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Yan Wei

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Date

Population dynamics of *Vibrio cholerae* and its bacteriophage

by
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Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
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Population dynamics of *Vibrio cholerae* and its bacteriophage

by
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M.Sc., University of Bath, 2004

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An abstract of
a dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
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Abstract

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Cholera is a diarrheal disease caused by toxigenic strains of *Vibrio cholerae*. Individuals contract cholera by consuming water or food contaminated with virulent *V. cholerae* from the aquatic reservoir. Field studies and epidemiological observations suggest bacteriophage that specifically infect *V. cholerae* may limit the severity of cholera outbreaks by killing bacteria present in the reservoir and in infected individuals. My dissertation is intended to enhance our understanding of how vibriophage modulate cholera outbreaks by combining laboratory experiments and mathematical modeling. My research shows that when *V. cholerae* was cultured with single species of phage, bacterial populations were only temporally limited by phage due to the rapid evolution of phage-resistant bacteria (chapter2). After phage-resistant bacteria emerged and dominated the community, bacterial populations were limited by resource rather than phage. However, these bacterial mutants exhibited an extreme fitness disadvantage relative to the wild-type, suggesting that resistant bacteria may not play an important role in the ecology and epidemiology of cholera. In chapter 3, I present results for conditions under which *V. cholerae* population was controlled by co-culturing with two different phage species over extended time (~ 650 hours). One of the two phage species displayed features that were not observed in well-studied lytic and lysogenic phage. Mathematical simulation of the population dynamics of this particular phage and its bacterial hosts suggests that this

phage might induce a persister-like of bacterial sub-population as some antibiotics do. Taken together, the above-described results support the hypothesis that phage predation plays an important role in the waning of cholera outbreaks. Chapter 4 discusses the topic "evolution of bacterial motility", which, while not directly related to bacteria-phage dynamics, was inspired by some of the findings from chapter 2. I hypothesize that motility is evolutionarily favored because it enables bacteria to move apart from each other, thereby allowing individual bacteria to obtain greater shares of limiting resources. Using a mathematical model and experiments with motile and non-motile strains of *E. coli* and *V. cholerae*, I test this hypothesis and explore the conditions under which motility provides bacteria with a fitness advantage in initially homogenous nutrient-limited spatially structured habitats.

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I was planning to say something here in my native language. But, Chinese characters are too special to display correctly in an English-operated machine, and I really don't have time to deal with the language package installing. So I would like to go ahead to continue my acknowledgements in English. In the final year of my Ph.D, I experienced great emotional stress that I never experienced in my life previously. I would like to thank some friends. Although I haven't met them in the real life, their words encouraged me a lot and helped me go through the greatest pain during my Ph.D. Melic, Finely, Fuyao, thanks! I also want to thank TQ who was the main reason of my stress and pain. Those experiences, although not happy and sort of painful, made me think a lot about the meaning of life. From all these thoughts, I have a clearer view of my future life and I think I am a better person now. Thanks, TQ.

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CHAPTER 1

Introduction

1.1 Cholera: background and history

Cholera, a diarrheal disease, has been infecting humans for at least a thousand years based on descriptions of its unique symptoms in ancient Greek and Chinese literature (Mekalanos, Rubin and Waldor 1997). It is one of the most rapidly fatal human diseases known. Death can occur as fast as eight hours from the onset of symptoms. Before the development of intravenous and oral fluid replacement therapies, cholera was estimated to have killed over 100 000 people each year in India alone between 1900 and 1950. As the disease grew to pandemic proportions in the early 1800s, it killed millions worldwide (Mekalanos, Rubin and Waldor 1997). Although diarrhea can be caused by various bacterial pathogens, no other bacterium produces the repeated pandemics characteristic of *Vibrio cholerae*. *V. cholerae*, a gram-negative bacterium, was isolated as the causative organism of cholera by Robert Koch from rice water stools of patients in Egypt and India during the fifth pandemic (1883 - 1884). The bacteria exist as free-living organisms in coastal waters throughout the world. Most *V. cholerae* do not cause harm to humans. Out of more than 200 serogroups of *V. cholerae* reported so far, only two serogroups (O1 and O139) are toxigenic and are responsible for major pandemics and epidemics. Humans contract cholera by ingesting water or food contaminated with toxigenic *V. cholerae*. Two main weapons possessed by *V. cholerae* enable it to infect and grow viciously within human hosts: cholera toxin (CT) and toxin co-regulated pilus (TCP). Toxigenic *V. cholerae* carries one or more copies of CT genes that produce toxin causing secretion of

chloride ion into the lumen of the intestine while inhibiting sodium uptake (Mekalanos, Rubin and Waldor 1997). The result of this imbalanced secretion and uptake of ions occurring in the intestine is watery diarrhea that takes on the appearance of rice-water, a cloudy liquid with flecks of mucus. Up to 20 liters of diarrhea during a single infection have been reported in cholera infected patients and the fluid usually contains 10^8 bacteria per milliliter that will be disseminated to the aquatic environment and continue to infect more susceptible people in the absence of preventative measures. Without the help of TCP, a crucial colonization factor in the small intestine, CT alone does not cause extensive damage to the host. *V. cholerae* mutant strains without TCP are more than 1000-fold reduced in their capacity to colonize the infant mouse and essentially do not colonize the intestine of human volunteers (Taylor *et al.* 1987; Herrington *et al.* 1988; Mekalanos, Rubin and Waldor 1997).

Considering our extensive knowledge on how *V. cholerae* sickens people and the fact that we are familiar with the details of transmission, it is a shame that cholera remains a major threat to human health worldwide. The distribution of epidemic cholera cases is directly related to public health measures taken to provide clean water. For example, In Europe and North America, epidemic cholera is essentially non-existent as a result of sewage systems which chlorinate water and prevent drinking water contamination. Elsewhere in the world, primarily Asian, Africa and South America, clean drinking water is not accessible to all population and sanitation can be poor. As a result, cholera is most prevalent in these regions of the world. The true burden of cholera is estimated to reach

several million cases per year, predominantly in Asia and Africa (Nelson *et al.*2009). From 1995 to 2005, Africa reported the largest and most frequent cholera outbreaks, followed by Asia (Griffith, Kelly-Hope and Miller 2006). Zimbabwe probably offers the most recent example of the tragedy that befalls a country and its people when cholera strikes. The 2008-2009 outbreak rapidly spread across every province and brought rates of mortality similar to those witnessed as a consequence of cholera outbreaks a hundred years ago (Nelson *et al.*2009). This enormous human suffering caused by cholera earned cholera the distinction of being one of the "emerging and reemerging infections" threatening many developing countries (Satcher 1995).

1.2 Factors affecting cholera outbreaks

A distinctive epidemiological feature of cholera is its seasonal pattern in endemic regions, such as Bangladesh. Bangladesh is situated in the Ganges delta, the region where all except the seventh cholera pandemics started (Faruque, Albert and Mekalanos 1998). Epidemics usually occur twice a year, with the highest number of cases just after the monsoons from September to December. A smaller peak of cases also is observed during the spring, between March and May (Faruque *et al.* 2005.1 PNAS). Various factors, biological and environmental, have been hypothesized to shape the seasonal pattern of cholera outbreaks.

First, environmental factors, such as temperature or rainfall which can be broadly termed as "climate", undoubtedly play a decisive role, not only in cholera outbreaks, but in many infectious diseases caused by bacteria pathogens (Colwell 1996; Pascual *et al.* 2000). *V. cholerae* is characterized by an increased growth rate at warm temperatures, which is evident in higher rates of isolation in the environment during warm months. Between epidemics in areas of endemicity, it is exceedingly difficult to isolate toxigenic *V. cholerae*; however, during periods of warmer water temperatures, success in isolation of toxigenic *V. cholerae* rises substantially (Lipp, Huq and Colwell 2002).

Climate also triggers a series of changes in other biological factors interacting with *V. cholerae* in the environment. Marine bacteria, including Vibrio spp., have been found to consume chitin as carbon and nitrogen source using their chitinase. Because seasonal changes in climate can influence populations of chitinous organisms, such as copepods, amphipods and other small crustaceans, climate may also influence the presence of cholera in the environment. Approximately 10^{11} metric tons of chitin per year is produced in aquatic environments; $>10^{9}$ metric tons is produced by copepods alone (Lipp, Huq and Colwell 2002). Nalin first suggested that *V. cholerae* might use the strategy of its sister species, *V. parahaemolyticus*, and adsorb onto copepod zooplankton to survive the unfavorable environment between the epidemics (Nalin 1976). Nalin then demonstrated *V. cholerae* is able to adhere and grow on the chitin particles (Nalin *et al.* 1979). After this, many studies, both in the laboratory system and in the field, showed that the presence of crustacean copepods enhance the survival of *V. cholerae* (Huq *et al.* 1983;

Huq *et al.* 1984; Tamplin *et al.* 1990; Chiavelli, Marsh and Taylor 2001). Direct observation in Bangladesh strongly supports an important role for zooplankton in cholera outbreaks. During spring and late summer in Bangladesh, phytoplankton blooms occur, followed by zooplankton, with heaviest blooms occurring in September and October, which are then followed by cholera outbreaks (Colwell *et al.* 2003). Based on the association between zooplankton and *V. cholerae*, a simple procedure, using folded sari cloth to filter water before consuming, was developed in Bangladesh. Cholera bacteria associated with zooplankton copepods will be removed by simple filtration. Cholera cases in regions practicing water filtration were half as frequent compared with the regions where filtration was not performed (Colwell *et al.* 2003).

Apart from the biological factors stated above, host immunity and bacterial virulence (hyper-infectious state) have also been suggested to be involved in shaping cholera outbreaks (Koelle *et al*. 2005; Hartley, Morris and Smith 2006; Nelson *et.al* 2009). All these factors are non-exclusive, interacting with each other and influencing cholera outbreaks on different scales and at different stages. Mathematical models accounting for extrinsic factors, such as temperature, as well as some biological factors, have shown that both non-biological and biological factors shape the epidemic cycle of cholera (Koelle *et al.* 2005; Hartley, Morris and Smith 2006). However, knowledge about one distinctive feature of cholera outbreaks, the rapid collapse of the epidemic, still remains elusive. In this dissertation, I will discuss one biological factor, the presence of phage, which could be responsible for the rapid collapse of cholera outbreaks. Interestingly, phage has been

associated with cholera since it was first observed in 1896 (Hankin 1896), but little is known about its role in bacterial population dynamics.

1.3 Bacteriophage : history and the role in cholera epidemics

1.3.1 General information about Bacteriophage

Bacteriophage are small viruses that specifically infect and lyse bacteria. Although extremely diverse, all phage share several common steps during their life cycle: adsorption, separation of genetic materials from protein coat, expression and replication of the genetic material within bacterial host, virion assembly, release and transmission (Weinbauer 2004). Phage first adsorb to the surface of a bacterium through a two-step process. The first step of adsorption is reversible but specific to a particular cell surface component. This is followed by an irreversible binding to a receptor, through which the genetic material is injected into the cells. After the injection, genetic material of the phage is either integrated into host genome or remains free in the cytoplasm depending on the nature of phage and physiological state of the cells. The genomes of a particular group of phage, the lytic or virulent phage, always stay outside the bacterial genome and turn their bacterial hosts into a phage factory in which new progeny are produced. The new progeny phage are then released through the lysis of the host and seek new hosts to infect. Another phage group of phage, lysogenic or temperate phage, do not kill their hosts rapidly like lytic phage. Lysogenic phage instead replicate with the hosts by inserting their genome into the bacterial host genome. The harmonious state in which the

phage remains in the bacteria without killing it can be interrupted physically or chemically and lysogenic phage will then behave like lytic phage. Finally, pseudolysogenic phage are similar to lysogenic phage except their genomes remain in cytoplasm than being inserted into host genome (Weinbaur 2004). These pseudolysogenic phage are characterized by the ability of the phage to induce a chronic infection in the bacteria during which phage progeny are constantly released into the environment by budding or extrusion, without having to lyse the bacterial host cell (Weinbaur 2004). To illustrate life cycles of different bacteriophage, a figure (Figure 1.1) is presented below.

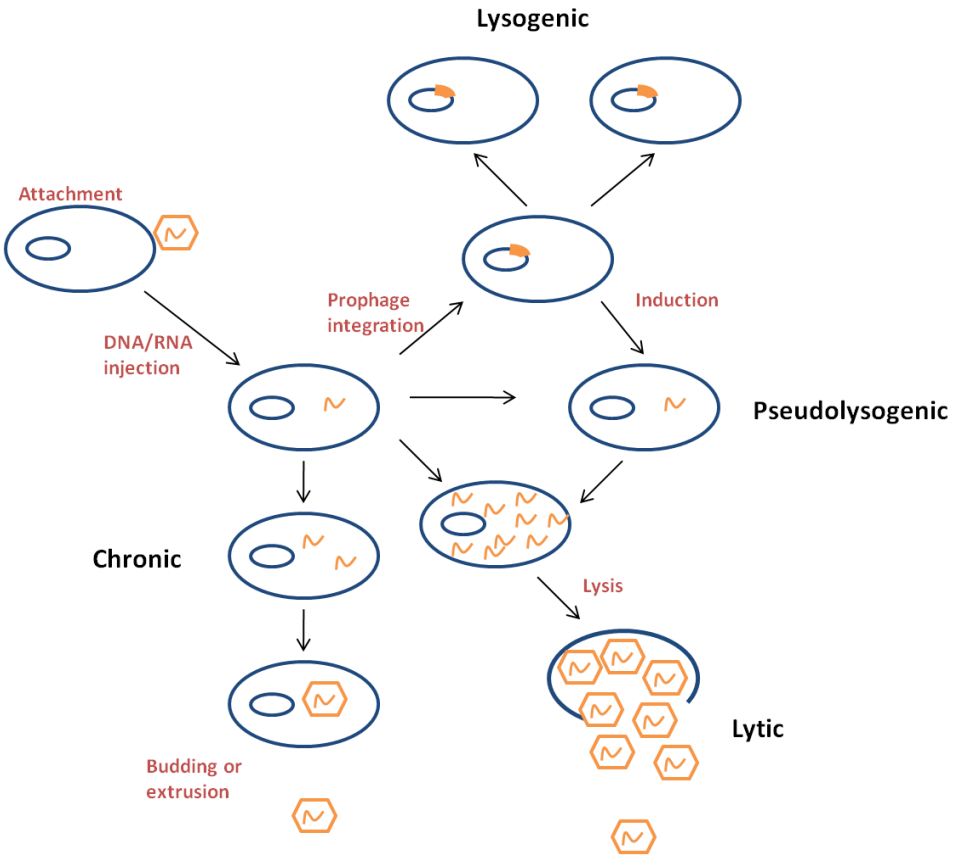

Figure 1.1 Types of viral life cycles

1.3.2 History of phage

The scientific discovery of bacteriophage is attributed to Twort (1915) and d'Herelle (1917). However, the first observation of phage was made 20 years earlier by Ernest H. Hankin in 1896 (Hankin 1896). Intrigued by the difference between the cholera outbreaks along the Ganges and Jumna rivers in India, Hankin found an unknown source of antibacterial activity against *V. cholerae* in the river and then suggested that this unidentified substance, which passed through fine porcelain filters and was heat labile, was responsible for limiting the spread of cholera epidemics (Hankin 1896). Despite the capability for killing bacteria of this mysterious source, no one, including Hankin himself, used it for controlling bacterial populations until 1919 when d'Herelle championed their use in controlling the spread of infectious diseases. D'Herelle named them bacteriophages and eventually, he and his colleagues implemented phage therapy throughout the world, with major efforts in India, Egypt, the United States and the Soviet Union. Bacterial infections that have been successfully controlled using phage therapy include *Shigella*, *Staphylococcus*, *Streptocooccus*, *Klebsiella*, *E. coli*, *Salmonella*, *V. cholerae*, *Pesudomonas* and *Proteus* (Sulakvelidze, Alavidze and Morris 2001). Although most human phage therapy studies show positive results, research of phage therapy gradually declined in the west in the 1940s due to the failure to establish rigorous proof of efficacy and increasing availability of antibiotics (Levin and Bull 2004). The Republic of Georgia, however, survived the "anti-phage" times, and the George Eliava Institute of Bacteriophage, located in the capital city of Georgia, has actively employed phage therapy since the 1930s and is now the global center of phage therapy expertise.

As mentioned above, phage therapy was also used to fight cholera. The first therapy trial compared 244 untreated cholera patients with 219 patients who were treated with vibriophage; the untreated group had a 20% mortality rate whereas mortality in the treated group was 6.8% (Nelson *et al.* 2009). Several other studies showed similar results. Although suffering from the same limitations as other early phage therapy studies did (poor controls and inconsistent therapeutic results), these studies demonstrated enough success to continue the use of phage to treat cholera on a large scale at a later time. From 1928 to 1934, over a million vibriophage doses were prepared and disseminated in specific study communities in India. This application was novel because, for the first time, vibriophages were also disseminated into drinking water sources as a means of prophylaxis. The triennial death rates from cholera fell from 30 to 2 per 10,000 in communities that were treated with phage (Nelson *et al.* 2009).

At the same time, an interesting relationship between *V. cholerae* and vibriophages in the environment was also reported. Influenced by d'Herelle and Malone's suggestion that the cessation of cholera epidemic was due to the spread of bacteriophage from convalescent cases, Pasricha *et al.* studied the prevalence of vibriophage in nature and its relationship to cholera in Calcutta (Pasricha, de Monte and Gupta 1931). They found that vibriophage in nature vary with the incidence of the disease and that the mortality rate, which is high at the beginning of the cholera season, falls rapidly once vibriophage have become widely distributed in nature. It was therefore concluded that bacteriophage plays an important role in lowering mortality and in bringing an epidemic to a close (Pasricha, de

Monte and Gupta 1931). It is understandable that the phage biology in these early experiments were rather crude considering that phage had only been discovered 15 years ealier. However, this is the first scientific study, to our knowledge, clearly reporting the relationship between cholera and vibirophages from the natural environment and also from the patients. Subsequently, advances in rehydration and antibiotic therapy made cholera phage therapy, either as individual treatment or in the environment as a prophylaxis, insignificant.

The story of cholera and phage certainly did not end. In the 1990s, as more and more efforts emphasized the molecular pathology of *V. cholerae*, John Mekalanos and colleagues found that cholera toxin (CT), the key virulent factor, was actually carried by a filamentous phage (Waldor and Mekalanos 1996). They showed experimentally that a toxigenic *V. cholerae* strain under certain conditions, is able to transmit genetic elements containing CT genes to other strains which lack CT. This transfer, which does not require cell-cell contact, could be accomplished by co-culturing recipient cells with filtered culture supernatant from the donor. Electron microscopic studies later showed that culture supernatants from the donor strain contained structures similar in morphology to filamentous bacteriophage (Waldor and Mekalanos 1996). The phage was then designated CTX phage. What is more surprising is that CTX phage uses TCP as a receptor to infect naïve bacteria; as previously mentioned, TCP is another virulence factor in cholera pathogenesis (Waldor and Mekalanos 1996). This finding illustrates how

a non-pathogenic bacterium becomes a highly virulent pathogen by horizontal transfer mediated by lysogenic bacteriophage.

1.3.3 Rediscovery of the important relationship between cholera and bacteriophage

About 60 years after Patrisha's report of the intriguing relationship between cholera and vibriophages, Shah and colleagues reported a similar association, but with a more systematic sampling method and much better knowledge of both bacteria and phage (Faruque *et al.* 2005.1). Over a three-year period, they systematically analyzed water samples collected from two major rivers and a lake in Dhaka. The majority of water samples suggested an inverse relationship between the presence of vibriophages capable of lysing a given serogroup of *V. cholerae* and the presence of a strain of that same serogroup (Faruque *et al.* 2005.1). Further, the number of cholera patients varied seasonally during the study period and frequently coincided with the presence of pathogenic *V. cholerae* strains in water samples that lacked detectable vibriophages (Faruque *et al.* 2005.1). During interepidemic periods, water samples were found to contain vibriophages but no bacteria (Faruque *et al*. 2005.1). In another smaller scale study, the environmental prevalence of the epidemic *V. cholerae* O1 strain and a particular vibriophage JSF4 was monitored during a local outbreak. In addition, excretions of the same phage were also monitored from cholera patients during the study period (~17 weeks) (Faruque *et al.* 2005.2). This closer look at the cholera-phage dynamics showed that the peak of epidemic was followed by high JSF4 levels in the environment. Furthermore, the buildup of the phage coincided with increasing excretion

of the same phage (JSF4) from the cholera patients (Faruque *et al.* 2005.2). Thus, these systematically collected data supported the original hypothesis presented by Patrisha and suggested that vibriophage play an important role in cholera outbreaks, particularly in ending them. To quantify this hypothesis, Jensen *et.al* developed a mathematical model combining the epidemiology of cholera and *V. cholerae* population dynamics in the presence of its bacteriophage (Jensen *et al.* 2006). The model predicts that under reasonable biological parameters, vibriophage can ameliorate cholera outbreaks. When the outbreak is initiated by an interruption of bacteria-phage equilibrium in the reservoir, the density of phage remaining in the reservoir affects the severity of the outbreak. Even if the outbreak is initiated directly by increased bacterial growth, in the absence of phage in the reservoir, the introduction of phage will reduce the severity of the outbreak and promote its decline. The major limitation of this model is bacterial resistance to phage, if developed, would essentially render phage useless on limiting the density of bacteria, producing no effect on the cholera epidemic.

Are populations of *V. cholerae* limited by bacteriophage? Or do *V. cholerae* develop resistance to phage? These questions, though important, were ignored or unanswered in the published reports speculating on the role of vibriophage in limiting cholera outbreaks. The goal of my dissertation is to fully characterize the effect of vibriophage on population dynamics of *V. cholerae*. Based on the laboratory results, which will be reviewed later, phage-resistance can easily develop in many other bacteria, including *E.*

coli, Pseudomoas, Salmonella etc. In natural environments that are of real interest to many scientists, data are quite scarce.

1.4 Population dynamics of bacteria and phage

1.4.1 Population dynamics of bacteria and phage in the laboratory

Bacteria-phage systems have been of long-standing interest to many biologists: not only to microbiologists but also to ecologists and evolutionary biologists. Microbial research systems are appealing because microbes can be routinely grown in the laboratory, have short generation times and large populations that can be easily maintained, allowing for rapid evolutionary change and the chance to study communities on both ecological and evolutionary time scales (Bohannan and Lenski 2000). As such, bacteria-phage systems provide an ideal model system for the study of processes in ecology and evolution. Most of the studies in this field investigate population dynamics and interactions between one bacterial species and one phage. Almost all of these studies have shown that phage-resistant bacteria evolve and replace the majority of sensitive ancestors, regardless of the bacterial or phage strain under study (Chao, Levin and Stewart 1977; Lenski and Levin 1985; Middelboe 2000; Mizoguchi *et al*. 2003). In some studies, phage have also evolved mechanisms to infect resistant bacteria. However, in this evolutionary arms-race, the bacteria have always overcome the phage (Lenski and Levin 1985; Chao, Levin and Stewart 1977).

In laboratory studies, as well as mathematical models, the fate of the phage-bacteria culture is greatly influenced by the fitness costs of resistance mutations to the bacteria. Populations of bacteria are only temporarily controlled by phage and later controlled by resources (limiting nutrients) after the emergence of phage-resistant bacteria. One study is an exception. While studying the long-term co-evolutionary arms race between *Pseudomonas fluorescens* and one of its naturally associated phage, Buckling & Rainey found that the phage persisted after 300 generations of co-cultivation with bacteria (Buckling and Rainey 2002). Furthermore, they also could infect the co-existing bacterial populations, indicating that phage-resistant bacteria never evolved or never dominated the population. This study failed to report bacterial or phage density, thus, it is hard to make the conclusion that it is a phage-limited condition, though the resistance data suggests that this is the case.

It is undisputed that evolutionary change occurs in both the bacterial and phage populations, even in instances where population density of bacteria and phage no longer change after a certain period of time. Occasionally, phage were found to persist at a much lower density than the bacteria, indicating the persistence of minority populations of partially resistant cells (Bohannan and Lenski 2000). In theory, as long as there are trade-offs between resistance and fitness, maintenance of phage does not require emergence of host-range mutants in the phage (Levin, Stewart and Chao 1977). Schrag and Mittler also found that the glass wall of laboratory equipment can act as spatial refugia for populations of sensitive bacteria (Schrag and Mittler 1996). As long as there are spatial refuges which can support a subpopulation of sensitive bacteria, phage can be maintained

in the co-culture. In one study where the population of *E. coli* O157:H7 and lytic phage PPO1 was investigated in continuous cultures, the density of phage was maintained at a level quite similar to the bacteria even in the presence of multiple different phage-resistant mutants (Mizoguchi *et al*. 2003). A mucoid bacterial mutant, which can support the growth of both the ancestral and evolved phage without being lysed, was found in the bacterial populations, which may explain the maintenance of phage population in that particular study.

