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Utilizing Amoeba as Hosts to Investigate Bacteria-Host Interactions: A Model System with *Vibrio cholerae*

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ABSTRACT

Vibrio cholerae, the causative agent of cholera, is a versatile bacterium with multiple strains exhibiting several mechanisms of survival. It is crucial to understand this mechanism for grasping how *V. cholerae* survives in a range of different environments. This study leverages *Acanthamoeba castellanii* as a host to investigate the interactions between *V. cholerae* and its environmental hosts, aiming to uncover the strategies employed by the bacterium to thrive.

In this study, Acanthamoeba castellanii was exposed to various strains of Vibrio cholerae, including the El Tor biotype, which is responsible for cholera outbreaks, and several mutants (Δ*hapR*, Δ*makA*, Δ*makB*, and Δ*makE*). The experimental procedures included regular passage of amoebae, bacterial plate streaking, measurements of optical density to monitor bacterial growth, and infection assays. For data analysis, holography and fluorescence cytometry were employed, alongside colony-forming unit (CFU) counting to quantify intracellular bacterial load.

This study highlights the critical role of nutrient availability in influencing bacterial growth, persistence, and host interactions between *V. cholerae* and *A. castellanii*. Under nutrient-depleted conditions in DASW, a seawater mimic, *A. castellanii* exhibited encystment as a protective response to osmotic stress, while the nutrient-rich PYG medium supported increased bacterial growth and intracellular persistence. The Δ*hapR* strain of *V. cholerae* formed biofilms specifically in PYG medium, indicating its potential role in defense, colonization, or symbiosis within the host environment. CFU counts confirmed significant bacterial loads in *A. castellanii*, with a notable absence of intracellular counts for the Δ*makA*, Δ*makB*, and Δ*makE* mutants, demonstrating that these genes are essential for the intracellular survival of *V. cholerae*. Additionally, the *V. cholerae* O1 El Tor strain A1552 displayed efficient invasion and survival within A. castellanii, particularly in the nutrient-abundant PYG medium.

These findings underscore the impact of nutrient conditions on bacterial behavior, with specific nutrient environments driving the survival strategies of *V. cholerae*. Higher encystation rates in DASW and enhanced bacterial survival in PYG medium highlight how these conditions shape *V. cholerae*'s adaptation to its host. Moreover, the Δ*hapR* strain's ability to induce amoeba cell death through biofilm formation emphasizes its strategic response to host interactions.

The study also underscores the significant influence of nutritional stress on antibiotic susceptibility. Nutrient depletion led to reduced growth rates, activation of stress response pathways, and changes in gene expression, which collectively increased antibiotic tolerance and supported the persistence of *V. cholerae* under adverse conditions.

Overall, these results advance our understanding of *V. cholerae*'s survival mechanisms and its complex interactions with the host, *A. castellanii*, providing crucial insights into bacterial-host dynamics and potential targets for controlling infections.

Contents

Abbreviations

Viable but non-culturable (VBNC)

toxin co-regulated pilus (tcp)

pore-forming toxin (PFT)

pore-forming proteins (PFP)

motility-associated killing factor A (makA)

colony forming unit (CFU) count

Multiplicity of Infection (MOI)

1 Introduction

1.1 Background

1.1.1 Introduction to *Vibrio cholerae* and its significance

Vibrio cholerae is a Gram-negative, comma-shaped bacterium that is the source of cholera, a severe diarrheal disease. This pathogen is mainly found in aquatic environments, both marine and freshwater, and is often associated with plankton and shellfish. Cholera outbreaks are often linked to contaminated water sources, causing it to become a major health concern for the public. Most notably in regions with poor water treatment and sanitation facilities. This bacterium has been classified into more than 200 serogroups due to its O antigen. However, only two such serogroups, O1 and O139, are known to cause cholera epidemics. The bacterium *V. cholerae* serogroup O1 has two subtypes: Classical and El Tor. Notably, El Tor has been the driving force behind recent cholera pandemics (Maheshwari et al., 2011).

Cholera, marked by rapid-onset, severe watery diarrhea, is caused by *V. cholerae*. The bacteria's cholera toxin (CT) disrupts intestinal ion transport, leading to massive fluid loss and potential death without prompt treatment. Another critical factor is the toxin-coregulated pilus (tcp), aiding in small intestine colonization. (Broeck et al., 2007). The primary virulence factor of *V. cholerae* is the CT which causes severe diarrhea associated with cholera. Genomic advances have provided us with insights into the evolution, diversity, and adaptation of *V. cholerae*. Comparative genomics has revealed genetic variations that end up contributing to the pathogenicity and environmental fitness of the bacterium (Heidelberg et al., 2000). These studies have allowed us to trace the origins and spread of different cholera strains, helping to understand the dynamics of cholera pandemics and informing public health strategies (Heidelberg et al., 2000).

The environmental significance of *V. cholerae* extends beyond its role in human disease. The bacterium’s ability to form biofilms on chitinous surfaces, such as zooplankton exoskeletons, enhances its survival and resistance to environmental stresses (Pruzzo et al., 2008). This biofilm formation is a crucial factor in the environmental persistence and transmission of the bacterium, highlighting the ecological interactions that influence the epidemiology of cholera. Moreover, the

study of *V. cholerae* in environmental reservoirs provides valuable insights into the mechanisms of bacterial survival and adaptation in natural habitats, contributing to a broader understanding of microbial ecology. Vaccination campaigns in endemic areas have shown promising results in preventing outbreaks (Bhattacharya et al., 2013). Furthermore, studying *V. cholerae* has provided insights into bacterial resistance mechanisms and the impact of environmental factors on bacterial virulence, which are critical for developing effective public health interventions.

1.1.2 *Acanthamoeba castellanii* as a Model System

A. castellanii is a free-living, versatile amoeba commonly found in various environments, including soil, freshwater bodies, and even air, making it ubiquitous in nature. Due to its ability to harbor bacterial pathogens, including Legionella pneumophila and Mycobacterium avium, A. castellanii serves as an excellent model for understanding survival mechanisms, pathogenesis, and the ecological dynamics of bacterial infections.

There are two stages in the life cycle of *Acanthamoeba* **(see Fig. 1).** The first of these is the trophozoite stage which is active and shows vegetative growth. The other stage is the dormant cyst stage which has low metabolic activity (Siddiqui & Khan, 2012). In the active trophozoite stage, *Acanthamoeba* feeds on microbes and other organic particles while undergoing division by mitosis under favorable conditions (food supply, neutral pH, and adequate temperature). It is only under harsh conditions which allow the *Acanthamoeba* to switch to double-walled cyst form.

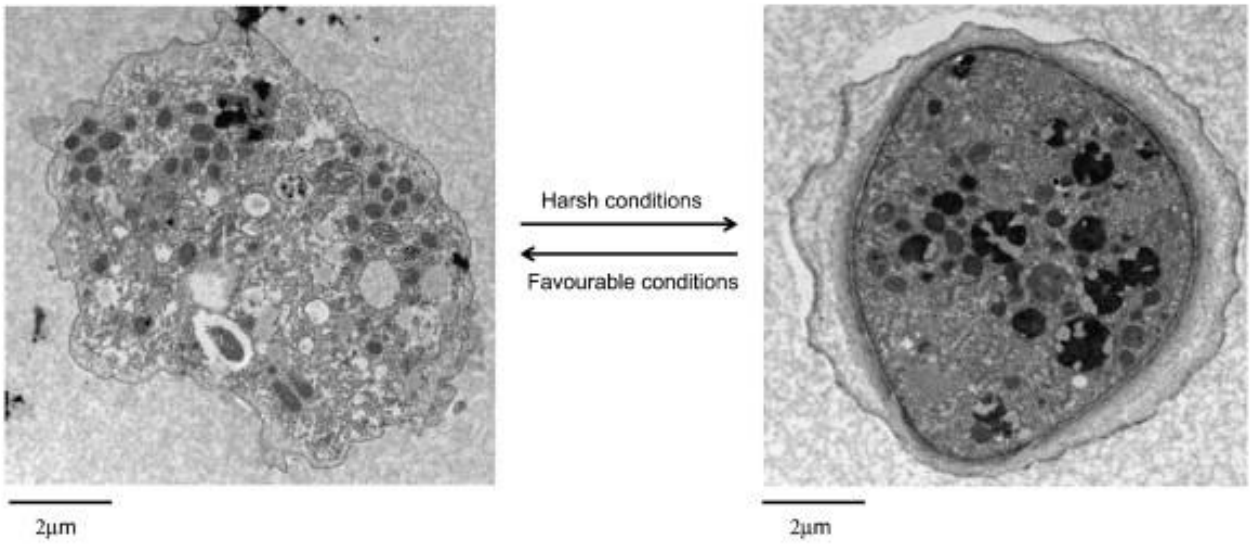

Figure 1: Transitioning of stages from trophozoite (left) to cyst (right). (Siddiqui & Khan, 2012)

Acanthamoeba has been found in a vast array of environments, from extreme conditions like hot springs, salt lakes, and Antarctica to common places such as air, water, and soil. These organisms have been isolated from various sources, including natural bodies of water, industrial waste, household items, food, animals, and even humans. They have been detected in both healthy and unhealthy individuals, across multiple bodily systems. Based on these sources, it is a fact that *Acanthamoeba* has ubiquitous presence in the environment and that we commonly encounter this organism in our daily lives. Culturing this microorganism is useful when classifying the organism, in order to test its virulence with human macrophages, to determine its antibiotic susceptibility, to use it in antigen or serological testing, and to produce monoclonal or polyclonal antibodies (Greub & Raoult, 2004). *A. castellanii* can be cultured easily in the laboratory under controlled conditions. This makes it an ideal model for researchers in order to study various aspects of microbial interactions.

1.1.3 Importance of Studying Bacteria-Host Interactions

Studying how hosts respond to bacterial infections provides us with better understanding of immune defense mechanisms. Amoeba, as primitive phagocytes exhibit defense strategies resembling those of more complex organisms. They serve as valuable models for understanding immune cell functions like macrophages due to their fundamental role in engulfing and digesting

proteins. The ability of V. cholerae to induce encystment in response to nutrient depletion and form biofilms for defense against amoebae mirrors similar tactics that pathogens employ to evade macrophage-mediated killing in higher organisms. Therefore, by investigating how *V. cholerae* interacts with amoeba, researchers can draw parallels to human cells. In particular, how macrophages and other immune cells enhance our understanding of innate immunity and how our bodies are able to evade bacterial attacks (Greub & Raoult, 2004). *V. cholerae*'s ability to survive and replicate within *Acanthamoeba castellanii* mirrors its interactions with human cells. Studies have also shown that *V. cholerae* can enter a viable but non-culturable (VBNC) state within amoeba. This allows bacteria to become persistent in the amoeba environment (Abd et al., 2004). This particular model involving amoeba and bacteria helps us to understand how bacteria evade host defenses and persist within amoeba. This persistence in turn leads to outbreaks.

Amoeba have co-evolved with bacteria for millions of years, making them valuable model organisms for studying the evolutionary dynamics for host-pathogen interactions. The interactions between *V. cholerae* and amoeba provide insights into the evolutionary pressures that shape pathogenicity and host defense mechanisms, offering a broader understanding of microbial evolution (Molmeret et al., 2005). Insights gained from bacteria-amoeba studies can assist in drug development method. Understanding how *V. cholerae* survives within amoeba could help identification of novel drug target aimed at disrupting these interactions, potentially reducing bacterial persistence in environmental reservoirs and human hosts (Barker & Brown, 1994). In fact, the role of amoeba is further seen in the maintenance of pathogens in the environment (Thom et al., 1992) and it was found that *V. cholerae* multiplied after ingestion by *Acanthamoeba* species. Free-living amoeba play a crucial role in maintaining *V. cholerae* in populations in natural water bodies worldwide, even in the absence of active cholera cases (Colwell et al., 1977). This makes them acting reservoirs for possible cholera infections.

Understanding how *V. cholerae* live in the environment and spread illness is key to preventing cholera outbreaks. If we can find ways to stop bacteria from surviving inside amoeba, it may be able to limit the spread of *V. cholerae* in the environment, which could significantly reduce the frequency of cholera outbreaks (Colwell et al., 1996). Historically, cholera outbreaks have driven technological advancements, enabling the use of tools such as remote sensing and computational modeling. By integrating of ecological, environmental, epidemiological, and spatial data, we can

develop predictive models for cholera outbreaks. By anticipating environmental and epidemiological conditions similar to those preceding past outbreaks, public health interventions can be deployed proactively, rather than reactively.

1.2 Research Objectives

1.2.1 Current Environmental Persistence of *V. cholerae*

The primary goal of this study is to investigate the pathogenesis and environmental persistence of *V. cholerae,* a bacterium that has profound global health implications. I shall be investigating its interactions with *A. castellanii,* a common environmental host organism.

