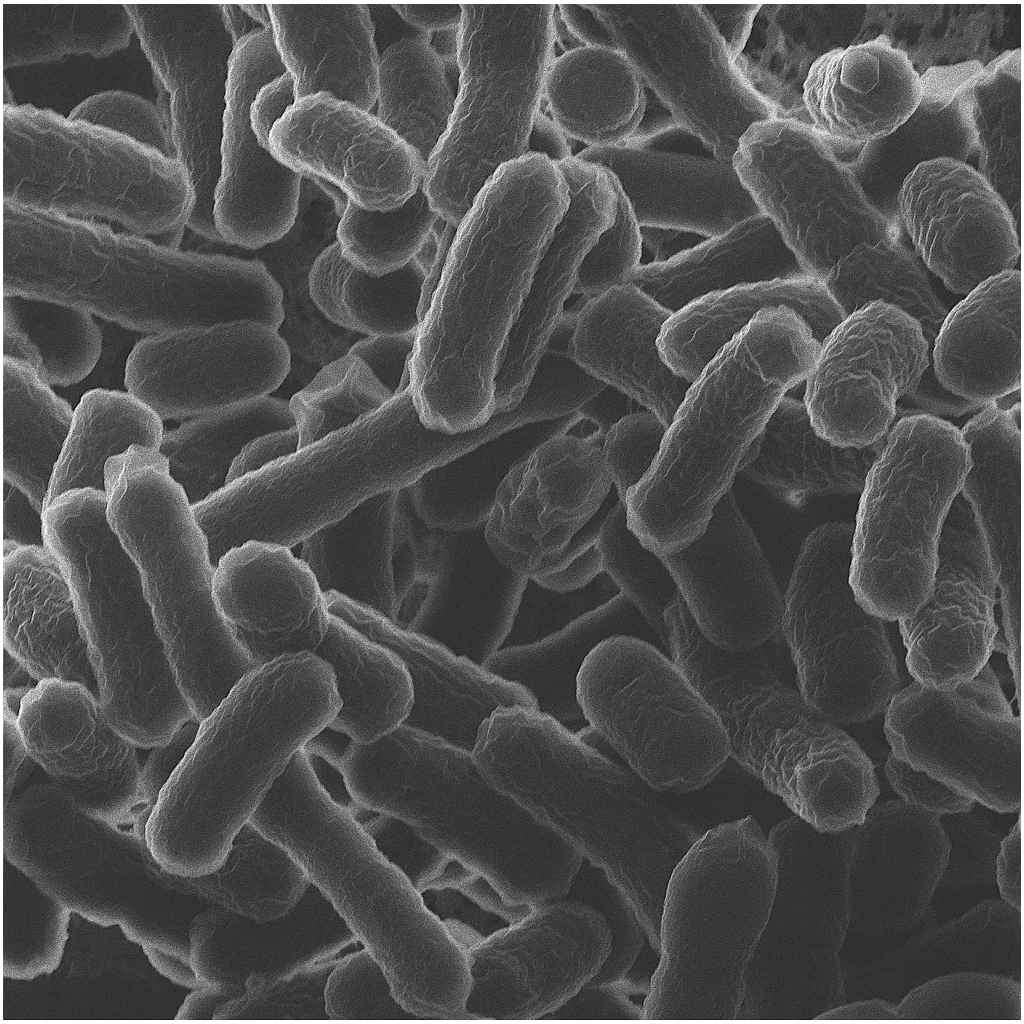
JYU DISSERTATIONS 413

Katariina Koskinen

# Interacting Microbes, a Source for Antimicrobial Resistance Propagation



UNIVERSITY OF JYVÄSKYLÄ
FACULTY OF MATHEMATICS
AND SCIENCE

JYU DISSERTATIONS 413

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# ABSTRACT

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Diss.

Microbial communities are highly abundant part of our biosphere and act as a source for the vast interactional network. This web of interactions not only affects the microbial behavior but also extends its causation to the human life as well. One of the most urgent threats microbes possess globally is antimicrobial resistance. Microbial communities consist of multiple participants, their metabolites, and the surrounding environment. In this thesis contribution of bacteria, their conjugative resistance plasmids, bacteriophages, and protozoa is studied in both microbial community settings and simplified assemblies. The effect of microbial interactions to the spread of antibiotic resistant bacteria and antibiotic resistance gene carrying conjugative plasmid persistence are examined in the thesis as well as the potential of bacteriophage therapy in overcoming antimicrobial resistance crisis. One of the main findings is the effect of both protozoan predation and leakiness of antibiotic resistance mechanisms that promote antibiotic resistance plasmid persistence in the multi-trophic community rather than the surrounding antibiotic pressure. Also, the both genomic and phenotypic characteristics were evaluated, to investigate the differences in the distribution patterns of multi-drug resistant bacteria. Found drought tolerance highly associated with the epidemical successfulness status of the studied strains. The interactions between bacteria and bacteriophages were further studied and the host spectrum of tectiviruses was expanded to consider four additional genera. Also, three novel phages with possible therapeutic potential against clinical host sample were characterized both genetically and morphologically. Furthermore, this group of phages was found to interact between each other throughout the susceptibility-shifting host. For its part this thesis broadens up the vision of microbial community relevance in antimicrobial resistance prevention and cure, as well as gives an insight to overcome the crisis antimicrobial resistance cause.

Keywords: Antimicrobial resistance; bacteria; bacteriophages; conjugative plasmids; microbial communities; protozoa.

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# TIIVISTELMÄ

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Mikrobiyhteisöt ovat keskeinen osa biosfääriämme. Mikrobien väliset vuorovaikutukset vaikuttavat sekä suoraan mikrobien toimintaan ja välillisesti ihmisten hyvinvointiin. Mikrobiyhteisöt koostuvat useista jäsenistä, jotka ovat jatkuvassa vuorovaikutuksessa toistensa ja ympäristönsä kanssa. Mikrobiyhteisöjen jäsenistä tässä väitöskirjassa esitellään bakteerit ja niiden sisältämät konjugatiiviset plasmidit, bakteriofaagit sekä alkueläimet. Antibioottivastustuskyvyn leviäminen on yksi merkittävimmistä mikrobien aiheuttamista ongelmista. Tässä väitöskirjassa tutkin antibiooteille vastustuskykyisten bakteerien leviämistä, antibioottivastustuskykygeenejä kantavien konjugatiivisten plasmidien pysyvyyttä sekä mikrobiyhteisön sisäisten vuorovaikutusten vaikutusta antibioottivastustuskyvyn leviämiseen. Lisäksi väitöskirja käsittelee antibioottivastustuskykyisten bakteerien ja niitä infektoivien bakteriofaagien vuorovaikutusta bakteriofaagiterapian näkökulmasta. Antibioottivastustuskykymekanismin ja alkueläinten aiheuttaman yhteispaineen havaittiin vaikuttavan antibioottivastustuskyvyn pysyvyyteen ympäristön antibioottipitoisuutta voimakkaammin. Myös bakteerien leviämiseen liittyviä geno- ja fenotyyppisiä piirteitä karakterisoidessa kuivuuden siedon havaittiin linkittyvän bakteerikantojen leviämiskaavan kanssa. Bakteerien ja bakteriofaagien vuorovaikutuksia genomitasolla tutkimalla onnistuttiin laajentamaan tektivirusten isäntäkirjoa uusiin bakteerilajeihin. Osana yhtä osajulkaisusta karakterisoitiin uusia bakteriofaageja, joilla havaittiin olevan yhtäläisyyksiä bakteriofaagiterapian kannalta potentiaalisten bakteriofaagien kanssa. Lisäksi bakteriofaagien havaittiin vuorovaikuttavan toistensa kanssa isäntäbakteerin välityksellä. Omalta osaltaan väitöskirja laajentaa näkemystä mikrobiyhteisöjen merkityksestä antibioottiresistenssikriisin torjunnassa ja sen aiheuttamien ongelmien ratkaisemisessa sekä uusien keinojen kartoittamisessa.

Avainsanat: Antibioottivastustuskyky; bakteerit; bakteriofaagit; konjugatiiviset plasmidit; mikrobiyhteisöt; alkueläimet.

*Katariina Koskinen, Jyväskylän yliopisto, bio- ja ympäristötieteiden laitos*
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## LIST OF ORIGINAL PUBLICATIONS

I Jalasvuori M. & Koskinen K. 2018. Extending the hosts of Tectiviridae into four additional genera of Gram- positive bacteria and more diverse Bacillus species. *xx*

II Cairns J., Koskinen K., Penttinen R., Patinen T., Hartikainen A., Jokela R., Ruusulehto L.,Viitamäki S., Mattila S., Hiltunen T. & Jalasvuori M. 2018. Black Queen Evolution and Trophic Interactions Determine Plasmid Survival after the Disruption of the Conjugation Network. *Msystems.* 3:10.1128/mSystems.00104-18. eCollection 2018 Sep-Oct

III Koskinen K., Penttinen R., Örmälä-Odegrip A.M., Giske C.G., Ketola T. & Jalasvuori M. 2021. Systematic comparison of epidemic and non-epidemic carbapenem resistant *Klebsiella pneumoniae* strains. *Front.Cell.Infect.Microbiol.* 11:599924

IV Koskinen K., Ylänne M., Penttinen R., Jalasvuori M. & Ketola T. 2021. Characterization of *Acinetobacter baumannii* phages and the shifting host-phage dynamics. Manuscript

RESPONSIBILITIES OF KATARIINA KOSKINEN IN THE ARTICLES OF THE THESIS

I I contributed in Rapid Annotation System comparisons and writing the article with Matti Jalasvuori.

II I designed and executed the conjugation efficiency and cheater experiments and wrote the manuscript in collaboration with Johannes Cairns, Teppo Hiltunen and Matti Jalasvuori.

III I designed and executed most of the experimental and analytical work excluding RAST annotations, statistical analyses and phyton script. Designing and experimental work in colicin E3 study was performed together with Matti Jalasvuori and Reetta Penttinen. Writing was done in collaboration with Matti Jalasvuori, Tarmo Ketola and Reetta Penttinen

IV I designed and executed most of the experiments together with Matti Ylänne, who also did the genetical analyses of the viruses. TEM imaging was done with Matti Ylänne and Reetta Penttinen. Writing the manuscript was done in collaboration with Tarmo Ketola and Matti Ylänne.

# CONTENTS

## ABBREVIATIONS

| | |
|---|---|
| AI | Artificial Intelligence |
| AMR | Anti-Microbial Resistance |
| BLAST | Basic Local Alignment Search Tool |
| ECDC | European Center for Disease Prevention and Control |
| ESBL | Extended Spectrum β-lactamase |
| ESKAPE | Group of pathogens commonly carrying antibiotic resistance genes (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) |
| HAI | Hospital Acquired infection |
| MDR | Multidrug Resistant |
| MIC | Minimum Inhibitory Concentration |
| LUCA | Last Universal Cellular Ancestor |
| ORF | Open Reading Frame |
| RAST | Rapid Annotation Subsystem Technology |
| ST | Sequent Type |
| XDR | Extensively Drug Resistant |
| WHO | World Health Organization |

# 1 INTRODUCTION

Microbiological world had perplexed human mind for a long until Robert Hook and Antoni van Leeuwenhoek started to open the curtain to the previously unseen part of our world (Gest 2004). In 1665, Hook published his first findings of micro fungus and later Leeuwenhoek reported his observations on protozoa and bacteria (Gest 2004). Ever since, scientist have been overwhelmed while explaining the countless roles of microbes, as not only disease-causing agents, but also symbionts, recyclers of chemical compounds, and workhorses of modern-day industry and science (Mohajeri *et al*. 2018, Lillington *et al*. 2020, Weimer *et al*. 2020). One by one multiple phenomena, previously linked with mystique, revealed to be performed by different kind of micro-organisms and knowledge of microbial function and utilization has accumulated in formidable speed. Though our understanding of microbial world differs from the 17th century's view, still every now and then new overwhelming discoveries are made that revolutionize our aspect. During the last decades the undisputedly most notable microbial finding has been the discovery of CRISPR-Cas system, first described already in 1987 (Ishino *et al*. 1987). Later CRISPR-Cas was identified as bacterial defense mechanism against viral infections (Barrangou *et al*. 2007) and its potential as a tool for eukaryotic genome editing was introduced by Doudna and Charpentier (Doudna and Charpentier 2014). Now genome editing properties of these CRISPR-Cas systems are in center of multiple novel applications and their further development (Xu and Li 2020). This is just one example of opportunities which research on microbial world can offer.

Currently only a fraction of microbial world has been explored and one major area that we do not know that much are interactions, both intra- and inter-species, between the microbes. In its own way CRISPR finding opened researchers minds, not only for further applications, but also incited us to look closer the genetics behind the interactions of microbes. In this thesis all the original publications tackle the microbial interactions from their own perspective. Original publications are numbered chronologically by their publication dates but since their themes interlace with each other, they are presented as grouped subjects. The original publication III focuses on the characterization of 14 strains

of the same bacteria and beside their property determination also their interactions between each other and their surroundings were observed. Original publication I focuses on extending the virus family host range from assumed hosts to new additional bacterial genera by looking back the genomes of possible hosts and the traits that interactions with phages have left into them. Original publication IV continues the theme of bacteria-phage interactions by studying the phage-phage interactions throughout the bacterial host. The most complex web of interactions in this thesis can be found in the original publication number II which describes the multitrophic environment consisting of protozoa, bacteria, conjugative plasmids and phages. All of the original publications do their own part in order to round up the consensus of which these important interactions form, within the microbial species, between the species, and even species and their surroundings, which we humans share with them.
As microbial world is an intrinsic part of our daily routines, it reflects the activities of our macro-sized reality and vice versa. One of the most concerning collision in this harmony is a vast progression of antimicrobial resistance (AMR). AMR is an urgent and accumulative global scale problem threating the health of a mankind (World Health Organization 2017, Majumder *et al*. 2020). Bacterial sensitivity to the clinically used antibiotic drugs has declined causing a progressive health crisis that science has not yet overcome. AMR is in a center of the original publications II, III and IV. Original publication III studies the highly antibiotic resistant bacteria and the reasons behind their spread and epidemical successfulness trying to answer the question why some antibiotic resistant strains conquer the world rather than others. The original publication IV studies another highly antibiotic resistant bacteria and evaluates the potential phage candidates to treat the bacteria instead of the antibiotic drugs. Original publication II in turn determines the role of antibiotic pressure in a microbial multitrophic community as an AMR driving force compared to the interaction dynamics between the community members. Although AMR is a biological phenomenon, its consequences reflect to health, economics, industry, and equality as well (Gyssens and Wertheim 2020, World Health Organization, 2018, Bartsch *et al*. 2017, Pokharel *et al*. 2019). While doing the biological research it is common for themes to extend onto areas not quite covered by biologist's expertise. For that reason, in this thesis I only point out the interfaces without thorough examining of fields other than biology but instead intend to broaden up the biological phenomena to touch these highly important issues.

## 1.1 History and current state of antimicrobial resistance

Since the discovery of penicillin in 1928 (Fleming 1929), several previously fatal bacterial infections became treatable and triumph of antibiotic drugs begun. Shortly multiple new antimicrobial agents were found and, in few decades, antibiotics were established as an infection treatment procedure (Hutchings *et al*. 2019). Soon after the introduction of antibiotics first signs of AMR towards clinically used antibiotics proclaimed itself (Lobanovska and Pilla 2017).

However, at the time clinical signs of AMR emerged, antibiotics yet appeared to be an inexhaustible resource (Hutchings *et al*. 2019), thus not raising the concerns of a global scale AMR crisis which we are facing today. The main reason behind the antibiotic resistance and its spread is unconcerned use of antibiotics (Machowska and Stalsby Lundborg 2018). At first resistance development did not seem to be such a great concern since new effective antibiotics were constantly found and fatal bacterial infections really seemed to be history, at least for those who could afford the healthcare services and antibiotic drugs. Today AMR has been estimated to globally cause 700,000 deaths annually and AMR infection treatments alone have remarkable costs (Majumder *et al*. 2020). Even though the AMR problem shakes the whole world, the most strained are the developing areas and nations with low- to middle-income (Pokharel *et al*. 2019). Unfortunately, not everyone is able to rely on healthcare and for those the options are limited in either remaining untreated, to support the illegal drug markets, or self-medication (Anstey Watkins *et al*. 2019, Moise *et al*. 2017, Nguyen *et al*. 2019, McGettigan et al. 2019). Irresponsible antibiotic drug markets thrive mainly in areas of lower gross domestic product, high class distinction, or poor public healthcare and in some places all the medication is legally sold without prescription (Pokharel *et al*. 2019, Moise *et al*. 2017, Nguyen *et al*. 2019). However, irrational and illegal antibiotic consumption have been reported also from the areas with strong social security, public healthcare, and the principles of antibiotic prescription have also raised criticism (Machowska and Stalsby Lundborg 2018). This highlights the importance of global overall stewardship and surveillance of rational antibiotic usage and increase of awareness of AMR related problems (World Health Organization 2018, Majumder *et al*. 2020). The human health-related consumption of antibiotics is only one example of the vast antimicrobial drug usage. Antibiotics are commonly used in agriculture, i.e. livestock husbandry and fish farming, and the amount of antibiotics used is remarkable (Manyi-Loh *et al*. 2018, Limmathurotsakul *et al*. 2020). The antibiotic molecules used in farming also increase the environmental antibiotic burden, which is one driving factor for AMR spread (Manyi-Loh *et al*. 2018). The negligent usage of antibiotics in agriculture, such as preventive dosing, is still a thriving custom (Manyi-Loh *et al*. 2018). However, corrective acts have been established in order to sever the detrimental antibiotic utilization towards necessary infection treatment (Limmathurotsakul *et al*. 2020, O'Neill 2015).

The more antibiotics are used the risk of resistance development rises (Machowska and Stalsby Lundborg 2018). For that reason, unnecessary antibiotic treatments or misdiagnosed infections that cannot be cured with antibiotics must be avoided (Machowska and Stalsby Lundborg 2018). The world in which antibiotics were first discovered less than 100 years ago was different in multiple ways. On average person-to-person contacts were limited compared the modern days as far-distance travel was a privilege for rare. If we add the modern global traveling rates to the transmission and spread of AMR, it is clear that local outbreaks and epidemics can expand to global pandemics (Bokhary *et al*. 2021). Also, other global crises can indirectly affect the AMR incidence. For example, the precautions used to prevent the transmission of covid-19 has also

hindered the spread of AMR pathogens but also increased the amount of local AMR infections since the increased amounts of antibiotics used in secondary treatment (Knight *et al*. 2021). The effect of these precautionary actions transpires in different timespans and the conclusive effect is hard to estimate (Knight *et al*. 2021).

As said, in early years of antibiotic research problems with emerging resistance could have been resolved by new type of antibiotic drug. However, the last novel antibiotic group was found in 1980s (Hutchings *et al*. 2019) and since then the drug discovery and development have been centered upon synthetic molecules or modifying the already existing drugs (Hutchings *et al*. 2019). In fact, our now-a-days last line antibiotic, colistin, already approved in clinical use in USA at 1962, was almost abandoned by its toxicity profile shortly after its approval (Nation and Li 2009). However, since the hindered discovery of new antibiotics and constantly accelerating AMR levels, colistin has become the last and valuable option in treating multidrug-resistant (MDR) infections today (Nation and Li 2009). Though it is undeniable that bacteria have took their win over antibiotics multiple times, all hope is not lost. We are sliding towards the post-antibiotic era, the time when existing antibiotic drugs as we traditionally have considered them, can neither cure nor prevent the spreading of infectious AMR pathogens. However, AMR and antibiotic usage is widely studied and both national and international surveillance are guiding the research, giving the best chance to overcome this threat either with novel antibiotic drug research or from related fields (World Health Organization 2018, Kirchhelle *et al*. 2020). Besides the conventional antibiotic drug research also other approaches must be conducted to fight against AMR. Since the golden years of traditional antibiotic discovery are gone, science is enforced to expand onto other possibilities. Already before antibiotic drugs took over the infection treatment bacteriophages were studied as a prospect for treatment (Wittebole *et al*. 2014). Phages, as natural enemies of bacteria, have been revived into focus and they can be considered as one remarkable trend for future AMR infection treatment. Though phages have high potential, there is a lot of uncertainty in the implementation to clinical usage (Nikolich and Filippov 2020). These themes with bacteriophages are considered in original publications I, II, and IV and the phage therapy itself is evaluated from multiple aspects as well in the original publication IV. Also, other approaches, such as re-sensitizing the bacteria with the factors that promote plasmid loss in community are studied in the original publication II.

## 1.2 Bacteria in clinical settings

We are co-living in the world full of microbiota, organisms so small our eyes cannot catch but without which we could not survive. One essential part of this microbiota are bacterial cells. Even though bacteria have a reputation as disease causing agents, our very own existence depends on them (Mohajeri *et al*. 2018). Most of the bacteria surrounding us are living in coexistence either as symbionts or as inconspicuous neighbors and only a small portion of bacteria is

dangerous for our health (Mohajeri *et al*. 2018, World Health Organization 2017). Bacteria habit all niches suitable for life including human body and remarkable amount of our body mass actually consist of bacterial cells (Sender *et al*. 2016). Despite most of the bacteria are harmless, there is a group of bacteria that are pathogenic and capable of infecting people. Severity of those infections vary from mild to lethal depending on the bacterial species and strain, virulence factors, and genes these strains carry (World Health Organization 2014). The threat status of one bacterial species is not fixed, and in many cases, same bacterial species contain both harmless and severe health problem causing strains. The balance between these groups of strains evolve and otherwise harmful strain can become a threat through antibiotic resistance gene acquisition (Lee *et al*. 2017a).

Bacteria circulate in the same settings in which we humans live our daily life routines. For that reason, there are different routes of acquiring infection causing pathogenic bacteria, represented in schematic Figure 1. Some bacterial strains transmit within the community, mainly in person-to-person contacts (Casewell and Phillips 1977) and some are acquired in hospital settings and thus called as hospital acquired infections (HAIs) (Jarvis *et al*. 1985, Rediwala *et al*. 2012). The problematics of HAIs are extensive and they are reason behind notable deaths around the globe annually (Koch *et al*. 2015). Especially worrisome with HAIs is the antibiotic resistance genes these strains carry within them. Since the certain strains have established their ground in hospitals and for generations adapted to the environment with antimicrobial agents, such as sanitizer and antibiotics, it is more likely that these strains are both carriers of resistance genes and extremely persistent in the harsh conditions (Fournier and Richet 2006). The original publication number III focuses on the themes on which certain bacterial strains seem to transmit between the hospitals more effectively than others. Reasons behind the epidemical successfulness are discussed later in more detail.

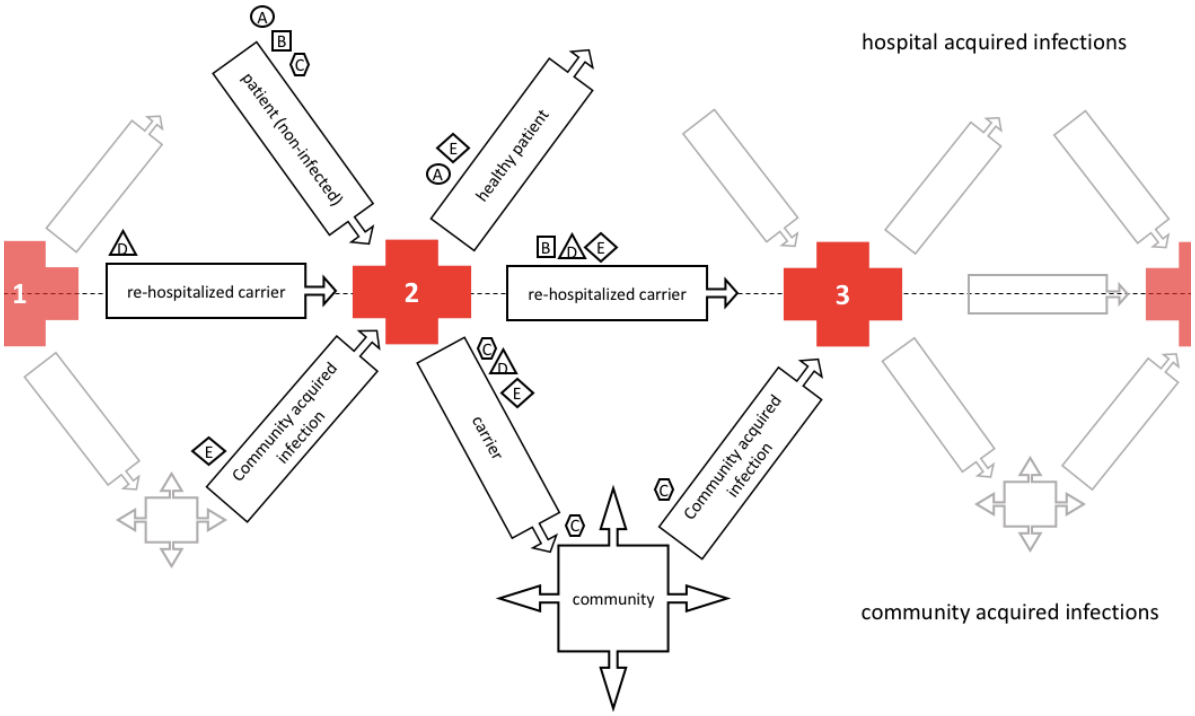


FIGURE 1 **Transmission of AMR bacterial infections in both hospital settings and community**. This scheme collects major transmission routes **(A-E**) of AMR related bacteria. In cases **A, B,** and **C** patient with no antibiotic resistant infection enters the hospital. In case **A**, the ideal case, patient leaves the hospital without the carriage of antibiotic resistant strain. In cases **B** and **C** patient receive HAI and become a carrier thus either showing symptoms and evidently becoming hospitalized again (**B**) or carrying the antibiotic resistant strain and transmitting it inside the community (**C**). In cases **E** and **D** patient already infected with antibiotic resistant bacteria, either being the symptomatic carrier (**E**) or after being exposed in community (**D**) enter the hospital. Since the carriage of antibiotic resistant strains are rarely reversible, carrier state of the patient continues after hospitalization (**D**) contrary to patients without carriage which can either be treated or become carriers as well (**E**).

Though every bacterial cell is an individual and capable to potentially cause infections and even to spark the epidemic, it must be kept in mind that usually bacteria exist as a community. Bacteria can either form multi-cellular community-like structures called biofilms, which are highly abundant in nature, or continue in single cellular planktonic state still communicating with other cells nearby (Gloag *et al*. 2019). Biofilms consist of both living and dead bacteria, which are bound together by proteins, polysaccharides and other small molecules secreted by bacterial cells (Gloag *et al*. 2019). These biofilms are extremely robust and can tolerate unfavorable conditions, such as drought, lack of nutrients or presence of bacteriocidic molecules (Gloag *et al*. 2019). Sometimes these biofilm structures occur during the pathogenic infection thus elongating the infection by protecting bacterial cells from drug molecules, which are unable to penetrate into biofilm structure (Magana *et al*. 2018). Bacterial cells can detach themselves from the structure and continue planktonic life and again form biofilms when needed (Magana *et al*. 2018). This, in turn complicate infection

treatment and increases the risk of developing antibiotic resistant secondary infections during the treatment (Roy *et al*. 2018). Biofilms can also enhance the persistence of vital bacteria on surfaces, which in turn enable bacterial strains to transmit for longer periods and gives an opportunity to spread for multiple targets (Magana *et al*. 2018). This is a major concern especially in a transmission of HAIs pathogens from inanimate surfaces, such as desks, instrumentation or catheters and other invasive tubes (Fig. 1, Traits B and C).

Bacterial species focused on this thesis are two pathogenic bacterial species *Klebsiella pneumoniae* and *Acinetobacter baumannii* which both play crucial roles in out spread of AMR. *K. pneumoniae* and *A. baumannii* are both gram-negative bacteria which belong to the ESKAPE pathogens, the six prominent nosocomial pathogens corresponding AMR (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) (Mulani *et al*. 2019). Common feature for all these pathogens is their wide and rapidly evolving Multi Drug Resistance (MDR), thus enabling their successfulness in causing HAIs. This group is addressed to have most significant impact both clinically and economically (Mulani *et al*. 2019). In 2017 World Health Organization (WHO) released a report of 12 bacterial species or groups of bacteria on which research and development of drugs should be focused on (World Health Organization 2017). All ESKAPE pathogens place either in priority class 1, which is considered as critical, or in class 2 designated with high priority status (World Health Organization 2017). On the top of this listing are carbapenem resistant bacteria, especially *A. baumannii* and also Enterobacteriaceae, including *K. pneumoniae*.

### 1.2.1 Klebsiella pneumoniae

*Klebsiella pneumoniae* is a gram-negative, rod shaped facultative anaerobic bacteria, which belongs to the normal and healthy human gastrointestinal flora (Podschun and Ullmann 1998). *K. pneumoniae* itself is an opportunistic bacterium that cause mild infections when entering the body part other than gastrointestinal tract (Podschun and Ullmann 1998). For that reason, *K. pneumoniae* is considered as minor threat and infections are easily treatable. However, the antibiotic resistance genes carried by *K. pneumoniae* strains can pose a major, even lethal, risk to human life as MDR related *K. pneumoniae* strains are common (World Health Organization 2017). Since in healthcare *K. pneumoniae* is commonly isolated as an infection causing pathogen, these strains are routinely identified based on their genomic sequence and thereafter grouped into sequence types (STs). Certain *K. pneumoniae* STs have been noted to dominate HAIs and their spreading through the hospitals globally is under surveillance (World Health Organization 2018). Anyhow, it seems that no reason why certain STs seem epidemically more successful than other has been discovered. Usually characterization of these infection-causing strains is restricted to the determination of ST and antibiotic resistance pattern. Though multiple studies focus on genetic comparisons of *K. pneumoniae* strains (Benulic *et al*. 2020), little is known about the phenotypic differences and how they affect to the epidemical successfulness. The original publication III in this thesis tackles this question,

how does the phenotypic features link with epidemical successfulness. The dataset of total 14 clinically isolated extensively drug resistant (XDR) *K. pneumoniae* strains, from which half are widely disseminated in hospitals globally whereas the other half is not, is characterized from multiple aspects.

### 1.2.2 Acinetobacter baumannii

*Acinetobacter baumannii* was named after Paul Baumann who isolated and identified the first *A. baumannii* strain (Baumann 1968). Much like *K. pneumoniae*, *A. baumannii* is also gram-negative and short rod-shaped bacterium, which lack flagella (Baumann 1968). A little more than decade ago, *A. baumannii* was considered as a minor threat to human health, predominantly causing opportunistic infections (Lin and Lan 2014). However, unexpected emergence of MDR *A. baumannii* and its takeover as a major nosocomial pathogen is an alarming example of unpredictability of the microbial world (Lee *et al*. 2017b). Occasionally *A. baumannii* infections are community-acquired and transmitted from person-to-person but these strains are mostly antibiotic susceptible and do not cause as severe health risks as does the highly resistant HAIs strains (Lin and Lan 2014). The carbapenem resistant *A. baumannii* strains are considered one of the most worrisome infection causing agents thus selected in the center of the original publication IV (World Health Organization 2017).

By enhanced pathogenicity, *A. baumannii* strains have found their way to permanently settle down in hospital settings. *A. baumannii* is known to cause multiple different kind of infections, varying from the wound infections to urinary tract infections (Lin and Lan 2014). The most severe infections are usually nosocomial infections that are acquired in intensive care units or by patients with severe health burdening issues (Wong *et al*. 2017, Lee *et al*. 2017b). Especially patients with invasive tubes, such as catheters or respirator related tracheal tubes, or patients with surgical wounds are in high risk for acquiring *A. baumannii* infections (Lin and Lan 2014). The major threat with wound infections is the high risk of developing a septicemia which in turn is often lethal if not responsive to antibiotic treatment (Wong *et al*. 2017). Behind the conquest of hospital environment are *A. baumannii's* exceptional capability to endure harsh conditions, such as long-term drought, to possess exceptional metal homeostasis system, and capability to hoard antibiotic resistance genes (Lee *et al*. 2017b). These properties are essential while surviving in hospital settings and ensuring the persistence and transmission inside and between the hospitals and patients. Similar to *K. pneumoniae*, also *A. baumannii* have found to be resistant to multiple antibiotic classes including third generation beta-lactams and even to the last line antibiotic colistin (Deveson *et al*. 2018) and the first isolate resistance to all clinically relevant antibiotics at the time was isolated in 1998 (Hsueh *et al*. 2002). In clinical settings *A. baumannii* isolates are routinely sequence typed and the distribution of certain hazardous strains is surveilled (World Health Organization 2017). AMR bacterial strains currently circulating globally share similar set of resistance genes and i.e. both *K. pneumoniae* and *A. baumannii* have noted to share the same MDR genes (Evans and Amyes 2014).

## 1.3 Resistance genes and mechanisms

Multiple different kind of AMR genes are circulating globally and novel resistance genes are developing as new antibiotic drugs are released to clinical use. These genes can reside either in chromosome of the host cell or to be coded into plasmids. There are two ways for bacterial cell to become resistant to antibiotic drugs, either by spontaneous mutation or via horizontal gene transfer (Lederberg and Tatum 1946, Soucy *et al*. 2015). Horizontal gene transfer itself can be divided into different classes from witch conjugation is the most relevant in dissemination of AMR genes (Munita and Arias 2016, Soucy *et al*. 2015). Most worrisome in the AMR dispersal are indeed conjugative plasmids, which encase resistance genes. These conjugative plasmids can be copied and transferred between bacterial cells, giving the new host cell an opportunity to resist otherwise lethal antibiotic pressure (Zwansig 2020). Accumulation of different resistance genes in the same conjugative plasmid has been observed, giving the recipient cell broad range resistance at once (Zwansig 2020). The persistence of these conjugative resistance plasmids in bacterial communities is a driving force in AMR spread (Wang and You 2020). Multiple interactions inside the community either promote or reduce the amount or transmission of the conjugative plasmids and these interactions are further studied in original publication II. Since bacterial cell can gather multiple different resistance plasmids, the definitions of resistance level can vary. Commonly used classifications are multi-drug resistance (MDR) bacteria that are known to be able to resist multiple different antibiotic drug and by literal definition strains are resistant to more than one antibiotic drug (Magiorakos *et al*. 2012). The extremely drug resistant (XDR) strains instead are defined to be resistant to all or almost all clinically used antibiotics from multiple antibiotic classes (Magiorakos *et al*. 2012). One more important classification is extended spectrum β-lactamase (ESBL) genes carrying strains. These strains have terrorized the healthcare system as β-lactam antibiotics form the base of infection treatment (Paterson and Bonomo 2005). These ESBL genes give a wide spectrum of resistance towards β-lactam antibiotics also presenting the third-generation drugs but not to carbapenems. However, ESBL strains, still susceptible to carbapenems, have found their way to develop resistance against carbapenems as well and the ultimate β-lactam resistant pathogens have been born (World Health Organization 2017). With these strains the treatment options are limited to last resort antibiotic colistin, towards witch resistance has also been reported (Bradford *et al.* 2015).

### 1.3.1 Resistance mechanisms

Resistance towards antibiotics can be acquired in multiple ways as described previously. Under antibiotic pressure bacterial cell usually undergo mutations, which any now and then, develop resistance against antibiotic drug and gives the head start in spreading under otherwise lethal antibiotic concentration (Munita and Arias 2016). Sometimes these genes are associated in conjugative

plasmids ensuring the spread between the bacterial cells (Soucy *et al*. 2015). The antibiotic resistance genes can protect cell from antibiotic drugs in different ways. Enzymatic defenses degrade the effective site of antibiotic molecule, thus preventing the lethal effect of the molecule (Benveniste and Davies 1973). These enzymes can either be intracellular or secreted to surroundings (Benveniste and Davies 1973, Yurtsev *et al*. 2013). If enzymes are intracellular the antibiotic is degraded after entering the cell (Peterson and Kaur 2018). When secreted, these enzymes are active in the surroundings of cells which also protects the other susceptible bacteria in close distance (Yurtsev *et al*. 2013). Hereby secreted enzymes allow the leakiness of resistance system. The antibiotic susceptible bacteria that benefit from antibiotic resistance enzyme secreting cell are often called cheaters and their role in microbial communities are discussed in more detail in original publication II. Enzymes are not the only way for bacteria to protect itself from the antibiotic pressure. Beside the enzymatic approach antibiotic molecules can be ejected from the cell by efflux pumps and spontaneous mutations in the target site of the antibiotic molecules protects the cell from bacteriocidic effect (Peterson and Kaur 2018). The significance of the leakiness of resistance mechanisms and their effect on resistance persistence in microbial communities is discussed in original publication II.

### 1.3.2 Carbapenem resistance

Carbapenems are group of last line β-lactam antibiotics which all share structural similarity in their backbone and differ on their active site (Craig 1997). Carbapenems are classified as highly effective with wide target spectrum for both gram-negative and gram-positive bacteria. For that reason, carbapenems are often used to treat ESBL infections (Vardakas *et al*. 2012). However, their efficacy has unfortunately been derogated by the fast accumulation of carbapenem resistance genes, especially abundant in hospital environment (World Health Organization 2017). The first clinically approved carbapenem, imipenem, was approved for trading in 1980's (Rodolf *et al*. 2006). However, imipenem was not the first carbapenem discovered since it is a derivate of the thienamycin, a compound found from *Streptomyces cattleya* in 1976 (Wilson *et al*. 1983). Thienamycin itself is not practical in clinical use due to its molecular properties. Since thienamycin is a zwitterion and undergo a degradation in the presence of water it is not suitable (Kahan *et al*. 1979). Other clinically approved carbapenems are meropenem, ertapenem, and doripenem (Lister 2007). Approval of some carbapenems varies between the countries and their usage might be allowed only in combination with other substances altering the pharmacology of the drug itself (Pei *et al*. 2016). One example of these carbapenems is panipenem, which is currently approved in clinical use only in Japan, and it can be used only together with betamipron (Kurihara *et al*. 1992). This combination reduces the observed nephrotoxicity of the panipenem thus improving the prognosis of the treatment (Kurihara *et al*. 1992). Currently some carbapenems are experimental, i.e. sulopenem, and also other old carbapenems, already abandoned by their toxicity profile, are studied as a candidate for combination treatments (Karlowsky *et al*. 2018). However, the rate

of new carbapenem discovery and research is falling behind the carbapenem resistance emergence and spread. Also, the risk of carbapenemase gene evolution to rapidly adapt to conquer new carbapenems, is high.

