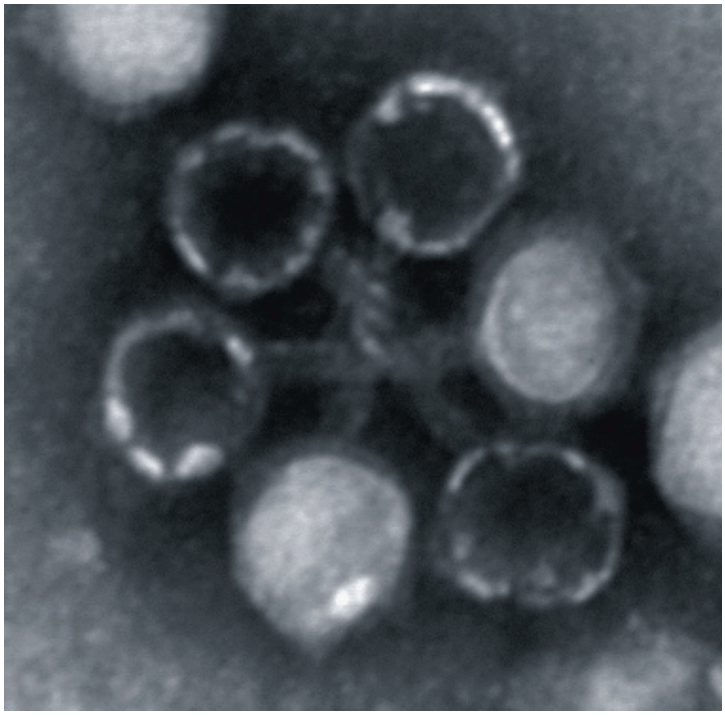
JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE
322

Sari Mattila

Small Things Matter

Of Phages and Antibiotic Resistance Conferring Plasmids



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UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2016

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Editors
Varpu Marjomäki
Department of Biological and Environmental Science, University of Jyväskylä
Pekka Olsbo, Ville Korkiakangas
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ABSTRACT

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Viruses and plasmids are small units of genetic material dependent on cells either transiently or continuously. Intriguingly, stories of these small entities intertwine in antibiotic resistance crisis. Horizontal gene transfer enables bacteria to respond rapidly to chances in their environment. Anthropogenic consumption of antibiotics induces the travel of resistance encoding genes mainly as passengers of conjugative plasmids. In this thesis, I demonstrate that clinically important resistance plasmids could evolutionarily rescue susceptible bacteria under lethal antibiotic concentrations. If mobile resistance genes are available in surrounding community, administration of high doses of antibiotic might not be enough to treat some bacterial infections – calling for alternatives to fight multi-resistant bacteria and interfere the spread and maintenance of resistance. Phage therapy, utilization of bacterial viruses against bacteria, could be one such avenue. In this thesis plasmid-depended phage PRD1 was studied in itself and as a tool to be utilized against resistance plasmid carrying bacteria. Blue native polyacrylamide gel electrophoresis revealed interaction between two virus entry related membrane proteins and zymogram analysis overruled a previous model of the lytic enzyme residing at the genome-packaging vertex. Bacterial resistance to PRD1 was linked with either lost or impaired conjugation ability, that could restore only when the initial resistance-conferring mutation was a dynamic tandem repeat insertion. Promisingly, the reversion also returned the susceptibility to the phage. Yet, as plasmid-dependent phages are currently available only against some resistance plasmids, an alternative approach, on-demand isolation of phages, was investigated against common nosocomial pathogens. *Staphylococcus*, *Acinetobacter* and *Enterococcus* phages were scarce in the environmental reservoir whereas phages against *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *Salmonella* strains could often be isolated as needed. Altogether, different manifestations of phage-therapy may provide answers to the current antibiotic resistance crisis.

Keywords: Antibiotic resistance; bacteriophage; conjugative plasmid; horizontal gene transfer; phage therapy; protein interactions.

Sari Mattila, University of Jyväskylä, Department of Biological and Environmental Science, P.O. Box 35, FI-40014 University of Jyväskylä, Finland

Author's address Sari Mattila
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland
sari.p.mattila@jyu.fi

Supervisors Professor Jaana Bamford
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland

Docent Matti Jalasvuori
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland

Reviewers Professor Tapani Alatossava
Department of Food and Environmental Sciences
P.O. Box 66
FI-00014 University of Helsinki
Finland

Docent Maija Pietilä
Department of Food and Environmental Sciences
P.O. Box 56
FI-00014 University of Helsinki
Finland

Opponent Professor Dennis Bamford
Department of Biosciences and Institute of Biotechnology
P.O. Box 56
FI-00014 University of Helsinki
Finland

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I-IV

I Mattila S., Oksanen H.M. & Bamford J.K. 2015. Probing protein interactions in the membrane-containing virus PRD1. *Journal of General Virology* 96: 453–462.

II Mattila S., Ruotsalainen P., Ojala V., Tuononen T., Hiltunen T. & Jalasvuori M. 2016. Conjugative ESBL-plasmids differ in their potential to rescue susceptible bacteria via horizontal gene transfer. Submitted manuscript.

III Ojala V., Mattila S., Hoikkala V., Bamford J.K., Hiltunen T. & Jalasvuori M. 2016. Scoping the effectiveness and evolutionary obstacles in using plasmid-dependent phages to fight antibiotic resistance. *Future Microbiology*. 11: 999-1009.

IV Mattila S., Ruotsalainen P. & Jalasvuori M. 2015. On-demand isolation of bacteriophages against drug-resistant bacteria for personalized phage therapy. *Frontiers in Microbiology* 6: 1271.

RESPONSIBILITIES OF SARI MATTILA IN THE ARTICLES OF THE THESIS:

I I performed the experiments and prepared the tables and figures. The data analysis and writing of the paper was done together with the co-authors.

II I isolated the plasmids and analyzed their sequence. Pilvi Ruotsalainen and I did the PCR analysis. All the authors participated in the conjugation experiments. I co-supervised the Master's thesis project involving part of these experiments. Ville Ojala and I analyzed the data from conjugation experiments. I wrote the manuscript with the co-authors.

III I performed the sequencing. I participated in the rest of the experiments, data analysis and writing of the article.

IV I did the experiments together with Pilvi Ruotsalainen. I prepared all the figures and data analysis and participated in the writing of the article.

ABBREVIATIONS

ATPase	adenosine triphosphatase
ATV	*Acidianus* two-tailed virus
BN-PAGE	blue native polyacrylamide gel electrophoresis
cryo-EM	cryo-electron microscopy
DDM	N-dodecyl-β-maltoside
DNA	deoxyribonucleic acid
ds	double stranded
ESBL	extended-spectrum beta-lactamase
GST	glutathione S-transferase
GTAs	gene transfer agents
HGT	horizontal gene transfer
Inc	incompatibility
kb	kilobase pairs
kDa	kilodalton
MGEs	mobile genetic elements
MIC	minimum inhibitory concentration
MPC	mutant prevention concentration
Mpf	mating pair formation
MRSA	methicillin-resistant *Staphylococcus aureus*
MSW	mutation selection window
OriT	origin of transfer
RNA	ribonucleic acid
rRNA	ribosomal RNA
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
ss	single-stranded
T	triangulation number
TEV	tobacco etch virus
T4CP	type IV coupling protein
wt	wild-type

1 INTRODUCTION

Not so long ago our world was unaware of the underlying properties of the chemical compound essential for something we call life. Finally, it was bacterial viruses that helped to unravel the role of deoxyribonucleic acid (DNA), which had long been thought to be too simple to carry the burden of inheritance (Hershey and Chase 1952). But the story got more complicated as great adventures tend to do: There is another nucleic acid, ribonucleic acid (RNA), which serves as a messenger (among other important tasks) and even works in some viruses, instead of DNA, as genetic material. Francis Crick formulated the central dogma of molecular biology in 1958 (Crick 1958), only five years after the discovery of the structure of the DNA (Watson and Crick 1953). The central dogma states that genetic information flows from DNA to RNA and finally to polypeptide chain of protein and rules out transfer from protein to nucleic acid.

It was thought that mysteries of life would be revealed after the DNA code was solved but it soon became clear that many secrets still remain to be untangled. All living things function through complicated interplay of nucleic acids, proteins, lipids and carbohydrates and they can also use various components from their surroundings. Most importantly they are not static but constantly changing. Behind the scenes work evolutionary forces, work of which are visible to us for example through the common ancestry of all current forms of life (Koonin and Wolf 2010) and constant emergence of new strains of influenza (Scholtissek 1995). Darwinian view where genetic information and variation are exclusively passed to the offspring's of an organism was first thought to be the only way to inherit genes (Boto 2010). However, microbes (and later turned out multicellular organisms too) had another surprise for us: horizontal gene transfer (HGT), where, instead of vertical inheritance, genes can be acquired from even an unrelated organism (Soucy *et al*. 2015). Viruses and plasmids are major players in these processes.

Word virus tends to have a threatening echo in peoples' minds exuding about illnesses like AIDS, Ebola and Influenza. Research on human viruses is undeniably an important part of virology, but viruses have also many other roles in life. Viral (and related) genetic material is part of genomes of all kinds

of living beings including humans (de Parseval and Heidmann 2005, Cortez *et al*. 2009). Evidence is accumulating that viruses were here before the divergence of three domains of life and have greatly influenced the evolution of modern cells (Forterre 2010). Even phages, viruses infecting bacteria, that may appear seemingly unimportant to us, have their part in this biosphere. They are the most abundant entities on earth: estimates reach 10^{31} virus particles (Hendrix *et al*. 1999). Only recently viruses have been recognized as important regulators in different ecosystems, including human gut and marine environments (Fuhrman 1999, Dalmasso *et al*. 2014). Phages also have had a major role in the development of molecular biology and biotechnology (Salmond and Fineran 2015).

Plasmids are depicted often as little travellers within cells, floating in the cytoplasm apart from the genome. They may seem mostly harmless at first glance. However, they are selfish genetic units that often ensure their own preservation by post-segregational killing or partitioning systems (Ogura and Hiraga 1983, Van Melderen 2010). Plasmids may also carry of beneficial traits that bring competitive advantage to their host cells and some of them are capable of self-inducible transfer to new host cells via conjugation (Norman *et al*. 2009). These qualities make plasmids more than just genetic bystanders, lifting them up as providers of diversity in microbial communities (Jain *et al.* 2003). They have also claimed a central role in antibiotic resistance crisis by spreading old and new resistance genes wherever antibiotics are present, wreaking terror especially in hospital settings (Norman *et al.* 2009).

We need to take a better look at viruses and plasmids to resolve the mysteries of their part in life. In this thesis, I have studied the structure of phage PRD1 as well as the possibility to utilize phages in practical applications. I introduce a story that unravels couple of pieces in the puzzle that attempts to decipher PRD1 protein interactions, an investigation that has been going on for over four decades. I also explore new aspects and evaluate old ideas for using phages against antibiotic resistant bacteria. Part of the thesis provides insight into the spread of antibiotic resistance via conjugation and how plasmid-depended phages could be utilized to prevent it. Additionally, isolation of new phages against common bacterial pathogens is investigated. In the introduction I wish to present the reader the interesting world of small genetic actors, phages and plasmids. These important tools of evolution direct the world in some cases to our misfortune as for instance by carrying and spreading antibiotic resistances. The idea of harnessing phages as our workhorses to benefit the society has been around for a while. Also, sometimes they should be appreciated as just interesting creatures challenging our imagination about what can be.

1.1 Bacteriophages

Lifestyle of viruses differs from that of cellular life: they have extracellular inactive state, virion, that is not capable of any functional activity on its own. Crenarchaea infecting virus *Acidianus* two-tailed virus (ATV) is a rare exception that forms a tail structure outside its host cell (Häring *et al*. 2005). All viruses are obligate parasites and often regarded as non-living. However, inside the host cell they are brought alive by the cellular machinery and take it over to produce new virions. At this stage virus is equal to all the other living entities capable of reproduction, metabolic activity and adaptation and can be described by virocell-concept (Forterre 2011). "A virocell" includes both the virus genome and the host genome functioning as a unit, i.e. viral infection turns the host to a novel type of virion-producing cellular organism.

Viruses present enormous variety both in genome and structure. Along with DNA, also RNA can carry the heritable material of a virus. Genomes may be double stranded (ds) or single stranded (ss), and in some cases something in the between (nicked). These molecules can exist in linear, circular or segmented configurations (ICTV 2016). Information stored in viral sequences are the most diverse in the biosphere, partly reflecting the constant birth of new genes driven by evolutionary forces acting in host-virus interactions (Forterre 2011). Virion, by a classical definition, is a virus genome enclosed within a proteinaceous capsid. In addition, some virions encompass host-derived lipid components as an external or internal part of the virus particle. Helical and icosahedral symmetries of the capsid are prevalent among viruses but also other morphologies exist, especially in extreme environments. These include spindle-, bottle-, droplet- and coil-shaped virions, among others (Pietilä *et al.* 2014).

None of the living entities appear to have been spared from the influence of viruses: Also bacteria have these parasites named by one of their founders, Felix d'Herelle, as eaters of bacteria i.e. bacteriophages (Sulakvelidze *et al.* 2001). Bacteriophages are currently classified in eleven families (ICTV 2016) after the recently approved phage genus *Gammasphaerolipovirus* was included in as a member of the novel family *Sphaerolipoviridae* (Pawlowski *et al.* 2014). Phages represent eight capsid morphotypes: icosahedral with either an inner or outer membrane or without a membrane, icosahedral with a contractive, non-contractive or short tail as well as filamentous and pleomorphic (Pietilä *et al.* 2014). For comparison, 16 morphotypes of archaeal viruses have been described so far. Viruses can also be classified according to their nucleic acid (RNA or DNA, single or double stranded) and type of replication intermediates (Baltimore 1971) or by structural comparison of their capsid proteins (Benson *et al*. 1999, Bamford *et al.* 2002, 2005). Latter scheme can reveal ancient evolutionary relationships that are not visible in the relatively rapidly changing nucleotide sequence.