This relationship is significant because amoebae provide a protective intracellular environment for V. *cholerae*. The interactions between *V. cholerae* and A. *castellanii* are complex and multifaceted. The amoeba can engulf the bacterium, but *V. cholerae* has developed several mechanisms to resist destruction within the amoeba. Studies have indicated that *V. cholerae* can manipulate the amoebic host's cellular processes to its advantage, allowing it to survive stressors such as starvation, temperature, and salinity fluctuations while also managing to survive within the amoeba (Lutz et al., 2013).

As stated earlier, *V. cholerae* can enter a viable but non-culturable (VBNC) state in response to environmental stress allowing it to persist in the environment and later on, revert to infectious form when conditions become favorable. This particular adaptability is crucial for the bacteria's ability to maintain its population during inter-epidemic periods and potentially resurge during outbreaks.

A. castellanii other amoebae serve as environmental reservoirs for *V. cholerae*, providing a protected intracellular niche where the bacteria can survive and multiply. This relationship is symbiotic, with amoeba offering a defense against environmental stressors while also acting as vectors for the bacteria's dissemination. The interaction between *V. cholerae* and amoebae highlights the importance of protozoa in the environmental life cycle of the bacteria, facilitating its survival and enhancing its infectious potential (Almagro-Moreno & Taylor, 2013).

The association of *V. cholerae* with various aquatic organisms, including zooplankton, shellfish, and fish, also underscores its ecological versatility. These associations enable the bacteria to form biofilms, which enhance its resistance to environmental stressors and contribute to its persistence. For instance, the chitinous exoskeletons of crustaceans, such as copepods, serve as a significant environmental reservoir for *V. cholerae*, providing both a substrate for biofilm formation and a source of nutrients.

The presence of *V. cholerae* in environmental reservoirs supports the hypothesis that such reservoirs play a major role in cholera transmission. Environmental monitoring and enrichment techniques could prove to be an important method to identify and understand dynamics of pathogenic *V. cholerae* populations. Vesicles serve as the mechanism for bacteria to engage with both prokaryotic and eukaryotic cells in their surroundings. Biochemical analysis and functional studies of outer membrane vesicles produced by pathogens have shown that this secretion method is utilized by pathogens to deliver active virulence factors into host cells (Kuehn & Kesty, 2005).

1.2.2 Investigating Amoeba-Bacteria Interactions

The second main objective of this study is to utilize *A. castellanii* to explore its interactions with *V. cholerae,* providing a broader model to investigate bacterium-eukaryote interactions. Given the limitations of existing animal model systems in fully capturing bacterium-host interactions, amoeba-bacterium interactions offer a simple, reproducible model system to study intracellular symbiosis (Shi et al., 2021). The study involves co-culturing *A. castellanii* and *V. cholerae* to observe and analysis of their interactions under controlled laboratory conditions. I will then employ microscopy techniques to visualize the process of bacterial uptake by amoeba, providing a clear view of how *V. cholerae* invades and resides within the host cells. Quantitative analysis of bacterial uptake will be performed to measure the efficiency of this process. Additionally, I will investigate cyst formation in *A. castellanii*, a critical survival strategy for amoeba under adverse conditions. Furthermore, I aim to assess the intracellular survival of *V. cholerae* within these cysts. These experiments will provide comprehensive understanding into the dynamics of amoeba-bacteria interactions, shedding light on how *V. cholerae* exploits *A. castellanii* for its survival in the environment.

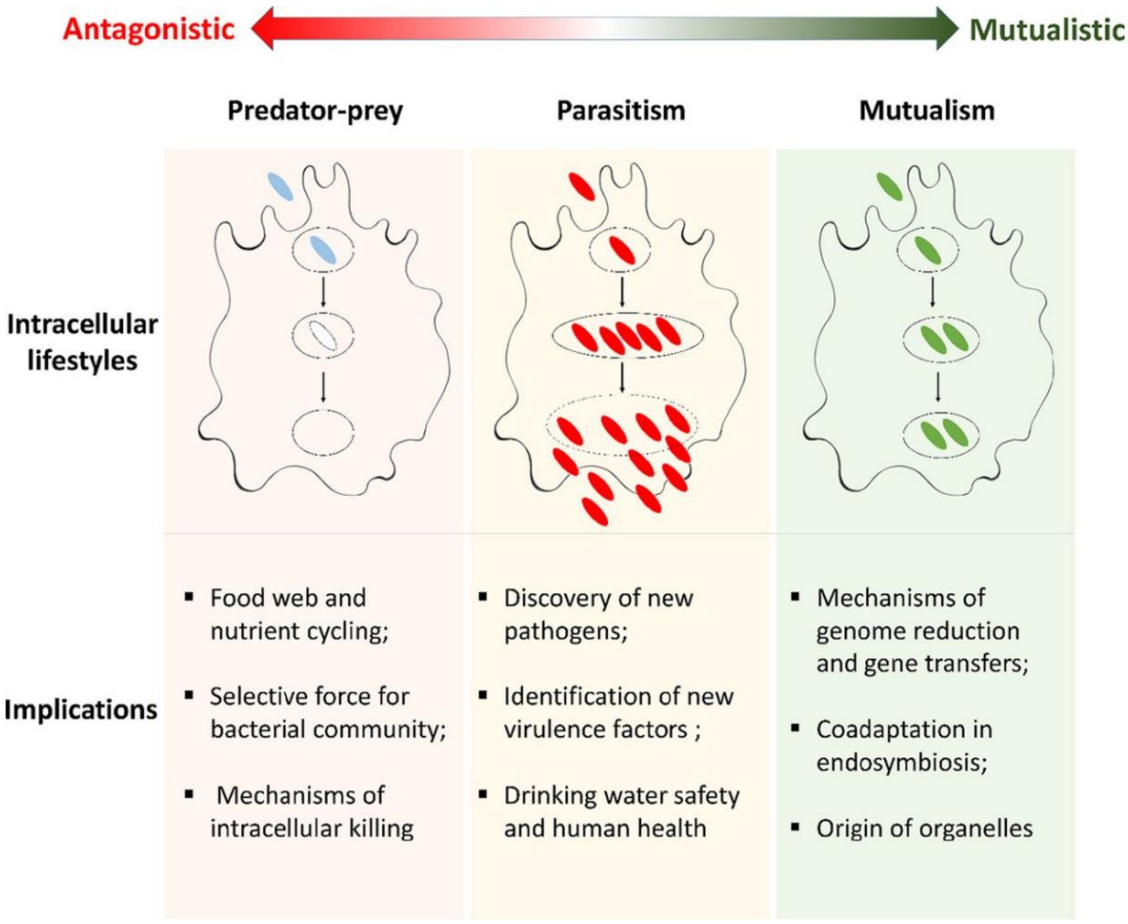

Figure 2: Ecological interactions between amoebae and bacteria (Shi et al., 2021)

As illustrated in **Fig. 2**, amoeba-bacterium interactions are of immense importance in understanding microbial evolution and host-pathogen dynamics. Before bacteria began interacting with animals and humans, they first coexisted and interacted with amoeba. Therefore, amoeba-bacterium interactions are highly complex, encompassing the full spectrum of symbiotic relationships, from mutualism to parasitism. Amoeba have evolved various mechanisms in order to locate, kill, and digest bacteria whereas bacteria have also developed strategies to resist amoebal predation, and in some cases, even infect and kill the amoeba.

Further analysis suggests that amoeba serves a melting pot which allows diverse bacteria to adapt to an intracellular lifestyle (Molmeret et al., 2005). Amoeba can also act as environmental reservoirs for many human pathogens as well. Evolutionary studies indicate that core mechanisms used by amoebae to kill and ingest bacteria have been conserved in human phagocytic cells,

demonstrating the evolutionary link between these ancient organisms and modern immune systems (Schmitz-Esser et al., 2008).

Therefore, my understanding of amoeba-bacterium interactions will allow me to understand better the role of amoebae in the origin, spread, and control of certain diseases.

1.2.3 Shedding Light on Mechanisms of Environmental Survival

Through this project, I will gain a deeper understanding of mechanisms underlying the environmental survival *of V. cholerae* by identifying key factors that facilitate the bacterium's interactions with environmental organisms, specifically amoeba. The study aims to uncover strategies that *V. cholerae* employs in order to endure and proliferate outside human hosts. This knowledge is crucial for identifying potential targets that could disrupt V. cholerae-amoeba interactions, thereby reducing environmental reservoirs and risk of cholera outbreak. Insights gained from this research will contribute to a broader understanding of bacterial survival mechanisms.

Free-living amoeba have evolved along the line of phagocytic cells where they have managed to engulf bacterial cells and use them as nutrients. They act as predators, keeping control on bacterial populations (Winiecka-Krusnell & Linder, 2001). Although some of the bacteria that are engulfed may show intracellular viability, occasionally infections may end up destroying the amoeba host. If the defense mechanisms of the host are compromised, internalized bacteria can then persist intracellularly and give rise to coadaptation due to establishment of a symbiotic relationship. Since gram-negative bacteria support the growth of *Acanthamoeba* as per existing literature, therefore a model utilizing *Acanthamoeba* and *V. cholerae* is ideal for this study (Marciano-Cabral & Cabral, 2003).

1.2.4 Development of Practical and Technical Skills

Finally, a major goal of this study is to equip myself with practical and technical knowledge essential for executing advanced microbiological research. Practical knowledge will consist of carrying out experiments, data analysis, and troubleshooting experimental issues, along with the application of various methodologies learned throughout the project. This hands-on experience is

vital for developing an adequate understanding of experimental procedures and interpreting results accurately. Technically, I aim to refine my skills in culturing *A. castellanii* and *V. cholerae*, ensuring optimal growth conditions for both organisms. Proficiency in microscopy will be important for visualizing interactions between amoeba and bacteria. This will allow me to observe and document my findings in detail. Furthermore, I will also learn bacterial colony counting methods in order to quantify the extent of bacterial uptake and survival within amoeba. Molecular biology techniques, those related to protein function analysis, will be central to this study, enabling me to identify the roles of specific proteins involved in interactions between *A. castellanii* and *V. cholerae*. These will be indispensable skills for successfully executing my planned experiments and correctly analyzing data generated during the study.

2 Literature Review

2.1 Components Involved in *V. cholerae* Virulence

V. cholerae infects humans primarily through the ingestion of contaminated water or food. In the small intestine, *V. cholerae* adheres to the epithelial lining and produces CT, leading to profuse watery diarrhea of cholera. The expression of the *ctx* gene, responsible for CT production, is regulated by the ToxR regulatory protein. The virulence of *V. cholerae* is primarily due to CT (Collin & Rehnstam-Holm, 2011). Collin and Rehnstam-Holm, isolates from the Swedish coast were positive for *toxR* gene but lacked the cholera toxin gene (*ctx*), indicating potential pathogenicity through other virulence factors. Both clinical and environmental *V. cholerae* strains showed similar cytotoxic effects, with a killing index (KI%) of 78% suggesting high pathogenic potential even in the absence of the *ctx* gene. This indicated that environmental strains can be highly pathogenic. Other *V. cholerae* genes are regulated in the same way, including the *tcp* (toxin co-regulated pilus) operon, which is responsible for fimbrial synthesis and assembly. The *ctx* operon and *tcp* are part of a regulon, with their expression controlled by the same environmental signals. Researchers have identified ToxR, ToxS, and ToxT as the proteins involved in this regulation (Awuor et al., 2022). ToxR, a transmembrane protein, has about two-thirds of its amino terminal part exposed to the cytoplasm. ToxS, a periplasmic protein, likely responds to environmental signals, changes conformation, and influences the dimerization of ToxR, which

then activates the transcription of the operon. ToxR activates the expression of ToxT, which in turn activates the transcription of tcp genes for the synthesis of tcp pili (Parsot & Mekalanos, 1990).

V. cholerae strains are divided into two subtypes: classical and non-classical serotypes. Classical ones represent O1 on their surface where classical serotypes are further divided into two categories: classical and El Tor. El Tor causes a milder form of cholera with infected individuals being frequently remaining asymptomatic early on during infection (Bart et al., 1970). *V. cholerae* encodes several virulence factors that regulate its survival, pathogenicity, and colonization. Having mentioned moments ago that *V. cholerae* virulence is due to CT, *V. cholerae* also requires the toxin co-regulated pilus virulence factor for pathogenesis (Klose, 2001). The ability to synthesize toxin co-regulated pilus is advantageous for *V. cholerae* in aquatic environments, as it improves *V. cholerae* fitness by making inter-bacterial interactions possible during colonization of host chitinaceous surfaces (Reguera and Kolter, 2005). There is another chitinase regulator of *V. cholerae* known as CytR (Watve et al., 2015). Since chitinases play a central role in pathogenesis, they may also be involved in controlling virulence cascade, which is ultimately attributed to the pathogenicity of *V. cholerae*, therefore causing cholera. Recently it has been discovered that among multiple roles, CytR is involved in the regulation of *V. cholerae* extracellular chitinase ChiA1 and ChiA2. Thereby, making CytR is a possible agent being involved in *V. cholerae* pathogenesis (Das et al., 2020). A *cytR* deletion mutant in this study demonstrated reduced pathogenicity, indicating that CytR plays a key role in the bacterium's virulence.