Carbapenem resistance enzymes, carbapenemases, were first found in 1996 (Yigit *et al*. 2001). As mentioned, the resistance development against carbapenems is especially alarming since they are used as last line β-lactams to treat infections resistant to other β-lactam antibiotics i.e. ESBL strains. Major carbapenem resistance genes circulating globally are KPC, VIM, NDM, and OXA genes. All of these genes are now often found in multiple different HAIs causing gram-negative bacteria (Lee *et al*. 2016, Giske *et al*. 2012, Hasan et al. 2014, Kitchel *et al*. 2010, Samuelsen *et al*. 2011, Vading *et al*. 2011). These genes are effectively transmitted in conjugative plasmids causing severe threat (Lee *et al*. 2016, Zwansig 2020). Common to all these mentioned carbapenemase genes is their ability to hydrolyze the beta-lactam structure of carbapenem molecule (Jeon *et al*. 2015). These carbapenemases can be divided into different groups, classes, based on their hydrolyzing structures and demand of metal cations in the hydrolyzing process (Jeon *et al*. 2015). The most known class A carbapenemase is KPC, which has first been isolated in *K. pneumoniae* and thus named Klebsiella Pneumoniae Carbapenemase (KPC). Common feature to this class A carbapenemases is that hydrolysis involves catalytic serine residue (Jeon *et al*. 2015, Ke *et al*. 2007). OXA-genes differ from their hydrolyzing activity and some of the genes, such as OXA-48, can function as carbapenemase. OXAs belong to class D, which also represent a two-step hydrolyzing effect not requiring metal cations (Jeon *et al*. 2015). VIM and NDM genes instead are metallo-beta-lactamases both belonging to class B. In cases of VIM and NDM zinc ion is needed in the hydrolyzing process, which separate them from the other beta-lactam classes (Jeon *et al*. 2015). All of these genes are highly abundant in *K. pneumoniae* clinical samples and especially OXA-genes are frequently found in clinical *A. baumannii* strains (Lee *et al*. 2016). Frequency of these carbapenemases are indeed the reason why both of these pathogens have reclaimed their placement in the top of the WHO priority listing (World Health Organization 2017). In original publication III all studied *K. pneumoniae* strains possess KPC, VIM, or NDM genes and are all thus associated with the severe HAIs and the reasons behind their spread between the hospitals are examined.

## 1.4 Microbial communities

Microbial communities are complex systems tightly bound to the AMR crisis but still often overlooked. If only focused on bacteria or on AMR spreading conjugative plasmids, as most of the studies do, remarkable part of the reality is neglected. All the places that bacteria habit are also inhabited by other bacteria and their plasmids, parasites such as phages, predators consuming bacterial cells, and all the secreted metabolites from community members (Cairns *et al*. 2018, Zwanzig 2020). One important pressure in these microbial communities is the trophic pressure. The multitrophic network and pressures between the

preys and predators has noted to link to the preservation of genes conferring the antibiotic resistance (Zwanzig 2020). Also, the relationships between the antibiotic concentration and level of AMR genes in population has been established and intensively studied (Barbosa and Levy 2000). However, not much is known of the role of multitrophic pressures on AMR gene preservation and horizontal gene transfer efficiency. The original publication II focuses on that intriguing question and results of the study are disentangled in the discussion section.

Even though focusing on the antibiotic resistance genes as an independent unit might be the simplest way to study AMR, the more accurate image can be received if microbial community members are added to the setup. Since these resistance genes are often encoded in conjugative plasmids, which can also be considered as their own entity, genes and conjugative plasmids are tightly bound together (Lee *et al*. 2016). The amount of AMR coding plasmids in the community is instead affected by not only the antibiotic pressure but also indirectly by the interactions and pressures plasmid harboring bacteria encounters (Bergstrom *et al*. 2000). Sometimes in the community bacteria can survive under lethal antibiotic pressure even without carrying resistance genes themselves due the leakiness of resistance mechanism as described earlier (Yurtsev *et al*. 2013, Morris *et al*. 2012). The interactions between the bacteria can be diverse and they can be either neutral, beneficial or harmful to surrounding cells. Some of these harmful interactions are studied in original publication III in which cross-infective prophages and growth inhibiting colicin are studied among the *K. pneumoniae* strains. Even though antibiotic resistance is a fitness-raising factor in the environment containing antibiotics, production of antibiotic resistance enzymes drain resources. If the living as a cheater is a pure coincidence or selected strategy to save resources is unclear (Morris 2015). However, the risks of this strategy cheaters are taking is notable in the conditions containing antibiotics (Morris 2015).

In microbial community bacteria encounter also various pressures from the behalf of phages and protozoa. Phages and their utilization in infection treatment is one major component of this thesis and thus separated as its own chapter. Protozoan predation is a key segment in the original publication number II. In microbial community protozoa is a predator feeding on bacterial cells. The protozoan feeding affects to the structure and metabolic activity of bacterial community (Cairns *et al*. 2016). These predation-based pressures have significant impact on how bacteria themselves behave and the effect to AMR plasmid persistence and thus community's ability to tolerate antibiotic pressure is studied in the original publication II. As described previously microbial communities are complex systems with endless dynamics all affecting to each other. The schematic Figure 2 illustrates the network of microbial community interactions studied in this thesis. In addition to interactions between the members, also the pressures from surrounding environment affects the whole community structure and dynamics. For example, temperature, nutrient levels, and inhibitory molecules, such as sanitizers or antibiotics, add their own pressures.

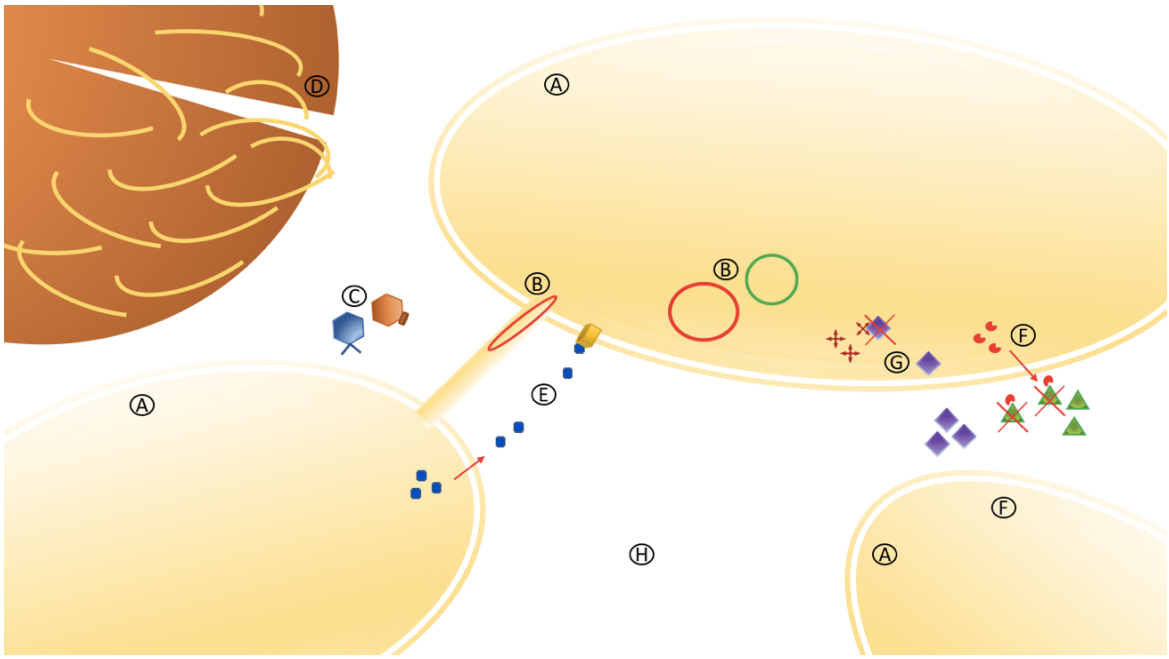


FIGURE 2 **AMR and the role of multitrophic microbial communities**. Schematic figure showing main features considered in this thesis affecting dynamics in multitrophic microbial communities and AMR transmission. Bacterial cells (**A**), resistance gene encoding conjugative plasmids (**B**), bacteriophages (**C**), and predators consuming bacteria (**D**) are the main characters in multitrophic community. Other features affecting the function of microbial community are molecules secreted between the bacterial cells, including biocides like colicin E3 (**E**), different strategies to fight against antibiotics (**F** and **G**) and environmental pressures (**H**). Original publications **I** and **IV** focus on the relationships between the bacterial cells and phages (**A** and **C**) while original publication **III** focuses on the bacterial cells (**A**) and their surrounding environment (**H**). Original publication **II** covers almost the whole community from bacteria (**A**), conjugative plasmids and their resistance genes (**B**), plasmid-dependent phages (**C**), protozoan predation (**D**) and antibiotic resistance mechanisms (**F** and **G**), and the leakiness of resistance inside the community (**F**).

## 1.5 Bacteriophages and phage therapy

The increasing antibiotic resistance has left an unmet need for the novel ways to treat infections. One way to meet this need is bacteriophages, simply phages, viruses selectively infecting bacterial cells. These phages are abundant in nature where ever their hosts can be found and a natural part of our microbial world. The discovery of these bacteria lysing agents, as they were first described, dates to late 1890's (Abedon *et al.* 2011). However, the first one to link these observations to viruses was Frederick Twort (Abedon *et al.* 2011, Duckworth 1976, Nikolich and Filippov 2020). Unfortunately, due the lack of funding, Twort was not able to prove his hypothesis and the one who first was able to prove the existence of bacteriophages was Felix d'Hérelle (Duckworth 1976,

d'Hérelle 1917). Already in 1917 phage therapy was born when d'Hérelle was administrating phages to patients suffering from dysentery and himself to prove the safety of his treatment method (d'Hérelle 1917, Nikolich and Filippov 2020). During the following years phage therapy studies expanded in several countries and to treat multiple different infections, continuing until the penicillin became clinically available in 1942 (Lobanovska and Pilla 2017). Even though most of the world abandoned phages as a treatment option after effective antibiotic treatments came available, in Soviet Union phage therapy research remained popular (Myelnikov 2018). From the modern perspective these studies establish the basement from which phage therapy approaches are now developed.

Nowadays when we are heading to the crisis of running dry the antibiotic treatment options, phages have been on display again. First phage therapy treatment to antibiotic resistant bacteria was conducted already in 1983 and new approaches are constantly developed (Smith and Huggings 1983, Gordillo Altamirano and Barr 2019). Phage therapy has significant advantages. First advantage is its ability to be personalized. Phages with therapeutic potential can either be isolated from natural sources or be stored and revived from the stocks in order to design custom dose of phages or their lysing enzymes (Hyman 2019, Mattila *et al*. 2015). Second advantage is the phages ability to penetrate into biofilm structures. As mentioned earlier, antibiotics are not able to diffuse into biofilm structures but phages instead can infect the living bacteria inside the structure and cause a cascade of phage infections throughout the biofilm (Pires *et al*. 2017). However, phage therapy still has disadvantages and challenges and the first critical review of the phage therapy trials dates already in 1933-1934 (Gordillo Altamirano and Barr 2019). Major problem is rapid resistance development against phages. Bacteria are quick to overcome phage pressure by either mutations, via CRISPR-systems, or by altering their metabolism to avoid either phage attachment or replication (Yang *et al*. 2020, Barrangou *et al*. 2007). Even though bacteria and phages have their own co-evolution, which eventually allows phage to develop infective again, the cycle is too slow for clinical usage (Yang *et al*. 2020). Other disadvantage is unpredictability of phage behavior, especially when multiple phages are used at once (Loessner *et al*. 2020). Phages also play their part in a microbial community member as mentioned previously. For that reason, the unpredictable dynamics inside the community can occur either between the host and a phage or between the phages. In original publication IV we describe the unexpected interactions between two phages through the host response, which in turn might alter the outcome of potential phage therapy treatment. For that reason, currently plenty of research is focusing on which phages should be used in therapy and should they be administrated as single phages or as a multi-phage cocktail (Yang *et al*. 2020).

One interesting group of bacterial viruses also presented in this thesis is the so-called plasmid-dependent viruses. One of these viruses is tectivirus PRD1, a model virus of the family *tectiviridae*. PRD1 have been found to infect enterobacteria that bear certain types of conjugative plasmids (Kotilainen *et al*. 1993). Especially the PRD1's ability to target bacteria harboring antibiotic resistance gene encoding plasmids have got the attention and it has been inten-

sively studied (Ojala *et al*. 2013). Multiple ideas to exploit these phages in order to selectively destroy antibiotic resistant bacteria has been proposed varying from direct exposure on infection site to the preventing AMR spread by altering the resistance gene persistence in bacterial community (Jalasvuori *et al*. 2011). These plasmid-dependent phages are studied in the original publications I and II, in which the ability to detect the traces of phages from the sequence data banks and the role of phages in the persistence of AMR in microbial communities are studied, respectively.

## 1.6 Future prospects to understand antimicrobial resistance

Since the world is heading to the post-antibiotic era and new naturally occurring antibiotics have not been found for decades, closer look must be taken towards the AMR problem itself and the focus points of actions against distribution of AMR has to be emphasized. As the likelihood of finding new naturally occurring clinical use suitable antibiotics, other approaches must be considered on a side. WHO has listed the priority list of pathogens for witch new drug research and development should be focused on (World Health Organization 2017). This announcement gives a frame for the target of drug research but does not highlight which approaches are preferred. A common way to bypass emerged antibiotic resistance is to alter the active site of drug molecule so that it cannot be recognized by bacterial cell (Klein and Cuncha 1995). This method has led to the circle of generating a new generation of existing antibiotic after resistance emergence and yet other generations after that. Unfortunately, even the brightest human mind cannot compete with the tremendous speed of resistance development in conditions where bacteria are exposed to the newest antibiotics. For the limitations of human mind capacity, the artificial intelligence (AI) has arouse interest in drug research (Camacho *et al*. 2018, Hessler and Baringhaus 2018). Computation-based research and designing of antimicrobial molecules have been part of drug development for several years (Hessler and Baringhaus 2018). Implementing AI techniques could also benefit phage therapy development. By extending the AI also from classic drug design to predict phage therapy outcomes, possible resistance formation, and host phage interactions could help to design more efficient phage cocktails (Leite *et al.* 2018).

Considering the threats AMR propel, it has not been as vigorously taken as it should have been. Now when it is evident that antibiotic resistance occurs, spread, and progress, we have no time to procrastinate. Even though there are differences in geographical distribution of AMR, present-day travelling rates and movability of prominent share of population flatten the differences and there are no places where AMR would not be a concern (Kantele *et al*. 2015). European Center for Disease Prevention and Control (ECDC) has estimated in 2018 that just in Europe occurs 670 000 infections that are caused by antibiotic resistant bacteria and from those infections 33 000 are lethal (European Center for Disease Prevention and Control 2018). From the *K.* pneumoniae isolates in

Europe 37.2 % have acquired resistance towards at least one antibiotic drug (European Center for Disease Prevention and Control 2018). Bacteria carrying AMR genes are not only transmitting between the humans but the network of transmission also cover animals and environment, linking the farming and industry into the focus when considering AMR prevention (Manyi-Loh *et al*. 2018). Most of the carbapenemase genes are indeed first discovered in *K. pneumoniae* and later found to be transmitted to *E. coli* strains (European Center for Disease Prevention and Control 2018). A short-term surveillance from 2015 to 2018 revealed significantly increasing trend in carbapenem resistance and the highest increases in national level followed the trend of overall antimicrobial resistance occurrence (European Center for Disease Prevention and Control 2018). Similar results showing increasing trend has reported also in US and parts of Asia (Martens and Devain 2017, Lai *et al*. 2014). These findings emphasize the importance of actions against AMR spread in global level. Multiple programs are now established and some are already in a level where conclusions and actions can be made to guide the direction of AMR crisis (World Health Organization 2018). However, the unpredictable events can also indirectly affect to the AMR spread and control. Current covid-19 pandemic provides an example of surprising chain of events that resonates with the AMR crisis. The total impact of covid-19 to the AMR occurrence in the future is hard to predict but lingering consequences are probable (Knight *et al*. 2021).

# 2 AIMS OF THE STUDY

Aim of this thesis is to focus on AMR crisis, its current status, future prospects, and on microbial community dynamics that intertwine with this global state emergency. All four publications included in this thesis seize their own share and aspect, each crucial, on understanding the scale of AMR phenomenon.

I Using the existing databases to find whether the host range of certain virus family can be extended and how well current tools in use can recognize the prophage elements from the host sequence.

II Investigate on how dynamics in multitrophic communities can affect the prevalence of antibiotic resistance genes in the community.

III Significance of phenotypic features in order to understand the epidemical successfulness of different carbapenem resistant *Klebsiella pneumoniae* strains responsible for global nosocomial infections.

IV Characterization of three novel phages with high therapeutic potential and further study of interactions of these phages through a shifting bacterial host.

# 3 OVERVIEW OF THE METHODS

Materials, sequences, bacterial strains, and isolated viruses as well as methods used are presented in detail in the original publications (numbered in roman numbers I-IV). To ease the search, methods are listed in Table 1 in which original publications are alluded by their roman numbers.

TABLE 1. Materials and methods used in the publications included in this thesis.

| Method | Publication |
|---|---|
| Evolutionary analyses | I, II, III, IV |
| Automated annotation tools | I, III, IV |
| Sequence processing | I, III, IV |
| Nucleotide and/or protein BLAST | I, IV |
| Bacterial metabolic activity assay | II |
| Conjugation ability and rate experiments | II |
| Plasmid persistence and bacterial cheaters measurement | II |
| Light microscopy | II |
| Growth density experiments | II, III, IV |
| Statistical analyses | II, III, IV |
| Spectrometric assays | II, III, IV |
| Community experiment | II, IV |
| Phage exposure experiments | II, IV |
| Acidic pH tolerance and compensation capacity | III |
| Alcohol tolerance assays | III |
| Confocal microscopy | III |
| Cross-strain interactions | III |
| Drought tolerance assay | III |
| Morphological characterization | III, IV |
| Phage exposure experiments | II, IV |
| Transmission electron microscopy | IV |

# 4 RESULTS AND DISCUSSION

This section of the thesis highlights the main results of all the original publications. Instead of presenting the results as publication by publication, the results are disseminated throughout the section and the related discussion is presented along with each theme. The original publications are attached in the end of the thesis providing the results, figures, and graphs for more detailed exploration. A common theme combining the all four publications is microbial interactions, which are scaled up from intra-species interactions to multitrophic community scale. The result and discussion section starts with the bacterial level and adds the phages and the phage-host interactions to the scheme. Ultimately, studies are expanded to the microbial community settings and the interactions of the whole community. Throughout the way these microbial interactions are linked to the surrounding environment and bound to the practical purposes as well.

## 4.1 Bacterial cells

Bacteria are crucial part of our ecosystems and cover remarkable portion of microbial communities. The bacterial cells themselves have been under extensive study for multiple aspects ever since their discovery. In this thesis, bacteria are studied in all original publications (directly in publications II, III, and IV, and indirectly as genetic sequences in publication I). This section features the main results and their significance of studied hosts, mainly the *K. pneumoniae* and *A. baumannii*, which are studied in original publications III and IV, respectively. The *E. coli* is discussed in chapter 4.3 Microbial communities and the sequence based bacterial studies of original publication I are presented in section 4.2.2 Tectiviruses.

As discussed in introduction section, the ESKAPE pathogens form a core for large scale AMR propagation (Mulani *et al*. 2019). The possibility to work with the two most acute AMR transmitting bacteria, classified as high priority path-

ogens, is important in order to construct fuller understanding of the AMR phenomena itself (World Health Organization 2017). Collecting the data from these acute AMR transmitting pathogenic bacteria, carbapenem resistant *K. pneumoniae* and *A. baumannii*, gives a valuable information which can be utilized in further research. Results represented in this thesis give an insight of the function and behavior of these bacterial species and help to understand the background of properties behind AMR proliferation.

### 4.1.1 Klebsiella and epidemical successfulness

*Klebsiella pneumoniae* is an opportunistic pathogen, which in recent years has become a considerable threat for the modern health care. Since the role of *K. pneumoniae* is established as crucial in dissemination of AMR, a lot of data about *K. pneumoniae* and their resistance evolution has accumulated over the years. However, the reasons behind the differences between global spread of the strains has not yet been established. In the original publication III we were given an opportunity to work with a collection of 14 globally isolated clinical samples of carbapenem resistant *K. pneumoniae*. These studied strains were classified either epidemically successful or non-successful depending on their ability to either effectively transmit between the hospitals or not, respectively. The dataset consisting of *K. pneumoniae* isolates were characterized by both their genotypic and phenotypic properties. The association of studied properties, strain's ST, and distribution pattern was analyzed. As the behavior of a cell is a combination of genetic traits and phenotypic expression of these features, both aspects must be combined to unveil the reasons behind the epidemic successfulness of the strain. Currently the standard procedure for identifying the infection causing strain is a ST analysis, in which the seven housekeeping genes are sequenced and analyzed (Urwin and Maiden 2003). This method is effective in grouping the strains in evolutionary branches that has proposed help to determine which strains are the most relevant disease-causing agents and thus must be kept an eye on (Urwin and Maiden 2003). However, as our original publication III indicate, the strains presenting same ST sometimes are total opposites in their behavior in certain circumstances. These phenotypic features are often overlooked, probably due to laborious experimental work it requires, but their significance might be important to overcome AMR spread.

In the genetic analyses of these 14 strains we observed that epidemically successful and non-successful strains did not group together. Epidemically successful strains did not show to share common ancestor either, indicating that the features corresponding on epidemic successfulness is unlikely inherited vertically but more likely evolved multiple times or transferred horizontally. For example, ST14 and ST11 seem to be genetically far distant even though both are broadly associated as carbapenem resistance spreading STs (Lee *et al*. 2016). However, some studies indicate that ST11 and ST258 share evolutionary history and based our genetic studies, our samples consolidate these findings (Lee *et al*. 2016, Kitchel *et al*. 2009, Samuelsen *et al*. 2009). Anyhow, since this sequence typing is based on the very conserved areas of the genome, it is applicable only to confirm the rough evolutionary relationships but not handy to confirm the

fast accumulating and behavior affecting mutations. For that reason, sequence type assays give a frame for more detailed genetic analysis such as i.e. Rapid Annotation Subsystem Technology (RAST) or further studies with short Open Reading Frames (ORFs), both performed in the original publication III. The RAST analysis did not reveal differences between the groups of different epidemical status thus implying the strains being consistent both functionally and metabolically. Neither the presence of prophages, plasmids, CRISPR loci, nor virulence factors explained epidemical successfulness, as these features did not combine with the epidemical status. Even the short ORFs, between 30-150 nucleotides in length, that were extracted from the genomes gave the equal count of these short putative genes in all of strains. Combining the ORFs with RAST results is important since the automatic annotations recognize only some of the possible gene products but latest applications to genome might stay unnoticed. Even though the designed short ORF recognizing algorithm do not classify or group the potential gene products, it gives a valuable information of the amount of potential gene-encoding areas in the genome. Adding these two tools, RAST annotation and short ORF algorithm, is a relatively effortless way to spot possible differences between the strains circulating around the world. Inasmuch as effort in genetic analyses in a field of clinical microbiology is done, these two sequel analyzes might help to spot minor differences in a larger set of samples that are routinely sequenced and sequence typed globally.

These sequence-based findings, showing the close to equal results regardless the epidemic successfulness status of the strain, emphasize the importance of extending the study from genomics to also phenotypical characters, or more grandiloquently to the behavior and regulation of bacterial cell. In the original publication III we focused on phenotypic features that could associate with spreading of the bacterial strains. As many traits and features were studied and they can be found listed in the original publication III, only the mainline observations and findings are featured in this thesis. All the data from phenotypic measurements was collected and analyzed with discriminant analysis. With discriminant function study data was separated into distinct groups of epidemic and non-epidemic strains. With this analysis likelihood of certain trait to associate with epidemical successfulness or non-successfulness was highly significant. One major feature to stand out in the discriminant analyzes was the drought tolerance which associated with the epidemically non-successful strains. First it might seem peculiar that in particular epidemically non-successful strains were superior in tolerating and recovering for up to 6 months dryness. However, when taken a closer look on the dissemination and infective routes of these strains the explanation might expound with the transmission of these strains. The HAIs caused by *K. pneumoniae* are mainly acquired by either person-to-person contact (Casewell and Phillips 1977) or by the contact with contaminated inanimate surfaces. These inanimate surface contacts are often surgical instrumentation or catheters entering the body and creating a passage between the body and environment (Jarvis *et al*. 1985, Revdiwala *et al*. 2012). In original publication III we declared the strains that have noted to transmit between the hospitals as epidemically successful and the ones that was not detected in multiple hospitals as epidemically non-successful. It is possible that stud-

ied strains indeed differ with their transmission strategy. Our theory is that those strains able to withstand extended periods of drought are the ones to most probably colonize inanimate surfaces thus causing frequent infections on the site of original occurrence. In contrary to this, those strains that are not as drought persistent might have evolved to favor person-to-person contact in their transmission. The found differences in drought tolerance could be a hint of the division of these two transmission strategies. As we already know, the *K. pneumoniae* are naturally habiting the gastrointestinal track (Podschun and Ullmann 1998). For that reason, sometimes AMR strains as well can settle down on the intestines without causing disease and transform a person to the carrier of strain in question (Gorrie *et al*. 2017). This means that pathogens have their way to hitch-hike without causing immediate or severe symptoms but later compel their carrier to the healthcare system. This is an effective and inconspicuous way for a pathogen to transmit inside the community or between the hospitals. Some *K. pneumoniae* STs have found to be hypervirulent and especially hypermucoviscosity has been found in many STs that our dataset suggests to be epidemically successful ones. However, hypermucoviscosity was not observed in any strains studied in our original publication III, hinting that the hitchhiking theory might be possible among the epidemically successful strains. The phenotypic analyses of recent studies mostly focus on hypermucoviscosity testing which can be estimated with string test (Ding *et al*. 2020). However, phenotypic analyses focusing on the spread associated traits seem absent in literature. Altogether, the current opinion of differences in epidemical successfulness of *K. pneumoniae* strains is not very coherent. Since *Klebsiella*s are opportunistic pathogens, relating studies mainly focus on the factors that promote ability to cause disease (Micozzi *et al*. 2017, Nordmann *et al*. 2011). Even though the ability to cause disease is admittedly an important spread promoting feature, our dataset suggest that both epidemically successful and non-successful strains are highly potential to cause disease. As our results implicate, one explanation might be the enhanced drought tolerance and the phenomenon itself has been studied, especially in association of plant epiphytes (Ullah *et al*. 2019). However, currently little is known about bacterial drought tolerance and its association with spread strategies which our results propose to exist.

Among the phenotypic characterizations the cross-inhibition of strains was evaluated. In order to prosper in microbial community, bacteria are often secreting molecules that suppress the growth of other strains. In our set of samples these, competition based, inhibiting molecules were observed and one was further examined. We found one of the epidemically non-successful strains, presenting ST 334, capable to produce inhibiting molecule, hypothesized colicin E3, which was effective against epidemically successful strain of ST group 11. These naturally occurring molecules that inhibit the growth of strains found to be problematic in healthcare are under intensive interest. Since we are heading towards the post-antibiotic era, every potential antimicrobial agent is in demand. Although the colicins are characterized already decades ago and their function is well described, we decided to perform an evolutionary study to investigate the inhibiting effect and resistance emergence against hypothesized colicin E3 (Akutsu *et al*. 1989). The 4-week evolutionary study yielded quick re-

sistance development and stability against colicin E3 which was not found reversible during the experiment. As discussed earlier, the enhanced drought tolerance might support the theory of epidemically non-successful strains indeed being more local. With further deduction, for those strains evolved to persist in one location the ability to produce other strains inhibiting molecule is highly beneficial. As the mechanisms of colicin E3 is based on the ribosomal cleavage inside the target cell, thus leading to the crippling the whole protein production, it is an effective way to verify producer's own position (Ng *et al*. 2010). Several publications have suggested naturally occurring antimicrobial peptides, such as colicins, as substituent for antibiotics (Hassan *et al*. 2012, Gradisteanu Pircalabioru *et al*. 2021). Nisin, prob ably the most known and best characterized representative of these antimicrobial peptides, is currently used as a food biopreservative and lately suggested as a candidate to treat antibiotic resistant infections as well (Hassan *et al*. 2012). However, as our results in original publication III propose, the fast and persistent resistance evolution against at least colicins limit the potential. Similar resistance development has occurred also in other studies related to other colicins and it has been associated with the iron metabolism (Inglis *et al*. 2016). This is a significant drawback that hinder the peptide based antimicrobial agent initialization (Inglis *et al*. 2016).

The carbapenem resistant *K. pneumoniae* spread has been a major concern among a vast group of researchers. The organized surveillance of emergence of these strains and globally shared knowledge have expedited the possibilities to tackle the crisis (World Health Organization 2018). However, the pace of the race against *K. pneumoniae*, and antibiotic resistance it disseminates, is fast and the head start AMR related bacteria possess is hard to stretch. The information of mechanisms propelling the spread of antibiotic resistant strains forms a basis for further studies. Identifying the key steps allows the development of new strategies to intervene and disturb the transmission network. Current actions underline prevention of AMR resistant bacteria transmission in the first place with early detection of possible sources and risks that enhance AMR infection incurrence (Gorrie *et al*. 2017, Lee *et al*. 2017a). The gradually gathering understanding of transmission details, in which our original publication III result also include, could push the development of novel transmission route disturbing approaches in future. This would help to tackle the AMR spread during the transmission and in combination with precautionary actions ease the AMR propagation interception.

### 4.1.2 Acinetobacter baumannii

Other clinically highly relevant MDR bacterial species regarded in this thesis is *Acinetobacter baumannii*, which is in the center of the original publication IV. The triumph of *A. baumannii* as a number one concerning bacterial pathogen and HAIs causing agent is an acute modern-day crisis (World Health Organization 2017). As mentioned in the introduction, *A. baumannii* has rapidly accelerated its status from a rare opportunistic pathogen to the severe and even untreatable infective agent testing the limits of our health care system. *A. baumannii* itself is an intriguing bacterial species. It is a perfect example of how properties refined

to answer the evolutionary pressures of certain habitat can turn out to be superior in a totally different environment, thus leading the newcomer quickly on the top of the niche. In previous studies *A. baumannii*'s exceptional machinery to imbibe resistance genes and exploit features evolved to harsh conditions has been established as a source for prospering in clinical settings (Lee *et al*. 2017b).

In original publication IV multiple *A. baumannii* clinical strains were studied from the perspective of phage therapy and during the phage experiments especially one particular strain caught our attention due its unusual behavior in phage susceptibility tests. The strain itself had a notable antibiotic resistance gene reservoir, consisting of resistance genes against beta-lactams ($_{bla}$OXA-66, $_{bla}$OXA-24 $_{bla}$, and $_{bla}$ADC-25), aminoglycosides (aph(3')-Via, aph(6)-Id, and aph(3'')-Ib), and tetracycline (tetB). This strain had also the high annotation percent with genes relating to high virulence, disease causing, and increased tolerance to compounds normally toxic to bacterial cells. The tolerance towards the sanitizers has been noted to increase due to repeated exposures and *A. baumannii* has found to effectively adapt to presence of sanitizers used in hospital settings (Dorsey *et al*. 2006). This is often explained by the capacity of *A. baumannii* to efficiently adapt to the challenging conditions and rapidly regulate its metabolism to survive in unfavorable conditions (Lee *et al*. 2017b). All these phenotypic features align with the notion of WHO that especially carbapenem resistant *A. baumannii* strains must be considered with the highest priority (World Health Organization 2017). With the found gene palette it is not amazement that similar strains literally terrorize the health care system. As the *A. baumannii* is often transmitted via surfaces and traditionally cause wound infections, the high variety of virulence and long persistence related genes makes it treacherous especially for those recovering from a surgery or spending extended times in a hospital wards with catheters or tubes (Fournier and Richet 2006).

During the phage exposure experiments an intriguing host susceptibility shift between two phage pressures was found and it is discussed in more detail in the chapter 4.2.1. Host-phage interactions. Briefly, we observed that after exposure to a phage 6P1 the host strain's, referred as AB6, phage susceptibility expand to phages 1P1 and 1P2, previously uncapable to infect the host, simultaneously giving the resistance against the phage 6P1. This phenomenon was found to be reversible leading to the shifting host phenotype between these two phage types. Although the clear reason for this reversible shifting susceptible phenotype was not fully retraced in original publication IV, the phenotypic observations and four-week evolutionary study characterize this extraordinary phage-phage interaction throughout the host. The host bacterium altering its phenotype in order to escape the phage pressure is a natural behavior. However, the minimalistic community consisting of two types of phages and a shifting host allowing these two parasites to interact with each other is diminutively noticed in microbial settings. The finding of clear phage-phage interaction is also important in a phage therapy approach in which multiple phages are combined as a cocktail to treat the antibiotic resistant bacterial infections.

## 4.2 Bacteriophages

Phages themselves are an intriguing study subject and have aroused a lot of attention. Since these bacteria infecting viruses are an important part of the original publications I, II, IV, (and briefly discussed in original publication III as well as prophage cross-infection assay), the results of the publications are curated from the phage point of view. However, as pointed out earlier, the existence and actions of phages resonate throughout the whole community and are thus mentioned in detail also in the chapter 4.3 Microbial communities.

### 4.2.1 Host-phage interactions

This chapter instead focuses on the results of original publication IV, the two phages pressure and the shifting host phenomena. All of the phages used in the original publication IV were isolated and named by the Mattila and colleagues (2015) in their earlier publication and these phages form a collection for further studies and characterization performed in our original publication IV. As briefly described previously, the original publication IV characterizes the *A. baumannii* host AB6 susceptibility shifting phenotype between the two phage pressures. Initially the host's susceptibility profile consists of a phage referred as 6P1 but not of the phages 1P1 or 1P2. However, the expose of AB6 to the phage 6P1 was found to cause resistance appearance in which shifted the host susceptibility range and allowed phages 1P1 and 1P2 both infect the host cell. Further expose with phages 1P1 and 1P2, either grouped or individually, yet again caused resistance towards these two phages. Intriguingly however, the 1P1 and/or 1P2 resistant phenotype was found to be again susceptible to the phage 6P1. This susceptibility shift leads to the circle in which the two phages alter the host in the middle, thus providing both of the phages seasonally susceptible host. In order to further study this, the 4-week evolutionary microbial community study, consisting of two groups of bacteria and a host, was performed and long term cyclicity of susceptibility was evaluated. Slightly surprisingly none of the individual community clones were colonized by the mutant resistant to all phages even though such mutants were observed in every community. Anyway, it seems that the emerging resistances enabling the cyclic susceptibility is rather an alteration of the behavior than genetic mutation based on the rapid change. The persistence of susceptible bacteria in a long-term cultivation indicates that the entirely resistant mutant is not preeminent. The combined resistance against both phage pressures probably cause a high fitness-cost that restrains the generalization of the resistant mutant.

When considered from the phage's aspect the found shifting phenomenon is an indirect interaction between the two phages, which is a minuscule studied phenomenon in microbial research. Most of the studies, which accentuate the parasite-parasite interactions, in which phage-phage interplay also falls into, usually focuses on sequentially arriving parasites or opportunistic pathogens (Halliday *et al* 2020). However, at least once similar phenomenon with shifting susceptibility of *A. baumannii* and two phages has been described (Regeimball *et*

*al*. 2016). There the initially infective phage was found to target the capsule structure of the host thus leading to the capsule lost as a defense mechanism. In turn, the loss of capsule exposed the cell to the infection of another phage. This capsule loss was found visible in the colony morphology as well (Regeimbal *et al*. 2016). However, in the case of our original publication IV similar relation to capsule and its suppression was not observed. In general, the both host-phage and phage-phage interactions and their unpredictability still hinder the phage therapy progress. Especially multiple phages interacting with the shared host simultaneously can lead to the undirect interaction between the parasites or quite contrary to elevated perform depending on the interactions. Nevertheless, the shifting host phenomenon and phage-phage interactions themselves are fascinating. The further studies might reveal certain traits that could predict synergistic phage-phage interactions that could be utilized in the phage combinations, which could ease the personalized phage treatment design.

