (Baker-Austin et al. 2018; Austin et al. 2020). As we discuss below, a multidecadal study recently demonstrated a steady increase in the abundance of pathogenic Vibrios, including *V. cholerae*, over the past half-century (Vezzulli et al. 2016). Furthermore, some water bodies are warming up faster than the global average such as the Baltic Sea, the White Sea, and those along the US east coast, posing a very high risk of *Vibrio* infections (Baker-Austin et al. 2013; Martinez-Urtaza et al. 2013; Rice and Jastram 2014). These patterns only exacerbate the problem of the emergence and reemergence of pathogenic Vibrios, the spread of virulence genes and their proliferation (Trinanes and Martinez-Urtaza 2021).

## 1.3 Life on a Warming Planet: Climate Change and the Global *Vibrio* Expansion

Human activity since the beginning of the industrial age has had an unprecedented impact on climate and on the future of life on the planet. The combustion of coal and other fossil fuels has generated levels of greenhouse gases that has caused a deep change on global climate patterns with impacts being perceptible at all ecological scales. The effects of climate change have a strong regional component, with geographical areas showing a faster rate of warming than others. In general terms, warming is having a greater effect on marine ecosystems because oceans capture more than 90% of all the heat (Zanna et al. 2019). In coastal areas, the most relevant impacts of climate change include the increase of temperatures, frequency of extreme weather events, and rise of sea level. Some areas are experiencing faster warming rates than others (Lima and Wethey 2012). For instance, the Baltic Sea, the Mediterranean Sea and the Northeastern USA are three marine regions with warming rate above the global average (Karmalkar and Horton 2021). Events of extreme weather, such as heat waves or torrential rains have a strong impact on coastal areas due to their shallow waters. Extreme weather rapidly influences temperature and salinity conditions in adjacent areas capturing the heat or assimilating the rainwater, causing a rise in temperature or sudden drops in salinity. The thawing of ice masses at the poles and large glaciers is mobilizing large masses of fresh water into the oceans with drastic consequences for oceanic currents, as well as generating a rise in sea water level that is causing the flooding of shorelines globally (Llovel et al. 2019).

Not every living organism is being affected negatively by climate change. For instance, some insects, such as mosquitoes, are being favoured by this new climatic situation with higher temperatures and higher humidity that facilitates the expansion towards the poles and they occupy new ecological niches at high latitudes that until recently were not suitable for these organisms. Interestingly, from the many examples of species benefitting from the conditions imposed by climate change, Vibrios have emerged as a barometer of climate change (Baker-Austin et al. 2017). *Vibrio* species have some of the fastest growth rates among bacteria (Joseph et al. 2008; Aiyar et al. 2002). This key characteristic shared by all members is critical to

understand their adaptive ecological success and pathogenic potential (Baker-Austin et al. 2017). Under favourable conditions, Vibrios can double their populations in a matter of minutes. This facilitates their expansion and rapid occupation of new niches, which provides the ideal conditions to trigger infections (Baker-Austin et al. 2013). The shift in ecological conditions has two major potential effects on *Vibrio* populations: (a) increase the seasonal abundance (occurrence for longer periods) and (b) an expansion of their distribution range towards the poles.

Recent studies demonstrate the impacts of climate change on *Vibrio* populations showing the steady expansion of these species across coastal areas worldwide during the last 30 years (Baker-Austin et al. 2018). Around 71% of the world's coastal areas are warming at different rates. In the waters of enclosed or nearly enclosed seas (e.g. Mediterranean Sea or Gulf of Mexico), the rate of warming is even greater than the one in the oceans (Dutheil et al. 2022). As a result of these changes, the number of days with suitable conditions for the presence of *Vibrio* in shorelines across the planet has increased since the 1980s by about 10%. Vibrios have been identified in areas located at high latitudes as suitable ecological conditions have been amplified toward the poles reaching areas near the Arctic Circle (Baker-Austin et al. 2013, 2016). Recent progress in our understanding of the ecology of *Vibrio* has been a key element in the development of new frameworks for the construction of models to generate epidemiological and predictive tools (Semenza et al. 2017). For instance, these tools aid at remotely identifying areas with favourable ecological conditions for *Vibrio* growth and dispersal based on environmental data obtained from satellites and other remote sensing technologies (Semenza et al. 2017). The use of environmental data that dates back to the pre-industrial period together with the application of advanced climate models, has been combined to build a new generation of monitoring systems that enable to reconstruct the past, understand the present and predict the future of the environmental conditions for *Vibrio* on the planet (Trinanes and Martinez-Urtaza 2021). These studies show that the extent of coastal zones favourable for *Vibrio* remained relatively stable until 1980. Since then, the expansion of Vibrios has been increasing rapidly and in parallel to the rate of global warming, with an expansion towards the poles. Suitable periods for the occurrence of *Vibrio* have been amplifying at a rate of 1 month every 30 years. Furthermore, the distribution of these bacteria is reaching new areas that were considered adverse for the presence of *Vibrio* only a few years ago (Fig. 1.1). In fact, at the current rate of warming, their distribution is expected to extend about 38,000 km by the year 2100 (Fig. 1.1) (Trinanes and Martinez-Urtaza 2021).

Global human populations living in coastal regions with suitable conditions for *Vibrio* grew over the past century and reached an estimated value of 610 million people by 1980 (Trinanes and Martinez-Urtaza 2021). The projection for 2020 duplicated the estimate for 1980, ranging from 1107 to 1133 million according to different scenarios (Trinanes and Martinez-Urtaza 2021). This trend is expected to continue to increase until 2050 and after this point simulations show a stabilization in the projections or even a slight decline (Trinanes and Martinez-Urtaza 2021). Population at risk for *Vibrio* infections in suitable regions almost doubled from 1980 to 2020 (from 610 million to 1100 million), although the increment will be more moderate in the future, and it is expected to reach stable conditions after 2050 at 1300 million (Trinanes and Martinez-Urtaza 2021). According to these estimates, the global estimate for non-cholera *Vibrio* infections would be around half a million of cases worldwide in 2020. Geographical areas with the largest population at risk are in coastal areas of the north of Europe, southeast Asia, the Gulf of Guinea, the Atlantic northeast, the Pacific northwest, and some specific hot spots in the Gulf of Venice, the south coast of the Black Sea, and coastal areas of Egypt (Trinanes and Martinez-Urtaza 2021). New regions for populations at risk identified in high latitudes in the northern hemisphere (Russia and Canada) are a clear indication of the poleward expansion of *Vibrio* infections (Fig. 1.1). However, projections indicate that the growth trend in the number of cases will be

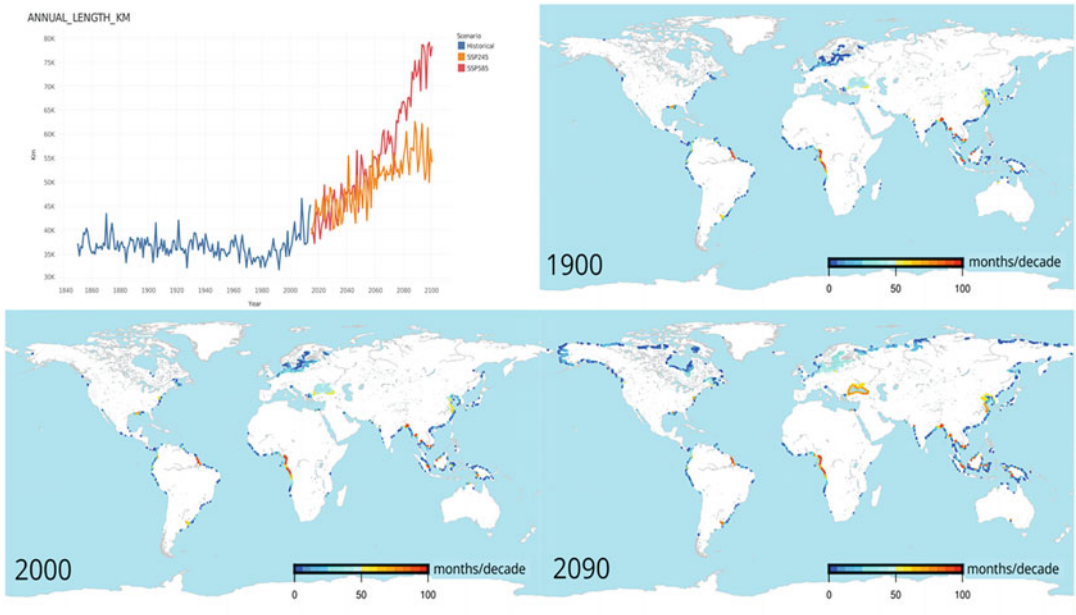


**Fig. 1.1** Suitable *Vibrio* habitats over time. Changes in the extent (in thousands of km) of coastal areas with suitable ecological conditions for *Vibrio* in the planet since the pre-industrial period (1840), and distribution of these areas in 1900, 2000 and projections for 2090 according to different climate scenarios. Adapted from J. Trinanes and J. Martinez-Urtaza, The Lancet Planetary Health, 5:e426–35

weakened for the next decades primarily due to (a) the stabilization of the world population in regions with *Vibrio* risk and (b) the low population in new areas at high latitudes reaching favourable conditions for *Vibrio*.

## 1.4 The Future of Cholera

Cholera is an ancient disease that remains a major scourge in places with limited access to clean drinking water, poor sanitation practices or social unrest (Kanungo et al. 2022; Lancet 2017; Grant et al. 2021). Estimates indicate that the disease continues to infect over 3 million people and kill over 100,000 per year (Kanungo et al. 2022; Islam et al. 2022). Nonetheless, the real disease burden is difficult to calculate due to the large number of cases that remain unreported. Currently, cholera remains endemic and continues to be reported from several countries in Asia (Bangladesh, India, Philippines, and Myanmar), Africa (Cameroon, Democratic Republic of Congo, Kenya, Somalia, Sudan, and Mozambique), the Caribbean (Haiti) and the Middle East (Yemen and Syria) (Kanungo et al. 2022; Islam et al. 2022). Recent outbreaks of epidemic cholera due to war and/or natural disasters have been reported in refugee camps in Bangladesh, Syria, Yemen, and Lebanon (Kanungo et al. 2022; Islam et al. 2022; Connolly et al. 2004). For instance, human displacement due to a civil war in Yemen led to the largest cholera outbreak recorded in human history (Qadri et al. 2017; Lancet 2017).

**The Global Roadmap to 2030** proposes to end the disease within this decade and suggests a comprehensive approach based on (a) early detection of cholera cases and prompt responses to contain outbreaks, (b) a targeted multisectoral approach to prevent disease recurrence, and (c) an effective and coordinated mechanism for technical support, mobilization of resources and partnerships at local and global levels (Kanungo et al. 2022; Islam et al. 2022). This approach must be cheap and must require limited expertise to be

widely implemented. Furthermore, it must be delivered and maintained on the ground by community health workers and should include rapid diagnostics, real-time reporting, and proper treatment for mild and severe cases (Islam et al. 2022).

There are two complementary approaches for the prevention and control of cholera: (1) **Short term**, Oral Cholera Vaccines (OCVs), as they provide faster but temporary protection, rapid diagnostics and real-time reporting, and (2) **Long term**, the WASH framework, which stands for improving water, sanitation, and hygiene. The latter lacks immediacy but can lead to sustained reductions in transmission of *V. cholerae* O1.

1. **Short term**
   (a) **OCVs.** Three types of OCVs are available: killed whole-cell vaccines (Shanchol and Euvichol), killed whole-cell vaccines with a recombinant B subunit (Dukoral), and a live attenuated vaccine (Vaxchora) (Clemens et al. 2017; Bhattacharya et al. 2013; Clemens et al. 1988; Sur et al. 2009, 2011; Baik et al. 2015; Bi et al. 2017). The latter two are primarily used by people travelling to cholera-endemic areas, whereas Shanchol and Euvichol are the OCVs used during cholera outbreaks. OCVs stockpiles were created after the cholera outbreaks in Zimbabwe and Haiti to facilitate and ease the supply of vaccines during emergencies. The number of doses has increased from 2 million per year (2013), to 25 million (2021); however, given the large demand of OCVs, vaccine supply must increase over the coming years to lead to a lasting effect on the disease.
   (b) **Rapid diagnostics and real-time reporting.** Rapid diagnostic tests should be used in the home of patients with suspected cases of cholera using some of the tests that are currently available, such as Cholkit (Incepta Pharmaceuticals) and Crystal VC (Arkray Healthcare) (Chowdhury et al. 2021). Even though these tests do not always provide 100% accuracy, they are inexpensive and widely accessible. The WHO's global task force has developed a cell phone-based app for cholera reporting: GTFCC cholera (Islam et al. 2022). The app acts as a real-time reporting method after a case is identified in the field and notifies health authorities helping map disease transmission and evaluate control strategies.
   (c) **Treatment.** Patients with mild to moderate signs of dehydration can be effectively treated at home with an oral rehydration solution plus zinc (Davies et al. 2017; Pietroni 2020; Sousa et al. 2020). If a patient is deemed to have severe dehydration, they must be referred to a local hospital and receive immediate intravenous fluid replacement over three hours for adults and six hours for children less than 1 year of age (Davies et al. 2017; Pietroni 2020; Sousa et al. 2020). Antibiotics should be used only in patients with severe dehydration, options including macrolides, fluoroquinolones, and tetracycline (Davies et al. 2017; Pietroni 2020; Sousa et al. 2020). Azithromycin can be used prophylactically for household contacts after cholera detection in a home as it is effective both for the treatment of cholera and in preventing colonization of *V. cholerae* in the gut.
2. **Water, sanitation, and hygiene framework (WASH).** Numerous basic characteristics of cholera outbreaks are shared among settings (e.g. the pathophysiology of the disease, the waterborne nature of transmission, etc.). Nonetheless, recent findings suggest that transmission within households in endemic settings may play a larger role in cholera outbreaks than previously appreciated (D'Mello-Guyett et al. 2020; Sugimoto et al. 2014; Meszaros et al. 2020). Focused interventions around the households of medically attended patients with cholera represent an efficient way of interrupting transmission (Ratnayake et al. 2020). Specifically,

approaches that include WASH interventions have been shown to reduce the duration of outbreaks at a community scale in Haiti (Michel et al. 2019). Furthermore, mathematical models of cholera that incorporate transmission within and between households show that variation in the magnitude of household transmission changes multiple features of disease dynamics, including the severity and duration of outbreaks (Meszaros et al. 2020). Importantly, integrating household transmission into cholera models influences the effectiveness of possible public health interventions (e.g. water treatment, antibiotics, OCVs) indicating vaccine interventions are more effective than water treatment or antibiotic administration when direct household transmission is present.

Approximately 1.6 billion people in the world live without safe water at home and 2.8 billion people without safe sanitation. Major infrastructure improvements, including piped water and sewage systems, are needed in order to achieve potential elimination of cholera as it was previously achieved in parts of Latin America and Europe (Balasubramanian et al. 2021). Nonetheless, while these are implemented, there are several smaller-scale WASH interventions that can be used to reduce cholera risk. For instance, safe storage of water systems and point-of-use water treatment, provision of sanitation facilities and campaigns targeted at increasing handwashing and other sanitary practices (Kanungo et al. 2022; Balasubramanian et al. 2021; Islam et al. 2022). These smaller-scale interventions can lead to sustainable reductions in cholera incidence and will ease the implementation of longer term ones that will lead to the control and eventual demise of this scourge.

## 1.5 Emergence of Novel Pathogenic Variants: *Vibrio vulnificus* and Aquaculture

Aquaculture is one of the fastest-growing global food industries, accounting for more than 50% of the world's fish supply. Most of this development has occurred in the past 50 years and is projected to rise significantly to meet the accelerating demand for seafood (Ahmad et al. 2021; Botta et al. 2020). However, the environmental implications of such a dramatic increase are far-reaching as the expansion of this industry has led to reduced land availability, nutrient over-enrichment, release of toxic chemicals into the ecosystem, and threats to the food chain (Ahmad et al. 2021; Botta et al. 2020). Moreover, the excessive use of antibiotics to control infections in fish farms has majorly influenced the occurrence and spread of antimicrobial resistance among many marine bacterial species (Elmahdi et al. 2016; Ibrahim et al. 2020). Heavy reliance on antibiotics, over-intensive exploitation of aquaculture, and unrestricted industrialized practices have ultimately contributed to the emergence of several aquaculture-associated diseases (Sanches-Fernandes et al. 2022; Sony et al. 2021; Deng et al. 2020).

Vibriosis is one of the most prevalent bacterial diseases affecting a diverse array of marine organisms (Sony et al. 2021; Chatterjee and Haldar 2012). The economic losses associated with diseases in aquaculture were estimated to have been over $3 billion per year by 1997 and have nearly tripled in the last two decades to over $9 billion per year (Sanches-Fernandes et al. 2022; Chatterjee and Haldar 2012; Novriadi 2016). Several members of the family Vibrionaceae, including *V. vulnificus*, *V. parahaemolyticus*, *V. harveyi, V. alginolyticus*, and *V. anguillarum*, have been linked to vibriosis in marine species (Deng et al. 2020; Chatterjee and Haldar 2012). For instance, over two-third of disease cases reported in the *Epinephelus* spp. of fish are due to *V. parahaemolyticus* and *V. anguillarum* infections (Deng et al. 2020). *V. alginolyticus* and *V. harveyi* infections in China, the largest aquaculture market in the world, exhibit mortality rates as high as 80% (Deng et al. 2020). *V. vulnificus* has been associated with drastic mortality rates in aquaculture-raised marine species including *Anguilla* spp., tilapia, and shrimp (Amaro et al. 2015; Rippey 1994; Fouz and Amaro 2003; Mahmud et al. 2010; Chen et al.

2006; Longyant et al. 2008). Overall, vibriosis has led to a significant decline in fish health and production globally, posing a significant threat to the aquaculture industry.

*V. vulnificus*, one of the most frequently isolated *Vibrio* spp. from diseased seafood, is also the leading cause of non-Cholera, *Vibrio*-associated infections in humans (Phillips and Satchell 2017; Jones and Oliver 2009; Cabanyero and Amaro 2020). The annual case counts of *V. vulnificus* infections in humans have steadily increased over the past 20 years in the USA (Phillips and Satchell 2017), over 75% of which occur during summer (Wright et al. 1996; Givens et al. 2014). This high incidence rate strongly coincides with increased prevalence of *V. vulnificus* in estuarine environments corresponding to the high sea surface temperatures (>20 °C) and low-to-moderate salinities (5–25 ppt) encountered during that season (Wright et al. 1996; Givens et al. 2014; Levine et al. 1993; Bisharat et al. 1999; Tilton and Ryan 1987). Recent reports further demonstrate an upsurge in the worldwide distribution and abundance of *V. vulnificus* in correlation with increasing sea surface temperature and climate change (Paz et al. 2007; Baker-Austin et al. 2017; Kaspar and Tamplin 1993). This has led to disease outbreaks in regions with no prior history of *V. vulnificus* infections (Paz et al. 2007; Baker-Austin et al. 2017; Kaspar and Tamplin 1993). Furthermore, recent studies underline a strikingly high diversity and recombination rates in *V. vulnificus* populations (Fig. 1.2) (López-Pérez et al. 2019, 2021). This is particularly worrisome as practices such as aquaculture can lead to the emergence of hybrid strains (Fig. 1.2). The most prominent example of this is the *V. vulnificus* outbreak in Israel stemming from a novel hybrid clade. Between the years 1996–1997, 62 cases of wound infection and bacteremia were recorded in Israel, the majority of which occurred during the summer months of Aug-Oct (Bisharat et al. 1999). Interestingly, all 62 patients reported contact with aquaculture-reared tilapia fish. Molecular typing and phenotypic characterizations showed that the causative agent was a new bio-group of *V. vulnificus*, Biotype 3 (BT3) (Bisharat et al. 1999; Zaidenstein et al. 2008). All cases reported in this period were caused by BT3 strains associated entirely with tilapia or carp aquaculture. Typing and molecular evolutionary analyses show that members of the new BT3 are hybrid organisms evolved through the acquisition of genes from two distinct and independent populations, BT1 and BT2 (Bisharat et al. 2005). Although BT3 strains exhibit a high degree of genetic homogeneity, they are distinct from BT1 and BT2. For the first time, it was evidenced that close contact between two distinct populations led to the emergence of an infectious outbreak caused by a new pathogenic variant.

Prior to the 1996 outbreak, no cases of *V. vulnificus* human infections were reported in Israel. However, a single strain of halophilic bacteria that caused wound infection in a male patient after handling fish was reported in 1981, which proved to be genetically identical to the BT3 strains isolated after the 1996 outbreak (Paz et al. 2007). This suggested that the pathogen has been circulating within these water reservoirs long before the disease outbreak in 1996. Investigations assessing changing trends of *V. vulnificus* infections in Israel have reported patterns of increasing disease severity with rising sea surface temperatures, with more than 55% of cases occurring in patients with no known underlying diseases (Zaidenstein et al. 2008). Rising water temperatures fueled by climate change in the area could have increased prevalence of *V. vulnificus* populations over time, ultimately leading to the emergence of the disease outbreak in 1996 (Paz et al. 2007). Overall, given the distinctively high genome plasticity of this pathogen paired with the unexpected outcomes associated with manmade environmental changes and practices such as aquaculture, makes *V. vulnificus* a major threat to human health for which no effective therapeutic or surveillance strategies are available. The emergence of highly pathogenic hybrid variants of other *Vibrio* spp. could be a clamoring hazard in the coming

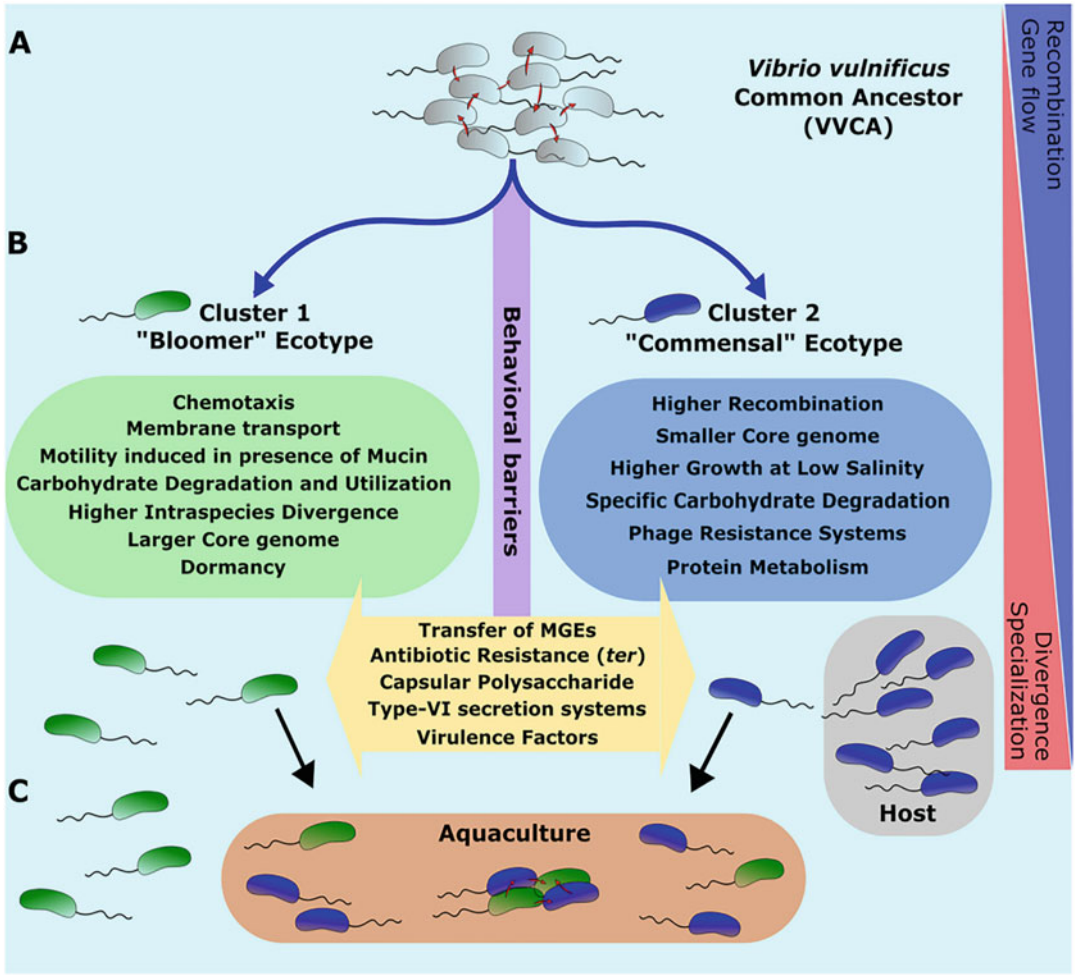


**Fig. 1.2** Evolutionary model of cluster divergence in *V. vulnificus*. (**a**) VVCA. Clonal lineages start diverging from the *V. vulnificus* common ancestor (VVCA). (**b**) Divergence. The acquisition of different ecological determinants allowed the development of diverse lifestyles within the same environment, which has led to a higher divergence. This divergence led to a recombination and gene flow decrease, although frequent exchange of mobile genetic elements is found within the species and with other species. (**c**) Convergence. With the advent of aquaculture, we have created an artificial environment that has led to colocalization of strains from the two major clusters. Adapted from M. López-Pérez et al., mBio, 2019, 10: e02852-18

decades that, as highlighted below, can only be exacerbated by the effects of climate change.

## 1.6 *Vibrio* Population Dynamics and Climate Change: The *Vibrio parahaemolyticus* Paradigm

To date, our understanding of the actual impacts that climate change has on Vibrios at the population and evolutionary level is still limited. For example it remains to be determined whether the colonization of new geographical areas is introducing any change on the effective population size of *Vibrio* populations. It is also possible that this expansion is the result of the dispersal of certain genetic variants that are adaptively successful in colonizing new areas, increasing the population census but with no effects on the effective population size. It remains to be

addressed whether any recent event in the planet linked to human activity or initiated by natural causes facilitated the restructuring of *Vibrio* populations with the consequent effects on the demography and evolution of these populations. A major limiting factor to address these questions is our inadequate knowledge on the demographic oscillations and evolutionary histories of *Vibrio* populations. *Vibrio* species are characterized by a high genetic diversity, a highly variable genome rich in accessory genes, one of the highest recombination rates among all bacteria, and poorly structured populations as a consequence of their unique evolutionary dynamics (Roux and Blokesch 2018; Yang et al. 2019a). The high genetic diversity and large pangenomes (the entire set of genes from all strains) are partly defined by their complex lifestyle. Their presence in habitats with very different conditions (e.g. seawater, plankton microbiome, human gut) requires a very large and diverse genetic repertoire to adapt effectively to these diverse environments and survive under highly variable conditions (Vázquez-Rosas-Landa et al. 2020).

Similar to other free-living organisms, Vibrios are characterized by their large pangenomes and effective population size, which typically correlates with the efficacy of natural selection. In particular, *V. parahaemolyticus* has an effective population size greater than $10^8$, which ranks it among the largest among all bacteria. This species also exhibits high recombination rates which progressively erase non-random associations between markers (linkage disequilibrium) and result in a less structured population in a near state of panmixia (in opposition to clonality characterized by very little or no genetic diversity among isolates) (Smith et al. 1993; Shapiro 2016; Yang et al. 2019b; Cui et al. 2020). The large availability of complete genomes of *V. parahaemolyticus* from global populations has enabled us to identify signals of the potential impacts of human activity on changes in demography or population structure of pathogenic Vibrios. The analysis of 1103 genomes revealed that the diversity patterns of *V. parahaemolyticus* populations are consistent with having arisen by progressive divergence through genetic drift during geographic isolation over most of its evolutionary history (Yang et al. 2019a). However, these analyses show that the genetic barriers keeping these populations isolated have been recently eroded by human-related activities or natural events that have enabled long-distance dispersals of local variants (Yang et al. 2019a). This dispersion has contributed to the introduction of new genetic variants in remote areas and the genetic exchange and overlap between different populations, consolidating a change in the biogeographical distribution of *V. parahaemolyticus*. Analyses based on time-calibrated divergence trees estimate that the processes of genetic mixing between the different populations occurred as recent as the past decades (Yang et al. 2019a).

Taken together these results indicate that human activity and/or recent profound ecological changes are responsible for the shift in the global distribution pattern of *V. parahaemolyticus* populations. Clearly certain human activities such as shipping, the global market of aquaculture products, or the increased migratory flows between continents may have contributed total or partially to the observed changes in these populations. All these activities have been intensified during the last decades and have originated a flow of water masses and living organisms from one continent to another. But natural causes, such as changes in plankton distribution patterns or ocean currents may also contribute to intensify long-distance migrations (Frémont et al. 2022). Climate change is restructuring the biogeography of plankton communities in the oceans at all scales, from viruses to mesozooplankton, and ocean currents are accelerating in response to warming (Richter et al. 2022). These complex and globally interconnected processes may be influencing a shift in the distribution of *Vibrio* populations given their planktonic nature and their connection to migratory process of other marine organisms. In the future, it is essential to introduce improvements into population analyses with the use of a more comprehensive collection of genomes and community structures (e.g. metagenomes) covering understudied areas

of the world in the existing repositories. Furthermore, the development of novel sets of tools to analyze bacterial populations is essential to have a more robust inference of the basic parameters of these population genetics. Another key area of research will be the study of the biological dynamics of *Vibrio* in offshore waters, including oceans, to explore the possible existence of cross-oceanic migrations. Oceanic biological corridors, similar to those that exist for other species of plankton or fish, would break the genetic isolation and contribute to the dispersal of *Vibrio* populations, including those with pathogenic potential, with major consequences towards the global burden of *Vibrio* diseases.

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# New Insights into *Vibrio cholerae* Biofilms from Molecular Biophysics to Microbial Ecology

2

Jung-Shen B. Tai, Micah J. Ferrell, Jing Yan, and Christopher M. Waters

**Abstract**

With the discovery that 48% of cholera infections in rural Bangladesh villages could be prevented by simple filtration of unpurified waters and the detection of *Vibrio cholerae* aggregates in stools from cholera patients it was realized *V. cholerae* biofilms had a central function in cholera pathogenesis. We are currently in the seventh cholera pandemic, caused by O1 serotypes of the El Tor biotypes strains, which initiated in 1961. It is estimated that *V. cholerae* annually causes millions of infections and over 100,000 deaths. Given the continued emergence of cholera in areas that lack access to clean water, such as Haiti after the 2010 earthquake or the ongoing Yemen civil war, increasing our understanding of cholera disease remains a worldwide public health priority. The surveillance and treatment of cholera is also affected as the world is impacted by the COVID-19 pandemic, raising significant concerns in Africa. In addition to the importance of biofilm formation in its life cycle, *V. cholerae* has become a key model system for understanding bacterial signal transduction networks that regulate biofilm formation and discovering fundamental principles about bacterial surface attachment and biofilm maturation. This chapter will highlight recent insights into *V. cholerae* biofilms including their structure, ecological role in environmental survival and infection, regulatory systems that control them, and biomechanical insights into the nature of *V. cholerae* biofilms.

**Keywords**

Biofilm · *Vibrio cholerae* · Biofilm structure · Biofilm regulation

## 2.1 The Structure and Developmental Process of *V. cholerae* Biofilms

The bacterial pathogen *Vibrio cholerae* is a worldwide pandemic that is responsible for millions of infections and 100,000 deaths (Hu et al. 2016; Ganesan et al. 2020). Disruptions to infrastructure or public health exacerbate cholera infections (Enserink 2010; Kuna and Gajewski 2017; Owoicho et al. 2021; Hassan and Nellums 2021). Here we review the role biofilm formation plays in the persistence and spread of *V. cholerae*. The developmental cycle of biofilms

Jung-Shen B. Tai and Micah J. Ferrell contributed equally with all other contributors.

J.-S. B. Tai · J. Yan
Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT, USA

M. J. Ferrell · C. M. Waters (✉)
Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI, USA
e-mail: watersc3@msu.edu



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is initiated by planktonic cells approaching and subsequently attaching to a solid surface (Hall-Stoodley et al. 2004). In the process, many factors including temperature, surface chemistry, nutrients, and environmental fluid flow can affect the mechanism and strength of bacterial adhesion to surfaces (Bos et al. 1999). The initial attachment of *V. cholerae* cells to a surface is mediated by the type IV mannose-sensitive hemagglutinin (MSHA) pilus and flagellum (Floyd et al. 2020; Utada et al. 2014). MSHA pilus is a dynamic extendable and retractable filamentous appendage, whose activity is controlled by cyclic di-GMP (c-di-GMP), a widespread second messenger in bacteria that is a linchpin cue for inducing a biofilm lifestyle (discussed more in detail in Sect. 2.3). MSHA pilus and flagellum synergistically modulate *V. cholerae* swimming motility near a surface and the eventual attachment. Frictional forces between MSHA pili and the surface result in two distinct surface-specific motility modes: "roaming," characterized by meandering trajectories due to low friction interactions, and "orbiting," characterized by repetitive high-curvature orbits and longer loiter time due to high friction interactions. Only orbiting cells eventually transition to irreversible attachment and develop into microcolonies (Floyd et al. 2020; Utada et al. 2014).

A key surface that *V. cholerae* interacts with in the environment is chitin, and several mechanisms are implicated in attachment to chitin surfaces. The Type IV ChiRP pilus, which is necessary for bacterial competence, contributes to attachment to chitin surfaces (Meibom et al. 2004; Adams et al. 2019). Another important colonization factor is GbpA, which mediates attachment to chitinous and epithelial cell surfaces by binding to the GlcNAc residues (monomer of chitin) and *V. cholerae* cell surface through separate domains. GbpA thus plays a significant role in colonization of chitin in the natural habitat and host intestine (Kirn et al. 2005; Wong et al. 2012). The toxin co-regulated pilus (TCP), another Type IV pilus encoded by *V. cholerae*, is most often associated with colonization of the small intestine as null mutations in TCP have a severe colonization defect (Thelin and Taylor 1996). However, TCP also mediates adherence of *V. cholerae* to chitin surfaces, although it does not appear to do so directly but perhaps by mediating cell-to-cell attachment (Reguera and Kolter 2005). Such an environmental role for TCP, which also functions as the receptor for the CTX phage that encodes cholera toxin (CT), suggests that this dual function is an evolutionary driver of *V. cholerae* pathogenesis (Reguera and Kolter 2005).

### *V. cholerae* Biofilm Matrix

Upon initial attachment, *V. cholerae* cells adhere robustly to both biotic and abiotic surfaces (Watnick et al. 1999; Fong and Yildiz 2007; Shikuma and Hadfield 2010) and the surface-attached cells begin to construct a three-dimensional (3D) structure through proliferation and production of extracellular polymeric substances (EPS), which form a highly hydrated polymer matrix in which cells are embedded. The structural integrity of *V. cholerae* biofilms is critically dependent on the production of the biofilm matrix (Flemming and Wingender 2010; Teschler et al. 2015). Multiple components in *V. cholerae* biofilm matrix have been identified, including the key polysaccharide, Vibrio polysaccharide (VPS), and three accessory matrix proteins, RbmA, Bap1, and RbmC (Fig. 2.1) (Teschler et al. 2015). When biofilm cells face environmental challenges such as nutrient limitation, they undergo dispersal such that the biofilm development cycle is reinitiated on a new favorable surface (Rumbaugh and Sauer 2020).

Among the matrix components of *V. cholerae* biofilm, VPS plays the dominant role in defining the biofilm structure of *V. cholerae* and the functioning of all accessary proteins is dependent on VPS (Fig. 2.1) (Fong et al. 2010). In response to environmental stresses, *V. cholerae* cells can develop phenotypically different colonies on agar: rugose and smooth colonial variants (Morris et al. 1996; Wai et al. 1998; Yildiz and Schoolnik 1999). Many studies suggest that the rugose phenotypes are associated with an elevated level of VPS production. The synthesis

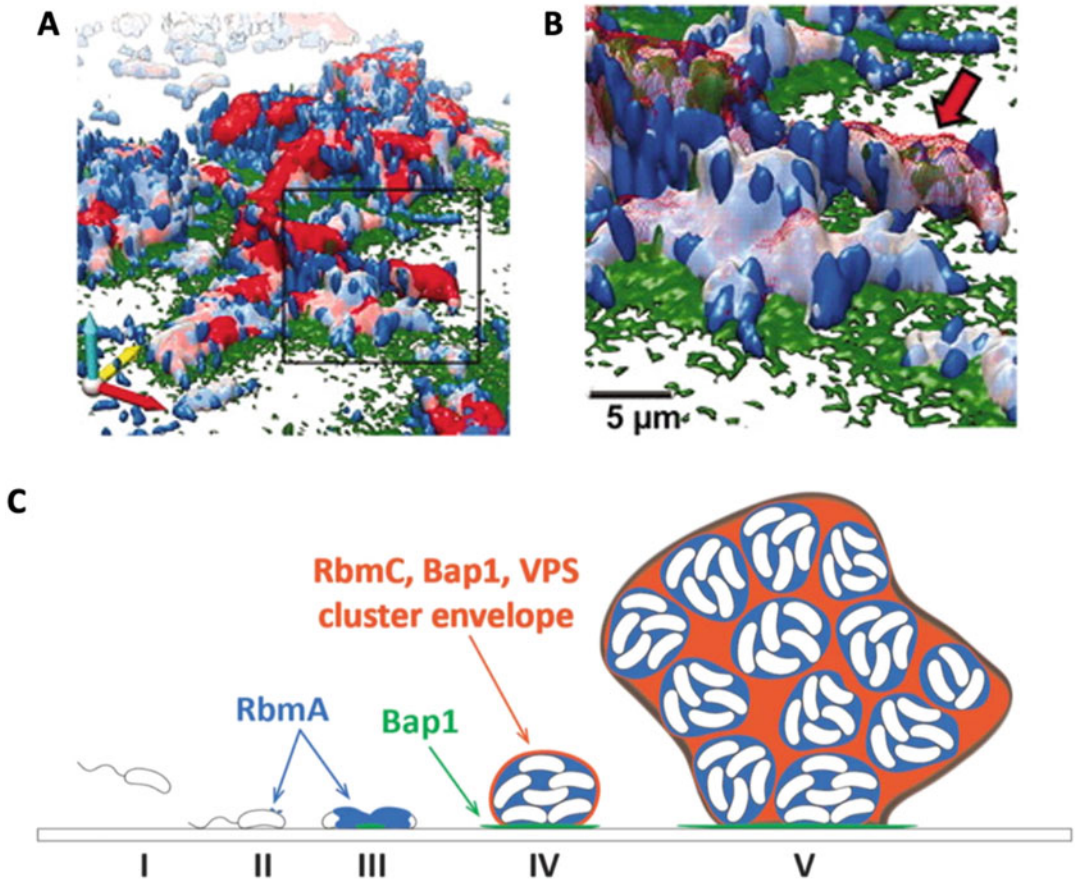


**Fig. 2.1** *V. cholerae* biofilm structure and matrix components. (**a**) 3D architecture of a *V. cholerae* biofilm obtained through high-resolution scanning confocal laser microscopy. Images are pseudo-colored in blue (cells), gray (RbmA), red (RbmC), and green (Bap1). RbmA localizes around and within the cell cluster, whereas Bap1 and RbmC encase the cell clusters. The Bap1 signal is also concentrated at the biofilm-substratum interface. (**b**) Zoomed-in view of the box region in (**a**). The red arrow indicates one cell cluster. (**c**) Proposed model of biofilm development in *V. cholerae*. This figure is adapted with permission from Berk et al. (2012)

and secretion of VPS are carried out by the products of *vps*-I and *vps*-II gene clusters (Fong et al. 2010). The chemical nature of VPS contains glucose, galactose, N-acetylglucosamine, and guluronic acid, and VPS is made of repeating units of an acetylated tetrasaccharide which is unique to *V. cholerae* (Yildiz et al. 2014; Reichhardt et al. 2015).

Among the other matrix components, RbmA is a well-characterized accessory protein, which was first discovered as a secreted protein that modulates the morphology of *V. cholerae* colonies on agar plates (Fong et al. 2006). RbmA was subsequently shown by *in situ* immunostaining and high-resolution microscopy to adhere biofilm cells to each other (Fig. 2.1) (Absalon et al. 2011; Berk et al. 2012). Structural and genetic work further demonstrated that RbmA binds VPS directly and uses a binary structural switch with its fibronectin type III (FnIII) domains to modulate its function (Giglio et al. 2013; Maestre-Reyna et al. 2013; Fong et al. 2017). During the late stages of biofilm formation, *in situ* proteolysis of RbmA promotes attachment of planktonic cells to existing biofilms (Smith et al. 2015). These foundational studies

have revealed the important role of RbmA in maintaining the structural integrity of *V. cholerae* biofilms.

Two proteins, biofilm-associated protein 1 (Bap1) and rugosity and biofilm structure modulator C (RbmC), have been found to contribute to cell-to-surface adhesion in *V. cholerae* biofilms as well as to biofilm strength (Fong and Yildiz 2007; Teschler et al. 2015; Yan et al. 2018). While single mutants possess similar colony morphology and adhesion capabilities to WT, double deletion of *rbmC* and *bap1* results in floating biofilm clusters and different colony morphology (Absalon et al. 2011). This observation suggests that RbmC and Bap1 are partially redundant in conferring adhesion to *V. cholerae* biofilm. High-resolution microscopy showed that the spatial distributions of Bap1 and RbmC are notably different at the interface between cell clusters and the glass substratum (Fig. 2.1) (Berk et al. 2012). Bap1 appears to act as an anchor between the biofilm and the solid surface as it is highly localized at the biofilm-substratum interface, whereas the signal from RbmC at the interface was much weaker (Fig. 2.1) (Berk et al. 2012; Yan et al. 2016). Together with VPS, both Bap1 and RbmC contribute to the formation of dynamic envelopes surrounding cell clusters (Berk et al. 2012).

Besides the key matrix components mentioned above, additional matrix proteins also contribute to *V. cholerae* biofilm architecture and development. Many of those factors are encoded in the *vps* intergenic region, downstream of *rbmA* (Fong and Yildiz 2007). Fong *et al*. demonstrated that in addition to *rbmA* and *rbmC*, *rbmB*, *rbmD*, *rbmE,* and *rbmF* all encode proteins that modulate *V. cholerae* rugose colony development and biofilm formation (Fong and Yildiz 2007). Among these genes, RbmB is suggested to function as a polysaccharide lyase since the Δ*rbmB* mutant developed into more wrinkled colony biofilm with higher VPS accumulation, and the Δ*rbmB* biofilm was defective in dispersal (Yan et al. 2017a; Singh et al. 2017). Recently, RbmD is suggested to contribute to biofilm formation by glycosylating other periplasmic proteins (Vorkapic et al. 2019; Jiang et al. 2021), but the mechanism is still unclear. Furthermore, extracellular DNA and extracellular nucleases are also suggested to contribute to biofilm architecture and other developmental processes (Seper et al. 2011). A more comprehensive understanding of the molecular mechanism underlying these matrix components and their functions in *V. cholerae* biofilm architecture and development is clearly needed.

**VPS-Independent Biofilms**

*V. cholerae* can also form biofilms independent of VPS. Formation of VPS-independent biofilms was found in sea water conditions and depends on calcium cations (Kierek and Watnick 2003). The development of $Ca^{2+}$-dependent biofilms is promoted by the *V. cholerae* O-antigen and capsule, where $Ca^{2+}$ is an integral part of the extracellular matrix and hypothesized to form salt bridges between O-antigen moieties. VPS-independent biofilms have also been reported under hydrodynamic conditions in a flow cell (Muller et al. 2007). Still, relatively little is known about the cellular structure and the developmental process of such VPS-independent biofilms or other aggregated multicellular forms of *V. cholerae* (Jemielita et al. 2018, 2021).

## 2.2 The Ecological Function of *V. cholerae* Biofilms

Toxigenic *V. cholerae* lives in two distinct environments. In between disease outbreaks, *V. cholerae* resides in aquatic reservoirs where it persists primarily in a biofilm or in a dormant state known as conditionally viable environmental cells (CVECs). Once inside a human host, *V. cholerae* transforms into an acute pathogen, undergoing rapid growth in the small intestine before dissemination back into the environment via cholerae toxin (CT) induced diarrhea (Faruque et al. 2006; Alam et al. 2007). Biofilms provide adaptive benefits for *V. cholerae* in both phases of its life cycle.

**Biofilms Shield *V. cholerae* from Predation**

A primary role of *V. cholerae* biofilms in the environment is protection from predation by protozoa, predatory bacteria, or phage infection (Fig. 2.2). *V. cholerae* can form two distinct colony types, a smooth colony which is associated with low biofilm formation and a rugose colony that is caused by high biofilm formation (Yildiz and Schoolnik 1999). Rugose colony formation is often caused by a null mutation in the quorum sensing (QS) master regulator, *hapR* (Hammer and Bassler 2003; Yildiz et al. 2004). QS regulation of biofilms is discussed in detail in Sect. 2.3. Planktonic cells of both smooth and rugose variants were effectively grazed by protozoa, but formation of biofilms by both morphologies completely inhibited grazing (Matz et al. 2005). This defense was mediated not only in part by the physical nature of the biofilm, but also by a HapR-dependent secreted factor that inhibited feeding activity of protozoa. Coculture of protozoa with smooth *V. cholerae* also selected for rugose variants. Such resistance was seen with both toxigenic and non-toxigenic *V. cholerae*, demonstrating that biofilm formation in *Vibrios* is a fundamental mechanism of environmental persistence not specifically associated with disease-causing strains (Matz et al. 2005). Resistance to protozoal grazing has been linked to formation of toxic ammonium by secreted chitinases for *V. cholerae* forming biofilms on chitin flakes (Sun et al. 2015), pyomelanin production (Noorian et al. 2017), and the actin cross-linking domain of the Type VI secretion system (Drebes Dorr and Blokesch 2020). Thus, defense against protozoal grazing is multifactorial, illustrating the importance of these traits for environmental survival. In addition to eukaryotic predators, *V. cholerae* biofilms also protect against the bacterial predator *Bdellovibrio bacteriovorus*, but only once a mature biofilm has developed (Wucher et al. 2021). Interestingly, predation by *B. bacteriovorus* alters the three-dimension architecture of the biofilm, demonstrating how environmental factors can shape the structure of *V. cholerae* biofilms (Wucher et al. 2021).

Another major predator of *V. cholerae* is bacteriophage. Like a wolf/rabbit predator/prey cycle, blooms of *V. cholerae* are often followed by subsequent phage outgrowth (Zahid et al. 2008). Three predominant lytic phage, known as ICP-1, ICP-2, and ICP-3, have been isolated both in the environment and stool samples from cholera patients (Seed et al. 2011), while other phage such as JA-1 (an O139-specific phage) (Faruque et al. 2000), JSF7 (Naser et al. 2017), and K139 (Molina-Quiroz et al. 2020) have also been observed. In general, bacterial biofilm formation and phage have intricate ecological interactions depending on the species and phage (Pires et al. 2021). Although the importance of phage for cholera outbreaks and *V. cholerae* ecology is well established, there has been surprisingly little research to elucidate how biofilms impact these interactions. Rugose variants of *V. cholerae* resist infection by the K139 phage, and coculture of smooth *V. cholerae* strains with this phage catalyzed the emergence of rugose variants (Nesper et al. 2001). It was speculated but never directly shown that the physical nature of biofilms prevented phage access to the bacterial cells (Nesper et al. 2001). This finding is consistent with a more recent study that showed biofilm formation inhibited infection by JSF3 (a JA-1 variant) and JSF4 (an ICP-1 variant) vibriophage isolated from patient stool samples (Naser et al. 2017). However, a novel phage designated JSF7 was able to disperse the biofilms of both O139 and O1 *V. cholerae* isotypes (Naser et al. 2017). As JSF7 can only infect O1 strains, this result indicates that dispersal did not rely on phage infection, but rather might be due to matrix degrading enzymes on the phage head (Naser et al. 2017). Another recent study found that WT biofilms were resistant to phage infection, but addition of antibiotics altered the architecture of the biofilm matrix such that phage could then infect biofilm-grown cells (Diaz-Pascual et al. 2019).

***V. cholerae* Biofilms Increase Stress Resistance**

In addition to protection from predation, bacterial biofilm formation increases the resistance of

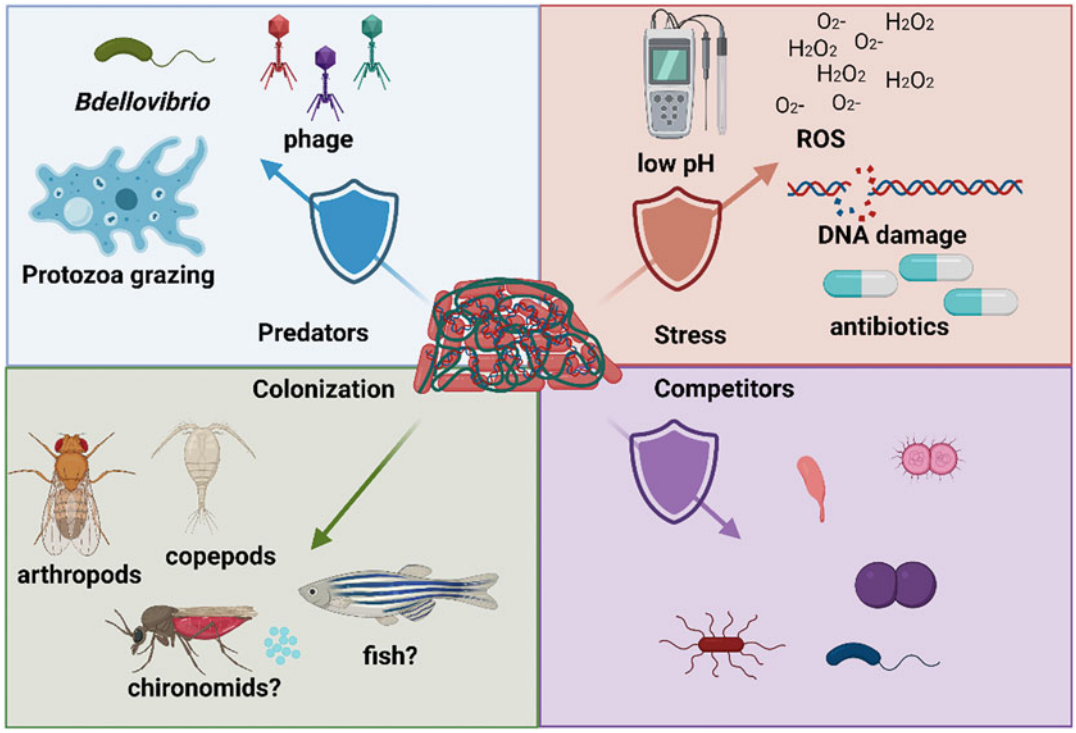


**Fig. 2.2** Environmental biofilms of *V. cholerae* protect the bacteria from predation, stresses, competitors, and promote colonization of animal hosts

bacteria to various stresses and antibiotics, and *V. cholerae* is no exception (Fig. 2.2) (Hall and Mah 2017). *V. cholerae* biofilms increased resistance to low pH up to 1000-fold, a trait which is proposed to be important for the hyper-infectious state of *V. cholerae* discussed further below (Zhu et al. 2002). Rugose colonies increased resistance to chlorine and oxidative stress (Morris et al. 1996; Wai et al. 1998). Null mutations in the methyl-directed mismatch repair system gene *mutS* were resistant to reactive oxygen species (ROS) during infection of infant mice (Wang et al. 2018). These *mutS* mutants evolved higher levels of rugose variants during colonization of the mice, and the biofilms formed by these rugose variants were 30-fold more resistant to ROS treatment (Wang et al. 2018). This result is consistent with c-di-GMP increasing resistance to ROS by activating the catalase gene *katB* (Fernandez and Waters 2019). Furthermore, c-di-GMP induces alkylation DNA repair pathways in *V. cholerae,* suggesting that elevated c-di-GMP in biofilms activates multiple stress response pathways (Fernandez et al. 2018). Consistent with other bacteria, biofilms of *V. cholerae* increased resistance to antibiotic treatment (Gupta et al. 2018), although the mechanisms of such antibiotic resistance in *V. cholerae* are largely unexplored.

### Biofilm Formation Contributes to the Colonization of Environmental Animal Reservoirs

*V. cholerae* biofilms also promote persistence in environmental reservoirs by mediating animal colonization in planktonic crustacean copepods, where *V. cholerae* attaches to the oral region and egg sac of both live and dead animals (Fig. 2.2) (Huq et al. 1983). Attachment to live copepods significantly increased survival of *V. cholerae* in environmental water samples, suggesting this is an important mechanism of environmental persistence (Huq et al. 1983). Copepods are encased in chitin, and chitin catabolism is a major nutrient source for *V. cholerae* in the environment

(Meibom et al. 2004). *V. cholerae* also attach to chitin via colonization factors such as GbpA as described above (Reguera and Kolter 2005; Drescher et al. 2014; Antonova and Hammer 2011). However, the role of VPS in colonization of these plankton is unclear as *V. cholerae* cells formed a monolayer and null mutations in VPS, MSHA, and other biofilm-related genes did not exhibit defects in colonization (Mueller et al. 2007). Rather, transposon mutations in genes necessary for motility and chemotaxis exhibited decreased colonization. Importantly, in this study, colonization of dead copepods was much greater than live animals, suggesting that dead organisms may be the natural reservoir for *V. cholerae* (Mueller et al. 2007). Cell morphology also impacts chitin colonization as a naturally filamentous strain of *V. cholerae* formed a VPS-independent biofilm on chitin surfaces that outcompeted VPS-dependent *V. cholerae* biofilms over short time scales or with frequent disturbance (Wucher et al. 2019). Chitin monomers are the environmental signal that induces natural competence, making biofilms on chitin key drivers in *V. cholerae* evolution (Antonova and Hammer 2011; Lo Scrudato and Blokesch 2012; Suckow et al. 2011). These studies highlight that *V. cholerae* has multiple mechanisms for colonizing different surfaces, and the molecular features elucidated for VPS-dependent laboratory-grown biofilms may not be critical for all surface interactions.

*V. cholerae* colonizes other animals in the environment, although the role of biofilms in these interactions is less clear. Arthropods have been implicated as an environmental reservoir for *V. cholerae* (Broza et al. 2005; Fotedar 2001). The fruit fly *Drosophila melanogaster* serves as a colonization model for *V. cholerae*, and production of VPS is essential for colonization of the rectum, potentially by mediating adherence of the bacteria to epithelial cells (Blow et al. 2005; Berkey et al. 2009; Purdy and Watnick 2011). Furthermore, HapR, the master high-cell-density QS regulator of *V. cholerae*, represses *vps* gene expression in *D. melanogaster* (Purdy and Watnick 2011). The female egg sacs of chironomids and fish are well-established environmental reservoirs of *V. cholerae*, but the role of biofilms in the colonization of these animals remains to be studied. (Halpern et al. 2004; Senderovich et al. 2010). A recently developed *Danio rerio* (zebrafish) colonization/pathogenesis model could serve as a valuable tool to explore the role of biofilms in interaction of *V. cholerae* with fish hosts (Runft et al. 2014).

### Study the Evolution of Cooperation Using *V. cholerae* Biofilms

Another function of biofilms is to maximize the use of public goods, which are defined as products that are made by cooperating producer cells whose benefits can be shared by the entire community (Nadell et al. 2008). Such public-goods can be evolutionary unstable as non-producing cheater cells, either caused by loss of the cooperative trait or invasion of a non-cooperative foreign species, gain a significant fitness advantages by benefiting from the public goods without incurring the production cost (Popat et al. 2012). Biofilm formation is proposed to be a solution to the public goods dilemma by localizing them to producing cells and clonal relatives, and *V. cholerae* has become a model system for testing this idea (Nadell et al. 2008). The formation of thick *V. cholerae* biofilms on a chitin surface limited chitinase products to producers located near the surface, shielding these public goods from cheater exploitation (Drescher et al. 2014). The matrix of *V. cholerae* biofilms also excludes invasion of other cells by RbmA tightly binding biofilm cells together, creating a dense, impenetrable material (Nadell et al. 2015). Moreover, osmotic pressure generated by the matrix also contributes to cheater exclusion by driving a densely packed biofilm (Yan et al. 2017b). RbmA is secreted and shared in a limited fashion within producer cells, conferring protection from exploitation. In contrast, the two other major matrix proteins, RbmC and Bap1, can diffuse outside producer cell clusters and therefore be exploited by cheater cells within a certain range (Absalon et al. 2011; Tai et al. 2022). Environmental conditions can

also impact the evolution of biofilms in *V. cholerae* as increased resources drove the evolution of hyper-biofilm forming *V. cholerae* rugose variants (Connelly et al. 2017). Thus, due to its experimental tractability, mechanistic understanding of biofilm formation, and ease of genetic manipulation, *V. cholerae* biofilm formation has become a model system to address key evolutionary questions about the role of biofilms in the maintenance of cooperative traits.

### *V. cholerae* Biofilms Minimally Contribute to Colonization of the Small Intestine

*V. cholerae* predominantly colonizes the small intestine where it rapidly grows, releasing CT to be dispersed into the environment. Two of the most significant factors in colonization are CT and TCP (Thelin and Taylor 1996; Peterson and Mekalanos 1988; Waldor and Mekalanos 1996). In contrast, the role of biofilms during colonization of humans and animal models of infection has been less clear. In support of the importance of biofilms during infection, studies using *in vivo* expression technologies showed that biofilm genes are induced during infection of humans or in animal models of infection (Lombardo et al. 2007; Lee et al. 2001). In addition, humans infected with *V. cholerae* have serum antibodies that recognize VPS proteins (Hang et al. 2003). Further evidence for the importance of biofilms during infection is the formation of biofilm-like aggregates in the stools of cholera patients that are highly infective in infant mice (Faruque et al. 2006). VpsR, a c-di-GMP-dependent master regulator of the VPS genes, also induces expression of AphA, a transcriptional activator in the virulence cascade, suggesting biofilm formation is linked to virulence gene expression (Fig. 2.4) (Lin et al. 2007; Srivastava et al. 2011). A direct examination of the role of VPS and VpsR in the colonization of infant mice showed a complicated relationship between these traits and infection depending on the time of analysis and whether the background strain was smooth or rugose (Rashid et al. 2004). Another study showed that a rugose variant of *V. cholerae* had 3.6-fold more CFUs than a smooth variant, and this increase was dependent on VPS and RbmA but not RbmC or Bap1, proposing that biofilm formation enhanced colonization (Fong et al. 2010).

Alternatively, other studies have demonstrated that biofilm formation does not contribute to infection of the small intestine, and in some cases, even inhibits colonization. Zhu et. al. found no significant differences in CFUs between WT or a *hapR* or VPS mutant in single-strain infection studies in the infant mouse model (Zhu and Mekalanos 2003). However, a dual-strain competition found that the hyper-biofilm forming *hapR* mutant had a tenfold reduction in colonization when competed with the WT strain. The authors suggest this reduction is due to reduced dispersal in the *hapR* mutant (Zhu and Mekalanos 2003). Similarly, a rugose O139 *V. cholerae* variant had decreased colonization in the infant mouse (Watnick et al. 2001). These findings have been confirmed recently as a mutant unable to produce VPS did not exhibit any defect in competition with its WT counterpart in the infant mouse model (Barrasso et al. 2022).

What conclusions can we draw from this broad collection of contradictory research? One consistent finding is that colonization differences attributed to biofilm formation, be they positive or negative, are relatively minor compared to the colonization defects observed in TCP or CT null mutants of *V. cholerae* (Thelin and Taylor 1996; Peterson and Mekalanos 1988; Waldor and Mekalanos 1996). Thus, the impact of biofilm during *in vivo* infection of animal models is more subtle. Another important point is that the details of these experiments are critical. Although colonization differences can be observed in single-strain infection studies, these differences are often mitigated in competition experiments, presumably due to complicated interactions between competitor strains in the context of the gut environment. (Fong et al. 2010). Moreover, the timing at which colonization is assessed is important and can lead to different conclusions (Rashid et al. 2004). A consistent finding supported by several studies is that hyperproduction of biofilms is detrimental to colonization of the small intestine. These results agree

with the requirement of motility and chemotaxis for *V. cholerae* colonization (Butler and Camilli 2004), and the migration of *V. cholerae* deep within epithelial cell crypts during colonization (Millet et al. 2014).

Recent studies might provide clues as to the incongruence in published literature. Infant mice are used to study *V. cholerae* colonization because they lack a mature immune system and do not have a complex microbiome (Sit et al. 2021). However, these factors are not representative of human infections. More recently, research has focused on the impact of the human intestinal microbiome on *V. cholerae* infection (Hsiao et al. 2014; Cho et al. 2021). For example, the Gram–species *Paracoccus aminovorans* was abundant in cholera stools, which was surprising as most other gut microbiome members are significantly decreased (Barrasso et al. 2022). *V. cholerae* formed dual-species biofilms with *P. aminovorans*, and the addition of *P. aminovorans* significantly enhanced *V. cholerae* colonization of infant mice in a VPS-dependent manner (Barrasso et al. 2022). This study suggests that VPS and biofilm formation during infection might drive interactions with the gut microbiome. If so, then the contradictory literature on the role of VPS and biofilms in animal models could be in part due to unappreciated differences in the microbiome of the subject animals. Biofilm-growing *V. cholerae* also upregulated virulence factors via enhanced expression of the virulence regulator ToxT, leading to increased colonization (Gallego-Hernandez et al. 2020). Therefore, differences in preparation of *V. cholerae* for colonization studies could significantly impact the outcomes. In addition, this study showed that planktonic *V. cholerae* colonizes the base of epithelial cell villi as previously described whereas biofilms form aggregates near the top of the villi. Moreover, biofilm-forming bacteria better colonize the medial region of the small intestine, while planktonic bacteria better colonize the proximal and distal small intestine (Gallego-Hernandez et al. 2020). Thus, crude quantification of colonization such as measuring total viable bacteria may not be sufficient to discriminate the more subtle *in vivo* impacts of biofilm formation. Importantly, the predominance of *in vivo* colonization studies probing biofilms has utilized the infant mouse model. Although this model has several advantages such as cost, other more physiological relevant models have been developed like infant rabbits, which actually exhibit the classical symptoms of cholera (Sit et al. 2021). Exploring the role of *V. cholerae* biofilms in additional models is key to understanding if the results observed with the infant mouse are broadly applicable.

### *V. cholerae* Biofilms Contribute Significantly to Hyperinfectivity

In 2002, Merrel et al. showed that *V. cholerae* isolated from human cholera stools was hyperinfectious, significantly outcompeting laboratory-grown bacteria up to 100-fold in infant mice colorizations (Merrell et al. 2002). This hyper-infectious state lasted for up to 5 h in pond water (Merrell et al. 2002). With the observation of biofilm-like aggregates in rice-water stools that are hyper-infective (Faruque et al. 2006) and resistance of biofilms to acidic pH (Zhu and Mekalanos 2003), it was hypothesized that *in vivo* derived biofilms could be critical for this transmission. Indeed, filtration of biofilm-like particles reduces transmission (Colwell et al. 2003). The hyper-infectious state could also be mimicked by recovery of *V. cholerae* from infected mice (Schild et al. 2007; Alam et al. 2005) and by growing *V. cholerae* in a biofilm (Tamayo et al. 2010). Interestingly, biofilm-grown cells maintained their competitive advantage, even when the biofilm was dispersed, suggesting it is not the physical structure of the biofilm itself that is critical but the physiological changes of cells within the biofilm (Tamayo et al. 2010). A search for these components identified the Pst2 phosphate transfer system as upregulated in biofilms and a contributing factor for the hyperinfectious state (Mudrak and Tamayo 2012). More recently, growth in a biofilm was found to significantly upregulate virulence factor expression via ToxT (Gallego-Hernandez et al. 2020). When expression of *toxT* was driven from a

promoter that was equivalent in planktonic and biofilm states, the hyperinfectivity of biofilms was lost, showing that virulence factor upregulation in biofilms is required for this state (Gallego-Hernandez et al. 2020).

Integration of the published studies described above suggests that *V. cholerae* can colonize both as planktonic cells and in a biofilm; however, the location of *V. cholerae* in epithelial cell crypts and in the small intestine for these two growth states is distinct. In addition, hyper-biofilm formation is determinantal to colonization. Perhaps most importantly for *V. cholerae* outbreaks, formation of biofilm aggregates during *in vivo* infection renders *V. cholerae* hyper-infective, increasing the chance of person-to-person spread (Fig. 2.3).

## 2.3 Regulation of *V. cholerae* Biofilms by Quorum Sensing (QS) and c-di-GMP Signaling

As discussed, the formation and dispersal of biofilms are essential to the success of *V. cholerae* as both a denizen of aquatic environments and an intestinal pathogen. Switching between these lifestyles occur in response to multiple environmental cues that feed into a complex regulatory network. These inputs must be integrated into common regulatory pathways that control the formation and dispersal of biofilms. Here, we focus on recent insights into the role of extracellular QS, intracellular second messengers, and key environmental signals that control biofilm gene regulation.

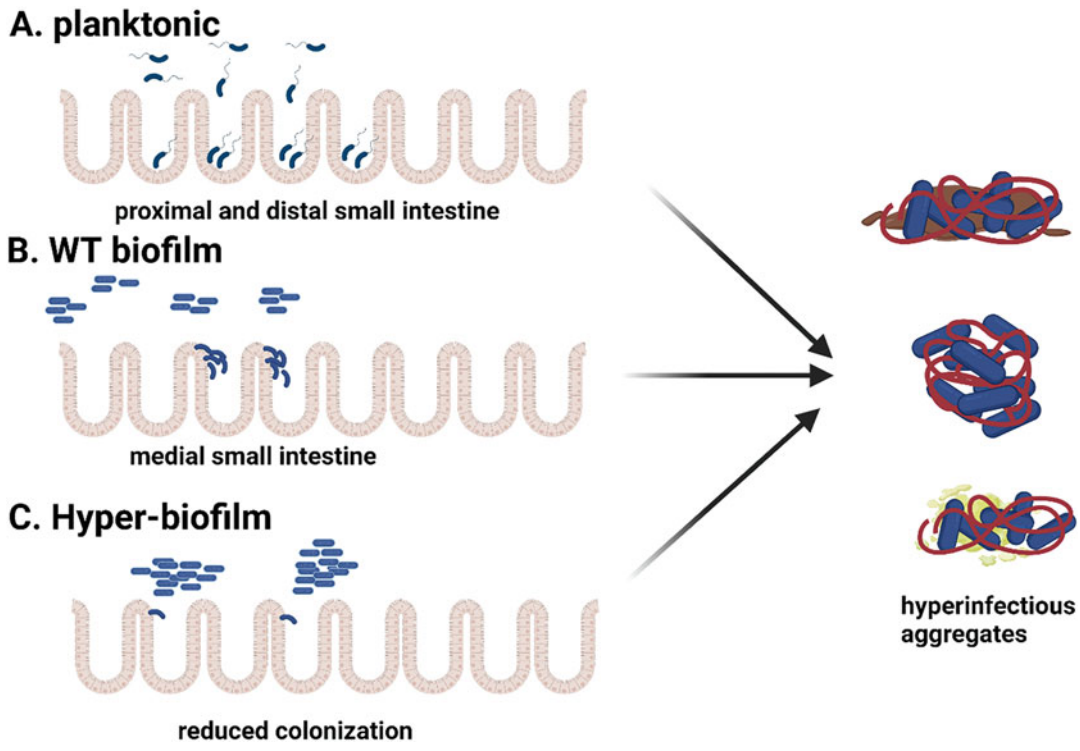


**Fig. 2.3** A summation of published literature on the role of colonization for *V. cholerae* inoculated as planktonic cells (**a**), biofilms (**b**), or hyper-biofilms (**c**)

**Quorum Sensing (QS) Regulates Biofilm Formation**

Upon the discovery of QS in *V. cholerae*, it was quickly realized that this cellular chemical communication regulates biofilm formation (Hammer and Bassler 2003; Zhu and Mekalanos 2003). However, unlike in *P. aeruginosa*, in *V. cholerae* biofilm formation is induced at low cell density (LCD) and is repressed at high cell density (HCD) via multiple regulatory pathways (Hammer and Bassler 2003; Zhu and Mekalanos 2003; Srivastava and Waters 2012). Two transcriptional activators, *vpsR* and *vpsT*, induce expression of the *vps* operons and extracellular matrix proteins at LCD, and they are directly activated upon binding to c-di-GMP as discussed below (Fig. 2.4) (Casper-Lindley and Yildiz 2004; Yildiz et al. 2001). The master LCD regulator, AphA, predominates for most of the biofilm lifespan and enhances biofilm formation by inducing transcription of *vpsT* (Srivastava et al. 2011; Rutherford et al. 2011; Yang et al. 2010). As the cells transition to HCD, HapR, the master HCD regulator, is increased immediately prior to biofilm dispersal and directly represses the transcription of *aphA* and *vpsT* (Fig. 2.4) (Lin et al. 2007; Srivastava et al. 2011).

Quorum sensing regulates AphA and HapR expression through a complex phosphorelay pathway that responds to multiple autoinducers. The

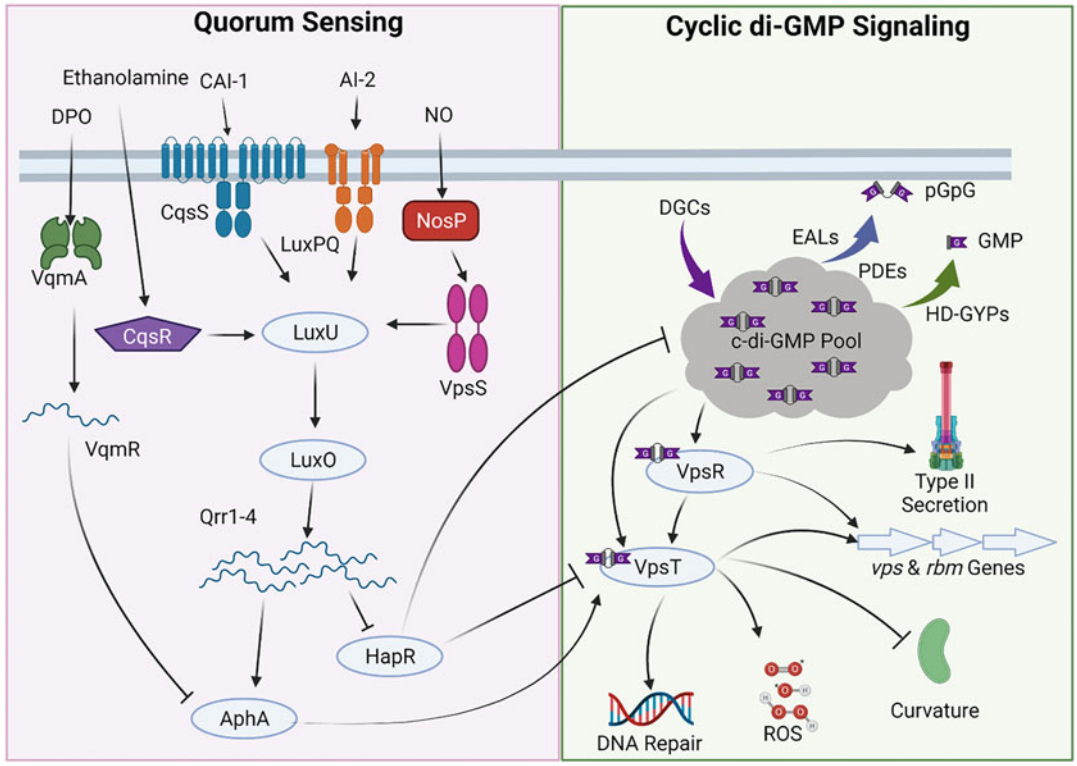


**Fig. 2.4** QS (left) and c-di-GMP (right) jointly regulate biofilm formation and biofilm-associated phenotypes. Four histidine kinase receptors recognize AIs or other signals to control phosphate flow through LuxU and LuxO to regulate the Qrr sRNAs, which induce AphA and repress HapR at LCD. The VqmAR QS pathway recognizes DPO to repress AphA and biofilm formation independently of the LuxUO pathway. AphA induces biofilm formation through activation of VpsT, while HapR inhibits biofilms by repressing VpsT and decreasing intracellular c-di-GMP levels, which are controlled by the concerted activity of DGCs and PDEs. c-di-GMP directly binds to and activates VpsR and VpsT, which regulate multiple phenotypes concurrently with the VPS and matrix protein genes necessary for biofilm formation

autoinducer (AI)/receptor pairs CAI-1/CqsS and AI-2/LuxPQ were the first discovered and have been extensively characterized, but more recently two additional kinase receptors, CqsR and VpsS, were shown to control phosphate flux into this pathway (Papenfort and Bassler 2016; Jung et al. 2015; Jung et al. 2016). Although not as well understood, CqsR directly binds and responds to ethanolamine, which is a common small molecule in the gastrointestinal environment (Watve et al. 2020), while VpsS responds to nitric oxide (Hossain et al. 2018). Whether these cues are self-produced, making these legitimate QS receptors, or are environmental inputs requires further study. Under LCD conditions, the ligand-free membrane receptors act as kinases to phosphorylate LuxU which in turn phosphorylates LuxO. Phospho-LuxO activates the transcription of the Qrr1-4 sRNAs which repress HapR and activate expression of AphA leading to the biofilm phenotype. Under HCD conditions, AI binding to the membrane receptors causes this system to work in reverse with the receptors now acting as phosphatases, halting Qrr sRNA transcription leading to the de-repression of HapR and loss of AphA expression (Papenfort and Bassler 2016).

Understanding how these QS systems each contribute to biofilm regulation has been complicated by the convergence of these systems at the phosphorylation of LuxU. Studies with transcriptional reporters employing Δ*cqsS* and Δ*luxS* deletion strains have suggested that the CAI-1/CqsS system is the dominant system (Hurley and Bassler 2017). However, recently developed live biofilm formation and dispersal assays suggest that AI-2/LuxPQ is more dominant. It has been proposed that positive feedback on *cqsS* transcription (and its absence in a Δ*cqsS* background) is responsible for these contradictory results (Bridges and Bassler 2019).

Recently, a new QS system controlled by the receptor VqmA, an orphan LuxR-type transcriptional regulator, was found to respond to extracellular concentrations of 3,5-dimethylpyrazin-2-ol (DPO) leading to the transcription of the VqmR sRNA and repression of biofilm formation (Fig. 2.4) (Papenfort et al. 2015, 2017). VqmR inhibits biofilm production by base pairing with the ribosome binding site of the *aphA* mRNA transcript providing a biofilm repression mechanism independent of the LuxUO-Qrr pathway (Papenfort et al. 2015). DPO is widely produced by bacteria including the normal flora of the intestine, and sensing DPO may be a mechanism to monitor the surrounding microbial population in the environment to control biofilm regulation (Papenfort et al. 2017). Since its initial characterization the structure of the DPO-VqmA-DNA complex has been reported (Wu et al. 2019) and the mechanism on ligand binding has been elucidated (Huang et al. 2020). Beyond its role in QS, VqmA has been shown to mediate response to low oxygen levels indicating a role as a signal integrator (Mashruwala and Bassler 2020). As the molecular underpinning of these regulatory circuits are elucidated, the next challenge is to understand their role in environmentally relevant conditions such as biofilm formation on chitin surfaces, upon host colonization, or aggregate formation in hyper-infectious cholera stools.

### Cyclic di-GMP Integrates Environmental Cues to Control Biofilm Formation

Working in concert with the AphA/HapR regulatory circuit is the second messenger c-di-GMP which regulates numerous cellular processes including biofilm formation (Fig. 2.4, reviewed in Conner et al. 2017). C-di-GMP is produced by diguanylate cyclase (DGC) enzymes containing the GGDEF domain and degraded by phosphodiesterase (PDE) enzymes containing either EAL or HD-GYP domains which degrade c-di-GMP to 5′-pGpG or GMP, respectively (Römling et al. 2013). Interestingly, *V. cholerae* encodes approximately 70 C-terminal DGCs and PDEs, most of which are fused to a N-terminal sensory domain that is predicted to recognize a specific environmental cue, illustrating that c-di-GMP signaling in *V. cholerae* is a complex regulatory pathway that integrates dozens of environmental cues to modulate the intracellular c-di-GMP pool and control biofilm regulation (Galperin 2004). QS

controls c-di-GMP in *V. cholerae*, and at LCD conditions c-di-GMP pools are elevated (Waters et al. 2008; Hammer and Bassler 2009), inducing the expression of biofilm biosynthesis genes through direct interactions with VpsR (Srivastava et al. 2011; Hsieh et al. 2018; Chakrabortty et al. 2021) and VpsT (Krasteva et al. 2010). Conversely, under HCD conditions c-di-GMP pools are reduced by HapR transcriptional regulation of about a quarter of all DGCs and PDEs, ultimately increasing net PDE activity to inhibit biofilm gene expression (Fig. 2.4) (Waters et al. 2008).

The mechanism of regulation by VpsT and VpsR has been examined in some detail. In vitro transcription assays have demonstrated that VpsR in the presence of c-di-GMP directly activates genes necessary for VPS synthesis and matrix proteins, functioning as both a Class I and Class II activator (Hsieh et al. 2018, 2020). C-di-GMP binding does not regulate VpsR binding to DNA or dimerization of the protein, but rather alters its interaction with RNA polymerase to drive open complex formation (Hsieh et al. 2018). Recently, the crystal structure of VpsR was solved, identifying the key residues necessary for binding to c-di-GMP (Chakrabortty et al. 2021). Furthermore, although VpsR has a conserved aspartate in its N-terminal receiver domain that is critical for activity, this transcription factor is not phosphorylated but rather directly senses phosphate to modulate its sensitivity to c-di-GMP (Hsieh et al. 2022). The mechanism of VpsT is less studied, but it has been shown to countersilence the abundant nucleoid organizing protein H-NS, which competitively binds promoters for biofilm biosynthesis genes blocking promoter access for RNA polymerase. Displacement of this binding by the transcription factor VpsT is enhanced in the presence of c-di-GMP (Ayala et al. 2015). An additional relationship between H-NS and c-di-GMP pools has been described with H-NS silencing the expression of the VieA phosphodiesterase in the classical biotype (Ayala et al. 2018). TrsA, a structural homolog of H-NS has a similar regulon and also regulates biofilm genes (Caro et al. 2020). A chromatin immunoprecipitation (ChIP) sequencing approach recently identified 23 VpsT bindings sites in *V. cholerae* and found that it upregulates c-di-GMP in a positive feedback loop through activation of the DGC VpvC. For unknown reasons, all VpsT binding sites were identified on Chromosome I while none were identified on Chromosome II (Guest et al. 2021).

In addition to regulation genes necessary for biofilm formation, VpsR and VpsT also control several other phenotypes in response to c-di-GMP (Fig. 2.4) VpsR bound to c-di-GMP induces expression of the Type II Secretion System, leading to the formation of a pseudopillus (Sloup et al. 2017). VpsT activates various stress responses including the DNA glycosylase *tag*, which repairs DNA damaged by alkylation, and *katB*, a catalase that counters reactive oxygen species (Fernandez and Waters 2019; Fernandez et al. 2018). Finally, VpsT also regulates the cell morphology of *V. cholerae*, driving cells to straighten at high c-di-GMP concentrations (Fernandez et al. 2020). These findings suggest that c-di-GMP, VpsR, and VpsT are not just responsible for inducing the genes directly responsible for biofilm formation, but also regulating behaviors that increase fitness for *V. cholerae* growing in a biofilm state.

Although still an active area of study, several environmental cues have been identified that impact QS and c-di-GMP signaling. The membrane permeable gas nitric oxide (NO) has been reported to inhibit biofilm formation (Rinaldo et al. 2018). However, by itself NO is unable to induce dispersal in a flow cell biofilm model (Singh et al. 2017). NO binding to the cytosolic heme protein NosP (VspV) activates kinase activity leading to the phosphorylation of VpsS which in turn phosphorylates LuxU (Hossain et al. 2018). *V. cholerae* lacks a known NO synthase making this a potential inter-kingdom signal from the human host. Polyamines can regulate biofilm formation through the NspS-MbaA signaling pathway (Sobe et al. 2017). MbaA functions as a DGC when NspS is bound to extracellular norspermidine (Young et al. 2021; Bridges and Bassler 2021) while it functions as a PDE when NspS is bound to spermine (Wotanis et al. 2017). Norspermidine is uncommon in the environment

but is produced by marine *Vibrio* species including *V. cholerae*. Conversely, spermine is widely distributed and abundant in the human intestine. Together these polyamines provide environment-specific cues for biofilm control via a single pathway. Other potential *in vivo* cues that regulate biofilm formation includes indole, ethanolamine, bile, and bicarbonate (Watve et al. 2020; Howard et al. 2019; Koestler and Waters 2014; Hay and Zhu 2015; Hung et al. 2006).

Much of the research on *V. cholerae* biofilms has focused on their formation and the regulatory networks that initiate VPS and matrix protein expression. The other aspect of *V. cholerae* biofilms that is much less studied is how they disperse. Transition to the HCD state via both AI-2 and CAI-1 function as a coincidence detector to induce biofilm dispersal (Bridges and Bassler 2019). Dispersal is also controlled via alterations in c-di-GMP through sensing of polyamines (Bridges and Bassler 2021). Finally, a genetic screen for dispersal mutants identified several new mechanisms that are required for dispersal including the novel DbfS/DbfR two-component sensing system, matrix degradation enzymes, and chemotaxis, all of which required further study to understand their contribution to dispersal (Bridges et al. 2020).

## 2.4 Biomechanical Properties of *V. cholerae* Biofilms

Of increasing interest are the mechanical phenomena during biofilm development arising from cell-cell, cell surface, and cell-matrix interactions (Persat et al. 2015; Maier 2021). Again, due to the vast knowledge on the biochemistry and regulation of the matrix, *V. cholerae* has emerged as a model organism to understand biophysical and biochemical principles underlying biofilm development, which we review below.

The extracellular matrix provides the mechanical properties necessary to protect the embedded cells from external forces such as fluid shear and to ensure that the biofilm community remains attached to a surface. The physicochemical properties of the biofilm matrix constituents and the interactions between them give rise to the global biofilm mechanical properties. Tools and concepts from the field of rheology have been adapted to quantitatively define biofilm mechanics. Rheology is the study of viscoelastic materials: materials that have both solid and liquid properties (Billings et al. 2015). For rheologic measurements, the material of interest (biofilm) is sandwiched between parallel plates and subjected to shearing. Such measurements determine the elastic modulus, which is the stiffness of the material at small deformation, and the yield strain, which is the degree of deformation a material can sustain before it fails (Kovach et al. 2017). The product of the elastic modulus and the yield strain determines the yield stress, which is the minimum force that causes a biofilm to fail.

Rheological measurements of *V. cholerae* biofilms with deletion of genes encoding matrix components enabled the association between these components and the mechanical properties of *V. cholerae* biofilms (Yan et al. 2018). The *V. cholerae* biofilm can be considered as a dual-networked hydrogel: one formed by the VPS reinforced by RbmC and Bap1, and the other formed by the cells connected by RbmA. Elimination of RbmA or RbmC/Bap1 weakens the dual network and reduces the elastic modulus. Elimination of all three matrix proteins causes the VPS to swell, resulting in an increased yield strain but at the expense of a highly reduced elastic modulus. All matrix components are required for *V. cholerae* biofilms to have a large enough yield stress (~100 Pa) that can withstand the typical fluid shear they experience in their natural habitats, for example, on sinking "marine snow" (marine detritus) (Alcolombri et al. 2021).

### *V. cholerae* Biofilm Architecture and Organization Principles Revealed by High-Resolution Imaging

Imaging plays an increasingly important role in studies of biofilms. Indeed, high-resolution imaging of biofilm internal structures has

revolutionized our understanding about how cells are organized in biofilms, how extracellular matrix components are distributed, and how biofilm structures respond to environmental challenges including shear flow (Hartmann et al. 2019), nutrient limitation (Yan et al. 2017a; Singh et al. 2017), and osmotic shock (Yan et al. 2017b; Wong et al. 2021). In particular, 3D confocal microscopy at single-cell resolution enables the segmentation of biofilms into individual cells. The ability to acquire 3D position and orientation of each cell in biofilms allowed researchers to apply concepts and tools from colloidal science and soft-matter physics to analyze, simulate, and theoretically model the observed cellular organization.

Recent progress in single-cell imaging reveals the important roles of matrix components in shaping the architecture and cell ordering of *V. cholerae* biofilms (Wong et al. 2021). At first, images of fixed *V. cholerae* biofilms obtained at different incubation times were acquired to follow how cell arrangements change as biofilms grow and mature (Drescher et al. 2016). The cell community transitions from a branched 2D morphology to a dense mature cluster that extends into 3D, where vertical cells reside at the biofilm center near the surface and radially orientated cells are at the rim. Enabled by improvements in confocal microscope design, availability of bright and photostable fluorescent proteins, and development of new computer algorithms, the entire sequence of biofilm structural transitions was subsequently visualized in living, growing *V. cholerae* biofilms from the founder cell to 10,000 cells (Fig. 2.5) (Yan et al. 2016). Mutagenesis coupled with immunostaining of matrix components showed that *V. cholerae* biofilms lacking cell-to-surface adhesion, due to deletion of RbmC and Bap1, exhibit cell density comparable to the rugose biofilm but has a random organization, suggesting the importance of cell-to-surface adhesion to facilitating biofilm cell ordering. In contrast, biofilms lacking cell-cell adhesion in the Δ*rbmA* mutant show reduced cell packing density but enhanced vertical cell alignment.

To explore the mechanism behind pattern formation in *V. cholerae* biofilms, agent-based simulations were performed to complement high-resolution imaging of biofilms to understand the effect of cell-to-cell and cell-to-surface interactions on cell reorientation and biofilm structural transition (Beroz et al. 2018). When a biofilm grows on a surface, it expands outward from the founder cell as a thin film (Fig. 2.5). During expansion, cells experience increasing mechanical stress as they divide and push against their neighbors. At the same time, the neighboring cells resist the pushing force via cell-to-surface adhesion. Subsequently, the pressure resulting from these opposing forces exceeds the cell-to-surface adhesive force and results in individual cells at the center of the biofilm to reorient, transitioning the cells from aligning in parallel to aligning perpendicularly to the substratum. The verticalized cells further project their offspring into the third dimension when they divide, enabling the biofilm to gradually transition from a 2D surface layer to a mature 3D community.

Notably, the basal layer of an expanding *V. cholerae* biofilm develops a striking radial order much like a blooming aster. This self-patterning process was elucidated recently using a combination of single-cell imaging, agent-based simulations, and active nematics modeling (Fig. 2.5) (Nijjer et al. 2021). During biofilm growth, growth-induced stress and surface adhesion jointly cause cells in the core to verticalize and remain anchored to the surface. These stably verticalized cells generate differentially directed growth, which drives the radial alignment of the cells at the rim, while the growing rim generates compressive stresses that further expand the vertical core. This interdependent reorientation cascade leads to the core-rim structure reminiscent of a blooming aster observed in *V. cholerae* biofilms (Fig. 2.5).

More recently, light sheet microscopy has been deployed to push the time resolution of 3D imaging of biofilms to a few minutes (Qin et al. 2020). Combined with puncta labeling to map the positions of all bacterial cells, the high spatial and temporal resolution imaging reveals a fountain-

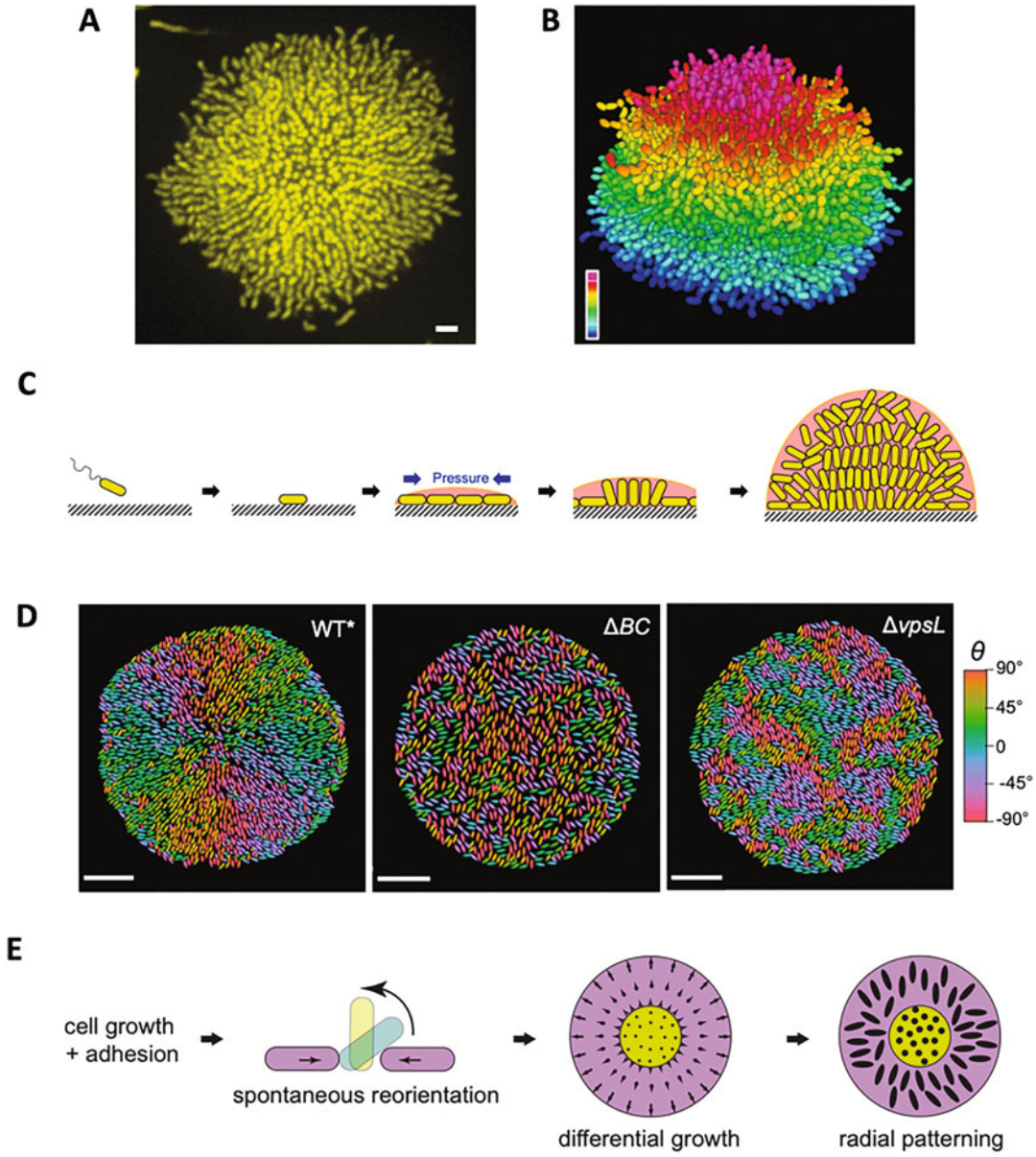


**Fig. 2.5** *V. cholerae* biofilm structural transition and pattern formation revealed by single-cell imaging. (**a**) Cross-sectional image of the bottom cell layer of a growing *V. cholerae* biofilm cluster at 18 h and (**b**) the corresponding segmented image with color coding according to the *z* position. Scale bar: 3 μm. (**c**) Schematic model of *V. cholerae* biofilm growth on solid substrata in an open geometry. (**d**) Cell orientation color-coded by each cell's azimuthal angle in the basal plane in *V. cholerae* biofilms of Δ*rbmA*, Δ*rbmA*Δ*bap1*Δ*rbmC*, and Δ*vpsL* strains, respectively, from left to right. Scale bars: 10 μm. (**e**) Schematic of the biofilm reorientation cascade and self-patterning process. Panels (**a**) and (**b**) are adapted with permission from Yan et al. (2016). Panels (**d**) and (**e**) are adapted with permission from Nijjer et al. (2021)

like flow and cell trajectories as the biofilm mature from a founding cell into a 3D community.

### Macroscopic Morphology of *V. cholerae* Biofilm Colonies

The mechanical instability at the microscopic scale that leads to biofilm structural transition and pattern formation can be extended to the macroscopic scale. Mechanical characterizations and modeling suggested that rugose colony morphologies on agar plates arise from a macroscopic mechanical instability (Yan et al. 2019). Specifically, it was shown that the mismatch in mechanical strain between the growing biofilm layer and the non-growing substratum (agar) causes mechanical instabilities that result in the transition in biofilms from a flat to a wrinkled film, and subsequently to a partially surface-detached film containing delaminated blisters. The mechanical compression required in the instability arises from RbmC/Bap1-modulated surface friction when a colony biofilm expands on the agar plate, as shown by a subsequent modeling study (Fei et al. 2020). RbmC and Bap1 thus play a critical role in determining the colony morphology, consistent with findings in the initial genetics work (Fong and Yildiz 2007): when they are absent, delamination occurs easily and the delaminated blisters collapse onto each other, while the blisters in wild-type rugose colonies are homogeneously distributed throughout the colony circumference (Yan et al. 2019).

### *V. cholerae* Biofilms Under Confinement

In addition to living on solid surfaces, bacterial communities also survive and thrive in soft, confining environments, such as bio-hydrogels. For example, during a gut infection, *V. cholerae* cells can swim through the mucus layer and form mucus-associated clusters (Gallego-Hernandez et al. 2020; Ritchie et al. 2010). To understand the biomechanics involved in such growth conditions, *V. cholerae* biofilms were recently studied in the 3D confinement of agarose gels (Fig. 2.6) (Zhang et al. 2021). Such constrained biofilms show stiffness-dependent morphologies: the biofilm develops an isotropic spherical shape at low gel concentration, while an anisotropic oblate shape emerges at higher gel concentration. The global morphology dependence is conceptually similar to the classical problem of elastic cavitation and is the consequence of minimization of total mechanical energy of the biofilm-gel system (Barney et al. 2020). At the single-cell level, cells display a well-defined pattern of nematic ordering reminiscent of the bipolar configuration of molecules in liquid crystal droplets, where the alignment field connects the two +1 "boojum" defects at the poles along constant longitudes. The bipolar order was found to be driven by the tensile stress at the biofilm-gel interface, created by the biofilm expansion and in turn, transmitted back to the biofilm by VPS and RbmC/Bap1.

### Summary

From the realization that rugose colonies were hyper-biofilm-forming variants over 20 years ago, our understanding of *V. cholerae* at both the molecular and ecological levels have grown tremendously (Wai et al. 1998; Yildiz and Schoolnik 1999). It is now appreciated that the matrix of *V. cholerae* is a complex mixture of VPS and matrix proteins, each component serving a specific role in the biofilm. Contact of surfaces to stimulate adherence is also a complex but beautiful molecular process whereby both flagella and the MSHA Type IV pili ultimately drive stable attachment. Multiple regulatory pathways control induction of biofilms, including QS and c-di-GMP, illustrating the importance of properly controlling this phenotype for the adaptation of *V. cholerae*. New imaging and biophysical studies have shed light on the physical properties of *V. cholerae* biofilms and continue to provide roadmaps for the broader understanding of biofilm formation in all bacteria species. Biofilms protect *V. cholerae* from predation and stress, and contribute to hyperinfectivity, but studies of *V. cholerae* biofilms in natural environments such as chitin surfaces in aquatic reservoirs or during colonization are less understood. Many fundamental questions remain to be

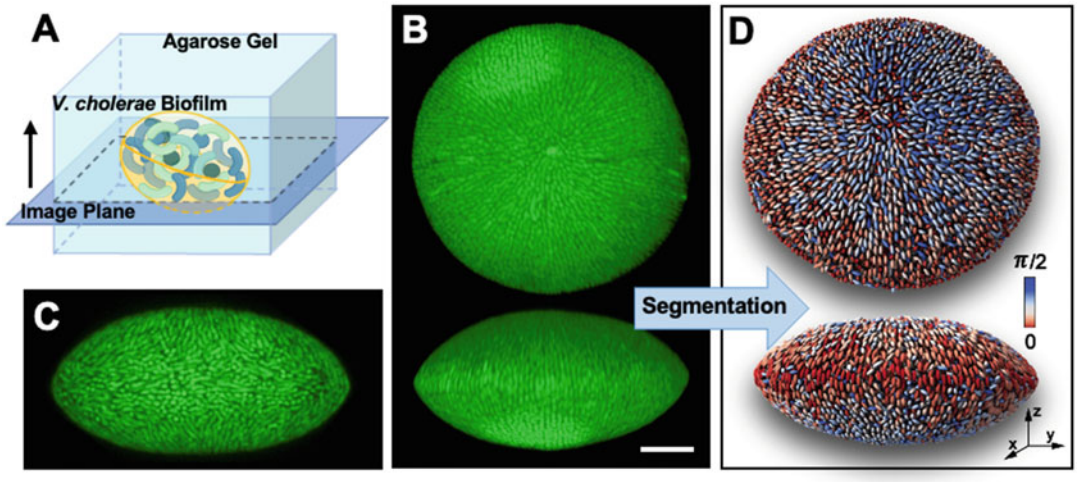


**Fig. 2.6** *V. cholerae* biofilm under confinement. (**a**) Schematic illustration of biofilm growth in an agarose gel. (**b**) 3D view (top and side) and (**c**) cross-sectional image of a *V. cholerae* biofilm embedded in a 2% agarose gel. Scale bar: 10 μm. (**d**) Single-cell 3D reconstruction of the embedded biofilm shown in **b**. Cells are color-coded according to their angles with respect to the *z*-axis. Adapted with permission from Zhang et al. (2021)

addressed such as the role of different matrix components in different environments, the integration of complex regulatory networks, and the differences in mechanical properties in divergent biofilms and the impact of these differences on biofilm function. Given its importance and large knowledge base, *V. cholerae* will continue to serve as a bountiful experimental system to gain fundamental insights into bacterial biofilm formation.

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# Type VI Secretion Systems: Environmental and Intra-host Competition of *Vibrio cholerae*

3

Francis Santoriello and Stefan Pukatzki

**Abstract**

The *Vibrio* Type VI Secretion System (T6SS) is a harpoon-like nanomachine that serves as a defense system and is encoded by approximately 25% of all gram-negative bacteria. In this chapter, we describe the structure of the T6SS in different *Vibrio* species and outline how the use of different T6SS effector and immunity proteins control kin selection. We summarize the genetic loci that encode the structural elements that make up the *Vibrio* T6SSs and how these gene clusters are regulated. Finally, we provide insights into T6SS-based competitive dynamics, the role of T6SS genetic exchange in those competitive dynamics, and roles for the *Vibrio* T6SS in virulence.

**Keywords**

*Vibrio cholerae* · Type VI secretion system · Bacterial competition

F. Santoriello
Department of Biology, The City College of New York, New York, NY, USA

Department of Molecular Biology, Princeton University, Princeton, NJ, USA

S. Pukatzki (✉)
Department of Biology, The City College of New York, New York, NY, USA
e-mail: spukatzki@ccny.cuny.edu

## 3.1 Introduction

The *Vibrio* genus is a diverse collection of bacterial species with various environmental niches, host preferences, and pathogenic potentials. Thus, these organisms cover a broad spectrum of lifestyles and include harmless environmental organisms and symbionts like *Vibrio fischeri* in the Hawaiian bobtail squid, opportunistic pathogens, and pathogens that are highly adapted to an animal host such as *Vibrio cholerae* in humans, *Vibrio parahaemolyticus* in shrimp, and *Vibrio anguillarum* in fish. Despite their diversity, a unifying theme for Vibrios is the need to compete for niche space in diverse environments like the chitinous surfaces of shellfish or the gastrointestinal tracts of infected hosts. Whether a *Vibrio* strain finds a host often depends on its abundance in the environment surrounding the host. An organism's arsenal of defense mechanisms can decide effective colonization and survival in both the environmental reservoir and host niche. One such defense system is the type VI secretion system (T6SS) (Pukatzki et al. 2006), a harpoon-like nanomachine encoded by approximately 25% of all gram-negative bacteria (Bingle et al. 2008; Boyer et al. 2009). The T6SS is evolutionarily, structurally, and functionally related to the contractile tail of a T4 bacteriophage (Pukatzki et al. 2007; Leiman et al. 2009; Pell et al. 2009; Basler et al. 2012). Bacteria use this system for contact-dependent translocation of proteinaceous effectors into neighboring



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competitor cells via membrane puncturing. Strains that encode the toxic effectors also encode cognate immunity proteins, so sister cells (kin) can neutralize their toxicity (Hood et al. 2010; Russell et al. 2011; Dong et al. 2013; Brooks et al. 2013; Fritsch et al. 2013; Miyata et al. 2013).

This chapter will focus on the *V. cholerae* T6SS, with a few examples from other *Vibrio* and non-*Vibrio* species. Here we summarize the current knowledge of *Vibrio* T6SS structure, the genetic loci that encode the structural elements that make up the *Vibrio* T6SSs, how these gene clusters are regulated, T6SS-based competitive dynamics, the role of T6SS genetic exchange in those competitive dynamics, and roles for the *Vibrio* T6SS in virulence.

## 3.2 The Structure and Mechanism of the T6SS

While the T6SS is functionally conserved across many gram-negative species, the core components of the T6SS vary. T6SSs can be phylogenetically classified into four types (T6SS-i, T6SS-ii, T6SS-iii, and T6SS-iv), with T6SS-i further divided into six subtypes (i1, i2, i3, i4a, i4b, and i5) (Boyer et al. 2009; Barret et al. 2013; Russell et al. 2014; Li et al. 2015; Böck et al. 2017). The T6SS-ii is an atypical T6SS solely found in the Francisella Pathogenicity Island of *Francisella novicida* and *Francisella tularensis* (de Bruin et al. 2007; Bröms et al. 2010). The T6SS-iii, to date, has only been identified in the phylum Bacteroidetes (Russell et al. 2014). The T6SS-i is the general proteobacterial T6SS described primarily in gammaproteobacteria (*V. cholerae*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Serratia marcescens, Acinetobacter baumanii*, among others) (Pukatzki et al. 2006; Mougous 2006; Shrivastava and Mande 2008; Murdoch et al. 2011; Carruthers et al. 2013). T6SS-i clusters can be identified across multiple phyla of gram-negative bacteria, including plant-associated organisms, marine bacteria, and medically-relevant human pathogens (Boyer et al. 2009).

The T6SS-i consists of approximately 13 conserved core genes (*tssA-M*) that are a mix between T4 bacteriophage-like tail and sheath components and type IV secretion system (T4SS) IcmF/DotU-type membrane anchoring proteins (Bingle et al. 2008; Boyer et al. 2009) (Fig. 3.1). The steps necessary to build a functional T6SS have been thoroughly examined in *V. cholerae* and *E. coli*. The T6SS-i first needs a pore for transit through the bacterial envelope. The pore through which T6SS components are secreted is known as the membrane complex and is formed by TssJ, TssL, and TssM; TssL and TssM are inserted into the inner membrane, and TssJ is inserted into the outer membrane (Felisberto-Rodrigues et al. 2011; Durand et al. 2012). TssJ and TssM interact to form a pore running through the bacterial envelope, allowing T6SS components to exit the cell (Felisberto-Rodrigues et al. 2011). It has been shown that TssM can also recruit peptidoglycan-degrading enzymes to its periplasmic domain to assist in forming the membrane complex through the peptidoglycan layer (Weber et al. 2016; Santin and Cascales 2017). The baseplate is built next and is composed of the bacteriophage structural proteins VgrG (TssI), TssE, TssF, TssG, and TssK. VgrG is the spike of the T6SS that allows for membrane puncturing of nearby cells, and it is homologous to the T4 phage-tail spike gp5/gp27 complex (Pukatzki et al. 2007; Leiman et al. 2009). In some cases, the VgrG spike is further sharpened by small proline-alanine-alanine-arginine (PAAR) repeat motif proteins (Shneider et al. 2013). TssE is homologous to the T4 bacteriophage wedge protein pg25 (Leiman et al. 2009; Lossi et al. 2011). TssE, -F, -G, and -K form a stable baseplate complex with VgrG in the cytoplasm. This structure is then recruited to the TssJLM membrane complex (Brunet et al. 2015; Logger et al. 2016; Nguyen et al. 2017). Next, the Hcp (TssD) tail and surrounding TssBC (VipAB) sheath extend from the baseplate as new subunits polymerize onto the distal end of the growing complex (Brunet et al. 2014, 2015; Vettiger et al. 2017) (Fig. 3.1). T6SS sheath-tube extension is primarily controlled by interactions between the membrane complex protein TssM,

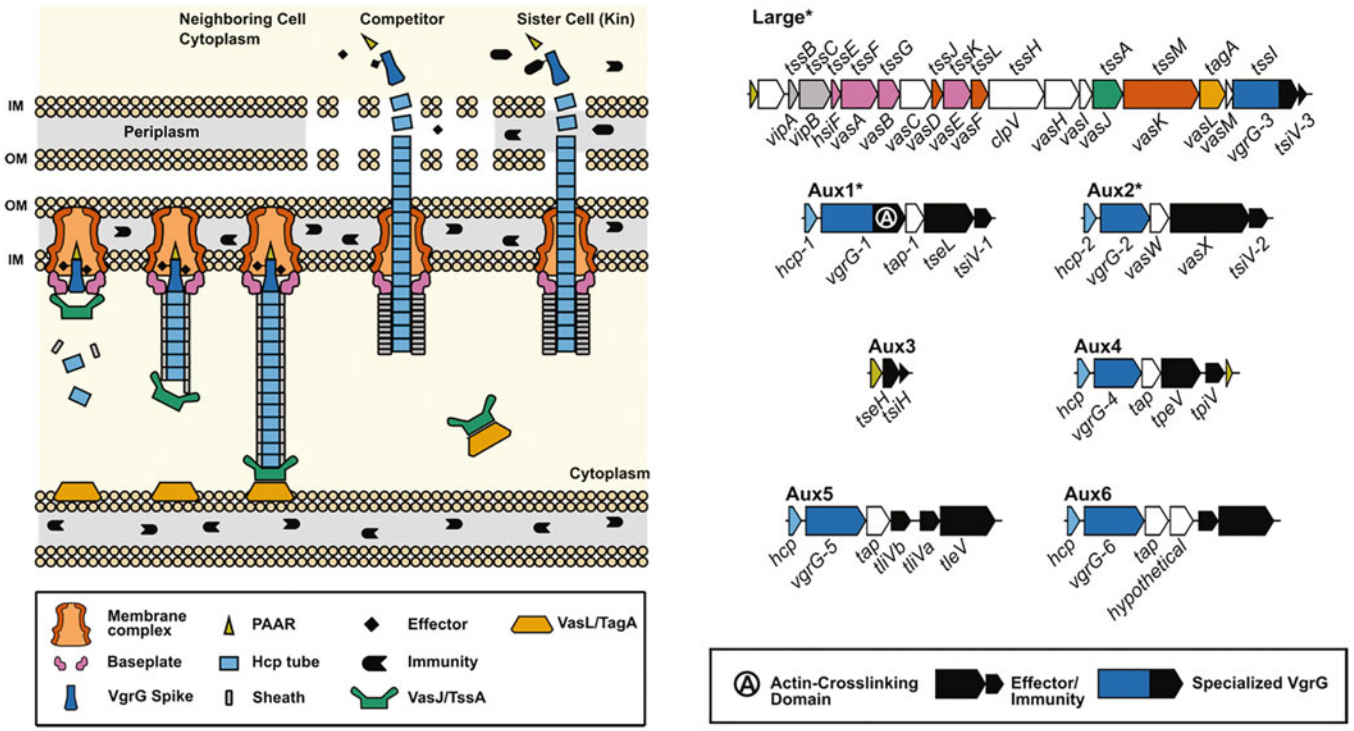


**Fig. 3.1** The T6SS of *V. cholerae*. (Left) Schematic of T6SS extension, stabilization, contraction, and delivery of toxic effector proteins into a neighboring cell. Proteins and complexes are color-coded to the genetic diagram on the right. (Right) Genetic diagrams of all identified T6SS gene clusters in the *V. cholerae* population. Clusters marked with an asterisk are the core loci present in all *V. cholerae* strains for which sequences are available. Gene cassettes are color-coded to their corresponding proteins/complexes on the left. Both the common T6SS structural gene names (top) and the *V. cholerae*-specific structural gene names (bottom) are shown for the large cluster

TssA, and TagA (Stietz et al. 2020). TssA stabilizes this process by binding to the baseplate and remaining associated with the distal end of the growing tail and sheath (Zoued et al. 2016; Schneider et al. 2019). TagA can counter this process by either binding TssM in the membrane complex and inhibiting extension or interacting with the TssA cap of the extending sheath on the opposite side of the cell to halt the extension (Santin et al. 2018; Schneider et al. 2019; Stietz et al. 2019, 2020). Dissociation of TssA results in the firing of the T6SS through contraction of the TssBC sheath (Basler et al. 2012; Zoued et al. 2016; Schneider et al. 2019).

The toxic effector proteins that diversify this conserved complex have been identified as covalent modifications to the VgrG spike, cargo effectors loaded onto the spike with adaptor proteins, or loaded within the lumen of the Hcp tube (Pukatzki et al. 2007; Brooks et al. 2013; Unterweger et al. 2015; Wood et al. 2019; Zhang et al. 2021). Cargo effectors with either DUF4123 chaperone proteins or PAAR repeat motif proteins specialized to their specific effectors attach onto the VgrG spike (Unterweger et al. 2015; Wood et al. 2019; Zhang et al. 2021). Loading effector proteins onto the T6SS is an essential step for efficient assembly and firing of the apparatus (Liang et al. 2019); cells expressing catalytically-inactive effectors assemble and fire the T6SS normally, while effector deletion strains are T6SS defective. The mechanical steps described above lead to the assembly of the T6SS, loading of toxic effectors, and translocation of these effector proteins into neighboring cells, resulting in contact-dependent killing of incompatible cells or effector neutralization and recycling of T6SS components in kin cells (Vettiger and Basler 2016) (Fig. 3.1).

## 3.3 Genome Organization of the *V. cholerae* T6SS

The term "T6SS" was coined for the system identified in the pathogen *Vibrio cholerae* (Pukatzki et al. 2006), and has since been found to be highly conserved in the *Vibrio* genus (Weber et al. 2009; Yu et al. 2012; Church et al. 2016; Huang et al. 2017; Kirchberger et al. 2017). *V. cholerae* and many closely related *Vibrio* species, including *V. metoecus*, *V. mimicus*, *V. fluvialis*, and *V. furnissii*, carry a single T6SS-i1 (Kirchberger et al. 2017). All *V. cholerae* strains minimally encode this T6SS-i1 in three genetic loci: a Large cluster and two auxiliary clusters (Aux1 and Aux2) (Pukatzki et al. 2009; Unterweger et al. 2014) (Fig. 3.1). The Large cluster encodes the majority of the system's structural components, including the membrane complex that anchors the system to the bacterial inner membrane (*vasDFK/tssJLM*), the baseplate complex (*hsiF/tssE*, *vasABE/tssFGK*) from which the contractile sheath (*vipAB/tssBC*) is extended, and two *tssA*-type proteins involved in the regulation of sheath extension and firing dynamics (*vasJ/tssA, vasL/tagA*) (Zoued et al. 2014; Cianfanelli et al. 2016; Schneider et al. 2019). The Large cluster also encodes an effector/immunity pair: a VgrG spike (*vgrG-3*) with a specialized bactericidal C-terminus and its cognate immunity factor (*tsiV3*) (Dong et al. 2013; Brooks et al. 2013). All Large cluster genes, except for two genes of unknown function (*vasI* and *vasM*) and the immunity gene *tsiV3*, are required for T6 secretion (Zheng et al. 2011). Two auxiliary T6SS clusters (Aux1 and Aux2) each encode an Hcp protein (*hcp-1* and *hcp-2*), an alternate VgrG spike (*vgrG-1*, *vgrG-2*), a DUF4123 domain-containing chaperone protein for effector loading (*tap-1*, *vasW*) (Unterweger et al. 2015), and a distinct effector-immunity pair (*tseL/tsiV1* and *vasX/tsiV2*, respectively) (Miyata et al. 2013; Unterweger et al. 2014) (Fig. 3.1). Aux1 and Aux2 are necessary to form a fully functional T6SS, as Hcp is required to form the central tube upon which the VgrG spike is fired from the cell. The VgrG-1 protein encoded by Aux1 is sometimes fused to a specialized C-terminal actin-crosslinking domain (ACD) with anti-eukaryotic properties (Pukatzki et al. 2007).

Four non-core auxiliary clusters have been identified in the *V. cholerae* population: Aux3, Aux4, Aux5, and Aux6 (Altindis et al. 2015; Labbate et al. 2016; Crisan et al. 2019, 2021;

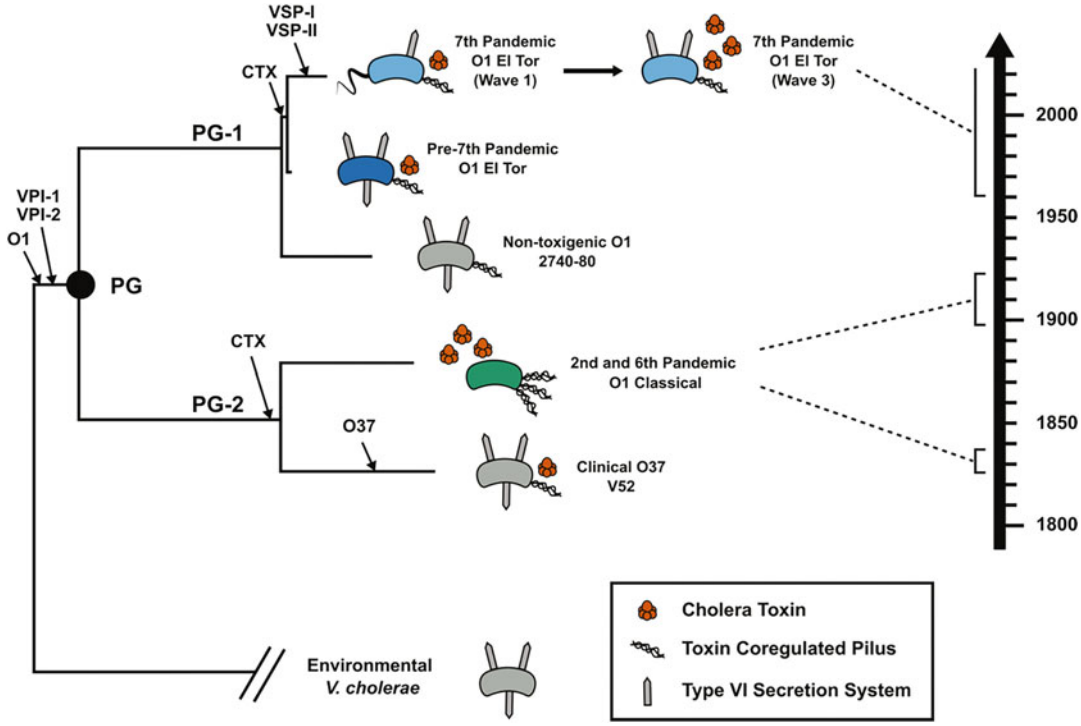


**Fig. 3.2** Evolutionary history of pandemic *V. cholerae* clades. Theoretical phylogenetic tree of environmental, O1, and pandemic *V. cholerae*. The black circle indicates the common ancestor of the O1 VPI-1(+) VPI-2(+) PG (phylocore genome) clade that diverged into PG-1 and PG-2, which independently gave rise to a pandemic clade. Black arrows indicate the acquisition of pandemic-associated genomic islands or other evolutionary events of note (e.g., acquisition of the O37 antigen). *V. cholerae* cells are colored and labeled according to the clade they represent: seventh pandemic O1 El Tors (light blue), pre-seventh pandemic O1 El Tors (dark blue), O1 Classicals (green), and close relatives of the seventh pandemic O1 El Tors and O1 Classicals (grey). Virulence factors expressed by each clade are represented. The number of each symbol represents the quantity of each factor expressed by the strains within a specific clade. On the right is a timeline from 1800 through today, indicating the periods in which strains from the different pandemic clades were isolated

Drebes Dörr and Blokesch 2020) (Fig. 3.1). Except Aux3, these auxiliary clusters carry an *hcp*, a *vgrG*, a DUF4123 chaperone, and an effector/immunity gene pair. T6SS clusters Aux4, Aux5, and Aux6 are absent from pandemic strains of *V. cholerae* and sporadically dispersed through environmental strains (Labbate et al. 2016; Carpenter et al. 2017; Crisan et al. 2019; Drebes Dörr and Blokesch 2020). Aux3 is unique in that it is a minimal T6SS cluster only encoding its effector/immunity pair and a PAAR-repeat chaperone protein (Shneider et al. 2013; Altindis et al. 2015; Hersch et al. 2020). The Aux3 cluster appears to be strongly associated with pandemic strains and a small number of environmental *V. cholerae* strains (Kirchberger et al. 2017; Santoriello et al. 2020). Most available *V. cholerae* sequences are from pandemic *V. cholerae,* as there is a bias towards collecting isolates from patients during outbreaks. Increasing efforts in whole-genome sequencing have identified an increasing number of genetic traits important for *V. cholerae* ecology. The Aux4, Aux5, and Aux6 clusters were identified by analyzing newly sequenced environmental *V. cholerae* strains, and so it is likely that undiscovered T6SS auxiliary clusters are circulating in *V. cholerae* populations.