Whatever the constituents of the virion, they need to be stable enough to protect the phage genome from the environment, but labile enough to release it

when a suitable host cell is encountered. Initial step in phage life cycle is the attachment of the virion onto the cell surface via highly specific receptor-binding protein. This step includes irreversible and reversible binding and may involve secondary receptors as in the case of T4 phage (Flayhan *et al.* 2012). Majority of known bacteriophages then deliver their genome through vertex associated tail structure inside the cell leaving the capsid outside (Bhardwaj *et al.* 2014). Exceptions include RNA phage phi6 and alike phages that internalize the nucleocapsid by fusion of their outer membrane with the host membrane, referred also an endocytosis-like pathway (Poranen *et al.* 1999) and DNA-phage PM2 that dissociates the coat at the cell envelope (Kivelä *et al.* 2004, Cvirkaite-Krupovic *et al.* 2010). Bacterial cells, both gram-negative and gram-positive, have peptidoglycan layer surrounding the cytoplasmic membrane (Brown *et al.* 2015). Gram-negative bacteria also have an outer membrane embedding glycolipids and lipoproteins. To enable genome delivery, bacteriophages use peptidoglycan-degrading enzymes to penetrate the cell wall (Loessner 2005).

In the productive cycle of phages, the genome is replicated, proteins translated and a capsid assembled. Most known phages package their genome into a preformed capsid but the packaging can also happen simultaneously with the building of the capsid (Russel 1991). Complete particles need to exit the host cell to start the life cycle again in another host. Encounter with a phage often leads to lysis of the host cell. A lytic enzyme cleaves the cell wall peptidoglycan layer while holin provides access to it by making holes in the cell membrane. Chronic infection of filamentous phages is one exception, instead of lysing the cell phages are released by extrusion (Rakonjac *et al.* 2011). Some phages, called temperate, can also enter another kind of life cycle. After entry, their genome is integrated to the host genome resulting in a bacterial state called lysogen. In this state, phage stays as a silent resident of bacterium (prophage) replicating alongside of the host genome, most of its gene expression suppressed. Another variation of dormant phage residency within the host cell is called pseudolysogeny, state in which phage stays episomal without actively entering either lysogenic or lytic cycle. It has been associated with the host's nutrient-depletion, possibly reflecting a strategy to avoid harsh outside conditions and/or to remain independent of host's damage responses (Cenens *et al.* 2013).

Like mentioned above, phages are the most abundant creatures on earth. Even human gut virome, i.e. collection of all the viruses that inhabit gut environment, has been estimated to be dominated by phages (Breitbart *et al.* 2003), although and in contrast to marine environment, majority of them are predicted to be temperate (Reyes *et al.* 2012). Most of the currently known phages belong to order *Caudovirales*, a group of viruses characterized by a tail and an icosahedral head. However, common laboratory culturing methods are not suitable for all phages and there has been a tendency to focus on phages of well-known study systems like *Escherichia coli*. This inevitably biases the sampling and can skew the obtained picture of the actual virosphere. Though, even highly prevalent, some of the viruses can only be detected by analysis of metagenomics data (Dutilh *et al.* 2014). Also, sometimes simultaneous isolation

of a phage and a host can provide novel findings as represented by recently found freshwater phage FLiP, which has a previously unknown combination of ssDNA genome and inner membrane within an icosahedral capsid (Laanto *et al.* unpublished). In addition, although phages have been studied for decades even the familiar ones still hold some secrets: for instance, understanding of the molecular mechanisms of the tail structures and functions increases in concert with the technical development (Chaban *et al.* 2015). Meanwhile, the function of many phage-encoded genes remains unknown despite increasing number of phage sequences (Pope *et al.* 2015), calling for classical characterization efforts. Also, phage ecology has only recently attracted notable interest from the research community. While it has already been confirmed that phages are everywhere, their distribution and influence on their hosts in environmental settings remain largely unknown (Thurber *et al.* 2009). We have indeed only scratched the surface both in terms of diversity and detailed analysis of these entities.

1.2 Plasmid-dependent phage PRD1

PRD1 represents one of the few known inner-membrane containing tailless phages. Other morphologically similar phages include PM2 (Kivelä *et al.* 2002) and Bam35 (Gaidelyte *et al.* 2005). PRD1 is a plasmid-dependent phage: It infects a wide range of gram-negative bacteria harbouring plasmids belonging to incompatibility (Inc) groups P, W and N (Olsen *et al.* 1974). One of them, RP4 encoding resistance for ampicillin, tetracycline and kanamycin, is an IncP type plasmid (Datta *et al.* 1971) that has widely been used to study interaction of PRD1 with its host cell. Structure called mating pair formation (Mpf) complex taking part in plasmid conjugation process (see below chapter 1.6) is required for PRD1 attachment to host (Grahn *et al.* 1997). It has been, however, suggested that rest of the infection process is independent of the conjugation machinery (Grahn *et al.* 2002).

PRD1 is the type member of *Tectiviridae* family and also, based on structural comparisons of major capsid proteins and virion architecture, belongs to a lineage of viruses with a double beta-barrel major capsid protein fold. Viruses with similar capsid proteins have been found to infect hosts in all three domains of life, possibly reflecting a common ancestry (Benson *et al.* 2004, Abrescia *et al.* 2012). PRD1 has been predicted to encode total of 31 proteins, of which 18 have been confirmed to be structural (Fig. 1). The capsid of PRD1 consists of major capsid protein P3 trimers organized in pseudo triangulation number (T) = 25 lattice (Butcher *et al.* 1995, Abrescia *et al.* 2004). Underneath, attached to the surface of the membrane and following the edges of capsomers, reside protein P30, which has been suggested to force the capsid into its shape (Abrescia *et al.* 2004). Protein complexes consisting of four different proteins (species) occupy vertices of the PRD1 virion: receptor-recognizing P2, trimeric spike P5, penton base P31 and five P16s linking the vertices to the inner

membrane (Jaatinen *et al.* 2004, Huiskonen *et al.* 2007). Attachment of the P2 to the receptor initiates a cascade leading to formation of membranous tube that delivers the genome into the cell. The tube is built of proteins and lipids and many membrane proteins play part in the virus entry: P18, P14 and P32 are essential for tube formation, transglycosylase P7 is involved in the entry process and membrane associated protein P11 is responsible of the initiation of DNA delivery (Grahn *et al.* 2002, Peralta *et al.* 2013). Membrane protein P34 is also part of the virion (Bamford *et al.* 1991). One of the 12 vertices of PRD1 capsid is different from the others. It has been shown to function in the packaging of the linear ~15-kilo basepair (kb)-long dsDNA genome (Gowen *et al.* 2003, Strömsten *et al.* 2003). The packaging occurs after protein P8 primed replication of the genome (Caldentey *et al.* 1993a) and the assembly of the virus particles. Non-structural proteins P10 and P17 are required for proper particle assembly, former of which is functioning as scaffolding protein and associated with a vesicle pinched off from cytoplasmic membrane (Mindich *et al.* 1982, Rydman *et al.* 2001). The unique vertex includes at least membrane proteins P20 and P22, packaging adenosine triphosphatase (ATPase) P9 and accessory factor protein P6 (Gowen *et al.* 2003, Strömsten *et al.* 2003). Muramidase P15, responsible of host cell lysis, is also a structural protein, being an exception to other known phages (Rydman and Bamford 2002). Despite extensive structural and functional studies on PRD1, organization and interactions of proteins associated with the lipid-membrane remain widely unknown. Most data on membrane proteins is derived from the studies on PRD1 amber mutants, which provide virions deficient of proteins that are encoded by the mutation-carrying gene. These mutations are conditional and are not manifested if the viruses are propagated in amber suppressor strains. Particle formation is undisrupted in the absence of most structural proteins except in the case of P3 and P30 forming the capsid (Mindich *et al.* 1982, Rydman *et al.* 2001) and mainly qualitative data on tube formation, absence of other protein species or membrane permeability during PRD1 entry is available. Neither cryo-electron microscopy (cryo-EM) nor X-ray crystallography has by far been successful approach in resolving the structure or interactions of inner-membrane proteins of PRD1 but the latter one solved the overall structure of the virion in ~4 Å resolution and brought detailed information on the outer capsid and the receptor recognizing vertices and how these structures are interconnected and associated with the membrane (Abrescia *et al.* 2004). This contradiction probably reflects the unsymmetrical distribution of the proteins, unsuitable to averaging used in these methods. In this thesis two different biochemical approaches were used to further resolve (membrane) protein interactions in PRD1 virion (I).

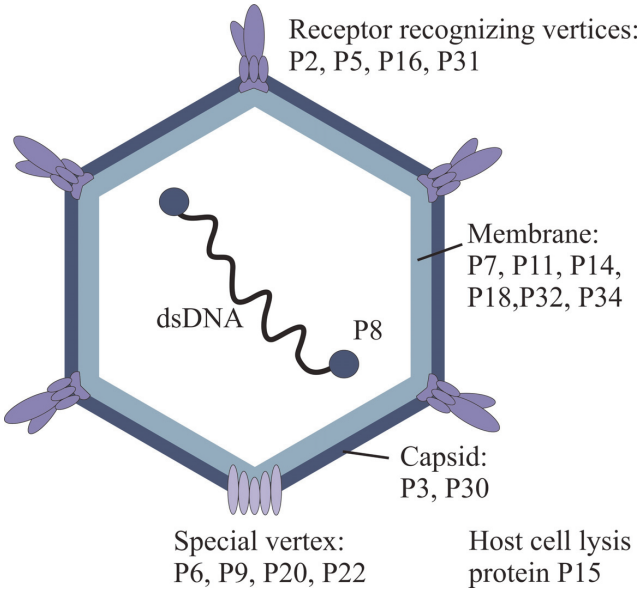

FIGURE 1 Schematic representation of PRD1 virion (I). The known structural proteins are indicated.

1.3 Bacterial resistance to antibiotics

In medicine, antibiotics are commonly synonyms for antibacterial drugs used to treat bacterial infections. They can either kill target organism (bacteriosidic) or inhibit their growth (bacteriostatic) assisting body's natural defence (U.S. National Library of Medicine 2016). Many microbes produce antibiotics and commercialized products can be these naturally occurring molecules but the majority are their semisynthetic versions. Also some entirely synthetic antibiotics such as quinolones are on the market (Fair and Tor 2014). Our excessive and empirical usage of antibiotics both in medicine and farming industry has led to an unfortunate outcome: decreased efficiency of these drugs orchestrated by the emergence of multi-resistant pathogenic bacteria all over the world (Blair *et al.* 2015). Synthetic drugs (quinolones) represent no exception (Ruiz *et al.* 2012). The progress has been fast since first commercial antibiotic, penicillin, was introduced in 1945 (Aminov 2010). Today in Europe, over 25,000 deaths are associated with antibiotic resistant infections every year and overall estimated costs of treatments and prolonged hospital stays reach 1.5 billion euros (ECDC/EMEA 2009). Similarly to antibiotic production, resistance to antibiotics is proven to be common and diverse among environmental microbes, leading to hypothesis that they are part of signalling and regulatory networks of microbes (Aminov 2009). Global anthropogenic antibiotic usage is in evolutionary scale a relatively sudden process, putting enormous selection

pressure for resistances, and in many cases microbes have responded by mobilization of these mechanisms (Wright 2010).

Most problematic species associated in antibiotic-resistant nosocomial i.e. hospital acquired infections include *Acinetobacter baumannii*, *Enterococcus faecium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa, Salmonella sp.* and *Staphylococcus aureus* among others (Bereket *et al.* 2012). They can all be considered as opportunistic pathogens (either part of common human microbiota or inhabitants of environment), which typically turn pathogenic when encountering somehow weakened (e.g. aged, wounded, on medication or carrying other infections) individuals. However, infections caused by these often multi-drug resistant pathogens are no longer an exclusive problem of the hospital-settings since community acquired infections are increasing (Rossolini *et al.* 2014). Also, reports on colonization of multi-resistant bacteria both in human and domestic animals portend worsening of the situation by predicting increasing number of untreatable infections (Bhattacharya 2013).

Development of new drugs cannot keep up with the current situation: in the last five decades only few completely new antibiotic classes have been introduced (Coates *et al.* 2011, Ling *et al.* 2015). Reasons underlying behind this course of development include financial and regulatory challenges (Fernandes 2015), that we might be able to overcome eventually, but the contemporary situation calls for immediate and extensive measures. One obvious avenue is sustaining the effectiveness of current antibiotics as long as possible. This requires radical changes in healthcare and antibiotic usage practises like more targeted treatment guided by thorough diagnostics (Ventola 2015). Also raising awareness by educating professionals and general public both in antibiotic usage and hygiene measures in order to prevent infections in the first place could lead to better prescription practices and infection control (Ventola 2015). In addition, according to some studies this situation is not only a problem of unawareness but partly reflecting the socioeconomical problems such as poverty and corruption (Planta 2007, Collignon *et al.* 2015). Also, majority of the antibiotics usage and release to environment occurs outside of healthcare, providing diverse platforms for the development and transmission of resistances (Andersson and Hughes 2012). For this reason, restricting the usage of antibiotics especially in growth promoting purposes in animal husbandry is one of the necessary actions to be globally adopted (Bartlett *et al.* 2013). Ultimately, however, these measures alone are not likely to solve the crisis in its entirety because the selection pressure favouring antibiotic resistances still remains. Deeper understanding of the dynamics of antibiotic resistances and alternative approaches to fight them taking account the mechanisms behind the emerging resistances have to be studied and put into action. One potential avenue to be explored is phage therapy (Golkar *et al.* 2014), which is one of the main focuses of this thesis (III, IV) and which will be discussed later in more detail.