Phage-resistance also occurs when several different phage are used to challenge the bacteria. In a study investigating the role of restriction-systems, Korona and Levin found that *E. coli* B populations gradually developed resistance to three different lytic phage within a short period of time (Korona and Levin 1993). Similar results were also reported in a study where four different marine bacteria were co-cultured with their respective lytic phage (Middelboe *et al*., 2001). When two bacterial species (*E. coli* B and *Salmonella enterica* serovar Typhimurium) were co-cultured and maintained stably together, introduction of lytic phage T5 or T7, which can infect *E. coli* B, lowered the density of *E. coli* B for extended time or even made it extinct (Harcombe and Bull 2005). This phenomenon was only observed in the presence of *Salmonella*, but not when *E. coli* B was singly cultured with its phage. Furthermore, long-term suppression or even extinction of bacterial populations caused by phage did not occur when phage specific to *Salmonella* was introduced to the two-bacteria system. The basis for this difference remains unclear. It could be that only *E. coli* B bacteria were affected by competition from Salmonella. Alternatively the results could be specific to the particular phages used

and the types of bacterial resistance for which they selected (Harcombe and Bull 2005). Whatever the reason, these data clearly show that phage is able of controlling the density of its host species in a multi-species community, which more closely resembles the natural environment.

1.4.2 Population dynamics of bacteria and phage in the environment

Studies of the dynamics of bacteria and phage in the natural environments have had quite different designs from the experimental studies, partly due to the difficulties of working with specific bacteria or phage in natural settings. Most studies done in aquatic habitats have explored the total number of virus-like particles in a specific environmental setting (Bergh *et al*. 1989; Heldal and Bratbak 1991; Suttle and Feng 1992; Hennes and Simon 1995). Factors, such as decay of viruses (especially by UV) and protozoan predation of bacteria, which are not considered in most laboratory studies, play a significant role in controlling phage populations in natural aquatic environments. According to several reports, phage-induced mortality of bacteria in the aquatic environment does not exceed 15%-20% of total bacterial mortality (Heldal and Bratbak 1991; Suttle and Feng 1992; Hennes and Simon 1995). However, bacterial resistance to phage was not explored in most of these studies, although selection for resistance might not be strong enough to enable resistant bacteria to dominate the population when the growth rate of bacteria is low, 0.1 to 0.25 day^{-1} (Hennes and Simon 1995). One study, however, presents compelling evidence that marine *Synechococcus* populations can be dominated by resistant bacteria, even when the phage are diverse and abundant in the same environmental settings (Waterbury and Valois 1993). These data support the hypothesis

that lytic phage have a negligible effect on regulating the bacterial populations, at least for marine *Synechococcus* populations. *V. cholerae* resistant to co-existing lytic phage were found in 5/10 of sampling sites (Faruque *et al*. 2005 PNAS.1). However, it is unclear whether those resistant bacteria comprised the majority of the *V. cholerae* populations because the sampling procedure employed could not provide a precise estimate of bacterial density.

Several studies also explored dynamic changes of viruses in soils and plants (Germida 1986; Stephens, O'sullivan and Ogara 1987; O'sullivan, Stephens and Ogara 1990; Ashelford *et al*. 1999). One study, investigating phage population predating on bacterial species living on the surface of sugar beet, reported fluctuation of phage populations infecting three different bacterial species in a 9-month period. All phage populations infecting specific bacteria consisted of multiple phage species. The phage population predating on *Serratia liquefaciens*, a bacterium indigenous to the phytosphere, consisted of six genetically distinct DNA phage (Ashelford *et al*. 1999). However, there was not enough information about the change of the corresponding bacterial populations because of similar technical difficulties suffered by the aquatic studies.

1.4.3 Mechanisms of phage resistance

As mentioned above, phage resistance plays a decisive role in the population dynamics of bacteria and phage in the laboratory. Bacteria are able to defend against phage infection,

almost in every step of the infection process. By blocking phage receptors, producing an extracellular matrix and competitive inhibitors, bacteria prevent the phage adsorbing to their surface. Bacteria also have superinfection systems to block the entry of phage DNA into their cells. Even after phage successfully insert their DNA into the cells, these superinfection systems protect bacteria by the production of restriction enzymes that cut phage nucleic acids. Furthermore, regularly interspaced short palindromic repeats (CRISPRs), which are widespread in the genomes of many bacteria, confer phage resistance (Sorek, Kunin and Hugenholtz 2008). These repeats are separated by short spacer sequences that match bacteriophage or plasmid sequences and specify the targets of interference (Marraffini and Sontheimer 2010). During phage infection, CRISPRs can acquire new repeat spacer units that match the challenging phage. Cells with this extended CRISPR locus will survive phage infection. The content of the spacer therefore reflects the many different phage and plasmids that have been encountered by the hosts (Marraffini and Sontheimer 2010). How CRISPRs works still remain elusive though it is hypothesized CRISPRs may targets RNA and act as a bacterial RNA interference mechanism (Sorek, Kunin and Hugenholtz 2008). Finally, bacteria can employ an abortive infection system that shuts down the replication of phage but will also lead to the death of the infected cell (Labrie, Samson and Moineau 2010). The mechanisms by which bacteria avoid the phage infection are truly fascinating and diverse.

In turn, phage also evolve to adapt to the bacterial changes. It has been shown that at least for phage receptors blocking, restriction enzyme and CRISPR systems, phage can easily

mutate to allow continuous infection of resistant bacteria. The evolutionary duration of this arms-race co-evolution based on a particular mechanism has been a long-standing question in biology. While the details of many resistance mechanisms are known, gaps in our knowledge remain. Further research should be focused on characterizing the fitness costs of a particular resistance mechanism to understand and predict the long term population dynamics of bacteria and phage. Such data will be very valuable to the studies of the population and evolutionary dynamics of bacteria and phage.

1.5 Summaries of chapter 2, 3 & 4

The hypothesis proposed by Pasricha in the 1930s and by Shah and Mekalanos more recently is clearly reasonable: bacteriophage play an important role in ending cholera epidemics. Mathematical models combining cholera epidemiology and bacterial population dynamics in the presence of phage suggest that the condition phage could contribute to the fast collapse of cholera outbreak is they can control the populations of *V. cholerae* continuously. This seems unlikely based on the results obtained from laboratory studies of population dynamics of many other bacteria and their bacteriophage. Once phage-resistant bacteria evolve, phage can no longer suppress the density of bacteria. To investigate population and evolutionary dynamics of *V. cholerae* and its phage, I first studied the El Tor O1 strain of *V. cholerae* N16961 and its naturally occurring phage JSF4 in continuous cultures. The results of this study were reported in the Proceedings of the Royal Society in a study entitled "An experimental study of the population and evolutionary dynamics of *Vibrio cholerae* O1 and the bacteriophage JSF4" (Wei,

Ocampo and Levin 2010). The paper is presented in chapter 2 and its supplementary information is presented in Appendix 1. Consistent with other population dynamics of bacteria and phage reported so far, JSF4 is only able to control populations of N16961 for a short period of time. After JSF4-resistant N16961 emerged and dominated the community, bacterial populations were limited by resource rather than phage. However, results from my other experiments suggest that relative to the phage-sensitive wild-type, the evolved JSF4-resistant *V. cholerae* experience fitness costs associated with resistance that impair their ability to compete. These fitness costs can prevent JSF4-resistant strains from dominating the population in the natural habitat and may be associated with avirulence to human hosts. The prediction based on these results can be examined using observation of natural populations of *V. cholerae* and infected patients.

In chapter 3, I studied a different scenario in which the density of *V. cholerae* N16961 was limited by two different bacteriophages in continuous cultures over an extended time (~650 hours). Initially, I intended to study bacterial dynamics in the presence of a single phage species. However, I later discovered that two distinctive phages were present in the chemostat and were responsible for the phage-limited dynamics. This was also confirmed by reconstruction experiments in which two phages were co-cultured with *V. cholerae* N16961. In three independent chemostats running for over 600 hours, the densities of *V. cholerae* co-cultured with two phages were maintained approximately one order of magnitude lower than that of *V. cholerae* cultured alone. In one chemostat, bacteria simultaneously resistant to both phages were isolated at around 500 hours.

However, the density of those resistant bacteria never increased to the level expected to occur in a resource-limited condition. While we do not currently have sufficient data to explain this phenomenon, we discussed several possibilities with some experimental results.

In chapter 4, I present a study that is not fully related to bacteria-phage dynamics but was inspired by some of the findings from chapter 2. In chapter 2, I discovered that some of JSF4-resistant bacteria are non-motile and the relative fitness of these non-motile strains is dependent on the conditions under which the competition tests are done. In liquid, non-motile JSF4-resistant strains are almost equally fit as the motile reference strain. However, when competition occurs on the surface of an agar plate, non-motile strains exhibit an extreme fitness disadvantage relative to motile reference strain. Inspired by these intriguing results, I asked the seemingly basic and simple question "Why are bacteria motile?". I hypothesize that motility is favored because it enables bacteria to move apart from each other, thereby obtaining greater shares of limiting resources. A mathematical model and experiments with motile and non-motile strains *E. coli* and *V. cholerae* were used to test this hypothesis and explore the conditions under which motility provides bacteria a fitness advantage in initially homogenous nutrient-limited spatially structured habitats. We found that in an agar matrix with diffusing nutrients, bacteria with the capacity to move have an advantage in the rate at which they saturate the environment and compete with populations of less motile bacteria. As predicted by the models, the magnitude of the advantage of being motile in our experiments is directly

proportional to the quantity of the limiting resource and the viscosity of the habitat. This study is finished and a manuscript is being prepared for submission.

CHAPTER 2

An Experimental Study of the Population and Evolutionary Dynamics of *Vibrio cholerae* O1 and the Bacteriophage JSF4

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2.1 Introduction

Not only was the lysis of *V. cholerae* by a heat-labile substance in river water the first evidence for the existence of bacteriophage (Hankin 1896), there is compelling field and patient evidence that suggests lytic bacterial viruses are responsible for the decline in the incidence of cholera during seasonal outbreaks (Pasricha *et al.*1931; Faruque *et al.* 2005.1; Faruque *et al.* 2005.2). The hypothesis that lytic phage are responsible for the waning of cholera outbreaks is also supported by the results of a theoretical study that employs a mathematical model that combines the epidemiology of cholera with the population dynamics of *V. cholerae* and a lytic phage (Jensen *et al.* 2006). In this model, for phage to modulate outbreaks of cholera, this bacterial virus rather than growth-limiting resources must limit the densities of *V. cholerae* in the environment.

Unfortunately, the proposition that predation by bacteriophage rather than resources limit the density of bacterial populations is inconsistent with the results of all the *in vitro*

studies of the population and evolutionary dynamics of bacteria and lytic phage of which we are aware. Although phage may limit the densities of bacteria for extended periods, e.g. Chao *et al.* (1977), eventually bacterial mutants resistant to the phage evolve and the bacterial population becomes limited by resources (Bohannan & Lenski 2000; Chao *et al.* 1977; Lenski & Levin 1985; Levin *et al.* 1977; Mizoguchi *et al.*2003). This occurs even when the bacteria are confronted with three phage "species" with different adsorption sites and when all three phage are sensitive to the restriction-modification system borne by these bacteria (Korona & Levin 1993). There may be a coevolutionary arms race, with mutations for bacterial resistance being countered by host-range phage mutations enabling them to replicate on the resistant cells. However, if the lytic phage of *E. coli* are at all typical, these arms races end within a couple of cycles. Resistant bacterial mutants are generated to which the phage cannot produce host-range mutants capable of replicating on them (Lenski & Levin 1985). For a possible exception to this limited arms race see Buckling & Rainey (2002), but even in that study no evidence was presented for the bacteria being limited by phage rather than resources.

To test the hypothesis that populations of *V. cholerae* are limited by phage rather than resources in batch, chemostat and serial transfer culture, we performed population dynamic, evolutionary and parameter estimation experiments with the El Tor strain of *V. cholerae* O1 (N16961) and a lytic phage from a natural population (JSF4). Our results indicate that, when cultured together, *V. cholerae* resistant to JSF4 rapidly emerge and continue to dominate the bacterial populations which are maintained at levels similar to that observed for phage-free, resource-limited cultures of bacteria.

While these observations are inconsistent with the hypothesis that predation by lytic phage, rather than resources, limits the densities of *V. cholerae* populations, the results of our other experiments suggest an alternative mechanism whereby lytic bacteriophage can contribute to the waning of outbreaks of cholera. The phage-resistant *V. cholerae* mutants obtained in these experiments are less fit than wild-type in laboratory culture, possess a number of characteristics that are likely to further reduce capacity to be maintained in natural population and may be avirulent in human hosts. We postulate that because of the impaired ecological fitness of phage-resistant *V. cholerae* mutants and their relative avirulence, lytic phage like JSF4 can modulate outbreaks of cholera. We discuss these hypotheses and the extent to which they are supported by current field, experimental animal and clinical studies and suggest how they can be tested in natural populations of *V. cholerae* and human hosts.

2.2 MATERIAL AND METHODS

Organisms: bacteria, phage, nematodes and crustaceans

The *V. cholerae* used in this study were derived from El Tor O1 N16961, a spontaneous streptomycin-resistant (Sm^r) mutant of a clinical isolate from Bangladesh. JSF4 is a naturally associated bacteriophage of *V. cholerae*. Table 2.1 lists the variants of *V. cholerae* employed in these experiments and their sources.

Two species of *Daphnia*, *D. pulex* and *D. magna* were used to explore the relative capacity of *V. cholerae* isolates to adhere to the surfaces of zooplankton. These crustaceans were purchased from Carolina Biological Supply Inc.

The *Caenorhabditis elegans* used for the virulence assays is the wild-type reference strain var. Bristol (N2). *E. coli* OP50 was used as a food source for *C. elegans*.

Table 2.1 *V. cholerae* strains used in this study

Strain	Relevant phenotype	Comments	References
N16961	Sm^r smooth wild-type	El Tor O1 *V. cholerae*	John J. Mekalanos
R-N16961	Sm^r rugose wild-type	Isolated from a batch culture	This study
Rif.R	Rif^r smooth N16961	spontaneous Rif^r mutant of smooth N16961	This study
RF	$JSF4^r$ smooth N16961	Isolated from a batch culture	This study
R1.1	$JSF4^r$ smooth N16961	Isolated from chemostat 1(Figure 2.1c) – 25 hours after introduction of phage	This study
R1.2	$JSF4^r$ smooth N16961		This study
R2.1	$JSF4^r$ smooth N16961	Isolated from chemostat	This study

R2.2	JSF4^r smooth N16961	2(Figure 2.1a) – 75 hours after introduction of phage	This study
RR	JSF4^r rugose N16961	Isolated from a batch culture	This study

Sm^r, streptomycin resistant; JSF4^r, JSF4 resistant; Rif^r, rifampin resistant

10 more JSF4 resistant mutants isolated from three additional chemostats were included in the fitness assay. Their source is noted in the legend to Figure 2.2. A comprehensive version of this table is in supplementary information to this chapter (Appendix 1, Table A2).

Media, Culturing and sampling procedures

Vibrio cholerae were grown in either Luria- Bertani Broth (LB) or a medium composed of 5% LB and autoclaved 95% tap water. The density of *V. cholerae* N16961 was estimated from colony count data on LB agar or LB agar with rifampin (25 µg/ml). The density of JSF4 was estimated from plaque counts on LB agar overlaid with lawns of *Vibrio cholerae* N16961. Unless otherwise noted, the experimental populations were maintained at 30^{o}C with aeration via shaking or (in chemostats) bubbling.

The chemostats used in these experiments were of a "homemade" design similar to that in the appendix to Chao *et al.* 1977 (see www.eclf.net for a description of the latest incarnation of these chemostats). *V. cholerae* N16961 and JSF4 were co-cultured in

chemostats with 5% LB and 95% tap water at dilution rates ranging from 0.07 h^{-1} to 0.24 h^{-1} for 120 to 340 hours (in a volume of 20 – 25 ml). Unless otherwise noted, estimates of bacterial density and phage titers from chemostats were made from samples taken directly from the chemostat vessels.

Assays for phage resistance.

Resistance of *V. cholerae* N16961 to JSF4 was first determined by spotting 10 - 20 µl of a ~10^8 PFU /ml lysate of JSF4 on lawns derived from single colonies of the sampled culture. Bacterial clones for which there was no evidence for phage lysis on lawns were further tested for the ability to support the replication of JSF4 in liquid cultures. Those unable to support phage replication were deemed resistant.

Ecological Fitness and Virulence experiments

Pairwise competition in liquid culture**:** The fitness cost associated with resistance to JSF4 was estimated by competing JSF4- sensitive wild-type *V. cholerae* N16961 with JSF4-resistant isolates. To facilitate these assays, a spontaneous rifampin-resistant mutant of N16961 (designated Rif.R) was used. The relative frequencies of JSF4-sensitive and -resistant strains were estimated by plating on LB agar and LB agar with rifampin. For each competing pair, at least three replicate experiments were done. The Malthusian selection coefficient *s* was calculated using the formula

$$s = \frac{\ln W_t - \ln W_0}{\ln\left(\frac{N_t}{N_0}\right)},$$

where W_t and W_0 are, respectively the ratio of the densities of JSF4-resistant and -sensitive N16961 at time t and the initial time 0, and N_t and N_0 the total densities of bacteria at those two times (Travisano & Lenski 1996). The relative fitness of each JSF4-resistant mutant to Rif.R was calculated as 1 / (1-s). As a control for the fitness cost of the rifampin resistance marker, pairwise competition experiments were performed with wild-type N16961 and Rif.R. *t-test* was used to analyze the fitness data and false discovery rate (FDR) was controlled at 0.05 to correct for multiple comparisons.

Fitness in surface culture: A membrane filter method similar to that employed by Fujikawa & Morozumi (2005) to study surface growth of *E. coli* was used to ascertain the relative fitness of the resistant and sensitive *V. cholerae* when they grow as colonies. 10µl of culture containing approximately equal densities (~ $3x10^5$ cells/ml) of the competing bacteria were spotted onto 25mm diameter 0.45 µm Tuffryn membrane filters (Pall, 66221). These filters were placed on Petri dishes containing 5% LB agar and incubated at 30°C for 24 hours. To estimate the relative frequencies of the competing pair, the filters were removed from the agar, placed in 1ml 0.85% saline and vigorously vortexed for 30 seconds to release the bacteria. The fitness effect of rifampin marker on the competing wild-type bacteria was also estimated in these surface cultures.

Colonization of *V. cholerae* N16961 on Daphnia: As a measure of the relative capacity of the JSF4-sensitive and resistant *V. cholerae* to colonize the chitinous surfaces of crustaceans we used *D. pulex* and *D. magna*. *V. cholerae* were grown to stationary phase in LB and washed three times with autoclaved pond water obtained from Lullwater Park (Emory University). After gently washing with sterile pond water, two to three *Daphnia* were incubated with 10^6-10^7 CFU of bacteria in 1.5 ml Eppendorf tubes at room temperature overnight in the dark.

After overnight co-incubation, the tubes were rinsed with autoclaved pond water 3-5 times to remove the unattached bacteria. The density of free bacteria after washing was determined by diluting and plating. The *Daphnia* in the tubes were then homogenized using a pellet pestle (Kontes, K749510). The difference between the post homogenizing and pre-homogenizing estimates was used as our estimate of the density of *V. cholerae* adhering to the *Daphnia*. To control for contribution of aerobic bacterial flora of the daphnia we used LB agar with streptomycin; N16961 is resistant to this antibiotic. *t-test* was used to analyze the colonization data and false discovery rate (FDR) was controlled at 0.05 to correct for multiple comparisons.

C. elegans mortality assay**:** Nematode growth medium (0.3% NaCl, 1.7% agar, 0.25% peptone, 0.001M $MgSO_4$, 0.001M $CaCl_2$, 0.005mg/ml cholesterol in ethanol, 0.025M pH6.0 K_3PO_4 buffer) agar plates were prepared and inoculated with 100µl of overnight

LB cultures of *V. cholerae*. These plates were incubated for 8 hours at 37 ^{o}C at which time distilled water washed *C. elegans* L4 stage (adults) were added to agar plates bearing *E. coli* OP50 or JSF4-sensitive or resistant *V. cholerae* and incubated at room temperature (~23^{o}C). Each day, the number of viable worms was estimated and every second day all of the viable worms were transferred to fresh plates containing the same strain of bacteria.

2.3 RESULTS

Population dynamics of *V. cholerae* N16961 and lytic bacteriophage JSF4 in chemostat culture

We open this section with the results of experiments addressing the question of whether populations of *V. cholerae* are limited by phage rather than resources. The changes in the density of bacteria and phage in three of the eight chemostat experiments of this type that we performed are presented in Figure 2.1.

Two to four hours after the introduction of phage into chemostats bearing wild-type N16961, the density of bacteria in the chemostats dropped by approximately four orders of magnitude while that of the phage rose to between 10^{9} and 10^{10} PFU/ml (Figure 2.1a). No substantial change in the density of bacteria was observed in the phage-free control chemostats. Similar dynamics were observed with the introduction of JSF4 to chemostats

with rugose colony variants of N16961 and mixtures of rugose and wild-type, smooth colony, N16961, albeit with less of a decline in the bacterial density than observed when the phage were added to chemostats bearing solely wild-type N16961 (Figure 2.1b, Figure 2.1c). Rugose variants, were examined this way because it had been proposed that these variants, which produce copious quantities of exopolysaccharide, have higher rates of survival in the environment (Ali *et al*. 2002) and are presumed to be more refractory to phage than smooth colony types (Beyhan & Yildiz 2007).

Most importantly in all three cultures depicted in Figure 2.1 (and in five similar chemostat experiments; supplementary information: Appendix 1, A1), the drop in bacterial density following the addition of phage was transient. The bacterial population recovered and by 24 hours the densities of the bacteria in these cultures with JSF4 were similar to those in the corresponding phage-free controls. The same results of resource- rather than phage-limited cultures were obtained in analogous experiments performed in 1/100 dilution 10 ml serial transfer cultures (data not shown).

While the density of JSF4 decreased after its initial bloom, the phage continued to persist at approximately 10^6 ~10^7 PFU/ml. We interpret this as evidence for phage replication. Were that not the case, with the dilution rates of the chemostats in Figures 2.1a-c, following a peak density of 10^9 PFU/ml the density of phage would be less than 10

PFU/ml by 184, 77 and 88 hours in the chemostats, Figure 2.1a, 2.1b and 2.1c, respectively.

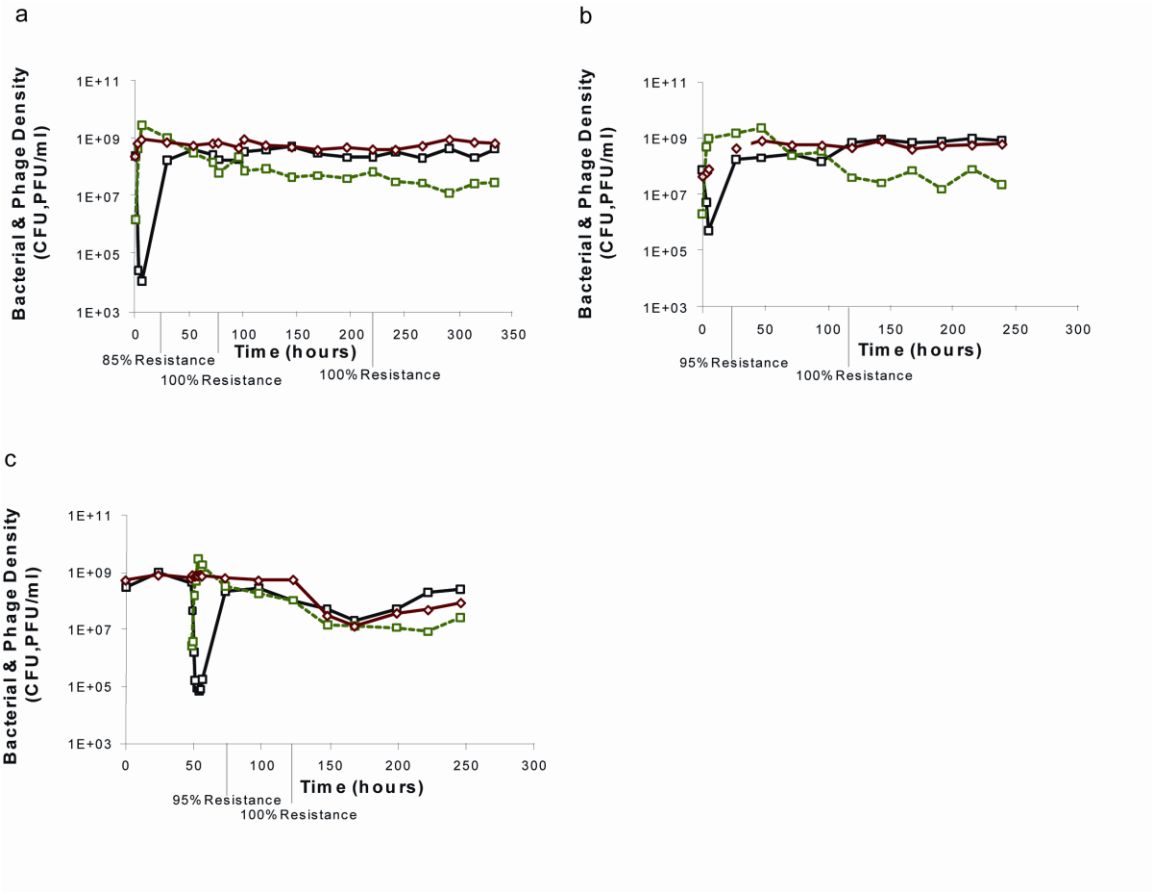

Figure 2.1 Change in the density of bacteria and phage in continuous culture, 5% LB,where the solid line and broken lines with open boxes are, respectively the densities of the bacteria and phage in mixed culture and solid line with diamond bars are the density of bacteria in the phage-free control. All chemostats were inoculated with bacteria from an overnight LB culture for an initial density of ~10^8 CFU/ml culture and ~ 10^6 PFU/ml of phage. The dilution rates, w, are fraction of the total volume of the chemostat change per hour. The line with "% resistance" denotes the percentage of JSF4-resistant mutants out of 20 randomly chosen colonies at specific time points.