2.2 Amoeba as Hosts in Bacteria-Host Interactions

Amoeba are widely used as non-mammalian model organisms in research. The focus of my study however is the interaction between *V. cholerae* and free-living amoeba *A. castellanii*, having both been found frequently in aquatic environments (Martinez & Vivesvara, 1997) and same water samples from cholera endemic areas (Valeru et al., 2014). This particular amoeba preys on bacteria and has been described as a pathogenic bacteria reservoir (Fouque et al., 2012). As mentioned earlier in section 1.1.2, *A. castellanii* is characterized by biphasic life cycle, with the metabolically active trophozoite form that feeds and the stress-induced, dormant 'cyst' form that resists various environmental hazards (Naveed Ahmed Khan, 2006). The transition from trophozoite to mature

cyst involves several encystment steps, including global cell rounding and increased cell-wall synthesis (Chavez-Munguia et al., 2013). This process results in cysts having a thickened cell wall, enhancing their resistance to disinfection treatments. While the impact of predatory flagellates on *V. cholerae* populations is known (Matz et al., 2005), there is less understanding of the interaction between *V. cholerae* and free-living amoeba, including the molecular mechanisms involved. Notably, both *V. cholerae* and *A. castellanii* have been detected in the same environmental habitats.

Single-cell experiment revealed that *V. cholerae* (i) can resist intracellular killing by *A. castellanii*; (ii) is released from trophozoites by exocytosis; (iii) can establish intracellular proliferation; (iv) is able to maintain its niche upon amoebal encystment; and (v) can promote the lysis of *A. castellanii* cysts. These deductions strengthen the concept of *V. cholerae* being a facultative intracellular pathogen and suggest that this intracellular environment enhances its survival and protection (Van der Henst et al, 2016). *V. cholerae* in combination with *Acanthamoeba* species has shown that it can grow and survive inside *A. castellanii* (Shanan et al., 2016). Interaction between *V. cholerae* and *A. castellanii* involves attachment of bacteria to amoeba cells by engulfment, intracellular growth and intracellular survival of the *V. cholerae* O1 El Tor strain A1552 and OmpA mutant (a mutant used for the particular study) (Shanan et al., 2016). This was consistent with earlier studies which showed that the interaction of *A. castellanii* with *V. cholerae* O1 El Tor strain A1552, the capsule mutant strain, and the capsule/LPS double mutant strain increased the survival of all these bacterial strains. Additionally, despite *V. cholerae* O1 El Tor having a mannose-sensitive haemagglutinin fimbria, unlike *V. cholerae* O1 classical, both strains demonstrated enhanced survival and similar levels of intracellular growth in *A. castellanii* (Abd et al., 2007).

2.3 Role of Pore-Forming Toxins

The most common bacterial cytotoxic proteins are pore-forming toxins (PFT). They are the largest class of bacterial toxins that are a major class of pore-forming proteins (PFP). These are expressed by virulence factors via pathogenic bacteria. PFTs undergo structural and functional transition from inactive monomers to active, multimeric transmembrane pores that insert into the membrane of target cells. Through the disruption of epithelial barriers and interactions with the immune

system, PFTs promote the growth and spread of pathogens. PFTs also alter the plasma membrane of their target cells which can potentially lead to cell death (Dal Peraro & van der Goot, 2015).

PFTs are further classified into two larger groups - α-PFTs and β-PFTs based on their secondary structures, which are composed of α-helices or β-barrels. In this study, we focus on α-PFTs, which consist of hydrophobic α-helices. These α-PFTs oligomerize at the surface of their targeted cells and transition from a soluble to a protomer state. This allows these toxins to expose their hydrophobic region and insert themselves into the membrane in order to form a pore (Herrera et al., 2022). These pores could either be composed of homooligomers of one component or heterooligomers with either two or three components, consequently resulting in bi- or tri-partite toxins, respectively. The multicomponent α-PFTs are expressed through a single operon. In Nadeem's study, an α-PFT, was discovered in *V. cholerae* known as the motility-associated killing factor A (makA). makA is part of a gene cluster **(see Fig. 3)** which encodes an additional four proteins: makD (vca0880), makC (vca0880), makB (vca0880), and makE (vca0880). This gene cluster is known as the mak operon.

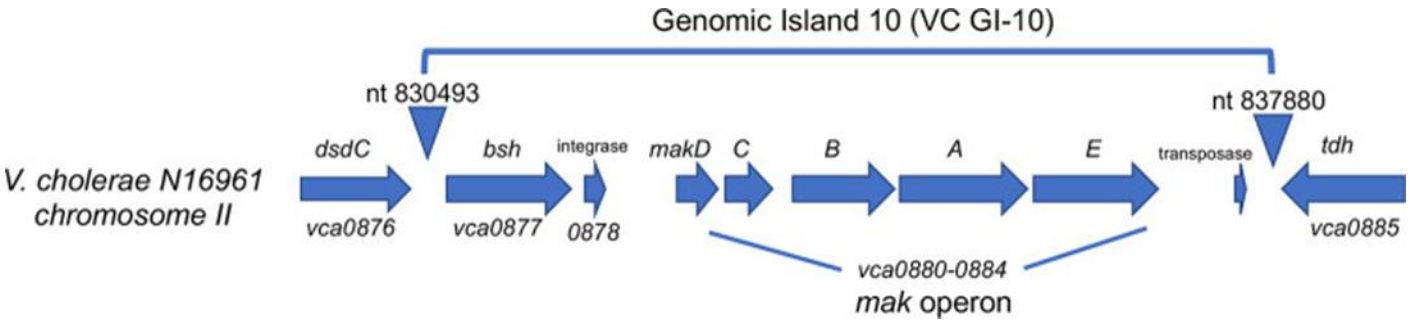

Figure 3: Genomic Island V. cholerae GI-10 which also depicts the mak operon (Nadeem et al., 2022)

The *V. cholerae* makDCBAE operon is positively regulated by hapR, a transcriptional regulator of quorum sensing that also represses cholera toxin expression. Quorum sensing is the communication between bacterial cells which allow bacteria to share information about cell density and therefore adjust gene expression as per its conditions (Rutherford & Bassier, 2012). makA is regulated tightly by hapR (Herrera et al., 2022). These mak proteins are expressed less during cholera. Instead, they are more likely to occur in natural aquatic environments where nutrition constraints challenge bacterial fitness (Nadeem et al., 2021). X-ray crystal structures for makA, makB, and makE tell us that they are structurally related to the α-PFT family. After testing myeloid linage cell lines with different conditions, one with makA, and one with a combination of

makA, makB, and makE, it was highlighted in Herrera's study that makA alone is cytotoxic at micromolar concentrations whereas combining makA with makB and makE is cytotoxic at nanomolar concentrations (Herrera et al., 2022). This data suggests that makA, -B, and -E are α-PFTs and when combined, show tripartite action. Nadeem's study further helps us to understand that these three proteins can contribute to Vibrionaceae fitness and virulence in different host environments and organisms.

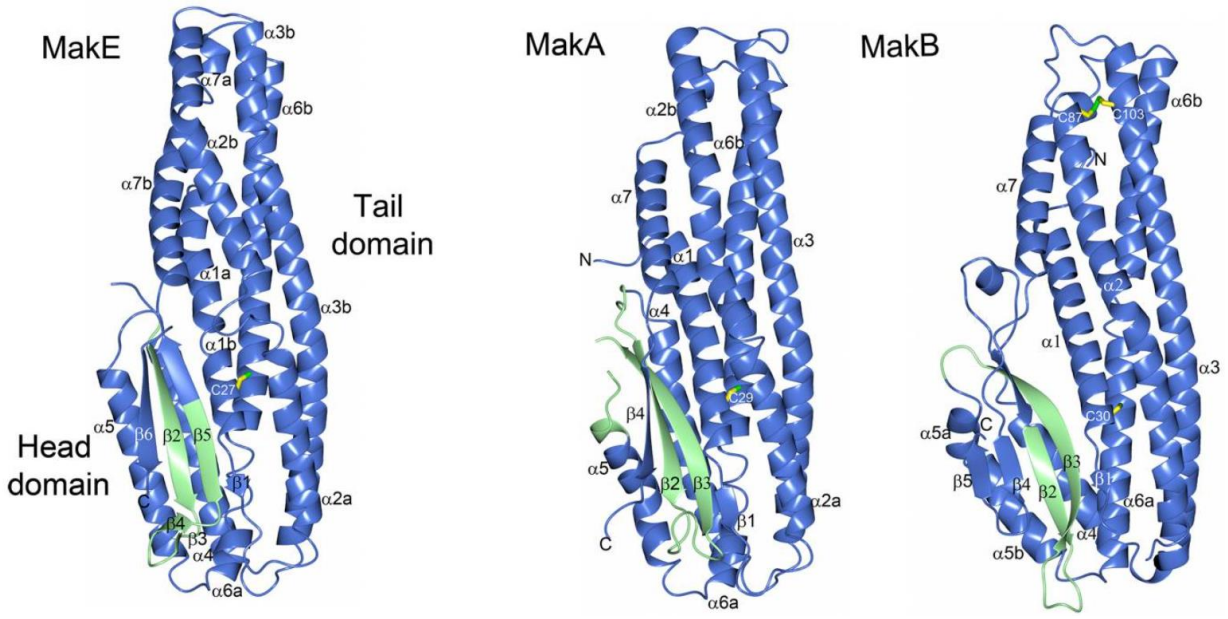

Figure 4: Crystal structures of makE, makA, and makB (Nadeem et al., 2021)

3 Aim of Thesis

I aim to analyze the interaction between *Acanthamoeba castellanii* and *V. cholerae* with the purpose of identifying the elements that make this bacterium capable of surviving in the environment. In this regard, the study employs co-culture techniques together with microscopy and quantitative analysis to establish factors that may be crucial for the survival of *V. cholerae* within the identified environmental hosts with the view of enhancing the understanding on the pathogenicity and environmental fitness of this bacterium.

4 Materials and Methods

The study design primarily centers on experimental methods complemented by observational analysis. The core of the experiments involved microbiological techniques conducted in the laboratory. Following the execution of these microbiological procedures, the results were analyzed using various methods, including holographic microscopy, cell detection via fluorescent labeling, and colony-forming unit assays.

Reagents

PBS (phosphate buffered saline) 1X sterile/EDTA 0.02%; Trypsin 0.05%/EDTA 0.02% (Gibco, Thermo Fisher Scientific; Lysogeny Broth (LB) (Miller) Medium (10 g/L NaCl) (Gibco, Thermo Fisher Scientific; Waltham, Massachusetts, USA); Defined artificial seawater (DASW); Peptone-Yeast-Glucose (PYG) medium (Thermo Fisher Scientific); Acridine Orange (AO) stain (Thermo Fisher Scientific); Calcofluor-white (CFW) stain (Sigma-Aldrich, USA); Propidium Iodide (PI) stain (Sigma-Aldrich, USA); Triton X-100 (Sigma-Aldrich, USA); Gentamicin (Thermo Fisher Scientific)

4.1 Multiplicity of Infection (MOI) and Amoeba Culture

Multiplicity of infection (MOI) is a crucial concept in virology, microbiology, and cell biology, including studies involving amoeba cells. It refers to the ratio of infectious agents (such as viruses, bacteria, or other pathogens) to target cells during an infection process. Specifically, in the context of amoeba cells, MOI is used to describe the ratio of the number of infectious units (e.g., bacteria or viruses) added to the number of amoeba cells in a given experiment. For my experiments, it is the number of bacterial cells added to amoeba cells.

For instance, an MOI of 1 indicates that one infectious unit is introduced per amoeba cell, whereas an MOI of 10 indicates ten infectious units are introduced per amoeba cell. In my experiments, the MOI was taken as 200. A high MOI, as used in my study, implies that nearly all amoeba cells, if not all, will be infected, resulting in a consistent infection rate across the culture.

MOI is calculated using the following formula:

MOI = Number of infectious units added / number of amoeba cells in the culture

Protocol

To seed *Acanthamoeba castellanii* effectively for infection studies, I used the prepared Peptone-Yeast-Glucose (PYG) medium, which served as the nutrient base for the amoeba. It served as one of the two infection mediums apart from defined artificial seawater.

