# IMPLICATIONS OF BACTERIAL VIRUSES ON PATHOGENIC BACTERIA

## FROM NATURAL MICROBIAL COMMUNITIES TO THERAPEUTIC APPLICATIONS

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V Örmälä-Odegrip, A-M. and Jalasvuori, M. (2013) Phage therapy: Should bacterial resistance to phages be a concern, even in the long run? *Bacteriophage* 3(1):e24219

VI Örmälä-Odegrip, A-M., Eriksson, H., Mikonranta, L., Ruotsalainen, P., Mattila, S., Hoikkala, V., Nilsson, A., Bamford, J. and Laakso, J. Evolution of virulence in *Klebsiella pneumoniae* treated with phage cocktails. *Manuscript*.

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ABSTRACT

Bacterial viruses (i.e. phages) are ubiquitous intracellular parasites of bacteria, that – along with protist grazers – account for majority of bacterial mortality in nature. Phages impose strong selection for bacterial phage-resistance, which is often coupled with fitness costs on bacterial traits such as growth ability, virulence or motility. Traditionally phage-host interactions have been studied with two species' systems in the laboratory, neglecting the complex web of interactions present in natural communities. The ability of phages to selectively kill bacteria has ignited an interest on phages as alternative antibacterials. However, in order to develop phage therapy, understanding of phage-host interactions in the eco-evolutionary context is essential. In this thesis I studied the implications of lytic phages on opportunistic pathogenic bacteria, as opportunists often have the ability reproduce and reside in outside-host environments, where they are predisposed to a variety of selection pressures. The role of phages in top-down control of bacterial biomass and the evolution of bacterial phage-resistance were studied in the presence of protist predators with differing feeding modes, in low-resource systems mimicking natural pond environment. Hypothesis of coincidental evolution suggests that virulence is a by-product of selection for traits that maximize bacterial fitness in environmental reservoirs. Yet, disease outbreaks by opportunists are relatively rare, suggesting that something constrains the selection for virulence. To assess the role of lytic phages on the evolution of virulence, bacteria were cultured in low-resource environment, accompanied with changes in temperature regime or changes in composition of the community of interacting bacterial enemy species, and the virulence of bacteria was measured *in vivo*. To study whether the potential phage-resistant bacteria surviving phage therapy would be coupled with lowered virulence, due to costs associated with phage-resistance, a clinical bacterial isolate was exposed to phage cocktails and the virulence of the phage-resistant bacteria was measured *in vivo*. Given the strong selection for phage-resistance, the prospects of phage therapy depend a great deal on whether new phages infecting pathogenic bacteria can be readily isolated from environment. To address this, an attempt was made to isolate phages against clinical bacterial isolates harboring resistance genes to multiple antibiotics.

A single lytic phage was shown to be a non-efficient top-down regulator of bacterial biomass. Rapidly emerging phage-resistant bacteria took over the bacterial populations after initial lysis by phages and protist grazers accounted for most of the long-term negative trophic effects on bacterial biomass. The presence of protist predators selected for bacteria that were less susceptible to infection by lytic phages, which suggests an overlap in the bacterial defense against a parasite and predatory protists. In general, the presence of lytic phages selected for lowered virulence in bacteria. High temperature selected for more virulent and more motile bacteria, but this was constrained by the presence of a lytic phage. In the multispecies communities the presence of all bacterial enemies led to decreased virulence *in vivo*. Altogether, these results contrast the hypothesis of coincidental evolution, and suggest that the presence of phages in natural reservoirs constrains the evolution of virulence, most likely through fitness costs associated with phage-resistance. Exposure to phage cocktails was also shown to be associated with decreased bacterial virulence in the phage-resistant bacteria. However, exposure to some individual phages resulted in more virulent bacteria, suggesting that the outcome of therapy could depend on the identity of the phage cocktail. Finally, a phage cocktail lysing a wide range of clinical strains was isolated from sewage. This, along with geographical patterns of phage infections suggest that new phages are available in environmental reservoirs for therapy, and the emergence of phage-resistance should not hinder the prospects of phage therapy in the global perspective.

# YHTEENVETO

Bakteerivirukset (faagit) ovat bakteerien solunsisäisiä loisia, jotka yhdessä alkueliösaalistajien kanssa aiheuttavat suurimman osan bakteerien kuolleisuudesta luonnossa. Faagit luovat voimakkaan valintapaineen faagivastustuskyvylle ja vastustuskyvystä seuraa usein kustannus esimerkiksi bakteerin kasvu-, taudinaiheuttamis- tai liikkumiskyvylle. Perinteisesti faagin ja isännän välisiä vuorovaikutuksia on tutkittu kahden lajin kokeissa laboratoriossa, mutta lähestymistapa jättää huomiotta luonnollisissa yhteisöissä vallitsevien vuorovaikutusten kirjon. Koska faagit tappavat kohdennetusti tiettyjä bakteereita, on ehdotettu, että faageja voitaisiin käyttää antibiootteina. Faagiterapian kehittäminen vaatii kuitenkin faagi-isäntä vuorovaikutusten tuntemusta niin ekologisesta kuin evolutiivisestakin näkökulmasta. Tässä väitöskirjassa olen tutkinut lyyttisten bakteerivirusten vaikutuksia opportunistisiin taudinaiheuttajabakteereihin. Osa opportunistisista bakteereista kykenee elämään ja lisääntymään isäntänsä ulkopuolisissa ympäristöissä, missä ne altistuvat lukuisille erilaisille valintapaineille. Tutkin faagien merkitystä bakteeribiomassan vähentäjinä, sekä bakteerien faagivastustuskyvyn evoluutiota matalaravinteisissa lampea muistuttavassa ympäristössä, joissa oli faagien lisäksi läsnä ravinnonhankintatavoiltaan toisistaan poikkeavia alkueliöitä. Sattumanvaraisen evoluution hypoteesin (coincidental evolution hypothesis) mukaan taudinaiheuttamiskyky on seurausta sellaisiin elinkiertopiirteisiin kohdistuvasta valinnasta, jotka parantavat kelpoisuutta isännän ulkopuolisissa ympäristöissä. Opportunistien aiheuttamat tautiepidemiat ovat kuitenkin verraten harvinaisia, mikä viittaa siihen että jokin tekijä ympäristössä toimii vastavoimana taudinaiheuttamiskykyyn kohdistuvalle valinnalle. Faagien vaikutuksia opportunistibakteerien taudinaiheuttamiskyvyn evoluutioon tutkittiin niinikään matalaravinteisessa ympäristössä, joissa vaihtelevina ympäristötekijöinä olivat joko lämpötila tai bakteereja ravinnokseen käyttävien vihollisten eri yhdistelmät. Kokeiden päätteeksi taudinaiheuttamiskyky mitattiin hyönteisissä. Kliinistä taudinaiheuttajabakteerikantaa altistettiin ‘faagikoktaileille’ (phage cocktails) kokeessa, jonka tarkoituksena oli selvittää onko faageille vastustuskykyisten bakteerin taudinaiheuttamiskyky alentunut faagipuolustuksen aiheuttamien kustannusten seurauksena, ja mahdolliset muutokset taudinaiheuttamiskyvyssä mitattiin hyönteisissä. Faagivastustuskykyyn kohdistuvan voimakkaan valintapaineen huomioonottaen faagiterapian onnistumisen kannalta on merkittävää, voidaanko uusia faageja tarvittaessa eristää ympäristöstä. Tavoitteeksi asetettiin eristää uusia faageja, jotka estävät kliinisten antibiooteille vastustuskykyisten bakteereiden kasvua.

Yksittäinen faagityyppi osoittautui tehottomaksi säätelemään bakteeribiomassan määrää pitkällä aikavälillä ja faageille vastustuskykyiset bakteerit palauttivat populaatiot nopeasti lähes kontrollitasolle. Alkueläimet näin ollen vastasivat pitkällä aikavälillä käytännössä kaikesta bakteeribiomassan vähentymisestä. Alkueliöt vaikuttivat myös bakteerien faagivastustuskyvyn evoluutioon: alkueliöille altistuneet bakteerit olivat vähemmän alttiita faagi-infektioille. Korkea lämpötila johti taudinaiheuttamiskykyvyn nousuun bakteereissa, mutta ilmiö kumoutui faagien vaikutuksesta. Monilajisissa yhteisöissä kaikkien bakteeripetojen läsnäolo taas alensi bakteerien taudinaiheuttamiskykyä. Nämä tulokset puhuvat sattumanvaraisen evoluution hypoteesia vastaan ja faagit näyttäisivät sen sijaan luovan valintapaineen vähemmän taudinaiheuttamiskykyisille bakteereille. Myös faagikoktailit aiheuttivat taudinaiheuttamiskyvyn laskua bakteereissa. Jotkut yksittäiset virukset näyttivät kuitenkin nostavan bakteerien taudinaiheuttamiskykyä vastustuskykyisissä bakteereissa, minkä vuoksi tuleekin noudattaa erityistä varovaisuutta valittaessa faageja ‘koktaileihin’ ja faagiterapiaan. Jätevedestä onnistuttiin eristämään kokoelma faageja, jotka estivät kasvua laajassa joukossa kliinisiä antibiooteille vastustuskykyisiä bakteerikantoja. Tämä tulos yhdessä faagi-infektioiden maantiedettä koskevien havaintojen kanssa antaa olettaa, että uusia faageja on eristettävissä ympäristövarannoista ja että laaja-alaisen faagivastustuskyvyn syntyminen ei ole este faagiterapian kehittämiselle tulevaisuudessa.

# 1 INTRODUCTION

In the late 1890's, after the birth of bacteriology, several bacteriologists all made the same observation – an unidentified substance was limiting the growth of their bacterial cultures. It was not until a couple of decades later when an English bacteriologist Fredrik Twort and French-Canadian microbiologist Felix d'Herelle independently presented the same hypothesis, that this unidentified substance was a virus multiplying within the bacteria (Sulakvelidze et al., 2001; Twort, 1915). Twort's research was not pursued due to the lack of funding brought about the onset of the Word War I (Sulakvelidze et al., 2001), however, in 1917 d'Herelle was able to identify "an invisible, antagonistic microbe of the dysentery bacillus" forming clear areas on bacterial lawns, later referred to as plaques. He called it a "bacteriophage" (from the Greek word "phagein" meaning "to eat" or "devour") and since then the name has become established to describe all bacterial viruses to date. Since Twort and d'Herelle's days scientists have isolated vast amounts of bacteriophages, or phages for short, and phages have had an essential role in the development of modern molecular biology.