(a) Chemostat inoculated with wild-type *V. cholerae* N16961, w=0.07-0.12 (b) Chemostat inoculated with rugose variant of *V. cholerae* N16961, w=0.24, (c) Chemostat

inoculated with wild-type *V. cholerae* N16961. The phage JSF4 was introduced after the rugose variants in this chemostat reached a level of 9.4% of total bacteria, w=0.21.

Resistant bacteria and host range phage: The majority of bacteria recovered from these cultures were resistant to the original phage by approximately 25 hours after the phage were introduced. This was also the case for all the colonies tested in later samples (Figure 2.1). We interpret this as evidence that the bacterial populations in these cultures with phage were dominated by phage-resistant cells. No JSF4-resistant colonies were observed in the samples taken from the phage-free control cultures. Presumably, the phage are maintained in these chemostats by replicating on a minority population of planktonic sensitive bacteria (Levin *et al.* 1977) or on sensitive cells adhering to the walls of the chemostats (Schrag & Mittler 1996). To ascertain whether the JSF4 phage produced host-range mutants capable of replicating on the resistant N16961, the lysates from final samples of the chemostat were mixed with lawns of resistant mutants from the corresponding chemostat; no plaques were observed.

Competitive performance and virulence of the JSF4-Resistant Mutants of *V. cholerae* N16961

If phage rather than resource limitation is a necessary condition for these viruses to be responsible for the waning of outbreaks of cholera (Jensen *et al.* 2006), the results of these chemostat and serial transfer experiments are inconsistent with that hypothesis. However, as we now show, the results of our experiments characterizing the resistant

mutants suggest that in natural habitats, JSF4-resistant mutants may not be significant players in the ecology of *V. cholerae* and epidemiology of cholera.

1-Most but not all JSF4-resistant mutants have a fitness disadvantage when competing with wild-type in liquid culture. Using procedures similar to those used by Travisano & Lenski (1996), we estimated the Malthusian fitness of 12 JSF4-resistant smooth strains and 4 rugose resistant strains relative to a phage-sensitive rifampin resistant mutant, Rif.R. The results of these pair-wise competition are presented in Figure 2.2a.

Relative to wild-type, the fitness of the JSF4- sensitive Rif.R, was 0.71±0.03, indicating that the rifampin resistance marker engenders a substantial fitness cost. To facilitate our comparison of the relative fitness of the different resistant mutants and wild-type, in Figure 2.2a, we assign a unit value (1.0), the horizontal line, for the fitness of the Rif.R common competitor, which relative to wild-type has a fitness of 1/0.71=1.41. By this criterion, 7 resistant mutants were significantly more fit than the Rif.R (*t-test*, $p<0.05$) and 11 of the 12 resistant mutants were significantly less fit than wild-type (*t-test*, $p<0.005$). While the JSF4-sensitive rugose variant was more fit than Rif.R, all four JSF4- resistant rugose variants were significantly less fit than Rif.R (*t-test*, $p<0.005$).

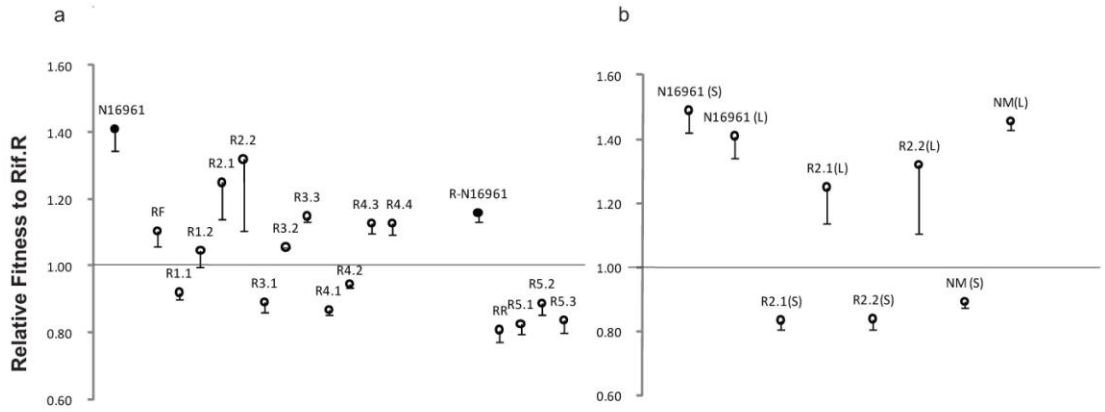

Figure 2.2 (a) Relative Malthusian fitness of a Rif-resistant JSF4-sensitive common competitor in pair-wise competition with Rif-sensitive JSF4-sensitive wild-type N16961, rugose variant of N16961and 16 Rif-sensitive JSF4-resistant mutants (12 are smooth colony and 4 are rugose colony types). The horizontal line denotes the fitness of the common competitor, the Rif-resistant mutant. The error bars are the standard deviations of the mean estimated relative fitness of at least 3 independent pair-wise competition experiments.

RF, R1.1, R1.2, R2.1, R2.2, RR: JSF4-resistant mutants (see Table 2.1 for the source)

R3.1, R3.2, R3.3, R4.1, R4.2, R4.3, R4.4 are smooth JSF4-resistant mutants. R5.1, R5.2, R5.3 are rugose type JSF4-resistant mutants. All of them were isolated from 3 different chemostats. The source of these mutants is listed in supplementary information to this chapter (Appendix 1, Table A2).

(b) Relative fitness of different *V. cholerae* N16961 in competition with Rif- resistant JSF4-sensitive common competitor in liquid and in surface culture. (S): competition on surface, (L): competition in liquid; R2.1, R2.2: non-motile JSF4-resistant mutants; NM: a non-motile JSF4-sensitive isolate.

2- The resistant strains of similar fitness to wild type in liquid culture are less fit than wild-type in surface culture. As observed in liquid, in surface culture the rifampin-resistant, JSF4-sensitive common competitor, Rif.R, is less fit than the wild-type N16961 (Figure 2.2b). On the other hand, when competing as colonies in surface culture, the

phage-resistant mutants R2.1 and R2.2 were less fit than the Rif-R common competitor, rather than substantially more fit as they were in liquid (Figure 2.2b). We interpret this to mean that in surface culture these resistant mutants are substantially less fit than wild-type.

3- JSF4-resistant *V. cholerae* N16961 are less motile than wild-type *V. cholerae* N16961.

The swarming motility of more than 200 smooth phage-resistant mutants separately isolated from the batch and chemostat with N16961 and JSF4 was examined. These resistant mutants were either non-motile or somewhat motile, but all were less motile than wild-type. The average swarming distance of the 209 phage-resistant mutants examined was 6.1±4.6 mm (mean ± s.d.). Of these 209, 77 of them were non-motile, with an average swarming distance of 1.4 ± 0.16 mm. The remaining 132 resistant mutants were at least somewhat motile, with an average swarming distance of 9.0 ± 3.2 mm. All of these JSF4-resistant mutants are significantly less motile than wild-type N16961, which have an average swarming distance of 23.2 ± 4.74 mm (*t-test*, $p<10^{-7}$). To exclude the possibility that the reduced motility observed among JSF4-resistant mutants is associated with the adaptation of *V. cholerae* to chemostat culture, rather than phage resistance, 252 isolates from two JSF4-free control chemostats were tested for motility. Of these 252 isolates, 20 appeared to be less motile than the wild-type. All of 20 of the reduced motility colonies were isolated from the later samples of the chemostat cultures. None of the clones isolated at similar time points as those above described JSF4-resistant mutants had a reduced motility phenotype. Since the JSF4-sensitive rugose

variants were substantially less motile than wild-type (swarming distance of about 4 mm rather than 23mm), we did not assay for the difference between JSF4-sensitive and -resistant rugose variants.

4- JSF4-resistant mutants are more likely to form clumps and switch to rugose at lower frequencies than wild type. The methods and results of the experiments demonstrating this are in the supplementary information to this chapter (Appendix 1, A2-A3).

5- JSF4-resistant mutants have a disadvantage relative to wild-type in colonizing crustaceans. In their natural aquatic habitat, *V. cholerae* are associated with zooplankton such as copepods, where they colonize the carapace and other parts of the chitin exoskeleton (Huq *et al.* 1983; Cottingham *et al.* 2003; Levy 2005).The relative ability of JSF4-sensitive and resistant *V. cholerae* to colonize zooplankton was examined by culturing single clones of JSF4- resistant and -sensitive *V. cholerae* N16961 with *D. magna* and *D. pulex* (Chiavelli *et al.* 2001; Kirn *et al.* 2005). In the course of these experiments, we noted that for any given strain of bacteria, the more intensely red daphnia supported higher densities of colonizing *V. cholerae* than those that were more colorless (i.e.had less hemoglobin; Pirow *et al.* 2001). To control for this hemoglobin effect, we used the same batch of daphnia for each colonization experiment.

In Figure 2.3a we present the results of these colonization experiments with *D. pulex* and in Figure 2.3b those with *D. magna*. For both JSF4-sensitive and JSF4-resistant *V. cholerae*, the number of bacteria recovered from *D. magna* was substantially greater than that from *D. pulex*, presumably because of the greater size and thus greater surface area of *D. magna*. In all cases significantly fewer JSF4-resistant cells were recovered from both species of daphnia than the wild-type (*t-test*, $p<0.05$).

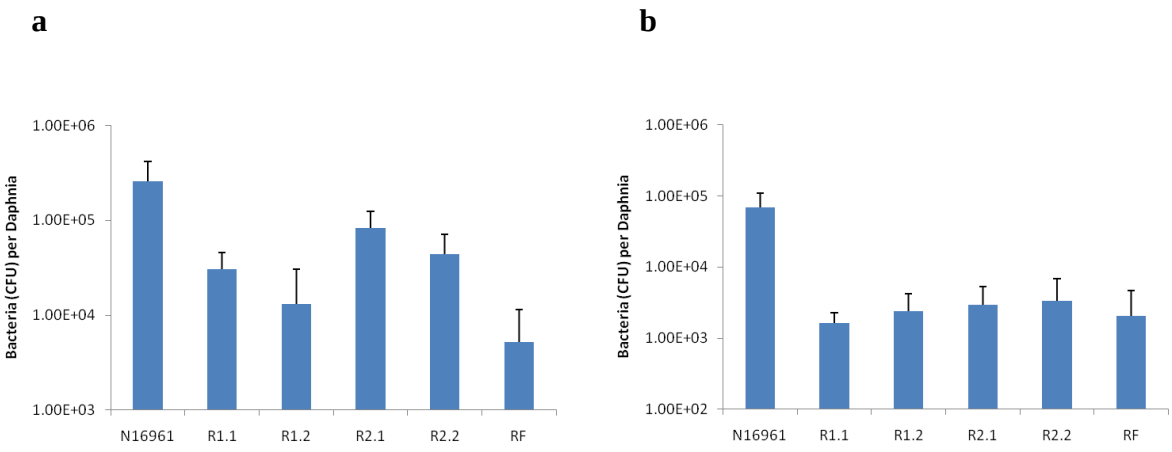

Figure 2.3 Colonization of wild-type *V. cholerae* N16961 and JSF4-resistant mutants on Daphnia

(a). Colonization on *Daphnia magna* **(b).** Colonization on *Daphnia pulex*

RF, R1.1, R1.2, R2.1, R2.2: JSF4-resistant mutants Error bar represents standard deviation of bacteria (CFU) attached to single daphnia.

Although it is beyond the scope of this report to ascertain the reasons that *V. cholerae* are more successful in colonizing dead crustaceans than live ones (Mueller *et al.* 2007), for this study it seemed reasonable to ascertain whether the differences in the relative ability of JSF4-sensitive and -resistant *V. cholerae* to colonize viable *Daphnia* can be attributed

to their relative capacity to adhere to chitin. To determine if this is the case, we examined the ability of JSF4-sensitive and -resistant strains to colonize chitin in an aqueous suspension (Appendix 1, A4). Among 5 smooth JSF4-resistant mutants, only two mutants, which are non-motile, were significantly less capable of colonizing chitin than wild-type (*t-test*, $p<5\times10^{-7}$). This result is consistent with the observation that motility is positively associated with the ability of these bacteria to colonize dead crustaceans (Mueller et al. 2007). As noted above and contrary to this observation, there seemed to be no association between motility and the colonization of viable *Daphnia*. There was also no significant difference in the colonization on chitin between JSF4-sensitive and -resistant rugose mutants (*t-test*, $p>0.1$).

6- JSF4-resistant mutants are less virulent than wild-type when ingested by *C. elegans* .

Using a procedure similar to that in Vaitkevicius *et al.* (2006) we estimated the relative rate of mortality of *C. elegans* feeding on six independent JSF4-resistant mutants, a wild-type N16961 and *E. coli OP50*. At a qualitative level, our survival results are consistent with those of Vaitkevicius and colleagues; the rate of decline in viable *C. elegans* ingesting wild-type N16961 was more than those fed of *E. coli* OP50 (Figure 2.4). Most importantly for this study, the decline in viable *C. elegans* feeding on JSF4-resistant *V. cholerae* was significantly less than those ingesting phage-sensitive, wild-type, *V. cholerae* (Log-rank test, $p<0.01$).

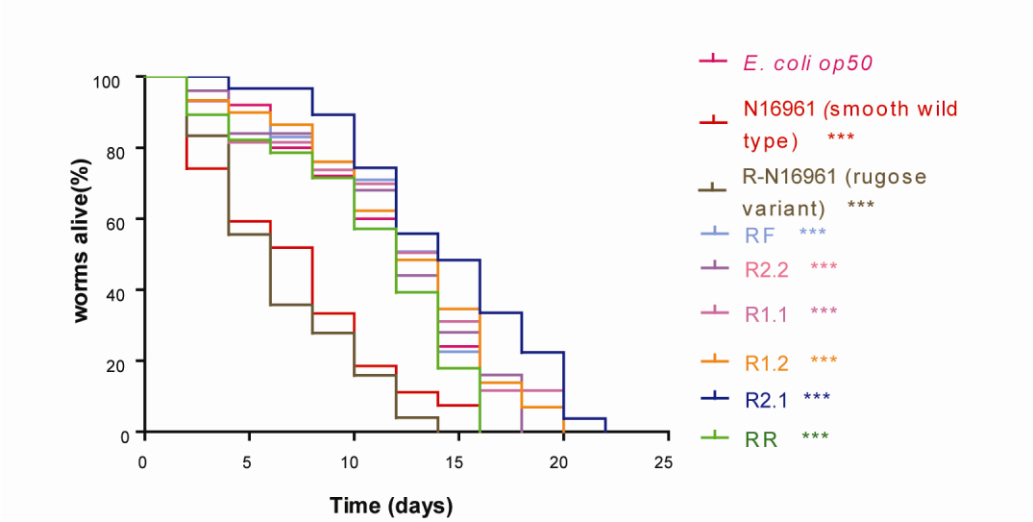

Figure 2.4 Rate of mortality of *C. elegans* feeding on phage-sensitive and resistant *V. cholerae* and an *E. coli* OP50 control. RF, R1.1, R1.2, R2.1, R2.2 are smooth phage-resistant mutants and RR is rugose phage-resistant mutant.

*** indicates a significant p value of Log-rank test for comparison of survival curves of JSF4- resistant relative to JSF4-sensitive

2.4 Discussion

If, as postulated in Jensen *et al.* (2006), the necessary condition for phage to modulate outbreaks of cholera is that these viruses, rather than resources, limit the densities of *V. cholerae* populations, the results of our chemostat and serial transfer experiments are inconsistent with this hypothesis. When the lytic phage JSF4 are introduced into chemostat and serial transfer cultures of the El Tor O1 *V. cholerae* N16961, JSF4-resistant cells rapidly ascend to dominance. The densities of these experimental populations of *V. cholerae* and the phage JSF4 are no different from that of phage-free, resource-limited, control cultures. Presumably, by replicating on minority populations of phage-sensitive *V. cholerae* (Levin *et al.* 1977), the phage persist in these mixed

experimental cultures. These results are what would be anticipated from studies of the population dynamics of *E. coli* and its phage (Bohannan & Lenski 2000; Mizoguchi *et al.* 2003) as well as those of *Pseudomonas syringae* and its phage Phi6 (Lythgoe & Chao 2003) and *Pseudomonas aeruginosa* with phage PP7 (Brockhurst *et al.* 2005).

Although our *in vitro* population dynamic experiments with *V. cholerae* N16961 and the phage JSF4 reject the hypothesis that this bacterial virus limits the density of *V. cholerae*, the other experiments we performed are not inconsistent with the epidemiological/clinical hypothesis that lytic phage contribute to the waning of outbreaks of cholera. We postulate that because of their lower fitness in their natural habitat and possible avirulence in humans, phage-resistant *V. cholerae* play a relatively insignificant role in the ecology of these bacteria and the epidemiology of cholera. In support of this interpretation are the following observations:

(1) In pair-wise competition between JSF4-resistant mutants and the JSF4-sensitive *V. cholerae* in liquid culture, the majority of the resistant mutants are significantly less fit than the sensitive cells from whence they were derived. Although one of the phage-resistant clones we tested was not less fit than wild-type in liquid, it had a profound fitness disadvantage relative to wild-type in surface culture.

(2) All the JSF4-resistant mutants we examined were less motile than wild-type. We believe that this reduced motility may account for why some phage-resistant mutants were less fit than wild-type in surface but not in liquid culture (Figure 2.2b). We

postulate that in surface and semisolid culture, motility enables bacterial colonies to spread and, by diffusion, sequester more resources than non-motile colonies. As a result, colonies of motile bacteria produce more cells than non-motile colonies and thereby have a competitive advantage in a manner analogous to the way the diffusion of bacteriocins provides a selective advantage to producing colonies in competition with bacteriocin-sensitive, non-producing populations (Chao & Levin 1981). We are currently testing this resource sequestration hypothesis for the advantages of motility in physically structured habitats.

(3) In their natural habitat, *V. cholerae* colonize copepods and other crustacean (Mueller *et al.* 2007). *In vitro*, the JSF4-resistant cells appear less able to colonize viable crustaceans than the sensitive. The number of JSF4-resistant *V. cholerae* recovered from *Daphnia pulex* and *Daphnia magna* was significantly lower than the corresponding number of phage-sensitive, wild-type.

(4) The rate of mortality of the nematode *C. elegans* feeding on JSF4-resistant *V. cholerae* was significantly less than that when these worms feed on susceptible *V. cholerae*. This too may be a reflection of the reduced motility of these bacteria. There is evidence for a direct relationship between motility and the virulence of *V. cholerae*, but that appears to depend on the nature of the mutations responsible for the non-motile phenotype (Gardel & Mekalanos 1996). We have not yet ascertained whether the mutations responsible for the decreased motility of JSF4-resistant *V. cholerae* are of this virulence – reducing type.

Predictions, current evidence from natural population and clinical isolates, and suggestions for testing hypotheses

Model systems, like mathematical models, provide a way to generate hypotheses about natural population but cannot be used to test those hypotheses

One prediction of these experimental results is that if *V. cholerae* and its lytic phage are together and bacterial densities are high enough for the phage to replicate, the majority of the bacteria would be resistant to the phage. This prediction is at least somewhat consistent with the results reported by Faruque and colleagues (Faruque *et al.* 2005.2). Of 15 samples from river and lake water where both *V. cholerae* and its lytic phage were present, bacteria resistant to the co-isolated phage were found in 10 of the samples. Whether the *V. cholerae* were eliminated by the phage in the 87 samples, where only *V. cholerae*-specific phage but no bacteria were isolated, is not clear, although these phage must have replicated on *V. cholerae*. Whether the *V. cholerae* in their 49 phage-free samples were resistant to the phage in the same lake or river is not reported.

One problem in interpreting the results of existing field studies is that we do not have good estimates of the densities of the bacteria and phage populations. Samples are commonly enriched and even then the densities of bacteria in those samples are relatively low ($<10^3$) which is too low to maintain a phage population that has even a modest rate of loss (see the Appendix 1, A5). Obviously, there are habitats or sub-habitats where the densities of bacteria are sufficiently high to support the replication of the phage. One

goal of a field study testing the predictions generated from this experimental model would be to identify the habitats or sub-habitats, where the densities of bacteria are sufficiently high to support the replication of phage. In these habitats we would anticipate that (1) the bacterial population would be dominated by cells resistant to the co-existing phage, and (2) if a novel phage that can replicate on the bacteria in that habitat is introduced, resistance will evolve and these resistant cells will ascend to dominance. Presumably because of the infective dose requirement (Cash *et al.* 1974), these sub-habitats with high densities of *V. cholerae* are those from whence humans obtain symptomatic cholera.

A second prediction is that *V. cholerae* resistant to lytic phage are ecologically less fit than wild-type. We are unaware of evidence in support of or in opposition to this prediction, but believe it can be tested in natural populations. Phage-sensitive and -resistant bacteria *V. cholerae* can be released in habitats where phage are not present, and their relative rates of recovery can be monitored in open water and colonizing on copepods and other crustaceans in these natural communities. Although there have been studies of the colonization of different strains of *V. cholerae* on viable and dead crustaceans in the field and *in vitro* (Huq *et al.* 1983; Mueller *et al.* 2007), to our knowledge there have not been field studies that have explored the relative abilities of otherwise isogenic phage-sensitive and -resistant *V. cholera* to colonize viable and dead crustaceans.

A third prediction made from these *in vitro* experimental results is that phage-resistant *V. cholerae* are less virulent in human hosts than otherwise isogenic sensitive cells. There are at least two lines of evidence in support of this hypothesis. First, a recent report presented evidence that the minimum infective dose of *V. cholerae* O1 in infant mice was ten times greater when the bacteria were mixed with JSF4 than when they were not (Zahid *et al.* 2008). In that study, as in our own study, while JSF4-resistant *V. cholerae* C6706 emerged and dominated *in vitro*, LB culture, in the mice only JSF4-sensitive *V. cholerae* were recovered from the stools. Second, the *V. cholerae* recovered from stools of cholera patients infected with both bacteria and phage were susceptible to the phage isolated from these stools (Zahid *et al.* 2008). If these results are general, we would expect that if mixtures of *V. cholerae* and JSF4 are introduced into human volunteers (i) the phage would replicate and (ii) the bacteria recovered from the stools would be primarily, if not exclusively, sensitive to these phage.

CHAPTER 3

The Population and Evolutionary Dynamics of *Vibrio Cholerae* and its Bacteriophage: Conditions for Maintaining Phage-Limited Communities

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3.1 Introduction

Bacteriophages have been reported to be the most abundant organisms on earth (Suttle 2005; Weinbauer 2004) and thereby have population densities that exceed that of their host bacteria. Does this mean that phage, rather than resources or other predators, limit the densities of bacteria in oceans and other natural communities? This is what has been observed in phage-limited experimental populations of *E. coli* and its phage T7 (Chao, Levin and Stewart 1977) and anticipated from mathematical models (Levin, Stewart and Chao 1977). However, to our knowledge, the question of whether phage rather than resources or other predators regulate the densities of specific species of bacteria or bacteria at large has not been addressed for natural communities.