	Number of cells	Volume per well
12-well	$5x10^4$	1 mL
24-well	$5x10^4$	1 mL
96-well	$5x10^3$	0.1 mL

Table 1. Cells seeding scheme for Amoeba

The *A. castellanii* cells were cultured in T25 flasks using PYG medium (Proteose peptone, 2% [w/v]; yeast extract, 0.1% [w/v]; 0.4 mM CaCl2; 4 mM MgSO4·7H2O; 2.5 mM Na2HPO4·7H2O; 2.5 mM KH2PO4; sodium citrate·2H2O, 0.1% [w/v]; 0.05 mM Fe(NH4)2(SO4)2·6H2O; 100 mM glucose; pH 6.5) The flasks are incubated at 30°C, the optimal temperature for *A. castellanii*. The reason for incubating amoeba at 30°C is because slightly warmer temperature promotes higher metabolic activity and faster growth. Periodically, usually every 2-3 days, cell counting is conducted using a Bürker chamber. When the culture reaches high density, typically indicated by 70-90% confluency, subculturing is necessary. This involves transferring a portion of the culture to fresh wells containing new PYG medium, diluting the culture as needed to maintain optimal growth conditions and prevent overcrowding.

The Bürker chamber grid consists of several squares with the central large square containing 25 smaller squares, used for cell counting. Calculation of the cell concentration in cells per mL is performed using the following formula:

Cell concentration (cells/mL) = (Average cell count per square x Dilution factor x 10^4) / Volume factor of the chamber

In this formula:

The average cell count per square refers to the mean number of cells counted in the specified squares within the chamber grid. The dilution factor accounts for any dilution made to the sample before loading into the chamber. Finally, the multiplication by an exponent of 4 converts the count from the chamber's volume to a concentration per mL. Each square in the Burker chamber holds a volume of 0.1 mm^3.For 1 mL of culture, I require 50,000 amoeba cells when counted using the Bürker chamber. Therefore, when scaling down the volume, I should have 5,000 cells for every 100 µL of culture. For a smaller volume, such as 100 µL (0.1 mL), this would correspond to 5,000 cells, aligning with the target density when the concentration is correctly diluted to 50,000 cells/mL.

The following calculations are carried out using the Bürker chamber where the 10 µL volume is inserted into the Bürker chamber:

Volume of cells required

= (Cells required in a single well / number of cells counted in Burker chamber) x vol. of bacterial culture

Once the required cell count is obtained, the amoeba cell culture is mixed with PYG medium and then transferred to multi-well plate. This is done to allow *A. castellanii* cells to adhere to the surface of culture vessel, overcome stress, and for cell proliferation and growth. The plate is then incubated overnight at 30°C.

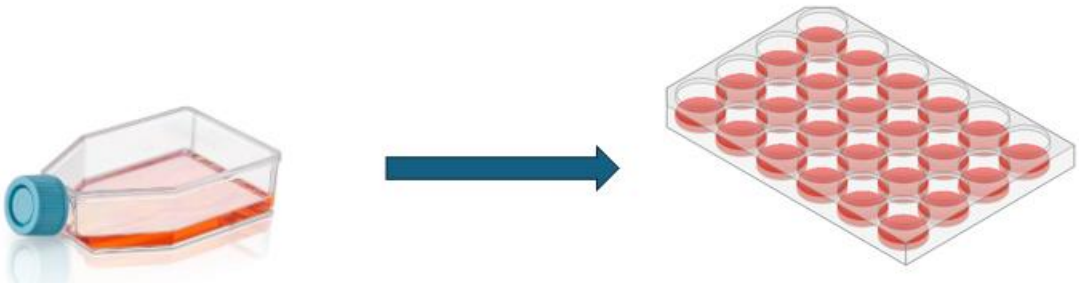

Figure 5: Transfer of amoeba from culture flask to plate

4.2 Bacterial Plate Streak

After amoeba culture, we streak our bacterial strains onto the plate. This includes the wild-type strain **A1552**, the mutants **Δ*makA***, **Δ*makB***, and **Δ*makE***, and the quorum sensing-regulated transcription factor, **Δ*hapR***. I begin by preparing the LB plates, which served as the growth medium. The preparation of these plates involves dissolving LB powder in distilled water according to the manufacturer's instructions. Once sterilized, the molten LB agar is carefully poured into sterile Petri dishes near a lit Bunsen burner to minimize the risk of contamination. The plates should be filled to a uniform depth, typically about 15-20 mL of agar per plate using a serological pipette. After pouring, the plates are allowed to rest on the workbench under sterile conditions until the agar solidifies, which generally takes around 30 minutes. Once solidified, the plates are ready for use.

The bacterial strains used in this protocol are sourced from glycerol stocks stored at -80°C. These stocks are retrieved and handled with care to prevent thawing. A plastic single-use sterile wire loop is used to scrape a small amount of the frozen bacterial culture from the glycerol stock. This loopful of bacteria is then streaked onto the LB agar plate using the quadrant streak method **(see Fig. 6)**. The first plate is divided into three sections for A1552*,* Δ*makA*, Δ*makB*, whereas the second plate is divided into two sections for Δ*makE* and Δ*hapR*.

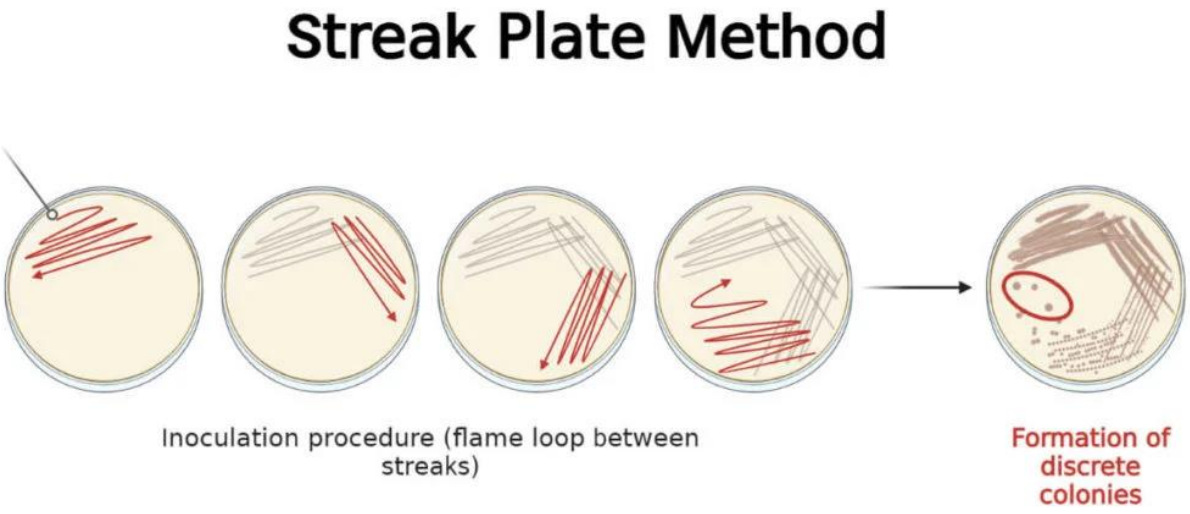

Figure 6: Streak Plate Method (Microbe Notes)

The quadrant streaking technique is employed to dilute the bacterial sample across the surface of the agar, enabling the isolation of single bacterial colonies. The first quadrant is streaked by gently gliding the loop across the agar in a back-and-forth motion as illustrated in Figure 5. Subsequent quadrants are streaked by dragging the loop from the edge of the previous streaks into fresh areas of the agar, progressively diluting the bacteria. As the bacterial cells are spread across the agar surface during streaking, they are gradually diluted, reducing the number of cells in each subsequent streak. This dilution leads to the formation of distinct, well-separated colonies in the final quadrant after incubation. Each isolated colony originates from a single bacterial cell or a small group of genetically identical cells, ensuring that the colony represents a pure culture.

Precaution is critical, especially when opening Petri dishes or handling sterile materials. The plates are immediately covered after streaking and placed in an overnight incubator set to 37°C. The following day, the optical density (OD) of bacterial cultures is measured to assess the growth phase and density of the bacteria.

4.3 Measuring Optical Density (OD) of Bacteria

After the overnight incubation period, once bacterial growth was seen, I proceeded to measure the optical density (OD) of the bacteria using a spectrophotometer. For each strain, approximately 5-6 colonies are carefully picked from the bacterial plates using a sterile wire loop and then suspended in 2 mL of PBS buffer. From this bacterial suspension, 100 µL is transferred into a cuvette, which is then filled with 900 µL of PBS to bring the total volume to 1 mL. Therefore, giving a dilution of 1:10. This process is repeated for all bacterial strains. Additionally, a blank cuvette containing 1 mL of PBS is used to calibrate the spectrophotometer before measuring the OD of the bacterial samples.

Once the absorbance is measured, the required infection volume is calculated using a set of calculations.

I consider the required amoeba cell concentration, the measured bacterial OD, and the MOI to correctly calculate the volume of infection. For example, my goal is to infect amoeba cells with a particular bacterial strain within a well of a 96-well plate. The OD measured for the bacterial strain makA is OD3.39 and we need to calculate the volume of infection.

A 10X dilution is made where 100 µL of the bacterial solution is mixed with 900 µL PBS. Therefore,

OD1= 1x10^9 CFU/mL

OD3.39/100 = 0.0339

0.0339 x 1x10^9 = 33900000

For MOI = 200 and 96-well plate (5000 cells/well)

(200 x 5000) / 33900000 = 0.0294 mL

= 29.4 µL

Therefore, in this example, bacterial volume required to infect amoeba cells for *makA* strain is 29.4 µL.

This procedure is repeated for the WT1552, *makA*, *makB*, *makE*, and *hapR* strains depending on the OD measured as well as the respective multi-well plate being used for infection.

4.4 Infection of Amoeba Cells with Bacterial Strains

Once the bacterial cultures were prepared, I then transferred the respective volume of the amoeba cells into a falcon tube and mixed it with the PYG media to make up the total volume of the amoeba culture that I used for infection.

For instance, if the multi-well plate is a 24 well plate, then my total volume for the amoeba culture would be 25 mL It is due to the fact that each well in a 24-well plate contains 1 mL volume and 24 mL of amoeba culture would be used. This total volume of 25 mL includes amoeba cells, and the PYG medium.

At the end of section 4.1, cells were transferred to a multi-well plate and kept overnight for amoeba cells to adhere to the wells. After overnight incubation, the infection protocol was be carried out.

Protocol

To prepare for infection, two types of media were used; the seawater mimic is employed to simulate natural seawater conditions, is used for all the schemes – 12-well, 24-well, and 96-well, whereas PYG medium was used only in the 96-well scheme. The reason PYG is used only in a 96-well scheme is because uniformity of 96-well format facilitates easier data reproduction and analysis. Furthermore, smaller volume reduces the risk of cross-contamination between wells, which is important when using a nutrient-rich media like PYG. The optical density (OD) of the bacterial suspension is measured using a spectrophotometer to ensure accurate adjustment of bacterial concentration to achieve the desired MOI, as explained in 3.3.

The MOI for the experiment is set at 200, meaning that for each amoeba cell, 200 bacterial cells will be introduced. The volume of bacterial suspension needed to achieve this MOI is calculated based on the OD measurements and the amoeba cell counts obtained from the Bürker chamber. Once the appropriate volumes are determined, the amoeba culture is infected with bacterial suspension in the multi-well plates. The plates are then covered with a plastic case and layered with damp paper towels to maintain humidity and prevent drying. The infection is allowed to proceed overnight at 25°C.

Experiments were carried out in replicates involving an appropriate negative control to ensure the feasibility of results. The negative control wells contained the growth medium only with the amoeba. Negative controls provide a baseline to compare the experimental data against. In this

particular setup, the negative control wells contained only the growth medium (either DASW or PYG) along with *Acanthamoeba castellanii*, without the addition of any bacterial strains. This helps determine the activity of amoeba when it is not exposed to bacterial infection. The following schemes utilized for the given multi-well plates:

12-well plates

For the 12-well plate, two wells were assigned for the negative control (CT), *makA*, *makB*, *makE*, and *hapR*.

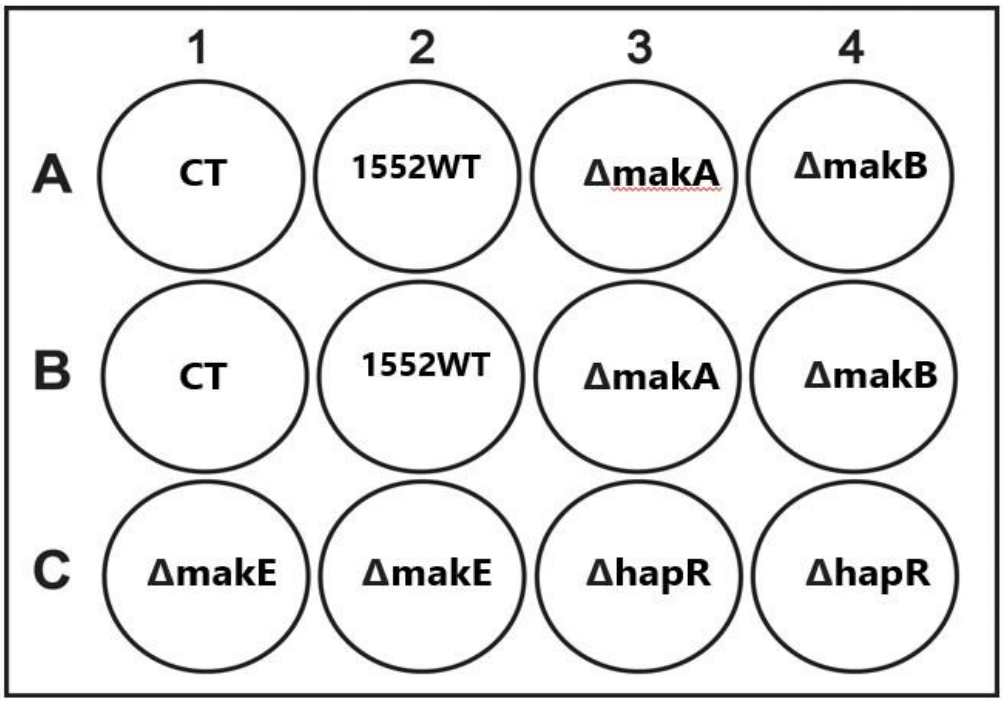

Figure 7: 12-well plate scheme for DASW

24-well plates

In the 24-well plate, one column of four wells was designated for the negative control (CT), while the other columns were allocated to WT1552, *makA*, *makB*, *makE*, and *hapR* respectively.