1.4 Acquired antibiotic resistance

Antibiotic resistance can be either intrinsic or acquired. In the former, microbes inherently possess qualities that prevent the drug from targeting them and thus the presence of the antibiotic does not affect them. Examples include the inability of vancomycin to penetrate the outer membrane of gram-negative bacteria (Zhou *et al.* 2015) and β-lactamase resistance in enterococci caused by low affinity of the drug to cell wall targets (Hollenbeck and Rice 2012). Acquired antibiotic resistance means that a previously susceptible strain turns resistant either via mutations or HGT. When it comes to antibiotic resistance crisis acquired resistance is mainly considered in discussions, because emerging resistances and multi-resistant strains complicate diagnostics and may lead to treatment failure whereas intrinsic resistances are known beforehand and thus are easily avoided. However, there have recently been attempts to expand the activity of some drugs by circumventing the intrinsic resistances with combination therapy (Liu *et al.* 2010).

Commonly described mechanisms of acquired resistance are alteration of the drug's intracellular target, inactivation or modification of the drug and restriction of the drug's access to the target (cell wall modification, efflux enhancement or target bypass). Examples of the first two include rifampicin resistance acquired via mutations in RNA polymerase (Rabussay and Zillig 1969) and production of enzymes breaking the beta-lactam ring (Livermore 1995), respectively. Alternative mechanisms can also modify target without mutation in itself or alter the drug molecule, as represented by chloramphenicol–florfenicol resistance (cfr) methyltransferase targeting 23S ribosomal RNA (rRNA) (Long *et al.* 2006) and acetyltransferases, phosphotransferases and nucleotidyltransferases capable of modifying aminoglycoside antibiotics (Shaw *et al.* 1993). Limited access to the antibiotic target can be achieved in several ways: for instance, by limiting porin production or introduction of mutations to porins (Wozniak *et al.* 2012), which provide channels across the outer cell membrane for passive trafficking of molecules. In addition, overexpression of efflux pumps, that are active molecule transporters of the cytoplasmic membrane, moves antibiotics away from the target (Kosmidis *et al.* 2012).

Many studies have shown that once established resistances do not diminish easily, even when the selection pressure is released (Andersson and Hughes 2011). Additionally, co-resistance to another class of antibiotics is often present because these genes often accumulate to resistance cassettes. For these reasons, preventing or at least delaying bacteria to acquire resistance is one of the key challenges of our time. Recently, below minimum inhibitory antibiotic concentrations have been on a spotlight and shown to contribute in development of antibiotic resistances in a stepwise manner in environments where low concentration gradients of antibiotic are present (Andersson and Hughes 2012). Main driver of resistance development in current situation is

anthropogenic drug pollution, which could be limited for example by ozone treatment of wastewaters containing pharmaceuticals. However, in human and animal healthcare antibiotics have to be used in order to eradicate bacterial infections.

In resistance development, mutation selection window (MSW) is a widely applied concept. It is usually defined as antibiotic concentration range between minimum inhibitory concentration (MIC) and mutant prevention concentration (MPC). In theory, above MSW of antibiotic should eliminate all susceptible bacteria and also prevent the emergence of one-step mutants. It has been suggested to remarkably reduce development of antibiotic resistances during the treatment of bacterial infections (Canton and Morosini 2011). HGT, where elsewhere-evolved resistance can transfer to susceptible cells, complicates the situation. Avoidance of traditional mutation selection window does not prevent evolutionary rescue of susceptible subpopulation via HGT as was demonstrated by Ojala *et al.* (2014). In this thesis, I mainly concentrate on horizontal gene transfer as a mechanism by which bacteria become resistant to antibiotics.

1.5 Horizontal gene transfer

Phylogenetic relationships are used to reflect the evolutionary history of species and their relation to one another (Doolittle 1999). However, on the level of individual genes there can be numerous inconsistencies in the branching patterns (Poptsova and Gogarten 2007) or differences in actual gene content (oligonucleotide ratio or codon usage) compared to core genome (Langille *et al.* 2010). These genes can be homologues of genes found even very distantly related organisms and if they are absent from the closest relatives they draw a picture of their own, a web of life. These gene relationships echo potential HGT events, where instead of inheriting genes directly from predecessor, they are acquired from outside of the organism (Olendzenski and Gogarten 2009). There are three main mechanisms of HGT: transformation, transduction and conjugation. In transformation genetic material is taken into the cells directly from the environment (Johnston *et al.* 2014). Phages are responsible of transduction by packaging unrelated genetic material inside the virion and delivering it to the next host cell. Transduction can be specialized, referring to an imprecise excision of the genome of a temperate phage from that of the host, or generalized, in which part of host DNA is randomly packaged into the virion instead of the phage genome (Ebel-Tsipis *et al.* 1972, Morse *et al.* 1956). Conjugation is a unidirectional process where donor cell harbouring the conjugative genetic element is connected to the recipient cell by a channel, through which the replicative transfer of the element occurs (Norman *et al.* 2009).

Couple of recently represented mechanisms of HGT include cell fusion and gene transfer agents (GTAs). Both bacteria and archaea encode GTAs transferring small incidental pieces of genome inside a protein coat, usually too

small to carry all GTA encoding genes (Lang *et al.* 2012). Bidirectional gene transfer via cell fusion has been demonstrated only in archaea, but unidirectional also occurs in bacteria (Naor and Gophna 2013). Additionally, in eukaryotes, endosymbiosis or repeated backcrosses after interspecies hybridization can lead to HGT (Timmis *et al.* 2004, Harrison and Larson 2014).

Genetic sequences mediating their own HGT are collectively called mobile genetic elements (MGEs). Conceptual distinction can be made between translocative elements like transposons, integrons and insertion sequences physically moving from one DNA location to another and dispersive elements like plasmids and other between cells travelling parties of HGT. Translocative elements often inhabit dispersive elements (Norman *et al.* 2009). However, there are elements representing both categories called integrative and conjugative elements including conjugative transposons and transposable prophages (Burrus *et al.* 2002). In addition to aforementioned division there have been other attempts to classify these elements for instance according to their modular structure (Leplae *et al.* 2004). Even though often labelled as selfish entities, genetic elements prone to HGT actually represent variety of symbiotic behaviours within the paratisism-mutualism continuum, and they can also be classified accordingly while acknowledging their different forms of intercellular mobility (Jalasvuori and Koonin, 2015). This classification of genetic replicators includes chromosomes and lytic viruses and could offer some advantages when the impact of HGT in different aspects of bacterial evolution is being considered.

It has been suggested that HGT is the main driver for diversity among prokaryotes providing a fertile ground for rapid adaptation and genome innovation (Jain *et al.* 2003). Some limitations, however, have been recognized: a study on prokaryotic genomes showed that the closer the relative the more frequently HGT occurs (Popa *et al.* 2011). This was traced back to barriers in genome sequence and GC content similarity. Also, environmental conditions, interspecies interactions and composition of MGEs alongside with their transfer mechanisms contribute extensively to the occurrence of HGT (González-Candelas and Francino 2011). Translocative elements or their combinations often accommodate accessory genes and gene clusters offering selective advantage in some environments. There is a bias towards certain types of genes in their frequency of transfer: Genes encoding informational processes (transcription and translation for instance), which usually are part of complicated regulatory networks, are transferred rarely (Jain *et al.* 1999). More often transferred operational genes typically include those encoding cell envelope and regulatory functions and cellular processes. Last category comprises mainly DNA transformation, pathogenesis, toxin production, and resistance conferring genes (Nakamura *et al.* 2004). There are ongoing discussions in the scientific community on how much HGT actually affects and has affected (bacterial) evolution. Mutual agreement exists that its impact on antibiotic resistance crisis is remarkable. Antibiotic usage induces global environmental change, tracing back to anthropogenic pollution, which is vastly faster and wider than most naturally occurring gradual and disperse changes in

the environment. Furthermore, resistances to all commonly used antibiotic classes have been found to reside in plasmids, many of which are conjugative (Bennett 2008), thus making them one of the key mediators of antibiotic resistance crisis.

1.6 Conjugative plasmids

Conjugative plasmids are extra-chromosomal autonomously replicating DNA molecules that carry, at minimum, genes required for their multiplication and transfer from a donor to recipient cell. They are usually depicted as circular molecules but also linear conjugative plasmids exist (Meinhardt *et al.* 1997). Plasmids can also be non-transmissible or mobilizable, leading to their spread by cell division or conduction with conjugative elements. According to Smillie *et al.* (2010), about one quarter of the known plasmids in proteobacteria are conjugative and the same portion mobilizable, leaving half of the plasmids non-transmissible. They classified the plasmids based on conjugation and mobilization modules instead of traditional Inc groups that reflect the replication mechanism of a plasmid (Couturier *et al.* 1988). Host-range of conjugative plasmids can be wide as exemplified by IncP-1 and IncPromA-type plasmids that have recently shown to be able to transfer and express genes in all classes of *Proteobacteria* and transfer between gram-negative and gram-positive bacteria in a soil community (Klumper *et al.* 2015) providing platforms for interspecies gene spread. In contrast, narrow host-range plasmids belonging to IncF, IncI and IncX groups are limited mainly to *Enterobacteriaceae* (Norman *et al.* 2009). Conjugation always requires cell-to-cell contact and can occur even across phylogenetic kingdoms (Bates *et al.* 1998). Also, bacteria have shown to be able to exchange genetic material inside mammalian cells (Ferguson *et al.* 2002, Lim *et al.* 2008). The process itself requires type IV secretion system, which consists of Mpf complex providing mating channel, relaxosome and the type IV coupling protein (T4CP) (Smillie *et al.* 2010). The relaxase protein recognizes origin of transfer (*oriT*) and leads to subsequent formation of relaxosome. T4CP attaches relaxome to the Mpf complex.

Conjugation is considered to contribute most broadly to genetic exchange in bacteria, thus playing major part in bacterial evolution (Halary *et al.* 2010). As mentioned above, MGEs, including plasmids, are often more than just plain parasites: they might carry accessory genes that help the host to adapt to prevalent conditions. For instance, virulence genes, antibiotic resistance genes or genes providing metabolic functions are frequently part of conjugative plasmids. Furthermore, these genes are often co-localized on plasmids within translocative elements (Rahube *et al.* 2014). In addition to adaptation, propagation and replication related functions, conjugative plasmid can harbour stability modules. Genes involved in vertical stability include partitioning systems (*parAB*), which distribute plasmids equally between dividing cells, as well as toxin-antitoxin systems (Ogura and Hiraga 1983, Van Melderen 2010).

Latter are also called post-segregational killing systems and they encode two components: stable toxin and labile antitoxin. If the plasmid is not present in the daughter cell after cell division, antitoxin molecules break down before the toxins molecules, leading to cell death. Also, establishment of plasmid in a new cell might induce stability mechanisms that protect the plasmid from restriction endonucleases or inhibit SOS response (Althorpe *et al.* 1999). These modules are clearly beneficial for the preservation of the genetic units themselves and plasmids accommodating them, but can also work as defence systems for the cell or cell community (Dy *et al.* 2014).

It has been reported that as a result of compensatory mutations plasmids persist in bacterial populations also in the absence of positive selection. This could help to explain why these complex extra-chromosomal elements are not lost to purifying selection or why the translocation of accessory genes to chromosome rarely happens (Harrison *et al.* 2015). Plasmids may also speed up the evolution of accessory traits by providing gene multiplication via increased copy number and thus increased opportunity for beneficial mutations. Plasmid mobility is a key variable in dissemination and prevalence of adaptive traits in bacterial populations. It has been shown that plasmid transfer might be affected not only by physical conditions but also by the presence of plasmid-free cells (Lundquist and Levin 1986). Additionally, biofilms i.e. structures formed by adherent bacterial cells are hot spots for conjugation (Hausner and Wuertz 1999). Protozoan predation might also promote conditions for plasmid mobility (Cairns *et al.* 2016).

Relevance of conjugation on antibiotic resistance dissemination is thought to be remarkable since it can occur over 10^5 times more frequently than spontaneous mutations (Waters 1999) and also because single plasmids can carry multiple resistance genes providing one major avenue for emergence of multi-drug resistant strains (Barlow 2009). It is known that resistance genes are everywhere including natural environments that have not had contact with modern medicine (Bartoloni *et al*. 2009). However, anthropogenic action has created areas such as hospitals, wastewater treatment plants, drug factories and farmlands where high selection pressures enrich resistance-carrying elements/strains. Influence of this drastic change in the environment is evident when plasmid samples from pre-antibiotic era pathogens are compared to those of post-antibiotic era, confirming appearance of various types of resistance genes and thus the mobilization of antibiotic resistances in response to global selection pressures (Datta and Hughes 1983). Metagenomic approaches also show clear correlation between abundance of resistant pathogens and high antibiotic usage (Forslund *et al.* 2014). It is however difficult to trace back where resistance genes originate from and how they have ended up in human pathogens (Davies and Davies 2010). Plasmids are indeed complicated platforms of gene transfer and in antibiotic crisis they represent an important route of antibiotic resistance dissemination. A well-documented case are extended spectrum beta-lactamases (ESBLs) where their dissemination via plasmids has extended to global epidemics of gram-negative bacteria carrying different variants of these enzymes.