Phages are viruses infecting only bacterial hosts, and like all viruses, they lack the features required for autonomous replication. In order to reproduce, a phage needs to take over the metabolism of the bacterial cell and an infection by a phage often results in death of the bacterial cell, accompanied with the release of progeny phage particles. The idea of host-specific, self-replicating bacterial killers is well suited for targeting pathogenic bacteria. Indeed, there has been a newfound interest on bacteriophages, due to the growing problem of antibiotic resistance against virtually all known antibiotics. Using bacteriophages as antibacterial agents against harmful bacteria, though, is far from new, and the concept of "phage therapy" was first proposed by d'Herelle already in the late 1910's. The world's leading phage therapy facility Eliava Institute was established in 1923 in Tbilisi, Georgia and phages have been continued to be used therapeutically in Eastern Europe and in the former Soviet Union to this day. The discovery of penicillin by Sir Alexander Fleming in 1928 led to the mass production of antibiotics in the 1940s and 1950s. This, along with the onset of Cold War and the subsequent lack of scientific collaboration between USA and its' allies with the Soviet Union, caused phage therapy to become more or less forgotten in the West.

The story about the discovery of phages and conflicts between the "Eastern" and "Western" medicine is an intriguing one, and the concept of phage therapy a good example of the potential of these tiny bacterial parasites. In nature phages are present in very high numbers, and thus strong drivers of bacterial evolution. In this thesis I introduce the reader to evolutionary implications of bacteriophages on environmentally growing opportunistically pathogenic bacteria, that reside and reproduce in environments apart from their hosts. In the process I will cross

boundaries across the fields of molecular biology, ecology, evolution, medical biology and applications from all of the above. Due to the parasitic nature of phages, this is as much a story about bacteria, as the lives of phages and their hosts are inseparably tangled and shaped through some billions of years of coevolution. It should not go unnoticed that if phages are the headliner of this thesis, the evolution of bacterial virulence should be recognized as the best supporting act, as implications of phages on bacterial virulence form one of the cornerstones of this thesis. Moreover, I would like to remark that understanding the *ecology* and *evolution* behind phage-host interactions is crucial when visioning phage therapy as a potential solution for fighting bacterial infections that are no longer susceptible to conventional antibiotics.

## 1.1 Bacteriophages are obligate intracellular parasites

Bacteriophages are viruses that use exclusively bacterial cells as their hosts and like all viruses, they consist of a DNA or RNA genome packed within a protein capsid. Phages are obligate intracellular parasites, meaning that they cannot replicate without entering the host cell and taking over its metabolism. Phages recognize phage-binding receptors on the surface of the bacterial host cell and inject their genetic material into the host cell. Phages show two distinct types of life cycles; in a lytic life cycle, infection by a phage results in death of the host cell, accompanied with the release of phage progeny. A lysogenic life cycle, in contrast, results in integration of the phage into the host genome, or alternatively, the phage genome can exist as an extrachromosomal plasmid within the host cell (Waldor et al., 2005). An obligatory lytic phage is capable of only lytic life cycles, and could thus be defined as a parasitoid, as an infection with obligatory lytic phages always results in death of the host cell (Godfray, 1994). As opposed to obligatory lytic phages, temperate phages are able to display both lysogenic cycles and lytic cycles. In a lysogenic cycle, following the integration of the phage into the host genome, the host cell is a called a lysogen and the integrated phage a prophage. Lysogenic cycle can be stable for thousands of bacterial generations or result in lysis of the host, depending on environmental conditions and the state of the host cell. Different life cycles of a phage with a DNA genome are presented in Figure 1.

## 1.2 Phage-host interactions

Bacteriophages have been stated to be the most prevalent entities on Earth, as they outnumber their hosts at least by a tenfold (Bergh et al., 1989; Whitman et al., 1998). Most estimates for phage abundance include archaeal viruses. Here, phages are defined strictly as viruses with bacterial hosts, and discussing viruses infecting both bacteria and archaea (together comprising the domain prokaryotes), are referred to as prokaryotic viruses. Most prokaryotes are found in the open ocean, soil, in ocean

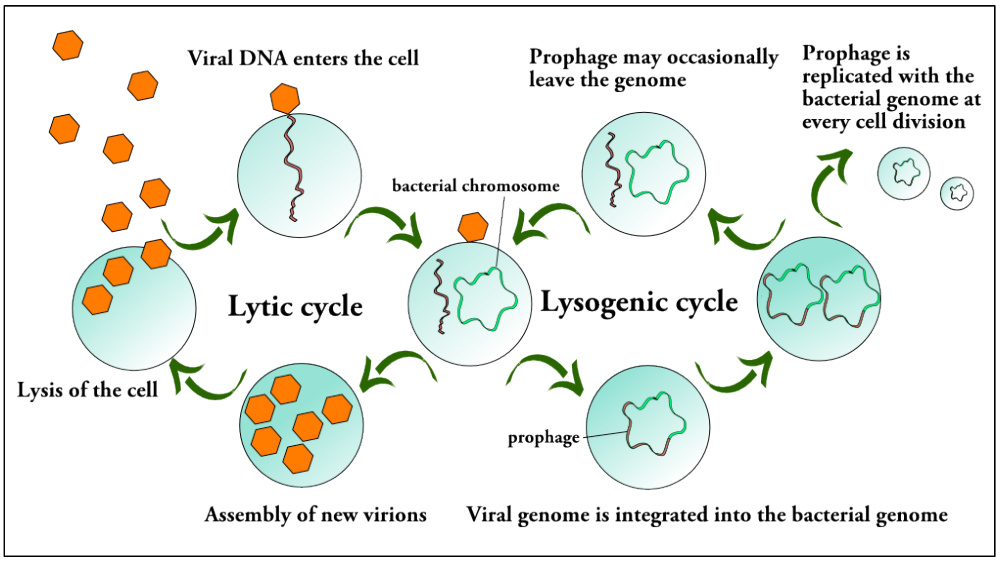


**Figure 1.** Lytic and lysogenic life cycles of a phage.

sediments, and terrestrial sub-surfaces where there are an estimated $1.2 \times 10^{29}$, $2.6 \times 10^{29}$, $3.5 \times 10^{30}$ and $0.25–2.5 \times 10^{30}$ prokaryotic cells, respectively, and the estimated number of prokaryotic viruses reaches up to $10^{31}$ (Bergh et al., 1989; Borsheim et al., 1990; Whitman et al., 1998).

Phages are known to affect competition within and between bacterial populations and species (Joo et al., 2006; Koskella et al., 2012), induce and maintain bacterial diversity (Buckling and Rainey, 2002; Williams, 2013), and mediate horizontal gene transfer among bacteria (Canchaya et al., 2003). Furthermore, phages have major global ecological implications as they are involved in cycling on organic matter in oceans (Fuhrman, 1999). Substantial efforts have been made to estimate quantitatively the degree of virus-induced bacterial mortality and assess the ratio at which phages are accountable for bacterial mortality. The extensive literature has been summarized to an estimated 15 % daily bacterial mortality by phages and this proportion is higher in heterotrophic bacteria than autotrophic bacteria (Chibani-Chennoufi et al., 2004; Suttle, 1994). In ecological context, however, rather than considering the absolute numbers of phage-mediated global bacterial mortality, it is more relevant to look at the impacts of phages within a community or population, as the selection acts locally, and the prevailing biotic and abiotic factors are likely to affect the outcome of selection by phages.

### 1.2.1 Antagonistic coevolution between bacteria and phages

Parasites impose selection for resistant hosts, and reversed, resistant hosts select for more infective parasites, which may result in rapid reciprocal evolution between host resistance and parasite infectivity (Thompson, 1998). Antagonistic coevolution is believed to cause rapid between-population differentiation of both parasites and hosts, which may be critical to the maintenance of genetic variation and the whole process of coevolution in nature (Thompson, 1999). There are two non-mutually exclusive types of selection modes acting on antagonistic coevolutionary relationships. The first is directional selection, resulting in so-called "arms-race" dynamics. In this mode of coevolution both host defense and parasite counter-defense are favored, resulting in increasingly resistant hosts accompanied with increasingly infectious parasites (Dawkins and Krebs, 1979; Gandon et al., 2008). Directional selection is not likely to be continued indefinitely but rather be constrained either genetically or metabolically. With bacteria and phages, directional selection seems to act mostly on populations under laboratory conditions cultured in nutrient rich media (Bohannan and Lenski, 2000; Brockhurst et al., 2007a; Brockhurst et al., 2007b; Brockhurst et al., 2003; Lenski and Levin, 1985).

The second mode of selection is called fluctuating selection. It is characterized by initial arms-race coevolution that eventually changes into oscillations of bacterial and phage genotypes with different resistance and infectivity characteristics, respectively (Gandon et al., 2008; Hall et al., 2011). These oscillations are driven by negative frequency-dependent selection; phages evolve to infect more abundant bacterial genotypes, giving an advantage to rare bacterial types. This results in the rare types becoming more abundant and so on, and the fluctuations of parasite and host phenotypes could potentially continue indefinitely (Burdon and Thrall, 1999). Fluctuating dynamics is assumed to dominate in natural microbial populations and has been shown to act in natural microbial communities in soil (Gomez and Buckling, 2011). One explanation for why directional selection seems to dominate in laboratory and fluctuating selection in natural microbial communities is that resistance is more costly in natural environments due to competition and access to fewer resources, and therefore resistance to previous phage types is lost in favor of specific resistance to contemporary phage (Gomez and Buckling, 2011; Lopez-Pascua and Buckling, 2008). This increased cost of resistance is likely to both constrain the continual arms-race selection towards increased host resistance and phage infectivity, and lead to fluctuating dynamics as new means of resistances are gained and old ones lost (Gomez and Buckling, 2011; Koskella and Brockhurst, 2014).

#### 1.2.1.1 Bacterial resistance against phages

Succesful infection by a lytic phage inevitably leads to death of the bacterial host cell, as host lysis is required for the release of phage progeny and transmission of phage particles to new susceptible hosts. This imposes a strong selection pressure for phage-resistance in bacteria. Bacteria can evolve resistance against phages with a number of mechanisms. One common way of avoiding infection by a phage is by preventing the adsorption of the virus on bacterial surface. These adsorption-blocking mechanisms can be divided into three categories: (i) blocking of phage receptors, (ii) production of extracellular matrix and the (iii) production of competitive inhibitors (Labrie et al., 2010). However, if a phage still successfully binds on the cell surface, there are a number of mechanisms directed at terminating the infection post phage adsorption. In the superinfection exclusion system (Sie), phage binds on the bacterial receptors but the entry of the phage genome into host cell is inhibited. The genes encoding Sie-proteins are often found in prophages, suggesting that in many cases Sie systems are important for phage–phage interactions rather than phage–host interactions (Labrie et al., 2010). Many bacteria harbor restriction-modification systems, which recognize and degrade unmethylated phage DNA after it has entered the cell, thus aborting infection (Pingoud et al., 2005). Bacteria can also gain adaptive immunity via clustered regularly interspaced short palindromic repeats (CRISPRs) and the associated Cas genes (Barrangou et al., 2007). In CRISPR-Cas system, the bacteria incorporate short sequences from invading genetic elements (such as a phage) into a region in the genome including CRISPRs. In conjunction with host Cas genes, these sequences encode multifunctional protein complexes that recognize and cleave incoming foreign genetic material (Bhaya et al., 2011).