### 4.2.2 Tectiviruses

Tectiviruses are the only members of the virus family *tectiviridae*. Tectiviruses are icosahedral tailless bacteriophages with inner membrane coating on their protein capsid and around 15kb double stranded linear DNA genome (Caldentey *et al*. 1992). Tectiviruses can be divided into different genera depending on weather they infect gram-positive or -negative bacteria or certain bacterial group (Saren *et al*. 2005, Gillis and Mahillon 2014). The model virus of tectiviruses is PRD1, since it has been intensively studied after its discovery in 1970's (Olsen *et al*. 1974). PRD1 is plasmid-dependent phage that has a broad host range and its infectivity is determined by the existence of certain plasmids (Olsen *et al*. 1974). Due to PRD1s exceptional plasmid-dependency it has been suggested to be utilized in re-sensitizing the bacterial populations carrying certain antibiotic resistance genes. In original publication II this phenomenon was used as a phage pressure that pushed bacterial cells to dispose their antibiotic resistance bearing RP4 plasmid, targeted by PRD1. The results of this microbial community experiment are presented in the section 4.3.2 Resistance evolution in microbial communities.

Inspired by a plasmid-dependent phage PRD1 and its potential in re-sensitizing the AMR plasmid bearing bacterial community, we conducted a study in which existing databases were used to screen traces of tectiviral elements on possible bacterial host genomes. In original publication I the known tectivirus major capsid protein coding sequences (from tectiviruses AP50, Gil16, Bam35, and pBClin15) were used to find prophage traces in bacterial genomes. This experiment resulted four new bacterial host genera, previously unassociated with tectiviruses. These four new genera, *Streptococcus*, *Clostridium*, *Brevibacillus* and *Exiguobacterium*, all belong to gram-positive bacteria. Since the gram-negative bacteria, especially enterobacteria, is the driving force of AMR spread, our results did not give any signs of undetected interactions between tectiviruses and gram-negative bacteria. However, tectiviruses that infect gram-negative bacteria seems to be rare and plasmid-dependent phages that could be utilized in AMR studies are even more infrequent. Even though the resulting new hosts

turned out gram-positive, our findings still remarkably expand the host spectrum of these tectiviruses that itself have significance in virus research (Caveney *et al*. 2019, Fornelos *et al*. 2018). The accumulating knowledge of viruses and their host spectrum are also utilized in other studies. One is an enormous research project aimed to build a picture of the last universal cellular ancestor (LUCA) (Krupovic *et al*. 2020). The central idea is that the LUCA was also a host to viruses and by projecting back, with modern bacterial and archaeal virus genomes, the traits of extinct LUCA virome can be traced (Krupovic *et al*. 2020). Traces of several virus families, including tecticvirus elements, have found to associate with LUCA virome (Krupovic *et al*. 2020). As the number of viruses in our planet has been estimated to exceed $10^{31}$ virus particles and their distribution and host spectrums have found to cover every corner found to sustain life, the virosphere builds a frame in which other entities can be studied (Koonin *et al*. 2020). The genetic parasites, which includes viruses, plasmids and transposons, have found to be an intrinsic driving force for coevolution of cellular organisms and parasites themselves (Berezovskaya *et al*. 2018). As we demonstrated in original publication I, the host-phage interactions can be successfully projected back from the genomic traits of viruses have found to leave in the host sequences. Though the LUCA virome research is a massive and complicated project it shares similar study approaches with the original publication I and our results have been noted in the project as well. The association of tectiviruses and LUCA virome indicates that tectiviruses, or rather their ancestors, date back to the ancient steps of cellular organism development. The persistence of phages throughout the time is a staggering study opportunity to patch the gaps on our knowledge, even if with just a handful of new host species.

The techniques used in original publication I highlight the limitations of automatic annotation systems to recognize provirus elements. Basic annotation systems were found to have very limited or non-existent completeness to detect elements without specific tool application. Utilization of these tools enhanced the probability of prophage element recognition dramatically. Thus, in many cases specific tool apply might be laborious and might demand a manual revision, it increases the likelihood to gain more specific data. This shortcoming of automated annotation tools might give misleading conclusions especially in studies focusing on phages and potential prophages as it might implicate falsely, the lack of the viral elements. In the original publication I the automatic annotation systems recognized and categorized the known tectivirus capsule protein coding genes as “hypothetical proteins” or “cytoplasmic proteins”. This indicates that annotation system recognizes these sequences as protein coding areas and since phage capsid proteins accumulate in cytoplasm these annotations are not completely incorrect (Strömsten *et al*. 2003). However, the implementation of specified tools with phage sensitive configuration results more detailed provirus labeling for these genes and provides more accurate results.

### 4.2.3 Phages as therapy solution

In phage therapy, certain features in phages are preferred in order to accomplish functional therapy outcome. When considering the therapeutic

potential of a phage the effectiveness, host range, possible lytic enzymes, and phage-host interactions are important aspects (Nikolich and Filippov 2020). Chosen phages must be suitable to effectively infect and lyse infection-causing bacteria in question. Since the pathogenic bacteria differ case-by-case, used phage or phage combinations must be personalized (Schooley *et al*. 2017). The personalized therapeutic phages can be extracted from natural sources or selected from the existing collections (Mattila *et al*. 2015, Schooley *et al*. 2017). Today phage therapy is more organized than its early days and international phage collections can already be found (Yerushalmy *et al*. 2020, Gordillo Altamirano and Barr 2019). These collections consist of therapy suitable well-characterized phages that can be easily introduced to clinical use (Gordillo Altamirano and Barr 2019, Yerushalmy *et al*. 2020). Since the extracting phages to some clinical bacterial strains from natural sources can be challenging, as seen in the original publication IV in which the phage source fluctuated through the years, the already characterized therapy suitable phages existing in collections can be advantageous option.

Phages can be administrated in infection treatment in multiple ways. In original publication IV single phages, multi-phage cocktails, and same multiple phages as serial exposure model were studied. Four clinical *A. baumannii* strains were all exposed to a set of phages either as single phage exposure, all phages simultaneously as a phage cocktail or serially. However, any of these exposure models did not turn out to be highly efficient, thus eventually leading to the resistance evolution in bacterial hosts. Even though these results did not yield significant triumphant configuration, a lot can be done to adjust the used cocktails. For instance, the interactions between the phages can be utilized to assemble single dose phage cocktails in which the phages infect host serially thus decreasing the phage resistant mutant development (Gordillo Altamirano and Barr 2019). The earlier described finding of *A. baumannii* strains susceptibility shift under two phage pressures is one example of unpredicted actions that challenges the consistence of therapy efficiency. These features are very scarcely understood. In future linking the certain genetic features into phage-phage interaction behavior could help prepare high-performance cocktail design. In this the AI techniques could be utilized to extract the sequences responsible for these interactions, which in turn could be used to collect phage cocktails based on collections sequence databases. This would be the novel way to personalize phage therapy and utilize the capacity of collected data to ensure forward development of the phage therapy.

The characterized three novel phages in original publication IV referred to belong in *myoviridae* and *podoviridae* or *autographiviridae* families and had similarities with phages with known therapeutic potential (Nir-Paz *et al*. 2019, Pulkkinen *et al*. 2019, Jansen *et al*. 2018, Jin *et al*. 2012). As the unsuccessful isolation attempts indicate the varying potential of new phage discovery, the value of characterization of phages that exist in our collections is high. Two of the studied phages, 1P1 and 6P2, showed the clear similarities with phage successfully used in an *A. baumannii* caused bone infection treatment in combination with antibiotics (Nir-Paz *et al*. 2019). The third characterized *A. baumannii* phage did not seem to result any consequential sequence hits with used databases indicat-

ing that the phage differs remarkable from already characterized ones. Even though the phage therapy most likely will not be as straightforward treatment option as antibiotics, the increasing AMR crisis has constricted us to take our chances. Multiple approaches are currently studied to increase the effectiveness and suitability phage therapy. The immobilization of phages into surfaces and controlled uncover has lately been on topic and its potential in phage therapy has been studied (Donati *et al*. 2021, Vinner *et al*. 2019). For example, coating the body penetrating tubes and catheters with antibiotic resistance gene bearing plasmid-dependent phage, such as PRD1, could prevent passage of selectively antibiotic resistant bacteria from inanimate surfaces to human body. These phage immobilization-based techniques are versatile and they can be exploited for instance in agriculture like fish farming, or other antibiotic consuming industry suffering the high rates of resistance emergence (Donati *et al*. 2021).

## 4.3 Microbial communities

Organisms, regardless their size or place as life as a whole of all organisms, tend to interact with each other. Like any other communities, the microbial communities are abundant throughout our biosphere. In addition to interest towards microbial communities themselves, their ability to reflect other community structures and phenomena found in nature has recently allured scientists (Brooks *et al*. 2017). As tackled in the introduction, the microbial communities are highly complex systems. When broken into simple interactions of two participants, some hints of the community dynamics can be estimated. However, as we noticed in our original publication II, in which the study takes place in a multitrophic microbial community, the whole community functions can alter the final outcome of the research. However, the full impact of factors rising from microbial community interactions are not yet intensively studied or well characterized. This leads to the unexpected results that continuously adjust the current opinion of microbes and their relationships (Örmälä-Odegrip *et al*. 2015, Ketola *et al*. 2016, Cairns *et al*. 2016).

Besides studying microbial communities themselves, these settings are often adjusted to fulfill larger studies in a cost-effective way. When studying ecological or evolutionary phenomena in laboratory grown communities, microbes are the most convenient study subjects (Blount 2015). Micro-sized communities can offer simplified study systems and fast generations. However, there are downsides using microbes and one of the most limiting factors is the lack of sexual exchange of genetic material, excluding the horizontal gene transfer i.e. plasmid conjugation (Buckling *et al*. 2009). On the other hand, this feature is sometimes desired when system needs to be simplified to the minimum. Since the complexity of dynamics accumulate by every addition, the microbial communities are hard to study in their natural composition. Simplifying is essential while doing the research but recent studies indicate that there is also still need for more complex microcosm studies. However, as the original publication II demonstrates, even modest looking microbial community setup, with just a

handful of participants, can turn out to become overwhelmingly complex web of dynamics with unexpected outcomes.

### 4.3.1 Multitrophic environments

Multitrophic environments consist of different organisms interacting with each other by consuming other organisms, by being consumed by others, or both. These different trophic levels exist throughout our biosphere and microbial communities are not an exception. Predation-prey composition is a remarkable pressure that can alter the behavior of all the community members linked into either prey or predator on a cascade-like fashion (Northfield *et al*. 2017). Some community members can act both as a consumer and become consumed in multitrophic communities, creating a web of consuming based pressures that can resonate not only in two ways but also in other interactions as well. These resonations can alter the final outcome of certain studied features as we found out in original publication II, from which the central findings are presented in this section. The trophic relationships of the original publication II are presented in the schematic Figure 3. The primary consumer in this study is bacteria Escherichia coli strain HMS174 that consumes the primary resource, which is nutrients derived from the broth. The secondary consumer is protozoan Tetrahymena thermophila, which consumes the primary consumer, namely bacteria.

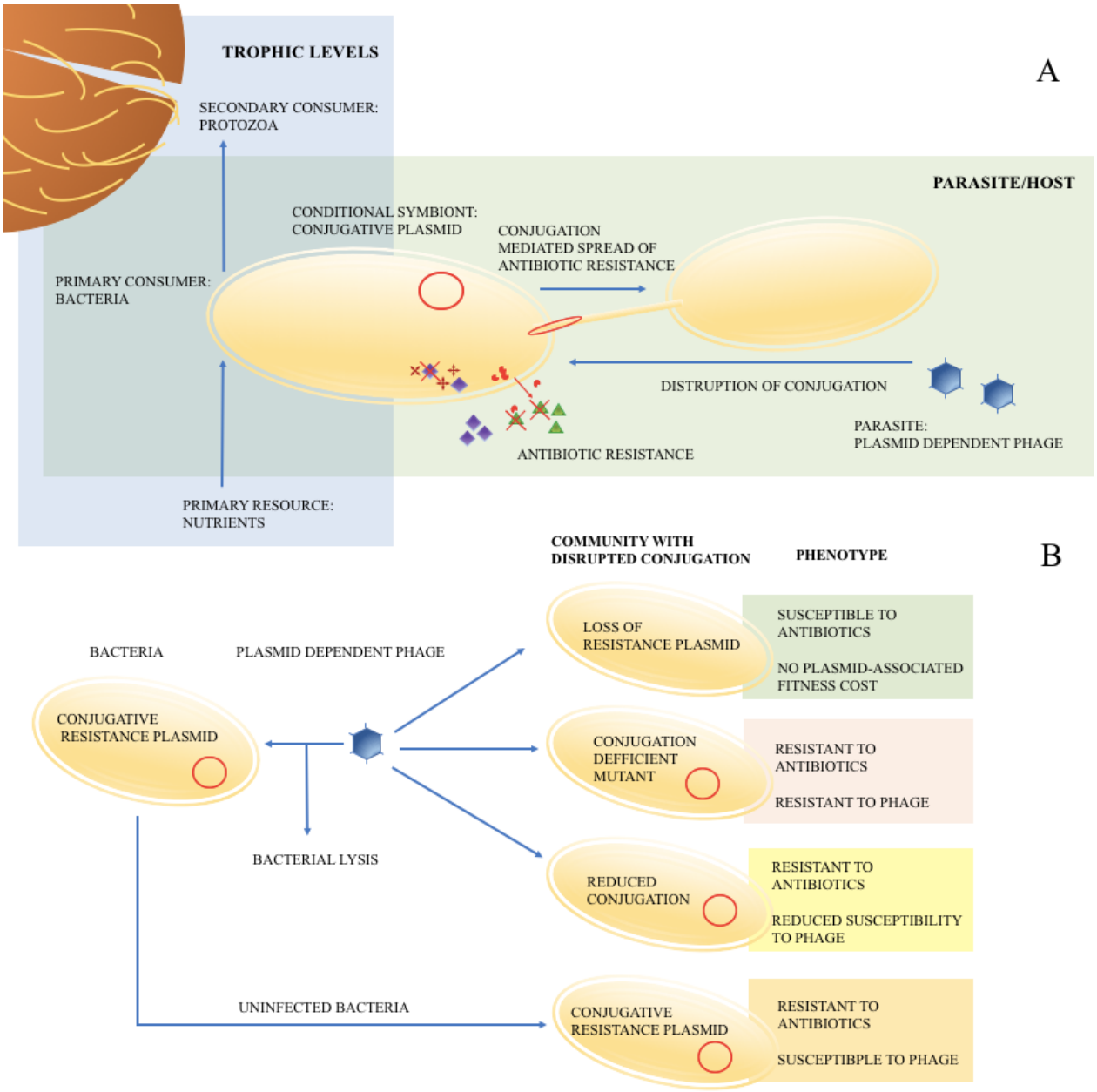


FIGURE 3 **Schematic figure of microbial interactions in multitrophic community assay.** Figure reproduced from the original article II. **(A)** Multitrophic community assembly consisting of two consuming levels, the parasite-host interactions with plasmid-dependent phage, and the environmental antibiotic pressure. **(B)** Conjugation disrupted with plasmid-dependent phage and phenotype options appeared in a disrupted community.

The study combines both multitrophic environment and parasite-host setup with conjugative plasmid and plasmid-dependent phage (Fig. 3). The primary consumer bacterium HMS174 contains the plasmid RP4 that gives a resistance towards both ampicillin and kanamycin. This plasmid-bacteria relationship can also be described as symbiotic relationship in which the plasmids inhabit, replicate, and spread within the bacterial host simultaneously giving the host ability to persist under lethal antibiotic condition. In our study setup, this symbiotic

relationship is disturbed by the plasmid-dependent phage PRD1, which function as a parasite selectively towards those bacteria carrying the plasmid RP4. Besides the interaction between the community members the multitrophic communities are affected by the surrounding environment as well. In the case of our original publication II, the effect of either environment with no antibiotics, increasing concentration of kanamycin, or increasing concentration of ampicillin, was studied. The community's ability to develop, sustain, and spread antibiotic resistance is discussed in the next chapter 4.3.2 Resistance persistence in microbial communities.

In the original publication II the population sizes of each community members were observed as well as the main effect of each member to each other's behavior. When the effects of different pressures were examined from an aspect of population densities of each community members, intriguing observations were made. The bacterial population size was affected by the community composition rather than the type or concentration of antibiotic. Introduction of protozoan decreased the bacterial population density irrespective the presence of a phage, excluding the slight increase caused by the addition of phages simultaneously with protozoan. The decrease in bacterial population density is explained by the feeding of the protozoan. The synergistic effect of the both protozoan and phage unexpectedly slightly increased the bacterial population size, however the increase remained diminutive. The only effect to the protozoan population size was observed when co-existing phages increased the protozoan density. Little is known of the interactions between protozoan and phages. Although these two inhabit the same community, their interactions are most likely indirect via their common target, bacteria. Some hints of these kind of interactions are described as the existence of protozoan have found to reduce the bacterial population susceptibility to phages (Örmälä-Odegrip *et al*. 2015). Possible explanations for the found increase in protozoan population size could thus be related to the competition between predator and parasite or reflections of the bacterial behavior. The co-existence of different protozoan species has indeed found to alter protozoan behavior, cell size, and predation related traits (ter-Horst 2011). The similar effect with protozoan and a phage competing from a common resource seems possible, yet not confirmed.

Neither bacterial or protozoan population sizes or dynamics presented above were affected by the presence of either ampicillin or kanamycin or the increasing concentration of these molecules. However, exception to this was the phage population density. The phage population size peaked in the beginning of the experiment in each antibiotic concentrations and antibiotic type but subsequent decrease of the phage population size varied between the antibiotic treatments. With kanamycin, the phage extinction was decelerated in lethal kanamycin concentration (1.5 MIC) without presence of protozoan predation. However, in sub lethal kanamycin concentration and combined lethal kanamycin concentration with protozoan predation this effect was not observed. In the case of lethal ampicillin concentration (1.5 MIC) in turn, the effect was opposite compared to kanamycin. The ampicillin concentration itself did not elongate the existence of the phage but in a presence of protozoa the extinction of the phage population was delayed. This observed opposite effect of protozoan presence

and high concentration of antibiotic on phage extinction delay seems surprising example of multiple dynamics that has its roots in plasmid co-existence. As the phage PRD1 is a conjugation-disturbing agent, it is strongly linked to the presence of plasmid in the community. As plasmid both enhances and decreases the fitness of its host, depending on the existence and type of antibiotics and plasmid-dependent phages in the environment, it is logical that antibiotic pressure and phage existence are bound together. In an environment with no antibiotics, bacteria were found to lose their plasmids in presence of PRD1 as a defense mechanism against phage infection. Similar effect has been found in other studies as well (Ojala *et al*. 2013). Since the ampicillin resistance has an altruistic behavior, it was observed to allow major part of the bacteria to lose the resistance plasmid, still keeping the bacterial population protected from the antibiotic. Contrary to ampicillin, the kanamycin resistance promoted the plasmid persistence even in a presence of PRD1. Since the kanamycin resistance has no altruistic characteristic, the mechanism does not enable leakiness of resistance. The case of elongated PRD1 existence in lethal kanamycin can be explained by the higher tendency of bacteria to keep their resistance plasmids, in order to survive the kanamycin pressure, as cells cannot rely on the resistance enzymes produced by neighboring cells. However, intriguingly adding the protozoan predation flipped the results. In a lethal kanamycin concentration, the similar effect of elongated PRD1 existence did not occur in presence of protozoan but interestingly it was observed in increasing ampicillin concentration combined with protozoa. The reason behind this is most probably linked to the indirect interactions of protozoan and the plasmid spread. The bacterial population size and type of bacterial behavior were dramatically affected in the presence of both protozoan and plasmid-dependent phage. As our original publication II shows, the metabolic activity of bacteria and conjugation frequency was elevated in the conditions where protozoan predation was present. The protozoan predation caused lower bacterial density most likely increased the metabolic activity of bacterial cells, thus also promoting conjugation. The increased conjugation activity instead leads to antibiotic resistance conferring plasmid spread in the community. The protozoan predation and conjugative plasmid survival have previously linked together (Cairns *et al*. 2016). Under protozoan predation, conjugation-deficient mutants were found more infrequent compared to communities lacking the protozoa (Cairns *et al*. 2016). However, the explanation for both increased conjugation and higher metabolic activity might relay in the decreased bacterial density caused by predation. As it has been shown, the released space and spare resources promote bacterial division (Hibbing *et al*. 2010). When protozoan is present, bacterial population do not reach stationary phase as easily as without predation, thus pushing the population towards more metabolically active early log-phase (Robador *et al*. 2018). In early log-phase many building blocks needed to conjugation are produced inside the cell and thus might enable enhanced conjugation rates (Robador *et al*. 2018).

Other consuming-based interaction alteration was observed between the plasmid-dependent phage PRD1 and the conjugative resistance plasmid RP4. When PRD1 was introduced to community setup, it produced four possible outcomes in the phage-disturbed community (Fig. 3). Those bacteria able to

avoid the PRD1 infection in a first place were able to continue with conjugation capable plasmid and retained their resistance towards antibiotics. However, this approach leaves the bacteria susceptible to further phage infections, thus being a pernicious strategy in the course of time. For those bacteria not able to avoid contact with phage there were three possible outcomes to overcome the phage predation pressure. First strategy was bacterium to lose the resistance plasmid. Since the infectivity of PRD1 is bound to the plasmid existence, this has permanently found to prevented the phage infection (Ojala *et al*. 2013). However, this strategy also leads to the resistance loss and expose bacterial cell to lethal effect of antibiotics. Second way to evade phage predation is to develop conjugation deficient mutant of the plasmid and thus protect the bacterium. This gives bacterial cell opportunity to benefit the antibiotic resistance genes but restricts the plasmid's opportunity to spread in the bacterial population via horizontal gene transfer. In the third strategy the conjugation efficiency is reduced thus compromising between the total secure from infection and ability to conjugate. In cases when conjugative bacteria were not able to escape the encounter with a phage, bacterial cell was lysed and the phage replicated. The amounts of bacteria falling into these three survival strategy categories, mentioned above, were observed in altered environment, which either contained or did not contain protozoan predation and two types of antibiotic pressures, aminoglycoside kanamycin and beta-lactam ampicillin. Introduction of PRD1 was found to favor conjugation deficient mutants, despite of the antibiotic pressure. The next chapter focuses more detail on the antibiotic resistance persistence in these evolutionary studies featured in original publication II. These different survival strategies are often studied in cultures with bacteria, plasmid, phage and antibiotic pressure but rarely studied in the presence of protozoan (Ojala *et al*. 2013, Colom *et al*. 2019). However, the presence of the community interaction has found to shake the community dynamics in other studies as well (Örmälä-Odegrip *et al*. 2015, Ketola *et al*. 2016, Cairns *et al*. 2016). The research extending into community scale undeniably define our understanding of microbial behavior. The coincidental occurrence of interactions that microbes encounter in the microbial community seem to mold certain traits of a pathogen (Ketola *et al*. 2016). For example, in life-history evolution studies the multitrophic pressures have noticed to reduce the virulence of bacterial pathogen (Zhang *et al*. 2014, Friman *et al*. 2009). Beside virulence, the protozoan predation was observed to have a crucial contribution in evolution of bacteria (Hiltunen *et al*. 2017). Interactions inside the microbial community also have an impact on how the communities themselves evolve. Microbial community composition and interactions affect also themes such how invasive species are introduced to the community (Ketola *et al*. 2017). It has been shown that in the bacterial communities under invasion the community construction, phylogeny, and interactions on their part determine the extent of the invasion (Ketola *et al*. 2017). In the context of AMR propagation this might also affect the invasion of AMR bearing species. These findings, among many others, emphasize the effects that rise from the complex dynamics microbial communities endow.

### 4.3.2 Resistance persistence in microbial communities

Interactions between the microbes and their relationship to AMR abundance has only recently gathered interest and it is not yet widely studied (Bottery *et al* 2020). One of the reasons why it is often overlooked is the complexity of the studies. Antibiotic susceptibility and resistance persistence tests are still often performed in monocultures, which lack the multispecies interactions that are present in the natural habitats (Bottery *et al*. 2020). For example, the antibiotic susceptibility of studied bacterial strain has found to remarkably weaken when the experimental strain has been embedded to microbial community (Klümper *et al*. 2019). In original publication II we conducted a study in which community dynamics under the antibiotic pressure were investigated. In the study 50-day evolutionary experiment was performed to examine contributions of each microbial community members to the antibiotic resistance persistence. Also, the altruistic behavior among resistance plasmid bearing bacteria was examined with two different antibiotics with two different resistance mechanisms, either altruistic in nature or not. In the original publication II the microbial community assays consisted of two treatments, a community composition treatment and an antibiotic treatment. In a community composition treatment, always consisting of the bacterium *E. coli* enclosing the ampicillin and kanamycin resistance plasmid RP4, was altered to either have a plasmid-dependent phage PRD1 and/or the protozoan *Tetrahymena thermophila* (Fig. 3). In a second treatment ampicillin or kanamycin antibiotics were added to the first treatment settings as 0.0, 0.1, or 1.5 x MIC (Minimum Inhibitory Concentration). RP4 plasmid gives two different kinds of resistances towards these antibiotics. For ampicillin the resistance mechanism can be altruistic in nature when the antibiotic degrading enzyme is secreted to cellular surroundings, whilst the kanamycin resistance mechanisms are located inside the cell thus not permitting altruistic behavior. This altruism was found to be even more significant in plasmid prevalence than used antibiotic concentration.

Black Queen evolution is an evolution theory, sometimes called 'race to the bottom', and it describes ecological situation in which genes beneficial in environment are loss by natural selection (Morris *et al*. 2012). From the aspect of individual, even necessary genes can be lost if other members of the community provide these gene products (Morris *et al*. 2015). In our study in original publication II this Black Queen evolution hypothesis spring from the leakiness of antibiotic resistance inside the system through the community members. The name Black Queen hypothesis is an analog for the playing cards in which the queen of spades (also known as the Black Queen) is useful during the game but in the end point the one who bears the card pays the price (Morris *et al*. 2012). In our original publication II the Black Queen refers to the plasmid which, indeed as in early stages of a card play, protects the bacterial cell from antibiotic pressure. As a card game continues, the pressure of disposing the Black Queen increases similar to a plasmid carriers' risk to being infected by PRD1. Altruistic mechanism of ampicillin resistance allows shortcut in the game for cheaters, which lose their plasmid but savor the resistance enzymes secreted by the unfortunate cells having the plasmid (or, the Black Queen) in the endpoint of the

game. The found phenomenon of altruistic behavior, following the theme of Black Queen hypothesis, was more significant than the concentration of ampicillin and counteracted by the protozoan predation. Consequently, both the mechanisms of antibiotic resistance and co-existence of protozoa are prominent features in the total resistance persistence. As the producing of antibiotic degrading enzymes is a fitness cost and bearing the resistance plasmid expose the bacterial cell to the phage predation, there is a pressure to either lose the plasmid and live as a cheater or become a conjugation deficient mutant. These conjugation deficient mutants still bear the fitness costs of producing the resistance enzymes but are safe from the phage infections. However, protozoan ability to induce the metabolic activity surprisingly balanced the bacterial community in such a way that conjugation deficient mutants did not take over the community. These two features, altruism and protozoan predation, ended up as the most significant factors to sustain the antibiotic resistance in our microcosm experiment.

The addition of plasmid-dependent phage into antibiotic resistance plasmid possessing bacterial community has been proposed to help re-sensitizing the bacterial population (Jalasvuori *et al*. 2011). Multiple *in vitro* studies indicate that this re-sensitizing method could be useful to locally lower the resistance burden (Jalasvuori *et al*. 2011, Harrison *et al*. 2015). However, as the results of original publication II denote, simple phage-host interactions can be embedded in the network of other interactions and reactions inside the community. The similar altruistic behavior observed with ampicillin resistance did not occur with the kanamycin resistance. This plasmid-dependent phage related re-sensitizing attempts could be applied to bacterial communities with non-altruistic antibiotic resistance, such as kanamycin resistance. However, we must bear in mind that introduction of PRD1 into our community structure induced the occurrence of conjugation deficient mutants. This in turn keeps part of the bacterial population resistant and safe from the phage predation regardless the non-altruistic nature of resistance. In our 50-day microcosm experiment in original publication II, the phage PRD1 was found to alter the bacteria and plasmid behavior as mentioned in previous section (Fig. 3). Even though the conjugation deficient plasmids cannot transmit via horizontal gene transfer, they still exist in the community since the preeminent advantage of resistance they offer for the host cell. In conditions with resistance plasmid-bearing, PRD1 and kanamycin the conjugation levels were slightly higher compared to antibiotic free or ampicillin having environment, but still remarkably reduced. The conjugation deficiency has found to rarely revert even when the phage pressure is departed (Ojala *et al*. 2013). This may lead to long term hindering of antibiotic resistant spread (Ojala *et al*. 2013). However, it will not totally eradicate the resistance problem. Even worse the situation is in communities with protozoan predation since our study found protozoan induce the conjugation capability regardless the PRD1 in every environment tested.

The existence of protozoan predation in human body is quite rare in countries with high living standards and of high hygiene levels (Chabe *et al*. 2017). Nonetheless, intestinal carriage of protozoan has found to be remarkable factor in several areas also combating with antibiotic resistance problem (Chabe *et al*.

2017, Gilchrist *et al*. 2017, Laxminarayan *et al*. 2013). The simultaneous existence of protozoan and resistance plasmid might promote antibiotic resistance persistence in individuals and in the community. Besides the human body protozoan also exist in surrounding environment i.e. wastewater (Dubber and Gray 2009). Wastewater combines all the community members introduced in the original publication II, thus creating the optimal settings for similar effects found in the publication. Even though wastewater itself scarcely interact with the humans it is potential that wastewater treatment plants act as a reservoir to antibiotic resistant strains. These reservoirs maintain the total antibiotic resistance, which in a global scale is a significant problem. As the results of original publication II indicate, the antibiotic resistance evolution and spread in the multitrophic communities is a sum of notable large set of factors. There are indications of existence of antibiotic resistance genes resembling modern resistance genes that dates prior to antibiotic era (Aminov and Mackie 2007). This in turn indicates that phenomenon of resistance development occurs naturally in the microbial communities in which bacteria fight for their persistence. In one theory resistance genes against the naturally occurring clinically used antibiotics have their origins in nature but their abundance and accumulation have been accelerated by the human antibiotic usage (Aminov and Mackie 2007, Barbosa and Levyx 2000). The Baas-Becking hypothesis, in which "everything is everywhere, but the environment selects", fits to this theory of antibiotic resistance originating prior to antibiotic drug usage (Baas-Becking 1934, Fondi *et al*. 2016). Similarly Baas-Becking hypothesis also offers a platform to Black Queen theory-based resistance development in a community that have multi-trophic network and outer antibiotic pressure, such as the on in original publication II.

## 4.4 Other crises and AMR problem

As the AMR phenomenon is not restricted individual instance, neither are the AMR related features studied in this thesis, and many themes circle around the AMR crisis. While writing this thesis in the spring 2021, world is still regaining from a microbe-based pandemic. This grievous example of microbial world's potential hazard is coronavirus caused covid-19 pandemic, still circulating around the globe. covid-19 itself is an unsettling reminder of the society scale crippling potential of a single human infecting virus that also resonates on the microbial community scale as well. Secondary treatment of covid-19 patients often requires antibiotics, thus linking also viral infections not treatable with antibiotics, to the antibiotic usage (Penalva *et al*. 2021). Acute and unexpected circumstances, such as covid-19 note, can considerably accelerate the AMR crisis (Pelfrene *et al*. 2021). However, there are ways to avoid, hinder, or treat the antibiotic resistance spread and secondary infections. The clearest way is effective vaccination of prominent part of the population. However, the availability and distribution of vaccines may provoke inequality, which is linked to the multiple health care issues. Nevertheless, vaccination has been considered to indirectly reduce antibiotic resistance by lowering the antibiotic usage in the

secondary treatment in many viral diseases (Klein *et al*. 2020). Precautional actions are the best way to keep the spread of any pathogens in minimum and the elevated hand wash hygiene has hindered the transmission of AMR associated pathogens as well (Pelfrene *et al*. 2021). Also, the potential phages for covid-19 based secondary infection treatments are under research thus providing alternative treatment options (Wu *et al*. 2021).

Not only the microbial crises are linked to the AMR issues. As mentioned, the inequality of people is one major dividing factor globally. Especially in developing countries or countries with large social class differences it is usual that remarkable part of the population struggle to get a professional health care treatment when needed (Anstey Watkins *et al*. 2019). The non-prescription antibiotic usage and inappropriate utilization of antibiotics are a major factor in the spread of AMR (World Health Organization 2019). Though the unconcerned antibiotic use can be found globally, the areas in which the problem peaks do exist. These areas are mainly in the countries of lower cost domestic product (Pokharel *et al*. 2019, Moise *et al*. 2017, Nguyen *et al*. 2019). One probably unthought-of crises that has suggested to link with both AMR crisis and increasing equality is the climate change (King and Harrington 2018). The warming climate has found to increase the appearing rate of infectious diseases thus offering AMR a stepping-stone to propagate (MacFadden *et al*. 2018). Climate change effects also restrict the agricultural capacity in which the hindered production is sometimes boosted with the preventive use of antibiotic drugs (Manyi-Loh *et al*. 2018). As the population on the planet earth keep on growing, the resources eventually run out (Schneider *et al*. 2011). The lack of resources strikes first on the areas already struggling with many themes related to inequality thus giving the AMR an even more potential to prosper (Schneider *et al*. 2011). As the novel antibiotic discovery has collapsed, we must consider existing and functional ones as a limited resource and act accordingly.

Although the linkage of AMR in vast global crises might seem discouraging, through the crises sprouts the new novel innovations as well. New antimicrobial agents and surfaces have been developed in accelerated speed during the covid-19 pandemic and many of them are already in use (Behzadinasab *et al*. 2020). Several novel approaches on the antibiotic resistant infection treatment has also been reported, i.e. light activated molecular drills has found to help to fight against meropenem resistant *K. pneumoniae* (Galbadage *et al*. 2019). Though the AMR crisis undeniably is one of the most arduous tasks to overcome in the upcoming years, the mankind has shown its perseverance in fighting for its own existence. The combined knowledge and collaboration, also in science including open access publishing, allow the full potential to excel in this task.

## 4.5 From them to us

Much of the fascination springing from the microbial research comes from the limitations of visual observation what comes to the behavior of the individual cells. Interacting with microbes is effortless in our daily lives, as it mostly occurs unnoticed and mainly the imbalances or pernicious actions are discerned. In research instead, the right words, that are to say study questions, must be found to ask from the microbial community and listen the answer, the results. Often these results are hints that lead to the further questions and answers, quite like many discussions. After sufficient amount of questions asked and answers listened the story of microbes can be written in words, from them to us. Similar to any discussion, each participant, regardless if it is a microbe interacting with microbe or human trying to figure the purpose of these companions, the conversation leads to interpretations, altered thoughts or behavior, and maybe further actions that are influenced by the discussion. The more there are participants in the discussion the wider is the web of interactions. A monolog of an organism is mainly affected by the past encounters and the surrounding environment. This describes the function of a cell isolated from the surrounding companions and their interactions. However, when the monolog is heard by others, the listeners might be impressed and alter their behavior, i.e. responding to the signaling molecules secreted by the speaker. Contrary to the monolog, in a dialog between two participants the involved ones are adjusting to both their own and other's perspectives simultaneously. Similar to the monolog these dialogs can also be observed. In the conversations between multiple participants different roles can be taken and the track of conversation meander around each individual's interests. The outcome of the conversation is hard to predict, much like the results of microbial community functions. However, by interacting more the standards of one's behavior and reactions start to perceive, which is also the purpose of this thesis. With each original publication the questions asked and answers listened have led to the conclusions presented in this thesis. My humble wish is that I have managed to act as a decent interpreter from microbes to us.

# 5 CONCLUSIONS

I Since the immense accumulation of sequencing data, new information can be secluded using databases and combination of both automated tools and manual extraction. In this example we were able to expand the host range of Tectiviruses into four additional genera, including non-sporeforming *Brevibacillus* and *Streptococcus*.

II Dynamics inside the multi-trophic microbial communities are complex and, as our study shows, in microbial communities the interaction-based pressures, such as protozoan predation, can promote antibiotic resistance gene spread. Also, the type of resistance mechanism in the community combined to protozoan presence enhance antibiotic persistence rather than environmental antibiotic pressure.

III By broadening up the characterization of nosocomial *Klebsiella pneumoniae* isolates from genetic features into phenotypic features can explain differences in epidemical successfulness of different strains. Despite the limited capacity of phenotype testing, we were able to associate drought tolerance and recovery from dryness with the epidemic status of the strains. This could be one of the key features guiding the initial establishment of certain strains and development of their virulence factors.

IV The study of host-phage interactions might also reveal unexpected phage-phage interactions that are unfrequently noted in literature. As evaluating phage therapy potential, these interactions, such as the found cyclic host susceptibility shift under two phage pressures, might give an insight to improve the future personalized phage cocktail assemblies and lead to the more successful therapy outcomes.

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# YHTEENVETO (RÉSUMÉ IN FINNISH)

## Mikrobien väliset vuorovaikutukset antibioottivastustuskyvyn leviämistä ajavana voimana

Mikrobien väliset vuorovaikutukset ovat keskeinen osa biosfäärimme toimintaa. Sekä yksittäiset mikrobit että mikrobiyhteisöt vaikuttavat arkeemme usealla tavalla. Yksi keskeisimmistä mikrobiyhteisöjen tuomista ongelmista on antibioottivastustuskyky, joka on yleistymisensä myötä noussut yhdeksi akuuteimmista terveyteen liittyvistä kriiseistä maailmanlaajuisesti. Antibioottivastustuskykykriisin myötä antimikrobiaalisten lääkkeiden vaikutus infektiohoidossa on heikentynyt ja ongelman ratkaisemiseksi ilmiön ymmärtäminen ja uusien menetelmien kehittäminen on välttämätöntä. Tämä väitöskirja pureutuu neljän osajulkaisun voimin sekä yksittäisten mikrobien, että mikrobiyhteisöjen sisäisten ja ulkoisten vuorovaikutusten osuuteen antibioottivastustuskyvyn ylläpitämiseen ja leviämiseen.