If we extrapolate from laboratory studies of the population dynamics of lytic phage and bacteria, the answer to this question would be "no". In all studies of this type we know of with single phage and bacteria, the bacterial population may be limited by the phage initially but eventually bacteria resistant to the co-existing phage emerge, ascend to

dominance and the bacterial population becomes limited by resources, rather than phage predation (Chao, Levin and Stewart 1977; Levin, Stewart and Chao 1977; Middelboe 2000; Mizoguchi *et al*. 2003). To be sure, there can be and usually is a co-evolutionary arms race with resistance in the bacteria countered by host range mutant phage capable of replicating on the resistant bacteria. Even in bacterial communities with single bacteria and phage species, this arms race can promote considerable diversity in both populations (Forde *et al.* 2008; Weitz, Hartman, and Levin 2005). However, to our knowledge, in almost if not all cases, these arms-race between these viruses and bacteria terminate with resistant bacteria for which the phage are unable to generate host range mutants (Chao, Levin and Stewart 1977; Lenski and Levin 1985; Levin, Stewart and Chao 1977). In theory, the adaptive immune system known as CRISPR (Barrangou *et al.* 2007; Jansen *et al.* 2002) could promote an almost indefinite arms race between bacterial immunity and host-range mutations in the phage (Vale and Little 2010; Levin 2010). In practice, however, we are aware of only one report of an extended genetic arms race between bacteria and phage (Buckling and Rainey 2002) and in that study it is unclear whether the phage rather than resources limit the bacterial population.

This question of whether phage can limit the densities of bacteria is not a purely academic one, but also has practical implications. For instance, phage therapy has been used in the treatment of bacterial infections (Levin and Bull 2004; Skurnik and Strauch 2006). As is the case for antibiotics, if resistance evolves and the resistant strains are virulent, which may not be the case, treatment may fail (Smith and Huggins 1982; Levin

and Bull 1996). Also, bacteriophage may play a role in limiting the densities of bacteria in natural communities. It has been proposed that phage are responsible for the waning of outbreaks of cholera (Pasricha *et al.*1931; Faruque *et al.* 2005a; Faruque *et al.* 2005b). In theory, for this to be the case, the bacteria have to be limited by the phage rather than resources (Jensen *et al.* 2006). When we tested this phage-limited hypothesis with a virulent El Tor O1 strain of *V. cholerae* N16961 and its naturally occurring phage JSF4 in experimental communities, we obtained negative results (Wei, Ocampo and Levin 2010). In our chemostats, mutant bacteria resistant to the phage rapidly evolved and the cultures went from a phage - to a resource-limited state. Host range JSF4 mutants capable of replicating on these resistant *V. cholerae* were not observed in our chemostat experiments and could not be isolated in the additional experiments we performed.

What about multiple phages? On first consideration, it may seem that with multiple phages each with different receptors and thereby requiring independent resistance mutations, it would be possible for phages to continue to limit the densities of the bacteria. That is certainly the strategy employed to prevent multiple resistance to chemotherapeutic agents in long-term infections, like that of HIV (Hammer *et al.* 1997) and tuberculosis (Onyebujoh *et al.* 2005). We are aware of only one formal experimental test of this hypothesis and the results were negative (Korona and Levin 1993). In that investigation, three phages with different receptors (independent resistance) were introduced into cultures of sensitive bacteria bearing a restriction-modification system to which all three phages were sensitive. Within 48 hours, bacteria mutants with

envelope resistance to all three phage ascended to dominance and bacterial population went from a phage- to a resource- limited state.

In this report we present the results of an experimental study of the population and evolutionary dynamics of *V. cholerae* N16961 and two of its phages and computer simulations of these processes. We interpret the results of these experiments and associated modeling as support for the hypothesis that when two or more phages are present these viruses can limit densities of bacterial populations for extended periods of time. We discuss the implication of these experimental and theoretical results for natural populations of phage and how the predictions made from this study can be tested in natural populations of bacteria and phage.

3.2 MATERIAL AND METHODS

Bacteria & Phage

The *V. cholerae* used in this study were derived from El Tor O1 N16961, a spontaneous streptomycin-resistant (Sm^r) mutant of a clinical isolate from Bangladesh. John J. Mekalanos and Shah Faruque generously provided these bacteria. John Mekalanos also provided us with lysates of naturally associated bacteriophage designated Peru-2.

Isolation and Analysis of Phage DNA

High titer phage lysates were treated with RQ1 DNase (Promega Corp.) for two hours at 37°C to destroy contaminating bacterial DNA. Phage were pelleted by ultracentrifugation in SW50.1 rotor (Beckman) at 50,000 rpm and 4°C for 45 min. Phage pellets were resuspended in 0.2M NaCl and the DNA was released by four consecutive extractions with an equal volume of pH-equilibrated phenol (Sigma). The DNA was precipitated with isopropanol, resuspended in sterile distilled water, and stored at 4°C.

Two micrograms of phage DNA was digested with each of the following enzymes in the manufacturer's (New England Biolabs) suggested buffers: *Bam*HI, *Sac*I, *Kpn*I, and *Hin*DIII. Digested DNA was separated by electrophoresis in a 0.8% TAE agarose gel, stained with ethidium bromide and imaged using a GelDoc 2000 Imaging System(Biorad).

Media, Culturing and sampling procedures

Liquid cultures of *V. cholerae* were maintained in either Luria- Bertani Broth (LB) or a medium composed of autoclaved 5% LB and 95% tap water at 30 °C. Long-term population dynamics of N16961 and phage were carried in continuous cultures, "home-made" chemostat of a design first presented in the Appendix to (Chao, Levin and Stewart 1977), for a modern version see www.eclf.net. The density of *V. cholerae* N16961 was estimated from colony count (CFU) data on LB agar. The density of phage was estimated

from plaque counts on LB agar overlaid with ~ 3ml 55^{o}C soft agar "lawns" of *V. cholerae* N16961. Unless otherwise noted, estimates of phage titers from these chemostats were made from samples taken directly from the chemostat vessel. In a few cases we added a drop of chloroform to samples to distinguish free-phage from infected cells. We found no difference in the estimated phage titers with the same sample with and without chloroform (data not shown). We interpret this to suggest that the majority of phage present in the culture were free-phage.

Assay for Phage resistance

Resistance of *V. cholerae* N16961 to phage was determined in two ways. First by spotting 10 µl to 20 ul 1e9 PFU /ml of phage lysate on 500ul soft agar lawns on 100 mm diameter Petri dishes containing LB agar (four lawns per plate). For bacterial samples taken from the chemostats, colonies were passage (streaked) on LB agar to minimize the likelihood of contamination with coexisting phage. In some cases, when these plaque assays were negative, there was no evidence for phage lysis, and the cells were tested to determine if they could support phage growth in liquid. For this, approximately ~1e5 PFU phage particles were added to exponentially-growing 5% LB cultures of the bacteria. After 8 to 10 hours, the densities of phage were estimated.

Assay for lysogeny

To test for lysogeny, samples were taken from the middle of the plaques with high densities of phage and streaked for single colonies on LB agar. After three colony to

colony passages, single colonies were taken and grown in 5% LB at 30 ^{o}C. When the OD was ~1/2 maximum the cultures in 12-well plates were exposed to UV (Long wave UV-366NM) at a distance of 5-10 cm for 45 seconds then allowed to grow overnight. A drop of chloroform was added to these cultures of potential lysogens, and spotted onto lawns of sensitive cells after centrifugation. In this assay, we assume that if the cells from the center of the plaques were lysogens for the phage, there would be free phage in the UV induced culture (Lwoff 1966).

Relative Malthusian fitness of phage-resistant *V. cholerae* N16961

Pair-wise competition experiments were performed to estimate the Malthusian fitness of phage-resistant bacteria relative to sensitive. For the sensitive cell line in these experiments we used a spontaneous, rifampin resistant mutant of the wild-type N16961. Approximately equal frequencies of the resistant strain and this Rif-r common competitor from overnight cultures were mixed 1:100 in 5% LB, cultured at 30^{0}C, and their densities estimated from CFU data at two time points. The Malthusian selection coefficient s was calculated using the formula,

$$s = \frac{\left(\ln\left(W_t\right) - \ln\left(W_0\right)\right)}{\ln\left(\frac{N_t}{N_0}\right)},$$

where Wt and W_0 are respectively the ratio of the densities of phage-resistant and sensitive N16961 at time t and the initial time 0, and N_t and N_0 the total densities of bacteria at those two times (Travisano and Lenski 1996). The relative fitness of each

individual phage-resistant mutant to Rif.R was calculated as 1 / (1-s). As a control for the fitness cost of the rifampin resistance marker, pair-wise competition experiments were performed with wild-type N16961 and Rif.R. To control for the adaptation to the chemostat growth, the relative fitness of bacteria which were isolated at the time as those phage-resistant mutants, but from a chemostat where no phage was present, was also estimated by competing with Rif.R.

Fitness of the phage

To estimate the growth of specific phage on N16961, we used a method similar to that employed by Wichman *et al* (Wichman, Wichman and Bull 2005). The bacteria were grown in 12-well Macrotiter plates in 2 ml 5%LB at 30 ^{o}C to a density ~10^{7} CFU/ml. Between 10^{3} -10^{4} PFU/ml of phage were added to these agitated, exponentially growing bacterial cultures. The phage densities were estimated immediately (P0) and after an hour of incubation (P1). As our estimate of the fitness of the phage, we use the doubling time during that period, $T2=Log_2 (P1)-Log_2 (P0)$.

Mathematical Model – See the Appendix 2

3.3 RESULTS

In Figure 3.1A we present the results of the first of our experiments where we introduced our original lysates of Peru-2 into an equilibrium chemostat of Peru-2 sensitive *V.*

cholerae N16961. As can be seen by comparing the densities of bacteria with these phage and parallel phage-free control, these bacterial viruses are able to limit the densities of bacteria for an extensive period and the phage continue to be maintained.

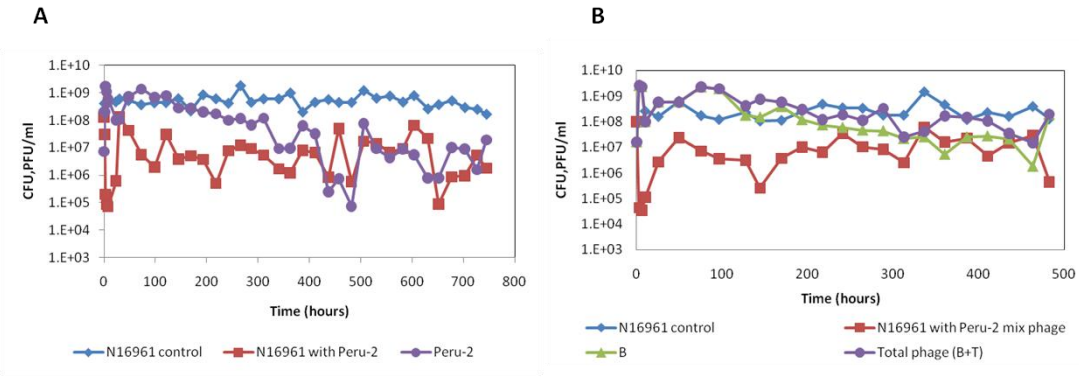

Figure 3.1 The population dynamics of *V. cholerae* N16961 and the phage(s) Peru-2 in chemostat culture. Changes in densities of bacteria and phage in bacteria only (control) cultures (blue) and cultures containing bacteria and phage (red total bacteria) and purple (total phage) and A. Original experiment dilution rate w=0.33 per hour. B. Replica experiment, dilution rate w=0.40 per hour. In this experiment, the densities of big, B , (green) and turbid, T, plaque phage were monitored,purple plot is the total phage(B+T).

In Figure 3.1B, we present a replicate of the Figure 3.1A experiment where we kept track of the densities of two plaque morphology types of phage, big and turbid, B and T, respectively. The T plaque phage were noted several hours after the introduction of phage , following which phage with both plaque morphologies were maintained, with T increasing in frequency but not monotonically so.

Hypotheses, tests and explanations:

The simplest hypothesis for why the culture in Figure 3.1 remained phage-limited and did not become dominated by resistant bacteria is that bacterial mutants resistant to Peru-2 could not be generated or were generated at too low a rate to have emerged during the course of the experiment. Another hypothesis is that seen in a study by (Chao, Levin and Stewart 1977); the original resistant mutant was countered by a host-range phage that could grow on both the sensitive and resistant bacteria and during the course of the experiment no bacteria resistant to this host range phage emerged. A third hypothesis is that bacterial mutants resistant to the wild-type and host-range phage were generated but grew at too low a rate to be maintained in the face of flow.

Multiple Phages: The results of the additional experiments we performed support the second hypothesis, but with a twist. While we initiated the Figure 3.1 experiment with a phage lysate derived from a single plaque, what we thought were mutants of the same phage with different plaque morphologies, B and T in Figure 3.1B, turned out to be multiple distinct phage "species". Upon further examination, we found the original Peru-2 lysate to include phages with three distinct plaque morphologies: small (S), big (B) and turbid (T) (Figure 3.2 A). As can be seen from the restriction digests in Figure 3.2, B, S, T and B are genetically three distinct phages: S is the only one cut with BamH1, T is the only one cut with KpnI and B was not cut by any of these enzymes. While B and T were observed in the samples from the chemostat depicted in Figure 3.1B, S plaque phage were not observed in any of the chemostat experiments and was only picked up in batch

cultures of N16961 in full concentration of LB maintained at 37 ^{o}C. When we attempted to grow the S phage with sensitive N16961 at 30 ^{o}C in the 5% LB used in our chemostat experiments, it failed to replicate. Moreover, in an experiment initiated with a lysate containing B and S, the dynamics observed were effectively the same as with B alone (see the following section).

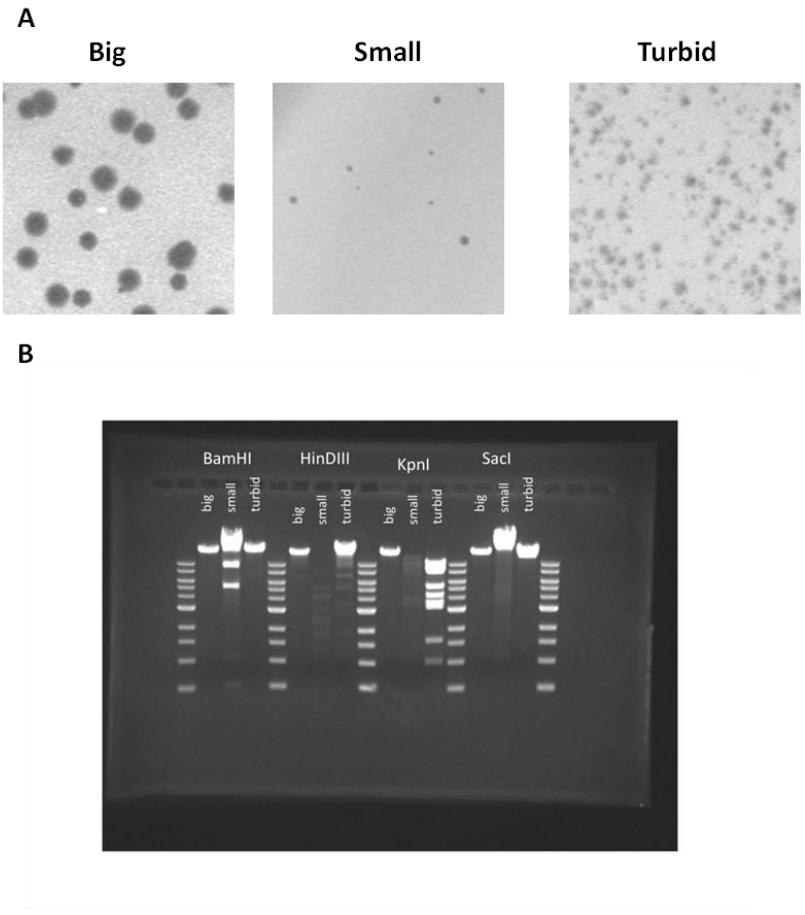

Figure 3.2 A. Plaque morphology of the Big, B-, Small, S- and Turbid, T- plaque phages on soft agar lawns of sensitive *V. cholerae* N16961. B. Restriction digest of the DNA of the B-, S and T phages with four restrictive enzyme (BamHI, HinDlll, Kpnl and Sacl). Lane 1, 4, 8, 12 and 16 are the 1 kb ladder.

Single Phage Dynamics: To better understand the processes responsible for the dynamics observed in Figure 3.1, we introduced pure, single plaque lysates of big plaque B and turbid plaque T phage into chemostats with established populations of *V. cholerae* N16961.

The results of our experiments with the big plaque, B phage are straightforward. Within the first 48 hours, mutants resistant to B emerged, ascended to dominance and the phage continued to persist (Figure 3.3). While we can't exclude the existence of minority populations of host-range phage mutants capable of growing on the resistant bacteria, we have not been able to isolate host-range phage mutants from plaques on lawns of B-resistant bacteria. We have also not been able to get a net increase in the density of B plaque phage in liquid cultures of the B-resistant mutant isolated from these chemostats. We interpret this to indicate that the bacteria designated to be "resistant" in our plaque assay are unable to support the growth of the phage from these chemostats in liquid culture. Although we have no evidence for host range B phage capable of growing on the resistant N16961, the B-plaque phage clearly evolved during the course of these experiments. Its rate of replication on the ancestral wild-type sensitive N16961 exceeds that of the original B lysates (Figure 3.4). No turbid or small, T or S plaques were observed in these cultures with B phage alone nor did we see any other obvious variation in plaque morphology of the phage recovered.

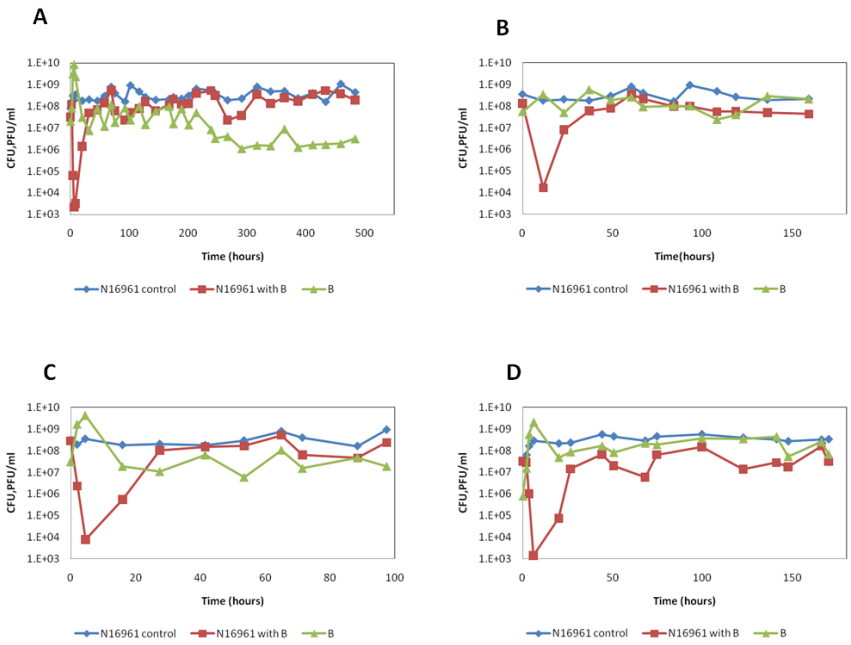

Figure 3.3 Population dynamics of big plaque phage in chemostats with N16961: Changes in the densities of bacteria phage-free controls (blue) and in mixed cultures with bacteria (red) and and B-plaque phage (green). Dilution rates (w) in A, B, C and D are 0.3-0.4/hr , 0.5-0.6/hr, 0.3-0.4/hr, 0.5-0.6/hr respecitvely.

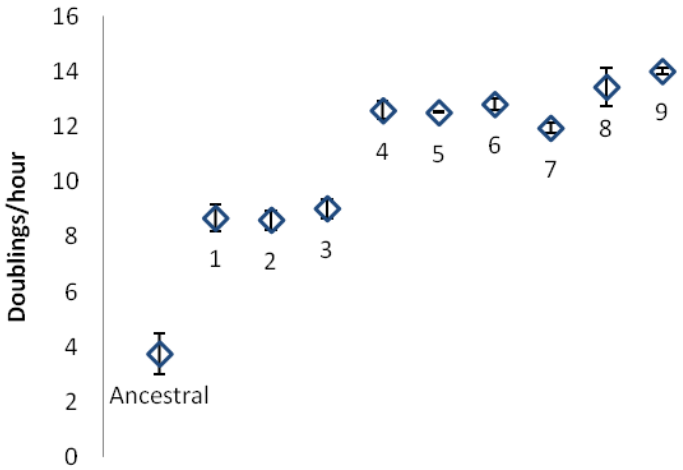

Figure 3.4 Evolution of higher fitness in B plaque phage on the ancestral, wild-type N16961. Mean and standard error of the of doubling per hour: 1, 2, 3 : phage lysates collected from 3 different chemostats around 30 hours after the introduction of big plaque

phage. 4, 5, 6, 7: phage lysates from growing single plaques isolated from 2 different batch cultures ~50 hours after the introduction of big plaque phage. 8, 9 : phage lysates were used from growing single plaques isolated from 2 different chemostats ~24 hours after the introduction of big plaque phage.

This evolution of higher fitness for the B phage was not restricted to the chemostats and was observed within 30 hours in batch culture (data not shown). At this time, we have not determined which of the the phage infection parameters – latent period, adsorption rate or burst size have changed in this evolution.

The results of our analogous experiments with turbid plaque, T phage were more variable than those with B. In three of the five chemostats we initiated with sensitive N16961 and the same turbid plaque phage lysate, the cultures were clearly limited by resources (Figures 3.5A, B and D). This may also be the case for the bacterial population in Figure 3.5E, albeit less compellingly so. In the experiment presented in Figure 3.5C, however, the density of bacteria in the culture with phage was substantially less than that of the phage-free control. This can be seen from the harmonic means in the densities of bacteria calculated from ~200- 400 hours.

Resistant bacteria replacing the sensitive as the dominant population of bacteria cannot readily account for the difference between these chemostats. In the case of the clearly resource-limited cultures depicted in Figures 3.5A and 3.5D, as measured by spot tests, a substantial fraction of colonies tested at the end of the experiment and some time before

were resistant to the co-existing turbid plaque and ancestral phages, but not all. For example, at 525 hours in Figure 3.5A 5/10 colonies were resistant to the ancestral and co-existing phage, at 417 hours the corresponding fractions in Figure 3.5D were 5/10 and 4/10. High frequencies of resistance were also noted in the samples taken from the culture presented in Figure 3.5C, respective 9/10 and 8/10 for the ancestral and coexisting phages at 417 hours. We failed to detect cells resistant to the ancestral (or coexisting) T-plaque phage in the samples taken from the culture in Figure 3.4B. In all the experiments, however, when first confronted with phage the density of bacteria dropped substantially. If not because of the ascent of resistant mutants, how did these bacteria populations recover? Why was the frequency of sensitive bacteria so high?