## 3.4 Regulation of the T6SS Across the *V. cholerae* Species

The *V. cholerae* population can currently be broken into 206 serogroups based on the presence of the major surface O-antigen (Yamai et al. 1997). A single serogroup expressing the O1 surface antigen gave rise to a monophyletic clade known as the pandemic-generating lineage or phylocore genome (PG) clade (Feng et al. 2008; Chun et al. 2009; Islam et al. 2017) (Fig. 3.2). The PG clade was founded by an O1 strain carrying two *Vibrio* pathogenicity islands (VPI-1 and VPI-2); VPI-1 encodes the major virulence factor toxin co-regulated pilus (TCP) and its regulator ToxT, and VPI-2 encodes a Type-I restriction modification system, an operon for sialic acid catabolism, and a neuraminidase (Thelin and Taylor 1996; Karaolis et al. 1998; Jermyn and Boyd 2002). The O1 VPI-1(+) VPI-2(+) founder then diverged into the two subclades: PG-1 and PG-2 (Fig. 3.2). These subclades then acquired cholera toxin independently en route to pandemicity (Chun et al. 2009). The PG-2 clade gave rise to the Classical biotype of *V. cholerae* that is believed to have caused the first six cholera pandemics. The PG-1 clade gave rise to Pre-7th pandemic isolates denoted El Tor (Chastel 2007) and eventually the El Tor biotype strains that cause the current 7th pandemic. The ongoing seventh pandemic started in Indonesia in 1961 and is caused exclusively by El Tor biotype strains (Hu et al. 2016). An ancestor of the seventh pandemic isolates within the PG-1 clade acquired two more unique genomic islands on the path to pandemicity: VSP-I and VSP-II (Dziejman et al. 2002; Chun et al. 2009). While the roles of VSP-I and VSP-II are less clear than the VPIs, they have been shown to modulate the cyclic di-nucleotide pool and regulate chemotaxis in response to zinc starvation, respectively (Davies et al. 2012; Murphy et al. 2021). The seventh pandemic has persisted significantly longer than any of the previous Classical pandemics, and in that time, there have been multiple distinct, overlapping waves of transmission with shifting physiology. Physiological shifts over the El Tor waves are typically defined by acquiring SXT/R391 family integrative conjugative elements, encoding multiple antibiotic resistances, and acquiring variant cholera toxin subtypes (Hochhut and Waldor 1999; Garriss et al. 2009; Wozniak et al. 2009; Mutreja et al. 2011; Kim et al. 2015). Wave 1 El Tor strains initiated the 7th pandemic and have since been displaced by Wave 2 and subsequently Wave 3 El Tor strains, which can further be divided into "Early Wave 3" and "Current Wave 3" isolates (Mutreja et al. 2011; Kim et al. 2015). A novel pathogenic serogroup of *V. cholerae*, O139, was identified in India and Bangladesh in 1992 (Ramamurthy et al. 1993; Chun et al. 2009). This serogroup likely occurred due to a horizontal acquisition of a new O-antigen cluster by a close relative of the Wave 2 El Tors (Blokesch and Schoolnik 2007; Mutreja et al. 2011). O139 strains are still endemic in India and Bangladesh but have not exhibited pandemic spread.

The T6SS of *V. cholerae* is differentially regulated across the different lineages of environmental and pandemic *V. cholerae* (Fig. 3.2). Environmental *V. cholerae* strains express their T6SS constitutively (Unterweger et al. 2012; Bernardy et al. 2016), likely lending a survival advantage in the variable environment of the aquatic reservoir. Constitutive T6SS activity was conserved into the O1 lineage. It can be observed in close relatives of both the pandemic El Tor clade (pre-seventh pandemic El Tor strains and non-toxigenic US Gulf Coast isolate 2740-80) and the Classical clade (O37 clinical strain V52) (Pukatzki et al. 2006; MacIntyre et al. 2010; Unterweger et al. 2012; Bernardy et al. 2016) (Fig. 3.2). Classical strains, however, have an inactive T6SS due to four separate mutations in the large cluster: *vipA/tssB*, *hsiF/tssE*, *vasE/tssK*, and *vasK/tssM* (Miyata et al. 2010; MacIntyre et al. 2010; Kostiuk et al. 2021) (Figs. 3.2 and 3.3).

Early studies of Wave 1 El Tor strains suggested that the T6SS was also inactive in seventh pandemic strains (Pukatzki et al. 2006; MacIntyre et al. 2010). It has since been shown that the El Tor T6SS is tightly regulated in a pathoadaptive manner (Fig. 3.3). The transition

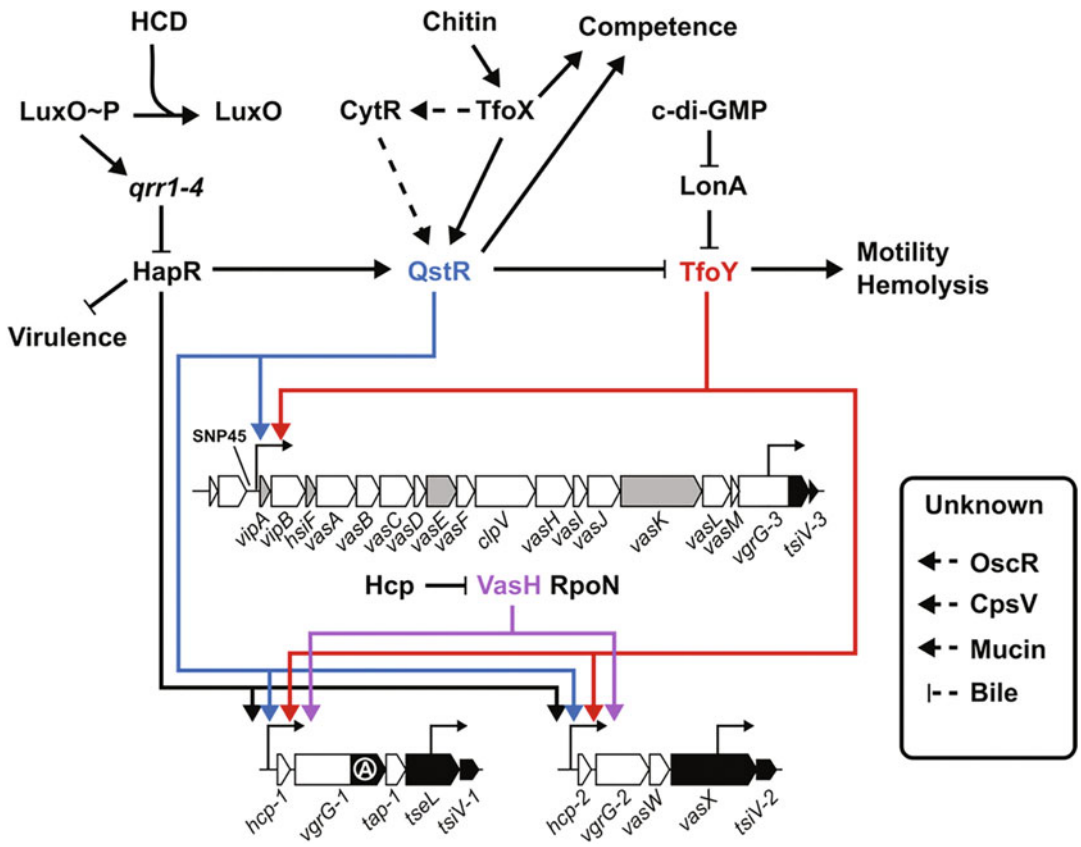


**Fig. 3.3** Regulation of the T6SS in pandemic strains of *V. cholerae*. Regulatory network controlling T6SS activation in pandemic *V. cholerae* and other co-regulated systems (virulence, motility, hemolysis, competence). Solid arrows indicate confirmed interactions. Dashed arrows equal observed interactions that have not been established as direct. Blunt-end arrows indicate repression. T6SS clusters are shown. Small arrows associated with clusters represent promoters. Black genes indicate effector genes/domains and their corresponding immunity genes (small cassettes). White A in a circle indicates the ACD of *vgrG-1*. Grey genes represent the genes mutated in O1 Classical strains

from constitutive T6SS activity in environmental *V. cholerae* strains to pathoadaptivity in pandemic strains is decided by a single nucleotide transversion (T to G) upstream of the *tssB/vipA* gene in the Large cluster (Drebes Dörr et al. 2022). Conversion from G to T at this site, dubbed SNP45, is sufficient to confer constitutive T6SS activity upon pandemic strains of *V. cholerae*, but the regulators affected by SNP45 are unknown. Common laboratory Wave 1 El Tor strains such as N16961, C6706, and A1552 do not express their T6SS under laboratory conditions but activate the system upon passage through infant mice, rabbits, and human volunteers (Lombardo et al. 2007; Mandlik et al. 2011; Fu et al. 2013; Bachmann et al. 2015). Multiple host signals have been shown to directly regulate the O1 El Tor T6SS directly. Passage of Wave 1 O1 El Tor isolate C6706 through the infant mouse small intestine activated the T6SS and triggered the C6706 cells to kill a mutant lacking its cognate immunity genes (Bachmann et al. 2015). This study further demonstrated that the T6SS of C6706 and N16961 was activated in response to mucin and repressed by exposure to commensal bacterium-generated bile acids, indicating that host and microbiome-derived signals in the small intestine influence T6SS

activation during infection. It was recently shown that T6SS activation by mucin results in slower, more directed flagellar motility indicating a killing-independent role for the T6SS in intestinal colonization (Frederick et al. 2020). Activation of the T6SS in the intestine has been partially supported in human volunteer studies showing that an O1 El Tor isolate highly upregulates the T6SS regulator VasH during passage through the human gastrointestinal tract (Lombardo et al. 2007). VasH is a RpoN (sigma 54)-dependent transcriptional regulator encoded in the Large T6SS cluster that is necessary for T6SS function due to its activation of the Aux1 and Aux2 clusters (Miyata et al. 2010; Kitaoka et al. 2011; Unterweger et al. 2012) (Fig. 3.3). Hcp protein produced from these clusters has been shown to bind to VasH directly and inhibit the activation of Aux1 and Aux2 (Manera et al. 2021) (Fig. 3.3). This VasH-Hcp negative feedback loop likely functions as an energy conservation mechanism, tuning expression levels of T6SS genes to the protein levels of its most abundant structural component. Another critical regulatory protein at the mucosal surface is the quorum sensing master regulator HapR, which is repressed upon mucosal penetration (Liu et al. 2008). HapR has been shown to activate the T6SS and likely plays a role in *in vivo* T6SS expression for O1 El Tor strains at the mucosal surface and potentially during the mucosal escape response (Nielsen et al. 2006; Tsou et al. 2009; Ishikawa et al. 2009; Shao and Bassler 2014) (Fig. 3.3). These results support a model in which the presence of mucus components triggers the T6SS to compete with the resident host gut microbiome residing upon the mucus layer of the gastrointestinal tract. The T6SS is then repressed once cells penetrate the mucus to reach the epithelial cell layer and initiate pathogenesis. Regulation of the T6SS by host signals like viscous mucous surfaces is not unique to *V. cholerae*. The T6SS is upregulated in both *V. fischeri* in response to squid host-like viscosity and *V. corallilyticus* in response to coral mucus (Speare et al. 2021; Gao et al. 2021).

Environmental conditions faced by *V. cholerae* in the aquatic reservoir have also been investigated for their modulatory effects on the T6SS (Fig. 3.3). In the estuarine environment where fresh and saline ocean water meet, *V. cholerae* cells regularly experience shifts in osmolarity. The T6SS of O1 El Tor strain A1552 is activated in response to increasing concentrations of various salts and other osmolytes through the repression of the osmoregulatory protein OscR (Shikuma and Yildiz 2009; Ishikawa et al. 2012). A1552 was also shown to activate its T6SS after the shift from human body temperature (37 °C) to temperatures mimicking the aquatic reservoir (25 and 15 °C) through activation of the cold shock regulatory gene *cspV*, indicating that leaving the host and re-entering the marine environment can trigger T6SS killing (Townsley et al. 2016). Regulation of the T6SS by osmolarity and temperature is a common theme across several *Vibrio* species. The pathogenic species *V. parahaemolyticus* activates its two T6SSs differentially, with its T6SS1 activated in response to warm, marine-like conditions and its T6SS2 activated in response to low salt conditions (Salomon et al. 2013). The fish pathogen *V. anguillarum* also carries two T6SSs that respond differentially to temperature, with its T6SS1 activated at high temperatures and its T6SS2 activated at low temperatures (Lages et al. 2019). After re-entry into the estuarine environment, O1 El Tor *V. cholerae* cells can often be isolated in association with chitinous surfaces. It has been shown that chitin metabolism triggers T6SS expression by activating the competence regulators TfoX and QstR (Meibom 2005; Borgeaud et al. 2015; Watve et al. 2015; Jaskólska et al. 2018) (Fig. 3.3). Quorum sensing also feeds into this regulatory circuit, as HapR activates the T6SS clusters directly and through activation of QstR (Lo Scrudato and Blokesch 2013). Quorum sensing regulation of the T6SS in the environment indicates a potential role for the T6SS in high cell density states like late-stage biofilm formation. The TfoX-homolog TfoY can also activate the *V. cholerae* T6SS clusters. Still, it does so in a quorum sensing and chitin-independent manner (Metzger et al. 2016) (Fig. 3.3). TfoY is instead activated by c-di-GMP, which directly binds

LonA protease and inhibits the degradation of TfoY (Rogers et al. 2016; Metzger et al. 2016; Joshi et al. 2020). TfoY co-regulates the T6SS, motility, and hemolysis, indicating that TfoY may be particularly important for competition with predatory eukaryotes like grazing amoeba (Metzger et al. 2016). Outside of O1 El Tor strains, TfoY is particularly important for T6SS activation in the constitutive T6SS-producing strain V52 (Metzger et al. 2016). Further, homologs of both TfoX and TfoY have been shown to modulate the activation of T6SS clusters in *V. parahaemolyticus*, *V. alginolyticus*, and *V. fischeri*. The genomic structure of the T6SS encoding gene clusters in these species is different from *V. cholerae*, so the TfoX and TfoY regulatory circuits are wired differently in each species. Still, these circuits are likely generally conserved across the *Vibrio* genus (Metzger et al. 2019).

Most studies on T6SS regulation in *V. cholerae* have focused on regulators that bind the main promoters upstream of the first gene in each T6SS cluster (*hcp-1*, *hcp-2*, *vipA*). It is important to note that each *V. cholerae* T6SS cluster has a second promoter internal to its specific effector gene (Brooks et al. 2013; Miyata et al. 2013) (Fig. 3.3). These internal promoters regulate the downstream immunity genes, resulting in a dual-expression profile for cognate immunity proteins.

## 3.5 The Role of the *V. cholerae* T6SS in Interbacterial Competition

Many effector proteins delivered by the T6SS into neighboring cells are toxic to non-kin, gram-negative bacteria (Hood et al. 2010; MacIntyre et al. 2010; Russell et al. 2011). Gram-positive species are largely resistant to T6SS attacks. Still, a recent study has shown that an *A. baumannii* T6SS-secreted peptidoglycanase can kill gram-positive prey cells after inducing a local rise in pH (Le et al. 2021). In the case of bactericidal effectors, the effector-secreting predator cell also encodes a cognate immunity protein to neutralize the effectors killing capacity and protect against attacks from sister cells (Hood et al. 2010; Russell et al. 2011; Dong et al. 2013; Brooks et al. 2013; Fritsch et al. 2013; Miyata et al. 2013). It is important to note that physical puncturing by the T6SS spike is not enough to kill a prey cell. The bactericidal effector protein is necessary for killing to occur (Kamal et al. 2020). Every known T6SS locus in the *V. cholerae* population encodes a bactericidal effector protein immediately next to a cognate immunity protein that neutralizes its activity (Figs. 3.1 and 3.3). Effector genes at the core loci in pandemic *V. cholerae* strains (*tseL*, *vasX*, and *vgrG-3*) encode a lipase, a pore-forming toxin, and a peptidoglycanase, respectively (Dong et al. 2013; Brooks et al. 2013; Miyata et al. 2013). At the auxiliary clusters, the Aux3 effector TseH is a peptidoglycan hydrolase, the Aux4 effector TpeV is a membrane permeabilizing toxin, and the Aux5 effectors TleV1-4 are putative lipases (Altindis et al. 2015; Crisan et al. 2019, 2021; Hersch et al. 2020). The Aux6 effector has been shown to kill non-immune prey bacteria, but no specific function for the effector protein has been reported (Drebes Dörr and Blokesch 2020). The only *V. cholerae* T6SS effector with no described function against bacteria is the ACD of VgrG-1. This effector has solely been shown to be toxic to eukaryotes and the intestinal epithelium of the host (Pukatzki et al. 2006, 2007; Logan et al. 2018).

Studies on the antibacterial properties of the *V. cholerae* T6SS have primarily focused on four different scenarios: (1) T6SS-active predator against a T6SS(−) or T6SS-inactive prey, (2) interspecies competition between incompatible T6SS-active strains, (3) intraspecies competition between incompatible T6SS-active strains, and (4) species-specific competition (Fig. 3.6a). In a one-sided competition between two bacterial strains, the T6SS-active cells can clear the niche of non-self bacteria while protecting kin cells and expanding outward clonally (MacIntyre et al. 2010; Unterweger et al. 2012). However, how well this clearance occurs depends upon the lysis kinetics of the involved bactericidal

effectors, as insufficient lysis of the dead prey cells after killing can lead to "corpse barriers" between the predator and prey population and failure to clear the niche (Smith et al. 2020). In the case of bidirectional competition between two different T6SS-active species with incompatible effector/immunity pairs, mathematical modeling and laboratory experiments show that T6SS killing drives phase separation of the two strains into local clusters of kin cells with distinct borders (Wong et al. 2016; McNally et al. 2017). Again, this phase separation is limited by accumulating dead cells at the interface of killing between clonal clusters (Steinbach et al. 2020). Strains within a species can have incompatible effector sets, leading to intraspecies competition with the same dynamics described for interspecies competition with two T6SS-active strains (Unterweger et al. 2014). Finally, the *V. cholerae* Aux3 effector TseH is the only *V. cholerae* T6SS effector protein to exhibit species-specific killing. It will not kill non-immune *V. cholerae* or *E. coli,* but it will kill *Aeromonas* and *Edwardsiella* species (Hersch et al. 2020). Killing by TseH depends on the presence or absence of specific bacterial envelope stress response systems, highlighting the importance of immunity gene-independent T6SS defenses (Hersch et al. 2020). This species specificity, however, can be overcome by local cation concentrations (Tang et al. 2022), indicating that species-specific competition can be decided by the balance of immunity-independent T6SS defenses and local abiotic factors.

## 3.6 Effector and Immunity Gene Diversity and Intraspecific Competition in the *V. cholerae* Population

The structural and regulatory components of the *V. cholerae* T6SS gene clusters are highly conserved between strains, but effector/immunity gene cassettes are variable between *V. cholerae* strains (Unterweger et al. 2014; Kirchberger et al. 2017; Hussain et al. 2021). The environmental *V. cholerae* population encodes 20 different effector/immunity types across the three core T6SS loci (Fig. 3.4, Table 3.1). In contrast, pandemic *V. cholerae* strains all encode an identical set of three distinct effectors referred to as A-type (*tseL*, *vasX*, *vgrG-3*) (Unterweger et al. 2014; Kirchberger et al. 2017). While the A-type effectors can be found sporadically in environmental strains of *V. cholerae*, no environmental strains have been identified with an AAA effector set (Kirchberger et al. 2017; Crisan et al. 2019). It is important to note that different effector types at the Aux1 (type A and C) and Aux2 (types A–E) loci encode distinct proteins rather than different alleles of a homologous gene. In contrast, variable types at the Large cluster (types A–M) are different C-terminal extensions on a conserved VgrG spike (Unterweger et al. 2014; Kirchberger et al. 2017) (Table 3.1). High variability of effector sets between environmental strains and a conserved effector set in pandemic strains mirrors the clonal nature of the rest of the PG clade outside the mobile pathogenicity islands. The complete A-type effector set is present in both PG-1 and PG-2 but lacking from the O1 CAA Sister clade (Kirchberger et al. 2017; Crisan et al. 2019), indicating that the full set of these effectors was acquired after the foundation of the PG clade but before the divergence of the PG clade into PG-1 and PG-2 (Fig. 3.4). This pattern extends to closely related species such as *V. mimicus*, *V. metoecus*, *V. fluvialis*, and *V. furnissii*, which all carry the three core *V. cholerae* T6SS loci encoding effector/immunity pairs with types homologous to those in the *V. cholerae* population. None of these species, which cause localized disease but not pandemic outbreaks, carry an AAA effector set (Kirchberger et al. 2017).

As each T6SS cluster in *V. cholerae* encodes an effector/immunity gene pair, the total complement of T6SS gene clusters in a given strain makes up its effector set. We previously discovered that the effectors encoded within these conserved gene clusters differ widely among *V. cholerae* strains (Unterweger et al. 2012, 2014; Thomas et al. 2017). Kin bacteria produce immunity proteins that protect them from the effectors of genetically-identical sister cells. Strains with the same effector-immunity gene

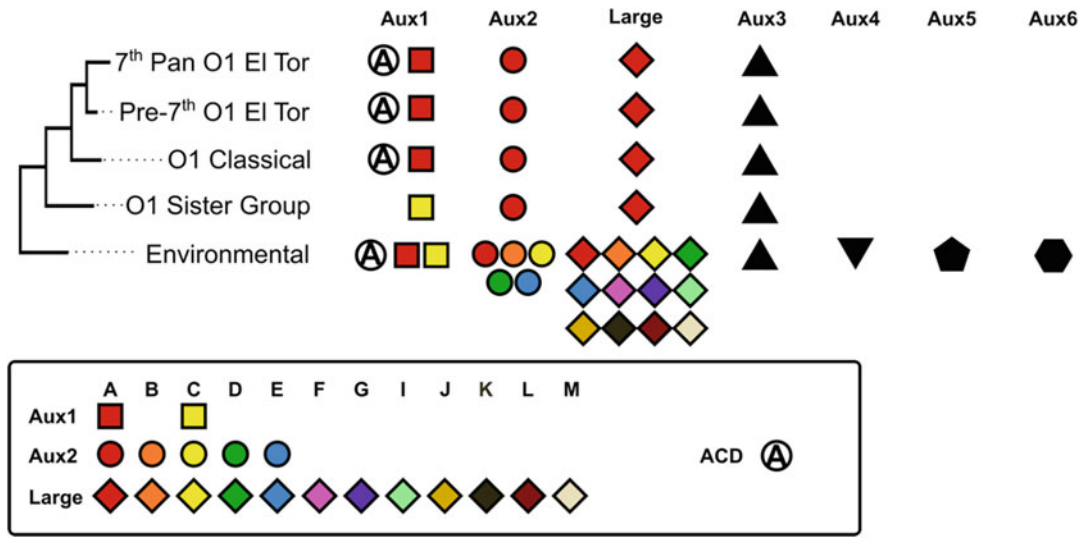


**Fig. 3.4** T6SS effector/immunity type distribution in the *V. cholerae* population. Effector/immunity protein diversity of the *V. cholerae* T6SS clusters across the O1 clade and environmental *V. cholerae* strains. A theoretical phylogenetic tree is broken into the subpopulations discussed in this chapter: 7th pandemic O1 El Tor strains (PG-1), Pre-7th pandemic O1 El Tor strains (PG-1), O1 Classical strains (PG-2), the O1 Sister Group (closest known ancestors of the PG clade), and environmental strains. Effector type is indicated by color, and locus is characterized by shape. T6SS clusters, Aux3, Aux4, Aux5, and Aux6, do not have associated types.

sets are said to belong to the same compatibility group and can coexist. In contrast, those with different sets compete against each other. There is evidence for the dominance of the AAA effector set of pandemic strains over other effector sets found in the environmental *V. cholerae* population, particularly at human host physiological temperature (Unterweger et al. 2014; Hussain et al. 2021; Tang et al. 2022). However, it is important to consider that the dynamics of T6SS-dependent intraspecies competition may not be limited to differential effector sets. Speed of T6SS firing, number of T6SS sheaths generated per cell, effector lysis kinetics, conditional toxicity driven by abiotic factors, the synergy between effectors, and immunity-independent protective mechanisms vary between T6SS-active strains and influence whether a strain wins in a competitive interaction (LaCourse et al. 2018; Toska et al. 2018; Hersch et al. 2020; Smith et al. 2020; Bernal et al. 2021; Hussain et al. 2021; Tang et al. 2022). Abiotic factors are critical because *V. cholerae* lives a dual lifestyle between the aquatic reservoir and the host gastrointestinal tract, two niches with variable conditions ranging from pH to temperature to salt concentration.

## 3.7 The Cost-Benefits Trade-Off of Exchanging T6SS Effectors and the Role of Orphan Immunity Genes in Interbacterial Competition

The variable T6SS effector/immunity types in the environmental population of *V. cholerae* are horizontally transferred between strains, as a strain's given effector set does not align with the whole-genome phylogeny of environmental *V. cholerae* strains (Kirchberger et al. 2017). T6SS predation itself is likely the catalyst for the transfer of effector/immunity modules, as interbacterial predation by the T6SS and acquisition of new genetic material with the competence pilus are linked functions in *V. cholerae* (Borgeaud et al. 2015). Binding to a chitinous surface triggers chitin metabolism, which induces the competence

**Table 3.1** *V. cholerae* effector gene types and functions

| T6SS cluster | Gene name | Effector/ immunity type | Knowna/predicted function | Ref. |
|---|---|---|---|---|
| *Aux1* | *vgrG-1* | | Actin crosslinking* | Pukatzki et al. (2007) |
| | *tseL* | A | Lipase* | Dong et al. (2013) |
| | | C | Putative lipase | Unterweger et al. (2014) |
| *Aux2* | *vasX* | A | Pore formation* | Miyata et al. (2011) |
| | | B | Pore formation | Unterweger et al. (2014) |
| | | C | No prediction | Unterweger et al. (2014) |
| | | D | Peptidoglycanase | Unterweger et al. (2014) |
| | | E | Pore formation | Unterweger et al. (2014) |
| *Large* | *vgrG-3* | A | Peptidoglycanase* | Brooks et al. (2013) |
| | | B | Cell adhesion | Unterweger et al. (2014) |
| | | C | Peptidoglycanase | Unterweger et al. (2014) |
| | | D | Peptidoglycanase | Unterweger et al. (2014) |
| | | E | Peptidoglycanase | Unterweger et al. (2014) |
| | | F | Peptidoglycanase | Unterweger et al. (2014) |
| | | G | Peptidoglycanase | Unterweger et al. (2014) |
| | | H | None reported | Kirchberger et al. (2017) |
| | | I | None reported | Kirchberger et al. (2017) |
| | | J | None reported | Kirchberger et al. (2017) |
| | | K | None reported | Kirchberger et al. (2017) |
| | | L | None reported | Kirchberger et al. (2017) |
| | | M | None reported | Hussain et al. (2021) |
| *Aux3* | *tseH* | | Peptidoglycanase* | Altindis et al. (2015) |
| *Aux4* | *tpeV* | | Membrane permeabilization* | Crisan et al. (2021) |
| *Aux5* | *tleV1* | | Lipase* | Crisan et al. (2019) |
| | *tleV2* | | Putative lipase | Crisan et al. (2019) |
| | *tleV3* | | Putative lipase | Crisan et al. (2019) |
| | *tleV4* | | Putative lipase | Crisan et al. (2019) |
| *Aux6* | | | None reported | Drebes Dörr and Blokesch (2020) |

regulator TfoX (Meibom 2005; Lo Scrudato and Blokesch 2013). TfoX activates a second competence regulator QstR, and both of these factors regulate co-expression of the T6SS and the natural competence machinery (Borgeaud et al. 2015; Watve et al. 2015). Interbacterial predation by the T6SS lyses competitive cells, releasing their genetic material into the local environment. Simultaneous expression of the competence pilus allows for the immediate uptake of the nearby DNA and diversification of the predator's genome (Borgeaud et al. 2015). Chitin also induces some lysogenic vibriophages from the genome of certain *V. cholerae* strains that can then lyse neighboring prey cells, allowing for DNA uptake by uninduced members of the lysogen population (Molina-Quiroz et al. 2020). Based on the bacteriophage origin of the T6SS, it is possible that the T6SS-natural competence relationship is a domesticated form of this bacteriophage-natural competence linkage. Further, this phenomenon of interbacterial predation linked to natural competence is not unique to *V. cholerae* and has also been demonstrated in *Acinetobacter baylyi* and *Streptococcus pneumoniae* (Steinmoen et al. 2002, 2003; Cooper et al. 2017; Ringel et al. 2017).

The linkage of T6SS predation and natural competence is likely an important factor in transferring of T6SS effector/immunity modules. It has been shown that naturally competent *V. cholerae* can acquire new functional T6SS effector/immunity modules that can be secreted by the T6SS (Thomas et al. 2017). The exchange

of effector/immunity types in a strain with an activated T6SS is inherently dangerous for the recipient cell. This modular exchange event puts the recipient strain at immediate odds with all of its surrounding T6SS-active ex-kin cells (Unterweger et al. 2014; Thomas et al. 2017). *V. cholerae* potentially decreases the risks of effector exchange by encoding extra immunity genes without their cognate effectors (orphan immunity genes) downstream from the main effector/immunity module at the T6SS loci (Kirchberger et al. 2017) (Fig. 3.5a). These orphan immunity genes are typically different from the neighboring effector immunity module and are likely maintained from the previous effector/immunity module during effector/immunity module exchange. Maintenance of the previous immunity gene would protect a diversified strain from ex-kin attacks and circumvent the costs of effector/immunity module exchange, allowing the new strain to reap the benefits of the newly acquired effector (Fig. 3.5a). The Aux1 locus of pandemic *V. cholerae* encodes a single orphan C-type immunity gene, leading us to hypothesize that this A-type effector was acquired by an ancestor of the *V. cholerae* pandemic clade with a C-type effector/immunity module at Aux1 (Kirchberger et al. 2017) (Figs. 3.4 and 3.5a).

Orphan immunity genes have been identified in several other T6SS positive species, including *Salmonella enterica*, *Proteus mirabilis*, common bee symbiote *Snodgrassella alvi*, and several

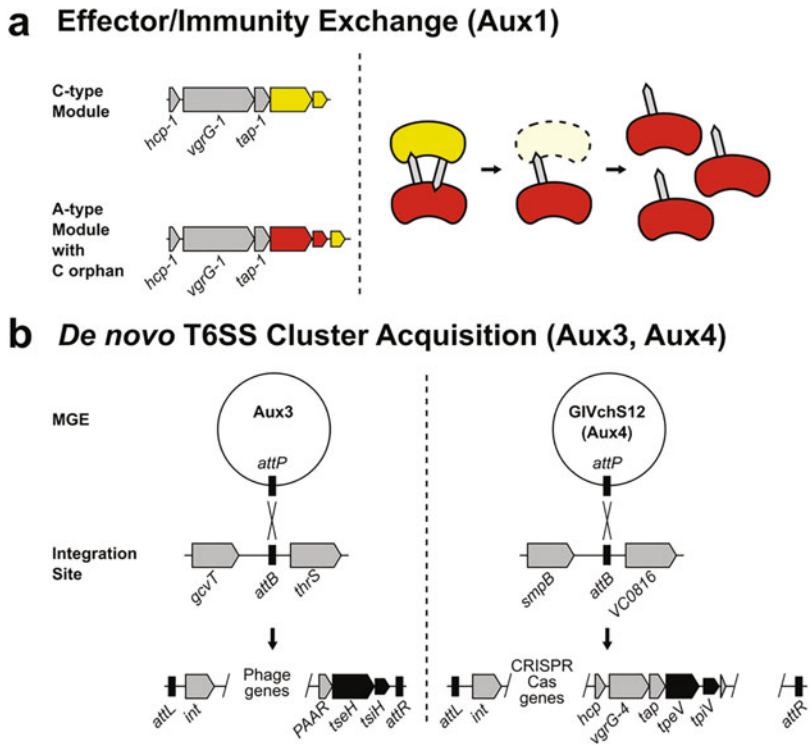


**Fig. 3.5** Common mechanisms for T6SS effector/immunity gene acquisition. (**a**) (Left) Schematic diagrams showing the orphan C immunity gene at the Aux1 locus. Effector/immunity genes are colored by type (Fig. 3.4). Grey cassettes indicate T6SS structural genes. (Right) Diagram of orphan immunity protection against attacks from ex-kin cells. (**b**) Schematic diagrams showing the mechanism of Aux3 and Aux4 acquisition. Black rectangles indicate the attachment (*att*) sites where site-specific integration of these clusters occur. Integration of Aux3 occurs between the *gcvT* and *thrS* genes. Integration of the GIVchS12 element carrying Aux4 occurs in the *ssrA* gene between *smpB* and *VC0816*, the site of VPI-1 integration in pandemic *V. cholerae* strains. Crosses indicate reversible recombination

species in the order Bacteriodales (Alteri et al. 2017; Steele et al. 2017; Ross et al. 2019; Sibinelli-Sousa et al. 2020; Barretto and Fowler 2020). Orphan immunity genes in Bacteroidales are unique. They are located on large genomic islands called AIDs that do not carry T6SS structural genes or effectors but carry long arrays of orphan immunity genes originating from diverse bacterial species (Ross et al. 2019). These orphan immunity gene arrays have been shown to confer broad protection against inter- and intraspecies T6SS attacks. All other highlighted studies, including those on *V. cholerae*, show a small number of orphan immunity genes directly downstream from or within a T6SS gene cluster with structural and effector genes (Kirchberger et al. 2017).

## 3.8 Mobile T6SS Clusters in the *V. cholerae* Population and Beyond

Two *V. cholerae* T6SS clusters, much like the majority of the other *V. cholerae* virulence genes, have been identified on mobile genetic elements (MGEs) that can readily excise themselves from the chromosome to form a plasmid: Aux3 and Aux4 (Fig. 3.5b). The Aux3 cluster is encoded on a long, prophage-like element in a subset of environmental *V. cholerae* strains (Santoriello et al. 2020). This element encodes an integrase and a recombination directionality factor that allow it to excise from the host chromosome and insert into a naïve chromosome in a site-specific manner. A shorter form of the Aux3 cluster is conserved in pandemic strains of *V. cholerae*, indicating that this mobile prophage-like element underwent a large deletion during the evolution of the PG clade (Santoriello et al. 2020). The Aux3 cluster is potentially an example of T6SS evolution from prophage to bacterial secretion system. The Aux4 cluster is encoded along with a CRISPR-Cas system on an MGE named GIVchS12 that is unique to environmental strains of *V. cholerae* (Labbate et al. 2016; Carpenter et al. 2017; Crisan et al. 2019). GIVchS12 is horizontally transferred between non-pandemic strains of *V. cholerae* where it integrates at a defined *att* site in the transfer-messenger RNA *ssrA*, the same site as VPI-1 in pandemic strains (Labbate et al. 2016; Carpenter et al. 2017). This element carries its own recombinase and inserts into the chromosome by site-specific recombination, priming it for efficient transfer between strains (Labbate et al. 2016; Carpenter et al. 2017). T6SS components carried on MGEs likely provide both a selective pressure and selective advantage for maintaining the element in the new host. From a T6SS evolutionary perspective, acquiring a T6SS locus de novo on an MGE, rather than replacing the current effector/immunity module in an existing cluster, equips a strain with a new toxin that can kill surrounding ex-kin cells without any potential vulnerability to ex-kin attacks. Cluster acquisition in this manner lacks a cost-benefits dilemma and should be highly favorable to *V. cholerae* cells. Again, this type of T6SS cluster acquisition is not unique to *V. cholerae* and can be identified in other *Vibrio* species. The T6SS2 of *V. vulnificus* appears to be transmitted horizontally between *V. vulnificus* strains (López-Pérez et al. 2019). This cluster from *V. vulnificus* is 97% identical to a T6SS cluster found in the *V. anguillarum* population, further supporting the presence of this T6SS cluster on a mobile genetic element (López-Pérez et al. 2019). Minimal clusters of T6SS genes composed of only effector and immunity genes have also been shown to move horizontally between *Vibrio* species like *V. alginolyticus*, *V. anguillarum*, *V. parahaemolyticus*, and *V. campbellii* (Salomon et al. 2015). These minimal clusters were occasionally associated with recombinase genes like integrases and transposases, indicating their status as MGEs (Salomon et al. 2015). In support of these concepts, T6SS clusters have been identified on MGEs in many gram-negative organisms, such as *Cronobacter sakazakii*, *Campylobacter jejuni*, and the species of the order *Bacteroidales* (Franco et al. 2011; Ross et al. 2019; Marasini et al. 2020; García-Bayona et al. 2021).

## 3.9 The Role of the *V. cholerae* T6SS in Inter-kingdom Competition

Many bacterial pathogens use their T6SSs to deliver toxic effectors into the cells of infected hosts and other competitive eukaryotic cells, including fungi and predatory protozoa (Monjarás Feria and Valvano 2020). The first description of the T6SS in *V. cholerae* highlighted the ability of the system to protect against grazing by the predatory amoeba *Dictyostelium discoideum* (Pukatzki et al. 2006). Since that discovery, multiple anti-eukaryotic roles have been identified for the *V. cholerae* T6SS through the action of the ACD of the VgrG-1 spike protein and the effectors TseL and VasX (Fig. 3.6b). The ACD of VgrG-1 was shown to induce cytotoxicity in both *D. discoideum* and murine macrophages in a T6SS-dependent manner by crosslinking cytosolic actin (Pukatzki et al. 2007). Both the pore-forming toxin VasX and the lipase TseL were shown to contribute to protection against grazing by *D. discoideum*, but not to the same level as VgrG-1 (Miyata et al. 2011; Zheng et al. 2011). This T6SS-dependent *D. discoideum* cytotoxicity has been shown to require endocytosis of T6SS-active *V. cholerae* cells (Ma et al. 2009). TseL and VasX are the only *V. cholerae* T6SS effectors to date to show trans-kingdom toxicity, as they kill both eukaryotic cells and bacteria (Miyata et al. 2011, 2013; Zheng et al. 2011; Unterweger et al. 2014). The action of these effectors does not

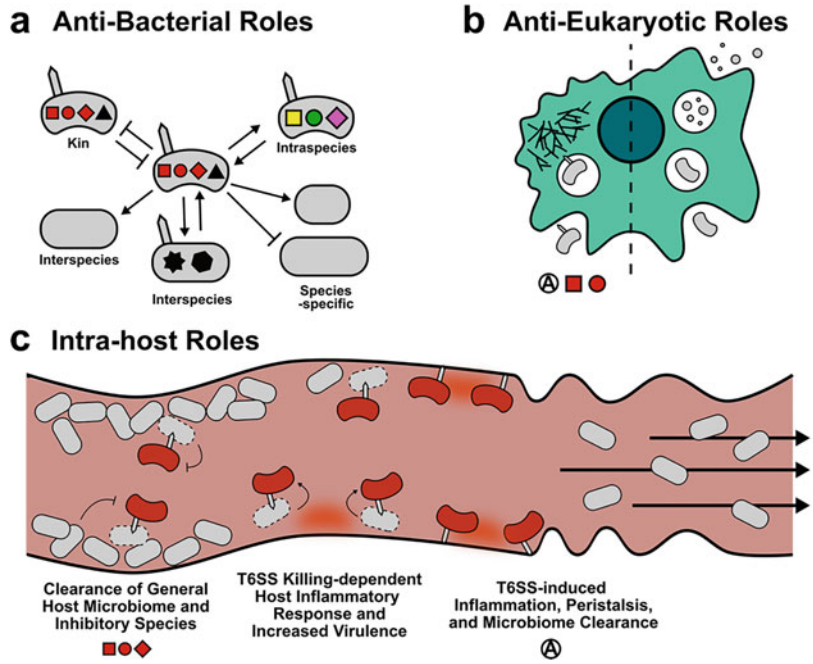


**Fig. 3.6** The various roles of the *V. cholerae* T6SS in competition and pathogenesis. (**a**) Dynamics of interbacterial killing by the *V. cholerae* T6SS. Curved rods indicate *V. cholerae*, and straight rods indicate other gram-negative species. Effector sets are coded according to Fig. 3.4. Black star and hexagon indicate T6SS effectors not found in the *V. cholerae* population. Arrows represent T6SS killing dynamics for each scenario. (**b**) The role of the *V. cholerae* T6SS in protection from protozoan grazing. T6SS-active cells secrete toxic effectors into the predatory eukaryote after endocytosis and trigger actin-crosslinking, cell rounding, and death. T6SS-inactive cells are digested. (**c**) Known roles for the T6SS in the host intestine. Red curved cells indicate *V. cholerae*. Grey rods show commensal bacteria. A red glow means host inflammation. Small blunt-end arrows indicate repression from the host microbiota. Small arrows indicate unknown signal feedback. Large arrows indicate efflux due to peristalsis. (**b–d**) Grey spike indicates active T6SS. Effectors implicated in processes are indicated

broadly extend to all species of grazing amoeba, as the *V. cholerae* T6SS is dispensable for resistance against grazing by the aquatic protozoan *Acanthamoeba castellanii* (Van der Henst et al. 2018). The Large cluster effector VgrG-3 is a peptidoglycan-degrading enzyme and is unlikely to affect eukaryotic cells (Brooks et al. 2013). The majority of anti-eukaryotic studies have used O1 El Tor *V. cholerae* or the O37 strain V52, meaning these studies have focused entirely on the capacity of the A-type effector proteins to inhibit protozoan grazing. One recent study has assessed the inhibition of grazing *D. discoideum* by environmental *V. cholerae* strains with CAE and CEJ effector sets, but the findings of this study focused on the ACD of VgrG-1 (Drebes Dörr and Blokesch 2020). A better understanding of the activity of non-A-type effectors against predatory eukaryotic cells would be of great interest to the field, considering non-A-type effectors are widespread in strains that spend all or most of their time in the aquatic reservoir competing with such cells.

## 3.10 Known Roles of the T6SS in Host Colonization and Virulence

Its antibacterial and anti-eukaryotic activities make the *V. cholerae* T6SS an important system in the host intestinal tract (Fig. 3.6c). Initial studies of the T6SS in the infant mouse infection model focused on its bacteria-targeted and host-targeted functions separately (Ma and Mekalanos 2010; Fu et al. 2013; Bachmann et al. 2015). The ACD of VgrG-1 was the first *V. cholerae* T6SS effector shown to have a role in *V. cholerae* pathogenesis, as T6SS activity and secretion of the VgrG-1-ACD effector led to increased fluid accumulation, induction of innate immune responses, and higher recovered *V. cholerae* cell counts from the mouse intestine (Ma and Mekalanos 2010, p. 2). The role of the VgrG-1-ACD in pathogenesis is likely carried over from protozoan defense mechanisms and fine-tuned for the mammalian gastrointestinal tract. It was later shown in the zebrafish model of *V. cholerae* infection that the VgrG-1-ACD could trigger peristaltic waves in the intestinal muscles leading to the expulsion of the native gut microbiome and facilitating *V. cholerae* colonization, but the exact mechanism of peristalsis and any links to VgrG-1-ACD induced inflammation were not discussed (Logan et al. 2018).

Initial studies of the role of antibacterial activity of the T6SS in the host gut were focused on whether or not the system was activated. These studies used the Wave 1 O1 El Tor strain C6706, which is T6SS-inactive under laboratory conditions. They showed that the T6SS is on and can mediate cross-killing of non-immune *V. cholerae* in the infant mouse and infant rabbit intestines (Fu et al. 2013; Bachmann et al. 2015). The pandemic-associated T6SS effector VgrG-3 is particularly important for interactions between *V. cholerae* strains. A strain carrying a transposon insertion in *tsiV3* was unable to co-colonize the infant rabbit intestine with a wildtype strain of C6706 but was able to co-colonize with a T6SS-deficient strain (Fu et al. 2013). T6SS-dependent cross-killing of non-immune *V. cholerae* and interactions between *V. cholerae* and the local microbiota in the host gastrointestinal tract were shown to be primarily localized to the proximal and middle small intestine and are important for colonization of these regions (Fu et al. 2018). Specific human gut commensal species have been shown to restrict *V. cholerae* colonization (Hsiao et al. 2014; Chen et al. 2022), highlighting the importance of T6SS-dependent clearance of the host gut microbiota (Fig. 3.6c). Outside of mammalian hosts, the *V. cholerae* T6SS has also been shown to significantly modulate the intestinal microbiota of laboratory infected zebrafish (Breen et al. 2021). Later studies in mice and gnotobiotic flies demonstrated a link between microbiome predation via the T6SS, signals from dead prey cells, and the induction of virulence in *V. cholerae* cells (Zhao et al. 2018; Fast et al. 2018) (Fig. 3.6c). In each study, *V. cholerae* was significantly more virulent towards its host after the T6SS-mediated killing of vulnerable, gram-negative commensals. In this three-part system of T6SS-active predator, prey, and host, it was shown that inactivating the T6SS in the

predator or removing the T6SS-vulnerable prey was sufficient to reduce the symptoms of cholera such as inflammation and fluid accumulation (mice) as well as host viability (flies) (Zhao et al. 2018; Fast et al. 2018). In mice, the T6SS-dependent increase in virulence appears to be caused by some link between the death of commensal bacteria and the induction of higher levels of CT and TCP in *V. cholerae* (Zhao et al. 2018). In flies, it was shown that T6SS killing of commensal bacteria by *V. cholerae* triggers polymicrobial interactions within the gut microbiome that result in a lack of cellular differentiation to repair *V. cholerae*-induced damage to the intestinal epithelium (Fast et al. 2020). These studies demonstrate that the role of the T6SS in the host is more complex than simply killing commensal bacteria to allow for colonization and that the T6SS is a virulence factor that drives pathogenesis directly by targeting the host and indirectly by targeting the host microbiota.

## 3.11 Summary

The T6SS is a versatile defense mechanism used by *Vibrio* species and many other gram-negative bacteria to contend with bacterial and eukaryotic competitors in constantly shifting environments. Across *Vibrio* species, this structurally conserved system shows great diversity in its genomic organization, toxic effector repertoire, regulatory circuits, and specific biological purpose. In this chapter, we aimed to summarize the current knowledge surrounding the *Vibrio* T6SS to identify uniting themes, including regulation by common host and environmental signals, evolutionary mechanisms that allow a change in a system under constant selective pressure, the T6SS-dependent and -independent factors that shape competitive dynamics, and roles of the T6SS in pathogenesis across several model organisms. There is still much to learn about the T6SS in *V. cholerae* and the *Vibrio* genus. Due to advancements in sequencing and a concurrent push to sample a greater portion of the microbial world, new T6SS gene clusters that encode diverse effector/immunity proteins are regularly identified. These discoveries will open the door for a deeper understanding of the environmental and pathogenic roles of the T6SS as well as genetically engineered tools for designer protein secretion and new antimicrobial technologies (Liang et al. 2019; Hersch et al. 2021; Jana et al. 2021).

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# 4 Motility Control as a Possible Link Between Quorum Sensing to Surface Attachment in *Vibrio* Species

Sandra Sanchez and Wai-Leung Ng

**Abstract**

In this chapter, we discuss motility control as a possible link between quorum sensing (QS) to surface attachment in *Vibrio* species. QS regulates a variety of behaviors that are important for the life cycle of many bacterial species, including virulence factor production, biofilm formation, or metabolic homeostasis. Therefore, without QS, many species of bacteria cannot survive in their natural environments. Here, we summarize several QS systems in different *Vibrio* species and discuss some of emerging features that suggest QS is intimately connected to motility control. Finally, we speculate the connection between motility and QS is critical for *Vibrio* species to detect solid surfaces for surface attachment.

**Keywords**

*Vibrio* cholerae · Quorum sensing

## 4.1 Introduction to Quorum Sensing

It is now accepted that bacteria live a social life (Parsek and Greenberg 2005). To act collectively as a group, bacterial cells in a population use quorum sensing (QS) to communicate with each other and alter their behaviors in response to changes in density as well as species composition in the vicinity. QS regulates a variety of behaviors that are important for the life cycle of many bacterial species, including bioluminescence, virulence factor production, biofilm formation, metabolic homeostasis, and genetic competence development. Therefore, without QS, many species of bacteria cannot survive in their natural environments. In this chapter, we will summarize several paradigmatic QS systems in different *Vibrio* species and discuss some of emerging features that suggest QS is intimately connected to motility control. We speculate the connection of these two regulatory networks is critical for *Vibrio* species to use QS to detect a solid surface for surface attachment.

## 4.2 QS Systems That Use Acyl Homoserine Lactones for Communication

Several types of QS systems have been identified in *Vibrio* but the general mechanistic steps in responding to changes in cell density are the same across these systems (Lupp et al. 2003; Cao and Meighen 1989; Chen et al. 2002; Miller et al. 2002; Henke and Bassler 2004a; Lenz et al. 2004; Papenfort et al. 2017). Intercellular communication via QS relies on production,

S. Sanchez · W.-L. Ng (✉)
Department of Molecular Biology and Microbiology, School of Medicine, Tufts University, Boston, MA, USA
e-mail: Wai-Leung.Ng@tufts.edu



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secretion, detection, and response of small signaling molecules called autoinducers (**AIs**). AIs are first synthesized intracellularly and then either passively released or actively secreted into the environment. As the number of cells increases in a population, the levels of AIs in the environment also increase proportionally. Once a concentration threshold is reached, binding of AIs to their cognate receptors occurs, triggering a signal transduction cascade that results in changes in gene expression across the population. This cell-cell communication system allows the bacteria cells in the same population to act collectively as a group.

Acyl homoserine lactones (**AHSLs**) represent the first chemical class of autoinducers identified in any bacteria (Nealson et al. 1970; Fuqua et al. 1994; Moré et al. 1996; Parsek and Greenberg 2000; Nealson and Hastings 1979) and AHSL-based QS systems unequivocally are the most studied QS signaling network in Gram-negative bacteria. To date, AHSL is found to be recognized by two types of receptors: the cytoplasmic LuxR-type transcriptional regulator (e.g., in *Vibrio fischeri*) and the transmembrane LuxN-type histidine sensor kinase (e.g., in *Vibrio harveyi*).

## 4.3 LuxI-LuxR Type QS Systems

*Vibrio fischeri* is a bioluminescent marine bacterium that colonizes the light organ of the Hawaiian Bobtail Squid *Euprymna scolopes*. The relationship between the Hawaiian Bobtail squid and *V. fischeri* has been studied for decades as a model of symbiosis (Nyholm and McFall-Ngai 2021). The nutritious environment inside the light organ of the squid allows the bacteria to grow efficiently. In turn, the light (bioluminescence) produced by the bacteria inside the light organ provides counter-illumination and eliminates the squid's shadow at nighttime which helps conceal the squid from predators and preys.

Interestingly, *V. fischeri* activates bioluminescence only at high cell density (**HCD**) via QS. Two proteins are essential for QS control in *V. fischeri*: LuxI is the synthase for the AI *N*-3 (oxo-hexanoyl)- homoserine lactone (3OXOC6HSL) (Nealson et al. 1970; Eberhard et al. 1981) which is small enough to freely diffuse in and out of the cell (Kaplan and Greenberg 1985). Therefore, at low cell density (**LCD**), most extracellular 3OXOC6HSL diffuses away and is diluted in the environment. However, as the density of the bacterial cells increases in the population, the concentration of 3OXOC6HSL accumulates and once a threshold is reached, 3OXOC6HSL re-enters the cell and binds to its cytoplasmic receptor LuxR (Fig. 4.1a). When the LuxR-3OXOC6HSL complex is formed, it recognizes a consensus binding sequence upstream of the *luxICDABE* operon and activates the expression of these genes (Devine et al. 1989; Kaplan and Greenberg 1987; Antunes et al. 2008). The genes *luxCDABE* encode the enzymes for luminescence production and *luxI* is also activated which increases the production of 3OXOC6HSL, acting as a positive feedback loop to ensure all cells switch to HCD gene expression (Devine et al. 1989). QS in *V. fischeri* not only regulates bioluminescence but other activities, including motility, competence, and biofilm formation (Antunes et al. 2007; Qin et al. 2007).

The LuxI/R type of QS system in *V. fischeri* is very common and found in a variety of Gram-negative bacteria including many known pathogens. AHSL-type QS systems are usually highly specific and respond only to the AHSLs produced by the species themselves, suggesting that it is used for intra-species communication (Schuster et al. 2013).

## 4.4 LuxM-LuxN Type AHSL QS Systems

The second type of QS system that uses AHSL as a signal has been thoroughly studied in *Vibrio harveyi* which does not possess the archetypical *luxI* and *luxR* genes. As opposed to the LuxI/R system where the AI receptor is a cytoplasmic DNA-binding transcriptional regulator, the AI receptor in *V. harveyi* is a membrane-bound histidine kinase called LuxN (Bassler et al. 1993).

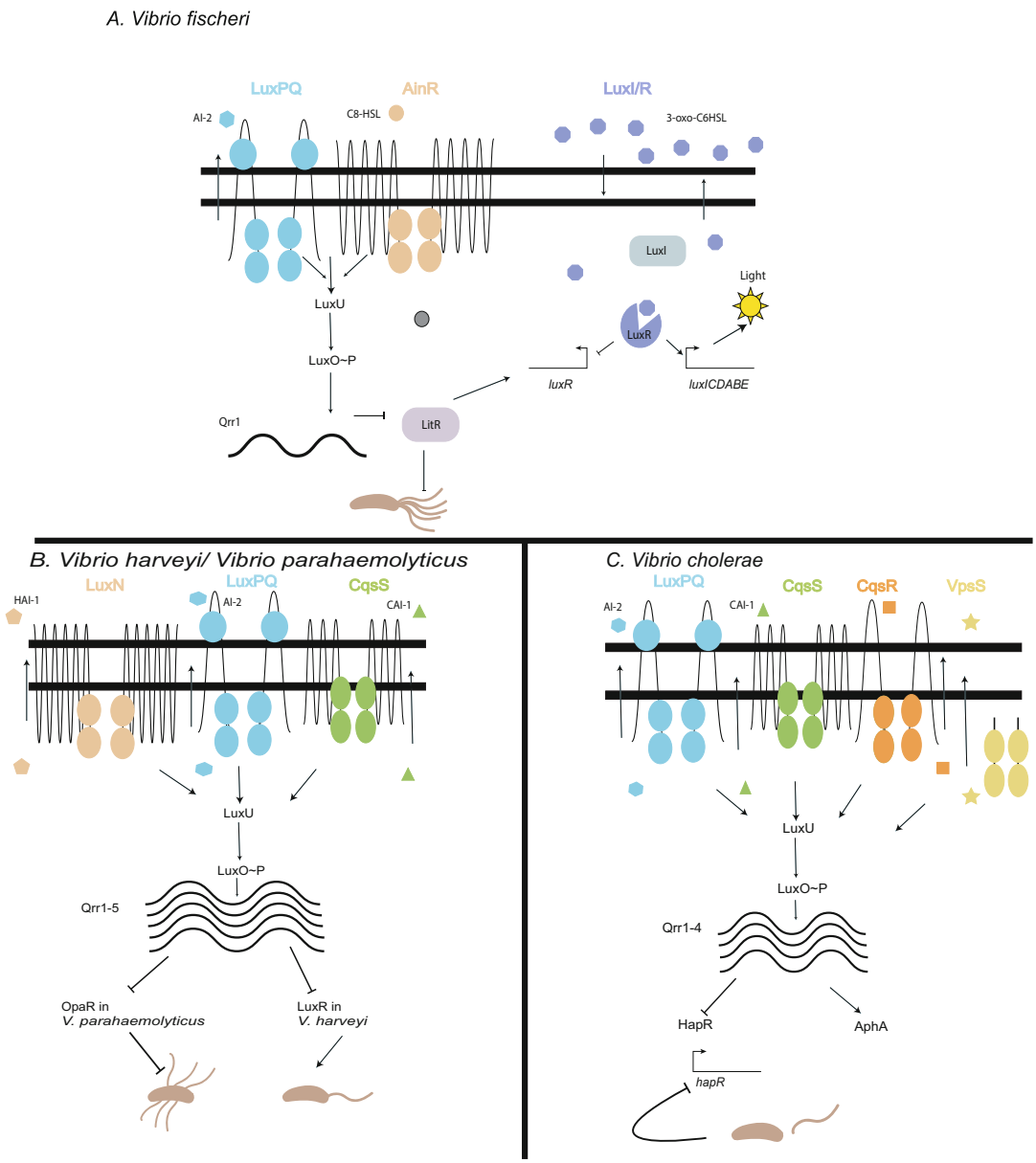


**Fig. 4.1** Quorum sensing pathways and their connection to motility in different *Vibrio* species. (**a**) Three QS systems operate in *V. fischeri*: LuxI-LuxR, AinS-AinR, and LuxS-LuxP/Q. At LCD, QS regulator LuxR is inactive and luciferase operon is not expressed. Furthermore, AinR and LuxP/Q function as kinase to phosphorylate LuxO, which activates the expression of the sRNA Qrr1. Qrr1 prevents the production of LitR, resulting in limited *luxR* gene expression. At HCD, LuxR/3-oxo-C6HSL complex is formed, resulting in activation of the luciferase operon. Accumulation of the two autoinducers recognized by AinR and LuxP/Q results in dephosphorylation of LuxO and increased level of LitR. LitR further activates the transcription of *luxR*. LitR negatively regulates the expression of several flagellar genes. It is hypothesized that the QS is involved in motility repression once the *V. fischeri* cells reach the deeper crypt tissue in the bob tail squid. (**b**) Three QS systems operate in *Vibrio harveyi/Vibrio parahaemolyticus,* CqsA-CqsS, LuxM-LuxN, and LuxS-LuxP/Q. At LCD, QS receptors LuxN, CqsS, and LuxPQ autophosphorylate and transfer the phosphate to LuxO via LuxU. Phosphorylated LuxO activates the transcription of the five regulatory small RNAs: Qrr1-5. These sRNAs destabilize the transcript of the QS master regulator LuxR in *V. harveyi* or OpaR in *V. parahaemolyticus*. In *V. harveyi*, QS positively regulates motility by affecting flagellar gene expression. In *V. parahaemolyticus,* OpaR represses the lateral flagellar genes (thus represses swarming) and induces *cps* expression at HCD. (**c**) Four QS systems operate in *V. cholerae,* CqsA-CqsS, LuxS-

*V. harveyi* is a free-living marine bacterium and is an important pathogen of marine organisms. *V. harveyi* uses QS to activate bioluminescence and repress type III secretion and the QS regulon of *V. harveyi* is estimated to be more than several hundred genes (Waters and Bassler 2006; van Kessel et al. 2013).

Like other Gram-negative QS bacteria, *V. harveyi* produces, secretes, and detects an AHSL autoinducer called HAI-1 (3OHC4HSL) (Cao and Meighen 1989). In addition, two other autoinducers called AI-2 and CAI-1 are produced and detected by *V. harveyi* for QS (Fig. 4.1b) (Henke and Bassler 2004a). HAI-1 is synthesized by the LuxM synthase which shows no significant sequence similarity with members in the LuxI family (Bassler et al. 1993). CAI-1 is synthesized by the CqsA synthase (Miller et al. 2002; Henke and Bassler 2004a; Papenfort et al. 2017; Bassler et al. 1993; Higgins et al. 2007; Ng et al. 2011) and AI-2 is synthesized by LuxS (Surette et al. 1999; Schauder et al. 2001; Bassler et al. 1997). These additional AIs are also detected by membrane-bound histidine kinases (LuxPQ for AI-2 and CqsS for CAI-1) as opposed to the LuxR-type cytoplasmic receptors (Henke and Bassler 2004a; Bassler et al. 1993; Miller and Bassler 2001). At low cell density (**LCD**) when the extracellular concentrations of these autoinducers are low, these receptors, which are hybrid histidine kinases that carry both the histidine kinase domain and an additional receiver domain, are mostly ligand-free and their kinase activities are predominant, resulting in the autophosphorylation of the receptors at a conserved histidine in the kinase domain. The phosphate group is subsequently transferred to the conserved aspartate residue of the receiver domain in the same receptor. The phosphate group from all three receptors is then passed onto a histidine transfer protein called LuxU, which ultimately transfers the phosphate to a master response regulator called LuxO (Freeman and Bassler 1999; Henke and Bassler 2004b).

Together with RNA polymerase/Sigma 54 complex, phosphorylated LuxO (LuxO-P) activates transcription of five small regulatory RNAs (sRNAs) Qrr1-5 (Lenz et al. 2004). The main target of these sRNAs is the mRNA encoding the QS master transcriptional regulator LuxR (which is different from the *V. fischeri* LuxR). At LCD, these sRNAs block the ribosome binding site and destabilize the *luxR* mRNA and prevent the production of LuxR (Lenz et al. 2004). At HCD, AI binding inhibits the kinase activity of these receptors, resulting in dephosphorylation and inactivation of LuxO, therefore Qrr1-5 sRNAs are not made and *luxR* mRNA is translated and LuxR is made. LuxR functions both as an activator and repressor to regulate over several hundred genes (van Kessel et al. 2013). All of these QS components are identified in the human pathogen *Vibrio parahaemolyticus,* and a similar QS circuit has been proposed to operate in this bacterium. However, recent study shows that expression of small RNA Qrr2 is independent of Sigma 54 and this sRNA can function alone to regulate QS. Even with this singular exception, at LCD, the master QS regulator OpaR (homolog of LuxR in *V. harveyi*) is not made (Tague et al. 2022). At HCD, in contrast, OpaR is made and it induces the expression of genes required for extracellular polysaccharide production and represses the expression of one of the two Type III secretion systems (Henke and Bassler 2004b; McCarter 1998; Gode-Potratz and McCarter 2011).

**Fig. 4.1** (continued) LuxP/Q, CqsR, and VpsS. The autoinducers for CqsR and VpsS are unknown but their activity can be modulated by ethanolamine and nitric oxide, respectively. At LCD, kinase activities of CqsS, LuxPQ, VpsS, and CqsR are dominant. LuxO is phosphorylated via LuxU and promotes the trascription of the four small RNAs, Qrr1-4, which activate translation of AphA and inhibit production of HapR. As cells reach the small intestine and travel through the mucus layer, it is hypothesized that the flagella are lost which allows for secretion of FlgM (anti-sigma factor) resulting in an increased activity of FliA (the alternative sigma factor for late flagellar genes). With mechanism currently unkown, FliA further represses *hapR* trasncription. This ensures that the small number of cells that reach the small intestine are primed for virulence factor production and colonization

The presence of multiple QS systems within a single cell is not uncommon. Indeed, there are two other histidine kinase-based QS systems that indirectly control bioluminescence in *V. fischeri* by modulating *luxR* expression*:* AinS and AinR, which respectively produces and responds to N-octanoyl-homoserine lactone (C8-HSL), are homologous to LuxM and LuxN in *V. harveyi; V. fischeri* also possesses LuxS and LuxPQ, which respectively synthesizes and responds to AI-2 (Fig. 4.1a). These two additional QS systems are both used to regulate the activity of LuxO in *V. fischeri*. At LCD, LuxO-P activates transcription of *qrr1*, which encodes a single sRNA that prevents the production of the transcription factor LitR (Homologous to LuxR in *V. harveyi* and OpaR in *V. parahaemolyticus*) (Fig. 4.1a). At HCD, the phosphorelay is halted which allows the production of LitR to enhance *luxR* expression, further contributing to increased light production (Lupp and Ruby 2005).

## 4.5 Non-AHSL Type QS in *Vibrio cholerae*

The organization of the *V. cholerae* QS pathway is similar to that of *V. harveyi* but with several distinctions. First, *V. cholerae* does not make HAI-1 and does not have the LuxN sensor; instead, the *V. cholerae* QS system is composed of four signaling pathways (CqsS, LuxPQ, CqsR, and VpsS) (Fig. 4.1c) (Miller et al. 2002; Jung et al. 2016). Second, there are 4 Qrr sRNAs in *V. cholerae* but each of the Qrr sRNA appears to be sufficient to mediate a full QS response (Lenz et al. 2004). Third, even though the *V. cholerae* CqsS/CqsA system is homologous to that from *V. harvey*i, the *V. cholerae* system is less specific and responds to both CAI-1 with either C8 or C10 hydrocarbon tails, while the *V. harveyi* system only responds to CAI-1 with a C8 tail (Ng et al. 2011; Miller and Bassler 2001). LuxPQ in *V. cholerae* also responds to AI-2, but the identity of the AIs sensed by VpsS and CqsR is unknown. Interestingly, the activity of VpsS and CqsR can be modulated by nitric oxide and ethanolamine, respectively (Hossain et al. 2018; Watve et al. 2020). Finally, the functional homolog of *V. harveyi* LuxR is called HapR in *V. cholerae.* HapR functions both as transcriptional repressor and activator of many genes. At LCD, Qrr1-4 sRNAs facilitate the production of AphA, which are essential for expression of biofilm and virulence genes (Lenz et al. 2004; Rutherford et al. 2011). In contrast, at HCD, HapR activates the gene *hapA* that encodes the Hap protease and many genes that are needed for genetic competence (Zhu et al. 2002; Hammer and Bassler 2003; Lo Scrudato and Blokesch 2013).

A new class of autoinducer, called DPO, was identified in *V. cholerae* and some other *Vibrio* species. DPO is produced by the enzyme Tdh and is sensed by a cytoplasmic transcriptional regulator called VqmA. VqmA/DPO complex activates transcription of an sRNA called VqmR and is involved in regulation of biofilm formation (Papenfort et al. 2017). Interesting, some phages also encode VqmA; and it is proposed that these phages use their VqmA receptor to detect the DPO produced by the bacterial host to control its lytic-lysogeny switch (Silpe and Bassler 2019).

## 4.6 The Link Between QS and Motility in *Vibrio* Species

Although QS was first described in *V. fischeri* and *V. harveyi* to regulate bioluminescence (Nealson et al. 1970; Bassler et al. 1994), as discussed above, another very common process that is controlled by QS is surface attachment. As part of their life cycle, bacteria are required and capable to attach to a variety of solid surfaces, including eukaryotic cells, other bacteria, plants, and other abiotic surfaces (Tuson and Weibel 2013; Verstraeten et al. 2008; O'Toole and Wong 2016; López et al. 2010). QS communication has been identified as one of the main pathways that regulates the production of adhesion factors necessary for attachment to various surfaces (e.g., host adhesins, exopolysaccharides, biosurfactants, etc.) in both Gram-negative and Gram-positive bacteria (reviewed in Parsek and Greenberg 2000; Rutherford and Bassler 2012;

Koutsoudis et al. 2006; Labbate et al. 2007). However, for bacteria to switch between a motile to a sessile life, the cell has to sense the presence of a surface. It has been shown in several organisms that the flagellar apparatus not only allows the cell to attach to the surface, but the flagella, or in turn motility, can also act as a sensor to alert the cell it has reached a surface (O'Toole and Wong 2016; Belas 2014; Laventie and Jenal 2020).

Thus, connecting QS and motility/flagellar synthesis could function as a potential mechanism for integrating spatial cues from surface sensing into QS to precisely regulate surface attachment. Indeed, motility has been shown to be connected to QS in *V. cholerae*, *V. fischeri*, *V. alginolyticus* and *V. parahaemolyticus,* where it appears that QS represses motility in these species (Butler and Camilli 2004; Millikan and Ruby 2004; Tian et al. 2008; Gode-Potratz et al. 2011). Interestingly, motility appears to be positively regulated by QS in *V. harveyi*. In the following sections, we discuss the current knowledge of the connection between QS and motility and how these two cellular processes are wired together differently across various *Vibrio* species.

## 4.7 *Vibrio fischeri* QS and Motility Control

As described earlier, *V. fischeri* forms beneficial symbioses in various marine animals (Nyholm and McFall-Ngai 2021). Motility is one of the essential factors that allows the bacteria to initiate symbiosis and successfully colonize the host (Millikan and Ruby 2004; Graf et al. 1994; Millikan and Ruby 2002, 2003). Motility in *V. fischeri* is achieved by a tuft of 2–7 polar sheathed flagella which allow the cells to swim (Allen and Baumann 1971). Details of the structure, function, and regulation of each flagellar components have been reviewed in other chapters of this book. In brief, the flagellum is composed of three major structural components: the basal body, the hook, and the filament. The Ain QS system appears to negatively regulate flagellar genes including several flagellin genes as well as some flagellar basal body genes (Lupp and Ruby 2005). It is hypothesized that although motility is required for the cells to reach the light organ of the squid, once they have reached the deeper end of the crypt, flagella biosynthesis is repressed by QS. Repression of flagellar synthesis inside the host may allow the bacteria to evade the attack by the host immune system (Aschtgen et al. 2019). Moreover, while host colonization studies have been traditionally performed with strain ES114, another strain called KB2B1 outcompetes ES114 for colonization and exhibits a slower migration pattern (Dial et al. 2021). It was recently found that LitR inhibits motility in KB2B1, since a *litR* and other LCD-locked QS mutants showed decreased motility (Dial et al. 2021). It is unclear how exactly the *V. fischeri* QS system represses the flagellar synthesis and motility; however, QS could function as an important pathway to ensure that motility is coordinated during colonization of the host light organ.

## 4.8 *Vibrio harveyi* QS and Flagellum Synthesis Regulation

*Vibrios* belonging to the *harveyi* clade are among the major pathogens of aquatic organisms which include fish, crustaceans and mollusks (Austin and Zhang 2006). *V. harveyi* pathogenicity requires biofilm formation, swimming motility and production of virulence factors (Austin and Zhang 2006). Virulence factor production has been shown to be negatively regulated by QS in most *Vibrio* species, but motility appears to be positively regulated in *V. harveyi*. Genetic analyses showed that LCD QS-locked mutants (e.g., mutants defective in autoinducer production or *luxR* deletion mutant) display decreased motility in *V. harveyi* compared to that of wild type. In contrast, mutation in the LuxO response regulator, resulting in a genetically locked HCD QS state, increases motility rates than that of wild type which suggests positive regulation of motility by QS (Yang and Defoirdt 2015).

The effect of QS on *V. harveyi* motility may be due to its role in flagellar gene expression. *V. harveyi* possesses a dual flagellar system that allows cells to move under different environments. The single polar flagellum is used to swim in liquid media, whereas the lateral flagellar system is responsible for movement in viscous environments. Over 50 genes are required for construction of the polar flagellum and 30 for the lateral flagella (Yang and Defoirdt 2015). Like many bacterial species, in many *Vibrio* species, the flagellum is assembled in a hierarchical manner and there is a temporal order of expression of three classes of flagellar genes: early, middle, and late flagellar genes (Echazarreta and Klose 2019; Kutsukake et al. 1990; Jones and Macnab 1990). In *V. harveyi*, transcription of middle and late flagellar structural genes is higher in a QS overexpressing strain than that of the wild type. QS appears to regulate not only these flagellar structural genes but also that of *flaK*, a gene that encodes the master regulator of flagellar genes. Expression levels of *flaK* are lower in a *luxR* deletion mutant than that of wild type (Yang and Defoirdt 2015). In contrast to what is previously found, it is shown that some *V. harveyi luxS* mutants, presumably producing less LuxR (Fig. 4.1b), display better swimming and swarming motility (Zhang et al. 2021). These mutants also produce more lateral flagella and have high levels of transcripts of both polar and lateral flagellar genes (Zhang et al. 2021). Further studies are required to identify the molecular link between QS and motility in this bacterium.

## 4.9 *Vibrio parahaemolyticus* QS and Swarming

*V. parahaemolyticus* is a leading worldwide cause of seafood-borne gastroenteritis. This organism encodes a variety of potential virulence factors, including proteases, hemolysins, two type VI secretion systems T6SS1 and T6SS2 and two type III secretion systems, T3SS1 and T3SS2 (Letchumanan et al. 2014). This organism has a high ability to colonize a surface due to its vigorous capacity to swarm and form robust biofilms.

Genetic alteration in the QS master regulator OpaR (homologs of LuxR in *V. harveyi* and HapR in *V. cholerae*) leads to two different colony morphologies, opaque (OP) and translucent (TR). The capsular polysaccharide (CPS) determines colony opacity and stickiness. OP isolates with the wild-type allele of *opaR* produce thick capsule that does not swarm. In contrast, TR isolates with defective OpaR are less sticky and are swarming proficient. The associated phenotypes with these two morphotypes are due to repression of the expression of the lateral flagellar genes and activation of *cps* expression by OpaR. OpaR has been shown to bind to the promoter regions of several operons involved in motility (Lu et al. 2019, 2021). In addition, OpaR also regulates directly or indirectly about 5.2% of the genome of *V. parahaemolyticus* including the surface sensing regulon (Kernell Burke et al. 2015). While swarming is considered a group behavior in most other organisms, it is interesting that in *V. parahaemolyticus* swarming is initiated at LCD where OpaR is not made (Gode-Potratz and McCarter 2011). Thus, the physiological roles of QS in the regulation of motile/sessile lifestyle in *V. parahaemolyticus* need additional investigation.

## 4.10 Reciprocal Control of *Vibrio cholerae* QS by Motility

*Vibrio cholerae* is the causative agent of the diarrheal disease cholera, it can lead to severe dehydration and result in death if left untreated (Faruque et al. 1998). *V. cholerae* is a highly motile organism that uses its single polar flagellum to swim (McCarter 2004; Butler and Camilli 2005). Motility has been shown to be important for host colonization and infection (Lee et al. 2001). After passage through the stomach, cells need to transit from the lumen of the small intestine to the mucosal layer of the epithelium surface for successful colonization (Lee et al. 2001; Almagro-Moreno et al. 2015; Liu et al. 2008). The current model suggests that LuxO activation and AphA production (Fig. 4.1c) is critical for *V. cholerae* to activate virulence gene expression

at LCD when it first initiates colonization of the small intestine. In contrast, the pathogen uses QS master regulator HapR at HCD to shut down virulence expression to exit the host to restart its aquatic life cycle (Miller et al. 2002; Jung et al. 2016; Watve et al. 2020; Zhu et al. 2002; Jung et al. 2015).

While the studies discussed above suggest that QS can either directly and indirectly control motility in different *Vibrio* species, previous studies in *V. cholerae* elucidated a reciprocal connection between motility and QS in which flagellar synthesis intimately controls QS via the regulation of *hapR* expression (Liu et al. 2008). The flagellar rod is made up of several proteins including FlgD. Unexpectedly, deletion of *flgD* gene results in a decreased level of *hapR* transcription (Liu et al. 2008). It was further shown that mutants with defective FlgD secret an increased amount of FlgM to the extracellular milieu. FlgM is the anti-sigma factor that physically interacts with FliA (Sigma 28), the alternative sigma factor that controls expression of the late flagellar genes. Thus, the *flgD* mutant has an increased intracellular activity of FliA. Interestingly, deletion of *fliA* in the *flgD* mutant restores HapR production, suggesting *hapR* repression in the *flgD* mutant is due to increased activity of FliA (Liu et al. 2008). While it is evident that there is a link between FliA and QS, it is unknown if the repression of *hapR* expression is due to FliA directly or indirectly. Regardless of the exact mechanism, it is suggested *V. cholerae* cells lose their polar flagellum upon migrating through the mucus layer during the initial stage of host colonization, and therefore this mechanism ensures full repression of *hapR* to allow for proper colonization by activating the virulence gene expression program (Liu et al. 2008).

## 4.11 Conclusion and Outlook

While the interconnection between motility and QS has been studied for over a couple decades, the exact mechanism by which these two processes link together is still poorly understood. Moreover, the physiological significance of the connection of these two networks is poorly defined. We speculate that how these two processes are connected could be variable in different *Vibrio* species and are mostly likely influenced by their specific niches. For example, some species may use QS to repress motility to allow successful colonization of the host as in *V. fischeri* and *V. cholerae*, while others such as *V. harveyi* may use QS to promote motility for foraging to overcome nutrient limitation. From a mechanistic standpoint, are flagellar/motility genes regulated by QS using a universal mechanism? What is the connection between QS and the hierarchical flagellar synthesis program controlled by regulators such as FlrA, FlrB, and FliA? Interestingly, certain *Vibrio* species deviates their polar flagellar transcriptional regulatory network from the canonical hierarchical model (Simpson et al. 2021). Investigation into the flagellar structure and how it might interact with QS is needed to answer these questions. The genus *Vibrio* contains many of the main pathogens to fish, shellfish, and humans. They use their flagella to access their preferred colonization niches and use QS to precisely regulate the temporal dynamic of virulence gene expression, we believe that understanding the connection between QS and motility can potentially lead to new therapeutic strategies.

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# The *Vibrio* Polar Flagellum: Structure and Regulation

5

Cameron J. Lloyd and Karl E. Klose

**Abstract**

Here we discuss the structure and regulation of the *Vibrio* flagellum and its role in the virulence of pathogenic species. We will cover some of the novel insights into the structure of this nanomachine that have recently been enabled by cryoelectron tomography. We will also highlight the recent genetic studies that have increased our understanding in flagellar synthesis specifically at the bacterial cell pole, temporal regulation of flagellar genes, and how the flagellum enables directional motility through Run-Reverse-Flick cycles.

**Keywords**

*Vibrio* flagellum · Flagellar structure · Flagellar synthesis

Vibrios are Gram-negative marine bacteria with characteristic curve-shaped bodies. Also characteristic of this genus is their motility, which is typically achieved by rotation of flagella. Most Vibrios have a flagellum (or flagella) at a single pole (polar flagellum) which is used for swimming in liquid environments. Some Vibrios additionally synthesize multiple peritrichous flagella (lateral flagella) under certain conditions which are mostly used for swarming motility on solid surfaces.

Flagellar motility is integral to the *Vibrio* lifestyle. Vibrios are ubiquitous in the marine environment, which is a relatively nutrient-poor environment where chemotactic motility facilitates access to nutrients. Moreover, motility contributes to the ability of Vibrios to colonize various surfaces and persist in the environment. There are many different *Vibrio* species found in the marine environment, and several of these are known to cause disease in humans, for example *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus,* and *V. alginolyticus.* Additional Vibrios cause disease in various marine hosts, such as shrimp, fish, and corals (e.g., *V. campbellii, V. anguillarum,* and *V. coralliilyticus*). Some Vibrios also form close symbiotic relationships with their host, most notably *V. fischeri* with the bobtail squid. In all of the *Vibrio* species mentioned here, polar flagellar motility has been shown to play a role in interactions with their host. For example, non-motile *V. fischeri* are unable to colonize squid (Graf et al. 1994), and non-motile *V. vulnificus* are defective for adherence, biofilm formation, and virulence in mice (Lee et al. 2004). In fact, the basis of immunity against cholera provided by anti-O Antigen (OAg) antibodies against *V. cholerae* is their ability to prevent the bacteria from swimming, due to the antibodies binding the OAg in the

C. J. Lloyd · K. E. Klose (✉)
South Texas Center for Emerging Infectious Diseases, University of Texas San Antonio, San Antonio, TX, USA

Department of Molecular Microbiology and Immunology, University of Texas San Antonio, San Antonio, TX, USA
e-mail: Karl.Klose@utsa.edu



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flagellar sheath (Wang et al. 2017; Bishop et al. 2010; Charles et al. 2020). Additionally, flagellar-based motility enables resistance to predators, e.g., *Bdellovibrio* spp. (Duncan et al. 2018), and facilitates the initial steps of biofilm formation (Utada et al. 2014). Moreover, the flagellum is a Type 3 secretion system that is used to deliver non-flagellar proteins, such as cytotoxins (2008) and biofilm matrix proteins (Jung et al. 2019), and its rotation creates outer membrane vesicles that are used to signal to hosts (Aschtgen et al. 2016).

This review will focus on the polar flagellum (or flagella) of *Vibrio* species. Tremendous insight into the structure of this nanomachine has recently been enabled by cryoelectron tomography (cryoET). Moreover detailed genetic studies have enlightened our understanding of how the flagellum is synthesized specifically at the bacterial cell pole, how the flagellar genes are temporally regulated to maximize efficiency, and how the polar flagellum enables directional motility through Run-Reverse-Flick cycles. Chemotaxis controls flagellar rotation and is critical to drive directional motility; great strides have also been achieved in dissecting the *Vibrio* chemotaxis system. However chemotaxis will not be discussed and instead readers are directed to an excellent recent review of this topic (Ortega et al. 2020). Likewise the contributions of flagellar motility to biofilm formation (Teschler et al. 2015), virulence (Echazarreta and Klose 2019), and *V. fischeri* symbiosis (Aschtgen et al. 2019) have been recently covered in comprehensive reviews.

## 5.1 Flagellar Structure

The structure of the *Vibrio* polar flagellum is divided into three main parts: the basal body, the hook, and the filament (Fig. 5.1). The basal body spans from the cytoplasm inside the cell, across the cytoplasmic membrane and periplasmic space, to the hook on the outer membrane. The hook is the flexible linker that connects the basal body to the filament (that mediates the "flick" during reversal of flagellar rotation than reorients the cell, as discussed below). The filament is composed of flagellin subunits and is the largest part of the flagellum, whose rotation propels the bacterium. Unique to *Vibrio*s and a few other bacteria, the hook and filament are coated by a layer of membrane referred to as a sheath; this is an extension of the outer membrane resulting in LPS coating the entire filament (Fuerst and Perry 1988; Chu et al. 2020). The basal body is anchored in the cytoplasmic membrane by the MS ring and is associated with the C ring within the cytoplasm, which is involved in torque generation and directional switching. Within the basal body, the rod passes from the MS ring in the inner membrane through the P ring within the periplasmic space (associated with the ***P***eptidoglycan layer), and the L ring associated with the outer membrane (normally associated with ***L***ipopolysaccharide in other Gram-negative bacteria). *Vibrio* spp have two additional ring structures within the flagellar basal body, the T ring and the H ring, which sandwich the P ring within the periplasm. The core of the basal body, hook, and filament is hollow, because this is the conduit through which nascent components of the flagellum are secreted, to allow the flagellum to be built at the distal end of this tube in a step-wise fashion. Entrance of components into this hollow core is controlled by a secretion apparatus related to Type III secretion systems that sits inside the C ring within the cytoplasm (Chen et al. 2011). The stator surrounds the basal body structure within the periplasm, extending to the C ring in the cytoplasm, and facilitates rotation of the entire flagellum via the $Na^+$ gradient (Fig. 5.1).

**Outer Membrane Complex** Exquisite detail of the *in situ* structural components of the *Vibrio* basal body has been achieved through cryoelectron tomography, mostly of the *V. alginolyticus* flagellum, but also including imaging of *V. fischeri* and *V. cholerae* flagella (Fig. 5.2). The flagellar sheath can be seen in tomograms as an extension of the outer membrane that coats the hook and the filament (Fig. 5.2a, b). A structure referred to as the "O ring" can be seen at the base of the flagellum on the external side of the membrane that is perhaps involved in sheath formation; it is unclear which

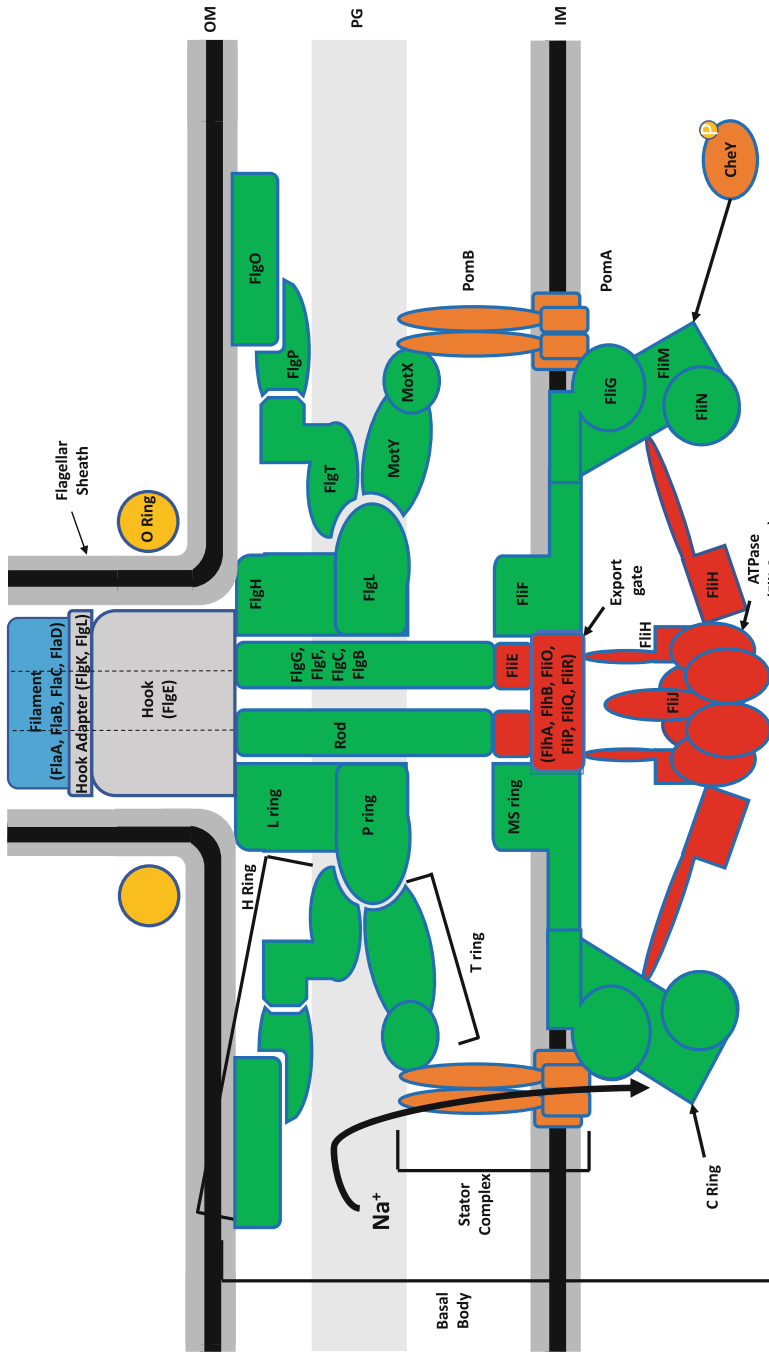


**Fig. 5.1** Schematic representation of the *Vibrio* polar flagellum

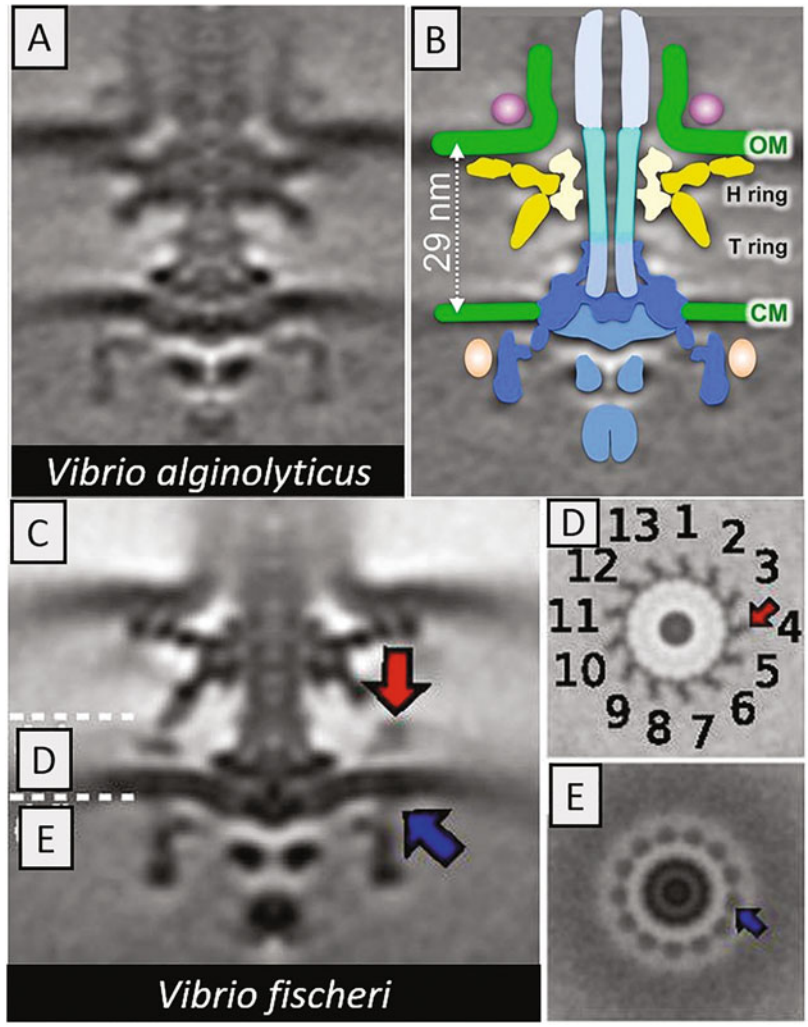


**Fig. 5.2** The flagellar complex of *V. alginolyticus* and *V. fischeri*. The flagellar complex was imaged using cryoelectron tomography (Cryo-ET) combined with genetic subtomogram analysis (Beeby et al. 2016; Zhu et al. 2017). Final reconstructed images were generated via superimposed density maps. (**a**) and (**b**) from Zhu et al. (2017). (**a**) Electron microscopy rendering of the sheathed *V. alginolyticus* flagellar complex. (**b**) Schematic of the sheathed *V. alginolyticus* flagellar complex, *Vibrio*-specific attributes are depicted by colors: the sheath (green), H and T rings (yellow) and the O ring (purple). (**c**–**e**) from Beeby et al. (2016). The stator complex of *V. fischeri* surrounding the flagellar rotor exhibits 13-fold symmetry. Red arrow highlights position of MotB, while blue arrow highlights FliG-MotA interface. The planes for D and E are shown by dotted lines in C. Images reproduced and modified with permission from the *Proceedings of the National Academy of Sciences USA*

protein(s) constitute the O ring. The L ring, composed of FlgH, occupies a space close to the outer membrane, but it does not appear to puncture it, as the equivalent protein does in other Gram-negative bacteria (Cohen and Hughes 2014; Zhu et al. 2020). The H ring lies underneath the L ring and extends outward to make contact with the periplasmic side of the outer membrane.

The H ring (Fig. 5.2a, b) is composed of three proteins: FlgO, FlgP, and FlgT (Zhu et al. 2018). FlgO is located in the distal portion of the H ring that contacts the underside of the outer

membrane, while FlgT is located in the proximal portion of the H ring next to the basal body, and FlgP appears to be a bridge between FlgO and FlgT (Beeby et al. 2016). Based on the crystal structure of FlgT (Terashima et al. 2013) there are 13 FlgT subunits within the H ring (Zhu et al. 2017). Tomograms reveal that a *flgT* mutant lacks the entire H ring; *Vibrio* strains lacking *flgT* appear non-motile (Cameron et al. 2008; Terashima et al. 2013; Martinez et al. 2010) despite synthesizing a flagellum. The flagella of *flgT* strains have been reported to be released into the medium due to weaker attachment to the cells (Martinez et al. 2010) or localized in the periplasmic space due to poor penetration of the outer membrane (Zhu et al. 2018). FlgP is a lipoprotein that is associated with the outer membrane (Morris et al. 2008), although lipidation does not appear to be critical for its role in motility. Cells lacking *flgP* are also non-motile yet flagellated (Cameron et al. 2008; Morris et al. 2008; Martinez et al. 2010), and tomograms reveal that a *flgP* mutant lacks the outer portion of the H ring (presumably both FlgP and FlgO) (Beeby et al. 2016). Interestingly, *V. cholerae* strains lacking *flgO* are still motile, although exhibiting decreased motility (Martinez et al. 2009; Petersen et al. 2021), indicating that lack of FlgO is less detrimental to motility than lack of FlgP or FlgT. The flagella of *flgO* and *flgP* cells are shorter, but only in the presence of a functional motor, which suggests that rotation of the flagellum in the absence of FlgO or FlgP results in shorter flagella being synthesized (Martinez et al. 2009). However an alternate interpretation might be that the flagella are shorter on average because of the instability of the flagellum leading to release of flagella into the supernatant and constant regrowth of new flagella. The P ring, composed of FlgI, lies underneath the L and H rings and is presumed to contact the peptidoglycan layer (Hizukuri et al. 2008) as well as make contact with the rod, the H ring above, and the T ring.

**The T Ring and Stators** The T ring (Fig. 5.2a, b) is a structure unique to the *Vibrio* flagellum, composed of proteins MotY and MotX (Zhu et al. 2017; Terashima et al. 2006). MotY makes up the bulk of the T ring, and based on the MotY crystal structure (Kojima et al. 2008), there are 13 MotY subunits within the T ring. MotX is located at the tip of MotY and involved in stator assembly and function. The $Na^+$ gradient across the cytoplasmic membrane drives flagellar rotation in *Vibrio* spp (Atsumi et al. 1992), (unlike the $H^+$ gradient that drives flagellar rotation in other bacteria), and MotX and MotY are essential components of *Vibrio* flagellar rotation, along with homologues of the motor proteins MotA and MotB, referred to as PomA and PomB in *Vibrio*s (Li et al. 2011). The T ring appears to provide the scaffold that recruits these stator units (PomAB) to assemble around the flagellum (Zhu et al. 2017). PomA and PomB form a 4A:2B complex in the cytoplasmic membrane that has sodium conducting activity (Sato and Homma 2000). PomA has four transmembrane domains and a large cytoplasmic domain that interacts with FliG in the C ring, (Asai et al. 1997), while PomB has a single transmembrane segment and a periplasmic peptidoglycan binding motif that anchors it to the cell wall. The transmembrane segments form the channel that facilitates $Na^+$ flux through the membrane that generates the torque required to rotate the C ring (Li et al. 2011). PomA and PomB are the stator units that dynamically assemble around the basal body to facilitate rotation of the flagellum. Visualization of the stator complex by cryo ET has been challenging due to the dynamic nature of this structure, but based on electron densities, a model of the *Vibrio* flagellar motor containing 13 stator units has been developed (Fig 5.2c–e), in symmetry with the 13 MotYX components of the T ring (Zhu et al. 2017; Beeby et al. 2016)

**MS and C Ring** The MS ring, composed of FliF, anchors the flagellum in the cytoplasmic membrane, and FliF is the first component of the flagellum assembled (see below). The MS ring is composed of two distinct substructures, the M ring embedded in the membrane and the S ring which lies above it in the periplasm (Kojima et al. 2021). 34 subunits of FliF form the MS ring, and a combination of cryoET and crystal structure

analyses of the *S. enterica* MS ring revealed 34-fold symmetry within the S ring, whereas substructures within the M ring lead to an inner ring of 23-fold symmetry surrounded by cogs with 11-fold symmetry, due to two distinct orientations of two domains within this portion of FliF (Takekawa et al. 2021a; Kawamoto et al. 2021). This alternate symmetry is postulated to correctly accommodate the export apparatus that sits within the MS ring, while still maintaining the 34-fold symmetry for interaction with the C ring within the cytoplasm, which is known to exhibit 34-fold symmetry. Although the exact structure of the *Vibrio* MS ring has not been solved, it is presumed to have a similar structure to the *S. enterica* MS ring (Kojima et al. 2021).

The MS ring is not only the platform for building the flagellum through the export apparatus embedded within, it also is connected to the C ring in the cytoplasm, which interacts with the PomAB stator to rotate the flagellum (Fig. 5.2c–e). The C ring is also responsible for switching the direction of flagellar rotation, and is composed of FliG, FliM, and FliN (Terashima et al. 2020). The C-terminus of FliF interacts with the N-terminus of FliG, to form the C ring below the MS ring (Ogawa et al. 2015). FliG interacts with the stator complex (PomAB) localized in the membrane surrounding the MS ring. The middle domain of FliM binds to FliG, and the C-terminus binds FliN, and importantly the phosphorylated form of the chemotaxis protein CheY (CheY-P) binds the N-terminus of FliM to change the direction of rotation (Takekawa et al. 2021b). Elegant cryoET studies utilizing directionally locked mutant forms of C ring proteins have illuminated how CheY-P binding to FliM causes structural remodeling of the C ring (Carroll et al. 2020). The large conformational rearrangement of the rotor causes FliG to interact differently with the stator complex, causing the flagellum to change from counterclockwise (CCW) to clockwise (CW) rotation. The precise mechanism of the directional switch caused by CheY-P binding to FliM was seen in the *Borrelia burgdorferi* rotor-stator interaction, in which the conformational change in FliG allows it to interact with the opposite side of the stator protein MotA (PomA), thus leading to a change in direction of rotation (CCW>CW) (Chang et al. 2020) ; this mechanism likely pertains to flagellar directional switching in other bacteria, including Vibrios.

**Flagellar Export Machinery** The type III flagellar export apparatus secretes the components of the rod-hook-filament through the MS ring. FlhA, FlhB, FliP, FliQ, and FliR are membrane proteins that interact with each other and form a complex in the central pore of the MS ring that is called the export gate (Minamino et al. 2020). Cryo ET analyses of the *V. mimicus* export gate indicate that $FliP_5Q_4R_1$ forms a complex that is embedded within the MS ring above the cytoplasmic membrane, and FlhB wraps around this complex, including covering the entrance to the pore on the cytoplasmic side; it is proposed that this allows for conformational changes in FlhB to trigger opening of the export gate (Kuhlen et al. 2020) . FlhA (from *V. parahaemolyticus* lateral flagella) forms a nine-member ring directly underneath the FlhBFliPQR complex with a large cytoplasmic portion that is predicted to switch between a closed and open state, and a transmembrane portion that is thought to conduct protons to energize secretion (Kuhlen et al. 2021). FliH, FliI, and FliJ form a ring complex below the export gate (Minamino et al. 2020). In *S. enterica*, it has been shown that FliI is an ATPase that forms a hexamer ring, and FliJ binds to the center of this ring, while FliH binds to this ring and also to FliN in the C ring and FlhA in the export gate, thus aligning the ATPase ring in the cytoplasm below the export gate (Fig. 5.1). ATP hydrolysis by FliI activates the export gate through an interaction of FliJ with FlhA, and FliI with FlhB, and the complex couples protein export to an inward flow of $H^+$.(Minamino et al. 2011, 2014, 2016; Kinoshita et al. 2021; Morimoto et al. 2016). These observations have led to the "ignition key" mechanism for flagellar export in *Salmonella*, in which ATP hydrolysis is used to activate the export gate, but the membrane potential is used to export the flagellar proteins (Fig. 5.3) (Minamino et al. 2014); this mechanism is likely

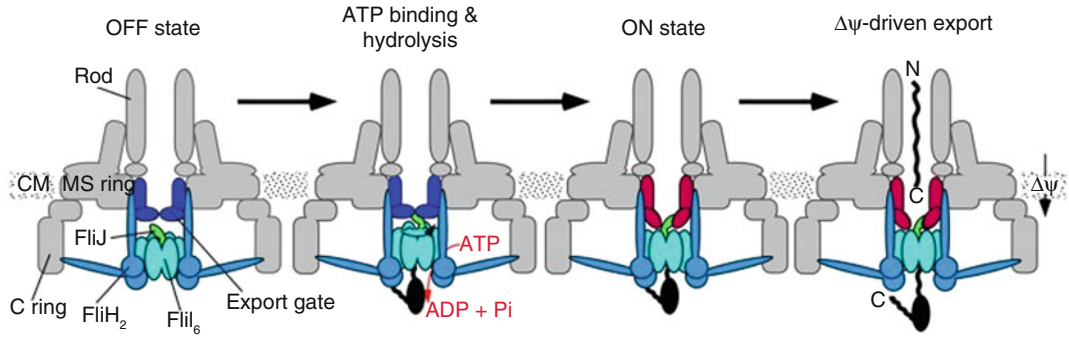


**Fig. 5.3** Model for "ignition key" mechanism of flagellar protein export. Reproduced with permission from Minamino et al. (2014). ATP hydrolysis by the $FliI_6$ ring induces the FliJ rotation within the ring to cause a conformational change of the export gate to activate it, allowing entry of the substrates into the gate. The export gate utilizes membrane potential ($\Delta\Psi$) of proton motive force to unfold and transport substrates into channel

conserved in the *Vibrio* flagellum. An enigmatic "hat-like" structure has been visualized in the inner membrane of *Vibrio* spp separate from the flagellum that appears to contain components of the export machinery (Kaplan et al. 2022), but the function and purpose of this structure are currently unknown.

**Filament** The filament is attached to the hook via the adaptor proteins FlgK and FlgL (Homma et al. 1990). The filament makes up the bulk of the flagellum, and in many bacteria (e.g., *S. enterica*) it is composed of thousands of copies of a single flagellin polymerized into a helical structure with a hollow core (Yonekura et al. 2003). In contrast, the *Vibrio* filament contains at least four different flagellins with relatively high homology to each other (Klose and Mekalanos 1998a; Mcgee et al. 1996; Kim et al. 2014; Mccarter 2001). *Vibrio* genomes typically contain two flagellin loci, one downstream of *flgL* that encodes two to three flagellins (*flaAC* in *V. cholerae*), and one upstream of *flaG* that typically encodes three flagellins (*flaEDB* in *V. cholerae*). Each flagellin gene has its own promoter, and the genes are differentially regulated such that one flagellin in *V. cholerae* is expressed as a Class III gene (*flaA*), and the other four are expressed as Class IV genes (*flaBCDE*) (Klose and Mekalanos 1998a). FlaA, FlaB, FlaC, and FlaD can be detected in purified *V. cholerae* flagella (Yoon and Mekalanos 2008; Jung et al. 2019). The fifth flagellin, FlaE, can be detected among the extracellular matrix in biofilms (Jung et al. 2019), and it has been proposed that this represents a flagellin-homologous protein that plays a role in biofilm formation. Although the genes are named differently in *V. vulnificus* and *V. parahaemolyticus*, the equivalent four flagellins were found in the filament, and the equivalent flagellin to FlaE was found in the biofilm matrix; *V. vulnificus* and *V. parahaemolyticus* have a sixth flagellin gene located downstream of the *V. cholerae flaAC* equivalent, and this flagellin-homologous protein (FlaF) was also found in the biofilm matrix (Jung et al. 2019). Thus it appears that the *Vibrio* flagellar filament is typically composed of four different flagellin subunits, with a fifth and sometimes sixth flagellin-like protein being secreted through the flagellum into the biofilm matrix.

The flagellin subunits in the *V. cholerae* filament are not all equivalent. Loss of FlaA prevents flagellar synthesis and motility, despite the other flagellins being synthesized (Klose and Mekalanos 1998a); the *flaA* bacteria appear to synthesize only the hook, and the other flagellins are secreted into the supernatant (Klose and Mekalanos 1998a; Xicohtencatl-Cortes et al. 2006). Overexpression of FlaA in the absence of all the other flagellins allows a filament to be formed, whereas overexpression of any of the

other flagellins does not. It is still not entirely clear why FlaA is so critical for filament formation, given the high level of homology between the flagellins, but the other flagellins have a strategically located lysine residue within a beta sheet in the D1 domain; when a lysine is introduced into FlaA at this location (A145K) it fails to allow filament formation (Echazarreta et al. 2018). Other *Vibrio* spp do not appear to have a single critical flagellin; inactivation of the FlaA equivalent flagellin in *V. parahaemolyticus*, *V. vulnificus*, *V. campbellii,* or *V. anguillarum* does not cause a non-motile phenotype, although the bacteria are noticeably reduced in motility (Mccarter 1995; Mcgee et al. 1996; Kim et al. 2014; Petersen et al. 2021). Interestingly, not all the other flagellins in these species have the strategically located lysine residue, so perhaps there are redundant "critical" flagellins in these bacteria. In *Shewanella putrefaciens*, the flagellum is composed of two distinct flagellins that spatially occupy different locations within the filament and impart different swimming capabilities on the bacterial cell; localization of one specific flagellin close to the base of the filament with the other flagellin more distally located stabilizes the filament and improves motility through a variety of different environments (Kuhn et al. 2018). A similar mechanism may explain the importance of the FlaA (or equivalent) flagellin in the *Vibrio* filament.

**Flagellar Sheath** The flagellar sheath is an extension of the outer membrane that coats the entire filament (Fuerst and Perry 1988; Chu et al. 2020). How the sheath forms around the filament remains mysterious, but an "O ring" complex of unknown origin is visible at the base of the flagellum in cryoET images (Fig. 5.2a, b) that has been hypothesized to be involved in sheath formation (Zhu et al. 2017). Interestingly, a few unsheathed flagella were visualized in this same study, but it is unclear whether these arose as a result of the hyperflagellated state of the background *V. alginolyticus flhG* strain, or whether unsheathed flagella can be found at some percentage in otherwise wildtype Vibrios with a single polar flagellum. The sheath has been implicated in shielding the flagellins from recognition by TLR5 in the host (Yoon and Mekalanos 2008), and in acting as a cap to prevent flagellin secretion during filament growth (Mccarter 2001). Rotation of the sheathed flagellum generates OMVs that signal to host tissues, which is important for triggering morphogenesis in the squid host by *V. fischeri* (Aschtgen et al. 2016). The presence of the sheath coating the filament has important consequences for cholera disease: antibodies against the OAg of *V. cholerae* LPS (anti-O1) protect against *V. cholerae* infection in both animal models and in humans (Wang et al. 2017; Bishop et al. 2010; Charles et al. 2020), and these antibodies specifically function to prevent bacterial motility by binding to the sheathed flagellum, which is coated with LPS. Further insights into bacterial flagellar sheaths can be found in an excellent review covering this topic (Chu et al. 2020).

## 5.2 Flagellar Polar Localization

The localization of the flagellum at the cell pole is a distinct difference between the *Vibrio* flagellum and the flagella of *S. typhimurium*. Polar localization is critical for flagellar rotation to push the bacterial cell forward. Vibrios (and other polarly flagellated bacteria like *Pseudomonas* spp, *Shewanella* spp, and *Campylobacter jejuni*) control flagellar location and number through two interacting proteins: FlhF and FlhG (also called FleN) (Kojima et al. 2020). FlhF and FlhG have opposing activities: When FlhF is overexpressed or FlhG is removed, the cell becomes hyperflagellated. Likewise when FlhF is removed or FlhG is overexpressed, the cell becomes non-flagellated (Kusumoto et al. 2006; Correa et al. 2005). Thus FlhF is a positive regulator and FlhG is a negative regulator of flagellar number. FlhF is a signal recognition particle (SRP) type GTPase, and some of the insights into structure/function of FlhF have been derived from studies of the orthologue in other bacteria, most notably the crystal structure of *Bacillus subtilis*

FlhF (Bange et al. 2007). FlhG is a MinD type ATPase, and likewise insights have been gained on FlhG structure/function from the study of orthologues, especially from the crystal structures of *P. aeruginosa* FleN (FlhG) (Chanchal et al. 2017) and *Geobacillus thermodenitrificans* FlhG (Schuhmacher et al. 2015) . FlhF and FlhG interact with each other (Kusumoto et al. 2008), so how do they regulate polar flagellar number and placement? (Fig. 5.4)

FlhF dimerizes when bound to GTP, but is a monomer when bound to GDP (Kondo et al. 2018). FlhF binding to GTP is critical for flagellar biogenesis, but GTPase activity *per se* is not (Green et al. 2009; Kondo et al. 2018) FlhF intrinsically localizes to the old bacterial cell pole without any other factors (Kusumoto et al. 2008) and the FlhF central domain is required for localization. However in the absence of FlhG, more FlhF is localized to the old cell pole and some can also be found at the new pole (Arroyo-Perez and Ringgaard 2021) indicating that FlhG can suppress FlhF polar localization to some extent. FlhF recruits the earliest flagellar structural component, FliF (MS ring) to the cell pole, consistent with FlhF establishing the site of flagellar assembly at the old cell pole (Green et al. 2009; Terashima et al. 2020).

FlhG also localizes to the cell pole, but it requires the polar landmark protein HubP (Yamaichi et al. 2012; Arroyo-Perez and Ringgaard 2021). FlhG localization at the cell pole also requires FlhF (Arroyo-Perez and Ringgaard 2021), and FlhG is unstable in the absence of FlhF. The structure of the orthologue from *P. aeruginosa*, FleN, revealed that the protein undergoes structural rearrangement when bound to ATP that allows it to dimerize (Chanchal et al. 2017). In *V. alginolyticus*, FlhG needs to bind ATP to localize to the cell pole and suppress hyperflagellation, but it does not need to hydrolyze ATP for these functions (Ono et al. 2015), similar to what was found for *P. aeruginosa* FleN (Chanchal et al. 2017). FlhG appears to exert its negative effects on *Vibrio* flagellar synthesis at both the transcriptional and post-translational levels (Fig. 5.4).

FleN (FlhG) from *P. aeruginosa* was found to directly bind to the master regulator of flagellar transcription, FleQ (FlrA) and downregulate transcriptional activation (Dasgupta and Ramphal 2001) by inhibiting the FleQ ATPase activity required for $\sigma^{54}$-dependent transcription initiation (Chanchal et al. 2017). The structure of the FleQ ATPase (central) domain complexed with FleN indicates that FleN inhibits ATP binding to FleQ and remodels the $\sigma^{54}$ contact site to prevent transcription activation. FlhG from *Shewanella putrefaciens* also directly binds FlrA and inhibits transcriptional activation, but it binds to the C-terminal HTH domain of FlrA rather than to the central ATPase domain (Blagotinsek et al. 2020). Since the *flhG* (*fleN*) gene is transcribed from a FlrA (FleQ)-dependent promoter, this provides a feedback loop whereby FlhG negatively controls its own expression. This mechanism has not yet been demonstrated for *Vibrio* FlhG-FlrA interactions, but one of these mechanisms seems likely because a *V. cholerae flhG* mutant has increased transcription of all classes of flagellar genes (Correa et al. 2005) and a direct interaction between the *Vibrio* FlhG and FlrA has been observed but not yet reported in the literature (S. Kojima and M. Homma, personal communication). FlhG of *S. putrefaciens* also interacts with the flagellar C ring protein FliM to promote its assembly, and switches between binding FlrA and FliM to restrict flagellar number (Blagotinsek et al. 2020); this mechanism has not been identified in *Vibrio* spp.

FlhG also interacts with FlhF at the cell pole and is hypothesized to inhibit FlhF initiation of the biogenesis of multiple flagella post-translationally. FlhG stimulates the GTPase activity of FlhF (Homma et al. 2022), as has been seen in *C. jejuni* (Gulbronson et al. 2016). The current model (Fig. 5.4) that incorporates known activities of FlhF and FlhG from *Vibrio* spp as well as from other bacteria indicates that GTP-bound FlhF dimerizes and localizes to the cell pole, where it facilitates the initiation of flagellar synthesis by stimulating the insertion of FliF into the membrane. FlhG bound to ATP dimerizes and also localizes to the cell pole by

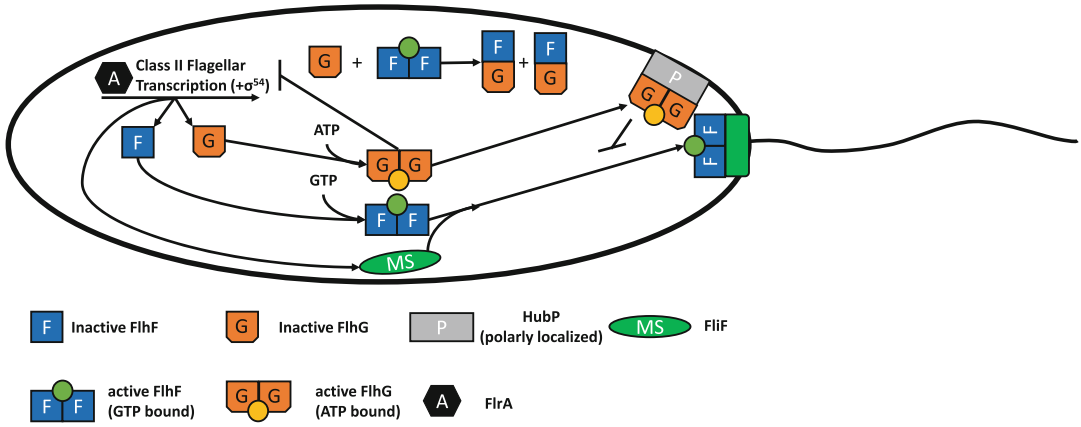


**Fig. 5.4** Involvement of FlhF and FlhG in flagellar number and polar placement. Adapted from Kojima et al. (2020). FlhG bound to ATP forms a homodimer, FlhF bound to GTP forms a homodimer. GTP-bound FlhF localizes to the cell pole, where it facilitates insertion of FliF at the cell pole and promotes MS-ring formation. GTP-bound FlhG is recruited to the cell pole by interaction with HubP, where it stimulates FlhF GTPase activity, which in turn removes FlhF from the cell pole. Inactive FlhF and FlhG interact with each other in the cytoplasm. ATP-bound FlhG is predicted to also interact with the master regulator FlrA to downregulate transcription of Class II flagellar genes

association with HubP. At the pole, FlhG associates with FlhF and stimulates its GTPase activity, which causes it to monomerize and localize to the cytoplasm. ATP-bound FlhG is also predicted to interact with FlrA and inhibit Class II gene transcription in *Vibrio* spp, as it has been shown to do in other bacteria and described above. Thus FlhG acts at both the transcriptional and post-translational levels to inhibit flagellar synthesis to achieve a single flagellum, while FlhF ensures the flagellum is localized to the cell pole. Several aspects of this model remain to be demonstrated in *Vibrio* spp, including the exact mechanism involved in FlhG-dependent post-translational flagellar regulation, and especially FlhG interaction with and inhibition of FlrA-dependent transcription.

SflA is another enigmatic protein associated with polar flagellar synthesis in *Vibrio* spp. A *V. alginolyticus* strain lacking both FlhF and FlhG is non-flagellated, but suppressor mutations arise that allow for the formation of multiple peritrichous flagella (Kojima et al. 2011). These mutations inactivate SflA, a transmembrane protein that is unique to *Vibrio* spp with a cytoplasmic DnaJ-like domain (Kitaoka et al. 2013). Inactivation or overexpression of SflA in a wildtype (i.e., FlhFG+) strain has no effect on flagellar synthesis. SflA localizes to the cell pole in FlhFG+ cells via HubP, and the DnaJ-like cytoplasmic domain is sufficient to suppress peritrichous flagellar formation in the absence of FlhFG (Inaba et al. 2017). The structure of the periplasmic domain of SflA revealed a domain-swapped dimer that likely interacts with a partner protein(s) (Sakuma et al. 2019). Exactly how SflA suppresses peritrichous flagellar synthesis, and how this is initiated in the absence of FlhFG, remains to be determined. Another protein, FapA, was identified in *V. vulnificus* that localizes to the cell pole via HubP and promotes flagellar synthesis; this protein is sequestered from the cell pole in the presence of glucose by the PTS system, which prevents flagellar synthesis (Park et al. 2016). Two proteins, MotV and MotW that associate with HubP in *V. cholerae*, affect tumbling frequency and swimming speed (Altinoglu et al. 2022). It remains to be determined whether SflA, FapA, MotV, and/or MotW have similar functions in all *Vibrio* spp.