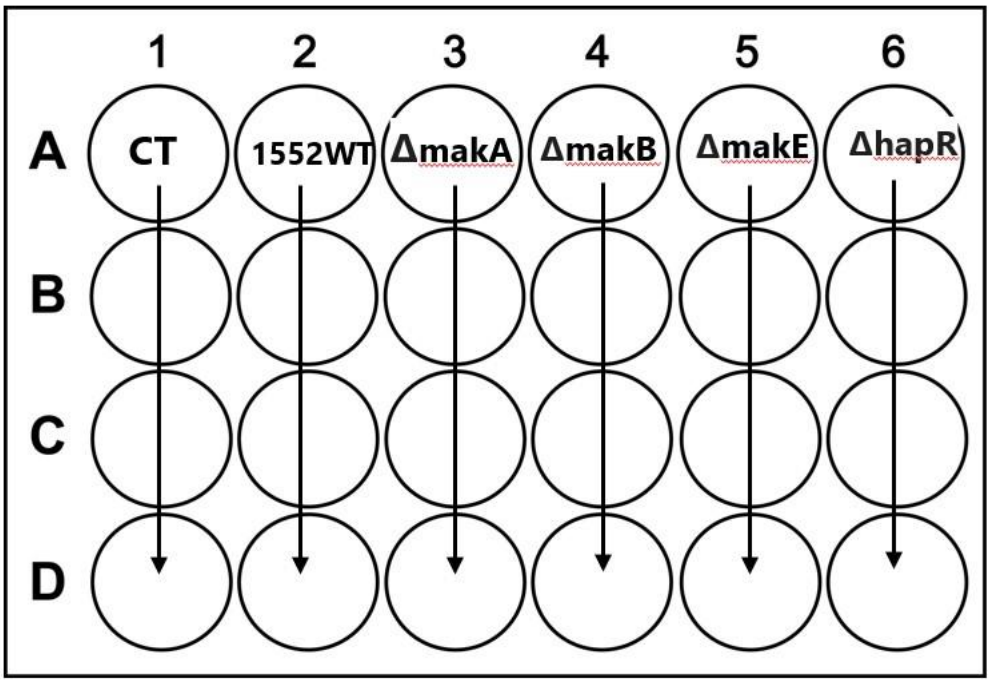

Figure 8: 24-well plate scheme for DASW

96-well plates

The 96-well plate was divided into two sections: six rows for infections in DASW medium and six rows for PYG medium. Each row within these sections was dedicated to a specific bacterial strain, with additional rows serving as untreated negative controls (CT).

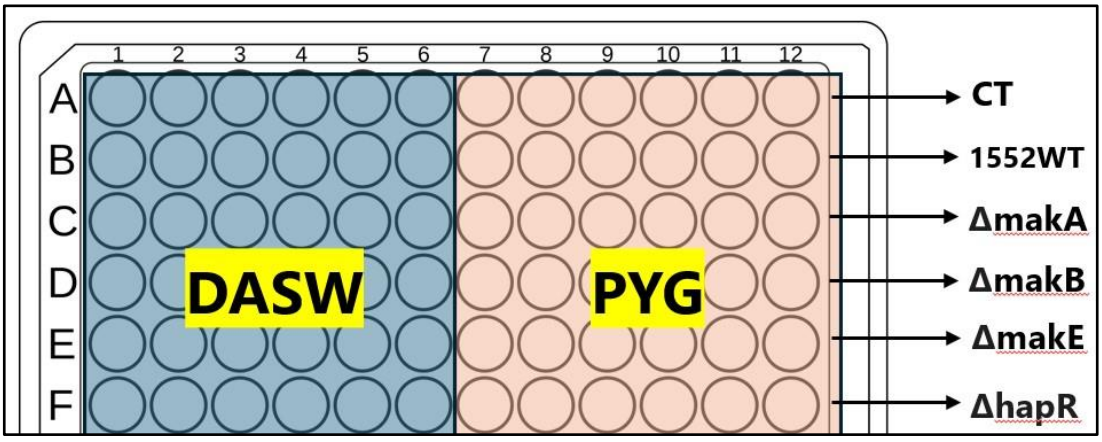

Figure 9: 96-well plate scheme for DASW and PYG

The following day, the infection is stopped by washing the amoeba cells with PBS to remove any unbound bacteria. The samples are then immediately visualized under a holographic microscope.

This step is crucial for observing amoeba motility and conducting non-invasive visualization without compromising cell integrity. After holographic microscopy, the samples undergo fluorescent labeling and are analyzed using flow cytometry. This allows for the quantification and further assessment of the infection at the single-cell level.

Using this protocol, a thorough and well-structured study can be conducted to examine the infection of *A. castellanii* by multiple *V. cholerae* strains. The experiment was replicated several times in order to make sure that the generated data is reliable.

4.5 Holographic Microscopy

Holographic microscopy of *A. castellanii* was carried out post-infection in order to assess their motility and morphology without compromising cell integrity. For this, I used HoloMonitor® Live Cell Imaging System and its software.

Operating this imaging system is simple and user-friendly. I placed my plate directly on the microscope stage, which automatically adjusts to position the sample. After powering on and allowing the microscope to calibrate automatically, we can fine-tune the stage focus of the microscope in order to visualize the amoeba cells and its features. No coverslips or extra tools are necessary—the plate remains covered with its plastic lid, allowing for easy and efficient microscopy.

The HoloMonitor® Live Cell Imaging System used the “Kinetic Motility Assay” function to observe amoeba cells. Areas of the sample with low density counts were observed in order to properly visualize the cells and avoid microscopic drift.

4.6 Flow Cytometry

The primary goal of flow cytometry is to assess cell viability, observe the presence of cysts, and determine the extent to which each bacterial strain has caused cell death.

Protocol

Following the infection, the cells were washed twice with PBS to remove unbound bacteria and debris. The cells were then stained using a combination of Acridine Orange (AO) and Calcofluor-White (CFW), which allowed for the detection of trophozoite cells and cysts, respectively.

Specifically, 20 µL of AO, containing Calcofluor-White, was added to each well in the 24-well and 96-well plates. The plates were incubated at 25°C for 1 hour to allow the dye to stain the cells.

After incubation, the plates were placed in the Tecan Spark® Cyto live cell plate reader, where the cells were visualized, and images were captured. The live cell reading focused on detecting green fluorescence for trophozoite cells and blue fluorescence for cysts, and the data was exported for further analysis.

Following the live cell reading for AO, Propidium Iodide (PI) was added to the wells to detect dead cells. PI was directly added at a volume of 3.3 µL per well in both the 24-well and 96-well plates. The plates were then incubated at 25°C for 40 minutes, allowing the PI to stain dead cells without additional washing steps. After the incubation, the plate reader was used again to visualize and capture images of non-viable cells, which were detected as red fluorescence. The data from this step was also exported for analysis.

AO stains live cells by binding to their DNA, causing them to emit green fluorescence, while PI stains non-viable cells by penetrating damaged membranes, resulting in red fluorescence. Together, they allow for the distinction between live and dead cells, helping to identify cell viability and potential apoptosis.

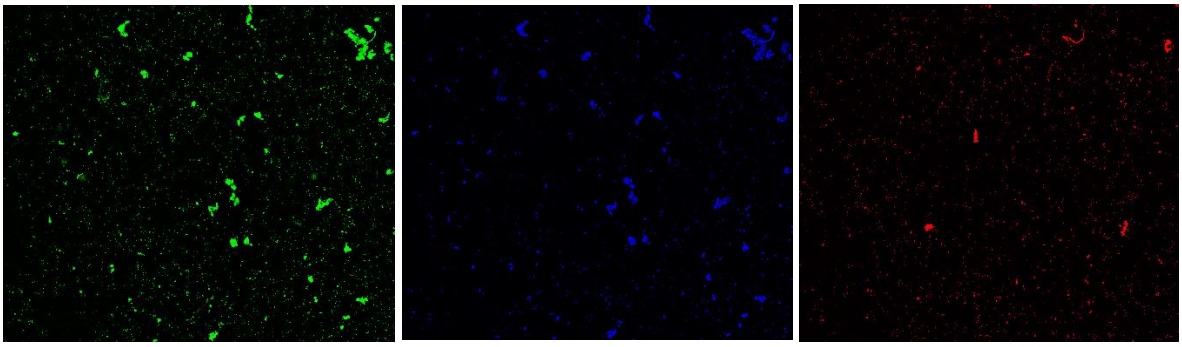

Figure 10: Visualization of stains displaying; (i) Trophozoites, (ii) cysts, (iii) non-viable cells (L to R)

Controls were included throughout the experiment to ensure the reliability of the results. Negative controls, which were not exposed to bacterial strains, served as baseline references, validated the staining and detection processes. The experimental setup also included replicates within the multi-well plates.

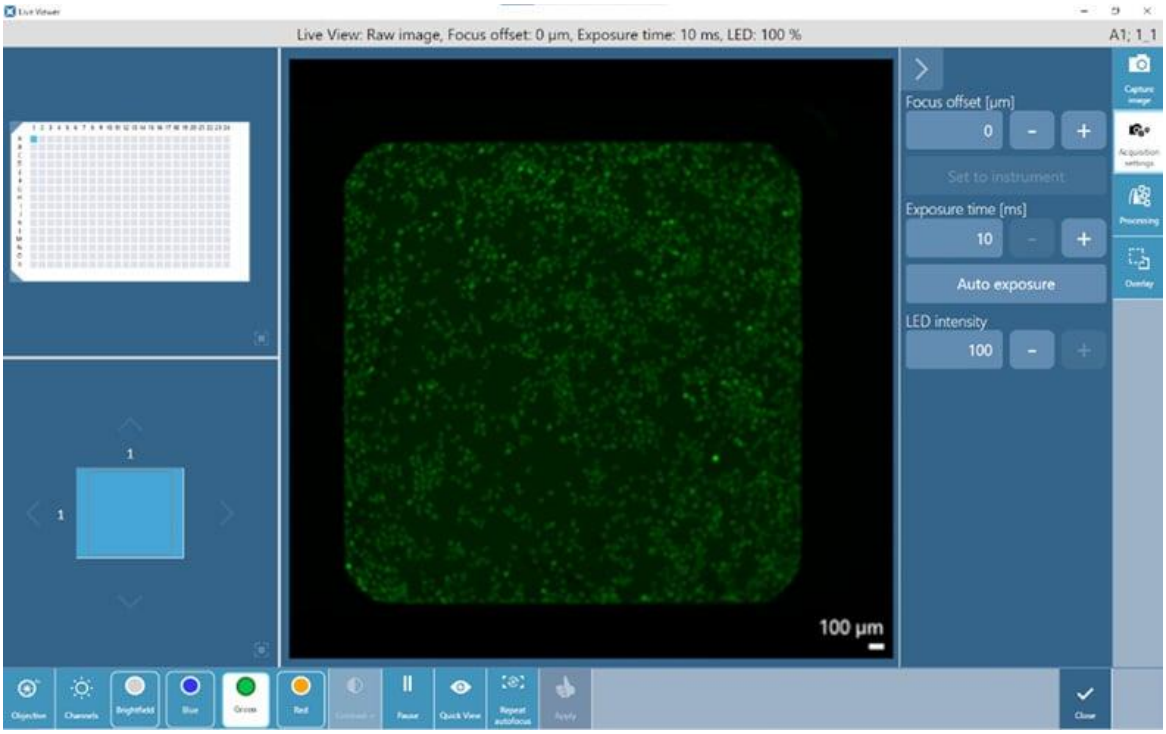

Figure 11: A screenshot of Tecan Spark® Cyto live cell plate reader software

The Tecan Spark® Cyto live cell plate reader, which was used for both imaging and data collection, was set up to operate at 25°C, the standard condition for this experiment. The excitation and emission wavelengths for AO are 500 and 525 nm respectively whereas for PI, it is 535 nm for excitation and 617 nm for emission. Focus offset was kept within the range of -10 µm to 10 µm for better visualization. Going beyond this range resulted in higher background noise. The software configured the plate layout according to the sample arrangement in the 24-well and 96-well plates, allowing for accurate detection and analysis of the stained cells. Acridine Orange was always added first, followed by Propidium Iodide. This is to ensure that Propidium Iodide absorbed the Acridine Orange signal in non-viable cells, preventing double-positive results.