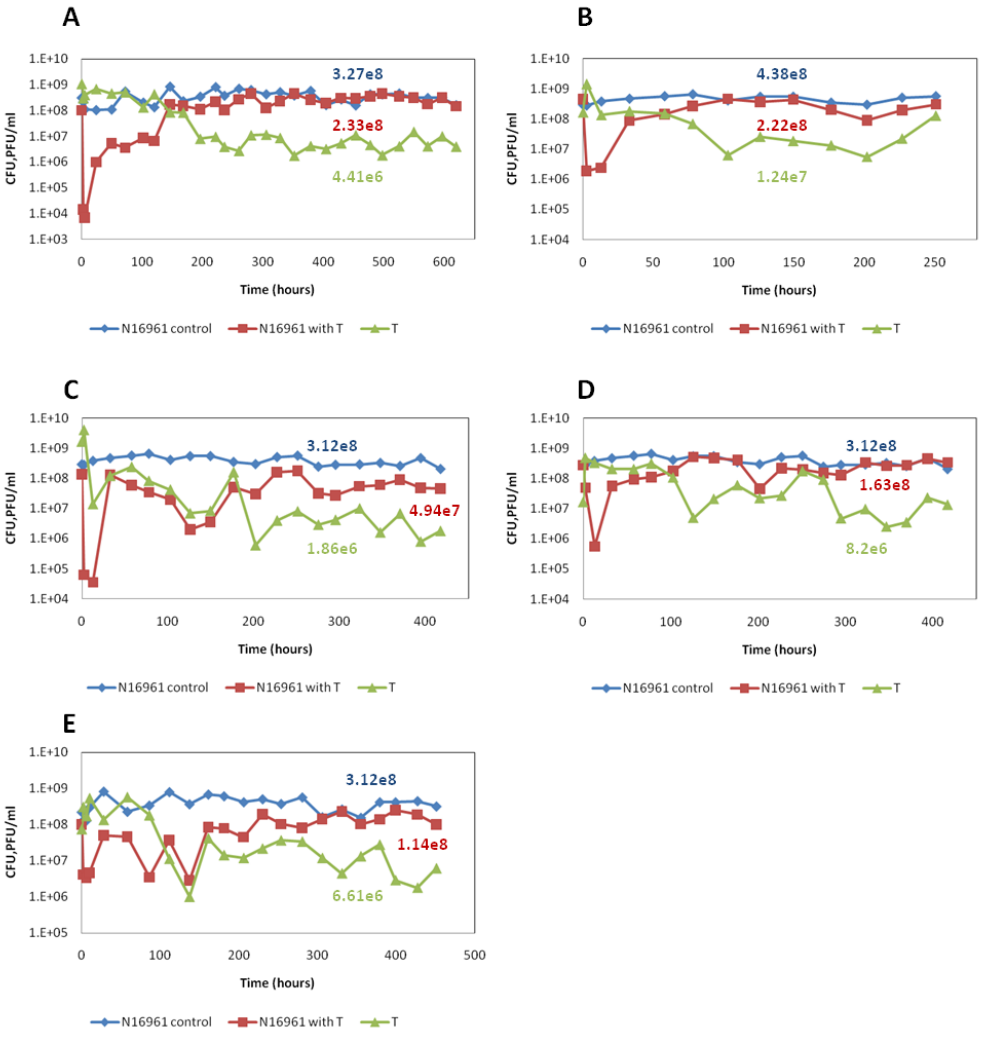

Figure 3.5 *V. cholerae* in chemostat culture with turbid plaque phage. Changes in the densities of bacteria in phage-free controls, (blue), and bacteria (red) and phage (green) in mixed culture. Five independent chemostats, dilution rates (w) in A, B, C, D and E are 0.36-0.44 /hr , 0.2-0.3/hr, 0.2-0.3/hr, 0.2-0.3/hr, 0.5-0.6/hr respecitvely. The numbers on these figures are the harmonic mean densities of the bacteria and phage estimated from ~ 200 hours to the end of the experiments (B:~100 hours to the end).

One possibility is that the bacteria exposed to the phage had been modified in some phenotypic way to change their susceptibility to these viruses and at the same time support their replication. This is what would be anticipated for lysogeny, where the

surviving bacteria carry the genomes or part of the phage genomes and the expression of those genes provide some immunity to super-infection with that phage (Stewart and Levin 1984). In the case of classical lysogens like those of the phage Lambda, the density of the lysogenic population would be similar to that of a phage-free, resource-limited, culture (Stewart and Levin 1984). However, when the lysogenic population was plated onto fresh medium, the density would be much lower than the phage-free culture, because there would be a high rate of induction and the majority of the lysogens would not form colonies.

Lysogeny would also account for the turbid nature and the high density of viable bacteria within these plaques. As estimated with the plug- suspension assay described in the Materials and Methods, the estimated densities of viable bacteria within these plaques and from the phage-free part of the lawn, were respectively, $4.02 \pm 0.15 \times 10^7$ and $7.47 \pm 0.34 \times 10^8$ CFU per ml (mean ± standard error of 6 samples). The density of the phage in the plaques sampled was $2.35 \pm 0.17 \times 10^9$ particles per ml. To determine whether the bacteria in these plaques were classical lysogens in the sense of carrying an inducible prophage, we used the protocol described in the Methods section for 5 colonies from a turbid plaque. The results were inconsistent with the hypothesis of classical lysogeny. There was no evidence for free phage in the UV-treated cultures.

If not lysogeny, is there another way sensitive bacteria exposed to the T-plaque phage could survive? Although current evidence suggests that CRISPR does not exist in *V. cholerae* N16961 (Grissa, Vergnaud and Pourcel 2007) it is conceivable that *V. cholerae* N16961 has an analogous mechanism where a fraction of bacteria exposed to phage are not killed and become physiologically refractory to those phage. To determine if this is the case, we performed phage fitness assays with the T plaque phage in culture with naïve bacteria and bacteria isolated from the plaques and purified by streaking. We failed to detect differences in the capacity of the T-plaque phage to replicate on Naïve N16961 and those derived from turbid plaques (data not shown). We interpret this to suggest that the exposure to T-plaque phage did not result in an epigenetic (CRISPR-like) or genetic resistance in *V. cholerae* N16961.

Another possibility is a phenomenon analogous to persistence observed for antibiotic treated bacteria (Balaban *et al.* 2004; Bigger 1944), a subpopulation of non-growing cells that are refractory to T-plaque phage. Were this the case, when an exponentially growing population of N16961 are exposed to T-plaque phage, killing would not be complete, but level off and a substantial fraction of the bacteria would survive (Wiuff *et al.* 2005). To test this hypothesis, we followed the changes in phage and bacterial density in batch culture with different initial multiplicities of infection (MOI's). The results of this experiment are consistent with the persister hypothesis. Even when there were more than 100 phage particles to each cell, at least 10^4 bacteria survived. As seen in the Appendix 2, the dynamics observed in these experiments are consistent with that anticipated if a

subpopulation of non-growing and thereby phage refractory cells were produced from the sensitive population. As we also demonstrate in the same Appendix, a persister population would also contribute to stabilizing the bacteria and phage communities.

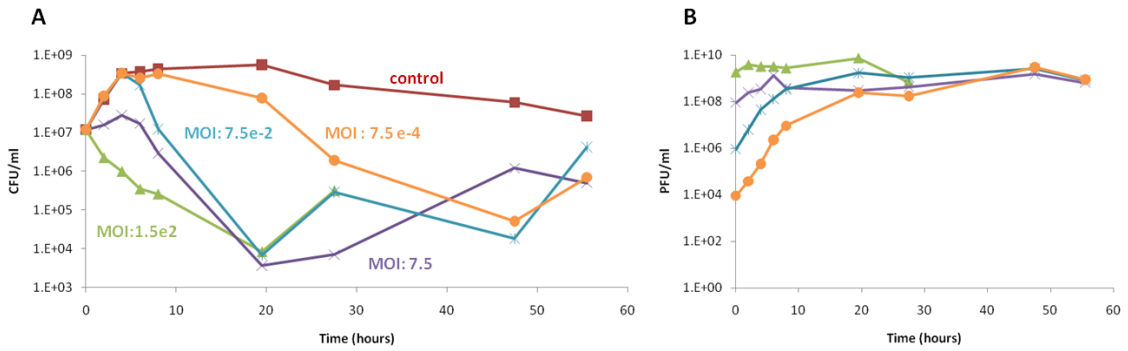

Figure 3.6 Changes in the density of turbid-plaque, T-, phage with naïve *V. cholerae* N16961 in batch culture with different multiplicities of infection (MOI) A. Change in the density of bacteria B. Corresponding change in the density of phage

Two phages: It is clear from the preceding consideration that the big and turbid plaque phages, B and T, by themselves cannot account for the dynamics presented in Figure 3.1, where the density of bacteria is limited by the phage, rather than resources. When we performed the Figure 3.1 experiments we had not yet known that multiple phages were present in the culture. We can demonstrate with reconstruction experiments that when we introduce pure lysates of B and T into equilibrium chemostats with N16961 susceptible to both, we get phage-limited cultures very much like that depicted in Figure 3.1. The results of three independent chemostat experiments of this type are presented in Figure 3.7.

The densities of the N16961 in all three cultures remained limited by the phage. And, in all three, for most of the experiment both the B and T phages were present at substantial frequencies, although as time proceeded, the frequency of T increased whilst that of B declined. This increase in the frequency of T is correlated with the recovery of bacteria resistant to B. In two of these chemostats we were unable to isolate bacteria resistant to T, much less simultaneously resistant to B and T. In one chemostat (that depicted in Figure 3.7c), however, starting at around 500 hours, strains resistant to B and T were obtained in the dominant population of bacteria. Despite this high frequency of strains resistant to both phages in this one chemostat, the population remained limited by the virus rather than nutrients. Why?

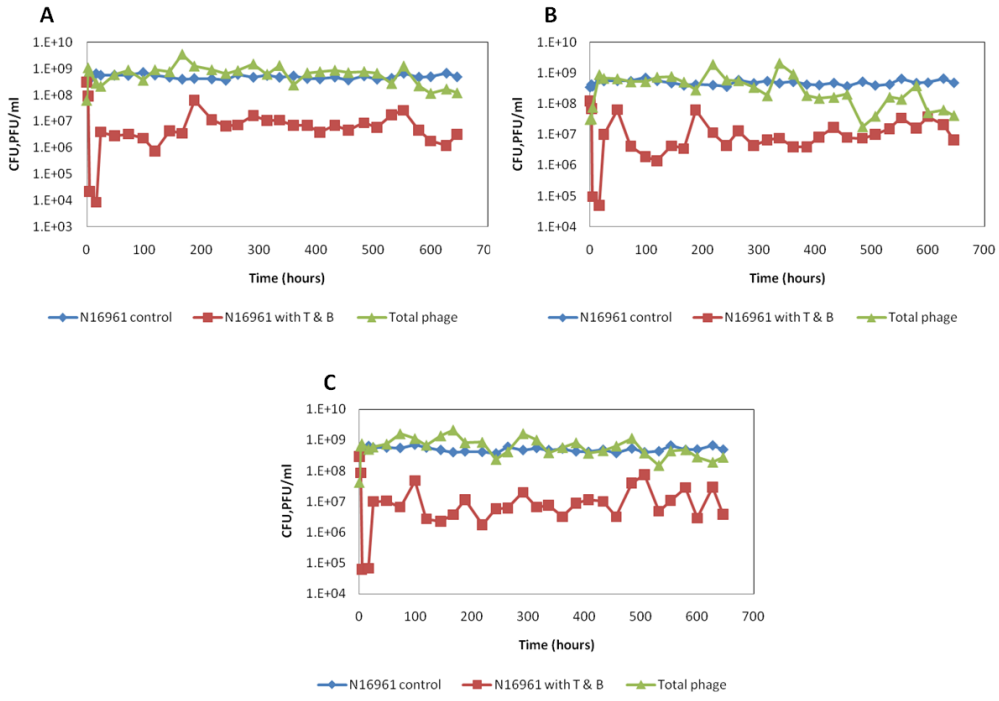

Figure 3.7 Dynamics of N16961 with a mixture of T and B phage in the chemostat cultures. Changes in the densities of bacteria in phage-free culture (blue) and total bacteria and phage in mixed culture, the red and green lines, respectively. Dilution rates in A, B and C are 0.29-0.35/hr, 0.44-0.52/hr, 0.36-0.44/hr respectively.

One possible reason for the failure of the doubly resistant strain to ascend to a resource-limited level is that the doubly resistant mutant did not arise until later in the experiment and these cells were impaired in such a way that their rate of growth was not much greater than the rate at which they were washed out. To test this low growth rate hypothesis, we first undertook a competition assay with 10 resistant mutants randomly chosen from the third chemostat (Fig.3.7c) at ~500 hours and 10 at the end of the experiment (20 isolates in total). As can be seen from Figure 3.8, all of the doubly resistant isolates were less fit than the reference strain Rif.R which is about 20% less fit than the wild-type. The average relative fitness of these isolates is approximately ~55% of the wild-type N16961. The wild-type N16961 grows at a rate of about 0.8 per hour. Calculated from these competition results, the growth rates of these mutants are quite close to the dilution rates of the chemostats, 0.36 to 0.44 per hour. These competition assays measure the overall competitive ability (lag phase, maximum growth rate and stationary phase) of two strains. To ensure that what we observed from competition assay truly represented the differences in maximum growth rate of the competing strains, we also examined the maximum growth rate of several mutants (data not shown). Although they grew at lower rates as the competition results suggested, approximately 0.6 per hour, this rate is not low enough for them to be washed out from chemostat. Thus, the results so far are inconsistent with low growth rate hypothesis. At this juncture, we do not know why these doubly resistant mutants did not ascend to dominance. That is certainly what we would anticipate soon after they emerged, see Figure A3 (B) in the Appendix 2.

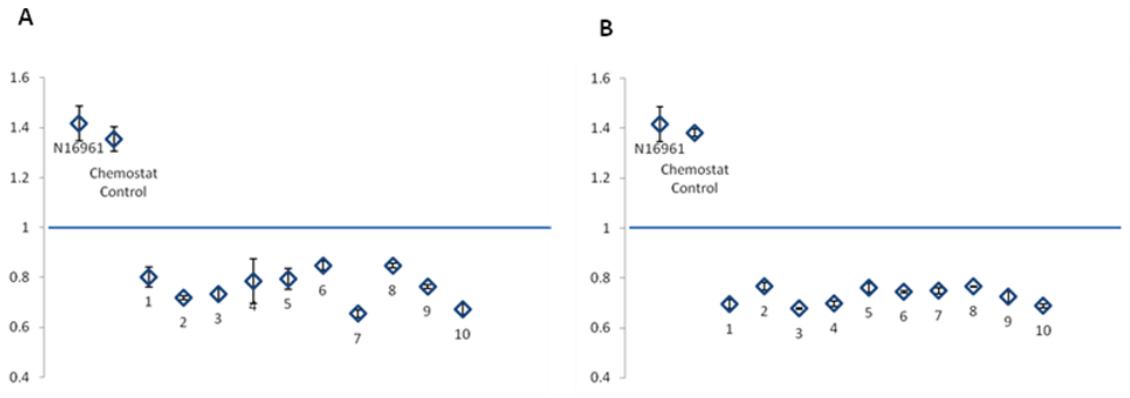

Figure 3.8 Means and standard errors of the relative fitness 1/(1-s) of bacteria relative to a Rif-resistant, phage-sensitive mutant of N16961 (Rif.R). The control population was isolated a phage-free chemostats. A. Double phage resistant and control bacteria isolated from the chemostat depicted in Figure 6C at ~500 hours. B. Double phage resistant and control bacteria isolated from the chemostats depicted in Figure 6C at ~650 hours.

3.4 Discussion

To paraphrase the famous George Box quote, 'all models and model systems are wrong, some are useful'. We, of course, believe the *V. cholerae* – Peru-2 experimental model described above and the mathematical models presented in the appendix are useful to make inferences about the role of phage in limiting the densities of bacteria in natural communities. We interpret the results of this study as support for the hypothesis that in their natural habitat, bacterial populations can be limited by predation by multiple, if not single phage species.

Of the two phage species in our chemostat experiments, the clear plaque phage, B was more typical of that observed in similar experimental studies of the population dynamics

of bacteria and single species of phage (Chao, Levin and Stewart 1977; Lenski and Levin 1985; Levin, Stewart and Chao 1977; Middelboe 2000; Wei, Ocampo and Levin 2010) Mutants resistant to B phage evolved and within short order the bacterial community becomes phage limited. We have not observed or been able to isolate host range B phage capable of growing on these resistant bacteria. On the other hand, despite the failure to generate host-range phage mutants, the B-plaque phage confronting *V. cholerae* evolved to become better able to replicate on N16961. At this juncture we do not know whether the B-plaque phage-susceptible N16961 are co-evolving with respect to this phage.

The turbid, T-plaque phage is more interesting (read new-to-us) than the B-plaque phage or any other phage we have studied recently. The results of our experiments with this phage suggest that either, (i) the interactions between *V. cholerae* and this phage generate a subpopulation of cells that are phenotypically refractory to this virus, or (ii) predation by the T-plaque phage selects for and thereby reveals a subpopulation of phenotypically immune cells that was already present. The latter would be anticipated from what is known about non-replicating persisters from antibiotic treatment studies (Balaban 2004; Bigger 1944; Wiuff *et al.* 2005) and could account for these results if the T-plaque phage do not kill or replicate on this kind of non-growing bacteria (see the Appendix 2, A1).

Our observation that mixtures of B and T can maintain a phage limited population of *V. cholerae*, despite neither phage being able to do so alone is consistent with what we

would anticipate from our models, as long as mutants resistant to both phage are not produced or grow too slowly to maintain their population (see the Appendix 2, A3). These models also account for the continued persistence of the two populations of phage. And, if we allow a phage-refractory wall population, (Schrag and Mittler 1996), as noted in Appendix 2 (A2), a persistent subpopulation would also contribute to the stability of these phage and bacterial communities.

An important result we cannot explain is why *V. cholerae* mutants simultaneously resistant to both the B and T-plaque phage that were found in one of the three phage chemostats did not ascend to a level expected for a resource-limited community. A simple explanation would be these mutants grow at a rate less that that at which they were washed out. The results of our pairwise competition (fitness) assay were roughly consistent with this hypothesis; their fitness was almost half that of their sensitive ancestor which grew at a rate of 0.9 per hour. On the other hand, the exponential rates of growth of the bacteria resistant to B and T exceeded that required for them to be maintained in the chemostat.

Why in this study with two phages were we able to maintain a phage-limited population of bacteria, whilst in the (Korona and Levin 1993) study with *E. coli* and three phages with different receptors, triple resistance evolved within short order? We postulate that the reason for this is that in these *V. cholerae* phage, there exists the hierarchy of phage

replication noted in the (Korona and Levin 1993) and demonstrated earlier for *E. coli* and it phage (Weigle and Delbruck 1952). As a consequence of this hierarchy, selection favored resistance to single phages, which would not be the case here if, as we postulate, co-infection occurs.

In opening this report we suggest that the information gleaned from this study with experimental populations will increase our understanding of the role of phage in limiting the densities of bacteria in natural communities. We stand by that assertion. These experimental results and theoretical analysis support the hypothesis that phage may indeed play a substantial role in determining the structure of natural communities of bacteria by regulating the densities of different populations of bacteria. We doubt, however, that these viruses regulate the densities of bacterial communities at large. Resistant mutants of the sorts considered here are not the only source of competitors for the different members of the bacterial community. Resources made available by phage limiting the densities of one species of strain of bacteria could be consumed by other bacteria not susceptible to those phage. In addition, bacteriophage are not only species-specific, but are also very strain specific (Skurnik and Strauch 2006). Other predators like grazing protozoa, nematodes, insect larvae, and various kinds of zooplankton are less discriminatory than bacteriophage. The life of a bacterium is hard and almost certainly short and no matter how important phage seem to those of us who work on them.

Chapter 4

The Population and Evolutionary Dynamics of Bacteria in Physically Structured Habitats: The Adaptive Virtues of Motility

Yan Wei, Xiaolin Wang, Jingfang Liu, Ilya Nememan, Amoolya H. Singh, Howie Weiss, and Bruce R. Levin

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4.1 Introduction

If we accept that the presence of genes coding for flagella indicates the phenotype of self-propulsion, motility is an ancient and almost ubiquitous character in the eubacteria. Flagellar genes are significantly overrepresented in environmental samples of DNA (Dinsdale *et al*. 2008; Harrington *et al.* 2007), and almost two-thirds of sequenced bacteria with phenotypic annotations are motile (NCBI Prokaryotic Genomes) . Why is motility so widespread? At a qualitative level the obvious answer is almost certainly true: in most of the habitats in which bacteria reside, nutrients are not evenly dispersed. Cells with the facility to propel themselves have more access to resources than those that move solely at the whim of external forces. This qualitative answer, however, raises a number of quantitative questions that have to be addressed for a comprehensive understanding of the ecological conditions under which natural selection will favor the evolution and maintenance of motility. Under what conditions will the relatively weak propulsion ability of a bacterium enable it to overcome the turbulence or the viscosity of its environment for its movement to be effective? Under what conditions will movement in random directions provide a bacterium with an edge in the acquisition of resources when competing with bacteria that are non- or less- motile? In contemporary bacteria, motility

is commonly coupled with chemotaxis, i.e. the sensory and signaling machinery needed to direct their motion towards nutrients (Falke *et al*. 1997; Wadhams and Armitage 2004). Logic, retrospective evidence and, as we show here, mathematical models and experiments, all suggest that chemotaxis evolved in bacteria that were already capable of self-propulsion in random directions.

For natural selection to favor motility for the acquisition of nutrients by bacteria, their environment must be physically structured in such a way that nutrients are not equally available to all cells. Thus, to address questions about the conditions for the evolution and maintenance of motility theoretically (with mathematical models) and experimentally we cannot use the traditional modeling and experimental tools of bacterial population biology. These tools consist of ordinary differential equations (ODEs) (Stewart and Levin 1973), and experiments performed with bacteria in liquid medium in the well-agitated flasks and chemostats of laboratory culture (Lenski 2003; Chao, Levin and Stewart 1977), both of which consider bacterial populations as arrays of planktonic cells cavorting about in an environment that, from the perspective of an individual bacterium, is dimensionless.

Experimentally addressing questions about the role of the physical structure of the environment in the ecology and evolution of bacteria is relatively straightforward. At least two methods have been successfully used: one is with the bacteria embedded in a semisolid, like soft agar (Chao and Levin 1981), the other is with the bacteria spread on the surface of harder agar media (Kerr *et al*. 2002; Simonsen 1990). More complex and

realistic experimental systems to address these questions can also be employed to address these questions. For example, in a recent study the relative ability of strains of *V. cholerae* to colonize zooplankton was examined in laboratory culture with Daphnia (Wei, Ocampo and Levin 2010). However, there are limits to the inferences one can draw about natural populations from purely experimental studies. It has been proposed that "studying population biology without mathematical or computer simulations is like playing tennis without a net or boundary lines" (www.eclf.net). Although some readers may not agree with this perspective, most will appreciate the utility of mathematical models for the design and interpretation of empirical studies and generalizing on their results.

Analyzing models of bacteria in physically and spatially structured habitats is more challenging than the same for planktonic bacteria. The most widely used spatial population models are partial differential equations (PDEs) (Dinsdale *et al.* 2008) and agent-based or cellular automata simulations (Harrington *et al*. 2007). PDEs allow modeling at the level of populations, whereas agent-based and cellular automata simulations model individuals. PDEs with explicit diffusion terms have been used to explore the factors contributing to the shape of bacterial colonies (Adler and Dahl 1967; Mimura, Sakaguchi and Matsushita 2000; Matsushita *et al*. 1999), the diffusion and uptake of resources in two-dimensional habitats by growing populations of bacteria (Lauffenburger, Aris, and Keller 1981;Berg 1998; Golding *et al.* 1998; Berg 2003), and the population dynamics of allelopathy (Frank 1994). Agent-based and cellular automata models have also been used to study the growth and population dynamics of bacteria in

physically structured habitats (Kerr *et al.* 2002; Wimpenny and Colasan 1997; Durrett and Levin 1997; Prado and Kerr 2008; Majeed *et al.* 2010). In general, the advantages of mathematical formalism and mathematical analysis evaporate with agent-based and cellular automata models.

Here we consider the conditions for the evolution and maintenance of random motility in bacteria using theory and experiments. We use a system of reaction-diffusion PDEs to model the resource-limited growth and competition between bacteria with different motilities in a physically structured habitat. Our model predicts that the extent to which purely random motility will be favored is proportional to the viscosity of the environment and the rate at which the bacteria propel themselves through that environment. To test the predictions of our model, we use experimental populations of motile and non-motile strains of *E. coli* in liquid and soft agar culture. The results of our experiments are quantitatively as well as qualitatively consistent with the predictions of our model; knowing where they are going is not a necessary condition for motile bacteria to be favored in physically structured habitats. Our results indicate that the capacity for self-propulsion in a random direction is favored because it enables bacteria to move away from each other and thereby increase their individual access to limiting resources. The results of our theoretical and experimental analyses also support the hypothesis that the sensory and signaling apparatus associated with chemotaxis further augments the fitness of bacteria in physically structured, resource limited communities.

4.2 Results

In Figure 4.1(A), we plot the ratio of total number of motile and non-motile bacteria, M/N, predicted by our model after four days of competition with two initial concentrations of resource and different rates of diffusion. The prediction is clear; the advantage of motility increases with the amount of resource, R_{MAX}, and the viscosity of the environment, as measured by the decline in the diffusion coefficient D_R. In Figure 4.1(B), we consider the effect of the rate of diffusion of the motile strain, d_M on the M/N ratio with constant rates of diffusion of the resource and the non-motile strain, d_r and d_N. Over a broad range of values of the diffusion coefficient of the motile strain, d_M, the M/N ratio increases. This increase is not monotonic, however, and declines as d_M gets larger. The reason for this decline is that the rate at which the environment is saturated by the motile strain increases with its diffusion coefficient. Once the environment is saturated, the M/N ratio no longer changes. This saturation effect can be seen by following the M/N ratio over the first 2 days (data not shown).