## 5.3 Flagellar Loss

*Vibrio* bacterial cells have been observed to lose their polar flagella under certain conditions, including nutrient depletion and cessation of growth (Zhuang et al. 2020; Ferreira et al. 2019), as well as movement through mucin (Liu et al. 2008). Visualization of the disassembly process in *V. alginolyticus* revealed that flagellar ejection initiates with an apparent "break in the rod" that liberates the MS/C ring structure to freely diffuse in the inner membrane, followed by ejection of the hook/filament (Zhuang et al. 2020). In stationary phase, ejected flagella composed of hook and filament accumulate in the media (Ferreira et al. 2019). A subcomplex is visible in the outer membrane of *V. cholerae*, *V. fischeri*, and *V. harveyi* following flagellar loss that appears to be the relic P and L rings (decorated with the H and T rings), referred to as the PL-subcomplex (Kaplan et al. 2020; Ferreira et al. 2019). The PL-subcomplex is coated with outer membrane on the outer surface, and contains a proteinaceous plug that occupies the otherwise hollow core. The authors speculate that the PL-subcomplex functions to seal the outer membrane after motor disassembly.

## 5.4 Run-Reverse-Flick Motility

The run-and-tumble motility of peritrichously flagellated bacteria, e.g., *E. coli*, has been well-studied. This type of motility results from counterclockwise rotation of the peritrichous flagella leading to all the flagella forming a bundle at one pole that pushes the bacterium forward in a straight line ("run"), and clockwise rotation of the flagella resulting in the flagella unbundling and the cell tumbling and reorienting itself (Fukuoka et al. 2012). The reorientation of the bacteria between straight "runs" via clockwise flagellar rotation is a critical component of directional movement. Chemotaxis controls the length of time the flagella spin counterclockwise vs clockwise in response to chemotactic cues, which leads to directional motility.

Counterclockwise rotation of the single polar flagellum on Vibrios pushes the cell forward in a relatively straight line, similar to the "runs" of peritrichously flagellated bacteria. But clockwise rotation of the flagellum only causes the bacterial cell to be "pulled" by the flagellum in the reverse direction without reorientation, which would lead to the cells only able to travel backwards and forwards in a relatively straight line. However, Vibrios are observed to undergo a run-reverse-flick (RRF) motility, in which the cells swim in a forward direction, then in reverse direction, followed by a "flick" that reorients the bacterial body (Xie et al. 2011). Amazingly, the flagellar hook at the base of the flagellum facilitates this reorientation by buckling during spinning of the flagellum. This leads to the "flick" of the bacterial cell body, which changes the direction of swimming about 90°; analyses of individual cells indicate that the flick occurs shortly after cells have resumed forward swimming following reverse swimming (Fig. 5.5) (Son et al. 2013). The hook undergoes compression during rotation of the flagellum and the hydrodynamic load leads to the buckling event (flick) that allows a change of direction. Reducing the speed of flagellar rotation (by altering $Na^+$ concentration) suppresses this reorientation by reducing the viscous load on the flagellum (Son et al. 2013), and because the flicking frequency increases with increased speed, faster cells are more chemotactic (Son et al. 2016).

The RRF motility pattern mediated by the polar flagellum was initially described in *V. alginolyticus* (Son et al. 2013, 2016; Xie et al. 2011) and subsequently confirmed in *V. cholerae* (Frederick et al. 2020; Grognot et al. 2021), and is likely a common motility pattern for all Vibrios with a single polar flagellum. The swimming speed of *V. cholerae* has been calculated as approximately 90 μm/s in both forward and reverse, and the forward runs are approximately 3.6 times longer than the reverse runs (Grognot et al. 2021). However, *V. cholerae* also undergoes observable "deceleration" events during runs where the swimming speed is temporarily decreased (Grognot et al. 2021).

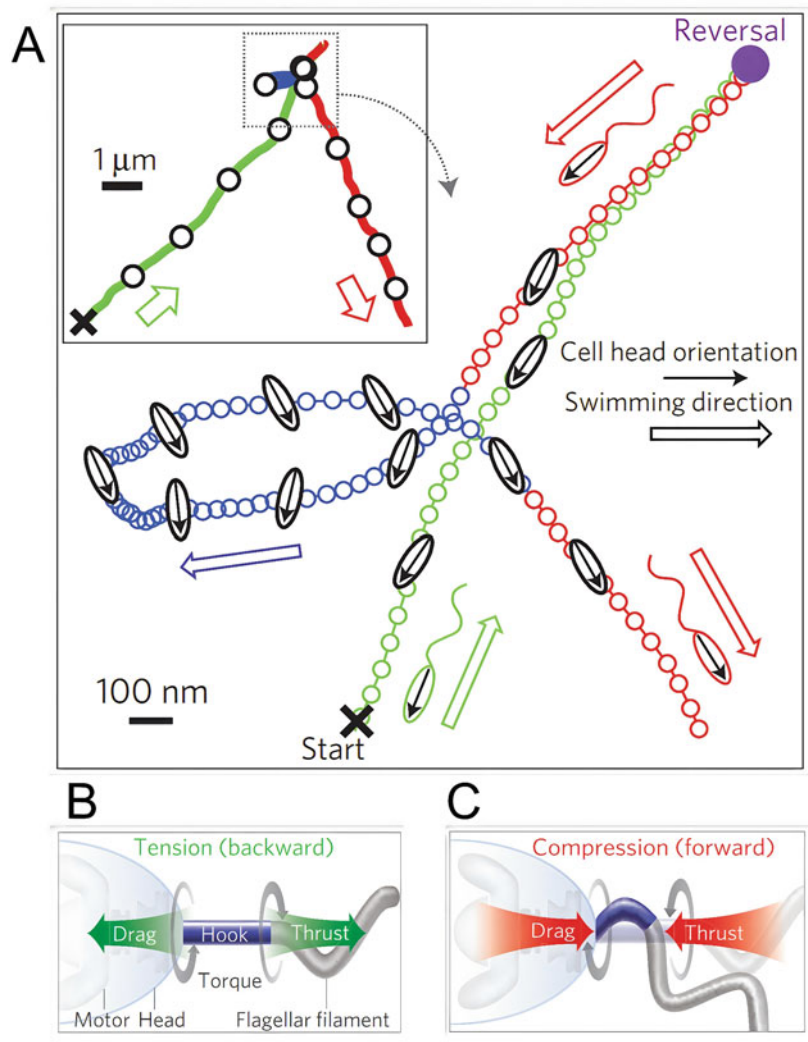


**Fig. 5.5** *Vibrio* run-reverse-flick motility. Reproduced with permission from Son et al. (2013). High-speed video microscopy of *V. alginolyticus* was used to characterize the flick that reorients the swimming direction of cells. The flick (blue) occurs during the initiation of forward swimming (red) following reverse swimming (green). (**a**) Cell trajectory containing a flick, captured with high-speed imaging. Cell head positions are shown by circular markers at 1 ms intervals. A schematic of the head orientation at selected times is overlaid (not to scale). The inset (**b**, **c**) shows the entire trajectory. Schematics (not to scale) of the flagellar filament, hook and rotary motor during backward swimming (**b**), when the hook is in tension, and during forward swimming (**c**), when the hook is in compression

*V. cholerae* must swim through mucin in the human gastrointestinal tract to colonize the crypts, and observations of *V. cholerae* swimming within mucin or polymers revealed that while swimming speed is reduced with increasing viscosity, cells maintain similar RRF frequency (Grognot et al. 2021). These results contrast with a previous report that observed suppression of flicking when *V. cholerae* swam in mucin, which was postulated to facilitate *V. cholerae* arrival at the intestinal epithelial cell surface (Frederick et al. 2020). The previous study found that mucin induced the expression of the Type VI secretion apparatus (T6SS), the injection device typically used to kill neighboring bacteria, and the T6SS contributed to the suppression of flicking (Frederick et al. 2020). The discrepancy between these two studies may be explained (in part) by the *V. cholerae* strains used in these experiments: the classical biotype *V. cholerae* O395 strain used in the later study (Grognot et al. 2021) is a representative 6$^{th}$ pandemic strain and it lacks T6SS due to multiple mutations (Kostiuk et al. 2021), whereas the El Tor biotype *V. cholerae* N16961 strain used in the previous study (Frederick et al. 2020) is a representative seventh pandemic strain that is T6SS competent.

## 5.5 Flagellar Transcription Regulation

Flagellar synthesis is controlled at the transcriptional level by a hierarchy of gene expression that facilitates the temporal transcription of flagellar genes in the order in which they are assembled into the flagellar structure. The flagellar transcription hierarchy of *S. enterica* is a paradigm for the coupling of gene expression to a functional secretion apparatus to achieve the correct timing of late flagellar gene transcription. Specifically, the flagellins, which make up the bulk of the flagellum in the filament, are not transcribed until the basal body genes are expressed and assembled correctly, which prevents a build up of flagellins inside the cell. This is achieved by secretion of the anti-$\sigma^{28}$ factor FlgM through the basal body, which frees $\sigma^{28}$ to transcribe the flagellin gene (Hughes et al. 1993). This event divides most of the *S. enterica* flagellar genes into either Class II genes (transcribed prior to FlgM secretion), or Class III genes (transcribed after FlgM secretion from sigma28-dependent promoters). The Class II genes are transcribed by the master regulator FlhDC, so the *flhDC* operon is considered the sole Class I gene; in the absence of FlhDC none of the flagellar genes are expressed.

The *Vibrio* flagellar transcription hierarchy (specifically *V. cholerae*) is more complicated than that of *S. enterica* because it involves a four-tiered hierarchy, rather than a three-tiered hierarchy (Prouty et al. 2001; Syed et al. 2009) (Fig. 5.6). The master regulator of the flagellar transcription hierarchy is FlrA, a $\sigma^{54}$-dependent transcriptional activator (also called FlaK in some *Vibrio* spp, FleQ in *P. aeruginosa*) (Klose and Mekalanos 1998b; Arora et al. 1997; Brennan et al. 2013). FlrA activates $\sigma^{54}$-dependent transcription of Class II genes. Microarray and promoter expression analyses in *V. cholerae* identified the *fliE* operon, *flhA*, and the *flrBC* operon as being expressed from Class II promoters; an additional $\sigma^{54}$ binding site upstream of *flhF* (Dong and Mekalanos 2012) indicates that a second FlrA-dependent promoter drives expression of *flhFGfliAcheYZABW* immediately downstream of *flhA*, which was not identified in the original analysis (Prouty et al. 2001). Class II genes encode the MS ring (the first component of the flagellum assembled), FlhF and FlhG (which dictate flagellar placement and number, see above), a component of the C ring (FliG), components of the ATPase ring complex (FliH, FliI, FliJ), a component of the secretion complex (FlhA), and regulatory proteins (FlrB, FlrC, $\sigma^{28}$), as well as chemotaxis proteins. Interestingly, we originally identified the Class II promoter upstream of *fliE* as driving transcription of an operon that included all the genes from *fliE* through *flhB* (Prouty et al. 2001). However, another $\sigma^{54}$-dependent promoter was identified upstream of the *fliKLMNOPQRflhB* genes (Dong and Mekalanos 2012), and microarray analysis and promoter expression studies indicated these genes are Class III genes, not Class II genes (Syed et al. 2009). It is still unclear what intermediate in flagellar synthesis is required to progress to Class III gene transcription (see below) (Burnham et al. 2020), but components of the C ring (FliM, FliN) and the secretion complex (FliP, FliQ, FliR, FlhB) appear to be expressed only after transcription has progressed to Class III expression.

FlrA is a $\sigma^{54}$-dependent transcriptional activator with a central domain that oligomerizes to induce the ATPase activity required to stimulate open complex formation by the $\sigma^{54}$-containing RNA polymerase; it also contains an amino-terminal regulatory domain and a C-terminal DNA binding domain (Klose and Mekalanos 1998b; Srivastava et al. 2013). The FlrA binding site in the *flrBC* promoter is approximately 27 bp upstream of the $\sigma^{54}$ binding site (Srivastava et al. 2013). CytR also positively stimulates *flrBC* transcription (Das et al. 2020), but whether CytR modulates FlrA activity is not yet clear. FlrA transcriptional activity is modulated in other closely related species. FlrA/FleQ from *Shewanella* and *Pseudomonas* has been shown to bind to FlhG (FleN), which inhibits $\sigma^{54}$-dependent transcriptional activation of Class II genes (Dasgupta and Ramphal 2001; Chanchal et al. 2021; Blagotinsek et al. 2020); the mechanism involves either inhibition of ATP binding to

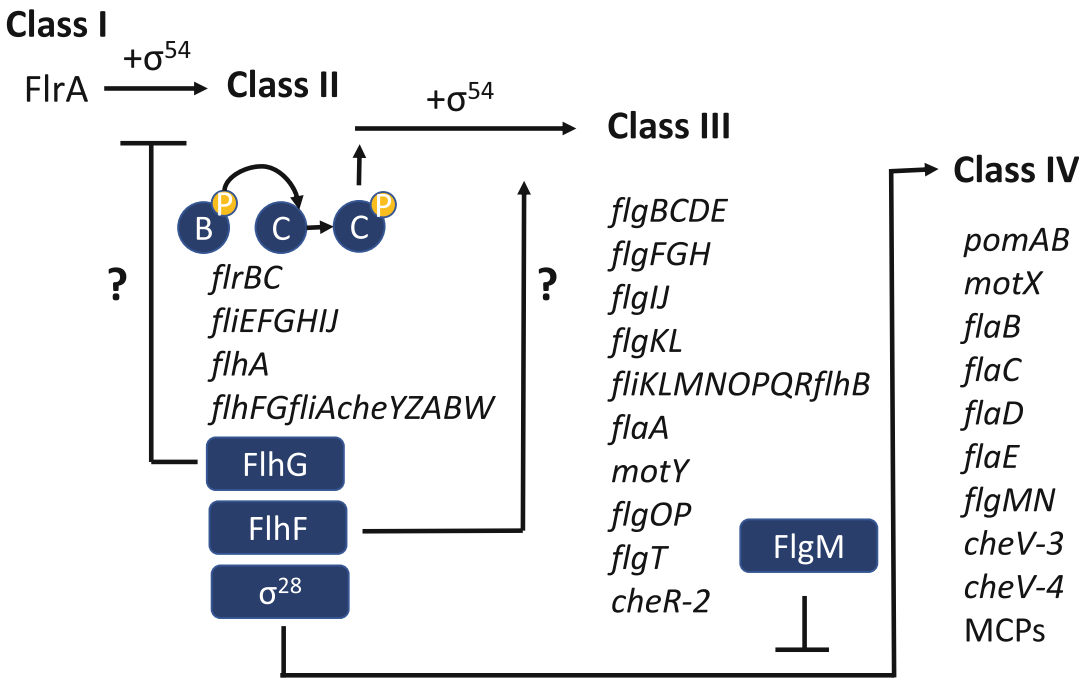


**Fig. 5.6** The *V. cholerae* flagellar transcription hierarchy. FlrA is the master regulator of flagellar transcription. FlrA activates $\sigma^{54}$-dependent Class II flagellar genes, which include *flrBC*. FlrB is a histidine kinase that autophosphorylates itself then transfers the phosphate to FlrC. FlrC-P activates $\sigma^{54}$-dependent Class III flagellar genes. $\sigma^{28}$ (FliA) is bound to an anti-sigma factor FlgM, which is secreted through the flagellum to allow activation of Class IV genes by $\sigma^{28}$-RNA polymerase. FlhG is suspected to interact with FlrA to inhibit Class II transcription, while FlhF enhances Class III gene transcription by an unknown mechanism. The *flhF* operon contains three additional orfs not listed

FlrA (*P. aeruginosa*), or inhibition of DNA binding by FlrA (*S. putrefaciens*). This provides a negative feedback loop whereby the FlrA-dependent factor FlhG downregulates FlrA activity; disruption of FlhG binding to FlrA leads to a multiflagellate phenotype in *S. putrefaciens*, similar to a deletion of *flhG* (Blagotinsek et al. 2020). FlhG has not yet been demonstrated to modulate *Vibrio* FlrA activity, but it is likely given that in the absence of *flhG,* transcription of the different *V. cholerae* flagellar gene Classes increases (Correa et al. 2005) . It has however been shown that cdGMP directly binds to *V. cholerae* FlrA (Srivastava et al. 2013) and negatively modulates its DNA binding activity, similar to *P. aeruginosa* FleQ (Hickman and Harwood 2008), partially explaining how high levels of cdGMP repress flagellar synthesis and motility. Arginine residues at positions 135 and 176 in FlrA contribute to cdGMP binding, but a *V. cholerae* strain expressing FlrA that no longer binds cdGMP is still repressed for motility at high cdGMP levels (Srivastava et al. 2013), which is due, at least in part, to *Vibrio* PolySaccharide (VPS) expression. Additionally, the *V. cholerae* biofilm regulator VpsT, which binds cdGMP and dimerizes to activate the VPS genes, also inhibits flagellar gene transcription (Krasteva et al. 2010), and because Class II gene transcription is inhibited, this implicates VpsT in modulating FlrA activity. Little is known about the regulation of FlrA expression, but transcription of *flrA* is negatively modulated by the anaerobiosis regulatory protein ArcA in *V. cholerae* (Li et al. 2022) and by the quorum sensing autoinducer LuxS in *V. harveyi* (Zhang et al. 2022).

The two-component regulatory proteins FlrB and FlrC, which are expressed from a Class II

(i.e., FlrA-dependent) promoter, control expression of Class III flagellar genes (Klose and Mekalanos 1998b). The Class III genes/operons positively regulated by FlrC include the *flgBCDE* operon, the *flgFGH operon*, the *flgIJ* operon, the *flgKL* operon, the *fliKLMNOPQRflhB* operon, *flaA*, the *flgOP* operon, *flgT*, *cheR-2*, and *motY* (Prouty et al. 2001; Burnham et al. 2020; Dong and Mekalanos 2012; Syed et al. 2009) These encode components of the C ring (FliMN), components of the secretion complex (FlhB, FliOPQR), components of the rod (FlgBCFG), H ring (FlgOPT), L ring (FlgH), P ring (FlgI), T ring (MotY), hook and hook junction (FlgEKL), and one of the filament proteins (FlaA) as well as a chemotaxis protein (CheR-2). The *flrC* gene is a target of the RNA binding protein CsrA (Butz et al. 2021), which positively regulates motility; presumably CsrA enhances post-transcriptional expression of FlrC to enhance Class III gene transcription.

FlrB is a histidine kinase (HK) that phosphorylates FlrC at a conserved aspartate residue (D54) in its N-terminal receiver domain (Correa et al. 2000); phospho-FlrC stimulates $\sigma^{54}$-dependent transcription at Class III flagellar gene promoters. FlrB is a cytoplasmic protein with no transmembrane domain, indicating that modulation of its activity occurs entirely within the cytoplasm, unlike many other bacterial HKs in which a periplasmic domain modulates activity of a cytoplasmic-localized HK. The structure of the central ATPase domain of FlrC has been solved (Dey et al. 2015), which revealed that it forms a heptameric ring whether or not ATP is bound, and it can interact with $\sigma^{54}$ in either state. This heptamer structure is critical for ATPase and transcriptional activity, and cdGMP binding destabilizes the heptamer and abrogates ATPase activity (Chakraborty et al. 2020), illuminating that cdGMP negatively regulates Class III gene transcription, in addition to Class II through binding to FlrA. Because these studies were performed with the isolated central domain of FlrC, it is not yet clear whether phosphorylation of the FlrC N-terminus results in heptamer formation, or stimulation of ATPase activity within a pre-formed heptamer. The C-terminus of FlrC contains the DNA binding domain (Klose and Mekalanos 1998b), and interestingly FlrC binds to sites located downstream, rather than upstream, of the transcription startsites of the *flaA* and *flgK* promoters (Correa and Klose 2005). The FlrC binding site within the *flaA* promoter (at +24 to +85) functions as a true enhancer, because it can be moved upstream of the $\sigma^{54}$ binding site (at –371 to –310) and still function, whereas the FlrC binding site within the *flgK* promoter cannot (Correa and Klose 2005).

*V. cholerae* strains expressing FlrC that cannot be phosphorylated (D54A) are unable to transcribe Class III flagellar genes and are non-motile (Correa et al. 2000), whereas strains lacking FlrB acquire mutations within FlrC that enhance its transcriptional activity and regain motility (Correa et al. 2000; Klose and Mekalanos 1998b), indicating that phosphorylation of FlrC is critical to transition from Class II to Class III gene transcription. The exact checkpoint that controls phosphorylation of FlrC is still unknown, but lack of the MS ring (FliF), C ring (FliG), or secretion components (FlhA, FlhB, FliPQR) abrogates Class III gene transcription; the authors hypothesize that formation of an MS ring-rotor-T3SS complex is the trigger that is sensed by FlrB and stimulates phosphorylation of FlrC (Burnham et al. 2020). Interestingly, the division of the flagellar hierarchy into Class II (FlrA-dependent) and Class III genes (FlrC-dependent) discussed here for *V. cholerae* is not found in all *Vibrio* spp. In *V. campbellii*, FlrA is not strictly required for motility, and FlrA and FlrC coregulate most of the genes designated as Class III in *V. cholerae*, leading to a three-tiered, rather than four-tiered, transcriptional hierarchy model where Class I is not dependent on $\sigma^{54}$ and includes both *flrA* and *flrBC* (Petersen et al. 2021). This is likely to be the same in *V. parahaemolyticus* (Mccarter 2001), and is reminiscent of the situation in *Shewanella oneidensis*, where FlrA and FlrC coregulate the majority of Class III genes, and in this case FlrC is dispensable for motility because FlrA can substitute for FlrC (Gao et al. 2018). Reprogramming rod and hook genes (*flgBCDEFGHIKL*) to be expressed from Class II rather than Class III promoters in *V. cholerae*

did not affect motility or flagellation, but this was dependent on a functional FlhG (Burnham et al. 2020). Thus there are likely to be more variations in the division of Class II and Class III genes in the flagellar hierarchies of Vibrios yet to be discovered.

Class IV flagellar genes are transcribed from $\sigma^{28}$-dependent promoters. Class IV genes include the *pomAB* operon, *motX*, *flaB*, *flaC*, *flaD*, *flaE*, the *flgMN* operon, *cheV-3*, *cheV-4*, and different methyl-accepting chemoreceptors (MCPs) (Prouty et al. 2001; Syed et al. 2009; Klose and Mekalanos 1998a). These encode components of the stator (PomAB) and T ring (MotX), the filament (FlaB, FlaC, FlaD) and biofilm matrix (FlaE), the anti-sigma factor FlgM, and chemotaxis proteins. The control of $\sigma^{28}$-dependent transcription by secretion of the anti-sigma factor FlgM through the flagellum has been well-characterized in *S. enterica* (Hughes et al. 1993). In *V. cholerae*, the FlgM anti-sigma factor is also secreted through the flagellum, which allows $\sigma^{28}$ to associate with RNA Polymerase and Class IV genes to be expressed (Correa et al. 2004), but details of this event that characterizes the transition to Class IV gene transcription are lacking. For example, how does secretion occur through a filament that is coated with sheath? It is not clear at what stage of flagellar assembly the anti-sigma factor is secreted, but *V. cholerae* mutants lacking the filament gene *flaA*, which is clearly a Class III gene (Correa and Klose 2005; Echazarreta et al. 2018), are not blocked for Class IV gene transcription (Klose and Mekalanos 1998a), indicating that the anti-sigma factor is likely secreted at an earlier stage of flagellar assembly prior to FlaA incorporation. Enhanced levels of secreted FlgM can be detected in strains with mutations in *flgD* (Liu et al. 2008), the hook-capping protein, which leads to enhanced Class IV gene transcription, but also repression of HapR, the quorum sensing regulator, by $\sigma^{28}$ through an unknown mechanism. When *V. cholerae* cells swim through mucus, many have lost their flagella and have enhanced levels of secreted FlgM, which leads to repression of HapR by $\sigma^{28}$ (Liu et al. 2008); because HapR represses virulence factor expression, the authors speculate that the loss of flagella as bacteria cross the intestinal mucus layer leads to enhanced virulence factor expression. $\sigma^{28}$ is rapidly degraded by the Lon protease (Pressler et al. 2016), revealing another layer of regulation over $\sigma^{28}$-dependent transcription.

## 5.6 Future Directions

The application of cryo ET to *Vibrio* cells has led to exquisite new details of the *in situ* structure of the polar flagellum, but many questions remain about its structure and function. How the sheath forms around the filament remains mysterious, and it is unclear how secretion of non-flagellar components (e.g., cytotoxin, flagellin-like matrix proteins) occurs through the sheath. The enigmatic "O ring" at the base of the hook/filament on the outside of the cell awaits identification and characterization. It is still not known why the filament is composed of different flagellins and what their contributions are to the swimming behavior of Vibrios. There remain many questions regarding regulation of flagellar transcription, including understanding the signals and mechanisms that regulate the transitions from Class II to Class III to Class IV expression, and why some Vibrios regulate flagellar gene transcription differently than others. Details on flagellar ejection, and the purpose of the PL and hat-like subassembly relics in the outer and inner membrane, respectively, are also questions that will undoubtedly be addressed in future research. Discoveries on how rotation of the flagellum influences VPS expression (Lauriano et al. 2004; Wu et al. 2020), and generally how cdGMP (Liu et al. 2010) and other factors like MotV and MotW (Altinoglu et al. 2022) control motility will drive further research into these areas. Further study on these and other questions will ultimately provide a better understanding of how the polar flagellum facilitates the virulence and environmental persistence of *Vibrio* spp.

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# 6 Environmental Reservoirs of Pathogenic *Vibrio* spp. and Their Role in Disease: The List Keeps Expanding

Parisa Noorian, M. Mozammel Hoque, Gustavo Espinoza-Vergara, and Diane McDougald

**Abstract**

*Vibrio* species are natural inhabitants of aquatic environments and have complex interactions with the environment that drive the evolution of traits contributing to their survival. These traits may also contribute to their ability to invade or colonize animal and human hosts. In this review, we attempt to summarize the relationships of *Vibrio* spp. with other organisms in the aquatic environment and discuss how these interactions could potentially impact colonization of animal and human hosts.

**Keywords**

*Vibrio* · Predation · Pathogen persistence · Chitin · Plankton

## 6.1 Introduction

The interactions of bacterial communities and the physical and biological environments where they exist are complex and affect the structure of communities (Materna et al. 2012). Work by Colwell et al. (1977) showed that pathogenic *Vibrio* spp. are natural inhabitants of aquatic environments worldwide (Vezzulli et al. 2010; Lutz et al. 2013). To date, 194 species of *Vibrio* (https://lpsn.dsmz.de/genus/vibrio) have been identified in aquatic systems and some possess pathogenic traits that are associated with disease of human and aquatic animals (Sawabe et al. 2013). Various phenotypes contribute to the fitness of these microorganisms in environmental niches and may also play roles in the infection of animal and human hosts (Keymer et al. 2007; Sun et al. 2018).

The best-studied member of this genus, *Vibrio cholerae,* is the causative agent of cholera, a severe diarrhea with signature rice water stools. *V. cholerae* enters the human body through the consumption of contaminated water or food. Cholera infections can lead to death due to dehydration, electrolyte imbalance, and shock (Carpenter 1971) and remains a global threat due to poor hygiene and lack of basic health infrastructure (Huq et al. 1990; Colwell and Huq 1994; Kaper et al. 1995; Ali et al. 2015).

There are more than 200 serogroups of *V. cholerae* identified to date, but only serogroups, O1 and O139 are known to be responsible for pandemic cholera (Feeley 1965; Chatterjee and Chaudhuri 2003). The O139 serogroup has alterations in both phenotypic and genetic characteristics when compared to the *V. cholerae* O1 serogroup that was responsible for previous epidemics (Swerdlow 1993). The O1 El Tor biotype acquired the O139 antigen by

P. Noorian · M. M. Hoque · G. Espinoza-Vergara · D. McDougald (✉)
Australian Institute for Microbiology & Infection (AIMI), University of Technology Sydney, Sydney, NSW, Australia
e-mail: diane.mcdougald@uts.edu.au



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horizontal gene transfer, becoming the current O139 strain (Waldor et al. 1994; Bik et al. 1995). These strains produce cholera toxin (CT), an enterotoxin responsible for rapid fluid loss from the intestinal epithelium (Kaper et al. 1995). However, some *V. cholerae* O1 strains are CT-negative (Kaper et al. 1981, 1995). Non-toxigenic *V. cholerae* non-O1/non-O139 strains are isolated predominantly from aquatic environments. They are also isolated from patients with gastritis (Hasan et al. 2015) as these strains carry other virulence genes and cause sporadic disease. However, the severity of disease from these strains is less when compared to O1/O139 serogroups (Reidl and Klose 2002).

*Vibrio vulnificus* is an opportunistic, ferrophilic pathogen responsible for acute gastroenteritis and septicemia following ingestion of contaminated seafood (especially raw or undercooked oysters) or wound infections after exposure to seawater (often from puncture by fish spines or crustacean shells). Even with aggressive antibiotic therapy, mortality rates can be as high as 75% for septicemia and 50% for wound infections (Blake et al. 1979; Johnston et al. 1985; Klontz et al. 1988; Depaola et al. 1994; Hlady and Klontz 1996; Shapiro et al. 1998; Strom and Paranjpye 2000; Belkin and Colwell 2006; Jones and Oliver 2009). High mortality rates for *V. vulnificus* infections result in excessive annual costs which are estimated to be ten times higher than any other seafood-borne illness. *V. vulnificus* infections account for 66% of seafood-related illness health costs and 26% of the total health costs in the United States (Ralston et al. 2011; Heng et al. 2017).

*V. vulnificus* strains exhibit considerable variation in genotype and phenotype, hence various attempts have been made to develop classification schemes. *V. vulnificus* strains are classified into three biotypes based on biochemical characteristics. Human infections are mainly caused by biotype 1 strains, while biotype 2 strains are primarily eel pathogens (Tison et al. 1982; Amaro and Biosca 1996). Biotype 3 strains cause human wound infections and are geographically limited to Israel. Genomic analysis indicates that biotype 3 is a hybrid of biotypes 1 and 2 (Bisharat et al. 1999; Naiel et al. 2005).

Further work based on the alignment of eight housekeeping and virulence loci of *V. vulnificus* clustered strains based on genotypes (C- or E-genotype), suggesting possible different ecotypes. The authors speculated that the E-genotype strains grew better under conditions present in the environment, whereas the C-genotype strains survived the stressful transition from seawater/oyster to humans better than the E-genotype. Therefore, it was speculated that the evolution of strains in different niches gave rise to the two genotypes (Rosche et al. 2010). One of the significant differences in the physiology of the E and C-genotype strains is the ability of C-genotypes to resist the bactericidal effects of human serum, whereas E-genotypes strains are sensitive (Bogard and Oliver 2007). However, genotypes do not strictly predict the pathogenicity of *V. vulnificus* biotype 1 strains (Thiaville et al. 2011)

*Vibrio parahaemolyticus* is also responsible for seafood-borne outbreaks. It is the causative agent of acute gastroenteritis in humans after the consumption of contaminated raw or undercooked seafood. Even though acute gastroenteritis is usually self-limiting, *V. parahaemolyticus* can cause life-threatening wound infections or septicemia in individuals with pre-existing medical conditions (Joseph et al. 1982; Daniels et al. 2000; Depaola et al. 2000; Ceccarelli et al. 2013; Zhang and Orth 2013). For example, the first reported outbreak of *V. parahaemolyticus* killed 20 individuals out of the 272 infected after consumption of semi-dried juvenile sardines (Fujino et al. 1953). Seafood industries in the United States, China, and Japan are heavily impacted by frequent outbreaks of *V. parahaemolyticus* (Su and Liu 2007), which mostly occur in the summer months, causing a heavy toll on communities that rely on seafood industries. For example, an outbreak occurred in a Chilean region with usually cold water temperatures that was experiencing higher-than-normal temperatures from January to March 2004

(González-Escalona et al. 2005). Between 2004 and 2006, Chile recorded 1000 cases annually and reached a peak of 3600 clinical cases in 2005 (Harth et al. 2009; Velazquez-Roman et al. 2014; Bonnin-Jusserand et al. 2019).

*V. parahaemolyticus* pathogenic strains are commonly identified by the presence of the thermostable direct hemolysin (*tdh*) and *tdh*-related hemolysin (*trh*) which have been associated with severe diarrhea in humans, although strains lacking these factors may still cause illness (Miyamoto et al. 1969; Takeda 1982; Kothary et al. 2000; Matsuda et al. 2010). *V. parahaemolyticus* detected by PCR in seawater and organic material collected in Japan showed the *tdh* and *trh* genes were positive in 55% and 20% of samples, respectively (Jahangir Alam et al. 2002). Another study from northern Gulf of Mexico sites found *tdh* and *trh* positive *V. parahaemolyticus* in 44% and 56% of oyster and 30% and 78% of water samples (Zimmerman et al. 2007). TDH and TRH have hemolytic activity and cause cardiotoxicity and enterotoxicity. Other important factors include two non-redundant type III secretion system (T3SS) proteins (Park et al. 2004; Matsuda et al. 2010; Shimohata and Takahashi 2010).

*Vibrio* spp. have a high capacity to evolve via the acquisition of new genetic information that may increase their survival in the environment (Seitz and Blokesch 2013). The emerging strains of *V. cholerae* and *V. vulnificus* are not only proof that these bacteria are constantly evolving, adapting, and proliferating in the environment, but that the environment is driving these adaptations. Furthermore, these changes are not always benign with respect to their interactions with human hosts (Igbinosa and Okoh 2008) as with the case of the newly emerging *V. cholerae* O139 strains (O'shea et al. 2004) and *V. vulnificus* biotype 3 (Bisharat et al. 1999). Here, we review: (1) the reservoirs of *Vibrio* species, with a major focus on the three pathogenic vibrios, *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus* and (2) the role of reservoirs/environment in the emergence of virulence traits.

## 6.2 Impact of Environment on Occurrence of *Vibrio* spp.

*Vibrio* spp. are found in brackish and estuarine water systems from the tropics to temperate waters worldwide where temperature and salinity affect the abundance of vibrios (Lutz et al. 2013; Baker-Austin et al. 2018). For example, *V. cholerae* survives for extended periods in nutrient-deplete warm water at salinities between 0.25 and 3% at a pH of 8.0 (Miller et al. 1984) and can persist under low nutrient and temperatures (i.e., 10 °C) for long periods if supplemented with sodium (Singleton et al. 1982). The occurrence of *V. vulnificus* is strongly correlated with temperature, turbidity, dissolved oxygen, and numbers of estuarine and coliform bacteria. *V. vulnificus* has been recovered from water with salinities between 0.4 and 3.7% with an optimum between 1.0 and 2.5% and a temperature range of 7–36 °C with an optimum of 20 °C. At temperatures below 8.5 °C, survival decreases (Kaspar and Tamplin 1993, Høi et al. 1998, Motes et al. 1998, Pfeffer et al. 2003). *V. parahaemolyticus*, has been detected during the summer months when water temperatures are above 17 °C and salinities below 13 ppt (Kelly and Stroh 1988) and is detected in higher numbers when temperatures are higher (Depaola et al. 1990).

*V. cholerae, V. vulnificus*, and *V. parahaemolyticus* are known to enter a viable but nonculturable (VBNC) state under stressful conditions (i.e., nutrition deprivation, high salinity, and low temperature) wherein the cells are no longer culturable on routine media but can be shown to be viable and metabolically active (Colwell et al. 1985; Nilsson et al. 1991; Jiang and Chai 1996; McDougald et al. 2002; Wong and Wang 2004). This is in contrast to starved bacteria which can grow in or on normal media after a period of inactivity (Colwell et al. 1985). *V. cholerae* biofilms have been shown to enter a VBNC state and to resuscitate to a culturable state after being passaged in animals (Alam et al. 2007). It has been shown by Huq et al. (1990) in a rabbit ileal loop infection model as well as in human volunteers that VBNC cells are capable of

resuscitation and of causing cholera, and thus, VBNC cells are a potential health threat (Huq et al. 1990; Colwell and Huq 1994). *V. vulnificus* and *V. parahaemolyticus* also remain virulent, at least for some time, when in the VBNC state and following *in vivo* resuscitation (Oliver and Bockian 1995; Baffone et al. 2003).

VBNC cells have been shown to express pathogenicity factors. For example, constitutive transcription of the gene encoding a hemolysin, *vvhA,* was detected in VBNC *V. vulnificus* cells (Saux et al. 2002) as well as the global stress regulator, RpoS (σS) (Smith and Oliver 2006), and perhaps other stress-related genes that provide cross-protection against multiple stresses (Nowakowska and Oliver 2013). In fact, VBNC cells of *V. vulnificus* show increased resistance to high temperature, low and high pH, oxidative and osmotic stress, and exposure to ethanol, zinc, chloramphenicol, and ampicillin when compared to culturable cells (Nowakowska and Oliver 2013).

With climate change leading to the warming of sea surface temperatures, *Vibrio* spp. are being reported in regions that typically do not support their growth, resulting in outbreaks of vibrio infections on a worldwide scale (Vezzulli et al. 2016; Baker-Austin et al. 2017). Alarmingly, the increase in temperatures has resulted in vibrio infections in areas of the world that were previously not of concern such as the Baltic Sea and its estuaries (Brehm et al. 2021) and Sweden and Finland (Baker-Austin et al. 2016). A modeling projection estimated that thousands of new coastal areas will be suitable for vibrios by the end of the century, which would dramatically increase the number of people who are exposed to infections (Trinanes and Martinez-Urtaza 2021). Moreover, various models show that the number of warm days in the year is increasing and will lead to shellfish beds meeting the temperature conditions for vibrio growth (Ferchichi et al. 2021).

In addition to increasing sea surface temperatures, the concentration of manmade poorly degradable particles in ocean waters is increasing. These particles serve as surfaces for microbial colonization and biofilm formation (Oberbeckmann and Labrenz 2020). It has been proposed that marine microplastics are now potential reservoirs and vectors for the transport of pathogenic *Vibrio* spp. For example, scientific evidence shows that microplastics collected during multiple studies from various locations (e.g., Southern Brazil, Bay of Brest in France, North and Baltic Seas) were colonized by *Vibrio* spp., including *V. vulnificus, Vibrio mimicus, V. cholerae*, and *V. parahaemolyticus* (Kirstein et al. 2016; Frère et al. 2018; Silva et al. 2019; Kesy et al. 2021). Most importantly, *Vibrio* spp. are part of the aquatic food web, thus conditions that favor their growth will result in increases in numbers and further affect the range where they occur (Fig. 6.1).

## 6.3 Heterotrophic Protozoa and Interactions with *Vibrio* spp.

Protists are a highly diverse group of unicellular eukaryotic microorganisms. They are abundant in aqueous and soil environments and exhibit a wide array of trophic states with a majority being heterotrophic (Porter et al. 1985; Sherr and Sherr 2007). Predation by bacterivorous protozoa is a major limiting factor for bacterial biomass in the environment (Sherr and Sherr 1994). For example, heavy grazing mortality has been shown to play a significant role in regulating numbers of *V. cholerae* in coastal marine waters (Worden et al. 2006). In light of this, it has been stated that predation by bacterivorous protists in aquatic habitats shapes the taxonomic composition and physiological status of bacterial communities (Hahn and Höfle 2001). Therefore, the prevalence of bacterial predation defense mechanisms determines overall bacterial mortality rates (Matz and Kjelleberg 2005; Pernthaler 2005).

Several defense mechanisms of *V. cholerae* have been identified. The biofilm lifestyle protects *V. cholerae* from predation by some protozoa (Matz and Kjelleberg 2005). Secretion of quorum sensing (QS)-regulated proteases such as PrtV protects against predation by the flagellate *Cafeteria roenbergensis* and the ciliate

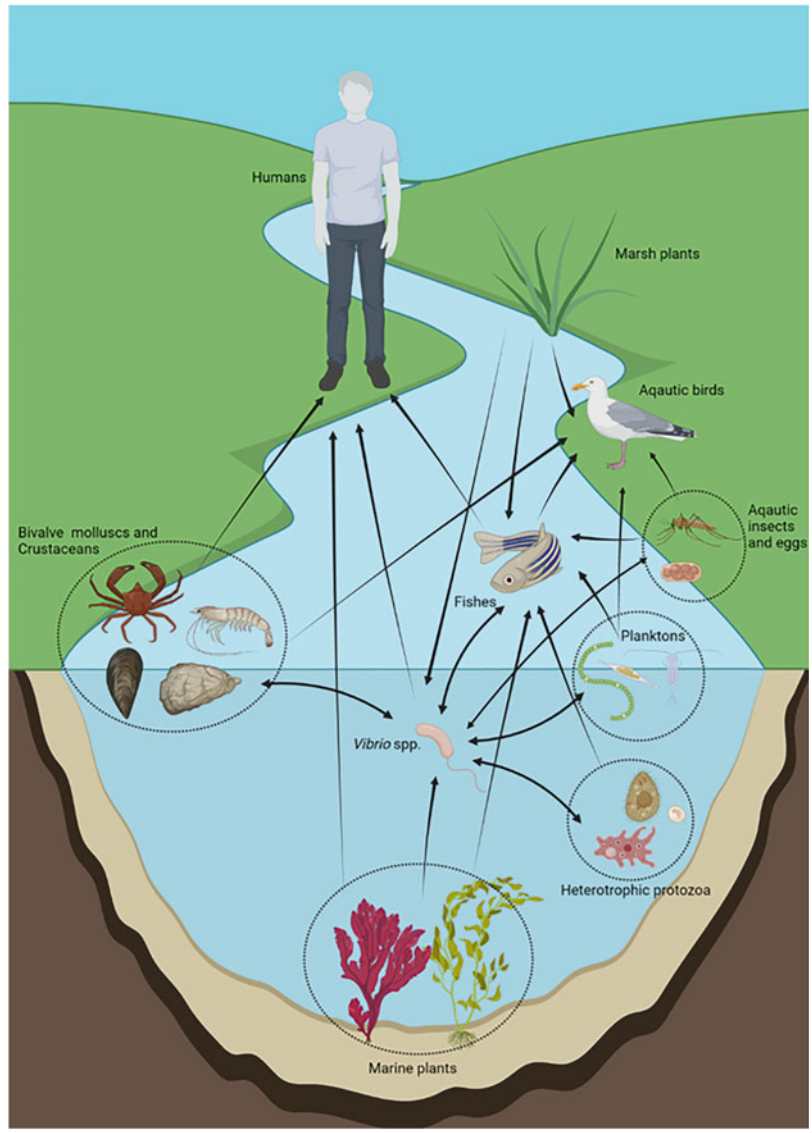


**Fig. 6.1** Simplified representation of interactions of *Vibrio* spp. and associated organisms in food webs. Black arrows indicate flow of nutrients. Pathogenic Vibrios are an integral part of the natural food web and dependent on the interactions between the members of the food web that produces opportunity for growth, transmission, and survival of *Vibrio* spp. (Matz et al. 2005; Trombetta et al. 2020)

*Tetrahymena pyriformis* (Vaitkevicius et al. 2006). In addition, ammonium production (Sun et al. 2015) and by-products of pyomelanin formation (Noorian et al. 2017) were shown to limit *V. cholerae* predation. Another predation resistance mechanism involves the type VI secretion system (T6SS), which secretes toxins that kill host cells such as the amoeba, *Dictyostelium discoideum* (Pukatzki et al. 2006; Pukatzki et al. 2007; Leiman et al. 2009; Miyata et al. 2011; Basler et al. 2012; Dong et al. 2013; Ho et al. 2014). However, this killing effect seems to differ among *Vibrio* spp., since a *V. vulnificus* strain that possessed T6SS did not exhibit anti-eukaryotic effects against *D. discoideum* (Hubert and Michell 2020). This is likely due to the fact regulation and expression of the T6SS in vibrios varies. Some strains have constitutively active T6SS systems while in others it is tightly regulated and expressed only under certain conditions (Unterweger et al. 2012; Metzger et al. 2016, 2019; Manera et al. 2021).

Grazing resistance mechanisms expressed by other *Vibrio* spp. are not as extensively studied.

For example, a *V. vulnificus* multifunctional autoprocessing repeats-in-toxin, (MARTX) type III was effective against the amoeba, *Neoparamoeba pemaquidensis* that was isolated and purified from the same turbot (*Scophthalmus maximus*) gill (Lee et al. 2013). Another report showed a *V. vulnificus* oyster isolate rewired central carbon metabolism during predation resulting in the production of excess organic acid which was toxic to a variety of ciliates, including *T. pyriformis* (Noorian et al. 2018; Rasheedkhan Regina et al. 2021).

Interactions of bacteria with protozoa are sometimes beneficial to bacteria. For example, *Acanthamoeba castellanii* does not prey on *V. parahaemolyticus* but instead secretes an unknown factor that promotes the survival of *V. parahaemolyticus* (Laskowski-Arce and Orth 2008). *V. cholerae* are known to survive intracellularly in amoebae such as *Naegleria* and *Acanthamoeba* (Thom et al. 1992; Abd et al. 2005; Abd et al. 2007; Van Der Henst et al. 2016) although strains have varying ability to survive internally (Shanan et al. 2016). *Vibrio harveyi* has been shown to survive in the marine ciliate *Cryptocaryon irritans* (Qiao et al. 2017). For this reason, protozoa have been referred to as "Trojan horses" (Barker and Brown 1994) or training grounds for pathogens where they are not only protected from adverse environmental stresses when internalized but also undergo selection for traits that contribute to infections in accidental human and animal hosts (Harb et al. 2000; Sun et al. 2018; Espinoza-Vergara et al. 2020). For example, long-term *in-vitro* co-incubation of *V. cholerae* O1 with the amoeba host, *A. castellanii*, resulted in phenotypic and genotypic changes associated with pathogen survival and fitness. The report showed that mutations in conserved regions of the flagellar transcriptional regulator, *flrA,* resulted in enhanced colonization of zebrafish (Hoque et al. 2021).

It has also been reported that *V. cholerae* can resist intracellular digestion in protozoa and escape in expelled food vacuoles (EFVs) (Espinoza-Vergara et al. 2019). The authors showed that *V. cholerae*-EFVs are better able to survive acidic environments, antibiotics, long-term starvation and have an increased capacity to colonize infant mice when compared to their planktonic counterparts. This finding has been established as the third hypervirulent state reported for *V. cholerae* in the literature (Mitterer et al. 2020), highlighting the impact that the interaction protozoa-bacteria has on the infective potential of pathogenic vibrios. In addition, OmpU was identified as an important factor for EFV production, suggesting that anti-grazing strategies displayed by bacteria can act as virulence factors for infecting a host.

The interaction between protists and vibrios is not only limited to aquatic environments. For example, protist parasites co-occur with *Vibrio* spp. within the digestive tract and in circulating hemocytes of the oyster, *Crassostrea virginica*. A protease produced by the pathogenic oyster protozoan, *Perkinsus marinus,* was initially shown to suppress the bactericidal activity of oyster hemocytes against *V. vulnificus* (Tall et al. 1999). However, no meaningful correlation has yet been established between the abundance of the parasitic protist with levels of either *V. vulnificus* or *V. parahaemolyticus.* Interestingly, oysters infected by *P. marinus* did not correlate with a higher abundance of pathogenic vibrios whereas oysters infected with another protozoan parasite, *Haplosporidium nelson,* had higher levels of *V. vulnificus* (Carnegie and Burreson 2012; Bienlien et al. 2021).

MARTX and the T6SS of *Vibrio splendidus* causes virulence in oysters but does not protect against grazing by the amoeba, *Vanella*. Rather, a region (*wbe*) involved in O-antigen synthesis was shown to be necessary for resistance to predation by amoebae (Oyanedel et al. 2020). In contrast, *Vibrio tasmaniensis* showed resistance to phagocytosis by oyster immune cells and grazing by the amoebae, *Vannella* sp. collected from oyster farms. A secreted metalloprotease, Vsm, and copper efflux p-ATPase, CopA, are known virulence factors affecting oysters and are also involved in the defense against predation by amoebae (Oyanedel et al. 2020). These examples highlight that the interactions between protozoa and *Vibrio* spp. are complex and not easily predicted.

## 6.4 Chitin and Vibrios

Chitin is the second most abundant organic polymer in nature and is composed of long a chain of *N*-acetylglucosamine (GlcNAc). Chitin is the major component of marine snow and is the main component of the exoskeletons of crustaceans such as copepods, shrimp, and crabs. Chitin is an excellent source of carbon and nitrogen for marine bacteria including vibrios (Rinaudo 2006; Martínez et al. 2014). One study tested 54 Vibrionaceae strains for growth on chitin and all tested strains, including *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* strains utilized N-acetylglucosamine (GlcNAc), the monomer of chitin. Most of the strains possessed chitinase A (*chiA*) that also degraded α (crab shell) and β (squid pen) chitin (Hunt et al. 2008). *V. cholerae* possesses two main extracellular chitinases, ChiA1 and ChiA2 (Meibom et al. 2004) and the expression of *chiA2* is also maximal in the host intestine (Mondal et al. 2014).

Clinical and environmental isolates of *V. cholerae* possess molecular mechanisms for the colonization of chitinous surfaces, including the exoskeletons of zooplankton and phytoplankton (Tamplin et al. 1990; Vezzulli et al. 2010). Both O1 and non-O1 strains of *V. cholerae* show increased colonization of dead plankton compared with colonization of live plankton (the dinoflagellate *Lingulodinium polyedrum* and the copepod *Tigriopus californicus*) (Mueller et al. 2007) and VBNC *V. cholerae* O1 attached to the cell envelope of the dinoflagellate *Noctiluca scintillans* (Akselman et al. 2010).

Many reports have shown that chitin metabolism is linked to pathogenicity in vibrios as similar factors are involved in interactions with chitin and the human host. However, the expression and role of these factors varies depending on strains and environmental conditions. *V. cholerae, V. vulnificus*, and *V. parahaemolyticus* all possess a variation of N-acetylglucosamine binding protein (GbpA), a type IV pilus mannose-sensitive hemagglutinin (MSHA) and a type IV pilus also known as the chitin-regulated pilus (ChiRP) (Aagesen and Häse 2012). *V. cholerae* uses GbpA for attachment to human intestinal cells and to chitin particles and chitin-containing plankton organisms (Kirn et al. 2005; Zampini et al. 2005). Similarly, *V. vulnificus* GbpA is also a mucin-binding protein, essential for pathogenesis in a mouse model of infection (Jang et al. 2016).

The MSHA pilus of *V. cholerae* is involved in surface attachment and colonization of zooplankton, chitin beads and the chitinous exoskeleton of the crustacean, *Daphnia pulex,* biofilm formation on non-nutritive abiotic surfaces and interactions with bivalve hemolymph (Finn et al. 1987; Jonson et al. 1991; Chiavelli et al. 2001; Meibom et al. 2004; Zampini et al. 2005). Colonization of the squid, *Euprymna tasmanica,* light organ by *V. fischeri* is directly linked to the expression of *mshA* (Ariyakumar and Nishiguchi 2009), however, a direct role in the pathogenesis of *V. cholerae* is debated (Heidelberg et al. 2000). A *V. parahaemolyticus* MSHA pilin mutant formed aggregates and exhibited a reduction in attachment to abiotic surfaces (Shime-Hattori et al. 2006). In addition, MSHA is also a significant factor in adherence of *V. parahaemolyticus* to human intestinal epithelial cells, thereby enabling pathogenesis (O'boyle et al. 2013).

*V. cholerae* MSHA is needed for attachment to chitin and biofilm formation and expression of *gbpA* and *mshA* increases with temperature (Stauder et al. 2010). Further studies of *V. vulnificus* show a strain-dependent attachment. At 20 °C, *V. vulnificus,* E-genotype strains attached significantly more to chitin than C-genotype strains while the reverse was true at 37 °C. E-genotypes had a higher level of Type IV pili (*pilA*, *pilD*, and *mshA*) even in the absence of chitin whereas *gbpA* was expressed significantly higher in C-genotype strains (Williams et al. 2014). Type IV pilin production was significantly downregulated whereas *gbpA* was upregulated in the C-genotypes during detachment in comparison to E-genotypes and C-genotypes produced more autoinducer-2 molecules in both aerobic and anaerobic conditions at 20 °C (Phippen and Oliver 2015).

A ChiRP mutant of *V. parahaemolyticus* attached to the surface of a coverslip but did not form aggregates, suggesting that ChiRP plays a

role in bacterial agglutination during biofilm formation (Shime-Hattori et al. 2006). ChiRP is expressed by chitin-attached *V. cholerae* (Meibom et al. 2004) and competence is induced, a process requiring the type IV pilus assembly complex (Meibom et al. 2005). *Vibrio* spp. display several factors involved in the uptake of exogenous DNA when associated with chitin surfaces (Antonova and Hammer 2015). Since chitin is widely distributed in aquatic environments (Beier and Bertilsson 2013), it has been hypothesized that chitin-induction of natural competency mediates the acquisition of genes (Meibom et al. 2005), potentially including those contributing to virulence.

Chitin-induced competency has been reported for many vibrios, including *V. vulnificus, V. parahaemolyticus*, and *V. cholerae* (Debnath et al. 2020). Despite the fact that some virulence factors play a role in attachment and biofilm formation of vibrios on chitin surfaces (e.g., MSHA and GbpA), factors directly involved in natural competence have not yet been related to virulence (Pruzzo et al. 2008). However, the activation of natural competence by the regulator, TfoX, has a positive effect on the activation of the T6SS in vibrios which has been implicated in virulence (Metzger et al. 2016; Joshi et al. 2017).

The T6SS is a contact-dependent bacterial system that translocates toxins into target hosts including bacteria and eukaryotes. In *V. cholerae*, different toxins, including VgrG1 (actin crosslinking protein in amoeba), VasX (pore-forming protein), TseL (lipase), VgrG3 and TseH (lysosyme) (Pukatzki et al. 2007; Jobichen et al. 2010; Basler and Mekalanos 2012; Dong et al. 2013; Hachani et al. 2014), have been described as effectors of the T6SS. It has been proposed that the T6SS in *V. cholerae* plays a role in both the aquatic environment and human host. In the environment, the formation of a biofilm on chitin in *V. cholerae* activates the T6SS which kills predators such as amoeba and competitor neighboring cells, while in the human host the T6SS enables competition with the intestinal microbiome (Joshi et al. 2017). For example, in *V. cholerae*, it is reported that at early stages of infection, the antagonistic effects of the activation of the T6SS toward the microbiota has a significant effect on the activation of two of the main virulence factors, *tcpA* and *ctxA*, suggesting an important role of the T6SS in infection (Zhao et al. 2018).

## 6.5 Association with Planktons

Many organisms that share niches with *Vibrio* spp. contain chitin and are part of the marine food web (Polis and Strong 1996). Vibrio concentrations correspond to the relative abundance of particular planktons, e.g., chitin containing diatoms and copepods correspond to high numbers of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* (Baffone et al. 2006; Turner et al. 2009; Rehnstam-Holm et al. 2010; Asplund et al. 2011; Turner et al. 2014; Diner et al. 2021).

Generally, *V. cholerae* has greater survival when attached to copepods, cyanobacteria and chironomid egg masses in comparison to free-living planktonic cells (Huq et al. 1983; Halpern et al. 2004; Islam et al. 2004). *V. cholerae* was found to be largely associated with copepods (Magny et al. 2011) the cases of cholera in Bangladesh coincided with the presence of rotifers, cladocerans, and copepods (Magny et al. 2011). In places where access to clean water is limited, it has been proposed that filtration of contaminated water through used sari cloth removes 99% of the plankton and consequently attached *V. cholerae* cells, resulting in a reduction in the incidence of cholera (Huq et al. 1996; Colwell et al. 2003).

*V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* associate with both the surface and gut of marine copepods (Sochard et al. 1979; Rawlings et al. 2007; Gugliandolo et al. 2008; Toubiana et al. 2019). Copepods are found in fresh and salt waters and feed on protozoa such as ciliates (Berk et al. 1977). Other aquatic species in turn feed on copepods and they are also used as live feed for larval and juvenile fish in commercial agricultural settings (Ajiboye et al. 2011; Rasdi and Qin 2016). Many vibrios colonize copepods. For example, the fish pathogen,

*V. anguillarum* colonizes the surface and the intestinal tract of the copepod, *Acartia tonsa* (Rasmussen et al. 2018). *Vibrio alginolyticus* and *V. anguillarum* have been isolated from *A. tonsa* eggs (Zidour et al. 2017) which is a concern for transmission and spread of disease for fish farms. *V. parahaemolyticus* numbers were also shown to be positively correlated with abundance of copepods while for total *Vibrio* spp. numbers, the diatom abundance was the most important (Rehnstam-Holm et al. 2014). Diatoms or unicellular photosynthetic algae are a key energy-rich component of phytoplankton communities and serve as 'food' in the oceans, lakes, and rivers. They all have an outer cell wall of biogenic silica and some species produce chitin as a component of the silica cell wall or as extracellular fibrils (Durkin et al. 2009).

Several studies have shown association of vibrios with different species of diatoms. *V. cholerae* VBNC cells have been shown to associate with *Nitzschia palea* and *Stigeoclonium* (Seeligmann et al. 2008). *V. parahaemolyticus* was shown to attach to estuarine strains of *Navicula* (diatom alga) (Kumazawa et al. 1991) and *Thalassiosira weissflogii* (Frischkorn et al. 2013) while the type IV pili was important for biofilm formation and adherence to *T. weissflogii*. The adherence to diatoms is controlled by increased chitin production that occurs in later stages of growth (Frischkorn et al. 2013). In contrast, some diatom species have been shown to inhibit the growth of *Vibrio* spp. such as *V. anguillarum*, *V. alginolyticus, V. campbelli,* and *V. harveyi* (Naviner et al. 1999; Molina-Cárdenas and Sánchez-Saavedra 2017).

Associations of vibrios also occur with dinoflagellates, a highly diverse group of single-celled phytoplankton. Some dinoflagellate species are not dangerous while others have been associated with harmful algal blooms (HABs). Since HABs produce toxins that can kill fish and accumulate in filter-feeding shellfish, they are of human concern. Some dinoflagellates can grow in numbers such that a visible coloration of the water, known as red tide can be seen in surface water (Smayda and Reynolds 2003; Bravo and Figueroa 2014). Different dinoflagellates respond differently to the presence of vibrios as some prey on vibrios while some are harmed by them (Seong and Jeong 2013).

The dinoflagellate *Cochlodinium ploykrikoides* is killed by *V. parahaemolyticus* while *Amphidinium carterae* and *Prorocentrum micans* prey on *V. parahaemolyticus* (Seong and Jeong 2011). In another study, two blooms during relatively warmer months (a cyanobacteria bloom and dinoflagellate bloom) led to increases in both *V. vulnificus* and *V. parahaemolyticus,* respectively, whereas three blooms that occurred during cooler months caused by dinoflagellates and euglenophytes were not associated changes in vibrio abundances (Greenfield et al. 2017). In a microcosm experiment, dissolved organic matter released by a bloom of the dinoflagellate *Lingulodinium polyedrum* supported explosive growth of *V. cholerae* (Mouriño-Pérez et al. 2003) while *V. parahaemolyticus* strains caused decreases in diatom, dinoflagellate and coccolithophore biomass (Klein et al. 2018).

*Vibrio* spp. also associate with Gram-negative photosynthetic cyanobacteria (blue-green algae) (Islam et al. 1999, 2004; Berg et al. 2009). The abundance of *Vibrio* spp. in microcosms increased in response to dissolved organic matter produced by *Nodularia spumigena* (Eiler et al. 2007), while other cyanobacteria have antibacterial activity, especially against vibrios. For example, the marine cyanobacterium *Leptolyngbya* sp. LT19 showed antibacterial activities against *V. harveyi* and *V. parahaemolyticus* (Maneechote et al. 2017). The cyanobacterium *Anabaena variabilis* has been identified as a possible long-term reservoir for *V. cholerae* (Islam et al. 1990) and mucinase, a soluble hemagglutinin protease, is important for the association. The *V. cholerae* mucinase degrades mucin which serves as a nutrient source (Islam et al. 2002) and a chemoattractant (Islam et al. 2006).

*V. cholerae* O1 increased production of CT when co-cultured with a green alga, *Rhizoclonium fontanum* (Islam et al. 1990) and toxigenic *V. parahaemolyticus* and *V. vulnificus* attach to some species of macro-algae and coastal seaweed (Hood and Winter 1997; Hayat

Mahmud et al. 2006; Mahmud et al. 2008; Gonzalez et al. 2014). The macroalga, *Gracilaria vermiculophylla*, found in the mid-Atlantic coastal region, USA, was identified as a potential reservoir for *V. parahaemolyticus*, and *V. vulnificus* (Gonzalez et al. 2014). However, as mentioned previously not all associations are predictable. For example, vibrio abundance was positively correlated with the microscopic algae, *Heterosigma akashiwo* but negatively correlated with *Fibrocapsa japonica* (Main et al. 2015). These negative relationships have led to research on antimicrobial factors produced by aquatic plants, cyanobacteria, diatoms, and algae as potential novel inhibitory drugs (Hassan et al. 2022; Molina-Cárdenas et al. 2022).

## 6.6 Association with Higher Organisms

Vibrios interact with many higher organisms and here we describe organisms that act as hosts, reservoirs, and vectors for the dissemination of vibrios. Some species of vibrios cause disease in animal hosts like the zoonotic pathogens *V. vulnificus* and *V. parahaemolyticus* (Austin 2010).

### 6.6.1 Chironomids

One of the most abundant insects in water habitats are chironomids (Diptera; Chironomidae) or non-biting midges that range from fresh to brackish water, estuaries, and marine environments. Chironomid egg masses contain hundreds of eggs embedded in a gelatinous matrix composed mainly of glycoprotein and chitin. These egg masses are a natural reservoir for *V. cholerae* and it has been reported that many of the *V. cholerae* inhabiting egg masses are in the VBNC state (Broza and Halpern 2001; Halpern et al. 2007; Broza et al. 2008; Thorat and Nath 2010; Armitage et al. 2012). Non-O1 and non-O139 *V. cholerae* have been isolated from chironomid egg masses in several countries highlighting their potential as an environmental reservoir (Halpern et al. 2004). Furthermore, adult midges collected in the air after emerging from water carried non-O1 and non-O139 *V. cholerae* on their inter-segmental membranes. *In vitro* studies showed that chironomid adults transport *V. cholerae* between water bodies (Broza et al. 2005) and a metagenomics analysis revealed pandemic O1/O139 serogroups in chironomid larvae (Laviad-Shitrit et al. 2020).

The *V. cholerae* hemagglutinin/protease (HA/P) degrades the gelatinous matrix of chironomid egg masses and has roles in human infection, including modification of CT and degradation of the protective mucus barrier in the intestines, thereby allowing access to the underlying microvilli (Halpern et al. 2003; Silva et al. 2006; Benitez and Silva 2016). *V. cholerae* HA/P production is QS-regulated and signals from different bacterial species isolated from chironomid egg masses were shown to induce HA/P in QS-deficient O1 El-Tor *V. cholerae.* this suggests possible interactions between insect gut microbiota which may also occur in the human gut microbiota (Sela et al. 2021).

### 6.6.2 Bivalve Molluscs

Contaminated seafood is one route for human infection by vibrios and higher numbers of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* were isolated from oysters (Pacific (*Crassostrea gigas*) and Atlantic or Eastern (*Crassostrea virginica*)) than from surrounding water (Hood et al. 1981; Kaysner et al. 1989; Tamplin and Fisher 1989; Depaola et al. 1990; Froelich and Noble 2016). *V. vulnificus* has been isolated from oyster shell biofilms, homogenates of whole oyster meat and tissues including the hemolymph, digestive region, gills, mantle, and adductor muscle (Tamplin and Capers 1992). In contrast to high densities of *V. cholerae, V. vulnificus, V. parahaemolyticus* detected in oysters, lower densities were detected in clams (Tobin-D'angelo et al. 2008; Froelich et al. 2017) and mussels (Ottaviani et al. 2005; Bauer et al. 2006; Normanno et al. 2006; Blanco-Abad et al. 2009).

The abundance of *V. parahaemolyticus* and *V. vulnificus* in oysters is positively associated with higher water temperatures (O'neill et al. 1992; Motes et al. 1998; Cook et al. 2002; Depaola et al. 2003; Randa et al. 2004). Specifically, the abundance of *V. parahaemolyticus* in oysters at room temperature increased rapidly to 50–790-fold within 24 h (Gooch et al. 2002). *V. vulnificus* survived in shucked oysters stored at 10 °C and below, demonstrating that they survive refrigeration (Kaysner et al. 1989). Although pandemic *V. cholerae* O1 has been isolated from oysters (Tamplin and Fisher 1989), non-O1 strains are more common, but these can still cause severe diarrhea after consumption of raw oysters, often requiring hospitalization (Tobin-D'angelo et al. 2008). Differences in sampling time, temperature, water quality, region, and host species have resulted in much conflicting data, hence pathogenicity and abundance of vibrios in bivalve organisms cannot be predicted (Flynn et al. 2019).

Based on the classification scheme for *V. vulnificus* using the virulence-correlated gene (*vcg*) that was discussed above, 84.4% of isolates recovered from oysters contained the *vcgE* allele (E-genotype). In contrast, isolates from waters surrounding the oyster sites revealed an almost equal distribution of E- and C-genotypes. Interestingly, the percentage of C-genotype strains from both sources increased when the water temperatures increased (Warner and Oliver 2008). E-genotype *V. vulnificus* strains formed more aggregates than C-genotype strains and consequently their uptake by *C. virginica* was higher than for C-genotype and other non-aggregated controls (Froelich et al. 2013; Froelich Brett et al. 2014). This formation of aggregates may partly explain the distribution of E- and C-genotype strains in oysters. Free-living vibrios are up taken by filter-feeding oysters. *In vitro* studies have shown fast uptake of *V. vulnificus* strains but also fast depuration after inoculation, while the oyster-adapted strains found naturally in oysters show resistance to depuration (Kelly and Dinuzzo 1985; Srivastava et al. 2009; Froelich et al. 2010; Froelich and Oliver 2013).

The *V. vulnificus* type IV pilus structural protein, PilA and to a greater degree the pre-pilin peptidase, PilD, contribute to binding to abiotic surfaces and to human epithelial cells (Paranjpye et al. 1998; Paranjpye and Strom 2005). PilA and PilD are also necessary for *V. vulnificus* and *V. parahaemolyticus* prolonged attachment to oysters (Paranjpye et al. 2007; Aagesen et al. 2013). The tight adherence (*tad*) pilus locus, generally found in Vibrionaceae, was shown to be involved in biofilm formation and colonization of oysters (Pu and Rowe-Magnus 2018) and deletion of the tad pilin gene (*flp*) led to decreased initial surface attachment and less robust biofilms (Pu and Rowe-Magnus 2018). Interestingly, in the mouse model of infection all three *V. vulnificus, tad* loci were required for septicemia, cell adhesion, and biofilm formation leading to lethality (Duong-Nu et al. 2019).

### 6.6.3 Crustaceans

Aquaculture and seafood industries are becoming popular around the world due to the health benefits of their products. However, these industries have a growing problem with contamination by bacterial pathogens, including *Vibrio* species. Mono-cultures of farmed products are susceptible to infectious disease caused by *V. harveyi, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus*, and *V. splendidus,* particularly in post-larvae and juvenile shrimp populations (Karunasagar et al. 1994; Lee et al. 1996; Vaseeharan and Ramasamy 2003; Jayasree et al. 2006; Longyant et al. 2008). Furthermore, VBNC *V. parahaemolyticus* has been detected in shrimp samples (Cao et al. 2019) which raises the concern over detection methods and safety of seafood for human consumption.

*V. parahaemolyticus* has acquired a unique 70 kb plasmid that contributes to a relatively new acute disease in the black tiger (*Penaeus monodon*) and whiteleg shrimp (*Penaeus vannam*), hepatopancreatic necrosis disease (AHPND). The plasmid encodes a binary toxin,

the Photorhabdus insect-related toxins (PirAvp and PirBvp) responsible for the destruction of host cells (Tran et al. 2013; Lee et al. 2015). The $PirB^{vp}$ subunit is important for recognizing the beta-hexosaminidases and mucin-like glycoproteins from the shrimp hepatopancreas (De Los Santos et al. 2022). *V. harveyi, Vibrio punensis*, and *Vibrio campbellii* strains have been isolated that carry the *pirVP* gene. However, not all strains of *V. parahaemolyticus* containing toxin genes, *pirA* and *pirB* genes display AHPND symptoms, while some *V. harveyi and V. campbellii* isolates produced toxins and cause AHPND (Kondo et al. 2015; Dong et al. 2017; Restrepo et al. 2018; Muthukrishnan et al. 2019; Vicente et al. 2020). Other crustaceans have also been shown to carry potentially pathogenic vibrios. For example, *V. cholerae. V. vulnificus, and V. parahaemolyticus* were isolated from the hemolymph and external carapace of the blue crab, *Callinectes sapidus* (Krantz et al. 1969; Colwell et al. 1975; Davis and Sizemore 1982) and a *Vibrio* sp. was isolated from the rock crab, *Cancer irroratus* (Newman and Feng 1982). *V. vulnificus, V. alginolyticus, V. mimicus, V. parahaemolyticus*, and *V. harveyi* were detected in lobsters and crabs from the Persian gulf (Raissy et al. 2012).

### 6.6.4 Fish

Humans consume many species of fish that in turn consume chitinous zooplankton, including copepods, chironomids, and crustaceans, all of which serve as hosts for *Vibrio* spp. Fish intestines also contain chitinous materials which favor the growth and persistence of *Vibrio* spp. (Tang et al. 2015).

Vibriosis is one of the most prevalent fish diseases and is characterized by septicemia, dermal lesions, ascites, and necrosis (Ina-Salwany et al. 2019). The common signs of vibriosis include red spots on the ventral and lateral areas of the fish and swollen and dark skin. Several stress factors contribute to vibriosis in fishes, including high water temperatures, overcrowding, pollution of the water, poor nutrition and improper handling. The common route of infection is penetration through skin, gills, and gastrointestinal tract (Frans et al. 2011). Vibriosis in fishes is commonly caused by *V. anguillarum*, *V. parahaemolyticus*, *V. harveyi*, *V. alginolyticus*, *V. vulnificus*, and *V. splendidus*. Among these, *V. anguillarum* is the most common cause of vibriosis and has been documented to affect more than fifty fresh and salt-water fishes, including fishes with economic importance to the aquaculture industry (e.g., salmon, rainbow trout, sea bream, cod, and eels) (Frans et al. 2011).

*V. cholerae* has also been associated with fishes in endemic cholera regions. Malka Halpern and her group reported that *V. cholerae* has been isolated from 30 different freshwater and marine fish species (Senderovich et al. 2010; Halpern and Izhaki 2017). These studies showed that approximately 87% of fishes are positive with *Vibrio* spp. with *V. cholerae* O1 reported in a few cases. For example, *V. cholerae* O1 and O139 have been detected using *ompW* and *ctxA* specific PCR in Hilsha fish (*Tenualosa ilisha*) and Tilapia (*Oreochromis niloticus*) in two separate studies conducted in Bangladesh and Tanzania respectively (Hossain et al. 2018; Hounmanou et al. 2019). Non-O1/non-O139 serogroups of *V. cholerae* have been isolated from different fishes, including lorna fish (*Sciaena deliciosa*) (Carvajal et al. 1998), turbot (*Scophthalmus maximus*) (Xing et al. 2013), tra fish (*Pangasius hypophthalmus*) (Tong Thi et al. 2014), bulls eye (*Priacanthus hamrur*) and hard tail scad (*Megalaspis cordyla*) (Sujatha et al. 2011).

There are several reports of cholera disease being linked to consumption of fish and their related products. The first records date back to 1951 where Pandit and Hora postulated that consumption of hilsa fish leads to cholera endemicity in India (Pandit and Hora 1951). Several *Vibrio* spp. outbreaks in Thailand, Tanzania, and Italy (Morgan et al. 1960; Killewo et al. 1989; Maggi et al. 1997) were linked to the consumption of raw fish and related products. Improper fish handling also leads to cholera cases. For example, 3 *V. cholerae* O1 cases occurred in Sydney, Australia after handling of raw whitebait

imported from Indonesia (Forssman et al. 2007) and one case was reported in Berlin, Germany after handling of fish imported from Nigeria (Schürmann et al. 2002).

*V. vulnificus,* biotypes are very heterogeneous, and some are shown to be zoonotic. Biotype 2 is subdivided into different serovars from which E and A are mainly eel pathogens. Serovar E can also infect humans producing severe wound infections and septicemia (Amaro et al. 1992; Amaro and Biosca 1996; Fouz et al. 2010). An investigation of the toxicity of lipopolysaccharides (LPS) showed a binding affinity for both eel and human erythrocyte membranes that led to the agglutination of the cells. However, the injection of pure LPS only caused endotoxic effects and death in rats but not in eels (Biosca et al. 1999).

## 6.7 Aquatic Birds

Aquatic birds live around bodies of water and are good indicators of the ecosystem they inhabit. Aquatic birds typically consume both aquatic and terrestrial food sources, depending on weather and season. For example, gulls eat crustaceans such as small crabs, small squilla, and other shellfish as well as fish. Mallards, European widgeons and common teals also consume aquatic vegetation such as seaweed, while some water birds feed on small invertebrates such as copepods and chironomids (Miyasaka et al. 2006; Green and Elmberg 2014).

Both O1 and non-O1/non-O139 strains of *V. cholerae* have been isolated from fecal samples of aquatic birds (Bisgaard and Kristensen 1975; Schlater et al. 1981; Lee et al. 1982; Ogg et al. 1989; Ismail et al. 2021). The presence of toxigenic *V. cholerae* (*ctxA*) was detected in the microbiome of 5 wild cormorant's intestines (Laviad-Shitrit et al. 2017). Although CT is not frequently found in these samples, other virulence factors are more prevalent. For example, from 23 *V. cholerae* strains isolated from aquatic birds all were positive for *toxR* but negative for *ctxA*, *tcpA*, *tcpI*, *zot*, and *ace* genes (Laviad-Shitrit et al. 2018). Another four non-O1/non-O139 *V. cholerae* strains isolated from domestic ducks in Germany with serious disease symptoms revealed a number of virulence factors, including the T3SS, cholix toxin (elongation factor 2-specific ADP-ribosyltransferase toxin) and MARTX which was closely related to MARTX of O1 strain N16961 (Hirsch et al. 2020). Forty *V. cholerae* and 34 *Vibrio metschnikovii* strains were isolated from migratory birds from the Inner Mongolia autonomous region of China. All *V. cholerae* were non-O1/non-O139 serotypes that encoded T6SS and *hlyA* but did not encode for CT, TCP fimbriae nor the extracellular matrix protein RbmA. Interestingly a strain of *V. metschnikovii* isolated from ill migratory birds carried the T6SS system contained the gene encoding the spike protein for T6SS while bacterial strains that carry T6SS without a spike protein are not pathogenic (Zheng et al. 2021).

Other *Vibrio* spp. have been found to be associated with aquatic birds. *V. cholerae*, *V. fluvialis*, *V. alginolyticus*, *V. mimicus*, *V. vulnificus*, *V. parahaemolyticus*, and *V. metschnikovii* have been detected in migratory birds in Romania (Páll et al. 2021). In Japan during winter when vibrio numbers were low in seawater, fecal samples from several aquatic wild birds such as various species of gulls, mallards, widgeons, and teals contained *V. parahaemolyticus* and *V. vulnificus* (Miyasaka et al. 2006). Fecal samples of aquatic birds from two coastal areas in Venezuela carried *V. cholerae* and *V. vulnificus* in one site along and strains from the Harveyi clade in the other (Fernández-Delgado et al. 2016).

Migratory aquatic birds have long been an area of concern as vectors and reservoirs for *Vibrio* spp. (Lee et al. 1982). Halpern et al. (2008) hypothesized that migratory birds feeding on copepods or chironomids contaminated with *V. cholerae* can disseminate the bacterium between continents to previously uninfected water systems. Waterbirds have been shown to carry living copepods and chironomids internally or externally from one waterbody to another (Frisch et al. 2007; Laviad-Shitrit et al. 2019). Furthermore, a lab experiment showed that *V. cholerae* could be detected in feces of

hand-reared cormorants even 72 h after switching from *V. cholerae* contaminated tilapia to a non-contaminated diet (Laviad-Shitrit et al. 2017). *Vibrio* spp. were detected in high abundance in sediment samples as well as from the common cockle *Cerastoderma edule*. Both *C. edule* and *V. splendidus* were detected in the feces of shorebirds especially in the warmer months (Albuixech-Martí et al. 2021).

Short-distance transmission investigated by whole genome sequencing showed a strain of *V. parahaemolyticus* and three strains of *V. mimicus* isolated from waterbirds along a river in China were clonally related to that of sediments and mollusks in the stream. Evidence of long-distance transmission was shown from two birds carrying the same clone of a *V. parahaemolyticus* strain isolated 1150 km apart and another two that were found 50 km apart (Fu et al. 2019). An analysis of fresh feces from migratory birds in Bangladesh identified *Vibrio* spp. (Saiful Islam et al. 2021).

## 6.8 Conclusion

War-torn Yemen's manmade crisis has led to more than 1.3 million suspected cases of cholera and 3000 deaths as of May 2020 (World-Health-Organization 2020). Contamination of water sources in Somalia by flash flooding due to heavy Gu rains led to 3858 suspected cholera cases and 27 associated deaths in the first 6 months of 2021 (World-Health-Organization 2021). Interestingly, one of the hypotheses on how the cholera epidemic in Yemen started is that it was due to a combination of strong El Niño rains in Somalia and southwestern winds over the Gulf of Aden in 2016 that disseminated cholera-contaminated chironomids from the Horn of Africa to Yemen (Paz 2019).

Cholera had been absent for over a century in Haiti, but the cholera outbreak after the earthquake in 2010 resulted in more than 531,000 cases (5% of the population) and more than 7050 deaths as of March 2012 (Chin et al. 2011; Sontag 2012). The suspected source of the outbreak was *V. cholerae* South Asian type introduced into the longest river in Haiti by the United Nation's peacekeeping troops from Nepal after contamination of the water system with human fecal material (Piarroux et al. 2011; Frerichs et al. 2012). A surveillance study using whole genome sequencing detected the outbreak of *V. cholerae* strains in Haiti Rivers more than 2 years after the onset of the epidemic (Kahler et al. 2015). Another surveillance study from Haiti surface waters and Rivers from 2013 to 2014 showed a fivefold increase in the number of water samples containing culturable *V. cholerae* O1 compared to the previous year (Alam et al. 2014, 2015).

A more recent metagenomics study on Haiti water samples by Roy et al. (2018) showed toxigenic *V. cholerae* O1 and O139 strains were not detected, consistent with the decline in cholera cases; however, environmental *V. cholerae* strains as well as cholera and Shiga toxin converting phages were detected. Interestingly, it has been shown that phage transduction with the CT-encoding phage CTXφ can convert the non-toxigenic environmental strains to CT-positive strains in the gastrointestinal environment (Waldor and Mekalanos 1996). This raises the concern that environmental strains that are consistently present are still potentially capable of becoming toxigenic, and these strains are not usually incorporated in reports regarding public health.

While mechanisms of *Vibrio* spp. infections of humans have been extensively researched, the information on the molecular interaction of vibrios and environmental hosts is lacking. Most reports on the associations between *Vibrio* spp. and various organisms are descriptive only. This has resulted in difficulties in the identification of the origin of outbreaks of various infections and predictions and monitoring of the spread of disease.

As mentioned in several sections, survival, growth, and dissemination of *Vibrio* spp. as members of the food web are complex. There are many factors that *Vibrio* spp. share (Table 6.1) but they do not necessarily have the

**Table 6.1** Critical or major interacting factors required for *Vibrio* spp. interactions with different host/reservoirs

| Host organism | Main interacting *Vibrio* spp. | Main virulence/interacting factor | Related disease or types of interactions |
|---|---|---|---|
| Human | *V. cholerae* | CT<br>HlyA<br>HAP<br>TCP<br>Zot<br>ChxA<br>Ace<br>GbpA<br>$MARTX_{Vc}$ | Host (diarrhea, gastroenteritis wound, infection, and septicemia) |
| | *V. parahaemolyticus* | TDH<br>TRH<br>MshA<br>T3SS1 and T3SS2 effectors | |
| | *V. vulnificus* | CPS<br>pilA<br>pilD<br>$MARTX_{Vv}$<br>VvhA<br>Vvp<br>GbpA<br>$LPS_{vv}$ | |
| Protozoa | *V. cholerae* | T6SS effectors<br>PrtV<br>HmgA<br>OmpU<br>flrA | Predator-prey<br>Host<br>Vector |
| | *V. vulnificus* | MARTX<br>Acetate metabolism | |
| | *V. splendidus* | MARTX<br>T6SS<br>*Wbe* | |
| | *V. tasmaniensis* | Vsm<br>CopA | |
| Copepod | *V. cholerae*<br>*V. parahaemolyticus*<br>*V. vulnificus*<br>*V. anguillarum*<br>*V. alginolyticus* | | Host |
| Diatom | *V. cholerae* | Type IV pili<br>VBNC | Host<br>Antagonistic Relationship |
| | *V. parahaemolyticus*<br>*V. anguillarum*<br>*V. alginolyticus*<br>*V. campbelli*<br>*V. harveyi* | | |
| Dinoflagellates | *V. cholerae*<br>*V. vulnificus*<br>*V. parahaemolyticus* | | Host<br>Antagonistic Relationship |
| Algae (micro and macro) | *V. cholerae* | Mucinase | Host<br>Antagonistic Relationship |
| | *V. parahaemolyticus*<br>*V. vulnificus* | Unknown compound | |
| Chironomids | *V. cholerae* | HAP | Host<br>Vector |

(continued)

**Table 6.1** (continued)

| Host organism | Main interacting *Vibrio* spp. | Main virulence/interacting factor | Related disease or types of interactions |
|---|---|---|---|
| Bivalves (mussels, oysters, and clams) | *V. cholerae* | | Host |
| | *V. parahaemolyticus* | | |
| | *V. vulnificus* | Tad pilus<br>PilA<br>PilD<br>GbpA | |
| Crustaceans (shrimp, crab, and lobster) | *V. cholerae* | ChiA<br>GbPA<br>MshA | Host |
| | *V. vulnificus* | ChiA<br>GbPA<br>MshA | |
| | *V. parahaemolyticus* | ChiA<br>GbPA<br>MshA<br>VBNC<br>PirAvp and PirBvp | Host<br>Shrimp: Acute hepatopancreatic necrosis disease (AHPND) |
| | *V. alginolyticus*<br>*V. anguillarum*<br>*V. splendidus*<br>*V. harveyi*<br>*V. campbellii* | | Host |
| Fish | *V. vulnificus* | LPS-vv | Host (Vibriosis)<br>Vector |
| | *V. cholerae*<br>*V. anguillarum*<br>*V. parahaemolyticus*<br>*V. harveyi*<br>*V. alginolyticus*<br>*V. mimicus*<br>*V. punensis*<br>*V. splendidus* | | |
| Birds | *V. cholerae*<br>*V. metschnikovi*<br>*V. fluvialis*<br>*V. alginolyticus*<br>*V. mimicus*<br>*V. vulnificus*<br>*V. splendidus*<br>*V. parahaemolyticus* | | Vector |

CT, cholera toxin; HlyA, hemolysin A; HAP, hemagglutinin protease; TCP, toxin-coregulated pilus; Zot, zonula occludens toxin; ChxA, cholix toxin; Ace, accessory cholera enterotoxin; GbpA, *N*-acetylglucosamine-binding protein A; TDH, Thermostable direct hemolysin; TRH, TDH-related hemolysin; T3SS ,type III secretion system; T6SS ,type VI secretion system CPS, antiphagocytic capsular polysaccharide; PilA and PilD, type IV pilins; MARTX, multifunctional autoprocessing repeats-in-toxin; VvhA, Cytosolin; Vvp, thermolysin-like zinc metalloprotease; T6SS, type VI secretion system; PrtV, *Vibrio* metalloprotease; HmgA, homogentisate-1,2-dioxygenase gene; OmpU, outer membrane protein; FlrA, flagellar regulatory protein A; wbe, O-antigen biosynthesis pathway; Vsm, metalloprotease; CopA, copper efflux p-ATPase; ChiA, chitinase A; MSHA, mannose-sensitive hemagglutinin; Tad, tight adherence pilus; PirAvp and PirBvp, Photorhabdus insect-related toxins; VBNC, viable but nonculturable

same function in different strains or may behave differently under different conditions. Nonetheless, it is important to study the interactions of vibrios in the environment to have a better understanding of this genus and of how pathogens may evolve in the environment.

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# Cholera Dynamics and the Emergence of Pandemic *Vibrio cholerae*

7

Deepak Balasubramanian, Mario López-Pérez, and Salvador Almagro-Moreno

**Abstract**

Cholera is a severe diarrheal disease caused by the aquatic bacterium *Vibrio cholerae*. Interestingly, to date, only one major clade has emerged to cause pandemic disease in humans: the clade that encompasses the strains from the O1 and O139 serogroups. In this chapter, we provide a comprehensive perspective on the virulence factors and mobile genetic elements (MGEs) associated with the emergence of pandemic *V. cholerae* strains and highlight novel findings such as specific genomic background or interactions between MGEs that explain their confined distribution. Finally, we discuss pandemic cholera dynamics contextualizing them within the evolution of the bacterium.

**Keywords**

*Vibrio cholerae* · Cholera · Pathogen emergence · Cholera pandemics · Pathogen evolution

## 7.1 Cholera

Cholera is a severely dehydrating diarrheal disease that affects over three million people worldwide resulting in ~100,000 deaths annually (Kanungo et al. 2022). It is one of the most rapidly fatal infections if not treated immediately and it disproportionately affects children <5 years of age (Baker-Austin et al. 2018; Kanungo et al. 2022). Cholera primarily occurs in regions with inadequate sanitation or access to clean water due to either poor infrastructure, natural disasters, or civil unrest (Baker-Austin et al. 2018; Barnett 2019). The disease remains endemic in numerous countries in Africa, Latin America, and Asia, where it causes seasonal outbreaks linked to regional weather patterns (Kanungo et al. 2022).

Cholera has been a major human scourge for centuries and has resulted in seven pandemics since 1817. The seventh and ongoing pandemic poses a health threat to 175 countries, affecting vulnerable populations in Haiti, Yemen, and the

D. Balasubramanian · S. Almagro-Moreno (✉)
Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, Orlando, FL, USA

National Center for Integrated Coastal Research, University of Central Florida, Orlando, FL, USA
e-mail: samoreno@ucf.edu

M. López-Pérez
Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, Orlando, FL, USA

National Center for Integrated Coastal Research, University of Central Florida, Orlando, FL, USA

Evolutionary Genomics Group, División de Microbiología, Universidad Miguel Hernández, Alicante, Spain



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Democratic Republic of Congo, among others (Baker-Austin et al. 2018). Furthermore, the recent epidemics have increased in duration, frequency, and intensity, underlining the pressing need for intervention (Emch et al. 2008).

Cholera is primarily spread via the fecal-oral route by consumption of water or food contaminated with choleragenic strains of *V. cholerae*. Bacterial pathogenesis results in a profuse, watery diarrhea leading to "rice water" stools that, if left untreated, can lead to severe dehydration, rapid circulatory collapse and, ultimately, death within 24 h. Timely administration of oral rehydration fluids typically resolves the fatal consequences of the disease, allowing patient recovery. Administration of antibiotics reduces the time of bacterial residence in the intestine, limiting the period of diarrhea but the emergence of resistant strains is of growing concern (Kanungo et al. 2022).

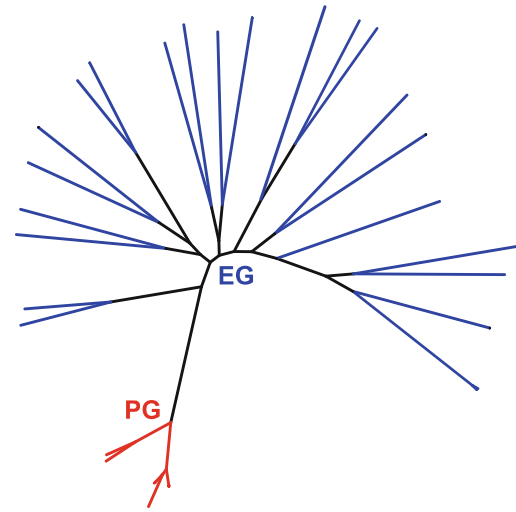


**Fig. 7.1** Phylogeny of *Vibrio cholerae* species. Simplified phylogeny of the *V. cholerae* species highlights the marked dichotomy between pandemic strains (PG, red) and non-O1 non-O139 environmental strains (EG, blue). Adapted from Shapiro et al. (2016)

## 7.2 *Vibrio cholerae*

Cholera is caused by the Gram-negative, comma-shaped bacterium *V. cholerae*, a natural inhabitant of brackish environments such as coastal waters and estuaries (Colwell et al. 1977; Huq et al. 1983). *V. cholerae* is commonly found in association with aquatic zooplankton (e.g., copepods) and phytoplankton (e.g., cyanobacteria) where it uses chitin and mucilaginous surfaces a major carbon and nitrogen source for proliferation (Nahar et al. 2011; Pruzzo et al. 2008). These metabolic adaptations, in addition to others such as resistance to protozoal grazing or phage predation, could contribute to preadaptations to the human host, as suggested by lipopolysaccharide (LPS) modifications. The structure of the LPS of *V. cholerae* allows classification of the strains into over 200 serogroups. Interestingly, only strains belonging to the O1 and O139 serogroups are known to cause epidemic and pandemic cholera and they form part of a confined phylogenetic clade: the pandemic group (PG) (Fig. 7.1) (Faruque et al. 1998; Kanungo et al. 2022). Many other serogroups, collectively termed as non-O1/non-O139, are generally non-pathogenic, although some isolates are associated with gastroenteritis (Morris 2003). The O1 serogroup is divided into two main serotypes, Ogawa and Inaba that primarily differ from each other in a methyl group in the LPS that is present only in the former (Wang et al. 1998). Both serotypes can cause severe disease and the dominant serotype fluctuates seasonally and geographically (Longini et al. 2002; Morris 1990). The O1 serotype is also divided into two biological variants (biotypes), classical and El Tor. The first six cholera pandemics were caused by the classical biotype of *V. cholerae* O1 but have been replaced by the El Tor biotype since 1961 and are responsible for the seventh, ongoing pandemic (Faruque et al. 1998). Acquisition of two pathogenicity islands (PAIs), the *Vibrio* Seventh Pandemic Islands I and II (VSP-I and -II), is thought to have resulted in the emergence of the El Tor variant, conferring pathogenic features distinct from the classical strains (Fig. 7.2; discussed below).

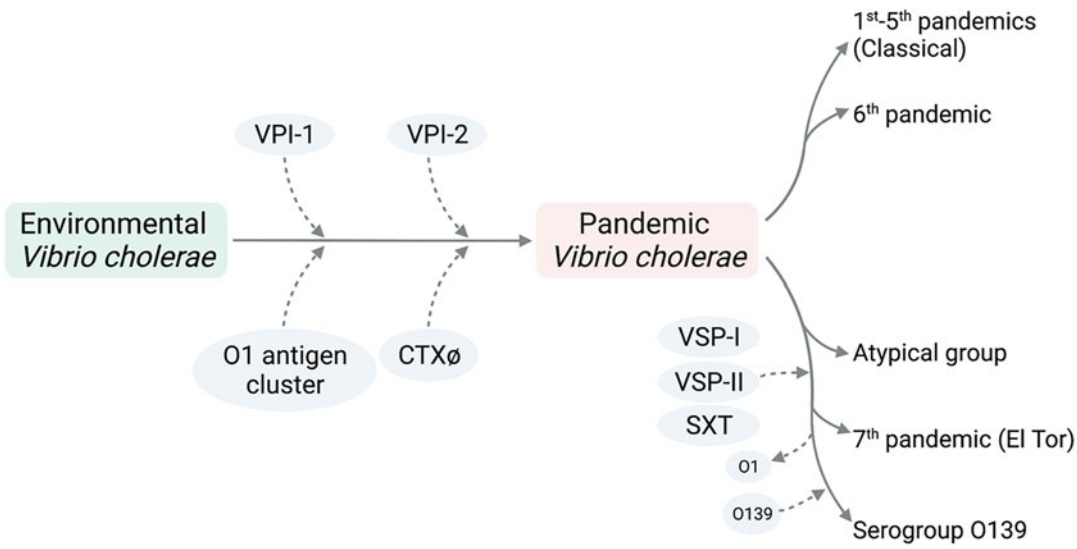


**Fig. 7.2** Evolution and emergence of pathogenic *Vibrio cholerae*. Schematic of the various steps involved in the acquisition of major virulence factors by environmental strains of *V. cholerae* to gain pandemic potential. VPI-1, *Vibrio* pathogenicity island-1; VPI-2, *Vibrio* pathogenicity island-2; CTXΦ, CTX phage; VSP-1, *Vibrio* seventh pandemic island-1; VSP-2, *Vibrio* seventh pandemic island-2; SXT, *Vibrio cholerae*-derived integrative and conjugative element

## 7.3 Pandemic Spread of *V. cholerae*

Cholera has been endemic in Asia, specifically in the Ganges delta of the Bay of Bengal, Bangladesh, and India for centuries (Barnett 2019). From there, the disease has spread sporadically to other parts of the world at various times since the early 1900s, primarily mediated by human activities (Fig. 7.3) (Orata et al. 2014; Poirier et al. 2012). Cholera is thought to have been introduced in Africa, Latin America, the Caribbean, Europe, and North America via infected humans, resulting in seven pandemics to date (Fig. 7.3) (Domman et al. 2017; Weill et al. 2017). In endemic regions, cholera shows seasonal peak patterns, typically associated with the monsoon rains and subsequent flooding (Sack et al. 2003). It is well established that climate is a major driver of the disease, with several abiotic and biotic factors such as temperature of the water bodies, precipitation, flooding, and plankton blooms influencing the frequency of outbreaks (Jutla et al. 2013; Koelle 2009; Pascual et al. 2000).

Phylogenomic analyses reveal that the seventh pandemic El Tor strains can be subdivided into three major groups that likely represent different waves of pandemic transmission (Mutreja et al. 2011). Correlating genomics data with the global spread of the disease reveals that the seventh pandemic originated from a distinct geographical location in Asia but has subsequently spread in three overlapping waves ((Mutreja et al. 2011); Fig. 7.3). Wave 1 isolates (1938–1961) encode the canonical CTX El Tor (CTX-1) and lack the Integrative conjugative element (ICE) SXT/R391 that encodes antibiotic resistance genes. The cholera outbreak in several countries in South America occurred during this period (Balasubramanian et al. 2021). The South American isolates form a discrete cluster with a single Angolan isolate that harbor novel, uncharacterized genes in the VSP-II and a novel genomic island WASA1 (Mutreja et al. 2011; O'Shea et al. 2004a). The transition from wave 1 to wave 2 was likely mediated by the acquisition of the SXT/R391 ICE (Mutreja et al. 2011). Wave 2 isolates (1978–1984) also encode a variant of the cholera toxin, CTX-2.

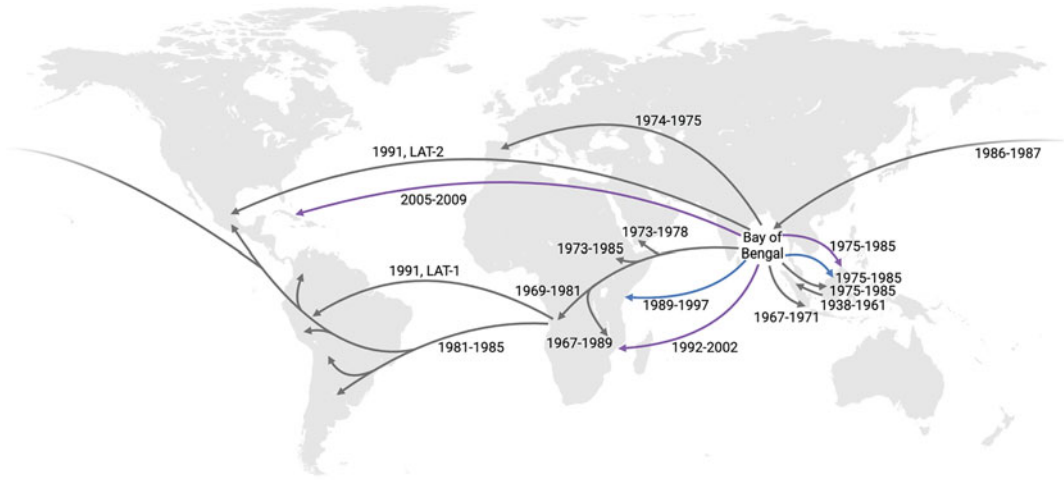


**Fig. 7.3** Global spread of cholera. The El Tor biotype of *Vibrio cholerae*, the causative agent of the seventh and current cholera pandemic, originated in the 1960s in the Bay of Bengal and spread out to the rest of the globe in three distinct but overlapping waves, as determined by phylogenetic analyses; wave 1 (gray), wave 2 (blue) and wave 3 (purple)

Interestingly, there were a high number of SNPs (3161 SNPs) within the SXT locus between the various wave 2 isolates compared to the SNPs defining the whole genome phylogeny (1757 SNPs), suggesting either a higher rate of recombination within the SXT locus or independent acquisition events from different sources (Mutreja et al. 2011). Wave 1, which spread globally, was replaced by the more geographically restricted waves 2 and 3 in more recent years and have been limited to nations in Africa and south Asia. The more recent outbreaks in Haiti, for example, are part of wave 3, where the strains share a common ancestor with south-Asian strains, suggesting direct and recent transmission (Chin et al. 2011; Mutreja et al. 2011). Interestingly, El Tor was replaced for a brief period in 1992 by a non-O1 serogroup strain, designated O139 Bengal that affected mostly coastal villages around the endemic regions of the Bay of Bengal (Alam et al. 2006; Nair et al. 2002; Ramamurthy and Sharma 2014). Despite two further outbreaks by O139 in 2002 and 2005, the El Tor biotype has remained dominant worldwide (Alam et al. 2006).

## 7.4 Cholera Pathogenesis

Upon entry into the human host, the first major barrier that *V. cholerae* encounters is the acidic environment of the stomach, which likely explains the high dose needed for successful infection (Almagro-Moreno et al. 2015). The cells that survive this barrier enter the small intestine and utilize chemotaxis to rapidly move toward the epithelial cells (Almagro-Moreno et al. 2015). The small intestine represents the primary site of bacterial replication and pathogenesis. Colonization of the small intestine requires the production of the toxin co-regulated pilus (TCP) (Taylor et al. 1987). TCP likely recognizes an unknown receptor on the intestinal epithelial cells and mediates microcolony formation, which is essential for intestinal colonization. Additionally, TCP also plays a role in bacterial survival in the environment by mediating attachment to the surface of chitin, enabling biofilm formation (Reguera and Kolter 2005). TCP production is coupled with the secretion of the cholera toxin (CT), a prototypical and potent $AB_5$-type toxin with one enzymatically active A subunit surrounded by a pentameric B subunit (Beddoe

et al. 2010). The CT holotoxin binds the GM1 monoganglioside receptors on the epithelial cell surface of the small intestine. The binding of CT triggers endocytosis of toxin-containing vesicles through the endoplasmic reticulum, where the catalytic A subunit dissociates from the B pentamer and is transferred to the cytosol. In the cytosol, A subunits of CT trigger the activity of adenylate cyclase, a regulatory G-protein, resulting in elevated levels of intracellular cAMP and hypersecretion of water and electrolytes into the intestinal milieu (Holmgren et al. 1973; Van Heyningen et al. 1971). The excess fluid secretion far exceeds the reabsorption capacity of the intestine, resulting in the characteristic rice water diarrhea that is typical of cholera (Field et al. 1972). Interestingly, CT also provides nutrients to the bacterium such as fatty acids, fostering its proliferation and growth (Rivera-Chavez and Mekalanos 2019).

## 7.5 Genetic Determinants of Cholera Pathogenesis

The genome of *V. cholerae* N16961 El Tor O1 was first sequenced in the year 2000 (Heidelberg et al. 2000). It consists of two circular chromosomes, 2.9 and 1 Mb in size (Heidelberg et al. 2000). The chromosomes are non-homologous and functionally divergent. For instance, chromosome I encodes genes involved in several critical aspects of cell survival such as DNA replication and repair, transcription and translation, metabolism pathways and cell wall biosynthesis, and those involved in pathogenesis (Heidelberg et al. 2000). Chromosome II also encodes several essential genes such as those involved in metabolism, and ribosomal and tRNA biosynthesis, but the function of a majority of these genes remains unknown (Heidelberg et al. 2000).

The ability to acquire and exchange genetic information is a crucial feature driving the emergence and success of bacterial pathogens (Arnold et al. 2022; Balasubramanian et al. 2022). Genetic exchange is facilitated by mobile genetic elements (MGEs), segments of DNA that mediate the movement of genes within and between bacteria, and include PAIs, plasmids, transposons, ICEs, and prophages, many of which encode genes involved in virulence (Balasubramanian et al. 2022). Interestingly, both *V. cholerae* El Tor N16961 chromosomes harbor acquired genes that were horizontally acquired and are integral to the pathogenic success of the bacterium. Some of these elements, as discussed below, encode critical virulence genes such as CT and TCP.

## 7.6 CTXΦ Phage

Bacteriophages are major vehicles for the acquisition of genetic material by numerous bacterial pathogens and are commonly associated with the gain of toxin-encoding genes among other horizontally acquired genes (Svab et al. 2015; Waldor and Mekalanos 1996; Wirtz et al. 2009). In the context of pandemic *V. cholerae*, lysogenic conversion by a M13-like filamentous phage named CTXΦ, led to the acquisition of the structural genes for CT: *ctxAB* (Fig. 7.4) (Waldor and Mekalanos 1996). Additionally, CTXΦ also encodes *zot* (zonula occludens toxin) and *orfU,* both being involved in phage morphogenesis, *ace*, which encodes an accessory cholera enterotoxin, and *cep,* encoding a core-encoded pilin that forms the virion capsid (Waldor and Mekalanos 1996). The CTXΦ phage is structured like a compound transposon with the toxin-encoding genes flanked by one or more copies of a 2.7 kb repetitive sequence (RS) (Mekalanos 1983; Pearson et al. 1993). The genes within the repetitive sequences (*rstABCR*) encode a site-specific recombination system that mediates integration at the attRS1 site on the chromosome of non-toxigenic *V. cholerae* strains, thus facilitating lysogenic conversion. Interestingly, TCP is the bacterial receptor recognized by CTXΦ, suggesting a sequential emergence of pathogenic features in *V. cholerae*.

The differences between the classical and El Tor strains of *V. cholerae* can be partly explained by variations in CTXΦ genes (Robins and Mekalanos 2014). For instance, mutations in the

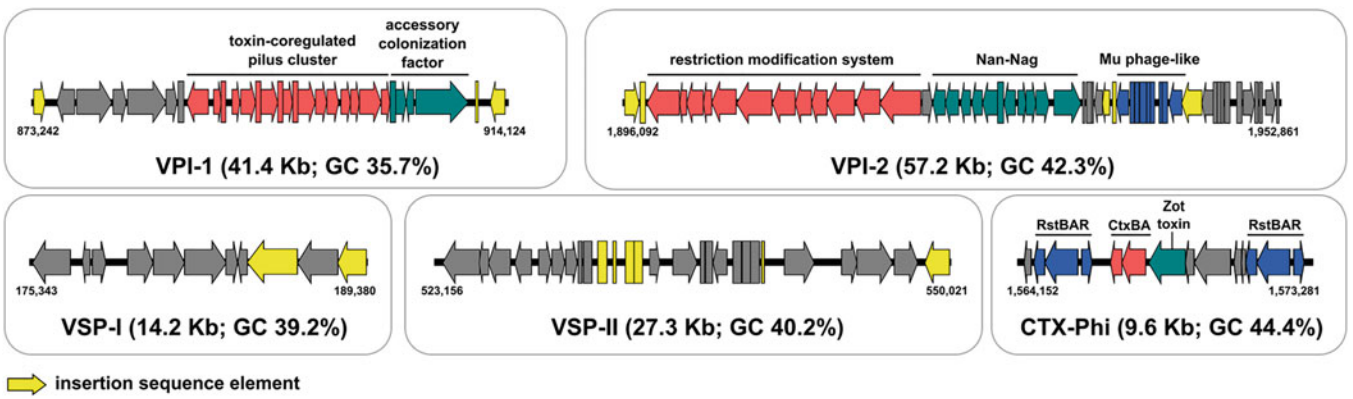


**Fig. 7.4** Mobile genetic elements responsible for *Vibrio cholerae* pathogenicity. Genetic organization and functional annotation of the *V. cholerae* MGEs that encode the major pathogenic features of the bacterium, based on our analysis of 1500 sequenced genomes of *V. cholerae*. Yellow arrows: IS element, gray arrows: unknown function

repressor coded for by *rstR* within CTXΦ is sufficient to confer biotype specificity (Kimsey and Waldor 1998). Several nonsynonymous SNPs in the *ctxB* gene allow for the differentiation between classical and El Tor genomes (Robins and Mekalanos 2014). Furthermore, although CTXΦ can be integrated at the *dif* site either as a single copy or in tandem in strains of *V. cholerae*, integration is limited to chromosome I in El Tor but can be found in either chromosome in the classical 0395 strain (Mekalanos 1983). This differential distribution of CTXΦ between the chromosomes of classical and El Tor strains can be attributed to variations in the *dif* integration sites of the bacterial chromosome (Das et al. 2010). Sequence variations have also been identified in the RS1/RS2 regions flanking the toxin-encoding genes on the phage genome. Both RS1 and RS2 regions are important for the stable acquisition of CTXΦ and encode genes involved in phage integration, replication, and regulation. Whereas El Tor strains have both RS1 and RS2, unlike classical strains that have only RS2, the El Tor RS1 region codes for an additional anti-repressor gene (*rstC*) that significantly increases CTXΦ production and *ctxAB* expression (Davis et al. 2002). Irrespective of these sequence variations, acquisition of CTXΦ by non-toxigenic strains of *V. cholerae* represents a critical step in the emergence of pathogenicity and has contributed significantly to their pandemic potential.

## 7.7 Pathogenicity Islands

PAIs are large mobile genetic elements that encode genes for chromosomal integration and excision in addition to cargo genes that contribute to bacterial virulence such as secretion systems, hemolysins, transporters, or pili (Hochhut et al. 2005). PAIs form a distinct lineage of MGEs and can integrate specifically at tRNA loci on the bacterial chromosome, precisely excise and form non-replicative, non-self-mobilizable circular intermediates (Boyd et al. 2009). Toxigenic *V. cholerae* strains harbor four PAIs that have played a crucial role in its emergence (Fig. 7.2): *Vibrio* pathogenicity island-1 (VPI-1), *Vibrio* pathogenicity island-2 (VPI-2), *Vibrio* seventh pandemic island-I (VSP-I) and *Vibrio* seventh pandemic island-II (VSP-II). The latter two are only encoded by strains from the seventh pandemic.

### 7.7.1 *Vibrio* Pathogenicity Island-1

The 31 genes encoded within *Vibrio* pathogenicity island-1 (VPI-1) lie at the heart of the pathogenicity of toxigenic *V. cholerae*. The 39.5 kb island has typical characteristics of other genomic islands: a distinct G+C content of 35% (rest of the genome: 47%), is located downstream of a tmRNA locus, is flanked by direct repeats (*attL* and *attR*) and encodes a transposase and an integrase (Fig. 7.4) (Kumar et al. 2020). Importantly, VPI-1 encodes genes that mediate bacterial colonization including the *tcp* genes required for the synthesis and assembly of TCP, and accessory colonization factors (*acf*) (Karaolis et al. 1998; Kovach et al. 1996). VPI-1 also encodes several critical virulence regulators such as ToxT, TcpP, and TcpH that, together with the regulator ToxR found on the core genome, are central to CT production and the virulence regulatory cascade in *V. cholerae* (Boyd et al. 2000; Karaolis et al. 1998; Kovach et al. 1996; Taylor et al. 1987). Transmission of VPI-1 between *V. cholerae* strains has been demonstrated. The integrated VPI-1 can excise from the chromosome and circularize but cannot self-mobilize (Rajanna et al. 2003). Nonetheless, it can be transferred between *V. cholerae* O1 strains by generalized transduction mediated by phages, allowing dissemination within the population, and represents an interesting example of the interaction between MGEs in shaping the evolution of bacterial pathogenicity (O'Shea and Boyd 2002).

Interestingly, the VPI-1 locus is widely conserved in the genomes of the epidemic and pandemic strains but is only sporadically found in the non-O1-non-139 environmental strains (Chun et al. 2009; Domman et al. 2017; Weill et al. 2019). Although found in both classical and El Tor strains, over 480 single nucleotide variations

have been identified within VPI-1 between the biotypes, especially among the TCP-encoding genes such as *tcpA* that codes for the major pilin protein (Karaolis et al. 2001). Overall, the acquisition of VPI-1 and subsequently the lysogenic CTXΦ were likely the two most important steps that led to the emergence of pathogenic traits in non-toxigenic environmental *V. cholerae* strains.

### 7.7.2 *Vibrio* Pathogenicity Island-2

The *Vibrio* pathogenicity island-2 (VPI-2) is a 57.3 kb island with a G+C content of 42% (rest of the genome: 47%). VPI-2 is located at the tRNA-serine locus, and codes for two putative integrase/excisionase (Fig. 7.4) (Jermyn and Boyd 2002; Murphy and Boyd 2008). Like VPI-1, VPI-2 can excise from the genome to form circular, non-self-mobilizable intermediates (Murphy and Boyd 2008). VPI-2 encodes 52 ORFs that can be functionally divided into three major regions (Fig. 7.4) (Jermyn and Boyd 2002). The first region comprises several genes that encode a type-1 restriction-modification system as well as chemotaxis proteins (Jermyn and Boyd 2002). The second major region encodes a Mu-phage-like (Jermyn and Boyd 2002). The third region, the *nan-nag* region, encompasses 12 genes and is responsible for sialic acid scavenging, uptake, and catabolism (Fig. 7.4). VPI-2 is present in all *V. cholerae* O1 isolates and variants of the PAI can be found in some non-toxigenic ones (Dziejman et al. 2005; Schwartz et al. 2019). Interestingly, most O139 serogroup strains exhibit major deletions in VPI-2 (Jermyn and Boyd 2002, 2005). This has been linked with their demise as the main source of cholera and virtual extinction (Jermyn and Boyd 2002, 2005). Some non-O1-non-O139 isolates that can cause gastroenteritis encode a truncated version of VPI-2 that retained the *nan-nag* region and also harbor genes encoding a type-3 secretion system that is absent in the O1 El Tor strains (Chen et al. 2007).

The *nan-nag* region of VPI-2 plays numerous roles in the virulence and colonization of toxigenic *V. cholerae*. For instance, the NanH neuraminidase is involved in the scavenging of sialic acid and converts higher-order sialogangliosides found on the intestinal epithelial cell surface into GM-1 gangliosides. As aforementioned, these gangliosides act as the receptors of CT, thus, NanH increases the number of available toxin receptors on host cell surfaces (Jermyn and Boyd 2002). The gut mucosal environment is rich in sialic acids (Almagro-Moreno and Boyd 2009a, 2010). The use of sialic acid as a carbon source allows the bacterium to exploit the host environment during the early stages of colonization and confer a survival advantage during infection (Almagro-Moreno and Boyd 2009b). Specifically, mutants unable to produce the aldolase that is necessary for the first step of sialic acid catabolism (NanA), exhibit colonization defects during the early stages of intestinal colonization of a vertebrate animal model (Almagro-Moreno and Boyd 2009b). Besides carbon usage, a possible reason for the colonization defect of the *nanA* mutants is reduced chemotactic motility in response to oligosaccharides found in mucin, specifically N-acetylneuraminic acid and N-acetyl glucosamine (Reddi et al. 2018). The catabolic pathways of these compounds converge in *V. cholerae* to produce glucosamine-6-phosphate (GlcN-6P), and mutants unable to produce GlcN-6P show reduced chemotactic motility toward mucin (Reddi et al. 2018). Furthermore, the production of GlcN-6P is required for the induction of motility in the presence of environmental reservoirs such as crustaceans or cyanobacteria, functioning as common signals in the host and environment that dictate the lifestyles of *V. cholerae* (Reddi et al. 2018).

Some insights have been gained into the molecular mechanisms of VPI-2 dissemination within *V. cholerae* populations. Recombination directionality factors (RDFs) are transcriptional repressors that mediate PAI excision by suppressing integrase expression (Carpenter et al. 2015). VPI-1 does not encode an RDF and can excise efficiently even without it. However, the RDFs VefA and VefB encoded on VPI-2 can also efficiently excise VPI-1, revealing crosstalk between separately acquired MGEs that influence their spread to the non-pathogenic strains within

the population (Carpenter et al. 2015). Recent studies have also identified the fascinating roles of VPI-2 in regulating foreign DNA uptake (discussed below) that highlights the non-canonical roles of MGEs in shaping the evolution of virulence in pathogens.

### 7.7.3 *Vibrio* Seventh Pandemic Island I

The *Vibrio* seventh pandemic island I (VSP-I) is a 16 kb region that has a G+C content of 40% (Fig. 7.4). Similar to the other PAIs encoded by toxigenic *V. cholerae*, VSP-I can excise from the genome and form circular intermediates allowing for horizontal gene transfer of the island (Murphy and Boyd 2008). VSP-I is found only in O1 El Tor and O139 strains isolated from the seventh pandemic and is absent in isolates from the sixth pandemic (O1 classical), pre-pandemic or non-toxigenic strains (Dziejman et al. 2002; O'Shea et al. 2004b). VSP-I, together and VSP-II, are thought to be responsible for the success of the seventh pandemic clone of toxigenic *V. cholerae* (Dziejman et al. 2002; Grim et al. 2010; Taviani et al. 2010).

VSP-I encodes 11 genes including a transposase, an integrase, a transcriptional regulator VspR (VC0665), a patatin-related protein, and several encoding hypothetical proteins (Dziejman et al. 2002). Although the roles of many of the genes in influencing pathogenesis are not known, recent studies have shed light on the importance of VSP-I. VspR regulates the expression of several VSP-I genes including one that encodes a novel class of di-nucleotide cyclase, DncV (Davies et al. 2012). DncV is required for efficient intestinal colonization and downregulates chemotaxis facilitating host adaptation (Davies et al. 2012). Interestingly, the virulence regulator ToxT, which is encoded within VPI-1, activates the expression of a VPI-1-encoded small RNA TarA that represses *vspR* expression affecting DcnV levels, and consequently host colonization (Davies et al. 2012). Such elegant mechanisms of crosstalk between MGEs and the core genome not only attest to the successful integration of acquired DNA within the regulatory networks of the bacterium but also for its crucial role in the pandemic success of *V. cholerae*.