After data acquisition, the fluorescence images were analyzed to assess the viability of the *A. castellanii* cells, the presence of cysts, and the extent of cell death across different bacterial strains. The data was then quantified, comparing the results between different strains and controls to evaluate the relative pathogenicity of each strain. Replicates ensured statistical validity, and the results were subjected to statistical analysis to determine the significance of any differences observed between treatments.

Data analysis of images exported from cell plate reader were analyzed using ilastik segmentation toolkit. This software helped us to differentiate trophozoites, cysts, and non-viable cells, consequently allowing us to deduce the effects the different bacterial strains had on amoeba. The Pixel Classification tool in ilastik enabled us to distinguish different regions within an image by training a machine-learning model to identify pixels representing cell boundaries and interiors. In contrast, the Object Segmentation tool facilitated the separation of objects of interest from the background and other structures in our raw images. Using these tools together allowed me to accurately quantify the objects based on their numbers. Throughout the experiment, safety protocols were followed, including handling all dyes and biological materials with appropriate personal protective equipment (PPE) and adhering to institutional guidelines for biosafety and chemical handling. Waste disposal, including used dyes and cell cultures, was carried out according to the laboratory's hazardous waste disposal protocols.

4.7 CFU Counts for Total and Intracellular Bacteria

CFU counts are performed in 96-well plates using two different media: DASW and PYG. The results helped the differences in bacterial survival and replication between the wild-type and mutant strains. The *A. castellanii* cells were infected with bacterial strains at a multiplicity of infection (MOI) of 200. Following infection, the 96-well plates were incubated at 25°C under two different time points: one set for 3 days and another for 5 days to assess the temporal dynamics of infection.

Protocol

Total Bacterial Count

After the 3-day and 5-day incubation periods, 100 µL of Triton X-100 (0.5M) was added to each well of the 96-well plate to lyse the host cells and release all bacteria (both intracellular and extracellular). The plate was kept at room temperature for 30 minutes to ensure effective lysis.

Following lysis, 800 µL of the sample was collected from each well (quadruplets for each bacterial strain) and transferred to Eppendorf tubes. The samples were centrifuged at 5000 rpm for 10 minutes, after which the supernatant was discarded. The bacterial pellet was resuspended in 300 µL of PBS and vortexed to ensure uniform distribution. A 6-fold serial dilution was prepared from

the resuspended sample to accurately count the CFUs. 5 µL of each dilution was dropped onto Luria agar plates.

The plates were incubated at 37°C for 24 hours. After 24 hours of incubation, the colonies were manually counted and recorded. The results helped determine the total bacterial load present post-infection.

Intracellular Bacterial Count

To assess intracellular bacteria, 15 µL of Gentamicin was added to each well of the 96-well plate post-infection and incubated at 30°C for 1 hour. Gentamicin killed extracellular bacteria, leaving only intracellular bacteria viable.

After the Gentamicin treatment, the wells were washed with 150 µL of PBS to remove any remaining extracellular bacteria and Gentamicin. The PBS was discarded, and 100 µL of Triton X-100 (0.25M) was be added to each well to lyse the host cells and release the intracellular bacteria. The plate was be kept at room temperature for 30 minutes to ensure complete lysis.

Similar to the total CFU protocol, 800 µL of the lysate was collected from each well and transferred to Eppendorf tubes. The samples were centrifuged at 5000 rpm for 10 minutes, and the supernatant was discarded. The bacterial pellet was resuspended in 300 µL of PBS and vortexed. A 6-fold serial dilution was prepared from the resuspended sample to enable accurate CFU counting. 5 µL of each dilution was dropped onto Luria agar plates.

The plates were incubated at 37°C for 24 hours. After incubation, colonies were manually counted and recorded. This count represented the intracellular bacterial load post-infection. For each bacterial strain and medium (DASW and PYG), duplicates of each fold of dilution were prepared. This ensures reliable and reproducible results.

Uninfected *A. castellanii* cells and non-treated wells served as controls to validate the effectiveness of the Gentamicin treatment and the overall infection process. The results were compared between the wild-type and mutant strains to assess the role of specific genes in intracellular survival and replication.

The bacteria counted were then plotted with their log values on a bar graph with error bars in Prism software.

5 Results and Discussion

5.1 Presence of Cyst in Seawater Media

Growth of *Acanthamoeba* in the seawater mimic is not optimal for the reproduction and growth of trophozoites. This has also been confirmed by a study from Boonhok where encystation took place in *Acanthamoeba triangularis* due to nutrient starvation (Boonhok et al., 2022). Nutrient deprivation is a well-known trigger for encystment in *Acanthamoeba* species. As the seawater mimic provides amoeba conditions similar to sweater therefore it forms cysts as a protective response to osmotic stress.

The following set of images obtained from the **Kinetic Motility Assay** from holographic microscopy in Fig. 12, 13, and 14 show us cysts **(circled red)** of amoeba in DASW media when infected with the following strains: WT1552, *makA*, and *hapR*.

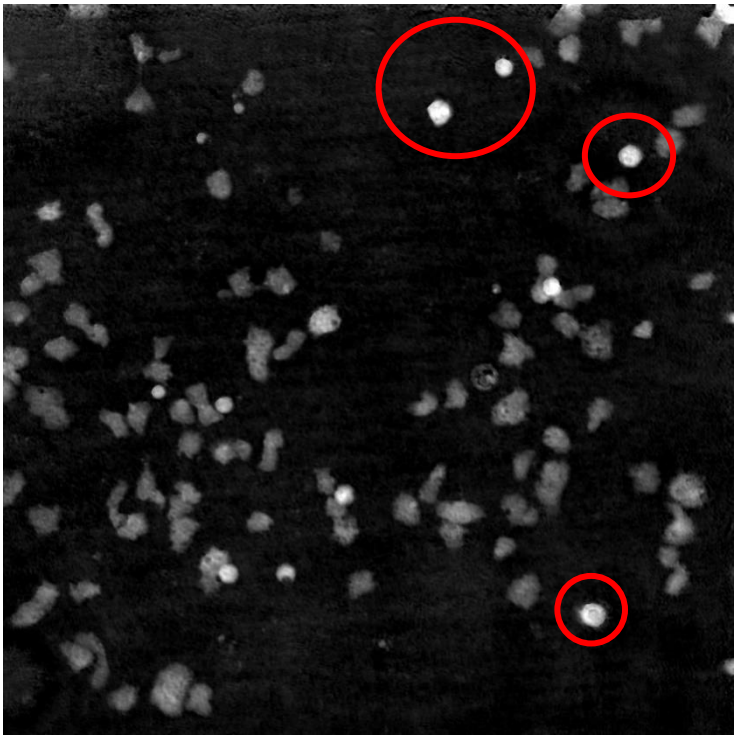

Figure 12: Amoeba infected with WT1552 strain

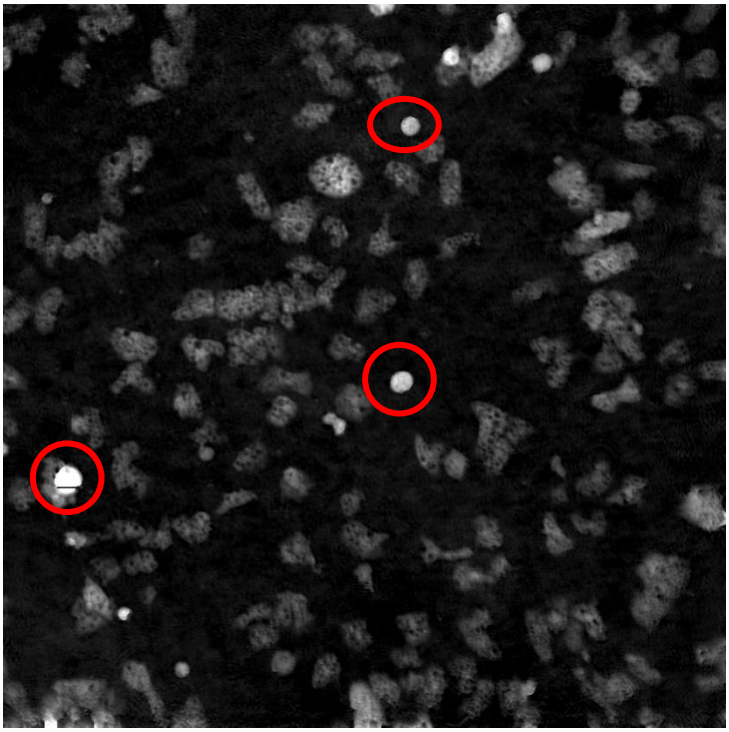

Figure 13: Amoeba infected with makA strain

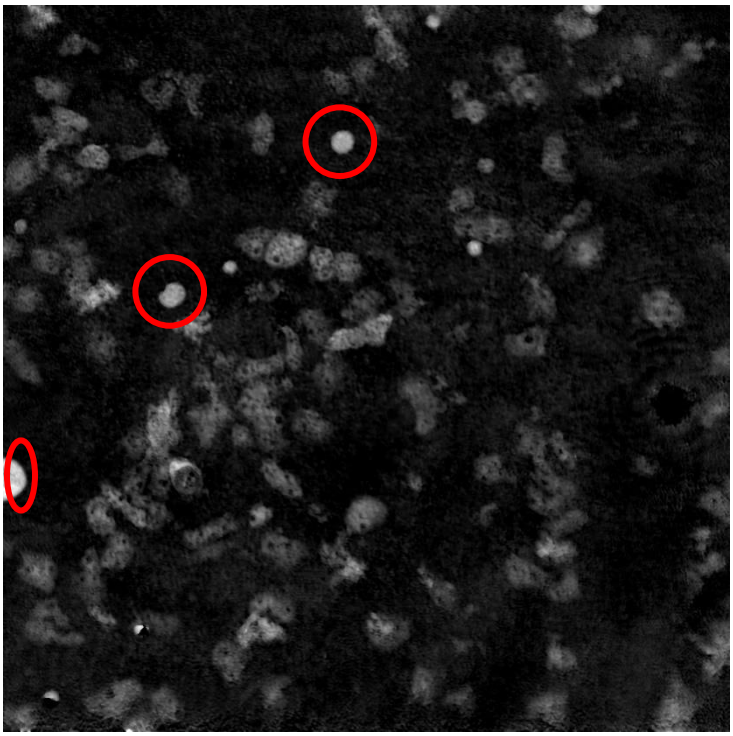

Figure 14: Amoeba infected with hapR *strain*

Observing these images does show us the presence of some cysts in these images, which I have managed to identify by their appearance under a microscope as well as their contrasting features from trophozoites. Cysts are non-motile, rounded, thick, and generally much smaller in size than trophozoites (Corliss, 2001).

However, observing the set of images (Fig. 12, Fig. 13, and Fig. 14) above gives us another important insight, which is that we can see a reasonably large number of trophozoites in the Δ*makA* and *hapR* strains compared to the wild-type strain. This could explain that the *makA* and *hapR* strains might be less effective at inducing stress in *A. castellanii* compared to the wild-type strain. Furthermore, there could also prove to be a difference of metabolites where *makA* and *hapR* mutants produce fewer stress-inducing metabolites due to which amoeba might find it much easier to sustain itself as a trophozoite. There is also reason to believe that mutations in *makA* and *hapR* may lead to a reduced ability of bacteria to trigger defensive responses in amoeba. Since amoeba's defense doesn't activate therefore it continues to grow and divide in its trophozoite phase without any disruption. In contrast, the wild-type strain might be more aggressive in its interactions with trophozoites, potentially leading to reduced numbers.

Finally, the *hapR* gene is a key regulator of quorum sensing in *V. cholerae*, which might control the expression of multiple virulence factors. Disruption of *hapR* could lead to an altered expression profile of these factors, resulting in a less harmful interaction with amoeba. Consequently, this could result in less interference for the trophozoites from the bacteria, allowing them to be present in higher numbers.

5.2 Biofilm Formation by *hapR* Strain

A significant result which was seen in PYG medium was the formation of a biofilm by the *hapR* strain. Biofilms are structured communities of bacteria that are attached to surfaces and embedded within a self-produced extracellular matrix. Numerous environmental elements, such as the availability of nutrients and interactions with other species like amoebae, might affect the formation of biofilms. It is well understood that the bacterial process such as biofilm formation are critical for bacterial survival (Ng & Bassler, 2015). Quorum sensing allows bacteria, or in our specific case, the *hapR* strain to carry out a socially grouped behavior allowing for bacterial survival for nutrient competition with amoeba.