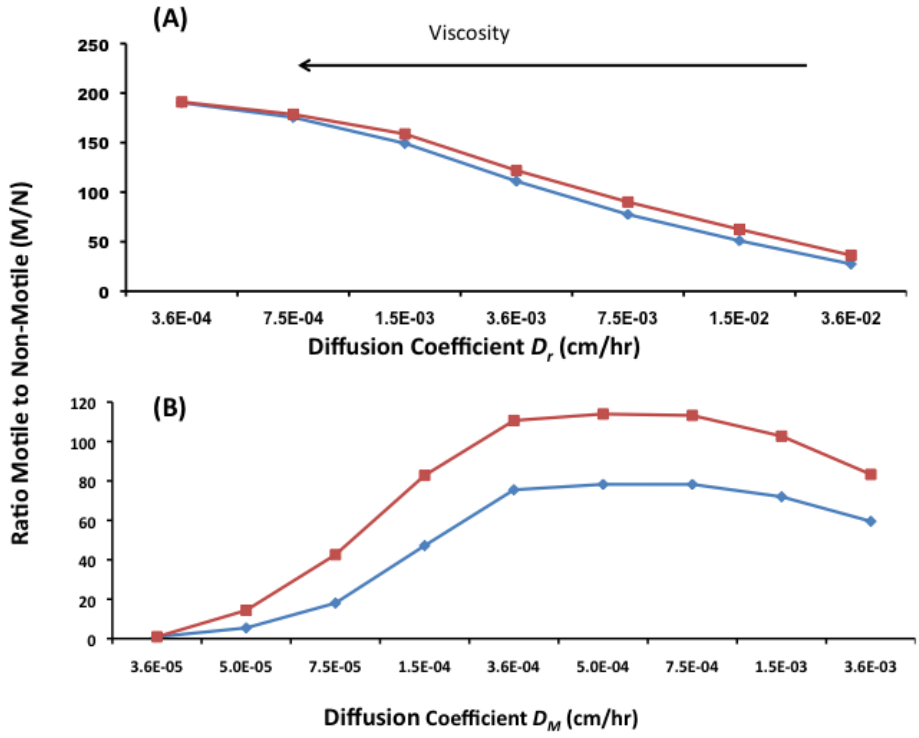

Figure 4.1 Simulation results: ratio of motile to non-motile cells after four days of competition in homogenous habitats with two different levels of resource 5µg/ml (lower lines with diamond ticks) and 50µg/ml (upper lines with square ticks). Common parameters, α=0.90 hr^{-1}, k=1.0 µg/ml, ν=4.75X10^{-7}µg, dt=0.1hr^{-1} and the matrix assumed 0.1 cm squares. (A) Ratio of motile to non-motile cells for different diffusion coefficients for the resource (the higher the rate of diffusion the greater the motility. D_M =3.19x10^{-4} and D_N =3.6x10^{-3} cm/hr. (B) Ratio of motile to non-motile bacteria with a constant rate of diffusion for the resource and non-motile cells, D_r = 3.6x10^{-3} and D_N =3.6x10^{-5} cm/hr, and different rates of diffusion for the motile strain, D_M.

To empirically test the validity of the conditions predicted by the model, we used two strains of *E. coli* K-12 from the KEIO collection, a Nal^r non-motile strain (JW1059) and a wild type motile strain, (JW5072), N, and M, respectively. All experiments were performed in medium that contained distilled water with 1% or 0.1% tryptone without agar (liquid culture controls) or with 0.175% and 0.35% agar.

In Figure 4.2(A), we show the progression of population growth and dispersion over a four-day period for the non-motile and motile bacteria in 35 mm diameter Petri dishes. By the end of the second day, the M population is already distributed throughout the Petri dish whereas the N population remains in the center of the dish. Although the volume covered by the N population increases with time, even after four days its population remains in the center of the dish.

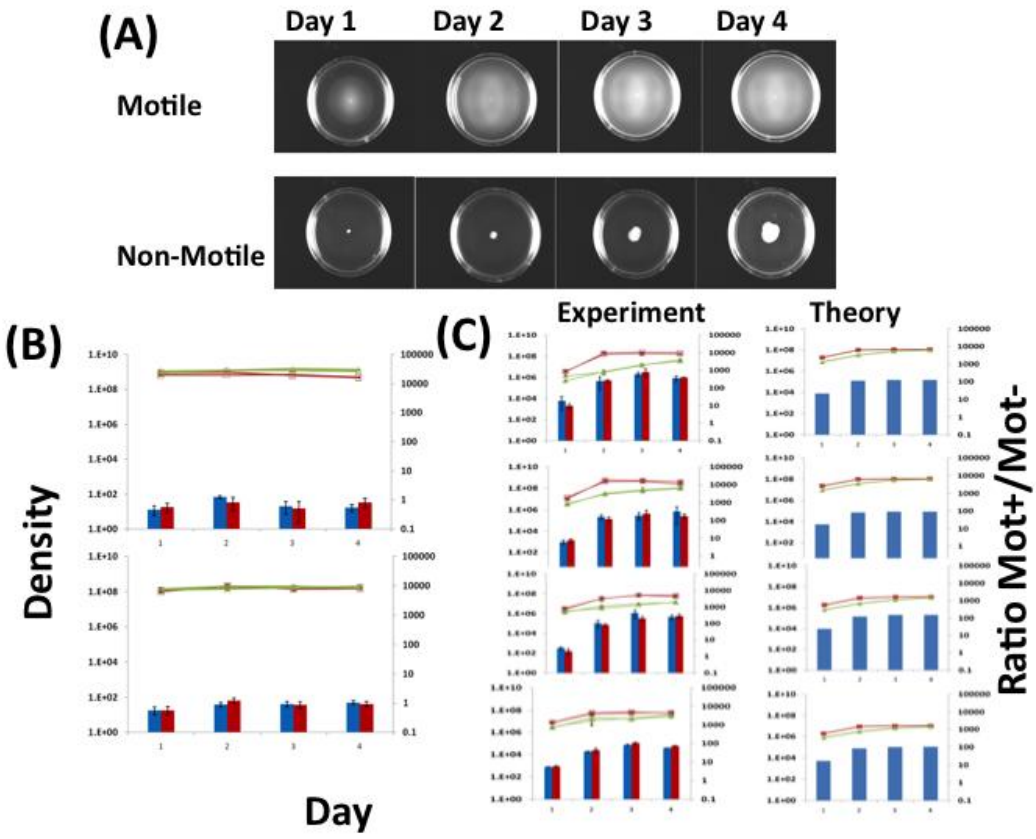

Figure 4.2 (A) Growth of the non-motile and motile strain in 35 mm diameter Petri dishes with a 1% tryptone, 0.35% agar medium. The estimated densities of cells in 4 plugs taken 5 mm and 15 mm from the edges from separate Petri dishes of the sort depicted in this figure are given in the below Table (means and standard errors of estimates from samples).

	Day 1	Day 2	Day 3	Day 4
N 5mm	0	0	0	0
N 15 mm	0	0	0	$7.3 \pm 0.7 \times 10^6$
M 5 mm	0	$5.5 \pm 0.6 \times 10^6$	$5.5 \pm 0.8 \times 10^6$	$2.6 \pm 0.3 \times 10^6$
M 15 mm	$9.9 \pm 0.4 \times 10^5$	$6.8 \pm 0.7 \times 10^6$	$8.2 \pm 1.1 \times 10^6$	$4.1 \pm 0.3 \times 10^6$

(B) and (C) Growth and cell densities and ratio of motile to non-motile cells in liquid culture and soft agar culture. Means and standard deviations for densities estimated for three separate dilutions of from each of two independent experiments. Initially 20-50 cells per ml and M/N ratios of ~ 0.7 and 0.6. The lines are the total cell densities with the square ticks the motile, and the triangles the non-motile. The bars are the ratio motile to non-motile (Blue and Red bars are two independent experiments). Means and standard errors (n=3) for two independent experiments.
(B) Liquid culture: top, 1% tryptone, bottom 0.1% tryptone.
(C) Soft agar culture Experiments and theory.

Position	Experimental	Theoretical (µg/ml)
Top	1.0% Tryptone - 0.35% agar	$R_{MAX} = 50 - D_r = 3.6 \times 10^{-3}$
Line 2	1.0% Tryptone - 0.175% agar	$R_{MAX} = 50 - D_r = 7.2 \times 10^{-3}$
Line 3	0.1% Tryptone - 0.35% agar	$R_{MAX} = 5 - D_r = 3.6 \times 10^{-3}$
Bottom	0.1% Tryptone - 0.175% agar	$R_{MAX} = 5 - D_r = 7.2 \times 10^{-3}$

$\alpha = 0.9\ hr^{-1}$, k= 1.0 mg/ml, $\nu = 4.75 \times 10^{-7}$ µg/hr, $D_M = 3.19 \times 10^{-4}$ cm/hr, $D_N = 3.6 \times 10^{-3}$ cm/hr
Simulation step size $dt = 0.1 hr^{-1}$ and the matrix assumed 0.1 cm squares.

In Figure 4.2 (B) and (C) we present the results of experiments with N and M alone and in mixed culture. In liquid by the end of the first day the cultures initiated with about 50 cells are nearly at their maximum densities. There was no evidence for the motile strain having an advantage over the non-motile in either the rate at which it saturates the environment in single clone culture or its competitive performance in mixed culture (Figure 4.2(B)). The outcome is very different in the structured habitat of soft agar. Here the M strain has a considerable advantage over the N in both the rate at which it saturates the habitat in single clone culture, and in competition with N in mixed culture

(Figure 4.2(C)). Because the bacteria saturate these habitats, the *M/N* ratio ceases to increase after the third day. As predicted by the model (Figure 4.1(A)) and seen by the increase in the M/N ratio, these advantages of motility are greater in medium with a higher concentration of the resource (the 1% tryptone) and greater viscosity (the 0.35% agar). Not only are these experimental results and theoretical predictions qualitatively coincident, they are reasonably consistent quantitatively (compare the Experiment and Theory columns in Figure 4.2(C)).

In the experiments illustrated in Figure 4.2, the soft agar cultures were initiated with a needle placing a low density of bacteria into the center of the agar. Although this procedure is the experimental analog of our mathematical model, it is not the only way structured communities can be colonized by bacteria. A more likely situation would be for these habitats to be initially composed of bacteria that are dispersed throughout. To mimic this, we performed experiments similar to those in Figure 4.2(C) with single clones or mixtures of motile and non-motile *E. coli,* introduced into the agar while it was still liquid. The cultures were immediately vortexed to distribute the bacteria. The results of this experiment (Figure 4.3) are virtually identical to the corresponding experiments with the needle inoculation procedure (Figure 4.2(C)).

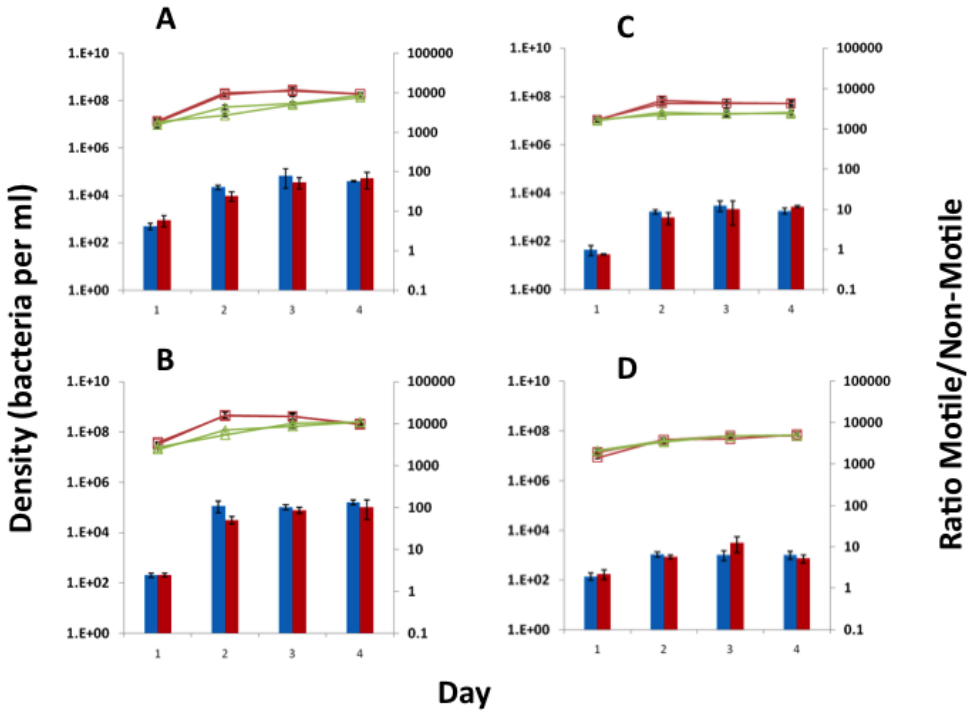

Figure 4.3 Single clone growth and mixtures of motile and non-motile *E. coli* in agar media. Cultures initiated with approximately 20-50 cells randomly dispersed in agar. The initial ratio motile/non-motile in these experiments was between 0.6 and 1.0. The lines are the total cell densities with the square ticks the motile, and the triangles the non-motile. The bars are the ratio motile to non-motile. Means and standard errors (n=3) for two independent experiments. A- 1% tryptone, 0.35% agar, B-1%tryptone, 0.175% agar. C- 0.1% tryptone 0.35% agar, D- 0.1% tryptone 0.175% agar.

We stress that our model does not require directed movement to regions of greater resource concentration (chemotaxis) for motility to be favored. However, the experiments presented in Figure 4.2 do not exclude chemotaxis from contributing to the advantage of motility. The wild type motile strain used in these experiments is capable of chemotaxis (Mot+Che+). On the other hand, in single clone culture motile but chemotactic negative strains of *E. coli* (Mot+Che-), "swarm" in soft agar (Wolfe and Berg 1989). Therefore it seems reasonable to anticipate that even without knowing where they are going, motile *E. coli* would have an advantage over non-motile. To

determine whether this is the case, we repeated the experiments presented in Figure 4.2 with a Mot+Che- construct of *E. coli*, PS2001(Δ*cheB, cheZ* and *cheY*) bearing a low copy number pLC576 plasmid carrying a *cheY* under *lac* promoter that was inducible for motility (Alon *et al.* 1998; Korobkova *et al.* 2004).

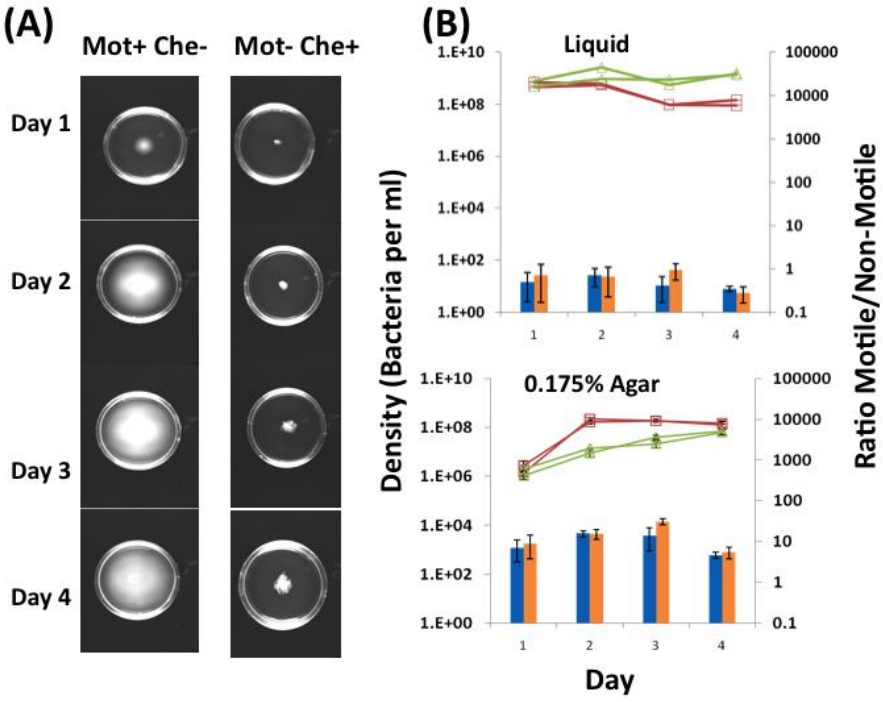

Figure 4.4 Growth and competitive performance of a Motile Chemotactic negative *E. coli* PS2001 and a non motile Chemotactic positive strain in 1% tryptone liquid and in 0.175% soft agar medium. (A) Growth of the non-motile and motile strain in 35 mm diameter Petri dishes. (B) Growth of motile and non-motile strains in single clone culture and in competition in liquid and soft agar. Means and standard deviations for densities estimated for three separate dilutions of from each of two independent experiments. Initially there were 20-50 cells per ml and M/N ratios of ~ 0.6- 0.7. The lines are the total cell densities with the square ticks the motile and the triangles the non-motile. The bars are the ratio motile to non-motile for two independent experiments.

As anticipated from the studies of Wolfe and Berg (Wolfe and Berg 1989), the Mot+Che- strain diffuses through the habitat faster and further than the Mot-Che+ strain (Figure 4.4(A)). In single clone agar culture the motile strain saturates the habitat faster than the non-motile. There are no differences in this saturation rate in liquid, although in liquid, the motile strain seems to die off more rapidly than the non-motile. As with the Mot+Che+ strain experiments depicted in Figures 4.2 and 4.3 in agar the motile strain is more fit than the non-motile. As measured by M/N ratio, the extent of this fitness advantage of the Mot+Che- strain in agar is less than the Mot+Che+ strain.

4.3 Discussion

We interpret the results of this study as support for the hypothesis that even without the directional information associated with chemotaxis, motility provides bacteria with an advantage in the rate at which they saturate and compete in resource-limited physically structured habitats. Motile bacteria are able to move away from each other faster than non-motile bacteria and thereby increase their likelihood of entering regions of more abundant resources.

Although in our model and in our experiments, the initial concentration of resources was homogenous throughout the habitat, as the bacterial population grows it consumes resources in its vicinity faster than that from more distant regions. Within short order, resource concentration gradients are formed. Although the bacteria are equally likely to move towards regions where resources are depleted as they are to regions where they are abundant, the consequences moving in these two directions are different. As assumed by

our model and manifest in our experiments, bacteria entering an area of greater resource concentration divide more rapidly than those entering areas of lower resource concentration and colonize those richer regions. As predicted by our model, this motility advantage increases with the viscosity of the environment and the extent to which the populations grow (the resource concentration effect).

Whereas our model does not explicitly account for chemotaxis, it does so implicitly. From the population dynamic perspective considered in our model and experiments, all of the elegant machinery of chemotaxis (Berg 1993; Berg 2003) can be subsumed into the diffusion coefficient of the bacteria. In essence, chemotaxis increases the net rate of bacterial movement to regions of higher resource concentration. In this interpretation, chemotaxis further augments the fitness of already motile bacteria in physically structured, resource-limited habitats. Chemotaxis augmenting the fitness of motile bacteria can be seen by comparing the extent of the advantage of the Mot+Che+ strain (Figure 4.2) and the Mot+Che- construct (Figure 4.4). At this juncture, however, we are not overwhelmed by this consistency. Factors other than chemotaxis could be responsible for this observation. These are different strains and although the nutrient levels and agar concentrations in the media are the same as those in the corresponding experiment presented in Figure 4.2, chloramphenicol was added to the media to prevent the loss of the plasmid and IPTG was added to induce the motility of the PS2001 construct.

In our experiments we mimicked a physically structured habitat with the bacteria embedded in soft agar (Chao and Levin 1981) rather than on the surfaces of hard agar (Kerr *et al.* 2002; Simonsen 1990).Recent experiments with *V. cholerae* however, provide evidence that motility would also be favored for bacteria growing on surfaces. In a study of the fitness costs of phage resistant mutants of *V. cholerae*, Wei and colleagues found that although the motile strain used as a common competitor had a disadvantage in liquid culture, it had a considerable advantage over the non-motile strain when grown on filters suspended on agar (Wei, Ocampo and Levin 2010).

Since the PDE model used here explicitly accounts for spatial heterogeneity in resource availability and the movement of bacteria, it is a far better analog of resource limited population growth and competition in physically structured habitats than ODE models. As with ODE models, this PDE model can (and will) be extended to other ecological and clinical situations that may be influenced by the physical structure of the habitat, like phage and protozoan predation, plasmid transfer and the pharmacodynamics of antibiotics. Moreover, the solutions of the PDE used here models are robust, and errors in their predictions models can be evaluated analytically.

On the other side, the model used here is a simplistic caricature of bacterial growth and competition in physically structured habitats. It is not expected or even desired to be a quantitatively precise analog of these processes. The role of this model was to facilitate the design and generate hypotheses for our experiments, and interpret and provide generality to their results in a qualitative way. But as noted in Figure 4.2, the agreement

between the predictions of the model and the results of our experiments are quantitatively as well as qualitatively coincident. While this is certainly gratifying, we see it as a lagniappe rather than something we anticipated. Although the model's parameters are in a realistic range and the solutions are robust with respect to changes in these parameters, this model is for a two dimensional habitat whereas the experimental habitat is three-dimensional. Moreover, as far as this model goes, motility will be favored no matter how viscous the habitat may be. Taken to the extreme this cannot be true. The power of the flagella and other motility machinery needed to propel bacteria are modest (Sowa and Berry 2008); bacteria would not be motile if embedded in very viscous substrates. Indeed, in the experiments with the *E. coli* used here, motility provides bacteria no advantage when competing in medium containing 1.5% or even 1% agar (data not shown).

Our hypotheses that motility evolved and is maintained because it enables bacteria to get away from competing cells is not inconsistent with the hypotheses that this capacity to move is favored because of chemotaxis (Berg 1993), to escape the clutches of predatory protozoa (Matz and Jurgens 2005), or to facilitate adhesion to tissues by fimbriae (Flores and Okhuysen 2009). This move to get away from peers provides broader and more ecologically general conditions for motility to be favored than these other mechanisms. It does not require the bacteria to recognize gradients in nutrient concentrations, or to confront predators, or to live in specific habitats. All that is needed is a physically structured environment of the sort in which most real-world bacteria live.

Our assertion that motility evolved before chemotaxis is based on logic and inductive inference rather than an eye witness account of this evolution. We have, however, added plausibility to our assertion. Here we have demonstrated the necessary condition for this evolutionary progression, motility can be favored in the absence of chemotaxis. Phylogenetic data are also consistent with our assertion for an evolutionary progression from motility to chemotaxis. Whereas the flagellar genes are highly conserved among motile species, the sensing, signal transduction, and transcription factors associated with chemotaxis vary among species and habitats, and are used with modifications for diverse cellular tasks (Zhulin 2001; Szurmant and Ordal 2004; Singh *et al*. 2008; Zhulin *et al.* 2003; Girgis *et al*. 2007; Kirby 2009).

4.4 Methods

The Model:

We assume a two dimensional matrix with two populations of bacteria of different levels of motility and a limiting resource. The latter is taken up by and converted into bacteria as a hyperbolic function of its concentration (Monod 1949) at different points in this matrix. With these definitions and assumptions, the rates of change in the densities of bacteria and concentration of the resource in this matrix are given by a set of partial differential equations:

$$\frac{\partial b_M}{\partial t} = D_M\left(\frac{\partial^2 b_M}{\partial x^2} + \frac{\partial^2 b_M}{\partial y^2}\right) + \frac{\alpha r}{r+k} b_M \quad (1)$$

$$\frac{\partial b_N}{\partial t} = D_N\left(\frac{\partial^2 b_N}{\partial x^2} + \frac{\partial^2 b_N}{\partial y^2}\right) + \frac{\alpha r}{r+k} b_N$$

$$\frac{\partial r}{\partial t} = D_r\left(\frac{\partial^2 r}{\partial x^2} + \frac{\partial^2 r}{\partial y^2}\right) + \frac{\nu r}{r+k}(b_M + b_N) \quad (2)$$

where $b_M = b_M(x,y,t)$ and $b_N = b_N(x,y,t)$ are, respectively, the densities of the motile and a less or non-motile bacteria, *M* and *N*, at a point (x, y) at a time t and r=r(x,y,t) is the corresponding concentration of the resource. We assume that *M* and *N* have the same maximum rate of cell division, α hr^{-1} and the same concentration of the nutrient at which their rate of cell division is half its maximum value, the Monod constant, *k µg/ml*. We also assume that the rate of bacterial death is negligible. The parameter ν µg/hr is the maximum per-capita rate at which bacteria consume the resource. D_M, D_N and D_r *cm/hr* are, respectively, the diffusion coefficients for the M and N bacteria and the resource.