Bacterial resistance against lytic phages has been found to come with substantial fitness costs for bacteria (Bohannan et al., 2002; Lenski, 1988b), including an increased cost of deleterious mutations (Buckling et al., 2006), decreased ability to metabolize carbon (Middelboe et al., 2009), decreased growth rate (Bohannan et al., 2002; Bohannan and Lenski, 1999; Buckling et al., 2006; Lenski, 1988b), changes in competitive ability (Brockhurst et al., 2005; Lennon et al., 2007; Quance and Travisano, 2009) and increased susceptibility to other phages (Avrani et al., 2011; Marston and Sallee, 2003). A study conducted with *E. coli* and phage T2 showed that the costs associated with bacterial phage-resistance vary across environments when bacteria are cultured in batch cultures or chemostats with glucose or trehalose as a limiting nutrient (Jessup and Bohannan, 2008). This suggests that the pleiotropic costs associated with phage-resistance are mediated by the biotic and abiotic selection also in natural microbial communities. The cost of phage-resistance on bacterial virulence is discussed in Chapter 1.2.1.

### 1.2.2 Phage-host interactions in multispecies communities

Traditionally phage-host interactions have been studied with two species' systems in the laboratory. The differences observed in the coevolutionary dynamics in laboratory vs. natural systems (See Chapter 1.2.1) show how abiotic environment has major implications on the evolutionary outcome of phage-host interaction. Against this background it seems justified to expect that the presence (or lack) of additional biotic factors, such as interacting species, should shape the interaction between phages and bacteria.

There are only a handful of studies investigating phage-host interactions in multi-species communities. Gomez and Buckling (2011) studied the phage-host dynamics of *Pseudomonas fluorescens* SBW25 and its' associated lytic bacteriophage inoculated in natural soil microcosms (with and without the natural microbial community). They found that coevolutionary dynamics observed in the systems was fluctuating, as opposed to the arms race dynamics previously seen in the laboratory settings with the same organisms (Brockhurst et al., 2007b). Interestingly, the coevolutionary dynamics between the phage and bacteria were very similar in the presence and absence of natural microbial community. However, there were significant differences in the population dynamics, especially regarding bacterial densities; phages decreased bacterial population size in the presence of the natural soil community, whereas in the absence of the natural community, phages increased the amount of bacteria in the system. A study by Friman and Buckling (2013) showed that the presence of a predatory protist *Tetrahymena thermophila* changed the coevolutionary dynamics between bacteriophage Φ2 and *Pseudomonas fluorescens* from the arms race dynamics observed in the laboratory settings (Buckling and Rainey, 2002) towards fluctuating dynamics. This was presumably resulting from bacterial diversification due to conflicting selection for defense specialists against the two enemies, as there was a trade-off with allocating into one or the other. The study concluded that strong pairwise coevolutionary interactions take place in more complex communities, but the presence of additional interacting species could qualitatively alter these interactions. Also the presence of additional phages has been shown to alter the coevolution with a phage and its' host: Koskella et al. (2012) assessed the fitness costs on *Pseudomonas syringae* in communities with a single lytic phage or multiple lytic phages and bacteria exposed to heterogenous phage populations were found to have lower fitness in terms of growth when measured without the phages.

## 1.3 Environmentally growing opportunistic pathogenic bacteria

There are a variety of slightly differing definitions for a pathogen, but here we define pathogen as a micro-organism capable of causing damage to its' host

and this damage can result from either direct microbial action or from the host immune response (Casadevall and Pirofski, 1999). Virulence, in turn, is defined here as the relative capacity of a microbe to cause damage in a host (Casadevall and Pirofski, 1999). Traditional theories on virulence evolution state that virulence is an unavoidable consequence of selection for maximizing parasite fitness in a given environment (de Roode et al., 2008; Frank, 1996; Levin, 1996). Parasites must replicate within hosts and this consumes host resources, damages host tissues and provokes host immune response, thereby shortening the infectious period during which parasite transmission can take place. Parasites thus face a trade-off between the benefits of increased replication and virulence, often resulting in highest fitness at intermediate levels of parasite replication (de Roode et al., 2008). This is referred to as the transmission-virulence trade-off. While obligate pathogens are dependent on their hosts and thus subjected to the transmission-virulence trade-off, environmentally growing opportunistic pathogens reside and reproduce in outside-host environments, occasionally causing disease outbreaks. In fact, many well-known bacterial pathogens co-exist with their host relatively peacefully, or reside in environments completely separated from their hosts (Brown et al., 2012). Common opportunist environmentally growing bacteria include e.g. *Vibrio cholera*, *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Listeria monocytogenes*, *Cryptococcus neoformans* and many species from *Mycobacterium*, *Flavobacterium*, *Serratia* genus and *Klebsiella* genus (Freitag et al., 2009; Friedman et al., 2002; Grimont and Grimont, 1978; Hall-Stoodley and Stoodley, 2005; Hilbi et al., 2007; Kunttu et al., 2009; Leclerc et al., 2002; Mahlen, 2011; Rahman et al., 2008; Restuccia and Cunha, 1984; Soto et al., 2008).

The diverse selection pressures in the environmental reservoirs arising from e.g. predation, parasitism and ecological changes may have correlated effects on the evolution of pathogen virulence. The coincidental virulence evolution hypothesis (Levin and Svanborg Eden, 1990; Read, 1994) suggests that bacterial virulence could be a by-product of selection acting on a pathogen's life-history traits that increase its fitness in environmental reservoirs and coincidentally also contribute to its virulence in a host. The outcome of selection in outside-host environments on bacterial virulence, though, is dependent on whether the traits that are selected for are correlated with virulence. For example, the competitive and co-operative traits of bacteria increase bacterial fitness and survival in natural microbial communities. However, these same traits also affect the severity of an infection by affecting how fast bacteria can proliferate within their hosts (Harrison et al., 2006; Inglis et al., 2009). Predation by protist predators is also known to contribute to bacterial virulence, as traits acting as defence mechanisms against protist predators can be used to invade the hosts, or after successful colonization, evade the host immune system (Cirillo et al., 1999; Harb et al., 2000; Lainhart et al., 2009; Matz and Kjelleberg, 2005; Rasmussen et al., 2005; Steinberg and Levin, 2007). Temperature is known to be important environmental factor for the expression of several bacterial virulence

factors (Konkel and Tilly, 2000) and elevated growth temperature leads to more virulent phenotypes in e.g. and *Legionella pneumophila* (Mauchline et al., 1994) and some *Shigella* species (Maurelli et al., 1984). Elevation in temperature has also been connected to evolution of virulent strains of *Flavobacterium columnare* in fish farms (Pulkkinen et al., 2010), altogether suggesting that high temperature environments could indirectly select for more pathogenic bacteria.

Even though there are studies showing positive correlations between bacterial virulence and traits improving survival in environmental reservoirs, the disease outbreaks driven by opportunistic bacterial pathogens are relatively rare. This suggests that some selective forces in environmental reservoirs are also likely select for lowered bacterial virulence. In this thesis I have studied the effects of phages on environmentally growing opportunistic bacteria. Implications of phages on bacterial virulence in general are discussed in the next chapter.

### 1.3.1 Bacteriophages and bacterial virulence

Temperate phages showing lysogenic life cycles are known to contribute to bacterial virulence. Brussow et al. (2004) have listed five factors how temperate phages can affect bacterial virulence through bacterial fitness; (i) by acting as anchor points for genome rearrangements, (ii) via gene disruption, (iii) by protection from lytic infection, (iv) by lysis of competing strains through prophage induction, and (v) by introduction of new fitness factors. The phage-mediated horizontal gene transfer occurs either via transduction or lysogenic conversion. Transduction refers to phage-mediated transfer of bacterial genes from one bacterium to another, whereas lysogenic conversion is characterized by an altered bacterial phenotype, resulting from a prophage-encoded genes. Lysogenic conversion is often coupled with increased virulence in pathogenic bacteria (Abedon, 2008; Abedon and Lejeune, 2005; Boyd et al., 2001). There are many bacterial virulence factors (VF) contributing to bacterial virulence that are known to be encoded by temperate phages (Brussow et al., 2004). Examples of pathogenic bacteria that produce specific phage-encoded exotoxins (acting as the causative agent of a specific disease) include *Vibrio cholerae* (Waldor and Mekalanos, 1996), Shiga toxin-producing *Escherichia coli* (Shaikh and Tarr, 2003), *Corynebacterium diphtheria* (Freeman, 1951), and *Clostridium botulinum* (Barksdale and Arden, 1974). The associated phage-encoded toxins are cholera toxin, Shiga toxin, diphtheria toxin and botulism toxin, respectively. *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Salmonella enterica* serovar Typhimurium, in turn, are examples of bacteria harboring multiple prophages where each phage-encoded virulence or fitness factor contributes incrementally to the fitness of the bacterium (Brussow et al., 2004). The studies currently available on the production of phage-linked VFs focus solely on lysogens (including induced lysogens) and there are no

studies reporting virulence factors encoded by obligatory lytic phages (Abedon and Lejeune, 2005).

As opposed to temperate phages, lytic phages are not commonly associated with increased bacterial virulence. However, lytic phages have been shown to select for more co-operative bacteria producing extracellular products ('public goods') that are crucial for growth and virulence in *Pseudomonas fluorescens* (Köhler et al., 2009; Morgan et al., 2012). A recent study with *Pseudomonas aeruginosa*, ciliate *Tetrahymena thermophila* and a lytic phage showed that predation by ciliates selected for lowered bacterial virulence, and this was constrained by lytic bacteriophages (Friman and Buckling, 2014), however, phages alone did not select for increased virulence. Interestingly, selection by heterogeneous lytic phage populations has been shown to result in elevated cytotoxicity in cultured mammalian cells with *Pseudomonas aeruginosa* (Hosseinidoust et al., 2013). However, whether these effects on cell cultures translate to virulence *in vivo* calls for future research.