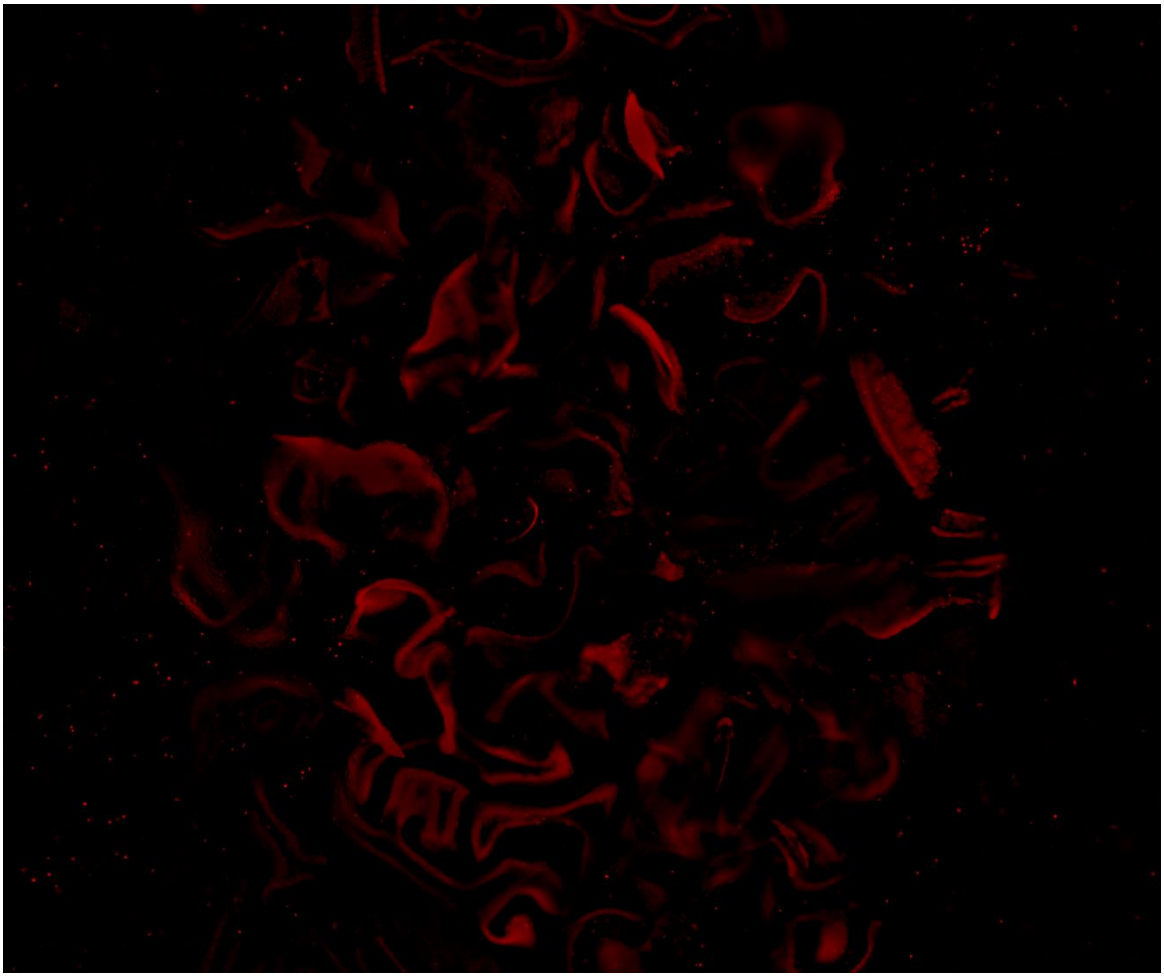

Figure 15: Biofilm formation by hapR strain in PYG medium

The formation of a biofilm by the *hapR* mutant strain could very likely be a defensive strategy against amoebal predation. Here, biofilms may have potentially provided a protective barrier that can shield bacterial cells from amoeba engulfment. The extracellular matrix of the biofilm prevents amoeba's ability to phagocytose the bacteria, allowing the bacteria to persist within the biofilm.

Furthermore, the biofilm also allows for efficient nutrient capture and retention (Loffler et al., 2023), which can be particularly advantageous for the bacteria in a competitive setting, such as that with amoeba. By forming a biofilm, the bacterium is able to outcompete amoeba for essential nutrients present in the PYG medium, something that was not seen in the seawater medium. Hence, further supporting bacterial survival in the presence of the host.

Generally, biofilms are resistant to environmental stresses, primarily managing to evade the host immune response (Martin et al., 2020). Biofilm formation by the *hapR* mutant could indicate a

shift towards a more chronic infection model where the *V. cholerae* bacteria persists in association with the *A. castellanii*.

5.3 Differences Observed Between DASW and PYG Media

The two mediums which we've carried out our experiments in have also shown differences over a wide scale. When comparing the two mediums, higher encystation is seen in seawater medium (DASW) in the wild-type strain compared to all the other strains. The first and second infection experiments carried out in the DASW after a three-day incubation period, give the following cyst percentages:

Bacterial Strain	Exp. 1 Encystation (%)	Exp. 2 Encystation (%)
WT1552	31.7	20.6
makA	5.65	9.3
makB	6	11.7
makE	4.8	7.74
hapR	8.1	12.6

Table 2. Encystation percentages for each bacterial strain infected in DASW

The highlighted values in Table 2 show us that in both these experiments, the highest cyst % was given by the wild-type *V. cholerae* strain. This could be due to the fact that the wild-type strain retains all of its virulence factors that cause higher amounts of stress to *Acanthamoeba* cells. This helps to understand that while wild-type strain is able to cause significant stress on the amoeba, it doesn't necessarily kill it. This could be due to the virulence factors, metabolic disruption, or other kinds of cellular stresses.

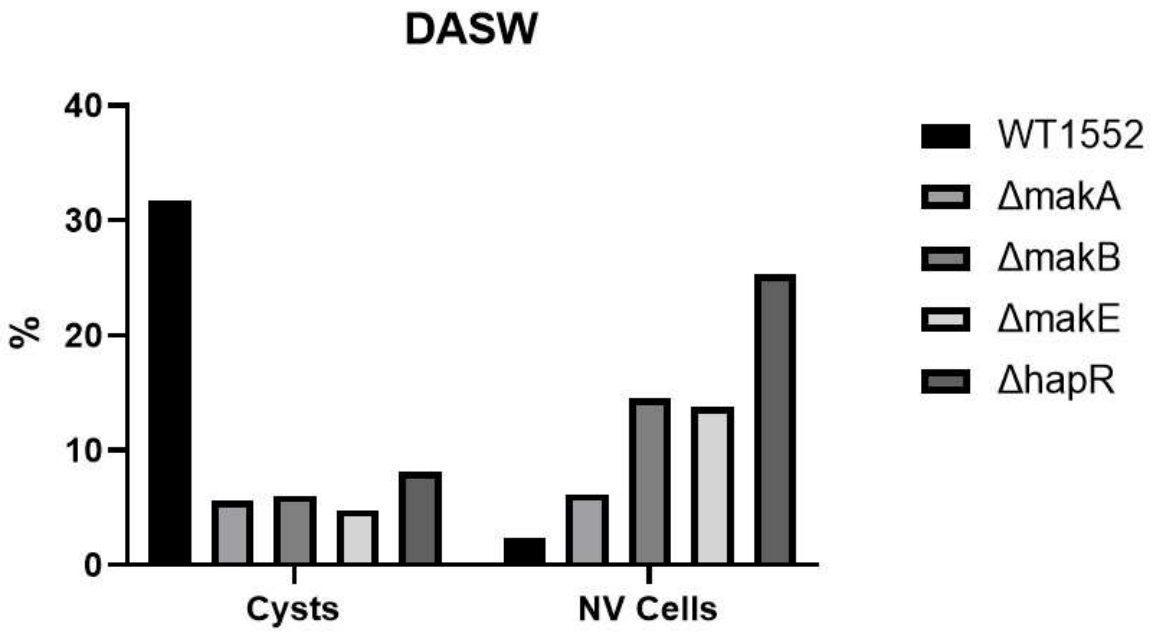

Figure 16: Percentages of cysts and non-viable cells in DASW

As reflected in **Fig 16.**, we can quite clearly observe that the highest % of cysts are seen in the wild-type strain whereas the highest % of non-viable cells are seen in *hapR* strain. This is true for the DASW medium whereas for the PYG medium, the highest killing of amoeba cells was carried out by *makA* strain. In fact, all the mak strains killed amoeba but the difference between *makA* and the other mak strains was quite significant. The fact that the mak mutant strains exhibit strong amoeba-killing activity in the PYG medium indicates that other virulence factors may be compensating for the loss of function in the mak operon.

Focusing on the PYG medium itself, this nutrient-rich medium is beneficia for both *Vibrio cholerae* and *Acanthamoeba*. In such an environment, bacteria are able to grow and produce virulence factors with much more ease. Consequently, a higher number of bacteria results in more activity and increasing killing of amoeba.

The mak operon is crucial in regulating activity of the bacteria in different media, like any other operon carrying out fine-tuning activity for specific prokaryotic functions (Li et al., 2015). In the DASW medium, the operon may adjust things depending on the bacterial response and interactions

with the host, whereas the PYG medium allows bacteria to proliferate easily due to abundance of nutrients.

5.4 Quantification of Bacteria Using CFU Counts

Gentamicin is the antibiotic used in intracellular bacterial quantification because it kills extracellular cells while helping to retain intracellular cells (Hamrick et al., 2003). The CFU counting experiments carried out in the DASW and PYG are shown in figures **17 (a) and 17 (b).**

The conditions were similar for both media, most importantly with both of them having an incubation period of **96 hours**. The gentamicin treatment for the intracellular counts was carried out for 60 minutes.

For DASW, the total counts show a relatively high bacterial load whereas the *makA*, *makB*, and *makE* strains show zero intracellular counts. This suggests that these strains are essential for intracellular survival of *V. cholerae* in *A. castellanii*. The total counts of *hapR* strain in DASW are consistent with wild-type levels which could suggest that this strain has a variable role in intracellular survival or invasion.

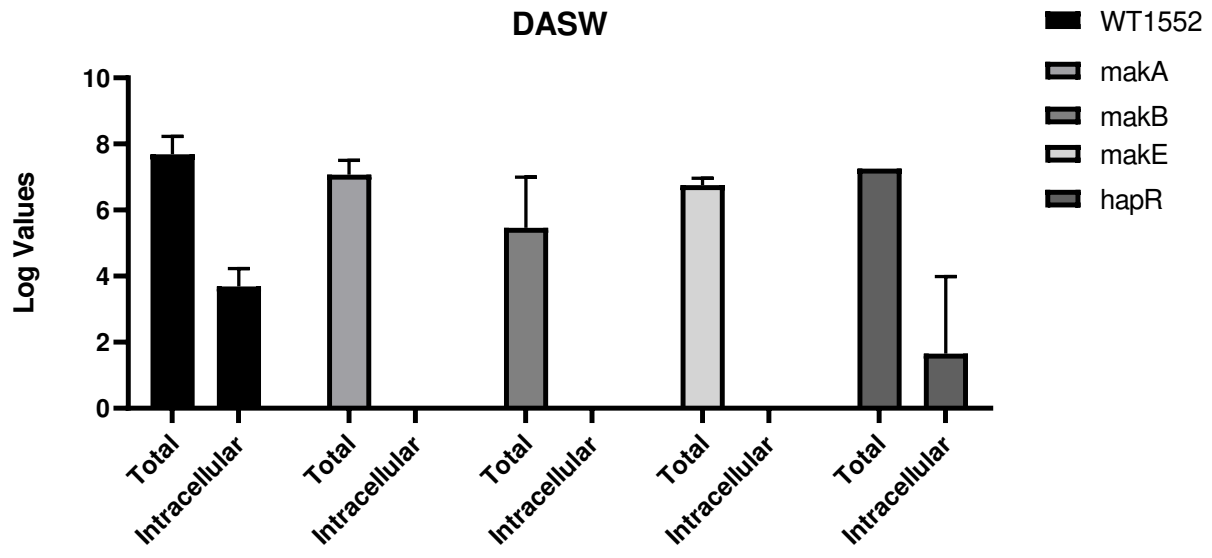

Figure 17 (a): Total and Intracellular CFU Count of each strain in DASW medium

For the PYG medium, the wild-type strain invades and survives well within the amoeba. In PYG medium, the *makA* gene is crucial for intracellular survival because its deletion leads to reduced intracellular counts. On the other hand, *makB*, *makE*, and *hapR* strains have minimal impact on

intracellular survival as their count isn't significantly reduced when compared with the total bacterial CFU count.