Recent work also suggests that VSP-I may function as a phage defense system and warrants further investigation into the fascinating 'repurposing' of existing processes for novel applications by the bacterium (Cohen et al. 2019; Hsueh et al. 2022). For instance, a second gene (*dcdV, VC0175*) that co-occurs with *dncV* (discussed above) on VSP-I, is involved in phage defense. A major challenge faced by lytic phages is to rapidly replicate multiple copies of its genome in a short window of time, a process that requires sufficient nucleotide substrates (Kreuzer and Brister 2010). DcdV functions as a deoxycytidylate deaminase that depletes cellular nucleotide concentrations of dCTP and dCMP, and thus protects the bacterial population from phage infection (Hsueh et al. 2022).

### 7.7.4 *Vibrio* Seventh Pandemic Island II

The *Vibrio* seventh pandemic island II (VSP-II) is a 26 kb island that contains 27 annotated ORFs (Fig. 7.4) (Taviani et al. 2010). Even though its function remains poorly understood, VSP-II can excise from the genome and form a circular intermediate (Murphy and Boyd 2008). The roles of some VSP-II genes have been recently elucidated including integrases, endopeptidases, and the DdmABC system, which is involved in defense against incoming MGEs such as plasmids and phages (discussed below) (Jaskolska et al. 2022; Murphy and Boyd 2008; Murphy et al. 2019). Most genes within the PAI are hypothetical or uncharacterized and include transcriptional regulators, methyl-accepting chemotaxis proteins, and a phosphodiesterase (O'Shea et al. 2004a). Interestingly, VSP-II genes are not induced under standard laboratory conditions and recent studies have begun to identify the conditions that favor their expression (Mandlik et al. 2011). For instance, many of the VSP-II genes are induced during zinc starvation and

play a role in chemotaxis (Murphy et al. 2021). However, the relationship between zinc starvation and host infection remains unclear (Kamp et al. 2013; Sheng et al. 2015). Interestingly, there is a variation in the cargo of different VSP-II variants even among the El Tor strains (Murphy et al. 2021). For instance, the *V. cholerae* O1 El Tor strain isolated from Peru in 1991 does not encode *vc0511-vc0515* whereas the Haiti isolate (2010) lacks *vc0495-vc0512* (Murphy et al. 2021). Nevertheless, the presence of VSP-II exclusively in the seventh pandemic strains, and their recently identified roles in preventing the uptake of MGEs and shaping the evolution of *V. cholerae* El Tor (see Sect. 7.10 below), warrants further research into the role of this island in pathogenicity and the emergence of the seventh cholera pandemic.

## 7.8 Super Integron on Chromosome II

Integrons are 100–200 kb genetic elements that can capture and promote the expression of horizontally acquired gene cassettes. They are structurally simple and are composed of a proximal recombination site, an integrase and one or more promoters that drive the expression of the captured MGE (Mazel et al. 1998; Partridge et al. 2018). The *V. cholerae* super integron (a large integron island) is 120 kb in size, comprising of ~3% of the bacterial genome, and is located on chromosome II (Marin and Vicente 2013). It consists of an integrase gene, a cassette promoter (Pc) and the primary recombination site (*attI*). Approximately 207 ORFs have been identified in the super integron that are arranged in several cassettes, which generally consist of a promoterless ORF flanked by two repeats that function as recombination sites (Marin and Vicente 2013). The super integron consists of 21 core genes that are conserved between the strains, most of them present in multiple copies and distributed along the entire element with no positional conservation. On the other hand, the presence and number of the non-core genes is variable, largely dependent on the niche and type species (Marin and Vicente 2013), and likely facilitate adaptation. Functional categorization of the core genes within the super integron suggests that they play a varied set of roles such as transcription, replication, recombination and repair, translation, or ribosomal structure and biogenesis. Although the exact roles remain largely uncharacterized, some insights have been gained over the past decades. For instance, the super integron encodes genes involved in secondary metabolism and cell surface modification (Boucher et al. 2011; Chun et al. 2009). Specifically, (1) capsular biosynthetic proteins that have been directly implicated in virulence, (2) plasmid *Achromobacter* secretion factors that facilitate toxin secretion, and (3) lipocalins that enable host colonization (Chun et al. 2009). Additionally, the super integron also confers resistance to several antibiotics including aminoglycosides and fosfomycin, as well as some virulence factors such as the heat-stable toxin gene (*sto*), mannose-fucose-resistant hemagglutinin (*mrhA*) (Mazel 2006). Additionally, the super integron of *V. cholerae* N16961 codes for 13 toxin-antitoxin systems, six of which are part of the core SI genes. Of these, *higBA* system that encodes mRNA cleaving enzymes and can stabilize plasmids, and *higBA-1* TA locus (uncharacterized) are found in all clinical *V. cholerae* strains and plays a role in stabilizing acquired plasmids and the super integron cassettes (Christensen-Dalsgaard and Gerdes 2006; Marin and Vicente 2013; Rowe-Magnus et al. 2003). Generally, the integrons are recognized for their association with antibiotic resistance. However, in *V. cholerae*, antibiotic resistance is mostly associated with the SXT element (discussed below) and the super integron is primarily linked to bacterial fitness, phage resistance, and survival under stressful conditions as well as virulence, expanding the role of integrons in bacterial evolution. Given the large size and interactive nature of the super integron, future research will uncover novel features and functions of this fascinating and crucial element in toxigenic *V. cholerae* evolution.

## 7.9 The Integrative and Conjugative Element, SXT

Integrative and conjugative elements (ICEs) are a class of MGEs primarily found integrated on the chromosome and are related to conjugative transposons (Ryan et al. 2016). They can excise from the chromosome, are non-replicative, and can be transferred to neighboring cells via conjugation (Botelho et al. 2020). Like PAIs and conjugative transposons, they encode an integrase and excisionase that mediates mobility, chromosomal attachment sites (*att*), an origin of transfer (*oriT*), and genes encoding conjugal transfer proteins (Botelho et al. 2020). *V. cholerae* strains from the seventh pandemic display enhanced levels of resistance to sulfamethoxazole, trimethoprim, streptomycin, and furazolidone (Johnson et al. 1994; Nair et al. 1994; Waldor and Mekalanos 1994). Resistance to three of these antibiotics, the former two being some of the most widely used, is due to the acquisition of the ICE element SXT. SXT is a ~100 kb ICE originally identified in a *V. cholerae* O139 isolate from India (Cholera Working Group 1993). Conjugative transfer between strains is mediated by a 25 kb region that encodes the *tra* genes (Beaber et al. 2002). SXT integrates at the 5′ end of *prfC*, a gene located on chromosome I that encodes a peptide chain release factor 3 (Hochhut and Waldor 1999). Integration involves a site-specific recombination event between the 17 bp nearly identical *attP* (SXT) and *attB* (chromosome) sites. Chromosomal integration and excision of the SXT element is mediated by an SXT-encoded tyrosine recombinase Int (Burrus et al. 2006). In some cases, tandem SXT arrays are formed during conjugation that can use the same integration site (*prfC*) (Hochhut et al. 2001). Interestingly, SXT can also mobilize the transfer of other plasmids RSF1010 and CloDF13 in *trans* as well as chromosomal DNA flanking the integration site (Hochhut et al. 2000). Although little is known about additional roles of SXT outside of antibiotic resistance, they appear to play a wider role in horizontal gene transfer among Gram-negative bacteria.

## 7.10 Inhibition of DNA Uptake

*V. cholerae* must balance the acquisition and expression of potentially beneficial traits against the indiscriminate uptake and assimilation of costly and/or deleterious exogenous genetic material. The bacterium has intricate adaptive mechanisms to address this complex trade-off (Almagro-Moreno 2022; Balasubramanian et al. 2022). Whereas integrons (discussed above) favor the successful maintenance and expression of acquired genes, DNA assimilation is inhibited by either restriction of incoming DNA via clustered regularly interspaced short palindromic repeat (CRISPR)-associated proteins (Cas proteins) systems or by preventing expression via **xenogeneic silencers** (Fig. 7.5). Furthermore, recently, novel mechanisms that prevent the acquisition and establishment of foreign DNA have been unearthed.

### 7.10.1 CRISPR-Cas

CRISPR-Cas systems are an adaptive immunity mechanism that degrades incoming foreign DNA, such as those acquired by HGT (Barrangou and Marraffini 2014). CRISPR-Cas systems can interfere with HGT as their spacer sequence-mediated 'immune recognition' mechanism cannot differentiate foreign DNA based on their functional role (Fig. 7.5). CRISPR-Cas systems appear to provide cells with a "check and balance" mechanism to avoid indiscriminate uptake of foreign DNA. Interestingly, in *Vibrio* species, CRISPR-Cas systems have been predominantly identified within MGEs such as PAIs, plasmids, and transposon-like elements and this mobility could have led to novel variant CRISPR subtypes (Mcdonald et al. 2019). However, although functional CRISPR-Cas systems are prevalent in the Classical O1 and in non-choleragenic strains, they

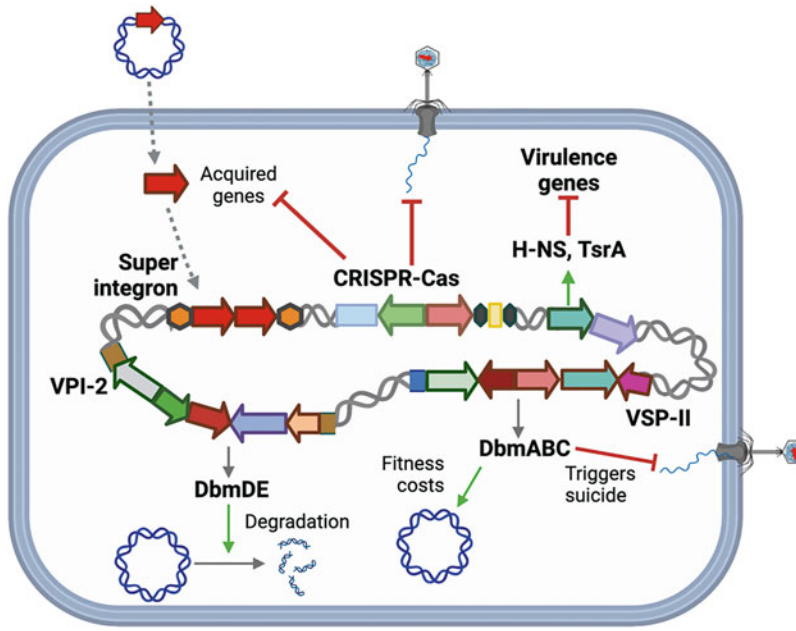


**Fig. 7.5** Mechanisms of expression and inhibition of acquired DNA in *Vibrio cholerae*. The uptake of non-native DNA can be prevented by CRISPR-Cas systems. The fate of DNA that has been taken up by the cell can have one of several outcomes: (1) successful assimilation and expression of foreign DNA is facilitated by the super integron on chromosome II of *V. cholerae* that has promoters (orange hexagons) driving the expression of downstream integrated DNA, (2) indiscriminate expression of non-native DNA can be temporally silenced by xenogeneic silencer proteins such as H-NS and TsrA, or (3) foreign DNA can be degraded or diluted out rapidly over generations by one of several mechanisms encoded on MGEs such as DdmABC or DdmDE. Together, the systems maintain a healthy balance between uptake and indiscriminate expression, enabling bacterial survival

are largely absent in some representative El Tor O1 strains of *V. cholerae* (Box et al. 2016; Mcdonald et al. 2019). This suggests that CRISPR-Cas systems might have played a role in the emergence of pathogenesis in El Tor O1 but were eventually selected out of the current pandemic strains, although the timelines involved are not clear. The evolutionary forces that resulted in the CRISPR-Cas counterselection in El Tor strains might provide additional insights into the gene mobility and the emergence of pathogenic traits in non-pathogenic bacteria.

### 7.10.2 Xenogeneic Silencers

Xenogenic silencer (XS) proteins offer a means for the cell to reversibly repress the expression of incoming genes (Fig. 7.5) (Singh et al. 2016). XS homologs are found in different bacteria where they bind the foreign DNA that is typically AT-rich, preventing indiscriminate expression of acquired DNA (Singh et al. 2016). In *V. cholerae*, the histone-like nucleoid structuring protein, H-NS, negatively regulates gene expression at multiple phases of the bacterial life cycle and represses numerous critical promoters of its virulence cascade including *tcpP*, *toxT*, *tcpA*, and *ctxAB* (Ayala et al. 2017; Kovacikova and Skorupski 2001; Nye et al. 2000; Wang et al. 2015). As discussed above, many virulence-associated phenotypes in *V. cholerae* are encoded on MGEs. Given that H-NS silences acquired DNA, it is not surprising that this XS protein regulates the expression of ~20% of genes associated with virulence, surface attachment,

biofilm formation, motility, and chemotaxis (Wang et al. 2012, 2015). Additionally, H-NS also silences the expression of ancillary toxins produced by *V. cholerae* outside of the ToxR regulon such as hemolysin (*hlyA*) and the repeat toxin (RTX) (Olivier et al. 2007). Consequently, transcriptional activation from these silenced promoters requires displacement of H-NS and "derepression" (Ayala et al. 2017). Similarly, TsrA is an H-NS homolog that was recently found to transcriptionally silence virulence genes including genes coding for CT and TCP, and the type-VI secretion system (T6SS) (Caro et al. 2020). Overall, XS proteins offer a rapid and reversible means of targeting and silencing the expression of non-native DNA until the expression of these genes becomes advantageous for the specific physiological state of the cell.

### 7.10.3 PAI-Encoded Systems for Plasmid Degradation

Recently, two novel defense systems encoded within VSP-II and VPI-2, named DdmABC and DdmDE, respectively (Jaskolska et al. 2022). These two systems cooperate to degrade and rapidly eliminate small multicopy plasmids from the cells. The DdmDE system encoded within VPI-2 is composed of two proteins that can eliminate small plasmids from most cells within ten generations by degrading them (Jaskolska et al. 2022). DdmABC, encoded on VSP-II, enhances this activity either by clustering the plasmids or by directly degrading them. In addition, DdmABC confers protection against phage infection by the classical abortive infection (altruistic cell death) model. Interestingly, DdmABC also eliminates larger conjugative plasmids by counterselecting against them in a manner similar to abortive infection (Jaskolska et al. 2022). Together, these two systems explain why the seventh pandemic strains of *V. cholerae* lack plasmids and represent a robust mechanism for preventing indiscriminate DNA uptake.

## 7.11 Genomic Preadaptations to Pathogenesis: Virulence Adaptive Polymorphisms

For decades it has remained enigmatic why only O1 and O139 strains from the pandemic group emerged to cause pandemic cholera in humans. As exemplified above, (a) the essential virulence factors of toxigenic *V. cholerae* are encoded within MGEs and (b) numerous non-pathogenic environmental strains encode virulence traits. It is therefore not feasible that this phylogenetically confined distribution is due to the presence/absence of virulence genes. Recently, we hypothesized that toxigenic *V. cholerae* must possess a unique genomic background that encodes preadaptations to virulence rendering it susceptible to emerging as a pandemic pathogen. Specifically, we determined that pandemic strains encode these genomic preadaptations in the form of what we term virulence adaptive polymorphisms (VAPs), which appear to occur in the environment prior to host selective pressures (Shapiro et al. 2016). VAPs are allelic variations in core genes that confer preadaptations to virulence (Shapiro et al. 2016).

One gene encoding VAPs, *ompU*, codifies a major outer membrane porin that plays a critical role in intestinal colonization and resistance to antimicrobials (e.g., bile) (Provenzano et al. 2001; Shapiro et al. 2016; Sperandio et al. 1995). Expression of a variety of environmental *ompU* alleles in an isogenic *V. cholerae* O1 background indicates that alleles that do not encode VAPs cannot confer these virulence properties. Interestingly, strains with VAP-encoding environmental *ompU* alleles exhibit a similar phenotype as clinical ones, indicating that these adaptations to virulence are circulated in the environment and are present in the genomic background of this pathogen prior to host colonization (Shapiro et al. 2016). Expression of *ompU* is regulated by the master virulence regulator ToxR, which is encoded within the core genome (Crawford et al. 1998). Interestingly, ToxR controls the expression of virulence regulator ToxT, encoded within VPI-1, and CT,

encoded within the CTXΦ phage, demonstrating common regulatory pathways between VAPs and MGEs encoding virulence genes (Dirita and Mekalanos 1991; Krukonis et al. 2000). ToxR is also repressed by the histone-like nucleoid structuring protein H-NS, which typically silences acquired DNA (Ayala et al. 2017; Crawford et al. 2003; Krukonis et al. 2000). Overall, these recent findings suggest an intricately orchestrated molecular regulatory network involving the genomic background, including preadaptations in the form of VAPs, and elements acquired via HGT. Understanding the different layers involved in these processes will help decipher the forces that dictate the pandemic potential and emergence of toxigenic *V. cholerae*.

## 7.12 Emergence of Novel Serogroups

In addition to the pandemic-causing O1 serogroup, a new serogroup of *V. cholerae*, O139, emerged in Bangladesh and India in 1992 causing localized cholera outbreaks (Nair et al. 1994). The LPS of the O139 serogroup was very different from *V. cholerae* O1 and emerged due to the replacement of a 22 kb *wbe* locus that codes for the O-antigen polysaccharide in the O1 strains with a *wbf* region encoding the O139 antigen (Comstock et al. 1995; Mooi and Bik 1997; Stroeher et al. 1997). Interestingly, evidence suggests that the O139 *wbf* locus arose due to genetic rearrangements of DNA from several donors because parts of *wbf* have been identified in other non-O1 *V. cholerae* serotypes such as O22 (Stroeher et al. 1997; Yamasaki et al. 1999). Detailed molecular epidemiological analyses indicate that O139 strains are closely related to O1 El Tor strains in terms of virulence potential and disease severity ((Berche et al. 1994; Bhattacharya et al. 1993; Faruque et al. 2003), Morris et al. 1995). However, unlike the *wbe* locus of O1 strains, the *wbf* locus of O139 strains encodes a capsule (O-antigen capsule) and a modified core polysaccharide of the LPS that resulted in the seroconversion (Johnson et al. 1994; Waldor et al. 1994). Since the emergence of *V. cholerae* O139, several new genetic and phenotypic variants have emerged, including new ribotypes, CTX genotypes, and altered antibiotic resistance (Faruque et al. 1997, 1999; Mitra et al. 1996). The emergence of O139 represents the continued competition among two different serogroups and the evolution of fitter strains for enhanced survival.

## 7.13 Climate Change and Cholera

The rising global temperatures are a pressing threat to human and animal health and is leading to an increase in the propensity of emergent and reemergent infectious diseases (Rossati 2017; Semenza et al. 2022). Critically, climactic changes add another layer of unpredictability to modeling disease epidemiology and severity. *Vibrios* have been proposed as microbial indicators of a changing global climate (Lipp et al. 2002; Vezzulli et al. 2016). Specifically, cholera is an ideal model to understand how global warming increases the spread and severity of spatially and temporally confined diseases (Lipp et al. 2002; Vezzulli et al. 2016). Furthermore, abiotic conditions that lead to the proliferation of aquatic dwellers such as crustaceans, or cyanobacteria, indirectly promote the growth of *V. cholerae*, as the bacterium establishes close associations with them (Almagro-Moreno and Taylor 2013; Colwell 1996; Huq et al. 1984). For instance, fluctuations in the surface water temperature result in varying distribution of phytoplankton species, such as algal blooms, which strongly correlate with the incidence and number of cholera cases (Lipp et al. 2002). Analyses of the temporal cycles of cholera over 32 years suggest a role for environmental and climactic factors in increasing the frequency and duration of cholera outbreaks (Emch et al. 2008). Furthermore, metanalyses and modeling studies have identified a strong correlation between the sea surface temperature, weather events such as the El Niño-Southern Oscillation, and cholera incidence and severity (Anyamba et al. 2019; Asadgol et al. 2020; Pascual et al. 2000). Additionally, increased incidence of some abiotic factors such

as UV light due to ozone depletion can promote excision rates of lysogenic phages like CTXΦ resulting in lysogenic conversion of non-toxigenic environmental *V. cholerae* (Faruque et al. 2000). Higher temperatures can also serve as a selective pressure for strains possessing enhanced virulence potential (Vezzulli et al. 2020). Overall, several complementary lines of evidence suggest that we are already witnessing the effects of anthropogenic influence on disease outcomes (Brumfield et al. 2021).

## 7.14 Concluding Remarks

Despite cholera being largely preventable, the disease has remained a major source of human morbidity and mortality for centuries, primarily in regions of the world with limited access to clean water and sanitation infrastructure or due to natural or man-made disasters. This problem is further magnified by the nature of its causative agent, *V. cholerae*, which represents a quintessential example of adaptive evolution toward a disease phenotype. Toxigenic strains of the bacterium have evolved after a series of sequential (e.g., VAPs) and non-linear acquisition of several virulence factors on MGEs (e.g., VPI-1, CTXΦ) by non-pathogenic environmental isolates (Fig. 7.2). Several lines of evidence suggest the continued evolution of the pandemic strains, with the emergence of novel pathogenic serotypes, such as the atypical group and serogroup O139. Additionally, we are only beginning to uncover fascinating insights into the extent of crosstalk and novel modes of regulation allowing the bacterium to fully harness the acquired genes, far beyond the conventional modes of virulence gene expression.

Currently, the most effective means of controlling outbreaks are coordinated and multidisciplinary approaches focusing on robust public health monitoring and sanitary practices, and the effective use of vaccines and therapeutics, as proposed in a recent "blueprint" for the eradication of cholera (Islam et al. 2022). However, novel molecular insights into the mechanistic aspects of the bacterium could potentially offer new therapeutic targets. For instance, small molecule inhibitors targeting crosstalk of virulence gene expression show promise for being developed as therapeutic agents (Hung et al. 2005). Additionally, natural phage populations have been proposed to be an effective means of cholera control (Bhandare et al. 2019; Hsueh and Waters 2019). There is promise in exploring the manipulation of vibriophage populations to prevent or shorten the duration of cholera outbreaks. Therefore, the future of mitigation of cholera outbreaks will likely involve a combination of strategies (Islam et al. 2022) and serve as a model system for a robust public health infrastructure in infectious disease control.

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# Role of Bacteriophages in the Evolution of Pathogenic Vibrios and Lessons for Phage Therapy

8

Roberto C. Molina-Quiroz, Andrew Camilli, and Cecilia A. Silva-Valenzuela

**Abstract**

Viruses of bacteria, i.e., bacteriophages (or phages for short), were discovered over a century ago and have played a major role as a model system for the establishment of the fields of microbial genetics and molecular biology. Despite the relative simplicity of phages, microbiologists are continually discovering new aspects of their biology including mechanisms for battling host defenses. In turn, novel mechanisms of host defense against phages are being discovered at a rapid clip. A deeper understanding of the arms race between bacteria and phages will continue to reveal novel molecular mechanisms and will be important for the rational design of phage-based prophylaxis and therapies to prevent and treat bacterial infections, respectively. Here we delve into the molecular interactions of *Vibrio* species and phages.

**Keywords**

*Vibrio cholerae* · Bacteriophages · Phage therapy · Vibrio evolution

## 8.1 History of *Vibrio* Bacteriophages

More than 100 years ago, Nikolai Gamaleya described the first "bacteriolysin" of *Bacillus anthracis* that was able to dissolve (lyse) bacteria. Interestingly, this effect was different when compared to other antimicrobial agents, since it required 6–12 h for lysis and it was also transmissible as observed by the ability to recover this "lytic ferment" by serial passages (Gamaleya 1898; Bardell and Ofcansky 1982).

One of the first observations of a "substance" showing the ability to kill the cholera bacterium was reported in 1896. In this paper Hankin ME, meticulously showed by colony counting that cholera germs are killed in distilled water but they die faster when grown in the presence of filtered water samples collected from different locations of Ganges and Jamuna rivers (India). He did not observe the same killing effect when water samples were boiled. However, he was not able to discover the nature of the entity responsible for the microbial killing effect. Today we may confidently say that he was observing the activity phages present in the river waters of India (Hankin 1896).

R. C. Molina-Quiroz
Stuart B. Levy Center for Integrated Management of Antimicrobial Resistance (Levy CIMAR), Tufts Medical Center and Tufts University, Boston, MA, USA

A. Camilli
Department of Molecular Biology and Microbiology, Tufts University, School of Medicine, Boston, MA, USA

C. A. Silva-Valenzuela (✉)
Microbes Lab SpA, Valdivia, Los Ríos, Chile
e-mail: cecilia@microbes-lab.com



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The first designation of the virus of bacteria concept was assigned by Twort during the early 1900s based on his observations of a "transparent material that is able to stop the growth of a micrococcal colony." He proposed that this ultramicroscopic agent might correspond to a living organism with a lower organization than bacteria or amoeba (Twort 1915). However, it was only 2 years later that Félix d'Herelle observed "circles on which culture is non-existing." In this work, he elegantly described that by dilution, he was able to quantify the "live germ" which he propagated and could recover after reinnoculation. Finally, he determined "(1) that this microorganism was specific for shiga [*Shigella*] culture," (2) "allowed the immunization [not in the modern sense] of rabbits that otherwise were killed in 5 days and he was able to recover this invisible microbe from patients recovering from dysentery" (d'Herelle 2007). A more definitive report of the phage concept was later published in 1921 (d'Herelle 1921).

Inspired by the work of Louis Pasteur, d'Herelle started to travel around the world (1914–1927) studying phages in their natural habitats. He collected samples from different sources such as animal's feces and water and isolated phages that were specific for different bacteria such as *Escherichia coli*, *Salmonella* spp., *Proteus vulgaris*, *Corynebacterium diphtheriae*, *Bacillus subtilis*, *Agrobacterium tumefaciens,* and others. He documented this travel and his findings in a more than 700 typed pages memoir that he named "Les peregrinations d'un Microbiologiste" or "The Pilgrimage of a Microbiologist" (d'Herelle).

Using serial passages of the lysate (or ferment as he named it), d'Hérelle observed that he could lyse cultures (dissolve bacterial cultures in his words) and established that: "The bacteriophage corpuscle is a living ultramicroscopic being" and "A bacteriophage is, therefore, of necessity a virus, a parasite of bacteria." Importantly, based on his work, he stated: "Absent during the disease, bacteriophage appears constantly in convalescents. Bacteriophagy is thus contemporary with recovery." Altogether, these observations led him to propose that "phages are a critical element of immunity [not in the modern sense] (d'Herelle 1931)." These beliefs generated constant discussions with Jules Bordet (1870–1961), who also contributed to the phage and immunology fields. Bordet was awarded the Nobel Prize in 1919 for the discovery of the complement system (Schmalstieg and Goldman 2009).

In 1927 d'Hérelle was in Punjab, India trying to treat Asiatic cholera. He administered phages to patients and their families. Those people who refused to receive the treatment were considered as the control group. This treatment was so successful (8.1% mortality compared to 62.9 in the untreated population), that the Indian Medical Service decided to replicate this in another location (Assam) where they obtained similar results (8–11% mortality in the treated population compared to 60–80% in untreated people). A decrease to 3% mortality was observed in patients who received the treatment endovenously, which is a curious finding considering that cholera is a disease of the small intestinal lumen. Conversely, a complete failure was reported by three other scientists in parallel, who seemed to have used avirulent bacteriophages in their treatments. For these results d'Hérelle said "I have always emphasized that any attempt of treatment with such a [avirulent] phage would lead to complete failure" (d'Herelle 1931). At the time, these words were intended to promote the better design of bacteriophage therapies. He may not have suspected the significance that such disparate results would have regarding the bacteria-phage co-evolutionary arms race.

During 1930–1940, several studies were conducted regarding the resistance of *Vibrio cholerae* to vibriophages. The first observations revealed morphological changes in the colonies after cultures were treated with phages (smooth vs. rough). Rough variants displayed different agglutination patterns, motility, and salt tolerance compared to the smooth strains. Similar studies showed that a phage-mediated modification led to drastic changes in phenotypes such as turning an agglutinable into a non-agglutinable strain or switching a non-hemolytic into a hemolytic strain. Importantly, these variants exhibited

different patterns of phage sensitivity. These aspects of phage biology represent the first insights into the arms race between *Vibrio cholerae* and its phages (Pollitzer 1955).

## 8.2 Phage-Based Therapies of Pathogenic *Vibrios*

Phages were discovered 105 years ago and the research efforts to understand their biology led to significant contributions to the fields of molecular biology and microbial genetics. Nowadays, phages provide a bevy of uses and potential uses in biomedicine and biotechnology such as (1) treatment of infections caused by multidrug-resistant bacteria, (2) biocontrol agents to enhance food safety, (3) tools for epitope identification during the design of novel vaccines by phage-display technology, (4) vaccine carriers, (5) tools for molecular biology research, (6) surface disinfectant agents, (7) bacterial biosensing strategies, (8) nanodevices for drug delivery, and (9) corrosion control strategy (Harada et al. 2018).

Infections generated by *Vibrio* spp. represent a global public health concern. These can be divided into cholera and non-cholera infections (vibriosis). *V. cholerae* is the etiologic agent of cholera which presents as acute secretory diarrhea that in severe cases may lead to death. Vibriosis is caused mainly by ingestion of raw and/or undercooked contaminated seafood, but can also manifest as skin or invasive infections following exposure. Clinical outcomes can range from mild self-limiting gastroenteritis and wound infections to septicemia and death depending on the causing agent (Baker-Austin et al. 2018).

Since pathogenic and non-pathogenic *Vibrios* inhabit marine and estuarine environments, they are usually associated with fish and marine invertebrates such as lobsters, crabs, and shrimps. Hence, the presence of these bacteria generates a threat to food security and a tremendous negative impact on production of seafood for human consumption (de Souza Valente and Wan 2021).

The use of phage cocktails—mixes of different phages that ideally recognize different bacterial receptors—for the treatment and/or prophylaxis of *Vibrio* infections, is a promising alternative to antibiotics since it diminishes the probability of selecting resistant mutants that may limit their use. In this context, a comprehensive understanding of the biology of each type of phage in a therapeutic cocktail is critical. In the next section, we describe virulent phages, which are phages that reproduce exclusively via the lytic cycle (Fig. 8.1), that have been recently isolated and characterized. These phages may potentially be used to generate phage cocktails against major pathogenic *Vibrios.*

### 8.2.1 *Vibrio alginolyticus*

*V. alginolyticus* are halophilic Gram-negative bacteria that are commonly found in warm sea water. This bacterium causes soft tissue and skin infections that are non-healing but which respond to topical treatments. Rare complications have also been reported as otitis, gastroenteritis, and bacteremia (Sganga et al. 2009). Several virulent phages of *V. alginolyticus* have been isolated that potentially could be used for phage therapy (Flemetakis 2016; Sasikala and Srinivasan 2016; Kokkari 2018; Luo et al. 2018; Li et al. 2019, 2021a, b; Kim et al. 2019b; Goehlich et al. 2019; Thammatinna et al. 2020; Gao et al. 2020; Chen et al. 2020). In addition, since this bacterium is considered a pathogen of oyster larvae, the prophylactic use of phages in oyster farms has also been evaluated (Le et al. 2020a, b).

### 8.2.2 *Vibrio cholerae*

*V. cholerae* inhabits warm estuaries and is the causative agent of cholera, an acute and severely dehydrating diarrheal disease caused by ingestion of contaminated water or food. *V. cholerae* is a highly motile toxigenic bacteria that colonize the small intestine. By the action of its cholera toxin, permeability of intestinal epithelial cells is altered generating excretion of fluids to the intestinal lumen with elevated concentrations of sodium, potassium, chloride, and bicarbonate. Due to the

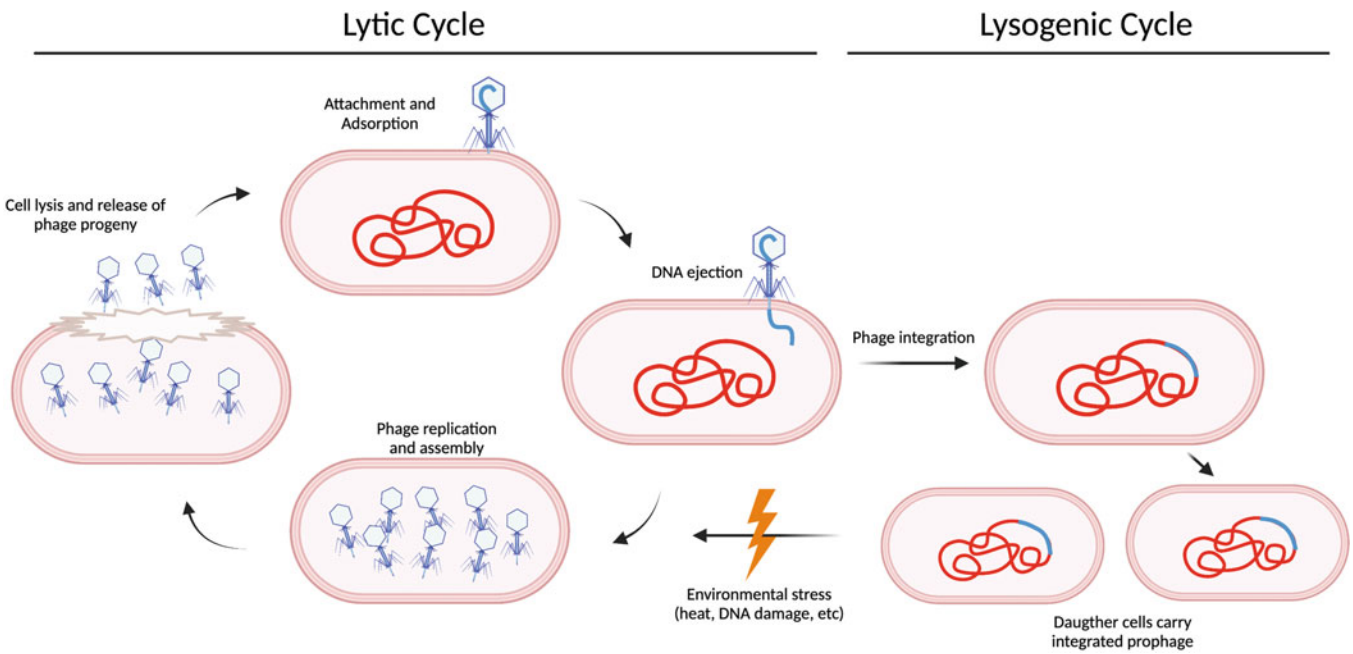


**Fig. 8.1** Bacteriophage life cycle. Bacteriophages can infect and lyse their bacterial host (lytic cycle) or incorporate their phage genome into chromosomal bacterial DNA to ensure their maintenance within a bacterial population (Lysogenic cycle). Upon stress, some temperate phages can resume their lytic cycle and infect neighboring bacteria. Bacteriophage K139 is able to replicate assembly and lyse *V. cholerae* chromosome during growth on chitin. After K139 release, newly formed viral particles can infect and kill susceptible *V. cholerae*. In addition, free *V. cholerae* DNA from lysed cells becomes available for HGT on naturally competent bacteria *V. cholerae*

loss of large volumes of watery stool, the fast and severe dehydrating effect is up 60% lethal if not treated properly with re-hydration therapy and in some cases with antibiotics. Cholera is a global health problem in many regions lacking safe drinking water. The burden of cholera is becoming greater due to the rapid rise and spread of multidrug-resistant strains. For these reasons, several virulent phages have been isolated and studied that could potentially be used for phage prophylaxis or therapy. Their effects have been evaluated *in vitro* and *in vivo* (during bacterial infection of a host) (Das and Ghosh 2017; Al-Fendi et al. 2017; Naser et al. 2017a; Bhandare et al. 2017a, b; Sarkar et al. 2018; Angermeyer et al. 2018; Yen et al. 2019; Maje et al. 2020).

### 8.2.3 *Vibrio parahaemolyticus*

*V. parahaemolyticus* can be found attached to marine plankton in warmer estuarine and marine water. The infection is generated by ingestion of contaminated raw shellfish and in some cases by contact of an open wound with contaminated seawater. The main virulence factor of *V. parahaemolyticus* is the thermostable direct hemolysin (TDH). It has been proposed that the molecular mechanisms leading to the clinical outcome of self-limiting gastroenteritis may be related to the ability of TDH to form pores and to the presence of a type III secretion system that injects effector proteins into host cells. However, this phenomenon requires additional study to attain a clearer understanding (Baker-Austin et al. 2018; Rezny and Evans 2021).

Vibriosis is a major disease in shrimps caused by *V. parahaemolyticus* and other *Vibrio* spp. *V. parahaemolyticus* is responsible for the acute hepatopancreatic necrosis disease (AHPND) that has generated a USD 43 billion loss on the shrimp industry. It has been shown that different virulence factors such as $PirA^{VP}$/$PirB^{VP}$ toxins, serine proteases, enterobactin, flagellin, metalloproteases, vibrioferrin, Type I Secretion System (T1SS), Type II Secretion System (T2SS) and Type VI Secretion System (T6SS) might have a role in toxicity of AHPND (Kumar et al. 2021). For these reasons, different virulent phages have been isolated and proposed as biocontrol strategies (Wang et al. 2016; Lal et al. 2016; Stalin and Srinivasan 2016; Delli Paoli Carini et al. 2017; Jun et al. 2017; Yu et al. 2018a, b; Onarinde 2018; Zhang et al. 2018; Richards 2019; Yang et al. 2019, 2020a, b; Ren et al. 2019; Matamp and Bhat 2019; Maje et al. 2020; Ding et al. 2020; Cao et al. 2020; Tan et al. 2021; Dubey et al. 2021; Wong et al. 2021; Li et al. 2021a; You et al. 2021).

### 8.2.4 *Vibrio harveyi*

*V. harveyi* infects marine vertebrates and invertebrates generating an impact on the aquaculture industry. Infected fish develop gastroenteritis and display skin ulcers, eye lesions, tail rot disease and muscle necrosis. It has been shown that *V. harveyi* pathogenicity is mediated by phospholipase B, an extracellular hemolysin that might kill fish cells by inducing apoptosis.

Shrimps infected with *V. harveyi* (or so-called luminous vibriosis) glow in the dark. Also, shrimp exhibit a second manifestation of the disease which generates sloughed-off tissue in the digestive tract. A role for extracellular proteases and endotoxin have been proposed as mechanisms of pathogenicity in this host (Zhang et al. 2020). Different virulent phages have been studied and proposed as bacterial control strategies for the aquaculture industry (Delli Paoli Carini et al. 2017; Stalin and Srinivasan 2017; Choudhury et al. 2019; Misol et al. 2020).

### 8.2.5 *Vibrio coralliilyticus*

*V. coralliilyticus* is one of the major pathogens inducing severe damage to the coral holobiont, which concomitantly generates a serious ecological imbalance. Bacterial infection generates death of Symbiodinium, coral bleaching, tissue lysis and necrosis (Ramphul et al. 2017; Rubio-Portillo et al. 2020). This pathogen also generates high mortality in oyster hatcheries (Richards et al. 2021). The use of virulent phages has been

proposed as an means to prevent deterioration of corals, and to avoid loss of production in oyster industry generated by bacterial infection (Ramphul et al. 2017; Kim et al. 2018, 2019a; Jacquemot et al. 2018, 2020; Richards et al. 2021).

### 8.2.6 *Vibrio anguillarum*

*V. anguillarum* infects more than 50 species of fresh and salt-water fish, crustaceans and bivalves, generating massive losses in the aquaculture industry. Molecular mechanisms related to its pathogenicity are not fully understood. However, a role for virulence genes related to iron uptake, extracellular hemolysins and proteases, motility, chemotaxis and lipopolysaccharide (LPS) has been established (Frans et al. 2011). Some phages have isolated and characterized during the past few years for this pathogen (Kalatzis et al. 2017, 2019; Rørbo et al. 2018).

### 8.2.7 *Vibrio splendidus*

*V. splendidus* inhabits marine and estuary water. It causes infections of different aquatic animals such as fishes, echinoderms, crustaceans, and bivalves. *V. splendidus* is one of the most relevant pathogens in the bivalve aquaculture, responsible for severe financial losses annually. In addition, in the fish industry, it has been linked to high mortality in turbot. Pathogenicity mechanisms have not been thoroughly studied, however, a role for the Vsm extracellular metalloprotease has been shown (Zhang et al. 2019). Only a few *V. splendidus* virulent phages have been recently isolated (Li et al. 2016; Katharios and Kalatzis 2017).

### 8.2.8 *Vibrio vulnificus*

*V. vulnificus* causes fatal septicemia, limited gastroenteritis and severe wound infections. This pathogen colonizes fish, shellfish (primarily oysters) and shrimps where the shrimp industry economic loses reach US$3 billion annually. It is transmitted to humans by ingestion of contaminated seafood or via direct contact of wounds with contaminated water (Haftel and Sharman 2021). For this reason, some phage-based therapies have been evaluated (Srinivasan and Ramasamy 2017; Kim et al. 2021).

### 8.2.9 *Vibrio campbellii*

*V. campbellii* are luminous bacteria that inhabit marine environments. It is an opportunistic pathogen of fishes, squids, shrimps, and other invertebrates that generates AHPND in its hosts. Molecular mechanisms related to the virulence of this pathogen remain understudied. However, it was recently shown that the BtsS/BtsR two-component system for the sensing/uptake of pyruvate is required to regulate chemotaxis, resuscitation from the viable but nonculturable state, and virulence in shrimp larvae (Göing et al. 2021). Some phage-based therapies to prevent shrimp infection have been proposed (Li et al. 2020a; Lomelí-Ortega et al. 2021).

### 8.2.10 *Vibrio ordalii*

*V. ordalii* causes vibriosis characterized by hemorrhagic septicemia in different species of aquacultured fish, mainly salmonids. This disease generates a severe impact in economies dependent on Salmon production like Chile (Echeverría-Bugueño et al. 2020). A recent report has characterized a phage able to infect this fish pathogen (Echeverría-Vega et al. 2020).

### 8.2.11 Challenges of Using Phage Therapy

The urgent need to develop novel therapies or prophylactic strategies is reaching a critical point due to the antibiotic-resistance crisis. Phage therapies should be designed in ways that minimize the emergence of bacterial resistance to

the product. Moreover, in the case of invasive bacterial infections, phage therapies should be tested for possible contribution to septic shock. In these contexts, there are some important aspects that should be considered in the design of phage therapies and prophylaxes:

- The use of non-transducing or at least poorly transducing phages and avirulent host strains to avoid the transfer by Horizontal Gene Transfer (HGT) of genetic material that may contain virulence and/or antibiotic-resistance genes.
- The use of a mixture of phages (cocktail), ideally with phages utilizing different receptors to decrease the chances of generating strains resistant to the cocktail.
- A comprehensive understanding of phage biology and the dynamics of interaction of these with their bacterial hosts during infection of animals.
- The use of phages that minimize bacterial lysis in order to reduce the release of LPS and intracellular virulence factors that may induce inflammation or even septic shock.
- A deeper understanding of the evolutionary forces phages and bacteria have on each other in the environment and during infection of animals.
- A better molecular level understanding of the evolutionary arms-race between phages and their hosts that can lead to phage-resistant strains and spread of anti-phage defense mechanisms (Table 8.1).

Phage-based approaches have proven to be effective for the treatment of extracellular pathogens in different settings. However, current knowledge on the use of phage therapy for intracellular pathogens is still scarce. Intracellular bacteria have the advantage of surviving inside host cells, thus evading humoral immunity, some classes of antibiotic, and most likely phages. In this context, the improvement of the invasive abilities of phages using synthetic biology and genetic engineering represents an attractive strategy (Lu and Collins 2009; Moradpour et al. 2009; Yehl et al. 2019; Al-Anany et al. 2021).

Isolation of diverse phages should be addressed using different protocols for phage isolation. As it was shown for the non-tailed dsDNA double jelly roll lineage phages, minimal modifications to the classical protocols for phage isolation generate critical differences in the enrichment of phages with special morphological traits (Kauffman et al. 2018b). Also, the constant development and improvements in sequencing tools for data analysis will contribute to our understanding of phage biology and taxonomic classification and their use as therapies (Kauffman et al. 2018a).

## 8.3 The Role of Temperate Phages in *Vibrio* Evolution

Phages are the most ubiquitous biological entities in the biosphere (estimated $10^{31}$ in aquatic environments) (Suttle 2007), where they co-exist in dynamic equilibrium with their bacterial hosts. Phages can be found extracellularly in the environment (virulent or temperate phages) or integrated within bacterial genomes (temperate phages). Remarkably, vibriophage DNA is among the most abundant phage DNA in sediment from the Kathiawar Peninsula and Arabian Sea (Nathani et al. 2021).

Temperate phages can undergo the lytic cycle or remain integrated into the host genome as prophages (the host strain is then named a lysogen) (For an extensive review on lysogeny see (Howard-Varona et al. 2017). When lysogens encounter stressful conditions, temperate phages can excise and replicate to complete the lytic cycle and infect a new host. Typically, this happens in a small fraction of the lysogen population, thus maintaining vertical transmission of the prophage within the bacterial population (Howard-Varona et al. 2017) (Fig. 8.1).

For some phages, the mechanisms that trigger the lytic-lysogeny switch are well known, as in the lambda phage of *E. coli* (Howard-Varona et al. 2017). One trait that promotes phage integration is the presence of a specific sequence in the host genome called the attB site. Integrases and recombinases can act on phage (attP) and

**Table 8.1** Bacterial defense mechanisms against phage predation

| Bacterial defense mechanisms | | |
|---|---|---|
| Phage | Bacterial mechanism | Effect |
| 919TP phage cholerae | Mutational change of receptor (*V. cholerae*) | Mutations in LPS-biosynthesis *wbe* cluster (Shen et al. 2016) |
| ICP1 | Mutational change of receptor (*V. cholerae*) | Frameshift of phase LPS variable and biosynthesis genes (Seed et al. 2012; Silva-Valenzuela and Camilli 2019) |
| ICP1 | BREX (*V. cholerae*) | Prevents ICP1 replication (For extensive review see Boyd et al. 2021) |
| ICP1 and other phages | Restriction modification (*V. cholerae*) | Degradation of phage genome (For extensive review see Boyd et al. 2021) |
| ICP1 | Prevention of virus assembly (*V. cholerae*) | Excision and circularization of PLE which prevents ICP1 replication and assembly (For extensive review see Boyd et al. 2021) |
| ICP2 | Mutational change of receptor (*V. cholerae*) | Mutation of aminoacidic residues within two external loops of OmpU (Seed et al. 2014) |
| ICP1, ICP2, and ICP3 | Phage bait (*V. cholerae*) | Secretion of membrane vesicles (OMVs) containing the phage receptors (Reyes-Robles et al. 2018) |
| JSF environmental phages | Downregulation of receptor (*V. cholerae*) | Production of a hemagglutinin protease (HAP) and downregulation of the O1-antigen phage receptor (Hoque et al. 2016) |
| Mix of environmental phages | Restriction modification (*V. lentus*) | Degradation of phage genomes (Hussain et al. 2021) |
| KVP40 | Mutational change of receptor (*V. anguillarum*) | Premature stop codons, frameshifts, and amino acid changes in the protein OmpK (Castillo et al. 2019a, b) |
| CHOED | Genetic diversification (*V. anguillarum*) | Mutations in LPS, hypothetical outer membrane protein, impaired growth, decreased motility, and increased protease production (León et al. 2019) |
| Mix of environmental phages | Genetic diversification (*V. alginolyticus*) | Mutations in flagellar, LPS, and EPS genes (Zhou et al. 2021) |
| OWB | Mutational change of co-receptor (*V. parahaemolyticus*) | Polar flagella rotation (Zhang et al. 2016) |
| Several phages | CRISPR-Cas (non-O1/non-O139 *V. cholerae)* | Adaptive immunity against phage genomes (Labbate et al. 2016; Carpenter et al. 2017; McDonald et al. 2019) |
| Several phages | CRISPR-Cas (O1 *V. cholerae)* | Adaptive immunity against phage genomes (Box et al. 2016; Bourgeois et al. 2020) |
| Several phages | CRISPR-Cas (*V. parahaemolyticus)* | Adaptive immunity against phage genomes (Baliga et al. 2019) |
| Several phages | CRISPR-Cas (*V. metoecus)* | Adaptive immunity against phage genomes (Grüschow et al. 2021) |
| Several phages | Abortive infection (*V. cholerae*) | TA module, MosAT encoded within the SXT/ICE (Dy et al. 2014) |
| Several phages | Abortive infection-like (*V. cholerae*) | cGAMP-cGAS signaling cascade which leads to cell death before completion of phage reproduction (Cohen et al. 2019) |
| Several phages | Phage DNA modification (*V. cyclitrophicus*) | Phosphorothioate (PT) DNA modifications of phage genome which impairs phage replication (Xiong et al. 2020) |
| Aphrodite1, phiSt2, and Ares1 | Metabolic reprogramming (*V.* alginolyticus) | Modulate levels of surface receptors, nutrient uptake and availability (Skliros et al. 2021) |

bacterial (attB) attachment site sequences that determine specificity for the integration locus (Howard-Varona et al. 2017). Over time, temperate phages can be subject to degradation and lose the ability to undergo the lytic cycle, becoming defective prophages which are fixed within bacterial genomes. Prophages are widely distributed among bacterial species and can carry virulence determinants, antibiotic-resistance genes, metabolic pathways or other genes that confer beneficial traits on their host, thus promoting their maintenance within bacterial genomes. Recently, many prophages have been identified among *Vibrio* spp. Some of these where further characterized for their excision ability and fitness advantages/cost to their bacterial hosts, including *V. cholerae* (Anandan et al. 2017; Dutta et al. 2017; Levade et al. 2017; Takemura et al. 2017; Langlete et al. 2019; Verma et al. 2019; Molina-Quiroz et al. 2020; Santoriello et al. 2020), *V. parahaemolyticus* (Ahn et al. 2016; Vázquez-Rosas-Landa et al. 2017; Castillo et al. 2018; Garin-Fernandez and Wichels 2020; Garin-Fernandez et al. 2020; Yang et al. 2020a, b; Yu et al. 2020), *V. harveyi* (Kayansamruaj et al. 2018; Deng et al. 2019; Thirugnanasambandam et al. 2019), *V. natriegens* (Pfeifer et al. 2019; Yin et al. 2020), *V. alginolyticus* (Wendling et al. 2017; Goehlich et al. 2019; Chibani et al. 2020; Qin et al. 2021), *V. anguillarum* (Castillo et al. 2017, 2019a; Tan et al. 2020), *V. campbellii* (Lorenz et al. 2016), *V. fluvialis* (Zheng et al. 2017), *V. mimicus* (Neogi et al. 2019), and *Salinivibrio* (Olonade and Trindade 2021).

One well-studied example is the temperate, filamentous phage CTXΦ of *V. cholerae* which encodes cholera toxin (CT) and has been linked to the acquisition of antibiotic resistance. Both traits actively enhance the fitness of this pathogen (For extensive reviews of CTXΦ and pathogenic traits of *V. cholerae* see (Sakib et al. 2018; Pant et al. 2020b). The receptor for CTXΦ is the toxin-coregulated pilus, TCP (Waldor and Mekalanos 1996), were TcpB-mediated retraction facilitates CTXΦ uptake (Gutierrez-Rodarte et al. 2019). CTXΦ can transit across the bacterial periplasm by binding its coat protein pIII to a bacterial inner-membrane receptor, TolA. TolA is a receptor for the pIII protein from at least three other *Vibrio* species: *V. alginolyticus, V. anguillarum,* and *V. tasmaniensis.* CTXΦ is widely distributed among *V. cholerae* strains belonging to the O1 and O139 serogroups (Houot et al. 2017).

The CTXΦ locus in *V. cholerae* is flanked by prophages RS1Φ (upstream) and TLCΦ (downstream) (Hassan et al. 2010; Das 2014; Sinha-Ray et al. 2019). TLCφ and RS1φ recognize the MSHA and MSHA/TcpA pilus as receptors, respectively (Faruque and Mekalanos 2012; Das 2014; Sinha-Ray et al. 2019). TLCφ, RSIφ and CTXφ have been shown to integrate sequentially in a site-specific manner into the *V. cholerae* genome (Hassan et al. 2010; Faruque and Mekalanos 2012; Sinha-Ray et al. 2019). The *V. cholerae* RecA protein helps CTXΦ to evade host defenses and allows for its replication within the host (Pant et al. 2020a). Besides phage infection, it has been proposed that *V. cholerae* can acquire the entire RS1-CTX-TLC prophage array by chitin-induced natural transformation (Sinha-Ray et al. 2019).

There are two biotypes of *V. cholerae* O1, classical and El Tor, with the El Tor being the predominant cause of cholera since the 1960s. The CTXΦ from El Tor differs from CTXΦ found in classical (CTX-cla) *V. cholerae*. CTX-cla was thought to be defective for replication. However, atypical El Tor strains harboring a CTX-cla-like (CTX-2) element suggest that CTX-cla and CTX-2 are able to replicate and mobilize between *V. cholerae* strains (Kim et al. 2017). Additionally, recombination experiments between CTX prophages in laboratory conditions might explain the generation of CTX-2 (Yu et al. 2018a, b). In this context, several recent studies have shown atypical El Tor strains linked to recent cholera outbreaks harbor: (1) variants of cholera toxin (CT), (2) altered CTX or CTX-RS1 arrangements, (3) different CTXΦ copy numbers and (4) different integration loci (Rezaie et al. 2017; Mironova et al. 2018; Pham et al. 2018; Bundi et al. 2019; Dorman et al. 2019; Hounmanou et al. 2019; Neogi et al. 2019; Verma et al. 2019; Irenge et al. 2020; Li et al. 2020b; Safa et al. 2020; Ochi et al. 2021; Thong

et al. 2021). However, copy number of CTXΦ does not seem to affect CT production in El Tor *V. cholerae* (Rezaie et al. 2017). Conversely, it has been shown that biofilm formation upregulates TCP and CT, enhancing *V. cholerae* infectivity (Gallego-Hernandez et al. 2020). Furthermore, newer epidemic isolates are constantly evolving by cycles of CTXΦ excision and integration of new CTXΦ sequences. These cycles are mediated by a Xer recombination factor encoded in a TLCΦ phage satellite which facilitates CTX integration (Hassan et al. 2010; Midonet et al. 2019). A recent software tool named VicPred was used to classify the distribution of the CTX prophage and related genetic elements in available *V. cholerae* genomes (Lee et al. 2021).

The presence of prophages can impact the fitness of their hosts. This is the case for the prophage protein VpaChn25_0724 which is proposed to modulate glycine betaine levels, that in turn maintain the integrity of cell membranes in *V. parahaemolyticus*. This can protect this pathogen against excessive salt, cold, heat and freezing among other stressful conditions (Yang et al. 2020a, b). In *V. harveyi*, prophage regions are thought to encode proteins involved in evolution and virulence. However, further characterization is needed (Thirugnanasambandam et al. 2019). In *V. fluvialis* several prophage regions are hypothesized to improve cell adhesion and HGT. In addition, prophage sequences from *V. fluvialis* were found to be similar to prophages found in other *Vibrio* species suggesting phage-mediated HGT drives virulence and diversification of bacteria (Casjens 2003; Zheng et al. 2017). Yet, these HGT-mediated arrangements found in prophage sequences are not exclusive to *V. fluvialis*. In *V. cholerae*, strains isolated in Northern Vietnam carry a phage-like sequence with a mosaic structure of two different *Vibrio* phages (KSF-1Φ, VCYΦ), and unknown foreign DNA at the CTX integration site (Takemura et al. 2017). Similarly, some environmental *V. cholerae* strains carry a prophage-like element encoding one of the Type 6 secretion system (T6SS) gene clusters (Aux3) (Santoriello et al. 2020; Santoriello and Pukatzki 2021). T6SS of *V. cholerae* has been linked to interbacterial competition and this could represent one of many examples of prophage or prophage-like element acquisition that conferred increased competitive fitness to pre-pandemic *V. cholerae* strains, which later became fixed in the population of pandemic *V. cholerae* (Santoriello et al. 2020; Santoriello and Pukatzki 2021).

Prophages benefiting their host is not always the case. In *V. alginolyticus,* prophages have been shown to slow growth in stressful environmental conditions such as low salinity (Goehlich et al. 2019). Additionally, in *V. natriegens* nucleotide variations in prophages have been proposed to decrease growth rate (Yin et al. 2020). A prophage-free variant is able to outcompete the wild type in competitive growth. Interestingly, the prophage-free strain also seemed to have improved survival to DNA-damage and hypo-osmotic stress conditions (Pfeifer et al. 2019).

Prophages can impact their host fitness by carrying virulence genes. Prophage genes have been found to encode potential zonula occludens toxin (zot) (Garin-Fernandez et al. 2020) or zot and RTX toxins (Castillo et al. 2018) in *V. parahaemolyticus*, *V. harveyi* Y6 (Kayansamruaj et al. 2018), *V. anguillarum* (Castillo et al. 2017) and *V. alginolyticus* (Chibani et al. 2020). For the latter species, it was confirmed that the presence of only vibriophage VALGΦ6 increased *V. alginolyticus* virulence in a juvenile pipefish infection model (Chibani et al. 2020). This further suggests that the presence of prophage-encoded toxin genes might contribute to bacterial virulence in different *Vibrio* spp. Prophages have also been found among *V. parahaemolyticus* linked to acute hepatopancreatic necrosis ($VP^{AHPND}$) disease (Yu et al. 2020). Interestingly, $VP^{AHPND}$ genomes that carried prophages lacked the anti-phage defense CRISPR. The authors propose that the absence of CRISPR allowed for prophage insertion which in turn led to acquisition of virulence genes, enhancing the virulence of $VP^{AHPND}$ strains (Yu et al. 2020). A similar phenomenon was observed in *V. alginolyticus*, where phage-susceptible strains were more pathogenic in a

juvenile pipefish infection model (Wendling et al. 2017).

As mentioned, temperate phages can drive *Vibrio* evolution by HGT. A *V. cholerae* close-relative, *V. mimicus* has been found to carry CT encoded by *ctxA* and variant *ctxB* genes. The CT production among *V. mimicus* isolates was variable due to differential transcription of the virulence regulon (Neogi et al. 2019). It has been proposed that *V. mimicus* could act as a reservoir of genes that *V. cholerae* can obtain by HGT, including genes which might contribute to the evolution of hybrid *V. cholerae* strains (Neogi et al. 2019).

Spontaneous or chemical induction of prophage excision and lytic growth was detected for about 50% of *V. anguillarum* strains in a small pilot study (Castillo et al. 2019a). The produced phage particles had diverse host range patterns and were able to reintegrate in non-lysogenic strains, reinforcing the idea that prophages contribute to a rapid and efficient spread of genes within *Vibrio* species (Castillo et al. 2019a). In this framework, two prophages from *V. campbellii* (HAP1Φ-like and Kappa Φ-like) were shown to be induced not only by mitomycin C, but also by heat stress (Lorenz et al. 2016). *Vibrios* are constantly subject to temperature shifts and with the ongoing global warming, phages could be released from *Vibrios* in their natural habitats promoting the rise of new or more virulent variants.

Temperate phage excision can also be controlled by quorum-sensing (QS) signaling. In *V. anguillarum*, bacterial high cell densities correlated with increased lysogeny (Tan et al. 2020). The authors proposed that this would not only be a phage transmission strategy, but could also be a host tactic to control the lytic-lysogeny switch to promote its own fitness (Tan et al. 2020). Phage VP882 a non-integrating temperate phage which infects *V. cholerae* and *V. parahaemolyticus* and also exploits host QS to control its lytic-lysogeny switch. VP882 encodes a QS receptor $VqmA_{Phage}$ that can bind to a bacterial-produced autoinducer involved in QS signaling. This in turn, induces the expression of Qtip which sequesters the phage cI repressor, activating the phage lytic cycle (Silpe and Bassler 2019; Silpe et al. 2020). Thus, tight control of $VqmA_{vc}$ is essential for adequate regulation of gene expression and to ensure the survival of both *V. cholerae* and Phage VP882 (Duddy et al. 2021).

One intriguing example of the relevance of prophages in *V. cholerae* evolution is the Kappa- family member, phage K139 (Reidl and Mekalanos 1995) (Fig. 8.3). Its receptor is the O1-antigen (Nesper et al. 2000) and it is widely distributed among *V. cholerae* strains reaching a prevalence up to 50% (Reidl and Mekalanos 1995). However, this prophage is absent from the most recent Haiti strains from the 2010 cholera outbreak (Levade et al. 2017). K139 DNA has been detected in extracellular vesicle fractions during *in vitro* growth of *V. cholerae* (Langlete et al. 2019) and this phage has been found to excise and form viable viral particles during growth on chitin (Molina-Quiroz et al. 2020). As mentioned, phages have long been thought to mediate HGT solely due to their ability to transfer DNA from their bacterial host to newly infected bacteria (transduction) (Fig. 8.1). However, a recent study showed that temperate phage-mediated lysis also leads to HGT by neighbor predation and natural transformation. *V. cholerae* lysogenic strains carrying temperate phage K139, were able to kill susceptible (non-lysogenic) neighboring bacteria and promote the transfer of DNA unidirectionally from susceptible to lysogenic bacteria (Figs. 8.1, 8.2, and 8.3). This confers an evolutionary advantage and might explain why the K139 prophage has been maintained in a large fraction of the *V. cholerae* population (Molina-Quiroz et al. 2020) (Fig. 8.3). The role of temperate phages in increasing host fitness and HGT are primary examples of selective pressures that have driven their maintenance in bacterial genomes, although there could be other mechanisms as well.

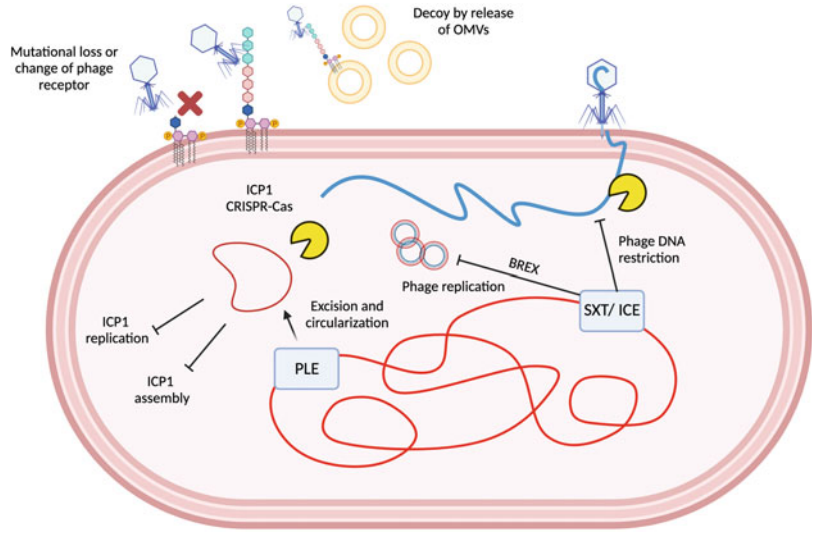


**Fig. 8.2** Arms-Race between *V. cholerae* and its vibriophage ICP1. *V. cholerae* evades ICP1 infection by mutational change or loss of ICP1's receptor, LPS or release of OMVs carrying LPS molecules on the surface. Once the phage DNA is injected, *V. cholerae* can degrade ICP1 DNA by restriction-modification systems. If the phage lytic cycle continues, *V. cholerae* can prevent viral replication and assembly through the BREX system and the induction of the viral satellite PLE. For a successful infection, ICP1 utilizes an endonuclease and acquired its own CRISPR-Cas system against *V. cholerae* defense mechanisms

## 8.4 Phage-Escape Mechanisms and Co-evolutionary Arms-Race

Bacterial species are constantly exposed to selective pressure by phage predation. This is one of many cases where the red queen, also called evolutionary arms race hypothesis applies (Stern and Sorek 2011; McLaughlin et al. 2017; Rostøl and Marraffini 2019). The unceasing threat of phage predation drives bacterial evolution by selecting for organisms able to avoid or overcome infection. Bacteria must constantly develop defense mechanisms against these predatory phages which are ubiquitous in environmental reservoirs where both co-exist in dynamic equilibrium (Table 8.1). On the other hand, phages must counter-adapt to these changes to maintain their infectivity by means of mutating or capturing new genes to counteract anti-phage mechanisms (Table 8.2). Thus, the constant battle between phages and bacteria helps to drive the evolution of both by acquisition of new traits that increase bacterial or phage fitness.

Once phages recognize their bacterial surface receptor and bind irreversibly (adsorption), they inject their DNA, subvert bacterial machinery for their own replication, assemble new viral particles and typically lyse the infected cell to release newly formed phage particles to predate on neighboring bacteria. Some phages, particularly filamentous phages, can extrude progeny phage in a non-lytic manner. Therefore, bacterial species must counteract each stage of viral infection to survive.

### 8.4.1 First Step: Evading Phage Attachment

A well-known mechanism of resistance to phages is mutational change or loss of the receptor recognized by a specific-phage. However, in the case of bacterial pathogens, often these mutations

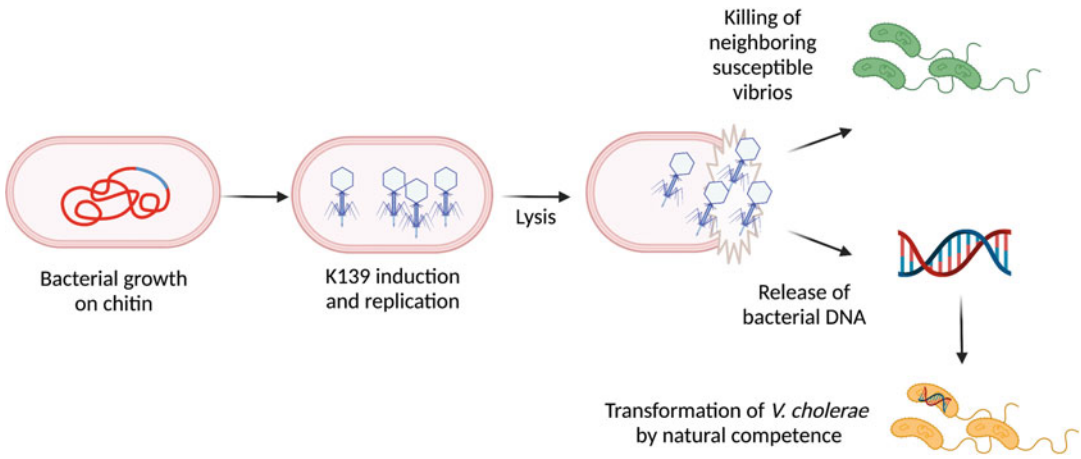


**Fig. 8.3** Role of phage K139 in *Vibrio* killing and transformation. A recent study showed that temperate phage-mediated lysis also leads to HGT by neighbor predation and natural transformation. *V. cholerae* lysogenic strains carrying temperate phage K139, are able to kill susceptible (non-lysogenic) neighboring bacteria and promote the transfer of DNA unidirectionally from susceptible to lysogenic bacteria. This confers an evolutionary advantage and might explain why the K139 prophage has been maintained in a large fraction of the *V. cholerae* population

**Table 8.2** Phage mechanisms to avoid bacterial resistance

| Phage counter-attack mechanisms | | |
|---|---|---|
| Phage (host) | Mechanism | Effect |
| ICP2 (*V. cholerae*) | Mutational change of tail fibers | Recognition of non-wild-type OmpU (Seed et al. 2014; Lim et al. 2021) |
| ICP1 (*V. cholerae*) | Epigenetic modification | OrbA protein counteracts the BREX system (LeGault et al. 2021) |
| ICP1 (*V. cholerae*) | Endonuclease | Endonuclease that mimics the PLE-encoded replication initiation factor RepA (Barth et al. 2021; Boyd et al. 2021) |
| ICP1 (*V. cholerae*) | CRISPR-Cas | CRISPR-Cas system against *V. cholerae* PLE (Seed et al. 2013) |
| Several phages | tRNA acquisition | tRNAs support translation of late genes during phage infection (Yang et al. 2021) |

render bacteria avirulent and thus unable to cause infection (Mangalea and Duerkop 2020). A common receptor for many phages is the LPS, a structural component of the gram-negative bacterial outer membrane. One example is phage 919TP which is a typing phage of *V. cholerae* strains. Isolates with mutations in the LPS synthesis gene cluster were found to be 919TP-resistant (Shen et al. 2016). Interestingly, besides mutational changes in the phage receptor, many 919TP-resistant isolates were of unknown nature or carried a temperate phage that might avoid superinfection by 919TP (Shen et al. 2016).

Of three phages commonly associated with O1 *V. cholerae* isolated from patient diarrheal stool samples in Bangladesh (ICP1, ICP2, and ICP3) (Seed et al. 2011), ICP1 was found to be the most prevalent. This phage unlike the other two has the ability to kill *V. cholerae* in many niches including nutrient-poor aquatic microcosms that mimic conditions found in the environment (Silva-Valenzuela and Camilli 2019). The receptor for ICP1 is the *V. cholerae* O1-antigen (Seed et al. 2012), and a mechanism to avoid phage infection is to shut off O1-antigen expression in a reversible manner by phase variation. Examples of such

phase variable mutations include single nucleotide polymorphisms within O1-antigen biosynthetic genes *wbeL* and *manA* (Seed et al. 2012; Silva-Valenzuela and Camilli 2019) as well as in other LPS biosynthetic genes. However, such mutations render *V. cholerae* avirulent (Seed et al. 2012) (Fig. 8.2).

The receptor for ICP2 is the outer membrane protein and virulence factor OmpU (Seed et al. 2014). To avoid ICP2 recognition within a cholera patient, *V. cholerae* can spontaneously mutate to change aminoacidic residues within two external loops of OmpU. With this, *V. cholerae* was able to diminish or abolish phage recognition while maintaining expression of a functional OmpU protein and thus virulence (Seed et al. 2014). Conversely, to overcome these bacterial mutations, ICP2 counter-adapts its tail fibers to recognize mutational variants of OmpU (Seed et al. 2014; Lim et al. 2021). Changes in the OmpU sequence showed a mild competitive defect after multiple passaging in growth medium, suggesting a slight decrease in fitness. However, these mutants were enriched in the presence of ICP2 in a rabbit model of infection, demonstrating the strong selective pressure that phage predation imposes on *V. cholerae* during infection (Seed et al. 2014). In addition to OmpU mutations, several ICP2-resistant isolates carried mutations in the *toxR* gene. ToxR is a transcriptional activator of many virulence factors, including OmpU. In this case, ICP2-resistant ToxR mutants were attenuated for infection (Seed et al. 2014).

For the fish pathogen *V. anguillarum*, resistant isolates to phage KVP40 encoded premature stop codons, frameshifts, and amino acid changes in the outer membrane protein OmpK, which is the KVP40 receptor. In addition, all resistant isolates tested had reduced virulence in a cod larval model (Castillo et al. 2019b). Predation by phage CHOED selects for *V. anguillarum* phage-resistant mutants, some of which retain virulence. CHOED-resistant mutants showed a range of phenotypic differences compared to the wild-type strain. Besides changes in the LPS profile and mutations within a hypothetical outer membrane protein (the proposed phage receptor), some resistant isolates showed impaired growth, decreased motility, and increased protease production. The majority of these phage-resistant mutants were avirulent, but not all (León et al. 2019). A similar diversity was observed in *V. alginolyticus*, where phage-resistant mutants showed abundant phenotypic variations (Zhou et al. 2021). However, it is important to note that the experimental design included a mix of phages from wastewater samples. Therefore, in this case one might expect diversity among the selected phage-resistant mutants. The mutations the authors identified between resistant isolates include those affecting flagella, LPS, and extracellular polysaccharide, with the latter two proposed as phage receptors (Zhou et al. 2021). It has been recently shown that rotation of the *V. parahaemolyticus* polar flagella, reduced absorption of phage OWB (Zhang et al. 2016). The authors propose that rotation but not spatial interference can protect *V. parahaemolyticus* from the phage (Zhang et al. 2016). However, polar flagella rotation in *V. parahaemolyticus* acts as a mechanosensor (Belas 2014). Therefore, phage resistance could be due to altered bacterial surface properties that affect phage attachment.

Mutational changes or loss of phage receptor by the bacterial host may impair bacterial fitness or virulence (Seed et al. 2012, 2014; Castillo et al. 2019b; León et al. 2019). In the case of *V. cholerae*, there is pressure to evolve phage-escape mechanisms that do not involve surface receptors, since these receptors serve as critical virulence factors. A number of phages have been isolated from cholera patient stools where, by definition, the presence of the phage clearly was not sufficient to prevent or clear the bacterial infection (Seed et al. 2011). These observations suggest additional mechanisms of phage resistance not related to mutation of the surface receptor are operative during human infection. One example of such mechanisms is to inactivate attacking phages by secreting outer membrane vesicles (OMVs) containing the phage receptor (Reyes-Robles et al. 2018) (Fig. 8.2). However, the release of OMVs only partially reduces phage infection.

Another conserved mechanism among bacteria is to make the receptor unavailable to infecting phages through the construction of a physical barrier in the form of biofilms. Biofilms are typically controlled by QS. In addition to the barrier mechanism of phage resistance, it has been shown that the presence of auto-inducers promotes the emergence of phage-resistant *V. cholerae* by means of production of a hemagglutinin protease and by downregulation of the O1-antigen phage receptor, leading to impaired phage adsorption at high cell densities (Hoque et al. 2016). Biofilm formation is common amongst *Vibrio* species and it is likely that vibriophages have evolved mechanisms to counter this defense mechanism.

### 8.4.2 Second Step: Battling Phage DNA

Bacteria can recognize and degrade foreign DNA, including phage DNA, primarily through two mechanisms, restriction enzyme-mediated cleavage and CRISPR-Cas cleavage, where CRISPR is short for clustered regularly interspersed short palindromic repeats (CRISPR), and its CRISPR-associated (Cas) proteins. Both of these phage resistance mechanisms as well as a third mechanism termed abortive infection can be found in *Vibrio* species (For extensive reviews see and (Stern and Sorek 2011; Rostøl and Marraffini 2019).

#### 8.4.2.1 Restriction-Modification Systems

Restriction-modification (RM) systems are present in around 90% of prokaryotic genomes. They act by cleaving unmethylated or differentially methylated foreign DNA while host DNA having the correct methylation is protected (Reviewed in Stern and Sorek 2011). Anti-phage RM systems in *V. cholerae* have been found in mobile genetic elements belonging to the SXT family called integrative and conjugative elements (ICEs) (LeGault et al. 2021). The SXT element encoded in pandemic O1 *V. cholerae* carries genes needed for conjugation and for resistance to sulfamethoxazole, chloramphenicol, trimethoprim, and streptomycin, among other features (Dalia et al. 2015). Variable genes within the SXT element also include: (1) a DNase that inhibits natural transformation (Dalia et al. 2015), and (2) RM systems that have been recently linked to anti-phage defense against ICP1 and other phages (LeGault et al. 2021). Interestingly, a variety of RM systems can be found in the same locus of SXT, called hotspot 5. In addition, a recently characterized phage exclusion (BREX) system (Goldfarb et al. 2015) was also found in hotspot 5 of some SXT elements (Slattery et al. 2020; LeGault et al. 2021). This presents a challenge for ICP1 and other *V. cholerae* phages to overcome (Boyd et al. 2021; LeGault et al. 2021). To counteract RM systems, phages need to either acquire the methylase or make inhibitory proteins (Reviewed in Stern and Sorek 2011). ICP1 has been suggested to evade restriction through epigenetic modification (LeGault et al. 2021) and to evade BREX through an anti-BREX protein, namely OrbA (LeGault et al. 2021) (Fig. 8.2).

A recent study of the pangenome of 22 *V. lentus* strains identified 26 mobile genetic elements carrying at least one gene for phage defense. Among these phage defense elements (PDEs), PDE1 encoded a Type-I RM system. However, only a single gene (restriction enzyme) was found to have anti-phage function (Hussain et al. 2021). It is important to note that each strain carried 6–12 PDEs, and these PDEs could be rapidly acquired or lost within the population through HGT. The authors warn that rapid acquisition of phage resistance mobile elements within microbial populations might hinder longer term use of phages in therapy, analogous to what is seen with antibiotic therapies (Hussain et al. 2021).

#### 8.4.2.2 CRISPR-Cas Systems in *Vibrio* Species

CRISPR-Cas systems provide sequence-specific immunity against foreign nucleic acids. This immunity is adaptive since fragments of foreign DNA are incorporated into the CRISPR loci in the form of "spacers," providing bacteria immune memory to respond faster and more decisively to a second attack (Barrangou et al. 2007). CRISPR-cas loci have been found in a number

of classical biotype O1 as well as non-O1/non-O139 *V. cholerae* strains at the *Vibrio* pathogenicity island 1 (VPI-1) insertion site (Labbate et al. 2016; Carpenter et al. 2017; McDonald et al. 2019). These CRISPR-cas arrays contained spacers originating from several phage genomes, suggesting an active immune role. One CRISPR-Cas system was found within genomic island 24 (GI-24) that if mobilized to other strains, would confer immediate immunity to several phages (Labbate et al. 2016; Carpenter et al. 2017; McDonald et al. 2019). Indeed, it has been proposed that exchange of CRISPR-Cas systems on mobile genetic elements—elements that are widely distributed among *Vibrio* spp.—can lead to novel strain types with enhanced phage resistance phenotypes (McDonald et al. 2019).

On the other hand, within pandemic O1 *V. cholerae* strains, the presence of CRISPR-Cas systems appears to be restricted to the classical biotype, which is thought to be extinct in nature (Faruque et al. 1993; Alam et al. 2012; Harris et al. 2012; Bourgeois et al. 2020). This Type I-E CRISPR-Cas system, which is located within GI-24, could be transferred into an O1 El Tor strain by natural transformation *in vitro* (Box et al. 2016). Type I-E CRISPR-Cas has not been found in O1 El Tor sequenced genomes. It has been suggested that this CRISPR-Cas system could act as a barrier, preventing the acquisition of beneficial traits such as antibiotic resistance by HGT that are crucial for the evolution of pandemic *V. cholerae* strains (Box et al. 2016). Nevertheless, a recent study identified the Type I-E CRISPR/Cas system among some non-toxigenic environmental isolates, suggesting that it continues to play a role in the defense of *V. cholerae* against phages(Bourgeois et al. 2020).

A bioinformatic study showed that out of 570 *V. parahaemolyticus* genomes only 35% carry a CRISPR-Cas system, suggesting this strategy is not widely distributed within this species (Baliga et al. 2019). A type III-B CRISPR-Cas loci was recently found within a prophage in *V. metoecus* (Grüschow et al. 2021), the closest known relative of *V. cholerae* (Orata et al. 2015). It has been proposed that this type III-B CRISPR-Cas system could play a role in inter-phage competition (Grüschow et al. 2021).

#### 8.4.2.3 Abortive Infection

Abortive infection (Abi) is considered to be a form of bacterial innate immunity against phages. Upon phage DNA entry, the infected cell induces its own death, preventing phage replication and thus protecting neighboring cells (Stern and Sorek 2011; Rostøl and Marraffini 2019). Some Abi systems have been linked to toxin-antitoxin (TA) modules (For an extensive review of TA modules in phage resistance, see (Song and Wood 2020).

*V. cholerae* carries 18 TA gene pair of which 17 are located in the superintegron within chromosome 2 (Iqbal et al. 2015). One TA module, MosAT has been identified to promote maintenance of the SXT ICE (Wozniak et al. 2009). Further analyses of the MosAT suggest it could be a system similar to AbiE which has been linked to stabilization of mobile elements and phage resistance (Dy et al. 2014). Some TA modules encode Abi systems (Stern and Sorek 2011) and the fact that *V. cholerae* encodes 18 TA modules suggests this mechanism of phage defense is of extreme importance. However, further characterization of the role of *V. cholerae* TA modules in phage resistance is needed.

### 8.4.3 Step Three: Preventing Virus Assembly

Phage-inducible chromosomal islands (PICIs) excise upon helper-phage infection to ensure the dissemination of their genetic material. However, the excision of these elements interferes directly with the helper-phage life cycle and has consequently been classified as an anti-phage mechanism (Rostøl and Marraffini 2019). An 18-kb inducible PICI-like element (PLE) from *V. cholerae* is excised upon ICP1 infection by the PLE-encoded recombinase Int (Seed et al. 2011; McKitterick and Seed 2018), replicates (O'Hara et al. 2017; Barth et al. 2020; Netter

et al. 2021) and hijacks ICP1 virions for PLE transduction (Netter et al. 2021) thus inhibiting ICP1 replication (Seed et al. 2013). PLE+ *V. cholerae* die by blocking helper-phage -in this case ICP1- replication, protecting the population at large, similar to Abi systems. There are at least four different PLEs found among pandemic O1 El Tor *V. cholerae* strains. To overcome this phage defense mechanism, ICP1 (Seed et al. 2013) and some environmental phages from the JSF collection (Naser et al. 2017b) encode two distinct strategies that target PLE. One mechanism, encoded in approximately 40% of ICP1 isolates is Odn, an endonuclease that mimicks the PLE-encoded replication initiation factor RepA (Barth et al. 2021; Boyd et al. 2021). A second mechanism present in about 60% of ICP1 isolates (Boyd et al. 2021) is to encode a CRISPR/Cas system which harbors spacers targeting one or more PLEs (Seed et al. 2013). Although the PLEs can spread amongst *V. cholerae* strains via transduction, the ICP1 CRISPR/Cas can respond by acquiring spacers targeting new PLEs thus restoring the ICP1 life cycle (Seed et al. 2013). However, spacers of unknown origin have been identified within some ICP1 CRISPR arrays, suggesting other advantages may be conferred to ICP1 besides destruction of PLEs (McKitterick et al. 2019)For an extensive review of the arms race between *V. cholerae* and ICP1 see(Boyd et al. 2021).

Additionally, a conserved mechanism among phages to overcome phage defenses is the acquisition of tRNA genes. Phage-encoded tRNAs support translation of phage late genes as the host cell shuts down during toward the end of the infection (Yang et al. 2021).

### 8.4.4 Other Phage Escape Mechanisms

Metabolic changes, phage DNA modification and second messengers have also been linked to phage resistance among *Vibrio* spp. (Cohen et al. 2019; Xiong et al. 2020; Skliros et al. 2021). A cGAMP-cGAS signaling system comprised by a four-gene operon has been recently linked to an anti-phage defense system found in diverse bacteria (Cohen et al. 2019). The cGAMP-cGAS signaling system encoded within the *V. cholerae* genome conferred defense against multiple phages. This pathway acts by compromising host membrane integrity through the action of a cGAMP-activated phospholipase leading to cell death before completion of phage reproduction, akin to Abi (Cohen et al. 2019).

An unusual mechanism of phage resistance is a recently described phosphorothioate-dependent DNA modification system that causes a sulfur replacement in the non-bridging oxygen of the sugar-phosphate backbone (Xiong et al. 2020). *V. cyclitrophicus* encodes a phosphorothioate-dependent defense system SspABCD-SspE which impairs phage DNA replication (Xiong et al. 2020).

Lastly, metabolic changes have also been found to play a role in phage defense (Skliros et al. 2021). Virulent phage infection of *V. alginolyticus* induced metabolic reprogramming which downregulates surface receptors and nutrient transporters (Skliros et al. 2021). These findings would represent a novel and understudied bacterial adaptation strategy to limit phage predation.

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# *Vibrio vulnificus*, an Underestimated Zoonotic Pathogen

9

Carmen Amaro and Héctor Carmona-Salido

**Abstract**

*V. vulnificus*, continues being an underestimated yet lethal zoonotic pathogen. In this chapter, we provide a comprehensive review of numerous aspects of the biology, epidemiology, and virulence mechanisms of this poorly understood pathogen. We will emphasize the widespread role of horizontal gene transfer in *V. vulnificus* specifically virulence plasmids and draw parallels from aquaculture farms to human health. By placing current findings in the context of climate change, we will also contend that fish farms act as evolutionary drivers that accelerate species evolution and the emergence of new virulent groups. Overall, we suggest that on-farm control measures should be adopted both to protect animals from Vibriosis, and also as a public health measure to prevent the emergence of new zoonotic groups.

**Keywords**

*Vibrio vulnificus* · Human and fish vibriosis · Emergent pathogens

C. Amaro (✉) · H. Carmona-Salido
Departamento de Microbiología y Ecología, & Instituto Universitario de Biotecnologia i Biomedicina (BIOTECMED), Universitat de València, Burjassot, Valencia, Spain
e-mail: carmen.amaro@uv.es

## 9.1 Generalities

*Vibrio vulnificus* is a pathogenic bacterial species of the genus *Vibrio* that inhabits brackish water ecosystems located in temperate or warm geographical areas. The geographical distribution of *V. vulnificus* is changing due to global warming, so that it is increasingly being isolated from cold water ecosystems such as the Baltic Sea area (Europe) in summer (Deeb et al. 2018). For this reason, this bacterium is considered one of the biological barometers of climate change (Baker-Austin et al. 2017).

In water, *V. vulnificus* survives either in free-living form as a swimming cell motile by a single polar flagellum or in sessile form on different types of organic and inorganic surfaces (Pfeffer et al. 2003; Jones and Oliver 2009). This species is strongly attracted to the mucosal surfaces of fish and colonizes them by forming biofilms (Carda-Diéguez et al. 2017). In addition, *V. vulnificus* can be accumulated by filtering organisms such as different species of bivalves with which it establishes a commensal relationship (Froelich and Oliver 2013). For these reasons, fish and filter-feeding mollusks are considered to be the main environmental reservoirs of the pathogen. Under adverse conditions, such as those imposed by the absence of nutrients and/or low temperatures, this bacterium can enter a viable non-culturable state, a form of resistance that allows it to survive until favorable conditions



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return (Oliver et al. 1995; Biosca et al. 1996; Smith and Oliver 2006).

Figure 9.1 shows the life cycle of this pathogen in the aquatic ecosystem. This cycle was proposed from the results of a series of transcriptomic studies in which the expression of all the genes of the pathogen was analyzed at different temperatures and iron concentrations chosen for their relevance in the transmission and severity of the diseases it causes in animals and humans (Pajuelo et al. 2016; Hernández-Cabanyero et al. 2019, 2020; Hernández-Cabanyero and Amaro 2020). Globally, a rise in temperature increases the transcription of genes related to metabolism, colonization (chemotaxis, flagellum biosynthesis, motility), and resistance to innate immunity (O antigen biosynthetic genes and several plasmid genes), while iron controls the genes involved in the same processes plus genes involved in disease severity (*vvhA*, *rtxA1* and *vvp*, encoding the two main toxins and a protease, respectively) (Hernández-Cabanyero and Amaro 2020). Consequently, the increase in water temperature prepares the pathogen for successful infection, contributing not only to the spread of the bacterium to areas where it had never been isolated before, but also facilitating the colonization of aquatic animals coexisting in the same ecosystems and, therefore, increasing the probability of infection. In the case of iron, the process is more complex and will be briefly discussed in the next section.

## 9.2 Human and Animal Diseases: Epidemiology

*V. vulnificus* is the most versatile pathogenic species of the genus *Vibrio* since it can cause different types of diseases in multiple species of aquatic animals and in humans (Ceccarelli et al. 2019; Amaro et al. 2020). All these diseases receive the generic name of vibriosis, considering that there are other animal vibriosis caused by other species of the genus. The relevance of this species as an animal pathogen in its natural habitat is unknown, since the available data only come from fish farms. However, the affinity of *V. vulnificus* for the mucosal surfaces of fish supports the hypothesis that it is an animal pathogen that accidentally infects humans when they interfere with its habitat.

Interestingly, human and fish vibriosis can be transmitted by both contact and ingestion and, in all cases, the most severe form of the disease is an acute septicemia with a high probability of rapid death by sepsis in susceptible hosts (Ceccarelli et al. 2019; Amaro et al. 2020). For this reason, *V. vulnificus* is considered a pathogen of interest in both animal and human health.

**Human Vibriosis** The diseases that *V. vulnificus* causes in humans occur in two main forms depending on the route of infection. When vibriosis is acquired by ingestion of raw seafood, this bacterium causes gastroenteritis or, directly, primary septicemia (Heng et al. 2017; Baker-Austin and Oliver 2018), and when is acquired by contact with seawater or carrier/diseased animals, the pathogen causes severe wound infections that may require debridement and even amputation of the infected limb (Heng et al. 2017; Baker-Austin and Oliver 2018; Coerdt and Khachemoune 2021). Remarkably, wound infections can also converge into secondary septicemia, and both primary and secondary septicemia can cause death by sepsis in patients at risk in less than 24–48 h (Baker-Austin and Oliver 2018). It should be noted that sepsis by primary or secondary septicemia presents the same clinical signs, which makes differential diagnosis difficult (Fig. 9.2a). *V. vulnificus* also stands out as the only truly zoonotic vibrio (Lehane and Rawlin 2000; Gauthier 2015). Consequently, *V. vulnificus* as a human pathogen is extraordinarily versatile since it can be classified as a food-borne pathogen, as a pathogen of the group of flesh-eating bacteria, such as *Streptococcus pyogenes* (Quirk and Sternbach 1996), or as a zoonotic pathogen depending on the etiology of the disease. However, since there are very few documented cases of transmission of the disease from diseased animal to human, this pathogen is mostly considered as a food-borne pathogen or a marine flesh-eating bacterium.

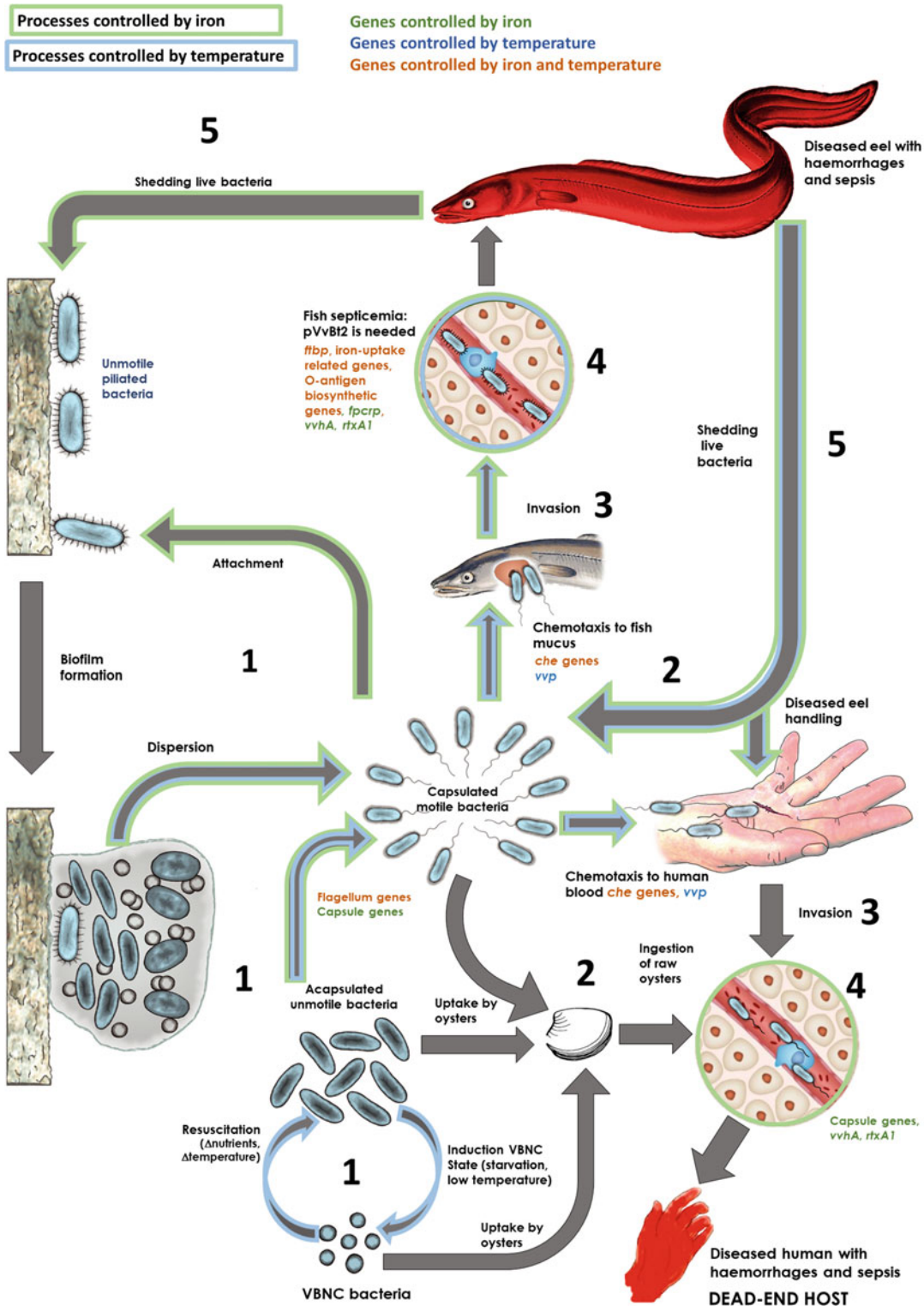


**Fig. 9.1** Life cycle of *V. vulnificus*: role of temperature and iron. This figure summarizes the life cycle of *V. vulnificus* and the role of temperature and iron concentration in the surrounding environment in determining its

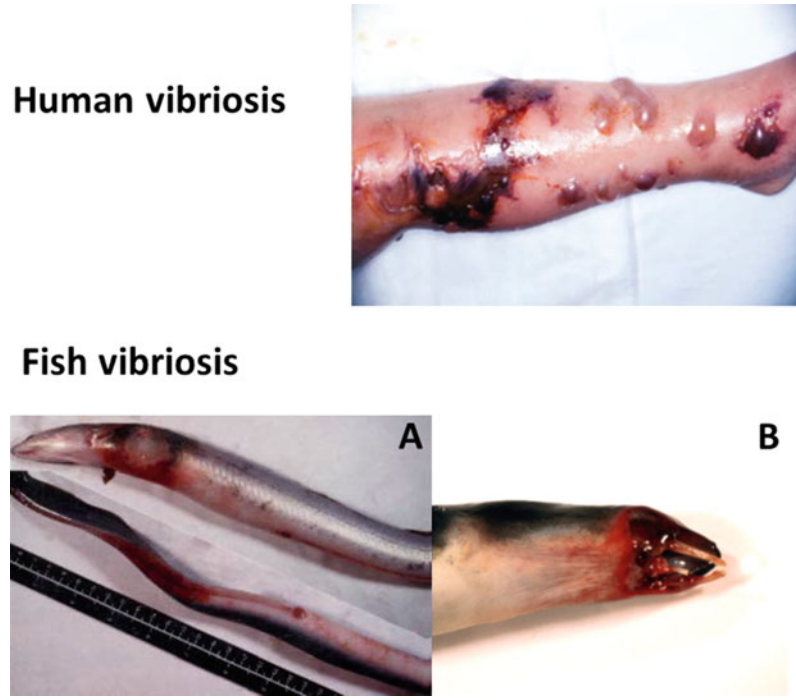


**Fig. 9.2** Clinical signs of human and fish vibriosis caused by *V. vulnificus*. Human vibriosis: A patient showing typical clinical signs of primary and secondary sepsis caused by *V. vulnificus*: swelling, erythema, development of vesicles or bullae and tissue necrosis (picture credits to Dr. Ching-Chuan Liu, Department of Pediatrics, National Cheng Kung University Hospital, College 2653 of Medicine, National Cheng Kung University, Tainan City, Taiwan). Eel vibriosis: hemorrhagic septicemia caused by contact (**a**) or by the oral route (**b**) (picture credits to C. Amaro and B. Fouz)

***Epidemiology*** The majority of reported human vibriosis are sporadic cases occurring in areas where the pathogen naturally inhabits (Fig. 9.3a). As a food-borne pathogen, *V. vulnificus* is mostly relevant in the USA, where it is responsible for most human deaths transmitted by ingestion of seafood (Baker-Austin and Oliver 2018). As a marine "flesh-

**Fig. 9.1** (continued) life strategy (Pajuelo et al. 2016; Hernández-Cabanyero et al. 2019, 2020). Temperature- and iron-controlled processes are surrounded by a blue or green line, respectively and the main genes involved are represented by different colors (see the head of the figure). Globally, a rise in temperature increases the transcription of genes related to metabolism, colonization (chemotaxis, flagellum biosynthesis, motility) and resistance to fish innate immunity (O-antigen biosynthetic genes, and several plasmid genes), while iron controls genes involved in the same processes plus the two main toxins VvhA and RtxA1 and the protease Vvp. (1) *Survival in water. V. vulnificus* survives in water either as a free-living cell or by forming biofilms on biotic or abiotic surfaces. Low temperatures induce the entry of the bacterium in a "dormant" state known as VBNC (viable but not culturable) state, while warm temperatures activate resuscitation and biofilm dispersion. (2) *Colonization of the host*. Bacteria are attracted by blood and/or mucus from their susceptible hosts and colonize them, a process enhanced at warm temperature and also controlled by iron. Bacteria can also be taken up by filtering organisms and these are ingested by humans. (3) *Local lesions and invasion*. From the colonized tissue, the pathogen invades the bloodstream of the host. (4) *Resistance to innate immunity in blood and sepsis.* To resist the innate immunity in human blood, the pathogen produces a capsule whose synthesis is increased under iron excess conditions in risk-patients. To resist innate immunity in fish blood, the pathogen produces an outer membrane enriched in O-antigen plus two proteins (Fpcrp [fish phagocytosis complement resistance protein] and Ftbp [fish transferrin binding protein]) encoded in the plasmid, whose synthesis is increased under iron starvation and at warm temperatures. Only the cells that resist the innate immunity multiply and secrete the toxins RtxA1 and VvhA that will cause the death of the host by a toxic sepsis, a complex process regulated by iron throw master regulators (Choi and Choi 2022). (5) *Transmission to new hosts and the environment*. Diseased fish can infect humans (zoonosis) by contact and shed live bacteria in the water as well. Transmission of the pathogen is increased at warm temperatures. Figure from Hernández-Cabanyero and Amaro (2020)

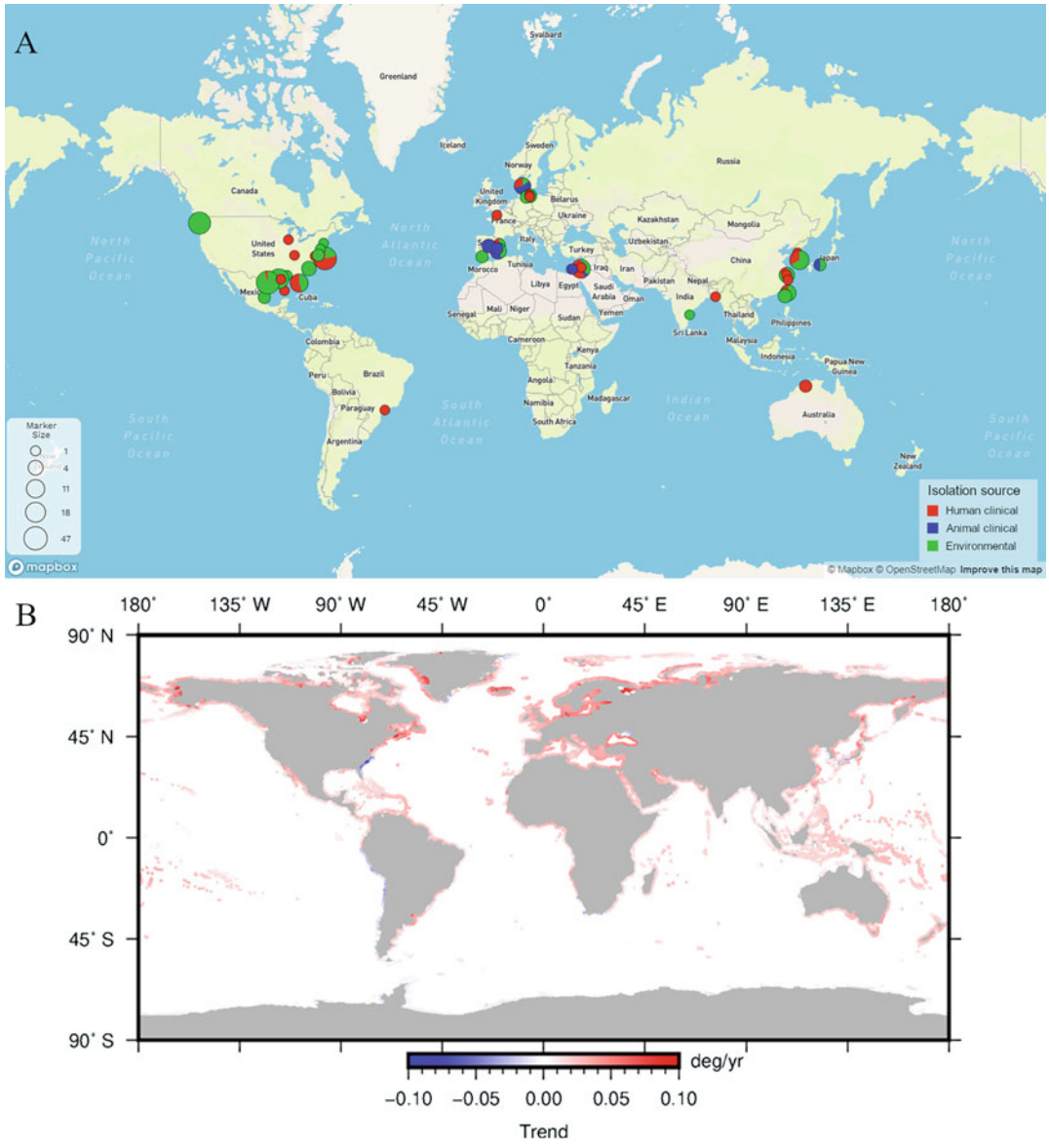


**Fig. 9.3** Geographical distribution of *V. vulnificus* and increase in coastal water temperature. World map showing the isolation points of *V. vulnificus* strains of sequenced genome (**a**) as well as evidence of global warming (**b**). (**a**) Spatial distribution of *V. vulnificus* reported genomes. Metadata for each strain was retrieved when available from NCBI database (https://www.ncbi.nlm.nih.gov/) and map was plotted with microrreact (Argimón et al. 2016). Note that each point is just an approximation since only the country or the state (for USA isolations) was available. Points are colored by isolation source. Red: Human clinical, blue: animal clinical, green: environmental (water, healthy marine animal, sediment, etc.). The size of each point represents the number of isolations. (**b**) Global warming trend in coastal areas. Data from superficial seawater temperature were taken from 1982 to 2020 and a lineal regression was performed. The map indicates in color code the difference in number of weeks of temperature above 18 °C between the periods 2006–2015 and 1982–1991 (figure elaborated by Joaquín Triñanes and Jaime Martínez-Urtaza)

eating" bacterium, it is also relevant in the USA and, in addition, in Asia and Europe (Oliver 2005; Frank et al. 2006; Kim and Chun 2021). In Europe, reported cases in humans are associated with severe wound infections and secondary septicemia acquired by contact (Brehm et al. 2021). There have also been human vibriosis associated with fish farms reported mainly in Europe and in the Eastern Mediterranean, all of them of the type of wound infections and secondary contact-acquired septicemia (Dalsgaard et al. 1996; Bisharat et al. 1999; Danin-Poleg et al. 2015). According to the scientific literature, the European infections have been sporadic cases of zoonosis, while those in the eastern Mediterranean have been non-zoonotic outbreaks in which healthy fish acted as carriers. All these cases underline the role of fish as a common source of human infections. Finally, if we look at the world map presented in Fig. 9.3a, we will see how the Baltic Sea appears as an area from which *V. vulnificus* can be isolated and where cases of human vibriosis have been recorded, all clearly associated with climate change (Fig. 9.3b).

***Risk and Virulence Factors*** Epidemiological data suggest that the main risk factors predisposing to death from septicemia caused by *V. vulnificus* are related with a malfunctioning immune system (Horseman and Surani 2011). Of note, however, is the dependence between blood iron levels and disease severity: the higher the level, the greater the severity (Bullen et al. 1991). Nutritional immunity due to iron sequestration by serum transferrin thus appears to be one of the key defense mechanisms to keep the pathogen under control. Diseases such as hemochromatosis and cirrhosis cause elevated iron levels and subvert or override nutritional immunity predisposing the patient to death by sepsis caused by this pathogen (Nazir et al. 2016). However, serum complement and phagocytosis are mechanisms of innate immunity as important, if no more, as iron sequestration in the control of sepsis (Minasyan 2019). Early studies on the virulence factors in *V. vulnificus* by using *ex vivo* and/or *in vivo* assays in animal models suggested that the capsule protected the bacterium from complement and phagocytosis (Simpson et al. 1987; Wright et al. 1990; Amaro et al. 1994). Later, a transposon insertion sequencing study (see Carda-Diéguez and Amaro 2022 for a detailed description of the methodology) performed in an *ex vivo* model of human septicemia confirmed that the bacterial capsule was the only virulence factor essential for resistance to the bactericidal effect of serum while none of the iron uptake mechanisms produced by this pathogen was sufficient to reverse nutritional immunity if serum comes from healthy patients (Carda-Diéguez et al. 2018). Complementary transcriptomic studies performed in human serum with and without iron along with *in vitro*, *ex vivo*, and *in vivo* assays suggested that the key to explain the susceptibility to death from sepsis in patients with elevated blood iron levels is that the production of the protective capsule is enhanced by iron (Pajuelo et al. 2016; Hernández-Cabanyero et al. 2019). The conclusion of all these studies is that the bacterium becomes septicemic in patients with hemochromatosis or cirrhosis because only under these conditions the pathogen can produce sufficient capsule to resist the mechanisms of innate immunity and proliferate in the blood. In excess iron, moreover, the bacterium can incorporate iron directly, so it would not need more sophisticated mechanisms for its uptake such as the production of siderophores and the set of membrane proteins necessary for iron-siderophore transport and internalization. Figure 9.1 summarizes the role of iron throughout the life cycle of the pathogen. The figure was constructed from several transcriptomic studies in which the pathogen was grown under iron excess (artificial medium or iron-supplemented human serum) and iron deficiency (artificial medium supplemented with an iron chelator or human serum from healthy donors) supplemented with mutagenesis of differentially expressed genes (Pajuelo et al. 2016; Hernández-Cabanyero et al. 2019). The global results show that iron directly or indirectly controls the transcription of genes also controlled by temperature plus the genes for the main toxins of the species (VvhA and RtxA1) and the Vvp protease (Fig. 9.1). According to the model, under

iron excess both toxins are expressed in early stages of the infection and in parallel. Consequently, rapid death of the patient can occur (more details in Hernández-Cabanyero and Amaro 2020).

**Vibriosis of Fish** *V. vulnificus* causes a disease known as warm water vibriosis in different fish species of interest in aquaculture (Amaro et al. 2015, 2020). It is so named because the disease is recorded at temperatures above 22 °C. Warm water vibriosis appears as outbreaks or epizootics of varying mortality that mainly affect the tilapia and eel species of interest in aquaculture (*Anguilla anguilla*, *A. japonica*, *Oreochromis niloticus* and *O. mossambicus,* among others). As with human diseases, warm water vibriosis is transmitted by contact or ingestion. When transmitted by contact with animals or water, the pathogen is chemoattracted towards the mucus covering the gills, colonizes the mucosal surface by forming a biofilm and, depending on the state of the animal's defenses, it can either produce only superficial lesions or invade the bloodstream and reach internal organs causing death by hemorrhagic septicemia (Marco-Noales et al. 2001). When transmitted by ingestion, the pathogen can also be attracted by intestinal mucus, colonize the intestine and, depending on the state of the defenses, pass into the blood and cause hemorrhagic septicemia (Fouz et al. 2010). The clinical signs in both cases are very similar, as in human septicemias, with the main clinical signs being abdominal petechiae, hemorrhages at the base of the dorsal and anal fins and redness in the operculum region (Fig. 9.2b).

***Epidemiology*** Outbreaks of warm water vibriosis occur in extensive fish farms located in areas where the pathogen is endemic (Fig. 9.3a). In addition, outbreaks and epizootics can also occur in any intensive fish farm that uses recirculating water at the appropriate temperature and salinity for pathogen transmission and infectivity, regardless of its geographic location. The most susceptible farmed hosts for the pathogen are the eel and tilapia species of commercial interest. These species can be cultured at different salinities, but always at temperatures over 20 °C, both in closed and open circuits (https://www.fao.org/3/S5407E/S5407E05.htm, El-Sayed 2020). When fish are grown in open culture, the culture areas coincide with the distribution area of the pathogen. Finally, this pathogen can also cause septicemia in different species of fish kept in zoo aquariums (clownfish, pompano, grouper...) and even in mammalian species also kept in aquariums (Li et al. 2018; Gibello et al. 2019; Liu et al. 2019; Sumithra et al. 2019).

***Risk and Virulence Factors*** In the case of animal vibriosis, risk factors have to do not only with the immune status of the fish but also with external factors affecting the survival and virulence of the pathogen (Amaro et al. 2020). Thus, stressful situations in fish farms, such as those due to overcrowding of animals in tanks and water physicochemical parameters far from the optimal ones for culture, can have an immunosuppressive effect, predisposing fish to the most severe forms of vibriosis and other infectious diseases (Smith 2019). Furthermore, since the causative agent of vibriosis is an aquatic bacterium, culturing fish under conditions that favor pathogen virulence, survival and transmission can be lethal to fish even under optimal culture conditions in farms (Amaro et al. 2020). The effect of water salinity and temperature on *V. vulnificus* virulence and vibriosis transmission has been extensively studied (Kaspar and Tamplin 1993; Amaro et al. 1995; Marco-Noales et al. 1999, 2001). In the case of salinity, the results obtained showed that the pathogen although survives for a long time in artificial seawater microcosms it is only infective trough water at salinities from 0.5 to 3%, with a maximum in virulence at 1–1.5% (Amaro et al. 1995). Regarding temperature, the survival studies carried out in microcosms maintained at different temperatures together with virulence tests carried out at the same temperatures showed that, although the pathogen remains viable under starvation between 10 and 30 °C for months, it is only infectious between 20 and 28 °C (Amaro et al. 1995; Marco-Noales et al. 1999). Subsequent transcriptomic studies carried out at temperatures between 20 and 37 °C showed that the

transcription of genes involved in the fish colonization and invasion process is significantly increased at 25–28 °C, which would prepare the pathogen for infection at warm temperatures (Hernández-Cabanyero et al. 2020). Among the genes that are activated are those involved in iron uptake and resistance to microcidal peptides (Hernández-Cabanyero et al. 2020) (Fig. 9.1). Of the two physico-chemical factors, temperature is absolutely limiting. Thus, when the temperature is below 20 °C the disease is not transmitted at any salinity (Amaro et al. 1995). But when the temperature is between 22 and 28 °C the disease is transmitted even at a salinity of 0.2–0.3%, although in this case, the pathogen is much less virulent and the preferred route of infection does not appear to be contact but ingestion (Amaro et al. 1995, and unpublished results). All these results correlate with field data: the disease is much more severe and virulent when salinity and temperature are optimal for both pathogen viability and expression of colonization and invasion genes.

Unlike human vibriosis, excess iron content in the blood has not been reported as a risk factor, probably because fish do not appear to be susceptible to hemochromatosis or cirrhosis (Smith 2019). However, iron deficiency, as found in the serum of healthy fish, activates the transcription of chromosomal and plasmid genes involved in resistance to innate immunity in fish (*ftbp*, genes for O antigen biosynthesis, etc.), as well as the transcription of VvhA and Vvp (Fig. 9.1). VvhA is a hemolysin whose expression is subject to a complex regulatory process that is not exclusively iron-dependent and is in fact transcribed early in fish. The hypothesis is that VvhA would lyse erythrocytes releasing enough iron to trigger RtxA1 transcription and thus host death (more details in Hernández-Cabanyero and Amaro 2020).

**Features Common to Human and Animal Vibriosis**

From the above it is clear that there are several features that are common to human and animal vibriosis despite the obvious physiological differences between their hosts. The first is the mode of transmission since both can be transmitted by contact or ingestion. The second is that the most severe form of both vibriosis is a septicemia, reason by which *V. vulnificus* is also considered a multihost septicemic pathogen. The third is the rapidity in which the pathogen can produce the host death under risk conditions, and the fourth is that hemorrhages are common clinical signs, as shown in Fig. 9.2.

## 9.3 Intraspecific Variability: Mobile Genetic Elements and Virulence

One of the most important challenges in studying pathogenic bacterial species is to find out whether they are genetically variable and whether this variability affects virulence genes. Taking as an example the type species of the genus *Vibrio*, the human pathogen *V. cholerae*, the genes coding for cholera toxin (the main virulence factor involving in cholera) are present in a lysogenic phage, so only strains lysogenized by the phage will be able to cause cholera (Faruque and Mekalanos 2012). But, considering *V. vulnificus*, a multihost pathogen, would the genes involved in virulence and host specificity also be present in mobile genetic elements?

Historically, the species was formally defined in 1980 to group a series of lactose-positive vibrios from environmental and human clinical samples taken in the USA (Farmer 1980) and, as early as 1982, it was divided into two biotypes, one to group the original strains (biotype 1) and the other to group strains from diseased eels (*Anguilla japonica*) cultured in Japan (biotype 2) (Tison et al. 1982). The eel strains were phenotypically homogeneous and easily distinguished from biotype 1 by their negative character in some biochemical tests (indole production and mannitol fermentation among others). Subsequently, biotype 2 was found to constitute a new serogroup O within the species (serovar E (Amaro and Biosca 1996)), and serological identification of biotype 2 by ELISA or

immunoblot (Amaro et al. 1997; Biosca et al. 1997) was proposed. At that time, biotype 1 included human and environmental clinical strains and biotype 2 clinical strains of animal origin (eel). But in the following decade, the taxonomic situation became more complicated. First it was found that biotype 2 also included human clinical strains, some of which were clearly zoonotic (Amaro and Biosca 1996; Dalsgaard et al. 1996), and later new serological variants were described within biotype 2, all of them from cases of vibriosis in European eel (serovars I and A) (Høi et al. 1998; Dalsgaard et al. 1999; Fouz et al. 2006). These new serovars were phenotypically indistinguishable from biotype 1 but shared with biotype 2 their virulence to fish. In the same decade, a third biotype was described in Israel (eastern Mediterranean) to group the only strains of the species associated with outbreaks of human vibriosis among fish farm workers and tilapia consumers (Bisharat et al. 1999). These strains were described as a clonal group virulent for humans (Bisharat et al. 1999). The virulence for fish of biotype 3 was not assessed and fish were seen as carriers but not as hosts for this new group. Finally, as more and more strains of the species were isolated, greater variability was found in all groups and a time came when it was no longer possible to ascribe a given isolate to a particular biotype. It was then that a new classification system was needed, if possible, an evolutionary system that would facilitate the understanding of the evolution of the species and the emergence of the virulent groups.

The first phylogenomic study on *V. vulnificus* was published in 2018 (Roig et al. 2018). This work had both a taxonomic and evolutionary objective. The authors studied the intraspecific variability of the species by analyzing the single nucleotide polymorphisms present in the core genome of 80 strains of the three biotypes isolated from all over the world and from all sample types (human clinical, animal clinical, water, seafood, fish…) and reconstructed the phylogenetic tree for chromosomes I and II. Figure 9.4 shows the phylogenetic tree reconstructed from the two chromosomes. According to this study, the species was divided into five evolutionary lineages (L) that did not correspond to the three biotypes except for biotype 3, in the study L3. The two major lineages, L1 and L2, were of worldwide distribution, while the rest were restricted to specific geographic areas, L4 to Europe and L3 and L5 to Israel. L1 grouped the majority of isolates from cases of primary septicemia in humans and L2 those from secondary septicemia and wound infections in humans together with all biotype 2 isolates which in turn were grouped into three serovar-related sublineages (L2-serE, L2-serA, and L2-serI). Interestingly, the strains used by Tison et al. (1982) for the description of biotype 2 and all the zoonotic strains related to eel farms in Europe belonged to serovar E (Tison et al. 1982; Veenstra et al. 1993; Amaro and Biosca 1996; Dalsgaard et al. 1996; Fouz et al. 2006). Finally, L1 and L4 were formed by independent clones, L2 by independent clones plus clonal groups related to eel vibriosis in farms (L2-serE, L2-serA and L2-serI) and L3 and L5 by clonal groups related to tilapia-farms linked human vibriosis, apparently not zoonotic (Bisharat et al. 1999; Efimov et al. 2015). In conclusion, the authors proposed that the species be subdivided into five lineages instead of three biotypes plus a pathovar including all virulent groups for fish (pv. *piscis* from fish in Latin). The problem with this subdivision is that practical systems would be needed to ascribe a particular isolate to a lineage without the need for genome sequencing and phylogenomic analysis. Carmona-Salido et al. have designed two PCRs using comparative genomics that allow the identification of L3 (Carmona-Salido et al. 2021b) and L4 (unpublished results). Future studies will allow the identification of the rest of the lineages by PCR.