Selection by phages has also been associated with lowered virulence in bacteria. Selection by lytic bacteriophages imposes a strong pressure for phage-resistance. Resistance to phages, in turn, has been shown to be often negatively correlated with bacterial traits linked to virulence, such as growth efficiency (Bohannan and Lenski, 1999; Brockhurst et al., 2004; Lenski, 1988a; Lenski and Levin, 1985) and motility (Brockhurst et al., 2005; Heierson et al., 1986; Paruchuri and Harshey, 1987). Growth efficiency and motility are both virulence factors, as motility can help pathogens colonize suitable niches within the host (Josenhans and Suerbaum, 2002; Lane et al., 2007) and growth efficiency can determine how fast bacteria can exploit their hosts (de Roode et al., 2005; Frank, 1996; Harrison et al., 2006). Indeed, phage-resistance in bacteria has been shown to be associated with decreased virulence in many bacterial species, such as *Serratia marcescens* (Flyg et al., 1980), *Salmonella enterica* (Santander and Robeson, 2007), *Staphylococcus aureus* (Capparelli et al., 2010), *Bacillus thuringiensis* (Heierson et al., 1986), *Vibrio cholera* (Zahid et al., 2008), *Yersinia pestis* (Filippov et al., 2011), *Klebsiella pneumoniae* (Gu et al., 2012) and *Flavobacterium columnare* (Laanto et al., 2012). Altogether, if phage-resistance is traded off with traits contributing to bacterial virulence, phages could potentially select for lowered virulence in environmentally growing opportunistic bacteria.

Possible costs on virulence and the subsequent lowered virulence is especially intriguing in the context of phages having been championed as an alternative antibacterial agent in treating bacterial infections. In fact, the appearance of less virulent or avirulent phage-resistant bacteria has been observed in association with phage based antibacterial therapy trials with *Escherichia coli* and *Vibrio cholerae* (Smith and Huggins, 1983; Zahid et al., 2008)

## 1.4 Phage therapy

Phage therapy, the use of bacterial viruses in treating bacterial infections, has received attention during the past decades, triggered by the increasing problem of antibiotic resistance in pathogenic bacteria. While the primary focus of phage therapy is on treating human infections, the concept of phage therapy can be extended to eliminating undesirable bacteria e.g. in agriculture, aquaculture and food products (Abedon, 2008). The use of phages to treat bacterial infections dates back to the early 1900s and, despite the failure of phage therapy becoming established in the western medicine, phage therapy has persisted in some countries, particularly in the former Soviet Republic of Georgia, where phages are often used as the standard treatment for bacterial infections (Abedon et al., 2011; Kutter et al., 2010; Miedzybrodzki et al., 2012). In the phage therapy center in Wroclaw, Poland, phages are used to treat especially chronic bacterial infections that are proven to be resistant to antibiotic treatment (Kutter et al., 2010). Given the Georgian and Polish experiences, there is indisputable evidence of the medical potential of phages to treat antibiotic-resistant bacterial infections. However, whether the potential of phage therapy can be well integrated into most western models of drug development, regulation and clinical implementation is uncertain (Chan et al., 2013).

Phages can be applied locally or systemically and the process of using phages for therapy is conceptually simple, although rather complex pharmacokinetics could be associated with phage therapy, due to the ability of phages to replicate and thus increase in numbers during the course of therapy (Abedon, 2011; Chan et al., 2013). The use of phage preparations consisting of a single phage isolate is called monophage therapy, whereas the use of preparations with multiple phages is referred to as polyphage (or multiphage) therapy (Chan and Abedon, 2012; Hall et al., 2012; Levin and Bull, 2004). The primary motivation for the use of cocktails consisting of multiple phage types, is their broad host range, in comparison to individual phage isolates. Phage cocktails can also affect more bacterial types, or work under a larger variety of conditions. Combining phages into cocktails can also facilitate better targeting of multiple strains making up a bacterial species or target multiple species that might be responsible for the same disease. Importantly, the use of cocktails can also delay the appearance of phage-resistant mutants (Tanji et al., 2004). A number of studies have investigated the potential of phage cocktails in treating infections caused by i.e. *Pseudomonas aeruginosa* (Alemayehu et al., 2012; Fu et al., 2010; Hall et al., 2012; Hawkins et al., 2010; McVay et al., 2007; Wright et al., 2009), *Klebsiella pneumoniae* (Gu et al., 2012), *Vibrio cholerae* (Jaiswal et al., 2013), *Escherichia coli* (Abuladze et al., 2008; Anany et al., 2011; Callaway et al., 2008; Denou et al., 2009; Maura et al., 2012; Oliveira et al., 2010; Rozema et al., 2009; Viazis et al., 2011), *Listeria monocytogenes* (Anany et al., 2011), *Salmonella enterica* (Andreatti Filho et al., 2007; Borie et al., 2008; Hooton et al., 2011; Wall et al., 2010), *Enterococcus faecalis* (McLean et al., 2011), *Campylobacter jejuni* (Carvalho et al., 2010) and *Clostridium perfringens*

(Miller et al., 2010). The results from these studies vary from very promising reductions in bacterial densities to quite modest reductions. To optimize phage cocktails for improved efficacy, several techniques have been proposed; a procedure with *Staphylococcus* phage K was described by Kelly et al. (2011), where previously resistant bacterial strains were used to select for broad-range phage mutants. Gu et al. (2012) have described a similar selection based method for composing phage cocktails; they isolated phages for the cocktail using wild-type and phage-resistant mutants as hosts for phage isolation. Filippov et al. (2011), in turn, identified *Yersinia pestis* receptors for eight bacteriophages and phages attaching to at least seven different *Y. pestis* receptors were postulated to be promising for formulation of phage cocktails. In addition to reducing bacterial densities, most phage-resistant bacterial variants showed attenuated virulence when tested in a mice model. This suggests that the use such engineered phage cocktails could be coupled with lowered virulence in the bacteria that would potentially survive exposure to phage cocktail.

Phage therapy is mostly criticized for the lack of double-blinded clinical trials that are essential in the process of developing new therapeutics in western medicine (Abedon et al., 2011; Chan et al., 2013). These randomized controlled studies are expensive, and the lack of such studies on phage therapy is most likely due to the relative lack of funding, rather than a result of phages failing to show evidence of efficacy (Chan et al., 2013). The challenges of phage therapy are not obviously limited to this, but at the moment development of phage therapy in western countries seems to be slowed down by the legislation, rather than the research itself (or the lack of it). However, despite the current lack of breakthroughs in human medicine, there are some commercially available phage based food additives approved by the United States Food and Drug Administration (FDA), such as LISTEX™P100 (FDA, 2006) and SALMONELEX™ (FDA, 2013) (MICREOS Food safety, Inc., Wageningen, The Netherlands) targeting *Listeria monocytogenes* and *Salmonella enterica*, respectively, along with "ListShield™", "EcoShield™" and "SalmoFresh™") (Intralytix, Inc., MD, USA) (FDA, 2014), targeting *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella enterica*, respectively .

Potential problems associated with phage therapy as a treatment include the capacity of some phages to carry genes encoding toxins and the potential to transfer bacterial genes between bacteria (transduction) (Skurnik et al., 2007). This calls for careful characterization of the phages, in order to minimize secondary pharmacodynamics (Abedon, 2011). The narrow host range of phages could also be a disadvantage, although the lytic spectrum of phage cocktails can be much broader than the spectrum of activity of individual phage types (Loc-Carrillo and Abedon, 2011).

There are also advantages associated with the use of phages as therapeutics in comparison to traditional chemical antibacterials. First, the success of phage therapy is not affected by the rapidly spreading resistance against traditional

antibiotics (Loc-Carrillo and Abedon, 2011). Secondly, phages are abundant, diverse, easily isolated and, in some cases, readily characterized (Chan et al., 2013). In addition, a substantial fraction of phages are not inherently toxic to life forms other than their target bacteria (Curtright and Abedon, 2011) and collateral damage to normal microbiota, which can be associated with the use of less specific chemical antibacterials, (Rea et al., 2011) could be avoided.

# 2 AIMS OF THE STUDY

The aim of this study was to assess implications of lytic bacteriophages on environmentally growing opportunistic pathogenic bacteria facing diverse selection pressures in natural microbial communities. The main themes of this thesis are i) phage-bacteria interactions within multispecies communities, ii) implications of phages on the evolution of bacterial virulence in environmentally growing opportunistic bacteria and iii) whether the emergence of phage-resistant bacteria annihilates the hopes of developing effective phage therapy to fight pathogenic bacteria. The more specific aims of each chapter were:

I To investigate the top-down effects of a lytic bacteriophage on biomass of opportunistic bacterium in microbial consumer-resource communities with multiple interacting species.

II To study how the presence of protist predators affects the susceptibility of bacteria to infection by lytic bacteriophages.

III To study how outside-host selection by a lytic phage and different temperature regimes in environmental reservoirs shape the evolution of virulence in an opportunistic pathogen.

IV To study how outside-host selection by communities consisting of a lytic phage and two predatory protists affect the evolution of bacterial virulence in an opportunistic pathogen.

V To discuss the potential emergence of global scale phage-resistance associated with phage therapy.

VI To study the virulence evolution of opportunistic bacteria that would potentially survive exposure to phage cocktails.

VII To isolate phages for constructing a phage cocktail lysing a wide range of multi-resistant clinical isolates of *Klebsiella pneumoniae*.

## 3 SUMMARY OF THE MATERIALS AND METHODS

An overview of the methods used in this thesis is presented below. All study species are listed in Table 1. More detailed descriptions of methods and study species used can be found in the corresponding chapters.