1.7 Beta-lactamases and the rise of ESBLs

Beta-lactam antibiotics target cell wall synthesizing enzymes, carboxypeptidases and transpeptidases, eventually causing cell lysis. Penicillin belongs to this broad class of antimicrobials that also includes cephalosporin, carbapenem and monobactam groups. Resistance against beta-lactam antibiotics in clinically relevant gram-negative pathogens is primarily mediated by beta-lactamases (Bush and Jacoby 2010). Beta-Lactamase Data Resources of NBCI currently lists 1154 different allozymes of these enzymes along with their resistance profiles (http://www.ncbi.nlm.nih.gov/pathogens/beta-lactamase-data-resources/). This tells a story of a serious global problem. The most problematic variants called extended-spectrum beta-lactamases are capable of hydrolysing third-generation cephalosporins and monobactams, leaving very few options to fight bacteria producing them (Paterson and Bonomo 2005). The most prevalent types of ESBLs are TEM, SHV, and CTX-M (Bush and Jacoby 2010). The former two types are derivatives of SHV-1 or TEM-1 β-lactamase. They have been mainly associated with hospital-acquired infections of *Klebsiella pneumoniae* (Paterson and Bonomo 2005). CTX-M enzymes are not related to TEM or SHV but apparently originate from *Kluyvera* spp. (Humeniuk *et al.* 2002, Poirel *et al.* 2002). They were first reported at the end of 1980s and have been a "success story" ever since. They are now the most frequent ESBL type (Canton *et al.* 2012) and considered the biggest *E. coli* associated threat outside the clinic (Livermore *et al.* 2007, Coque *et al.* 2008, Pitout and Laupland, 2008). Currently over 300 variants of ESBL genes are known (Gniadkowski 2008).

One of the reasons why ESBL producing organisms cause major concern globally is that their prevalence leads to increased usage of last resort drugs like carbapenems and polymyxins. This promotes the spread and emergence of resistances against them and eventually might lead to development of pathogen strains resistant to all known drugs. This is already happening as mobilized colistin (polymyxin and one of the last resort drugs against some *E. coli* and *Klebsiella* infections) resistance gene was identified in China last year (Liu *et al.* 2016). Presence of wide variety of ESBLs also requires detailed knowledge of the resistance gene to avoid treatment failure because resistance profiles can differ remarkably even if variants differ only by a couple of amino acids. Proper diagnostics of ESBL producing bacteria is highly important in early stages of the infection, since empirical treatment can be inadequate in as often as half of the cases and lead to severe consequences, even death (Peralta *et al.* 2012). Furthermore, ESBL-genes are commonly located in large plasmids that often carry other resistance genes as well, including aminoglycosides, fluoroquinolones, and tetracyclines (Canton *et al.* 2012, Tacao *et al.* 2014). This further complicates the situation by reducing treatment options and providing alternative targets for selection to act on.

Increasing trend in the number of ESBL infections has been reported all over Europe during this millennium (ECDC, 2015). Many eastern and southern

countries reach over 25 % prevalence in samples from bacterial infections, whereas in northern countries under 10 % of isolates produce ESBLs, at least for now. In USA a study covering 26 hospitals reported 6.4 % prevalence of ESBL among bacteremia patients (Castanheira *et al.* 2013), whereas study from Latin America found ESBLs from 36.7% of *K. pneumoniae* and 20.8% of *E. coli* isolates (Rossi *et al.* 2008). Even more worrisome numbers come from Asia: over 50 % *E. coli* and *Klebsiella spp.* isolates from ten medical centres in India collected in 2000 were ESBL positive (Mathai *et al.* 2002). More recently ESBL producing bacteria were shown to be responsible of nearly 80 % of blood stream infections in a hospital intensive care unit in New Delhi (Nasa *et al.* 2012). Also, ESBLs have been found from healthy human volunteers (Ben Sallem *et al.* 2010) and animals (Bortolaia *et al.* 2010, Girlich *et al.* 2007, O'Keefe *et al.* 2010), including wild life (Guenther *et al.* 2010, Poirel *et al.* 2012). These findings reflect the dispersal potential and possible reservoirs of ESBL genes and the strains they reside in. Given the constant mobility of people, animals and food, worsening of the epidemiological status might be ahead. Complexity of the situation becomes evident when all the different levels of the problem are considered including clonal enrichment and dispersal of antibiotic resistant bacteria, dissemination and modular structure of MGEs carrying resistance genes, mutation of these genes and the variety of selection pressures in healthcare settings and environment. Detailed knowledge of the resistance genes and plasmids and other MGEs carrying them is essential part of understanding and intervening the emergence and maintenance of antibiotic resistances. Numerous studies map the presence of different variants of ESBL-enzymes and the epidemiological status based on resistance profile. Though, molecular screening methods and sequencing are increasingly used (Brolund *et al.* 2013). These actions alone, however, are not enough for instance to predict which emerging plasmids and resistance genes are the most probable to cause problems. In the second manuscript of the thesis (II) we isolated plasmids from hospital *E. coli* strains, sequenced them as well as estimated their dispersal potential under lethal antibiotic concentrations to evaluate their implications for treatment of ESBL infections and resistance transfer and maintenance.

1.8 Phage therapy

The potential of bacteriophages for treating bacterial infections was recognized already by d'Herelle and trialled in many European countries and Soviet Union. Due to the discovery and establishment of antibiotics and the end of World War II phage therapy was soon forgotten in Western countries, but its development continued in Soviet Union and Eastern Europe (Sulakvelidze *et al.* 2001). Today phage therapy has once more caught world's attention: there are hopes that it could help us to manage with the unintentional but definitely self-inflicted situation of global emergence of multidrug resistant bacteria.

Phages differ from conventional antibiotics in many aspects. Their parasitic life style, including multiplication in the host bacteria, results in complicated pharmacokinetics (Skurnik *et al.* 2007), which requires adjustment of the current drug administration practices like reducing amount of dosages and more customized treatment compared to antibiotics. Also, as bacteriophages form a variable group of biological entities, part of them have life styles prone to spread harmful genes encoding resistances or virulence-associated factors (see above chapter 1.1). Therefore, care should be taken when considering appropriate phages for applications. Strictly lytic (also called virulent) phages that destroy their hosts to complete their life cycle should be chosen for therapeutic purposes because they are rarely associated with horizontal gene transfer (Skurnik *et al.* 2007). Furthermore, the specificity of phages often limits their usage only to some strains of bacterial species. However, this also leads to minimal disturbance of the normal bacterial flora (Loc-Carrillo and Abedon 2011). Compared to the wide target spectrum of antibiotics it is highly advantageous when recovery of patient is considered. Bacteria develop resistance also to phages and their lysis releases endotoxins *in situ*. Phage cocktails (polyphage therapy) are combinations of different phages considered partly to overcome the problem of host specificity and the emergence of resistance, when their components are carefully selected (Chan *et al.* 2013). In addition, they potentially maintain better their efficiency at least partly since each phage has their own optimal conditions.

It is often stated that bacteriophages are enormously diverse and abundant, and they are easy to isolate. All bacteria probably have specific viruses infecting them and geographical distribution of infection patterns suggest that it is at least theoretically possible to find almost anywhere applicable phages for any pathogen, provided that related hosts are present in the environment (Wolf *et al.* 2003, Örmälä and Jalasvuori 2013). Because isolation success can be one of the limiting steps of phage therapy it was explored in this thesis. We evaluated probability of finding phages infecting various medically significant pathogens from an environmental reservoir (IV).

Early days' research lacking controlled and standardly reported trials earned phage therapy a bad reputation (Kutateladze and Adamia 2010). Currently limitations on intellectual property rights as well as regulatory challenges probably hinder the interest of pharmaceutical industry on phage therapy (Chan *et al.* 2013). Nevertheless, in recent years there have been several studies evaluating different aspects of the polyphage therapy, some reaching also human clinical trials (Wright *et al.* 2009). Both promising and less promising effects on both infection clearance and density reduction of many clinically relevant pathogens have been reported including *E. coli* (Bach *et al.* 2009, Rozema *et al.* 2009, Oliveira *et al.* 2010, Rivas *et al.* 2010, Maura *et al.* 2012), *K. pneumoniae* (Kumari *et al.* 2009b, Gu *et al.* 2012), *P. aeruginosa* (McVay *et al.* 2007, Kumari *et al.* 2009a, Hawkins *et al.* 2010, Alemayehu *et al.* 2012, Hall *et al.* 2012) and *Salmonella* sp. (Andreatti Filho *et al.* 2007, Borie *et al.* 2008, Wall *et al.* 2010, Hooton *et al.* 2011). There is also some evidence that synergistic usage of

antibiotics and phage therapy could be beneficial in infection control (Comeau *et al.* 2007, Kaur *et al.* 2012, Kirby 2012).

The scope of phage therapy research has widened beyond treatment of bacterial infections. There are already phage products for food safety applications both commercially available, such as Listex™ P100 (FDA 2006, Sharma 2013), and under research (Anany *et al.* 2011, Viazis *et al.* 2011a). Phages are also considered to be used as surface disinfectants (Roy *et al.* 1993, Abuladze *et al.* 2008, Fu *et al.* 2010, McLean *et al.* 2011, Viazis *et al.* 2011b). In other applications, they are engineered to carry antibiotics to specific targets (Vaks and Benhar 2011) or boosted with enzymes promoting access to their receptors (Lu and Collins 2007, Scholl *et al.* 2005). Phage lytic enzymes are used as such or as modified versions against gram-positive pathogens (Fischetti 2010).

Given that plasmids are often vehicles of resistance dissemination they have been suggested to be potential targets for counteractions (Williams and Hergenrother 2008). Plasmid-dependent phages provide additional strategy to fight antibiotic resistances. They specifically recognize plasmid-encoded proteins to enter the cell (Caro and Schnös 1966) and thus in principle share the host-range of the plasmid. Plasmid-dependent phage PRD1 has been shown to select for loss of antibiotic resistances conferring conjugative plasmid RP4 or in some cases for losing its' conjugation ability (Jalasvuori *et al.* 2011). In a subsequent study PRD1 was able to reduce the spread of plasmid-mediated resistances even in the presence of sublethal antibiotic concentrations favouring the transfer of plasmid (Ojala *et al.* 2013). In this thesis an evaluation of evolutionary consequences of phage and antibiotic induced contradicting selection pressures was conducted by determining if the resulting loss of plasmid conjugation ability is irreversible and if not, is the phage susceptibility restored as well (III). Like the use of antibiotics, phage therapy might have consequences outside the clinic. Therefore, and for above discussed reasons, it has to be thoroughly evaluated before put into action.

2 AIMS OF THE STUDY

The aim of this study was to investigate small genetic replicators, viruses and plasmids, from structural level to practical aspects. Spread of antibiotic resistances among pathogenic bacteria motivated us to explore different approaches to the phage therapy and to analyze conjugative plasmids isolated from multi-resistant *E. coli* strains. Virus particles of well-characterized membrane-containing phage PRD1 were studied to unravel its (membrane) protein interactions. Specific aims and questions set for each chapter were:

I To study protein interactions in PRD1 using Blue Native PAGE (BN-PAGE) and zymogram analysis of packaging deficient mutant particles

II To characterize nosocomial ESBL strains derived plasmids genetically and to study their horizontal transfer dynamics under lethal antibiotic selection

III To assess sustainability and evolutionary obstacles of using plasmid-dependent phages to restrict the spread of plasmid-encoded antibiotic resistance

IV To study feasibility of on-demand phage isolation against different species of hospital derived antibiotic resistant bacteria

3 MATERIALS AND METHODS

The bacterial strains, viruses, and plasmids used in this thesis are found in the original publications and manuscript (Roman numerals). The methods used in this thesis are summarized in Table 1. More detailed description of each method is found in the original publications and manuscript (Roman numerals).

TABLE 1 Methods used in this thesis.

Method	Publication
Antibody production	I
Biochemical dissociation of virus particles	I
BN-PAGE	I
Bradford assay	I
Molecular cloning and polymerase chain reactions	I
N-terminal amino acid sequencing and mass spectrometry	I
Production of recombinant proteins	I
Protein purification	I
Purification of viruses	I
SDS-PAGE	I
Viral genome isolation	I
Western Blotting	I
Zymogram analysis	I
Plaque assay	I,III,IV
Propagation of viruses	I,III,IV
Virus isolation	IV
Agarose gel electrophoresis	I,II
Nucleotide sequencing and annotation	I,II,III
In vitro evolution experiments	II,III
Comparative genomics	II,III
Colony assay	II,III
Colony PCR	II,III
Conjugation assays	II,III
Statistical analysis	II,III
Phage resistance tests	III

4 RESULTS AND DISCUSSION

4.1 Updating our view of protein interactions in PRD1 (I)

4.1.1 Cloning of gene *XV* for expression and purification of GST-P15 and production of polyclonal anti-P15

The aim was to express and purify protein P15 of PRD1 for producing a polyclonal antibody against it. First purified DNA of PRD1 wild-type (wt) was used as a template to amplify gene *XV* in PCR. The gene sequence was cloned into vector pGEX-4T-3 (GE Healthcare) provided with tobacco etch virus (TEV) cutting site. Sequencing of the cloned gene revealed a point mutation changing the sixth amino acid of the end product from tryptophan to leusine. Because polyclonal antibodies recognize many epitopes of the target protein this was not considered to remarkably interfere the production of anti-P15. Mutations might affect the folding (Lorch *et al*. 1999) of the protein but the activity of purified recombinant-P15 was confirmed in zymogram analysis (see below), thus indicating preserved functionality and potentially the correct folding of the protein.

Plasmid pGEX-4T-3 TEV provided a glutathione S-transferase (GST) tag for the P15 and enabled purification by binding to glutathione sepharose matrix. TEV linker region combined the components of recombinant-P15. Removal of the tag with TEV protease was confirmed in SDS-PAGE and followed by gel filtration. Purity of the product was estimated to be over 90 % from an SDS-PAGE gel using automated software (Quantity One 1-D analysis software, Bio Rad). Mass spectrometry analysis confirmed the identity of the protein prior to commercial antibody production. Immunization was done in a rabbit and the resulting serum was tested for its activity against purified PRD1 wt virus particles and mutant virus particles lacking P15. The former was the only one to give a signal in a Western blot at ~15 kilodalton (kDa) which is close to the estimated ~17 kDa size of P15. Also, preimmune serum collected before immunization did not give any signal when used at similar ratio as immunized serum. These analyses confirmed the functionality of anti-P15 in Western blot

assay. Given the 90 % purity of the original protein preparation it is possible that also other components (remnants of GST tag or impurities from *E. coli*) raised antibody production in the rabbit. However, a purified virus was used in all of the following experiments, minimizing the possibility of false positive results.