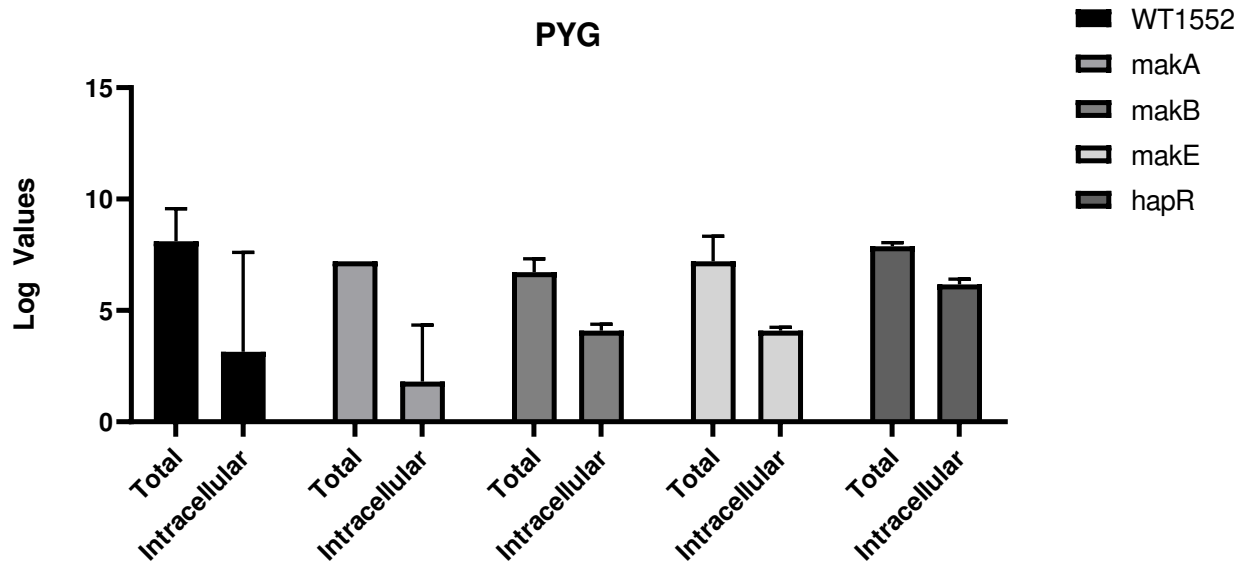

Figure 17 (b): Total and Intracellular CFU Count of each strain in PYG medium

A second experiment was also conducted only in **DASW** medium shown in figures 17 (c) and (d) where there were two different incubation times and there were two time-based treatments for intracellular bacteria. The incubation time was **3-days** post infection and **5-days** post infection whereas the Gentamicin treatments were for 15 minutes and 60 minutes, respectively. Shorter exposure times ensure correct interpretation of results (Sharma & Puhar, 2019). By treating bacteria for these time points, we can compare the extent of bacterial killing over short versus longer exposure times. This helps us to understand how quickly gentamicin exerts its bactericidal effect and whether or not prolonged exposure significantly increasing killing of bacteria.

An exposure time of 15 minutes can kill extracellular bacteria making sure bacteria are rapidly killed outside the host cells whereas for a time of 60 minutes can ensure all extracellular bacteria has been effectively killed, especially if bacteria have a way of evading the antibiotic.

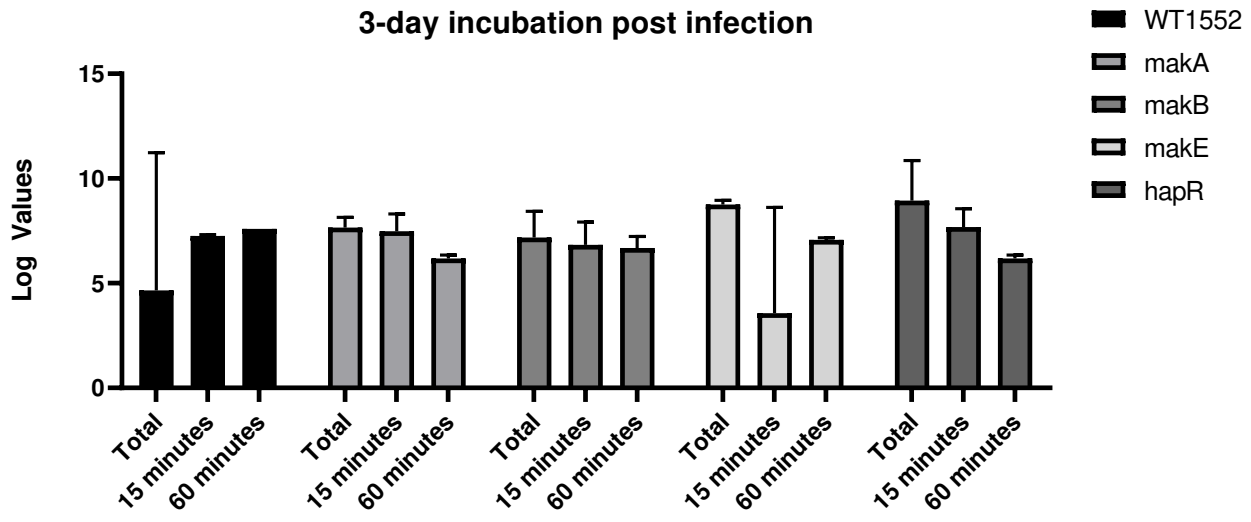

Figure 17 (c): Total and Intracellular (15m, 60m) CFU Count of each strain in DASW medium with 3-day incubation period

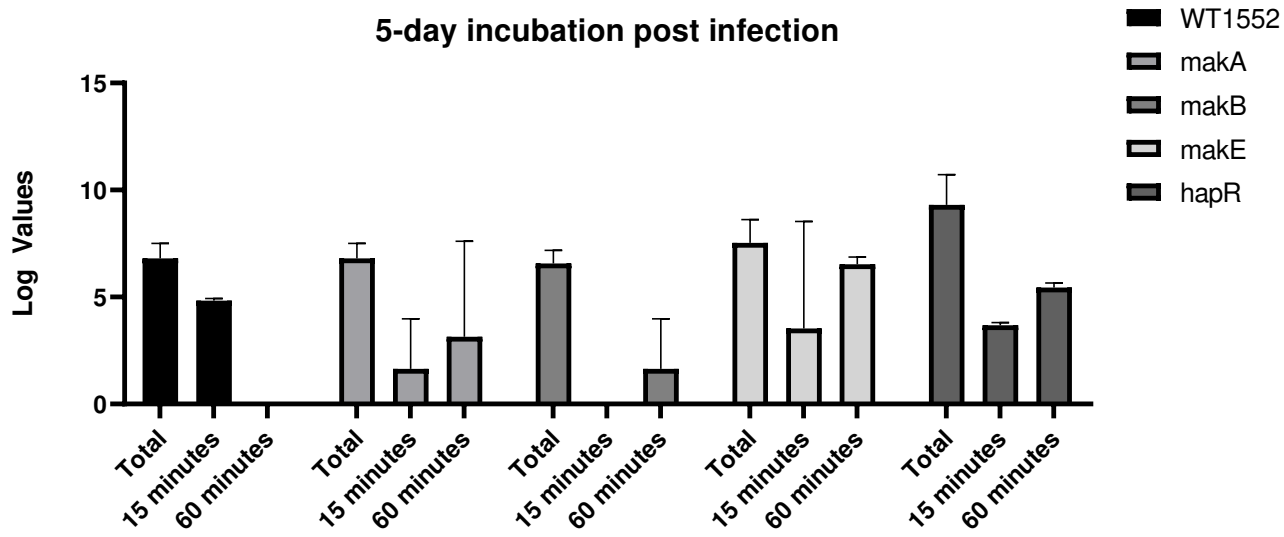

Figure 17 (d): Total and Intracellular (15m, 60m) CFU Count of each strain in DASW medium with 5-day incubation period

In figs. 17 (c) and (d), we can observe that 5-day incubation gives us significantly less intracellular counts compared to that of the 3-day incubation period. This could be due to bacteria entering stationary or death phase or a higher response elicited by amoeba.

Furthermore, if we focus on 17 (c), we can see that the wild-type intracellular count after 15 minutes and 60 minutes of gentamicin treatment is nearly the same. This suggests that most of the

bacteria are likely to be intracellular due to nearly similar log values. Total value being lower than the intracellular count for the wild type indicates experimental errors. As for the makA strain, we can see that it does not rely heavily on intracellular niche for its survival in the presence of *Acanthamoeba*. The initial treatment of 15 minutes managed to kill most of the bacteria, indicating that they were extracellular.

The results for *makE* strain in fig. 17 (c) tell us that a higher count at 60 minutes could mean that bacteria recovered from antibiotic stress which they might have faced at 15 minutes due to the antibiotic. Stress responses from the make might allow some bacteria to survive and become detectable after a longer exposure period. For *hapR* values, we can see that bacteria are eventually killed with extended exposure as gentamicin exposure of 60 minutes reduces the bacterial count compared to 15-minute exposure.

Looking at fig. 17 (d), we can clearly see that a prolonged infection period may have led to the death of host cells. If amoeba cells die and lyse, any bacteria that was previously intracellular could have been released into the extracellular environment. The bacteria would then come into contact with gentamicin and be killed, leading to absence of intracellular bacteria.

Another possible explanation is that the 5-day incubation period might have eliminated much of the bacterial population due to factors such as limited nutrition and immune response from host cell.

CFU counting helped us conclude that a 3-day incubation period is when intracellular bacterial activity is at its highest inside host cells while longer infection times result in lesser bacterial counts.

In PYG medium, the *makA* gene plays a crucial role in supporting intracellular survival, indicating its importance in nutrient-rich environments. However, other genes like *makB*, *makE*, and *hapR* appear less critical for intracellular survival in PYG, suggesting that their roles may be more relevant under different conditions. In DASW medium, where nutrient stress is high, the dependency on these genes for survival might increase, as they could be involved in stress adaptation or survival strategies that are less relevant in nutrient-rich conditions.

6 Conclusion and Future Perspectives

The medium in which *Acanthamoeba* resides plays a crucial role in determining whether it will continue to thrive or transition into cysts. This was clearly demonstrated by the significant differences observed between DASW and PYG media. Encystation was markedly higher in the nutrient-poor DASW medium which mimics natural environmental stress such as nutrient deprivation and osmotic pressure; both acting as driving factors of encystation. Our CFU counts also evidenced the fact that DASW led a greater tendency to form cysts. In contrast, in the PYG medium, bacteria successfully killed a large number of amoebae. The enriched PYG medium provided an advantageous environment for bacterial survival and proliferation within the amoeba leading to amoeba cell death. Consequently, this also results in an increase in bacterial virulence.

In seawater, which mirrors the natural environment of *Acanthamoeba*, the wild-type strain of *Vibrio cholerae* was significantly more lethal to amoebae compared to the mutant strains. Bacterial survival within *Acanthamoeba* was notably enhanced in the nutrient-rich PYG medium, where the availability of resources supported bacterial growth and replication. The presence of a biofilm further indicates suggest that under specific conditions, *Acanthamoeba* and *Vibrio cholerae* may benefit from each other's presence. More experimentation is required in this area to prove that a symbiotic relationship between amoeba and bacteria exists.

Incorporating another medium, such as LB medium, into the infection protocol could provide valuable insights into the role of *Acanthamoeba* in yet another different environment. LB medium is a more general bacterial growth medium, offering a middle ground between the nutrient-poor DASW and the nutrient-rich PYG medium. By using LB medium, future experiments could explore how moderate nutrient levels influence the dynamics of *Acanthamoeba-Vibrio* cholerae interactions, potentially affecting both bacterial virulence and amoeba survival. Observing how *Acanthamoeba* responds to these intermediate conditions could help in understanding its adaptability and defense mechanisms.

Future experiments could also focus on enhancing the amoeba's response as a host to neutralize bacterial virulence, which could contribute to developing strategies for combating cholera. This could involve exploring genetic factors that regulate the amoeba's defense mechanisms or using

chemical compounds to boost its immune-like response. By identifying genes or signaling pathways that strengthen *Acanthamoeba's* resistance, or screening for compounds that enhance its stress responses and phagocytic abilities, researchers could develop strategies to limit bacterial survival and biofilm formation. These findings could also have broader implications for creating host-targeted therapeutic interventions in other organisms.

If we can understand the conditions that encourage Vibrio cholerae to thrive within *Acanthamoeba* or trigger its release into the environment, we could develop targeted strategies to disrupt this interaction. This research has significant implications for public health, particularly in regions where Vibrio cholerae is endemic, such as parts of South Asia, Africa, and Latin America. By targeting the amoeba-bacteria interaction, we could potentially reduce the environmental reservoirs of Vibrio cholerae, thereby decreasing the frequency and severity of cholera outbreaks.

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Finally, I dedicate this dissertation to my sister Moomal, my brother Waris, my father Zafar, and above all, my mother Shabana. You are the ones whom I do it for.

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