Returning to the question of genetic variability affecting virulence genes, Roig et al. (2018) demonstrated that nearly 80% of the human virulence genes described to date were present in the core genome and suggested that all strains of the species should be considered potentially virulent to humans. But what did the pv. *piscis* strains have in common that distinguished them from the rest in their ability to cause vibriosis in fish?

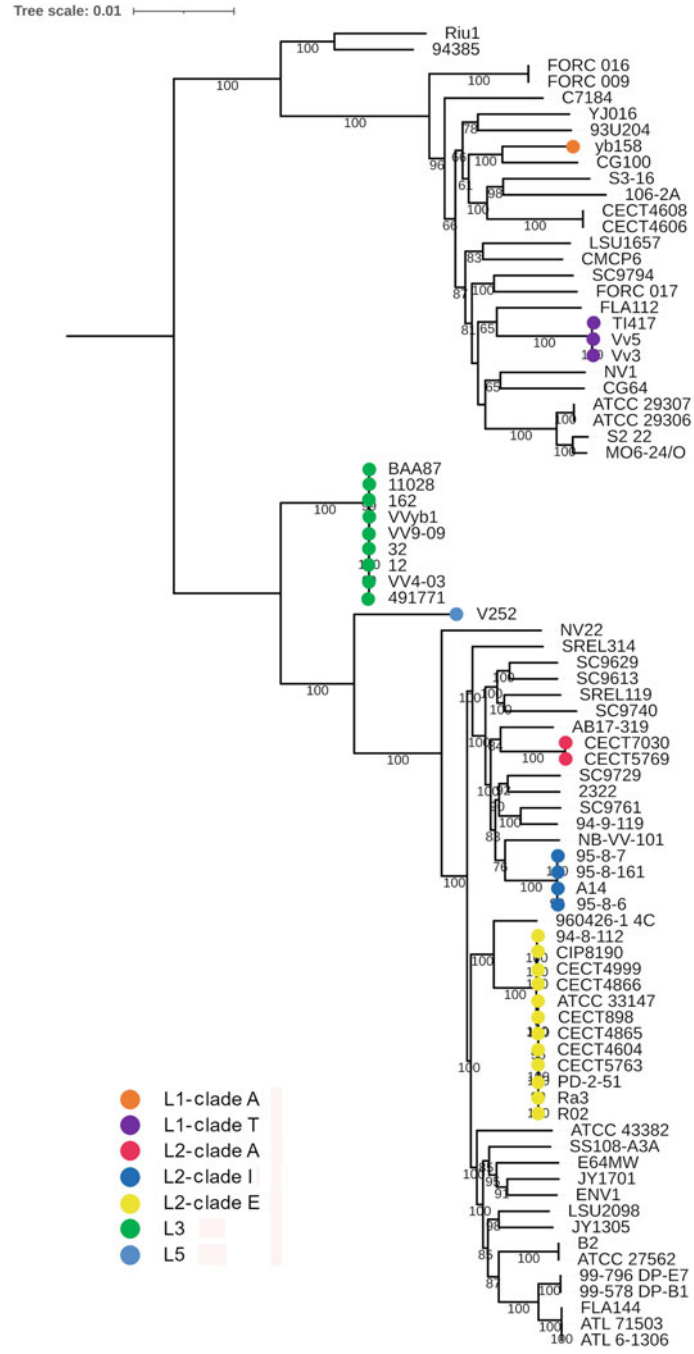


**Fig. 9.4** *V. vulnificus* phylogeny**.** The phylogenetic tree was reconstructed from the SNPs present in the core genome using the maximum-likelihood method and the generalized time-reversible model (GTR + F + R5) of evolution. Bootstrap support values from 1000 replicates are indicated in the corresponding nodes as percentages. L, lineage. Figure from Carmona-Salido et al. (2021a, b)

**Plasmids** Lee et al. attempted to answer the above question by comparing DNA from fish virulent strains (tester DNA) with DNA from avirulent strains (driver DNA) by subtractive hybridization (Lee et al. 2005). This methodology allows PCR amplification of DNA fragments that are specific to the tester DNA after discarding those that are common with the driver DNA after two successive hybridizations (see Rebrikov et al. 2004 for more details). In this study, the

authors compared the DNA from a strain of L2-ser E (from now L2-clade E) with an equimolar mixture of DNA from three L1 strains from human clinical and environmental origin. The result obtained suggested that the fish virulent strains contained unique information of plasmid origin. To demonstrate the link between plasmids and fish virulence, Lee et al. (2008) isolated the plasmids from two L2-clade E strains, one containing a single ~68 Kb-plasmid (strain R99) and the other one containing two plasmids, one of ~67 Kb and the other one of ~57 Kb (strain CECT4602). The authors sequenced the plasmids and closed and annotated the genomes. Figure 9.5 shows the main genetic features of the three sequenced plasmids. The plasmids pCECT4602-2 and pR99 were virtually identical and corresponded to a putative virulence plasmid, as they contained a complete gene cluster for one of the major toxins of this species, the toxin RtxA1, its post-transcriptional modification and its transport and secretion (Woida and Satchell 2018). In contrast, pCECT4602-1 was a putative conjugative plasmid, as it contained a complete set of genes for conjugative transfer. In addition, pR99 and pCECT4602-1 also contained two genes for a toxin/antitoxin system that would be responsible for plasmid maintenance in the host cell. Finally, all three plasmids contained genes for hypothetical proteins or with very low homology to known proteins, genes for transposases, and genes related to DNA recombination with a putative viral origin. Notably, the three plasmids possessed identical sequences that the authors named ID1 and ID2 (Fig. 9.5). To demonstrate that the 68–69 kb-plasmid was a virulence plasmid, Lee et al. first deleted the gene encoding the MazF toxin in pR99 and then obtained the cured strain by growing the Δ*mazF* mutant at limiting temperature in the presence of acridine orange, a classic procedure. The cured strain was then subjected to *ex vivo* (resistance to fish serum and human serum) and *in vivo* (virulence for eel by immersion and for mice by injection) pathogenicity tests and was found to be sensitive to the bactericidal effect of eel serum and avirulent to eel, while maintaining its resistance to human serum and its virulence to mouse. Consequently, pR99 was shown to be a virulence plasmid encoding host specificity by means of a resistance system to innate immunity in eel blood. Later, Roig and Amaro (2009) demonstrated by southern blotting with probes against marker genes for each plasmid that the virulence plasmid was present in all pv. *piscis* strains, whereas the putative conjugative plasmid was present in 90% of L2-pv. *piscis* strains, 100% of L3 strains and 50% of L1 strains.

**Plasmid Virulence Genes Involved in Host Specificity** To determine which virulence genes were involved in the resistance to the eel immune system, a series of mutants in plasmid genes were obtained both by allelic exchange and by transposition (Lee 2008; Lee et al. 2013; Pajuelo et al. 2015; Hernández-Cabanyero et al. 2019). The first selected gene was the only known virulence gene present in the plasmid, *rtxA1,* which had been implicated in resistance to phagocytosis and eel death (Lee et al. 2013). First, Roig et al. found that the whole *rtxA1* cluster was duplicated on chromosome II in all the pv. *piscis* strains (Roig et al. 2011). Later, Lee et al. (2013) showed that the plasmid gene (p*rtxA1*) was not involved in host specificity as the mutant deficient in p*rtxA1* was as resistant to eel serum and virulent for eel as the wild-type strain. Of the remaining mutants analyzed, the most interesting ones were found to be those in *vep07* and in *vep20* genes. Firstly, deletion of each gene significantly reduced growth in fish serum and virulence to fish as well as the ability to colonize and invade animals when infected through water, while did not affect resistance to human serum and virulence to mice (Pajuelo et al. 2015; Hernández-Cabanyero et al. 2019). Secondly, the inhibitory effect of serum on mutant growth was found to be reversed by adding exogenous iron, in the case of Δ*vep20,* or by inactivating the eel alternative complement pathway, in the case of Δ*vep07* (Pajuelo et al. 2015; Hernández-Cabanyero et al. 2019). From there, it could be hypothesized that Vep07 would be involved in serum complement resistance and Vep20 in iron uptake in serum. The

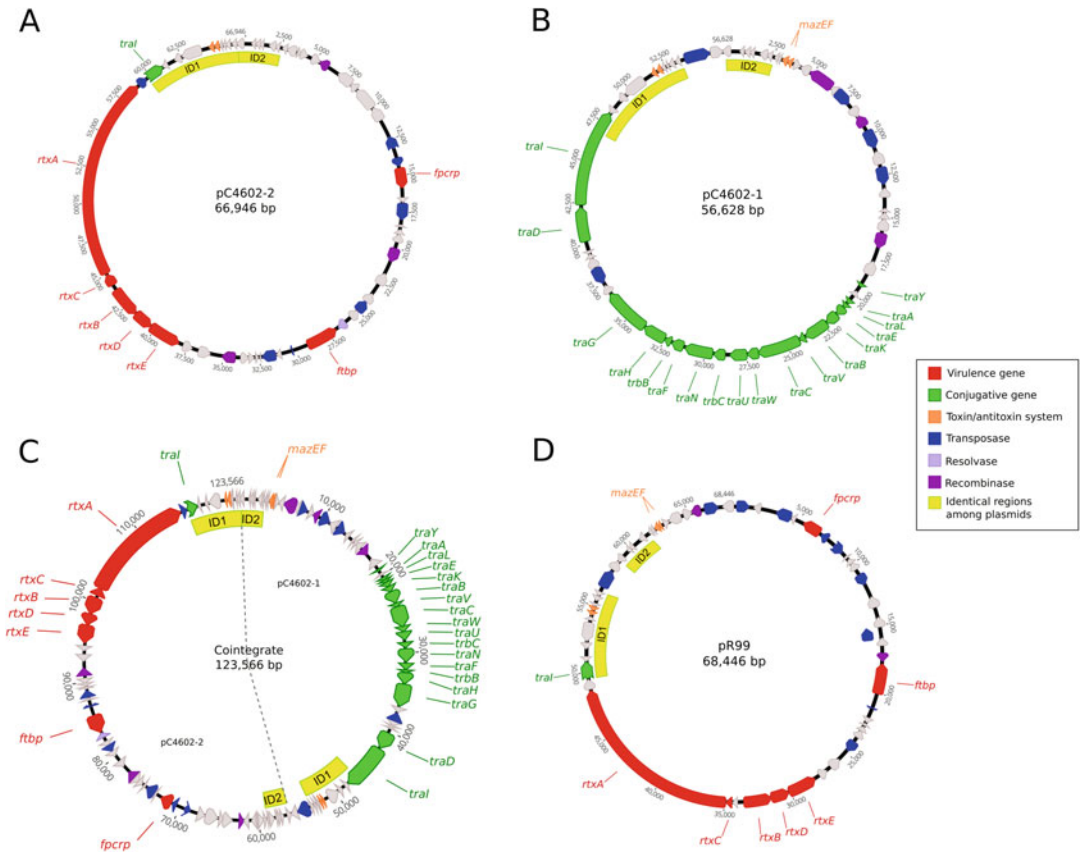


**Fig. 9.5** Plasmids in pv. *piscis* strains from L2. Circular representation of the virulence plasmids pC4602–2 (Genbank accession: AM293860.1) (**a**) and pR99 (Genbank accession: NZ_CP014638.1) (**d**), the conjugative plasmid (pC4602-1, Genbank accession: AM293859.1) (**b**) and the cointegrate (hypothetical model) (**c**). Each annotated gene is represented by an arrow. Virulence genes are in red, conjugative genes in green, toxin/antitoxin genes in orange, transposases in blue, resolvases in mauve, recombinases in purple and the rest in grey. Identical regions among virulence and conjugative plasmid are represented with yellow boxes

proteins were localized in the bacterial outer membrane and their transcription was shown to be iron-regulated being optimally expressed under the iron-restrictive conditions imposed by serum from healthy fish (Pajuelo et al. 2015; Hernández-Cabanyero et al. 2019). The in cursive analysis and modeling of both proteins showed that both were β-barrel type outer membrane proteins, Vep20 sharing 28% identity with the human transferrin receptor of *Neisseria meningitidis,* and Vep07 being actually a lipoprotein that did not resemble any known protein (Pajuelo et al. 2015 and unpublished results). The fragments predicted to be exposed in the external face of the outer membrane were obtained in recombinant form to identify whether they bound some component of eel serum under the hypothesis that Vep20 would bind eel transferrin and Vep07 any component with an inhibitory effect on complement activation and phagocytosis. The hypothesis was confirmed for Vep20 as the recombinant protein specifically bound eel transferrin (Pajuelo et al. 2015) but not for Vep07 as no serum component bounded to recombinant Vep07 was identified by liquid chromatography coupled to mass spectrometry (Hernández-Cabanyero et al. 2019). Additional experiments performed by Hernández-Cabanyero

et al. demonstrated that Vep07 was also involved in resistance to eel phagocytosis (Hernández-Cabanyero et al. 2019). From these results, it can be concluded that Vep20 is a fish transferrin-specific receptor involved in iron uptake in serum and Vep07 an outer membrane lipoprotein involved in protection against complement activation and phagocytosis by an unknown mechanism. Vep07 was named Ftbp (*f*ish *t*ransferrin *b*inding *p*rotein) and Vep07 Fpcrp (*f*ish *p*hagocytosis and *c*omplement *r*esistance lipo*p*rotein). Therefore, the virulence plasmid confers resistance to eel innate immunity in blood because it encodes for a "survival in fish blood kit" that facilitates multiplication of the bacterium and, consequently, production of the virulence factors (mainly the toxin RtxA1) that cause death. Thus, when the bacterium infects a human, since it does not have a specific complement resistance system, it needs the serum to contain free iron to activate the production of the capsule and to survive, but in fish, the bacterium containing the virulence plasmid expresses the survival kit that specifically provides iron from transferrin and protects it from complement and phagocytosis. These results support the hypothesis that *V. vulnificus* is a fish-adapted pathogen that only causes severe disease in humans when their immune system is somehow compromised.

**Horizontal Gene Transfer and Evolution** Since the genes encoding the specific innate immunity resistance system in blood are located on a plasmid, the next question would be, is it a mobilizable plasmid? That is, could the virulence plasmid be transmitted by conjugation by parasitizing the transfer machinery encoded in the conjugative plasmid, a process that has been described for multiple non-conjugative plasmids? (Lang et al. 2014; Zechner et al. 2017). To demonstrate that the 56 kb-plasmid was a conjugative plasmid and, at the same time, to answer this question, Lee et al. (2008) performed a series of experiments of conjugation. In those experiments they used a L2-clade E strain containing the two plasmids as donor strain, and a cured strain derived from another L2-clade E strain harboring a cassette of resistance to chloramphenicol (to facilitate transconjugants recovery) as recipient strain. They found that the 56 kb-plasmid was a conjugative plasmid that could be transferred between strains of the same clade with 30% efficiency. More importantly, they found that the virulence plasmid could also be transmitted along with the conjugative plasmid with an efficiency of 3%. In addition, the authors found a third plasmid in the transconjugants presenting both plasmids. The third plasmid turned out to be a cointegrate probably formed by recombination between ID1 and ID2 present in both plasmids (Fig. 9.5). They analyzed the transconjugants and found evidence of sequence variability in the individualized plasmids. The authors proposed this variability had probably been generated after resolution of the cointegrate. In conclusion, the virulence plasmid is a mobilizable plasmid that can be transmitted by parasitizing the conjugative plasmid and this transmission could generate variability and consequently plasmid evolution.

Another interesting question is to know at what point of the evolution of *V. vulnificus* the virulence plasmid was acquired. To answer this question, Roig et al. (2018) constructed the phylogenetic tree from the plasmid core and compared it with the phylogenetic tree for the two chromosomes. They found that the trees were not congruent each other, that is, they did not tell the same evolutionary story. This finding is compatible with the hypothesis that the plasmid would have been acquired several times by different clones probably in fish farms. Subsequently, amplification of the transconjugants after successive outbreaks of vibriosis would have given rise to the clonal groups known today as clades A, E, and I within pv. *piscis*.

In consequence, unlike *V. cholerae*, all strains of *V. vulnificus* should be considered potentially virulent for humans and only those that have acquired the virulence plasmid by conjugation should be considered virulent for fish. This situation raised an interesting question, could fish

farms be acting as evolutionary engines that facilitate the transfer between clones of the virulence plasmid, thus contributing to the genetic variability of the species and the emergence of new virulent groups?

## 9.4 Fish Farms as Evolutionary Drivers in *V. vulnificus*

The results obtained by Lee et al. (2008) and Roig et al. (2018) supported the hypothesis that fish farms could act as accelerators of the evolution of this species by favoring the emergence of new virulent groups. To confirm this hypothesis, Carmona-Salido et al. analyzed a series of recent isolates from putative cases of vibriosis in fish and subjected them to *in vitro*, *ex vivo* and *in vivo* assays, including genome sequencing using the Illumina and MinIon platforms (Carmona-Salido et al. 2021a). The most relevant results were obtained with a series of strains from cases of vibriosis recorded in extensive fish farms located in the eastern Mediterranean between 2016 and 2019. At the time of the epizootics, the fish were kept in water of salinity between 0.2 and 0.42% and at a temperature between 26 and 27 °C. Although the temperature was adequate for the fast transmission of vibriosis caused by *V. vulnificus*, the salinity was below that necessary for the disease to have been particularly virulent. In fact, the mortality rate recorded in the various outbreaks was low (always less than 4%), although the losses were harsh because the outbreaks had affected the most valuable specimens in the companies.

**Identification of a New Zoonotic Clade Within pv. *piscis* That Belongs to L1** The new fish isolates from fishfarms located in the eastern Mediterranean were found to be virulent for tilapia (its original host), being able to reproduce the clinical signs of natural vibriosis, which confirmed that they belonged to pv. *piscis*. In addition, they were serologically homogeneous and constituted a new O-serovar within the species that the authors named serovar T (from tilapia). The analysis of their genomes demonstrated that they constituted a new clade within pv. *piscis* as the ANI (*a*verage *n*ucleotide *i*dentity) values among them were almost 100%. This new clade was designated as clade T. The isolates of the new clade were also virulent for mice, resisted the bactericidal action of human serum complemented with iron (iron-overloaded serum) and were positive in the PCR that determines public health hazard based on the amplification of a polymorphism in the *pilF* gene (Roig et al. 2010). All these results clearly indicated that this new pv. *piscis* clade was potentially zoonotic. However, the isolates were negative in both PCR targeting the pathovar marker gene (plasmid gene *fpcrp*) and PCR targeting the zoonotic clade (clade E) marker (*seq61* found by subtractive hybridization (Lee et al. 2005)). To find out whether the strains of the new clade really had neither *fpcrp* nor *seq61*, the authors analyzed their genomes. They found that *seq61* was not present while *fpcrp* was actually present, although showing variability just in the area selected for one of the primers used in the original PCR (Sanjuán and Amaro 2007). The authors redesigned the PCR to distinguish all strains of pv. *piscis*, including the new clade, and validated it with more than 150 strains belonging to all lineages in their collection.

Phylogenomic analysis based on the core genome in the context of the species located the pv. *piscis* clade T in L1 (Fig. 9.4), the lineage that presumably included isolates from primary septicemia in humans following consumption of raw shellfish. It has been recently proposed that the species is subdivided into four clusters (C1 to C4, equivalent to L1 to L4). From those, C1 and C2 represent two different ecotypes in the process of speciation, the first one (bloomer) better adapted to a free-living form and the other adapted to a host-associated life form (López-Pérez et al. 2019). The results obtained by Carmona-Salido et al. (2021a, b) suggest that L1 includes both sessile and free-living adapted isolates adding complexity to the evolutionary model proposed by Almagro et al. Remarkably, in both works, it has been pointed out that fish farms are acting as

genetic drivers in the evolution of the species, favoring genetic exchange and thus giving rise to new virulent variants. One clear example of this fact is that the recurrent outbreaks of vibriosis in tilapia had been caused by a new clade within pv. *piscis* that belonged to L1 and was potentially zoonotic.

**A Family of Fish Virulence Plasmids Present in Four of the Five Lineages of the Species** The next step was to find out whether this new clade harbored the virulence plasmid present in the pv. *piscis* strains described to date by analyzing their genomes. Indeed, the clade T isolates contained an identical plasmid of about 56 Kb carrying the genes encoding the survival in blood kit, *fpcrp* and *ftbp*. The new virulence plasmid appeared to be a hybrid between the original one and the conjugative plasmid since the site occupied by the *rtxA1* cluster in the original plasmid was occupied in the clade T plasmid by several *tra* genes identical to that of the conjugative plasmid (Fig. 9.6). Consequently, the clade T strains belonged to pv. *piscis* and possessed a virulence plasmid of the same family as the original one that contained the survival in blood kit but lacked the *rtxA1* cluster. This new virulence plasmid had been transferred to L1 and the transconjugant clone had amplified in eastern Mediterranean tilapia farms resulting in recurrent outbreaks over a period of at least 4 years.

The authors also determined the genomic relatedness of clade T with the different lineages and groups within the species by determining ANI values and found that clade T was genomically closer to the groups that had emerged in similar environments (tilapia farms) located in the same geographical area (L3, L5 and L1-clade A) than to the rest of the pv *piscis* groups, all of which emerged in eel farms in Europe and Asia, and, in case of L1-clade A even to the phylogenetically closest strains within L1 (Fig. 9.4). Remarkably, L3, L5 and L1-clade A were clonal groups that included human clinical and environmental strains, apparently unrelated to cases of fish vibriosis or zoonosis. Carmona-Salido et al. hypothesized that these groups might be zoonotic and belong to pv. *piscis*, so they performed the same tests as they did with the strains of clade T with representative strains of the three previously described clonal groups. The results they obtained confirmed this assumption: the isolates of the three groups were virulent for tilapia and multiplied in tilapia serum. Moreover, all of them were positive in the PCR redesigned to detect pv. *piscis*, which confirmed that all these groups belonged to the pathovar and had probably emerged by acquisition of the virulence plasmid and amplification of the transconjugant clone after successive outbreaks of vibriosis in tilapia farms. To demonstrate it, Carmona-Salido et al. analyzed the genomes of representative strains of the different clonal groups and found that L3, L5 and the L1-clade A contained a plasmid very similar (pL3) or virtually identical (pL5 and pL1-clade A) to that of the clade T (Fig. 9.6). Thus, all these plasmids contained the genes for survival kit in fish blood, which would classify them as virulence plasmids. Moreover, all these plasmids lacked the cluster encoding the RtxA1 toxin, its post-transcriptional modification and transport and instead of it presented genes for conjugative transfer probably derived from the original conjugative plasmid. The only one that contained a complete cluster of *tra* genes was pL3, suggesting that this virulence plasmid might be conjugative. In conclusion, a fish virulence plasmid family is already present in four of the five lineages of the species, implying that *V. vulnificus* is a more important zoonotic species than previously suspected.

**Plasmid Gene Variability and Adaptation to Fish Species** According to the results obtained by Carmona-Salido et al. (2021a, b), pv *piscis* would include seven clades, three associated with eel farming and emerged in Asia and Europe (L2-clade E, L2-clade A and L2-clade I) and four associated with tilapia farming and emerged in the eastern Mediterranean (L1-clade A, L1-clade T, L3 and L5). Analysis of the plasmid gene *fpcrp* had revealed an area of variability in the gene that could be associated with the origin of the isolate, either eel or tilapia.

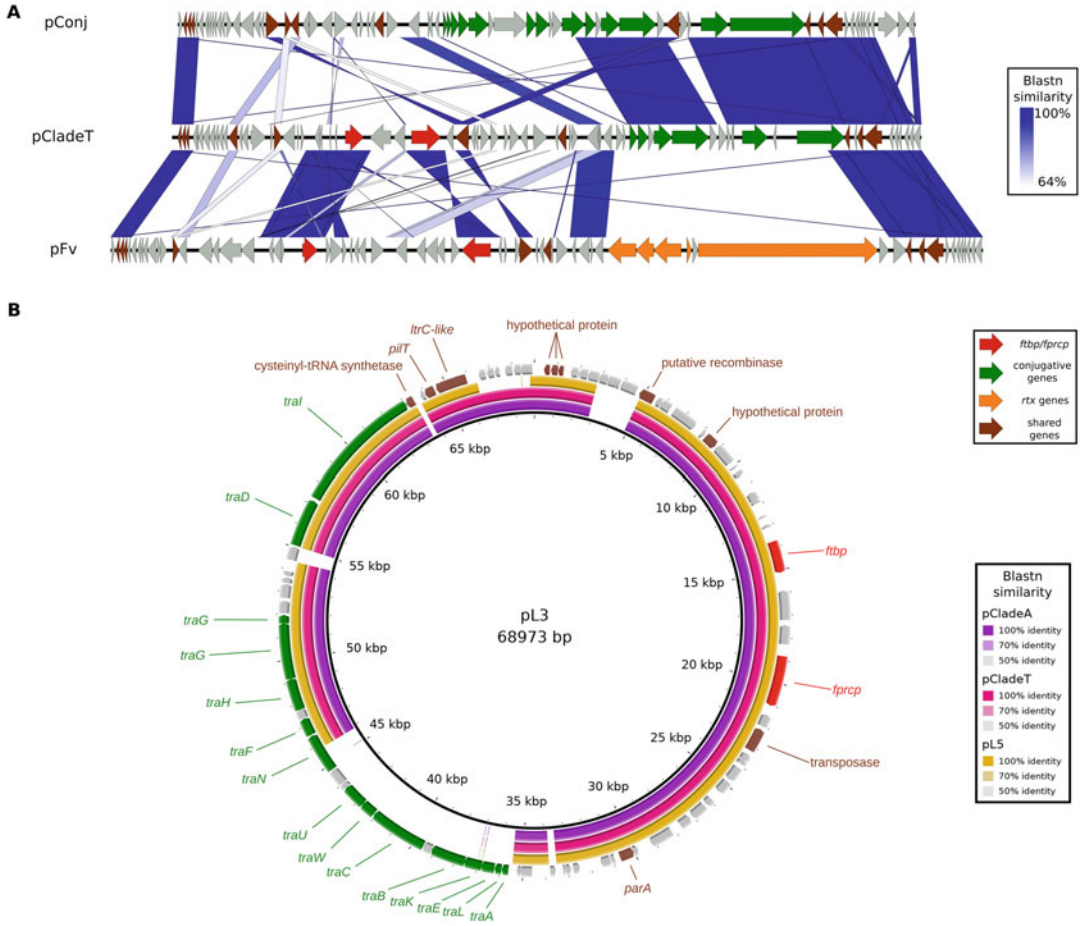


**Fig. 9.6** Plasmids in pv. *piscis* strains from L1, L2, L3, and L5. (**a**) Linear comparison among pConj, pCladeT and pFv performed with Easyfig (https://mjsull.github.io/Easyfig/) and (**b**) ring representation of the plasmids from clades and lineages emerged in the Eastern Mediterranean (from inside to outside, pL3 (used as reference), pClade A, pCladeT, and pL5). Genes are represented in arrows and color scale indicates nucleotide Blastn homology. The genes encoding the survival in fish blood kit are in red (*ftpb* and *fprcp*), conjugative genes in green and *rtx* genes in orange. The additional genes shared by all plasmids, including pFv, are colored in brown. Figure from Carmona-Salido et al. (2021a, b)

Consequently, the next question to be answered was: Is there a relationship between plasmid virulence gene variability and adaptation to fish species? To find it out, the authors tested the virulence of eel strains in tilapia and tilapia strains in eel and found host specificity: tilapia strains were not virulent for eel and eel strains were not virulent for tilapia. In parallel, they analysed the phylogeny of *ftbp* and *fpcrp* and found that strains clustered by infected host and not by phylogenetic relatedness. It appears, therefore, that the host adaptation may lie in the variability of proteins involved in resistance to the innate immune system in blood. This host adaptation affecting proteins involved in iron acquisition and complement resistance has been described in important human pathogens also involved in septicemia such as *N. meningitidis* and *N. gonohorroeae* (Baumler and Fang 2013). Nevertheless, this hypothesis should be demonstrated by obtaining deleted mutants in one of the gene variants (eel variant vs. tilapia

variant), by complementing the deleted mutant with the other variant and testing the mutants in virulence (eel-derived mutants in tilapia and vice versa). In conclusion, the results obtained by Carmona-Salido et al. demonstrate that the emergence of zoonotic groups related to vibriosis is occurring in fishfarms associated with the acquisition of a family of virulence plasmids encoding a protein and a lipoprotein that confer resistance to the innate immune system of certain fish species of interest in aquaculture that are cultured under conditions that favor the transmission and virulence of the pathogen. These results also suggest that small differences in these proteins may be related to adaptation to the infected host contributing to the spread of *V. vulnificus* to new habitats and host species.

## 9.5 Concluding Remarks

*V. vulnificus* is a pathogenic species of human and animal health concern. This species is mostly known as a human food-borne pathogen or as a marine flesh-eating bacterium. The results obtained in the above-mentioned studies suggest that its zoonotic pathogenic character is being underestimated and, consequently, the importance of fish farms as drivers of its evolution. Firstly, four of the five lineages of the species contain zoonotic groups or are themselves zoonotic, such as L3 and L5. Secondly, L3 and L5 arose in fish farms probably associated with outbreaks of vibriosis in tilapia, as is the case for the L1 clade T. Thirdly, the emergence of all these clades is associated with the acquisition of a family of virulence plasmids that are transmitted by conjugation and encode a kit for survival in fish blood. There is multiple recent evidence that variants of this survival kit are already present in the genomes of other fish pathogens such as *Pasteurella piscicida* and *V. harveyi*, and that their acquisition, at least in *V. harveyi*, is associated with increased outbreak virulence (Fouz et al. MS in prep.). All these results, in the context of climate change, strongly point to the role of fish farms as evolutionary drivers accelerating species evolution and the emergence of new virulent groups which, in the case of *V. vulnificus*, may be zoonotic. Special on-farm control measures, such as vaccination of animals, should be adopted in the coming years, not only to protect animals from vibriosis, but also to protect ourselves from infection and to prevent the emergence of new zoonotic groups.

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# The Role of Nutrients and Nutritional Signals in the Pathogenesis of *Vibrio cholerae*

10

N. D. McDonald, J. R. Rosenberger, S. Almagro-Moreno, and E. Fidelma Boyd

**Abstract**

*Vibrio cholerae*, the agent of cholera, is a natural inhabitant of aquatic environments. Over the past decades, the importance of specific nutrients and micronutrients in the environmental survival, host colonization, and pathogenesis of this species has become increasingly clear. For instance, *V. cholerae* has evolved ingenious mechanisms that allow the bacterium to colonize and establish a niche in the intestine of human hosts, where it competes with commensals (gut microbiota) and other pathogenic bacteria for available nutrients. Here, we discuss the carbon and energy sources utilized by *V. cholerae* and what is known about the role of nutrition in *V. cholerae* colonization. We examine how nutritional signals affect virulence gene regulation and how interactions with intestinal commensal species can affect intestinal colonization.

**Keywords**

*Vibrio cholerae* · Carbon utilization · Nutrient uptake · Host colonization · Host–pathogen interaction

*Vibrio cholerae* is a natural inhabitant of the aquatic environment, found in brackish and marine environments either as planktonic swimming cells or attached to biotic and abiotic surfaces, such as filamentous green algae, copepods, crustaceans, insects, and egg masses of chironomids (Colwell et al. 1977, 1981; Colwell 1992; Halpern et al. 2006; Raz et al. 2010). In the marine environment, *V. cholerae* lifecycle includes a planktonic stage and forms biofilms on biotic and abiotic surfaces as well as enter into a viable but non-culturable state in response to nutrient deprivation (Reguera and Kolter 2005; Yildiz and Schoolnik 1999; Colwell et al. 1985; Colwell and Haq 1994). The bacterium is also an extracellular intestinal pathogen that can colonize the human gut causing the pandemic infectious disease cholera an explosive water diarrhea (Balasubramanian et al. 2021; Barua 1972, 1992). The key virulence factors for *V. cholerae* strains that cause cholera are cholera toxin (CT), an AB5 exotoxin encoded on the CTX filamentous phage and the type IV pilus named the toxin co-regulated pilus (TCP) encoded on Vibrio pathogenicity island-1 (VPI-1), required for intestinal colonization (Holmgren et al. 1975; Herrington et al. 1988;

N. D. McDonald · J. R. Rosenberger · E. F. Boyd (✉)
Department of Biological Sciences, University of Delaware, Newark, DE, USA
e-mail: fboyd@udel.edu

S. Almagro-Moreno
Burnett School of Biomedical Sciences, University of Central Florida, Orlando, FL, USA

National Center for Integrated Coastal Research, University of Central Florida, Orlando, FL, USA



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Taylor et al. 1987; Waldor and Mekalanos 1996; Karaolis et al. 1998). A second pathovar of *V. cholerae* lacking CT and TCP exists that causes inflammatory diarrhea and uses a different mechanism of pathogenesis (Dziejman et al. 2005; Chen et al. 2007). These strains encode a type 3 secretion system (T3SS) that delivers effector proteins into host cells to cause inflammatory diarrhea (Dziejman et al. 2005; Chen et al. 2007; Tam et al. 2007).

For a long time, it was thought that *V. cholerae* could only be spread through water contamination by human carriers, however, it is known that *V. cholerae* colonizes zooplankton and phytoplankton with no necessity for a human host (Colwell et al. 1977; Huq et al. 1990). The ability to exist in a VBNC state may also allow *V. cholerae* to persist in the aquatic environment between cholera epidemics (Reidl and Klose 2002). The survival and spread of *V. cholerae,* due to its association with plankton, can be affected by changes in water temperature, salinity, and pH, conditions dependent on global climate change (Pascual et al. 2000; Alam et al. 2006; Colwell 1996; Huq et al. 1984). When suitable environmental conditions are present, increases in the abundance of *V. cholerae* populations occur, which may provoke large outbreaks of cholera in places like Africa and southern Asia, where the bacterium is endemic (Pascual et al. 2000; Alam et al. 2006; Colwell 1996; Huq et al. 1984).

The importance of metabolism in the environmental survival, host colonization, and pathogenesis of *V. cholerae* has only recently been reexamined and shown to be a neglected aspect and significant contributor to the spread and transmission of disease. For instance, *V. cholerae* has evolved inventive mechanisms that allow the bacterium to colonize and establish a niche in the intestine of human hosts to utilize of specific nutrients and micronutrients. In this niche, the bacterium must compete with the gut microbiota and other pathogens for available resources, which it does highly effectively. Here, we examine the carbon and energy sources utilized by pathogenic *V. cholerae* and the role of metabolism in establishing a niche in the human intestine. We discuss how nutritional signals affect virulence gene regulation and how interactions with the intestinal microbiota influences intestinal colonization.

## 10.1 Carbon and Energy Sources Utilized by Pathogenic *V. cholerae*

*V. cholerae* is a facultative anaerobic heterotroph and, as such, its metabolism requires at least one carbon source for energy and the production of intermediates for the biosynthesis of larger molecules. Various genomics, genetics, biochemical, and phenotypic analyses demonstrated that *V. cholerae* has the ability to grow on a wide range of carbon sources (Albert 1996; Albert et al. 1997; Heidelberg et al. 2002; Almagro-Moreno and Boyd 2009a, 2010; Reddi et al. 2018). Genome analysis of *V. cholerae* identified the major pathways for the metabolism of glycolysis metabolites (D-glucose-6-phosphate, D-fructose-6-phosphate, D-glucose, pyruvate), Tricarboxylic acid (TCA) metabolites (succinate, acetate, α-ketoglutarate, citrate, fumarate, and malate), alcohol sugars (D-mannitol), aldose sugars (D-mannose, D-galactose, D-ribose, D-glucose), disaccharides (trehalose, sucrose, maltose, dextrin) as well as at least nine different amino acids (Heidelberg et al. 2002; Shi et al. 2006; Patra et al. 2012), using glycolysis (Embden–Meyerhof–Parnas), Entner–Doudoroff (ED), pentose phosphate (PP), glyoxylate, and the tricarboxylic acid (TCA) pathways (Heidelberg et al. 2000).

Cholera is responsible for approximately 2.8 million cases of illness and 100,000 deaths, annually (Barua 1972, 1992; Alam et al. 2006; Karaolis et al. 1995). Two O1 serogroup biotypes of *V. cholerae* are important in pandemic cholera; the classical biotype which was the cause of the first six pandemics of cholera and the El Tor biotype that emerged in 1961 and the cause of current seventh pandemic (Barua 1972, 1992). In 1993, a serogroup O139 strain emerged in India and supplanted the O1 El Tor isolates as a leading cause of cholera (Hisatsune et al. 1993; Johnson

et al. 1994; Berche et al. 1994; Bhattacharya et al. 1994). However, by the late 1990s, the El Tor had re-emerged as the predominant cause of epidemic cholera (Faruque et al. 2003). The El Tor and classical biotypes are thought to have evolved independently of each other given their significant differences in their physiology and expression of virulence factors (Karaolis et al. 1994, 1995). Indeed, comparative genome sequence analysis of CTXΦ that contains the cholera toxin genes *ctxAB* from 13 *V. cholerae* strains revealed that there are distinct CTXΦ lineages in classical and El Tor strains. This data indicates that CTXΦ was acquired multiple times by *V. cholerae* confirming independent evolution of pathogen strains (Boyd et al. 2000).

In Fig. 10.1a, we examined the growth patterns of six *V. cholerae* strains in M9 minimal medium (M9) supplemented with D-glucose as a sole carbon source to determine whether differences exist among pathogenic strains in their ability to breakdown this carbon source. Three choleragenic strains were examined, two pandemic O1 serogroup strains (N16961, an El Tor biotype strain, and O395, a classical biotype strain), and an O139 serogroup strain (MO2) that emerged in 1993 as a predominant cause of cholera in India (Albert 1996; Berche et al. 1994; Bhattacharya et al. 2006). A *V. cholerae* strain NRT36S was also examined, a pathovar that causes inflammatory diarrhea and contains a type 3 secretion system (T3SS), but not cholera toxin (CT) or the toxin co-regulated pilus (TCP) (Chen et al. 2007). Two non-pathogenic strains (SG7 and 1528-79) were also included, which do not contain CT, TCP nor a T3SS (O'Shea et al. 2004). The growth curves showed differences existed among the strains, with some growing significantly better than others (Fig. 10.1a). For example, the O395 classical strain showed a significantly lower final biomass than the El tor N16961 strain. Indeed, it is known for some time that classical and El Tor strains grow very differently on carbohydrate rich media, and these differences may explain why the El Tor biotype supplanted the classical biotype as the predominant cause of pandemic cholera. Mekalanos and colleagues demonstrated that classical strains grown in the presence of glucose produced organic acids that resulted in deadly acidification of the media, whereas El tor strains when grown in glucose did not accumulate organic acids but instead produced acetoin and 2,3-butanediol and grew significantly better than classical strains (Yoon and Mekalanos 2006). Furthermore, El Tor strains with mutations in the acetoin pathway exhibited defects similar to classical strains when grown on glucose. The studies by Yoon et al also showed that an El Tor *alsS* mutant, which encodes acetolactase synthase, was defective in intestinal colonization in an infant mouse model. Thus, the ability to produce the neutral compounds acetoin and 2,3-butanediol and prevent organic acid production was an evolutionary adaptation that likely played a significant role in the emergence of the El Tor biotype. Prior work by Skorupski's group also revealed that acetoin production was essential for *V. cholerae* survival when grown in the presence of glucose by preventing lethal acidification (Kovacikova et al. 2005). They found that the quorum-sensing regulator AphA directly regulated the genes required for acetoin production. Interestingly, AphA is also a positive regulator of CT and TCP genes in *V. cholerae* thus connecting metabolism and virulence gene expression (Kovacikova et al. 2005; Kovacikova and Skorupski 2000, 2001, 2002a, b). Kovacikova and Skorupski's results suggest that AphA can mutually regulate acetoin production and virulence gene expression in response to changes in pH, which may also explain the differences in virulence gene expression between the two biotypes.

Growth pattern analysis of four *V. cholerae* pathogenic isolates using phenotypic array analysis identified 56 carbon sources that strains were able to catabolize efficiently (Fig. 10.2). However, there were significant differences among the strains in their ability to utilize different carbon sources. All strains showed growth on trehalose, sucrose, mannose, maltotriose, and glucose with a notable exception, strain NRT36S, did not utilize mannose, but grew significantly better on galactose compared to the other pathogenic strains examined. The El Tor strain N16961 and strain NRT36S both showed significantly better

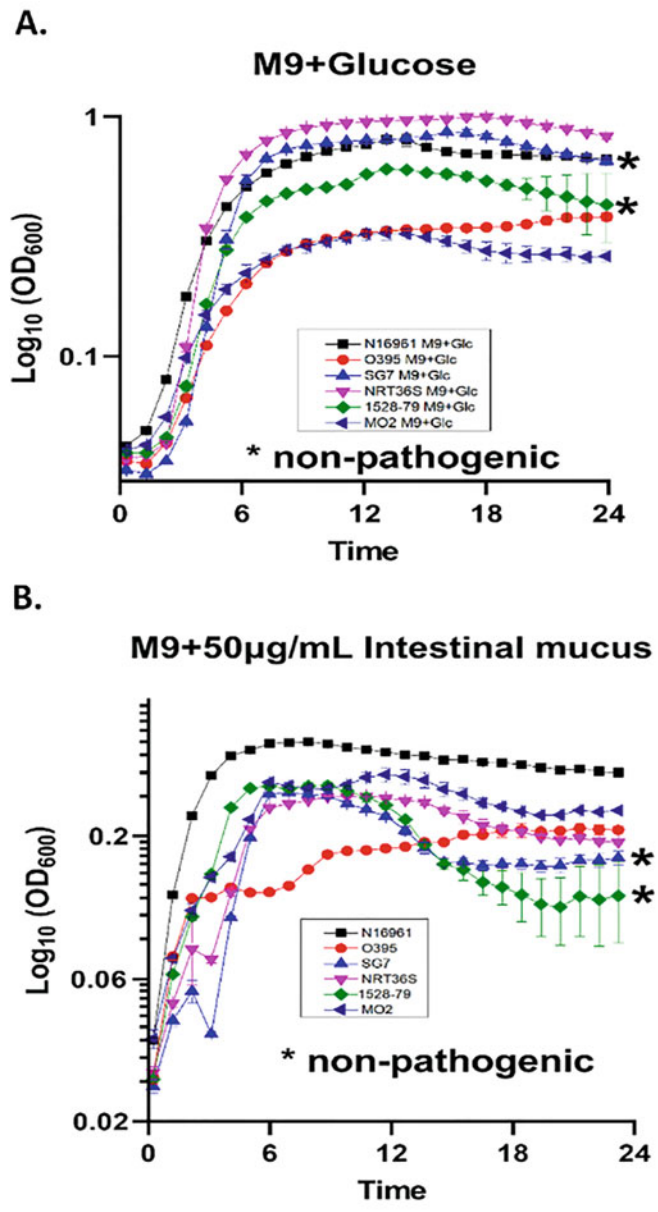


**Fig. 10.1** Growth pattern analysis of pathogenic and non-pathogenic *V. cholerae* strains. (**a**) Growth curves of V. cholerae strains grown in M9 media with glucose as the sole carbon source at 37 °C for 24 h. (**b**) Growth curves of *V. cholerae* strains grown in M9 media with mouse intestinal mucus as the sole carbon source at 37 °C for 24 h

growth on most carbon sources compared to the classical strain O395 and the serogroup O139 strain MO2—pathogenic clones that are no longer circulating. This analysis also demonstrates that pathogenic strains have acquired some specialized pathways, the most notable of which is sialic acid, an amino sugar present in all mucus membranes (Almagro-Moreno and Boyd 2009a, b; Chowdhury et al. 2012; Haines-Menges et al. 2015; McDonald et al. 2016).

## 10.2 Central Metabolism and Its Role in *V. cholerae* Intestinal Colonization

The importance of central metabolism for pathogenic *V. cholerae* in vivo fitness was investigated in a few studies mainly examining biotype El Tor strains. Van Alst and DiRita demonstrated that by deleting the pyruvate dehydrogenase (PDH) complex, mutant strains exhibited defects in

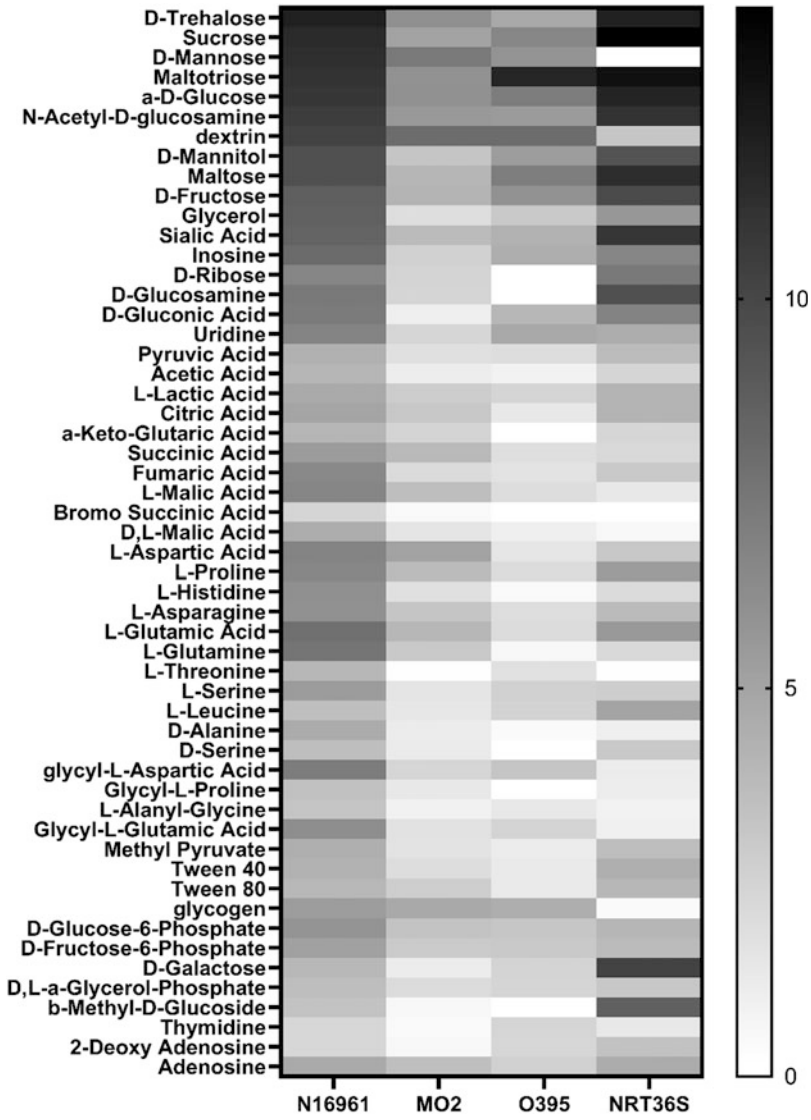


**Fig. 10.2** Heat map of four *V. cholerae* strains grown on 56 carbon sources. The heat map was generated from growth curves for each carbon source after growth for 24 h at 37 °C on phenotypic array plates PM1 and PM2. Dark boxes indicate robust growth and lighter color indicates poor or weak growth. White boxes indicates no growth

colonization in an infant mouse model of infection and had growth defects when grown on mucin (Van Alst and DiRita 2020). The PDH complex converts pyruvate to acetyl coenzyme A (acetyl-CoA), a precursor to citrate, and the first step in the TCA cycle. Their data suggests that oxidative metabolism of host mucin contributes to *V. cholerae* population expansion in vivo. Van Alst and DiRita determined that the phenotypes displayed by the mutant were due to metabolism defects and not virulence factor production, which was unaffected (Van Alst and DiRita 2020). This is in contrast to a study that demonstrated that central metabolism controlled ToxT, a positive regulator of both CT and TCP (Minato et al. 2013). Mutants defective in the respiration-linked sodium pump NADH:ubiquinone oxidoreductase (NQR) and the TCA cycle exhibited increased *toxT* expression. The data suggested that the effects on virulence expression were regulated by acetyl-CoA (Minato et al. 2013).

Additional studies have determined that strains lacking glucose phosphoenolpyruvate-dependent

phosphotransferase system (PTS) components have defects in colonization in an infant mouse model and a germ-free mouse model of infection (Houot et al. 2010; Wang et al. 2015). Waldor and colleagues showed that strains lacking EI, Hpr, or EIIA(Glc) components of the PTS system did not form TCP, secreted less CT, and had a reduced ability to colonize the infant mouse intestine (Wang et al. 2015). However, glucose PTS mutants can have pleiotropic effects since these mutants alter $EIIA^{Glc}$ dependent control of adenylate cyclase activity and thus cAMP levels and activation of the global regulator cAMP receptor protein (CRP). A more recent study by Dalia and colleagues examined the substrate specificity of the 13 distinct PTS transporters in *V. cholerae*. Their work indicates that carbohydrate transport by PTS plays a limited role in vivo in an infant mouse model and that glucose PTS transport only has a modest defect in vivo in strains with a functioning PTS (Hayes et al. 2017). In addition, Wang and colleagues found that the PP pathway did not play a significant role in host colonization (Wang et al. 2018). They proposed that gluconeogenesis was an important trait for the ability of *V. cholerae* to compete against the host microbiota (Wang et al. 2018). Specifically, a *ppsA*/*pckA* double deletion mutant exhibited impaired colonization of the adult mouse intestine and had growth defects on minimal media supplemented with mucin (Wang et al. 2018).

To date, studies examining specific carbon requirements for in vivo fitness remain limited, leaving a rich window open for future research. A study by Liu et al. showed that *V. cholerae* El Tor uses citrate in vivo as deletion of *citAB* resulted in a loss of fitness in a citrate supplemented mouse model (Liu et al. 2019). But the authors suggested that citrate metabolism likely had a limited role in initial stages of infection, but could be more important later in infection. Citrate fermentation is a key biochemical test for the identification of *V. cholerae* and this ability is also present in closely related species such as *Vibrio mimicus*, but is not a widespread phenotype within the genus. As Fig. 10.2 and other studies have demonstrated, classical biotype strain O395 cannot grow on citrate as a sole carbon source, which may also make this biotype less fit (Brumfield et al. 2018).

## 10.3 Intestinal Mucus and Sialic Acid Catabolism Essential for *V. cholerae* Host Colonization

*V. cholerae* can survive and multiply very effectively in the human intestine, with cholera patient and their contacts shedding cells for several days (Weil et al. 2014). Epithelial cells that line the gastrointestinal tract are covered in a mucus layer, the thickness of which varies considerably along the intestine (McGuckin et al. 2011). Continuous mucus production provides a rich source of nutrients for commensal and pathogenic bacteria that can forage glycans and glycoproteins (McGuckin et al. 2011; Tailford et al. 2015; Bell and Juge 2021). Intestinal mucin, both membrane and secreted mucins, are highly glycosylated, consisting of approximately 50–80% (w/w) carbohydrates, primarily composed of *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), fucose (Fuc), galactose (Gal) and *N*-acetylneuraminic acids (Neu5Ac), and relatively small amounts of mannose (Man) (Tailford et al. 2015; Bell and Juge 2021). In Fig. 10.2b, we examined growth of *V. cholerae* strains in M9 media supplemented with mouse intestinal mucus as a sole carbon source. In this analysis, *V. cholerae* N16961, the El Tor biotype strain, grew significantly better than all other strains, whereas the non-pathogenic strains had the lowest final biomass (Fig. 10.2b). This could suggest that El Tor strains are better adapted for growth in vivo utilizing mucus component more efficiently. There is limited information available on the mechanisms *V. cholerae* use to forage nutrients from the mucus layer, and only a handful of *V. cholerae* enzymes have been shown to act on mucin and epithelial cells (Fig. 10.3). Further understanding of this could provide novel insights into the intestinal colonization dynamics of the bacterium and novel targets to prevent infections.

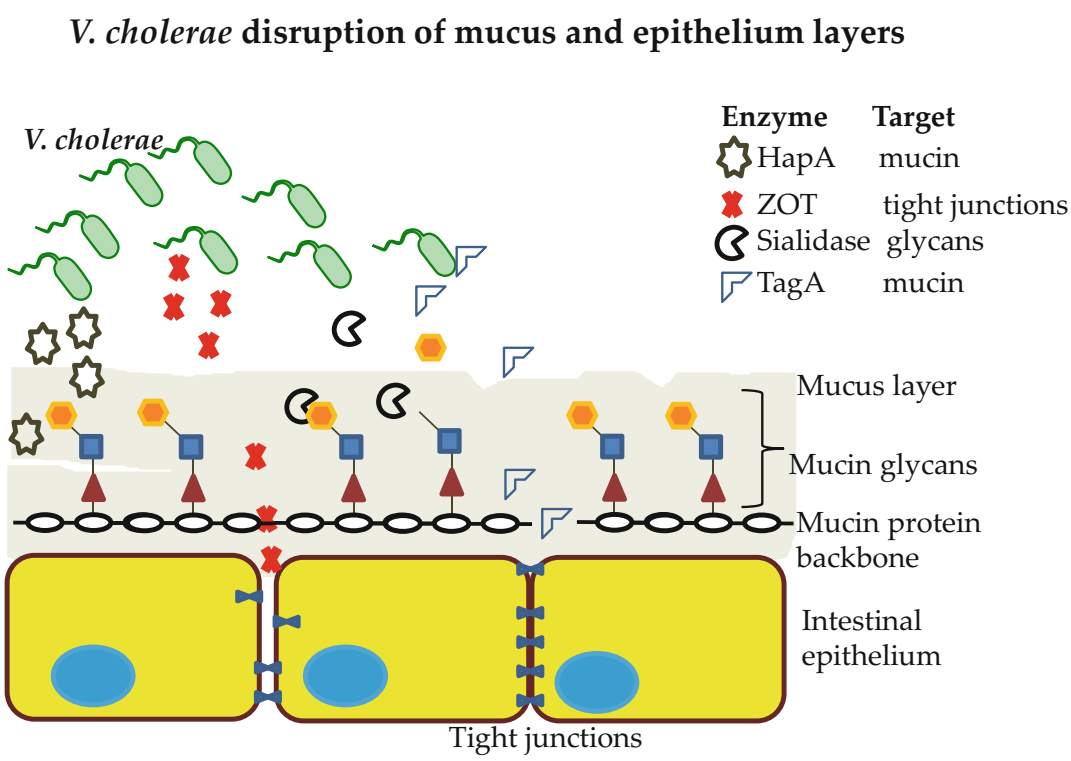


**Fig. 10.3** *V. cholerae* factors involved in interactions with host intestinal mucus layer. HapA is an enzyme that breaks down mucin. Zot was shown to disrupt epithelium cell tight junctions. Sialidase/neuraminidase cleavages high order gangliosides to expose the GM1 receptor for cholera toxin and release free sialic acid. TagA degrades mucin. Together these proteins allow the bacterium access to the epithelium surface layer with the concomitant release of compounds that can be catabolized

*V. cholerae* produces a hemagglutinin/protease (HA/Protease) HAP encoded by *hapA* that is secreted by the general secretory pathway into culture media and is often described as a mucinase (Häse and Finkelstein 1991; Finkelstein et al. 1983, 1992; Overbye et al. 1993). Benitez and colleagues demonstrated that HapA is under the control of the quorum-sensing regulator HapR and *hapA* expression is repressed in the presence of glucose, but induced in the presence of mucin (Benitez et al. 1999, 2001; Silva et al. 2003; Wang et al. 2011). HapA can breakdown mucin, fibronectin, and lactoferrin and plays an important role in penetration of the mucus barrier in vivo (Häse and Finkelstein 1991; Finkelstein et al. 1983, 1992; Benitez et al. 1999, 2001; Silva et al. 2003; Wang et al. 2011). Thus, HAP could be considered an important factor for in vivo survival and colonization. Similarly, a gene within the CTX phage named *zot*, which encodes the zonula occludens toxin (ZOT), interacts with occludin and zonula occludens 1 protein (ZO1) to disrupt intercellular tight junctions of epithelium cells (Fig. 10.3) (Fasano et al. 1991). This permits the bacterium to overcome the mucosal barrier allowing access to the basal membrane. TagA, which is contained within VPI-1, is a homolog of the StcE mucinase of *Escherichia coli* O157:H7 (Szabady et al. 2011). Szabady and colleagues showed that TagA was indeed a mucinase and proposed that it may directly modify host cell surface during infection (Szabady et al. 2011). The genome of *V. cholerae* N16961 contains 20 proteins annotated as a protease, including two Zn-proteases and two serine proteases that could potentially be involved in release of glycan

proteins from mucin for use as nutrients. Chemotaxis studies have demonstrated that N16961 can respond specifically to mucin and increased motility was shown in the presence of *N*-acetylneuraminic acid (Reddi et al. 2018).

*N*-acetylneuraminic acid, commonly known as sialic acid, is a nine carbon amino sugar ubiquitously present in the mucus layers of all mammalian cells mainly as a terminal sugar (Varki 1992, 2008; Varki et al. 2015; Varki and Varki 2007). Sialic acids are found in most lineages of metazoans and perform a wide range of functions in eukaryotes (Varki 1992, 2008; Varki et al. 2015). Within the sialic acid family, there are over 40 different structural variations including *N*-glycolylneuraminic acid (Neu5Gc) and 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid (KDN) (Varki 1992, 2008; Varki et al. 2015). Sialic acids are an essential component of all mucous membranes, where the negatively charged molecules will repel one another resulting in a sliding effect as well as providing protection particularly against bacteria (Varki 1992, 2008; Varki et al. 2015). Many commensal and pathogenic species including pathogenic *V. cholerae* carry neuraminidases (sialidases)—an enzyme that cleaves sialic acids from host glycans (Fig. 10.4) (Corfield 1992; Roggentin et al. 1989, 1993; Lewis and Lewis 2012). Select bacteria, again both commensal and pathogenic species, can catabolize free sialic acid and use it as a nutrient (Almagro-Moreno and Boyd 2009a, b, 2010; Haines-Menges et al. 2015; McDonald et al. 2016). For example, it has been demonstrated that the ability to catabolize sialic acid is widespread among human commensal and pathogenic species and this ability is an important phenotype for host–bacterial interactions (Almagro-Moreno and Boyd 2009a, b, 2010; Haines-Menges et al. 2015; McDonald et al. 2016). In pathogenic strains of *V. cholerae*, sialidase serves two functions. The first is that cleavage of sialic acid that exposes the GM1 gangliosides on the intestinal epithelium surface, which is the receptor for cholera toxin (Holmgren et al. 1975; Galen et al. 1992). Second, upon cleavage, the free sialic acids can be taken up and catabolized as a carbon source by pathogenic *V. cholerae* (Fig. 10.4) (Almagro-Moreno and Boyd 2009a, b, 2010; Haines-Menges et al. 2015; McDonald et al. 2016). Studies have revealed that *V. cholerae* and other enteric bacteria encode specialized transporters for sialic acid uptake into the bacterial cell (Almagro-Moreno and Boyd 2009a; Chowdhury et al. 2012; McDonald et al. 2016; Kelly and Thomas 2001; Fischer et al. 2010; Mulligan et al. 2011; Thomas and Boyd 2011; Thomas 2016; Bell et al. 2020; Severi et al. 2021).

The five genes that encode the enzymes required to catabolize sialic acid are present in the *V. cholerae* genomes of pathogenic strains on a pathogenicity island named Vibrio pathogenicity island- 2 (VPI-2) (Fig. 10.5) (Almagro-Moreno and Boyd 2009b; Jermyn and Boyd 2002). Neu5Ac lyase (encoded by *nanA*) breaks down sialic acid into *N*-acetylmannosamine (ManNAc) and phosphoenolpyruvate (PEP). ManNAc kinase (NanK) adds a phosphate group to carbon six of ManNAc generating *N*-acetylmannosamine-6-phosphate (ManNAc-6-P). ManNAc-6-P epimerase (encoded by *nanE*) converts ManNAc-6-P into *N*-acetylglucosamine-6-P (GlcNAc-6-P). In *V. cholerae*, the genes for the first three enzymes (*nanA, nanK,* and *nanE*) are found together forming the Nan cluster (Almagro-Moreno and Boyd 2009b; Jermyn and Boyd 2002). GlcNAc-6-P deacetylase (NagA) and glucosamine-6-P deaminase (NagB) convert GlcNAc-6-P into fructose-6-P (Fru-6-P), which is a substrate for general glycolysis (Fig. 10.4). The genes encoding NagA and NagB cluster elsewhere on the genome, although a second homolog of NagA is encoded within the Nan cluster along with *nanH* on VPI-2. There is a novel mutarotase (NanM) encoded within the Nan cluster, however, its specific role in *V. cholerae* remains unknown (Severi et al. 2008). Sialoglycoconjugate associated sialic acids are found in the α-anomer form; but bacteria can only utilize the β-anomer. Severi and colleagues found that *E. coli* produced an extracellular mutarotase (NanM) that converts α-Neu5Ac into β-Neu5Ac allowing the organism to utilize it as a carbon source (Severi et al. 2008). It is likely that *V. cholerae* can perform a similar reaction using

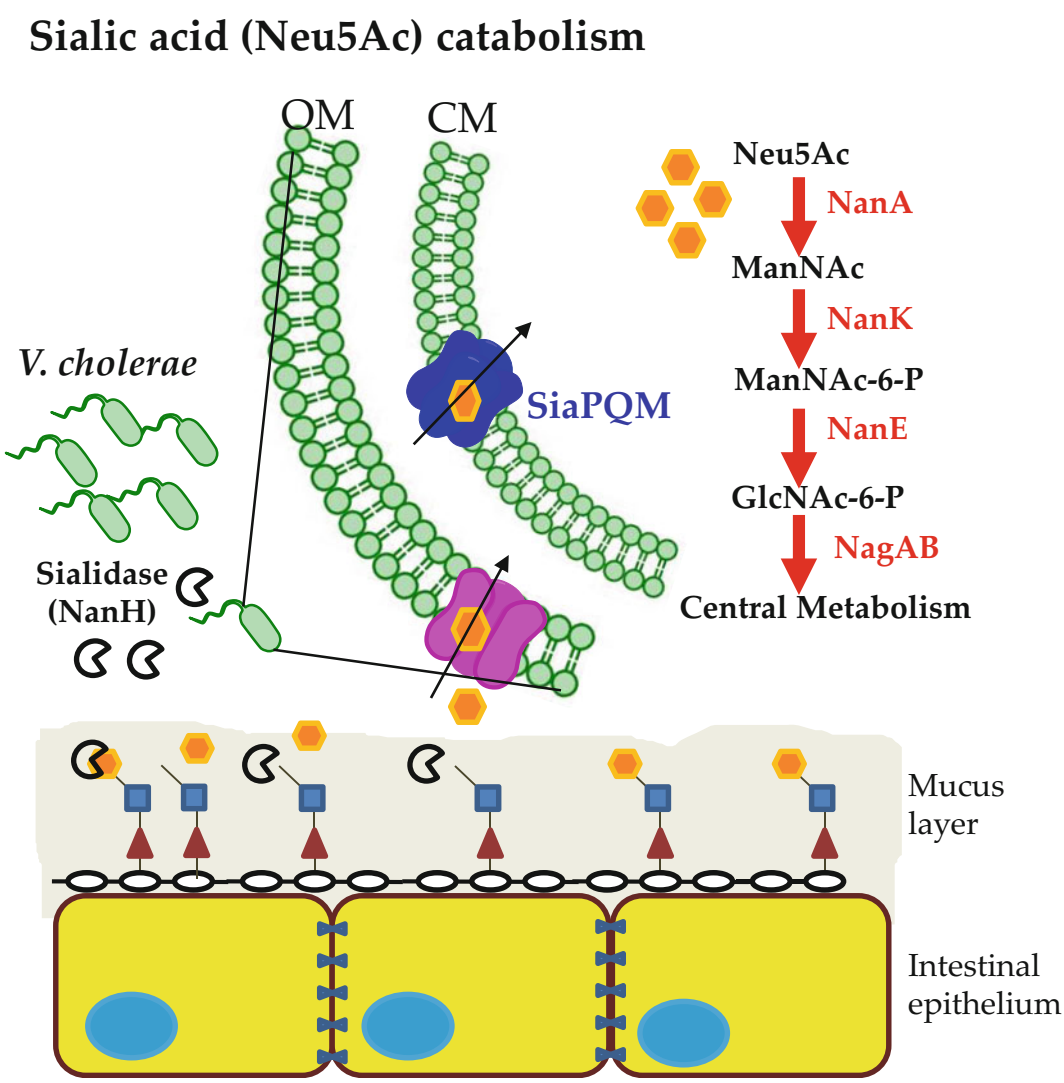


**Fig. 10.4** *V. cholerae* scavenging of host sialic acids for use as a carbon and energy source. Sialidase/neuraminidase releases terminal sialic acid, which is transported into the cell by a specific high affinity TRAP transporter and catabolized by the *nan-nag* genes present within VPI-2 in pathogenic isolates

its NanM homolog. Almagro-Moreno and colleagues demonstrated that the ability to utilize sialic acid as a carbon and energy source conferred an advantage to *V. cholerae* in the mucus-rich environment of the gut. Using the infant mouse model of infection, it was demonstrated that a *V. cholerae* Δ*nanA* mutant was defective in intestinal colonization (Almagro-Moreno and Boyd 2009b, 2010). In addition, the Δ*nanA* mutant showed a decreased competitive index during in vivo competition assays in the infant mouse model against the wild type strain. These analyses uncovered the important relationship between the catabolism of nutrient sources and bacterial pathogenesis, for *V. cholerae* fitness in vivo (Almagro-Moreno and Boyd 2009b, 2010).

Pathogenic strains, both those that cause cholera and strains that cause inflammatory diarrhea contain the Nan cluster along with the genes that

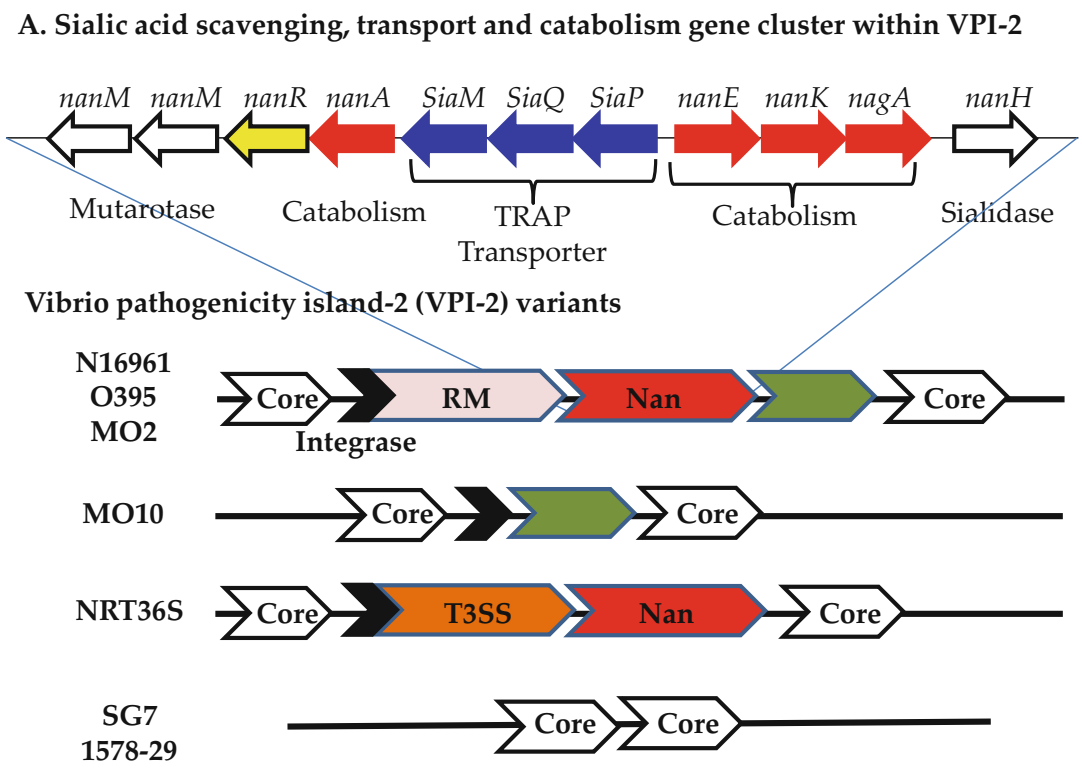


**Fig. 10.5** Sialic acid scavenging (*NnanH*), transport (*SsiaPQM*), and catabolism (*nanEKA, nagA*) genes within VPI-2. Variant VPI-2 regions are present among pathogenic strains. The canonical VPI-2 is present in classical and El Tor biotype strains, which also contains a type I restriction modification (RM) system. O139 serogroup strains that first emerged in the early 1990s contained a complete VPI-2, however later isolates contained a truncated version with the loss of the *nan-nag* region and the RM region. Isolates of *V. cholerae* that cause inflammatory diarrhea contain a type 3 secretion system (T3SS) adjacent to the *nan-nag* region. Non-pathogenic isolates do not contain VPI-2. Core refers to the ancestral genome present in all isolates of the species. The black arrow represents the integrase genes required for site-specific integration of the island at the tRNA-serine site

encode a specialized sialic acid transporter. In strains that cause inflammatory diarrhea the T3SS gene cluster is present along with the Nan region in a modified VPI-2 region (Fig. 10.5). It was demonstrated that the tripartite ATP-independent periplasmic (TRAP) transporter, SiaPQM is solely responsible for the transport of the sialic acid in *V. cholerae* (Chowdhury et al. 2012; Thomas and Boyd 2011). To examine whether sialic acids uptake played a significant role in *V. cholerae* intestinal colonization, in vivo competition and persistence assays were performed using a *V. cholerae* streptomycin-pretreated adult mouse model of colonization (McDonald et al. 2016). It is well established that conventionally reared mice contain over 500 diverse species of commensals, predominantly from phyla Firmicutes and Bacteroides that prevent infection by many enteric pathogens. This phenomena is known as colonization resistance (CR) and was first described in the 1960s and the 1970s (Freter 1955, 1962; Freter et al. 1983; van der Waaij et al. 1971; Vollaard and Clasener 1994). Streptomycin-treatment of adult mice favors expansion of Proteobacteria at the expense of Bacteroides with the total number of Firmicutes remaining the same (Miller and Bohnhoff 1963; Stecher and Hardt 2011). McDonald and colleagues showed that wild type *V. cholerae*

could colonize streptomycin-pretreated mice and that *V. cholerae* could grow efficiently on intestinal mucus and its component sialic acid as sole carbon sources (McDonald et al. 2016). Their study showed a *siaPQM*-deficient mutant strain was attenuated for colonization using the streptomycin-pretreated adult mouse model. In in vivo competition assays, the transporter mutant was outcompeted by wild type up to 3 days post infection indicating that sialic acid uptake is essential for fitness.

## 10.4 L-ascorbate as a Nutrient Source In Vivo

In addition to sialic acids, another carbon source that is abundant within the GI tract is L-ascorbate (vitamin C). L-ascorbate is a ubiquitous six-carbon carbohydrate known for its role as an antioxidant and is an essential nutrient required for enzyme function and tissue repair in humans and other animals (Sies and Stahl 1995). Some higher order eukaryotes, such as primates, have lost the ability to biosynthesize ascorbate (Chatterjee 1973; Padayatty and Levine 2016). In these species, the requirement for ascorbate is met through dietary intake.

L-ascorbate fermentation has been demonstrated in *E. coli* and *Klebsiella pneumoniae* and the genes required have been well characterized (Campos et al. 2007, 2008). In *E. coli*, two systems exist for utilization of L-ascorbate, the Ula system and the YiaK-YiaS system. The Ula system is required for the fermentation of L-ascorbate under anaerobic conditions whereas under aerobic conditions, both the Ula system and the YiaK-YiaS system are required (Campos et al. 2007, 2008). However, neither *E. coli* nor *Klebsiella pneumoniae* can aerobically catabolize L-ascorbate as a sole carbon source due to the reactivity of this molecule and the generation of oxidative stress (Campos et al. 2007, 2008). Thus, aerobic catabolism of L-ascorbate requires the presence of specific amino acids (proline, threonine, or glutamine) in the culture medium that are proposed to decrease L-ascorbate oxidation (Campos et al. 2007, 2008). Recently, we identified the presence of L-ascorbate fermentation genes in pathogenic *V. cholerae* isolates and found that *V. cholerae* was capable of L-ascorbate fermentation. A non-polar deletion of the *ulaG* (*vca0248*) gene homolog in *V. cholerae* indicates that the gene is required for L-ascorbate fermentation. Furthermore, the transcriptional pattern of *ula* genes shows that they were induced in the presence of L-ascorbate and when cells are grown on mouse intestinal mucus as a sole carbon source (Rosenberger et al. 2020). Competition experiments between wild type and an *ula* mutant showed that the ability to utilize ascorbate gave a competitive advantage when cells were grown on intestinal mucus as a nutrient (Rosenberger et al. 2020). In *V. cholerae*, we identified a putative pyridoxal phosphate phosphatase homolog encoded by *vca0243* within the *ula* operon that is not present in enteric species. A deletion mutant of this gene showed significant overall growth defects suggesting a wider role beyond ascorbate metabolism (Rosenberger et al. 2020).

Studies aimed at identifying the key *V. cholerae* genes essential for colonization identified several of the ascorbate metabolism genes as important for intestinal colonization (Fu et al. 2013; Kamp et al. 2013). A whole genome Tn-seq library of *V. cholerae* mutants was constructed and competed against the wild type strain in the rabbit intestine. The authors found that the majority of mutants that were defective in colonization were associated with metabolism. Specifically, *ulaEDP*, *ulaR*, and *ulaG* mutants from the ascorbate cluster were all found to be outcompeted by wild type (Fu et al. 2013). A second study, also utilizing Tn-seq to identify key genes associated with the *V. cholerae* lifecycle found an association with L-ascorbate utilization and survival (Kamp et al. 2013). This study demonstrated that the putative transcriptional regulator, *ulaR*, is associated with fitness in both the rabbit small intestine as well as cecal fluid. Interestingly, the *ulaR* mutant is also defective in transitioning into aquatic environmental conditions (Kamp et al. 2013).

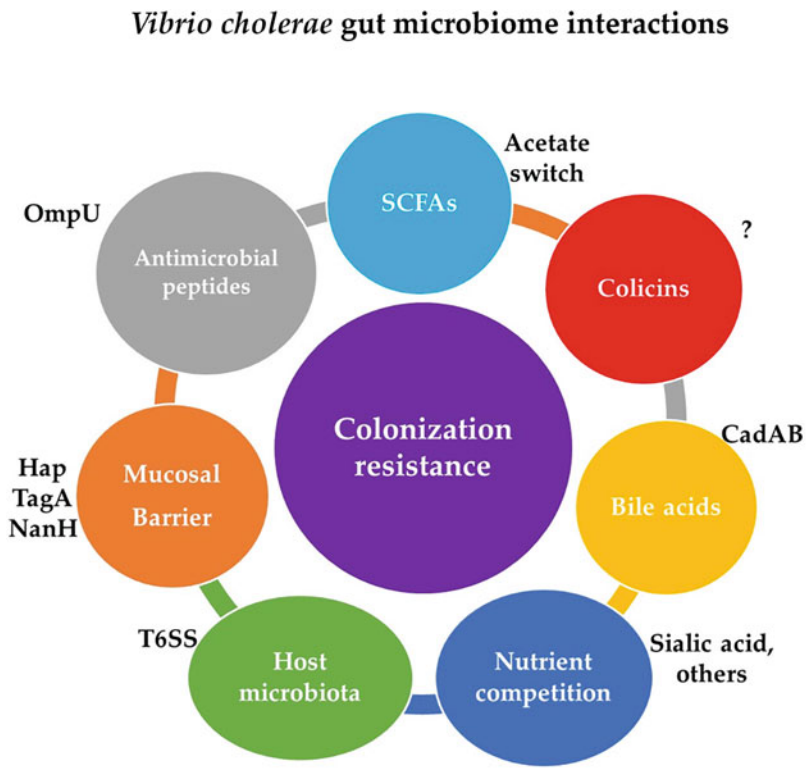


**Fig. 10.6** Simplified diagram of *V. cholerae*'s interactions with the gut microbiome and known bacterial factors used to overcome colonization resistance—an important mechanism to prevent pathogen infection. Intestinal commensals produce antimicrobial peptides, bacteriocins, and short-chain fatty acids (SCFAs) that all have antibacterial effects. SCFAs include acetate, butyrate, and propionate, which lower the gut pH. Bacteriocins (colicins or nisins) are small peptide toxins produced by commensal bacteria with various mechanisms of action. The gut microbiota are also important for nutrient competition for both macronutrients and micronutrients such as iron. Gut microbiota encode genes for type 6 secretion systems (T6SS), which have antibacterial activities. Although, many pathogens also contain these systems, including *V. cholerae*. The inner mucus layer forms an important physical barrier for bacteria, disruption of which can result in pathogen colonization

## 10.5 Mechanisms Used by *V. cholerae* to Overcome Colonization Resistance

One of the major barriers that pathogens face in the gastrointestinal tract is colonization resistance (Fig. 10.6). Colonization resistance is the ability of the host microbiome to inhibit pathogen survival and establishment. It is now known that colonization resistance is in large part the result of the gut microbiota preventing pathogen establishing a niche within the host by generating antimicrobial peptides, bacteriocins, short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate, as well as nutrient competition (Fig. 10.6) (reviewed in (Ducarmon et al. 2019)). In addition, the microbiota helps sustain the gut mucosal barrier by occupying this niche to prevent newly introduced pathogens from colonizing the intestine via the niche exclusion principle. Bacteriophages produced by the microbiota may also play a role in preventing pathogen colonization by targeting these bacterial hosts. One of the first studies to describe the importance of the microbiota in colonization resistance was preformed using *V. cholerae* and a guinea pig model of infection. This study showed that *V. cholerae* could colonize only

after inhibition of the normal flora of the animal that is removing microbial competitors (Freter 1955). Later Freter introduced the nutrient-niche hypothesis, which states that any species of bacteria must use at least one limiting nutrient better than another species for successful colonization (Freter 1962; Freter et al. 1983).

*V. cholerae* has adapted to in vivo survival and colonization by the acquisition of several traits that are key to overcoming colonization resistance (Fig. 10.6). Since many species of the microbiota produce SCFAs, the ability to consume these is important for *V. cholerae* survival because it is not highly acid resistant. The acetate switch, which provides the ability of bacteria to cease acetate production and instead uptake and catabolize it, is important for virulence of *V. cholerae* in the fly model of infection. However, the mechanism of virulence was not related to colonization, but rather altered host cell signaling that ultimately caused cell death (Hang et al. 2014). An earlier study showed that a *V. cholerae pta* mutant had a colonization defect in an infant mouse model and produced less TCP (Chiang and Mekalanos 1998). PTA converts acetyl-CoA to acetyl-P that is converted to acetate by ACKA. Acetyl-P is known to modulate virulence gene expression in several enteric species by activating response regulators (RR). In *V. cholerae* the *pta* mutant in vivo phenotype was rescued by over expression of *toxT*, which encodes a major positive regulator of CT and TCP expression suggesting that Acetyl-P controls a RR that regulates ToxT, a master regulator of virulence.

## 10.6 Conclusions

The role of carbon nutrition is becoming increasingly appreciated as one of the most critical aspects surrounding pathogenesis. As highlighted in the studies above, a wide array of flexible carbon metabolism enables pathogens such as *V. cholerae* to gain a competitive advantage in the gastrointestinal tract, such as the consumption of sialic acid or other mucus components and its derivatives. As our knowledge on the specific nutrients and their particular role in colonization, competition against the microbiota, and disease onset increases we will identify novel approaches to tackle human threats like the dreaded agent of cholera.

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# Stress Responses in Pathogenic Vibrios and Their Role in Host and Environmental Survival

11

Jay K. Akolkar and Jyl S. Matson

**Abstract**

*Vibrio* is a genus of bacteria commonly found in estuarine, marine, and freshwater environments. *Vibrio* species have evolved to occupy diverse niches in the aquatic ecosystem, with some having complex lifestyles. About a dozen of the described *Vibrio* species have been reported to cause human disease, while many other species cause disease in other organisms. *Vibrio cholerae* causes epidemic cholera, a severe dehydrating diarrheal disease associated with the consumption of contaminated food or water. The human pathogenic non-cholera *Vibrio* species, *Vibrio parahaemolyticus* and *Vibrio vulnificus*, cause gastroenteritis, septicemia, and other extra-intestinal infections. Infections caused by *V. parahaemolyticus* and *V. vulnificus* are normally acquired through exposure to sea water or through consumption of raw or undercooked contaminated seafood. The human pathogenic Vibrios are exposed to numerous different stress-inducing agents and conditions in the aquatic environment and when colonizing a human host. Therefore, they have evolved a variety of mechanisms to survive in the presence of these stressors. Here we discuss what is known about important stress responses in pathogenic *Vibrio* species and their role in bacterial survival.

**Keywords**

*Vibrio cholerae* · Stress responses · Host colonization · Environmental survival

## 11.1 Introduction

*Vibrio* species are ubiquitous, Gram-negative, comma-shaped bacteria that are natural inhabitants of aquatic and marine environments. There are more than 100 described species of *Vibrio*, however only about a dozen species have been reported to cause disease in humans. Of the human pathogenic *Vibrio* species, three cause the vast majority of disease: *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* (Baker-Austin et al. 2018; Newton et al. 2012). Pathogenic *Vibrio* species share several biological, clinical, and environmental characteristics. The genome of *Vibrio* species is contained on two circular chromosomes, which are shaped by recombination events and horizontal gene transfer. *Vibrio* species typically grow in warm (≥15 °C) sea water and brackish water, while *V. cholerae* can also grow in fresh water. *Vibrio* species can survive in multiple states in the aquatic environment, including planktonically growing, colonizing fish and marine

J. K. Akolkar · J. S. Matson (✉)
Department of Medical Microbiology and Immunology, University of Toledo College of Medicine and Life Sciences, Toledo, OH, USA
e-mail: Jyl.Matson@UToledo.Edu



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invertebrates, or associating with plankton and algae (Nelson et al. 2009). These bacteria can also form biofilms on biotic and abiotic surfaces, which plays an essential role in their survival and environmental persistence.

### 11.1.1 *V. cholerae*

*V. cholerae* is the causative agent of epidemic cholera, a severe diarrheal disease caused by the ingestion of contaminated food or water. An estimated 1.3–4 million people contract cholera worldwide annually, resulting in an estimated 95,000 deaths (Ali et al. 2015). The disease is characterized by voluminous watery diarrhea and severe dehydration, which can lead to death within hours if untreated (Kaper et al. 1995). Annual seasonal outbreaks occur in areas of the world where cholera is endemic, mainly in the Ganges delta of the Bay of Bengal. However, large outbreaks of the disease can occur in other parts of the world due to natural disasters, war, or other circumstances that impact infrastructure and the ability to acquire clean drinking water (Faruque et al. 1998). Individuals with cholera shed very high numbers of *V. cholerae* in their stool, and these bacteria exhibit a hyperinfectious phenotype, exacerbating a cholera epidemic (Merrell et al. 2002a).

*V. cholerae* has >200 serogroups that are distinguished by the structure of the lipopolysaccharide (LPS) O-antigen. Of these serogroups, only two (O1 and O139) cause pandemic cholera (Kaper et al. 1995). Non-O1 and non-O139 *V. cholerae* strains are the causative agents of sporadic gastrointestinal and extraintestinal infections (Baker-Austin et al. 2017). O1 strains are further divided into the classical and El Tor biotypes based on several phenotypic differences. *V. cholerae* of the classical biotype caused the first six recorded cholera pandemics, while the El Tor biotype is responsible for the ongoing seventh pandemic. Classical strains are thought to cause more severe disease than El Tor strains, while El Tor strains are thought to have increased fitness in the environment (Kaper et al. 1995; Faruque et al. 1998).

### 11.1.2 *V. parahaemolyticus*

*V. parahaemolyticus* is a common inhabitant of temperate and tropical coastal areas around the world. *V. parahaemolyticus* typically causes sporadic cases of infection along these coastal areas, and anomalous warm weather events can initiate outbreaks of disease (Baker-Austin et al. 2017). *V. parahaemolyticus* is recognized as one of the leading causes of seafood-derived gastroenteritis throughout the world. Infection is generally associated with the consumption of raw or undercooked contaminated seafood, although wound exposure to contaminated water can also cause occasional infections. *V. parahaemolyticus* does not spread via person-to-person transmission or the fecal–oral route (Baker-Austin et al. 2010). Patients infected with *V. parahaemolyticus* typically present with symptoms of gastroenteritis including headache, abdominal cramping, nausea, vomiting, and fever. Patients with underlying conditions such as diabetes or liver disease sometimes go on to develop septicemia.

*V. parahaemolyticus* was discovered in 1950 as the cause of a large outbreak of gastroenteritis in Japan, and since that time it has been isolated in both large outbreaks and sporadic cases worldwide (Nair et al. 2007). Serotyping of *V. parahaemolyticus* is based on the antigenic properties of the somatic (O) and capsular (K) antigens, and there are currently more than 80 described serotypes (Iguchi et al. 1995). The first pandemic isolate of *V. parahaemolyticus* emerged in 1996 in Kolkata, India and was from the O3:K6 serotype (Nair et al. 2007). This serotype then went on to spread to other countries in Asia, Europe, and the Americas over the next several years. By 2016, there were an estimated 49 *V. parahaemolyticus* serotypes worldwide (Han et al. 2016).

### 11.1.3 *V. vulnificus*

Like *V. parahaemolyticus*, *V. vulnificus* infections originate from two different sources: consumption of contaminated seafood, resulting in

gastroenteritis or primary septicemia, or exposure of broken skin to contaminated sea water, resulting in wound infections and secondary septicemia. However, *V. vulnificus* infection is typically far more serious than infection with *V. parahaemolyticus*. *V. vulnificus* is responsible for >95% of seafood-related deaths in the USA and it has the highest case fatality rate (~50%) of any foodborne pathogen (Bross et al. 2007; Jones and Oliver 2009). Wound infections associated with *V. vulnificus* are usually contracted during recreational activities such as swimming or fishing and have a substantial mortality rate (~25%) (Oliver 2005a). These severe wound infections are characterized by necrotizing fasciitis or soft tissue infection (Jones and Oliver 2009). *V. vulnificus* infections are characterized by an average 48-hour incubation period between the ingestion and onset of symptoms for gastrointestinal infections, and an average 16-hour incubation period in the case of wound infections (Jones and Oliver 2009). Unlike *V. cholerae* and *V. parahaemolyticus*, *V. vulnificus* is primarily an opportunistic pathogen. Most people who present with a *V. vulnificus* infection also have underlying liver disease (≥80%).

*V. vulnificus* is categorized into three different biotypes based on genomic, biochemical, and serological characteristics, and host range (Jones and Oliver 2009). Biotype 1 is responsible for the majority of human ingestion cases that lead to septicemia, as well as most wound infections. Biotype 2 is responsible for a zoonotic infection of farmed eels that leads to a rapidly fatal septicemia in that species (Fouz et al. 2007). Biotype 3 causes human wound infections and has been reported in Japan and Israel to date (Bisharat et al. 1999; Hori et al. 2017).

Due to their similar lifestyles as bacteria that inhabit aquatic environments and human gastrointestinal pathogens, these *Vibrio* species need to appropriately respond to a number of similar stress-inducing conditions. The mechanisms by which pathogenic *Vibrio* species sense and respond to stress have generally been studied in far more detail in *V. cholerae* than any other species. However, we aim to highlight what is known about these stress response mechanisms in *V. parahaemolyticus* and *V. vulnificus* as well.

## 11.2 Responses to Environmental Stress

Adaptation to changing environmental conditions is critical for the survival of bacterial pathogens. Vibrios are normal inhabitants of aquatic environments, where they must appropriately sense and respond to numerous biotic and abiotic stresses to survive and reproduce. In the aquatic environment, Vibrios must adapt to changes in temperature, salinity, and nutrient availability. In addition, they also encounter significant stress from predation by bacteriophage and protozoa that share their environment. Pathogenic *Vibrio* species have evolved mechanisms to successfully adapt to physiological and biological changes in the aquatic environment, leading to survival, increased dissemination, and occasional transmission to a human host. One of the many strategies employed by Vibrios to survive these environmental challenges is the formation of biofilms on biotic and abiotic surfaces. The molecular basis of biofilm formation and the regulatory events that govern this process in Vibrios has been reviewed elsewhere (Conner et al. 2016; Jiang et al. 2021; Pazhani et al. 2021; Silva and Benitez 2016).

### 11.2.1 Temperature

In the aquatic environment *Vibrio* species experience a wide range of temperatures, including both seasonal and interannual temperature changes. These aquatic habitats typically exhibit a temperature range of 12 °C to 30 °C (Gil et al. 2004; Huq et al. 2005), where *Vibrio* species that colonize humans must be able to survive at 37 °C. Temperature is hypothesized to be a key signal to differentiate between the environment and the host, stimulating the production of virulence factors when colonizing the human gastrointestinal tract and initiating environmental survival strategies when exiting the body.

Ecological studies have shown that water temperature above 15 °C is a good predictor for the presence of *V. cholerae* (Lama et al. 2004; Lipp et al. 2002, 2003; Louis et al. 2003). High water temperature stimulates the production of the MSHA and GpbA adhesins in *V. cholerae*, which promotes bacterial attachment to chitin in the environment (Stauder et al. 2010). This adherence may allow *V. cholerae* to better persist as well as increase its transmission to a human host. Low temperatures cause numerous challenges to bacterial cell physiology. As temperatures decrease, the lipid composition of the cell membrane changes, transitioning from a liquid crystalline state to a more rigid state (Hebraud and Potier 1999). Low temperatures also negatively impact translation, causing the poor ribosome assembly and the formation of extensive RNA secondary structures (Chen and Shakhnovich 2010). Cold-shock proteins produced by bacteria are thought to counteract the negative impact of temperature on RNA structure by acting as chaperones (Phadtare and Severinov 2010). *V. cholerae* encodes four predicted cold-shock genes, *cspA*, *cspV*, *vc1142*, and *vca0184*. However, only CspA and CspV are highly induced when *V. cholerae* was shifted to low temperatures (Datta and Bhadra 2003). More recent studies demonstrated that CspV regulates biofilm formation and expression of the T6SS (Townsley et al. 2016). However, how this contributes to survival in low temperatures is not fully understood.

*Vibrio parahaemolyticus* can grow at a wide range of temperatures (16 °C–42 °C) with an optimum growth temperature of 37 °C (Beuchat 1982). Transcriptomic studies show strong temperature-dependent regulation of gene expression in *V. parahaemolyticus* (Urmersbach et al. 2015). The expression of cold-shock protein CspA is highly elevated at 10 °C, a temperature below where bacterial growth is arrested. This suggests CspA plays an important role in the bacterial cold-stress response (Yang et al. 2009). The growth of *cspA* mutants is repressed at 10 °C, further reinforcing the importance of the CspA protein in *V. parahaemolyticus* cold-shock stress response (Zhu et al. 2017). Transcriptomic studies demonstrated that CspA also significantly alters the expression of genes in several metabolic pathways in *V. parahaemolyticus* at 10 °C (Zhu et al. 2017). Another cold-shock protein, CspD, was shown to repress growth at 10 °C (Zhu et al. 2017).

*V. parahaemolyticus* increases expression of several genes at high temperatures, including those that encode for heat-shock proteins Hsp60 and Hsp70 (Segal and Ron 1998). Temperature also impacts the activity of Type VI secretion systems crucial for bacterial survival and pathogenicity in a variety of environmental and host conditions (Salomon et al. 2013). The two Type VI secretion systems in *V. parahaemolyticus* (T6SS1 and T6SS2) operate optimally at differing temperature and salinity conditions. T6SS1 is most active at higher temperatures and high salinity, while the T6SS2 operates best in both cold and high temperatures in low salinity conditions (Salomon et al. 2013). Higher temperatures also increased urease activity in *V. parahaemolyticus* through the regulation of transcription factor UreR, which assists survival in host gastric acids (Park et al. 2009). Heat-shock conditions also significantly alter the fatty acid composition of *V. parahaemolyticus* cells resulting in decreased tolerance to organic acids and high salinity environments (Chiang et al. 2005).

*V. vulnificus* can grow at a wide range of temperatures with an optimum growth temperature of 35 °C (Panicker et al. 2004). Analysis of global gene expression during cold shock (conditions below 10 °C) showed upregulation of cold-shock genes *cspA* and *cspB* (Wood and Arias 2011). Cold-shock temperatures also have an important role in the initiation of the VBNC state in *V. vulnificus*. Higher temperatures alter the expression of other survival genes in *V. vulnificus*. The iron acquisition heme receptor protein, HupA, was increased at 40 °C compared to 30 °C (Oh et al. 2009). HupA activity is necessary for both survival and virulence in *V. vulnificus* host infection (Oh et al. 2009). *V. vulnificus* also utilizes temperature-dependent regulation of metalloprotease (VVP) and cytolytic toxin (VVH) (Elgaml and Miyoshi 2015; Elgaml et al. 2014). VVP and VVH expression in *V. vulnificus* is controlled by the global

regulator histone-like nucleoid structuring protein (H-NS). H-NS exhibits increased activity at higher temperatures, resulting in subsequent upregulation of VVP expression and down-regulation of VVH expression (Elgaml and Miyoshi 2015). Temperature-dependent H-NS expression is also important for *V. vulnificus* cross-protective survival in the presence of acidic pH, hyperosmotic, and oxidative stress (Elgaml and Miyoshi 2015).

### 11.2.2 Salinity

As aquatic pathogens, *Vibrio* species must be able to survive large shifts in salinity due to fluctuating environmental conditions such as rainfall, varying river input, and tidal changes (Lalli and Parsons 1993). Additionally, pathogenic Vibrios need to adapt to salinity shifts when entering and exiting the human host. *V. parahaemolyticus* can survive a wide range of NaCl concentrations ranging from 0.5% to 10.5%, with optimal growth concentration at 3% NaCl or 0.5 M (Wong and Wang 2004). However, most *Vibrio* species grow preferably at salinities of less than 25 ppt.

Several osmolytes are utilized by pathogenic Vibrios for survival in varying salinities. Ectoine is an important osmolyte in the bacterial response to osmotic stress. Ectoine is synthesized by *V. cholerae* and *V. parahaemolyticus* by the *ectABC* operon, which is conserved in Gram-negative and Gram-positive bacteria (Ongagna-Yhombi and Boyd 2013; Louis and Galinski 1997; Czech et al. 2018). Expression of *ectABC* is regulated by osmolarity and is controlled by the MarR-type repressor CosR in *V. cholerae*. The CosR homolog (VP1906) in *V. parahaemolyticus* is also a direct repressor of the *ectABC-asp* operon under low salinity. Ectoine production contributed to *V. parahaemolyticus* growth on high salinity media (Ongagna-Yhombi and Boyd 2013).

Glycine betaine (GB) is another important osmoprotectant in bacteria. *V. cholerae* lacks the genes required for GB synthesis, but uses the OpuD transporter to import GB produced by other organisms. Dimethylglycine (DMP), a GB intermediate, is also a common osmolyte used by *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae* (Gregory et al. 2020). The synthesis of both ectoine and GB in *V. parahaemolyticus* is induced by NaCl (Naughton et al. 2009). Dimethylsulfoniopropionate (DMSP) is an osmoprotectant in *V. parahaemolyticus* (Gregory and Boyd 2021). Both *V. cholerae* and *V. vulnificus* can use DMSP as an osmolyte. In environments with high salinity, *V. cholerae* increases the production of the pigment melanin that provides resistance to UV radiation (Coyne and al-Harthi 1992; Valeru et al. 2009).

Exposure to different osmotic conditions can stimulate *Vibrio* species to become more resistant to other stresses. *V. parahaemolyticus* grown in high salt conditions demonstrated a strong degree of cross-protection against acid shock and extreme temperature conditions (Kalburge et al. 2014; Whitaker et al. 2010). *V. vulnificus* introduced to prior osmotic shock was cross-protected to both heat and oxidative stresses (Rosche et al. 2005). The level of the alternate sigma factor RpoS was significantly induced by osmotic shock and is necessary to achieve full cross-protection to oxidative stress, but not heat stress. Furthermore, RpoS expression was necessary for the survival of *V. vulnificus* in the presence of hyperosmotic stress (Hulsmann et al. 2003).

### 11.2.3 Viable but Non-culturable State

The viable but non-culturable (VBNC) state is where bacteria enter near dormancy, while metabolic activity is maintained at a minimal level. The VBNC state refers to bacterial cells that are alive but cannot be cultured using standard laboratory techniques. This is an important stress response to harsh environmental conditions and is reversable, as bacteria revert to a culturable state once conditions are more favorable (Oliver 2005b). Changes in temperature, salinity, pH, oxygen concentration, and starvation are all conditions that can induce the VBNC state (Oliver 2005b; Colwell 2000; Jayakumar et al. 2020).

It is a common strategy of survival employed by many bacteria, including *Vibrio* species.

In *V. cholerae*, nutrient deprivation is one of the most common signals that induce the VBNC state, where the lipid, DNA, RNA, carbohydrate, and protein content of the bacterial cell are diminished (Hood et al. 1986). Studies comparing the proteome profiles of *V. cholerae* in the culturable, VBNC, and recovery states showed several differences in levels of proteins associated with stress. The expression of l-ectoine synthase EctC (required for the biosynthesis of the osmoprotectant ectoine) is not produced by VBNC bacteria but is during recovery (Debnath et al. 2019). This indicates that it might be an essential protein for growth after recovery from the VBNC state. AhpC (alkyl hydroperoxide reductase subunit C) is produced by *V. cholerae* in both the VBNC and recovery state (Debnath et al. 2019). The expression of AhpC was greater during recovery from the VBNC state, possibly due to the enhanced metabolic activity needed to support bacterial growth, which further increases oxidative stress. The expression of AhpC during VBNC induction in *V. cholerae* is hypothesized to play a role in protection against the oxidative stress produced in bacteria incubated at low temperature under nutrient limitation.

Several genes continue to be expressed in the VBNC state by *V. vulnificus*. Genes necessary for stress responses (*rpoS*) and virulence (*vvhA* and *wza*) were shown to be expressed in environmentally collected VBNC *V. vulnificus* (Smith and Oliver 2006). *V. vulnificus* in the VBNC state is highly resistant to numerous, unrelated environmental stressors (Nowakowska and Oliver 2013). This includes resistance to heat challenge where VBNC cells subjected to 50 °C heat shock showed almost no reduction in viability. VBNC cells also show great resistance to ethanol, which may be related to the significant morphologic changes to the cell wall and membrane, thus affecting permeability (Day and Oliver 2004). Decreased expression of catalase also plays an important role in the VBNC state of *V. vulnificus*. *V. vulnificus* exhibits continued expression of the *oxyR* gene during entry into VBNC state, but also decreased *katG* transcription and decreased catalase activity under low temperature stress (Kong et al. 2004). It is theorized that the subsequent accumulation of hydrogen peroxide is involved in initiating the VBNC state under low temperature conditions. *oxyR* mutants, which lack catalase activity at ambient temperature, showed subsequent nonculturability on routine media under ambient temperature conditions. This demonstrates the importance of hydrogen peroxide in the entry into VBNC state. Furthermore, it is theorized that resuscitation from VBNC in *V. vulnificus* requires reactivation of *katG* transcription and the re-synthesis of catalase under permissive temperatures (Kong et al. 2004). Both *V. vulnificus* environmental and clinical genotypes demonstrate the ability to withstand several stressors, while maintaining the ability to resuscitate to full culturability (Nowakowska and Oliver 2013).

Like other *Vibrio* species, *V. parahaemolyticus* is reported to enter the VBNC state under a variety of harsh environmental stresses (Chen et al. 2009; Lai et al. 2009; Yoon et al. 2017). Thirteen genes involved in transcription, translation, ATP synthase, gluconeogenesis-related metabolism, and antioxidants are upregulated during the induction of VBNC state (Lai et al. 2009). Changes to cellular ultrastructure and increased tolerance toward various stresses (heat, bile salts, low salinity) have been reported in *V. parahaemolyticus* upon entering the VBNC state (Su et al. 2013). One structural change observed is the increasing thickness of the cell wall with increased VBNC induction time. *V. parahaemolyticus* also shows resistance to heat challenge at 47 °C in the VBNC state, similar to *V. vulnificus* (Wong and Wang 2004). *V. parahaemolyticus* shows significant resistance to acidic (pH 4) conditions in the VBNC state. *V. parahaemolyticus* exhibits several antioxidant defenses against ROS in the VBNC state including alkyl hydroperoxide reductase, catalase, and KatG (Lai and Wong 2013).

### 11.2.4 Predation by Protozoa

In the aquatic environment, *Vibrio* species interact with numerous organisms including heterotrophic protozoa. Protozoa are specialized eukaryotic cells that live in a wide variety of environments and are competent grazers of bacteria. Protozoa engulf bacteria and package them into phagosomes where the bacterial cells are exposed to numerous stressors, including low pH, antimicrobial peptides, reactive oxygen/nitrogen species, proteolytic enzymes, and low iron concentrations. In order to survive predation, *V. cholerae* has evolved both anti-grazing strategies and factors that enable the bacteria to survive in the phagosome, followed by escape into the extracellular environment. In addition, *V. cholerae* expressing the T6SS can kill the model amoeba *Dictyostelium discoideum* through direct contact with predator cells (Miyata et al. 2011; Pukatzki et al. 2006).

The formation of biofilms is one of the strategies that *V. cholerae* uses to protect itself from grazing by protozoa (Matz et al. 2005; Sun et al. 2015). In addition, *V. cholerae* is able to kill the flagellate *Cafeteria roenbergensis* and the ciliate *Tetrahymena pyriformis* when grown in coculture. The secreted protease PrtV and quorum sensing were required for this activity (Vaitkevicius et al. 2006). Ammonia produced during *V. cholerae* chitin metabolism is also toxic to protozoa (Sun et al. 2015). Expression of the pigment pyomelanin and the production of reactive oxygen species (ROS) aids in protection of *V. cholerae* from grazing in the environment (Noorian et al. 2017).

*Vibrio* species are capable of intracellular survival in several species of amoeba, including *Acanthamoeba castellanii*, *A. polyphaga*, and *Naegleria gruberi* (Abd et al. 2007; Thom et al. 1992). In *A. castellanii*, *V. cholerae* can grow in the trophozoites and cysts and can access the contractile vacuole in order to escape into the extracellular environment (Abd et al. 2005, 2007; Thom et al. 1992; Van der Henst et al. 2016). The mechanisms by which *V. cholerae* survives within the protozoal cell and ultimately escapes into the environment are starting to be revealed. Flagella-based motility and extracellular enzymes play key roles in the replication and transmission of *V. cholerae* in *A. castellanii* (Van der Henst et al. 2018). In addition, the outer membrane porin OmpU is important for survival within protozoa (Espinoza-Vergara et al. 2019). Since OmpU is also required for *V. cholerae* resistance to other stressors such as bile, antimicrobial peptides, and organic acids, this suggests that this protein may be required to resist factors within the phagosome that aid in bacterial digestion.

The *V. vulnificus* MARTX (Multifunctional Autoprocessing Repeats-in-Toxin) plays a key role in bacterial virulence and cell survival as a cytopathogen (Lee et al. 2007). *V. vulnificus* produces four different MARTX systems (Types I–IV) (Kwak et al. 2011). *V. vulnificus* biotype 2 produces MARTX type III (encoded by RtxA13), which is involved in cell lysis of various eukaryotic cell lysis including amoebae (Lee et al. 2013). Recent studies showed an additional strategy used by an environmental strain of *V. vulnificus*, ENV1, to resist predation by *T. pyriformis*. These bacteria have a change in their central carbon metabolism that allows them to continuously produce excess organic acids that are toxic to protozoa (Rasheedkhan Regina et al. 2022).

In *V. parahaemolyticus* the type III secretion system (T3SS-2) serves an important role in bacterial survival from various aquatic, bacterivorous protists (Matz et al. 2011). This enhanced survival was mediated through 2 mechanisms, T3SS-2 mediated cytotoxicity and enhanced facultative parasitism on coexisting protists. The T3SS-2 effector protein VopC is involved in allowing *V. parahaemolyticus* to escape from eukaryotic vacuoles (de Souza and Orth 2014). This function is crucial to the intracellular survival of *V. parahaemolyticus* in eukaryotic cells. Additionally, the T3SS-2 effector protein VopL acts to counteract the production of ROS (de Souza et al. 2017). While many of these studies outline bacterial cell defenses within host cells, there is evidence to suggest that several bacterial virulence factors serve a dual role in

providing resistance to protozoal predation (Sun et al. 2018).

### 11.2.5 Phage Predation

Bacteriophages, or phages for short, are viruses that infect bacteria and therefore play a critical role in controlling bacterial populations. Naturally, vibriophages are found in aquatic environments where *Vibrio* species also reside. Long-term studies investigating the dynamics of phages and *V. cholerae* in aquatic environments found an inverse correlation between the presence of vibriophages and the presence of viable *V. cholerae* (Faruque et al. 2005). It was also determined that this correlated with the number of reported cholera cases in the regions studied. Therefore, it is hypothesized that phages play an important role in cholera outbreaks, with increased phage levels leading to a decline in cases and vice versa.

The long-term interactions between *Vibrio* species and vibriophages in the environment have led to the evolution of multiple strategies to evade phage infection. *V. cholerae* can become resistant to infection by multiple environmental phages by down-regulation of cyclic AMP (cAMP) and the cyclic AMP receptor protein through mutations in the *cyaA* or *crp* genes (Zahid et al. 2010). One of the major targets of vibriophages is the O antigen, as phage binding to the cell surface using this antigen is typically the first step of phage infection. Therefore, O antigen modification is a common strategy used as defense (Seed et al. 2012). However, the requirement of O1 strains of *V. cholerae* needing this receptor for human infection imposes limitations in advantageous mutations. Alternatively, shedding outer membrane vesicles (OMVs) that serve as decoys may protect intact bacteria from phage infection (Reyes-Robles et al. 2018). *Vibrio* species also have strategies to inhibit phage replication once they have entered the cell. Restriction-modification (RM) systems are a commonly used bacterial defense against phage DNA. RM systems in *V. cholerae* are generally carried on integrative and conjugative elements (ICEs) belonging to the SXT family (LeGault et al. 2021; Waldor et al. 1996) and recognize phage DNA to be foreign due to its lack of methylation. These RM systems act to protect *V. cholerae* against infection by diverse phage. In contrast, phage-inducible chromosomal island-like elements (PLEs) provide *V. cholerae* with specific defense against the ICP1 phage (O'Hara et al. 2017). At least five different PLEs have been circulating in epidemic *V. cholerae* strains for more than 70 years, highlighting the evolutionary importance of maintaining a defense against this specific vibriophage (Boyd et al. 2021).

There is limited understanding of bacteriophage-induced stress responses in *V. parahaemolyticus* and *V. vulnificus*. One proposed response is using an abortive infection (Abi) system whereby phage-infected bacteria can enter programmed cell death, thus preventing the spread of phage infection to neighboring cells (Kalatzis et al. 2018). One proposed mechanism of an abortive infection involves a toxin–antitoxin (TA) system. Phage infection results in reduced production of both toxin and antitoxin components, however, the antitoxin degrades faster. This imbalance within the TA-system allows the toxin to enter the bacterial cell into programmed cell death (Kalatzis et al. 2018). Several TA systems have been identified in *V. parahaemolyticus*, but their potential function as an Abi system still needs to be verified (Hino et al. 2014). Other mechanisms of resistance including preventing viral attachment, blocking viral DNA injection, phage DNA degradation, and DNA methylation have also been reported in other, non-pathogenic *Vibrio* species (Kalatzis et al. 2018).

## 11.3 Responses to Stress Encountered in the Host

When pathogenic *Vibrio* species are ingested by a human, they must adapt to a dramatically different set of stress-inducing conditions than they were exposed to in the aquatic environment. The bacteria first enter the stomach and must respond

to the extremely acidic conditions there before passage to the small intestine. Again, biofilms play a role in this stress response by providing some physical protection for ingested Vibrios, and they are thought to increase survival of *V. cholerae* in the stomach (Zhu and Mekalanos 2003). Once the bacteria reach the small intestine they encounter a new set of stressors including bile acids and antimicrobial peptides. In addition, they need to overcome the physical barrier provided by the mucus layer in order to access the epithelial surface of the intestine, which is the primary colonization site. Once the bacteria reach the epithelium, they attach, replicate, and produce the virulence factors responsible for the symptoms of disease.

### 11.3.1 Acid Stress

The stomach is the first line of defense encountered when an enteric pathogen is ingested. Therefore, it is important that *Vibrio* species that infect the human gastrointestinal tract be able to survive passage through the extremely acidic environment of the stomach in order to reach the small intestine. The pH of the stomach typically ranges from 1 to 3, although it can be raised by the presence of food. The infective dose of *V. cholerae* is between $10^3$ and $10^6$ bacteria when ingested with water and between $10^2$ and $10^4$ cells if consumed with food (Colwell et al. 1996). *V. cholerae* has a relatively low tolerance for acidic conditions, with an estimated 4–6 log reduction in viable bacteria during passage through the stomach (Spagnuolo et al. 2011).

The acid tolerance response (ATR) is defined as the induced resistance to normally lethal low pH (acid challenge) following growth at moderately low pH or following exposure to mildly acidic conditions (acid adaptation) (Merrell et al. 2002a). Many of the genes induced by exposure to acid in *V. cholerae* are heat-shock proteins and chaperones. CadC, a transcriptional regulatory protein, is activated in response to low pH by the LysR-type regulator AphB (Kovacikova et al. 2010). This is also relevant to the pathogenesis of *V. cholerae* as AphB is also a virulence gene regulator. CadC directly activates the expression of the *cadBA* operon, which encodes a lysine/cadaverine antiporter (CadB) and lysine decarboxylase (CadA) (Merrell and Camilli 2000). Lysine decarboxylases act by pumping H + ions out of the cell, producing cadaverine and carbon dioxide. Cadaverine is transported out of the cell by CadB in exchange for lysine. This raises the pH inside the bacterial cell, relieving stress. *cadA* expression is critical for the ATR in *V. cholerae*, as *cadA* mutants are not able to survive acid shock after acid adaptation (Merrell and Camilli 1999). *cadA* expression is induced during *V. cholerae* infection of infant mice and rabbit ileal loops, indicating that the acid stress response is important for host infection (Merrell and Camilli 1999).

*V. vulnificus* utilizes lysine decarboxylation and the lysine/cadaverine antiporter in its ATR in a manner very similar to that of *V. cholerae* (Rhee et al. 2002, 2005, 2006). Further studies showed that Lrp, a leucine-responsive regulatory protein, is also involved in transcriptional regulation of the *cadBA* operon. Lrp functions independently of the CadC regulatory pathway by directly binding to the *cadBA* promoter (Rhee et al. 2008). This suggests that CadC and Lrp function cooperatively to activate *cadBA* expression. The RpoS regulator is also required for *V. vulnificus* survival in acidic conditions, among other stress conditions (Hulsmann et al. 2003). *V. parahaemolyticus* also utilizes lysine decarboxylation in its ATR, with some similarities to the systems in *V. cholerae* and *V. vulnificus* (Gu et al. 2021; Tanaka et al. 2008). Studies in *V. parahaemolyticus* have also demonstrated that acid stress confers a degree of cross-protection to high salinity and temperature stress (Kalburge et al. 2014; Wong et al. 1998; Huang and Wong 2012). Transcriptomic studies showed that CadC is involved in the upregulation of several heat-shock proteins, suggesting a potential role in cross-protection against varying environmental stressors in *V. parahaemolyticus* (Gu et al. 2021).

A large-scale screen revealed additional genes that are important for the ATR in *V. cholerae*. Included in these genes is *gshB*, which encodes

glutathione synthetase, the enzyme that catalyzes the last step of glutathione synthesis (Merrell et al. 2002b). Glutathione regulates the Kef system, which is important for $K^+$ transport. pH homeostasis involves intracellular fluxes of $Na^+$ and $K^+$ ions, so the inability of a *gshB* mutant to regulate intracellular $K^+$ levels may result in altered pH homeostasis in *V. cholerae*. GshB also plays a role in colonization of the infant mouse intestine, again linking virulence to the ATR. Two other genes showed similar defects in this study, *hepA* and *recO* (Merrell et al. 2002b). Both of these genes are thought to play a role in DNA repair, and both were required for colonization of the infant mouse intestine.

### 11.3.2 Bile

Intestinal bile is an important defense mechanism that pathogenic bacteria must overcome to successfully colonize the gastrointestinal tract. Bile is a fluid synthesized by the liver, mainly composed of bile acids and cholesterol, which acts as a biological detergent to solubilize lipids in food, thereby playing an essential role in fat digestion. Bile is a well-recognized virulence inducing signal in Vibrios. However, due to their amphipathic nature, bile acids are also toxic for bacterial cells, causing membrane damage and disrupting cellular homeostasis. Therefore, it is not surprising that the *Vibrio* species that colonize the human intestinal tract have developed numerous strategies to overcome stress induced by bile acids.

In *V. cholerae*, resistance to bile stress is largely dependent on the activity of efflux pumps and porins. Tripartite efflux pumps are typically comprised of an outer membrane pore protein, a periplasmic membrane fusion protein, and an inner membrane transporter. These systems act to pump toxic compounds from the cytoplasm or periplasmic space to the outer environment. In *V. cholerae*, TolC is hypothesized to be the pore protein for its efflux pump systems as it is required for bile resistance and intestinal colonization (Bina and Mekalanos 2001). Two RND (resistance-nodulation-division) family efflux pump systems, VexAB and BreAB (also known as VexCD) are required for bile resistance in *V. cholerae* (Bina et al. 2006). While the BreAB system is more specific for resistance to bile acids and certain detergents, the VexAB system confers resistance to a wider range of antimicrobial agents. Similarly, *V. vulnificus* also encodes a VexAB efflux pump that is required for resistance to bile and several other substrates (Lee et al. 2015a). The *V. cholerae* porins, OmpU and OmpT also play important roles in bile resistance. OmpT has a larger pore size than OmpU, which makes the membrane more permeable to certain small molecules like bile acids. Upon bile exposure, *V. cholerae* upregulates expression of *ompU*, replacing OmpT and increasing resistance (Wibbenmeyer et al. 2002). *V. parahaemolyticus* also produces a ToxR-regulated outer membrane protein that is required for resistance to bile, although its exact identity is unknown (Provenzano et al. 2000).