4.1.2 Zymogram analysis excludes P15 from the special vertex

Zymogram gel analysis provides a qualitative method to identify peptidoglycan degrading enzymes (Bernadsky *et al.* 1994). Denatured protein samples are run into a standard SDS-PAGE gel containing peptidoglycan isolated from a bacterium of interest. After a gel run, proteins are renatured in potassium phosphate buffer containing 0.2 % Triton X-100. Staining with 0.1 % (w/v) methylene blue /0.01 % (w/v) KOH leaves clear zones as indication of peptidoglycan hydrolysing enzyme activity. As the proteins are originally separated according to their size, identification is possible according to adequate controls and/or standards.

We produced and purified PRD1 mutant viruses devoid of the membrane protein P20 to investigate if the presence of the packaging vertex is needed for P15 incorporation. These virus particles, Sus400 (amber mutation in gene XX) and Sus526 (amber mutation in gene XX), are known to miss also other proteins localized to the DNA packaging vertex: the ATPase P9 and packaging associated protein P6 (Strömsten *et al.* 2003). Consequently, they are incapable of packaging DNA. Amber mutation introduces an Amber stop codon within a gene sequence resulting in disruption of translation of messenger RNA representing the gene in question (Belin 2003). The previous publication suggested that P15 interacts with P20 and is part of the packaging vertex (Rydman and Bamford 2002). However, later it was revealed that mutant viruses used in the experiment contained mutations in other genes than *XX* affecting this result (Strömsten *et al.* 2003). The Amber mutants we used (*sus400* and s*us526*) in our zymogram analysis have been shown to have amber mutations only in gene *XX*. Zymogram analysis revealed that both P20 mutant particles contain two peptidoglycan-degrading proteins, identified as P7 and P15. This strongly suggests that P15 is not part of the special vertex complex because interaction with P20, P9 or P6 is not needed for its inclusion in the particle. Recent single particle electron cryo-microscopy analysis of PRD1 packaging mutant virus particles also supports this conclusion (Hong *et al.* 2014). Comparison of mutant and whole particle density maps suggested that membrane proteins P20 and P22 as well as ATPase P9, packaging assisting protein P6 and terminal protein P8 form the packaging vertex. Zymogram analysis might sometimes give false positive results if proteins without lytic activity bind to peptidoglycan, thus preventing dye binding (Kohler *et al.* 2007). Previous mutant analyses confirm that this is not the case with PRD1 particles since mutants devoid of lytic enzymes also lack clear zones at corresponding size in the gels (Rydman and Bamford 2002).

4.1.3 BN-PAGE can be used to unravel protein interactions in PRD1 particles

Membrane proteins are challenging to study for several reasons. Production of membrane proteins in bacterial cells often results in aggregates accumulating in cytoplasm (Seddon *et al.* 2004). Due to their hydrophobicity membrane proteins are often insoluble in aqueous solutions, which has hampering consequences on sample preparation for common structural and functional techniques such as nuclear magnetic resonance and X-ray crystallography. Many viruses contain a membrane component (Atanasova *et al.* 2015). Commonly, many of the membrane proteins are not symmetrically distributed even if the virions contain ichosahedral capsid outside or inside the membrane. Additionally, the complexity, heterogeneity and dynamicity of these environments limits the application of the above-mentioned techniques for studying these parts of virus particles. One of the aims set in this thesis was to study protein interactions in PRD1 virion with focus on membrane proteins using BN-PAGE. We started from intact purified virions and tried to disrupt them partly to preserve interactions within detached protein complexes in order to use them in further analyses.

BN-PAGE is a method originally used to study mitochondrial membrane protein interactions (Schägger and von Jagow 1991). It is based on disruption of protein membrane structures using detergents and separation of the resulting complexes by size in a native polyacrylamide gel. Coomassie brilliant blue dye is used to negatively charge the molecules. A second dimension gel is applied to separate constituent proteins of the complexes in denaturing conditions (SDS-PAGE). We used Western blotting for recognition of the proteins, because there are antibodies available against most of the PRD1 structural proteins (P2, P3, P5, P6, P7/P14, P9, P11, P16, P22 and P31, see Fig. 1 for schematic representation of PRD1 virion). Alternatively, mass spectrometry could have been used (Wessels *et al.* 2009). In order to gain variable protein complexes three different detergents were used in virus disruptions: SDS, Digitonin and N-dodecyl-β-maltoside (DDM). First of them is an anionic detergent also used in complete denaturation of proteins for instance in protein gel sample preparation. We used very low concentrations of SDS because this way it has been shown to be applicable for BN-PAGE sample preparation (Klodmann *et al.* 2011). Digitonin and DDM are non-ionic milder detergents and more commonly used for BN-PAGE applications (Wittig *et al.* 2006). We used previous disruption studies of PRD1 as a reference in planning suitable conditions for sample preparation (Caldentey *et al.* 1993b, Luo *et al.* 1993) in addition to the adjustment procedures previously described for BN-PAGE (Reisinger and Eichacker 2007). Maximal visible protein complex amounts in the first dimension gel were obtained with 20 min 0.1 % SDS treatment, 10 min 1.0 % Digitonin and 10 min 2.0 % DDM treatments (at 4 °C, 70 °C and 70 °C, respectively).

Two main findings of this study were the detection of the receptor recognizing vertex complex after SDS treatment and putative complex of the transglycosylase P7 and membrane protein P14 after the DDM treatment. First

one indicated that our method is working, since the structure of the receptor recognizing vertex has been resolved before (Abrescia *et al.* 2004). Sizes of the vertex protein complexes ranged from 372 to 694 kDa. Distribution of antibody labels in the second dimension gels indicated that the smallest of these complexes contained only the proteins shown to build the receptor recognition vertex structure i.e. the receptor-recognizing protein P2, spike protein P5 and membrane proteins P16 and P31, the latter one forming the pentameric base of the vertex. Protein sizes and numbers in this complex are known and its estimated size is about 300 kDa which is very near the sizes of the smallest two complexes detected by BN-PAGE (372 and 398 kDa). Capsid forming protein P3 was part of the bigger vertex complexes, which have been detected in previous disruption studies to detach partly from the virion along with the surrounding P3 (Luo *et al.* 1993). This loss of peripentonal trimers from nearby vertices has recently been suggested to be part of the natural process of tube formation (Peralta *et al.* 2013). Disruptions with other detergents also resulted in complexes containing part of these proteins in different combinations. This most probably reflects differing effects of treatments in protein interactions in the vertex structure. Complex of P7 and P14 was detected after DDM treatment. These proteins are encoded by the same gene *VII*. Gene *XIV* resides at the gene's 3′ terminal. This has been suggested to result from capturing a transglycosylace-encoding region from a host cell (Rydman and Bamford 2000). The proteins encoded by these genes predictably have multimerization signal encoded by the 3′ end of the gene *VII* leading to heteromultimer formation. This is in accordance with the gained results and also the estimations of 20 copies of P7 in each PRD1 virus particle as well as similar amount of P14 that could form 84-kDa complexes containing two of each protein fit well into the estimated size of 75 kDa for the obtained complexes.

We did not find other obvious protein complexes. Proteins of unique packaging vertex appeared in monomer zone (size under 66 kDa) after each disruption. This might be an indication of the bi-functionality of the capsid as a vehicle of the virus genome between the intracellular phases of the life cycle: capsid has to protect the genome from the environment but on the other hand it must be labile enough to deliver the genome when an appropriate host is encountered. Consequently, it could be that some protein interactions cannot be preserved outside the whole virion structure. Knowledge of the assembly process could give indication of this. For instance, studies on PRD1 amber mutants suggest that P6 and P9 are the final proteins (along with the terminal protein) to be added to the virus particles. Therefore it is possible that they only interact with membrane proteins P20 and P22 during the packaging and in the complete virion. In this case it would probably be difficult to detach them from the particle as a complex and thus detect this interaction using BN-PAGE. Other feasible explanation might be that the obtained complexes were so small that the resolution of the used gels was not enough to resolve them. Smaller gradient range for the gels could help to overcome this.

Another interesting result was that proteins P3 and P11 were found in almost all of the obtained complexes. This is logical because both proteins

surround the membrane and then probably have interactions with many of the membrane proteins either directly or indirectly. P3 forms the outer capsid and P11 predictably covers the whole membrane underneath the capsid, since it causes aggregation of the membranes (Bamford and Mindich 1982). Also, P15 was only seen as a monomer after SDS disruption. No further information of its localisation in the virus particle was thus recovered from BN-PAGE analysis. Given that we did not have antibodies against all structural proteins, it is possible that the observed protein complexes contained additional proteins that could not be detected here.

The obtained results show that BN-PAGE can be utilized to resolve protein interactions in PRD1 virion. However, stability of the capsid poses an interesting problem: Detergent treatments harsh enough to reach the membrane might be too harsh to preserve the membrane protein interactions. It could be possible to circumvent this problem by analysing the isolated membrane vesicles by using PRD1 mutants missing the aggregation factor P11 (Bamford and Mindich 1982). This is accomplished by guanidine hydrochloride treatment and gradient centrifugation of purified mutant virus particles. Then of course interactions of capsid proteins with the membrane would be lost. Downsides of BN-PAGE include enormous multiplication of gel runs and especially Western blotting procedures whenever one additional condition is added. Even though analysis of first dimension complexes by mass spectroscopy could ease the job, it might still take a lot of time to gather relevant information because this method is based on screening. Combination of different methods is therefore essential to eventually solve protein puzzle of PRD1. Additional methods to be used in the future could utilize recombinant proteins for fluorescence resonance energy transfer technique (Sourjik *et al.* 2007) or bacterial two-hybrid system (Karimova *et al.* 1998). These approaches, however, have their own downsides because virus proteins would then be removed from their natural context unable to interact all the other virus-encoded proteins present during the infection. Additionally, cryo-EM imaging techniques currently take leaps towards better resolution (Baker *et al.* 2013, Zhou 2014, Sun *et al.* 2015) and might eventually be used to reveal new protein structures and interactions in PRD1 virion.

4.2 Plasmids and plasmid-dependent phages in antibiotic resistance

4.2.1 Efficiency of evolutionary rescue via conjugation under lethal antibiotic selection depends on plasmid type (II)

It has been estimated that human body carries 10^{14} bacteria (Sekirov *et al*. 2010). In addition to opportunistic pathogens these include symbionts and commensals. Human gut microbiota is a major reservoir of antibiotic resistances and thus a potential source of these genes for pathogens (van Schaik 2015).

During the treatment of bacterial infections, despite the usage of above MSW antibiotic concentrations to prevent mutations, the existing resistance determinants can provide problems in several ways: Pathogens already harbouring resistance genes have to be eradicated with antibiotics that they are susceptible to, sometimes limiting treatment options. In some cases the surrounding microbial community can rescue otherwise susceptible bacterial pathogens. For instance, β-lactams can be inactivated by co-operating β-lactamase-producing bacteria leaving alive a population of so-called susceptible cheaters. The two populations reach equilibrium in the initial antibiotic concentration in cell-density dependent manner (Perlin *et al*. 2009, Yurtsev *et al*. 2013). Furthermore, a conjugative plasmid carrying a β-lactamase gene can transfer to susceptible strains even in the presence of lethal antibiotic selection (Ojala *et al*. 2014). This leads to generation of genuine novel co-operators (i.e. resistant bacteria) in an evolutionary rescue situation. Evolutionary rescue means adaptive evolution that prevents extinction of a population in a new (altered) environment (Gomulkiewicz and Holt 1995). In order to extend our previous study, we investigated if conjugative plasmids derived from hospital ESBL-strains could rescue susceptible bacteria in the presence of lethal β-lactam concentrations.

Conjugative plasmids are selfish genetic elements that can act against the interests of their host cell to enhance their own survival in a bacterial community (Jalasvuori 2012). Their life-style exposes them to multilevel selection: plasmid maintenance induces a fitness-cost. Between-cell competition selects for reduced cost whereas within-cell competition promotes strategies to ensure the plasmid survival (discussed above in chapter 1.6) often leading to increased burden for cell reproduction. To balance these opposing demands plasmids can carry accessory genes providing the host bacteria means to survive in specific environmental conditions. Sometimes these genes also contribute to the common good of the bacterial community thus enabling the survival of competing bacteria like for instance in the case of β-lactamases. This contradiction could be explained several ways. First, it might be of selfish interest of the plasmid to provide existence conditions for future hosts. Secondly, the genes themselves might benefit from locating in transferable elements because they provide increased assortment (Dimitriu *et al*. 2014). Thus selection favors cooperation on community level promoting conjugative transfer of plasmids.