Mikrobiyhteisöt koostuvat useista mikroskooppisen pienistä eliöistä, jotka vaikuttavat yhdessä ympäristönsä kanssa. Keskeisimpiä mikrobiyhteisöjen jäseniä antibioottivastustuskyvyn yleistymisen kannalta ovat bakteerit, antibioottivastustuskykygeneejä sisältävät konjugatiiviset plasmidit, bakteriofaagit ja alkueläimet. Osajulkaisu III tutkii karbapeneemivastustuskykyisten *Klebsiella pneumoniae* -bakteerien leviämistä globaalisti sairaaloista toiseen. Osajulkaisussa kartoitettiin sekä geneettisiä, että fenotyyppisiä ominaisuuksia, joiden arveltiin liittyvän tiettyjen kantojen tehokkaampaan kykyyn levitä sairaalasta toiseen. Suoritettujen geneettisten analyysien perusteella mikään yksittäinen piirre ei noussut esiin selittävänä tekijänä, mutta tutkituista fenotyyppisistä piirteistä pitkäkestoinen kuivuuden sieto ja siitä toipuminen linkittyi leviämisstatuksen kanssa. Tutkimuksessa havaittiin, että paikallisia infektioita yksittäisissä sairaaloissa aiheuttavat *K. pneumoniae* kannat selviytyivät globaalisti sairaalasta toiseen leviäviä kantoja paremmin kuivuudesta. Havainnon pohjalla saattaa olla kantojen erikoistuminen erityyppisiin leviämisstrategioihin, joissa paikallisia infektioita aiheuttavat kannat hyötyvät erinomaisesta kuivuuden kestävyydestä ihmisvälitteisesti leviäviä kantoja enemmän.

Bakteriofaagit ovat bakteerisoluja luontaisesti selektiivisesti infektoivia viruksia, joita on jo pitkään tutkittu keinona hoitaa antibioottivastustuskykyisiä infektioita. Osajulkaisut I ja IV keskittyvät bakteriofaagien ja niiden isäntäbakteerien välisten vuorovaikutusten tutkimiseen. Osajulkaisussa I tutkittiin yleisesti saatavilla olevan geneettisen sekvenssidatan avulla *tectiviridae* virusperheeseen kuuluvien virusten isäntäkirjoa. Tektivirukset ovat faagiterapian kannalta mielenkiintoinen ryhmä, sillä niihin lukeutuu myös osajulkaisussa II käytetty plasmidiriippuvainen bakteriofaagi PRD1. Tutkimalla ja hyödyntämällä vapaasti käytettävissä olevaa sekvenssidataa sekä automatisoituja että manuaalisia proteiinitunnistusmenetelmiä osajulkaisussa I onnistuttiin laajentamaan tektivirusten isäntäkirjoa neljään uuteen bakteerisukuun. Osajulkaisussa IV puolestaan karakterisoitiin kolme laajasti antibioottivastustuskykyistä *Acineto-*

*bacter baumannii* bakteerin kliinistä kantaa infektoivaa bakteriofaagia. Näillä uusilla bakteriofaageilla havattiin olevan yhtäläisyyksiä jo ennalta karakterisoitujen faagiterapiapotentiaalisten bakteriofaagien kanssa, mikä lisää niiden mielenkiintoa terapeuttisen käytön kannalta. Toinen mielenkiintoinen seikka löytyi kuitenkin sattumalta, kun kahden bakteriofaagiryhmän havaittiin vuorovaikuttavan toistensa kanssa isäntäbakteerin välityksellä. Syklisen isäntäbakteerin herkkyyden vaihtelun havaittiin mahdollistavan molempien bakteriofaagityyppien vuorottaisen infektiivisyyden. Vastaavia bakteriofaagien välisiä vuorovaikutuksia on kuvattu harvakseltaan ja niiden vaikutusta esimerkiksi useita bakteriofaageja yhdistelevien hoitomuotojen onnistumiseen ja tehokkuuteen ei juurikaan ole tutkittu.

Bakteerien ja bakteriofaagien lisäksi mikrobiyhteisöissä vuorovaikuttavat bakteerisoluista toiseen siirtyvät konjugatiiviset plasmidit, jotka usein levittävät mukanaan antibioottivastustuskykygeenejä sekä alkueläimet. Osajulkaisu II tutkii mikrobiyhteisön sisäisten vuorovaikutusten sekä ulkoisen antibioottipaineen vaikutusta antibioottivastustuskykygeenien säilymiseen ja leviämiseen mikrobiyhteisössä. Bakteereita ravinnokseen hyödyntävien alkueläinten läsnäolon havaittiin parantavan antibioottivastustuskykygeenien leviämistä bakteeriyhteisössä. Myös antibioottivastustuskykymekanismilla havaittiin olevan antibioottivastustuskyvyn säilymisen kannalta merkittävä vaikutus, kun yhteisöön lisättiin konjugatiivisen plasmidin omaavia bakteereita selektiivisesti infektoiva bakteriofaagi PRD1. Yhteisön sisäisten vuorovaikutusten, kuten alkueläinten saalistuksen, sekä antibioottivastustuskykymekanismin vaikutuksen havaittiinkin olevan merkittävämpi antibioottivastustuskyvyn säilyvyyden kannalta koeasetelman mikrobiyhteisössä, kuin ulkoisen antibioottipaineen.

Tämä väitöskirja kokoaa edellä mainittujen osajulkaisujen keskeisimmät tulokset ja arvioi niiden merkitystä antibioottivastustuskyvyn säilyvyyden ja leviämisen kannalta. On selvää, että mikrobien väliset vuorovaikutukset ovat moniulotteisia ja tässä väitöskirjassa pystyttiin kartoittamaan vain niistä muutamia. Kuitenkin jo näiden tulosten perusteella voidaan todeta, että mikrobiyhteisöjen synergiset vaikutukset todellakin vaikuttavat antibioottivastustuskykygeenien olemassaoloon ja voivat muuttaa koko yhteisön antibioottivastustuskyvyn määrää. Tutkittaessa uusia ratkaisuja antibioottivastustuskyvyn voittamiseksi onkin tärkeää muistaa laboratorioiden ulkopuolella vallitsevien vuorovaikutusten verkon ennalta arvaamattomat vaikutukset. Esimerkiksi bakteriofaagien hyödyntäminen rutiininomaisesti antibioottiresistenttien infektioiden hoidossa omaa suuren potentiaalin, mutta kuten tämän väitöskirjan osajulkaisut osoittavat, vaatii se vielä tarkempaa ymmärrystä isäntä-bakteriofaagi vuorovaikutuksista. Kumuloituva tieto, niin sekvenssidatan kuin julkaisujenkin muodossa, auttaa kuitenkin pala palalta kasaamaan eheämpää kuvaa sekä itse mikrobiyhteisöistä itsestään että myös niiden vuorovaikutuksesta ympäristönsä kanssa. Tämän ympäristön muut eliöt, mukaan lukien me ihmiset, jakavat mikrobien kanssa ja se toimiikin kuin rajapintana koko biosfäärin kattavan vuorovaikutusten verkossa.

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# ORIGINAL PAPERS

# I

## EXTENDING THE HOSTS OF *TECTIVIRIDAE* INTO FOUR ADDITIONAL GENERA OF GRAM- POSITIVE BACTERIA AND MORE DIVERSE BACILLUS SPECIES

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# Extending the hosts of *Tectiviridae* into four additional genera of Gram-positive bacteria and more diverse Bacillus species

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ABSTRACT

*Tectiviridae* are composed of tailless bacteriophages with an icosahedral capsid and an inner membrane enclosing a double-stranded 15 kb linear DNA genome. Five of the seven previously studied Tectivirus isolates infect bacteria from *Bacillus cereus* sensu lato group (Betatectivirus), one distantly related member (PRD1) infect *Enterobactericeae* (Alpatectivirus) and one recently discovered virus infect *Gluconobacter cerinus* (Gammatectivirus). Here we expand the host spectrum of Betatectivirus elements to four additional genera (*Streptococcus*, *Exiguobacterium*, *Clostridium* and *Brevibacillus*) and to more distantly related *Bacillus* species (*B. pumilus* and *B. flexus*) by studying the genomes of fourteen novel tectiviral elements. Overall, the genomes show significant conservation in gene synteny and in modules responsible for genome replication and formation of the virion core (including DNA packaging). Notable variation exists in regions encoding host attachment and lysis along with the surrounding area of a site in which mutations are known to alter phage life cycle.

## 1. Introduction

The virus family *Tectiviridae* contain a single genus, Tectivirus, that comprises tailless bacteriophages with an icosahedral protein capsid of approximately 70 nm in diameter. The protein coat encloses an inner membrane, which during infection extrudes from the capsid to penetrate the cell wall and membrane(s) of the host. The ~15 kb double stranded linear DNA genome harbors around 30 genes and is replicated in protein-primed manner by a phage-encoded polymerase (Caldentey et al., 1992; Berjón-Otero et al., 2016). After genome replication, DNA is packed with an ATPase into the capsid and the host cell is lysed with an endolysin.

The type virus of the *Tectiviridae* is PRD1, Gram-negative bacteria infecting plasmid-dependent phage (Olsen et al., 1974). As PRD1 binds to a plasmid-encoded receptor on the host cell, its host-range aligns with that of the plasmid (Olsen et al., 1974). PRD1 has been studied intensively for several decades, making it one of the best characterized viruses in terms of genome, structure and function (Abrescia et al., 2004; Cockburn et al., 2004). Yet, PRD1 is the sole representative of the family in the Gram-negative clade (excluding a handful of other isolates that are genetically almost 100% identical to PRD1, Saren et al., 2005). The other previously known members of *Tectiviridae* infect Gram-positive bacteria of *Bacillus cereus* sensu lato group (*Bacillus anthracis*, *Bacillus cereus* and *Bacillus thuringiensis*, Gillis and Mahillon, 2014a). However, very recently, a novel Tectivirus GC1 infecting *Gluconobacter cerinus* was characterized and proposed to form a new genus, "Gammatectivirus" (Philippe et al., 2018). There are clear differences among the clades of the family. PRD1 is a strictly lytic virus whereas the *Bacillus* infecting representatives and GC1 can establish lysogeny (in case of tectiviruses, the genome is replicated within the host cell without integrating into the host genome). Lysogenic cycle of Gram-positive bacteria infecting tectiviruses, however, is prone to switch into strictly lytic cycle when the host-encoded LexA repressor binding site on the virus genome gets disrupted by mutations (Fornelos et al., 2011, 2015). Genetically the clades are very different and there is little homology on the level of DNA (Ravantti et al., 2003; Philippe et al., 2018). However, the products of the core genes of these viruses (namely the genes coding for major capsid protein and DNA packaging ATPase) share approximately 20–30% similarity on the level of amino acids and all tectiviruses are structurally very similar.

Currently identified members of *Bacillus cereus* sensu lato infecting *Tectiviridae* share 60–100% identity on DNA level. These phages are Bam35 (or almost identical Gil01 that differs by eleven nucleotides, Verheust et al., 2003; Ravantti et al., 2003), Gil16 (Verheust et al., 2005), AP50 (Sozhamannan et al., 2008), Wip1 (Kan et al., 2013) and pBClin15 (Stromsten et al., 2003). One of early tectiviral isolates infecting *B. acidocaldarius*, phiNS11 (Sakaki et al., 1977), is no longer available in the laboratory and has not been sequenced. A large-scale

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PCR-based screening for Tectivirus-related elements among 2000 *B. cereus* isolates confirmed their presence in only 2.7% of the strains, indicating that they are relatively rare but also genetically conserved (Gillis and Mahillon, 2014a, 2014b). It is also notable that only around half of the annotated open reading frames (ORFs) of these tectiviruses have identifiable homologs in databases (Sozhamannan et al., 2008; Berjón-Otero et al., 2017). Given that the currently identified phages infect relatively closely related hosts (and PRD1 and GC1 infect extremely distant hosts), it has not been possible to study the evolution of (Gram-positive bacteria infecting) tectiviruses beyond *B. cereus* sensu lato group. In this paper, we analyze the genomes of fourteen new Tectivirus-related elements from four new genera (*Streptococcus*, *Exiguobacterium*, *Clostridium* and *Brevibacillus*), from more distantly related *Bacillus* species (*B. pumilus* and *B. flexus*) in addition with elements from new strains of *Bacillus cereus* sensu lato group.

## 2. Results and discussion

Tectivirus genomes were used as a query to search homologous viral elements in databases. Genomes of twelve novel tectivirus-like elements were discovered from the GenBank. Four of these elements originate from novel genera, namely *Streptococcus*, *Clostridium*, *Brevibacillus* and *Exiguobacterium*, none of which have been previously known to host tectiviruses. Six additional elements were discovered from different *Bacillus* species: *B. cereus*, *B. thuringiensis*, *B. pumilus* and *B. licheniformis*. The latter two are not part of the *Bacillus cereus* sensu lato group, and thus provides examples of these elements in more distant *Bacillus* hosts. Further, there are two very incomplete environmental metagenomics sequences from Red Sea in which clear Tectivirus-like elements exists (GenBank ids KX984138 and KX984131). However, we left them out from detailed analysis due to the lack of information on hosts and the incompleteness of the sequences. Nevertheless, the sequences studied here double the number of Tectivirus-like elements for which nearly-complete genome sequence is available (listed in Table 1). Note that the two elements in *Streptococcus* are the same and the element in *Bacillus cereus* strain FSL M8-0473 is identical to previously studied element pBClin15 because the same strain was sequenced twice, first in 2003 (Ivanova et al., 2003) and later in 2017 (direct deposit).

All of these elements were manually checked for their (automated) annotations. Unsurprisingly, many of the likely coding domains had not been identified. We curated the genomes by adopting the following criteria to locate putative genes: the open reading frames (ORFs) should be preceded by a potential Shine-Dalgarno site (AGGAGG sequence allowing some permutations) to which ribosomes bind, and the ORFs should fill the apparent "non-coding" regions in the genome (as tectiviruses are known to be tightly packed with coding areas and thus existence of long regions without genes is unlikely). Five to ten new putative genes were identified from each element and most had homologs in other tectiviral elements, suggesting that they are likely to be genes. Further, analysis of the deposited annotations revealed a short-coming of these automated tools as they generally failed to describe the genetic content accurately enough in order to identify them as potential proviruses without either utilizing specific tools (such as the improved Phage Search Tool, PHASTER, Arndt et al., 2016, which indeed successfully recognized the elements studied here as potential tectiviruses) or conducting targeted analysis of individual genes on sequence level. The problem in identification derives from the annotations of the known viruses in databases as most of them describe the coding domains to produce "hypothetical proteins" or "cytoplasmic protein". Yet, in reality, many of these hypothetical proteins have been demonstrated to produce structural components of the virions (see e.g. Strömsten et al., 2003; Sozhamannan et al., 2008) or to have interactions with other proteins in the genome (Berjón-Otero et al., 2017). Accurate identification of the elements appear relevant for deriving useful information from whole genome sequencing projects given that members of *Tectiviridae* are known to influence growth rate, biofilm formation, swarming motility, sporulation (Gillis and Mahillon, 2014c) and they continuously produce viral particles in liquid cultures (that infect other hosts in their environment, Jalasvuori et al., 2009, 2013).

The evolutionary distances of the elements were inferred by studying the major capsid protein (MCP, Fig. 1) along with the packaging ATPase (Supplementary Fig. 1) as they have been repeatedly argued to define the identity of the virus and are considered to be evolutionarily tightly linked (and thus likely to be co-inherited to viral offspring from the same virus, Bamford et al., 2002; Krupovic and Bamford, 2010; Sinclair et al., 2017); the same approach has been utilized to group relatively distant elements in other viruses with icosahedral capsids and inner membranes (Jalasvuori et al., 2010; Pawlowski et al., 2014). Both proteins indeed produce comparable trees (Fig. 1 and Supplementary Fig. 1), although there are some uncertainties (low bootstrap values) with more distantly related elements. Yet, in the case of Gil16c and the elements in *B. cereus* strains VD166 and VD184, a clear recombination between the genes for ATPase and MCP has occurred. On amino acid level, Gil16 ATPase is 96% identical to that of VD184 but only 79% identical to VD166 while the Gil16c MCP is 98% identical to MCP of VD166 but only 72% identical to VD184. This reveals that even the areas between tightly linked "virus identifying" genes may sometimes serve as sites for recombination among *Tectiviridae*. Overall, the most divergent members of the group are the elements in the hosts that were previously not known to harbor tectiviruses, i.e. *Brevibacillus* sp., *Clostridium* sp., *Exiguobacterium antarcticum*, *Bacillus pumilus* and *Bacillus flexus*, all sharing around 32–40%

**Table 1**
Tectiviridae related elements in databases.

| Tectiviridae element | Tectiviridae element in bacterium | Sequence length | Putative genes | GC% | RefSeq id | Putative genes in original annotation |
|---|---|---|---|---|---|---|
| BThuPhage1 | Bacillus thuringiensis serovar sumiyoshiensis strain BGSC 4AO1 | 14329 | 31 | 39.9 | NZ_NFCM01000003 | 23 |
| BCerPhage1 | Bacillus cereus strain MOD1_Bc143 | 14642 | 27 | 33.9 | NZ_NBNG01000017 | 20 |
| BCerPhage2 | Bacillus cereus strain FSL M8–0473 | 15258 | 26 | 38.1 | NZ_MUAP01000079 | 21 |
| BCerPhage3 | Bacillus cereus strain MOD1_Bc67 | 12062 | 25 | 37.4 | NZ_MIFF01000080 | 19 |
| BCerPhage4 | Bacillus cereus VD184 | 15259 | 30 | 40.1 | NZ_KB976851 | 25 |
| BCerPhage5 | Bacillus cereus VD166 | 14445 | 29 | 36.1 | NZ_JH791864 | 22 |
| BPumPhage1 | Bacillus pumilus strain CB01 | 14220 | 29 | 42.2 | NZ_LYXP01000014 | 24 |
| BLicPhage1 | Bacillus licheniformis strain B4092 | 13295 | 24 | 44.1 | NZ_LQYK01000004 | 19 |
| ClosPhage1 | Clostridium sp. HMSC19B11 | 8527 | 18 | 38.6 | NZ_KV785301 | 13 |
| EAntPhage1 | Exiguobacterium antarcticum DSM 14480 | 14829 | 28 | 42.2 | NZ_JMKS01000002 | 23 |
| BFlexPhage1 | Bacillus flexus T6186-2 | 13112 | 27 | 36.0 | NZ_JANV01000006 | 22 |
| SPneuPhage1 | Streptococcus pneumoniae strain 6B | 14440 | 31 | 35.4 | NZ_CWJH01000041 | 21 |
| SpneuPhage2 | Streptococcus pneumoniae strain 38 | 14558 | 31 | 38.9 | NZ_CMPS01000060 | 21 |
| BreviPhage1 | Brevibacillus sp. CF112 | 13935 | 31 | 45.9 | NZ_AKKB01000094 | 25 |

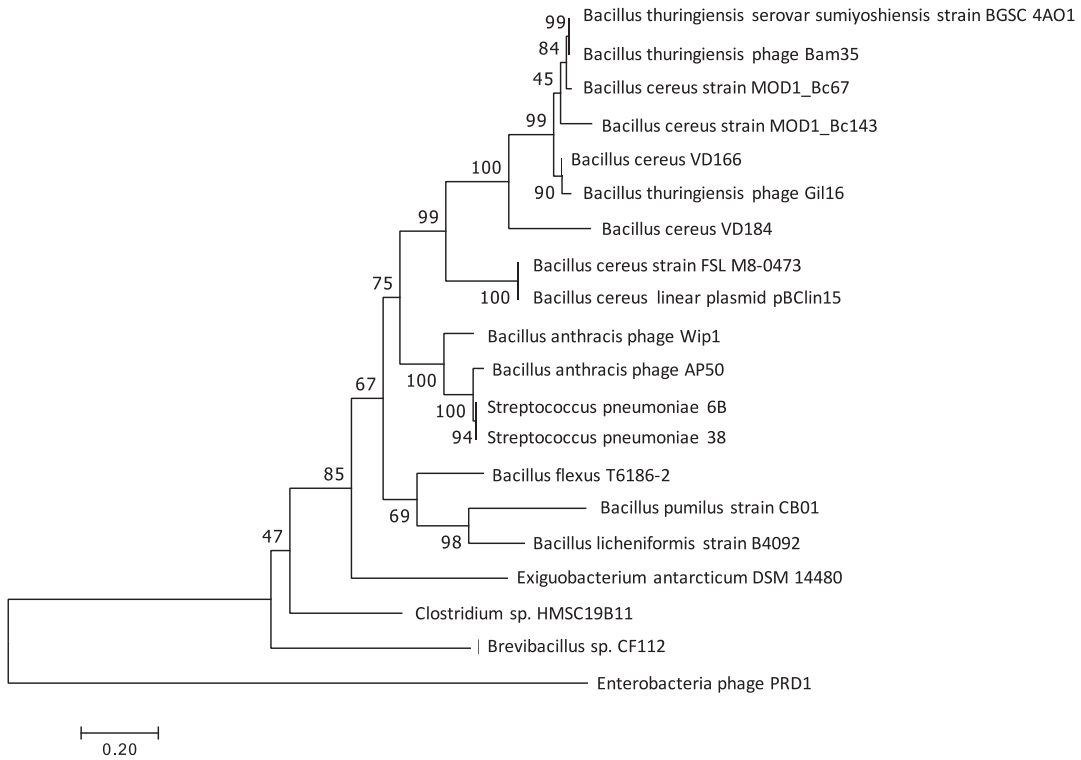


**Fig. 1.** Molecular Phylogenetic analysis of the major capsid protein sequences of the Tectivirus-like elements. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of trees in which the associated taxa clustered together is shown next to the branches. There was a total of 348 positions in the final dataset.

similarity on DNA level with the others (Table 2).

Overall, the divergence of tectiviruses appears to follow that of their host organisms when the 16sRNA trees of Gram-positive bacteria is used as a reference (Onyenwoke et al., 2004). This is expected, given the host specificity of bacteriophages in general and tectiviruses in particular (Jalasvuori et al., 2013; Gillis and Mahillon, 2014b; Mattila et al., 2015), making jumps to taxonomically distant hosts unlikely. However, there is single exception. The element in *Streptococcus* is closely related to *Bacillus anthracis* viruses of AP50 and Wip1 despite of the more distant relationship of their hosts. This suggest that a Tectivirus-like element of probably *Bacillus* origin has recently invaded *Streptococcus*.

We compared the genomes of all the elements to each other (Fig. 2) and scored the number of (potential) homologs in other members of the group (including PRD1). Hits to identical elements were ignored (pBClin15 and element in *Bacillus cereus* strain FSL M8-0473; and both *Streptococcus pneumoniae* elements). Overall gene synteny among tectiviruses or Tectivirus-like element in Gram-positive bacteria is very conserved and no major shifting of the modules is observed, excluding the previously denoted localization of DNA polymerase in Wip1 in the 3′ end of the genome (Kan et al., 2013). The genome replication machinery of Wip1 in general shows notable divergence in comparison to other elements. Otherwise, several genomic regions are conserved, including those for DNA polymerase and the genome terminal protein, both of which are necessary for the protein-primed replication, along with those genes encoding for key structural components of the virion and DNA packing. Most variability, on the other hand, is observed around the previously demonstrated host encoded LexA suppressor binding site, where mutations often result in virus to be unable to suppress its lytic life cycle (Fornelos et al., 2011). Such mutations can be favourable in certain ecological situations, such as when there is large susceptible host population for lytic mutants to proliferate in. Yet, in the absence of new hosts, reversion to temperate cycle can be necessary for the long-term survival of the phage (Jalasvuori and Koonin, 2015). These possible alterations in ecological conditions have been suggested to contribute in the genetic diversity of the region (Jalasvuori et al., 2013). However, while previously most of the genes in this region had homologs neither in databases nor among other tectiviruses, now we were able to identify potential homologs for majority of the genes in other elements. This suggest that the region, while variable, is not evolving as rapidly as was formerly considered. Also, relatively long putatively non-coding sequences are more common within this region than elsewhere in the genome. Second region where variability abounds is the one in the 3′ end of the genome encoding for host recognition features along with endolysins responsible of successful release of virions to the environment. Given that both functions act either on cell surface or murein components, both of which are in itself highly divergent (Scott and Barnett, 2006; Davis and Weiser, 2011), the variability is possibly best explained by host-parasite evolutionary arms race.

In most tectiviral elements, the polymerase is immediately followed by a short and conserved gene coding for a protein that binds to LexA (a host-encoded repressor activated upon bacterial stress, see e.g. Fornelos et al., 2016) enhancing its binding to the promoter that activates the operon for lytic functions. Further, this protein in sufficiently high concentrations can interfere with bacterial SOS response (Fornelos et al., 2015), suggesting a phage-adaptation to control normal bacterial regulation pathways under stress. *Brevibacillus* element lacks the gene for LexA binding protein, but at the same loci it harbors a gene encoding a putative protein with distant similarity to DNA unwinding helicases, DNA binding proteins and zinc finger proteins. Further, in both *B. flexus* and *B. pumilus* elements, the gene is similarly missing but has been replaced with two and three genes, respectively, without any similarity to proteins in databases. Given the genomic location, it is possible that these genes code for proteins that have functions in controlling normal gene expression and/or stress response of the host and

**Table 2.**
DNA identity matrix of complete Tectivirus-elements.

| Element in / virus | Bacillus pumilus strain CB01 | Bacillus licheniformis strain B4092 | Clostridium sp. HMSC19B11 | Bacillus flexus T6186-2 | Bacillus cereus strain FSL M8–0473 | Bacillus cereus phage pBClin15 |
|---|---|---|---|---|---|---|
| Bacillus pumilus strain CB01 | | 55.8 | 48.0 | 45.5 | 37.2 | 37.2 |
| Bacillus licheniformis strain B4092 | | | 46.9 | 44.3 | 36.3 | 36.3 |
| Clostridium sp. HMSC19B11 | | | | 44.3 | 37.5 | 37.5 |
| Bacillus flexus T6186-2 | | | | | 38.4 | 38.4 |
| Bacillus cereus strain FSL M8–0473 | | | | | | 100.0 |
| Bacillus cereus linear plasmid pBClin15 | | | | | | |
| Bacillus cereus strain MOD1_Bc67 | | | | | | |
| Bacillus cereus VD166 | | | | | | |
| Bacillus thuringiensis serovar sumiyoshiensis strain BGSC 4AO1 | | | | | | |
| Bacillus thuringiensis phage Bam35 | | | | | | |
| Bacillus thuringiensis phage Gil16 | | | | | | |
| Bacillus cereus strain MOD1_Bc143 | | | | | | |
| Bacillus cereus VD184 | | | | | | |
| Streptococcus pneumoniae strain 6B | | | | | | |
| Bacillus anthracis phage AP50 | | | | | | |
| Bacillus anthracis phage Wip1 | | | | | | |
| Exiguobacterium antarcticum DSM 14480 | | | | | | |
| Brevibacillus sp. CF112 | | | | | | |

| Element in / virus | Bacillus cereus strain MOD1_Bc67 | Bacillus cereus VD166 | Bacillus thuringiensis serovar sumiyoshiensis strain BGSC 4AO1 | Bacillus thuringiensis phage Bam35 | Bacillus thuringiensis phage Gil16 | Bacillus cereus strain MOD1_Bc143 |
|---|---|---|---|---|---|---|
| Bacillus pumilus strain CB01 | 37.4 | 36.4 | 36.6 | 37.3 | 36.8 | 36.8 |
| Bacillus licheniformis strain B4092 | 37.4 | 35.9 | 36.3 | 36.3 | 36.1 | 35.9 |
| Clostridium sp. HMSC19B11 | 37.9 | 36.1 | 36.1 | 36.8 | 36.3 | 36.5 |
| Bacillus flexus T6186-2 | 40.1 | 38.1 | 38.9 | 38.0 | 37.8 | 37.4 |
| Bacillus cereus strain FSL M8–0473 | 54.1 | 52.0 | 52.3 | 54.8 | 53.7 | 53.6 |
| Bacillus cereus linear plasmid pBClin15 | 54.1 | 52.0 | 52.3 | 54.8 | 53.7 | 53.6 |
| Bacillus cereus strain MOD1_Bc67 | | 82.4 | 83.4 | 73.9 | 73.5 | 68.7 |
| Bacillus cereus VD166 | | | 82.9 | 69.3 | 70.3 | 71.9 |
| Bacillus thuringiensis serovar sumiyoshiensis strain BGSC 4AO1 | | | | 79.6 | 69.8 | 66.3 |
| Bacillus thuringiensis phage Bam35 | | | | | 82.3 | 79.9 |
| Bacillus thuringiensis phage Gil16 | | | | | | 80.4 |
| Bacillus cereus strain MOD1_Bc143 | | | | | | |
| Bacillus cereus VD184 | | | | | | |
| Streptococcus pneumoniae strain 6B | | | | | | |
| Bacillus anthracis phage AP50 | | | | | | |
| Bacillus anthracis phage Wip1 | | | | | | |
| Exiguobacterium antarcticum DSM 14480 | | | | | | |
| Brevibacillus sp. CF112 | | | | | | |

| Element in / virus | Bacillus cereus VD184 | Streptococcus pneumoniae strain 6B | Bacillus anthracis phage AP50 | Bacillus anthracis phage Wip1 | Exiguobacterium antarcticum DSM 14480 | Brevibacillus sp. CF112 |
|---|---|---|---|---|---|---|
| Bacillus pumilus strain CB01 | 36.1 | 37.5 | 36.8 | 36.3 | 35.7 | 33.1 |
| Bacillus licheniformis strain B4092 | 35.1 | 36.2 | 35.8 | 35.1 | 34.4 | 32.2 |
| Clostridium sp. HMSC19B11 | 35.4 | 37.1 | 36.7 | 35.3 | 34.7 | 34.2 |
| Bacillus flexus T6186-2 | 37.2 | 38.1 | 37.8 | 37.5 | 36.6 | 35.3 |
| Bacillus cereus strain FSL M8–0473 | 56.9 | 54.4 | 53.5 | 45.6 | 38.6 | 36.4 |
| Bacillus cereus linear plasmid pBClin15 | 56.8 | 54.4 | 53.5 | 45.6 | 38.6 | 36.4 |
| Bacillus cereus strain MOD1_Bc67 | 63.4 | 49.0 | 49.2 | 46.9 | 38.0 | 35.7 |

Table 2. *(continued)*

| Element in / virus | Bacillus cereus VD184 | Streptococcus pneumoniae strain 6B | Bacillus anthracis phage AP50 | Bacillus anthracis phage Wip1 | Exiguobacterium antarcticum DSM 14480 | Brevibacillus sp. CF112 |
|---|---|---|---|---|---|---|
| **Bacillus cereus VD166** | 58.5 | 48.0 | 47.6 | 46.3 | 36.9 | 34.7 |
| **Bacillus thuringiensis serovar sumiyoshiensis strain BGSC 4AO1** | 63.9 | 47.3 | 47.4 | 46.6 | 37.3 | 34.7 |
| **Bacillus thuringiensis phage Bam35** | 67.2 | 50.6 | 50.7 | 45.4 | 39.0 | 35.5 |
| **Bacillus thuringiensis phage Gil16** | 63.3 | 50.4 | 50.7 | 44.7 | 38.3 | 35.4 |
| **Bacillus cereus strain MOD1_Bc143** | 64.3 | 50.0 | 50.2 | 43.9 | 38.6 | 35.2 |
| **Bacillus cereus VD184** | | 52.5 | 52.4 | 43.2 | 39.1 | 34.7 |
| **Streptococcus pneumoniae strain 6B** | | | 83.5 | 60.4 | 40.3 | 37.0 |
| **Bacillus anthracis phage AP50** | | | | 58.5 | 39.7 | 36.1 |
| **Bacillus anthracis phage Wip1** | | | | | 37.3 | 33.9 |
| **Exiguobacterium antarcticum DSM 14480** | | | | | | 34.6 |
| **Brevibacillus sp. CF112** | | | | | | |

may thus be of value for future research in these species.

*Brevibacillus* element in general has most genes without resemblance to other members of the group. The gene in the beginning of the virion assembly region encodes a possible TonB-domain (COG0810), which is known to link inner and outer membranes in Gram-negative bacteria. Given the inner membrane in Tectivirus virion, the protein can be speculated to be a structural component of the capsid. None of the putative proteins encoded from genes in the 3′ end of the element has significant similarity to proteins in databases suggesting that the element encodes a novel receptor binding spike. Further, the second last gene in *Exiguobacterium antarctica* element is dissimilar to other tectiviral elements but similar to peripherally T4-related phage Pf16 infecting *Pseudomonas putida*, indicating potential gene exchange between a Tectivirus and a member of *Caudovirales*. Previously similar recombination was identified in PRD1-like tectiviruses that apparently acquired a lysis cassette from *Caudovirales* (Krupovic et al., 2008). Also, its notable that around half of the elements contain putative genes of below 100 nucleotides in length (sometimes being as short as 63 nts), a limit utilized by many algorithms for detecting open reading frames. These putative genes are shared by multiple elements, suggesting that they are not annotation artefacts. In PRD1, short genes encode peptides with transmembrane helices and are known to be involved in linking DNA packing complex to the virion (Strömsten et al., 2003). Yet, most of the short peptides analyzed for this study have no predictable transmembrane regions (although some do) and possibly carry out different functions. Many of the genes indeed have identical counterparts in certain plasmids of the same or closely related species suggesting that they do provide fitness benefits for several types of extrachromosomal genetic elements that are not likely to form virions. Nevertheless, as short genes are likely to go unnoticed by automated tools and thus, even if potential homologs exist in sequences per se, their translations are likely to be absent from curated non-redundant protein databases that lack majority of directly deposited sequences (such as BLAST). As has been argued previously (Samayoa et al., 2011; Andrews and Rothnagel, 2014), it is still a somewhat overlooked area of research to scan existing (bacterial and bacteriophage) genomes solely for putative short genes that show characteristics of being actually translated into peptides (such as having significant Shine-Dalgarno sites, encode structurally stable peptides and the ORFs having counterparts in whole genome alignments with closely related species) and then associate them with different phenotypic, ecological and environmental qualities.

To conclude, tectiviral elements in Gram-positive bacteria are conserved for their gene synteny and for genes necessary for building up the core of the virion. Genomic variability is observed for regions that are responsible of controlling the life cycle as well as mediating host attachment and lysis. All elements reside in bacteria known to be present in soil (along with various other environments). *Brevibacillus* and *Streptococus* are not known to form spores, thus implying that *Tectiviridae* in Gram-positive bacteria are not restricted to spore-formers. In the future, whole-genome sequencing projects are likely to produce more Tectivirus-like elements, thus expanding the known host spectrum even further. Yet the complete absence of new elements from Gram-negative hosts despite of the exponentially increasing amount of sequences from all possible samples suggests that they may indeed be very rare.

## 3. Materials and methods

Amino acid sequence of major capsid proteins of known Tectivirus members AP50, Gil16, Bam35 and pBClin15 (complete genome RefSeq ids NC_011523, NC_006945, NC_005258 and NC_00472, respectively), were used as seeds for BLAST (https://blast.ncbi.nlm.nih.gov/). Significant hits to unknown sequences were manually checked for the size and the presence of other Tectivirus-like genes. Fourteen sequences of likely tectiviral origin were obtained and thereafter handled with


**Fig. 2.** Genomic comparison of known tectiviruses and Tectivirus-like elements. The translations of all annotated open reading frames (ORFs) were compared to the translations of annotated ORFs in other elements. The whole genome alignment was conducted via ClustalW. The stated functions for genes in the bottom of the figure are those determined or suggested for phage AP50, but the functions of genes in similar aligned positions in other phages may still differ.

Geneious 11.0.2 software (www.geneious.com). PHASTER (Arndt et al., 2016; http://phaster.ca) was used to further investigate whether the elements can be recognized by automated tools as prophages by submitting the complete sequence for analysis (all of them being identified correctly as prophages related to Bam35). Whole genome alignments were conducted with ClustalW 2.1. (Larkin et al., 2007) with IUB cost matrix and gap open and extension costs of 7 and 4, respectively. The annotations of the sequences were checked manually for potential errors and unmarked putative genes, and were re-annotated accordingly (as described in Results and Discussion). All putative gene products of the novel elements along with (putative) genes in all previous Tectivirus sequences were translated into a local protein database. Then each protein from each element was BLASTed against this database. Two proteins were considered potential homologs when the selected E- values were considered positive hits using the following criteria. The E - value to a hit was below 1.0e-1 or in the case of short proteins (i.e. < 60 amino acids) the pairwise align to a similar sized protein including identical or similar amino acids. Naturally, in some cases where the similarity is relatively low, potential homology cannot be inferred in terms of the genes sharing a common ancestral gene in an ancestor virus, but may rather implicate different types of relationships such as being originated by duplication of partial genes or transposition of partial sequences elsewhere in the genome. A total of approximately 550 proteins were manually checked for their hits to putative proteins in other members of the family. Short proteins of below around 35 amino acids in size were investigated for the existence of putative transmembrane regions with TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The existence of potential homologs in databases to putative gene products of Tectivirus-like elements was conducted with BLAST. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). Divergence of MCPs and ATPases were inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). The trees in Fig. 1 and in Supplementary Fig. 1 are the ones with the highest log likelihoods (−7105.24 and −3586.80, respectively). Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. All positions containing gaps and missing data were eliminated.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2018.02.014.