**Table 1.**

| | Species | Strain | Source / Reference |
|---|---|---|---|
| I, II, IV | *Serratia marcescens* | Db11 | Flyg, Kenne, and Boman 1980 |
| I, II, IV | *Acanthamoeba castellanii* | CCAP 1501/10 | Culture Collection of Algae and Protozoa |
| I, II, IV | *Tetrahymena thermophila* | ATCC 30008 | American Type Culture Collection |
| I, II, IV | Phage Semad11 | | Zhang et al. 2014 |
| III | *Serratia marcescens* | ATCC 13380 | American Type Culture Collection |
| III | Phage PPV | | Friman et al. 2011 |
| III | *Parasemia plantaginis* | | Friman et al. 2011 |
| IV | *Drosophila melanogaster* | Oregon R | Christina Nokkala Univ. Of Turku |
| VI | Phage F524B | | Not published |
| VI | Phage F524H | | Not published |
| VI | Phage F524K | | Not published |
| VI | Phage F524X | | Not published |
| VI | *Galleria mellonella* | | Kreca, Ermelo, Netherlands |
| VII | *Klebsiella pneumoniae* | 07RAFM-KPN-501 | Barbro Olsson Liljequist (SMI) |
| VII | *Klebsiella pneumoniae* | 07RAFM-KPN-502 | Barbro Olsson Liljequist (SMI) |
| VII | *Klebsiella pneumoniae* | 07RAFM-KPN-503 | Barbro Olsson Liljequist (SMI) |
| VII | *Klebsiella pneumoniae* | 07RAFM-KPN-505 | Barbro Olsson Liljequist (SMI) |
| VII | *Klebsiella pneumoniae* | 07RAFM-KPN-506 | Barbro Olsson Liljequist (SMI) |
| VII | *Klebsiella pneumoniae* | 07RAFM-KPN-507 | Barbro Olsson Liljequist (SMI) |
| VII | *Klebsiella pneumoniae* | 07RAFM-KPN-510 | Barbro Olsson Liljequist (SMI) |
| VII | *Klebsiella pneumoniae* | 07RAFM-KPN-511 | Barbro Olsson Liljequist (SMI) |
| VII | *Klebsiella pneumoniae* | 07RAFM-KPN-512 | Barbro Olsson Liljequist (SMI) |
| VII | *Klebsiella pneumoniae* | 07RAFM-KPN-513 | Barbro Olsson Liljequist (SMI) |
| VII | *Klebsiella pneumoniae* | 07RAFM-KPN-514 | Barbro Olsson Liljequist (SMI) |
| VII | *Klebsiella pneumoniae* | 07RAFM-KPN-515 | Barbro Olsson Liljequist (SMI) |
| VI, VII | *Klebsiella pneumoniae* | 07RAFM-KPN-524 | Barbro Olsson Liljequist (SMI) |
| VII | *Klebsiella pneumoniae* | 07RAFM-KPN-525 | Barbro Olsson Liljequist (SMI) |
| VII | *Klebsiella pneumoniae* | 07RAFM-KPN-527 | Barbro Olsson Liljequist (SMI) |
| VII | *Klebsiella pneumoniae* | 07RAFM-KPN-534 | Barbro Olsson Liljequist (SMI) |

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| VII | *Klebsiella pneumoniae* | 07RAFM-KPN-537 | Barbro Olsson Liljequist (SMI) |
| VII | *Klebsiella pneumoniae* | 07RAFM-KPN-542 | Barbro Olsson Liljequist (SMI) |
| VII | *Klebsiella pneumoniae* | 07RAFM-KPN-549 | Barbro Olsson Liljequist (SMI) |
| VII | *Klebsiella pneumoniae* | 07RAFM-KPN-550 | Barbro Olsson Liljequist (SMI) |
| VII | *Klebsiella pneumoniae* | 07RAFM-KPN-552 | Barbro Olsson Liljequist (SMI) |
| VII | *Klebsiella pneumoniae* | 07RAFM-KPN-557 | Barbro Olsson Liljequist (SMI) |
| VII | *Klebsiella pneumoniae* | 07RAFM-KPN-559 | Barbro Olsson Liljequist (SMI) |
| VII | *Klebsiella pneumoniae* | 07RAFM-KPN-566 | Barbro Olsson Liljequist (SMI) |
| VII | *Klebsiella pneumoniae* | 70165 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 200825 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 1534 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 200832 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 71076 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 13663 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 10924 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 70708 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 2008022 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 14358 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 70415 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | IR8 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | N17 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 10ED | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | IR21 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | N6 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | OS506/08 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | IR25 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | N21 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | IR15 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | B357 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | N11 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | N26 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | K6 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | K1 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | K9 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | N12 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | N14 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | N13 | Christian G. Giske (KI) |

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| VII | *Klebsiella pneumoniae* | N27 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ED | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | Bolkan/84 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | IR27 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | IR34 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | HR10 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | HR4 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | K16 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | VPKP374 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | VPKP205 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | VPKP309 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | VPKP440 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | VPKP6 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | VPKP389 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | VPKP194 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | VPKP203 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | VPKP842 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | VPKP83 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | VPKP229 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | VPKP267 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | VPKP307 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | VPKP754 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET500127 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET500154 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET500164 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET500222 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET500312 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET500317 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET500219 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET500322 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET500325 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET500341 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET500392 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET500402 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET500492 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET500516 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET500529 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET500545 | Christian G. Giske (KI) |

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| VII | *Klebsiella pneumoniae* | 11ET500611 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET500626 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET500635 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET500727 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET500748 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET500756 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET500790 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET500874 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET500905 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET500950 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET501000 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET501093 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET501104 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET501154 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET501157 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET501159 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 11ET501161 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 12ET500034 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 12ET500040 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 12ET500058 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 12ET500059 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 12ET500062 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 12ET500080 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 12ET500088 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 12ET500094 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 12ET500102 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 12ET500130 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 12ET500136 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 12ET500146 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 12ET500149 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 12ET500155 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 12ET500168 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 12ET500169 | Christian G. Giske (KI) |
| VII | *Klebsiella pneumoniae* | 12ET500170 | Christian G. Giske (KI) |
| SMI = Smittskyddsinsitutet, Solna, Sweden | | | |
| KI = Karolinska Institute, Solna, Sweden | | | |

### 3.1 Isolation of phages (III, VI, VII)

Bacteriophages were isolated from samples taken from sewage plant effluent with enrichment method as follows. The collected samples were transferred to 500 ml Erlenmeyer flasks, each containing 100 ml of fresh Luria Broth (LB) (Sambrook and Russel, 2001). The flasks were inoculated with 200 µl of appropriate bacterial culture in the late logarithmic phase and maintained at room temperature or 37 °C for 24 hours with constant agitation of 220 rpm. Bacteria were collected by centrifugation (3 min, 17000 x g) and 100 µl of the supernatant was plated with 200 µl of host bacteria and LB-agar (0.7 %) with the overlay method. To establish genetically homogenous phage populations, single plaques were picked, suspended in 400 µl of $dH_2O$ and plated with 200 µl of host bacteria. Final phage stocks were prepared by collecting the soft agar layer from semiconfluent plates and incubating the agar culture for 3 h at 37 °C. The cell debris was removed from the culture by centrifugation (Sorvall SS-34 rotor, 9000 rpm, 20 min, 4 °C). Stocks were filtrated through a 0.45 µm filter (Whatman, Puradisc 30).

### 3.2 Isolation of phage DNA (III, VI)

1. Phage particles were precipitated with PEG (0.5 M NaCl, 10 % PEG 6000), and further purified in a caesium chloride density gradient (1.5 g/ml CsCl, 50 mM Tris-HCl pH 7.2, 100 mM $MgCl_2$, 150 mM NaCl). (III)

2. High titer phage stocks were treated with DNAse I overnight, followed by proteinase K treatment. DNA solution was then purified using phage lock gel and 25:24:1 phenol:chloroform:isoamyl alcohol. Finally DNA was precipitated with 96 % ethanol. (VI)

### 3.3 Sequencing of phages (III)

Phage DNA was purified as described above and digested with restriction enzymes. The resulting bands were observed using agarose gel electrophoresis. Three separate restriction fragments of the genomic DNA of PPV were cloned into the pSU18 plasmid. The inserts were sequenced with ABI Prism® 3130x1. We followed the manufacturer's protocol by using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

### 3.4 Transmission electron microscopy of phage particles (III, VI)

Purified particles were stained with 1 % phosphotungstic acid (PTA), pH 6.5 (III) or 1 % uranyl acetate on carbon-coated grids (VI), and examined with a transmission electron microscope (Jeol JEM-1200EX, 60 kV (III) or Tecnai G2, 80 kV (VI).

### 3.5 Selection experiments (I, II, III, IV, VI)

1. *Serratia marcescens* Db11 (B) was cultured alone or in a co-culture with *Acanthamoeba castellanii* (A), *Tetrahymena thermophila* (C) and bacteriophage Semad11 (P) in five combinations (B, BA, BC, BP and BACP) for eight weeks. There were four replicates for each treatment. The experiment was executed in 25 $cm^2$ polystyrene flasks with 0.2 µm hydrophobic filter membrane caps (Sarstedt) in NAS (New Cereal Leaf - Page's modified Neff's amoebae saline) medium (Page, 1988). The total volume of static liquid co-cultures was 15 ml. Flasks were incubated in 25 °C and 50 % of the cultures were renewed weekly. Before every renewal, four flasks from each treatment were randomly chosen for destructive sampling. (I and II)

2. *Serratia marcescens* Db11 (B) was cultured alone or in a co-culture with *Acanthamoeba castellanii* (A), *Tetrahymena thermophila* (C) and bacteriophage Semad11 (P) in eight combinations (B, BA, BC, BP, BAC, BAP, BCP and BACP) for eight weeks. There were eight replicates for each treatment. The experiment was executed in 25 $cm^2$ polystyrene flasks with 0.2 µm hydrophobic filter membrane caps (Sarstedt). The total volume of static liquid co-cultures was 15 ml. Flasks were incubated in 25 °C and 50 % of the cultures were renewed weekly. (IV)

3. *Serratia marcescens* (ATCC strain #13880) was cultured in two temperatures; 25 °C and 37 °C, in the presence and absence of phage PPV. The experiment was conducted in 250 ml plastic Erlenmeyer flasks (Corning), capped with membrane filters to maintain aerobic conditions. Bacteria was cultured in phosphate-buffered hay extract containing 2.15 mg $l^{-1}$ of plant detritus (Friman et al., 2008) in total volume of 30 ml. Ten microcosms were established in both low (25 °C) and high (37 °C) temperature regime; and five replicates from of each were inoculated with the phage. The microcosms were renewed at four-day intervals by transferring 90 % of the cultures to new microcosms containing fresh bacterial culture medium.

4. Continuous batch cultures with *Klebsiella pneumoniae* Kpn524 were conducted in 50 ml conical centrifuge tubes in $LB_{amp}$ (final concentration

of ampicillin 100 µg/ml) with total volume of 10 ml. Kpn524 was grown overnight at 30 °C on $LB_{amp}$ -plates (final concentration of ampicillin 50 µg/ml), a single colony was inoculated into $LB_{amp}$ and grown overnight at 37 °C. Phages F524B, F524H, F524K and F524X were added in ten different combinations with three replicates for each microcosm, resulting in 30 tubes in total. Microcosms were initiated by adding 10 µl of late log phase bacterial culture and 10 µl of each appropriate phage stock, depending on the microcosm combination. All phage stocks were adjusted to $10^6$ pfu/ml. The continuous cultures were renewed daily by transferring 10 µl of the culture to a fresh tube with fresh medium (to give a 99 % renewal). 10 µl of appropriate phage stock was added to all phage-containing tubes at every renewal.

### 3.6 Bacterial growth measurements (I, II, III, IV, VI)

Bacterial growth and biomass measurements were done using a Bioscreen C® spectrophotometer (420–580 nm broadband filter) on 100-well "Honeycomb 2" plates (Oy Growth Curves Ab Ltd). Bacteria were inoculated into 400 µl of or LB medium. When measuring bacterial susceptibility to infection by phages, inoculums of phage stocks were added in the wells simultaneously with the bacteria. Optical density was monitored at steady intervals.