We conjugated several plasmids from hospital derived *E. coli* strains, which had caused multi-resistant infections, first to HMS174 (Campbell *et al*. 1978) and then to JM109(pSU18), both of which are *E. coli* K12 strains. We selected five resulting strains that different plasmid contents based agarose gel electrophoresis for further characterization. According to the earlier given names of the strains (IV), plasmids were named pEC3, pEC13, pEC14, pEC15 and pEC16. Plasmids were conjugated once more to HMS174 to exclude the pSU18 before their isolation and sequencing. Sequence assembly revealed that pEC13 strain contained one plasmid, pEC14 three and rest three all had two plasmids. Annotation of plasmid genes was done automatically and their

sequences were compared to NCBI database. Highly similar sequences (≥ 89 %) were found for all but one plasmid, pEC14III. Plasmids pEC15II and pEC16I resembled those found earlier from retail meat in the Netherlands and chicken in Switzerland, respectively (Johnson *et al*. 2012, Wang *et al*. 2014). This might be an indication of transmission of the strains or plasmids between livestock and humans. All the plasmid sequences were screened for antibiotic resistance genes. One β-lactamase encoding gene per each strain was found. pEC3I, pEC14I and pEC15II carried genes TEM-1C, TEM-1B and TEM-52B, respectively. CTX-M-14 was found from plasmid pEC13. Plasmid pEC16I encoded gene SHV-12. Therefore, all prevalent types of ESBLs were represented in these plasmids (Bush and Jacoby 2010). We also determined the mobility (MPF) and relaxase (MOB) group of each plasmid using reference sequences (Shintani *et al*. 2015), BLASTx (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Geneious 9.0.5. More thorough analysis of plasmids is depicted in Table 2. I focus here mainly on discussing the transfer potential of the different plasmid combinations under lethal antibiotic selection.

TABLE 2 Plasmid features. Alterations to plasmid size due to shufflon area are indicated in parenthesis.

Plasmid	Plasmid size bp	Inc type	MPF type	MOB class	β-Lactamase identified	Other resistance genes
pEC3I	91 885	*IncB/O/K/Z*	MPFI	MOBP	*blaTEM-1C*	*strA, strB, sul2*
pEC3II	59 192 (59 192)	*IncI2*	MPFT	MOBP	-	-
pEC13	71 656	*IncFII*	MPFF	MOBF	*blaCTX-M-14*	-
pEC14I	143 590	*IncFII, IncQ1, IncP, IncFIB(AP001918)*	MPFF	MOBF	*blaTEM-1B*	*strA, strB, aadA1, mph(B), sul1, sul2, tet(A), dfrA1*
pEC14II	87 848 (87 666)	*IncI1*	MPFI	MOBP	-	-
pEC14III	80 057	*IncFII*	MPFF	MOBF	-	-
pEC15I	87 811 (87 767)	*IncI1*	MPFI	MOBP	-	-
pEC15II	38 611	*IncX1*	MPFT	MOBQ	*blaTEM-52B*	-
pEC16I	94 325 (95 380)	*IncI1*	MPFF	MOBP	*blaSHV-12*	-
pEC16II*	7 939	*ColRNAI*	-	MOBP	-	-

* non-conjugative mobilizable plasmid

We tested the transfer frequency of all five plasmid combinations to antibiotic susceptible cells under lethal ampicillin concentrations of 15 μgml^{-1}, 75 μgml^{-1} and 150 μgml^{-1}, and compared the results to a treatment without antibiotics (Fig. 2).

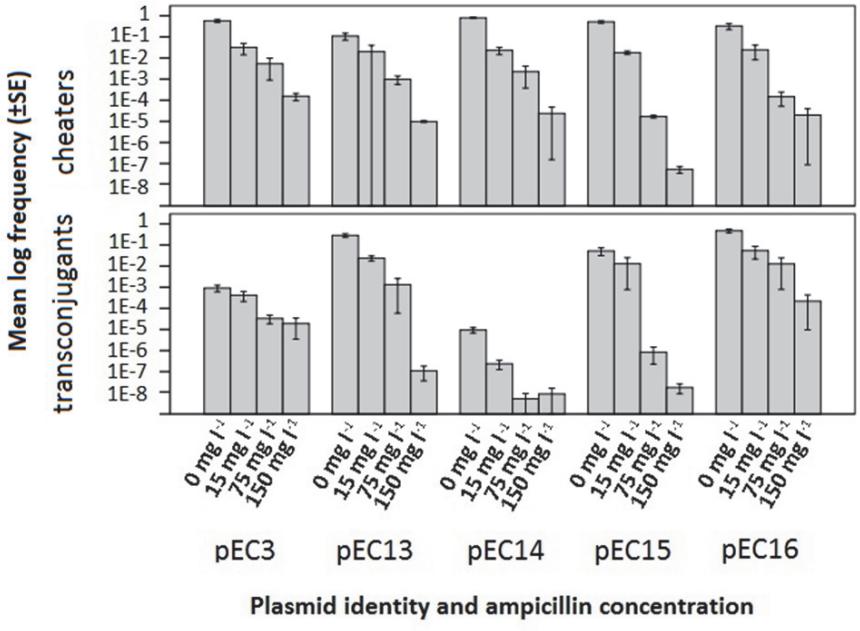

FIGURE 2 Frequency of transconjugants and cheaters (HMS174) after 24 h in different ampicillin concentrations at 37 °C (n=3).

We used *E. coli* strains JM109(pSU18) and HMS174 as a plasmid donor and recipient, respectively. The co-culture experiments were let to grow for 24 h. In the bacterial population originally harboring the plasmid, co-operators were able to sustain smaller cheater (bacterial cells not capable of producing β-lactamases themselves) population in all five experimental set-ups. Expectedly, this population was reduced when antibiotic concentration increased. Proportions of cheaters at the end of the experiment were not significantly different between the treatments, except that pEC15 provided lower levels of cheaters at higher antibiotic concentrations. This suggests that TEM-52B enzyme was not as efficient in degrading the antibiotic whereas all of the other β-lactamases provided higher hydrolyzing capacity. HGT frequency varied substantially between the plasmid combinations. Notably, pEC14 evolutionarily rescued susceptible bacteria very poorly in all of the treatments. This could reflect the fact that larger plasmids usually are less mobile (Smillie et al., 2010) and indeed three large plasmids were mobilized from strain EC14. Plasmid pEC3 also conjugated inefficiently without antibiotics but kept the frequency relatively well at higher antibiotic concentrations. The rest of the plasmids transferred efficiently in the absence of antibiotics but their evolutionary rescue potentials started to differ remarkably at above 15 $\mu g ml^{-1}$ ampicillin concentrations. Plasmid pEC15 provided substantially lower transconjugant

frequency in the higher antibiotic concentrations probably owing to its diminished ability to sustain cheaters. In the case of pEC16 conjugation as a rescue mechanism was enhanced in higher antibiotic concentrations whereas the rescue potential of plasmid pEC13 was almost abolished in the highest ampicillin concentration tested. These results suggest that neither the amount of cheaters was an indication of the evolutionary rescue potential of the plasmids nor did the classifiable plasmid backbone determinants, such as MOB or MPF family, correlate with it. Solving the underlying molecular mechanisms of conjugation could shed more light on evolutionary pressures acting on these plasmids. The regulatory aspects of the plasmid genes were not investigated in this study and their role in plasmid transfer calls for further research. Comparative genomics of transfer regions might give some insight to this.

Efficiency of transfer may vary remarkably between different bacterial strains. In a recent study resolving the plasmid transfer in soil bacterial community, phylogenetic distance or abundance of the recipient strain did not explain the immediate transfer range of the wide host-range plasmids (Klumper *et al*. 2015). The donor strain, however, had an effect. Additionally, ESBL-plasmids have shown not to affect the fitness of some strains belonging to pandemic *E. coli* lineages (Schaufler *et al*. 2016). Part of them also had an effect on chromosomally encoded virulence associated factors. This could partly explain their association with epidemics. Also, several factors can increase plasmid transfer frequency, like heterogeneous communities in the presence of efficient donors (Dionisio *et al*. 2002) and biofilms (Hausner and Wuertz 1999). Single-host environment can result in a fitness trade-off in an evolved plasmid. However, this can be circumvented in multi-host environment (Kottara *et al*. 2016). Thus it should be acknowledged that the behavior of each plasmid is always inflicted by its own evolutionary history coupled to that of its hosts following that our results might be applicable only in the current genetic background of the plasmids. It would be interesting to study this further by testing the conjugation efficiency and fitness cost of all the plasmid combinations in different donors and recipients. Also, these parameters could be measured for the original isolates.

Antibiotic as a concept is rather anthropogenic. Any molecule inhibiting bacterial growth can be determined as antibiotic. Therefore, when discussing the emergence and evolution of antibiotics and antibiotic resistances, it should be noted that suggestions of their biological roles in natural environments are variable including signaling, competition and predation (Sengupta *et al.* 2013). When the evolutionary paths of the spread of resistance genes are considered, variability may emerge simply because of the antibiotics' differing mechanisms of action. Therefore, resistance genes could provide very different ecological and evolutionary implications. For instance, ion pumps only protect the resistant individuals themselves because they do not inactivate the drug molecules. In the case of beta-lactamase enzymes it is possible that resistance-carrying bacteria quickly reduce concentrations of beta-lactams below lethal levels. Reason for this could be the relatively fast kinetics of the reaction leading to quick eradication of the antibiotic (Matagne *et al*. 1998), which probably was

reflected in our experiment by the small differences in survival of the susceptible cells. However, in *in vivo* situation it is possible that this happens at different rate which can change the observed dynamics. Also, antibiotics have different targets in the cells causing variable consequences. Same rescuing effect was not seen in kanamycin treatment using RP4 plasmid even though also this resistance was provided with enzymatic degradation of the antibiotic molecules (data not shown). For these reasons the results obtained here cannot be generalized but this kind of evolutionary rescue mechanism might well apply to some of the other antibiotic-resistance pairs and should be considered in treatment practices.

Our study highlights that preventing mutations leading to antibiotic resistance is not enough. The global resistome can be within the reach of the pathogens if these genes reside in conjugative plasmids harbored by the surrounding bacterial community. Since ESBL genes are often carried in these elements (Bush 2010) and hospital patients in turn might be colonized by ESBL-expressing bacteria on admission (Harris *et al*. 2007, Valverde *et al*. 2008) HGT could compromise the treatment success of some of these infections. All in all, knowledge of the dynamics of conjugative plasmids could help us to understand and interfere the spread and promotion of resistances. Alternative treatment options for antibiotic resistant infections are urgently needed. Bacteriophages could provide such an option both in terms of eradicating the infections or preventing the spread of resistance carrying conjugative plasmids as discussed below.

4.2.2 Plasmid dependent lytic phages could be a sustainable tool against the spread of plasmid encoded antibiotic resistances (III)

For reasons discussed above, it is impossible to entirely block antibiotic resistance development. Despite this it has been the main focus in medical research of antibiotic resistance almost the entire antibiotic era. However, the so-called eco-evo strategies that aim either to restore susceptibility of bacterial populations or to prevent the emergence of new resistant organisms could be more sustainable solution and should thus be increasingly investigated (Baquero *et al*. 2011). Limiting the horizontal gene transfer has been suggested as one such option (Williams and Hergenrother 2008).

Plasmid-specific phages recognize plasmid-encoded structures on the surfaces of bacterial cells. For instance, phages belonging to *Leviviridae* family with ssRNA genomes as well as filamentous DNA phages represented by f1 all use plasmid-encoded pili to attach to and infect cells (Rumnieks and Tars 2012, Caro and Schnös 1966). PRD1 has been shown to need the protein complex required for Mpf (maiting pair formation) for its entry but no individual receptor protein has been identified so-far (Grahn *et al.* 1997). All these phages provide selection pressure against both the plasmid encoding these structures and the host cells carrying the plasmids. Non-lytic filamentous phage M13 and its protein pIII have been successfully used to block the pilus from initiating conjugation (Lin *et al*. 2011). Jalasvuori *et al.* (2011) showed that the presence of

plasmid-dependent phage PRD1, whether added once or continuously (every 24 h), reduced the presence of plasmid harboring cells in a ten-day serial culturing experiment on average by 95 %. They used two wide host-range plasmids, RP4 and RN3, both providing receptors for PRD1. Bacterial cells still containing the plasmid at the end of the experiment were shown to be conjugation deficient. Ojala *et al*. (2014) set-up an evolutionary experiment to test how the presence of non-lethal but growth reducing antibiotic concentrations providing different selection pressures affects transconjugant emergence rate in the presence of PRD1 using *E. coli* K12 strains JE2571(RP4) and HMS174 as a donor and recipient, respectively. Levels of these multi-drug resistant transconjugants were reduced by multiple orders of magnitude even after three days under antibiotic selection favoring either donor or recipient bacteria, as well as in the absence of antibiotics. Even when double antibiotic treatment putting selection pressure for transconjugant formation and against both original strains was used, the presence of PRD1 kept the prevalence of multi-resistant bacteria significantly lower on first experimental day compared to that of control treatment without the phage. After this their quantities reached those observed in phage free treatment. Transconjugants from this treatment were further investigated. Minority (35 %) of them were able to conjugate to chloramphenicol-resistant *E. coli* strain, JM109(pSU19). This is in accordance with previous studies showing that PRD1 selection can induce RP4 mutants that have reduced or completely lost their conjugation ability (Kornstein *et al*. 1992, Grahn *et al.* 1997, Jalasvuori *et al*. 2011). Furthermore, non-conjugative clones were exposed to reversion favoring antibiotic selection for three days in the presence of recipient strain JM109(pSU19) (Bartolome *et al*. 1991). They produced very low levels of transconjugants in two of the five experiments while none was observed in the rest. Although these results are promising in terms of usage of plasmid-dependent phages to limit the horizontal spread of antibiotic resistances several open questions remain about the implications of phage-resistant plasmid-harboring phenotypes: Are the PRD1 induced conjugation-deficient mutants able to regain their conjugation ability? If so, what happens to phage susceptibility? How underlying genetic changes causing the conjugation deficiency affect the outcome? In this thesis we set to investigate these questions.