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# II

# BLACK QUEEN EVOLUTION AND TROPHIC INTERACTIONS DETERMINE PLASMID SURVIVAL AFTER THE DISRUPTION OF THE CONJUGATION NETWORK

by

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RESEARCH ARTICLE
Ecological and Evolutionary Science

# Black Queen Evolution and Trophic Interactions Determine Plasmid Survival after the Disruption of the Conjugation Network

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**ABSTRACT** Mobile genetic elements such as conjugative plasmids are responsible for antibiotic resistance phenotypes in many bacterial pathogens. The ability to conjugate, the presence of antibiotics, and ecological interactions all have a notable role in the persistence of plasmids in bacterial populations. Here, we set out to investigate the contribution of these factors when the conjugation network was disturbed by a plasmid-dependent bacteriophage. Phage alone effectively caused the population to lose plasmids, thus rendering them susceptible to antibiotics. Leakiness of the antibiotic resistance mechanism allowing Black Queen evolution (i.e. a "race to the bottom") was a more significant factor than the antibiotic concentration (lethal vs sublethal) in determining plasmid prevalence. Interestingly, plasmid loss was also prevented by protozoan predation. These results show that outcomes of attempts to resensitize bacterial communities by disrupting the conjugation network are highly dependent on ecological factors and resistance mechanisms.

**IMPORTANCE** Bacterial antibiotic resistance is often a part of mobile genetic elements that move from one bacterium to another. By interfering with the horizontal movement and the maintenance of these elements, it is possible to remove the resistance from the population. Here, we show that a so-called plasmid-dependent bacteriophage causes the initially resistant bacterial population to become susceptible to antibiotics. However, this effect is efficiently countered when the system also contains a predator that feeds on bacteria. Moreover, when the environment contains antibiotics, the survival of resistance is dependent on the resistance mechanism. When bacteria can help their contemporaries to degrade antibiotics, resistance is maintained by only a fraction of the community. On the other hand, when bacteria cannot help others, then all bacteria remain resistant. The concentration of the antibiotic played a less notable role than the antibiotic used. This report shows that the survival of antibiotic resistance in bacterial communities represents a complex process where many factors present in real-life systems define whether or not resistance is actually lost.

**KEYWORDS** antibiotic resistance, Black Queen evolution, conjugation, predation, trophic levels

Bacterial resistance to antibiotics has emerged as a serious concern for modern health care. The majority of resistant bacteria in hospitals harbor mobile genetic elements that provide the bacteria with their efficient resistance phenotype. Therefore, the maintenance of resistance in a bacterial community is in many cases tightly linked

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Protozoan predation improves the survival of antibiotic resistance plasmids

to the survival of the mobile elements themselves (1). Horizontal gene transfer is a dominant feature among bacteria as environmental selection can favor individual organisms in a population that have either acquired or lost a particular gene. Studies have shown that genes are exchanged readily even between taxa also in cases where anthropogenic selection has induced a notable fitness benefit with respect to a particular trait (e.g., cephalosporin resistance) only relatively recently (2). As such, the microbiome appears to conform to a great extent to the Baas-Becking hypothesis that "everything is everywhere, but the environment selects." In the case of resistance, the selection itself might appear to represent a relatively simple issue given that resistance should be beneficial only in the presence of antibiotics and costly to the bacterium in their absence. Based on this argument alone, the best solution to the resistance problem would be the careful stewardship of antibiotic use. However, in reality, the survival of resistance-conferring elements, such as conjugative plasmids, relies on various factors.

Bacterial hosts and their plasmids can coadapt, thus ameliorating plasmid-associated fitness costs (3–6). In *Pseudomonas fluorescens*, the underlying adaptive mutations in the host were shown to occur in the region encoding the bacterial *gacA*/*gacS* two-component global regulatory system (7). Mutations in the N terminus of the plasmid-encoded replication protein TrfA1 compensated for the cost of carrying a broad-host-range plasmid in *Shewanella oneidensis* (8). Loftie-Eaton and colleagues demonstrated that once a plasmid had acquired a transposon carrying a putative toxin-antitoxin system along with a resolvase gene, its persistence increased significantly in various hosts (5). The results of those recent studies imply that various adaptive changes in both the plasmid and the host can influence plasmid survival. However, pairwise adaptation itself is not likely to completely compensate for the costs, at least not in communities where the plasmid is continuously transferred into naive hosts. Therefore, interhost mobility itself is likely to play a role in plasmid maintenance (9), but the extent to which this is relevant has been debated (10–12). In a recent study, Lopatkin and colleagues conducted a meticulous analysis of the role of bacterial conjugation in maintaining resistance plasmids, showing that if the rate of plasmid loss and the costs of plasmid carriage are low enough compared to the frequency of horizontal transfer, then no selection (such as that associated with antibiotics) is required for plasmid survival (1). This was true also for communities consisting of several plasmids and bacterial strains, and it was therefore argued that disrupting conjugation and plasmid segregation can provide an avenue to limit the maintenance of resistance. Indeed, the presence of linoleic acid (inhibiting conjugation) and phenothiazine (promoting segregation loss) significantly reduced plasmid persistence in the community (1).

Naturally, plasmid survival in various environments (such as the human gut) is also heavily influenced by direct selection via antibiotics, since it is often impossible to completely avoid their administration. Antibiotic use results in environmental concentration gradients where they exert either inhibitory or subinhibitory effects on bacterial growth. In certain cases, plasmids may be transferred to susceptible but perhaps otherwise competitively superior hosts even after the initial exposure to a lethal antimicrobial component, thus restoring their positive growth (13). Further, subinhibitory concentrations can promote the maintenance of resistance plasmids by alleviating associated costs (14).

The "altruism" of the resistance mechanism also influences the selective landscape in the presence of antibiotics. Yurtsev and colleagues have shown that bacterial "cheaters" can stably persist in the presence of high concentrations of beta-lactam antibiotics provided that a fraction (depending on the initial concentration of antibiotic) still retains resistance (15). Similarly, altruism, or, more accurately, "leakiness," of genetically encoded functions plays an important part in biology as various processes generate products that can be "public" (i.e. accessible by other organisms) (16). As the production of public goods is often costly to the producer, competition favors those that can rely on others to provide the necessary functions. Such examples of "Black Queen evolution" lead to the "race to the bottom," that is, the loss of genes which are

not needed (17, 18). The existence of gene-depleted "beneficiaries," however, requires a fraction of the community to remain as "helpers." Here, beta-lactam resistance in a mixed population is a prime example (15, 13). However, other resistance mechanisms, such as enzymatic resistance to aminoglycosides, cannot be readily exploited by those not producing the enzyme themselves. Therefore, in the presence of antibiotics, the possibility to race to the bottom is yet another facet that determines the extent to which resistance plasmids are maintained. It is also notable that in natural environments, bacteria do not exist only in communities with other bacteria, plasmids, and parasites such as bacteriophages but also with organisms that feed on them. Trophic interactions between bacteria and their consumers such as protozoa have received little attention in the research concerning resistance plasmid maintenance, which may partly result from their seemingly irrelevant effect on plasmids that reside within bacteria. Yet in natural microbial communities such as seas and freshwater systems, protozoa are considered to be major consumers of bacteria (19–21). They are present in wastewater treatment plants (22) and sometimes also exist in the human gut (23). Therefore, they can modulate the ecological landscape of (potential) plasmid hosts in many different types of environments. Recently, it was shown that, in contrast to a wild-type conjugative plasmid, a conjugation-deficient mutant was unable to persist in a bacterial population under conditions of predation (24). In contrast, in the absence of predation, the conjugation-defective mutant had higher persistence than the conjugative plasmid, suggesting that the relevance of conjugation to plasmid persistence may become evident only in a more realistic trophic setup.

Theoretical and experimental advances have been made to understand the dissection of populations into beneficiaries and helpers in the presence of different antibiotics and, together with the studies on the role of conjugative transfer in the maintenance of resistance plasmids, are starting to clarify the complexity behind the general antibiotic resistance problem. However, the interventions in the network that would resensitize communities to antibiotics are less extensively studied. Given that the effectiveness of the disrupted network is likely to depend on all the various factors described above, we set out here to use a factorial experimental setup to investigate the maintenance of resistance plasmids and conjugative phenotypes in a multitrophic community supplemented with antibiotics against which the plasmid encodes either "leaky" or "nonleaky" resistance (Fig. 1). For the initial disruption of plasmid conjugation, we utilized a natural "antiplasmid agent," the bacteriophage PRD1, which specifically recognizes plasmid-encoded receptors on a bacterial cell. Exposing populations to PRD1 is known to select for cells that have lost either their plasmids (become resensitized) or, in part or completely, their conjugative ability (providing increased or full resistance to phage, respectively) (25, 26). In this study, we showed that two main factors have a major effect on plasmid prevalence: leakiness of antibiotic resistance and the modulation of the community by predation. These results suggest that even brief periods of exposure to low levels of antibiotics against which resistance is nonleaky can considerably increase the fraction of the population harboring a resistance plasmid. In contrast, with antibiotics against which resistance is leaky, such as beta-lactams, the effect is less notable even at inhibitory concentrations. Furthermore, the addition of a next level to the trophic network efficiently promoted plasmid persistence under all conditions. This was likely to result from lowered cell density, which in turn enhanced the per-cell metabolic activity and probably contributed to increased bacterial conjugation rates. Hence, these results give rise to the interesting possibility that protozoan predators may be playing a previously unrecognized role in promoting the prevalence of antibiotic resistance in various environmental reservoirs.

## RESULTS

**Community dynamics.** To understand the relative contributions of antibiotic concentration, altruism of resistance, and ecological interactions on plasmid persistence, we performed a 50-day microcosm experiment. We used a fully factorial experimental design with two treatments: (i) a community composition treatment, consisting of the

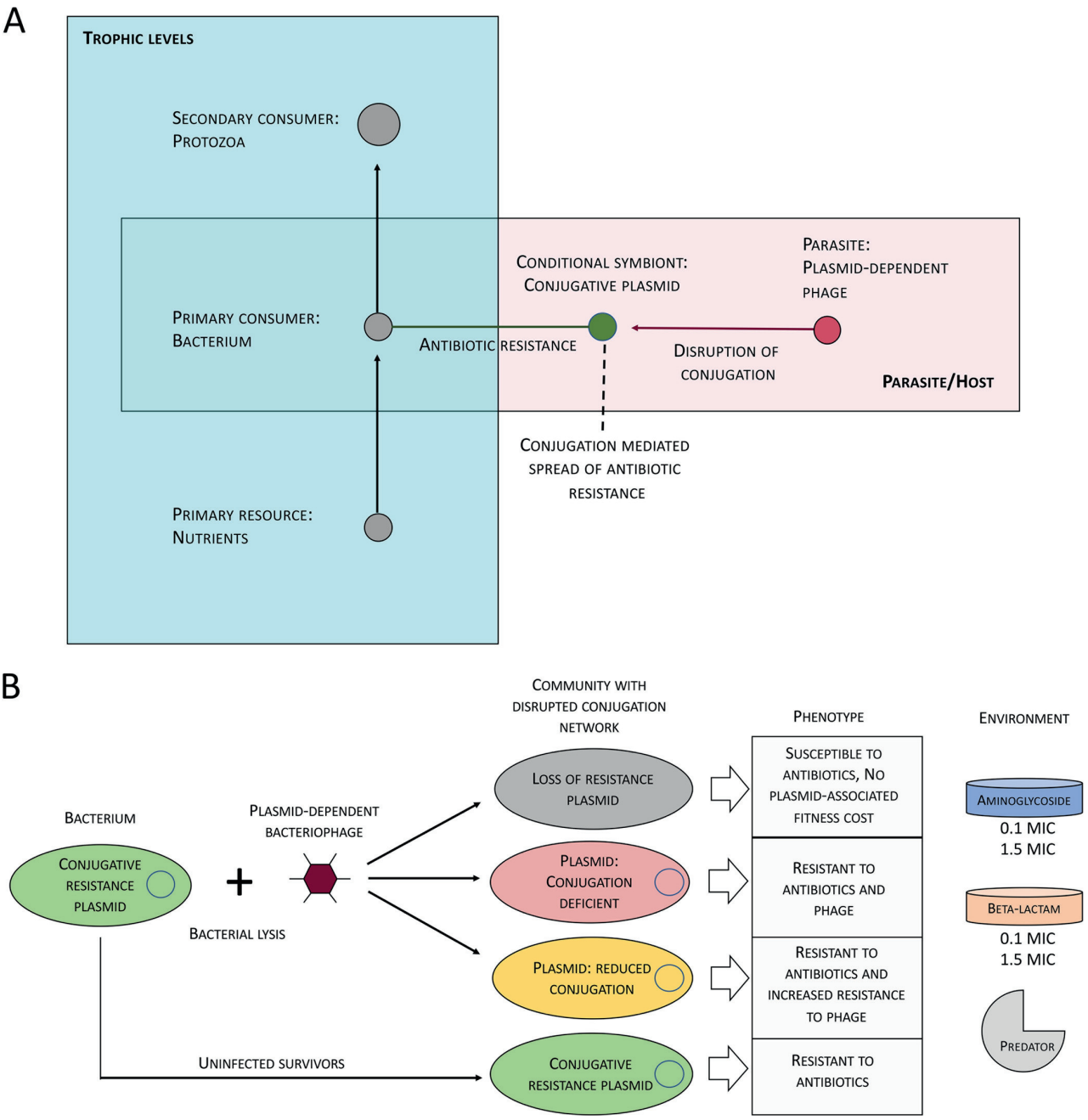


**FIG 1** Schematic presentation of the ecological and evolutionary interactions investigated in the factorial experimental setup. (A) The trophic contacts in the community experiment. (B) Disruption of the conjugation network induced by the presence of plasmid-dependent bacteriophage and the factors in the experiments (protozoa, phage, two antibiotics with different resistance mechanisms).

bacterium *Escherichia coli* K-12 HMS174(RP4) harboring the multidrug (ampicillin, kanamycin, tetracycline) resistance plasmid RP4 with or without the plasmid-dependent bacteriophage PRD1 (hereafter, phage) and/or the ciliated protozoan *Tetrahymena thermophila* CCAP 1630/1U, and (ii) an antibiotic treatment consisting of no antibiotic or 0.1× MIC of ampicillin or kanamycin and 1.5× MIC of ampicillin or kanamycin against which RP4 confers altruistic or selfish resistance, respectively.

We used an optical density (OD)-based method, light microscopy, and a plaque assay to track bacterial, protozoan, and phage population sizes, respectively, over time.

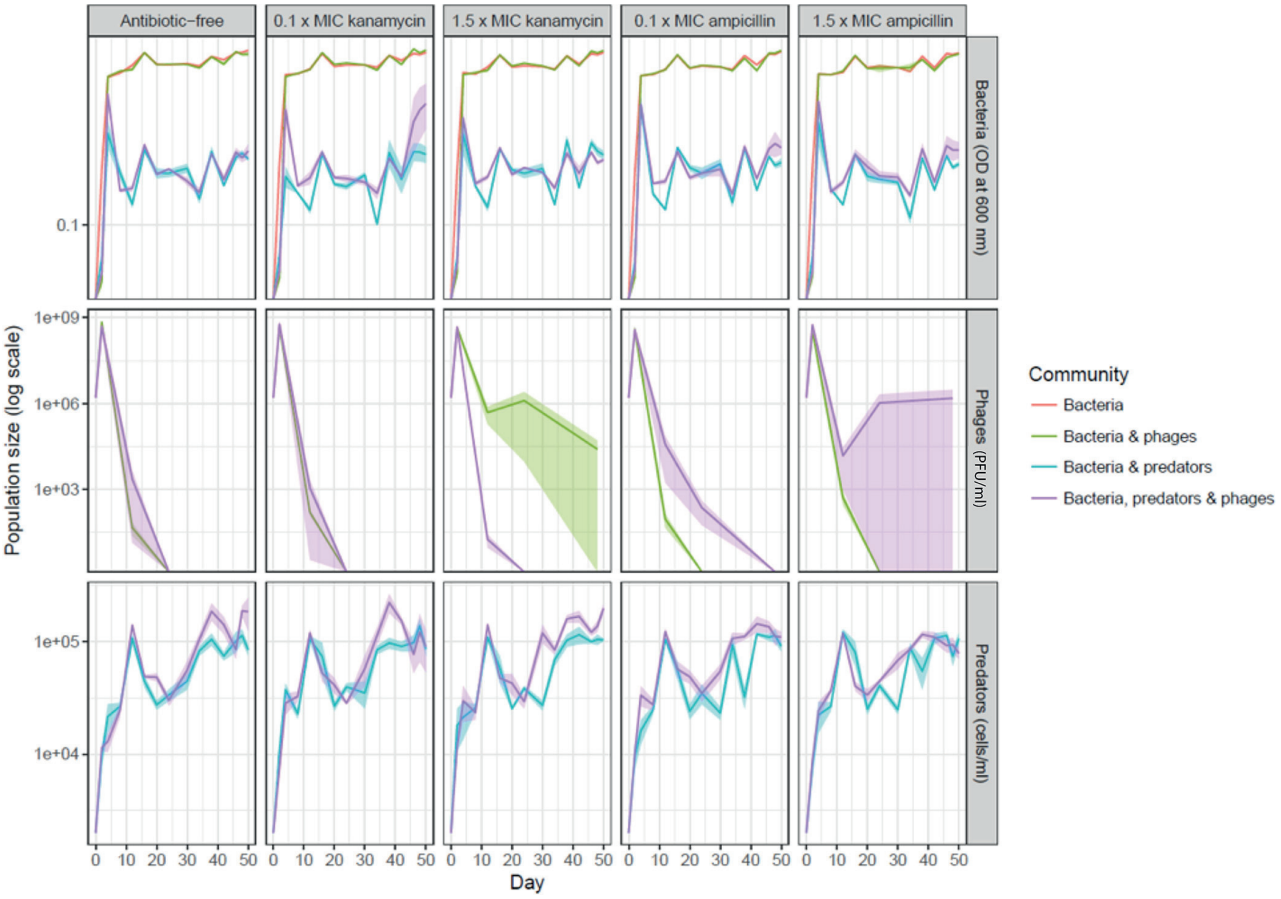


**FIG 2** Bacterial, predator, and phage population sizes over time in each treatment in a 50-day microcosm experiment (data represent means ± standard errors [SE]). All treatments were replicated four times.

Bacterial population size was not affected by antibiotic type or concentration but was affected by community composition (linear mixed models [LMM] antibiotics, $\chi^2 = 3.32$, df = 2, $P = 0.19$; kanamycin concentration, $\chi^2 = 0.44$, df = 2, $P = 0.80$; ampicillin concentration, $\chi^2 = 3.29$, df = 2, $P = 0.19$; community composition, $\chi^2 = 4{,}091.5$, df = 42, $P < 0.001$) (Fig. 2). Protozoan predation decreased the bacterial population size, regardless of the presence or absence of phage (general linear hypothesis test [glht], predator treatments versus predator-free treatments, $P < 0.001$ for all), although the population size was elevated slightly (1.08-fold on average, discounting the transient phase on days 2 to 4) in the simultaneous presence of phage (glht, predator alone versus predator with phage, $P = 0.003$).

Similarly to the results seen with bacteria, only community composition affected the protozoan population size, such that ciliate density was increased in the simultaneous presence of phages (LMM antibiotic, $\chi2 = 2.81$, df = 2, $P = 0.25$; kanamycin concentration, $\chi^2 = 2.19$, df = 2, $P = 0.33$; ampicillin concentration, $\chi^2 = 1.77$, df = 2, $P = 0.41$; community composition, $\chi^2 = 92.14$, df = 14, $P < 0.001$) (Fig. 2). The phage population size peaked early in the experiment and subsequently decreased to low levels (Fig. 2). Overall population sizes did not differ between the two antibiotic treatments (LMM, $\chi^2 = 3.72$, df = 2, $P = 0.16$), but the treatments affected the population sizes differently. With kanamycin, concentration and community composition interactively affected phage population size (LMM concentration, $\chi^2 = 64.13$, df = 16, $P < 0.001$; community composition, $\chi^2 = 63.05$, df = 12, $P < 0.00$; concentration × community composition, $\chi^2 = 64.13$, df = 16, $P < 0.001$), such that in the presence of a lethal kanamycin

concentration without predation, extinction of the phage population was delayed compared with the results seen with other environments (glht, 1.5× MIC versus 0/0.1× MIC [both], $P < 0.01$; 0 versus 0.1× MIC, $P = 0.99$). In contrast, with ampicillin, the concentration did not significantly affect the phage population size (LMM, $\chi^2 = 5.96$, df = 2, $P = 0.05$), and community composition had the opposite effect (LMM, $\chi^2 = 4.32$, df = 1, $P = 0.038$), such that the phage population size was increased or extinction of phage populations was delayed in the presence of protozoa.

**Leakiness of resistance and ecological interactions determine plasmid persistence more than lethal antibiotic concentrations.** While the altruistic/leaky nature of beta-lactam resistance (in this study, ampicillin resistance) is well studied (27), as is also the case for the strains utilized in this study (28), we first confirmed the selfish/nonleaky nature of aminoglycoside resistance (in this study, kanamycin resistance) provided by plasmid RP4. Therefore, the ability of a conjugation-defective HMS174(RP4) strain to support a susceptible strain in the presence of kanamycin (25 $\mu$g ml$^{-1}$ and 50 $\mu$g ml$^{-1}$) was measured. No surviving cheaters (i.e. bacteria that did not encode resistance themselves) were observed ($n = 5$).

We determined plasmid prevalence over time by isolating clones from three time points within the 50-day experiment and culturing on agar plates containing high concentrations of selective antibiotics. Plasmid loss was observed only in bacterial populations with plasmid-dependent phages selecting against the plasmid (LMM community composition, $\chi^2 = 306.1$, df = 31, $P < 0.001$; glht, phage alone versus other community compositions [all comparisons], $P < 0.02$) (Fig. 3A). Plasmid loss caused by phages was counteracted by the simultaneous presence of protozoa, such that the plasmid loss results did not significantly differ from those seen with phage-free treatments (glht [all comparisons], $P =$ not significant [NS]), despite individual replicate populations exhibiting considerable decreases in plasmid prevalence (Fig. 3A). Similarly, kanamycin—against which the plasmid confers selfish resistance—maintained the plasmid at high prevalence in populations in the presence of phages (LMM antibiotic, $\chi^2 = 335.8$, df = 30, $P < 0.001$; glht, kanamycin versus ampicillin/antibiotic-free environment, $P < 0.001$). In contrast, the plasmid reached a low frequency with ampicillin—against which the plasmid confers leaky (altruistic) resistance. The plasmid loss seen with ampicillin did not differ significantly from that seen with the antibiotic-free environment (glht, ampicillin versus antibiotic-free environment, $P = 0.33$). This result potentially is the consequence of high variability between replicate communities, as the mean prevalence reached was 0%, 20%, or 40% under conditions of no, sublethal, or lethal kanamycin selection, respectively, which is consistent with the Black Queen hypothesis of stable coexistence between helpers and beneficiaries. Increasing the antibiotic level from a sublethal to a lethal concentration did not have a significant effect on plasmid persistence with either antibiotic (LMM ampicillin concentration, $\chi^2 = 1.91$, df = 2, $P = 0.39$; LMM kanamycin concentration, $\chi^2 = 308.4$, df = 30, $P < 0.001$; glht, kanamycin at 0.1/1.5× MIC versus antibiotic-free environment, $P < 0.001$ [but for 0.1 versus 1.5× MIC, $P = 0.92$]).

**Plasmid-dependent bacteriophages select for defective conjugation counteracted by conjugation-selecting protozoa.** Phage PRD1 selects for various types of plasmid mutants whose conjugation ability is either reduced or lost (see Fig. S1 in the supplemental material). To measure the loss of wild-type conjugation ability during the 50-day microcosm experiment, we cocultured clones isolated from the end of the experiment with recipient strain *E. coli* K-12 JM109(pSU19). This was followed by culturing in agar plates containing antibiotics that allow only those recipient bacteria to grow that have acquired the conjugative plasmid from the clone and by rating cultures from 1 to 4, where 1 represents no growth and 4 represents normal growth (i.e. wild-type conjugation ability; see Fig. S2). The persistence of the wild-type plasmid conjugation ability at the end of the evolution experiment was not affected by antibiotic or concentration (beta regression antibiotic, $\chi^2 = 1.00$, df = 2, $P = 0.61$; kanamycin concentration, $\chi^2 = 1.03$, df = 2, $P = 0.60$; ampicillin concentration, $\chi^2 = 2.16$, df = 2, $P = 0.34$) (Fig. 3B). With both kanamycin and ampicillin (beta regression

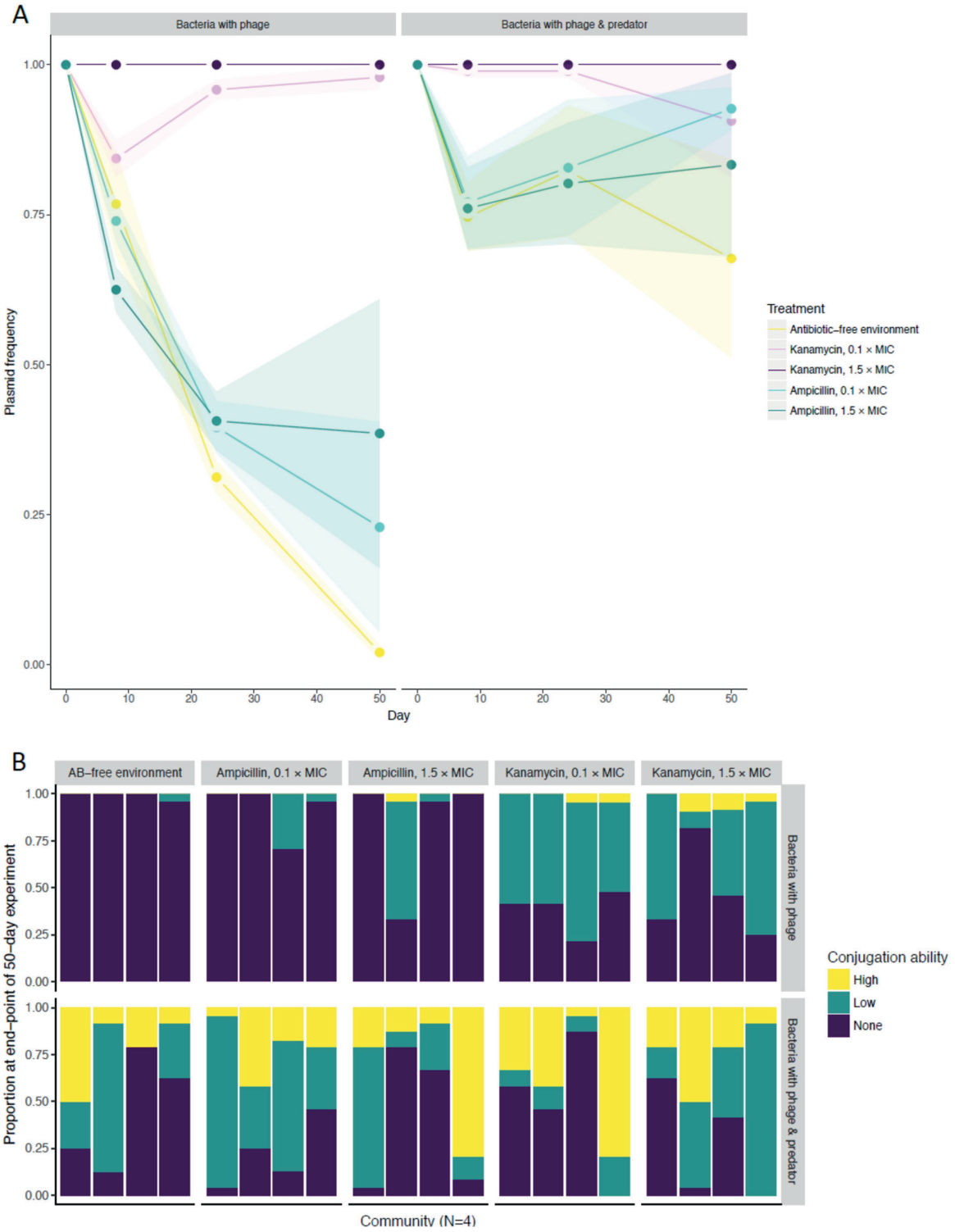


**FIG 3** (A) Frequency of plasmid harboring bacteria over the course of a 5-day community experiment in different environments (data represent means ± SE). Plasmid RP4-encoded resistance mechanisms against kanamycin and ampicillin represent selfish and altruistic resistance mechanisms, respectively. Altruism of resistance and protozoan predation, rather than antibiotic concentration, are implicated as major drivers of plasmid persistence in the presence of plasmid-dependent phage. Because plasmid loss was not observed in the absence of phage, only populations from treatments containing phage are shown. Each treatment was replicated four times. (B) Observed conjugation ability for plasmid-harboring clones of bacteria isolated from the end of the community experiment. 24 clones were isolated from all four experiments, and the phenotypes are depicted separately for each replicate. AB, antibiotic.

community composition, $\chi^2 = 130.1$, df = 3, $P < 0.001$), conjugation ability was almost completely lost with phages alone (glht, phage alone versus all other community compositions, $P < 0.001$), was decreased but significantly retained under conditions of phage selection by the simultaneous presence of protozoa (glht, phage with protozoa versus bacteria alone or with protozoa, $P < 0.001$), and was completely retained with predation alone (glht, bacteria alone versus bacteria with protozoa, $P = 0.85$).

**Bacterial metabolic activity is elevated under conditions of protozoan predation, potentially promoting conjugation activity and mediating plasmid persistence in bacterial communities.** We hypothesized that selection for plasmid conjugation in bacterial populations under conditions of protozoan predation might be caused indirectly by lower cell densities under conditions of predation, since low cell densities may maintain higher metabolic activity and, thereby, higher conjugation activity than high cell densities where cells assume the stationary phase. To test this, we conducted a separate 8-day microcosm experiment using a luminescence-based method to measure differences between the relative levels of ATP production by bacterial cells with or without predation. Bacterial metabolic activity per cell was higher in the presence of protozoan predation than in the absence of protozoa (LMM, $\chi^2 = 100.4$, df = 5) ($P < 0.001$) (Fig. 4A).

In addition, to test whether more-active cells are more likely to conjugate, we determined the conjugation rate of plasmid RP4 in different growth phases of the host bacterium HMS174 by culturing for 2 h, 4 h, 6 h, or 28 h, mixing with the recipient *E. coli* JM109(pSU19) strain, allowing bacteria to conjugate for 2 h, and plating on medium selective for transconjugated JM109(pSU19)(RP4) cells. The conjugation rates of the wild-type HMS174(RP4) plasmid differed depending on the growth phase (Fig. 4B). Each cell in a bacterial culture in five independent experiments that had been growing for 2, 4, 6, and 28 h conjugated (on average) with a probability of 2.8%, 3%, 1.1%, and 1.1%, respectively.

To further evaluate the influence of predation on plasmid persistence, an individual-based model was constructed (Fig. S3). In this model, populations containing both plasmid-harboring and plasmid-free bacteria were exposed to differing environmental conditions. Conjugation probability was adjusted based on the population density in the system such that the probability decreased from the wild-type probability level to one-third that level in relation to population density. The generation time and conjugation probability were adjusted based on the microcosm experiment, where around eight bacterial generations corresponded to a 48-h culture refreshment cycle (see Table S1 in the supplemental material). The fitness cost determined for plasmid carriage was based on the replication rates measured in the absence of antibiotics (Table S2). Similarly to *in vitro* experiments, simulated predation significantly improved plasmid survival over 175 generations (iterations of the model) by modulating the bacterial population size (Fig. 4C). While plasmid frequencies in simulations align with the observed frequencies in serial culture experiments when experimentally determined parameter values are used, the plasmid nevertheless disappears from the system when a much greater number of generations ($>10^4$) is simulated. This suggests that plasmid-host coadaptation and selective sweeps (such as the transient presence of antibiotics) may be necessary for ensuring the long-term survival of the plasmid in real-life settings even in the presence of predators. To investigate this, we explored how different factors influence plasmid persistence. Indeed, when the plasmid-associated fitness cost was decreased from the observed 16.7% to just 15%, the plasmid occasionally (3 of 5 individual simulations) survived for over $10^4$ generations (example simulations shown in Fig. S4). Also, exposure to 0.1× MIC antibiotics (against which the plasmid encodes nonleaky resistance) for 10 simulation cycles in every 100 cycles significantly improved plasmid survival (5 of 5 simulations) (Fig. S4C).

## DISCUSSION

In this study, we investigated the maintenance of a conjugative plasmid providing leaky (altruistic) and nonleaky (selfish) resistance against beta-lactams and aminoglycosides, respectively, in a multitrophic system consisting of bacterial prey, plasmid-

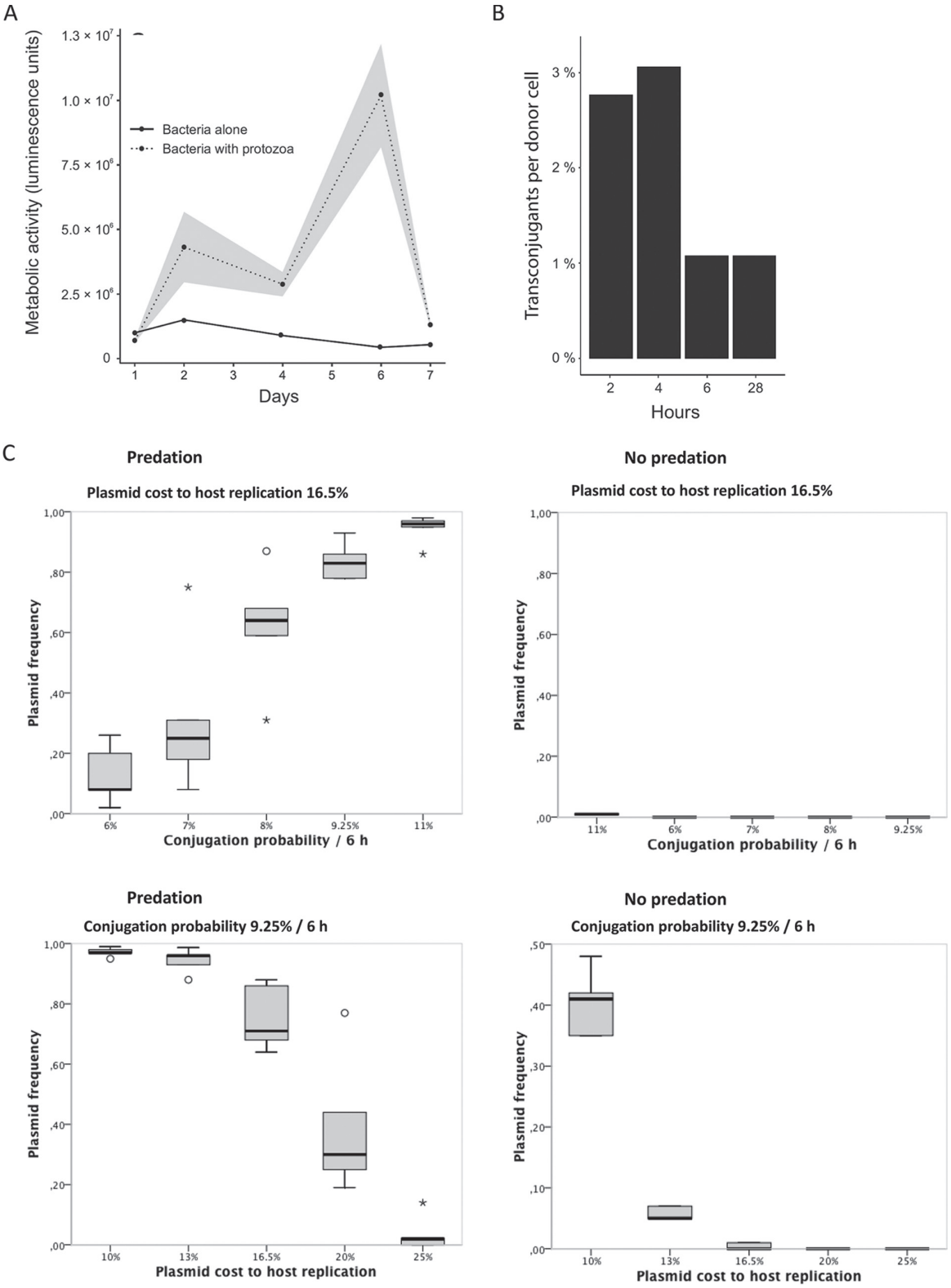


**FIG 4** Evidence regarding potential mechanisms by which protozoa maintain plasmid conjugation in bacterial populations. (A) Per-cell metabolic activity in bacterial populations in the presence (dashed line) or absence (solid line) of predation by the protozoan *T. thermophila* (data represent means ± SE). Both treatments were replicated four times. (B) Bacterial conjugation rates in different growth phases. The early to mid-logarithmic-growth phase is represented by the 2-h and 4-h time points, the late logarithmic-growth phase by the 6-h time point, and the stationary-growth

(Continued on next page)

dependent bacteriophage, and protozoan predator. The plasmid-dependent bacteriophage PRD1 was used to disrupt the conjugation network, and, indeed, its presence alone caused *E. coli* populations to lose their plasmids, thus rendering them susceptible to antibiotics. The altruistic nature of beta-lactam resistance was clearly seen when the phage-bacterium combination was cultivated in ampicillin as only a subpopulation of bacteria retained the plasmid. In terms of Black Queen evolution, this is a clear example of a race to the bottom, as the benefits of plasmid carriage were distributed among the community members but the cost was borne by individuals, thus favoring beneficiaries over helpers. In contrast to the ampicillin results, the presence of kanamycin caused the entire population to maintain the plasmid over the 50-day period, likely owing to the nonleaky nature of the resistance mechanism. Surprisingly, there were only minor differences between the results seen with lethal (1.5× MIC) and sublethal (0.1× MIC) antibiotic levels, suggesting that leakiness of resistance rather than antibiotic concentration is the defining factor in plasmid maintenance. Bottery and colleagues have observed similar outcomes with selfish tetracycline resistance (29).