To identify maximum growth rate (rmax) linear regressions were fitted to log-transformed data by using a sliding time window of 25 data points (measured at 5 minute intervals), and rmax was determined by finding the largest slope of linear regression within all fitted regressions for a particular clone.

Bacterial yield was determined as the highest arithmetic mean of untransformed OD values in the 25-point sliding time window data. In all the analyses, the OD of the background medium was subtracted from OD values.

The potential of a phage population to inhibit the growth of a particular bacterial clone was quantified as follows (II): Three replicate growth curves were drawn for each bacterial clone from each phage treatment (ancestral phage, coevolved phage, or no phage). The data obtained from the growth curves were permuted 1000 times to obtain mean estimates and SD per clone and treatment. Thus, in these 1000 created growth curves, a single value for a given time point could originate from any of the three replicate measurements. The data from phage-containing and phage-free treatments were subsequently superimposed, and the mean of maximum decrease in optical density caused by phage was designated the "phage effect" of a given phage type.

### 3.7 Staining and quantifying bacterial biofilm (I, III, IV, VI)

Biofilm production of each clone was measured on Honeycomb 2 microtitre plates (Thermo Electron Ltd). Bacteria were grown on the plates for 48 hours in 37 °C or 25 °C. The plates were drained and 1 % crystal violet solution was added into the wells and incubated for 10 minutes. After incubation the plates were rinsed with distilled water for three times, and subsequently 96 % ethanol was added to dissolve crystal violet from the walls. Plates were incubated for 24 hours and the amount of formed biofilm was quantified as the OD of crystal violet-ethanol solution at 460-580 nm with Bioscreen C® spectrophotometer (modified from O'Toole & Kolter 1998).

### 3.8 Verifying presence of phage and determining phage host range (I, II, III, IV, VI, VII)

200 µl of host bacteria was mixed in 3 ml of melted soft agar (0.7 %) and poured on a plate. 10 µl of phage lysate is placed on the plate, incubated in optimal conditions depending to the host bacterium in question and checked for bacterial lysis where the phage lysate was placed.

### 3.9 Virulence experiments (III, IV, VI)

It has been shown that virulence in invertebrate hosts correlates with virulence measured in mammals and mammalian cell cultures (Brennan et al., 2002; Insua et al., 2013; Jander et al., 2000; McLaughlin et al., 2014; Miyata et al., 2003; Mukherjee et al., 2010; Seed and Dennis, 2008; Wand et al., 2013). In this thesis we used three insect models to assess bacterial virulence.

1. Wood tiger moth larvae (*Parasemia plantaginis*, Arctiidae) were used in III as insect model hosts, to determine changes in bacterial virulence. Infection was performed by the injection method (needle). 5 µl of a well-mixed *Serratia marcescens* (ATCC #13880) solution grown to late logarithmic phase, or sterile 5 µl of $dH_2O$ (as a control), was injected between the second and third segments of the larvae with a Hamilton syringe. A total of 210 larvae were injected during the experiment: 27 control larvae with water, 46 larvae with the ancestral *S. marcescens* strain, 70 larvae with the *S. marcescens* strains that had evolved in the absence (N = 36) or presence (N = 34) of phages at 25 °C and 67 larvae with the *S. marcescens* strains that had evolved in the absence (N = 32) or presence (N = 35) of phages at 37 °C. Infected larvae were monitored at 1-3 hour intervals for 53 hours.

2. In study IV *Drosophila melanogaster* oral infection model (Nehme et al., 2007) was used to determine changes in bacterial virulence. *Serratia marcescens* Db11 was grown in LB for 24 h, at 25 °C without shaking, and 800 µl of the bacterial culture was mixed with 800 µl of 100 mM sucrose solution. The mixture was absorbed to cotton dental roll (Top Dent, Lifco Dental, Enköping, Sweden) folded on the bottom of a standard 75×23 mm fly vial (Sarstedt, Nümbrecht, Germany). 1600 µl of 100 mM sucrose solution was used as a negative control. Ten 2–3 days old *D. melanogaster* adults (kindly provided by Christina Nokkala from the University of Turku) were transferred to each vial and plugged with cotton. Survival of flies was monitored over four days at 3–6 h intervals. Infection experiment was performed for two clones from each of the 64 experimental replicate microcosms .

3. Wax moth larvae (*Galleria mellonella*, Lepidoptera; Pyralidae) were used in VI to determine changes in bacterial virulence. Each of the 240 bacterial clones was used to infect two *G. mellonella* larvae (Kreca, Ermelo, Netherlands) accounting for 480 larvae altogether. Additionally, seven larvae were injected with distilled water to control for the damage caused by the injection itself (total n = 487 larvae). Bacterial clones were grown for 48 h in 37 °C and 220 rpm agitation, and the larvae were injected with 5 µl of bacterial culture between segments six and seven using a Hamilton syringe. Infected larvae were monitored at 1-3 hour intervals for 44 hours.

# 4 RESULTS AND DISCUSSION

## 4.1 Phage-host interactions in microbial communities with multiple interacting species

### 4.1.1 Top-down effects of phages on bacterial biomass (I)

In natural consumer-resource communities predation by protist predators and lysis by parasitic bacteriophages are the most prominent causes of bacterial mortality (Fuhrman, 1999; Jurgens and Matz, 2002). However, the relative impacts of these bacterial enemies on top-down control of bacterial biomass are unclear. In the first study (I) of this thesis, I assessed the impacts of two protist predators and a lytic bacteriophage on bacterial biomass in free water and biofilms. An opportunist pathogenic bacterium (*Serratia marcescens* strain Db11) was exposed to two protists grazers (surface feeding amoeba *Acanthamoeba castellanii*, and open water particle feeding ciliate *Tetrahymena thermophila*), and a lytic bacteriophage (Semad11) in static aquatic microcosms with a low concentration plant detritus medium simulating pond water.

This study demonstrates that bacterial enemies with different feeding strategies had eminently different spatial and temporal effects on the bacterial population. Bacteriophages and protist grazers use relatively diverse strategies to utilize bacteria, for example, most ciliates prey on suspended bacteria, whereas amoebae often feed almost exclusively on biofilm (Molmeret et al., 2005; Rodriguez-Zaragoza, 1994). Lytic phages are in principle capable of attacking bacteria in the free water as well as in biofilms (Hanlon et al., 2001). However, the ability of a phage to infect biofilms (formed by an otherwise susceptible bacterial host), depends whether the phage has access to the surface of the bacterium, which in turn is determined by the structure of biofilm and the capability of bacteriophage to degrade the extracellular polymers forming the matrix of the biofilm (Sutherland et al., 2004).

The ciliates accounted for the strongest long-term negative effect in reducing bacterial biomass in the open water. Neither ciliates nor bacteriophages reduced bacterial biofilms when they were the only bacterial enemy in the system. However, the lowest amount of biofilm was found in the system where ciliates, amoebae and bacteriophages were all present. These results indicate that the effect of ciliates and bacteriophages on the amount of biofilm was of significance only when the amoebae were present. Amoebas were the most efficient single enemy type in reducing the bacterial biofilm. Moreover, this effect was most pronounced during the three final weeks of our experiment. Notably, amoebae were also able to reduce the bacterial biomass in the free water. One explanation for this could be that the amoebae consumed the suspended bacteria through pinocytosis (Bowers, 1977; Vogel et al., 1980). This niche overlap with ciliates is also consistent with the observed ciliate

population dynamics and competition between ciliates and amoeba seemed to be asymmetric in favor of the ciliates.

The lytic phages had the lowest long-term effect on bacterial biomass in the open water, and no effect on the amount of the bacterial biofilm when cultured with the bacteria alone. However, separate short-term experiments demonstrated a substantial negative effect of Semad11 on bacterial population dynamics: inoculation of phages was followed by a 93 % decrease of free-living bacterial biomass within 12 hours, in comparison to the control populations. However, the high mortality risk imposed by a lytic phage is likely to create a strong selection pressure for phage-resistant bacteria and indeed, the bacterial populations were restored close to control population sizes within 100 hours. Thus, in the ecological context, the appearance of phage-resistant bacteria weakened the trophic link between the parasitic phages and their host bacteria. However, the phage-resistant bacterial populations did not quite reach the population sizes of bacteria cultured without the phage, suggesting a cost on growth for the acquired phage-resistance and possible cryptic coevolutionary dynamics. The costs for phage-resistance have been well demonstrated (Bohannan et al., 2002; Lenski, 1988b) and the costs are likely to be higher in low-resource environments (Lopez-Pascua and Buckling, 2008). Thus, we suggest that the fitness cost associated with phage-resistance is accountable for the small but persisting long-term negative effect on free-water bacterial biomass. All in all, protist predators accounted for most of the negative long-term effects on bacterial biomass, whereas a single phage type seemed to account for fluctuations in bacterial genotypes within the population, rather than decrease the total amount of bacterial biomass.

### 4.1.2 Susceptibility to infection by lytic phages is affected by interacting species (II)

Consumer-resource interactions have been studied extensively and most experimental setups investigating these interactions consist of one consumer and one resource species. However, in natural communities, any given species interacts with multiple other species, and the potential interactions between species increase exponentially with the number of species. The outcome of pairwise antagonistic coevolution is expected to be altered by the presence of additional interacting species, depending on how the selection pressures imposed by multiple species on a given species are correlated (Iwao and Rausher, 1997; Strauss et al., 2005). There are plenty of studies showing negative correlations where the presence of one enemy reduces the evolutionary impact of another species (Berenbaum and Zangerl, 2006; Craig et al., 2007; Edeline et al., 2008; Friman and Buckling, 2013; Gomez et al., 2009; Iwao and Rausher, 1997; Koskella et al., 2011; Pitt, 1999; Siepielski and Benkman, 2008; Sih et al., 1998; Stinchcombe and Rausher, 2001; Thompson, 2005). This is likely to be due to trade-offs between defense mechanisms against multiple enemies, where a benefit

from a change in one life-history trait is overridden by the disadvantage introduced by a change in another trait in a given environment (Davies and Brooke, 1989; Stinchcombe and Rausher, 2001)

In the second study (II) of this dissertation, I investigated how the presence of protist predators affects the susceptibility of bacteria to infection by lytic bacteriophages in a low-resource environment, and whether there are associated costs on the competitive ability. To study this, we used two microbial systems; i) *Serratia marcescens*, ciliate *Tetrahymena thermophila*, surface-feeding *Acanthamoeba castellanii*, and a lytic parasitoid phage Semad11 and ii) *Pseudomonas fluorescens* with lytic parasitoid phage SBW25 Φ2.