We wanted to study both previously observed phage resistant plasmid phenotypes i.e. the so-called conjugation deficient and semi-conjugative in comparison to ancestral phenotype. We introduced plasmid carrying strains JE2571(RP4) to phage selection inducing loss of plasmids/loss of conjugation function accompanied with antibiotic selection that favoured the plasmid maintenance for 48 h. We then selected resulting conjugation deficient and semi-conjugative clones for further analysis. Latter of which were measured to have approximately 2.5 orders of magnitude lower conjugation rate compared to ancestral phenotype. Two representative plasmids of each type were sequenced to identify the mutations leading to the observed phenotype. Their conjugation efficiency and its reversibility were determined in a five-day serial-passage culturing experiment in the presence of growth reducing antibiotic

concentration that favoured the formation of transconjugants. This was compared to a treatment without antibiotics. All non-conjugative phenotypes resulted in very low levels or absence of transconjugants. Semi-conjugative clones produced transconjugant levels comparable to the ancestral plasmid in the presence of antibiotic selection. To check if this was a result of reversion rather than transconjugant generalisation due to selective advantage we tested the end-point transconjugant clones for their phage susceptibility and their conjugation rate. This was the case with one of the semi-conjugative plasmids. There was always a correlation between phage susceptibility and higher conjugation rate implying that reversion of mutation also restored sensitivity to the phage treatment and vice versa. Sequence analysis of the plasmids revealed changes in conjugal transfer Tra2 region encoding 11 genes, 10 of which are involved in Mpf complex in conjugation and PRD1 propagation (Haase *et al*. 1995, Grahn *et al*. 1997). Only non-essential component in this complex is the entry exclusion factor TrbK (Haase *et al*. 1996). Two non-conjugative clones both contained several nucleotide deletions in the gene *trbJ* encoding a periplasmic protein (Grahn *et al*. 2000). Semi-conjugative plasmids possessed a transposon insertion between genes *trbJ* and *trbK* and a 22-nucleotide duplication in gene *trbI* encoding a cytoplasmic MPF complex protein (Grahn *et al*. 2000). In the last case reversion of full plasmid conjugation rate was detected. This was also seen in sequence level since revertants had lost the tandem repeat reflecting a dynamic mutation (Treangen *et al*. 2009). This kind of mutation probably allows rapid enough change in geno- and phenotype in response to changing selection pressures. Thus in our study this type of mutation was the only one providing also possibility to revert back to previous genotype.

Previous study on bacteria developing resistance to PRD1 demonstrated a wide variety of mutations in RP4 Tra2 region, excluding *TrbK* (Grahn *et al.* 1997). Bacteria were plated with large amount of PRD1 and resistant colonies were then picked, grown and analysed. It is possible that all these mutations detected by Grahn *et al.* (1997) also arose in our experiment but because we did not screen more mutants this remains unknown. In the plating experiment mutations accumulated mainly in three genes (*trbC*, *trbE* and *trbL*). Interestingly, the mutations that we detected were in genes that rarely contained mutations in plating experiment. This might reflect the set-up of our experiment. Also, it is probable that our experiment would better reflect real-life situation, because plating restores any mutation providing the phage resistance since bacteria grow as localized colonies and no competition against the other bacteria happens. In natural environment, bacteria do not grow as individual colonies and within community competition quickly eradicates low fitness variants. This probably also happened, at least in some extend, during our experiment. In the future it might be interesting to investigate if mutants covering all the Tra2 region genes could be found in our set-up.

An obvious next step would be to study if these results could be replicated within an *in vivo* animal model and eventually followed by evaluation in actual patients. Of course it is also possible that our observations not necessarily reflect how other plasmid-dependent phages induce changes in their hosts and

for this reason it would be interesting to test this experimental set-up changing all of its components i.e. antibiotics providing counteracting selection pressures, plasmid, virus and bacterial strains. For instance, in above described M13 experiments the donor strains did not lose their conjugation ability. It is therefore uncertain if non-lytic phages could be used in a similar way as PRD1 and if the observed transient prevention of conjugation would be enough to provide time for the immune system to clear the infection. However, other studies with M13 show that a conjugative plasmid can be lost under phage selection and thus highlight the importance of optimal dosage and conditions (Palchoudhury and Iyer 1969). Also, our data suggests that simultaneous usage of antibiotics and phages, i.e. presence of counteracting selection pressures, often leads to non-conjugative and conjugative plasmid types resistant to phage infection whereas phage alone mostly induces plasmid loss (Jalasvuori *et al*. 2011). During the treatment of bacterial infections it would be of utmost importance that such therapeutic strategy would be chosen that minimizes adverse outcomes such as transfer of resistance genes to pathogenic bacteria. Therefore, based on our data it would be advantageous to administer plasmid-dependent phages alone or prophylactically. It is not however always possible to delay or avoid antibiotic exposure. Even in the presence of both phage and antibiotics, reversions in the resulting conjugation deficient and semi-conjugative plasmids were improbable, except in the case of dynamic tandem repeat insertion. However, in the case of reversions also the phage susceptibility returned. These findings indicate that utilization of plasmid-dependent phages could serve as a functional and sustainable anti-conjugation therapy.

4.3 Phage isolation success differs between bacterial host species (IV)

Phage therapy has been suggested as one potential alternative for conventional antibiotics in treatment of multi-resistant bacterial infections (Keen 2012). In current literature there are, to my knowledge, no reports of quantitative measurement of phage isolation success despite it can be one of the limiting steps in phage therapy. Premade cocktails and phage libraries aim to circumvent the need for on-demand isolation but inevitably strains resistant against the available phages are encountered either via phage resistance development (Lenski 1984, Labrie *et al.* 2010) or emergence of new pathogenic strains. Phages are often depicted as easy to isolate (Drulis-Kawa *et al.* 2012) but this rather non-scientific expression *per se* does not reveal how the probability to find appropriate phages to given bacterial strains may differ. Often, the number of isolation attempts required for acquiring phages have not been mentioned in the methods section of papers reporting phage isolations and cannot thus be derived from the existing data. We conducted a study where a wastewater plant in Jyväskylä, Finland (Nenäinniemi) was used as a source

for phage isolation against different strains from seven commonly encountered opportunistic pathogen genuses: *Escherichia, Salmonella, Klebsiella, Pseudomonas, Staphylococcus, Enterococcus, and Acinetobacer*. Most of the strains used in our study were multi-resistant hospital isolates. We used slightly adapted standard two-step isolation procedure for phage hunt (Van Twest and Kropinski 2009). We chose a wastewater plant as a source of isolations because it has already been used to find phages for all of the above-mentioned bacterial genuses (Sunagar *et al.* 2010, Santos *et al.* 2011, Alemayehu *et al.* 2012, Hong *et al.* 2014, Kesik-Szeloch *et al.* 2013, Kitti *et al.* 2014, Khalifa *et al.* 2015).

We obtained phages in almost all of the isolation attempts (from individual samples) for *E. coli* (90.6 % of 35), *K. pneumoniae* (83.3 % of 15), *Salmonella* sp (88.9 % of 11), *P. aeruginosa* (79.4 % of 44). However, among these bacteria there were a few strains that were not successful hosts for isolation. *Enterococcus* and *A. baumannii* phages were found in less than half of the trials, 33.9 % (of 27) and 38.9 % (of 34), respectively. Suprisingly, *S. aureus* phages were really difficult to be isolated, since only 6.1 % of the 117 attempts were successful. Currently known *S. aureus* phages are most commonly temperate (Deghorain and Van Melderen 2012). If this reflects the true proportions of different kinds of phages infecting *S. aureus* in the nature it could partly explain our results. However, lytic *S. aureus* phages capable of infecting various clinical strains have been isolated before: Synnott *et al.* (2009) reported recovery of as many as 52 *S. aureus* phages from influent collected from wastewater plant in Japan, so in theory it should be possible to find them easily. One relevant question is whether the wastewater plant (that we used) was an optimal reservoir for all investigated bacteria. This motivated us to conduct additional isolation attempts from alternative isolation sources of *S. aureus* phages. Also, variations of the isolation protocol were tested. Despite this phages for only two of 14 strains were found. This implies that *S. aureus* phages may be difficult to isolate despite the isolation source, at least for strains used here. To be thorough, the presence of each bacterial species at the sampling place (and time) in question should be resolved, because it is the ultimate limiting factor for the presence of phages. This was not possible in the time frame and resources of this thesis.

S. aureus is common on skin and in nares and its presence in communal wastewater has been studied in Sweden where it was shown that seasonal variation did not affect the presence of these bacteria and that they were found in all of the wastewater treatment steps (Börjesson *et al.* 2009). However, methicillin-resistant *Staphylococcus aureus* (MRSA) was found mainly in the early treatment steps. Also Rosenberg Goldstein *et al.* (2012) showed that MRSA levels drop in along processing of the sewage. One explanation might be that even though phages infecting bacterial strains isolated from distant locations and/or in the past can be found (Wolf *et al.* 2003, Flores *et al.* 2009) they probably are not everywhere. Biogeography and possibility of endemic phages have recently been discussed and should not be overlooked (Thurber 2009). Thus additional isolation sources should be investigated in the future to address this question thoroughly. Additionally, the isolation method itself is

biased enriching phages that are cultivable and able to persist in applied conditions. However, these conditions were chosen considering the possible application of treating human bacterial infections where persistence of viruses at body temperature of 37 °C is an evident requirement. All of our bacterial strains tolerated well these conditions. It is well documented that phages differ substantially in their ability to tolerate different external conditions (Jonczyk *et al.* 2011) and therefore it is possible that part of the phages present in the isolation source could not be isolated using our method. Also, enrichment method has been shown to favour faster multiplying phages (Dunbar *et al.* 1997) which could be desired feature in phage therapy but it might exclude phages that for instance tolerate better external conditions and reach better the target site. Metagenomics analysis of the virome could provide more detailed information on the actual viral diversity (Thurber 2009).

Phages considered for phage therapy trials have to be easily propagated in high titers and preserve their infectivity in storage. To address this, we determined the titers of the phage stocks immediately and after one-month storage. Both low and high titer phages were found for each host species and those with highest isolation success also provided highest mean titer. On average 0.5 log reduction in phage titers were observed after the storage period, with the exception of *Acinetobacter* and *Enterococcus* phages whose titer dropped 0.973 and 0.222 log, respectively. These observations call for more detailed characterization of individual viruses, which was not performed in this study. Application in phage therapy would also require their genomic analysis to exclude temperate phages that might carry toxin encoding and other harmful genes (Loc-Carrillo and Abedon 2011).

We determined the host ranges of the phages using spot test where diluted phage stock is spotted on a bacterial lawn and infection is detected as clear zones in the plate. According to this analysis *Pseudomonas* phages generally had the widest host range and also some of the *Salmonella* phages infected almost all strains available. Other phages had clearly narrower host ranges and some infected only their isolation host. Spot test, however, has its limitations in terms of giving faulty positive results caused by lysis from without (early bacterial lysis induced by high-multiplicity virion adsorption or exogenously supplied phage lysin), bacteriocins or abortive infection (Khan Mirzaei and Nilsson 2015). Despite these limitations it can be concluded that the majority of the found phages were quite specific for their hosts. Spot test could still be used for initial screening of potential wide-host range phages when larger amounts of phages are isolated.

All in all, with this information it is difficult to conclude whether here obtained isolation patterns truly reflect general features of the host species or they were dependent other factors like isolation source or bacterial strains used. In some cases the observed phage infection patterns were almost identical for certain strains possibly reflecting their genetical similarity. This could happen when the same strains circle around at the same area or even globally as an outbreak (Price *et al.* 2013). In addition to resistance profiles of some of the bacterial isolates we did not have more specific information about the used

strains available. Furthermore, because resistances are often carried by plasmids it is possible that despite the differing resistance profile the bacterial strains carrying them are otherwise very similar or the same. Typing of the used bacterial strains could shed some light on these open questions (Duc Cao *et al.* 2015).

This study has implications for the consideration of phage therapy as a treatment choice for multi-resistant bacterial infections. When phages are hard to find other research avenues should be explored. For instance, lytic enzymes of phages have shown promise against gram-positive bacteria (Fischetti 2010). Even if these results would not be applicable generally for phage isolation they highlight the importance of ready-to-use reservoirs. Wider application of phage therapy would require global access on phages with therapeutic potential and knowledge on where phages should be isolated if on-demand isolation is necessary. Thus above raised issues call for subsequent studies before any definitive conclusions can be made.

5 CONCLUSIONS

The main conclusions of this thesis were:

I BN-PAGE was used to probe protein interaction in PRD1 virion. The receptor recognizing vertex could be detected as well as interaction of two membrane proteins involved in virus entry. Additionally, zymogram analysis suggested that the lytic enzyme P15 is not part of the packaging vertex.

II ESBL-strains derived plasmids showed different levels of evolutionary rescue potential under lethal β-lactam concentration. This suggests that high doses of antibiotic might not always be enough to clear the infection if the surrounding bacterial community can provide resistance via conjugation.

III Conjugation ability of PRD1 resistant plasmids rarely return even under conjugation-favouring antibiotic selection. This is dependent on the type of mutation causing the resistant phenotype. Phage susceptibility is also restored among the conjugation ability.

IV On-demand isolation of phages from an environmental reservoir was a feasible approach against common pathogenic bacteria *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *Salmonella*. However, effects of isolation source and used strains on isolation success call for further investigations.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Pienillä asioilla on merkitystä - faageista ja resistenssiplasmideista

Geneettinen materiaali (DNA tai RNA) on elämän keskiössä, sillä se sisältää tiedon siitä millaiseksi eliö muodostuu, eli millaisia proteiineja ja RNA-molekyylejä tuotetaan. Darwinin mukaan eliöiden ominaisuudet periytyvät yksinomaan vanhemmilta jälkeläisille. Nykyään tiedetään, että geenit siirtyvät myös horisontaalisesti eliöstä toiseen, jopa eri lajien välillä. Tämä on pääosin mahdollista siksi, että jotkin geeneistä sijaitsevat kromosomin ulkopuolella, pienten itsenäisesti kopioituvien molekyylien tai molekyyliyhdistelmien matkassa. Nämä ovat usein elävistä soluista riippuvaisia viruksia tai plasmideja, joiden vaikutus isäntäsoluun voi olla joko haitallinen tai hyödyllinen. Sekä virukset että plasmidit ovat tärkeä osa biologiaa: ne ovat osallisina mm. merten ravinteiden kierrossa ja antibioottiresistenssikriisissä. Siksi niiden tutkimus sekä toiminnalliselta että eko-evolutiiviselta kannalta auttaa ymmärtämään, miten elollinen maailma ympärillämme toimii.