While antibiotics forced plasmid maintenance, bacteriophage selection still caused the vast majority of the plasmids to become conjugation defective. This phenotype was retained over the course of the experiment, disregarding the fact that phages rapidly disappeared from most of the communities. Notably, however, selection for selfish resistance hindered the complete loss of plasmid-dependent phage from the community in comparison to selection for altruistic resistance and to the absence of antibiotics, as the phage were present also at the end of the experiment. The addition of the protozoan predator *T. thermophila* to the system had a major impact on the frequency of plasmids maintaining a conjugative phenotype in both the absence and presence of antibiotics. This is in line with previous experiments denoting the importance of predation for conjugative plasmid persistence (24). Since a bacterial population reaches lower density under conditions of predation (Fig. 2), it is possible that the individual cells remain in a more active state throughout each step of the serial culture experiment, which, in turn, increases the dissemination of conjugative plasmids between individual bacteria. We investigated this further by showing that the bacteria conjugated approximately three times more frequently in the early logarithmic-growth phase than in the late logarithmic-growth and stationary-growth phases. Also, the metabolic activity of bacteria was shown to remain significantly higher in the presence of *T. thermophila*, with all of the results suggesting that predation has an indirect influence on the conjugation rate.

Lopatkin and colleagues demonstrated that the absence of antibiotic selection alone is not enough to cause bacterial communities to become sensitive to antibiotics (1). Yet they also showed that plasmid-mediated resistance is more likely to be lost if the conjugation network is disrupted by chemical agents. We observed here that a similar outcome can be achieved with biological agents, namely, plasmid-dependent phages. However, even subinhibitory concentrations of antibiotics can have a notable impact on plasmid prevalence, depending on the leakiness of the plasmid-encoded resistance mechanism. Simulations also suggested that the long-term survival of plasmids may depend on a minor decrease in the plasmid-associated fitness cost and/or periodical selection. Hence, bacterium-plasmid coadaptation and fluctuations in environmental conditions are factors that might nullify the outcome of resensitization applications which target the conjugation network. Even more intriguing is the notion that predation can effectively counter the loss of resistance plasmids after the disruption of the conjugation network. Since predation selects for conjugative plasmids over nonconjugative ones (24), and given that conjugation may be lost by several

**FIG 4** Legend (Continued)
phase by the 28-h time point. The bar height represents the mean of results from five technical replicates. (C) Effects of plasmid cost and conjugation probability on plasmid maintenance in simulated communities with and without predation. Plasmid cost indicates the relative decrease in replication frequency due to plasmid carriage. Bacterial population density was set to modify the conjugation probability. Since predation lowered the effective population density of the community and thus increased the conjugation rate, the prevalence of plasmid increased in the presence of protozoa after disruption of the conjugation network (i.e. the emergence of plasmid-free individuals) ($n = 5$).

types of mutations, of which only some are readily reversible (see Fig. S1 in the supplemental material), under oscillating selection pressures (for and against conjugation), mutations in these reversible sites could be considered contingency loci improving the survival of resistance plasmids. Therefore, taking trophic interactions into account in real-life systems may play a defining role in whether or not resistance is actually lost from the community.

Nevertheless, predation may not be of particular relevance in human carriage of resistance plasmid-harboring bacteria, at least not in Western societies where intestinal protozoa are rare among individuals (23). On the other hand, in many developing countries in sub-Saharan Africa and Southeast Asia, the majority of children (especially in slums) are infected with protozoa such as *Entamoeba histolytica* by the age of 2 years (30). These countries also experience a severe burden of antibiotic-resistant bacteria and resistance-associated effects on health care (31). It is possible that the trophic interplay between eukaryotic and prokaryotic microbes might be also furthering the overall persistence of resistant bacteria in these areas. However, other environments with low concentrations of antibiotics, such as farms and wastewater treatment plants, may be habitats where the role of predation in resistance maintenance is likely to be more prominent.

## MATERIALS AND METHODS

**Strains and culture conditions.** We used *E. coli* K-12 HMS174 as the bacterial host species (32). MICs of antibiotics were determined with the plasmid-free ancestral strain, and for the community experiment, the plasmid RP4 was transconjugated to HMS174 from *E. coli* K-12 JE2571 (33). Broad-host-range conjugative plasmid RP4 (incompatibility group P) has multiple genes for addicting the host and encodes resistance to the antibiotics kanamycin, ampicillin, and tetracycline (34). We used virulent double-stranded DNA (dsDNA) plasmid-dependent bacteriophage PRD1 (family *Tectiviridae*) (35) as the viral parasite and the ciliate *Tetrahymena thermophila* CCAP 1630/1U (axenic stock obtained from Culture Collection for Algae and Protozoa, United Kingdom) as the protozoan predator.

We followed previously established protocols for microcosm experiments with bacterium-phage and bacterium-ciliate systems (36–42). The culture medium for bacteria contained M9 salts and King's B (KB) nutrients at a 5% concentration compared to full-strength medium (concentrations used, 1 g peptone number 3 and 0.5 ml of 85% glycerol in 1 liter of $dH_2O$). All media and microcosm vials were sterilized by autoclaving prior to use and kept at 28°C (±0.1°C) during the experiments, with constant rotation at 50 rpm. In the conjugation ability and rate experiments, we used lysogeny broth (LB) medium (43) and *E. coli* K-12 JM109 harboring chloramphenicol resistance-encoding plasmid pSU19 (44) as the recipient strain.

**Community experiment.** In order to determine antibiotic MIC values for the community experiment, the ancestral HMS174 strain was cultured under experimental conditions in a concentration gradient of 0 to 1.9 $\mu$g ml$^{-1}$ ampicillin or 0 to 4.5 $\mu$g ml$^{-1}$ kanamycin (19 different concentrations with both antibiotics). Bacterial growth was measured as optical density (Bioscreen C spectrophotometer; Oy Growth Curves Ab Ltd.) using a 420-to-580-nm wideband filter, and the MIC was determined as the lowest concentration with no detectable bacterial growth after 96 h. The MICs were 1.1 $\mu$g ml$^{-1}$ and 2.5 $\mu$g ml$^{-1}$ for ampicillin and kanamycin, respectively.

To test for the interactive and relative contributions of trophic interactions, altruism of resistance, and antibiotic concentration to plasmid persistence in HMS174(RP4) with different concentrations and types of antibiotic, we performed a 50-day microcosm experiment. We used a community treatment consisting of the presence or absence of phage or protozoan and an antibiotic treatment consisting of no antibiotic, 0.1× MIC of ampicillin (0.11 $\mu$g ml$^{-1}$) or kanamycin (0.25 $\mu$g ml$^{-1}$), or 1.5× MIC of ampicillin (1.65 $\mu$g ml$^{-1}$) or kanamycin (3.75 $\mu$g ml$^{-1}$). All treatments were started from a clonal culture of HMS174(RP4) cultured overnight in KB. The initial bacterial density was approximately $5.4 \times 10^6$ CFU ml$^{-1}$ and the initial phage density approximately $1.6 \times 10^6$ PFU ml$^{-1}$, constituting a multiplicity of infection (MOI) value of 0.3. The initial protozoan density was $2 \times 10^3$ cells ml$^{-1}$. All treatment combinations were replicated four times in 25-ml glass vials containing 6 ml KB. Every 48 h, 1% (60 $\mu$l) of each culture was transferred to a new vial containing fresh KB. Every 96 h (or every 48 h for the first three transfers), bacterial density was estimated as optical density (OD) at 600 nm (UV-1800 spectrophotometer; Shimadzu, Japan), and *T. thermophila* density was enumerated directly from live samples using a compound microscope (Zeiss Axioskop 2 plus; Oberkochen, Germany), as described previously (36). A 1.0-ml subsample was frozen with 0.5 ml of 85% glycerol or without glycerol (for phage analyses) and kept at −80°C for later analysis. Phage abundances were estimated for days 2, 12, 24, and 48 from freeze-stored samples using plaque assay (44).

**Plasmid persistence.** To detect the loss of RP4 plasmid during the community experiment, we isolated 24 bacterial clones per population from freeze-stored samples from day 8, the middle (day 24), and the endpoint (day 50) of the experiment. Clones were inoculated in 200 $\mu$l of LB medium in a 96-well plate, cultured overnight, and frozen with 50 $\mu$l of 87% glycerol at −80°C for later analysis. To test for the presence of plasmid, a 10-$\mu$l subsample was cryo-replicated (45) on a large (140-mm-diameter) petri dish containing LB agar with high concentrations of all antibiotics to which the RP4 encodes resistance as follows: 150 $\mu$g ml$^{-1}$ ampicillin, 25 $\mu$g ml$^{-1}$ kanamycin, and 20 $\mu$g ml$^{-1}$ tetracycline.

**Bacterial cheaters in the presence of aminoglycoside kanamycin.** We investigated the capacity of JM109(pSU19) to survive at lethal concentrations of ampicillin (150 $\mu$g ml$^{-1}$ and 300 $\mu$g ml$^{-1}$) or kanamycin (25 $\mu$g ml$^{-1}$ and 50 $\mu$g ml$^{-1}$) in the presence of a conjugation-deficient HMS174(RP4) mutant. The conjugation-deficient mutant was created as described previously (26). Briefly, HMS174(RP4) was cultured overnight (37°C, 220 rpm) in the presence of kanamycin and plasmid-dependent phage PRD1 and plated on LB agar. Several clones were picked, and their ability to conjugate with JM109(pSU19) was investigated. A mutant producing no transconjugants was selected for the cheating experiment. Subsequently, 5-$\mu$l volumes of overnight cultures of JM109(pSU19) and HMS174(RP4) mutants were cultured together in 5 ml of LB medium with antibiotics at different concentrations for 21 h (37°C, 220 rpm). These cultures were plated on chloramphenicol (25 $\mu$g ml$^{-1}$) to select for JM109(pSU19) cells that had survived in the presence of ampicillin or kanamycin.

**Conjugation ability.** In order to measure the conjugation ability of bacteria at the endpoint of the evolution experiment (day 50), clones were isolated and transferred to 200 $\mu$l of KB medium in 96-well plates. Another 96-well plate was prepared with recipient strain *E. coli* K-12 JM109(pSU19). Both plates were cultured overnight at 37°C. A third 96-well plate was prepared with 200 $\mu$l of LB medium in each well. A plate replicator was used to transfer the clones from evolution experiments with the recipient bacterium to the third plate. This conjugation plate was cultured overnight at 37°C. A plate replicator was utilized to transfer samples from conjugation experiments to large petri dishes containing antibiotics that allowed only those recipient bacteria to grow that had acquired the conjugative plasmid from the clone. Petri dishes were transferred to 37°C conditions, and conjugation ability was inferred based on overnight growth in sampled wells. Each spot was assigned to one of the following three categories: (i) no growth, (ii) some growth, and (iii) normal growth (where "no growth" indicates complete loss of conjugation ability and "normal growth" indicates wild-type conjugation ability) (see Fig. S2 in the supplemental material).

**Bacterial metabolic activity.** To test for the effect of protozoan predation on bacterial metabolic activity, we conducted an 8-day experiment using a luminescence-based method to measure differences between the relative levels of production of ATP by bacterial cells with or without predation. The test was started from an overnight clonal culture of HMS174(RP4). The same initial bacterial and predator densities as those described for the community experiment were inoculated into 50 ml of experimental medium (5% KB) in a 250-ml screw-cap polyethylene terephthalate (PET) storage bottle (Corning, New York, USA), with two treatments: bacteria with live predator and bacteria with heat-killed predator (to eliminate any effect of predator cells or carryover medium). Both treatments were replicated four times. Culturing was performed for 8 days without transfers or rotation.

On days 1, 2, 3, 4, 6, and 8, aliquots of 2 ml were filtered through 5-$\mu$m-pore-size filters to remove ciliates. The filtrates were used to measure bacterial density (36) and metabolic activity (ATP production). Metabolic activity was measured with a well plate reader (Victor3 1420 Multilabel Counter; PerkinElmer, MA, USA) using the BacTiter-Glo microbial cell viability assay (Promega, Madison, WI, USA) according to manufacturer's instructions, except that the culture and BacTiter-Glo reagent were mixed at a 2:1 ratio instead of a 1:1 ratio (comparable results were observed). Bacterial metabolic activity was estimated as per-cell ATP production. Results were corrected for nonlinearity (a slight increase in per-cell ATP signal with decreasing cell density). The luminescence signals of sterile medium and filtered ciliate stock did not differ, demonstrating that the filtration had removed any effect of the ciliates on the results.

**Conjugation rate in different growth phases.** We determined the conjugation rate of plasmid RP4 at different growth phases of the host bacterium HMS174. HMS174(RP4) and recipient JM109(pSU19) were cultured overnight (37°C, 200 rpm). Subsequently, 5 $\mu$l of HMS174(RP4) was transferred to 5 ml of fresh LB medium and cultured at 37°C with constant rotation at 200 rpm. A 5-$\mu$l subsample was taken from this culture after 2 h, 4 h, 6 h, and 28 h and combined with the recipient bacterium at a 1:20 ratio along with the addition of 100 $\mu$l of LB. The bacteria were allowed to conjugate for 2 h, after which they were plated on LB agar containing 150 $\mu$g ml$^{-1}$ ampicillin, 25 $\mu$g ml$^{-1}$ kanamycin, and 25 $\mu$g ml$^{-1}$ chloramphenicol to select for transconjugated JM109(pSU19)(RP4) cells. Bacterial density was measured for bacteria before the conjugation experiment to determine the ratio of transconjugants per donor bacterium.

**Brief model description.** An individual-based model was constructed in an attempt to investigate whether the effect of predation on plasmid persistence can be replicated *in silico* and to what extent different variables influence the dynamics within the system. The source code for the model is freely available on Dryad, and a more detailed description is presented in Text S1 in the supplemental material. The model consists of the following interacting "biological" entities: bacteria, plasmids, plasmid-dependent bacteriophages, and protozoa (model entities and factors that influence their abundance are depicted in Fig. S4). Bacteria replicate in the system for as long as the environment maintains the carrying capacity. Each bacterium has an individual probability of being replicated during a single iteration of the model. The standard probability of 1.0 is lowered with plasmid carriage (owing to plasmid-associated fitness costs). The conjugation rate is adjusted based on bacterial population density such that as the population approaches the carrying capacity of the system, the conjugation rate decreases to one-third of the maximum rate. A protozoan requires a preset number of bacteria to be consumed before it can replicate, and a protozoan consumes a fixed number of bacteria during each iteration of the simulation. Aminoglycoside-like antibiotics can be introduced into the simulated community (using either constant or periodic exposure) (e.g., 0.1× MIC antibiotic kills the bacterium with a 10% probability). All parameters can be adjusted by the user. The values used in this study are listed in Table S1 in the supplemental material.

**Statistical analyses.** For the 50-day microcosm experiment, we conducted three analyses each for plasmid prevalence over time, population sizes over time, and proportion of wild-type conjugating plasmids at the end of the experiment. These consisted of separate analyses for kanamycin and ampicillin as well as a

combined analysis, disregarding concentration, to compare antibiotics results. All statistical analyses were performed in R v. 3.2.2. For correlations between plasmid prevalence or population size over time and experimental treatments (antibiotic/concentration, community composition, and time) in the 50-day experiment and between ATP production and the predation (presence/absence) treatment in the 8-day experiment, we used lme4 (46) to generate linear mixed models (LMM), with the treatment as the fixed effect and transfer within replicates as the random effect. Models with and without the fixed effect were compared to determine the significance of the correlations. To compare proportions of wild-type conjugating plasmids between treatments at the end of the experiment (day 50), we performed beta regression with the logit link function, which accommodates continuous proportion data, using betareg (47). Multiple comparisons were performed using the general linear hypothesis test (glht, function) in multcomp (48) with default parameters for each model type (i.e., custom *post hoc* contrasts testing whether pairwise differences differ significantly from 0).

**Data availability.** Data are available on the Dryad depository (https://doi.org/10.5061/dryad.10gk660). Data include the population sizes and plasmid frequencies from the evolution experiment, model files, and images from plasmid conjugation ability experiments.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSystems.00104-18.

**TEXT S1**, DOCX file, 0.01 MB.
**FIG S1**, TIF file, 1 MB.
**FIG S2**, PDF file, 1.2 MB.
**FIG S3**, TIF file, 0.05 MB.
**FIG S4**, TIF file, 0.2 MB.
**TABLE S1**, DOCX file, 0.01 MB.
**TABLE S2**, DOCX file, 0.01 MB.

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T.H., M.J., and J.C. designed the experiment. J.C. supervised the experimental procedures and analyzed data. A.H., R.J., L.R., S.V., and J.C. performed evolutionary experiments. J.C. performed the metabolic activity assay. K.K., T.P., R.P., M.J., and S.M. performed plasmid survival, plaque assay, and conjugation ability and cheater tests. M.J. is responsible for the individual-based model. J.C., M.J., K.K., and T.H. wrote the manuscript. All of us gave final approval for publication and accept accountability for the content and the work performed.

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# III

# SYSTEMATIC COMPARISON OF EPIDEMIC AND NON-EPIDEMIC CARBAPENEM RESISTANT *KLEBSIELLA PNEUMONIAE* STRAINS

by

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# Systematic Comparison of Epidemic and Non-Epidemic Carbapenem Resistant *Klebsiella pneumoniae* Strains

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Over the past few decades, extensively drug resistant (XDR) resistant *Klebsiella pneumoniae* has become a notable burden to healthcare all over the world. Especially carbapenemase-producing strains are problematic due to their capability to withstand even last resort antibiotics. Some sequence types (STs) of *K. pneumoniae* are significantly more prevalent in hospital settings in comparison to other equally resistant strains. This provokes the question whether or not there are phenotypic characteristics that may render certain *K. pneumoniae* more suitable for epidemic dispersal between patients, hospitals, and different environments. In this study, we selected seven epidemic and non-epidemic carbapenem resistant *K. pneumoniae* isolates for extensive systematic characterization for phenotypic and genotypic qualities in order to identify potential factors that precede or emerge from epidemic successfulness. Studied characteristics include growth rates and densities in different conditions (media, temperature, pH, resource levels), tolerance to alcohol and drought, inhibition between strains, ability to compensate pH, as well as various genomic features. Overall, there are clear differences between isolates, yet, only drought tolerance was found to notably associate with non-epidemic *K. pneumoniae* strains. We further report a preliminary study on the potential to control *K. pneumoniae* ST11 with an antimicrobial component produced by a non-epidemic *K. pneumoniae*. This component initially restricts bacterial growth, but stable resistance develops rapidly *in vitro.*

**Keywords: XDR *Klebsiella pneumoniae*, extended-spectrum beta-lactamase, epidemic, antibiotic resistance, virulence**

## INTRODUCTION

*Klebsiella pneumoniae* is a Gram-negative bacillus causing opportunistic infections outside of the gastrointestinal tract (Podschun and Ullman, 1998). Common conditions include pneumonia, urinary tract infections, wound infections, and less often liver abscess, meningitis, and septicemia. Some of the strains circulating in clinical settings are also showing increasingly virulent phenotypes (Pomakova et al., 2012; Shon et al., 2013). These strains are often characterized by hypermucoviscosity when cultivated on agar plates and they are more resilient against killing by serum or phagocytosis (Catalan-Najera et al., 2017). Moreover, extensively drug-resistant (XDR) among *K. pneumoniae* is increasing very rapidly compared to many other priority pathogens (World Health Organization, 2014). In particular, infections caused by *K. pneumoniae* strains which have developed resistance against newer generations of β-lactams, such as carbapenems, can be hazardous and often life-threatening. These carbapenemase genes are found to be abundant in bacteria originating from hospital environments although there are notable regional differences. Yet, sequence typing of the pathogens indicate that resistant *K. pneumoniae* strains can also disseminate globally between hospitals.

WHO has classified carbapenemase-producing *K. pneumoniae* as an urgent threat (World Health Organization, 2017). Often, *K. pneumoniae* isolates are typed by utilizing partial sequences from seven housekeeping genes. These genes are part of the core genome and hence unlikely to be horizontally transferred between different *K. pneumoniae* strains. As such, sequence typing provides a rudimentary approach to identify genetic similarity among isolates of different sources of origin. It appears that certain sequence types (STs) have been more successful in dispersing between hospitals compared to other equally resistant strains. NDM-1 metallo-β-lactamase producing ST11 and 14 have been noted to be responsible of epidemics in various countries (Yong et al., 2009; Pitout et al., 2015; Samuelsen et al., 2017). *Klebsiella pneumoniae* carbapenemase (KPC) producing ST258 have even been referred as hyperepidemic clone (Bowers et al., 2015) and its epidemic potential has been further investigated in several meta-analyses (Dautzenberg et al., 2016). ST512, a single-locus variant of ST258, is also highly associated in epidemics globally (Conte et al., 2016). ST147 is also hazardous with numerous virulence and resistance genes (Turton et al., 2018). Despite of the notion that certain STs appear to be more prone for inter-hospital dispersal, it is still unclear what qualities alongside of pathogenicity, if any, may be responsible for this epidemic success. Majority of the surveillance attempts focus on resistance profiles and genotypic features (Giske et al., 2012). Genomic data also accumulates rapidly as whole genome sequences of many strains have become available (Holt et al., 2015). Yet, the phenotypic characteristics of differently successful *K. pneumoniae* STs are rarely studied in detail, or the phenotypic analysis focus on specific traits such as hypermucoviscousity (Catalan-Najera et al., 2017). Comparison of the phenotypes of epidemic and non-epidemic strains could potentially reveal meaningful interactions between bacteria and their environment that contribute to the epidemic spread of XDR strains.

In this study, we selected 14 carbapenem resistant *K. pneumoniae* strains isolated from patients hospitalized in USA, Sweden, UK, Greece, or India (Kitchel et al., 2009; Samuelsen et al., 2009; Kitchel et al., 2010; Samuelsen et al., 2011; Vading et al., 2011; Giske et al., 2012; Hasan et al., 2014). Five of these STs are continuously being detected in hospitals in multiple countries and can be considered as epidemiologically successful or epidemic (**Table 1**). The rest of the STs have made only seldom appearances and have rarely if at all dispersed to other hospitals and may hence be considered as non-epidemic STs. Here, we systematically determined and measured potentially relevant characteristics for these strains in order to reveal differences that may correlate and perhaps partly explain the epidemic success.

## MATERIALS AND METHODS

### Strains

All 14 studied *Klebsiella pneumoniae* strains (see **Table 1**) were Illumina sequenced at Karolinska Institutet, Sweden. Genome sequences can be found from GenBank under BioProject id PRJNA680903. Sequences of EKP24 and NKP2 were also PacBio sequenced in University of Helsinki, Finland. Sequenced genomes were annotated by Rapid Annotation using Subsystem Technology (RAST, https://rast.nmpdr.org) and both secure and potential protein coding genes were mapped and the distribution of protein families were compared between the strains. Resistance genes were identified with ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/), prophages with Prophage Finder (https://omictools.com/prophage-finder-tool), and CRISPR-regions with CRISPR-finder (https://crispr.i2bc.paris-saclay.fr). An algorithm was written to identify unannotated short open reading frames (ORFs) from the genome files (**Supplementary File 1**). The algorithm scans the genome for ORFs that have a potential ribosome binding site upstream of the start codon and is not overlapping with annotated genes.

### Growth Experiments

Growth densities to each strain were measured in temperature of +37°C and room temperature with 230 rpm shaking or without shaking. Cells were grown overnight in 5 ml of LB media (+37°C, 230 rpm) and then transferred into 5 ml of fresh LB media in 1:5,000 ratio. Fresh cultures were grown in experimental settings of +37°C and 230 rpm or RT and 0 rpm for 20 h and growth densities were calculated as colony forming units (cfu)/ml. As a standard initial liquid culture for the growth curve experiments all the strains were cultured in 5 ml of 100% LB, +37°C, and 230 rpm overnight and then transferred into experimental settings. In order to test the effect of shaking, the initiating cultures were prepared then transferred into 5 ml of LB in 1:100 ratio and grown in experimental settings of +37°C and 230 rpm and +37°C

**TABLE 1** | K. *pneumoniae* isolates used in the study.

| | Isolate | ST from database | Isolation location | No. of CRISPR loci | No. of pro-phage regions | Reference | Virulence genes | Capsule types | Beta-lactamase |
|---|---|---|---|---|---|---|---|---|---|
| NKP01 | 1534 | 37 | USA | 0 | 10 | Kitchel et al., 2009 | *mrk* | K15K17K50K51K52 | blaTEM-1B, blaKPC-2, blaSHV-11 |
| NKP02 | 10924 | 334 | USA | 0 | 4 | Kitchel et al., 2009 | *mrK* | N/A | blaKPC-3, blaOKP-B-4, blaOXA-9, blaTEM-1A |
| EKP03 | 70165 | 14 | USA | 3 | 10 | Kitchel et al., 2009 | *irp, fyu, ybt, kfu, mrk* | K2 | blaTEM-1A, blaSHV-28, blaKPC-3 |
| EKP05 | 70708 | 258 | USA | 0 | 7 | Kitchel et al., 2009 | *mrk* | N/A (K15K17K50K51K52) | blaOXA-9, blaKPC-3, blaSHV-12 |
| EKP08 | 2008025 | 11 | USA | 0 | 11 | Kitchel et al., 2009 | *mrk* | K13 | blaKPC-2, blaSHV-11 |
| EKP10 | AO-8053 | 512 | Sweden (Israel*) | 0 | 10 | Samuelsen et al., 2009 | *mrk* | N/A | blaTEM-1A, blaOXA-9, blaSHV-11, blaKPC-3 |
| EKP11 | AO-15200 | 147 | Sweden (Greece*) | 2 | 6 | Samuelsen et al., 2011 | *mrk* | K64,K14 | blaSHV-11, blaVIM-1 |
| NKP18 | VPKP389 | 36 | Athens, Greece | 1 | 10 | Hasan et al., 2014 | *irp, mrk, fyu, ybt* | k27 | blaSHV-129, blaVIM-26 |
| NKP20 | VPKP229 | 17 | Athens, Greece | 0 | 8 | Hasan et al., 2014 | *irp, mrk, ybt* | k25 | blaSHV-129, blaVIM-1 |
| EKP22 | N6 | 14 | UK | 2 | 6 | Giske et al., 2012 | *mrk, ybt, kfu* | k2 | blaCTX-M-15, blaSHV-11, blaTEM-1A, blaOXA-1, blaNDM-1, blaOXA-9 |
| EKP24 | ED502873 | 11 | Sweden | 0 | 6 | Giske et al., 2012 | *irp, mrk, fyu, ybt* | N/A (K15K17K50K51K52) | blaSHV-11, blaCTX-M-15, blaNDM-1 |
| NKP25 | N12 | 231 | UK | 1 | 4 | Giske et al., 2012 | *kfu, mrk* | K51 | blaSHV-1, blaTEM-1B, blaNDM-1, blaOXA-1 |
| NKP28 | B357 | 43 | UK | 1 | 8 | Giske et al., 2012 | *mrk, kfu* | K30 | blaCTX-M-15, blaDHA-1, blaCMY-6, blaSHV-11, blaOXA-9, blaNDM-1, blaTEM-1A |
| NKP30 | IR34 | 624 | Chennai, India | 1 | 3 | Giske et al., 2012 | *mrk* | K12, K29 | blaTEM-1B, blaDHA-1, blaNDM-1, blaSHV-36, blaCTX-M-15, blaOXA-1 |

**Isolates associated with import from the country.*

and 0 rpm. In growth curve experiments the effect of different media and varying concentrations and compositions of nutrients on growth was determined for each strain. One hundred percent LB was used throughout the experiments unless mentioned otherwise. Growth curves were measured in 10 and 1% LB, 100% BHI, and 100% of pure DMEM by diluting the initial culture in 1:100. Growth curves were measured at +37°C, 595 nm wavelength with Multiscan FC (Thermo Scientific) for 20 h in 5 min intervals and maximum growth and average growth rate were calculated.

## Survival in Acidic pH and Compensation Capacity

Bacterial cells' ability to tolerate acidic surrounding pH and capability to compensate it by metabolism was measured for each strain in pH 3–7. Initial cultures were grown in 5 ml LB pH 7 at +37°C and 210 rpm overnight. Each strain was then transferred into 5 ml LB of either pH 3, pH 4, pH 5, pH 6, or pH 7 in 1:100 ratio and cultured in +37°C and 210 rpm. In pH 5–7 cultures were grown for 90 h and growth densities were calculated by plating in 16, 24, and 90 h. Then 1.5 ml of culture was filtered through 0.2 μm and supernatant pH was measured with Basic pH Meter (Denver Instruments) in 24 and 90 h. Cultures in pH 3–4 were shortened into 24 h experiment and growth densities were calculated in 16 and 24 h and supernatant pH was measured in 24 h. Growth curves were measured by diluting the initial pH 7 cultures in 1:100 ratio into LB pH of 3–7 and growth curves were measured at +37°C, 595 nm wavelength for 20 h in 5 min intervals.

## Cross-Strain Interactions

In aim to study the dynamics all the strains were cultured separately, and their metabolic products secreted into surroundings were tested against other strains in cross-strain inhibition experiments. Cross-strain interactions were tested by collecting the media after overnight culturing at +37°C and 210 rpm. Overnight cultures were centrifuged first with 7,000 × g for 4 min, and the supernatant was centrifuged again with 10,000 × g for 1 min. Each strain was cross-plated with all the supernatants (overnight, +37°C), and the inhibition of the growth of each strain was observed.

## Supernatant Inhibition and Prophages

A 4-week evolutionary experiment was designed to study appearance, persistence, and reversibility of putative colicin E3 resistance in sensitive EKP24 strain. For the first 2 weeks EKP24 was cultured with (n = 5) and without (n = 5) colicin E3 in 10% LB media supplemented with either NKP2 (containing colicin E3) or EKP24 (not containing colicin E3) supernatant filtrate (0.2 μm) in 1:4 ratio. Ten percent LB media was supplemented

with 25 µg/ml of kanamycin and 150 µg/ml of ampicillin. After 2 weeks EKP24 cultured with the presence of colicin E3 were divided into two sets of samples (both n = 5). Other set was continued with colicin E3 exposure as described earlier. In the other set of samples, colicin E3 containing supernatant was replaced with EKP24 supernatant. Cultures were refreshed in 1:100 ratio three times and samples stored once a week with glycerol at −80°C. Development and persistence of colicin E3 resistance was determined by plating. Samples were taken at the beginning of the experiment, before division of colicin E3 exposed EKP24, and at the end of the experiment and were used for DNA extraction. DNA was isolated with DNeasy Blood & Tissue Kit (Qiagen) and sequenced with Illumina HiSeq. The observed reads were mapped to original PacBio-sequenced genome of NKP2 (described above) in order to detect the genetic variants. The variants developed under the exposure of colicin E3 were identified by filtering out those variants that were already present in the beginning of the experiment. The genetic analysis was performed with CLC Genomics Workbench v11 (Qiagen).

Interactions between the putative colicin E3 producing NKP2 and susceptible EKP24 bacterial cells were also observed with confocal microscopy. Then 200 µl of 1% LB-agar was placed into a chamber of eight-chambered ibidi® ibiTreat µ-Slide (Ibidi GmbH) covered with CID lid for µ-dishes (Ibidi GmbH), and 3 µl of NKP2 and EKP24 were injected under the agar onto opposite sides. Encounter of the two strains was visualized with Nikon AR1 laser scanning confocal microscope with 60× water immersion objective and using Galvano scanner.

Production of putative colicin E3 was further studied by growing NKP2 strain in different medias. NKP2 was grown in LB concentrations of 100, 10, and 1%, LB without tryptone, 100% Shieh (Song et al., 1988) and in 100% DMEM in +37°C and 200–230 rpm overnight. NKP2 cultures were filtered through 0.2 µm filter and colicin E3 presence was determined by plating the supernatant with susceptible EKP24 strain.

## Alcohol Exposure

All studied strains were exposed to multiple concentrations of ethanol (20, 50, 75, and 90%) and their ability to survive the exposure were measured with spectroscopy. In 30 s exposure experiment all the strains were first grown in 5 ml of LB (+37°C, 230 rpm, overnight) and then transferred in 1:10 ratio into fresh LB and left to grow overnight at +37°C on 96-well plate 100 µl per well to form biofilm. On the following day media was gently removed and replaced with 200 µl of ethanol in concentrations of 20, 50, 75, and 90%. After 30 s incubation in RT ethanol was replaced with 100 µl of LB and growth at +37°C was measured in 595 nm wavelength for 20 h in 5 min intervals. In ethanol evaporation experiment, cultures for 96-well plate were prepared as described earlier but volume was lowered into 35 µl per well. Ninety-six-plate cultures were incubated overnight at +37°C without the lid to let the excess media evaporate. Dried biofilms were exposed to 50 µl of ethanol (either 20, 50, 75, or 90%) and left to fully evaporate before addition of 200 µl of LB per well. Growth curve measurements was performed as earlier described.

## Drought Tolerance

The capability to survive over long-lasting drought in a room air humidity was tested by culturing the strains on 96-well plate by transferring overnight grown culture (5 ml LB, +37°C, 210 rpm) in 1:10 ratio to LB. One hundred microliters per well was used with four replicates of each strain. Plates were incubated at +37°C for 3 days in order to grow biofilm. After 3 days, plates were relocated to RT and lids were removed to ensure total evaporation of media. After 12 days, 2 months and 6 months in drought, 200 µl of fresh LB was added into each well and the growth curves were measured at +37°C, 595 nm wavelength, 20 h in 5 min intervals.

## Morphological Characterization

Morphological characteristics of the colonies were analyzed. In order to get single colonies, all strains were cultured in 5 ml LB in +37°C, 210 rpm overnight. Cultures were diluted into $10^{-6}$ in water and plated onto LB-agar plates. Plates were incubated at +37°C for overnight and the colonies were photographed. Strains EKP5, EKP3 and EKP22 were found to produce translucent colonies along with the traditional colonies. These translucent colonies were further cultivated by transferring one colony to a fresh LB-agar plate and incubated at +37°C overnight or transferred into liquid culture of 5 ml LB and cultured at +37°C and 210 rpm overnight before plating in $10^{-6}$ dilution onto the new LB-plates. Growth densities were calculated from these plates. From the same liquid culture used for the growth density definition, 1:100 dilutions were made into LB and growth curves were measured in +37°C, 595 nm wavelength, 20 h in 5 min intervals.

## Statistical Analyses

To explore if epidemic or non-epidemic strains can be characterized by their capabilities, we performed discriminant analysis using MASS package (Venables & Ripley, 2002) in R (version. 3.3.2). Effects of individual variables on discriminant function were tested by regressing predicted values against original variables. Overall performance of discriminant function was addressed by Bayesian logistic regression of epidemic status against predicted values of discriminant function using Stan with R (McElreath, 2016).

# RESULTS

## Selection of Strains and Genomic Analysis

We selected 14 K. *pneumoniae* strains for detailed phenotypic and genomic analysis in an attempt to identify characteristics that may potentially associate with epidemic STs. The strains were abbreviated either as EKP or NKP for Epidemic and Non-epidemic *K. pneumoniae*, respectively, and the strain number was derived from an internal naming system. The strains and their key genomic traits are listed in **Table 1**. Note that two epidemic STs are represented twice (ST14 and ST11), but their genetic features differ from one another and were thus selected for phenotypic studies in order to evaluate whether the phenotypes of different strains of a single ST are similar.

Epidemic and non-epidemic STs are not grouping together when the genomic regions used for sequence typing are used to infer genetic relationship (**Figure 1**). As such, the epidemic strains do not appear to share a common ancestor that diverged from non-successful strains. Therefore, epidemic success is not likely to be linked to a single vertically inherited (genetic) trait, which evolved once. This however does not exclude the possibility that traits preceding epidemic spread are transferred horizontally between strains of different STs. Intriguingly, strains EKP3 and EKP22 (both ST14) and EKP8 and EKP24 (both ST11) are phenotypically different regardless of the same ST (**Supplementary Figure 1**), sometimes being even the opposite phenotypic extremes out of all strains. Colony morphologies of all strains are highly similar (**Supplementary Figure 2**).

The genomes of all the strains were annotated and their overall gene contents compared. Based on the annotation, epidemic and non-epidemic strains appear to be generally uniform metabolically and functionally, hence providing no apparent genome-level design differences to explain epidemic qualities (**Table 2**). Neither the presence or absence of CRISPR system or the number of CRISPR loci appear to be linked with epidemic successfulness. Also, the number of mobile elements such as prophages or plasmids do not associate specifically with either group. The most obvious potentially explanatory features, *i.e.* virulence genes and antibiotic resistance genes, are similar between epidemic and non-epidemic *K. pneumoniae* despite of differences among individual strains (**Tables 1** and **2**). We further speculated that some generally overlooked features such as short open reading frames (ORFs) of length 30 to 150 nucleotides could possibly be linked with epidemic qualities. These genes are rarely identified as coding regions with automated annotation algorithms despite of the fact that they are sometimes transcribed and translated and may reflect recent adaptations to new life strategies or specific conditions (that may be related to epidemic spread). We prepared an algorithm to extract all short ORFs which are preceded by a (near-)perfect ribosome biding site and which do not overlap with existing annotated ORFs (Python code is available in **Supplementary File 1**). On average, approximately 200 unannotated ORFs were extracted from the sequences. Yet, while putative short genes are common, their count is similar in epidemic and non-epidemic strains (data not shown).