The structure of the bacteria cell wall is also important for bile resistance in *V. cholerae*, as mutations in genes that are required for LPS biosynthesis can also increase sensitivity to bile. *waaF* and *wavB* encode proteins required for the synthesis of the core oligosaccharide of LPS. Mutations in these genes increase bile sensitivity (Nesper et al. 2002). In addition, mutation of *galU*, which is involved in UDP-glucose biosynthesis, causes *V. cholerae* to become more sensitive to bile (Nesper et al. 2001). UDP-glucose is a carbohydrate that is part of the LPS and its loss likely leads to membrane alterations that impact bile resistance. Recent work showed that a BolA-like protein, IbaG, is required for bile resistance in *V. cholerae*. An *ibaG* mutant strain showed altered cell morphology and membrane composition, which likely causes the increased sensitivity to envelope stressors such as bile (Fleurie et al. 2019).

Bile-adapted *V. vulnificus* strains are cross-protected to low salinity stress (Wong and Liu 2006). Bile-adapted bacteria also showed cross-protection to high pH, heat, high salinity, and detergents. The alternative sigma factor RpoS plays a role in *V. vulnificus* stress response to bile, as bile adaptation occurs significantly slower in *rpoS* mutant strains (Chen et al. 2010).

### 11.3.3 Mucus

The thick layer of mucus lining the small intestine serves as a significant barrier for pathogens that colonize the intestinal epithelium. Mucus is a complex hydrogel made of mucins, lipids, and DNA (Allen et al. 1984). Mucins are complex glycoproteins crosslinked by disulfide bonds and are responsible for the viscosity of mucus. The intestinal mucus layer is continuously renewed by secretion of highly O-glycosylated MUC2 mucin by goblet cells (Gustafsson et al. 2012) and varies in thickness along the length of the small intestine. The mucus layer is thinner in the proximal part (~200 μm) than the distal part (~500 μm), where *V. cholerae* preferentially colonizes (Atuma et al. 2001).

*V. cholerae* initially attaches to the mucus layer through the action of an adhesin, GbpA (Bhowmick et al. 2008). GbpA binds a monomer of chitin, N-acetyl-D-glucosamine (GlcNac), which is a common component of human intestinal mucins. GbpA binding to mucins results in increased mucus secretion by stimulating transcription of host pathways. In turn, mucin increases GbpA expression by *V. cholerae* in a dose-dependent manner (Bhowmick et al. 2008). This coordinated interaction leads to elevated levels of GbpA on the bacterial surface, resulting in increased adherence to the mucus layer.

*V. cholerae* produces a soluble zinc-dependent metalloprotease, called haemagglutinin/protease (Hap), encoded by *hapA* (Booth et al. 1983; Silva et al. 2003). Hap has both mucinolytic and cytotoxic activity and is required for translocation through mucin in a column assay. Hap expression and production is induced by mucin, which aids in mucus degradation and transit to the epithelial surface (Silva et al. 2003). In addition to breaking down mucin, Hap also degrades GbpA, which may allow the bacteria to move further through the mucus layer (Jude et al. 2009). HapR, the quorum sensing master regulator, represses *gbpA* expression while activating *hapA* expression, suggesting that cell density plays a role in regulating genes involved in mucus penetration (Booth et al. 1983; Silva et al. 2003). In addition to Hap, *V. cholerae* produces two other proteases that may play a role in breaking down mucus near the intestinal epithelium. TagA is a secreted metalloprotease that cleaves mucin glycoproteins attached to the host cell surface (Szabady et al. 2011). Neuraminidase (NanH) is a protease that cleaves two sialic acid groups from the $GM_1$ ganglioside, a sialic acid containing oligosaccharide on the surface of the intestinal epithelial cells, revealing the receptor for cholera toxin (Galen et al. 1992). Motility is also critical for transit through the mucus layer. Recent studies showed that alkaline pH increases *V. cholerae* swimming speed and penetration through mucus (Nhu et al. 2021).

VvpE is a zinc metalloprotease produced by *V. vulnificus* (Jeong et al. 2000; Kothary and Kreger 1987). VvpE is a 45-kDa protein, which consists of a 35-kDa N-terminal catalytic domain and a 10-kDa domain for substrate attachment (Miyoshi et al. 1997). Unlike *V. cholerae*, which directly degrades gastrointestinal mucin through the production of Hap, VvpE inhibits Muc2 expression by acting through the lipid raft associated ITLN. This is a unique pathway which stimulates the methylation of the Muc2 gene promoter through the ROS-dependent activation of PKC (sigma)/ERK pathway (Lee et al. 2015b). VvpE was found to induce the recruitment of NADPH oxidase 2 and neutrophil cytosolic factor-1 into membrane lipid rafts coupled with ITLN, which facilitates the production of reactive oxygen species. Both *V. vulnificus* and *V. parahaemolyticus* produce metalloproteases, which play an indirect role in pathogenicity by promoting intestinal wall attachment through the degradation of the mucus layer (Miyoshi 2013).

### 11.3.4 Antimicrobial Peptides

Antimicrobial peptides (AMPs) are small amphipathic molecules produced by organisms from all domains of life. Typically, AMPs are between 12 and 50 amino acids in length and have a net positive charge, allowing them to interact with negatively charged molecules on the bacterial cell surface. AMPs can kill bacteria via a number

of mechanisms and often disrupt membrane integrity or form pores. *Vibrio* species that infect humans will encounter AMPs produced in the small intestine as a part of the innate immune response. Like many other bacterial species, Vibrios have evolved a variety of mechanisms to avoid the toxic effects of AMP exposure, including cell surface modification and exporting AMPs that enter the bacterial cell. While the human pathogenic *Vibrio* species encounter AMPs during host colonization, it is worth noting that these bacteria likely encounter a variety of AMPs while in the aquatic environment as well. The stress resistance mechanisms discussed here likely serve to protect Vibrios from AMPs produced by numerous organisms.

Like many gram-negative bacteria, *V. cholerae* modifies its LPS as a mechanism to resist killing by AMPs. While other bacteria add polar groups such as phosphoethanolamine or L-Ara4N, *V. cholerae* adds glycine or diglycine residues to the Lipid A portion of the LPS (Hankins et al. 2012). This modification system is encoded by three genes (*almG*, *almF*, and *almE*), whose expression is increased in the presence of a sublethal concentration of the AMP polymyxin B (Hankins et al. 2012; Bilecen et al. 2015; Matson et al. 2017). Inactivation of this modification system in El Tor strains of *V. cholerae* results in increased polymyxin B sensitivity. However, in strains of the classical biotype, this system is inactive due to a nonsense mutation in *almF* that truncates the protein (Hankins et al. 2012). This provides an explanation for the use of polymyxin B sensitivity as a method to differentiate classical and El Tor *V. cholerae* in the clinical laboratory, with classical strains being more sensitive to the peptide than El Tor strains. Expression of *almEFG* is regulated by the CarRS two-component system. CarR, the response regulator, directly binds the *almEFG* promoter and activates transcription (Bilecen et al. 2015; Herrera et al. 2014). Deletion of *carR* aso results in polymyxin B sensitivity, due to decreased *almGEF* expression. However, how these systems contribute to survival in the host intestine is less clear. In the infant mouse intestine, a *carR* deletion colonizes less well in some *V. cholerae* strains, while *almGEF* deletion does not show a colonization defect (Bilecen et al. 2015).

Lipid A modification is also used by *V. parahaemolyticus* to resist AMP stress. The plasmid-mediated colistin resistance gene, *mcr*, encodes an enzyme that adds a phosphoethanolamine group to lipid A (Gunn et al. 1998; Poole 2012). This modification confers fewer negative charges to the cell surface. An *mcr*-encoding plasmid has been identified in at least one *V. parahaemolyticus* isolate (Lei et al. 2019). The VP_RS21300 gene encodes a phosphoethanolamine transferase in the *V. parahaemolyticus* ATCC33846 isolate, which is another mechanism of lipid A modification (Xin Tan et al. 2021a). Deletion of this gene resulted in increased susceptibility to polymyxin B (Xin Tan et al. 2021a). Polymyxin B stress also results in upregulation of the cellular antioxidant system in *V. parahaemolyticus*, suggesting the potential for cross-protection between AMP stress and other environmental and host stressors (Xin Tan et al. 2021b).

Appropriate acylation of *V. cholerae* lipid A is also required for resistance to AMPs. Deletion of the acyltransferase MsbB results in underacylated lipidA and increased sensitivity to multiple antimicrobial peptides (Matson et al. 2010). In addition, an *msbB* mutant shows a colonization defect in the infant mouse model of cholera, suggesting that it is important for resistance to AMPs produced by the infant mouse intestine (Matson et al. 2010). CRAMP (cathelin-related antimicrobial peptide) is the primary innate immune defense mechanism of the neonatal mouse intestine and is the mouse version of LL-37, a cathelicidin produced in the human intestine (Pestonjamasp et al. 2001). An *msbB* mutant of *V. cholerae* shows decreased survival in the presence of either peptide, suggesting that MsbB may play a role in infection of humans as well.

*V. cholerae*, like many gram-negative bacteria, regulates the rate of small-molecule diffusion across its outer membrane by altering the production of specific outer membrane porin proteins. OmpU and OmpT are *V. cholerae* porins that play a role in resistance to the AMPs polymyxin B and

BPI (bactericidal/permeability-increasing) derived peptide P2 (Mathur and Waldor 2004). BPI is a potent antimicrobial protein expressed as a surface protein on human gastrointestinal epithelial cells. OmpU mediates BPI resistance during the mid-log and stationary phases of bacterial growth and OmpT is responsible for resistance only during stationary phase (Mathur and Waldor 2004). OmpU and OmpT are connected to both virulence gene regulation and other stress response mechanisms in *V. cholerae*. OmpU and OmpT are members of the ToxR regulon, which is responsible for regulating the production of virulence genes, including the cholera toxin. ToxR positively regulates the expression of *ompU* and negatively regulates expression of *ompT*. OmpU is a member of the $\sigma^E$ extracytoplasmic stress response pathway. OmpU modulates the expression and activity of $\sigma^E$ and is required for the $\sigma^E$ pathway to be activated by AMP exposure in *V. cholerae* (Mathur et al. 2007).

A recent study demonstrated a role for increased outer membrane vesicle (OMV) production in resistance to AMPs and host adaptation in *V. cholerae* (Zingl et al. 2020). Hypervesiculation acts to accelerate modulation of the composition of the cell surface. This results in the fast accumulation of glycine-modified lipid A and accelerated removal of the OmpT porin. This exchange of cell surface components increases bacterial survival during mammalian infection (Zingl et al. 2020).

Another mechanism commonly used by bacteria to counter the effects of AMPs is to remove them from the cell using efflux pumps. *V. cholerae* encodes six RND (Resistance-Nodulation-Division) efflux systems that all use TolC as their outer membrane pore (Bina and Mekalanos 2001; Bina et al. 2008). Deletion of any of the RND transporter proteins results in increased sensitivity to multiple antimicrobials, including cationic AMPs (Bina et al. 2008). Wild-type *V. cholerae* treated with RND efflux inhibitors are also sensitive to AMPs (Bina et al. 2009). A *vexB* mutant was the most sensitive to polymyxin B, suggesting that the VexAB system is primary RND efflux pump associated with AMP resistance in *V. cholerae* (Bina et al. 2008). *V. parahaemolyticus* and *V. vulnificus* also use RND efflux transporters to resist AMP stress. The *vmeAB* transporter was the first RND-type efflux transporter characterized in *V. parahaemolyticus*, with a *vmeAB* mutant showing higher susceptibility to some antimicrobial agents (Matsuo et al. 2007). A further 11 RND efflux systems have also been characterized in *V. parahaemolyticus*. Disruption of these systems resulted in decreased MICs in response to several antimicrobial agents and reduced fluid accumulation in rabbit ileal loops (Matsuo et al. 2013). Eleven RND efflux pumps homologous to those identified in *V. cholerae* have also been identified in *V. vulnificus*. Specifically, three putative RND pumps show homology to *V. cholerae* VexAB and VexCD. The *V. vulnificus* VexAB homologs contribute to in vitro antimicrobial resistance to a broad array of substrates (Lee et al. 2015a). Exposure to antibacterial chemicals also enhanced expression of two putative outer membrane RND efflux pumps, *tolCV1* and *tolCV2* (Lee et al. 2015a).

Transcriptomic studies aimed at identifying all genes that are differentially regulated by polymyxin B exposure in *V. cholerae* revealed that expression of a conserved hypothetical protein was induced by sublethal concentrations of the AMP (Matson et al. 2017). Subsequent studies revealed that the protein (named SipA) acts to bind AMPs in the periplasmic space and, through its interaction with the outer membrane protein OmpA, inactivates the AMPs or remove them from the bacterial cell (Saul-McBeth and Matson 2019). This AMP-resistance mechanism is more critical for bacterial survival in classical strains that cannot modify their lipid A, as *sipA* mutants of the classical biotype are sensitive to AMP exposure while El Tor mutants do not show increased sensitivity (Saul-McBeth and Matson 2019). The exact mechanism by which this novel stress-responsive protein acts in *V. cholerae* is not yet well understood, however, it is likely to be broadly relevant to other bacterial stress responses as SipA is well conserved in *Vibrio* species and more distantly related bacteria.

Another AMP-resistance mechanism identified in *V. vulnificus* involves the $K^+$ uptake protein, TrkA, which is required for survival in the presence of polymyxin B and protamine (Chen et al. 2004). Polymyxin B functions to create large, ion-permeable pores across the bacterial cell membrane (Hancock and Chapple 1999). This mechanism is similar to that of the serum complement membrane attack complex, which also forms ion-permeable channels to disrupt inner membrane potential (Muller-Eberhard 1986; Dankert 1991). The TrkA channel is hypothesized to rapidly accumulate cellular $K^+$, which is protective against the ion-permeable channels generated by polymyxin B and the serum complement membrane attack complex (Chen et al. 2004).

## 11.4 Conclusions

Clearly, Vibrios encounter a variety of stressful conditions while living in the aquatic environment. However, the species that also infect human hosts must appropriately respond to an additional set of challenges in order to survive and be transmitted to new hosts. Some of these stress response mechanisms provide protection from a variety of stressors and some are unique to certain environments or conditions. In addition, exposure to some stress-inducing conditions confer cross-protection to other types of stress. While the three *Vibrio* species discussed here have been studied for decades, we continue to identify new strategies that these bacteria use to survive and overcome harsh conditions, revealing the complex interplay between these pathogens and their environment.

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# *Vibrio parahaemolyticus* Epidemiology and Pathogenesis: Novel Insights on an Emerging Foodborne Pathogen

12

Ronnie G. Gavilan, Junior Caro-Castro, Carlos J. Blondel, and Jaime Martinez-Urtaza

**Abstract**

The epidemiological dynamics of *V. parahaemolyticus´* infections have been characterized by the abrupt appearance of outbreaks in remote areas where these diseases had not been previously detected, without knowing the routes of entry of the pathogens in the new area. However, there are recent studies that show the link between the appearance of epidemic outbreaks of *Vibrio* and environmental factors such as oceanic transport of warm waters, which has provided a possible mechanism for the dispersion of *Vibrio* diseases globally. Despite this evidence, there is little information on the possible routes of entry and transport of infectious agents from endemic countries to the entire world. In this sense, the recent advances in genomic sequencing tools are making it possible to infer possible biogeographical patterns of diverse pathogens with relevance in public health like *V. parahaemolyticus*. In this chapter, we will address several general aspects about *V. parahaemolyticus*, including their microbiological and genetic detection, main virulence factors, and the epidemiology of genotypes involved in foodborne outbreaks globally.

**Keywords**

*Vibrio parahaemolyticus* · Foodborne disease · Genomic islands · Molecular epidemiology

## 12.1 Introduction

The *Vibrio* genus is composed by a group of Gram-negative bacteria that naturally inhabit aquatic environments. These species are active in various biological processes taking, in some cases, the role of saprophytes, constituting the commensal microbiota of fish, mollusks, and plankton (Parveen et al. 2008). In other cases, they behave as opportunistic pathogens of marine animals and humans. Within the *Vibrio* genus, 12 species are known to be involved in human infections, where *Vibrio cholerae, V. parahaemolyticus*, and *V. vulnificus* are the main species responsible for the majority of

R. G. Gavilan (✉)
Instituto Nacional de Salud, Lima, Peru

Escuela Profesional de Medicina Humana, Universidad Privada San Juan Bautista, Lima, Peru
e-mail: rgavilan@ins.gob.pe

J. Caro-Castro
Instituto Nacional de Salud, Lima, Peru

C. J. Blondel
Instituto de Ciencias Biomédicas, Facultad de Medicina y Facultad de Ciencias de la Vida, Universidad Andrés Bello, Santiago, Chile

J. Martinez-Urtaza
Department of Genetics and Microbiology, Faculty of Biosciences, Universitat Autonoma de Barcelona, Barcelona, Spain



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gastroenteritis and extraintestinal infections, which can also be dangerous in immunocompromised patients (Baumann et al. 1984; DePaola and Kaisner 2001).

For centuries, the most representative member of this genus was *V. cholerae*, due to its implication in various cholera pandemics that have plagued the world for generations. However, *V. parahaemolyticus* has recently become one of the best studied species of *Vibrio* due to the implication of some serotypes in human foodborne infections. *V. parahaemolyticus* has a mesophilic and halophilic nature, presenting a facultative anaerobic metabolism. This bacterium inhabits estuarine areas of temperate and tropical marine environments around the world (Baumann et al. 1984). Some strains of this organism can cause enteric infections in humans, which are mainly associated with the consumption of raw shellfish and undercooked fish products. In most cases, the disease resolves without the need for treatment. However, *V. parahaemolyticus* also can cause debilitating and dysenteric forms of gastroenteritis (Levin 2006). Less frequent infections are associated with open wound infections that come into contact with contaminated seawater, necrotizing fascitis, and septicemia in immunocompromised patients (Zhang and Orth 2013).

This microorganism has been associated with human infections since 1953, when Japanese researchers led by Fujino et al. (1953) identified *V. parahaemolyticus* as the cause of food poisoning. This infectious outbreak was detected in the province of Osaka, Japan, reporting 272 people affected and 20 deaths, being associated with the consumption of raw sardines. Since that date, *V. parahaemolyticus* infections have been reported in various parts of the world, causing outbreaks in various countries in Asia, Europe, and America. All these epidemic episodes made it possible to elucidate that the majority of strains associated with pathogenesis are restricted to certain serotypes, which are defined by somatic (O) and capsular (K) antigens (Klein et al. 2014).

Despite most information on *V. parahaemolyticus* is related to foodborne infections, little is known about its ecology. *V. parahaemolyticus* is ubiquitous in nearshore marine waters, with high counts reported in surface sediments and turbid waters with large loads of resuspended sediments, contributing to the cycling of carbon and other nutrients (Parveen et al. 2008). Clearly, human exposure to these pathogens cannot be completely eliminated, but disease incidence can be reduced if environmental conditions that significantly elevate risk are identified and monitored. The main point of monitoring involves bivalve mollusks, such as oysters and mussels, which can concentrate a large part of these microorganisms, being capable of producing an infection by ingestion. Virulent strains of *V. parahaemolyticus* are clearly a seafood safety concern, and their detection is important anywhere high levels of this organism are found (Zimmerman et al. 2007).

## 12.2 Microbiological Identification of *V. parahaemolyticus*

Several enrichment media have been used for the isolation, detection, and enumeration of *V. parahaemolyticus* (Paydar et al. 2013). Due to these bacteria inhabiting marine environments, which includes high salinity and alkaline conditions, selective media usually include between 1% and 7% NaCl, and a pH in the range 8.6–9.4 (Farmer et al. 2003). The U.S. Food and Drug Administration (FDA) has recommended alkaline peptone water (APW) as the enrichment broth for all *Vibrio* species including *V. parahaemolyticus*, having good recuperation of this microbes, and inhibits the growth of other bacteria (Kaysner and DePaola 2004).

On the other hand, selective media are another alternative to recover *V. parahaemolyticus* from marine environments. The most common media is thiosulphate citrate bile salts sucrose (TCBS), a highly selective differential medium widely used for pathogenic *Vibrios* (Bisha et al. 2012). TCBS is a selective system consisting of components (ox bile, NaCl, and alkaline pH) which suppresses the growth of Gram positive organisms, having the advantage to differentiate sucrose-positive *Vibrio* such as *V. cholerae* from other *Vibrio*

species colonies like *V. parahaemolyticus* (Mrityunjoy et al. 2013). However, sometimes colonies on TCBS agar are difficult to distinguish physically from other bacterial colonies, making it difficult to isolate and enumerate *V. parahaemolyticus* from marine samples (Pinto et al. 2011). To overcome this problem, CHROMagar Vibrio (CV), a new selective agar medium for detecting *V. parahaemolyticus,* was developed, which contains colorimetric substrates for β-galactosidase to differentiate ortho-nitrophenyl-β-galactoside-positive *V. parahaemolyticus* (mauve color) from other related *Vibrio* species, being more specific and accurate than TCBS (Hara-Kudo et al. 2001).

## 12.3 Pathogenesis of *V. parahaemolyticus*

*V. parahaemolyticus* primarily causes acute gastroenteritis when food contaminated by pathogenic variants of this organism are ingested. The disease is characterized by severe cramps, abdominal pain, vomiting, and watery-bloody diarrhea. These clinical characteristics of *V. parahaemolyticus* cause pronounced dehydration in the person; not requiring treatment in several cases, but does require hydration. The diagnosis of this disease is generally made by stool culture in order to isolate the etiological agent. Since the first records of *V. parahaemolyticus* infections, it was observed that strains isolated from clinical samples presented beta-hemolysis when they were cultured in a medium with human blood, which was called Kanagawa phenomenon. Since then, most isolates made from patients infected with gastroenteritis showed a positive Kanagawa phenomenon (KP+) (Nishibuchi and Kaper 1995), whereas most *V. parahaemolyticus* strains isolated from food and marine environments are negative (KP-). Because the Kanagawa phenomenon was evidenced by marked hemolytic activity, it was considered the main pathogenic factor.

Currently, three hemolysins have been described for *V. parahaemolyticus*: Thermolabile hemolysin (TLH), thermostable direct hemolysin (TDH), and TDH-related hemolysin (TRH). Taniguchi et al. (1986) reported that TL was found in all strains of *V. parahaemolyticus*, but not in other species of *Vibrio*, being used as a genetic marker for the genus. Subsequently, it was shown that this hemolysin does not confer a pathogenic capacity to this microorganism. On the other hand, TDH is a 23 kDa protein with hemolytic activity on a wide range of erythrocytes. The presence of this hemolysin was ultimately associated with the Kanagawa phenomenon. This protein exhibits enterotoxigenicity, cytotoxicity, cardiotoxicity, and increased vascular permeability (Nishibuchi and Kaper 1995). There is another hemolysin genetically related to TDH that is also involved in gastroenteritis, called TRH. This toxin was initially detected in strains from gastroenteritis cases that did not present the Kanagawa phenomenon (Taniguchi et al. 1986).

Infections caused by *V. parahaemolyticus* are mainly associated with strains that have the ability to produce TDH, but a 10% of cases are related to strains with the presence of TRH (DePaola et al. 2003). For this reason, these two hemolysins are used as universal markers of the pathogenic capacity of *V. parahaemolyticus*. While nearly all isolates from clinical sources exhibit hemolysins, less than 1% of *V. parahaemolyticus* isolates from the environment exhibited this feature (DePaola and Kaisner 2001).

To study the pathogenesis of *V. parahaemolyticus*, several animal models of infection have been developed throughout the years. These include rabbit ligated ileal loops, orogastric, intrapulmonary, and peritoneal mouse infection models as well as orogastric piglets and infant rabbit infection models (Hiyoshi et al. 2010; Pineyro et al. 2010; Ritchie et al. 2012; Park et al. 2004a; Yang et al. 2019a, b). While all these models have provided key information regarding different aspects of the infection caused by *V. parahaemolyticus*, in some cases they provide contradictory results in terms of the contribution of individual virulence factors to *V. parahaemolyticus* infection (Okada et al. 2014; Yang et al. 2019a, b). These differences

highlight the importance of integrative in vitro and in vivo approaches to determine the overall contribution of potential virulence determinants to *V. parahaemolyticus*` pathogenesis.

## 12.4 Virulence Factors and Fitness Traits

While the first studies focused on the contribution of the TDH, TRH, and TLH hemolysins to *V. parahaemolyticus'* pathogenesis, the appearance of the pandemic clone in 1996, and the advent of whole genome sequencing together with the development of high throughput functional genomics, led to the identification of multiple other factors which can contribute to the virulence and environmental fitness of *V. parahaemolyticus*.

While every *V. parahemolyticus* strain sequenced to date encodes a Type III Secretion System encoded within chromosome 1 (T3SS1), whole genome sequencing of the pandemic clone revealed the presence of a second, evolutionary distinct, T3SS encoded within its second chromosome (T3SS2) (Makino et al. 2003). T3SSs are multicomponent nanomachines which enable Gram-negative bacteria the delivery (translocation) of proteins, known as effectors, directly from the bacterial cytosol into the cytosol of eukaryotic cells. Delivery of these effectors allow pathogens to hijack host-cell signaling, thereby manipulating a variety of host cell functions (Lara-Tejero and Galán 2019).

The T3SS1 is a highly conserved system, responsible for the delivery of 3 known effector proteins (VopQ, VopS, and VopR), all of which contribute to the cytotoxicity and cell death of infected cells in vitro (Wang et al. 2015). The T3SS2, identified almost exclusively in clinical strains, is responsible for the delivery up to ten effector proteins (VopA, VopC, VopG, VopL, VopO, VopT, VopV, VopZ, VPA1380, and VgpA) which contribute to the subversion of multiple cellular processes, including those controlling actin cytoskeleton dynamics and innate inflammatory responses during infection (Ritchie et al. 2012; Hiyoshi et al. 2011, 2015; Kodama et al. 2007; Zhou et al. 2013; Zhang et al. 2012; Trosky et al. 2004; Tandhavanant et al. 2018; Hu et al. 2021). T3SS2 gene expression is induced by bile through the VtrA, VtrB, and VtrC signaling network (Gotoh et al. 2010; Peng et al. 2016), and both in vitro and in vivo data have shown that the T3SS2 is the major contributor to *V. parahaemolyticus'* virulence and pathogenesis, with TDH and T3SS1 playing a minor role during infection (Ritchie et al. 2012; Hubbard et al. 2016). Interestingly, the TDH toxin has also been shown to be secreted by the T3SS2 (Matsuda et al. 2019). Functional genomic studies have also revealed host factors facilitating T3SS cytotoxicity, including the importance of host-cell sulfation for bacterial adhesion, T3SS-dependent cytotoxicity, and the host-cell fucosylation of cell surface glycans for efficient insertion of the T3SS2 into host membranes (Blondel et al. 2016).

The T3SS2 has also been linked to the ability of *V. parahaemolyticus* to compete with environmental predatory amoeba (Matz et al. 2011), and several studies have identified T3SS2 gene cluster in species other than *V. parahaemolyticus* (Okada et al. 2014). This suggests that acquisition and transfer of the T3SS2 in the ocean could provide an ecological advantage to the bacteria which could also, coincidentally, promote human infection.

In addition to the T3SSs, *V. parahaemolyticus* also encodes two distinct Type VI Secretion Systems (T6SS1 and T6SS2). T6SS are contractile poison-tipped spears nanomachines that mediate the delivery of anti-bacterial or anti-eukaryote effector proteins, which contribute to bacterial niche competition and infection (Cherrak et al. 2019). While T6SS2 is found in all *V. parahaemolyticus* strains sequenced to date and has been shown to be responsible for host-cell adhesion, the T6SS1 is mostly found in clinical isolates and has been shown to mediate anti-bacterial activity (Fridman et al. 2020). The anti-bacterial activity of T6SS1 suggests that this system could also play a role in environmental survival of *V. parahaemolyticus*, including bacterial competition with the microbiota of infected animals, including humans.

In addition to these specialized protein secretion systems, at least three different adhesins (MAM7, MshA1, and VpadF) also been linked to the virulence of *V. parahaemolyticus* through their ability to mediate host-cell adhesion in a variety of different types of host cells, including macrophages, fibroblasts, and epithelial cells (Stones and Krachler 2015; Krachler and Orth 2011; Liu and Chen 2015; O'Boyle et al. 2013). The acquisition of nutrients, such as iron, has also been shown to be important for *V. parahaemolyticus*. Two independent mechanisms for iron acquisition have been described, including production of siderophores such as vibrioferrin, ferrichrome, and aerobactin, as well as the use of heme as a direct source of iron (Broberg et al. 2011).

Finally, the environmental adaptation and virulence induction in *V. parahaemolyticus* rely on several gene regulatory networks. In addition to the bile-regulated VtrA-C signaling network, a complex signaling network, involving the second messenger c-di-GMP, has also been shown to be critical for motility and biofilm formation in *V. parahaemolyticus* (Martínez-Méndez et al. 2021). The regulation of the switch between free-living planktonic or the surface adapted lifestyles in *V. parahaemolyticus* depends on the OpaR and SrC regulatory networks, which can regulate the motility and biofilm formation of *V. parahaemolyticus* through changes in the intracellular c-di-GMP levels (Zhang et al. 2021; Kimbrough et al. 2020).

## 12.5 Epidemiology of *V. parahaemolyticus*

The importance that *V. parahaemolyticus* has acquired is largely due to the progression of infections associated with this pathogen in recent years. This progression became relevant since the emergence of a new variant of *V. parahaemolyticus,* called pandemic clonal complex or pandemic clone, composed by serotypes O3:K6, O4:K68, O1:KUT, and O1:K25. The appearance of this clone was initially related to the appearance of a large number of infections in India associated with a single serotype O3:K6 during the year 1996, which rapidly spread throughout Southeast Asia (Okuda and Nishibuchi 1998). The sudden detection of a high number of infections associated with the pandemic clone of *V. parahaemolyticus* in Peru (Martinez-Urtaza et al. 2008) and Chile (González-Escalona et al. 2005) at the end of 1997, revealed the pandemic expansion of this clone, as well as its pathogenic and dispersal potential, showing a significant change in the epidemiology of *V. parahaemolyticus*. Since the appearance of the pandemic clone of *V. parahaemolyticus* in South America, infections associated with this group have been detected in the USA and Russia in 1998, and in Mozambique, Mexico, and Spain in 2004 (Nair et al. 2007).

Despite the presence of infections associated with the pandemic clone that predominates in many parts of the world, in recent years, a parallel increase in the number of cases associated with other serotypes and clonal groups characteristic or endemic to different geographical areas has been observed. In this way, it has been possible to establish that the dominant clone in infections on the Pacific coast of the USA characteristically belongs to the serotype O4:K12 (DePaola and Kaisner 2001), while in Peru the dominant clone is O4:K8 and in Spain is O4:K11 (Martinez-Urtaza et al. 2008). More information about outbreaks of *V. parahaemolyticus* in the last 20 years can be found in Table 12.1 and Fig. 12.1.

There is currently little information on the factors that are promoting the unstoppable geographic advance of infections. A study carried out on the coast of Peru has provided some evidence of the role of water movement in the dynamics of pathogenic clones of *V. parahaemolyticus*, demonstrating that the equatorial waters transported by the El Niño phenomenon were responsible for transporting the pandemic clone of *V. parahaemolyticus* from Asia to America in 1997, a journey of more than 14,000 km (Martinez-Urtaza et al. 2008). The dissemination of this pathogen has also been related in this case to the entry of tropical zooplankton trapped in the waters of El Niño. These observations coincide

**Table 12.1** Most important outbreaks of *V. parahaemolyticus* around the world in the last 20 years

| Country | Year | Source of infection | Serotype involved | ST or CC | References |
|---|---|---|---|---|---|
| Australia | 2021 | Raw oysters | N/A | N/A | NSW Government (2021) |
| Canada | 2020 | Shellfish | N/A | N/A | Public Health Agency of Canada (2020) |
| EEUU | 2019 | Raw oysters | N/A | N/A | CDC (2019) |
| EEUU | 2018 | Crab meat | N/A | N/A | CDC (2018) |
| Canada | 2015 | Raw oysters | O4:KUT | CC-36 | Taylor et al. (2018) |
| China | 2013 | Undercooked food | O3:K6 | CC-3 | Liu et al. (2015) |
| Spain | 2012 | Undercooked shrimp | O4:K12 | CC-36 | Martinez-Urtaza et al. (2017) |
| Peru | 2009 | Undercooked seafood | O3:K59 | CC-120 | Gonzalez-Escalona et al. (2016) |
| EEUU | 2006 | Raw shellfish | O4:K12 | CC-36 | CDC (2006) |
| Chile | 2004–2005 | Shellfish | O3:K6 | CC-3 | Harth et al. (2009) |
| United States (Alaska) | 2004 | Raw oysters | O6:K18 | CC-3 | McLaughlin et al. (2005) |
| Mozambique | 2004 | Contaminated food | O3:K6, O4:K68 | CC-3 | Ansaruzzaman et al. (2004) |
| France | 2004 | Contaminated food | O3:K6 | CC-3 | FAO and WHO (2021) |
| Mexico | 2003–2004 | Undercooked shrimp | O3:K6 | CC-3 | Cabanillas-Beltrán et al. (2006) |
| India | 2003 | Rice with meat | O3:K6 | CC-3 | Sen et al. (2007) |
| Peru | 1998 | Undercooked seafood | O3:K6 | CC-3 | Gil et al. (2007) |
| Chile | 1998 | Undercooked seafood | O3:K6 | CC-3 | Cordova et al. (2002) |
| USA | 1998 | Undercooked seafood | O3:K6 | CC-3 | FAO and WHO (2021) |
| Taiwan | 1996–1999 | Undercooked seafood | O3:K6 | CC-3 | Chiou et al. (2000) |
| Japan | 1996–1998 | Undercooked seafood | O3:K6 | CC-3 | IASR (1999) |

with the results obtained in previous studies that showed that the survival and growth of *V. parahaemolyticus* in the marine environment is closely linked to its association with zooplankton (Kaneko and Colwell 1973). According to these latest investigations, the movements of ocean waters from distant areas may be directly related to the introduction of pathogenic populations of *Vibrio* in areas where they had not previously been detected, being the zooplankton, and more specifically copepods, the most likely candidates to facilitate transportation. In this way, the arrival of oceanic populations to the coast constitutes a permanent source of *V. parahaemolyticus* that makes it difficult to differentiate population groups at the local level.

## 12.6 Genomics and Evolutionary Aspects of *V. parahaemolyticus*

The first sequenced genome of *V. parahaemolyticus* belonged to a strain of the pandemic group RIMD2210633 serotype O3:K6 causing outbreaks in Asia. The genome of strain RIMD2210633 has two circular chromosomes of 3,288,558 bp and 1,877,211 bp in size, containing a total of 4832 coding sequences. The presence of

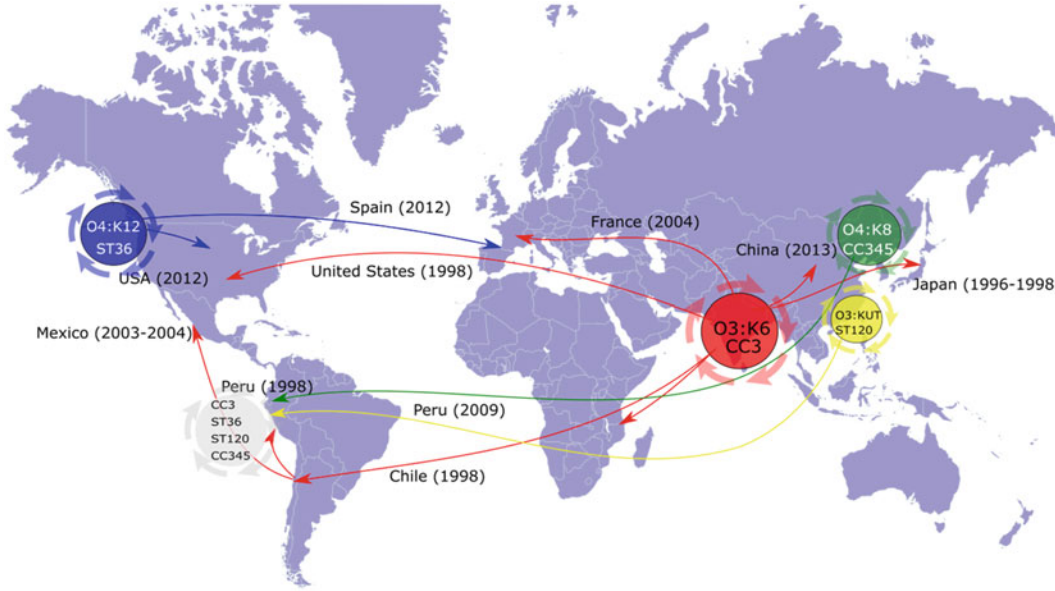



**Fig. 12.1** Dispersion of the most important serotypes of *V. parahaemolyticus* around the world. The circles indicate the first description of relevant serotypes, while the lines indicate the arrival of the countries and the year of the principal outbreaks. Pandemic clone O3:K6 (red) has the biggest distribution, while O4:K12 (blue) has caused the most recent outbreaks

two chromosomes in *V. parahaemolyticus* is common among members of *Vibrio* (Makino et al. 2003).

The complete sequence of the genome of *V. parahaemolyticus* revealed different aspects of the biology, and mainly of the genetic mechanisms that govern the pathogenesis that for several years was associated with the presence of two hemolysins, TDH and TRH, due to their epidemiological relationship with the strains of clinical origin. Analysis of the genome of strain RIMD2210633 showed that TDH genes flank the other genes encoding a type III secretion system (T3SS2), which together form part of a large pathogenicity island (VPAI-7) located on the second chromosome (Makino et al. 2003; Park et al. 2004b). This work showed that TDH was genetically linked to the presence of T3SS2, which explains its association with pathogenic strains, and its function as a marker of pathogenicity. More than mechanisms of pathogenicity *per se*, the presence of hemolysin genes was indicative of the presence of T3SS2, which was the true promoter of the bacteria's pathogenic capacity (Park et al. 2004b). Complete genome sequences of several *V. parahaemolyticus* strains are now available, revealing that pathogenicity systems in *V. parahaemolyticus* were much more complex than previously thought. Likewise, it was possible to demonstrate the presence of other homologous T3SS2 genes in strains of clinical origin with the presence of the TRH hemolysin, showing that hemolysins are genetically linked to these systems and that, in addition, the secretion systems are very widespread in populations of *V. parahaemolyticus* (Park et al. 2004b).

Clinical isolates of pandemic and non-pandemic *V. parahaemolyticus* showed the presence of other VPAIs. The first to be determined in common was VPAI-1, while the presence of VPAI-4, VPAI-5, and VPAI-6 was only found in pandemic strains (Hurley et al. 2006). Finally, it was determined that the isolates of the pandemic group carried the *tdh* gene, but not the *trh* gene, *orf8*, and seven VPAI, while the non-pandemic isolates are heterogeneous, but do

not have VPAI-7 (Chao et al. 2009). In addition, the O3:K6 pandemic strains could be detected with the *toxRS* sequence, which was useful in differentiating between pandemic and non-pandemic *V. parahaemolyticus* strains. The differences studied between the O3:K6 strains led to the definition of non-pandemic O3:K6 strains isolated in 1980–1990 in Asian countries such as India, Taiwan, Japan, Thailand, and Bangladesh (Ceccarelli et al. 2013).

Despite the information available at the genomic level of a few strains, little information is available on the genetic structure of *V. parahaemolyticus* isolated from both human infections and the environment. The study using Multilocus Sequence typing (MLST) has currently been revealed as a useful tool for the study of the characteristics of different pathogenic bacteria populations that allows studying the structure of a population in different habitats and, at the same time, establishing the genetic relationships and the interconnection between different world regions (Maiden 2006). To advance on this topic, an MLST scheme has been described, which has made it possible to determine the population characteristics of *V. parahaemolyticus* in the USA. This study analyzed the partial sequences of 7 housekeeping genes located on both chromosomes of *V. parahaemolyticus* corresponding to *recA, dnaE, gyrB, dtdS, pntA, pyrC*, and *tnaA*. During this study, clinical and environmental strains of different origins were investigated, focusing mainly on strains from the USA. The data obtained showed a great genetic diversity among the strains studied, and a high rate of homologous recombination was also observed. The presence of 3 clonal groups was described, one of them belonging to the pandemic clone, which highlights the semi-clonal structure in the group of strains studied (Gonzalez-Escalona et al. 2008).

Studies using whole genome sequencing revealed that *V. parahaemolyticus* undergoes high rates of homologous recombination as well as other members of the same Genus. In addition, evidence has been obtained that *V. parahaemolyticus* divides into several populations, whose members are not necessarily related at the clonal level. Despite this, there is a possibility that the strains may have recombined their entire genomes since sharing a common ancestor, but on average, they are more similar to each other than to members of other populations because they have DNA acquired from a common gene pool (Cui et al. 2015).

In a recent study, 1103 genomes of clinical and environmental strains were used to determine the global population structure of the species, finding four populations with different but overlapping modern geographic distributions: Asian population, US population, and two hybrid populations. Under the assumption that genetic exchange between strains is limited by geography, the current degree of overlap is too high to maintain populations as distinct entities, concluding that most of this recombination has taken place in recent decades, which would coincide with the recent emergence of pandemic clones (Yang et al. 2019a, b). Figure 12.2 is an updated representation of the phylogenetic relationships of *V. parahaemolyticus* globally, including a total of 1281 genomes. The high degree of recombination within the species is reflected by the high rate of nucleotide substitution between the different sequence types (STs) detected, in the form of long branches. Regardless of this, a high similarity between the strains belonging to a single clonal complex (CC); aspects that corroborate what was mentioned by Yang et al. (2019a). It is noteworthy the majority presence of genomes corresponding to the clones that have caused epidemics in recent years: CC-3 and CC-345, reported first by the end of the previous century; CC-36 and CC-120, detected in the recent century.

## 12.7 Genomic Island of *Vibrio parahaemolyticus*

Genomic islands are a group of chromosomal regions acquired by horizontal gene transfer, which can increase the fitness of the bacterium in a particular environment. For example, virulence genes present on islands of pathogenicity or

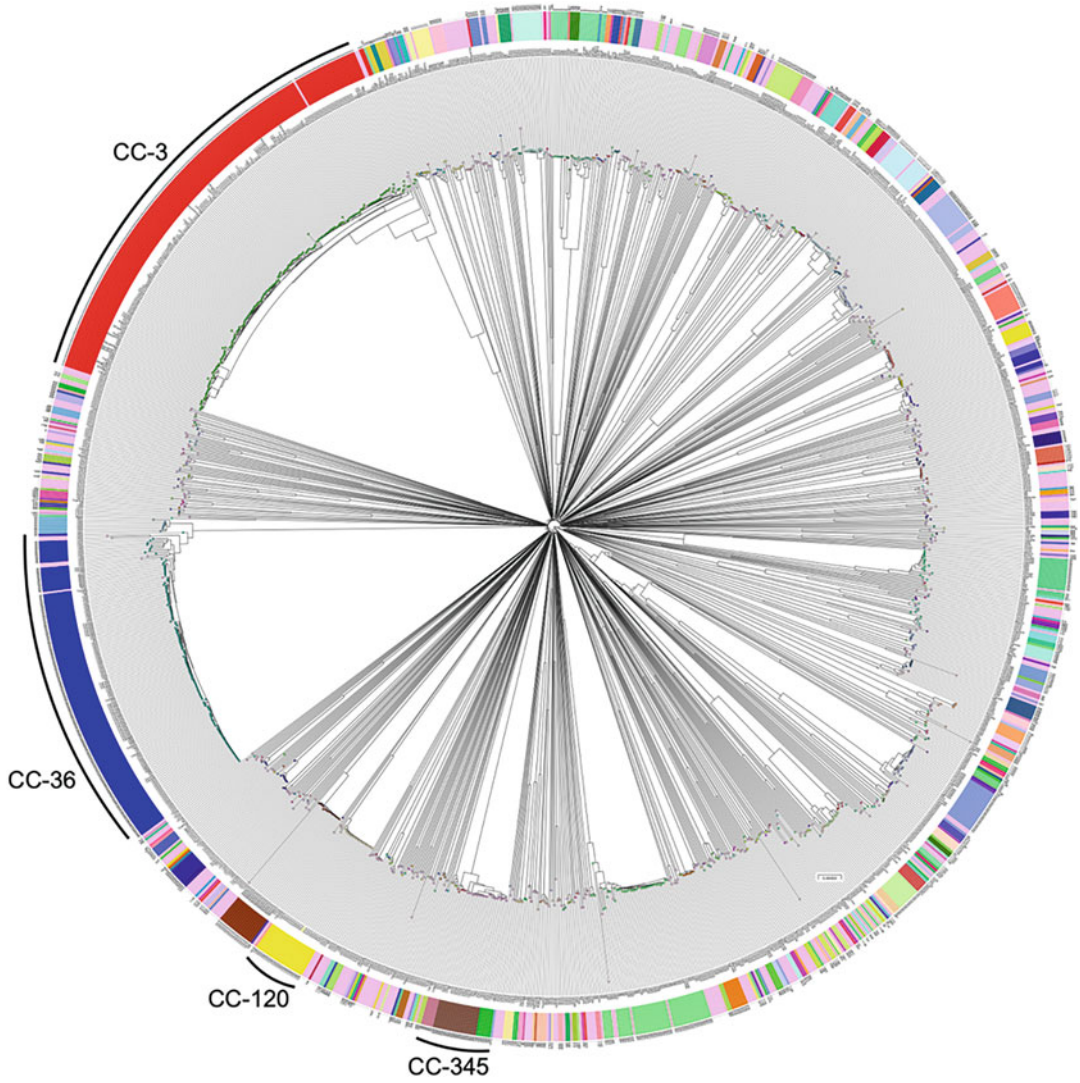



**Fig. 12.2** Global phylogenomic of *Vibrio parahaemolyticus*. Phylogenetic relationship of 1281 *V. parahaemolyticus* showing the main genotypes related to outbreaks and their clonal nature of diversification

genes that provide various metabolic capabilities on metabolic islands may play important roles in bacterial survival in various environments. The DNA sequences of these islands have aberrant characteristics of G + C content, dinucleotide frequency, and codon usage pattern different from those of the microbial genome. In addition to them, they have the characteristics to encode a bacteriophage-like integrase, flanked by repeat sequences, which are inserted adjacent to tRNA genes, probably indicating a similar mechanism of chromosomal integration (Dobrindt et al. 2004).

Not all *V. parahaemolyticus* strains have genomic islands, but are restricted to subpopulations. For example, the genome of *V. parahaemolyticus* strain RIMD2210633, belonging to the pandemic clone, has seven pathogenicity islands (VPaI-1 to VPaI-7). The 7 genomic islands, range in size from 10 to 81 kb, are flanked by direct repeats,

**Table 12.2** Genomic islands identified in the genome sequence of *V. parahaemolyticus* RIMD2210633 (Makino et al. 2003)

| Genomic island | Genomic position (bp) (ORFs) | Size (kb) | Integrase/transposase | %GC |
|---|---|---|---|---|
| VPaI-1 | 381054–403433 (VP0380–VP0403) | 24 | Integrase | 42 |
| VPaI-2 | 6660707–674355 (VP0635–VP0643) | 10 | Integrase | 45 |
| VPaI-3 | 1121252–1152668 (VP1071–VP1094) | 32 | Integrase | 42 |
| VPaI-4 | 2240007–2256166 (VP2131–VP2144) | 17 | Integrase | 39 |
| VPaI-5 | 3084846–3099979 (VP2900–VP2910) | 12 | Integrase | 38 |
| VPaI-6 | 1325821–1352643 (VPA1253–VPA1270) | 27 | Integrase | 43 |
| VPaI-7 | 1390967–1501509 (VPA1312–VPA1398) | 81 | Transposase | 39 |

and 6 of the 7 have lower G + C content (between 38% and 43%) than the 45% G + C content of *V. parahaemolyticus* genome. All VpaI encoded an integrase gene, with the exception of VPaI-7, which contains several transposase genes. In addition, VpaI-7 has two copies of the *tdh* gene. Five of these islands are located on the first chromosome, and two on the second chromosome (Makino et al. 2003, Table 12.2). On the other hand, the pre-pandemic strain AQ4037 has a pathogenicity island (trh-PAI), homologous to VpaI-7 on the second chromosome, with the main difference being the presence of urease genes and the one encoding *trh* gene. Furthermore, it is similar to the island found in *V. parahaemolyticus* TH3996. Interestingly, trh-PAI was found on the second chromosome of strain TH3996, but localized inside the first chromosome of strain AQ4037. This discrepancy in chromosomal location may provide a clue to the mobility of the pathogenicity island (Chen et al. 2011). Having some differences, the most relevant STs of *V. parahaemolyticus* possess VPaI-7 of its variants (like trh-PAI inside ST-36), but the other ones are exclusive to ST-3 (Fig. 12.3 and Table 12.2).

Additionally, several authors using comparative genomics have described the presence of other genomic regions that have probably been acquired by horizontal gene transfer (Hurley et al. 2006; Gavilan et al. 2013).

## 12.8 Molecular Detection of *V. parahaemolyticus*

Phenotyping and serotyping of *V. parahaemolyticus* from seafood and marine products are usually complex (Nishibuchi 2006). Due to this situation, PCR has become the most convenient technique for identification and detection of these bacteria (Drake et al. 2007). However, the variable genomic composition of *Vibrio* species makes the selection of targets the main point to characterize *V. parahaemolyticus* correctly, with high sensitivity and specificity (Nelapati et al. 2012). The PCR method to identify *V. parahaemolyticus* at the species level includes the target *toxR* gene, a gene related to the expression of *tdh* gene, being presented in both pathogenic and nonpathogenic *V. parahaemolyticus* strains (Sujeewa et al. 2009). Another alternative includes thermolabile hemolysin (*tlh*) to detect simultaneous virulent and non-virulent *V. parahaemolyticus*, being a reliable marker for the bacteria (Su and Liu 2007).

Conventional PCR methods for the detection of pathogenic *V. parahaemolyticus* include multiple protocols for the amplification of *tlh, tdh*, and *trh*, having high sensibility (Wei et al. 2014). In addition, real-time PCR has been used to detect pathogenic and nonpathogenic *V. parahaemolyticus*, having the ability to process a huge number of samples with speed and consistency in a single tube amplification targeting the genes (McKillip and Drake 2000). For example, Ward and Bej (2006) developed a multiplex real-time PCR assay that targeted four different genes, *tdh, trh*, and *orf8* for the detection of pathogenic *V. parahaemolyticus* (O3:K6 serotype), and *tlh* gene for the detection of total *V. parahaemolyticus*.

The modern methods also include loop mediated isothermal amplification (LAMP) based assays as an alternative to PCR, with the advantage of not using a thermal cycler (Notomi

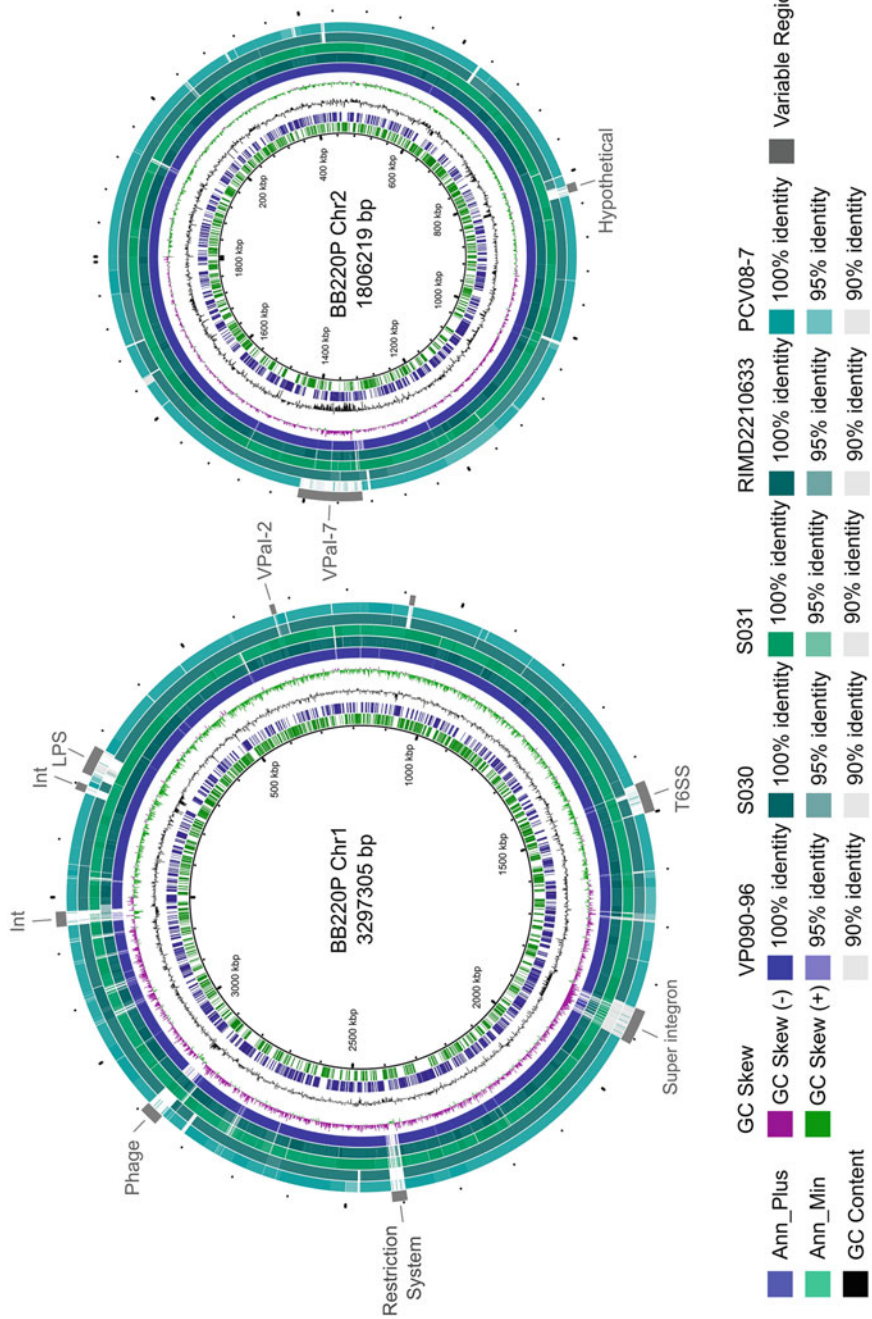



**Fig. 12.3** Genomic differences between the most important STs of *V. parahaemolyticus*. Excluding ST-36, VPaI-7 was detected in almost all STs, while pathogenic regions like VPaI-2, T6SS and several integrons are only found inside ST-3

et al. 2000). For example, a *toxR*-based LAMP assay was developed in 2010 (Chen and Ge 2010). Then, Zhou et al. (2016) extended the LAMP assay for *V. parahaemolyticus* identification into field-based detection. Besides, Wang et al. (2016) optimized LAMP by developing a multiple endonuclease restriction method. Finally, in recent years, a rapid detection of early-stage *V. parahaemolyticus* infection in humans using LAMP was developed (Li et al. 2020).

There are other methods that are also valid and are applied in several studies of *V. parahaemolyticus*, like the random amplified polymorphic DNA–PCR (RAPD-PCR) (Oakey et al. 1998) and RS-PCR, REP-PCR, and ERIC-PCR developed by Wong and Lin (2001). Another approach of detecting *V. parahaemolyticus* is through fluorescence in situ hybridization, developed by Sawabe et al. (2009), with the inconvenience of only detecting total *V. parahaemolyticus*.

## 12.9 Molecular Epidemiology of *V. parahaemolyticus*

Molecular epidemiology has become one of the newest methods in infectious pathogen research because it helps us identify patterns of disease distribution within microbial populations. Sequencing and bioinformatic analysis allow obtaining genomic assemblies for the application of techniques such as Multilocus Sequence Typing—MLST (Urwin and Maiden 2003). Genetic variants obtained by MLST identified as prevalent in different regions of the world can be mapped to determine connections between microbial populations from different geographic areas and delineate potential pathways for dispersal of pathogens (Gonzalez-Escalona et al. 2017).

The MLST scheme for *V. parahaemolyticus* and a centralized database (http://pubmlst.org/vparahaemolyticus) was established in 2008. This database has enabled researchers from around the world to compare their strains. Currently, more than 4000 strains from diverse regions of the world, belonging to more than 2500 Sequence Types (STs), are available for analyses. Genetic variants identified as prevalent in the different regions of the world can be mapped to identify potential connections between populations from diverse geographical areas, and delineate potential routes of dispersion. The MLST scheme described in the database for *V. parahaemolyticus* included the genes: *dnaE* (DNA polymerase III, α-subunit), *gyrB* (DNA gyrase, B-subunit), *recA* (recombinase A), *dtdS* (threonine 3-dehydrogenase), *pntA* (transhydrogenase, α-subunit), *pyrC* (dihydroorotase), and *tnaA* (tryptophanase).

The most important pathogenic variant of *V. parahaemolyticus* is ST-3. The history of this genotype began with its serological typing as O3:K6, widely distributed worldwide since 1996, receiving the name of the first pandemic clone of *V. parahaemolyticus*. With the arrival of the MLST, investigators determined that O3:K6 was comprised of several highly related genotypes, forming what is known as the CC-3 pandemic clone, which comprised four different genotypes (ST-3, ST -42, ST-27, and ST-51), finding ST-3 as the ancestral or founder type of this clonal complex using eBURST, unequivocally establishing the clonal relationship of the pandemic complex (Gonzalez-Escalona et al. 2008). Several years after that, non-pandemic strains of *V. parahaemolyticus* have been included within the same clonal complex, expanding the number of genotypes in this group, reaching to the date of this work the number of 68 genotypes, being the biggest clonal complex. CC-3 has a lot of available information from more than 20 countries deposited in the pubMLST database and various publications that indicate its continuous expansion (Chen et al. 2016). Furthermore, the clonal relationship between strains of different geographical origins is not in doubt, proven by the high degree of similarity between their genomes confirmed by techniques such as pulsed field gel electrophoresis (Wong et al. 2000), direct enzyme analysis genome restriction (Fuenzalida et al. 2007), arbitrarily primed PCR (Matsumoto et al. 2000), and MLST (Chowdhury et al. 2000).

A new pandemic clone of *V. parahaemolyticus*, corresponding to O4:K12,

is gaining importance in the last ten years, having a continuous expansion, since its initial detection in the Pacific Northwest region of the USA and Canada. This variant, ST-36, is currently expanding throughout the Atlantic until reaching Europe, causing several epidemics, especially in the east coast of Spain (Paranjpye et al. 2012). Investigators have more information about this clone, detecting two different populations: the Spanish population which presents low diversity of subpopulations and conserves the ancestral trait of a large genome and a greater number of genes, while the North America population presents a reduced genome size and number of genes, but a greater diversity of subpopulations compared to the Spanish strains (Martinez-Urtaza et al. 2017).

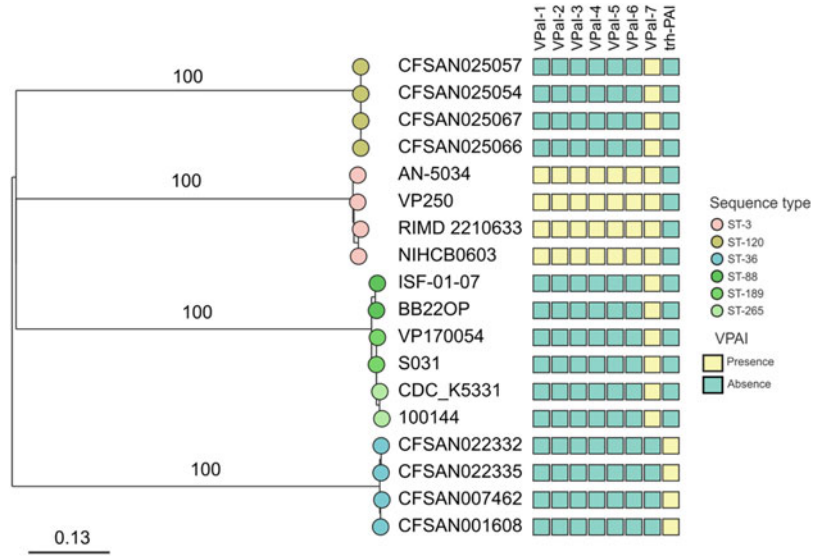
The O4:K8 is another interesting case inside molecular epidemiology of *V. parahaemolyticus*, because it contains several STs, that compound a big Clonal Complex (CC-345), which cause epidemics in countries like Bangladesh, Japan, and Peru before the arrival of ST-3 and ST-36, which is called the pre-pandemic period (Jensen et al. 2013; Gavilan et al. 2013). However, during the pandemic period, this Clonal Complex evolved to persist in the environment and cause other outbreaks like those reported between 2008 and 2014, especially in China (Ma et al. 2014; Li et al. 2015). The MLST has made it possible to determine that multiple STs correspond to the O4:K8 serotype, forming CC-345, whose most prominent members include ST-88, ST-189, and ST-265, having evidence that ST-88 is the precursor of the ST-189, and the latter in turn led to the ST-265. Finally, with the help of whole genome sequencing in the last decade, a genetic insertion of approximately 30 Kbp was detected in strains of Peruvian and Chinese origin belonging to the O4:K8 serotype, affecting the integrity of the *recA* gene (González-Escalona et al. 2015). On the other hand, sequencing has also made it possible to perform comparative genomics of O4:K8 with the pandemic clone O3:K6, showing that they share the regions of the T3SS, important virulent elements present within the pandemic serotype (Li et al. 2017).

The three previous serotypes and Clonal Complexes represent the main causes of *V. parahaemolyticus* outbreaks around the world; however, unusual STs sometimes can be involved in outbreaks that are only detected in a unique country or region. For example, in the summer of 2009, strains of serotype O3:K59 that presented the same genetic profile were related to an outbreak in several regions of Peru. Surprisingly, the strains of this outbreak had no genetic connection with strains recovered from previous years, forming a unique genetic group that was totally different from the other groups detected in the clinical cases of *V. parahaemolyticus* in Peru (Zamudio et al. 2011). Sequencing only corroborated the connections of all those strains, belonging to the ST-120, which were only reported in China (Gonzalez-Escalona et al. 2016).

A phylogenetic tree of the most important STs using referential genomes revealed big genetic distances between them, which is an indicative of their high recombination rates (Gavilan et al. 2013). ST-3, ST-120, and CC-345 are connected by the presence of the VPaI-7 almost identical between them, while ST-36 possesses trh-PAI, a variation of VPaI-7. Also, only ST-3 has the genes from the other genetic islands (Fig. 12.4). Another type of comparison using pangenome analysis revealed that the principal differences between strains are in fact virulence factors like genomic islands and other genes which helps strains to persist in the environment or their host, like TDH, TRH, *recA*, or carbohydrate metabolism gene clusters (Fig. 12.5).

## 12.10 Conclusion

*V. parahaemolyticus* is an important foodborne pathogen that causes several outbreaks since the appearance of CC3 pandemic strains and the emergence of other pandemic genotypes as ST36. However, there are many clues about *V. parahaemolyticus* that its necessary to unravel about the pathogenesis, ecology, and evolutionary history. Also, strengthening genomic epidemiology surveillance of microorganisms like



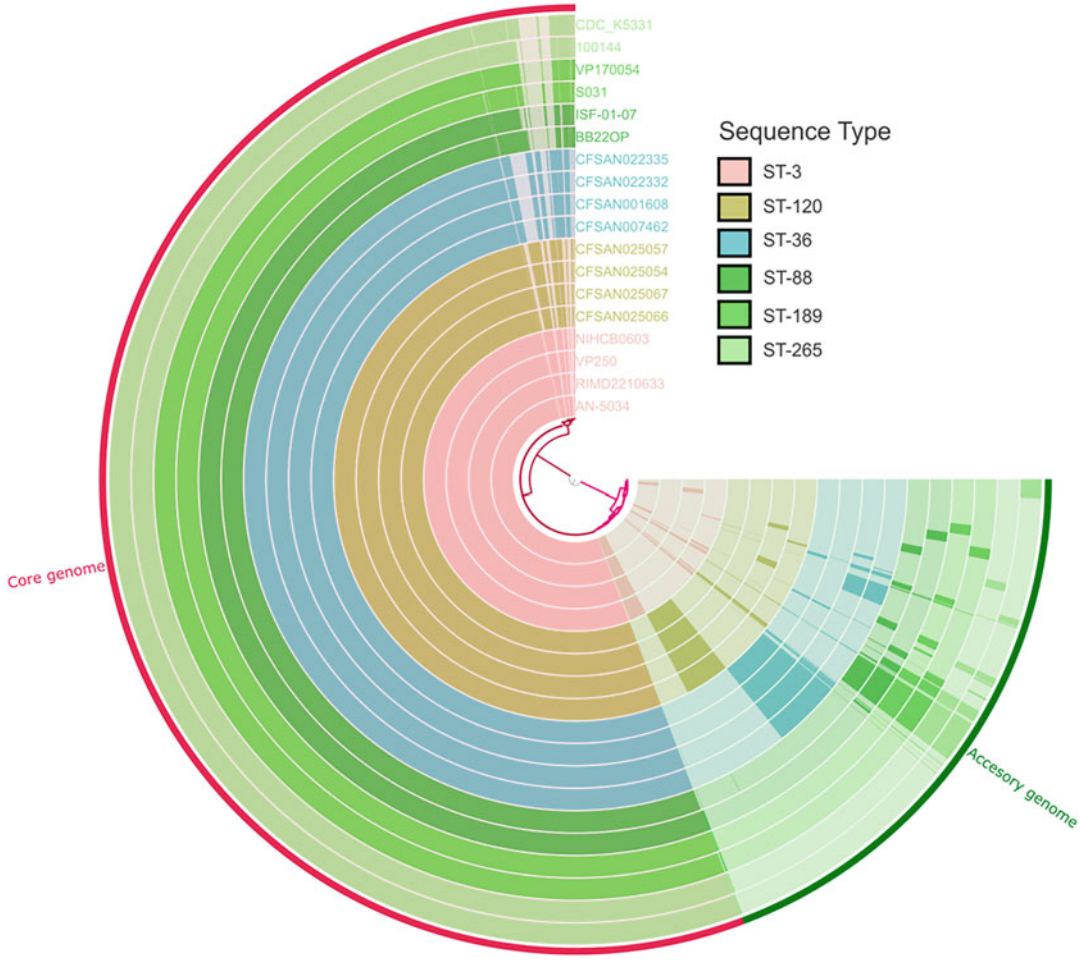




**Fig. 12.4** Phylogenomic relationship between *V. parahaemolyticus* genotypes related to outbreaks, using closed reference genomes. Information about detected pathogenic islands is located as blocks next to the strain names. Note the high homology between the most important virulence factors detected for each group

**Fig. 12.5** Pangenome analysis of *V. parahaemolyticus* related to outbreaks. Genetic differences between groups are mostly defined by the presence of pathogenic islands and other virulence factors like integrons, prophages, T6SS, and other non-classified proteins

*V. parahaemolyticus* will contribute to the timely detection and control of foodborne outbreaks.

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**Conflicts of Interest** None.

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# 13 The Viable but Non-Culturable (VBNC) State in *Vibrio* Species: Why Studying the VBNC State Now Is More Exciting than Ever

Sariqa Wagley

**Abstract**

During periods that are not conducive for growth or when facing stressful conditions, Vibrios enter a dormant state called the Viable But Non-Culturable (VBNC) state. In this chapter, I will analyse the role of the VBNC state in *Vibrio species* survival and pathogenesis and the molecular mechanisms regulating this complex phenomenon. I will emphasise some of the novel findings that make studying the VBNC state now more exciting than ever and its significance in the epidemiology of these pathogens and critical role in food safety.

**Keywords**

Vibrio · Dormancy · Viable but non-culturable · VBNC · Environmental survival

## 13.1 Introduction

The bacterial life cycle consists of 4 phases, the lag phase, the log phase (also known as the exponential phase), the stationary phase, and finally the death phase. The **lag phase** is where bacteria do not grow but begin to adjust to their environment; they will produce amino acids and vitamins needed to support their growth. The length of this phase can depend on what nutrients are available in the environment. The **log phase** is the period of the life cycle where bacteria are growing and dividing under their ideal environmental conditions. For most *Vibrio* species, these conditions are a warm temperature (>15 °C) and a minimal amount of salt (sodium ions) is needed to grow, and the rate of growth is enhanced by increases in salt concentration. Under these conditions, bacteria can divide exponentially. Bacteria such as *E. coli* have a doubling time of 20 mins but for *Vibrio* species, this is much faster and a doubling time of 12–14 min for *V. parahaemolyticus* has been recorded under ideal growth conditions (Dryselius et al. 2008; Ulitzur 1974). As bacteria divide in ideal growth environments; nutrients, vitamins, oxygen, and space become limiting factors and waste from bacterial growth begins to accumulate. The bacteria are now in **stationary phase** and growth starts to decline, as cells are unable to maintain their exponential division. If a new environment, fresh media, or a new host are found, bacteria can continue to rapidly grow. However, if the dwindling environment continues, the cells enter a period of stress, they begin to shut down, metabolic activity begins to reduce, cell division ceases, and many cells in stationary phase enter the **death phase**. The decline of cells in the death

S. Wagley (✉)
Biosciences, Faculty of Health and Life Sciences, University of Exeter, Exeter, Devon, UK
e-mail: S.Wagley@exeter.ac.uk



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phase can be as rapid as their growth seen in the log phase. Although many of the cells in a population will die during the death phase, a small proportion will remain alive but unable to grow. These cells are now in a **viable but non-culturable phase** a process that allows cells to save themselves from demise by entering a state of hibernation while their environmental conditions continue to be unfavourable.

Whether the viable but non-culturable state is accepted as a 'fifth state' in the bacteria life cycle remains to be seen. In this chapter, I will present why *Vibrio* species are an excellent model to study the viable but non-culturable phase, I will draw on the latest research in the field, the general controversies surrounding the viable but non-culturable state, and what next needs to be done to move this field forward. There are already several key reviews written on this subject, please see the works of Li et al (Li et al. 2014), Zhao et al (Zhao et al. 2017), Ramamurthy et al (Ramamurthy et al. 2014), Ayrapetyan and Oliver (2016), and Pinto et al (2015). The importance of the viable but non-culturable state in bacterial biology cannot be understated. If a small proportion of cells can save themselves from death, this is the ultimate way for a species to continue its existence. I hope to convince the reader that this is an area worth researching still.

## 13.2 The Significance of Viable but Non-culturable (VBNC) Cells

Bacterial cells that enter the viable but non-culturable (VBNC) state are dormant bacterial cells, which means they will resuscitate into an active population at some point in time and space. VBNC cells are dormant bacterial cells that are hard to detect in an environment because they change their morphology and structure compared to healthy cells. They have low metabolic activity and are unculturable on media on which they may normally grow, but can retain the ability to infect a host. Crucially, the VBNC state is reversible and dormant cells can reactivate to become culturable and cause disease once more. Below, I describe four reasons why the VBNC state is significant but this is not an exhaustive list.

### 13.2.1 Biology and Species Survival

Next generation sequencing reveals a wide range of bacterial taxa present in a marine ecosystem but only some of them will grow and establish into a population at one particular point in space and time, the majority of this diversity constitutes a huge seed-bank sitting dormant ready and waiting. Understanding what governs how bacterial species wake up and grow in the natural environment after a period of long-term senescence, is important to uncover the processes that determine ecosystem function and how biodiversity is structured. The VBNC state is fundamentally an important strategy for coping with long-term nutrient restriction in the natural environment until favourable conditions arise.

### 13.2.2 Environmental Reservoirs

*Vibrio* species are able to bioaccumulate and persist in nearshore ecosystems under a variety of conditions as part of a heterogeneous community within filter feeding shellfish. Filter feeding shellfish (e.g. oysters and mussels) and crustaceans (e.g. crabs and prawns) can take up microbes including pathogenic *Vibrio* species, which then accumulate in their tissues. *Vibrio species* are abundant in shellfish when sea temperatures exceed 15–18 °C, which coincides with elevated disease burden. In temperate regions, during the summer months and after heavy rainfall events where the salinity in the water is reduced, cases of *Vibrio* related infection have increased (Baker-Austin et al. 2013). When ideal growth conditions cease, *Vibrio species* cannot be detected in shellfish or water samples and it is thought that *Vibrio* species do not survive cold temperatures. **So is there evidence for environmental reservoirs of *Vibrio* species during non-permissible growth**

**conditions such as those seen in the winter months?** Studies have shown that clonal strains of *Vibrio* species appear seasonally indicating a senescent state for these bacteria (Wagley et al. 2009; Alam et al. 2011; Rashed et al. 2014). Secondly, during ad hoc testing of UK shellfish samples, researchers have shown that between 2001 and 2006, fifteen percent of samples were positive for *V. parahaemolyticus* only after an enrichment step was performed that allowed stressed or VBNC cells to be resuscitated (Wagley et al. 2008). *V. parahaemolyticus* is present at low or undetectable levels, using classical techniques, in environmental samples taken during the winter months (Coutard et al. 2007). A study showed that oysters harvested during the winter months contained *Vibrio* VBNC cells (Froelich and Noble 2014). In the Bay of Bengal, cholera endemics occur in two seasonal peaks, although little information is known about the reservoir of *V. cholerae* O1 between these peaks. There is evidence that non-culturable *V. cholerae* O1 cells are present between epidemics in samples collected from bodies of water that serve as a drinking water source in Bangladesh (Alam et al. 2007) indicating that the VBNC state plays a crucial role in the life cycle of *V. cholerae* in the environment. Dormant cells of *V. cholerae* cells are embedded in a biofilm matrix that can be recovered using enrichment culture techniques and regulation of these cells has been linked to quorum sensing (Kamruzzaman et al. 2010; Bari et al. 2013). Nevertheless, to date, no one has been able to isolate them from their unculturable form in the environment and there is a knowledge gap in information about what processes lead to their resuscitation.

### 13.2.3 Microbiological Testing

VBNC cells are unable to form colonies on standard culture media, which results in them evading standard testing methods. Testing of food and clinical samples typically involves measuring culturable bacteria that are actively growing and dividing. Consequently, dormant cells evade conventional tests, making it difficult to undertake epidemiological surveys or to diagnose disease from samples.

*Vibrio* VBNC cells in foodstuffs are not identifiable using the current culture methods used in testing laboratories. Efforts to use temperature and salinity models alone to predict the occurrence of *Vibrio* species in the ecosystem have not always been reliable indicators of *Vibrio* emergence. In some disease outbreaks, *Vibrio* species have appeared in shellfish where waters were less than $<18$ °C, indicating that simplistic environmental conditions and culture based techniques alone are not reliable indicators of disease prediction (McLaughlin et al. 2005).

### 13.2.4 Medical Context

The VBNC state has been reported to be responsible for the **latent**/dormant phase of *Mycobacterium tuberculosis* infections that can reactivate to cause recurrent infections (Young et al. 2009; Shleeva et al. 2004). With respect to *Vibrio* species, it was shown that *V. parahaemolyticus* VBNC cells can be converted to the culturable form when co-cultured with eukaryotic cells such as HT-29 or Caco-2 cells, indicating the potential for in vivo resuscitation (Senoh et al. 2010). *Vibrio* species can attach to surfaces and develop **biofilms** anchored to surfaces such as that of the human intestinal mucosa or to the chitinous exoskeleton of crustaceans (Silva and Benitez 2016; Vezzulli et al. 2015; Zhang et al. 2021; Lucero-Mejia et al. 2020; Yildiz and Visick 2009). Cells within a biofilm are supported by the generation of an extracellular polymeric substance (EPS) matrix, they have reduced growth rates, and up and down regulation of specific genes (Moorthy and Watnick 2004, 2005). *V. cholerae* enters biofilms-like aggregates that are involved in pathogenesis and disease transmission (Silva and Benitez 2016). Differential gene expression in *V. parahaemolyticus* VBNC cells compared

with those in either exponential or stationary phase (Meng et al. 2015) revealed that genes involved in biofilm maintenance were upregulated at least four-fold during the VBNC state. *Vibrio* VBNC cells existing within biofilms have not been widely documented but the concern that these dormant cells can revert to a culturable state is a concern for public health (Silva and Benitez 2016; Fu et al. 2021; Wang et al. 2021). Finally, VBNC cells are non-dividing cells and thus **antibiotics** are redundant against combatting biofilm associated infections or persistent latent infections where cells are present in a VBNC state.

## 13.3 How Is the VBNC State Induced in Vibrio Species?

The VBNC state has been described in over 115 bacterial species including over 70 human pathogenic bacteria either naturally occurring in different environmental habitats or in experimental conditions (Li et al. 2014; Zhao et al. 2017; Oliver 2010). *Vibrio* VBNC cells can be generated in the natural environment by a variety of stress inducing conditions including starvation, non-optimal temperature/salinity/pH/oxygen concentrations, and exposure to visible light (Table 13.1) (Jiang and Chai 1996; Mizunoe et al. 2000; Wong and Wang 2004; Wagley et al. 2021; Orruno et al. 2021; Almagro-Moreno et al. 2015). As environmental conditions fluctuate, it is possible that bacterial cells can enter and exit the VBNC state in response to favourable and non-favourable growth conditions that are naturally occurring in the surrounding environment. Food decontamination and industrial processes such as chlorination, freezing, and UV treatment have been shown to induce the VBNC state in foodborne pathogens such as *Salmonella enterica, Listeria monocytogenes, Helicobacter pylori*, and *Escherichia coli O157:H7* (Giao et al. 2010; Guo et al. 2017; Highmore et al. 2018). For *V. parahaemolyticus* nutrient restriction and temperature are the common known inducers of the VBNC state and recently industrial freezing of seafood samples was also shown to harbour *V. parahaemolyticus* cells in the VBNC state (Wagley et al. 2021).

**Problems** Reports to induce the VBNC state in laboratory experimental conditions produce inconsistent results. The lag period, before all

**Table 13.1** *Vibrio* species can be induced and resuscitated from the VBNC state

| Species | VBNC state inducing factor | Resuscitation condition | Resuscitation window | References |
|---|---|---|---|---|
| *Vibrio alginolyticus* | Low temperature | Temperature upshift | 8 days | Du et al. (2007b) |
| *Vibrio cholerae* | Starvation and low temperature Alkaline pH | Human intestine, eukaryotic cell lines, rabbit intestine | 110 days | Colwell et al. (1985) Senoh et al. (2010), Colwell et al. (1996) Almagro-Moreno et al. (2015) |
| *Vibrio fluvialis* | Starvation | Rich medium | 6 years | Amel et al. (2008) |
| *Vibrio mimicus* | – | – | | Oliver (2010) |
| *Vibrio parahaemolyticus* | Starvation, low temperature, low salinity, freezing | Temperature upshift | 50 days | Wong and Wang (2004), Wagley et al. (2021), Wong et al. (2004), Bates and Oliver (2004) |
| *Vibrio vulnificus* | Starvation, low temperature | Rich medium, temperature upshift, mice, clams | 3 days | Oliver and Bockian (1995), Nilsson et al. (1991), Oliver et al. (1995), Biosca et al. (1996), Wong and Liu (2008) |
| *Vibrio harveyi* | Starvation, visible light | – | | Orruno et al. (2021) |

*V. parahaemolyticus* cells become VBNC in a microcosm, has previously been shown to differ depending on the conditions used to set it up. For *V. parahaemolyticus* experiments, the time taken before cells enter into the VBNC state has been reported to be 9–35 days (Coutard et al. 2007; Meng et al. 2015; Mizunoe et al. 2000; Wagley et al. 2021) while Wong et al. showed that environmental strains of *V. parahaemolyticus* took between 35 and 49 days to become VBNC (Wong et al. 2004). Jiang et al. found it took 50–80 days for all cells to become VBNC when high salt concentrations were used in the starvation media (Jiang and Chai 1996). In our experiments, when fresh cultures (<5 days from culturing from freezer stocks) were used to prepare microcosms, we found that it took *V. parahaemolyticus* RIMD2210633 cells approximately 30–35 days to become VBNC and this became consistent between experiment. When older cultures were used to establish the microcosms, the lag phase was less, taking 20 days for all of the population to become unculturable. Thus, the published inconsistencies of lag periods between different laboratory groups studying the VBNC state could be due to how labs maintain strains prior to setting up the VBNC microcosms. We also found that during wash steps, dislodging the cells carefully with a sterile loop rather than pipetting up and down produced less injured cells (determined by live dead staining, data not published) and consequentially allowed more cells to enter the VBNC state. Thus, establishing microcosms that give consistent results between labs is important. Downstream resuscitation experiments, omics studies, and examination of phenotypic properties all rely on the setting up of the microcosms and therefore scientists should ensure they establish and publish reproducible methods, to move this field forward. Several papers describe the methodology to induce the VBNC state for laboratory cultures but two in-depth descriptions are referenced here (Mizunoe et al. 2000; Wagley et al. 2021). Once the VBNC microcosms have been established to produce consistent and reliable results, only then can you begin to study the molecular processes that underpin this state.

## 13.4 Molecular Level

To date, our understanding of the genetic control of the VBNC state is limited to a handful of genes that play a role in VBNC formation in *Vibrio* species (Li et al. 2014; Almagro-Moreno et al. 2015). Many of these genes are involved in repressing the entry of bacteria into the VBNC state and maintaining culturability of the cells (Table 13.2 and Fig. 13.1). When studying these genes using gene deletion knockouts, the data shows that the knockouts actively promote the entry of bacterial cells into the VBNC state compared to wild type cells. Whether bacterial cells use a self-regulatory system involving a network of genes and regulators that control the timing of when cells enter into the VBNC state, which is dependent on environmental conditions, has yet to been shown.

**OxyR** is an important transcriptional regulatory protein involved in the oxidative stress response. Bacteria synthesise superoxide dismutase and catalase to help mop up reactive oxygen species such as hydrogen peroxide ($H_2O_2$) and cope with oxidative stress. In *V. vulnificus* the oxidative stress regulator OxyR was shown to regulate the activity of catalase, which was required to degrade hydrogen peroxide generated in response to cold shock (Kong et al. 2004). In *V. cholerae*, OxyR was critical for antioxidation defence and in the presence of this protein *V. cholerae* was capable of scavenging environmental reactive oxygen species to facilitate growth (Wang et al. 2012). Alkyl hydroperoxide reductase subunit C (**AhpC**) is responsible for the detoxification of reactive oxygen species that form in the bacterial cell or from the host that the bacterial cell is infecting. In *V. parahaemolyticus* the *ahpC2* gene was shown to lengthen the time taken to induce the VBNC state by exhibiting antioxidative activities against $H_2O_2$ and organic peroxide (Wang et al. 2013).

**Table 13.2** Molecular mechanisms involved in the VBNC state

| *Species* | VBNC inducing factor | Molecular mechanism | Affect | References |
|---|---|---|---|---|
| *V. cholerae* | Nutrient starvation, alkaline pH<br>Nutrient starvation, low temperature | Loss of *ToxR* either by proteolysis or genetically<br>Quorum sensing, *HapR, RpoN*<br>*RelA* and *rpoS* | Proteolysis of ToxR under nutrient limitation and alkaline pH allows entry into VBNC state<br>Prolongs culturability during VBNC transition.<br>Prolongs culturability during VBNC transition. Allows them to adapt to environmental stress. | Almagro-Moreno et al. (2015)<br>Wu et al. (2020)<br>Asakura et al. (2007), Gonzalez-Escalona et al. (2006) |
| *V. parahaemolyticus* | Nutrient starvation, neutral pH<br>Nutrient starvation, low temperature<br>Nutrient starvation, low temperature | Loss of *ToxR* either by proteolysis or genetically lactate dehydrogenase (*lldD*)<br>*AhpC* is responsible for the detoxification of reactive oxygen species | Proteolysis of ToxR under nutrient limitation and alkaline pH allows entry into VBNC state<br>Prolongs culturability during VBNC transition.<br>Prolongs culturability during VBNC transition | Almagro-Moreno et al. (2015)<br>Wagley et al. (2021)<br>Wang et al. (2013) |
| *E. coli,*<br>*V. parahaemolyticus* | | *rpoS* | Prolongs culturability during VBNC transition. Allows them to adapt to environmental stress. | Coutard et al. (2007), Boaretti et al. (2003), Bhagwat et al. (2006) |
| *V. vulnificus and*<br>*V. cholerae*<br>*V. vulnificus* | | *OxyR*—Transcriptional regulator<br>Glutathione S-transferase (GST)—cellular detoxification enzyme | Regulates super oxidase dismutase and catalase that break down reactive oxygen species to cope with oxidative stress<br>Prolongs culturability during VBNC transition.<br>Modulate oxidative stress | Kong et al. (2004), Wang et al. (2012)<br>Abe et al. (2007) |
| *V. alginolyticus* | Nutrient starvation, alkaline pH | Proteolysis of *ToxR* | Allows entry into VBNC state | Zhou et al. (2022) |
| *V. cholerae* | Resuscitation<br>Resuscitation | Auto-inducer AI2<br>Proteases (Proteinase K) | Contributes to resuscitation of dormant cells<br>Contributes to resuscitation of dormant cells | Naser et al. (2021)<br>Debnath and Miyoshi (2021) |

**Glutathione S-transferase (GST)** is a cellular detoxification enzyme and in *V. vulnificus* GST was shown to modulate oxidative stress when the VBNC state was induced. Exogenously adding glutathione allowed cells being induced into the VBNC state to maintain their culturability (Abe et al. 2007). **ToxR** is a virulence regulator that influences the expression of more than 150 genes in *V. cholerae* including those involved in cellular transport, energy metabolism, motility, and iron uptake. The loss of ToxR either genetically or through proteolysis, from other bacteria, allows cells of *V. cholerae* and *V. parahaemolyticus* to enter a VBNC state similar to that which occurs in the natural environment (Almagro-Moreno et al. 2015). This mechanism was also identified more recently in *V. alginolyticus* where the loss of ToxR again through proteolysis allowed entry into the VBNC state (Zhou et al. 2022). There is also evidence that *V. cholerae O1* strains use a

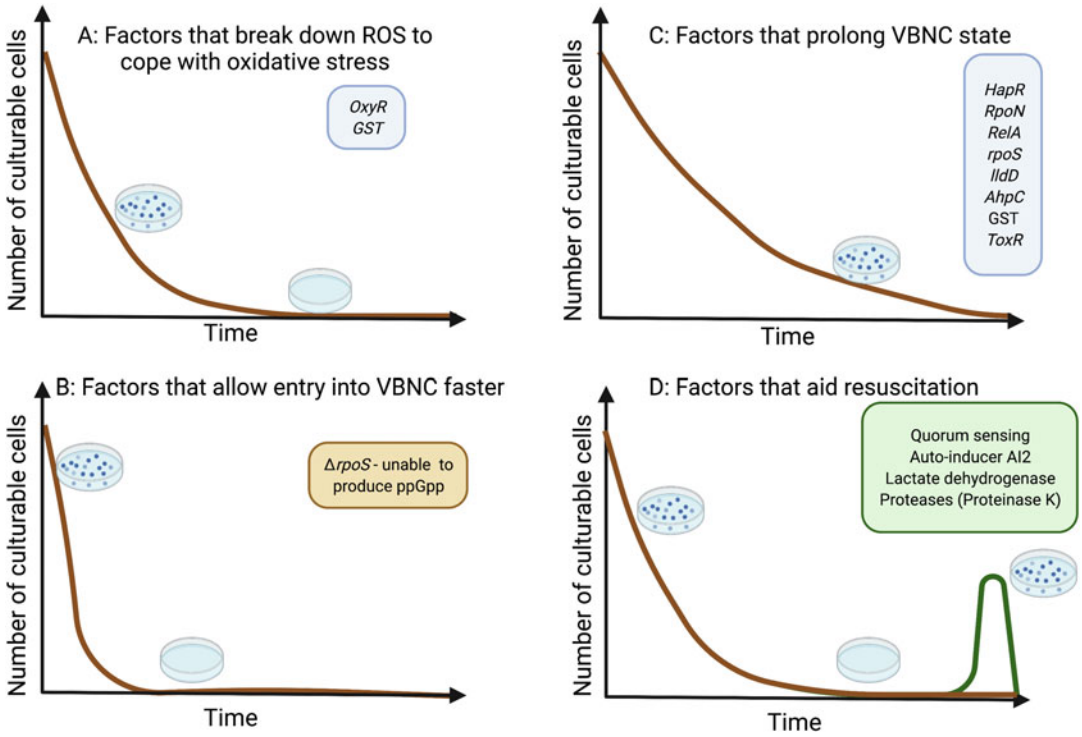


**Fig. 13.1** Molecular mechanisms that affect the VBNC induction and resuscitation: (**a**) shows factors that break-down reactive oxygen species to help mop up harmful free radicals that accumulate, (**b**) shows factors that allow cells entry into VBNC state faster, this currently only includes laboratory mutants that are unable to produce ppGpp a key signalling molecule, (**c**) shows factors that prolong the cells culturability for as long as possible, and (**d**) shows factors that aid resuscitation

quorum sensing system to prevent itself from entering the VBNC state under low nutrient and temperature conditions (Wu et al. 2020). **RpoS** (or sometimes referred to as $\sigma^S$ or $\sigma^{38}$) is a major stress regulator and is expressed to allow cells to survive under stationary phase in the bacterial cycle. In one study, RpoS was shown to mediate the expression of 10% of the *E. coli* genome (350 genes) when exposed to stress conditions and in *Pseudomonas aeruginosa* RpoS regulated 14% of the genome (772 genes) (Patten et al. 2004; Schuster et al. 2004). The production of guanosine 3′,5′-bispyrophosphate (ppGpp), which are small signalling molecules, are produced in response to various stress conditions (for the reviews, see (Potrykus and Cashel 2008; Magnusson et al. 2005; Dalebroux et al. 2010)) and activate *rpoS* expression. In Gammaproteobacteria, ppGpp levels are modulated by the protein enzymes RelA and SpoT. Mutants in *rpoS* are unable to produce ppGpp and this has resulted in cells entering the VBNC faster (Boaretti et al. 2003). Large transcriptomic studies of VBNC state in *V. cholerae* show that *relA* and *rpoS* were detected in cells entering the VBNC state indicating VBNC bacteria can adapt to their environment (Asakura et al. 2007; Gonzalez-Escalona et al. 2006). The *relA* and *spoT* and subsequent levels of ppGpp affect the 'stringent response' from bacterial cells where they reduce their growth and redirect their cells resources to promoting survival mechanisms in order to endure unfavourable conditions. The mediation of RpoS levels allows bacteria to survive under different environmental conditions, e.g. high osmotic pressures, changes to pH, nutrient starvation, and oxidation (Bhagwat et al. 2006). Studies showed that RpoS improves the

ability of bacterial cells to adapt to the environment and keeps cells that are undergoing stress in a culturable state. *E. coli* mutants that lacked *rpoS* produce little or no ppGpp and entered the VBNC state faster than wild type strains and showed a decreased ability of *E. coli* to remain in the VBNC state (Boaretti et al. 2003). In *V. parahaemolyticus* repression of *rpoS* expression was observed when cells could no longer be resuscitated at 37 °C (Coutard et al. 2007) and similarly in *E. coli rpoS* mutants lost culturability and died earlier. Therefore, RpoS must be tightly regulated in bacterial cells undergoing stress and the production of this gene must be vital for sustaining cells from entering the VBNC state.

**RpoN** activates the transcription of small regulatory RNAs and this in turn regulates the production of **HapR** and AphA which are master regulators of the quorum sensing pathway (Lilley and Bassler 2000). Expression of HapR causes *V. cholerae* to resist entering the VBNC state, which allows them to become the dominant strain in a population as the environment changes. Maintaining culturability under unfavourable conditions is a strategy that *V. cholerae* can use as its environment adversely changes. Another study showed that the enzyme **lactate dehydrogenase** (lldD) involved in respiration in *V. parahaemolyticus* prolonged the time needed for cells to enter the VBNC state ensuring they stay culturable for as long as possible (Wagley et al. 2021). Adding lactate exogenously to *V. parahaemolyticus* VBNC cells aided their resuscitation even after being dormant and unculturable for over 35 days (Wagley et al. 2021).

Other notable mentions have used mRNA (a good indicator of cellular function and viability) using RT-PCR to detect the transcript of particular genes in the VBNC state indicating that VBNC cells continue gene expression. Saux et al found that the virulence gene *vvhA* was expressed in *V. vulnificus* VBNC cells after 4.5 months of unculturability when the samples were concentrated indicating low levels of expression were 'ticking over' in VBNC cells (Fischer-Le Saux et al. 2002). The production of vvhA haemolysin in *V. vulnificus* is affected by temperature and salinity (Bang et al. 1999) and repressed by glucose (Lee et al. 2000) and controlled by the regulatory transmembrane transcription activator ToxRS (Lee et al. 2000). Vora et al used a microarray system to look at the expression of genes of *V. cholerae, V. parahaemolyticus*, and *V. vulnificus* in the VBNC state and found a number of virulence toxins (*ctxAB, rtxA, hlyA, tl, tdh, vvhA*) and virulence genes (tcpA, TTSS) continue expression when in the VBNC state (Vora et al. 2005). Conversely, Smith and Oliver showed that the expression of *vvhA* stopped being expressed as *V. vulnificus* cells entered the VBNC state and instead highlighted the detection of the major the regulator *rpoS* in VBNC cells (Smith and Oliver 2006).

## 13.5 Resuscitation Window

The **resuscitation window** is the period of time that *Vibrio* VBNC cells can be converted to a culturable form again. Many resuscitation experiments in VBNC studies are set up in bacterial microcosms under nutrient depravation and exposure to low temperatures. Evaluating this resuscitation period is difficult for two main reasons. Firstly, it is important that resuscitation experiments can distinguish between the resuscitation of unculturable and dormant cells (VBNC) and not the regrowth of one or more culturable cells that are undetectable cells. Secondly, the resuscitation of VBNC cells is usually determined after the whole population becomes unculturable and this lag period is inconsistent between studies as explained above. This inconsistent lag period among *Vibrio* VBNC cells and studies makes it difficult to interpret what the resuscitation window is for *Vibrio* VBNC cells. For *Vibrio* species the resuscitation window has been defined to be a long as 6 years for *V. fluvialis* (Amel et al. 2008), 110 days in *V. cholerae* (Senoh et al. 2010), and as little as 3 days for *V. vulnificus* (Oliver and Bockian 1995). Previous studies have shown that the resuscitation window for *V. parahaemolyticus* was generally in the region of 2 weeks (Wong and Wang 2004; Wong et al. 2004; Bates and Oliver

2004). Thirdly, the conditions or methods used to resuscitate the cells can differ from study to study and this can lead to differing resuscitation windows for the same species. If conditions that induce the VBNC state such as antibiotic pressure, high/low temperature, nutrient restriction, changes in pH or oxygen stress are removed, then cells in the dormant state of VBNC can begin to eek themselves out of dormancy. Depending on what conditions are used to induce the VBNC state may also affect the resuscitation window. In our studies, we found that the general resuscitation window *for V. parahaemolyticus* was 2 weeks which was similar to previous studies by others, i.e. by taking a portion of the microcosms and putting into a nutrient rich media and warming up the VBNC cells. However, we found that if we used flow cytometry methods to collect specific *V. parahaemolyticus* VBNC populations, cells could be resuscitated approximately 50 days after the microcosms turned unculturable extending the previously known resuscitation window of 2 weeks. These experiments are described in detail in the next section (Wagley et al. 2021).

## 13.6 Resuscitation Methods

*So what conditions or factors can help resuscitation of cells from the VBNC state*? Wong et al showed that induction and successful resuscitation of cells in and out of the VBNC state were strain dependent (Wong et al. 2004). The categories of the strains, i.e. whether they were clinical or environmental or a particular serotype did not appear to affect the induction or resuscitation period of the VBNC state (Wong et al. 2004). There is also evidence that incubating VBNC cells into rich media may be harmful to the cells. Incubating *V. vulnificus* VBNC cells into rich heart infusion medium at room temperature proved not to resuscitate the VBNC cells (Whitesides and Oliver 1997). Similarly, an introduction into a too high a temperature of VBNC cells may also prohibit resuscitation, where *V. parahaemolyticus* VBNC cells could only be resuscitated at 22 °C but not directly at higher temperatures of 37 °C (Wong et al. 2004). In our experiments, putting *V. parahaemolyticus* VBNC cells into rich media such as LB Broth or Marine Broth and at warm temperatures did not cause cells to become culturable (data not shown). Adding portions of the microcosm to phosphate buffered saline (PBS) (slightly higher in salt levels provides a salt gradient) and then incubating slowly over a temperature gradient (i.e. from room temperature to 37 °C over a 48 h period). *V. parahaemolyticus* cannot divide or grow in PBS and thus resuscitation of VBNC cells using PBS is real resuscitation and not just regrowth of a few culturable cells. These methods revealed that less than 1% of the cells in the microcosm went into the VBNC state.

Using flow cytometry *V. parahaemolyticus* VBNC cells could be separated into different subpopulations depending on size and shape (Fig. 13.2) (Wagley et al. 2021). One subpopulation (called P2) were phenotypically large coccoid in shape (6.3 μm/4.3 μm length/width), were hollow inside where cellular contents were pushed outwards to form protein blebs, and they had low levels of metabolic activity compared to the healthy *V. parahaemolyticus* cells (1.3 μm/1.0 μm length/width) (Fig. 13.2). When ~50,000 cells of this particular phenotype (P2 cells) were collected using flow cytometry and then resuscitated into PBS, all cells with this particular phenotype could be resuscitated. We observed this resuscitation was possible 50 days after the cells in the microcosm had turned unculturable. However, just taking a portion of the VBNC microcosms and placing it into PBS did not resuscitate cells suggesting resuscitation of VBNC cells was dependent on a critical mass of the P2 population of VBNC cells. This could only be achieved by sorting and collecting large numbers of VBNC cell populations using flow cytometry.

We also showed that the protein lactate dehydrogenase was abundant in *V. parahaemolyticus* VBNC cells and the addition of sodium lactate resuscitated cells regardless of any critical mass of a particular VBNC subpopulation. Thus, this led to us postulating where might *Vibrio* VBNC cells in the environment obtain a source of lactate that could support them in the VBNC state and

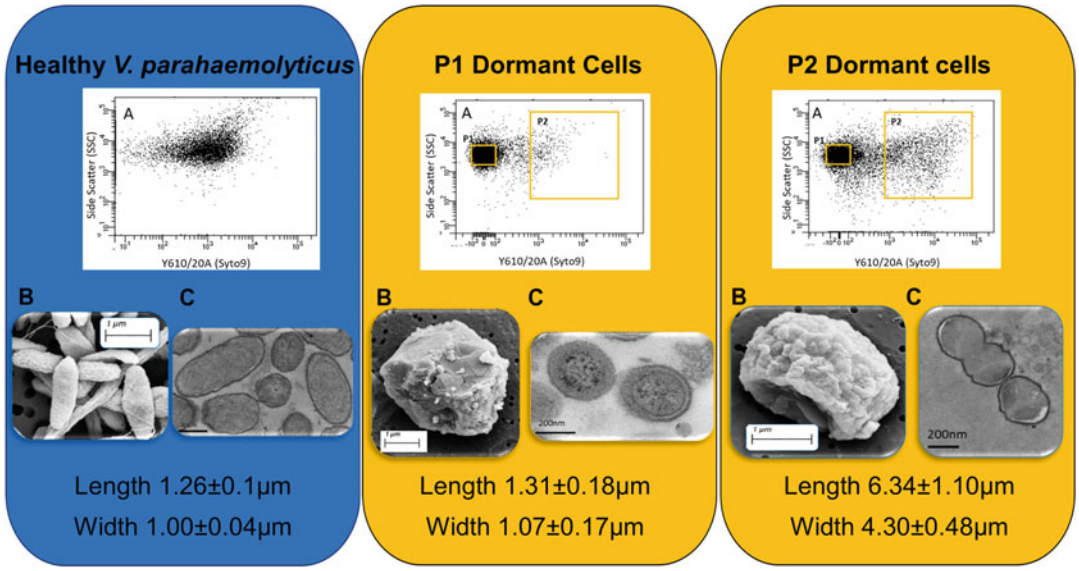


**Fig. 13.2** Morphological differences between healthy (log phase) and dormant P1 and P2 subpopulations of V. *parahaemolyticus* as described by Wagley et al. (2021). (**a**) Are fluorescently activated cell sorting dot plots, (**b**) are Scanning Electron Micrograph (SEM) images, (**c**) are Transmission Electron Micrographs (TEM). Average cell size are shown below SEM and TEM images. Image has been recreated from Wagley et al. (2021)

help resuscitate *Vibrio* cells in the environment? One such theory maybe in the ecological niches where *Vibrio* VBNC cells maybe found such as oysters. Oysters produce lactate as an energy source during the terminal step of glycolysis (Meng et al. 2018), oyster shells are high in calcium lactate (Hong et al. 2021), while lactic acid bacteria that produce lactate have been identified in shellfish species (Ringo et al. 2020). Studying *Vibrio* VBNC cells in their natural environment will be key to exposing how their surroundings help them resusicate to actively growing populations.

A study using in silico protein–protein interaction (PPI) network analysis identified that *CsrA* involved in motility switching could play a role in VBNC metabolic regulation (Wang et al. 2021). This work needs to be experimentally verified for a better understanding in the role of *CsrA* in controlling VBNC cells and decreasing biofilm tolerance. Naser and co-workers found that variants of *V. cholerae O1* overproduce a quorum sensing auto-inducer AI-2 and may contribute to the resuscitation of dormant cells (Naser et al. 2021). Another study showed proteases such as Proteinase K was found to promote the recovery of *V. cholerae* VBNC cells by decreasing the initial lag phase that cells take to enter the VBNC state (Debnath and Miyoshi 2021), which might indicate a role for bacteria to self-produce proteases to help in their resuscitation out of dormancy which needs to be investigated further. These possible sources of resuscitation factors need investigating in both laboratory and in situ models to deepen our understanding of how VBNC cells resuscitate in their natural environment.

## 13.7 Virulence

Understanding if dormant bacterial cells can become resuscitated in vivo is important for understanding the clinical significance of VBNC cells because there is extensive literature showing that some human pathogenic bacteria can retain the ability to cause disease while in the VBNC state (Shleeva et al. 2004; Highmore et al. 2018;

Alleron et al. 2013; Wilks et al. 2021; Pasquaroli et al. 2013). For example, one study reported that VBNC cells of *E. coli* O157:H7 continued to produce Shiga-like toxins (Liu et al. 2010). In another study it was shown that *Vibrio cholerae* non-O1/non-O139 and *V. parahaemolyticus* VBNC cells converted to the culturable form when co-cultured with eukaryotic cells such as HT-29 or Caco-2 cells (Senoh et al. 2012) indicating the potential for in vivo resuscitation. In the Bay of Bengal, cholera endemics occur in two seasonal peaks, although little information is known about the reservoir of *V. cholerae* O1 between these peaks. There is evidence that non-culturable *V. cholerae* O1 cells are present between epidemics in samples collected from bodies of water that serve as a drinking water source in Bangladesh (Alam et al. 2007). Nevertheless, to date, no one has been able to isolate them from their unculturable form and there is a knowledge gap in information about what processes lead to their resuscitation.

Due to the possibility that VBNC cells of pathogenic bacteria can retain their virulence, VBNC cells are a major public health concern in particular in food microbiological safety. The microbial contamination of a food sample is determined by plate count methods, and if VBNC cells are present, then they could go undetected during routine food microbiology testing due their inherent unculturability. This can lead to an underestimation of the disease potential of that sample. The detection of *V. parahaemolyticus* in the environment, and cases of disease in humans, typically peaks in the summer months when warmer sea temperatures allows bacterial proliferation. However, the pathogen is present at low levels, or undetectable using classical techniques, in environmental samples taken during the winter months (Coutard et al. 2007; Froelich and Noble 2014). If unculturable dormant cells are within food samples, then inadequate transportation, food handling and preparation by consumers could lead to their reactivation, regrowth and their potential to cause disease.

*Galleria mellonella* (wax moth) larvae can be used to assess virulence of *V. parahaemolyticus* (Wagley et al. 2018) including strain RIMD2210633. In our work we found that *V. parahaemolyticus* RIMD2210633 VBNC subpopulations cannot be resuscitated inside *G. mellonella*. This was supported by the lack of regulation of known virulence proteins including T3SS, TDH, capsular polysaccharide (CPS) proteins, and lipopolysaccharide (LPS) proteins, and we found only eight proteins significantly upregulated (4% of known virulence related proteins). Virulence of VBNC cells after resuscitation was similar to that of culturable RIMD2210633. If VBNC cells cannot be resuscitated inside the host under these conditions as shown in this study, then the pathogenic nature of the *V. parahaemolyticus* would appear to be curtailed. More tests need to be carried out to see if the virulence potential of VBNC cells is strain dependent. However, if there was a change in conditions, which allowed the VBNC cells in food product, time to resuscitate, this would restore virulence and would be a health risk. The evidence supports the suggestion that *V. parahaemolyticus* VBNC cells are a still a concern for public health once in their culturable form and further study is warranted.

## 13.8 Heterogeneous Populations of VBNC Cells and Future Omics Studies

Most studies on VBNC cells in other Gram-negative bacteria report cell dwarfing and/or rounding when in the VBNC state (Coutard et al. 2007; Jiang and Chai 1996; Thomas et al. 2002; Zeng et al. 2013; Du et al. 2007a; Inglis and Sagripanti 2006; Krebs and Taylor 2011a; Chaiyanan et al. 2001; Gray et al. 2019). Chaiyanan and authors reported that *Vibrio cholerae O1* and *O139* cells change from rod shaped to ovoid or coccoid morphology when in the VBNC state and became smaller with loss of outer cell wall rigidity (Chaiyanan et al. 2001). Furthermore, the cytoplasm of the cells condensed, resulting in a vacuole like spaces between the cytoplasmic membrane and the cell wall (Chaiyanan et al. 2001). Similar reports of

morphology changes of *Vibrio* spies were reported in *V. cholerae* and *V. parahaemolyticus* in other studies (Coutard et al. 2007; Krebs and Taylor 2011b). Our studies showed the presence of **distinct subpopulations of VBNC-like cells exist that have distinct cell morphologies and proteomic profiles.** Two subpopulations of VBNC cells called P1 and P2 were reported and the P2 subpopulation of VBNC cells exhibited as large coccoid in structure and are morphologically different to culturable rod-shaped cells, they had low metabolic activity and lacked cellular contents but proteomic analysis revealed a repertoire of proteins that protect the P2 population during dormancy. This P2 subpopulation was resuscitated fully after existing in the dormant state for >50 days, which is significantly longer than the other P1 subpopulation of VBNC-like cells identified (Wagley et al. 2021)**.** Coutard et al. also observed a heterogeneous population of *V. parahaemolyticus* VBNC cells in their microcosms using SEM where there were small coccoid cells as well as flattened larger cells (Coutard et al. 2007).

A recent transcriptomics study demonstrated differential gene expression in *V. parahaemolyticus* VBNC cells compared to exponential or stationary phase cells (Meng et al. 2015). The study revealed that genes involved in glutamate synthesis, biofilm maintenance, DNA repairing and transportation were upregulated at least four-fold during the VBNC state. Whilst the genes for virulence were identified within a larger group of upregulated genes, no distinction was made between them and genes of general and unknown function. Collectively, the transcriptome studies on VBNC state in *Vibrio* species (Meng et al. 2015; Asakura et al. 2007) are useful but do not identify genes, which play a role in VBNC formation. **A significant limitation of previous studies on VBNC cells is that they are carried out using whole populations, and failed to recognise the existence of different VBNC subpopulations**. Consequently, the transcriptome studies on VBNC *Vibrio* cells (Meng et al. 2015; Asakura et al. 2007) provide a global average dataset but may not identify genes, which play a role in VBNC formation. Imaging flow cytometry (IFC) combines flow cytometry and microscopy and, with advances in machine learning, has the advantage of capturing images of thousands of cells, thus allowing rapid and high-throughput quantitative analyses of cells in mixed populations (Wagley et al. 2021; Power et al. 2021). The ability to use flow cytometry methods to identify distinct VBNC/dormant subpopulations of cells, isolate them and resuscitate them in a controlled manner can allow scientists to characterise the molecular makeup of true VBNC/dormant cells. This will in turn open new opportunities to prevent, treat, and control disease.

## 13.9 Conclusion

*Vibrio* species are an excellent model organism to study the VBNC state because it is possible to control the switch between non-culturable and culturable cells. Recent studies using imaging flow cytometry have shown that VBNC cells induced within microcosms in the laboratory are not a heterogeneous population but in fact are a mixture of different cell types. Future studies on these different subpopulations, which include true VBNC cells that can revert to the culturable form, will move the bacterial dormancy forward. The use of single cell RNA sequencing experiments on true VBNC populations in the future may reveal genes involved in sustaining cells in dormancy as well as identifying genes involved in resuscitation. Identifying VBNC cells in the environment to see if laboratory induced VBNC cells are similar is also important. The World Health Organisation have a global strategy on cholera control, *'Ending Cholera: a global roadmap to 2030*', with a target to reduce cholera deaths by 90%. To fulfil this goal it will be key to understand how the VBNC state plays an important part of the life cycle of *V. cholerae* in the environment.

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# Structural Insights into Regulation of *Vibrio* Virulence Gene Networks

14

Charles R. Midgett and F. Jon Kull

**Abstract**

One of the best studied aspects of pathogenic Vibrios are the virulence cascades that lead to the production of virulence factors and, ultimately, clinical outcomes. In this chapter, we will examine the regulation of *Vibrio* virulence gene networks from a structural and biochemical perspective. We will discuss the recent research into the numerous proteins that contribute to regulating virulence in Vibrio *spp* such as quorum sensing regulator HapR, the transcription factors AphA and AphB, or the virulence regulators ToxR and ToxT. We highlight how insights gained from these studies are already illuminating the basic molecular mechanisms by which the virulence cascade of pathogenic Vibrios unfold and contend that understanding how protein interactions contribute to the host–pathogen communications will enable the development of new antivirulence compounds that can effectively target these pathogens.

**Keywords**

Vibrio · Virulence regulation · Transcription factors · Protein structure

C. R. Midgett · F. J. Kull (✉)
Chemistry Department, Dartmouth College, Hanover, NH, USA
e-mail: charles.midgett@dartmouth.edu; f.jon.kull@dartmouth.edu

## 14.1 Introduction

Enteric bacterial pathogens of the *Vibrio* spp. need to be able to properly regulate genetic networks to survive the harsh intestinal environment, colonize the host, produce virulence factors, and, in some cases, return to the external environment, e.g. (Almagro-Moreno et al. 2015a). These processes are regulated by a set of conserved transcription factors that respond by sensing environmental changes, such as oxygen level and pH, by binding directly to small molecular regulators, or via quorum sensing (Mey et al. 2012; Midgett et al. 2017; Rutherford et al. 2011; Li et al. 2016a; Lowden et al. 2010; Kovacikova et al. 2010). The complex regulatory networks have evolved to be temporally and spatially regulated in order to optimize virulence gene expression. One of the most well characterized regulatory systems of this type is from pandemic *Vibrio cholerae,* whose ingestion results in the diarrheal disease, cholera, for which much microbiological and structural information is known (Almagro-Moreno et al. 2015a; Clemens et al. 2017). In this chapter, we will review the protein regulators for which atomic structures are known, highlighting their structural features and what is known about their mechanism of regulation and activity, as well as outstanding questions related to their structure and function.

Induction of virulence in *V. cholerae* is controlled by a complicated regulatory cascade



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involving a number of transcription factors from diverse families (Fig. 14.1a). We will describe what is known about the structure and function of these proteins in the order they appear in this network, starting with HapR, which is expressed at high cell densities and is involved in quorum sensing (De Silva et al. 2007; Ball et al. 2017). At high cell density HapR has two main functions, inhibition of *aphA* expression, which subsequently reduces virulence gene expression, and induction of dispersal from biofilms (Zhu and Mekalanos 2003; Finkelstein et al. 1991; Kovacikova and Skorupski 2002). AphA, a member of the PadR family of transcription factors (De Silva et al. 2005), together with AphB, a LysR family transcription factors (Taylor et al. 2012; Kovacikova and Skorupski 1999), activates *tcpPH* expression (Kovacikova and Skorupski 1999; Skorupski and Taylor 1999). TcpP and TcpH are members of the ToxRS family of regulators, which consist of a transmembrane transcription factor and an integral membrane periplasmic binding partner. TcpPH, along with ToxRS, activates expression of the AraC family transcription factor ToxT (Lowden et al. 2010; Miller et al. 1987, 1989; Hase and Mekalanos 1998; Krukonis et al. 2000; Higgins et al. 1992; Matson et al. 2007). ToxT, the master regulator in *V. cholerae*, directly activates expression of the two main virulence factors, the toxin coregulated pilus (TCP) and cholera toxin (CT) (Matson et al. 2007).

Additionally, outside of this regulatory cascade, a virulence pathway in non-O1/O139 *V. cholerae* and *V. parahaemolyticus* that leads to the expression of the type 3 secretion system 2 (T3SS2). In this pathway (Fig. 14.1b), ToxRS works with another transmembrane transcription factor pair, VtrAC, in a bile dependent manner to activate the transcription of *vtrB,* a transmembrane transcription factor that activates transcription of the T3SS2 (Li et al. 2016a; Kodama et al. 2010; Gotoh et al. 2010; Hubbard et al. 2016; Miller et al. 2016). Through a combination of structural, biochemical, microbiological, and genetic information, we can start to understand and appreciate the exquisite dance of interactions and conformational changes that must occur for these transcription factors proteins to work together to respond to the environment and regulate virulence gene expression. Of course, such understanding also provides a foundation for manipulating their activity and designing inhibitors specifically targeting enteric pathogens and not the commensals around them, which could reduce the negative effects of antibiotic resistance (Cegelski et al. 2008).

## 14.2 HapR and Homologs Are Quorum Sensing Transcription Factors

HapR is part of the quorum sensing cascade in various *Vibrio* spp. where it is also known as SmcR (*V. vulnificus*) and LuxR (*V. harveyi*), which can all cross complement each other in their respective strains (Ball et al. 2017). They belong to the very large family of TetR transcription factors (Cuthbertson and Nodwell 2013). However, unlike many other members of the TetR family which are regulated only by directly binding small molecules, in *Vibrios* these proteins are primarily regulated via the action of autoinducers on other proteins such as autoinducer 2 though the LuxPQ system and cholera autoinducer 1 through the CqsS pathway (Ball et al. 2017; Cuthbertson and Nodwell 2013). Furthermore, unlike many other TetR family members, these proteins regulate many different genes and can act as activators and repressors (Ball et al. 2017).

While not all pandemic *V. cholerae* strains have a functional HapR, for those that do HapR plays a role in bacterial dispersion from biofilms at the beginning of infection, e.g. *V. cholerae* and *V. vulnificus*, and also at the end of infection (Zhu and Mekalanos 2003; Finkelstein et al. 1991; Stutzmann and Blokesch 2016; Zhu et al. 2002; Jobling and Holmes 1997; Kim et al. 2013). In *V. cholerae*, as well as other *Vibrios,* HapR and AphA make up a quorum sensing axis, with AphA expressed at low cell densities and HapR expressed at high cell densities (Rutherford et al. 2011; Ball et al. 2017). When expressed at high cell densities, HapR binds to the *aphA* promoter,

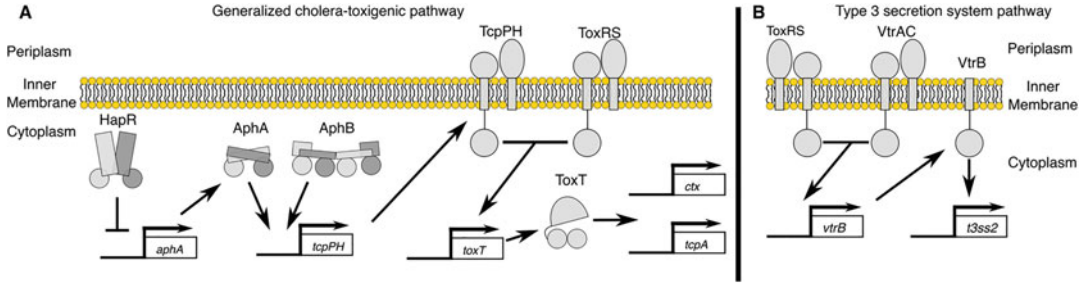


**Fig. 14.1** Virulence pathway for cholera toxin and type 3 secretion system 2. (**a**) Overview of the toxigenic pathway in pandemic *V. cholerae* focusing on the proteins and genes they regulate. (**b**) The pathway for type 3 secretion system 2 expression as determined in *V. cholerae* and *V. parahaemolyticus*, again focusing on the proteins and genes they regulate

inhibiting its transcription (Ball et al. 2017; Kovacikova and Skorupski 2002). In addition to inhibiting transcription, HapR also activates transcription of several genes, including the hemagglutinin protease, which degrades the putative intestinal cell surface receptors *V. cholerae* uses to attach to cells (Ball et al. 2017; Finkelstein et al. 1991).