Numerical solution and stability

For our numerical analysis of the properties of these models we assume: (i) the difference between the motile and non-motile bacteria is solely in their respective capacities to diffuse, $D_M > D_N$; (ii) initially the resource is homogeneously distributed throughout the matrix, *r(x,y,0)=constant*; (iii) low initial densities of bacteria are localized at the center of the matrix; (iv) there is no flow of bacteria or resources into or out of the matrix, (v) the bacterial growth α and resource uptake- conversion parameter ν, are in a range estimated for *E. coli* K-12 in glucose-limited minimal medium (Chao, Levin and Stewart

1977); and (v) the diffusion coefficients and parameters are similar to those estimated with a thin tube assay (Adler and Dahl 1967; Segel, Chet and Henis 1977; Kim 1996). Simulations commence with an initial resource concentration R_{MAX} and all the bacteria at the central point. The MatLab programs used for this analysis are available from the authors.

Since this nonlinear system of partial differential equations has no closed form solution, we use numerical methods to simulate solutions. Instabilities are a common problem when solving partial differential equations using finite difference schemes. To avoid this problem, we use a state-of-the art implicit scheme. We also prove that our algorithm possesses stability and consistency properties that make its application highly reliable.

Experimental Methods

The *E. coli* used in this study are from Keio collection (Baba *et al*. 2006). The Motile (M) strain is JW5702 *crp* and the non-motile (N), JW1059 *flgA*. To distinguish these strains by plating, we used spontaneous Nal^r mutant of the non-motile (N). The results of our competition experiments between the ancestral JW1059 and JW1059 Nal^r failed to detect fitness differences associated with this Nal^r marker. As is the case for all Keio strains, our M and N bacteria bear a Kan^r marker.

The PS2001 (ΔcheB, cheZ cheY) strain we used for our Mot+ Che- strain had been transformed with a low copy number pMS164 Cm^R plasmid carrying a cheYD 13K gene with an inducible lac promoter. We induced this gene and thereby motility by adding

0.5mM IPTG to the liquid and soft agar media. To insure the maintenance of the plasmid 25μg/ml chloramphenicol was also added to this media. By conjugation transferred an R1(Cm Km Am Str Sp) plasmid to JW1059 Nal^r so this Mot-Che+ strain would also be resistant to the chloramphenicol in the medium.

All of the motility experiments were done in 35mm diameter Petri dishes (Falcon, 351008) containing 5ml of medium, 1% tryptone or 0.1% tryptone and 0.5% NaCl with either 0.35% agar or 0.175% agar. The densities of bacteria were estimated in LB agar with 25μg/ml kanamycin (N and M) and LB agar with 20μg/ml naladixic acid (N only). To initiate the experiments, a needle was dipped into diluted 37^{o}C LB overnight cultures of N or M or mixed M and N cultures and then gently stabbed into the center of the agar surface of the Petri dishes. For each experiment, two independent replicate cultures were prepared for each day's sampling for a total of 8 plates. Three independent estimates of the densities of these cultures were made for each time point. The parallel liquid culture experiments were performed with 2 ml of agar-free 1% or 0.1% tryptone in the wells of 12 well plates, (Falcon, 353043), a total of 6 cultures for each experiment, 2 each *N* only, *M* only and *M* & *N*. All cultures were incubated at 30^{o}C at high humidity to minimize drying. To suspend the bacteria in the agar culture for sampling, the agar was put into tubes containing 10 ml 0.85% saline and vigorously vortexed for between 45- 60 seconds.

CHAPTER 5

Summary & Discussion

My dissertation work was initially driven by field observations and epidemiological data from the 1930s as well as more recent reports, which hypothesized that bacteriophage play a role in ending cholera outbreaks. Although the evidence was compelling, previous laboratory studies of population dynamics of bacteria in the presence of single phage are certainly inconsistent with this hypothesis due to the rapid emergence of bacterial mutant resistant to phage. The major goal of my dissertation was to test this hypothesis in the laboratory and to explore the conditions under which vibriophage limit bacterial host densities. By combining laboratory experiments and mathematical modeling, my research is intended to bring a clearer understanding of how vibriophage modulate cholera outbreaks.

To achieve this goal, I studied population and evolutionary dynamics of the El Tor O1 *V. cholerae* N16961 strain and three of its naturally occurring phage in continuous cultures (presented in chapter 2 & 3). All three phage, when singly cultured with the bacteria, fail to limit the bacterial densities over an extended time period. Although the dynamics of bacteria and these phage varied to some extent, the overall trend and outcomes are consistent and obvious: the densities of bacteria are only temporarily controlled by phage and, in the long run, are limited by the resources. This finding is consistent with other studies that have reported that single phage cannot control the bacterial populations in the laboratory. Does this mean that the hypothesis “bacteriophage play a role in ending

cholera outbreaks" is rejected? The results presented in chapter 2 certainly say no to this question.

During my studies, I found that phage-resistant mutants (JSF4-resistant *V. cholerae*) were less motile, grew slower, colonized less frequently on *Daphnia* and were less virulent in a *C. elegans* model, when compared to their phage-sensitive ancestor. These characteristics are likely to impair the ability of phage-resistant mutants in competing and maintaining their populations in their natural habitat and suggests that they may be avirulent in human hosts. My data from chapter 2 suggests that phage can contribute to the waning of cholera outbreaks even when resistant mutants emerge because these resistant mutants show an extreme fitness disadvantage relative to non-resistant strains, thus, phage-resistant strains of *V. cholerae* may not play an important role in the ecology and epidemiology of cholera.

In chapter 3, I found that the densities of *V. cholerae* can be controlled by phage for over 600 hours. The long-term phage-limited dynamics I observed was quite unexpected because I believed the phage population existing in the chemostats consisted of single phage species. Later, I isolated and identified two different phage species, which, when cocultured individually with *V. cholerae* N16961 in the chemostat, did not limit bacterial populations. However, when two phages simultaneously were inoculated with N16961, phage-limited dynamics occur. One of the two phages in these cultures exhibited features

(discussed in chapter 3) which are not found in the well-studied lytic T phage or lysogenic phage lambda. Mathematical simulation of the population dynamics of this particular phage and its bacterial hosts suggests that this phage might induce a persister-like of bacterial sub-population as some antibiotics do.

Although the contents are distinctive, chapter 2 & 3 directly address the question of "whether vibriophage can regulate cholera outbreaks by controlling the densities of *V. cholerae*". The scenarios presented in chapters 2 and 3 are not mutually exclusive and can occur in the environment. The works presented in chapters 2 & 3 are the first population study of *V. cholerae* and its bacteriophage. Because our current knowledge of population dynamics of bacteria and phage is largely based on *E. coli* and its T series phage, the data presented in this dissertation expands greatly on previous work.

The most intriguing result from chapter 3 is the demonstration that multiple phages can limit the densities of *V. cholerae*, even in the presence of bacterial mutants resistant to multiple phages. In theory, the emergence of phage-resistance would be much more difficult if there is more than one single phage present. Few studies have examined the dynamics of bacteria and multiple phages, and the only existing report to my knowledge does not support this theory (Korona, R. and B. R. Levin 1993). The results in chapter 3 clearly showed that as few as two phages are capable of limiting bacterial densities for an extended time in the laboratory. Whether any combination of two phages can produce

this dynamic is currently unknown to me and a topic of future study. Again, our observation was quite unexpected because I initially set out to examine the population dynamics of *V. cholerae* in the presence of a single phage. The capability for multiple phages to limit bacterial populations will be an important topic of study for the future because in the natural environment, it is almost certain to believe that multiple phages are present. My data suggest that the nature of the phage will greatly influence the infection process, as well as the interaction process with the other phage, both of which, coupled with environmental situations, will determine the overall dynamics.

Two main questions regarding the turbid phage remain elusive to us in chapter 3. As its name suggests, turbid phage only lyse a fraction of the bacterial population on a bacterial lawn, producing a turbid plaque which is indicative of its lysogenic nature. However, as shown in chapter 3, the traditional way of testing lysogeny rejects this possibility. Also, the turbid phage clears the bacterial density in liquid culture although quite slowly when the multiplicity of infection (MOI) is low. My basic population data from chapter 3 is not sufficient to characterize the nature of this turbid phage but there are future areas of investigation underway that can yield a more complete picture. Our collaborator Jim Bull will sequence the genome of this turbid phage and I expect that the sequence data, coupled with my classical population dynamics data and mathematical models will increase our understanding of this phage. As an atypical bacteriophage for T series lytic or lambda lysogenic phage, this type of phage may prevail in the natural environment and comparison to the genomes of other phage may reveal genomic regions associated with

their fitness advantage. The other unanswered question from chapter 3 is why the densities of bacterial mutants resistant to both phage do not increase to expected levels in a resource-limited condition. This particular question is important because it addresses the specific dynamics of *V. cholerae* and its multiple phage and could be a general trend for many other bacteria-multiple phage infection systems. Answering this question will be necessary to expand our understanding of the ecological role of phage in limiting bacterial populations and how to better use phage to treat and prevent bacterial infections.

Chapter 4 presents work somewhat unrelated to the major goal of this dissertation, however, it was inspired by some of the results acquired in chapter 3. I was interested in the behavior of bacteria on surface and performed a preliminary work competing phage-resistant and phage-sensitive *V. cholerae* on surface. Surprisingly, I found that some phage-resistant bacteria behave poorly on surface culture, but they grow as well as the phage-sensitive in liquid culture. All of these isolates were non-motile leading me to the question of why bacteria are motile. In chapter 4, I show that motility can be favored because motile bacteria can move apart from one another thereby obtaining greater shares of limiting resources. My data from this chapter suggest that the behavior of bacteria and phage in a physically structured environment is important for bacteria-phage population dynamics. Although most bacteria do not exist in a planktonic state most of the time in the natural environment, almost all of our knowledge of microbiology is obtained from experiments conducted in planktonic condition. What are the dynamics in a physically structured environment? How fast does phage-resistance evolve? How do the resistant

bacteria affect overall population dynamics? What is selection pressure on phage in such an environment? All these questions remain unknown. It won't surprise me if the results of the same dynamics of bacteria-phage differ in liquid and in physically structured environments. A few studies on population dynamics of bacteria and phage have been investigated in their natural habitat. The information gained from this type of study is valuable towards a better understanding of the interactions between bacteria and phage in the real word. In the meantime, systems and models can be developed in the laboratory to mimic physically structured environments. Although they may not be complex as the natural habitats of most bacteria and phage, general trends characterizing the dynamics could be obtained and can address the role of spatial structure in theoretical studies. These data can contribute insight to a diversity of long-standing research questions, including the ecology and evolution of allelopathy, the stability of predator-prey interactions and adaptive evolution (Bohannan, B. J. M. and R. E. Lenski 2000).

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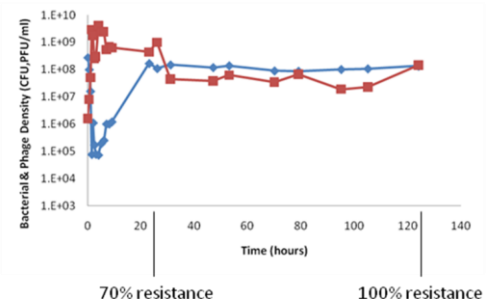
Appendix

Appendix 1: Supplementary information to chapter 2 - An Experimental Study of the Population and Evolutionary Dynamics of *Vibrio cholerae* O1 and the Bacteriophage JSF4

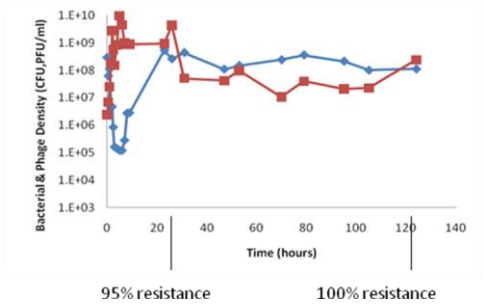
A1 - More chemostats results

In addition to 3 chemostats results shown in chapter 2 (Figure 2.1), 5 more chemostats with qualitatively the same results are presented as follows:

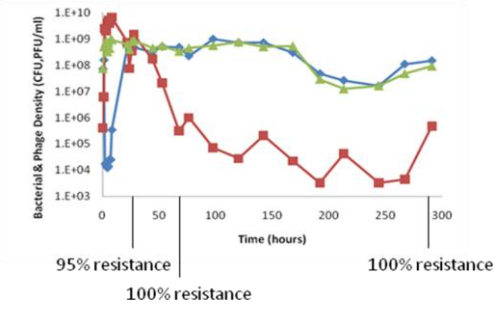
(a)



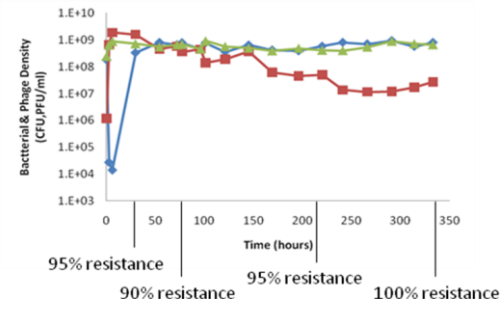
(b)



(c)



(d)



(e)

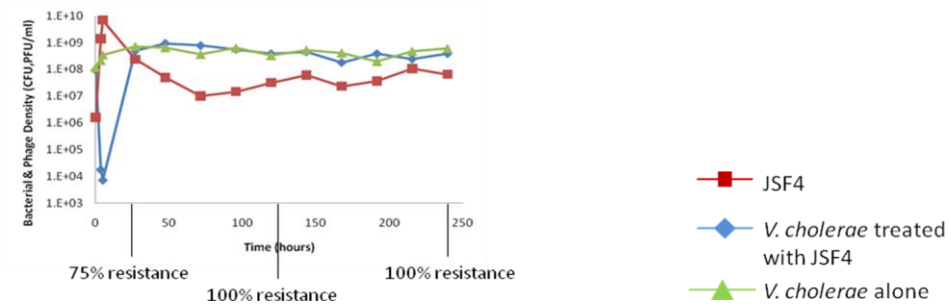


Figure A1 Change in the density of bacteria and phage in continuous culture

(a): Phage was introduced in the presence of rugose variants (~15% of whole bacterial population, estimated from plate counts). Dilution rate: ~0.11

(b): Phage was introduced into the bacterial population which was initiated with a single rugose variant (bacterial population was consisting of rugose variants). Dilution rate: ~0.11

For (*a) & (b)*, there were no bacteria-only controls.

(c): Phage was introduced when the bacterial population was consisting of smooth type (Plate counts can only detect rugose variants when they reached a high density, >1e6 cfu/ml. If no rugose variant was found on plate, the population was considered consisting of smooth type). Dilution rate: ~0.21

(d) & (e): Phage was introduced when the bacterial population was consisting of smooth type. Dilution rate: ~0.07-0.12

A2 - Clumping

Auto-agglutination, a macroscopic clumping phenomenon, is considered to be caused by high level expression of the Toxin Co-regulated Pili (TCP) and may be associated with the ability of *V. cholerae* to form microcolonies on the intestinal epithelium (Chiang & Mekalanos 1999). To determine whether phage-resistant isolates are different from wild-type *V. cholerae* in the extent of clumping, JSF4-sensitive and resistant bacteria were incubated overnight in LB in 3 ml glass tubes with shaking. These cultures were then left

on the bench for 30 minutes without agitation. We then compared the extent to which the cultures settled, our assumption being that clumping promotes settling.

As can be seen in Figure A2, some cultures remained turbid (presumably because the bacteria were planktonic), while the bacteria in others settled. It has been postulated that the sinking behavior is the result of auto-agglutination (clumping), due to high levels of expression of the toxin co-regulated pilus TCP (Chiang & Mekalanos 1999). Of the 175 JSF4-resistant clones isolated at different times from the same and different chemostats, 61 remained planktonic and others settled within 30 minutes. All of the JSF4-sensitive N16961, including the above described 252 isolates from two JSF4-free control chemostat, screened in this way remained turbid; the bacteria were planktonic. For the 175 JSF4-resistant clones tested for both motility in semi-solid agar and sinking in liquid, there appeared to be no association between these two phenotypes. The clumping phenotype was also observed in the JSF4-resistant rugose mutants but not the JSF4-sensitive rugose strain from which they were derived. Apparently, the wild-type *V. cholerae* strain used in this study, El Tor O1 N16961 does not auto-agglutinate *in vitro* presumably because of the low expression of the toxin-coregulated pilus (TCP) (Jonson *et al.*1992), but at this juncture we cannot say that sinking of the resistant mutants is due to an overproduction of TCP.

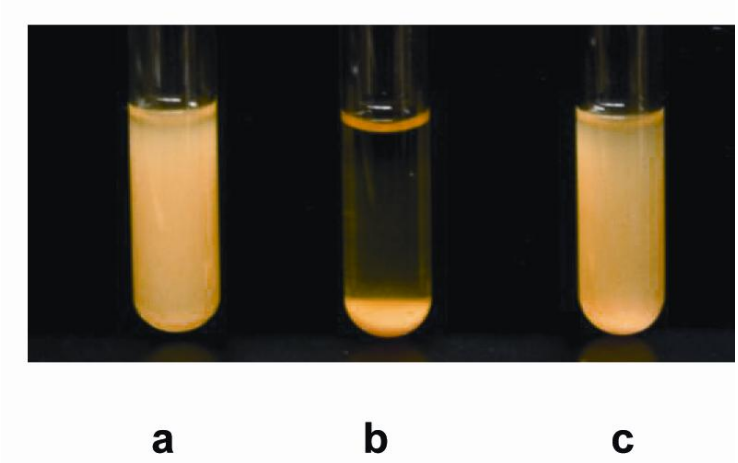


Figure A2 Clumping of JSF4-sensitive and resistant *V. cholerae* N16961 in unshaken broth: a .wild-type *V. cholerae* N16961; b. RF, JSF4^r *V. cholerae* N16961, c. R2.2, JSF4^r *V. cholerae* N16961.

A3 - Switch from smooth to rugose

The rugose colony type of *V. cholerae* are generated (either by a phenotypic switch or mutation) from wild-type smooth colony *V. cholerae* at a frequency that varies among different isolates (Beyhan *et al.* 2007; Beyhan & Yildiz 2007). It has been reported that the rate at which *V. cholerae* switch from smooth to rugose colony types, exopolysaccharide (EPS) production, is substantially greater in clinical than environmental isolates, switching at rates of up to 0.8 per division (Ali *et al.* 2002).

To ascertain if there are differences in the rate of rugose formation of JSF4-resistant and sensitive N16961, a smooth colony was inoculated into glass tubes containing 3 ml of peptone water and incubated without agitation for 48 hours at 37 ℃. The cultures were then plated onto LB agar and the relative frequency of rugose colonies determined. Five smooth JSF4-resistant mutants and wild-type N16961 were tested as the way mentioned

above. All 5 smooth phage-resistant mutants showed significantly lower frequencies of switching than wild-type (Table A1), <5% to >90% (Chi-square, $p<10^{-4}$).

Table A1 Percent of wild-type JSF4 sensitive and resistant strains shifting to rugose colony morphology

Strains	% Rugose colonies (mean ± standard deviation)
N16961	90.92 ± 7.61
R1.1	0 ± 0
R1.2	1.92 ± 1.76
R2.1	0 ± 0
R2.2	2.07 ± 0.5
RF	0.45 ± 0.342

Between 100 and 500 colonies were examined for each strain.

A4 - Colonization of *V. cholerae* N16961 on free chitin

To determine the extent to which differences in JSF4-sensitive and resistant N16961 observed in the above Daphnia experiments can be attributed to differential adhesion to chitin, we also performed colonization experiments with chitin from crab shells (Sigma, C7170). The protocol for this was similar to that described above for Daphnia colonization experiments. Chitin from crab shells was mixed with 0.85% saline to make a suspension of 0.5% (weight/volume) chitin. Overnight LB cultures of *V. cholerae* were washed 3 times and resuspended to the original volume in 0.85% saline. Next, 20 µl of these washed cultures were mixed with 2 ml 0.5% suspensions of chitin in 100x13mm glass tubes for a bacterial density of ~1e7/ml. These tubes were incubated between 18

and 24 hours at room temperature (~25 ℃). To remove bacteria not adhering to chitin particles, these suspensions were allowed to settle for about 15 minutes, at which time the supernatant was removed. This washing – settling process was repeated 5 times. Following the final wash, the density of bacteria in the supernatant was estimated from CFU data. The suspensions were then vigorously vortexted for 45-60 seconds and free bacterial density estimated again. The difference between the vortexed and prevortexted estimates of bacterial density was used as our measure of the extent to which the bacteria adhered to the chitin.

As can be seen in Figure A3 three out of five smooth JSF4-resistant mutants appeared to be as able to colonize chitin as their wild-type JSF4-sensitive ancestor. The remaining two mutants tested, R2.1 and R2.2, were significantly less capable of colonizing chitin than wild-type (t-test, $p<10^{-7}$). It is of interest to note that these two strains were non-motile, whilst the three JSF4-resistant strains that appeared to be as capable of colonizing chitin as wild-type were somewhat motile. This result is consistent with the observation that motility is positively associated with the ability of these bacteria to colonize dead crustaceans (Mueller *et al.* 2007). As noted above and contrary to this observation, there seemed to be no association between motility and the colonization of viable Daphnia. There was also no significant difference in the colonization on chitin between JSF4-sensitive and resistant rugose mutants (t-test, $p>0.1$). Finally, the JSF4-sensitive and -resistant rugose colony types were better able to colonize chitin than the wild-type, smooth (t-test, $p <0.01$).

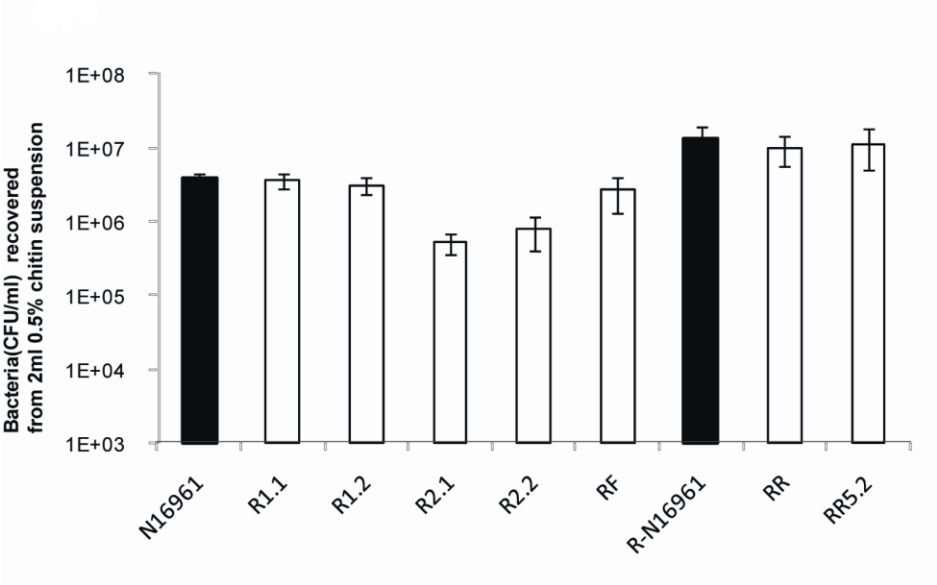


Figure A3 Colonization of wild-type *V. cholerae* N16961 and JSF4-resistant mutants on chitin

S5 - Minimum density of bacteria necessary to maintain a phage population in liquid culture

For the phage to maintain a population in a particular habitat, the rate at which they replicate has to exceed that at which they are lost (are killed - inactivated). In liquid culture the rate of replication of phage would be directly proportional to the density of susceptible bacteria. Assuming a mass-action process and neglecting the latent period the rate of replication of a phage population would be given by

$$\frac{dP}{dt} = \gamma\beta NP - dP$$

Where, N is the density of bacteria (cells per ml), P the density of phage (particles per ml), γ the adsorption rate constant (ml contacts per phage particle per cell per hour), β

the burst size (phage particles), and *d* the loss, death rate of the phage (particles per hour) (Levin *et al.* 1977).

For the phage to be maintained,

$$\gamma\beta NP > dP$$

or the minimum density of bacteria to maintain the phage is,

$$N_{MIN} \geq \frac{d}{\gamma\beta}$$

Using the methods in (Levin *et al.* 1977) we estimated γ and β for JSF4 and *V. cholerae* N16961 in 5% LB at 30^{o}C. They are, respectively, $\gamma \sim$ 2.9E-8 ml hr^{-1} and β = 36.7 particles per infected cell.

Thus if JSF4 had a loss rate of d = 0.01 per hour, the minimum density of *V. cholerae* needed to maintain the phage would be N_{MIN} = 9.4E3. The corresponding densities for d=0.1 and d=0.001 are, respectively, 9.4E4 and 9.4E2.