## Phenotypic Qualities of the Strains

It is possible that epidemic spread selects for or is preceded by specific phenotypic traits. These traits may not necessarily be linked with any particular genetic feature as there may be several mutational pathways to acquire the quality and hence, they may be difficult to identify with genetic or genomic comparisons. As such, we listed a number of measurable phenotypes that may be linked with epidemic success. The traits, their speculated association with epidemic dispersal, and the variables used in this study are listed in **Table 3**. The original data from measurements is available in **Supplementary File 2**.

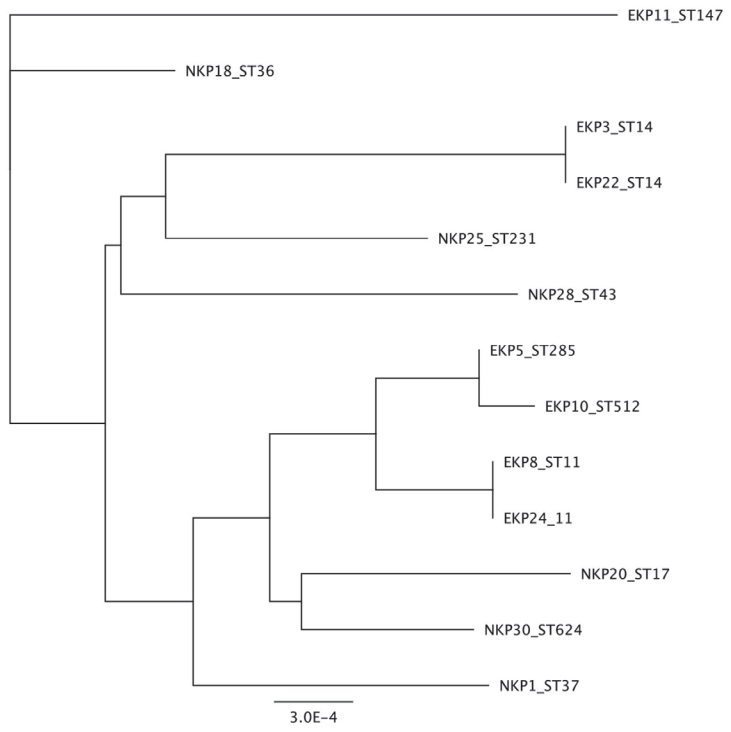


**FIGURE 1** | Phylogenetic distance based on Sequence Type sequences.

**TABLE 2 |** Genomic analysis of individual strains based on RAST-annotation.

| | EKP03 | EKP05 | EKP08 | EKP10 | EKP11 | EKP22 | EKP24 | NKP01 | NKP02 | NKP18 | NKP20 | NKP25 | NKP28 | NKP30 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Cell Wall and Capsule** | 217 | 230 | 218 | 230 | 234 | 214 | 220 | 216 | 196 | 214 | 217 | 217 | 193 | 227 |
| Capsular and extracellular polysacchrides | 38 | 54 | 42 | 54 | 57 | 38 | 41 | 38 | 42 | 36 | 37 | 37 | 39 | 49 |
| Gram-negative cell wall components | 91 | 90 | 91 | 90 | 90 | 38 | 90 | 89 | 66 | 90 | 88 | 92 | 69 | 92 |
| Cell wall and capsule—no subcategory | 88 | 86 | 85 | 86 | 87 | 85 | 89 | 89 | 88 | 87 | 88 | 88 | 85 | 86 |
| **Virulence, Disease, and Defense** | 148 | 150 | 141 | 155 | 156 | 154 | 136 | 146 | 143 | 154 | 144 | 168 | 134 | 129 |
| Adhesion | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 |
| Toxins and superantigens | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Bacteriocins, ribosomally synthesized antibacterial peptides | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 |
| Resistance to antibiotics and toxic compounds | 121 | 123 | 114 | 128 | 129 | 127 | 109 | 119 | 116 | 127 | 117 | 141 | 107 | 102 |
| Virulence, disease, and defense | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Detection | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Invasion and intracellular resistance | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 |
| **Phages, Prophages, Transposable elements, Plasmids** | 47 | 51 | 62 | 57 | 81 | 49 | 44 | 29 | 78 | 78 | 66 | 19 | 77 | 8 |
| Phage family-specific subsystems | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Transposable elements | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 7 | 0 | 0 | 0 | 0 | 5 | 0 |
| Phages, prophages | 47 | 50 | 57 | 57 | 80 | 48 | 43 | 20 | 77 | 77 | 66 | 19 | 70 | 8 |
| Phages, prophages, transposable elements, plasmids—no subcategory | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 2 | 1 | 1 | 0 | 0 | 1 | 0 |
| Pathogenicity islands | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Gene transfer agent | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Plasmid related functions | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Membrane Transport** | 231 | 267 | 214 | 246 | 289 | 346 | 238 | 293 | 247 | 310 | 270 | 325 | 279 | 234 |
| Protein secretion system, Type II | 19 | 19 | 19 | 19 | 19 | 19 | 19 | 19 | 19 | 19 | 19 | 19 | 19 | 19 |
| ABC transporters | 78 | 72 | 65 | 72 | 78 | 79 | 60 | 71 | 68 | 69 | 78 | 71 | 71 | 75 |
| Protein secretion system, Type VII (Chaperone/Usher pathway, CU) | 20 | 25 | 20 | 25 | 19 | 20 | 25 | 25 | 19 | 20 | 20 | 28 | 26 | 22 |
| Protein translocation across cytoplasmic membrane | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 |
| Protein secretion system, Type V | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 2 |
| Protein secretion system, Type I | 5 | 0 | 0 | 0 | 0 | 5 | 0 | 0 | 0 | 5 | 0 | 0 | 0 | 0 |
| Cation transporters | 24 | 24 | 22 | 24 | 24 | 24 | 24 | 24 | 23 | 24 | 24 | 25 | 22 | 22 |
| Protein secretion system, Type III | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Protein secretion system, Type VI | 19 | 17 | 16 | 17 | 14 | 19 | 15 | 14 | 0 | 14 | 15 | 14 | 13 | 22 |
| Protein secretion system, Type VIII (Extracellular nucleation/precipitation pathway, ENP) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Protein and nucleoprotein secretion system, Type IV | 21 | 68 | 28 | 46 | 92 | 137 | 49 | 97 | 73 | 114 | 67 | 125 | 87 | 29 |
| **Iron Acquisition and Metabolism** | 80 | 77 | 67 | 76 | 74 | 80 | 68 | 76 | 74 | 67 | 74 | 70 | 69 | 67 |
| Siderophores | 18 | 21 | 20 | 20 | 18 | 18 | 20 | 20 | 22 | 18 | 18 | 18 | 17 | 19 |
| Iron acquisition and metabolism—no subcategory | 62 | 56 | 47 | 56 | 56 | 62 | 48 | 56 | 52 | 49 | 56 | 52 | 52 | 48 |
| Iron transpot | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Motility and Chemotaxis** | 12 | 10 | 9 | 10 | 11 | 13 | 8 | 10 | 11 | 10 | 11 | 10 | 10 | 10 |
| Magnetotaxis | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Motility and chemotaxis—no subcategory | 12 | 10 | 9 | 10 | 11 | 13 | 8 | 10 | 11 | 10 | 11 | 10 | 10 | 10 |
| Flagellar motility in Prokaryota | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Social motility and nonflagellar swimming in bacteria | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Regulation and Cell Signaling** | 173 | 167 | 165 | 167 | 168 | 173 | 175 | 178 | 174 | 180 | 170 | 172 | 171 | 174 |
| Quorum sensing and biofilm formation | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 | 13 |
| Regulation of virulence | 9 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 |
| Programmed cell death and toxin-antitoxin systems | 16 | 18 | 15 | 17 | 17 | 18 | 23 | 23 | 17 | 19 | 20 | 18 | 19 | 24 |
| **DNA Metabolism** | 155 | 131 | 126 | 140 | 173 | 157 | 145 | 151 | 138 | 144 | 143 | 133 | 142 | 142 |
| CRISPs | 7 | 0 | 0 | 0 | 7 | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 7 |

**Supplementary Figure 1** summarizes the results for epidemic and non-epidemic strains.

We carried out a discriminant analysis for the measured phenotypic data. The analysis associated several phenotypic measurements with discriminant function. The discriminant function is a combination of linear effects of variables that give best separation of the data to distinct classes, in this study epidemic and non-epidemic strains. This approach revealed highly significant (posterior values did not overlap with zero) likelihood for a given trait to belong to either epidemic or non-epidemic group (resolved with Bayesian logistic regression between predicted values of discriminant function and epidemic status, Bayesian $R_2$ = 45%, **Figure 2**). Regressing predicted values of discriminant function against original variables indicated especially strong role of various measurements of drought tolerance in discriminant function (**Figure 3**) and non-epidemic strains with high discriminant function score were clearly more drought tolerant.

## Cross-Inhibition and Antibacterial Potential of a Putative Colicin

We further studied the cross-strain inhibition given that the epidemic successfulness could emerge from the ability of epidemic strains to suppress non-epidemic strains during spread between hospitals or hosts (**Figure 4**). The inhibition, when detected, was determined with a dilution series to be either due to molecular activity or prophage induction (diluted phages produce distinct plaques unlike inhibiting molecules). Pairwise inhibition was infrequent and no general pattern between epidemic and non-epidemic strains was identifiable.

As a curiosity, we selected one cross-inhibiting strain pair for more detailed analysis. NKP2 produces a component into its medium that inhibits EKP24 (ST11). Given the wide dispersal of ST11 *K. pneumoniae* and its association with NDM-1 encoding plasmids and hypervirulence (Gu et al., 2017), the inhibiting factor could provide a possible way to control these strains. However, the strain used in this study is not hypervirulent and therefore assessing direct applicability against hypervirulent strains was not conducted. Genomic comparison of NKP2 and EKP24 revealed the presence of genes for Colicin E3 in NKP2 that were absent from EKP24. Hence, Colicin E3 was hypothesized to be the inhibiting molecule. Co-culturing of these strains in the same medium demonstrates that EKP24 is unable to multiply. We further studied the adaptation of EKP24 to the continuous presence of the hypothesized Colicin E3 by serially culturing EKP24 in the presence of NKP2 medium extract for 4 weeks (n = 5). These cultures were refreshed three times a week. After 2 weeks, we removed the selection from five

**TABLE 3 |** Studied characteristics and their hypothesized association with epidemic capability.

| Trait/quality | Variables/factors used in this study | Relevance |
|---|---|---|
| Virulence genes | Number/type | Number of virulence genes may directly affect the strain's potential to cause infections |
| Antibiotic resistances | Number/type | Antibiotic resistance can compromise treatment, thus causing prolonged infections and increase the time during which bacteria disperse |
| Growth rate/density | Max growth rate (r), Max growth density (K) | Faster and more dense growth may increase the bacterial load in the surrounding environment and thus its epidemic potential |
| Growth temperature | 22°C (room temperature), 37°C | Growth differences in room temperature *vs* 37°C may reflect trade-offs in within- and outside-host environments and thus its adaptation to the environment vs the host |
| Growth in different media | LB, DMEM, BHI | Potential to grow in different nutrient environments may provide bacteria more opportunities to proliferate in alternative habitats and thus provide possibilities to survive outside the host |
| Growth in different nutrient levels | 1, 10, 100% L | Potential to grow in varying nutrient levels may provide advantage in different environments and hence affect its dispersal to new hosts |
| Growth in varying pH | pH 3, pH 4, pH 5, pH 6, pH 7 | Bacteria may be exposed to different pH in the environment and the host (phagocytosis, skin, gastrointestinal tract) and the sensitivity to pH may decrease the changes for dispersal |
| Potential to compensate surrounding pH | pH 3, pH 4, pH 5 | Potential to modify the surrounding microenvironment may play a crucial role in the bacterial chances to adapt to fluctuating environmental pH and hence affect its dispersal |
| Resilience in EtOH | 20, 50, 75, 90% EtOH | Survival in the presence of alcohol containing sanitizers may directly affect the persistence of the strain in the environment and hence influence its potential to get transmitted |
| Survival in the absence of water | 14 days, 2 months, 6 months | The potential to withstand drought can increase the timespan during which pathogen remains viable in hospital environment and hence affect its changes to get transmitted to new hosts |
| Recovery after drought | 14 days, 2 months, 6 months | Faster recovery after drought can provide bacteria increased potential to colonize or infect new hosts |
| Growth in mixed/ spatially structured population | 0 rpm, 230 rpm | Growth as a differently structured population may play a role in various stages of infection and persistence in the environment and may therefore affect the strain's potential to disperse |
| Number of plasmids | Number/Inc-type | Plasmids often carry genes that benefit the bacterium in specific conditions and hence their number may be related to survival in various conditions inside and outside the host |
| Genomic prophages | Number/Inc-type | Activation of prophages may cause infections in competing *K. pneumoniae* strains and may therefore provide the prophage carrying strain an advantage in situations where several strains occupy the same environment |
| Strain-specific inhibition | Pairwise inhibition | Production of bacteriocins or other antimicrobials may inhibit the growth of competing strains and thus may hinder the potential for sensitive strains to disperse into environments or hosts with other *K. pneumoniae* strains |
| Short ORFs in the genome | Number of ORFs | Number of short open reading frames in the genome may reflect bacterial adaptive history and hence may be linked to epidemic potential |

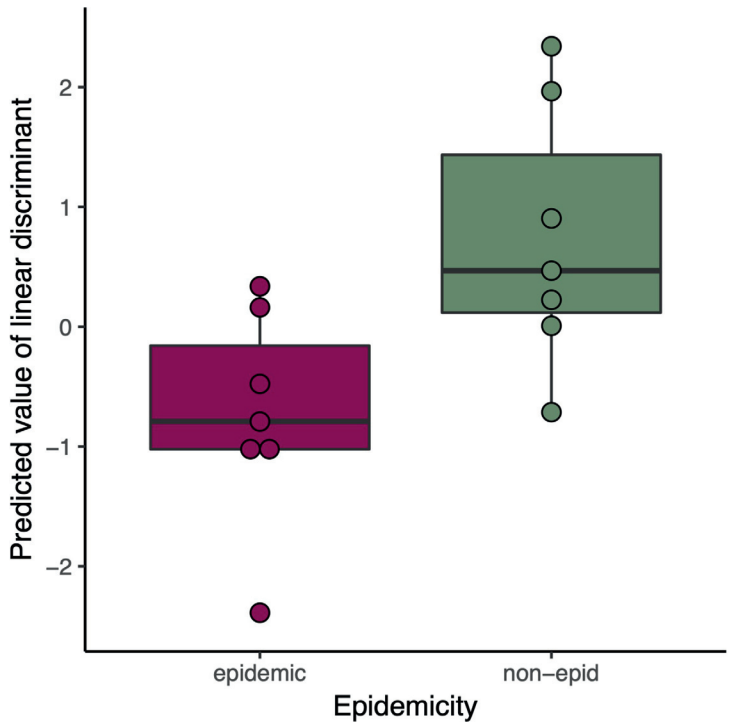


**FIGURE 2** | Predicted Linear discriminant values in epidemic (red) and non-epidemic (green) *K. pneumoniae* strains.

additional replicates. Resistance to NKP2 medium (with hypothesized Colicin E3) emerged already during the first culture transfer, and it remained stable even after the removal of selection. Re-sequencing of three putative Colicin E3 resistant samples revealed a prevalent mutation in Aerobactin siderophore receptor IutA. This mutation was absent in a culture that was not exposed to NKP2 extract. IutA has been shown to serve as a receptor for cloacin DF13 (Van Tiel-Menkveld et al., 1982), which is homologous to Colicin E6 and E3 (Akutsu et al., 1989). As such, we hypothesize that NKP2 extract rapidly selected for IutA mutants. Also, it is worth noting that NKP2 medium was not observed to inhibit the growth of the other ST11 strain EKP8, hence showing narrow activity. Altogether, the hypothesized Colicin E3 does not appear to provide efficient antimicrobial activity against drug-resistant *K. pneumoniae* strains even when the targeted strain is initially sensitive to the colicin.

## DISCUSSION

*K. pneumoniae* has become one of the priority drug-resistant pathogens in hospital settings worldwide. Some *K. pneumoniae* STs are more prevalent compared to others, which provokes the question whether there are qualities in these genetically related groups that have made them more potent for dispersal. Here, we studied multiple phenotypic and genotypic characteristics of seven epidemic and non-epidemic carbapenem resistant *K. pneumoniae* isolates that emerged from different parts of the world. None of the specific genetic qualities associated uniformly with epidemic or non-epidemic strains. Overall, this again suggests that sequence typing is not an optimal approach for inferring qualities of individual pathogens. In other words, there are *K. pneumoniae* strains that are relatively different from one another for their specific characteristics while still grouping together in ST-analyses. Phenotypic characterization revealed indication that, while genetic differences were minute based on ST-analyses, phenotypic differences that separate epidemic and non-epidemic *K. pneumoniae* strains do exist. Such apparently controversial result could emerge if phenotypes are strongly dictated by genetic differences other than indicated by core genome-based ST-analyses or even by epigenetic modifications (Casadesus and Low, 2006).

While most of the studied factors could not explicitly help explain epidemic qualities, the strong association of drought tolerance with epidemically non-successful strains could give some insights on the dissemination. All the isolates in this study were able to withstand 6 months of dryness. Interestingly, non-epidemic strains generally recovered faster and into higher density after the drought, which presents the possibility that non-epidemic strains may have qualities that provide them opportunities to cause infections in specific cases, for example, after long-term residence on surfaces (Kramer et al., 2006). Bacteria have multiple ways to protect itself during the drought. For example, effective biofilm formation is a major protection mechanism in bacterial

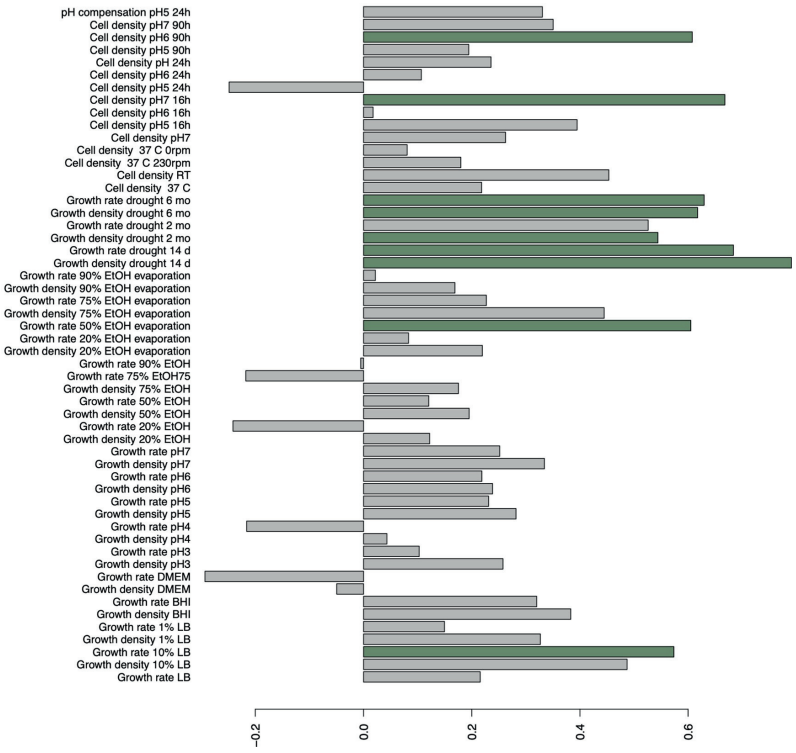


**FIGURE 3** | Correlations of original variables on discriminant function explaining the best linear combinations of variables in explaining epidemic status of strains. Bars highlighted with green indicate significant ($p < 0.05$) correlations.

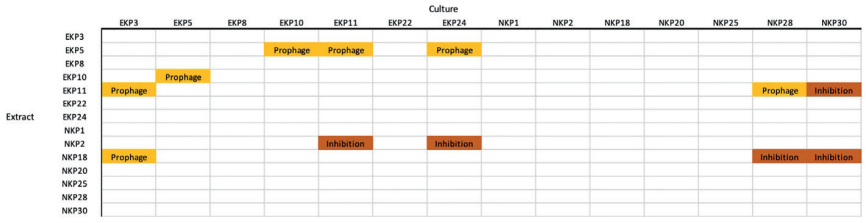


**FIGURE 4** | Cross-strain inhibition. "Prophage" indicates that the strain used for generating the extract produces a phage into the medium. "Inhibition" is a non-viral extract that inhibited the growth of the culture strain.

species incapable of endosporulation (Sunde et al., 2009; Reza et al., 2019) or cyst formation (Malinich and Bauer, 2018). Nosocomial carbapenem resistant *K. pneumoniae* infections are mainly acquired by two routes, either in person-to-person contact (Casewell and Phillips, 1977) or *via* contaminated surfaces and instrumentation (Jarvis et al., 1985; Revdiwala et al., 2012). Also, a gut carriage of carbapenem resistant *Klebsiella* have been associated with an elevated infection risk (Tischendorf et al., 2016; Gorrie et al., 2017). It is possible that drought tolerant strains are more likely to remain viable on inanimate surfaces, from which they are only rarely transferred to the patient. On the other hand, the epidemically successful strains could be more adapted to human host and tolerated as the colonizing pathogens. These might be two different strategies for the same species to survive in the shared environment and results of evolutionary trade-offs between inside host and outside host conditions (*e.g.* Mikonranta et al., 2015; Ashrafi et al., 2018). However, the reasons why certain STs seem to develop higher tolerance towards dryness remains unclear.

*K. pneumoniae* has also noted to be able to grow in acidic pH and even capable to compensate acidic environment of phagosomes (Cano et al., 2015). All the strains in this study

tolerated decreasing pH up to 4. In pH 3 no viable dividing cells were detected and for that reason no pH compensation was observed either. In the viable cultures with initial pH of 4, cells were able to increase the pH in the surrounding media by 1.04–1.62 in 24 h. Strain EKP10 was an exception as it was barely able to grow in pH 4 and could not compensate the low pH. Altogether, long-term exposure to environments that have pH of 3 or below serves as a potential barrier for *K. pneumoniae* viability. Increasing tolerance towards the alcohol-based hand rubs and sanitizers have been observed in multiple species of XDR bacteria. It has been suggested as one possible explanation to the increasing emergence of these pathogens in hospital environments (Pidot et al., 2018). The *K. pneumoniae* isolates utilized in this study, however, were not able to withstand ethanol at concentrations 50% (v/v) or above.

Overall, different *K. pneumoniae* strains vary in their phenotypic characteristics and hence may cause infections *via* differing routes. This highlights the importance of exerting precautionary steps in healthcare. Namely, these include early recognition of both carriers and infected patients (Borer et al., 2012; Gorrie et al., 2017; Liu et al., 2019), and controlling the spread of pathogens with apparently still effective hand hygiene and disinfection of inanimate surfaces (Kramer et al., 2006; Borer et al., 2012; Gorrie et al., 2017). Multiple studies also focus on novel approaches to prevent infections by coating endotracheal tubes and catheters with new antibacterial agents (Caratto et al., 2017; Bjorling et al., 2018; Seitz et al., 2019). Such multifrontal measures to control *K. pneumoniae* is likely to be necessary for tempering both epidemic and non-epidemic strains.

In conclusion, we were able to detect one specific quality that associated with epidemic status of *K. pneumoniae* isolates. Drought tolerance and recovery from dryness of up to 6 months significantly associated with the non-epidemic strains. Thus, we need to consider the possibility that non-epidemic strains may have unique advantages in specific conditions that let them occasionally show up as local epidemics in surveillance studies. Additionally, some non-epidemic isolates inhibited the growth of epidemic strains. This may allow these strains to cause infections instead of their epidemic contemporaries. However, it is possible although unlikely that pure coincidence may be behind the epidemic success of certain *K. pneumoniae* STs. The initial establishment, instead of any specific characteristic, may have provided certain strains chances to acquire new virulence factors and resistance elements, hence furthering their spread.

## DATA AVAILABILITY STATEMENT

All data is available either as **Supplementary Material** or in GenBank.

## AUTHOR CONTRIBUTIONS

KK and MJ conceived and planned the experiments. KK, RP, and MJ carried out the experiments. KK assembled and curated the results. MJ prepared the script for finding ORFs. TK conducted statistical analyses. A-MÖ-G and CG collected the bacterial strains. KK, MJ, RP, and A-MÖ-G conducted genetic analyses. All authors contributed to the interpretation of the results. First draft of the manuscript was written by KK and MJ and other authors provided critical feedback and helped shape the research, analysis, and the final manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.599924/full#supplementary-material

**Supplementary Figure 1** | Summary of all measurements.

**Supplementary File 1** | Phyton script for short ORFs.

**Supplementary File 2** | Original measurement data.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

# IV

## CHARACTERIZATION OF *ACINETOBACTER BAUMANNII* PHAGES AND THE SHIFTING HOST-PHAGE DYNAMICS

by

Koskinen K., Ylänne M., Penttinen R., Jalasvuori M. & Ketola T. 2021

Manuscript.

# Characterization of *Acinetobacter baumannii* phages and the shifting host-phage dynamics

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**Abstract**

Due the incremental antibiotic resistance, new options for the infection treatment are urgently needed. For a long, phage therapy has been a candidate as the next approach to overcome antibiotic resistant infections. In this study we characterize three novel *Acinetobacter baumannii* phages that successfully infect clinical samples of *A. baumannii*, a top priority multi-drug resistant pathogen. These three novel phages were found to relate other previously characterized phages with shown therapeutic potential, making our phages a valuable addition to therapy approaches. Furthermore, the infection tests revealed phage-phage interactions through the susceptibility shifting host. This phenomenon reveals important information about interactions during the virus co-infection potentially enhancing the successfulness of pre-designed phage cocktails.

**Introduction**

In the last decade multi-drug resistant *Acinetobacter baumannii*, a gram-negative, almost spherical, and rod-shaped pathogen, has become one of the most concerning threats in healthcare. Fast accumulation of resistance genes towards last line antibiotics has changed *A. baumannii*'s status from a low-risk opportunistic pathogen to the priority number one in the list of bacteria towards which new antibiotics and treatment options are urgently needed (World Health Organization 2014; World Health Organization, 2017). Though originating from the soil habitat (Baumann, 1968), *A. baumannii* quickly took its advantages and conquered hospital settings, now mostly causing hospital-acquired infections (HAIs) instead of community acquired infections. One of the most notable

reasons behind the enhanced spread capability in hospitals is *A. baumannii*'s exceptional competence to hoard antibiotic resistance genes (Lee et al., 2017). Especially notable is their ability to resist third generation beta-lactams, such as carbapenems, and other last line antibiotics like colistin (Deveson et al., 2018). This resistance gene accumulation combined with the ability to sustain in harsh conditions, which hospital environments offer, has made *A. baumannii* a genuine nuisance in the healthcare system (Lin and Lan, 2014).

Since the discovery of penicillin (Fleming, 1929) bacteriophages, or simply phages, were mostly abandoned as a treatment option for bacterial infections. However, the rising resistance levels against existing antibiotic drugs have steered the attention back towards the alternative solutions, including phages and phage endolysins (World Health Organization, 2019; Pirnay et al., 2015). Though the phages may first seem as an easy and effective solution with long research history, there is lot of uncertainties related to host-phage interactions. Phages are abundant in the presence of suitable host bacteria. For phage therapy, the naturally occurring phages can be found and isolated from the community waste water (Mattila et al., 2015). Isolated phages have found to also tolerate laboratory settings and preserve their vitality well during the storage (Mattila et al., 2015). However, there are drawbacks in phage therapy and one of the most concerning feature with this therapy option is the rapid resistance emergence against the phages (McCallin et al., 2019). Phage therapy has been suggested as both a self-reliant treatment option and also in a combination with other therapeutic agents, such as antibiotics (Jansen et al., 2018; McCallin et al., 2019). Phages as a treatment option allows construction of personalized treatment (Regeimbal et al., 2016), which in other hand is often also inevitable due the narrow host spectrum of the phages. Despite a lot of attention has been paid to different personalized administration of phages or designed phage cocktails, consensus on the optimal administration model has not yet been established (McCallin et al., 2019). It seems that the large variation in phage-host interactions are notable in the effectiveness of administration models, thus limiting the predictability of cocktails pre-designed based on the single phage-host effectivity. In this study we demonstrate three different options to administrate phages, examine the effects of phage exposures, and measure the growth and recovery of the hosts after exposure. In a single phage exposure model each strain was exposed to a one phage, while in other exposures a single bacterial population was exposed to all infectious phages either one at a time (serial exposure) or all at once (phage cocktail). In our experimental settings four of the previously isolated phages (1P1, 2P1, 6P1 and 6P2) (Mattila et al., 2015) and four multidrug resistant *A. baumannii* strains AB2, AB3, AB5, and AB6 were further studied.

Hence the complex dynamics, phage therapy treatment has significant benefits but also indisputable drawbacks and challenges. One remarkable challenge is the spontaneous reactions as the system is moved from *in vitro* to *in vivo*. The existence of companioning microbial community members and

environmental pressures can affect to the successfulness of the treatment regardless how effective it proved in the simplified *in vitro* studies (Carins et al., 2018; Bull et al., 2019). Dynamics between the microbial community members can alter the predicted response to the antibiotic treatment as well (Cairns et al., 2018). Even when stripped down to minimum, single phage-host interactions can have remarkable and unpredicted effect on total community response. Instead of full microbial community assays this study concentrates on phages and their host bacteria. However, as our results implicate, even addition of multiple phages into one community can result indirect interactions among phages. In order to figure out the therapeutic potential of three novel phages, the infection tests were conducted with clinical *A. baumannii* strains and phages were characterized morphologically and genetically. The phages 1P1 and 6P1 plus 6P2 showed a reversible host susceptibility shift. This phenomenon indicates that in microbial communities, also phages can interact between each other in an indirect cyclic manner that enables susceptible host reservoir regardless the short-term resistance appearance.

**Results**

Previously isolated phages 1P1, 1P2, 6P1, and 6P2 were characterized by their phenotypes (Figure 1) and genotypes suggesting them to belong into families of *myoviridae* and *podoviridae* or *autographiviridae*. 1P2 was found to be close to identical to a phage 1P1 thus potentially being one phage isolated twice. Capsid sizes of 1P1, and 6P2 was estimated to be ~100 μm and with 6P1 ~60 nm. Phages 1P1 and 6P2 were found to possess long contractile tail whereas 6P1 had short tail. Further RAST annotations and blasts indicated that all of them are novel phages having resemblance to the viruses with known therapeutic potential. The RAST Psi-Blast suggested capsid and tail protein coding genes of 1P1 and 6P2 (isolated by Mattila et al. 2015) gave high query cover (91-100%) and identity percent (75-99%) hits to phages existing in the database. However, no identical matches were found, thus indicating these viruses are not previously characterized and introduced in literature. Both phages 1P1 and 6P2 associated with *Acinetobacter pittii* phage vB_ApiM_fHyAci03 (GenBank id: MH460829.1) and *A. baumannii* phages KARL-1 (GenBank id: MH713599.1), ZZ1 (GenBank id: HQ698922.4), and AbTZA1 (GenBank id: MK278860.1) which all have shown high therapeutic potential (Pulkkinen et al., 2019; Jansen et al., 2018; Jin et al., 2012; Nir-Paz et al., 2019). Phage 6P1 did not offer any prominent hits with RAST database, thus not offering any consequential genes for further analysis.

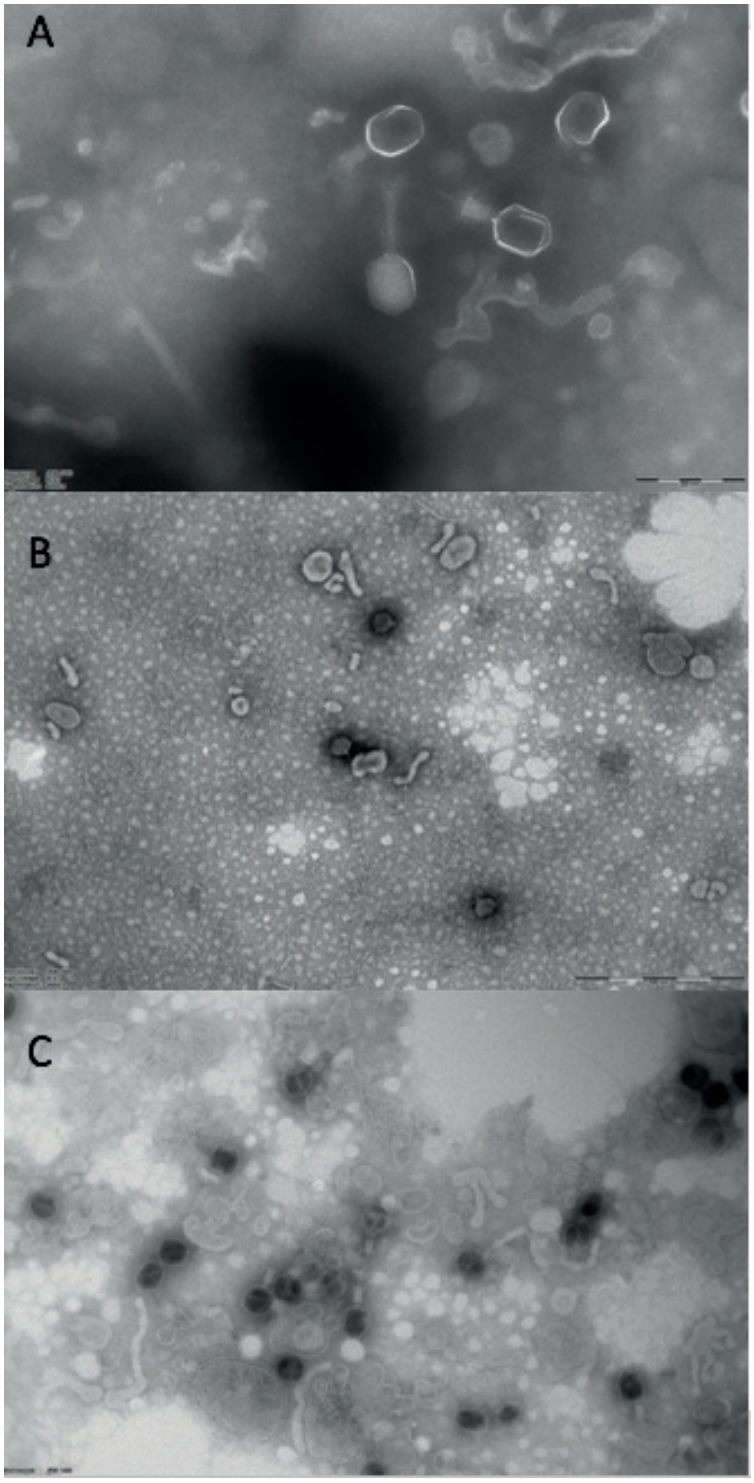


Figure 1. Morphological characterization of phages with transmission electron microscopy. Phages 1P1 (**A)** and 6P2 (**C**) was found to morphologically resemble *myoviridae* while the phage 6P1 (**B**) had structural similarities with *podoviridae* or *autographiviridae*. Capsid sizes of 1P1 and 6P2 were estimated to be ~100 μm with equally long tail. With phage 6P1 capsid size was estimated to be ~60 nm and tail to be short.

While measuring the fitness costs of the three different kind of exposure models, single phage, serial or phage cocktail, none of the administration models clearly stood out as a preeminent option for any four strains studied (Figure 2). Growth density of four bacterial

strains (AB2, 3, 5, and 6) in rich (100% LB) medium experiencing different phage treatments was sensitive to identity of bacterial strain ($F_{3,48}$=45.372, p<0.001), treatment ($F_{5,48}$=25.411, p<0.001) and their interaction ($F_{15,48}$=22.820, p<0.001). In poor (10% LB) medium the effects were bit less pronounced but alike, as growth density was sensitive to identity of bacterial strain ($F_{3,48}$=19.924, p<0.001) treatment ($F_{5,48}$=4.827, p=0.0012) and their interaction ($F_{15,48}$=3.492 <0.001). These results indicate that most effective phage treatment is sensitive to bacterial strain identity (Figure 2), pinpointing the importance of design of treatments. Effectiveness of treatments can range from the single most effective phage as in AB2, to serial administration as in AB3 or cocktail as in AB6 to a lack of effective treatment against AB5.