Analysis of *Vibrio* HapR protein family DNA binding sites has shown they bind to two motifs. Motif 1 has dyad symmetry with a variable spacer and is around 20–22 bp. While motif two is asymmetric with one half of the dyad on one side of the spacer and an incomplete dyad on the other side (Ball et al. 2017). The two motifs have generally been correlated with transcriptional repression (motif 1) and activation (motif 2) (Ball et al. 2017; Tsou et al. 2009). How these proteins interact with DNA is one question that structure has provided insight.

### 14.2.1 HapR Structure

The first high resolution crystal structure of a *Vibrio* HapR family member was solved in 2007, and confirmed it had an overall structure similar to other TetR family members (De Silva et al. 2007). As with other TetR family members, HapR was a homodimer, with each monomer containing 9 α-helices forming two domains, an N-terminal helix-turn-helix DNA binding domain, and a C-terminal dimerization domain containing a putative ligand binding pocket (Fig. 14.2) (De Silva et al. 2007; Cuthbertson and Nodwell 2013). The two domains are connected by a hinge region, which has been shown to be important for DNA binding (Dongre et al. 2011).

The structure contained an empty, solvent accessible pocket, suggesting that HapR is not only regulated at the expression level by the quorum sensing pathway, but also via direct binding of a small molecule ligand (De Silva et al. 2007). These features were also observed in the subsequently determined structures of *V. vulnificus* SmcR and *V. harveyi* LuxR (Kim et al. 2010; Zhang et al. 2021).

The presence of empty ligand binding pockets in these structures has naturally led to much speculation about the nature and identity of putative physiological regulatory ligands. In one study, SmcR activity was used to screen for inhibitory compounds, and 1-(5-bromothiophene-2-sulfonyl)-1H-pyrazole (qstatin) was shown to reduce the expression of elastase (Kim et al. 2018), and qstatin was also shown to inhibit homologs from other species, but not HapR (Kim et al. 2018). A structure of SmcR in complex with qstatin showed the molecule indeed bound to the previously identified pocket (De Silva et al. 2007), and that qstatin made SmcR less flexible as indicated by a decrease in the crystallographic B-factors when compared to the apo structure (Kim et al. 2018). Such ligand-induced stabilization of

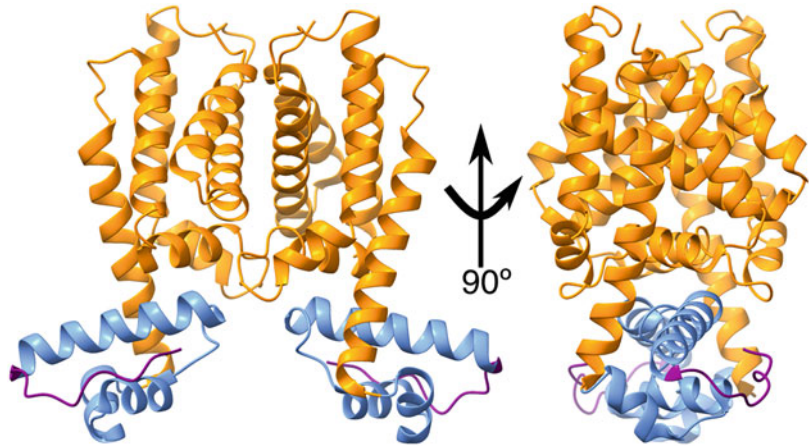


**Fig. 14.2** Overview of the HapR structure from *V. cholerae* (De Silva et al. 2007). The structure of HapR dimer from *V. cholerae* with the N-terminal tails that wraps back on to the DNA binding domain in purple, the DNA binding domains in blue, and the C-terminal dimerization domains in orange

proteins is not unusual and could of course serve to lock proteins into a particular conformation.

While qstatin was clearly inhibitory, its effects on SmcR DNA binding varied widely. For promoters which SmcR activates expression, including *vvpE,* there was almost no change in the affinity of SmcR to the promoter binding site. In contrast, for promoters that are repressed by SmcR, qstatin binding reduced the affinity for the promoter by six to eight fold (Kim et al. 2018). A stronger SmcR inhibitor that was identified from the same class of compounds also did not disrupt DNA binding to the SmcR activated *vvpE* promoter (Newman et al. 2021a). This suggests these inhibitors disrupt different processes depending on whether the transcription is activated or repressed by protein binding.

### 14.2.2 HapR DNA Binding

The ability of HapR and its homologs bind promoters and either repress or activate cognate gene expression is fascinating. Because they can bind to promoters of different lengths, it is thought they must be somewhat flexible in how they bind DNA (Newman et al. 2021b), and this characteristic is observed by crystal structures of SmcR in which the DNA binding domains are observed in narrow or wide conformations, which has also been confirmed by solution studies using small angle X-ray scattering (SAXS) (Newman et al. 2021b). Furthermore, in a natural HapR variant, HapRv2, where a small and flexible glycine is replaced by a less flexible and negatively charged aspartic acid, SAXS analysis produced a model in which the DNA binding domains were in an orientation unable to bind DNA (Dongre et al. 2011). However, a crystal structure of HapRv2 mutant protein determined in the absence of DNA showed the protein adopts a fold indistinguishable from the non-variant structure, suggesting DNA binding was blocked not by a conformational change but rather by a clash of the aspartic acid side chain with phosphates on the DNA backbone (Cruite et al. 2018).

The structures of *V. alginolyticus* LuxR bound to DNA from promoters it activates and represses were solved recently (Zhang et al. 2021). Both structures contained 21 bp of DNA and were similar in overall fold. Interestingly, the structure of the complex between LuxR and the activating promoter indicated that interactions with the protein extended beyond what was predicted to be the end of the shorter motif 2 (Zhang et al. 2021), raising the question of the nature of the observed lower binding affinity.

One possible explanation for this is the difference in interactions of the N-terminal tail of the protein, which forms contacts with the minor groove of the DNA. When bound to the repressed DNA, both N-terminal tails of the LuxR dimer are well ordered, while only one is visible in the activated DNA bound structure (Zhang et al. 2021). These contacts appear to be critical for function, as mutations in Arg9 and Arg11

severely disrupt DNA binding (Kim et al. 2010; Zhang et al. 2021). Another clue comes from the crystallographic B-factors, which for the LuxR-activated promoter structure are higher than that of the LuxR-repressed DNA structure This indicates increased flexibility of one complex in comparison to the other, which could be indicative of weaker affinity despite a similarly sized binding interface (Zhang et al. 2021). Regardless of the ultimate explanation of the disparate affinities, the observation that the structures are essentially the same when LuxR is bound to promoters it activates or represses is an important step in elucidating the detailed atomic interactions that must govern the strength of protein-DNA binding.

While the overall structures of LuxR bound to activated or repressed promoters do not significantly differ, a comparison of LuxR-DNA bound structures with the apo structure of *V. vulnificus* SmcR showed several significant changes these proteins undergo to bind DNA (Zhang et al. 2021). First, the DNA binding domains are drawn closer together by a several angstroms and are rotated with respect to each other. This movement generates significant rearrangements in the C-terminal domain, where new contacts are formed. In particular, Glu 124 moves 5 Å to form an amino acid cluster involving Arg60, Glu124, Arg122, and Glu116 (Zhang et al. 2021). Furthermore, An alignment of LuxR bound to an activated promoter (Zhang et al. 2021) with apo HapR (De Silva et al. 2007) also revealed binding DNA decreases the distance between helix 6 in the two monomers, suggesting that a compound which wedges the helices open would block the ability of these proteins to bind DNA (Fig. 14.3a, b).

Another interesting structural feature illuminated by the LuxR-DNA complex structures are differences in the proposed ligand binding domain. In the LuxR-DNA structures, while both subunits have a pocket (Zhang et al. 2021), the pockets are not connected by a solvent accessible tunnel, as observed in the apo HapR and SmcR structures (De Silva et al. 2007; Kim et al. 2010; Zhang et al. 2021). In addition, the pocket is mostly closed off from bulk solvent in the DNA bound structures, suggesting a potential ligand would be trapped inside, or unable to bind, until the protein releases from the DNA (Zhang et al. 2021) (Fig. 14.3c, d).

The HapR family of *Vibrio* proteins act in the quorum sensing pathway and therefore have garnered interest as antivirulence targets. However, targeting them is complicated as the role they play in pathogenesis differs at various stages of the process. In the beginning stages of infection, when dissemination is a problem, an inhibitor would be desired. However, in the late stages of infection, activating the proteins would be necessary to inhibit the virulence pathway. In any case, at this time the most promising approach is to use the available structural data to computationally screen for small molecule inhibitors that target the binding pocket to block the conformational changes necessary for DNA binding.

Among the outstanding questions related to the structure and function of HapR-like proteins, such as the identity of the physiological regulatory ligand and the temporal and functional effects it has on the pathway. Additionally, how does quorum sensing and ligand binding work together to regulate these proteins. And finally, a comprehensive understanding must also explain the details of how inhibitor binding modulates the binding affinity for some DNA sequences, but not others.

## 14.3 AphAB

AphA and AphB are two transcription factors from different families that are conserved in the *Vibrio* family. AphA is thought of belonging to a quorum sensing axis with HapR, where AphA is expressed at low cell densities and repressed by HapR at high cell densities (Rutherford et al. 2011; Kovacikova and Skorupski 2002). AphB is an environmental sensor that responds to changes in pH and oxygen (Kovacikova et al.

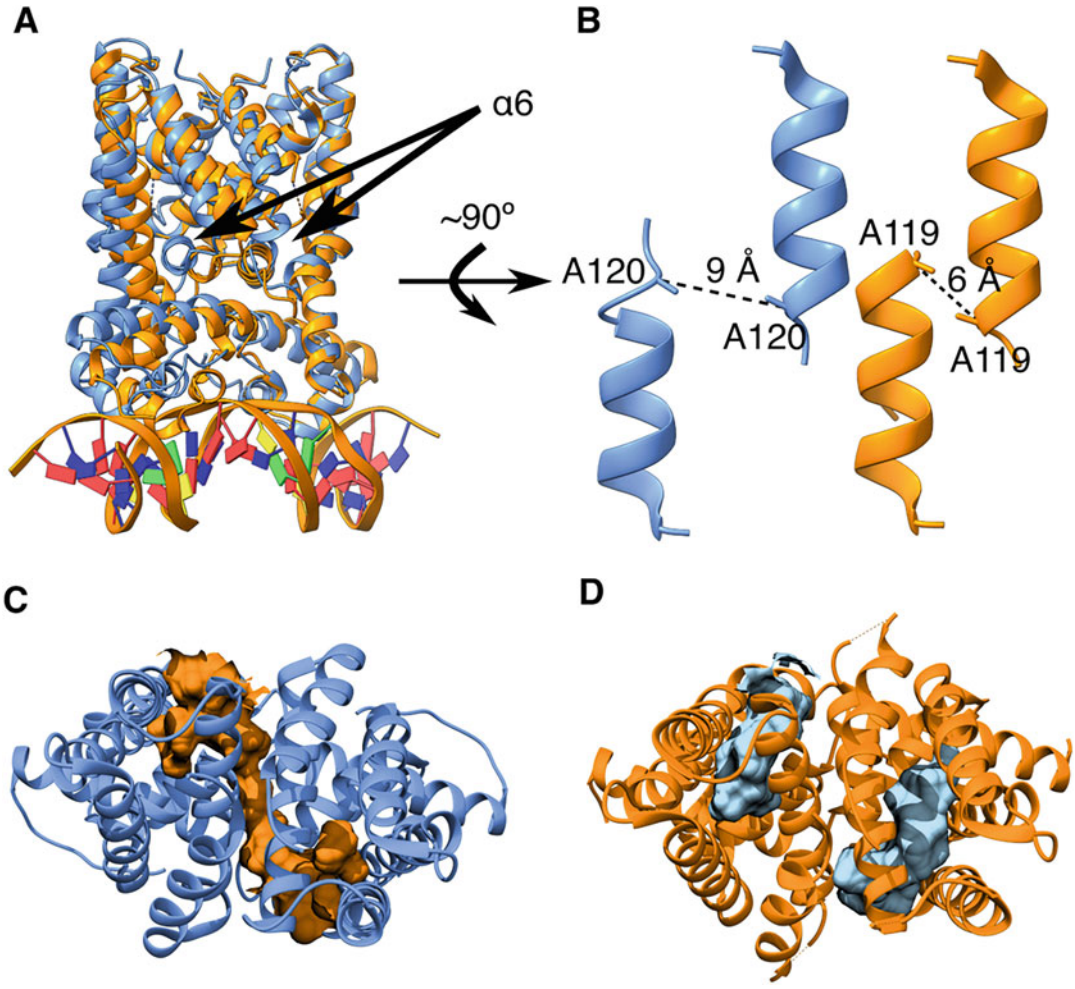


**Fig. 14.3** Comparison of LuxR bound to DNA (Zhang et al. 2021) and the HapR structure (De Silva et al. 2007). (**a**) Overlay of HapR (De Silva et al. 2007) in blue and LuxR bound to DNA (Zhang et al. 2021) in orange. The proteins are viewed from at an angle to emphasize the amount of movement that is necessary for the protein to undergo to bind to DNA. Arrows point to α6 for both the HapR and LuxR-DNA structures. (**b**) α6 undergoes significant movement from the unbound to DNA bound states. In blue is the α6 from the HapR (De Silva et al. 2007) structure and in orange is α6 from the LuxR-DNA structure (Zhang et al. 2021). This close approach is facilitated by the annotated alanines as the Cα distance between the monomers in the HapR apo structure is 9.4 Å and between the same residues in the LuxR-DNA bound structures is 6.1 Å as annotated in (**c**). The proposed HapR (De Silva et al. 2007) binding pocket as determined by Castp. The protein is in blue and the pocket is shown in orange and is continuous between the subunits. (**d**) The proposed binding pockets as determined by Castp of the LuxR-DNA structure (Zhang et al. 2021). The protein is in orange and the pockets are in blue

2010; Rhee et al. 2006; Liu et al. 2011). While both these proteins are global regulators of gene expression, they have been coopted to regulate virulence gene expression in several *Vibrio spp*. (Kovacikova and Skorupski 1999; Skorupski and Taylor 1999; Jeong and Choi 2008; Gao et al. 2017; Lim et al. 2014). In some *V. cholerae* strains, AphA and AphB work cooperatively to increase the transcription of *tcpPH* under virulence inducing conditions, which suggests they respond to an activation signal (Kovacikova and Skorupski 1999, 2000; Skorupski and Taylor 1999). It is hypothesized that AphB recruits AphA to the DNA as AphA mutants that can no

longer bind DNA have their activity rescued in the presence of AphB (Kovacikova et al. 2004). While these proteins can increase expression of virulence genes through *tcpPH* transcriptional augmentation, El Tor strains can carry a mutation in the promoter of *tcpPH* that reduces AphB binding and these strains remain virulent (Kovacikova and Skorupski 2000; Kovacikova et al. 2004).

### 14.3.1 AphA Structure

AphA is a member of the PadR family of environmental sensors (Rutherford et al. 2011; Kovacikova and Skorupski 2002; De Silva et al. 2005; Barthelmebs et al. 2000) and was one of the first of this family to have its structure determined (De Silva et al. 2005). These proteins function as a dimer composed of two monomers, with each monomers consisting of an N-terminal winged helix-turn-helix domain and an extended C-terminal dimerization domain composed of three helices (5–7). Rather than forming a standard 4-helix bundle, helices 6–7 from one monomer interact with those from an adjacent monomer in an antiparallel fashion, forming a relatively flat sheet of 4 helices (De Silva et al. 2005). Overall, the structure resembles a bridge with the pillars being the DNA binding domains and the top being the C-terminal helices (Fig. 14.4a).

The structure of PadR bound to ligand and PadR bound to DNA have been solved providing additional insight into the function and regulation of these proteins (Park et al. 2017a). Although no pocket was identified in AphA, in PadR, binds phenolic acids in between the N-terminal and C-terminal domain, and it seems likely AphA could also undergo conformation changes in this region to bind ligand (Fig. 14.4b) (Park et al. 2017a).

The structure of PadR bound to DNA shows PadR binds in the major groove in a diagonal relative to the DNA helix axis (Park et al. 2017a). Furthermore, comparing the PadR-DNA structure to the apo AphA structure shows the protein must undergo a conformational to contact DNA (Fig. 14.4c). These observations suggest AphA would bind AphB somewhere along its long axis most likely along the extended C-terminal dimerization domain (Fig. 14.4c).

### 14.3.2 AphB Structure

AphB belongs to the LysR-type transcriptional regulator family, which is the largest family of transcription factors in bacteria and examples are also found in archaea and eukaryotes (Taylor et al. 2012; Kovacikova and Skorupski 1999; Maddocks and Oyston 2008). LysR proteins are involved in a diverse set of processes, usually responding to environmental or metabolic cues, usually via ligand binding to the regulatory domain, although some are thought to respond directly to redox changes (Maddocks and Oyston 2008; Jo et al. 2019). AphB activity has been shown to increase under conditions of acidic pH and anaerobic conditions (Kovacikova et al. 2010; Taylor et al. 2012).

The structure of AphB was determined in 2012 and showed that it formed a tetramer, but has the two-fold symmetry of a dimer of dimers (Fig. 14.5) (Taylor et al. 2012). Each monomer consists of a helix-turn-helix DNA binding domain, a helical dimerization domain, and a C-terminal regulatory domain. The regulatory domain consists of two lobes, RD-I and RD-II, which formed a clamshell-like structure with a proposed binding pocket in the middle (Taylor et al. 2012). Each dimer is composed of two monomers, one in a compact conformation, and another in an extended conformation, which dimerize via antiparallel interactions of the helical domains, forming an L shape (Taylor et al. 2012). Two of the L-shaped dimers associate via their regulatory domains to form a tetramer through a two-fold symmetry rotation (Fig. 14.5a). This produces a complex with four DNA binding sites, two inner binding sites, from the compact monomers, and two outer DNA binding sites, from the extended monomers. Interestingly, the DNA binding helices are positioned such that they are too close to fit into major grooves without significant rearrangement (Taylor et al. 2012).

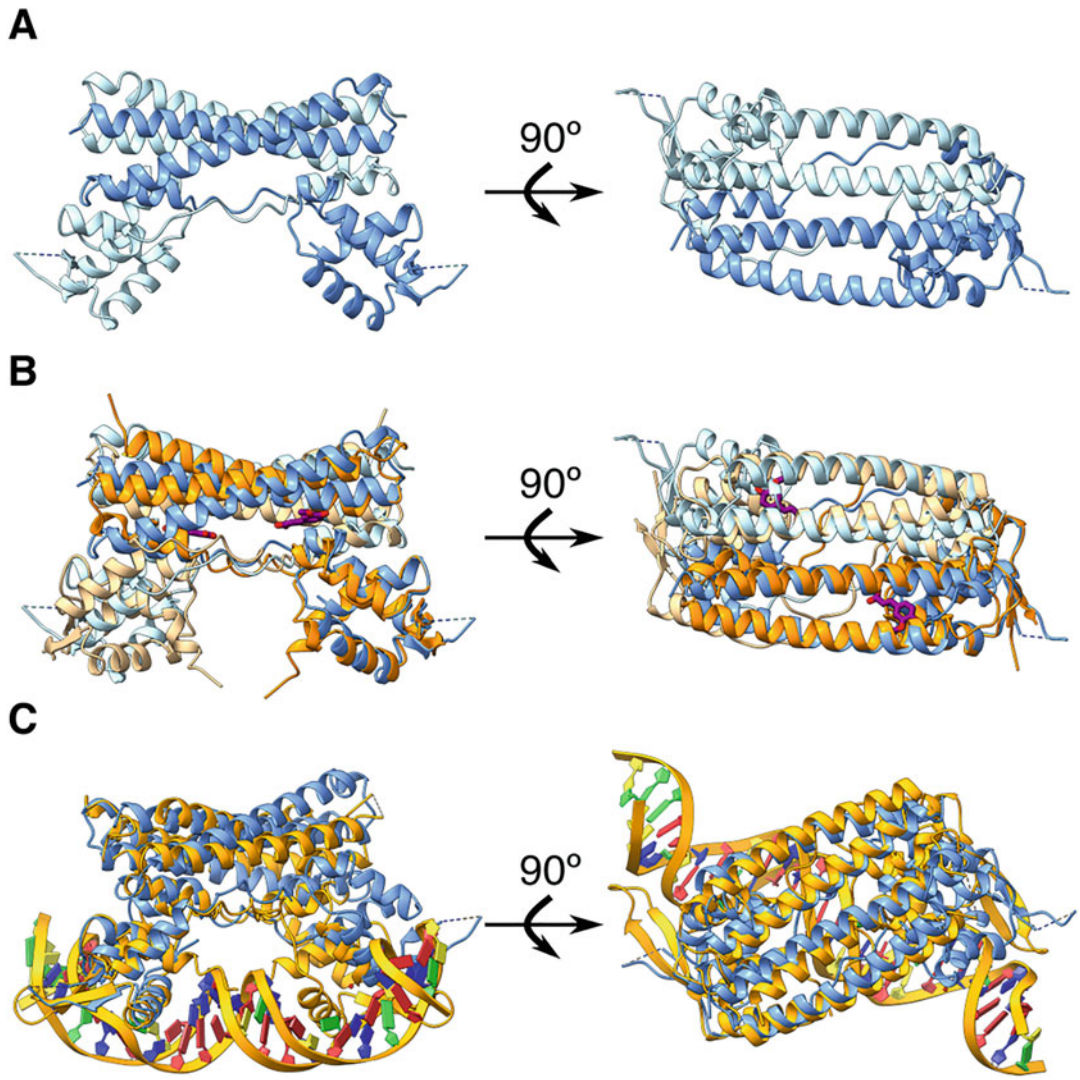


**Fig. 14.4** AphA overview and comparison to PadR bound to ferulic acid or DNA. (**a**) AphA (De Silva et al. 2005) in blue with side and top views. The two chains are in different shades for visibility. (**b**) AphA (De Silva et al. 2005), in blue, aligned with PadR bound to ferulic acid (Park et al. 2017a). The protein is in orange and the ferulic acid between the N- and C-terminal domain is colored purple. (**c**) AphA (De Silva et al. 2005) in blue aligned with PadR bound to DNA (Park et al. 2017a) in orange

Mutations in the regulatory domain have been shown to increase AphB activity by making it insensitive to response to alkaline pH and/or anaerobic conditions (Taylor et al. 2012). The crystal structure of one of these mutants, N100E, highlights some of the structural changes AphB likely undergoes upon activation (Fig. 14.5a). In N100E, while the compact and extended monomer conformations still form a dimer, the tetramer no longer showed two-fold symmetry, and the DNA binding domains became spaced further apart and therefore more able to accommodate DNA binding (Fig. 14.6) (Taylor et al. 2012). This suggests a model in which ligand binding activates AphB via a conformational change in the regulatory domain that is passed on to the DNA binding domains, separating them to allow DNA binding.

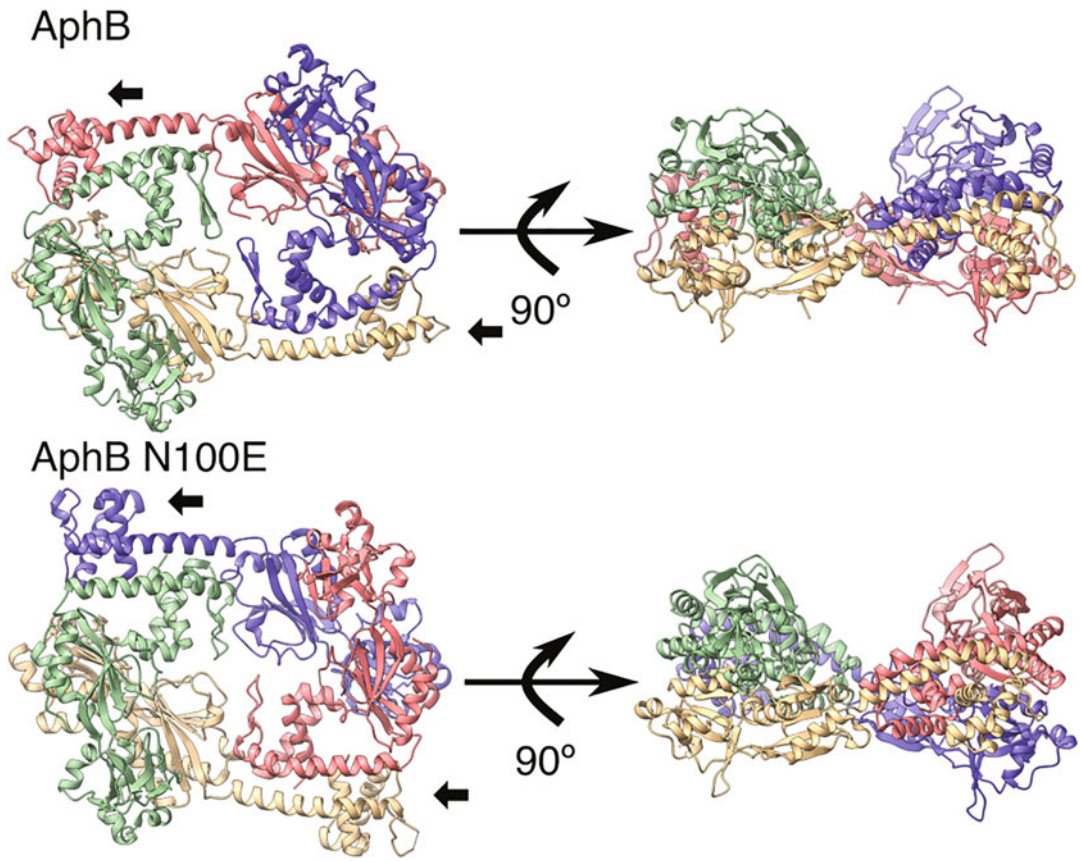


**Fig. 14.5** AphB and AphBN100E structures (Taylor et al. 2012). Top is the structure of AphB wildtype and bottom is the structure of AphBN100E. The complexes are colored by chain. The black arrows point to the extended monomers DNA binding domain. Note in AphBN100E how they are flexed away from the core structure

As the physiological ligand for AphB is unknown, effort has been put into identifying the nature of ligand, as well as finding potential inhibitors. To that end, our laboratory performed a virtual screen centered around the ligand binding pocket (Fig. 14.7a) (Taylor et al. 2012; Privett et al. 2017). A screen identified several potential ligands that were experimentally tested, and one was unexpectedly found to increase AphB activity. Furthermore, in silico modeling using AutoDock showed the ligand was unlikely to bind in the putative pocket, but rather binds in a secondary pocket between the dimer interface of the two regulatory domains, defined by K103, R104, and R224 (Fig. 14.7b) (Privett et al. 2017). A subsequent study found that K103 is acetylated in stationary phase, corroborating the importance of this secondary pocket for AphB function (Jers et al. 2018).

In another study, a screen identified a small molecule inhibitor, ribavirin, that presumably bound in the regulatory domain pocket as it was unable to bind the constitutively active AphB N100E mutant (Mandal et al. 2016). Ribavirin was able to inhibit in vitro production of virulence factors and was also able to inhibit *V. cholerae* colonization in mouse models (Mandal et al. 2016). The authors of this study noted that other LysR proteins have homologous residues within their binding pockets, suggesting ribavirin could inhibit other LysR family members, and subsequently ribavirin has been shown to inhibit

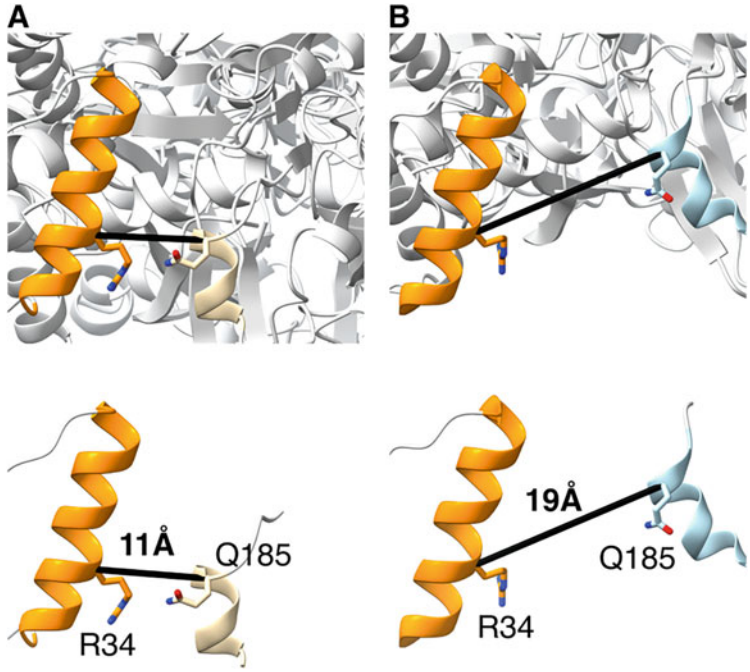


**Fig. 14.6** Flexing of the extended monomers DNA binding domain from the core of the protein. The DNA binding helix, in orange, from congruent AphB, and AphBN100E (Taylor et al. 2012) extended monomers were aligned. The distance from R34 on the DNA binding helix to Q185 from the partner regulatory domain was measured for each complex. (**a**) AphB protein with the DNA binding helix in orange and the helix with Q185 in tan with the rest of the protein in light gray. Top, the helices are shown with the rest of the protein and bottom only the helices are present for clarity. (**b**) Is the same view for AphBN100E with the DNA binding helix in orange, and the helix with Q185 in blue. Top is the helices with the rest of the protein in gray and bottom are the helices by themselves. The distance measured for each complex is shown and labeled

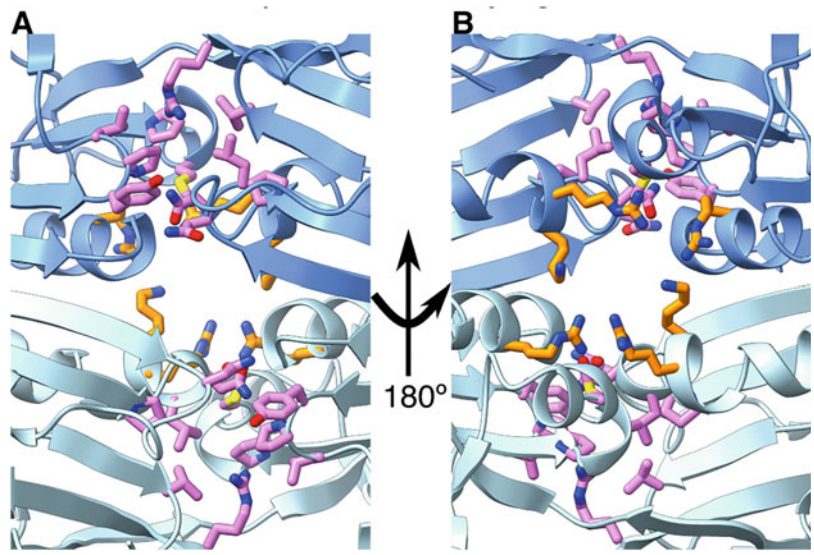


**Fig. 14.7** The different binding pockets in the *V. cholerae* AphB regulatory domain dimer (Taylor et al. 2012). The regulatory domain dimer is in blue with the chains in different shades. (**a**) The ligand binding pocket identified in each regulatory domain is shown with the amino side chains making up the pocket in violet. (**b**) The second pocket between the regulatory domain dimer shown with the side chains in orange

colonization of *Salmonella typhi* and enteropathogenic *E. coli* in mouse models, presumably through AphB homologs (Mandal et al. 2016).

In addition to *V. cholerae*, AphB has been implicated in *V. vulnificus* acid tolerance and indirectly in promoting pathogenesis (Rhee et al. 2006; Jeong and Choi 2008; Elgaml and Miyoshi 2017). To determine if there were changes in the regulatory domain in response to oxidative changes, the regulatory domain of *V. vulnificus* AphB (VvAphB-RD) was solved in the presence of various peroxides. VvAphB-RD formed a dimer that was structurally similar to that seen in the *V. cholerae* AphB full length structure, and the pocket formed by dimerization of the regulatory domains was preserved (Park et al. 2017b). The VvAphB-RD was incubated with peroxides to determine if C227 was involved in redox sensing, as proposed previously, by being converted to cysteine-sulfenic acid (Liu et al. 2011; Conte and Carroll 2013). While no changes in C227 were detected upon peroxide treatment, when the protein was incubated with cumene hydroperoxide electron density was observed in the secondary binding pocket described above (Privett et al. 2017; Park et al. 2017b). This further suggests the pocket formed by regulatory domain dimerization is important for modulating AphB activity.

There are two main outstanding questions related to AphA and AphB function. One is the mechanism by which AphB responds to low pH and anaerobic conditions. While it has been suggested that C227 is involved in sensing low oxygen levels (Liu et al. 2011), subsequent studies have been unable to replicate that finding (Taylor et al. 2012; Park et al. 2017b). A second is that because many LysR family proteins are activated by ligand binding, and as small molecules have been shown to influence AphB activity, it is reasonable to presume AphB has a physiological regulatory ligand or ligands that interact via one or both of the sites that have been identified in the regulatory domain or between the regulatory domain dimers (Fig. 14.7) (Taylor et al. 2012; Maddocks and Oyston 2008; Privett et al. 2017). Another major question is how AhpB and AphA interact with each other and with DNA (Kovacikova and Skorupski 1999, 2001). Modeling suggests there must be considerable distortion of either the DNA or proteins for both AphA and AphB to bind the promoter DNA determined by DNaseI footprinting (Kovacikova and Skorupski 2001). Finally, given AphA and AphB work together in *V. cholerae*, it is likely that other PadR-LysR protein pairs will be identified that work together to regulate transcription in other bacteria.

## 14.4 ToxRS

ToxR and ToxS are the founding members of the ToxR family of transmembrane transcription factors that work in concert with integral membrane periplasmic binding partners. Conserved across the Vibrionaceae, ToxR is responsible for adapting the bacteria to environmental stressors, such as bile salts, antimicrobial peptides, and acidic conditions (Miller et al. 1989; Provenzano et al. 2000; Mathur and Waldor 2004). ToxS binds to ToxR, leading to full transcriptional activation, and protects ToxR from protease degradation. In certain conditions, ToxS is required for ToxR activity (Mey et al. 2012; Midgett et al. 2017; Almagro-Moreno et al. 2015b), and *toxS* mutants are less competitive than wildtype in infant mouse models (Pearson et al. 1990). In addition to their role in environmental stress response, these proteins have also been coopted into regulating virulence in some species, including *V. parahaemolyticus* and *V. cholerae* (Hubbard et al. 2016; Herrington et al. 1988; Whitaker et al. 2012).

ToxR is essential for *V. cholerae* to transition from the aquatic environment to being pathogenic in the human intestine (Herrington et al. 1988). ToxR augments the activity of another transmembrane transcription factor, TcpP, at the *toxT* promoter (Hase and Mekalanos 1998; Krukonis et al. 2000; Krukonis and DiRita 2003; Morgan et al. 2011). ToxT then goes on to activate expression of the toxin coregulate pilus (TCP) and cholera toxin (CT), the two major *V. cholerae* virulence factors responsible for cell attachment and diarrhea (Almagro-Moreno et al. 2015a; Matson et al. 2007).

In *V. parahaemolyticus*, ToxR is required for colonization in various animal models (Hubbard et al. 2016; Whitaker et al. 2012), in part this is due to ToxR activating expression of the T3SS2. The secretion system is activated in a bile dependent manner requiring ToxR to augment the activity of VtrA, which then leads to expression of the transmembrane transcription factor VtrB (Kodama et al. 2010; Gotoh et al. 2010; Hubbard et al. 2016). VtrB subsequently activates the transcription of the genes encoding the T3SS2 (Kodama et al. 2010; Gotoh et al. 2010). Interestingly, *V. cholerae* also encodes these VtrA and VtrB, and in non-O1/O139 strains they are involved in the expression of a type three secretion system (Miller et al. 2016; Alam et al. 2010).

Structural studies of ToxR and ToxS seek to understand the mechanism by which the protein pair responds to environmental signals, including bile, as well as the manner in which ToxS activates and stabilizes ToxR, as well as protecting it from protease degradation.

### 14.4.1 ToxR Structure

ToxR is 34 kDa transmembrane transcription factor that has a winged helix-turn-helix (wHTH) DNA binding domain followed by a variable linker connecting to the transmembrane domain, and a C-terminal periplasmic domain (Miller et al. 1987; DiRita and Mekalanos 1991). Because of the inherent difficulties involved with solving transmembrane protein structures, initial ToxR structural studies have focused on individual soluble domains, and recent works have provided insight into some of the questions surrounding ToxR function (Midgett et al. 2020; Gubensäk et al. 2021a, b).

### 14.4.2 DNA Binding Domain

The ToxR DNA binding domain is homologous to the OmpR family of winged helix-turn-helix (wHTH) transcription factors (Miller et al. 1987; Aravind et al. 2005). These domains consist of a β-sheet domain that is followed by a helix-turn-helix domain (HTH) with a two β-strand wing following the third helix (Martínez-Hackert and Stock 1997; Sadotra et al. 2021; Blanco et al. 2002; Schlundt et al. 2017). Structures of ToxR homologs PhoB and OmpR bound to DNA show third helix and wing domain make the contacts with DNA. The third helix binds the major groove of the recognition site, and the wing domain binds in the minor groove (Sadotra et al. 2021; Blanco et al. 2002; Schlundt et al. 2017). Both OmpR and PhoB make head to tail contacts on the DNA, suggesting that in vivo these domains can form a curved filament like structure as proposed by Blanco et al. (2002). This is important as ToxR is known to bind stretches of DNA that can be over 100 bp long suggesting that up to 10 copies of ToxR can bind (Krukonis et al. 2000; Crawford et al. 1998; Li et al. 2000). Furthermore, ToxR DNA binding domain is thought to bind to TcpP through interactions with its wing domain (Morgan et al. 2019; Crawford et al. 2003).

Recently an NMR structure of the DNA binding domain of ToxR was solved, showing it forms a wHTH domain. Similar to the CadC DNA binding domain, the C-terminal end formed an extra strand in the β-sheet domain (Gubensäk et al. 2021b) (Fig. 14.8a). The structure also suggests that to bind DNA helix 3 must lengthen as seen in OmpR and the OmpR-DNA structures (Fig. 14.8c) (Sadotra et al. 2021).

The ability of the ToxR-DBD to bind DNA was assessed by NMR. While the binding was weak, in the μM range, the ToxR-DBD had the highest affinity to the *toxT* promoter being almost 100 fold better than the binding affinity to the *ompU* and *ompT* promoters. This was interpreted as a consequence of ToxR having to capture the promoter for TcpP to bind to activate *toxT* transcription (Gubensäk et al. 2021b). If this is the case a similar mechanism should play out at the *vtrB/vttRB* promoter (Hubbard et al. 2016; Miller et al. 2016). It is interesting that the ToxR-DBD exhibits the highest affinity to a promoter that it does not directly activate. In addition, there are two caveats that point the way to future studies. The first is the ToxR-DBD is isolated from the full-length protein and there could be there are other determinants to DNA binding. The second

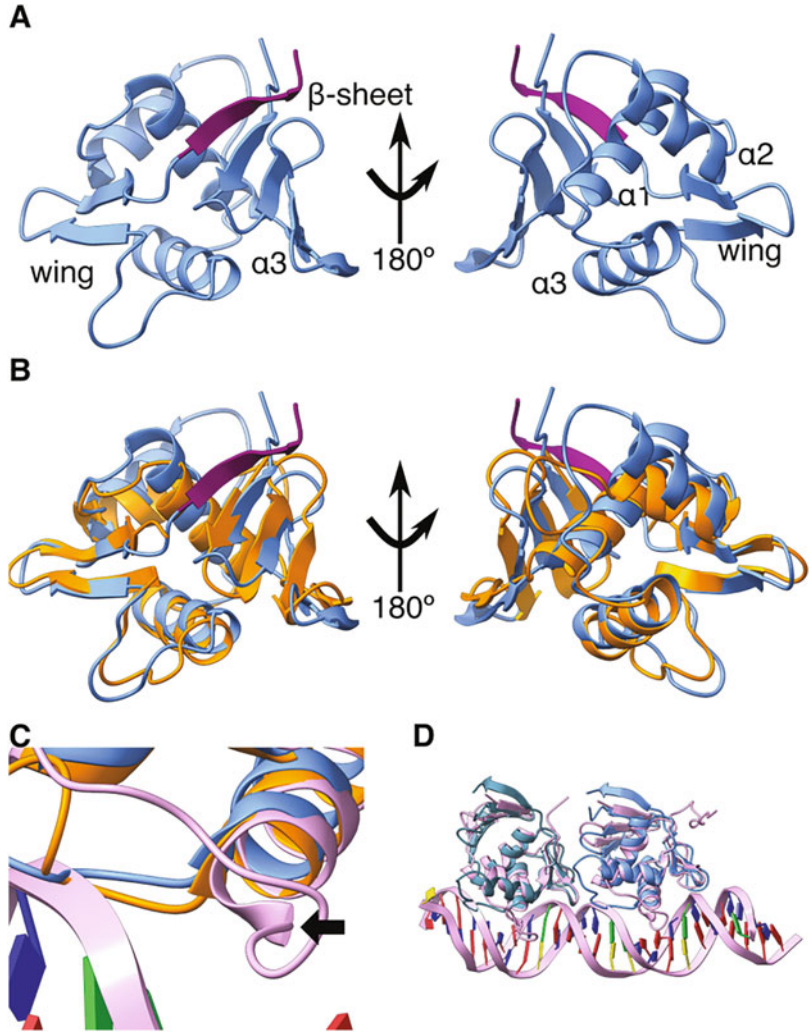


**Fig. 14.8** The NMR structure of the ToxR DNA binding domain. (**a**) In blue is the DNA binding domain of ToxR as determined by NMR (Gubensäk et al. 2021b), with the helices, wing, and β-sheet domain annotated. The extra β-strand is in purple. (**b**) Is an overlay of the ToxR (Gubensäk et al. 2021b), in blue, and the OmpR (Sadotra et al. 2021), in orange, DNA binding domains. Note α3 is about the same length in both structures. (**c**) Comparison of the ToxR (Gubensäk et al. 2021b), in blue, OmpR, in orange, and OmpR-DNA (Sadotra et al. 2021), in plum, DNA binding domains. The arrow points to the extension of α3 in the OmpR DNA bound structure, not present in OmpR alone or the ToxR DBD

is the DNA fragments were minimal binding domains and there are likely to be avidity effects with longer pieces of DNA.

It has been suggested that the DBDs of ToxR and TcpP interact using their wing domains (Krukonis and DiRita 2003; Morgan et al. 2019; Crawford et al. 2003). This view is supported by structures of OmpR, PhoB, and RstA bound to DNA in a head to tail fashion (Sadotra et al. 2021; Blanco et al. 2002; Li et al. 2014). However, because mutating residues in the wing domain can also impact DNA binding, it is difficult to distinguish changes in DNA binding from those involving protein–protein interactions. Arguing against direct contact, NMR experiments failed to observe direct interactions between the ToxR-DBD and the TcpP-DBD (Gubensäk et al. 2021b). Additional biochemical and structural studies are required to determine how ToxR and TcpP DBDs interact at the ToxT promoter.

### 14.4.3 Periplasmic Domain Structure

The role the ToxR periplasmic domain plays in activating ToxR remains unclear (Midgett et al. 2017, 2020; Lembke et al. 2020). The observation that ToxR activity increases in the presence of bile salts independent of an increase in protein expression levels has led to the hypothesis that the

ToxR periplasmic domain acts as a direct sensor of bile salts (Mey et al. 2012; Midgett et al. 2017). This idea is supported by the observation that the interaction of the ToxR periplasmic domain with ToxS is increased in the presence of bile salts even though the salts destabilize the ToxR periplasmic domain (Midgett et al. 2017). This led to hypothesis that destabilization of the ToxR periplasmic domain leads to increased binding to ToxS allowing for ToxR activation (Midgett et al. 2017). In addition to bile salts modulating the interaction between ToxR and ToxS, the ToxR periplasmic domain has two cysteines that primarily form an intrachain disulfide bond that increases the affinity of the ToxR periplasmic domain to ToxS (Midgett et al. 2020). While this would suggest ToxR and ToxS remain in contact throughout the infection cycle, there is a proposed model where ToxR is activated by forming disulfide linked homodimers, independent of ToxS (Lembke et al. 2020).

A thorough understanding of how the ToxR periplasmic domain changes conformation during activation could lead to the development of ToxR inhibitors which would block the ToxR-ToxS periplasmic domain interaction, leading to premature ToxR proteolysis. To visualize this interface, two structures of the ToxR periplasmic domain have been solved, one by X-ray crystallography using the *V. vulnificus* ToxR periplasmic domain, and the other by NMR using the *V. cholerae* ToxR periplasmic domain (Midgett et al. 2020; Gubensäk et al. 2021a) (Fig. 14.9a). The ToxR periplasmic domain structure from *V. vulnificus* consists of 5 β-strands and 2 α-helices. The 5 β-strands are arranged in a β-sheet with one face of the sheet facing the solvent and the other face packed against the two helices, which are connected by a disulfide bond. Interestingly, the loop connecting the last beta strand to the second helix (β5-α2 loop) is disordered, suggesting a role of the disulfide bond is to constrain the loop, α2, and the last β-strand (Midgett et al. 2020).

The structure of the *V. cholerae* ToxR periplasmic domain has a similar fold, though the last β-strand and α-helix are now part of a flexible C-terminal loop that was modeled in to wrap around the globular domain in two different directions to form the disulfide bond with the cysteine in helix 1 (Fig. 14.9b) (Gubensäk et al. 2021b). The exposed disulfide bond in these NMR structures would appear to be targets for DsbC cleavage to allow the protein to refold in a more stable configuration.

Both studies found that the ToxR periplasmic domain is a monomer in solution in both the oxidized and reduced forms (Midgett et al. 2020; Gubensäk et al. 2021a). Therefore, it seems likely that ToxR does not form dimers, even when in the active state. This helps to clarify a point of confusion, as previous microbiological and molecular studies have presented contradictory results involving dimerization, depending on the protein fusion construct and expression strains used (DiRita and Mekalanos 1991; Ottemann and Mekalanos 1995; Dziejman and Mekalanos 1994; Dziejman et al. 1999; Lembke et al. 2018; Kolmar et al. 1995). It is likely that other interactions drive ToxR proteins to come into proximity with each other, which the previous experiments were mimicking.

## 14.5 VtrAC

VtrA and VtrC are another transmembrane transcription factor, integral membrane periplasmic binding partner pair, like ToxRS, they are also conserved across the *Vibrio* family (Li et al. 2016a; Alam et al. 2010). VtrA is a structural homolog of ToxR and also responds to bile salts (Li et al. 2016a; Gotoh et al. 2010; Midgett et al. 2020). VtrC stabilizes VtrA, and both proteins are required for bile salt induction of the type 3 secretion system 2 expression (T3SS2) in *V. parahaemolyticus,* which is required for cytotoxicity (Li et al. 2016a; Kodama et al. 2010; Gotoh et al. 2010; Hubbard et al. 2016; Miller et al. 2016). However, regulation of expression of the secretion system is indirect, as VtrAC along with ToxR activates the expression of VtrB, a transmembrane transcription factor without a periplasmic domain, which activates transcription of the T3SS2 (Li et al. 2016a; Kodama et al. 2010; Gotoh et al. 2010; Hubbard et al. 2016; Miller et al. 2016; Alam et al. 2010). Interestingly, unlike ToxR, VtrAC is selective to which bile salts it responds. VtrAC has been shown to

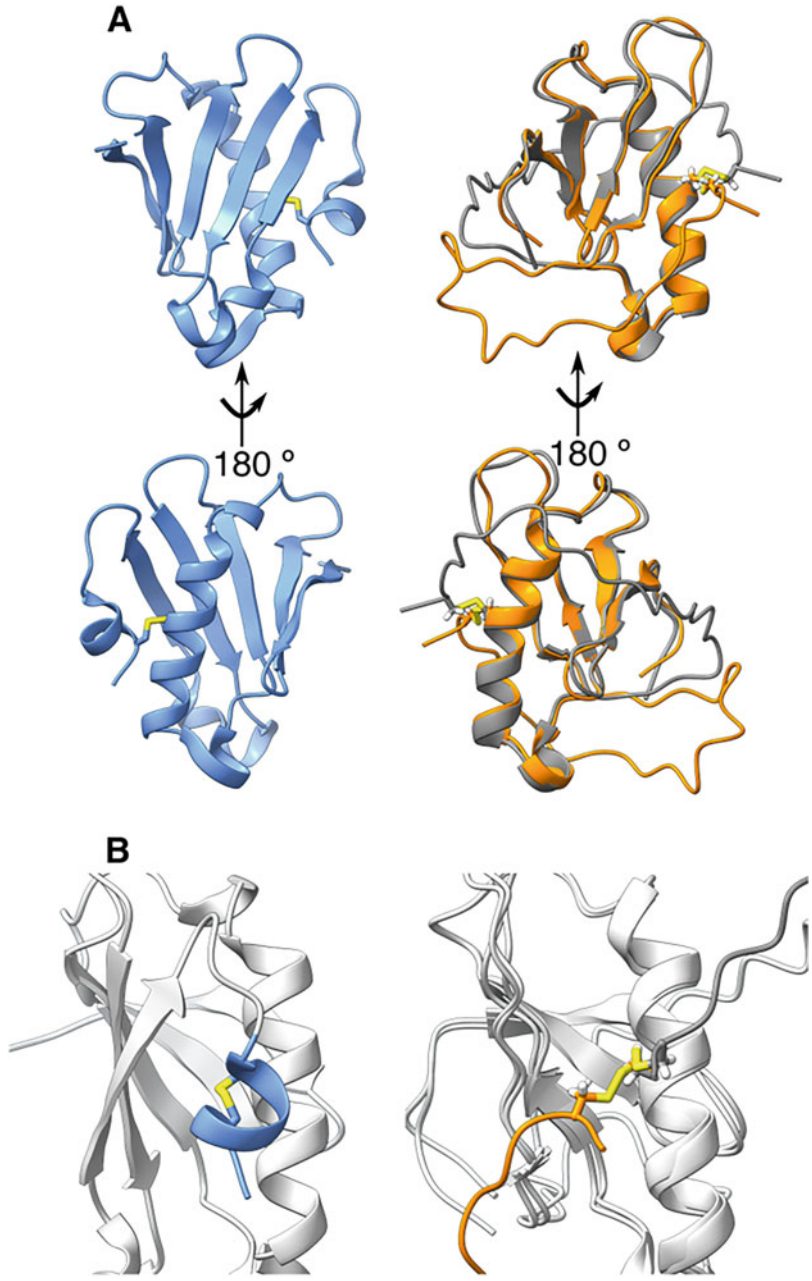


**Fig. 14.9** The structure of the ToxR periplasmic domain from *V. vulnificus* and *V. cholerae* determined by X-ray crystallography (Midgett et al. 2020) and NMR (Gubensäk et al. 2021a), respectively. (**a**) On the right in blue is the X-ray structure of the ToxR periplasmic domain (Midgett et al. 2020) from *V. vulnificus*. On the left, in orange and brown, are two of the NMR calculated structures of the ToxR periplasmic domain (Gubensäk et al. 2021a) from *V. cholerae*. Note that the C-terminal portion of this structure wraps around the protein from both directions. (**b**) Detail of the disulfide bond and the 7 C-terminal residues in each structure. In the X-ray structure, in blue, the C-terminal residues form a helix which helps shield the disulfide bond from the environment (Midgett et al. 2020). While the NMR structures (Gubensäk et al. 2021a), in orange and brown, have the disulfide exposed to the environment

respond most strongly to glycol- and taurodeoxycholate, then by deoxycholate, then glycol- and taurochenodeoxycholate, and finally the conjugated cholate salts (Li et al. 2016a; Gotoh et al. 2010). VtrAC activity is not induced by the unconjugated primary bile salts, which do activate ToxR (Midgett et al. 2017; Gotoh et al. 2010).

### 14.5.1 VtrA Periplasmic Domain Structure

The structure of VtrA in complex with VtrC was determined in 2016 (Li et al. 2016a). Interestingly, the VtrC periplasmic domain could not be expressed without the VtrA periplasmic domain, and the domains formed an obligate heterodimer. VtrC forms an 8-strand β-barrel that extends into

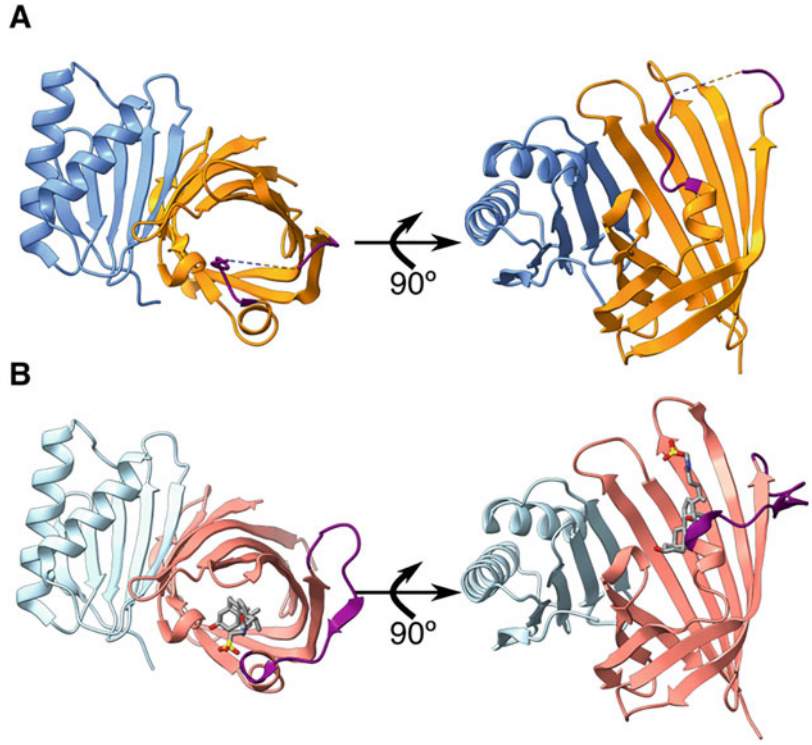


**Fig. 14.10** The structures of the apo-VtrAC and VtrAC-taurodeoxycholate bound periplasmic domains. (**a**) The apo-VtrAC periplasmic domain structure. VtrA is in blue and VtrC is in orange (Li et al. 2016a). (**b**) The VtrAC-taurodeoxycholate (TDC) bound periplasmic domain structures (Li et al. 2016a). VtrA is in light blue, VtrC is in coral, and the TDC is colored by element and shown in sticks. The residues (110–123) that are displaced in the TDC bound structure are colored in purple in both (**a**, **b**)

the last β-strand of the VtrA β-sheet. One side of the 5 stranded VtrA β-sheet interacts with VtrC and the other side with the two VtrA helices (Fig. 14.10) (Li et al. 2016a). Unlike the ToxR periplasmic domain, in which the two helices are held together with a disulfide bond, the two helices in VtrA are held together with non-covalent interactions (Li et al. 2016a; Midgett et al. 2020). VtrA and ToxR are clearly structural homologs despite the lack of sequence homology (Fig. 14.11) (Midgett et al. 2020), and a DALI search failed to find proteins with similar folds, indicating these periplasmic domains are part of a new family involved in environmental sensing (Li et al. 2016a; Midgett et al. 2020; Holm and Laakso 2016).

### 14.5.2 VtrC Periplasmic Domain Structure

A DALI search suggests VtrC is a member of the lipocalin family and therefore might bind a hydrophobic ligand, such as bile salts (Li et al. 2016a; Holm and Laakso 2016), and the structure of VtrAC in complex with bound taurodeoxycholate (TDC) was subsequently solved (Li et al. 2016a). Overall, the apo and ligand bound structures are similar, expect for a loop moves from the center of the β-barrel to the side of the barrel (residues 110–123), opening a pocket to bind TDC (Fig. 14.10b) (Li et al. 2016a). Despite the observed different activities of VtrAC for different bile salts (Gotoh et al. 2010), the structures do not provide much insight into this discrimination. For instance, deoxycholate and cholate only differ by cholate having a hydroxyl on C7 (Fig. 14.12b). Although the structure appears to be capable of accommodating such a difference, VtrAC is partially activated by the conjugated cholates and does not respond to cholate (Gotoh et al. 2010). In addition, the role of ligand conjugation in binding to VtrC is not clear, as the taurine conjugate does not make any contacts with VtrC (Fig. 14.12c). Given VtrAC is preferentially activated by conjugated bile salts (Gotoh et al. 2010) means there is more research to be done to understand how VtrAC discriminates between conjugated and unconjugated bile salts.

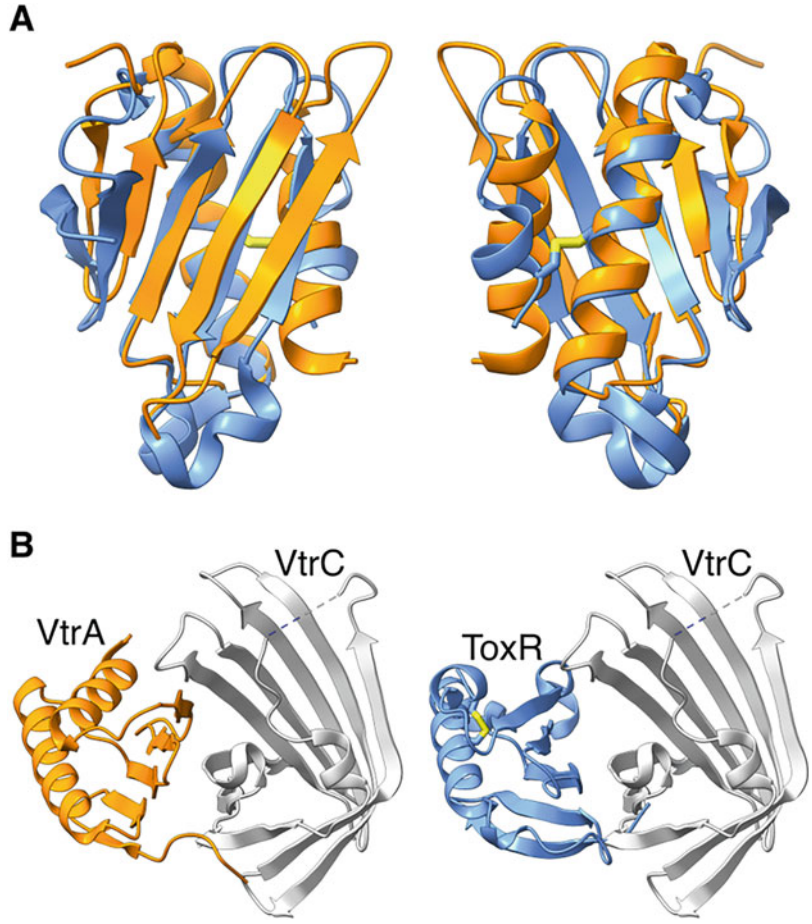


**Fig. 14.11** The ToxR and VtrA homology provide a model of how ToxR may interact with ToxS. (**a**) The X-ray structure of the ToxR periplasmic domain (Midgett et al. 2020), in blue, overlaid with the VtrA periplasmic domain structure (Li et al. 2016a), in orange. (**b**) On the left is the VtrAC structure (Li et al. 2016a) with VtrA in orange and VtrC in light gray. On the right the ToxR X-ray structure (Midgett et al. 2020), in blue, was aligned with the VtrAC structure (Li et al. 2016a). For clarity only ToxR, in blue, and VtrC, in light gray, are displayed

The structures also fail to clarify how VtrC passes information about its state to VtrA, as the apo and ligand-bound structures of VtrA are essentially the same, and there are no obvious changes to either the VtrA and VtrC interfaces (Li et al. 2016a) (Fig. 14.13). The question of how ligand binding to VtrC leads to VtrA activation remains unanswered.

These structures will allow us to address fundamental questions about virulence regulation, from the atomic level to organismal level to probe host–pathogen interactions. Chief among these questions, what is the role of the periplasmic domain interfaces in activating transcriptional regulation? Given the ToxRS periplasmic domains can be separately purified makes them the ideal model to investigate the relationship of individual residues to ligand mediated interactions, virulence gene expression, and intestinal colonization in animal models. Besides using genetic methods to determine if disrupting the periplasmic domain interface can interfere with virulence, these structures can provide the basis for small molecule screening to determine if pharmacological intervention is a viable method for inhibiting virulence, not only in *Vibrio*'s but also other bacteria genera with homologous systems, e.g. PsaEF from *Yersinia pseudotuberculosis* (Yang and Isberg 1997). Furthermore, the structures of the periplasmic and DNA binding domains provide a stepping stone to determining the full-length structures to understand how information is passed through the membrane, how ToxR oligomerizes on DNA, as well as how it functions with TcpP and VtrA to regulate gene expression.

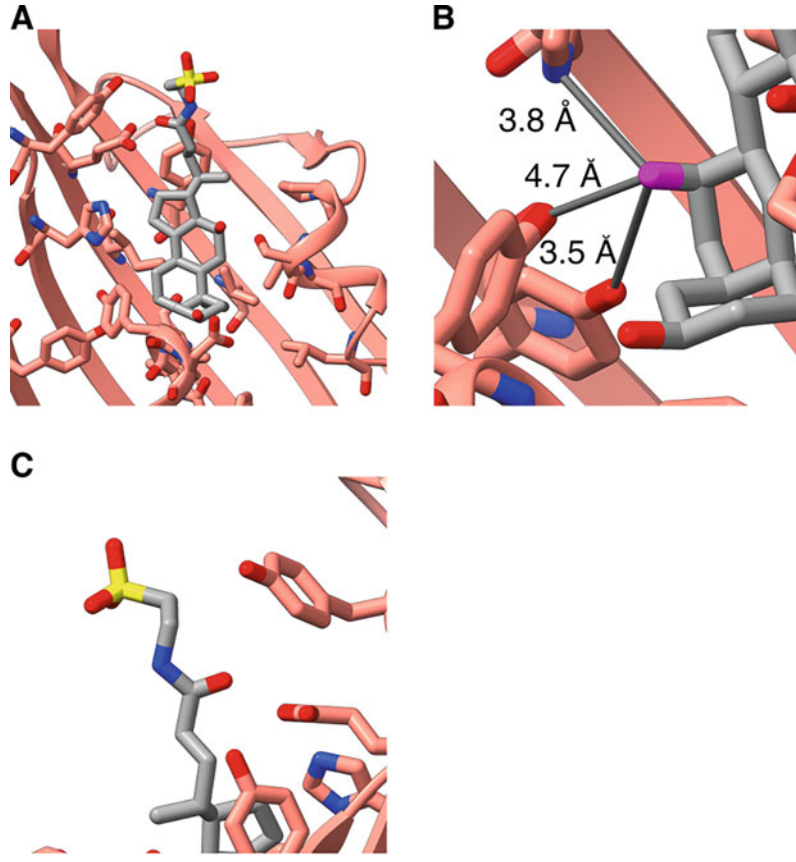


**Fig. 14.12** Exploring aspects of TDC binding to VtrC. (**a**) Overview of TDC binding to VtrC (Li et al. 2016a). The side chains of VtrC amino acids within 5 Å of TDC are displayed as sticks. (**b**) Taurocholate modeled in the binding pocket by adding an oxygen, in magenta, at the C7 position of TDC. Distances from the modeled oxygen to the closest side chains are shown and labeled. (**c**) Detail showing the taurine conjugate lack of interactions

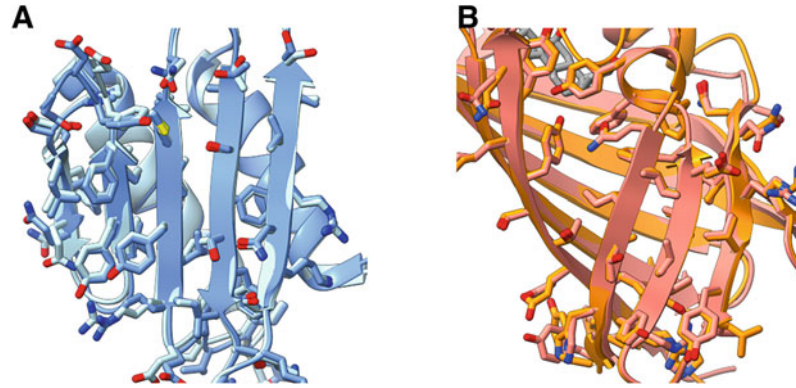


**Fig. 14.13** Detail of the interfaces used by VtrA and VtrC to bind each other in the apo and TDC bound state. (**a**) Overlay of VtrA in the apo state in blue and in the TDC bound state in light blue showing the interface used to bind VtrC (Li et al. 2016a). (**b**) Overlay of VtrC in the apo state in orange and the TDC bound state in coral showing the interface VtrC uses to bind VtrA (Li et al. 2016a)

## 14.6 ToxT

The transcriptional activity of ToxR and TcpP is regulated by bile salts and other stressors found in the intestine, and therefore it is activated fairly early in the colonization process (Mey et al. 2012; Midgett et al. 2017; Mathur and Waldor 2004; Miller and Mekalanos 1988; Fan et al. 2014; Yang et al. 2013). However, because virulence gene expression is a metabolically costly endeavor for *V. cholerae*, it makes sense to have virulence gene expression ready, but "on hold," until the environmental conditions are optimal. This is accomplished through the master regulator, ToxT, which activates expression of the two *V. cholerae* virulence factors, the toxin coregulated pilus (TCP) and cholera toxin (CT) (Higgins et al. 1992; DiRita et al. 1991). Early studies showed that ToxT was inhibited by the unsaturated fatty acid components of bile (Chatterjee et al. 2007; Gupta and Chowdhury 1997). This suggests while ToxT is being translated it is inhibited by the unsaturated fatty acids until the bacteria reach the intestinal surface. Inhibition of ToxT activity is thought to be achieved by blocking or destabilizing dimer formation (Shakhnovich et al. 2007; Cruite et al. 2019; Childers et al. 2011; Hung et al. 2005), because ToxT binds some promoters with two identified "Toxboxes," and other in vitro assays indicate ToxT activates transcription as a dimer (Withey and DiRita 2006; Bellair and Withey 2008; Shakhnovich et al. 2007; Cruite et al. 2019; Childers et al. 2011). The structural mechanism by which ToxT is inhibited by bile components was clarified when the structure of it was determined in 2010.

### 14.6.1 ToxT Structure

The crystal structure of ToxT shows a typical AraC protein fold with an N-terminal regulatory domain (NTD) containing a cupin fold composed of beta-strands, and three alpha helices making up the dimerization region. The C-terminal DNA binding domain (CTD) contains seven helices and two helix-turn-helix motifs (Fig. 14.14) (Lowden et al. 2010). Fortuitously, ToxT crystallized with a fatty acid ligand bound to its regulatory domain pocket. The ligand was identified as cis-palmitoleic acid (PAM), a fairly common, 16-carbon monounsaturated fatty acid (UFA). Analysis of the structure showed the negatively charged carboxylic acid head group of PAM bridged two positively charges lysine side chains, one from the NTD and the other from the CTD (Lowden et al. 2010). The presence of the PAM in the NTD pocket appears to stabilize ToxT in a closed conformation, in which the two domains are in close contact, burying the fatty acid along with the lysine side chains, and preventing the two DNA binding helices from assuming a parallel orientation necessary for DNA binding. It is hypothesized that upon release of the PAM ligand and its negatively charged head group, charge-charge repulsion of the two lysine side chains lead to an open conformation where the two domains separate, and the DNA binding helices are freed to assume a parallel orientation. Subsequent studies demonstrated that UFAs including PAM and oleic acid inhibit ToxT DNA binding, whereas saturated fatty acids do not (Lowden et al. 2010). A number of other ToxT crystal structures were subsequent solved, and they also contained UFA (Cruite et al. 2019; Li et al. 2016b).

### 14.6.2 ToxT Regulation

The model that emerged from these studies is that upon crossing the mucosal layer, the concentration of bile decreases, leading to a lower concentration of free UFA. Release of UFA from ToxT induces the open form, which is able to dimerize and bind to DNA, activating transcription of TCP and CT and inducing virulence. To date, efforts to crystallize ToxT in complex with DNA have failed, and unlike other AraC proteins with similar NTDs, ToxT did not crystallize as a dimer (Soisson et al. 1997; Shrestha et al. 2015; Midgett et al. 2021). Despite the lack of structural information on the ToxT dimer, a recent crystal structure of apo-ToxT provides some clues as to how

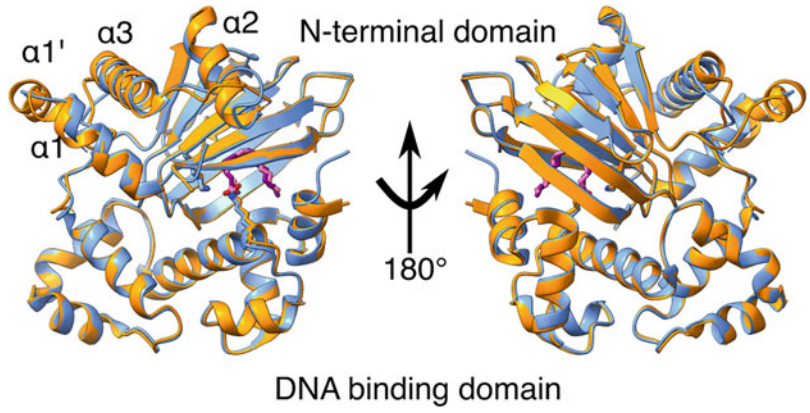


**Fig. 14.14** Overlay of two structures of ToxT. Overlay of the ToxT structure from (Lowden et al. 2010) in blue and (Li et al. 2016b) in orange. Note the high degree of similarity between the structures. The bound fatty acids are shown in purple, (Lowden et al. 2010), and pink, (Li et al. 2016b). The N-terminal and DNA binding domains are labeled. The alpha helices in the N-terminal domain that are potentially involved in dimerization are labeled. Note the absence of the α1' helix in the 3GBG structure

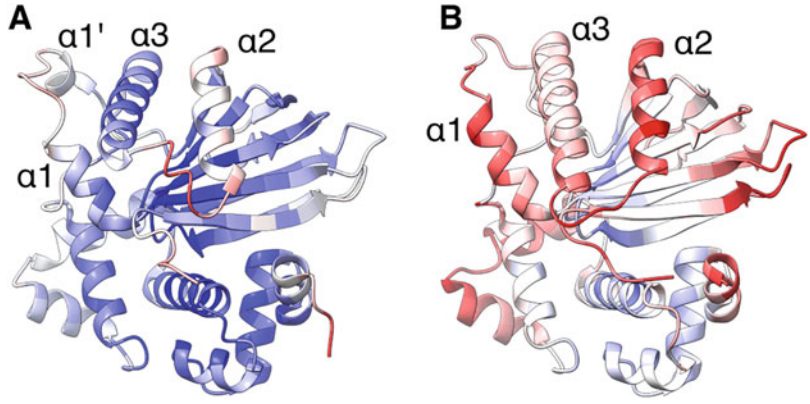


**Fig. 14.15** Comparison of the dimerization helices and overall B-factors of the ToxTenvK231A UFA bound structure to the apo-ToxTenvK231A structure (Cruite et al. 2019). (**a**) UFA bound and (**b**) apo $ToxT_{env}$K231A structures were overlaid in ChimeraX and colored by B-factor. The alpha helices in the N-terminal domain are numbered. Note α1' forms behind α3 in the apo-$ToxT_{env}$K231A structure and is no longer visible in this orientation

ligand binding might influence dimerization and lead to ToxT inhibition.

Given the critical role of the two lysine side chains in stabilizing the inactive form of ToxT, it made sense to characterize ToxT variants with these side chains altered, and it was shown that removing one of the positive charges by changing the C-terminal lysine to alanine reduced sensitivity to UFA (Cruite et al. 2019). Structural analysis of this mutant identified two different forms of ToxT, one resembling the previously determined wild-type structure and containing PAM, but importantly, another form without bound ligand. While the apo form was still monomeric, analysis of the structure showed significant changes in the crystallographic B-factors, particularly in the dimerization and DNA binding regions (Fig. 14.15). The model that emerged from this

work is that ToxT is regulated by a dynamics based allosteric mechanism in which loss of ligand leads to an increase in the overall flexibility of ToxT, enabling both the dimerization and DNA binding regions to assume their active conformations (Cruite et al. 2019).

### 14.6.3 ToxT Inhibitors

The UFA bound to the ToxT pocket assumes a distinct U-shape with the bulk of the fatty acid chain buried in the NTD pocket (Lowden et al. 2010). Interestingly, a known ToxT inhibitor, virstatin, somewhat resembles the folded configuration of the UFA, suggesting virstatin's inhibitory mechanism is similar to that of the natural ligand (Hung et al. 2005). Based on these observations, we hypothesized that molecules mimicking the U-shaped conformation of the bound fatty acid, that were also covalently constrained would bind more strongly to ToxT as they would be "prefolded" and not have to pay the thermodynamic cost in terms of the decrease in entropy associated with a dynamic, free fatty acid folding into a single conformation in the binding pocket. We therefore designed a series of inhibitors with bicyclic 6 carbon ring systems with different degrees of saturation. All contained both a methyl group and a carboxylic head group with different chain lengths attached to the rings (Woodbrey et al. 2017). These compounds outperformed virstatin in culture, and crystal structures showed the compounds bound in the pocket displacing the fatty acid, with the carboxylic acid forming ionic bonds with the two lysine side chains that bound the fatty acid carboxylate (Fig. 14.16) (Woodbrey et al. 2017). Analysis of the crystal structures indicated the pocket could accommodate a ligand with a longer tail, and subsequent compounds were shown to be even more effective than the initial series, and outperformed virstatin in mouse models of colonization at concentrations nontoxic to the bacteria (Woodbrey et al. 2018). While the fatty acids themselves are not chiral, the bound conformations they adopt are very specific and "chiral-like." By making use of this insight with different chemical scaffolds has led to even more potent inhibitors that have demonstrated the usefulness of mimicking constrained fatty acids to develop selective inhibitors to fatty acid binding proteins (Markham et al. 2021).

Studying ToxT has led to an exciting new hypothesis that fatty acids regulate virulence inducing ToxT homologs in many enteric pathogens, as has been shown to occur in *Salmonella enterica* and enterotoxigenic *Escherichia coli* (Midgett et al. 2021; Golubeva et al. 2016; Bosire et al. 2020). Interestingly, these proteins appear to bind fatty acids slightly differently (Lowden et al. 2010; Cruite et al. 2019; Midgett et al. 2021), suggesting there is flexibility in the binding pocket. This opens an opportunity to target these proteins specifically and individually, which would help to minimize the cross reactivity of any resulting antivirulence therapeutics. Moreover, the studies involving ToxT inhibitors provide proof-of-principle that mimicking constrained fatty acid conformations is a viable method to pharmacologically manipulate protein activity and is likely applicable to other diseases.

An outstanding question in this area is how ToxT, and indeed other AraC proteins, specifically and selectively bind to DNA. ToxT is thought to bind to adjacent Tox-boxes, which would require substantial rearrangements in the N-terminal domain and DNA binding domains (Cruite et al. 2019). While SAXS studies have provided some evidence of this (Cruite et al. 2019), a high-resolution ToxT-DNA complex structure would provide much needed insight into how these AraC-family proteins respond to ligand binding to regulate transcription.

## 14.7 Summary

This is an exhilarating time as great progress has been in understanding the structures of many of the proteins that contribute to regulating *Vibrio spp.* virulence. The insights gained from these studies and structures are stimulating further work to illuminate the basic molecular mechanisms by which the virulence cascade unfolds. This will enable the development of atomistic models of how protein interactions contribute to the host–pathogen communications that

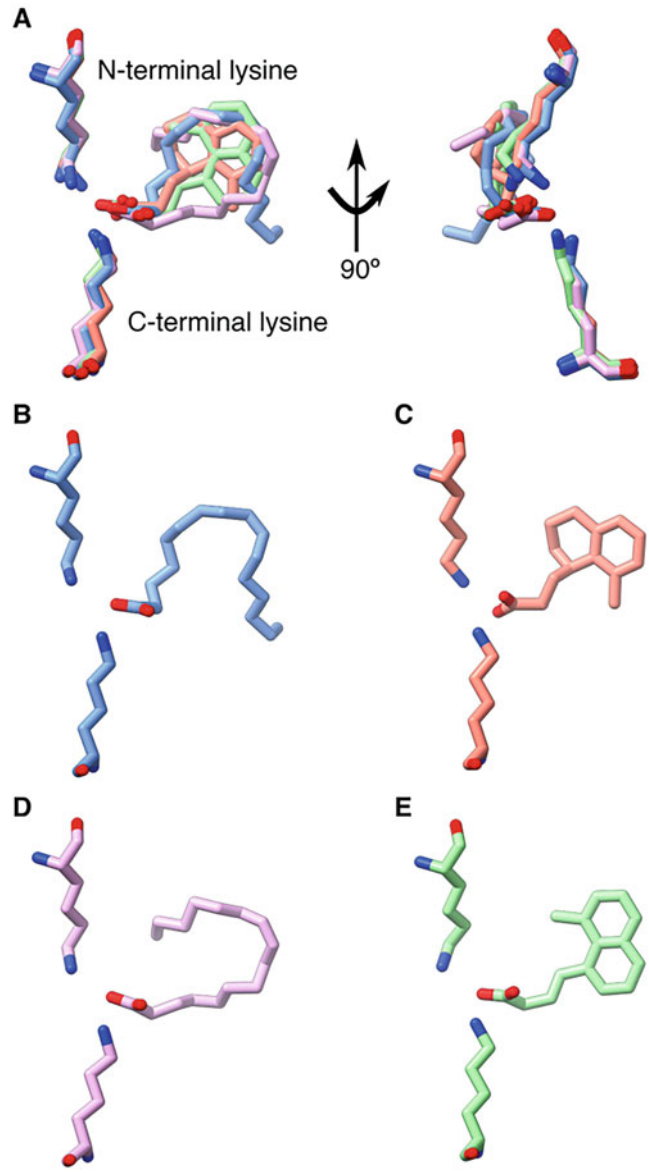


**Fig. 14.16** Conformations of various ligands bound to ToxT. (**a**) Overlay of the ligands with the two lysines from the N-terminal and C-terminal domains shown. (**b**) The palmitoleic acid bound to ToxT from the (Lowden et al. 2010) structure. (**c**) Compound 5a (Woodbrey et al. 2018) bound to ToxT. (**d**) UFA bound to $ToxT_{env}$ (Cruite et al. 2019). (**e**) Compound 3b (Woodbrey et al. 2018) bound to ToxT

leads to virulence. Additionally, such understanding will enable the development of new antivirulence compounds that can specifically target these pathogens and provide a foundation to target homologous proteins in other bacterial pathogens and in other disease states.

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# When *Vibrios* Take Flight: A Meta-Analysis of Pathogenic *Vibrio* Species in Wild and Domestic Birds

15

Andrea J. Ayala and C. Brandon Ogbunugafor

**Abstract**

Of the over 100 species in the genus *Vibrio*, approximately twelve are associated with clinical disease, such as cholera and vibriosis. Crucially, eleven of those twelve, including *Vibrio cholerae* and *Vibrio vulnificus*, have been isolated from birds. Since 1965, pathogenic *Vibrio* species have been consistently isolated from aquatic and ground-foraging bird species, which has implications for public health, as well as the One Health paradigm defined as an ecology-inspired, integrative framework for the study of health and disease, inclusive of environmental, human, and animal health. In this meta-analysis, we identified 76 studies from the primary literature which report on or examine birds as hosts for pathogenic *Vibrio* species. We found that the burden of disease in birds was most commonly associated with *V. cholerae*, followed by *V. metschnikovii* and *V. parahaemolyticus.* Meta-analysis wide prevalence of our *Vibrio* pathogens varied from 19% for *V. parahaemolyticus* to 1% for *V. mimicus.* Wild and domestic birds were both affected, which may have implications for conservation, as well as agriculturally associated avian species. As pathogenic *Vibrios* become more abundant throughout the world as a result of warming estuaries and oceans, susceptible avian species should be continually monitored as potential reservoirs for these pathogens.

**Keywords**

*Vibrio* spp. · Wild birds · Disease · Pathogenic · Ecology

## 15.1 Introduction

Waterborne pathogens around the globe are experiencing a period of unprecedented global change, with the Vibrionaceae categorized among the most climate-sensitive families of aquatic prokaryotes (Hofstra 2011; Lipp et al. 2002). Evidence continues to mount concerning the uptick in the abundance, distribution, and phenology of the Vibrionaceae, since rising temperatures, humidity, and precipitation have led to their increased survival and rates of replication (Wittman and Flick 1995; Montánchez and Kaberdin 2020; Vezzulli et al. 2020). Within this family resides the genus *Vibrio*, a genetically diverse group of gram-negative, motile, and facultatively anaerobic bacteria that are endemic to marine and estuarine waters (Pruzzo et al. 2005). With over 100 named species in the *Vibrio* genus,

A. J. Ayala · C. B. Ogbunugafor (✉)
Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT, USA
e-mail: andrea.ayala@yale.edu; brandon.ogbunu@yale.edu



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approximately twelve are known to be pathogenic to human hosts (Huehn et al. 2014). Specifically, eleven of the twelve, i.e., *V. alginolyticus*, *V. cholerae*, *V. cincinnatiensis*, *V. hollisae*, e.g., *Grimontia hollisae*, *V. furnissii*, *V. mimicus*, *V. parahaemolyticus*, *V. vulnificus*, *V. harveyi*, *V. scophthalmi,* and *V. metschnikovii,* are the causative agents of human vibriosis, a term that incorporates a broad range of clinical signs (Ramamurthy et al. 2014; Igbinosa and Okoh 2008; Morris and Acheson 2003). These pathogenic species arguably include some of the greatest public health burdens worldwide, and over the last 40 years, the incidence of *Vibrio* infections has strikingly increased (Rodrick 1991; Baker-Austin et al. 2010, 2017). The continued rise of the incidence and prevalence of *Vibrio* pathogens has contributed to an unprecedented worldwide health burden of enteric, diarrheal diseases (Levy et al. 2018; Semenza 2020). Yet, the Vibrionaceae are not only expanding their breadth throughout the human population—over the last one hundred and fifty years, but it also appears that the *Vibrio* genus is expanding its niche into avian hosts, with ensuing implications for the One Health paradigm, and how we contextualize "human" diseases (Sekyere et al. 2020; Destoumieux-Garzón et al. 2018; Jeamsripong et al. 2020; Sweet et al. 2021).

During the fifth pandemic of cholera (1881–1886), the bacteriologist Gamelaia reported a disease afflicting Rock Pigeons (*Columba livia*) and domestic chickens (*Gallus gallus*) in southern Russia. It was described as "a disease of fowls," of which the etiological agent was indistinguishable by morphological examination from *Vibrio cholerae* (Gamaleia 1888; Henze 2010). This etiological agent would eventually be classified as *Vibrio metschnikovii*, and by the early twenty-first century, it would be considered one of the twelve pathogenic *Vibrio* species that cause disease in human hosts (Huehn et al. 2014; Skerman et al. 1980; Tantillo et al. 2004). The occurrence of another pathogenic *Vibrio* isolated from birds would not be reported until 1966, when individual species from the Gifu and Higashiyama Zoos (Table 15.2) in Japan tested positive by culture for Biotypes 1 and 2 of *Vibrio parahaemolyticus* (Ose 1967). Pathogenesis in these zoo birds was not reported (Ose 1967). Based on the literature, it is possible that the bird that had cultured positive for Biotype 2 of *Vibrio parahaemolyticus* was in fact shedding *V. alginolyticus* (Sakazaki 1968; Fu et al. 2016).

Pathogenic *Vibrio* species can be lethal in human hosts. For example, *Vibrio vulnificus* is a causative agent of primary septicemia with a case fatality rate of up to fifty percent (Bross et al. 2007; Oliver et al. 2012). As one of the world's leading causes of seafood-related deaths, *Vibrio vulnificus* is an opportunistic pathogen which causes high morbidity and mortality among the immunocompromised and those with liver disease (Oliver and Sadowsky 2015; López-Pérez et al. 2021). *Vibrio cholerae*, specifically serotypes O1 and O139, is likely the most well-known member of the *Vibrio* genus. It is a pathogen which has generated seven pandemics since 1817, and whose ecology and pathogenesis has been covered in depth (Hu et al. 2016; Mutreja et al. 2011; Colwell 1996; Colwell and Spira 1992; Faruque et al. 2003; Almagro-Moreno and Taylor 2014). *Vibrio parahaemolyticus* is a leading cause of seafood-borne illness, with clinicians reporting gastroenteritis and septicemia as the primary causes of morbidity among patients (Li et al. 2019; Letchumanan et al. 2014). *V. alginolyticus* and *V. fluvialis* are considered emerging pathogens and have been linked to gastroenteritis and extraintestinal infections (Ramamurthy et al. 2014; Mustapha et al. 2013). The remaining *Vibrio* species, *V. cincinnatiensis*, *V. hollisae*, e.g., *Grimontia hollisae*, *V. furnissii*, *V. mimicus*, *V. harveyi*, *V. scophthalmi,* and *V. metschnikovii* have been linked to sporadic reports of disease in human hosts (Magalhães et al. 1996; Jean-Jacques et al. 1981; Jäckel et al. 2020; Edouard et al. 2009; Derber et al. 2011; Kay et al. 2012), however, that does not diminish their clinical, veterinary, or ecological importance.

The One Health paradigm is a collaborative endeavor that seeks to incorporate the health of the environment, animals, and humans, given the

understanding that the resilience of these individual components is integrated and intertwined (Patz and Hahn 2013; Conrad et al. 2009). Thus, the emergence of pathogenic *Vibrio* species in birds is not only of public health importance (Islam et al. 2020; Laviad-Shitrit et al. 2017), but also of significance to avian disease ecology, as little is known of the large-scale effects that members of the *Vibrio* genus may have upon species of conservation concern (Friend 2006; Friend et al. 2001). With few exceptions (Almagro-Moreno and Taylor 2014), little is also known concerning the role that birds may play in the maintenance or potentially cyclical contamination of the brackish, aquatic reservoirs they share with other susceptible vertebrates (Fukushima and Seki 2004; Ogg et al. 1989; West et al. 1983; Vezzulli et al. 2010; Meszaros et al. 2020). Therefore, in this chapter, we build on the work of prior investigators who have identified the presence of pathogenic *Vibrio* species in avian species to a) identify the avian taxas most likely to excrete the pathogens and b) assess the prevalence of individuals in each community or sample that do so. We further examine whether pathogenic *Vibrio* species are immunogenic and/or pathogenic to birds and the duration that they shed in experimental infection studies. We focus not just on studies that have identified the presence or absence of pathogenic *Vibrio* species in wild avian communities, but also include experimental infection and immunity studies. Our objective is to provide a baseline framework by which avian disease ecologists, wildlife management professionals, veterinarians, and One Health personnel can evaluate and/or mitigate the potential risks of emerging pathogenic *Vibrio* species within our wild birds.

## 15.2 Methods

Using Google Scholar and Web of Science (Wiethoelter et al. 2015; Murray et al. 2016), we searched for peer-reviewed studies, pre-prints, abstracts, and graduate theses in which the antibodies against or the antigens of pathogenic *Vibrio* species were isolated from birds or from the avian environment (e.g., the isolation of *Vibrio* pathogens from avian fecal matter or their nests) (Ayala et al. 2020). In our search strategy, we used the following search terms and Boolean operators: "*Vibrio* pathogen of interest" OR "*Vibrio* pathogen and disease" and "bird*" OR "wild bird*" OR "avian" ($n = 14{,}950$). In our search, we systematically searched for studies that examined evidence of infection by the following members of the *Vibrio* genus: *V. alginolyticus, V. cholerae, V. cincinnatiensis, V. furnissii, V. mimicus, V. parahaemolyticus, V. vulnificus, V. harveyi, V. scophthalmi,* and *V. metschnikovii.* Given the relatively recent taxonomic reclassifications of *Grimontia hollisae* (Thompson et al. 2003) and *Photobacterium damselae* (Smith et al. 1991) from the genus *Vibrio*, we also included these pathogens in our analysis. We included experimental infection studies, case reports, and cross-sectional studies published between 1966 and January 1, 2022. In our analysis, we excluded sources that did not serve as primary literature involving investigations of pathogenic *Vibrio* infections in domestic or wild birds, e.g., retrospective studies and review papers (retrospective and review papers, $n = 24$) as well as duplicates (duplicates, $n = 81$). We also excluded any literature without a clear diagnostic and physiological association between domestic or wild birds as hosts of our *Vibrio* species of interest (exclusion criteria, $n = 14{,}845$).

From each study, we extracted the following elements when available: avian species or taxonomic grouping, *Vibrio* species, country, year the study was conducted or published, the number of birds tested or infected, the number of birds from which *Vibrio* was isolated, and the method(s) by which pathogenic *Vibrio* species were identified. Where possible, we identified the prevalence of our *Vibrio* pathogens of interest, including presence and absence, to determine study-wide prevalence. We also reported serotypes and/or clinically important strains when that information was provided. We further identified whether our *Vibrio* pathogens of interest were associated with clinical signs or avian mortality events, however,

unless specifically stated in the text, we could not determine whether our *Vibrio* pathogens of interest were the causative agent(s) of reported morbidity or mortality.

## 15.3 Results

### 15.3.1 Literature Review

We identified 76 studies from the primary literature that met our inclusion criteria, resulting in 425 study records of avian species or taxonomic groups from which the presence or absence of pathogenic *Vibrio* species was recorded (identified species, $n = 171$, identified families, $n = 46$). In our meta-analysis, a study record ranges from a single examined bird to 565 examined birds, which reflects the same species or taxonomic group that was tested for a single pathogenic *Vibrio* species of interest that originated from within the same study (Tables 15.1, 15.2, 15.3, 15.4, and 15.5). In our meta-analysis, 29 countries were represented, constituting all continents except for Antarctica. Of the fifty-five years between 1966 and the start of 2022, studies were either published in or conducted in 41 of them. Sixteen study records did not provide sufficient information from which to identify *Vibrio* prevalence, as either the number of birds, flocks, nests, or sites were incompletely reported or collected samples were pooled. When the *Vibrio* pathogens of interest were either not named or not classified into species, it was categorized as "*Vibrio* spp.".

### 15.3.2 *Vibrio cholerae*

A total of 41 studies in the primary literature examined the role of *Vibrio cholerae* in wild or domestic birds (Laviad-Shitrit et al. 2017; Ogg et al. 1989; Aberkane et al. 2015; Aguirre et al. 1991, 1992; Akond et al. 2008; Bisgaard and Kristensen 1975; Bisgaard et al. 1978; Bogomolni et al. 2008; Buck 1990; Cardoso et al. 2014, 2018; Contreras-Rodríguez et al. 2019; Cox 1992; Fernández-Delgado et al. 2016; Hirsch et al. 2020; Ismail et al. 2021; Metzner et al. 2004; Laviad-Shitrit et al. 2018; Lee et al. 1982; Huamanchumo 2021; Mehmke et al. 1992; Myatt and Davis 1989; Páll et al. 2021; Rodríguez et al. 2010; Roges et al. 2010; Sack 1973; Salles et al. 1976; Sanyal et al. 1974; Schlater et al. 1981; Siembieda et al. 2011; Singh et al. 1975; Song et al. 1998; Strauch et al. 2020; Szeness et al. 1979; Watanabe et al. 2002; Watts et al. 1993; Sakazaki and Shimada 1977; Wobeser and Rainnie 1987; Zhang et al. 1996; Zheng et al. 2020, 2021). One hundred and fifty-six study records investigated the presence or absence of *Vibrio cholerae,* with the most common technique utilized being culture alone, followed by culture and PCR, or PCR coupled with sequencing. Twenty-five study records reported multiple serotypes from the same species, in the same study. Five of those 25 study records dealt with individual birds who either excreted or displayed multiple serotypes within the same fecal or blood sample or were sampled longitudinally and subsequently cultured positive for different serotypes at different times (Ogg et al. 1989; Singh et al. 1975). Four study records reported the detection of *Vibrio cholerae* O1 from within one or a flock of birds, with Inaba and Ogawa each reported at least once (Ogg et al. 1989; Rodríguez et al. 2010; Salles et al. 1976; Sanyal et al. 1974). Serotype distribution across species or taxonomic groups was not analyzed, since the number of birds positive for each serotype was usually not provided in the primary literature. We do, however, report the available data in Table 15.1. The most common "type" of *Vibrio cholerae* reported from birds was non-O1/O139, however, many study records did not identify or report the serotype of *Vibrio cholerae* that was isolated. *Vibrio cholerae* O139 was not reported from any study.

One hundred and seven ($n = 107$) species were examined for the presence of *Vibrio cholerae* antigens or antibodies. An additional sixteen records were extracted from the literature, but we were not able to identify those study records to species. The Anatidae (waterfowl) represented 49 study records, Laridae (gulls and terns) represented 20 study records, and the Ardeidae (shorebirds) represented 10 study records. Within our meta-analysis, 5492 reported

**Table 15.1** This table details the study records extracted from the 41 studies that investigated birds as hosts for Vibrio cholerae, both O1 El tor and Non-O1. In the table are provided the common name, the scientific name, the family, the number of birds that tested positive for Vibrio cholerae, the total number of birds examined for V. cholerae, and the prevalence for that record. We also provide the country that the study was performed in, as well as the year the study was conducted or published. For studies that reported clinical signs or mortality, we placed a "Yes" in that column. For serotypes or strains, there were several primary categories, for Non-O1, this involves any Vibrio cholerae that was tested for the properties specific to V. cholerae O1. The designation O999 was used when no typing was utilized beyond species, e.g., the serotype of V. cholerae was not reported or investigated further. NA was provided when the prevalence was zero, and no serotype was applicable. When multiple serotypes were reported, but not tied to records, we specified that in the column, and when serotypes were provided and could be tied to a record, we noted that in the column as well

| Avian species | Scientific name | Family | Number tested | Total examined | Prevalence | Country | Year | Clinical signs or mortality | Serotypes or strains | Citation |
|---|---|---|---|---|---|---|---|---|---|---|
| American Coot | *Fulica americana* | Rallidae | 0 | 13 | 0% | United States | 1990 | None reported | NA | Cox (1992) |
| American Coot | *Fulica americana* | Rallidae | 44 | 117 | 38% | United States | 1989 | None reported | O11, O14, O22, O23, O44, O106, O148, O176, O359, O999 | Ogg et al. (1989) |
| American Coot | *Fulica americana* | Rallidae | 1 | 2 | 50% | United States | 1989 | None reported | Non-O1 | Siembieda et al. (2011) |
| American Flamingo | *Phoenicopterus ruber* | Phoenicopteridae | 2 | 34 | 6% | Venezuela | 2012 | None reported | Non-O1 | Fernández-Delgado et al. (2016) |
| American Flamingo | *Phoenicopterus ruber* | Phoenicopteridae | 3 | 9 | 33% | Mexico | 1991 | None reported | Non-O1 | Aguirre et al. (1991) |
| American Oystercatcher | *Haematopus palliatus* | Haematopodidae | 4 | 15 | 27% | Peru | 2021 | None reported | Non-O1 | Huamanchumo (2021) |
| American Robin | *Turdus americana* | Turdidae | 0 | 13 | 0% | United States | 2007 | Unknown | NA | Siembieda et al. (2011) |
| American White Pelican | *Pelecanus erythrorhynchos* | Pelecanidae | 10 | 51 | 20% | United States | 1989 | None reported | O17, O19, O22, O999 | Ogg et al. (1989) |
| American Widgeon | *Mareca americana* | Anatidae | 0 | 41 | 0% | United States | 1990 | No | NA | Cox (1992) |
| Anas spp. | Unknown | Anatidae | 14 | 30 | 47% | Egypt | 2020 | None reported | Non-O1 | Ismail et al. (2021) |
| Aves spp. | Unknown | Unknown | 3 | 23 | 13% | Venezuela | 2012 | None reported | Non-O1 | Fernández-Delgado et al. (2016) |
| Aves spp. | Unknown | Unknown | 54 | 343 | 16% | Japan | 2000 | None reported | Non-O1 | Watanabe et al. (2002) |

(continued)

**Table 15.1** (continued)

| Avian species | Scientific name | Family | Number tested | Total examined | Prevalence | Country | Year | Clinical signs or mortality | Serotypes or strains | Citation |
|---|---|---|---|---|---|---|---|---|---|---|
| Aves spp. | Unknown | Unknown | 2 | 7 | 29% | Denmark | 1977 | None reported | Non-O1 | Song et al. (1998) |
| Aves spp. | Unknown | Unknown | 61 | 123 | 50% | China | 1994 | None reported | Non-O1 | Song et al. (1998) |
| Aves spp. | Unknown | Unknown | 7 | 8 | 88% | Australia | 1989 | None reported | Non-O1 | Myatt and Davis (1989) |
| Black Skimmer | *Rynchops niger* | Laridae | 0 | 1 | 0 | Venezuela | 2012 | None reported | NA | Fernández-Delgado et al. (2016) |
| Black-and-white Magpie | *Pica hudsonia* | Corvidae | 2 | 5 | 40% | Romania | 2021 | No | O999 | Páll et al. (2021) |
| Black-bellied Whistling Duck | *Dendrocygna autumnalis* | Anatidae | 3 | 110 | 3% | Mexico | 1992 | No | Non-O1 | Aguirre et al. (1992) |
| Black-crowned Night Heron | *Nycticorax nycticorax* | Ardeidae | 3 | 19 | 16% | United States | 1989 | None reported | O60, O999 | Ogg et al. (1989) |
| Black-crowned Night Heron | *Nycticorax nycticorax* | Ardeidae | 4 | 16 | 25% | United States | 2007 | None reported | Non-O1 | Siembieda et al. (2011) |
| Black-crowned Night Heron | *Nycticorax nycticorax* | Ardeidae | 7 | 8 | 88% | Israel | 2018 | None reported | O94 | Laviad-Shitrit et al. (2018) |
| Black-headed Gull | *Chroicocephalus ridibundus* | Laridae | 0 | 5 | 0% | Israel | 2018 | None reported | NA | Laviad-Shitrit et al. (2018) |
| Black-headed Gull | *Chroicocephalus ridibundus* | Laridae | 7 | 142 | 5% | United Kingdom | 1979 | None reported | Multiple, not reported | Lee et al. (1982) |
| Black-headed Gull | *Chroicocephalus ridibundus* | Laridae | 4 | 55 | 7% | China | 2018 | Yes | Non-O1 | Zheng et al. (2021) |
| Black-necked Stilt | *Himantopus mexicanus* | Charadriidae | 0 | 55 | 0% | China | 2018 | Yes | NA | Zheng et al. (2021) |
| Black-winged Stilt | *Himantopus himantopus* | Charadriidae | 1 | 55 | 2% | China | 2018 | Yes | Non-O1 | Zheng et al. (2021) |
| Blue-winged Teal | *Anas discors* | Anatidae | 1 | 80 | 1% | United States | 1990 | No | O999 | Cox (1992) |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Blue-winged Teal | *Anas discors* | Anatidae | 12 | 39 | 31% | United States | 1989 | None reported | O19, O22, O23, O44, O106, O355, O360, O999 | Ogg et al. (1989) |
| Brown Pelican | *Pelecanus occidentalis* | Pelecanidae | 1 | 6 | 17% | United States | 2007 | None reported | Non-O1 | Siembieda et al. (2011) |
| Bufflehead | *Bucephala albeola* | Anatidae | 2 | 12 | 17% | United States | 1990 | No | O999 | Cox (1992) |
| California Gull | *Larus californicus* | Laridae | 1 | 93 | 1% | United States | 1989 | None reported | O22, O31, O340, O999 | Ogg et al. (1989) |
| Canada Goose | *Branta canadensis* | Anatidae | 2 | 4 | 50% | Canada | 1985 | Yes | Non-O1 | Wobeser and Rainnie (1987) |
| Cattle Egret | *Bubulcus ibis* | Ardeidae | 1 | 6 | 17% | United States | 1989 | None reported | O312, O359 | Ogg et al. (1989) |
| Charadriiformes spp. | Unknown | Unknown | 1 | 7 | 14% | Venezuela | 2012 | None reported | Non-O1 | Fernández-Delgado et al. (2016) |
| Cinnamon Teal | *Spatula cyanoptera* | Anatidae | 1 | 2 | 50% | United States | 1989 | None reported | O106, O999 | Ogg et al. (1989) |
| Coal Tit | *Periparus ater* | Paridae | 0 | 4 | 0% | Germany | 1988 | None reported | NA | Mehmke et al. (1992) |
| Mourning Dove and Band-tailed Pigeon | *Zenaida macroura* and *Patagioenas fasciata* | Columbidae | 0 | 16 | 0% | United States | 2007 | None reported | NA | Siembieda et al. (2011) |
| Common Blackbird | *Turdus merula* | Turdidae | 0 | 1 | 0% | Germany | 1988 | None reported | NA | Mehmke et al. (1992) |
| Common Chaffinch | *Fringilla coelebs* | Fringillidae | 0 | 3 | 0% | Romania | 2021 | None reported | NA | Páll et al. (2021) |
| Common Goldeneye | *Bucephala clangula* | Anatidae | 0 | 1 | 0% | United States | 1990 | No | NA | Cox (1992) |
| Common Goldeneye | *Bucephala clangula* | Anatidae | 1 | 1 | 100% | United States | 2007 | Unknown | Non-O1 | Siembieda et al. (2011) |
| Common Greenshank | *Tringa nebularia* | Scolopacidae | 0 | 2 | 0% | Romania | 2021 | No | NA | Páll et al. (2021) |
| Common Kingfisher | *Alcedo atthis* | Alcedinidae | 0 | 1 | 0% | Romania | 2021 | No | NA | Páll et al. (2021) |

(continued)

**Table 15.1** (continued)

| Avian species | Scientific name | Family | Number tested | Total examined | Prevalence | Country | Year | Clinical signs or mortality | Serotypes or strains | Citation |
|---|---|---|---|---|---|---|---|---|---|---|
| Common Moorhen | *Gallinula chloropus* | Rallidae | 0 | 6 | 0% | United States | 1990 | No | NA | Cox (1992) |
| Common Murre | *Uria aalge* | Alcidae | 3 | 31 | 10% | United States | 2007 | None reported | Non-O1 | Siembieda et al. (2011) |
| Common Pochard | *Aythya ferina* | Anatidae | 1 | 55 | 2% | China | 2018 | Yes | Non-O1 | Zheng et al. (2021) |
| Common Snipe | *Gallinago gallinago* | Scolopacidae | 2 | 3 | 67% | Romania | 2021 | None reported | O999 | Páll et al. (2021) |
| Common Tern | *Sterna hirundo* | Laridae | 2 | 2 | 100% | Germany | 2019 | Yes | Non-O1 | Strauch et al. (2020) |
| Domestic chicken | *Gallus gallus* | Phasianidae | 24 | 250 | 10% | Bangladesh | 2008 | None reported | O999 | Akond et al. (2008) |
| Domestic chicken | *Gallus gallus* | Phasianidae | 4 | 22 | 18% | India | 1973 | None reported | O1 *El tor* Ogawa, Non-O1 | Sanyal et al. (1974) |
| Domestic chicken | *Gallus gallus* | Phasianidae | 2 | 7 | 29% | Unknown | 1977 | None reported | Non-O1 | Sakazaki and Shimada (1977) |
| Domestic chicken | *Gallus gallus* | Phasianidae | 27 | 60 | 45% | Egypt | 2020 | None reported | Non-O1 | Ismail et al. (2021) |
| Domestic chicken | *Gallus gallus* | Phasianidae | 134 | 212 | 63% | Ghana | 1973 | Yes | O1 *El tor* Ogawa | Salles et al. (1976) |
| Domestic chicken | *Gallus gallus* | Phasianidae | 1 | 1 | 100% | India | 1975 | None reported | O999 | Singh et al. (1975) |
| Domestic Duck | Unknown | Anatidae | 3 | 17 | 18% | Denmark | 1975 | Yes | Non-O1 | Bisgaard and Kristensen (1975) |
| Domestic Duck | Unknown | Anatidae | 51 | 80 | 64% | Denmark | 1978 | None reported | O34, O60, O34, O54, O2, O57 | Bisgaard et al. (1978) |
| Domestic Duck | *Anas platyrhynchos* | Anatidae | 1 | 1 | 100% | Germany | 2017 | Yes | Non-O1 | Hirsch et al. (2020) |
| Domestic Duck | *Anas platyrhynchos* | Anatidae | 1 | 1 | 100% | Germany | 2016 | Yes | Non-O1 | Hirsch et al. (2020) |
| Domestic Duck | *Anas platyrhynchos* | Anatidae | 1 | 1 | 100% | Germany | 2011 | Yes | Non-O1 | Hirsch et al. (2020) |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Domestic Duck | *Anas platyrhynchos* | Anatidae | 1 | 1 | 100% | Germany | 1996 | Yes | Non-O1 | Hirsch et al. (2020) |
| Domestic Goose | *Anser anser* | Anatidae | 1 | 2 | 50% | United States | 1980 | Yes | Non-O1 | Schlater et al. (1981) |
| Domestic Turkey | *Meleagris gallopavo* | Meleagrididae | 0 | 20 | 0% | Egypt | 2020 | None reported | NA | Ismail et al. (2021) |
| Domestic Turkey | *Meleagris gallopavo* | Meleagrididae | 1 | 23 | 4% | Germany | 2012 | Yes | Non-O1 | Metzner et al. (2004) |
| Double-crested Cormorant | *Phalacrocorax auritus* | Phalacrocoracidae | 32 | 84 | 38% | United States | 1989 | None reported | O12, O22, O23, O106, O360, O999 | Ogg et al. (1989) |
| Eared Grebe | *Podiceps nigricollis* | Podicipedidae | 1 | 2 | 50% | United States | 2007 | Unknown | Non-O1 | Siembieda et al. (2011) |
| Eastern Spot-billed Duck | *Anas zonorhyncha* | Anatidae | 3 | 55 | 5% | China | 2018 | Unknown | Non-O1 | Zheng et al. (2021) |
| Elegant Tern | *Thalasseus elegans* | Laridae | 0 | 51 | 0% | Mexico | 2012 | None reported | NA | Contreras-Rodríguez et al. (2019) |
| Eurasian Blue Tit | *Cyanistes caeruleus* | Paridae | 0 | 5 | 0% | Germany | 1988 | None reported | NA | Mehmke et al. (1992) |
| Eurasian Hobby | *Falco subbuteo* | Falconidae | 2 | 2 | 100% | Romania | 2021 | No | O999 | Páll et al. (2021) |
| Eurasian Nuthatch | *Sitta europaea* | Paridae | 1 | 5 | 20% | Germany | 1988 | None reported | Non-O1 | Mehmke et al. (1992) |
| Eurasian Sparrowhawk | *Accipiter nisus* | Accipitridae | 1 | 2 | 50% | Romania | 2021 | No | O999 | Páll et al. (2021) |
| Eurasian Tree Sparrow | *Passer montanus* | Passeridae | 0 | 1 | 0% | Germany | 1988 | None reported | NA | Mehmke et al. (1992) |
| Eurasian Tree Sparrow | *Passer montanus* | Passeridae | 2 | 2 | 100% | Romania | 2021 | No | O999 | Páll et al. (2021) |
| Eurasian Whimbrel | *Numenius phaeopus* | Scolopacidae | 0 | 9 | 0% | Peru | 2021 | None reported | NA | Huamanchumo (2021) |
| Herring Gull | *Larus argentatus* | Laridae | 1 | 55 | 2% | China | 2018 | Yes | Non-O1 | Zheng et al. (2021) |
| Herring Gull | *Larus argentatus* | Laridae | 5 | 88 | 6% | United Kingdom | 1979 | None reported | Multiple, not reported | Lee et al. (1982) |

(continued)

**Table 15.1** (continued)

| Avian species | Scientific name | Family | Number tested | Total examined | Prevalence | Country | Year | Clinical signs or mortality | Serotypes or strains | Citation |
|---|---|---|---|---|---|---|---|---|---|---|
| Franklin's Gull | *Leucophaeus pipixcan* | Laridae | 8 | 41 | 20% | United States | 1989 | None reported | O11, O22, O43, O999 | Ogg et al. (1989) |
| Gadwall | *Mareca strepera* | Anatidae | 10 | 200 | 5% | United States | 1990 | No | O999 | Cox (1992) |
| Gadwall | *Mareca strepera* | Anatidae | 2 | 3 | 67% | United States | 1990 | None reported | O22 | Ogg et al. (1989) |
| Great Black-backed Gull and Herring Gull | *Larus marinus* and *Larus argentatus* | Laridae | 23 | 45 | 56% | United States | 1990 | None reported | Non-O1 | Buck (1990) |
| Great Blue Heron | *Ardea herodias* | Ardeidae | 11 | 24 | 46% | United States | 1989 | None reported | O22, O1 *El tor* Ogawa | Ogg et al. (1989) |
| Great Cormorant | *Phalacrocorax carbo* | Phalacrocoracidae | 1 | 55 | 2% | China | 2017 | Unknown | Non-O1 | Zheng et al. (2021) |
| Great Cormorant | *Phalacrocorax carbo* | Phalacrocoracidae | 9 | 11 | 82% | Israel | 2017 | None reported | Non-O1 | Laviad-Shitrit et al. (2017) |
| Great Spotted Woodpecker | *Dendrocopos major* | Picidae | 0 | 1 | 0% | Germany | 1988 | None reported | Non-O1 | Mehmke et al. (1992) |
| Great Tit | *Parus major* | Paridae | 4 | 12 | 33% | Germany | 1988 | None reported | Non-O1 | Mehmke et al. (1992) |
| Greater Black-backed Gull | *Larus marinus* | Laridae | 3 | 15 | 20% | United Kingdom | 1979 | None reported | Multiple, not reported | Lee et al. (1982) |
| Greater Scaup | *Aythya marila* | Anatidae | 0 | 1 | 0% | United States | 1990 | None reported | NA | Cox (1992) |
| Greater Scaup | *Aythya marila* | Anatidae | 2 | 3 | 67% | United States | 2007 | Unknown | NA | Siembieda et al. (2011) |
| Greater Yellowlegs | *Tringa melanoleuca* | Scolopacidae | 6 | 6 | 100% | Venezuela | 2006 | None reported | O1 *El tor* Inaba | Rodríguez et al. (2010) |
| Green Heron | *Butorides virescens* | Ardeidae | 2 | 8 | 25% | United States | 2007 | Unknown | Non-O1 | Siembieda et al. (2011) |
| Green-winged Teal | *Anas carolinensis* | Anatidae | 2 | 35 | 6% | United States | 1989 | None reported | O22, O106, O999 | Ogg et al. (1989) |
| Green-winged Teal | *Anas carolinensis* | Anatidae | 15 | 255 | 6% | United States | 1990 | No | O999 | Siembieda et al. (2011) |

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|---|---|---|---|---|---|---|---|---|---|---|
| Grey Gull | *Leucophaeus modestus* | Laridae | 0 | 5 | 0% | Peru | 2021 | None reported | NA | Huamanchumo (2021) |
| Guanay Cormorant | *Leucocarbo bougainvilliorum* | Phalacrocoracidae | 0 | 1 | 0% | Peru | 2021 | None reported | NA | Huamanchumo (2021) |
| Heermann's Gull | *Larus heermanni* | Laridae | 0 | 44 | 0% | Mexico | 2012 | None reported | NA | Contreras-Rodríguez et al. (2019) |
| Heron spp. | Unknown | Ardeidae | 1 | 6 | 17% | Venezuela | 2012 | None reported | Non-O1 | Fernández-Delgado et al. (2016) |
| Hooded Merganser | *Lophodytes cucullatus* | Anatidae | 0 | 3 | 0% | United States | 1990 | No | O999 | Cox (1992) |
| House Crow | *Corvus splendens* | Corvidae | 3 | 3 | 100% | India | 1970 | None reported | O17, O23, O308 | Sack (1973) |
| Ibis spp. | Unknown | Threskiornithidae | 0 | 3 | 0% | Venezuela | 2012 | None reported | NA | Fernández-Delgado et al. (2016) |
| Indian Spot-billed Duck | *Anas poecilorhyncha* | Anatidae | 0 | 55 | 0% | China | 2018 | Yes | Non-O1 | Zheng et al. (2021) |
| Indian Spot-billed Duck | *Anas poecilorhyncha* | Anatidae | 2 | 2 | 100% | China | 1996 | Yes | Multiple, not reported | Zhang et al. (1996) |
| Kelp Gull | *Larus dominicanus* | Laridae | 5 | 11 | 45% | Brazil | 2009 | None reported | Non-O1 | Cardoso et al. (2018) |
| Killdeer | *Charadrius vociferus* | Charadriidae | 0 | 2 | 0% | Peru | 2021 | None reported | NA | Huamanchumo (2021) |
| Killdeer | *Charadrius vociferus* | Charadriidae | 13 | 15 | 87% | United States | 1989 | None reported | O22, O106, O999 | Ogg et al. (1989) |
| Large-billed Tern | *Phaetusa simplex* | Laridae | 1 | 1 | 100% | Venezuela | 2012 | None reported | Non-O1 | Fernández-Delgado et al. (2016) |
| Laughing Gull | *Leucophaeus atricilla* | Laridae | 1 | 7 | 14% | Peru | 2021 | None reported | Non-O1 | Huamanchumo (2021) |
| Least Sandpiper | *Calidris minutilla* | Scolopacidae | 0 | 1 | 0% | Venezuela | 2012 | None reported | NA | Fernández-Delgado et al. (2016) |
| Lesser Scaup | *Aythya affinis* | Anatidae | 0 | 8 | 0% | United States | 1990 | No | NA | Cox (1992) |

(continued)