Table A2 *V. cholerae* strains used in this study (This table is a more comprehensive version of Table 2.1 in chapter 2)

Strain	Relevant phenotype	Comments	References
N16961	Sm^r smooth wild-type	El Tor O1 *V. cholerae*	John J. Mekalanos
R-N16961	Sm^r rugose wild-type	Isolated from a batch culture	This study
Rif.R	Rif^r smooth N16961	spontaneous Rif^r mutant of smooth N16961	This study
RF	$JSF4^r$ smooth N16961	Isolated from a batch culture	This study
R1.1	$JSF4^r$ smooth N16961	Isolated from chemostat 1 (Figure 2.1c)– 25 hours after introduction of phage	This study
R1.2	$JSF4^r$ smooth N16961		This study
R2.1	$JSF4^r$ smooth N16961	Isolated from chemostat 2 (Figure 2.1a) – 75 hours after introduction of phage	This study
R2.2	$JSF4^r$ smooth N16961		This study
R3.1	$JSF4^r$ smooth N16961	Isolated from chemostat 3 (Figure A1.c)– 30 hours after introduction of phage	This study
R3.2	$JSF4^r$ smooth N16961	Isolated from chemostat 3 (Figure A1.c)– 70 hours after introduction of phage	This study
R3.3	$JSF4^r$ smooth N16961		This study
R4.1	$JSF4^r$ smooth N16961	Isolated from chemostat 4(Figure A1.d) – 30 hours after introduction of phage	This study
R4.2	$JSF4^r$ smooth N16961		This study
R4.3	$JSF4^r$ smooth N16961	Isolated from chemostat	This study

R4.4	JSF4^r smooth N16961	4 (Figure A1.d)– 70 hours after introduction of phage	This study
RR	JSF4^r rugose N16961	Isolated from a batch culture	This study
RR5.1	JSF4^r rugose N16961	Isolated from chemostat 5 (Figure 2.1b)– 48 hours after introduction of phage	This study
RR5.2	JSF4^r rugose N16961		This study
RR5.3	JSF4^r rugose N16961		This study
NM	JSF4^s non-motile smooth N16961	isolated from a JSF4-free chemostat	This study

Sm^r, streptomycin resistant; JSF4^r, JSF4 resistant; Rif^r, rifampin resistant

RF and RR were isolated from different batch cultures (24 hours after the introduction of JSF4).

Chemostat-1 (where R1.1 and R1.2 were isolated), was inoculated with N16961 and running for approximately 48 hours before the introduction of JSF4. Chemostats-2, 3, 4 and 5 (where remainder of mutants were isolated), JSF4 were introduced approximately 2 hours after the chemostat cultures were inoculated with N16961. NM is a JSF4 sensitive non-motile smooth N16961 and was isolated in the end of a control chemostat where JSF4 was absent.

Appendix 2: Supplementary information to chapter 3 - The Population and Evolutionary Dynamics of *Vibrio Cholerae* and its Bacteriophage: Conditions for Maintaining Phage-Limited Communities

The population dynamics of phage infection in batch and continuous culture: some theoretical considerations

One role of this Appendix is to facilitate, in the necessarily quantitative way, the interpretation of the experimental results reported in chapter 3. A second role is to provide a general perspective on the implications of these experimental results for natural communities of bacteria and phage. For this we use simple mathematical models of the population dynamics of the interactions between bacteria and phage in mass (liquid) culture. For our analysis of the properties of these models we use numerical solution to the differential equations. These computer simulations are programmed in Berkeley Madonna™. Copies of these programs can be obtained from www.eclf.net. The parameters used in these simulations are in realistic range and based on those estimated in our earlier studies with *E. coli* and *V. cholerae* and their phage, (Levin, Stewart and Chao 1977; Wei, Ocampo and Levin 2010) rather than in this study. Using these simulations we demonstrate in a semi-quantitative way how these models can account for the experimental observations. As complicated as they may seem, at best these models are simplistic caricatures of the interactions between bacteria and phage and even with precise estimates of these parameters, we would not anticipate or for that matter desire quantitative precision (Levins 1966).

A1- Persistence and the dynamics of the phage-bacterial interaction in batch culture

On page 64 we postulated that the failure of the T-plaque phage to totally eliminate the bacteria in flasks can be attributed to a minority population of cells that are genetically sensitive, but physiologically refractory to the phage. In accord with our hypothesis, this minority population is analogous and may be identical to the non-growing bacteria responsible for the phenomenon of persistence observed in studies of the pharmacodynamics of antibiotic treatment (Balaban *et al.* 2004; Bigger 1944; Wiuff *et al.* 2005). Here we use a model of bacteria and phage in batch culture to illustrate how this persistence mechanism can account for our observations.

A Model of single phage and bacteria with persistence in batch culture: In this model there are two bacterial populations, sensitive cells, N and persister cells X, and one phage population, P, where N, X and P are densities (cells or particles per ml) as well as their designations. The sensitive bacteria grow a rate proportional to the concentration of a limiting resource, R mg/ml via a Monod function (Monod 1949).

$$\psi(R) = \frac{vR}{k+R}$$

where v hr^{-1} is the maximum rate of replication and k mg/ml is the concentration of the resource where the growth rate is half its maximum. As in (Levin and Udekwu 2010) we assume that the resource concentration, which determines rate of cell division, is a measure of the physiological state of the bacteria. As the concentration of the resources

declines, the rate of adsorption δ and burst size β of the phage can also decline according to the formulae,

$$\delta(R) = (1-x)\delta_{MAX} + x\delta_{MAX}\frac{R}{R+k}$$

and

$$\beta(R) = (1-x)\beta_{MAX} + x\beta_{MAX}\frac{R}{R+k}$$

where x ($0 \leq x \leq 1$) is a coefficient to denote the magnitude of the resource effect on these phage infection parameters and δ_{MAX} and β_{MAX} are the maximum values of the adsorption rate parameter and burst size, respectively.

There is a latent period L (hours) between the time of infection and the burst. At any given time t there are M(t) infected phage cells per ml which burst and produced β(R) phage when time is equal to t+L. We assume that the bacterial population includes a subpopulation of density X cells per ml that do not replicate or support the growth of the phage. These "persister" bacteria are produced from the N population and return to the N population at a rates proportional to the resource concentration and coefficients, *nx* and *xn*, respectively. With these definitions and assumptions the rates of change in the density of bacteria and phage and concentration of the resource at a time t are given by,

$$\frac{dR(t)}{dt} = -\psi(R(t))N(t)e$$

$$\frac{dN(t)}{dt} = \psi(R(t))N(t) - \delta(R(t))N(t)P(t) - \frac{R(t)}{R(t)+k}\left[nxN(t) - xnX(t)\right]$$

$$\frac{dM(t)}{dt} = \delta(R(t))N(t)P(t) - M(t-L)$$

$$\frac{dX(t)}{dt} = \frac{R(t)}{R(t)+k}\left[-nxN(t) + xnX(t)\right]$$

$$\frac{dP(t)}{dt} = M(t-L)\beta(R) - \delta(R(t))N(t)P(t)$$

where M(t-L)=0 when t<L, and *e* is the conversion efficiency parameter (Levin, Stewart and Chao 1977).

In Figure A1, we present changes in the densities of bacteria and phage in batch culture anticipated for different multiplicities of infection. The parameters chosen for these simulations are in are a realistic range and intended to illustrate the properties of this properties, rather than fit specific situations.

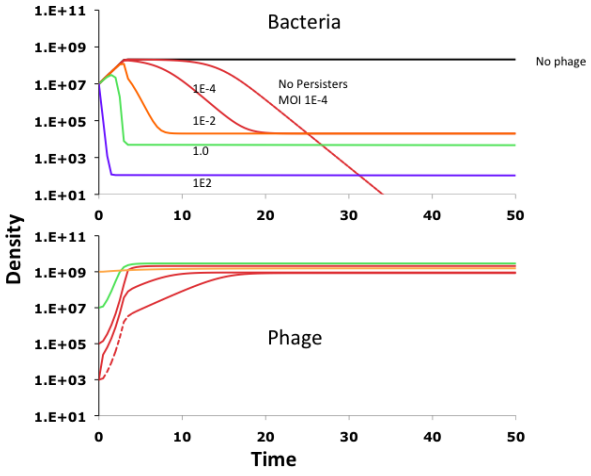


Figure A1 Changes in the density of total number of bacteria and phage in batch culture. Parameter values, v=1.0, k=0.25, e=$5x10^{-7}$, δ_{MAX}=1E-8, β_{MAX}=50, x=0.9, L=0.2, save for the simulation without persisters, nx=1e-4, xn=1E-3, initial R=100, initial N=1E7, and initial X = 1E3.

The phage continue to replicate until the density of bacteria becomes too low to sustain them. In the absence of persisters, the bacterial population dies off completely. With persistence subpopulations of viable cells are maintained. In this model, the density at which the bacterial population levels off is inversely proportional to the multiplicity of infection. The results of our batch culture experiments with the T-plaque phage and N16961 (Figure 3.5) are roughly consistent with the predictions of this model. This is more so for the multiplicity effect on the dynamics of the phage than the leveling off in the density of bacteria.

A2 - Population dynamics of single phage and bacteria in continuous culture

There are two outcomes of our experiments with the single B- or T-plaque phage in chemostat populations of N16961 that are, at least to us, striking and require explanation. One is the apparent stability of the phage bacterial community and the other is the continued maintenance of the phage following the ascent of resistance. We postulate that both of these results can be attributed to the existence of phage-refractory persister populations and/or wall growing populations. Here we use our models to illustrate this prediction.

A Chemostat model: One phage and one bacteria: This and the following model are extensions of the above batch model. Now, however, a limiting resources from a reservoir where it is maintained at a concentration of C µg/ml is continually added to a 1ml culture vessels at a rate w ml per hour which is the same rate at which planktonic bacteria are removed. In which in addition to the N and X bacterial populations there is a third planktonic population NR which is resistant to the phage, and a population bacteria *NW* adhering to the wall. This wall (biofilm) population is seeded by and seeds the planktonic, sensitive population *N* at rates *wx* and *xw* per cell per hour, respectively. For convenience, in this chemostat version of model we neglect the time delays associated with the later period. The wall (biofilm) population is washed out at a rate *ww* *(ww* << *w*), does not replicate and is totally refractory to phage infection. With these assumptions and the parameters and functions and defined above the batch model, the rates of change in the density of bacteria and phage and resources are given by,

$$\frac{dR}{dt} = (C - R) - \psi(R)Ne$$

$$\frac{dN}{dt} = \psi(R)N - \delta(R)NP - \frac{R}{R+k}\left[nxN - xnX\right] - nwN + wnNW - wN$$

$$\frac{dNW}{dt} = nwN - wnNW - wwNW$$

$$\frac{dX}{dt} = \frac{R}{R+k}\left[nxN - xnX\right] - wX$$

$$\frac{dP}{dt} = \delta(R)NP(\beta(R) - 1) - wP$$

.

In Figure A2 we follow the changes in the densities of the bacteria and phage under four conditions. In the absence of persistence or a wall population, the bacteria are rapidly

killed off, the phage ascend and then decline in density as they are washed out (Figure A2(A)). A planktonic persister subpopulation makes it possible for the phage and bacteria to co-exist, albeit with oscillations in their respective densities (Figure A2(B)). As demonstrated earlier (SCHRAG and MITTLER 1996), a phage- refractory wall population has a similar effect in stabilizing co-existence of the sensitive bacteria and phage (Figure A2(C)). If we allow for a resistant population with a somewhat lower fitness than the sensitive, it will ascend and all three populations continue to co-exist (Figure A2(D)).

Based on our semi-quantitative fit is good-enough criterion, we interpret these theoretical results to be consistent with observed in our experiments with single species of phage and bacteria (Figures 3.3 and 3.4).

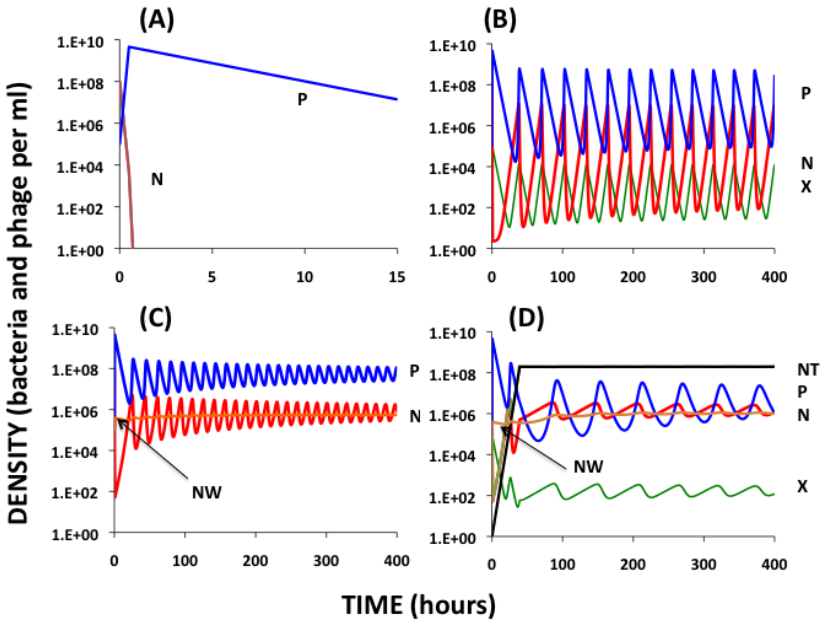


Figure A2 Changes in the density of bacteria and phage in continuous culture, N susceptible planktonic, P- free phage, X refractory persisters derived from N, and NW - phage-refractory wall population derived from N Standard parameters v=1.0, k=0.25, $e=5x10^{-7}$, δ_{MAX}=1E-8, β_{MAX}=50, x=0.5, w=0.4. C=100. (A) No persistence or wall population. (B) Persisters produced nx=xn=0.0001, no wall population (C) Wall population, nw=0.01, wn=0.005, no persisters, (D) Phage resistant population, v1=0.9, persisters and wall population nx=xn=0.0001, nw=0.01, wn=0.005.

A3 - Population dynamics of two phage and bacteria in continuous culture

In our experiments with a combination of B and T phage in chemostats with *V. cholerae* N16961 there were three results that require explanation. One, is the apparent stability of the phage-bacterial association, the second the persistence of both populations of phage, and the third the failure of bacteria resistant to both B and T to ascend. To address these issues we use an extension of the preceding model for this two phage situation.

Continuous culture model with two bacteria and two phage: In this model there are two populations of phages, with densities P1 and P2, four populations of planktonic, non-persister bacteria, N – sensitive to both phage, N1 – resistant to phage 1, N2- resistant to phage 2 and N12-resistant to both phages. For convenience once again, we assume there is no latent period and the rates of conversion of the bacteria to the corresponding persistent and wall population states are also the same, *nx, xn, nw, wn,* respectively. Although the their replication and rate of resource uptake are the same, we allow for differences in the maximum growth rates of the bacteria, v, vn1,vn2 and vn12, respectively for the N. N1, N2, and N12 populations. With these definitions and assumptions, and those of the preceding incarnations of this model, the rates of change in the densities of the bacterial and phage populations and concentration of the limiting resource are given by,

$$\frac{dR}{dt} = w(C-R) - \frac{R}{k+R} e(vN + v_1 N_1 + v_2 N_2 + v_{12} N_{12})$$

$$\frac{dN}{dt} = \psi(R)N - \delta_1(R)NP_1 - \delta_2(R)NP_1 - \frac{R}{R+k}\ nxN - xnX\ - nwN + wnNW - wN$$

$$\frac{dN_1}{dt} = \psi_1(R)N_1 - \delta_1(R)N_1P_1 - \delta_2(R)N_1P_1 - \frac{R}{R+k}\ nxN_1 - xnX_1\ - nwN_1 + wnNW1 - wN_1$$

$$\frac{dN_2}{dt} = \psi_2(R)N_2 - \delta_1(R)N_2P_1 - \delta_2(R)N_2P_1 - \frac{R}{R+k}\ nxN_2 - xnX_2\ - nwN_2 + wnN_{21} - wN_2$$

$$\frac{dN_{12}}{dT} = \psi_{12}(R)N_{12} - wN_{12}$$

$$\frac{dNW}{dt} = nwN - wnNW - wwN$$

$$\frac{dNW_1}{dt} = nwNW_1 - wnNW_1 - wwN_1$$

$$\frac{dNW_2}{dt} = nwN_2 - wnNW_2 - wwNW_2$$

$$\frac{dX}{dt} = \frac{R}{R+k}\left[nxN - xnX\right] - wX$$

$$\frac{dX_1}{dt} = \frac{R}{R+k}\left[nxN_1 - xnX_1\right] - wX_1$$

$$\frac{dX_2}{dt} = \frac{R}{R+k}\left[nxN_2 - xnX_2\right] - wX_2$$

$$\frac{dP_1}{dt} = P_1(\delta_1(R)(N+N_2) + \delta_1(R)N_2(\beta_1(R)-1)) - wP_1$$

$$\frac{dP_2}{dt} = P_2(\delta_2(R)(N+N_1) + \delta_2(R)N_1(\beta_2(R)-1)) - wP_2$$

In Figure A3 we illustrate the changes in density of the component bacterial phage populations the concentration of the resource for two situations: where there are no bacteria resistant to both phage, N12=0 (Figure A3 (A)), and where there are bacteria resistant to both phages (Figure A3(B)). Despite the difference in fitness (exponential growth rates) of the bacteria and infection parameters of the phage, in the absence of a population resistant to both phages, bacteria resistant to each phage, N1 and N2 continue

to be maintained, although the doubly sensitive population is eliminated ("data" not shown) in a phage limited state. Despite their differences in infection parameters, both phage co-exist in an apparently stable state as well (Figure A3(A)). Although a population of resistant bacteria ascends to dominance, and initially the phage density oscillates with great amplitude, these oscillations become increasingly damped and both phage continue to be maintained along with the N and N1 populations as well as the dominant N12. The lower fitness, N2 population, becomes extinct ("data" not shown).

Once again, by our semi-quantitative fit criterion, with a major exception we consider these theoretical results consistent with those observed in Figure 3.5. That exception is the failure to see the ascent of bacteria resistant to the two phages in our experiments. Whether this can be attributed to postulated too-low growth rate of the N12 population in the chemostat (V12 < w) is an empirical question we cannot answer at this time.

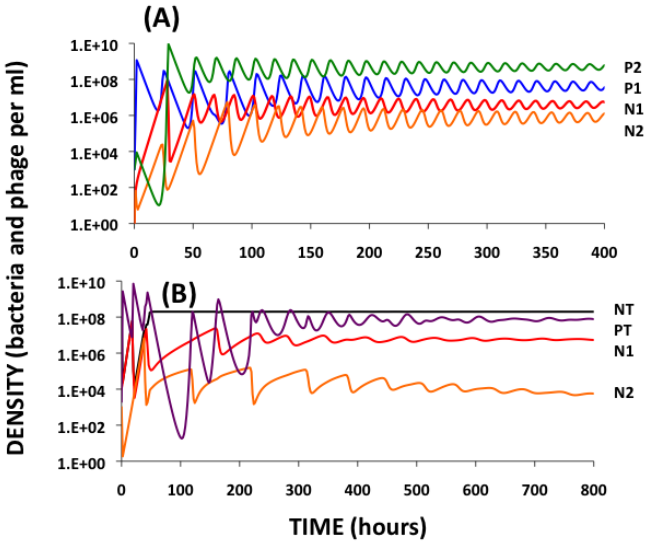


Figure A3 Changes in the densities of planktonic bacteria and phage in continuous culture: Ten possible populations of bacteria, N, N1, N2, N12, NW, NW1,NW2, X, X1,X2 and two populations of phage, P1 and P2. NT is the total density of bacteria and PT is the total density of phage. Standard parameters v=1.0, k=0.25, $e=5x10^{-7}$, δ_{MAX1}=1E-8, δ_{MAX2}=1E-9. β_{MAX1}=50, β_{MAX2}=100 x=0.5, w=0.4. C=100, nx=xn=0.0001, nw=0.01, wn=0.005. (A) No doubly resistant bacteria, v1=0.9, v2=0.85. (B) Doubly resistant bacteria present v1=0.9, v2=0.85, v12=0.80.

Appendix 3: Supplementary information to chapter 4 - The Population and Evolutionary Dynamics of Bacteria in Physically Structured Habitats: The Adaptive Virtues of Motility

What is true for *E. coli* is true for *V. cholerae*

In a study of the fitness costs of phage resistant *V. cholerae*, Wei and colleagues performed pair-wise competition experiments with *V. cholerae* sensitive and resistant to the phage JSF4 (Wei, Ocampo and Levin 2010). As part of those experiments, the relative fitness of the sensitive and resistant strains was examined when the bacteria were competing on surfaces, 0.45 micron filters on nutrient agar. The sensitive *V. cholerae*, N16961 and a spontaneous Rif-r mutant derived from this strain were motile, while all of the JSF4 resistant mutants we tested were non-motile. Although some of the JSF4 phage-resistant bacteria were more fit than this common competitor in liquid, in surface culture the motile strain had a considerable edge over the non-motile.

Our experiments indicated that this motility in *V. cholerae* is also favored when these experiments are performed with the soft agar protocol used in our article, see Figure A1. In these experiments, the non-motile strains are designated N1 - resistant to the phage JSF4 and N2, a JSF4-sensitive clone isolated from a chemostat culture. The procedures used for performing these experiments are identical to those depicted in Figure 4.2 in chapter 4.

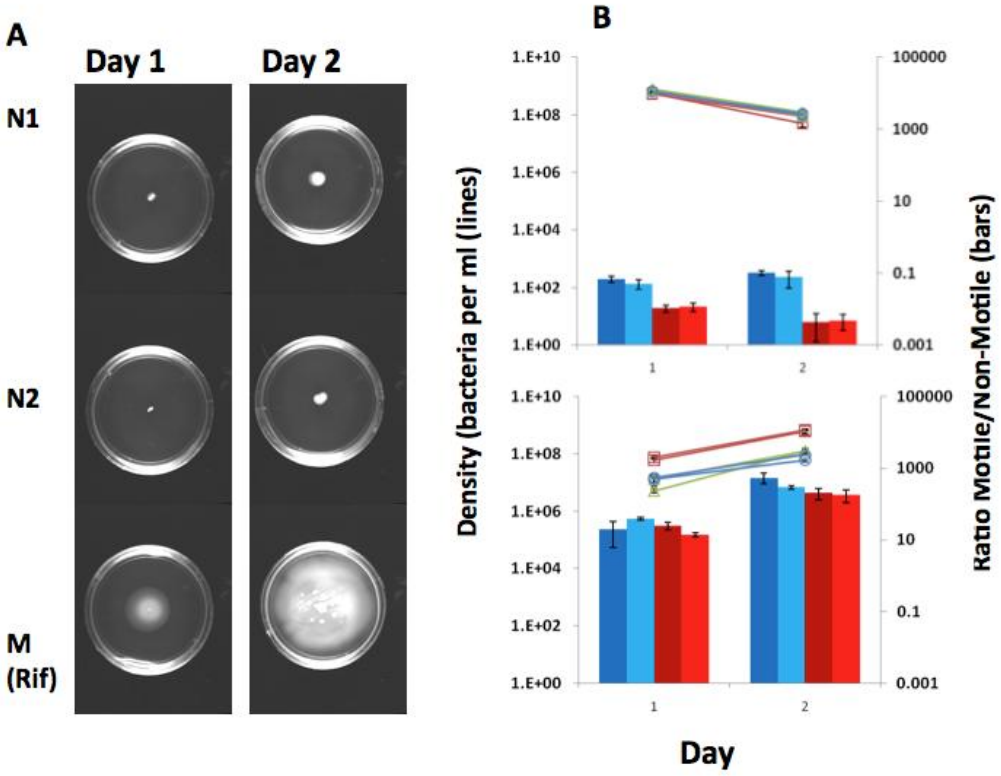


Figure A1 Motile and non-motile strains of *Vibrio cholerae* N16961 in 1% tryptone liquid and 0.35% agar culture. M(Rif) is the motile strain and N1 and N2 are the non-motile. The initial densities of these cultures were approximately 200 - 1000 cells and in the mixed cultures the initial ratio of Motile to Non-motile was 0.68 and 0.63 for the Rif/N1and Rif/N2 cultures, respectively. (A) Bacterial growth on motility agar. (B) Growth and competition in liquid culture, density and ratio of cells after 1 and 2 days of growth. (C) Growth and competition in motility agar, density and ratio of cells after 1 and 2 days of growth. The lines are the total cell densities with the square ticks the motile, and the triangles the non-motile. The bars are the ratio motile to non-motile. Means and standard errors (n=3) for two independent experiments.