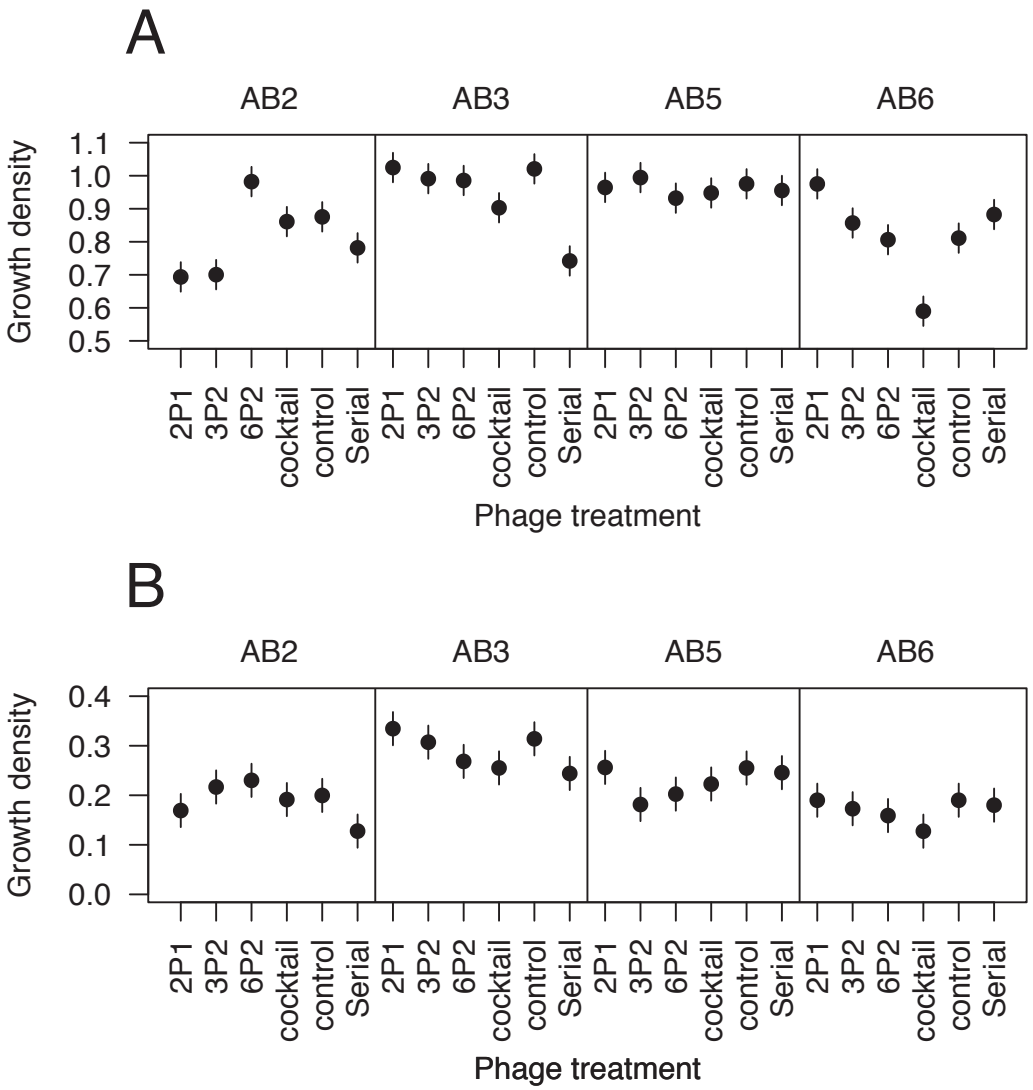


Figure 2. Effects of single phage, serial or phage cocktail exposure models on growth density measured as biomass yield of different *Acinobacter baumannii* -strains (AB2, 3, 5, 6), in rich (panel A) and low (panel B) nutrient medium.

By taking a further look to the host-parasite dynamics we found an intriguing phenomenon in which the pressure of phage 6P1 or 6P2 alters the host, which in turn become susceptible for phages 1P1 and 1P2, not previously capable of infecting the host (Figure 3). This was shown in simple plating assay. Figure 3 shows the wild type *A.baumannii* being infected by phage 6P1 but not by the phages 1P1 or 1P2. However, after 6P1 infection the same *A. baumannii* strain become resistant to 6P1 but susceptible to phages 1P1 and 1P2 (Figure 3). Later exposure to 1P1 or 1P2 was found to restore the original infection pattern leading the host resistant to 1P1 and 1P2, yet again susceptible to 6P1. When we conducted a 24-days microcosm experiment consisting of the wild type host AB6 and phages 1P1, 6P1 and 6P2, we found out that in six out of eight communities bacteria were able to develop mutants resistant to both type of phages but those mutants did not take over the whole community. The shifting host was preserved in all of the communities and neither type of the phages vanished during the experiment (Supplementary material1).

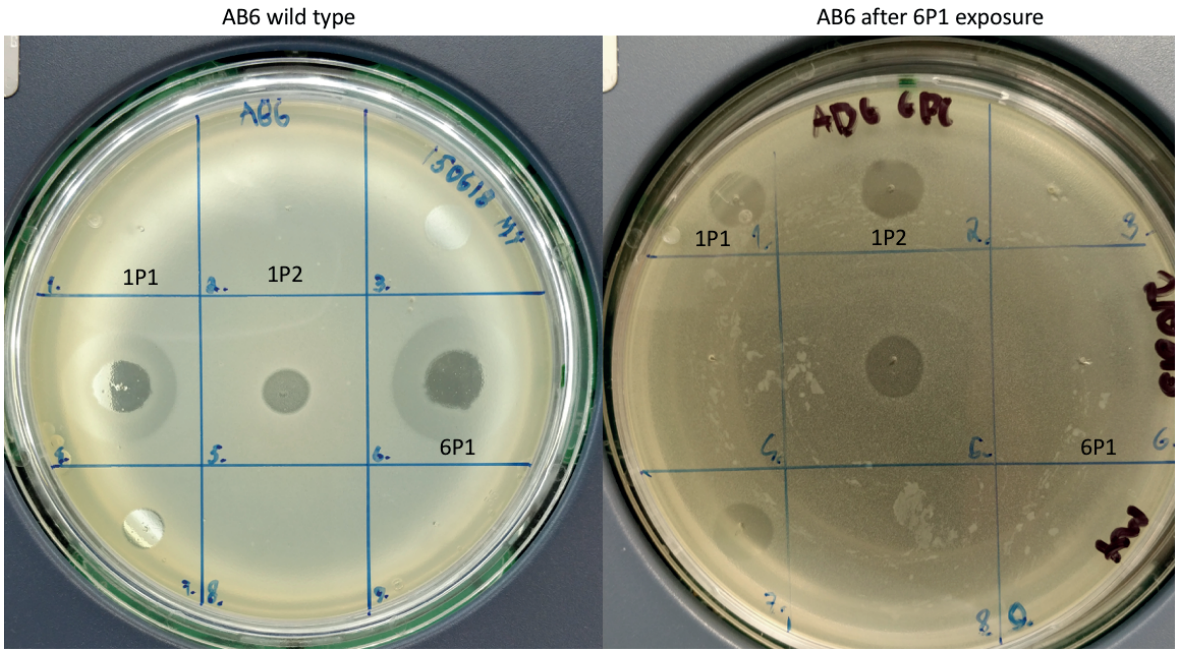


Figure 3. The shifting host susceptibility of host AB6 in phage droplet test. On the left AB6 wild type shows susceptibility to phage 6P1 but not to phage 1P1. On the right 6P1 exposed AB6 host shows the resistance against 6P1 but susceptibility to a phage 1P1. The emerging host susceptibility shift was found to be reversible thus leading to the cyclicity of infection pattern with these two group of phages. Similar effect was found as the 6P1 was substituted with 6P2 (not shown in figure). In further characterization phages 1P1 and 1P2 were found to be close to identical, indicating that the same phage had enriched twice from the same source.

Interestingly we also found surprising lack of phages in our repeated attempts to enrich more phages for *A. baumannii* strains used in previous isolations (Mattila et al., 2015). Even though earlier *A. baumannii* phage isolation attempts from the same Nenäinniemi waste water treatment plant yielded success rate of 38.9 % (Mattila et al., 2015) new isolation attempts did not enrich any phages (Table 1). It is noteworthy, that within the same isolation attempts phages for several other bacteria were

found, and hence this was not a methodological failure. Neither the phages previously enriched from the same source were observed indicating fluctuations of phage content in the waste water.

Table 1. Phage isolation attempts for clinical *A. baumannii* strains from Nenainniemi waste water treatment plant during five-year study period. Earlier isolation attempts yielded success rate of 38.9 % for *A. baumannii* samples, contrary to latest attempts which yielded no phages. The lack of phages indicates phage fluctuations in waste water treatment plant, thus challenging the on-demand isolation procedure.

| Isolate | Isolation date (month/year) | Source | Isolation hosts | Found phages | Phages for other bacteria |
|---|---|---|---|---|---|
| 1 | 9/13 | Nenäinniemi WWTP | AB1 | 2 | Yes |
| 3 | 12/13 | Nenäinniemi WWTP | AB3 | 1 | Yes |
| 4 | 12/13 | Nenäinniemi WWTP | AB6 | 1 | Yes |
| 5 | 6/18 | Nenäinniemi WWTP | AB2, AB3, AB5, AB6 | 0 | Yes |
| 6 | 6/18 | Nenäinniemi WWTP | AB2, AB3, AB5, AB6 | 0 | Yes |
| 7 | 6/18 | Nenäinniemi WWTP | AB2, AB3, AB5, AB6 | 0 | Yes |
| 8 | 7/18 | Nenäinniemi WWTP | AB2, AB3, AB5, AB6 | 0 | Yes |
| 9 | 7/18 | Nenäinniemi WWTP | AB2, AB3, AB5, AB6 | 0 | Yes |
| 10 | 7/18 | Nenäinniemi WWTP | AB2, AB3, AB5, AB6 | 0 | Yes |
| 11 | 7/18 | Nenäinniemi WWTP | AB2, AB3, AB5, AB6 | 0 | Yes |
| 12 | 7/18 | Nenäinniemi WWTP | AB2, AB3, AB5, AB6 | 0 | Yes |
| 13 | 7/18 | Nenäinniemi WWTP | AB2, AB3, AB5, AB6 | 0 | Yes |

A closer examination of the host strain AB6 itself gave multiple antibiotic resistance genes in ResFinder (Zankari et al., 2012) showing high identity (99.83 – 100 %) hits to sulphonamide resistance gene sul1, multiple beta-lactamase genes such as $_{bla}$OXA-66, $_{bla}$OXA-24 $_{bla}$, and $_{bla}$ADC-25, tetracyclin resistance gene tet(B), and aminoglycoside resistance genes aph(3')-Via, aph(6)-Id, and aph(3")-Ib. RAST annotations of AB6 genome gave total subsystem coverage of 31 % of the genome showing multiple genes related to virulence, defense and disease causing, especially those related to the increased tolerance towards multiple toxic compounds.

**Discussion**

While the traditional treatment options for multi-drug resistant bacteria like *A. baumannii* are limited, other approaches like phage therapy are suggested as the future candidate for infection treatment. We characterized three potential phages by their genotypic properties and morphology and estimated their therapy potential as well. Both phenotypic characterizations (Figure 1) and genetic analyses

support the idea of phages 1P1 and 6P2 belonging to the *myoviridae* and the phage 6P1 presenting either *podoviridae* or *autographiviridae*. Two of the studied phages highly associated with known phages vB_ApiM_fHyAci03, KARL-1, ZZ1, and AbTZA1, all successfully used in phage treatment either alone (Jin et al., 2012; Nir-Paz et al., 2019; Pulkkinen et al., 2019) or in combination a with antibiotics (Jansen et al., 2018). Hereby, our phages seem as promising candidates, yet not conducted with patients. With further studies estimating the optimal administration models for these phages, among the ones that were not characterized but found to efficiently infect the bacteria, none of the tested administration models (single, serial, or phage cocktail) seemed to be preeminent (Figure 2). However, the association with phages previously successfully used in combination treatment consisting of phages and antibiotic drugs could offer a possible approach in enhanced utilization of our phages as well (Jansen et al., 2018). Since 6P1 did not offer any prominent hits with RAST database it might be a novel phage and thus a valuable addition to *A. baumannii* phage collections. However, there are methods and databases that could help to classify these phages in more detail, such as Hidden Markov Model utilizing tools (Chibani et al., 2019).

Interestingly, we also detected a susceptibility shift of the *A. baumannii* host. This shift allowed the co-existence and replication of two group of phages when co-existent in the community. The naturally occurring wild type state of the host AB6 was sensitive to phages 6P1 and 6P2 but not to the phage 1P1. However, after the exposure of either phage 6P1 or 6P2 the host AB6 developed resistance against phages used in exposure. Simultaneously with emerging resistance host developed susceptibility to a phage 1P1 (Figure 3). The phenomenon was observed to be reversible, thus allowing the host to cyclically shift between these two phage pressures. Shift was found to lead to the community composition in which host provided a seasonal reservoir to each group of phages to replicate. These indirect phage-phage interactions are seldomly referred in literature (Regeimbal et al., 2016). However, as similar feature is previously detected among *A. baumannii* phages (Regeimbal et al., 2016), there are indications of shifting host phenomena not being uncommon with *A. baumannii*. Regeimbal and colleagues (2016) were able to link the shift with capsule regulation. However, analogous observations of the capsule loss and re-emergence was not found in our study though investigated.

As the community dynamics of shifting *A. baumannii* host AB6, phages 6P1, 6P2 and 1P1were further investigated in 24-day evolutionary experiment setup, all the original community members were observed to persist throughout the experiment (Supplementary material 1). During the community experiments most of the communities were able to develop mutants resistant to both group of phages. Interestingly and in some level against our predictions these mutants did not conquer the community, thus indicating that shifting the phenotype between the two phage pressures is not insuperable effort to the bacterial cell itself. In a phage therapy setup, comparable indirect

phage-phage interactions could lead to the unexpected therapy outcomes rising form the interactions either between phages used in the cocktail or with the phages residing on the infection site.

Since the discovery of novel *A. baumannii* infecting phages with high therapeutic potential can be considered challenging, it is important to grasp the opportunities that rise from the sifting infectivity. In our study the exact reason behind the shifting remains unsure. Detailed analysis of the phage genome could bring some insight of the possible regions responsible of the host shift. Further study of these phage genomes can reveal segments that are responsible for shifting the host susceptibility between the phages. Since the limited options for suitable phages against *A. baumannii* infections it is important to gather the knowledge of existing candidates. When considering the phage therapy, bacterial pathogens are routinely tested with single phages. However, our findings indicate the impact of previous phages might alter the susceptibility of the host cell. This causes a lot of uncertainty, especially when multiple phages are used at once, but also gives a potential to enhance the effectivity of the treatment. Since it is sure that at least in some cases previous phage infections alter the ways host interact with later arriving phages. Changing susceptibility could be further utilized to alter the host cell to become more vulnerable to different phages infecting in a sequential fashion. This approach emphasizes the potential of these susceptibility shifting phages as a basis for the new type of phage treatment.

It is known that *A. baumannii* can habit the human skin without causing major health problems and infrequent appearance may indicate a minor presence of *A. baumannii* within the community (La Scola and Raoult, 2004). Unexpectedly, *A. baumannii* strains previously successfully used in phage isolation did not yield any phages from the same source (Table 1). This indicates the fluctuations in community waste water, which in turn reflects the microbiome of surrounding community. Previous isolation attempts in 2013-2014 achieved 38.9 % success rate with the output of 9 enriched phages. Nine new samples, collected in 2018, from the same waste water treatment plant resource produced any phages (Table 1). Local reports of infectious diseases did not refer to known cases of *A. baumannii* infections in Central Finland, thus leaving source for the earlier phage findings open (Terveyden ja hyvinvoinnin laitos, 2019). However, overall amount of *A. baumannii* infections in Finland have decreased since 2014 (Terveyden ja hyvinvoinnin laitos, 2019). Since the isolation of new phages was unsuccessful regardless that the source was known to have suitable phages previously, it seems even more urgent to focus on the potential and dynamics of currently existing phages. The major problems hindering the phage therapy are limited host spectrum of existing phages and the fast resistance emergence against used phages. The tested three administration models, single, serial or cocktail, showed hardly any differences in laboratory settings and usually when upscaled to therapeutic scale these divergencies tend to narrow still or to differ from the predictions (Bull et al., 2019).

## Conclusions

As the acute crisis, caused by multi-drug resistant *A. baumannii* strains circulating in healthcare, is propelling, every therapy suitable phage increases the potential of creating effective phage therapy treatment options. In this study we characterized morphologically and genetically three novel *A. baumannii* infecting phages with high therapeutic potential. These phages presented the families of *myoviridae* and *podoviridae* or *autographiviridae* and shared similarities with already characterized phages with shown therapeutic potential. Both morphological and genetic characterization of new phages are valuable information while collecting phages for the possible phage therapy collections. Another fascinating feature observed was the phage-phage interaction through the host cell during the co-infecting cocktail assembly. Further analysis of phages themselves might give an insight of genetic background that enables this extraordinary behavior. These interactions can alter the functions of phages and their target host cell and further modify the outcome of phage therapy. Even though the phage therapy most likely will not become as straight forward treatment option as antibiotics, the more we know about functions of the phages, the more effective treatment outcomes it provides.

## Materials and methods

All *A. baumannii* strains, AB2, AB3, AB5, and AB6, were clinical isolates from Turku University Hospital. Bacteria were cultured in Luria-Berthani medium, + 37 °C and 210 rpm over-night if other not mentioned. DNA extraction of strain was performed with DNeasy Blood & Tissue kit (Qiagen) with starting volume of 2 ml of over-nigh culture. AB6 genome was sequenced with PacBio sequencing. Resistance genes of strain AB6 was identified by Center of Genomic Epidemiology ResFinder tool (www.cge.cbs.dtu.dk/services/ResFinder/; Zankari et al., 2012). AB6 genome was also annotated by Rapid Annotation using Subsystem Technology (RAST, www.rast.nmpdr.org).

Phages used in the experiments were isolated from community waste water by Mattila et al. (2015) and revived from the original phage stocks by co-plating with susceptible bacterial host strains proceeded with the plaque isolation technique described by Mattila et al. (2015). New isolation attempts followed the same procedure used by Mattila et al. 2015. Shortly, unprocessed wastewater was filtrated through 0.2μm filter to exclude bacterial cells and solid matter. Phage enrichment culture was prepared by mixing equal volume of filtrated wastewater and LB media and 1:400 stationary phase bacterial growth and let to incubate + 37 °C and 210 rpm over-night. After incubation bacterial cells were centrifuged down with 6000 g and supernatant was plated together with host bacteria and 0.7 % soft-agar on LB agar plate which was left in + 37 °C over-night. Single plaque was picked from the plate and vortexed with LB, then plated together with the same host used in enrichment and incubated in + 37 °C over-night. Plaque picking step was repeated twice to ensure

the purity of single phage. Phage stocks were prepared by scraping the soft-agar layer of semiconfluent plates incubated with 10 ml LB in + 37 °C and 210 rpm for 4 hours, then filtrated through 0.2μm and stored in + 4 °C. Possible host-related prophage cross-infection was tested to avoid false-positive results. New phage isolation and enrichment attempts were from the same Nenäinniemi wastewater treatment plant (Jyväskylä, Finland) as previous phages were isolated. It is noteworthy that the same waste water samples were also cultivated with *Klebsiella pneumoniae* strains which yielded new phages for *K. pneumoniae*.

Phage morphology was characterized by transmission electron microscopy (TEM). Phage semi-agar stocks were centrifuged with 25,000 g for 2 hours in + 4 °C. Phage pellet was suspended in 0.1M ammonium acetate (pH 7) and two consecutive centrifugations were performed with 25,000 g for 2 hours in + 4 °C. Pellet was suspended in 0.02M potassium phosphate (pH 7.5). Samples were allowed to dry 30 seconds to 2 minutes on 200-mesh Formvar carbon-plated grid and stained with 1 % phosphotungstic acid (PTA) for 30 seconds and observed with JEOL JEM-1400 microscope. Genotypic characterization of phages was performed by isolating the viral DNA with Norgen Phage DNA Isolation Kit, library was prepared using Nextera Flex kit and sequenced with Illumina MiSeq PE300. Genomes were assembled using CLC Genomics Workbench 12 software (Qiagen). Quality of reads was checked prior to trimming and host sequence removal. Assembled viral genomes annotated with Rapid Annotation Subsystem Technology (RAST, https://rast.nmpdr.org) and NCBI Microbial Nucleotide Basic Local Alignment Tool (BLAST) with discontiguous megablast and megablast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). NCBI Microbial nucleotide BLAST was also used to find similarities with existing phages in the database.

Community dynamics of AB6 and phages 1P1, 6P1 and 6P2 were explored in 24-day evolution experiment. Initial cultures were produced in eight replicas by adding in LB media 1:100 overnight AB6 culture and in total 1MOI of 1P1, and 1P2, and 1MOI of 6P1, and 6P2. Four of the community cultures were incubated in + 37 °C and still and the other four in + 37 °C and 120 rpm. All cultures were refreshed by substituting 2 mL of culture by fresh LB media. Samples from each community was taken in every four days and community's susceptibility toward each phage was determined via droplet test by plating heterogenic community with 0.7 % soft agar-LB on 1 % agar-LB plate and pipetting droplets of 1P1, 6P1, and 6P2 semi-agar stock over the soft agar layer. After culturing over-night in + 37 °C traces of infective efficacy of each phage was determined visually. Samples originating from each heterogenic community were taken and both amount of 1P1 or 6P1/6P2 susceptible bacterial cells and total amount of cells were counted by plating the either plain community culture or 1P1 or 6P1/6P2 treated culture on 1 % agar-LB plate and then incubated in + 37 °C over-night. From the same samples the amount of either phage 1P1 or both phages 6P1 and 6P2 together were measured by plating the 0.2 μm filtered community culture with either 1P1 or 6P1

and 6P2 susceptible host mutant and 0.7 % soft agar-LB on 1 % agar-LB plate. Plates were cultured over-night in + 37 °C and phage plaques were counted.

Administration study of phages was performed in three setups. Phage exposures were performed in two-step method to avoid excess phage accumulation in serial exposures. In single phage exposures all the bacterial strains were initially cultured in + 37 °C, 210 rpm over-night and then transferred into first step exposure by refreshing the culture in 1:5 in 100 % LB and 1:200 of each phage separately. Second step of exposure was performed by refreshing first step culture in 1:100 ratio on 100 % LB with 1:100 of the phage used in the first step and after incubating in + 37 °C, 210 rpm over-night culture was plated on 1 % agar plates and mutants were isolated and their resistance patterns towards phages were determined via droplet test described earlier. In serial exposures mutants from single phage exposures were exposed to the rest of the phages in two step sequential fashion one by one. In phage cocktail exposures all the phages were added in first step exposure culture in 1:200 ratio per phage and in 1:100 ratio on second step. Fitness costs caused by the phage encounters and emerged resistances towards phages were measured with spectrometric assays. Optical density of both untreated host and phage treated mutants were grown + 37 °C and 120 rpm over-night and then diluted in 1:100 ratio onto 96-well plate. Measurement was performed in + 37 °C with 595 nm laser in 5 minutes intervals for 20 hours. Maximum growth density of each exposed culture was measured and used in phage administration comparisons as the growth density of bacteria efficiently reflects the phage infection successfulness.

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Supplementary 1. Measured characteristics in 24-day community assembly studies with host AB6 and phages 1P1, 6P1 and 6P2. The amount of both 6P1/6P2 and 1P1 susceptible hosts, total amount of bacteria, amount of phage particles, and emerged resistances are listed in this table.

| Sample | Community | Shaking | Selection | Phenotype | Titer (pfu/ml) | Host total (cfu/ml) | Host with selection (cfu/ml) | 1P1 resistance | 6P1 resistance | 6P2 resistance |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | C1 | shaked | AB6 | 6P1 susceptible | 50000000 | 230000 | 265000 | Resistant | Resistant | Resistant |
| 1 | C2 | shaked | AB6 | 6P1 susceptible | 14000000 | 0 | 0 | Resistant | Susceptible | Resistant |
| 1 | C3 | shaked | AB6 | 6P1 susceptible | 53000000 | 0 | 5000 | Resistant | Resistant | Resistant |
| 1 | C4 | shaked | AB6 | 6P1 susceptible | 106000000 | 14000 | 96000 | Resistant | Susceptible | Resistant |
| 1 | C5 | still | AB6 | 6P1 susceptible | 33000000 | 32000 | 54000 | Resistant | Susceptible | Resistant |
| 1 | C6 | still | AB6 | 6P1 susceptible | 11000000 | 595000 | 1122000 | Resistant | Susceptible | Resistant |
| 1 | C7 | still | AB6 | 6P1 susceptible | 96000000 | 3400000 | 3260000 | Susceptible | Susceptible | Susceptible |
| 1 | C8 | still | AB6 | 6P1 susceptible | 2040000000 | 41000 | 57000 | Susceptible | Susceptible | Susceptible |
| 1 | C1 | shaked | AB6 6P1 | 1P1 susceptible | 317000000 | 230000 | 357000 | Resistant | Resistant | Resistant |
| 1 | C2 | shaked | AB6 6P1 | 1P1 susceptible | 66000000 | 0 | 0 | Resistant | Susceptible | Resistant |
| 1 | C3 | shaked | AB6 6P1 | 1P1 susceptible | 95000000 | 0 | 0 | Resistant | Resistant | Resistant |
| 1 | C4 | shaked | AB6 6P1 | 1P1 susceptible | 330000000 | 14000 | 25000 | Resistant | Susceptible | Resistant |
| 1 | C5 | still | AB6 6P1 | 1P1 susceptible | 181000000 | 32000 | 133000 | Resistant | Susceptible | Resistant |
| 1 | C6 | still | AB6 6P1 | 1P1 susceptible | 139000000 | 595000 | 1594000 | Resistant | Susceptible | Resistant |
| 1 | C7 | still | AB6 6P1 | 1P1 susceptible | 97000000 | 3400000 | 2864000 | Susceptible | Susceptible | Susceptible |
| 1 | C8 | still | AB6 6P1 | 1P1 susceptible | 238000000 | 41000 | 44000 | Susceptible | Susceptible | Susceptible |
| 2 | C1 | shaked | AB6 | 6P1 susceptible | 152400000 | 41300000 | 1478000000 | Resistant | Susceptible | Susceptible |
| 2 | C2 | shaked | AB6 | 6P1 susceptible | 28700000 | 8260 | 18000 | Resistant | Susceptible | Resistant |
| 2 | C3 | shaked | AB6 | 6P1 susceptible | 56700000 | 32880 | 49720 | Resistant | Susceptible | Resistant |
| 2 | C4 | shaked | AB6 | 6P1 susceptible | 27900000 | 4788000 | 5360000 | Resistant | Susceptible | Resistant |
| 2 | C5 | still | AB6 | 6P1 susceptible | 20700000 | 5076000 | 5485000 | Resistant | Susceptible | Resistant |
| 2 | C6 | still | AB6 | 6P1 susceptible | 66800000 | 1298000000 | 2284000000 | Resistant | Resistant | Resistant |
| 2 | C7 | still | AB6 | 6P1 susceptible | 51000000 | 3134000 | 2500000 | Resistant | Susceptible | Resistant |
| 2 | C8 | still | AB6 | 6P1 susceptible | 45800000 | 174000 | 25300 | Resistant | Susceptible | Resistant |
| 2 | C1 | shaked | AB6 6P1 | 1P1 susceptible | 276800000 | 41300000 | 1308000000 | Resistant | Susceptible | Susceptible |
| 2 | C2 | shaked | AB6 6P1 | 1P1 susceptible | 3300000 | 8260 | 18440 | Resistant | Susceptible | Resistant |
| 2 | C3 | shaked | AB6 6P1 | 1P1 susceptible | 22000000 | 32880 | 44560 | Resistant | Susceptible | Resistant |
| 2 | C4 | shaked | AB6 6P1 | 1P1 susceptible | 57600000 | 4788000 | 5920000 | Resistant | Susceptible | Resistant |
| 2 | C5 | still | AB6 6P1 | 1P1 susceptible | 129200000 | 5076000 | 4344000 | Resistant | Susceptible | Resistant |
| 2 | C6 | still | AB6 6P1 | 1P1 susceptible | 734400000 | 1298000000 | 2308000000 | Resistant | Resistant | Resistant |
| 2 | C7 | still | AB6 6P1 | 1P1 susceptible | 56800000 | 3134000 | 1328000 | Resistant | Susceptible | Resistant |
| 2 | C8 | still | AB6 6P1 | 1P1 susceptible | 54400000 | 174000 | 34100 | Resistant | Susceptible | Resistant |
| 3 | C1 | shaked | AB6 | 6P1 susceptible | 1200000 | 700000 | 6900000 | Not available | Not available | Not available |
| 3 | C2 | shaked | AB6 | 6P1 susceptible | 4200000 | 62000 | 76000 | Not available | Not available | Not available |
| 3 | C3 | shaked | AB6 | 6P1 susceptible | 900000 | 5988000 | 8632000 | Not available | Not available | Not available |
| 3 | C4 | shaked | AB6 | 6P1 susceptible | 3870000 | 35200000 | 18600000 | Not available | Not available | Not available |

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|---|---|---|---|---|---|---|---|---|---|---|
| 3 | C5 | still | AB6 | 6P1 susceptible | 12100000 | 102200000 | 45800000 | Not available | Not available | Not available |
| 3 | C6 | still | AB6 | 6P1 susceptible | 9100000 | 3300000 | 3700000 | Not available | Not available | Not available |
| 3 | C7 | still | AB6 | 6P1 susceptible | 16500000 | 34000000 | 21400000 | Not available | Not available | Not available |
| 3 | C8 | still | AB6 | 6P1 susceptible | 1100000 | 429000 | 541000 | Not available | Not available | Not available |
| 3 | C1 | shaked | AB6 6P1 | 1P1 susceptible | 5000000 | 700000 | 4700000 | Not available | Not available | Not available |
| 3 | C2 | shaked | AB6 6P1 | 1P1 susceptible | 20160000 | 62000 | 214000 | Not available | Not available | Not available |
| 3 | C3 | shaked | AB6 6P1 | 1P1 susceptible | 56300000 | 5988000 | 11179400 | Not available | Not available | Not available |
| 3 | C4 | shaked | AB6 6P1 | 1P1 susceptible | 1400000 | 35200000 | 42400000 | Not available | Not available | Not available |
| 3 | C5 | still | AB6 6P1 | 1P1 susceptible | 2800000 | 102200000 | 388800000 | Not available | Not available | Not available |
| 3 | C6 | still | AB6 6P1 | 1P1 susceptible | 108400000 | 3300000 | 3900000 | Not available | Not available | Not available |
| 3 | C7 | still | AB6 6P1 | 1P1 susceptible | 1200000 | 34000000 | 29100000 | Not available | Not available | Not available |
| 3 | C8 | still | AB6 6P1 | 1P1 susceptible | 1400000 | 429000 | 338000 | Not available | Not available | Not available |
| 4 | C1 | shaked | AB6 | 6P1 susceptible | 2600000 | 6600000 | 8100000 | Resistant | Susceptible | Resistant |
| 4 | C2 | shaked | AB6 | 6P1 susceptible | 1100000 | 1632000 | 259000 | Resistant | Susceptible | Resistant |
| 4 | C3 | shaked | AB6 | 6P1 susceptible | 1900000 | 32100000 | 24200000 | Resistant | Resistant | Resistant |
| 4 | C4 | shaked | AB6 | 6P1 susceptible | 1500000 | 23600000 | 33200000 | Resistant | Resistant | Resistant |
| 4 | C5 | still | AB6 | 6P1 susceptible | 29400000 | 9300000 | 15000000 | Resistant | Susceptible | Resistant |
| 4 | C6 | still | AB6 | 6P1 susceptible | 12500000 | 79300000 | 67000000 | Resistant | Resistant | Resistant |
| 4 | C7 | still | AB6 | 6P1 susceptible | 50400000 | 486000000 | 6000000 | Resistant | Resistant | Resistant |
| 4 | C8 | still | AB6 | 6P1 susceptible | 4600000 | 402000 | 480000 | Susceptible | Susceptible | Susceptible |
| 4 | C1 | shaked | AB6 6P1 | 1P1 susceptible | 7500000 | 6600000 | 13300000 | Resistant | Susceptible | Resistant |
| 4 | C2 | shaked | AB6 6P1 | 1P1 susceptible | 28240000 | 1632000 | 1552000 | Resistant | Susceptible | Resistant |
| 4 | C3 | shaked | AB6 6P1 | 1P1 susceptible | 68880000 | 32100000 | 24400000 | Resistant | Resistant | Resistant |
| 4 | C4 | shaked | AB6 6P1 | 1P1 susceptible | 61200000 | 23600000 | 27300000 | Resistant | Resistant | Resistant |
| 4 | C5 | still | AB6 6P1 | 1P1 susceptible | 33600000 | 9300000 | 15400000 | Resistant | Susceptible | Resistant |
| 4 | C6 | still | AB6 6P1 | 1P1 susceptible | 14100000 | 79300000 | 45600000 | Resistant | Resistant | Resistant |
| 4 | C7 | still | AB6 6P1 | 1P1 susceptible | 3000000 | 486000000 | 3482000 | Resistant | Resistant | Resistant |
| 4 | C8 | still | AB6 6P1 | 1P1 susceptible | 6600000 | 402000 | 274000 | Susceptible | Susceptible | Susceptible |
| 5 | C1 | shaked | AB6 | 6P1 susceptible | 290000 | 17100000 | 20100000 | Resistant | Susceptible | Susceptible |
| 5 | C2 | shaked | AB6 | 6P1 susceptible | 1580000 | 472000 | 615000 | Resistant | Susceptible | Resistant |
| 5 | C3 | shaked | AB6 | 6P1 susceptible | 1390000 | 9700000 | 9500000 | Resistant | Resistant | Resistant |
| 5 | C4 | shaked | AB6 | 6P1 susceptible | 820000 | 15400000 | 26900000 | Resistant | Resistant | Resistant |
| 5 | C5 | still | AB6 | 6P1 susceptible | 11300000 | 12800000 | 12400000 | Resistant | Susceptible | Resistant |
| 5 | C6 | still | AB6 | 6P1 susceptible | 1750000 | 59000000 | 64900000 | Resistant | Resistant | Resistant |
| 5 | C7 | still | AB6 | 6P1 susceptible | 2610000 | 12800000 | 22200000 | Resistant | Resistant | Resistant |
| 5 | C8 | still | AB6 | 6P1 susceptible | 18100000 | 231000 | 343000 | Resistant | Resistant | Resistant |
| 5 | C1 | shaked | AB6 6P1 | 1P1 susceptible | 700000 | 17100000 | 16800000 | Resistant | Susceptible | Susceptible |
| 5 | C2 | shaked | AB6 6P1 | 1P1 susceptible | 8280000 | 472000 | 695000 | Resistant | Susceptible | Resistant |
| 5 | C3 | shaked | AB6 6P1 | 1P1 susceptible | 3000000 | 9700000 | 12800000 | Resistant | Resistant | Resistant |
| 5 | C4 | shaked | AB6 6P1 | 1P1 susceptible | 9000000 | 15400000 | 31400000 | Resistant | Resistant | Resistant |
| 5 | C5 | still | AB6 6P1 | 1P1 susceptible | 800000 | 12800000 | 15400000 | Resistant | Susceptible | Resistant |
| 5 | C6 | still | AB6 6P1 | 1P1 susceptible | 2500000 | 59000000 | 47600000 | Resistant | Resistant | Resistant |
| 5 | C7 | still | AB6 6P1 | 1P1 susceptible | 500000 | 12800000 | 19400000 | Resistant | Resistant | Resistant |

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|---|---|---|---|---|---|---|---|---|---|---|
| 5 | C8 | still | AB6 6P1 | 1P1 susceptible | 800000 | 231000 | 322000 | Resistant | Resistant | Resistant |
| 6 | C1 | shaked | AB6 | 6P1 susceptible | 317000000 | 19200000 | 17800000 | Resistant | Susceptible | Susceptible |
| 6 | C2 | shaked | AB6 | 6P1 susceptible | 66000000 | 580000 | 900000 | Resistant | Susceptible | Resistant |
| 6 | C3 | shaked | AB6 | 6P1 susceptible | 95000000 | 12900000 | 23100000 | Resistant | Resistant | Resistant |
| 6 | C4 | shaked | AB6 | 6P1 susceptible | 330000000 | 25700000 | 44400000 | Resistant | Susceptible | Resistant |
| 6 | C5 | still | AB6 | 6P1 susceptible | 181000000 | 14800000 | 31900000 | Resistant | Susceptible | Resistant |
| 6 | C6 | still | AB6 | 6P1 susceptible | 139000000 | 45700000 | 35500000 | Resistant | Resistant | Resistant |
| 6 | C7 | still | AB6 | 6P1 susceptible | 97000000 | 61600000 | 49100000 | Resistant | Resistant | Resistant |
| 6 | C8 | still | AB6 | 6P1 susceptible | 238000000 | 1300000 | 1700000 | Resistant | Resistant | Resistant |
| 6 | C1 | shaked | AB6 6P1 | 1P1 susceptible | 50000000 | 19200000 | 19300000 | Resistant | Susceptible | Susceptible |
| 6 | C2 | shaked | AB6 6P1 | 1P1 susceptible | 14000000 | 580000 | 780000 | Resistant | Susceptible | Resistant |
| 6 | C3 | shaked | AB6 6P1 | 1P1 susceptible | 53000000 | 12900000 | 28700000 | Resistant | Resistant | Resistant |
| 6 | C4 | shaked | AB6 6P1 | 1P1 susceptible | 106000000 | 25700000 | 61800000 | Resistant | Susceptible | Resistant |
| 6 | C5 | still | AB6 6P1 | 1P1 susceptible | 33000000 | 14800000 | 15400000 | Resistant | Susceptible | Resistant |
| 6 | C6 | still | AB6 6P1 | 1P1 susceptible | 11000000 | 45700000 | 30500000 | Resistant | Resistant | Resistant |
| 6 | C7 | still | AB6 6P1 | 1P1 susceptible | 96000000 | 61600000 | 47200000 | Resistant | Resistant | Resistant |
| 6 | C8 | still | AB6 6P1 | 1P1 susceptible | 2040000000 | 1300000 | 2700000 | Resistant | Resistant | Resistant |