**Table 15.1** (continued)

| Avian species | Scientific name | Family | Number tested | Total examined | Prevalence | Country | Year | Clinical signs or mortality | Serotypes or strains | Citation |
|---|---|---|---|---|---|---|---|---|---|---|
| Little Egret | *Egretta garzetta* | Ardeidae | 5 | 11 | 45% | Israel | 2018 | None reported | O13, O16, O36, O128, O171, O40, O6, O21, O123, O193, O36 | Laviad-Shitrit et al. (2018) |
| Magnificent Frigatebird | *Fregata magnificens* | Fregatidae | 0 | 1 | 0% | Venezuela | 2012 | None reported | NA | Fernández-Delgado et al. (2016) |
| Mallard | *Anas platyrhynchos* | Anatidae | 0 | 32 | 0% | United States | 1990 | None reported | NA | Cox (1992) |
| Mallard | *Anas platyrhynchos* | Anatidae | 27 | 306 | 9% | United States | 1989 | None reported | O22, O48, O999 | Ogg et al. (1989) |
| Mallard | *Anas platyrhynchos* | Anatidae | 2 | 12 | 17% | United States | 2007 | Unknown | Non-O1 | Siembieda et al. (2011) |
| Mallard | *Anas platyrhynchos* | Anatidae | 17 | 29 | 59% | Hungary | 1979 | None reported | Non-O1 | Szeness et al. (1979) |
| Mallard | *Anas platyrhynchos* | Anatidae | 2 | 2 | 100% | China | 1996 | None reported | Multiple, not reported | Zhang et al. (1996) |
| Manx Shearwater | *Puffinus puffinus* | Procellariidae | 1 | 35 | 3% | Brazil | 2014 | None reported | Non-O1 | Cardoso et al. (2014) |
| Mottled Duck | *Anas fulvigula* | Anatidae | 2 | 123 | 2% | United States | 1990 | None reported | O999 | Cox (1992) |
| Mute Swan | *Cygnus olor* | Anatidae | 2 | 2 | 100% | United Kingdom | 1979 | None reported | O2 | Lee et al. (1982) |
| Neotropic Cormorant | *Phalacrocorax brasilianus* | Phalacrocoracidae | 0 | 3 | 0% | Venezuela | 2012 | None reported | NA | Fernández-Delgado et al. (2016) |
| Northern Pintail | *Anas acuta* | Anatidae | 0 | 53 | 0% | United States | 1990 | No | NA | Cox (1992) |
| Northern Pintail | *Anas acuta* | Anatidae | 5 | 56 | 9% | United States | 1989 | None reported | O22, O360, O999 | Ogg et al. (1989) |
| Northern Shoveler | *Spatula clypeata* | Anatidae | 3 | 37 | 8% | United States | 1990 | No | O999 | Cox (1992) |

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|---|---|---|---|---|---|---|---|---|---|---|
| Parus spp. | Unknown | Paridae | 4 | 22 | 18% | Germany | 1988 | None reported | Non-O1 | Mehmke et al. (1992) |
| Passerine spp. | Unknown | Unknown | Not reported | Not reported | NA | Germany | 1988 | None reported | Non-O1 | Mehmke et al. (1992) |
| Passerines | Unknown | Unknown | 0 | 24 | 0% | United States | 2007 | Unknown | NA | Siembieda et al. (2011) |
| Peruvian Booby | *Sula variegata* | Sulidae | 1 | 1 | 100% | Peru | 2021 | None reported | Non-O1 | Huamanchumo (2021) |
| Peruvian Pelican | *Pelecanus thagus* | Pelecanidae | 1 | 1 | 100% | Peru | 2021 | None reported | Non-O1 | Huamanchumo (2021) |
| Red-footed Falcon | *Falco vespertinus* | Falconidae | 2 | 2 | 100% | Romania | 2021 | None reported | Non-O1 | Páll et al. (2021) |
| Redhead | *Aythya americana* | Anatidae | 0 | 1 | 0% | United States | 1990 | None reported | O999 | Cox (1992) |
| Redhead | *Aythya americana* | Anatidae | 1 | 17 | 6% | United States | 1989 | None reported | O999 | Ogg et al. (1989) |
| Rhinoceros auklet | *Cerorhinca monocerata* | Alcidae | 1 | 1 | 100% | United States | 2007 | Unknown | Non-O1 | Siembieda et al. (2011) |
| Ring-billed Gull | *Larus delawarensis* | Laridae | 17 | 112 | 15% | United States | 1989 | None reported | O1 (not distinguished to Ogawa or Inaba), O16, O22, O102, O106, O999 | Ogg et al. (1989) |
| Ring-necked Duck | *Aythya collaris* | Anatidae | 4 | 37 | 11% | United States | 1990 | No | O999 | Cox (1992) |
| Rook | *Corvus frugilegus* | Corvidae | 1 | 28 | 4% | United Kingdom | 1989 | None reported | Multiple, not reported | Lee et al. (1982) |
| Ruddy Shelduck | *Tadorna ferruginea* | Anatidae | 2 | 5 | 40% | United States | 2007 | Unknown | Non-O1 | Siembieda et al. (2011) |
| Ruddy Shelduck | *Tadorna ferruginea* | Anatidae | 25 | 55 | 45% | China | 2018 | Yes | Non-O1 | Zheng et al. (2021) |
| Seabirds | Unknown | Unknown | 1 | 192 | 0.5% | United States | 2008 | None reported | Non-O1 | Bogomolni et al. (2008) |
| Seabirds | Unknown | Unknown | 4 | 116 | 3% | Brazil | 2010 | None reported | Non-O1 | Cardoso et al. (2018) |
| Seabirds | Unknown | Unknown | Unknown | 69 | NA | Brazil | 2010 | None reported | O999 | Roges et al. (2010) |

(continued)

**Table 15.1** (continued)

| Avian species | Scientific name | Family | Number tested | Total examined | Prevalence | Country | Year | Clinical signs or mortality | Serotypes or strains | Citation |
|---|---|---|---|---|---|---|---|---|---|---|
| Semipalmated Plover | *Charadrius semipalmatus* | Scolopacidae | 0 | 1 | 0% | Venezuela | 2012 | None reported | NA | Fernández-Delgado et al. (2016) |
| Semipalmated Sandpiper | *Charadrius pusilla* | Scolopacidae | 1 | 2 | 50% | Venezuela | 2012 | None reported | Non-O1 | Fernández-Delgado et al. (2016) |
| Snow Goose | *Chen caerulescens* | Anatidae | 0 | 22 | 0% | United States | 1990 | No | NA | Cox (1992) |
| Snowy Egret | *Egretta thula* | Ardeidae | 1 | 13 | 8% | United States | 1989 | None reported | O14, O340, O999 | Ogg et al. (1989) |
| Song Thrush | *Turdus philomelos* | Turdidae | 1 | 2 | 50% | Romania | 2021 | No | Non-O1 | Páll et al. (2021) |
| Squacco Heron | *Ardeola ralloides* | Ardeidae | 1 | 2 | 50% | Romania | 2021 | No | Non-O1 | Páll et al. (2021) |
| Surf Scoter | *Melanitta perspicillata* | Anatidae | 6 | 11 | 54% | United States | 2007 | Unknown | Non-O1 | Siembieda et al. (2011) |
| Wading bird spp. | Unknown | Unknown | 2 | 2 | 100% | United States | 2007 | Unknown | Non-O1 | Siembieda et al. (2011) |
| Wattled Jacana | *Jacana jacana* | Jacanidae | 2 | 6 | 33% | Venezuela | 2012 | None reported | Non-O1 | Fernández-Delgado et al. (2016) |
| Western Grebe | *Aechmophorus occidentalis* | Podicipedidae | 2 | 12 | 17% | United States | 2007 | Unknown | Non-O1 | Siembieda et al. (2011) |
| Western Gull | *Larus occidentalis* | Laridae | 1 | 7 | 14% | United States | 2007 | Unknown | Non-O1 | Siembieda et al. (2011) |
| Western Jackdaw | *Coloeus monedula* | Corvidae | 0 | 4 | 0% | Romania | 2021 | None reported | NA | Páll et al. (2021) |
| Western Scrub-Jay | *Aphelocoma californica* | Corvidae | 0 | 12 | 0% | United States | 2007 | Unknown | NA | Siembieda et al. (2011) |
| White Pekin Duck | *Anas platyrhynchos domesticus* | Anatidae | 2 | 187 | 1% | United States | 1993 | Yes | O999 | Watts et al. (1993) |

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|---|---|---|---|---|---|---|---|---|---|---|
| White-faced Ibis | *Plegadis chihi* | Threskiornithidae | 1 | 30 | 3% | United States | 1989 | None reported | O14 | Ogg et al. (1989) |
| Wilson's Phalarope | *Phalaropus tricolor* | Scolopacidae | 6 | 11 | 55% | United States | 1989 | None reported | O999 | Ogg et al. (1989) |
| Wilson's Plover | *Charadrius wilsonia* | Charadriidae | 16 | 16 | 100% | Venezuela | 2006 | None reported | Non-O1 | Rodríguez et al. (2010) |
| Wood Duck | *Aix sponsa* | Anatidae | 0 | 3 | 0% | United States | 1990 | None reported | NA | Cox (1992) |
| Wood Duck | *Aix sponsa* | Anatidae | 0 | 55 | 0% | China | 2018 | Yes | NA | Zheng et al. (2021) |
| Yellow-legged Gull | *Larus michahellis* | Laridae | 1 | 93 | 1% | France | 2013 | None reported | Non-O1 | Aberkane et al. (2015) |

**Table 15.2** This table details the study records extracted from the 20 studies that investigated birds as hosts for *Vibrio parahaemolyticus*. In the table are provided the common name, the scientific name, the family, the number of birds that tested positive for *Vibrio parahaemolyticus*, the total number of birds examined for *V. parahaemolyticus*, and the prevalence for that record. We also provide the country that the study was performed in, as well as the year the study was conducted or published. For studies that reported clinical signs or mortality, we placed a "Yes" in that column. For serotypes or strains, we identified the strain when it was available. NA was provided when the prevalence was zero, and no strain was applicable

| Avian Species | Scientific name | Family | Number positive | Total examined | Prevalence | Country | Year | Clinical signs or mortality | Serotypes or strains | Citation |
|---|---|---|---|---|---|---|---|---|---|---|
| American Coot | *Fulica americana* | Rallidae | 0 | 13 | 0% | United States | 1990 | None reported | Not specified | Cox (1992) |
| American Widgeon | *Mareca americana* | Anatidae | 3 | 41 | 7% | United States | 1990 | None reported | Not specified | Cox (1992) |
| Anas spp. | Unknown | Anatidae | 57 | 171 | 33% | Japan | 2005 | None reported | Not specified | Miyasaka et al. (2006) |
| Aves spp. | Unknown | Unknown | 0 | 298 | 0% | China | 2019 | None reported | Not specified | Zheng et al. (2020) |
| Aves spp. | Unknown | Unknown | 29 | 343 | 8% | Japan | 2000 | None reported | Not specified | Watanabe et al. (2002) |
| Aves spp. | Unknown | Unknown | 1 | 8 | 13% | India | 1986 | None reported | Not specified | Karunasagar et al. (1986) |
| Aves spp. | Unknown | Unknown | 4 | 8 | 50% | Australia | 1989 | None reported | Not specified | Myatt and Davis (1989) |
| Black-and-White Magpie | *Pica hudsonia* | Corvidae | 0 | 5 | 0% | Romania | 2021 | None reported | NA | Páll et al. (2021) |
| Black-crowned Crane | *Balearica pavonina* | Gruidae | 1 | 1 | 100% | Japan | 1965 | None reported | Biotype 1 | Ose (1967) |
| Black-crowned Night Heron | *Nycticorax nycticorax* | Ardeidae | 0 | 2 | 0% | Romania | 1989 | None reported | NA | Páll et al. (2021) |
| Black-headed Gull | *Chroicocephalus ridibundus* | Laridae | 68 | 125 | 54% | Japan | 2005 | None reported | NA | Miyasaka et al. (2006) |
| Black-tailed Godwit | *Limosa limosa* | Scolopacidae | 1 | 2 | 50% | China | 2005 | None reported | Not specified | Wang et al. (2021) |
| Blue-winged Teal | *Anas discors* | Anatidae | 0 | 80 | 0% | United States | 1990 | No | NA | Cox (1992) |
| Brown Pelican | *Pelecanus occidentalis* | Pelecanidae | 20 | 42 | 48% | United States | 1990 | None reported | Not specified | Buck (1990) |

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|---|---|---|---|---|---|---|---|---|---|---|
| Bufflehead | *Bucephala albeola* | Anatidae | 1 | 12 | 8% | United States | 1990 | No | Not specified | Cox (1992) |
| Charadriiformes spp. | Unknown | Unknown | 71 | 112 | 64% | China | 2019 | None reported | Not specified | Zheng et al. (2020) |
| Chinese Bamboo Partridge | *Bambusicola thoracicus* | Phasianidae | 1 | 1 | 100% | Japan | 1966 | None reported | Biotype 2 | Ose (1967) |
| Common Goldeneye | *Bucephala clangula* | Anatidae | 0 | 1 | 0% | United States | 1990 | No | NA | Cox (1992) |
| Common Greenshank | *Tringa nebularia* | Scolopacidae | 6 | 26 | 23% | China | 2017 | None reported | Not specified | Fu et al. (2019) |
| Common Greenshank | *Tringa nebularia* | Scolopacidae | 2 | 5 | 40% | China | 2015 | None reported | Not specified | Wang et al. (2021) |
| Common Loon | *Gavia immer* | Gaviidae | 1 | 434 | 0.2% | United States | 1994 | Yes | Not specified | Forrester et al. (1997) |
| Common Moorhen | *Gallinula chloropus* | Rallidae | 0 | 6 | 0% | United States | 1990 | No | NA | Cox (1992) |
| Common Teal | *Anas crecca* | Anatidae | 1 | 1 | 100% | Japan | 2005 | None reported | Not specified | Miyasaka et al. (2006) |
| Cormorant spp. | Unknown | Phalacrocoracidae | 0 | 23 | 0% | China | 2018 | None reported | NA | Zheng et al. (2020) |
| Crested Fireback | *Lophura ignita* | Phasianidae | 1 | 1 | 100% | Japan | 1965 | None reported | Biotype 1 | Ose (1967) |
| Crested Ibis | *Nipponia nippon* | Threskiornithidae | 1 | 1 | 100% | Japan | 1965 | None reported | Biotype 1 | Ose (1967) |
| Demoiselle Crane | *Grus virgo* | Gruidae | 0 | 125 | 0% | China | 2019 | None reported | NA | Zheng et al. (2020) |
| Elegant Tern | *Thalasseus elegans* | Laridae | 1 | 51 | 2% | Mexico | 2012 | None reported | Not specified | Contreras-Rodríguez et al. (2019) |
| Eurasian Blackcap | *Sylvia atricapilla* | Sylviidae | 0 | 1 | 0% | Romania | 2021 | None reported | NA | Páll et al. (2021) |
| Eurasian Blue Tit | *Cyanistes caeruleus* | Paridae | 0 | 2 | 0% | Romania | 2021 | None reported | NA | Páll et al. (2021) |
| Eurasian Curlew | *Numenius arquata* | Scolopacidae | 1 | 2 | 50% | China | 2015 | None reported | NA | Wang et al. (2021) |

(continued)

**Table 15.2** (continued)

| Avian Species | Scientific name | Family | Number positive | Total examined | Prevalence | Country | Year | Clinical signs or mortality | Serotypes or strains | Citation |
|---|---|---|---|---|---|---|---|---|---|---|
| Eurasian Sparrowhawk | *Accipiter nisus* | Falconidae | 0 | 2 | 0% | Romania | 2021 | None reported | NA | Páll et al. (2021) |
| Gadwall | *Mareca strepera* | Anatidae | 16 | 200 | 8% | United States | 1990 | No | Not specified | Cox (1992) |
| Garden Warbler | *Sylvia borin* | Sylviidae | 0 | 4 | 0% | Romania | 2021 | No | NA | Páll et al. (2021) |
| Great Argus | *Argusianus argus* | Phasianidae | 1 | 1 | 100% | Japan | 1965 | None reported | Biotype 1 | Ose (1967) |
| Great Black-backed Gull and Herring Gull | *Larus marinus* and *Larus argentatus* | Laridae | 23 | 45 | 56% | United States | 1990 | None reported | Not specified | Buck (1990) |
| Greater Scaup | *Aythya marila* | Anatidae | 0 | 1 | 0% | United States | 1990 | None reported | NA | Cox (1992) |
| Green Pheasant | *Phasianus versicolor* | Phasianidae | 1 | 1 | 100% | Japan | 1965 | No | Not specified | Ose (1967) |
| Green-winged Teal | *Anas carolinensis* | Anatidae | 18 | 255 | 7% | United States | 1990 | No | Not specified | Siembieda et al. (2011) |
| Grey-hooded Parakeet | *Psilopsiagon aymara* | Psittacidae | 0 | 1 | 0% | Germany | 2020 | Yes | NA | Reuschel et al. (2020) |
| Helmeted Guineafowl | *Numida meleagris* | Numididae | 1 | 1 | 100% | Japan | 1965 | None reported | Biotype 1 | Ose (1967) |
| Heron spp. | Unknown | Ardeidae | 26 | 89 | 29% | China | 2018 | None reported | Not specified | Zheng et al. (2020) |
| Herring Gull, Laughing Gull, Ring-billed Gull | *Larus argentatus*, *Leucophaeus atricilla*, *Larus delawarensis* | Laridae | 29 | 42 | 69% | United States | 1990 | None reported | Not specified | Buck (1990) |
| Herring Gull and Ring-billed Gull | *Larus argentatus* and *Larus delawarensis* | Laridae | 23 | 45 | 56% | United States | 1990 | None reported | Not specified | Buck (1990) |
| Herring Gull and Black-backed Gull | *Larus argentatus* and *Larus crassirostris* | Laridae | 216 | 320 | 68% | Japan | 2005 | None reported | Not specified | Miyasaka et al. (2006) |
| Hooded Crow | *Corvus cornix* | Corvidae | 0 | 7 | 0% | Romania | 2021 | None reported | NA | Páll et al. (2021) |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Hooded Merganser | *Lophodytes cucullatus* | Anatidae | 0 | 3 | 0% | United States | 1990 | None reported | NA | Cox (1992) |
| Icterine Warbler | *Hippolais icterina* | Acrocephalidae | 0 | 1 | 0% | Romania | 2021 | None reported | NA | Páll et al. (2021) |
| Japanese Quail | *Coturnix coturnix* | Phasianidae | Unknown | Unknown | Unknown | Korea | 2011 | None reported | ATCC 17802 | Kassim et al. (2011) |
| Lady Amherst's Pheasant | *Chrysolophus amherstiae* | Phasianidae | 1 | 1 | 100% | Japan | 1965 | None reported | Not specified | Ose (1967) |
| Lesser Scaup | *Aythya affinis* | Anatidae | 0 | 8 | 0% | United States | 1990 | None reported | NA | Cox (1992) |
| Mallard | *Anas platyrhynchos* | Anatidae | 2 | 32 | 6% | United States | 1990 | None reported | Not specified | Cox (1992) |
| Mallard | *Anas platyrhynchos* | Anatidae | 72 | 267 | 27% | China | 2018 | None reported | Not specified | Zheng et al. (2020) |
| Mallard | *Anas platyrhynchos* | Anatidae | 33 | 73 | 45% | Japan | 2005 | None reported | Not specified | Miyasaka et al. (2006) |
| Manx Shearwater | *Puffinus puffinus* | Procellariidae | 7 | 35 | 20% | Brazil | 2014 | None reported | Not specified | Cardoso et al. (2014) |
| Budgerigar | *Melopsittacus undulatus* | Psittaculidae | 1 | 2 | 50% | Germany | 2020 | Yes | Not specified | Reuschel et al. (2020) |
| Mottled Duck | *Anas fulvigula* | Anatidae | 5 | 123 | 4% | United States | 1990 | None reported | NA | Cox (1992) |
| Northern Pintail | *Anas acuta* | Anatidae | 4 | 53 | 8% | United States | 1990 | None reported | Not specified | Cox (1992) |
| Northern Shoveler | *Spatula clypeata* | Anatidae | 3 | 37 | 8% | United States | 1990 | None reported | Not specified | Cox (1992) |
| Red-backed Shrike | *Lanius collurio* | Laniidae | 0 | 1 | 0% | Romania | 2021 | None reported | NA | Páll et al. (2021) |
| Red-crowned Crane | *Grus japonensis* | Gruidae | 0 | 26 | 0% | China | 2018 | None reported | NA | Zheng et al. (2020) |
| Redhead | *Aythya americana* | Anatidae | 0 | 1 | 0% | United States | 1990 | None reported | NA | Cox (1992) |
| Reeve's Pheasant | *Syrmaticus reevesii* | Phasianidae | 1 | 1 | 100% | Japan | 1965 | None reported | Not specified | Ose (1967) |
| Ring-necked Duck | *Aythya collaris* | Anatidae | 0 | 37 | 0% | United States | 1990 | None reported | NA | Cox (1992) |

(continued)

**Table 15.2** (continued)

| Avian Species | Scientific name | Family | Number positive | Total examined | Prevalence | Country | Year | Clinical signs or mortality | Serotypes or strains | Citation |
|---|---|---|---|---|---|---|---|---|---|---|
| Seabirds | Unknown | Unknown | 0 | 192 | 0% | United States | 2008 | None reported | NA | Bogomolni et al. (2008) |
| Seabirds | Unknown | Unknown | 4 | 116 | 3% | Brazil | 2010 | None reported | Not specified | Cardoso et al. (2018) |
| Seabirds | Unknown | Unknown | Unknown | 69 | NA | Brazil | 2010 | None reported | Not specified | Roges et al. (2010) |
| Snow Goose | *Chen caerulescens* | Anatidae | 0 | 22 | 0% | United States | 1990 | None reported | NA | Cox (1992) |
| Snowy Egret | *Egretta thula* | Ardeidae | 1 | 2 | 50% | United States | 1990 | None reported | Not specified | Buck (1990) |
| Squacco Heron | *Ardeola ralloides* | Ardeidae | 1 | 2 | 50% | Romania | 2021 | None reported | Not specified | Páll et al. (2021) |
| Swinhoe's Pheasant | *Lophura swinhoii* | Phasianidae | 1 | 1 | 100% | Japan | 1965 | None reported | Biotype 1 | Ose (1967) |
| Wood Duck | *Aix sponsa* | Anatidae | 0 | 3 | 0% | United States | 1990 | None reported | Not specified | Cox (1992) |

**Table 15.3** This table details the records extracted from the eight studies that investigated birds as hosts for *Vibrio vulnificus*. In the table are provided the common name, the scientific name, the family, the number of birds that tested positive for *Vibrio vulnificus*, the total number of birds examined for the pathogen, and the prevalence for that record. We also provide the country that the study was performed in, as well as the year the study was conducted or published. For studies that reported clinical signs or mortality, we placed a "Yes" in that column. For serotypes or strains that were reported, we reported those as well, otherwise we designated the column as not specified

| Avian Species | Scientific name | Family | Number tested | Total examined | Prevalence | Country | Year | Clinical signs or mortality | Serotypes or strains | Citation |
|---|---|---|---|---|---|---|---|---|---|---|
| American Crow | *Corvus brachyrhynchos* | Corvidae | Unknown | Unknown | Unknown | United States | 2019 | None reported | NA | Zhao et al. (2020) |
| Barred Warbler | *Curruca nisoria* | Sylviidae | 0 | 3 | 0% | Romania | 2021 | None reported | NA | Páll et al. (2021) |
| Black-crowned Night Heron | *Nycticorax nycticorax* | Ardeidae | 0 | 2 | 0% | Romania | 2021 | None reported | NA | Páll et al. (2021) |
| Black-headed Gull | *Chroicocephalus ridibundus* | Laridae | 1 | 125 | 1% | Japan | 2005 | None reported | Not specified | Miyasaka et al. (2006) |
| Black-tailed Godwit | *Limosa limosa* | Scolopacidae | 1 | 2 | 50% | China | 2015 | None reported | Not specified | Wang et al. (2021) |
| Common Greenshank | *Tringa nebularia* | Scolopacidae | 0 | 5 | 0% | China | 2015 | None reported | Not specified | Wang et al. (2021) |
| Common Teal | *Anas crecca* | Anatidae | 0 | 1 | 0% | Japan | 2005 | None reported | NA | Miyasaka et al. (2006) |
| Domestic chicken | *Gallus gallus* | Phasianidae | 4 | 565 | 0.7% | Nigeria | 2017 | None reported | Not specified | Adebowale and Adeyemo (2018) |
| Eurasian Blue Tit | *Cyanistes caeruleus* | Paridae | 0 | 2 | 0% | Romania | 2021 | None reported | NA | Páll et al. (2021) |
| Common Teal | *Anas crecca* | Anatidae | 0 | 2 | 0% | Romania | 2021 | None reported | NA | Páll et al. (2021) |
| Garden Warbler | *Sylvia borin* | Sylviidae | 0 | 4 | 0% | Romania | 2021 | None reported | NA | Páll et al. (2021) |
| Herring Gull and Black-tailed Gull | *Larus argentatus* and *Larus crassirostris* | Laridae | 86 | 320 | 27% | Japan | 2005 | None reported | NA | Miyasaka et al. (2006) |
| Hooded Crow | *Corvus cornix* | Corvidae | 0 | 7 | 0% | Romania | 2021 | None reported | NA | Páll et al. (2021) |
| Japanese Quail | *Coturnix coturnix* | Phasianidae | Unknown | Unknown | Unknown | Korea | 2011 | None reported | KCTC 2959 | Kassim et al. (2011) |
| Laughing Gull | *Leucophaeus atricilla* | Laridae | Unknown | Unknown | Unknown | United States | 2021 | None reported | Not specified | Zhao et al. (2020) |
| Lesser Whitethroat | *Sylvia curruca* | Sylviidae | 0 | 2 | 0% | Romania | 2021 | Not reported | NA | Páll et al. (2021) |
| Mallard | *Anas platyrhynchos* | Anatidae | 0 | 73 | 0% | Japan | 2005 | None reported | NA | Miyasaka et al. (2006) |
| Muscovy Duck | *Cairina moschata* | Anatidae | Unknown | Unknown | Unknown | United States | 2019 | None reported | Not specified | Zhao et al. (2020) |

(continued)

**Table 15.3** (continued)

| Avian Species | Scientific name | Family | Number tested | Total examined | Prevalence | Country | Year | Clinical signs or mortality | Serotypes or strains | Citation |
|---|---|---|---|---|---|---|---|---|---|---|
| Seabirds | Unknown | Unknown | 2 | 116 | 2% | Brazil | 2010 | None reported | Not specified | Cardoso et al. (2018) |
| Seabirds | Unknown | Unknown | Unknown | 69 | NA | Brazil | 2010 | None reported | Not specified | Roges et al. (2010) |
| Wood Sandpiper | *Tringa glareola* | Scolopacidae | 0 | 2 | 0% | Romania | 2021 | Not reported | NA | Páll et al. (2021) |

**Table 15.4** This table details the records extracted from the 15 studies that investigated birds as hosts for *Vibrio alginolyticus*. In the table are provided the common name, the scientific name, the family, the number of birds that tested positive for Vibrio cholerae, the total number of birds examined for *Vibrio alginolyticus*, and the prevalence for that record. We also provide the country that the study was performed in, as well as the year the study was conducted or published. For studies that reported clinical signs or mortality, we placed a "Yes" in that column

| Avian species | Scientific name | Family | Number tested | Total examined | Prevalence | Country | Year | Clinical signs or mortality | Serotypes or strains | Citation |
|---|---|---|---|---|---|---|---|---|---|---|
| Kelp Gull | *Larus dominicanus* | Laridae | Unknown | Unknown | Unknown | Brazil | 2011 | None reported | Not specified | de Moura et al. (2012) |
| Lesser Scaup | *Aythya affinis* | Anatidae | 0 | 8 | 0% | United States | 1990 | None reported | NA | Cox (1992) |
| Magellanic Penguin | *Spheniscus magellanicus* | Spheniscidae | Unknown | Unknown | Unknown | Brazil | 2011 | None reported | Not specified | de Moura et al. (2012) |
| Mallard | *Anas platyrhynchos* | Anatidae | 0 | 32 | 0% | United States | 1990 | None reported | NA | Cox (1992) |
| Manx Shearwater | *Puffinus puffinus* | Procellariidae | 1 | 35 | 3% | Brazil | 2014 | None reported | Not specified | Cardoso et al. (2014) |
| Mauritius Kestrel | *Falco punctatus* | Falconidae | 1 | 6 | 17% | Mauritania | 1986 | None reported | Not specified | Cooper et al. (1986) |
| Mottled Duck | *Anas fulvigula* | Anatidae | 0 | 123 | 0% | United States | 1990 | None reported | NA | Cox (1992) |
| Mute Swan | *Cygnus olor* | Anatidae | 2 | 3 | 67% | United States | 1990 | None reported | Not specified | Buck (1990) |
| Northern Pintail | *Anas acuta* | Anatidae | 0 | 53 | 0% | United States | 1990 | None reported | NA | Cox (1992) |
| Northern Shoveler | *Spatula clypeata* | Anatidae | 1 | 37 | 3% | United States | 1990 | None reported | Not specified | Cox (1992) |
| Red-backed Shrike | *Lanius collurio* | Laniidae | 0 | 1 | 0% | Romania | 2021 | None reported | NA | Páll et al. (2021) |
| Red-footed Falcon | *Falco vespertinus* | Falconidae | 0 | 2 | 0% | Romania | 2021 | None reported | NA | Páll et al. (2021) |
| Redhead | *Aythya americana* | Anatidae | 0 | 1 | 0% | United States | 1990 | None reported | NA | Cox (1992) |
| Ring-necked Duck | *Aythya collaris* | Anatidae | 1 | 37 | 3% | United States | 1990 | None reported | Not specified | Cox (1992) |
| Seabirds | Unknown | Unknown | 8 | 192 | 4% | United States | 2008 | None reported | Not specified | Bogomolni et al. (2008) |

(continued)

**Table 15.4** (continued)

| Avian species | Scientific name | Family | Number tested | Total examined | Prevalence | Country | Year | Clinical signs or mortality | Serotypes or strains | Citation |
|---|---|---|---|---|---|---|---|---|---|---|
| Seabirds | Unknown | Unknown | 30 | 116 | 26% | Brazil | 2010 | None reported | Not specified | Cardoso et al. (2018) |
| Seabirds | Unknown | Unknown | 19 | 69 | 28% | Brazil | 2010 | None reported | Not specified | Roges et al. (2010) |
| Snow Goose | *Chen caerulescens* | Anatidae | 0 | 22 | 0% | United States | 1990 | No | NA | Cox (1992) |
| Song Thrush | *Turdus philomelos* | Turdidae | 0 | 2 | 0% | Romania | 2021 | No | NA | Páll et al. (2021) |
| Squacco Heron | *Ardeola ralloides* | Ardeidae | 1 | 2 | 50% | Romania | 2021 | No | Not specified | Páll et al. (2021) |
| Wedge-tailed Shearwater | *Ardenna pacifica* | Procellariidae | 24 | 246 | 10% | United States | 1994 | Yes | Not specified | Work and Rameyer (1999) |
| Wood Duck | *Aix sponsa* | Anatidae | 0 | 3 | 0% | United States | 1990 | None reported | NA | Cox (1992) |

**Table 15.5** This table details the records extracted from the 15 studies that investigated birds as hosts for *Vibrio fluvialis*. In the table are provided the common name, the scientific name, the family, the number of birds that tested positive for *V. fluvialis*, the total number of birds examined for the pathogen, and the prevalence for that record. We also provide the country that the study was performed in, as well as the year the study was conducted or published. For studies that reported clinical signs or mortality, we placed a "Yes" in that column

| Avian species | Scientific name | Family | Number positive | Total examined | Prevalence | Country | Year | Clinical signs or mortality | Serotypes or strains | Citation |
|---|---|---|---|---|---|---|---|---|---|---|
| American Oystercatcher | *Haematopus palliatus* | Haematopodidae | 2 | 15 | 13% | Peru | 2021 | None reported | Not specified | Huamanchumo (2021) |
| Aves spp. | Unknown | Unknown | 4 | 8 | 50% | Australia | 1989 | None reported | Not specified | Myatt and Davis (1989) |
| Aves spp. | Unknown | Unknown | 4 | 4 | 100% | Unknown | 1983 | None reported | Not specified | Shimada and Sakazaki (1983) |
| Brown Booby | *Sula leucogaster* | Sulidae | Unknown | Unknown | Unknown | Brazil | 2011 | None reported | Not specified | de Moura et al. (2012) |
| Brown Pelican | *Pelecanus occidentalis* | Pelecanidae | 20 | 42 | 48% | United States | 1990 | None reported | Not specified | Buck (1990) |
| Canada Goose | *Branta canadensis* | Anatidae | 1 | 289 | 0.3% | Germany | 2003 | None reported | Not specified | Bönner et al. (2004) |
| Common Kingfisher | *Alcedo atthis* | Alcedinidae | 0 | 1 | 0% | Romania | 2021 | None reported | NA | Páll et al. (2021) |
| Domestic chicken | *Gallus gallus* | Phasianidae | 10 | 10 | 100% | Iraq | 2014 | None reported | Not specified | Shnawa et al. (2014) |
| Domestic Duck | Unknown | Anatidae | 5 | Unknown | Unknown | Botswana | 2010 | Yes | Not specified | Moreki et al. (2011) |
| Eurasian Hobby | *Falco subbuteo* | Falconidae | 0 | 2 | 0% | Romania | 2021 | None reported | NA | Páll et al. (2021) |
| Eurasian Tree Sparrow | *Passer montanus* | Passeridae | 0 | 2 | 0% | Romania | 2021 | None reported | NA | Páll et al. (2021) |
| Great Black-backed Gull and Herring Gull | *Larus marinus* and *Larus argentatus* | Laridae | 23 | 45 | 56% | United States | 1990 | None reported | Not specified | Buck (1990) |
| Great Egret | *Ardea alba* | Ardeidae | 3 | 11 | 27% | United States | 2012 | None reported | Not specified | Jubirt (2012) |
| Great Tit | *Parus major* | Paridae | 0 | 2 | 0% | Romania | 2021 | None reported | NA | Páll et al. (2021) |
| Laughing Gull | *Leucophaeus atricilla* | Laridae | 2 | 15 | 13% | Peru | 2021 | None reported | Not specified | Huamanchumo (2021) |

(continued)

**Table 15.5** (continued)

| Avian species | Scientific name | Family | Number positive | Total examined | Prevalence | Country | Year | Clinical signs or mortality | Serotypes or strains | Citation |
|---|---|---|---|---|---|---|---|---|---|---|
| Magellanic Penguin | *Spheniscus magellanicus* | Anatidae | Unknown | Unknown | Unknown | Brazil | 2011 | None reported | Not specified | de Moura et al. (2012) |
| Mallard | *Anas platyrhynchos* | Anatidae | 3 | 38 | 8% | Canada | 1991 | Yes | Not specified | Wobeser and Kost (1992) |
| Manx Shearwater | *Puffinus puffinus* | Procellariidae | 2 | 35 | 6% | Brazil | 2014 | None reported | Not specified | Cardoso et al. (2014) |
| Red-backed Shrike | *Lanius collurio* | Laniidae | 0 | 1 | 0% | Romania | 2021 | None reported | NA | Páll et al. (2021) |
| Red-footed Falcon | *Falco vespertinus* | Falconidae | 0 | 2 | 0% | Romania | 2021 | None reported | NA | Páll et al. (2021) |
| Seabirds | Unknown | Unknown | 0 | 192 | 0% | United States | 2008 | None reported | NA | Bogomolni et al. (2008) |
| Seabirds | Unknown | Unknown | 9 | 116 | 3% | Brazil | 2010 | None reported | Not specified | Cardoso et al. (2018) |
| Seabirds | Unknown | Unknown | Unknown | 69 | NA | Brazil | 2010 | None reported | Not specified | Roges et al. (2010) |
| Western Jackdaw | *Coleus monedula* | Corvidae | 0 | 4 | 0% | Romania | 2021 | None reported | NA | Páll et al. (2021) |

birds were tested for *Vibrio cholerae*, and 864 reported birds tested positive, for an overall meta-analysis prevalence of 16%. The prevalence of various studies ranged from 100% in case reports to zero, for example, this often represented rarely captured species that did not yield evidence of exposure to the pathogen. Mallards (*Anas platyrhynchos*) ($n = 381$) (Ogg et al. 1989; Cox 1992; Siembieda et al. 2011; Szeness et al. 1979; Zhang et al. 1996) appeared to be the most captured and examined wild species, while domestic chickens ($n = 552$), both backyard and experimentally inoculated, were the most commonly examined domestic species (Akond et al. 2008; Ismail et al. 2021; Salles et al. 1976; Sanyal et al. 1974; Singh et al. 1975; Sakazaki and Shimada 1977). Wilson's Plover (*Charadrius wilsonia*), a species of shorebird that was examined in Venezuela ($n = 16/16$), had the highest cross-sectional study prevalence for any wild bird captured, with a prevalence of 100% (Huamanchumo 2021). This was followed by Greater Yellowlegs (*Tringa melanoleuca*), also in Venezuela ($n = 6/6$), with a prevalence of 100% (Huamanchumo 2021), and Killdeer (*Charadrius vociferus*) in the western United States ($n = 13/15$), with a prevalence of 86.7% (Ogg et al. 1989).

Clinical signs were reported from 20 study records and were most often associated with *V. cholerae* non-O1/O139 (Aguirre et al. 1991; Bisgaard and Kristensen 1975; Hirsch et al. 2020; Metzner et al. 2004; Salles et al. 1976; Schlater et al. 1981; Strauch et al. 2020; Watts et al. 1993; Wobeser and Rainnie 1987; Zheng et al. 2020, 2021). One study reported clinical signs, primarily edema and cellulitis of the gastrointestinal tract, with an experimental inoculation of O1 Ogawa in domestic chickens (Salles et al. 1976). Clinical signs from the literature ranged from respiratory signs to lethargy and sepsis; most infections were associated with other pathogens. However, in a mortality study of American Flamingoes (*Phoenicopterus ruber*), *V. cholerae* infection was associated with lead toxicity (Aguirre et al. 1991). The largest cross-sectional study to examine wild birds who had exhibited clinical signs in the wild was performed in China, whereby Ruddy Shelducks (*Tadorna ferruginea*) ($n = 25/55$) tested positive for *V. cholerae* non-O1 (Zheng et al. 2021). This study also examined other taxa of birds, such as waterfowl, gulls, shorebirds, and Great Cormorants (*Phalacrocorax carbo*) for the presence of *Vibrio cholerae*, however, study-wide prevalences were generally low when associated with clinical signs (Table 15.1).

### 15.3.3 *Vibrio parahaemolyticus*

We identified 20 studies in the literature that examined the role of wild birds as hosts for *V. parahaemolyticus* (Ose 1967; Bogomolni et al. 2008; Buck 1990; Cardoso et al. 2014, 2018; Contreras-Rodríguez et al. 2019; Cox 1992; Myatt and Davis 1989; Páll et al. 2021; Roges et al. 2010; Watanabe et al. 2002; Zheng et al. 2020; Forrester et al. 1997; Fu et al. 2019; Karunasagar et al. 1986; Kassim et al. 2011; Miyasaka et al. 2006; Reuschel et al. 2020; Wang et al. 2021). We extracted seventy-three study records from these papers that examined the prevalence of the pathogen, however, in an additional two study records, we were unable to determine the number of birds infected and/or the number of birds tested (Roges et al. 2010; Kassim et al. 2011). One paper examined the immunogenicity of *V. parahaemolyticus* and *V. vulnificus* in Japanese Quail eggs (*Coturnix coturnix*) and found that birds elicited a high humoral response to the antigens, as measured by ELISA and Western Blots (Kassim et al. 2011). Most studies utilized culture to determine the presence of *V. parahaemolyticus*, or suckling mice coupled with culture, however, PCR and sequencing were more commonly utilized in more recent works. The Anatidae were represented by 21 study records, the Phasianidae (turkeys, chickens, and pheasants) represented nine study records, and the Laridae represented six study records. We were able to identify 60 species that had been examined for *V. parahaemolyticus*, representing 22 families. For eleven study records, we were unable to identify the species or family of the birds involved in the study (Bogomolni et al. 2008; Cardoso et al.

2018; Myatt and Davis 1989; Roges et al. 2010; Watanabe et al. 2002; Zheng et al. 2020; Karunasagar et al. 1986). Common Loons (*Gavia immer*) were the most common species tested for *V. parahaemolyticus*, after a multi-year mortality event in Florida (Forrester et al. 1997), however the prevalence was only 0.23% (1/434).

Similar to *V. cholerae*, prevalences for *V. parahaemolyticus* ranged from 100% in the cases of individual study records that were examined, or zero when relatively cryptic and/or scarce species were assessed. Out of the seventy-five study records that we extracted, only 44 reported study records contained birds that tested positive for the pathogen. The highest prevalence for wild birds captured in a cross-sectional study was 68%, involving three species of gulls: Herring Gulls (*Larus argentatus*), Laughing Gulls (*Leucophaeus atricilla*), and Ring-billed Gulls (*Larus delawarensis*) captured off the coast of Florida (Buck 1990). This was followed by Herring Gulls and Black-tailed Gulls (*Larus crassirostris*) captured off the coast of Japan, with a prevalence of 67% (Miyasaka et al. 2006). Across our study records, we found that 3996 birds had been tested for the presence of the pathogen or for antibodies against the pathogen. A total of 761 birds were positive for *V. parahaemolyticus*, for a meta-analysis prevalence of 19%. Clinical signs were only reported for two studies, in both, co-infection with other organisms was noted (Forrester et al. 1997; Reuschel et al. 2020). Four studies were associated with other *Vibrio* spp. that were not identified to species (Buck 1990; Cox 1992; Páll et al. 2021; Wang et al. 2021). Few studies overlapped between reporting both *V. cholerae* and *V. parahaemolyticus* in birds (Buck 1990; Cox 1992; Roges et al. 2010).

### 15.3.4 *Vibrio vulnificus*

Eight studies reported examining wild or domestic birds for the presence of *V. vulnificus* or *V. vulnificus* antibodies in the literature, from which we were able to extract 21 study records (Cardoso et al. 2018; Páll et al. 2021; Roges et al. 2010; Kassim et al. 2011; Miyasaka et al. 2006; Wang et al. 2021; Adebowale and Adeyemo 2018; Zhao et al. 2020). At least 17 species were represented in this dataset, categorized into 10 families. We were unable to determine within-study prevalences for five of those 21 study records, however, due to the pooling of samples (Roges et al. 2010; Kassim et al. 2011; Zhao et al. 2020). The most commonly utilized method of determining exposure to *V. vulnificus* in birds was the use of a biochemical panel coupled with culture (Páll et al. 2021; Adebowale and Adeyemo 2018); similar to *V. parahaemolyticus*, PCR and sequencing were more commonly used in later papers (Wang et al. 2021; Zhao et al. 2020). The prevalence of relevant studies ranged from zero for rarely captured and/or examined species to 50%, which was attributed to one of the two Black-tailed Godwits (*Limosa limosa*) captured in China that was positive by PCR and sequencing (Wang et al. 2021). This was followed by a prevalence of 26% for Herring Gulls and Black-tailed Gulls sampled off the coast of Japan (Miyasaka et al. 2006). The largest cross-sectional study was performed in Ogun State, Nigeria, from which multiple farms, representing 565 domestic chickens, were sampled for the presence of exposure to *V. vulnificus* (Adebowale and Adeyemo 2018). The study-wide prevalence was 0.7%.

No clinical signs or mortality events were reported from any study. In a cross-sectional sampling of urban birds in Houston, Texas, Zhao et al. (2020) reported that Muscovy Ducks (*Cairina moschata*) and Laughing Gulls excreted more *V. vulnificus* (*vvh*) than American Crows in the winter as compared to the summer. The greatest diversity of pathogenic *Vibrio* species was reported from a study of stranded seabirds ($n = 17/69$, *Vibrio* spp., prevalence of 25%) in Brazil, from which *V. vulnificus* was isolated along with *V. cholerae, V. parahaemolyticus, V. cincinnatiensis, V. fluvialis, V. harveyi*, and *V. mimicus* (Roges et al. 2010). The individual prevalence of *V. vulnificus*, or the avian species afflicted in this study was not reported, however a follow-up study reported a *V. vulnificus*

prevalence of 1.7% in Brazilian seabirds (Cardoso et al. 2018). Overall, 1231 birds were examined for evidence of exposure to *V. vulnificus*, and 94 were positive, for a meta-analysis wide prevalence of 8%.

### 15.3.5 *Vibrio alginolyticus*

In the literature, we recovered 15 studies in which the role of domestic and wild birds as hosts for *V. alginolyticus* was examined, providing us with 49 study records (Bogomolni et al. 2008; Buck 1990; Cardoso et al. 2014, 2018; Contreras-Rodríguez et al. 2019; Cox 1992; Páll et al. 2021; Siembieda et al. 2011; Forrester et al. 1997; Kassim et al. 2011; Adebowale and Adeyemo 2018; Byrum and Slemons 1995; Cooper et al. 1986; de Moura et al. 2012; Work and Rameyer 1999). Two study records, involving seabirds off the coast of Brazil, did not identify the sampled birds to species (Cardoso et al. 2018; Roges et al. 2010). Forty-seven species were represented in this data subset, categorized into 18 families. Nineteen study records were attributed to the Anatidae, five study records to the Laridae, and three study records were represented by the Falconidae family, known for its small falcons and hawks. Culture, followed by biochemical panels, were the most commonly utilized methods to identify the pathogen. PCR was rarely utilized. The highest prevalence of *V. alginolyticus* recovered from a cross-sectional study of wild birds involved Herring Gulls, Laughing Gulls, and Ring-billed Gulls captured off the coast of Florida, with a prevalence of 68% (Buck 1990). The next highest prevalence of the pathogen was 55%, originating from Herring Gulls and Great Black-backed Gulls captured along coastal Connecticut (Buck 1990). Across the board, prevalences ranged from zero to 68%, no study record reached a prevalence of 100%. In a study performed in Ogun State, Nigeria, *V. alginolyticus* was isolated from 2% of domestic chickens (Adebowale and Adeyemo 2018).

Clinical signs and mortality were recorded by two studies, one involving a multi-year mortality event of Common Loons in Florida, and the second involved a mortality event off the coast of Oahu, Hawaii, of Wedge-tailed Shearwaters (*Ardenna pacifica*), which demonstrated a prevalence of 10% (Forrester et al. 1997; Work and Rameyer 1999). Clinical signs ranged from emaciation and lethargy to toxemia and sepsis; bacteremia in the case of the Wedge-tailed Shearwaters was strongly suspected (Forrester et al. 1997; Work and Rameyer 1999). In a diagnostic examination of critically endangered Mauritius Kestrels (*Falco punctatus*), a captive individual (1/6) was positive by culture for *V. alginolyticus,* yet no clinical signs were noted (Cooper et al. 1986). In general, waterfowl demonstrated the lowest prevalences for any group of birds, besides passerines, for the pathogen (Cox 1992; Páll et al. 2021). Throughout our dataset, 258 birds of 2967 sampled birds tested positive for *V. alginolyticus*, for a meta-analysis prevalence of 9%.

### 15.3.6 *Vibrio fluvialis*

From the literature, we found 15 studies that reported examining wild or domestic birds for the presence of *V. fluvialis* or *V. fluvialis* antibodies, from which we were able to extract 26 study records (Bogomolni et al. 2008; Buck 1990; Cardoso et al. 2014, 2018; Huamanchumo 2021; Myatt and Davis 1989; Páll et al. 2021; Roges et al. 2010; Kassim et al. 2011; de Moura et al. 2012; Bönner et al. 2004; Jubirt 2012; Moreki et al. 2011; Shimada and Sakazaki 1983; Shnawa et al. 2014; Wobeser and Kost 1992). At least 20 species were reported in this dataset, representing 15 families. Four records did not provide sufficient data from which to identify birds to species or family. Culture was the most commonly utilized method to identify *V. fluvialis*, followed by a biochemistry panel. The largest cross-sectional study examining the prevalence of *V. fluvialis* in wild birds was performed on Canada Geese in Germany, however, only one of 289 birds cultured positive for the pathogen (Bönner et al. 2004). *V. fluvialis* was associated with one mortality event—a die-off of overwintering Mallards in Canada which was

attributed to a Vitamin A deficiency (Wobeser and Kost 1992). The reported prevalence of the pathogen for these birds was 8%. In a study of captive study of Great Egrets (*Ardea alba*) captured from the Mississippi Delta, control birds shed *V. fluvialis* for four of seven days in captivity (Jubirt 2012). The highest prevalence was associated with a study performed in Connecticut involving Herring Gulls and Great Black-backed Gulls, with 55% culturing positive for *V. fluvialis*. Studies with a prevalence of 100% involved two experiments, one involving of avian-sourced strains, and a mitogenicity study on domestic chickens (Shimada and Sakazaki 1983; Shnawa et al. 2014). Overall, the meta-analysis prevalence, including experimental infection studies (88/834) was 11% percent (85/834).

Other Pathogenic Vibrio spp.: *V. cincinnatiensis*, *V. hollisae*, e.g., *Grimontia hollisae*, *V. furnissii*, *V. mimicus*, *V. harveyi*, *V. scophthalmi*, *V. metschnikovii*, and *Photobacterium damselae.*

The abundance of studies that reported on other pathogenic *Vibrio* species that were isolated from wild or domestic birds varied (Table 15.6). *V. cincinnatiensis* was examined by five studies and provided five study records (Jäckel et al. 2020; Cardoso et al. 2014, 2018; Roges et al. 2010; de Moura et al. 2012). Unspecified seabirds were the taxa that were examined most frequently (Cardoso et al. 2018; Roges et al. 2010), however overall prevalences were low across all studies for a mean prevalence of 3%. The presence or absence of *Photobacterium damselae* was examined by three studies (Buck 1990; Forrester et al. 1997; Colvile et al. 2012), and yielded three study records from the United Kingdom and the United States. Two studies involved mortality events, one of Common Loons in Florida, and the second of British passerines (Forrester et al. 1997; Colvile et al. 2012). Across these cross-sectional studies, the overall meta-analysis prevalence was approximately 5%. Our literature search of *V. furnissii* yielded four study records from three cross-sectional studies (Cardoso et al. 2018; Huamanchumo 2021; de Moura et al. 2012), two of those study records did not identify the number of birds positive or the number of individuals examined. The Laridae were the most prevalent species identified in association with *V. furnissii*, specifically Kelp Gulls (*Larus dominicanus*), Laughing Gulls, and Brown Boobys (*Sula leucogaster*). The overall meta-analysis prevalence for this *Vibrio* pathogen was approximately 2%.

The search for studies involving *V. harveyi* and avian species yielded four cross-sectional studies and four study records (Cardoso et al. 2014, 2018; Roges et al. 2010; Wang et al. 2021), involving seabirds and Manx Shearwaters (*Puffinus puffinus*). A single study involving a coastal sandpiper, the Common Greenshank (*Tringa nebularia*), had a prevalence of 0% out of five birds that were tested by PCR (Wang et al. 2021). From the two studies that provided individual birds positive in contrast to individual birds examined, we were able to calculate a *V. harveyi* prevalence of approximately 13%. No study that tested for this pathogen reported clinical signs or a mortality event. *Grimontia hollisae* was rarely detected in birds, as we found only two studies, resulting in three study records, that searched for the pathogen in avian hosts (Fu et al. 2020; Albuixech-Martí et al. 2021). One study examined the shared microbiota between wild Hooded Cranes (*Grus monacha*) and domestic geese (*Anser anser*) using MiSeq—*Grimontia hollisae* was identified as a potential pathogen, but the total number of birds colonized was not reported (Fu et al. 2020). A longitudinal microbiome study involving shorebirds off the coast of Cork, Ireland discovered *Grimontia hollisae* in fecal samples, however, the number of samples positive/examined was not enumerated (Albuixech-Martí et al. 2021). Clinical signs or mortality were not reported from either study.

*Vibrio metschnikovii* was reported from three studies, resulting in 10 study records (Páll et al. 2021; Zheng et al. 2021; Lee et al. 1978). Approximately half the study records examined passerines of Romania as hosts (Páll et al. 2021), including members of the Laniidae, Sylviidae, and Paridae families, all of which were negative for the pathogen by biochemical panels. The highest prevalence was reported from sites in

**Table 15.6** This table details the records extracted from the 18 studies that investigated birds as hosts for other pathogenic Vibrios, including *V. metschnikovii*, *V. mimicus*, *V. cincinnatiensis*, *V. scophtalmi*, *Grimontia hollisae*, formerly *V. hollisae*, *Photobacterium damselae*, formerly *V. damselae*, *V. furnissii*, and *V. harveyi*. In the table are provided the common name, the scientific name, the family, the number of birds that tested positive for the respective pathogen, the total number of birds examined, and the prevalence for that record. We also provide the country that the study was performed in, as well as the year the study was conducted or published. For studies that reported clinical signs or mortality, we placed a "Yes" in that column

| Avian species | Scientific name | Family | Number tested | Total examined | Prevalence | Country | Year | Clinical signs or mortality | Pathogen | Citation |
|---|---|---|---|---|---|---|---|---|---|---|
| Barred Warbler | *Curruca nisoria* | Sylviidae | 1 | 1 | 100% | Romania | 2021 | None reported | Vibrio metschnikovii | Páll et al. (2021) |
| Bearded Warbler | *Panurus biarmicus* | Paridae | 0 | 3 | 0% | Romania | 2021 | None reported | Vibrio metschnikovii | Páll et al. (2021) |
| Black-and-White Magpie | *Pica hudsonia* | Corvidae | 0 | 2 | 0% | Romania | 2021 | None reported | Vibrio metschnikovii | Páll et al. (2021) |
| Black-headed Gull | *Chroicocephalus ridibundus* | Laridae | 13 | 34 | 38% | China | 2018 | Yes | Vibrio metschnikovii | Zheng et al. (2020) |
| Black-necked Stilt | *Himantopus mexicanus* | Charadriidae | 1 | 34 | 3% | China | 2018 | Yes | Vibrio metschnikovii | Zheng et al. (2020) |
| Black-winged Stilt | *Himantopus himantopus* | Charadriidae | 0 | 34 | 0% | China | 2018 | Yes | Vibrio metschnikovii | Zheng et al. (2020) |
| Common Kingfisher | *Alcedo atthis* | Alcedinidae | 0 | 1 | 0% | Romania | 2021 | None reported | Vibrio metschnikovii | Páll et al. (2021) |
| Common Pochard | *Aythya ferina* | Anatidae | 1 | 34 | 3% | China | 2018 | Yes | Vibrio metschnikovii | Zheng et al. (2020) |
| Common Whitethroat | *Curruca communis* | Sylviidae | 0 | 5 | 0% | Romania | 2021 | None reported | Vibrio metschnikovii | Páll et al. (2021) |
| Domestic Chicken | *Gallus gallus* | Phasianidae | 1 | 1 | 100% | Unknown | 1978 | None reported | Vibrio metschnikovii | Lee et al. (1978) |
| Domestic Goose | *Anser anser* | Anatidae | 1 | 1 | 100% | Germany | 2001 | None reported | Vibrio cincinnatiensis | Jäckel et al. (2020) |
| Kelp Gull | *Larus dominicanus* | Laridae | Unknown | Unknown | Unknown | Brazil | 2011 | None reported | Vibrio cincinnatiensis | de Moura et al. (2012) |
| Manx Shearwater | *Puffinus puffinus* | Procellariidae | 2 | 35 | 6% | Brazil | 2014 | None reported | Vibrio cincinnatiensis | Cardoso et al. (2014) |
| Seabirds | Unknown | Unknown | Unknown | 69 | Unknown | Brazil | 2010 | None reported | Vibrio cincinnatiensis | Roges et al. (2010) |
| Seabirds | Unknown | Unknown | 2 | 116 | 2% | Brazil | 2010 | None reported | Vibrio cincinnatiensis | Cardoso et al. (2018) |

(continued)

**Table 15.6** (continued)

| Avian species | Scientific name | Family | Number tested | Total examined | Prevalence | Country | Year | Clinical signs or mortality | Pathogen | Citation |
|---|---|---|---|---|---|---|---|---|---|---|
| Brown Pelican | *Pelecanus occidentalis* | Pelecanidae | 20 | 42 | 48% | United States | 1990 | None reported | Photobacterium damselae | Buck (1990) |
| Common Loon | *Gavia immer* | Gaviidae | 1 | 434 | 0.2% | United States | 1994 | Yes | Photobacterium damselae | Forrester et al. (1997) |
| Great Tit | *Parus major* | Paridae | 1 | 5 | 20% | United Kingdom | 2011 | Yes | Photobacterium damselae | Colvile et al. (2012) |
| Kelp Gull | *Larus dominicanus* | Laridae | Unknown | Unknown | Unknown | Brazil | 2011 | None reported | Vibrio furnissii | de Moura et al. (2012) |
| Brown Booby | *Sula leucogaster* | Sulidae | Unknown | Unknown | Unknown | Brazil | 2011 | None reported | Vibrio furnissii | de Moura et al. (2012) |
| Laughing Gull | *Leucophaeus atricilla* | Laridae | 1 | 7 | 14% | Peru | 2021 | None reported | Vibrio furnissii | Huamanchumo (2021) |
| Seabirds | Unknown | Unknown | 1 | 116 | 1% | Brazil | 2010 | None reported | Vibrio furnissii | Cardoso et al. (2018) |
| Seabirds | Unknown | Unknown | Unavailable | 69 | Unknown | Brazil | 2010 | None reported | Vibrio harveyi | Roges et al. (2010) |
| Common Greenshank | *Tringa nebularia* | Scolopacidae | 0 | 5 | 0% | China | 2015 | None reported | Vibrio harveyi | Wang et al. (2021) |
| Manx Shearwater | *Puffinus puffinus* | Procellariidae | 7 | 35 | 20% | Brazil | 2014 | None reported | Vibrio harveyi | Cardoso et al. (2014) |
| Seabirds | Unknown | Unknown | 14 | 116 | 13% | Brazil | 2010 | None reported | Vibrio harveyi | Cardoso et al. (2018) |
| Aves | Unknown | Unknown | Unknown | 204 | Unknown | Ireland | 2019 | None reported | Grimontia hollisae | Albuixech-Martí et al. (2021) |
| Domestic Goose | *Anser anser* | Anatidae | Unknown | 20 | Unknown | China | 2017 | None reported | Grimontia hollisae | Fu et al. (2020) |
| Hooded Crane | *Grus monarcha* | Gruidae | Unknown | 20 | Unknown | China | 2017 | None reported | Grimontia hollisae | Fu et al. (2020) |
| Seabirds | Unknown | Unknown | Unknown | 69 | Unknown | Brazil | 2010 | None reported | Vibrio mimicus | Roges et al. (2010) |
| Bearded Reedling | *Panurus biarmicus* | Paridae | 0 | 2 | 0% | Romania | 2021 | None reported | Vibrio mimicus | Páll et al. (2021) |
| Black-tailed Godwit | *Limosa limosa* | Scolopacidae | 3 | 26 | 12% | China | 2018 | None reported | Vibrio mimicus | Fu et al. (2019) |
| Common Chaffinch | *Fringilla coelebs* | Fringillidae | 0 | 5 | 0% | Romania | 2021 | None reported | Vibrio mimicus | Páll et al. (2021) |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Common Kingfisher | *Alcedo atthis* | Alcedinidae | 0 | 1 | 0% | Romania | 2021 | None reported | Vibrio mimicus | Páll et al. (2021) |
| Common Snipe | *Gallinago gallinago* | Scolopacidae | 0 | 3 | 0% | Romania | 2021 | None reported | Vibrio mimicus | Páll et al. (2021) |
| Domestic Chicken | *Gallus gallus* | Phasianidae | 8 | 565 | 1% | Nigeria | 2017 | None reported | Vibrio mimicus | Adebowale and Adeyemo (2018) |
| Eurasian Blue Tit | *Cyanistes caeruleus* | Paridae | 0 | 2 | 0% | Romania | 2021 | None reported | Vibrio mimicus | Páll et al. (2021) |
| Eurasian Hobby | *Falco subbuteo* | Falconidae | 0 | 2 | 0% | Romania | 2021 | None reported | Vibrio mimicus | Páll et al. (2021) |
| Eurasian Stone Curlew | *Burhinus oedicnemus* | Burhinidae | 1 | 61 | 2% | Italy | 2018 | None reported | Vibrio mimicus | Foti et al. (2020) |
| Great Tit | *Parus major* | Paridae | 0 | 2 | 0% | Romania | 2021 | None reported | Vibrio mimicus | Páll et al. (2021) |
| Hawfinch | *Coccothraustes coccothraustes* | Fringillidae | 0 | 8 | 0% | Romania | 2021 | None reported | Vibrio mimicus | Páll et al. (2021) |
| Hooded Crow | *Corvus cornix* | Corvidae | 0 | 7 | 0% | Romania | 2021 | None reported | Vibrio mimicus | Páll et al. (2021) |
| Lesser Whitethroat | *Sylvia curruca* | Sylviidae | 0 | 2 | 0% | Romania | 2021 | None reported | Vibrio mimicus | Páll et al. (2021) |
| Seabirds | Unknown | Unknown | 8 | 116 | 7% | Brazil | 2010 | None reported | Vibrio mimicus | Cardoso et al. (2018) |
| Song Thrush | *Turdus philomelos* | Turdidae | 0 | 2 | 0% | Romania | 2021 | None reported | Vibrio mimicus | Páll et al. (2021) |
| Wood Sandpiper | *Tringa glareola* | Scolopacidae | 0 | 2 | 0% | Romania | 2021 | None reported | Vibrio mimicus | Páll et al. (2021) |
| Common Greenshank | *Tringa nebularia* | Scolopacidae | 1 | 26 | 4% | Romania | 2021 | None reported | Vibrio scophtalmi | Páll et al. (2021) |

Inner Mongolia, China, by Black-headed Gulls (*Chroicocephalus ridibundus*), from which an overall study prevalence of 38% was reported (Zheng et al. 2021). Clinical signs and a mortality event that spanned multiple waterfowl and waterbird species were documented in the study by Zheng et al. (2021). The number of total tested birds was not available for analysis in the latter study; thus, we could not report a meta-analysis prevalence of *V. metschnikovii* with confidence. *Vibrio mimicus* was examined by six studies, yielding 18 study records (Cardoso et al. 2018; Páll et al. 2021; Roges et al. 2010; Fu et al. 2019; Adebowale and Adeyemo 2018; Foti et al. 2020). Biochemical panels were the most common diagnostic tool used to identify the pathogen, however, overall prevalences were very low across studies. In a study of wading birds and songbirds performed along the Danube Delta of Romania, all sampled birds ($n = 38$) were negative for *V. mimicus* (Páll et al. 2021). On the other hand, wading birds and seabirds sampled in China, Brazil, and Italy demonstrated evidence of shedding the pathogen (Cardoso et al. 2018; Fu et al. 2019; Foti et al. 2020). The only study to examine the role of domestic birds as hosts was performed in Ogun State, Nigeria—this study yielded a prevalence of approximately 1% (Adebowale and Adeyemo 2018). Across studies, 806 birds were examined for the presence of the pathogen, with 21 testing positive, resulting in a meta-analysis prevalence of 2%. No clinical signs or mortality events were reported from any study that examined the role of birds as hosts for *V. mimicus*.

*Vibrio scophthalmi* was only reported from one study, resulting in a single study record (Fu et al. 2019). A Common Greenshank (1/26) that was sampled using whole genome sequencing was positive for the pathogen (Fu et al. 2019). This study was not associated with clinical signs or a mortality event. Uncategorized *Vibrio* spp. were reported from 14 studies (Bogomolni et al. 2008; Buck 1990; Cardoso et al. 2018; Cox 1992; Fernández-Delgado et al. 2016; Huamanchumo 2021; Páll et al. 2021; Watanabe et al. 2002; Zheng et al. 2020; Wang et al. 2021; Albuixech-Martí et al. 2021; White et al. 1973; Negruțiu et al. 2017; Saiful Islam et al. 2021). Two studies were associated with clinical signs and/or mortality events, however, these outbreaks were attributed to other causal pathogens (Zheng et al. 2020; White et al. 1973). Culture followed by biochemical panels were the most commonly utilized methods of identifying *Vibrio* spp. Given that many studies did not identify these *Vibrio* spp. to species or identify the number of birds excreting them, we were unable to calculate a meta-analysis wide prevalence.

## 15.4 Discussion

The question of pathogenic *Vibrio* spp. as the etiological agents of disease in birds remains only partially answered. Of the 76 studies that surveyed birds for pathogenic *Vibrio* species, 19 reported disease or death from individuals, scaling up to community-level events (Aguirre et al. 1991; Bisgaard and Kristensen 1975; Hirsch et al. 2020; Metzner et al. 2004; Salles et al. 1976; Schlater et al. 1981; Strauch et al. 2020; Watts et al. 1993; Wobeser and Rainnie 1987; Zheng et al. 2020, 2021; Forrester et al. 1997; Reuschel et al. 2020; Work and Rameyer 1999; Moreki et al. 2011; Wobeser and Kost 1992; Colvile et al. 2012; Lee et al. 1978; White et al. 1973). Yet, it remains uncertain whether these pathogenic *Vibrio* species are opportunistic pathogens that contribute to morbidity and/or mortality in already stressed individuals, or whether they can be the primary arbiters of disease (Zhao et al. 2020). Experimental inoculation studies reported contrasting results, if they reported clinical signs at all (Laviad-Shitrit et al. 2017; Salles et al. 1976; Zhang et al. 1996; Shnawa et al. 2014). In addition, avian susceptibility to pathogenic *Vibrio* species may also be conflated by host species, natural history, and prior exposure, resulting in an as yet-understood degree of immunity (Roche et al. 2009; Gamble et al. 2019). In our meta-analysis, disease was most commonly associated with *V. cholerae*, followed by *V. metschnikovii* and *V. parahaemolyticus*—notably, 11 of 39 study records were associated with domestic ducks (*Anas platyrhynchos* or *Anser anser*) or domestic chickens (Bisgaard and Kristensen

1975; Bisgaard et al. 1978; Hirsch et al. 2020; Metzner et al. 2004; Salles et al. 1976; Watts et al. 1993). This may have implications for agriculturally associated species in areas of the world where backyard birds are the primary protein source for pastoral families (Conan et al. 2012; Hamilton-West et al. 2012; Kariithi et al. 2021).

Of the 425 study records we extracted from the literature, interestingly, the Anatidae represented 105 of them, including wild and domesticated Mallards, which represented 16 study records. The Laridae represented 39 study records, prominently represented by Laughing Gulls, Herring Gulls, and Ring-billed Gulls. Shorebirds and waders, categorized into the Ardeidae family, represented 16 study records, primarily of egrets and herons. These bird species are often highly associated with coastal estuarine and marine environments (Barnes and Thomas 1987; Waldenström et al. 2002; Chatterjee et al. 2020), which are also inhabited by autochthonous and halophilic *Vibrio* species. These results are congruent with what is known of avian foraging ecology and *Vibrio* habitat specificity (Pruzzo et al. 2005; Almagro-Moreno and Taylor 2014; Vezzulli et al. 2010; Grimes et al. 2009; Johnson et al. 2012; Grimes 2020). What was unexpected were the number of ground-foraging birds that tested positive for pathogenic *Vibrio* species that are often not strictly associated with aquatic environments, such as Great Tits (*Parus major*), Garden Warblers (*Sylvia borin*), and Hooded Crows (*Corvus cornix*) (Mehmke et al. 1992; Páll et al. 2021).

For example, in a study of Egyptian backyard poultry (chickens, turkeys, and waterfowl), 36% of examined birds were positive for *V. cholerae,* including chickens and turkeys (Ismail et al. 2021). Domestic chickens accounted for 13 total study records, across geographical areas as varied as the United States, Bangladesh, Egypt, Ghana, Nigeria, Iraq, and India, and reported as early as 1972 (Akond et al. 2008; Ismail et al. 2021; Salles et al. 1976; Sanyal et al. 1974; Singh et al. 1975; Sakazaki and Shimada 1977; Adebowale and Adeyemo 2018; Byrum and Slemons 1995; Shnawa et al. 2014; Lee et al. 1978). On the other hand, another surprising result was the low prevalence of pathogenic *Vibrio* species cultured from seabirds that were sampled from the New England region of the United States, with only one of 192 birds testing positive for *Vibrio cholerae*, non-O1 (Bogomolni et al. 2008). This result may be due to several reasons, many of which are not mutually exclusive (Chatterjee et al. 2020). For one, as seabirds tend to spend more time in marine versus coastal habitats, they may be less susceptible to exposure from pathogenic *Vibrio* species that tend to congregate in lower salinity, brackish habitats (Hsieh et al. 2008). In addition, the northern Atlantic may harbor a lower abundance of pathogenic *Vibrios* during the cooler months as a result of low sea surface temperatures (Baker-Austin et al. 2010, 2012). Lastly, it may be possible that although pathogenic *Vibrio* spp. may cause disease in seabirds, that the recovery of carcasses or diseased individuals may be reduced due to minimal mortality, low carcass persistence, and increased distances from urbanized centers (Piatt and Ford 1996; Ford 2006; Ward et al. 2006).

Meta-analysis prevalence varied across pathogenic *Vibrio* species, but all were below 20% (e.g., 19% for *V. parahaemolyticus,* 16% for *V. cholerae*, 13% for *V. harveyi*, 11% for *V. fluvialis*, 9% for *V. alginolyticus*, 8% for *V. vulnificus*, 5% for *P. damselae*, 2% for *V. furnissii*, and 1% for *V. mimicus*). Given that we utilized experimental inoculation studies coupled with cross-sectional studies, there is likely a degree of reporting bias in our meta-analysis prevalences (Lachish and Murray 2018), however, we speculate that this reporting bias is likely offset by the reportedly few studies that have targeted these pathogens for investigation in wild and domestic birds. To determine true "prevalence," and avian susceptibility under ecological conditions, longitudinal studies that sought to recover these pathogens from a community of birds would be more informative (Wobeser 2007; Brown et al. 2013). In addition, these studies would need to utilize large sample sizes, as well as represent various ecological foraging guilds, in geographic locations with both low and high recovery rates of these pathogens from their aquatic, environmental reservoir

(Stallknecht 2007; Cardoso et al. 2021; Watsa and Wildlife Disease Surveillance Focus Group 2020; Sleeman et al. 2012).

With a meta-analysis *Vibrio* prevalence of 16% coupled with the reports of clinical signs, there is a possibility that pathogenic *Vibrio* species—specifically *V. parahaemolyticus, V. cholerae,* and *V. metschnikovii*—may be emerging pathogens of wild and domestic aquatic or wetland birds (Daszak et al. 2000; Robinson et al. 2010). Gire et al. (2012) defined emerging pathogens as falling into two categories: introduced microbes and existing microbes that rapidly increase in prevalence and/or incidence in a population. Given that so little is known of non-cholera *Vibrio* species in human hosts, however, it is difficult to distinguish between the two categories in our avian hosts given the currently available data. Speculation suggests that these *Vibrio* pathogens may have a long-standing relationship with aquatic birds. However, as climate change alters and influences the abundance and distribution of pathogenic *Vibrio* species in marine and estuarine environments, so too may the incidence of these pathogens in wild and domestic birds (Fuller et al. 2012).

In summary, we have offered a rigorous meta-analysis that examines the prevalence of *Vibrio* spp. across bird species. In doing so, we also reveal a plethora of data that fortifies the notion that birds are both an underappreciated object of study and potential reservoirs for pathogenic bacterial species. In the context of a dynamic ecology defined by climate change and human-associated activities, we suggest that avian reservoirs should be the focus of more rigorous study, as they may be an actor in *Vibrio* emergence events. Transcending the case of birds, our study proposes that more attention should be paid to animal species that may harbor pathogens of interest to human health.

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# What Whole Genome Sequencing Has Told Us About Pathogenic Vibrios

16

Dawn Yan Lam Lau, Jose Roberto Aguirre Sánchez, Craig Baker-Austin, and Jaime Martinez-Urtaza

**Abstract**

When the first microbial genome sequences were published just 20 years ago, our understanding regarding the microbial world changed dramatically. The genomes of the first pathogenic vibrios sequenced, including *Vibrio cholerae, Vibrio parahaemolyticus,* and *Vibrio vulnificus* revealed a functional and phylogenetic diversity previously unimagined as well as a genome structure indelibly shaped by horizontal gene transfer. The initial glimpses into these organisms also revealed a genomic plasticity that allowed these bacteria to thrive in challenging and varied aquatic and marine environments, but critically also a suite of pathogenicity attributes. In this review we outline how our understanding of vibrios has changed over the last two decades with the advent of genomics and advances in bioinformatic and data analysis techniques, it has become possible to provide a more cohesive understanding regarding these bacteria: how these pathogens have evolved and emerged from environmental sources, their evolutionary routes through time and space, how they interact with other bacteria and the human host, as well as initiate disease. We outline novel approaches to the use of whole genome sequencing for this important group of bacteria and how new sequencing technologies may be applied to study these organisms in future studies.

**Keywords**

*Vibrio cholerae* · *Vibrio vulnificus* · *Vibrio parahaemolyticus* · Genome · Bioinformatics · Virulence

## 16.1 Introduction

*Vibrio* has been one of the most extensively studied groupings of bacteria since historical times. With a first species described in 1854 (*Vibrio cholerae*) (Pacini 1854), the presence of vibrios can be tracked back to the very dawn of microbiology. When Ernst Haeckel described the first three-kingdom tree of life in his General Morphology of Organisms book, published in 1866

D. Y. L. Lau · C. Baker-Austin
Centre for Environment, Fisheries and Aquaculture (CEFAS), Weymouth, Dorset, UK

J. R. Aguirre Sánchez
Centre for Environment, Fisheries and Aquaculture (CEFAS), Weymouth, Dorset, UK

Centro de Investigación en Alimentación y Desarrollo (CIAD), Culiacán, Sinaloa, Mexico

J. Martinez-Urtaza (✉)
Centre for Environment, Fisheries and Aquaculture (CEFAS), Weymouth, Dorset, UK

Department of Genetics and Microbiology, Facultat de Biociències, Universitat Autònoma de Barcelona (UAB), Bellaterra, Barcelona, Spain
e-mail: jaime.martinez.urtaza@uab.cat



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(Haeckel 1866), among other innovations he introduced for the first time a new category to make reference to the no-plant/no-animal group and named this group Protista. His protists comprise all microscopic organisms known at that time, including one division called "Monera" described as "most simple organisms, without structure, homogeneous pieces of Plasma", which included prokaryotic microorganisms that we know today are distantly related (e.g. bacteria, cyanobacteria). Already in this tree proposing a new order for the biological world, *Vibrio* was explicitly mentioned as one of the five groups composing Monera (Kutschera 2016) (Fig. 16.1). Almost uniquely, vibrios are a group of microorganisms that have been studied continuously for almost two centuries.

*Vibrio* spp. are a group of common, Gram-negative rod-shaped bacteria that are natural constituents of freshwater, estuarine, and marine environments (Thompson et al. 2004; Baker-Austin et al. 2017). Vibrios grow in warm, brackish waters, and their abundance in the natural environment tends to mirror ambient environmental temperatures. *Vibrio* spp. are responsible for the majority of human diseases attributed to the natural microbiota of aquatic environments and seafoods (Faruque 2006). The genus *Vibrio* contains over 100 described species, and approximately a dozen of which have been demonstrated to cause infections in humans (Baker-Austin et al. 2018). From a clinical and epidemiological perspective, of these dozen species pathogenic to humans, the species *Vibrio parahaemolyticus, Vibrio cholerae* and *Vibrio vulnificus* are considered to be the most important (Thompson et al. 2004). Infections associated with these bacteria are usually initiated from exposure to seawater or consumption of raw or undercooked seafood produce (Altekruse et al. 2000; Potasman et al. 2002). Globally and historically, this group of bacteria are an important cause of morbidity and mortality and represent a significant group of human pathogenic bacteria. Human diseases caused by pathogenic bacteria of the *Vibrio* genus can be divided into two major groups: cholera and non-cholera infections. *Vibrio cholerae* is the etiological agent of cholera, a severe diarrheal illness, and has an ecological preference for brackish waters and is the unique *Vibrio* species that can be found in freshwater and can cause cholera. Globally, there are believed to be 3–5 million cholera cases each year (Zuckerman et al. 2017). Non-cholera species, such as *V. parahaemolyticus* and *V. vulnificus*, occupy habitats of moderate salinity and can be found in seawater and seafood and can cause different diseases based on the exposition, route of infection, and host susceptibility (Baker-Austin et al. 2018). *V. parahaemolyticus* and *V. vulnificus* are significant pathogens associated with the consumption of seafood products, particularly oysters and clams (Altekruse et al. 2000; Potasman et al. 2002; Jones and Oliver 2009). Unfortunately, epidemiology and surveillance systems for non-cholera *Vibrio* infections are poor, although in countries where this information is gathered systematically (such as the USA) it is likely that these bacteria represent an important and emerging public health risk (Newton et al. 2012).

There are now a wealth of studies that suggest how infectious diseases driven by pathogenic vibrios are likely to increase in the future. Because these bacteria tend to grow in warm water, cases of *Vibrio* infections have a marked seasonal distribution—with most cases occurring during warmer months. Growing interest has focussed on the role of climatic events in modulating the clinical impact of these bacteria across both regional and global scales (Martinez-Urtaza et al. 2010; Baker-Austin et al. 2013; Vezzulli et al. 2016). In this regard, climate warming is likely to be associated with an increase in the frequency and intensity of numerous extreme weather events, including general warming patterns, but also heat waves, hurricanes, tropical cyclones, droughts, and severe precipitation events. All of these climatic phenomena are likely to play some role in increasing disease risk (Baker-Austin et al. 2017). Furthermore, increased population densities in coastal regions, a greater future demand for global shellfish produce as well as a larger and older risk population (with underlying conditions that predispose them to *Vibrio*

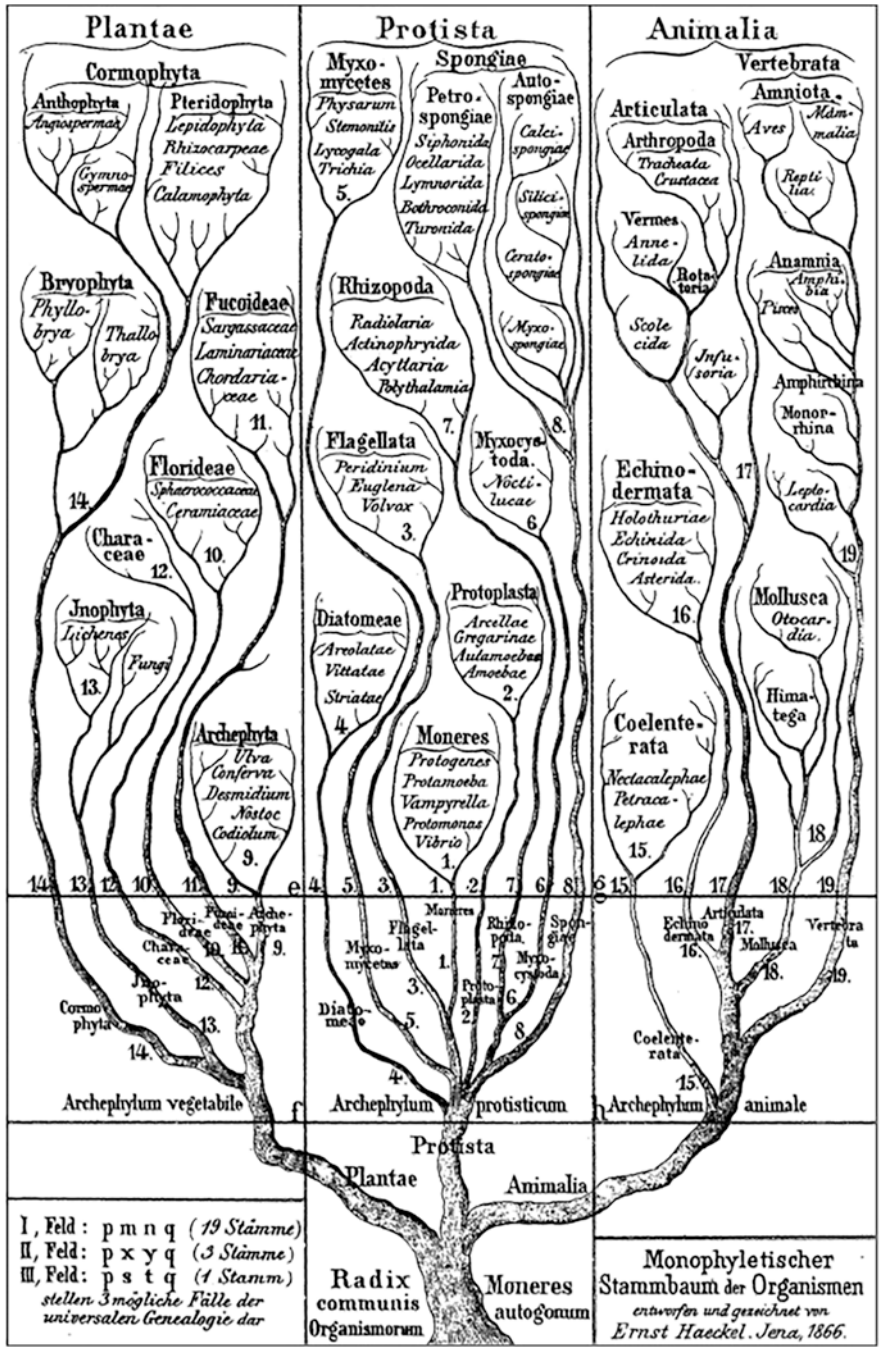


**Fig. 16.1** The first three-kingdom tree of life from Ernst Haeckel's General Morphology of Organisms (1866)

infections) may also enhance future risks associated with these bacteria (Baker-Austin et al. 2013; Scaglione et al. 2015; Baker-Austin and Oliver 2018).

Since the first two complete bacterial genome sequences were published in 1995, the study of microbiology has changed dramatically with the advent of whole genome sequencing (Land et al.

2015). In this review, we provide an overview on how our understanding of vibrios—and in particular the major pathogenic species such as *V. cholerae*, *V. parahaemolyticus,* and *V. vulnificus* has changed, using some of the key studies applied to these bacterial species. We discuss how the science of whole genome sequencing coupled to new and novel bioinformatic tools has developed over time and opened up a new and exciting opportunities to study these pathogens across a range of different environmental, clinical, and epidemiological contexts.

## 16.2 Initial Glimpses

There has long been interest in the genomic structure of vibrios. We now know that these fascinating bacteria share interesting and complex genomic structures, including two chromosomes, which are frequently shaped by recombination and intense horizontal gene transfer events. However, until the advent of tractable whole genome sequencing studies in the early 2000s, these insights remained elusive. Early studies had employed a physical map restriction enzymes and pulse-field gel electrophoresis (PFGE) to construct the physical map of *Vibrio* genomes and revealed that the key members of the *Vibrio* family possessed two chromosomes (Trucksis et al. 1998; Yamaichi et al. 1999). One of the first bacterial genomes sequenced and the first major *Vibrio* pathogen fully genomically sequenced was *Vibrio cholerae* El Tor N16961 in 2000 (Heidelberg et al. 2000). This landmark sequencing effort led by researchers at TIGR (The Institute for Genome Research) revealed a complex 4.03 Mb genome, comprising a large 2.96 and smaller 1.07 Mb chromosome. A myriad of interesting observations could be gleaned from this sequencing effort: the vast majority of genes associated with fundamental cell function (e.g. DNA replication, transcription, translation, and cell-wall biosynthesis) and pathogenicity (for example, toxins, surface antigens, and adhesins) were located on the large chromosome. The small chromosome also carried a gene capture system (the integron island) and host "addiction" genes that are typically found on plasmids. The authors noted that the small chromosome may have originally been a megaplasmid that was captured by an ancestral *Vibrio* species (Heidelberg et al. 2000). The observation of two functional chromosomes—but with vastly differing functions was clearly significant. What was perhaps one of the most noticeable observations from this study was that the entire genome of *V. cholerae* genome had numerous phage elements, insertion elements, and integrons had been shaped by previous horizontal gene transfer events. It was already known that key pathogenicity attributes in *V. cholerae* had emerged from distant sources. For instance, toxigenic strains of *V. cholerae* contain CTXφ, a lysogenic filamentous bacteriophage which carries the genes encoding the cholera toxin (Waldor and Mekalanos 1996). It became clear from this and subsequent sequencing efforts that the major virulence genes in *V. cholerae*—which are clustered in several chromosomal regions—appear to have been recently acquired from phages or through undefined horizontal gene transfer events (Faruque and Mekalanos 2012); indeed, it has been argued that horizontal gene transfer is an evolutionarily critical driver for these bacteria (Le Roux and Blokesch 2018). The authors noted that *V. cholerae*, "once a harmless environmental organism, has become pathogenic via multiple horizontal gene transfers" (Heidelberg et al. 2000). These initial studies suggested that horizontal transfer events have been a critical force in shaping this and other *Vibrio* pathogens.

Two further *Vibrio* pathogen genomes were published in the early 2000s, which again provided tantalizing insights into the function and genomic architecture of these pathogenic bacteria. The whole genome sequence of a clinical strain of *V. parahaemolyticus* (strain RIMD2210633) was published in 2003 (Makino et al. 2003). There were striking similarities to that of the El Tor cholera genome—the *V. parahaemolyticus* genome consisted of similarly sized two circular chromosomes of 3.28 Mb and 1.87 Mb, respectively. Again the structure and apparent function appeared to have been shaped by HGT events. However, some key

differences were noted between these two pathogens. Genes for a type III secretion system (TTSS) were identified in the genome of *V parahaemolyticus*, which were absent in the cholera genome structure. This authors noted the apparent difference in major virulence factors was probably important—it was suggested that this finding could explain clinical features of *V. parahaemolyticus* infections, which commonly include inflammatory diarrhoea and in some cases systemic manifestations such as septicaemia, distinct from those of *V. cholerae* infections, which are generally associated with non-inflammatory diarrhoea (Makino et al. 2003).

The first *V. vulnificus* genome was also published in 2003 (Chen et al. 2003), and these initial insights into the genomic structure of this sequenced strain also revealed a number of interesting findings. *Vibrio vulnificus* is a significant opportunistic human pathogen that can cause wound infections and primary septicaemia. The genome of biotype 1 strain, *V. vulnificus* YJ016 (a clinical isolate), was sequenced and included two chromosomes of estimated 3.37 Mb and 1.85 Mb in size, as well as a plasmid of 48,508 bp. Analysis of the genome identified a range of genes possibly associated with virulence, including an hemolysin (*vvhA*), the *rtx* gene cluster for MARTX and three complete secretion systems (Type I, II, and VI) as well as iron uptake-related genes (Chen et al. 2003; Baker-Austin and Oliver 2018). Interestingly, no single virulence marker (other than the haemolysin gene *vvhA,* which is found in all *V. vulnificus* strains) was identified. Again, a key finding from this sequencing study was the role of horizontal gene transfer (HGT) in shaping the evolution of the *V. vulnificus* genome, which like *V. parahaemolyticus* and *V. cholerae* genomes was scattered with insertion events, integrons, and conjugative elements. A gene capture system called a super integron, which allows the capture of genes and their subsequent expression, and commonly associated with antimicrobial resistance was identified in all three of these initially sequenced *Vibrio* genomes (Makino et al. 2003).

Despite the publication of the full genome sequences for the three major pathogenic *Vibrio* species, the routine use of Sanger method in the early 2000s was prohibitive for most microbiology laboratories. Sequencing based on this technology was expensive, cumbersome, and time-consuming restricting the use of massive application of WGS. However, the availability of information about the sequence and organization of the totality of the genes in the genome sequences created a new framework for sequencing-based studies of bacterial populations based on the sequence of a limited number of genes. These approaches, branded as Multi-locus Sequence Typing (MLST), typically used sequences of 7 housekeeping genes defined for each bacterial species and distributed along the chromosome, which were used to analyse bacteria at population level, defining the population structure and identifying the distinctive contribution mutation and recombination as driving forces of evolution. Contrary to other typing techniques used before, sequencing data could be easily shared about networks of collaborators and deposited in a single, centralized, and publicly accessible repository of data. Based on the publicly available data from the genomic sequencing, MLST schemes were developed for the three big pathogenic *Vibrio* (https://pubmlst.org/databases/) (Bisharat et al. 2005; Gonzalez-Escalona et al. 2008; Octavia et al. 2013) and with the help of the first generation of bioinformatic tools, scientists were able for the first time to compare isolates from different sources and regions and contribute to obtain a global picture of *Vibrio* populations and their incredible diversity in environmental sources. These initial studies were critical in more fully understanding the evolution of epidemic clones within a regional or global context. In the case of *V. parahaemolyticus*, MLST analysis revealed a high diversity within this species primarily driven by frequent recombination rather than mutation, with a semiclonal population structure and an epidemic structure similar to that of *Vibrio cholerae* (Gonzalez-Escalona et al. 2008).

## 16.3 From Genome to Pangenome: Refining the Species Concept

From the mid-2000s onwards, a variety of studies focussing on different aspects related to *Vibrio* genomics were subsequently published. What essentially changed and refocussed efforts across the *Vibrio* research community were the cost and speed of whole genome sequencing approaches, which made more ambitious sequencing projects and comparative analyses feasible. Several *Vibrionaceae* species have nearly identical 16S rRNA gene sequences. In these cases, the only alternatives for identification are the genomic fingerprinting, e.g. FAFL Pandrep-PCR, DNA-DNA hybridizations, or MLST (Thompson et al. 2004). The use of whole genome sequencing, particularly in the last decade has been instrumental in redefining the species concept and allowing taxonomists to differentiate *Vibrio* "species" based on a functionally and evolutionarily coherent framework. Furthermore, the follow-on sequencing efforts from the early 2000s onwards allowed the *Vibrio* scientific community to more fully understand the genetic attributes and dynamics that allow vibrios to survive and even proliferate in their ocean habitats, which include seawater, plankton, invertebrates, fish, marine mammals, plants, man-made structures (surfaces), and particulate matter (Grimes et al. 2009). Previous studies assessing the diversity of gene families in *Vibrio* have identified a hitherto unexplored genomic diversity. Several exciting studies utilizing genome sequencing to assess key ecological characteristics of vibrios in the open ocean have been published recently. These have addressed cooperation between conspecifics for antibiotic production and resistance (Cordero et al. 2017), spatial and temporal resource partitioning among *Vibrionaceae* strains coexisting in coastal bacterioplankton assemblages (Hunt et al. 2008), and the adaptive spread of functional genes in *Vibrio* communities (Hehemann et al. 2016).

As early as 2009, a first study analysed the genetic variation and revisited the taxonomy of *Vibrio* in the light of the sequenced genomes of different species (Thompson et al. 2009). The work applied a combination of different classical typing tools and genomic analysis to explore the relationship at taxonomic level between the available genomes at that time. Using information from core and pangenomes, the study provided novel insights of genomic differences between closely related Vibrio species. This work also provided a first estimate of the *Vibrio* pangenome which consisted of over 25,000 genes. More recently, Lukjancenko and Ussery compared the chromosome-specific genes in a set of 18 finished *Vibrio* genomes, and, in addition, also calculated the pan- and core-genomes from a data set of more than 250 draft *Vibrio* genome sequences (Lukjancenko and Ussery 2014). They found a massive gene diversity (~17,000 gene families) in the pangenome of vibrios and determined that many "housekeeping systems" encoded in chromosome 1, there are far fewer core functions found in chromosome 2, again highlighting the potential functional importance of the two-chromosome system in vibrios. How our understanding of the species concept and as applied to vibrios has also changed with the advent of whole genome sequencing.

WGS has been also recently applied to investigate how human activity could have shaped the population structure of *Vibrio* species. Using a global collection of 1103 clinical and environmental *V. parahaemolyticus* genomes, Yang et al. (2019) identified four diverse populations, with two populations with a distinctive geographical distribution, while other two had global presence. Divergence via genetic drift during geographical isolation was found as major driver shaping the diversity within and between populations. However, results showed that genetic mixture has taken place within the last few decades, suggesting that long-range dispersal may have been increased dramatically in the recent past. The observed change of distribution pattern of *V. parahaemolyticus* may have been consequence of a change in human activity over the last decades, such as shipping, aquatic products trade, and increased human migration between continents.

WGS has been proven helpful to infer phylogenomic analyses, and have been used to look retrospectively at issues related to vibrios. In 1996 an outbreak of *Vibrio vulnificus* occurred in Israel affecting fish farmers and consumers, the unusual pathogen was named Biotype 3 (Bisharat and Raz 1996; Bisharat et al. 1999). An investigation into the evolutionary relationship of Biotype 3 led to the revelation that it is a subtype of the E-genotype (a subset of "environmental" biotype 1 *V. vulnificus* strains). It has been suspected that these bt3 strains have virulence than other strains in aquaculture, and that a lateral gene transfer event and human behaviour may have led to the development of such distinct clone with the ability to cause disease in humans (Koton et al. 2015). While Biotype 3 was identified within the E-genotype, it formed a cluster separate from other E-genotypes strains; 1273 genes were also identified to be exclusive to Biotype 3 (Koton et al. 2015). Therefore, it was concluded that the distinct clone had originated from harmless environmental ancestors and acquired its pathogenic capabilities by lateral gene transfer from other vibrios—potentially due to the changes in fish marketing practices, allowing tilapia fish to be sold live in freshwater (Bisharat and Raz 1996; Koton et al. 2015). This study is noteworthy in that the study used WGS to elucidate a novel pathway in which epidemic populations can arise suddenly. Roig et al. (2018) used a similar WGS approach utilizing 80 *V. vulnificus* strains encompassing environmental and clinical strains, historical isolates and encompassing all three established *V. vulnificus* biotypes to infer their phylogenetic and evolutionary relationships. Based on their results, they proposed a new and updated classification approach for *V. vulnificus* utilizing phylogenetic lineages rather than on a previous biotype-based taxonomy that has been applied to study this species (Jones and Oliver 2009; Baker-Austin and Oliver 2018). A recent study on *Vibrio vulnificus* also applied genomic analysis to investigate genetic signatures associated with clinical and environmental strains and evolutionary driving force shaping the population structure of this species (López-Pérez et al. 2019). Comparative analysis of diverse set of 113 *V. vulnificus* genomes identified four different clusters among strain and found a decrease in the rate of recombination and gene flow between the two largest clusters, suggesting that these two clusters are diverging and evolving independently. Pangenome and phenotypic analyses were able to distinguish differences between lifestyles for these two clusters and identified frequent exchange of mobile genetic elements between and within species as major contributor of genetic diversity in the population.

In parallel to the expansion of the use of genomic data in all the field of biology, the extraordinary progress made over the last decade in the development of new algorithms and bioinformatic tools to analyse complex and large datasets of genomic sequences has enabled a more systematic scrutiny and improved visualization of the genomic landscape for these and other bacteria. These new tools, such as Anvi'o (Eren et al. 2015), an open-source platform for microbial omics, allow the identification of gene clusters for sets of *Vibrio* genomes, allowing the visualization of both shared and unique genes, as well as the overall genomic configuration and genomic composition of analysed strains. As shown in Fig. 16.2, referring to chromosome I (panel a) and chromosome II (panel b), 7 different pathogenic strains (encompassing both human and animal pathogens) were analysed to demonstrate the applicability of whole genome analysis approaches across this phylogenetically and evolutionarily diverse group. Pangenome reconstruction called for 20,989 genes and identified 6114 gene clusters for chromosome 1 and 10,792 genes and 4179 gene clusters for chromosome 2 (Table 16.1). Core genes for 7 *Vibrio* species primarily codified for central mechanism as metabolism, cell envelope or general regulatory functions. These tools also are able to discriminate among shell genome (genes present in 15–95% of *Vibrio* species) and accessory genome (genes which are unique for one genome),

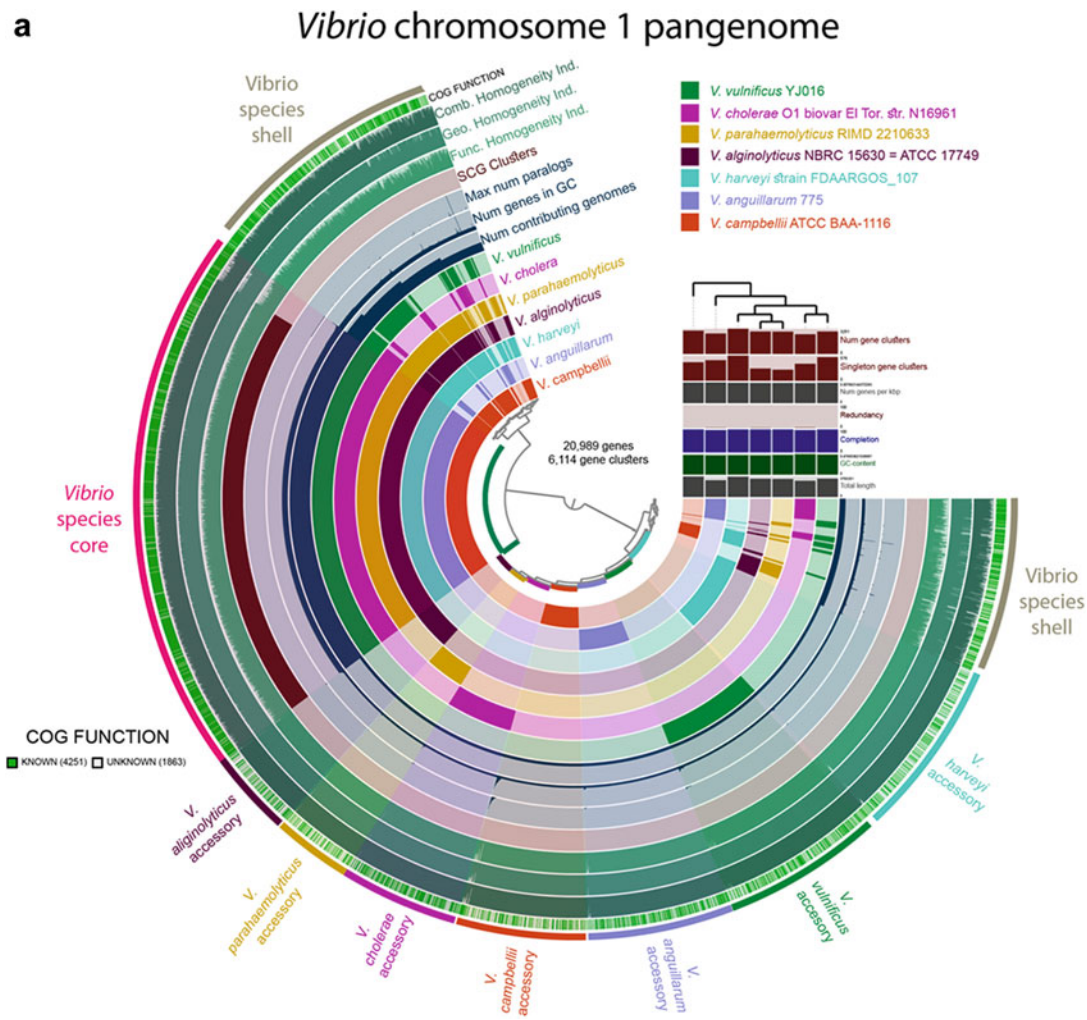


**Fig. 16.2** *Vibrio* species pangenome analysis using Anvi'o (Eren et al. 2015). Seven different *Vibrio* species were used to construct pangenome, which are denoted in different colours. (**a**) Pangenome analysis for chromosome 1 with a total of 20,989 genes and 6114 gene clusters were detected. The core genome is composed of 1776 gene clusters and 12,633 genes. (**b**) Pangenome analysis for chromosome 2 with a total of 10, 972 genes and 4179 gene clusters were detected. The core genome is composed of 307 gene clusters and 2219 genes. Accessory genes for each species were denoted using external rings. Central phylogenetic tree shows the species relationship according to gene frequency. Additionally, lateral cladogram was built according to the presence/absence gene clusters. COG functions are labelled with green and white stripes to indicate known and unknown function, respectively. COG: Clusters of Orthologous Groups

according to the frequency of the genes in the pool of genomes. These examples clearly demonstrate the advancement in data visualization approaches to study vibrios (as well as other bacteria). It is now possible to quickly and easily compare key aspects related to pathogenic vibrios—such as their respective chromosome structure and composition, comparative differences in inferred genetic function, core functional genes as well as accessory genes.

## 16.4 Reconstructing Past Outbreaks

One of the most exciting developments in the study of vibrios has been in the ability to utilize

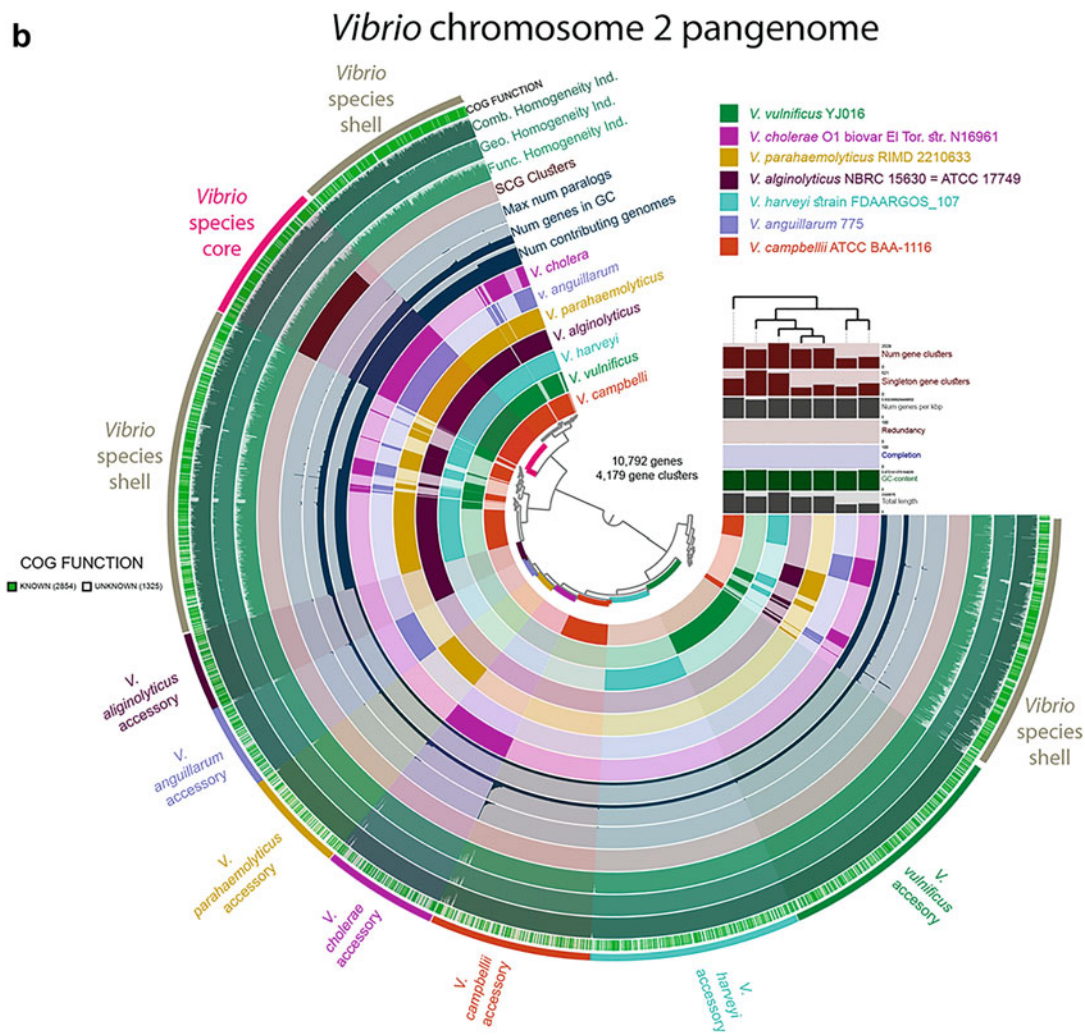


**Fig. 16.2** (continued)

whole genome sequencing approaches to retrospectively study outbreaks. However, the ability to piece together the phylogenetic and evolutionary history of bacterial pathogens from outbreak situations using high-throughput sequencing is relatively new concept (Harris et al. 2010; Köser et al. 2012). A key characteristic of *V. cholerae* and *V. parahaemolyticus* (that sets them apart in the vibrios) is their ability for pandemic expansion. For both pathogens, outbreaks have been observed emerging, then spreading globally in distinct "waves" (Mutreja et al. 2011; Baker-Austin et al. 2018). Mutreja et al. identified high-resolution markers (single nucleotide polymorphisms; SNPs) in 154 whole genome sequences of globally and temporally representative *V. cholerae* isolates. Using a phylogenetic analysis of these SNPs and coupled to a molecular clock approach, they were able to show that the seventh pandemic has spread from the Bay of Bengal in at least three independent but overlapping waves with a common ancestor in the 1950s and identify several transcontinental transmission events (Mutreja et al. 2011), (Fig. 16.3). Additionally, using whole genome sequencing they showed that the acquisition of the SXT family of antibiotic resistance elements was first acquired at least ten years before its

**Table 16.1** Size and genes for 7 reference genomes and core and accessory genes identified with Anvi'o

| | Whole genome | | Chromosome 1 | | | | Chromosome 2 | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Core genome | | Accessory genome | | Core genome | | Accessory genome | |
| *Vibrio* species | Genome size (bb) | Genes (total) | Gene calls | Gene clusters | Gene calls | Gene clusters | Gene calls | Gene clusters | Gene calls | Gene clusters |
| *V. alginolyticus* | 5146 Mb | 4680 | 12,633 | 1776 | 278 | 267 | 2219 | 307 | 180 | 172 |
| *V. parahaemolyticus* | 5165 Mb | 4732 | | | 254 | 249 | | | 231 | 226 |
| *V. cholerae* | 4033 Mb | 3737 | | | 401 | 386 | | | 287 | 255 |
| *V. campbellii* | 5969 Mb | 5592 | | | 637 | 425 | | | 527 | 347 |
| *V. anguillarum* | 4052 Mb | 3743 | | | 485 | 465 | | | 199 | 187 |
| *V. vulnificus* | 5211 Mb | 4858 | | | 562 | 537 | | | 523 | 518 |
| *V. harveyi* | 6038 Mb | 5571 | | | 574 | 564 | | | 461 | 457 |

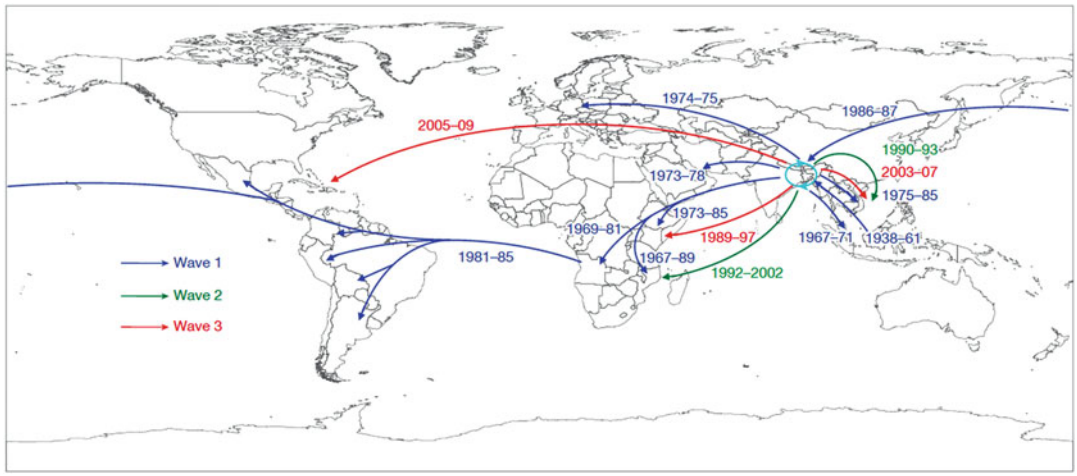


**Fig. 16.3** A key aspect of *V. cholerae* and *V. parahaemolyticus* (that sets them apart in the vibrios) is their ability for pandemic expansion. Utilizing a phylogenetic analysis of *V. cholerae* the seventh pandemic has spread from the Bay of Bengal in at least three independent but overlapping waves with a common ancestor in the 1950s. Figure courtesy Mutreja et al. (2011)

discovery in *V. cholerae*. Domman et al. similarly used WGS to characterize cholera strains obtained across the Americas over a 40-year time span, including the devastating cholera outbreaks that originated in Peru in 1991 and Haiti in 2010. They found that both epidemics were the result of intercontinental introductions of seventh pandemic El Tor *V. cholerae* strains and that at least seven lineages local to the Americas are associated with disease that differs epidemiologically from epidemic cholera (Domman et al. 2017). Similarly, to provide a more cohesive understanding of recent incidents of cholera in Africa, Weill et al. used genomic data from over 1000 *Vibrio cholerae* O1 isolates, across 45 African countries and over a 49-year period. They showed that past epidemics were attributable to a single expanded lineage (Weill et al. 2017). From their analysis they found that the lineage was introduced on multiple occasions since 1970, into two main regions, West Africa and East/Southern Africa, causing epidemics that lasted up to 28 years (Weill et al. 2017). The authors also noted that all the recent introductions were from Asia, and involved multidrug-resistant sublineages that replaced antibioticsusceptible sublineages after 2000. Whilst the current pandemic of *V. cholerae* emerged more than 50 years ago, the global expansion of *V. parahaemolyticus* is a recent phenomenon (Baker-Austin et al. 2018). Recent studies using WGS have been invaluable in piecing together the elusive spread of these pathogenic foodborne bacteria. Martinez-Urtaza et al. used genome-wide analyses of *V. parahaemolyticus* to reconstruct the evolutionary history of a highly pathogenic clone (ST36) over the course of its geographic expansion across the USA and into Europe (Martinez-Urtaza et al. 2013). The origin of this lineage was estimated to be in ~1985. They noted that by 1995, a new variant emerged in the region and quickly replaced the old clone, which has not been detected since 2000 (Martinez-Urtaza et al. 2017). The authors also suggested that after several introductions into the northeast coast of the USA, a new clone differentiated into a highly dynamic group that continues to cause illness on the northeast coast of the USA. Surprisingly, the strains detected in Europe in 2012 (Martinez-Urtaza et al. 2013) diverged from this ancestral group around 2000. More recently, an analysis of ST36 strains that have emerged in Peru (Abanto et al. 2020) showed the potential timeframe and route of the transcontinental

expansion of ST36 *V. parahaemolyticus* into South America. The identification of ST36 strains in both clinical and environmental sources is suggestive that these pathogens have now established themselves in Latin America, with potential for ongoing foodborne risk. In all of these studies, the unparalleled granularity afforded by WGS (compared to older subtyping methods) has allowed researchers to provide a more cohesive and integrated view of these pathogens in both time and space.

## 16.5 Novel Applications

The rate of technological change with regard to genomic sequencing—particularly in the last decade—has opened up new and exciting opportunities to study these important human pathogens. Numerous recent studies have shown that WGS can be used creatively for a variety of applications that are relevant in the field of epidemiology, clinical diagnosis, and ecology, among others. For example, *Vibrio* wound infections have a rapid onset of symptoms and can subsequently develop into necrotizing fasciitis and secondary septicaemia in a matter of hours. Correct clinical diagnosis coupled to the judicious use of antibiotics is therefore required. Unfortunately, routine examinations may fail to identify a pathogen, which was the case in a study by Li et al. (2019), where a suspected *Vibrio vulnificus* infection examined using routine wound and blood culture work did not lead to a correct clinical diagnosis. WGS was used for fast and accurate identification of *V. vulnificus*, with the use of PCR to confirm the subsequent results. This study demonstrates the effectiveness of WGS as a diagnostic method when routine examinations should fail (Alekseyev et al. 2018; Li et al. 2019).

WGS can be carried out on preserved historical specimens for phylogenomic investigations. A recent study analysed a clinically-preserved intestine sample from an 1849 cholera victim and provided crucial information regarding the evolutionary relationship of *Vibrio cholerae* at that time (Devault et al. 2014), Fig. 16.4. The current dominant *V. cholerae* biotype is the El Tor


**Fig. 16.4** Stored intestinal sample from a cholera victim from the second cholera pandemic, 1849 (Devault et al. 2014)

biotype, responsible for the 1900's cholera pandemics. It has been assumed that the Classical biotype was responsible for earlier pandemics, though no genetical characterization was carried

to confirm this hypothesis (Sack et al. 2004). Through extracting and reconstructing the genome from a victim of the second cholera pandemic, a phylogenetic analysis was carried out, which indicated a divergence from the El Tor biotype, while still sitting only a few SNPs away from the modern Classical biotype (Devault et al. 2014). To estimate evolutionary rates, a strict molecular clock proposed the El Tor biotype emerged in the mid-1900s, while the ancestor of the classical strains emerged in the mid-1800s, and the strain extracted from the sample occurred in the late 1700s to the early 1800s (Mutreja et al. 2011; Devault et al. 2014). However, factors such as site saturation and rapid recombination (37 detected between 2 linages by Devault et al. (2014)) increase the possible time frame of emergence to that of a millennia (Barua 1992; Feng et al. 2008; Devault et al. 2014). The high frequency of recombination made it challenging to identify if a specific recombination event led to the replacement of the Classical Biotype by the El Tor Biotype. However, a tandem CTX configuration was found on the genome, indicating a potential for pathogenic capacity, which could be interpreted as evidence against the hypothesis that the Classical Biotype was replaced due to lack of CTX virion production (Faruque et al. 2007; Devault et al. 2014).

The utilization of WGS in the investigation of the evolutionary relationship of biotypes in *Vibrio vulnificus* and *Vibrio cholerae* demonstrates the effectiveness of WGS in genomic analyses and better understanding the evolutionary development of pathogens. WGS was utilized to confirm that the Cholera outbreak in Haiti in 2010 was introduced by United Nations Stabilization Mission in Haiti (MINUSTAH) troops from Nepal (Orata et al. 2014). The troops were sent to Haiti to aid in humanitarian efforts to the aftermath of the catastrophic 7.0 magnitude earthquake in January 2010. However, a large cholera outbreak occurred shortly after with 8500 fatalities and 700,000 cases. When traditional epidemiological investigations into hospital case records established a route of entry and a spatiotemporal pattern but could not confirm the hypothesis that the MINUSTAH camp contaminated the longest and most important river in Haiti (Artibonite River), causing the outbreak, WGS was utilized (Piarroux et al. 2011; Ivers and Walton 2012). First, identical genetic profiles of the Haitian cholera strains were confirmed, indicating a clonal source of the outbreak, which was narrowed down to South Asia (Lam et al. 2010; Chin et al. 2011). Next, strains from the Nepal outbreak 3 months before the Haiti outbreak was examined with the Haiti *V. cholerae* O1 strains, it was observed that the Haitian and Nepalese strains formed a tight cluster and were almost identical, plus, no evidence of horizontal gene transfer was observed (Katz et al. 2013). Therefore, when the date of the most recent common ancestor (MRCA) matched the time frame of the Nepal cholera outbreak, arrival of the MINUSTAH troops, and the first Haiti cholera case, there was overwhelming evidence that the Nepalese soldiers had carried a *V. cholerae* O1 strain from Nepal. Through mishandling of sewage waste, it contaminated local river courses which are in close proximity to the first cholera cases identified at the time (Ivers and Walton 2012; Lantagne et al. 2014). The use of WGS in this context is unparalleled: as a result of these findings, human rights lawyers took legal action against the UN for damages and lack of compensation to the cholera victims.

## 16.6 Future Directions

Advances in sequencing technologies are moving at breakneck speed, and so too are analysis tools to scrutinize these datasets. Many of these approaches can now be achieved in almost real time. One of the most exciting developments is nanopore sequencing technologies—which can produce long read length sequences quickly and cheaply. Because these instruments are also portable, there is now the potential to use these methods in field-based applications, such as during outbreak situations (Quick et al. 2016). Data visualization approaches are also evolving quickly, to keep pace with the exponential increase in sequencing data and the inherent complexity therein. These approaches when applied

to pathogenic vibrios offer the potential to revolutionize the field of infectious diseases and microbiology by allowing us to unravel key aspects related to the evolution and spread of these diseases. Such approaches are incredibly exciting and will open a variety of applications, bridging the gaps between genomics, environmental microbiology, clinical infectious diseases, and epidemiology.

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