The presence of two protists, *T. thermophila* and *A. castellanii*, led to lowered susceptibility to infection by a lytic phage in *S. marcescens*, in comparison to a phage-only system, suggesting an overlap in bacterial defenses against parasitic and predatory enemies. These results were further supported by the other study species *P. fluorescens* and *T. thermophila*. Selection by bacterial enemies was costly in general and was seen as a lowered fitness in absence of phages, measured as a biomass yield.

*S. marcescens* from phage-only systems were more susceptible to the ancestral phage than to coevolved phages. This result supports a study by Gomez and Buckling (2011), demonstrating that bacteria are most resistant to their contemporary phages in a low-productivity environment. The bacteria could have acquired phage-resistance through minor mutations in their receptors, as these kinds of moderate structural changes have the potential to provide resistance against the specific phage genotypes present in the population (Bohannan and Lenski, 2000). The *S. marcescens* that had been exposed to phages and protists were less susceptible to infection by both contemporary and ancestral phages. This suggests that some of the evolved anti-predatory traits could be also beneficial against phage infection, and this mechanism was less selective to phage type. One such less selective mechanism to avoid infection by phages on bacterial population level could be the production of biofilm, allowing some bacteria to hide from phages as spatial refugees (Labrie et al., 2010)

This study demonstrates that the presence of additional interacting species can drastically shape the outcome of consumer-resource interactions; here, the interaction between a phage and its host bacterium. These results could also have implications beyond the eco-evolutionary community effects, as many bacteria that actively grow in natural multispecies reservoirs (such as *S. marcescens* (Grimont and Grimont, 1978) and *P. fluorescens* (Donnarumma et al., 2010; Madi et al., 2010) used in this study) can also opportunistically cause infections in multicellular organisms (Woolhouse et al., 2001). Competitive ability in bacteria is often linked to virulence, e.g. through the rate at which the host is colonized (de Roode et al., 2005; Frank, 1996; Harrison et al., 2006; Williams, 2013). If bacterial antipredatory defense traits

are traded off with competitive ability, the selection for antipredatory traits could select for lowered virulence in environmentally growing pathogens.

### 4.2 Lytic phages select for lowered virulence (III, IV, VI)

The coincidental evolution hypothesis (Levin, 1996; Levin and Svanborg Eden, 1990; Read, 1994) suggests that bacterial virulence could be a random by-product of selection on life-history traits that increase the fitness of a parasite in outside-host reservoirs. These traits would then coincidentally have implications on the virulence within the host. For example, the competitive and cooperative abilities of bacteria have probably evolved to increase bacterial fitness and survival in natural microbial communities. However, these same traits also affect the severity of an infection by determining how fast bacteria can proliferate within their hosts (Harrison et al., 2006; Inglis et al., 2009). Although previous studies have reported positive correlations between bacterial virulence and traits linked to survival in environmental reservoirs, the disease outbreaks driven by opportunistic bacterial pathogens are relatively rare, suggesting that there are some selective forces acting in environmental reservoirs that in all likelihood select for lowered bacterial virulence.

Lytic bacteriophages and protist predators are the two major causes for bacterial mortality in natural microbial communities (Fuhrman and Noble, 1995) and we conducted two studies investigating the implications of i) temperature regime and lytic phages and ii) presence of protist predators and phages, on virulence evolution in opportunistic pathogen *Serratia marcescens*. In the first study (III), *S. marcescens* (strain ATCC # 13380) was exposed to 25 °C and 37 °C temperatures in the presence and absence of a lytic bacteriophage PPV *in vitro*, and the changes in bacterial virulence were measured *in vivo* in *Parasemia plantaginis* model. The temperatures were selected to simulate the temperatures in outside-host (25 °C) and within-host (37 °C) environments. In the second study (IV) *S. marcescens* was exposed to the lytic phage Semad11 and two protist predators; *Tetrahymena thermophila* and *Acanthamoeba castellanii* in different combinations, and the virulence of the bacteria was assessed *in vivo* with *Drosophila melanogaster*. The results from both of these studies demonstrate that the presence of lytic phages select for lowered virulence in two strains of *S. marcescens*, contradicting the theory of coincidental evolution of virulence and rather suggesting that allocating to defense against phages is traded off with virulence.

In study III, the high temperature regime (37 °C) selection increased the motility and virulence of *S. marcescens* when the bacterium was cultured in the absence of the phage. However, past selection in high temperature did not affect bacterial virulence in the presence of the phage. These results suggest that high temperature could select for more virulent bacteria in environmental reservoirs, while selection by phages could constrain this effect. Phages impose a strong

selection for phage-resistant bacteria and this is likely to account for the observed lowered bacterial yield, as phage-resistance often comes with an associated fitness cost. Thus, phages could have constrained the temperature-mediated increase in bacterial virulence via a competitive growth cost (Bohannan and Lenski, 1999; Brockhurst et al., 2004; Lenski, 1988a; Lenski and Levin, 1985), resulting in less efficient host exploitation during the infection and, ultimately, in lowered bacterial virulence. (de Roode et al., 2005; Harrison et al., 2006) Alternatively, temperature-induced increase in bacterial motility could have been maladaptive in the presence of parasitic phages. This could result from increased probability of the more motile bacteria to encounter phages. Low motility has, indeed, been connected to resistance against phages (Brockhurst et al., 2005; Heierson et al., 1986; Paruchuri and Harshey, 1987), as many phages use the bacterial pilus or flagella (Brockhurst et al., 2005; Iino and Mitani, 1967; Malik-Kale et al., 2007; Schade et al., 1967) as a receptor for their attachment.

In study IV we found that the presence of all bacterial enemies (Semad11, *A. castellanii*, and *T. thermophila*) in all combinations decreased the virulence of *S. marcescens* after eight weeks of coevolution. The bacteria that had evolved with lytic bacteriophage had the lowest bacterial yield of all treatments. As lytic phages impose a strong selective pressure for phage-resistance, it is likely that phage-resistance was selected for and this was accompanied with a fitness cost on the growth (Bohannan and Lenski, 1999; Brockhurst et al., 2004; Lenski, 1988a; Lenski and Levin, 1985), accounting for the observed decrease in bacterial yield. As discussed with the previous study, this growth cost could then result in less efficient host exploitation during the infection and in decreased bacterial virulence in a multicellular host (de Roode et al., 2005; Frank, 1996; Harrison et al., 2006). The presence of lytic phages also decreased the biofilm production in bacteria in most phage-containing treatments, suggesting that phage-resistance is costly for the production of biofilm. Interestingly the coevolution with all enemies (phage, ciliate and amoeba) resulted in intermediate biofilm production in *S. marcescens*. This suggests that the joint selection by all enemies could have forced the bacteria to allocate to costly biofilm production anyway, if the selective advantage of allocating into the biofilm production would have overridden the costs in an environment with multiple enemies.

Together these results suggest that exposure to bacteriophages could select for lowered bacterial virulence in environmentally growing opportunistic bacteria, if defense against bacteriophages is costly in terms of the ability of the bacteria to cause disease. This is an important finding regarding the evolution of virulence in opportunistic environmentally growing pathogens. If virulence is coincidentally maintained in the outside-host environments, why are outbreaks by opportunists still relatively rare? And what constrains the virulence, as opportunists are not strongly subjected to the transmission-virulence trade-off? Based on the results of this thesis I suggest that lytic phages in environmental reservoirs are one potential

factor constraining the evolution of virulence in opportunistic pathogens, through trade-offs between defending against phages and virulence.

This is also interesting when considering phages as therapeutical agents. In addition to simply decreasing bacterial densities, phages have a unique relationship with bacteria, shaped through billions of years of coevolution. As lytic phages impose such a strong selection pressure on bacteria in natural communities, bacteria have evolved an array of means of overcoming the threat by phages, some being costly for the bacteria and thus resulting in lowered virulence. As far as designing phage based antibacterials go, understanding the eco-evolutionary antagonistic interactions between the medicine (phage) and its target (bacteria) could help us develop effective phage based antibacterial therapy, where even the appearance of resistant bacteria could be coupled with lowered virulence. This is discussed further in Chapter 4.3.

## 4.3 Phage therapy: the availability of phages and the emergence of phage-resistance in bacteria

Apart from the legislative problems and safety issues of phage therapy described in Chapter 1.3, more practical questions regarding phage based antibacterial therapy include i) can new phages be readily isolated for development of phage therapy and clinical trials and ii) how severe problem is the potential emergence of phage-resistance?

### 4.3.1 Isolating new phages lysing a wide range of clinical bacterial isolates (VII)

The aim of study VII was to assemble a phage cocktail lysing a wide range of clinical isolates of multi-resistant *K. pneumoniae* producing extended spectrum β-lactamases (ESBLs) or carbapenemases, conferring resistance to most penicillins and cephalosporins (Doern, 1995), and carbapenems (Jacoby, 1997), respectively. We isolated a total of 60 phages from sewage effluent and chose 6 lytic phages for a cocktail, based on their polyvalency against a set of 24 *K. pneumoniae* host strains. The cocktail was tested on 125 β-lactamase-producing clinical *K. pneumoniae* isolates and the cocktail lysed 99 isolates *in vitro*. This shows that isolating new phages against given bacterial isolates is relatively easy. In this study we did not further characterize the phages or assess whether these phages could be associated with some unwanted secondary pharmacokinetic characteristics. However, the magnitude of isolated phages against the *K. pneumoniae* strains, and the achieved wide host range associated with the assembled phage cocktail, are encouraging and our results indicate that it should not be a problem to find effective phages for further characterization and use in phage therapy trials.

### 4.3.2 On bacterial phage-resistance: a global perspective (V)

The emergence and fast spreading of resistance genes against the chemical antibacterials raises concerns regarding the potential emergence of global phage-resistance associated with phage therapy. Due to the strong selection pressure imposed by lytic phages on their bacterial hosts, the relatively quick emergence of phage-resistant bacteria is, in fact, expected to result from exposure to phages (Levin and Bull, 2004). It has been stated that the maintenance and development of functioning phage therapy will depend on the success of the constant expansion of the collection of therapeutic bacteriophages with lytic activity against newly arising phage-resistance of bacterial populations, as phage-resistant clones are expected to replace the previous hospital pathogens. However, from eco-evolutionary perspective, it is very unlikely that the use of phages should initiate the emergence and maintenance of similar multi phage-resistant bacteria than what we have seen associated with the use of chemical antibiotics.

In natural microbial communities bacteria are subject to competition, predation, parasitism and varying environmental conditions. Phage-resistance is known to be often associated with a fitness cost (Meyer et al. 2010, Inal 2003) and in an environment with diverse selection pressures, it could be disadvantageous for the bacteria to remain resistant against bacteriophages that are no longer present in their local environment (Gomez and Buckling, 2011). In fact, phage-resistance has been demonstrated to be a transient trait in bacteria in soil communities. This is likely to hold for many natural microbial communities, as allocation to phage-resistance often is associated with a fitness cost for the bacteria (Inal, 2003; Meyer et al., 2010). Moreover, these costs have been demonstrated to be more substantial in low-resource environments (Lopez-Pascua and Buckling, 2008), which most natural bacterial habitats represent.

As study VII shows, phages can be readily isolated from the environment. Thus, even if continuous use of phages forced a bacterial population to become permanently resistant to specific phage-cocktails, the prospects of isolating new phages from the environment looks promising. Moreover, biogeographical studies of phage infection patterns have shown that regardless of lack of recent contact, phages have remained infective to bacterial host cells on the other side of the world (Flores et al., 2011; Wolf et al., 2003). Altogether, a situation where new infectious phages for pathogenic bacteria can no longer be found seems extremely unlikely, and the theoretically infinite reservoirs of phages should warrant that the emergence of phage-resistance will not hinder the prospects of success of phage therapy.

### 4.3.3 Phage cocktails could select for lowered bacterial virulence but this could depend on the identity of the cocktail (VI)

If global phage-resistance associated with phage therapy should not become a problem, how about the expected emergence of resistance in the short-term? If phage-resistant bacteria are still expected to appear locally, are these resistant bacterial variants going to compromise the prospects of successful therapy in regards of a single patient or a single facility? As discussed in Chapter 1.3 the primary motivation for the use phage of cocktails is their broad range of activity but also the delay of phage-resistant mutants (Chan et al., 2013; Tanji et al., 2004). Fitness costs for phage-resistance have been shown with many bacterial species and the appearance of less virulent or avirulent strains in association with *in vivo* phage therapy trials has been demonstrated (Smith and Huggins, 1983; Zahid et al., 2008). In addition, bacteria exposed to an engineered phage cocktail where phages were known to use different receptors, resulted in attenuation of virulence in the bacteria surviving the phage treatment (Filippov et al., 2011). Thus, exposure to phage cocktails could lead to selection for lowered virulence, if defending against phages is costly in terms of virulence.

The aim of study VI was to assess whether the virulence of potential bacteria surviving a phage cocktail treatment would decrease due to allocation of resources into phage-resistance at the expense of virulence. We set up an experiment where a clinical isolate of ESBL-carrying *K. pneumoniae* (strain Kpn524) was exposed to phage cocktails for one week. Four lytic bacteriophages infecting a clinical isolate of *K. pneumoniae* Kpn524 were isolated and phages were administered in different combinations on Kpn524 for seven days. Subsequently, the remaining phage-resistant bacteria were isolated and the virulence of the bacteria was measured in *Galleria mellonella* wax moth larvae. In addition to virulence, bacterial growth rate and biofilm production were assessed for all isolated clones, as these traits are known to be linked to virulence (Antia et al., 1994; de Roode et al., 2005; Frank, 1996; Goto et al., 1999; Harrison et al., 2006; Kumoh, 1996; Paisley et al., 2005).

The administration of three out of five phage cocktails decreased the virulence of Kpn524 isolates surviving the phage cocktail treatment, in comparison to control treatment with no phage. Interestingly, two out of four phages caused an increase in the bacterial virulence, when administered alone. Bacteriophages are known to contribute to increased bacterial virulence by introducing genetic elements encoding bacterial virulence factors (VFs) (Brussow et al., 2004). However, the studies currently available on the production of phage-linked VFs focus on lysogens rather than lytic phage infections (Abedon and Lejeune, 2005; Ferrer et al., 2011). Exposure to lytic phages has been shown to select for upregulation of virulence factors when measured *in vitro* with mammalian cell cultures (Hosseinidoust et

al., 2013). However, to our knowledge there are no studies at the moment showing increased bacterial virulence *in vivo* resulting from exposure to lytic bacteriophages.

The increased virulence was accompanied by increased growth rates and bacterial virulence was positively correlated with the maximum growth rate of bacteria across all isolated bacterial clones. A study by Poisot et al. (2012) shows a behavioral response in bacteria resulting from exposure to lytic bacteriophages. The binding of phage triggers a phenotypically plastic response and as a result bacteria begin to divide earlier in their cell cycle, showing as increased growth rate (Poisot et al., 2012). This could be due to the faster replication possibly allowing bacterial cells to concentrate phage progeny in one of the daughter cells at cell division (Lee et al., 2009; Zeng et al., 2010), and thus some bacterial daughter cells could to avoid death by the phage (Poisot et al., 2012). This strategy of "terminal investment" could be accounting for the increased growth rate observed in our experiment. High growth rate can enable bacteria to exploit their hosts more efficiently, and is considered to be a virulence factor (Antia et al., 1994; de Roode et al., 2005; Frank, 1996; Harrison et al., 2006; Paisley et al., 2005). Subsequently, phage-induced increase in bacterial growth could result in increased virulence. Bacterial biofilm production *in vitro* was negatively correlated with both virulence and growth rate across all bacterial clones, suggesting a cost for biofilm production resulting from investing into phage-resistance.

These results indicate that the use of phage-cocktails and thus "attacking bacteria simultaneously from different fronts" increases the likelihood of decreased virulence, potentially through allocation to resistance against multiple phages. In addition, the increased virulence associated with the administration of individual phage types did not take place in the phage-cocktail treated bacteria, which further promotes the advantages of phage-cocktails over single phage isolates in phage therapy. The increased virulence resulting from exposure to two phages was not expected and calls for future research in order to find out the underlying mechanisms. Most of all, this result calls for careful assessment when composing phage cocktails for therapy trials, as poor choice of phages could results in adverse effects and vitiate the success of therapy. Our observations do ignite a need for more profound consideration about the nature of phage therapy and the selection criteria for phages used in therapy. Essentially, bacteriophage based antimicrobial therapy deals with microbial communities with reciprocal coevolutionary potential associated with both; the antimicrobial agent and the target, and ultimately, it is the selection in the prevailing biotic and abiotic environment that determines the outcome of therapy. Thus, understanding the underlying ecological and evolutionary processes is crucial in designing successful phage based therapeutics.

# 5 CONCLUSIONS

Listed below are the main conclusions from each individual chapter of this thesis:

I The trophic link between a lytic bacteriophage and its' host bacterium is significantly weakened by the appearance of phage-resistant bacteria. In a consumer-resource community with bacteria, phages and protist predators, the protists are likely to account for most of the long-term top-down control of bacterial biomass, whereas a single phage type is likely to account for fluctuations in bacterial genotypes within the bacterial population.

II The presence of interacting species within a consumer-resource community can shape the phage-host interaction. The presence of protist predators lowered bacterial susceptibility to infection by lytic bacteriophages, indicating an overlap against predatory and parasitic enemies in bacteria.

III Exposure to high temperature in an outside-host environment selects for increased motility and virulence in an opportunistic bacterium and this is constrained by the presence of a lytic bacteriophage.

IV Exposure to multispecies communities consisting of lytic phages and predatory protists in an outside-host environment selects for lowered virulence in an opportunistic bacterium.

V, VII The biogeographical infection patterns of phages suggest that the emergence of global phage-resistance associated with phage therapy is not likely, as infectious phages are readily isolated from the environment, even from geographically distant locations.

VI Bacteria surviving exposure to phage cocktails could be associated with lowered virulence, most likely through costs for phage-resistance against multiple phages. However, exposure to certain phages individually can even increase virulence in the phage-resistant bacteria and thus the implications of a given phage cocktail on bacterial virulence evolution is ultimately determined by the individual phage types included in the cocktail. Thus, the effects of individual phage types included in phage cocktails should be carefully assessed, as they could have detrimentally adverse effects on bacterial virulence and, as a consequence, on the success of the treatment.

The aim of this thesis was to study the implications of lytic bacteriophages on opportunistic pathogenic bacteria that reside and reproduce in outside-host

environments with multiple species interactions. I found that the presence of additional interacting species affects the antagonistic interaction between a phage and its' host bacterium. Moreover, there was an overlap in the bacterial defense against protist predators and parasitic phages. This is an intriguing finding from the eco-evolutionary perspective, as positive correlation between defending against parasitic and predatory enemies in microbial communities has not been shown earlier.

My findings on the effect of phages on the evolution of bacterial virulence contradict the theory of coincidental virulence evolution, and suggest that resistance to phages is rather associated with a cost on virulence, thus constraining the selection for virulence in environmental reservoirs. This could explain why environmental reservoirs do not generally select and maintain more virulent bacteria, which would be expected if survival in the environment would be positively correlated with virulence. It has to be noted, though, that selection by two individual phages in study VI caused an increase in bacterial virulence, which in turn supports the hypothesis of coincidental virulence evolution.

Understanding the eco-evolutionary aspects of phage-host interactions is extremely important in regards of development of phage-based therapeutics. In this context, the focus is on the evolution of phage-resistance and the consequences of this resistance on the success of therapy. In the studies presented in this thesis I found that in most cases phage-resistance was associated with lowered virulence in bacteria, most likely as a result of costs coupled with defending against phages. Moreover, even if resistance against phages should emerge, the existing literature, along with results from study VII suggest that new phages are nevertheless readily isolated from the environment. I hereby suggest that despite the strong selection for bacterial defense against phages, the emergence of phage-resistance should not hinder the prospects of utilizing phages as alternative antibacterials in the future.

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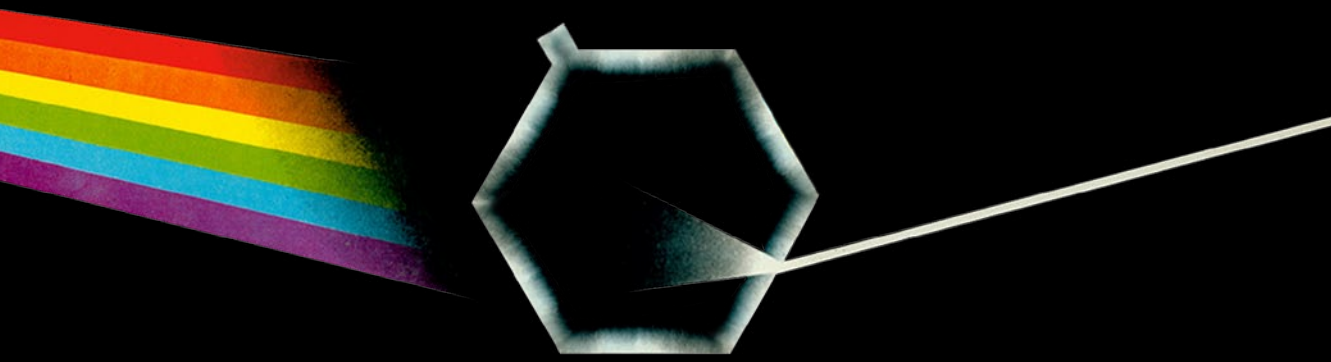

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