Virukset kuvataan usein genomista, proteiinikuoresta ja mahdollisesta rasvakalvosta koostuvina pienikokoisina partikkeleina. Näiden virioneiksi kutsuttujen partikkelien tehtävä on suojata viruksen perimää solun ulkopuolella sekä sopivan isäntäsolun tunnistettuaan siirtää perimä tavalla tai toisella solun sisälle. Virusten elinkierron vaiheisiin kuuluu kuitenkin paljon muutakin kuin virioni. Lähes poikkeuksetta virusten kaikki biologiset toiminnot (soluun tunkeutuminen, perimän monistaminen, proteiinien tuotto, uusien viruspartikkelien muodostaminen ja virionien vapautuminen) tapahtuvat isäntäsolun kanssa vuorovaikutuksessa ja ovat monilta osin myös riippuvaisia solun molekyyleistä. Virukset ovat hyvin monimuotoisia. Niiden genomit voivat olla yksi- tai kasijuosteista RNA:ta tai DNA:ta. Morfologialtaan virukset voivat olla mm. hännällisiä tai hännättömiä ikosaedreja, sauvoja, sukkuloita tai jopa pullon muotoisia. Lisäksi näyttäisi siltä, että kaikilla eliöillä on viruksia. Bakteereita infektoivia viruksia eli bakteriofageja (tai faageja) arvellaan olevan maapallolla 10^{31}, mikä tekee niistä maailman yleisimpiä geneettisesti lisääntyviä rakenteita. Faagitutkimus on lisännyt tietoamme mm. biologian perusmekanismeista ja luonut molekyylibiologian työkaluja. Silti nämä virukset ovat vielä monilta osin tuntemattomia. Esimerkiksi Jyväsjärvestä löydettiin hiljattain täysin uudenlainen faagi (yksijuosteisen DNA-genomin ja sisäkalvollisen kapsidin yhdistelmä). Emme myöskään vielä tiedä useimpien faagigeenien toiminnasta mitään.

Tämän väitöskirjan ensimmäisessä osatyössä tutkittiin plasmidiriippuvaista faagia PRD1, jonka virionit rakentuvat proteiinin ja rasvahappojen muodostamasta sisäkalvosta ja ikosahedraalisesta ulkokapsidista. Kapsidi ja kalvo ympäröivät kaksijuosteista DNA-genomia. Viruspartikkelin proteiinien vuorovaikutuksia selvitettiin kahdella biokemiallisella menetelmällä. Partikkelien hajotus detergentillä ja hajoamistuotteina syntyneiden proteiinikompleksien komponenttien selvittäminen Blue native PAGE -menetelmällä ja vasta-

aineleimauksella paljasti proteiinivuorovaikutuksen kahden viruksen soluun tunkeutumiseen osallistuvan proteiinin välillä. Myös ennestään tunnettu kulmaproteiinien muodostama kompleksi voitiin havaita tällä menetelmällä. Mutanttivirusten zymogrammianalyysillä pystyttiin osoittamaan, että virusinfektion lopussa solun hajottava entsyymi ei sijaitse genomin pakkauskulmassa, kuten aiemmin arveltiin.

Monet tautia aiheuttavat mikrobit ovat tulleet vastustuskykyisiksi antibiooteille, mikä vaarantaa terveydenhuollon perustana toimivian antibioottien tehon. Pitkään jatkunut antibioottien käyttö, oli se sitten asianmukaista tai tarpeetonta, suosii vastustuskyvyn syntymistä ja leviämistä bakteereiden keskuudessa. Erityisen ongelmalliseksi tilanteen tekee se, että uusia antibiootteja ei ole tarpeeksi kehitteillä paikkaamaan vanhojen tehon hiipumista. Käytännössä tämä voi johtaa ennen pitkää siihen, että yhä useampia bakteeri-infektioita on vaikea hoitaa tehokkaasti. Konjugatiiviset plasmidit ovat ongelman keskiössä: ne pystyvät levittämään bakteerista toiseen mm. vastustuskykyä aiheuttavia geenejä. Nämä pääasiassa bakteerigenomin ulkopuoliset DNA-elementit koodaavat konjugaatiokanavan muodostavia proteiineja ja siten säätelevät itse leviämistään. Vastustuskyvyn yleistyminen on globaali ongelma. Ymmärtämällä tekijöitä, jotka vaikuttavat konjugatiivisten plasmidien siirtymiseen bakteereiden välillä, voidaan pyrkiä kehittämään keinoja leviämisen estämiseksi. Samalla on olennaista etsiä vaihtoehtoisia tai täydentäviä keinoja antibiooteille vastustuskykyisten bakteerien tuhoamiseksi.

Tässä väitöskirjassa tutkittiin moniresistenteistä sairaalabakteereista eristettyjen konjugatiivisten plasmidien siirtymistä bakteerista toiseen kuolettavassa β-laktaami-antibioottipitoisuudessa. Osa näistä vastustuskyvyn tuottavista β-laktamaasigeenin sisältävistä plasmideista siirtyi hyvin tehokkaasti alttiisiin bakteereihin jopa korkeimmissa testatuissa pitoisuuksissa. Antibioottihoidon yhteydessä tämä voi johtaa epätoivottuun lopputulokseen, kun vastustuskyky siirtyy bakteerien välillä muodostaen mahdollisesti uusia moniresistenttejä yhdistelmiä. Yhtenä ratkaisuna horisontaalisen geenisiirron tuomiin ongelmiin on ehdotettu keinoja, joilla voitaisiin luoda valintapaine konjugatiivisten plasmidien siirtymistä vastaan. Yksi mahdollisuus olisi hyödyntää plasmidiriippuvaisia faageja. Ne tunnistavat konjugatiivisten plasmidien koodaamia reseptoriproteiineja ja ovat siten sekä plasmidien että niitä sisältävien bakteerisolujen luonnollisia vihollisia. Tässä väitöskirjassa käytettiin PRD1-virusta ja konjugatiivista plasmidia RP4 mallisysteeminä tutkittaessa, kuinka plasmidiriippuvaiset faagit voivat estää antibioottivastustuskyvyn leviämistä ja säilymistä bakteeriyhteisössä. Aiemmin on osoitettu, että PRD1:n läsnäolo johtaa plasmidien konjugaatiokyvyn menetykseen tai niiden häviämiseen bakteerisoluista. PRD1 myös tehokkaasti estää plasmidien siirtymistä bakteerisolusta toiseen erilaisten antibioottien aiheuttamien valintapaineiden alla jopa silloin kun ne suosivat moniresistenttien kantojen syntymistä. Tämän väitöskirjan osatyössä III keskityttiin tutkimaan, kuinka hyvin viruksen aiheuttama konjugaatiokyvyn menetys tai heikkeneminen palautuu suotuisissa olosuhteissa, eli konjugaatiota suosivassa antibioottiselektiossa. Tulokset viittaavat siihen, että palautuminen on harvi-

naista ja riippuu konjugaatiokyvyn menetykseen johtaneesta mutaatiosta. Palautuminen oli lisäksi aina yhteydessä uudelleenaltistumiseen virukselle, joten plasmidiriippuvaisten faagien käyttö voisi olla kestävä ratkaisu torjua plasmidivälitteisen antibioottivastustuskyvyn leviämistä.

Myös perinteinen faagiterapia, eli bakteereita infektoivien virusten käyttö bakteeritulehdusten hoidossa on otettu uudestaan laajemmin tutkimuskohteeksi. Tämä jo 1900-luvun alkupuolella kehitetty hoitokeino jäi syrjään antibioottihuuman myötä suurimmassa osassa maailmaa. Yhtenä rajoittavana tekijänä faagiterapiassa on ollut faagien spesifisyys ja sitä kautta niiden saatavuus tiettyä patogeeniä vastaan. Väitöskirjan yhdessä osatyössä selvitettiinkin, kuinka helposti faageja löydetään sairaalasta eristetyille infektioita aiheuttaville moniresistenteille taudinaihettajabakteereille. Eristyslähteenä toimi jätevesipuhdistamon jätevesi, jonka tiedetään yleisesti toimivan faagieristyksissä. *E. coli-, K. pneumoniae-, P. aeruginosa-* ja *Salmonella*-faageja löytyi lähes jokaisella eristysyrityksellä. *Staphylococcus, Acinetobacter* ja *Enterococcus* puolestaan olivat huonompia eristysisäntiä. Näiden tulosten perusteella olisikin ensiarvoisen tärkeää käyttää useita eri lähestymistapoja taistelussa antibiooteille vastustuskykyisiä bakteerikantoja vastaan, sillä kaikille patogeeneille ei välttämättä voida löytää faageja akuutin infektion aikana.

Tämän väitöskirjan tutkimukset lisäävät tietämystämme sisäkalvollisen PRD1-viruksen proteiinivuorovaikutuksista ja (plasmidiriippuvaisten) faagien soveltamisesta lääketieteen saralla. Plasmidien kuljettamat antibioottivastustuskykyä koodaavat geenit ovat uhka modernille lääketieteelle ja siten konjugatiivisten plasmidien tutkimus auttaa löytämään keinoja, joilla pystymme kääntämään pahenevan tilanteen suunnan. Faagiterapian rajoitukset tulee ottaa huomioon suunniteltaessa sen laajamittaista käyttöä. Tulokset kuitenkin viittaavat siihen, että faageja on helppo löytää monia yleisesti infektioita aiheuttavia bakteereita vastaan ja niiden avulla voitaisiin estää myös antibioottivastustuskyvyn leviämistä.

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ORIGINAL PAPERS

I

PROBING PROTEIN INTERACTIONS IN THE MEMBRANE-CONTAINING VIRUS PRD1

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Probing protein interactions in the membrane-containing virus PRD1

Sari Mattila,[1] Hanna M. Oksanen[2] and Jaana K. H. Bamford[1]

Correspondence
Jaana K. H. Bamford
jaana.bamford@jyu.fi

[1]Centre of Excellence in Biological Interactions, Department of Biological and Environmental Science and Nanoscience Center, PO Box 35, University of Jyväskylä, 40014 Jyväskylä, Finland

[2]Department of Biosciences and Institute of Biotechnology, PO Box 56, University of Helsinki, 00014 Helsinki, Finland

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PRD1 is a Gram-negative bacteria infecting complex tailless icosahedral virus with an inner membrane. This type virus of the family *Tectiviridae* contains at least 18 structural protein species, of which several are membrane associated. Vertices of the PRD1 virion consist of complexes recognizing the host cell, except for one special vertex through which the genome is packaged. Despite extensive knowledge of the overall structure of the PRD1 virion and several individual proteins at the atomic level, the locations and interactions of various integral membrane proteins and membrane-associated proteins still remain a mystery. Here, we demonstrated that blue native PAGE can be used to probe protein–protein interactions in complex membrane-containing viruses. Using this technique and PRD1 as a model, we identified the known PRD1 multiprotein vertex structure composed of penton protein P31, spike protein P5, receptor-binding protein P2 and stabilizing protein P16 linking the vertex to the internal membrane. Our results also indicated that two transmembrane proteins, P7 and P14, involved in viral nucleic acid delivery, make a complex. In addition, we performed a zymogram analysis using mutant particles devoid of the special vertex that indicated that the lytic enzyme P15 of PRD1 was not part of the packaging vertex, thus contradicting previously published results.

INTRODUCTION

Among the prokaryotic viruses infecting either archaea or bacteria, close to half of the described virus morphotypes contain lipid as a structural component (reviewed by Atanasova *et al.*, 2015). Membrane-containing viruses are the most characteristic virus types among the eukaryote-infecting viruses. One type of virus is the icosahedral internal membrane-containing morphotype. This type of virus is found infecting organisms of all domains of life, exemplified by bacteriophages PRD1, PM2 and P23-77 (Abrescia *et al.*, 2004, 2008; Jaatinen *et al.*, 2008), archaeal viruses SH1, *Sulfolobus* turreted icosahedral virus and *Haloarcula hispanica* icosahedral virus 2 (Jäälinoja *et al.*, 2008; Jaakkola *et al.*, 2012; Veesler *et al.*, 2013), and *Paramecium bursaria* chlorella virus 1 and mimivirus of eukaryotes (Xiao *et al.*, 2005; Zhang *et al.*, 2011).

Bacteriophage PRD1 is one of the best-known internal membrane-containing icosahedral viruses. It infects a variety of Gram-negative bacteria harbouring incompatibility P, N or W group plasmids (Olsen *et al.*, 1974). PRD1 is the type member of the family *Tectiviridae* (for review, see Grahn *et al.*, 2006; Oksanen & Bamford, 2012). The linear dsDNA genome of PRD1 (~15 kb) has inverted terminal repeats and covalently linked terminal proteins at the 5′ ends (Bamford & Mindich, 1984; Bamford *et al.*, 1983, 1991; Savilahti & Bamford, 1986). The internal membrane consists of both protein and phospholipids in ~1 : 1 mass ratio (Davis *et al.*, 1982). Eighteen out of the 31 genes encode structural proteins either associated with the membrane (half of the protein species) or with the capsid shell (Fig. 1). The major capsid protein P3 (43 kDa) is a trimer organized on the pseudo $T=25$ lattice forming the icosahedral capsid (Abrescia *et al.*, 2004). The minor capsid protein P30 is necessary for particle formation as a tape-measure protein defining the dimensions of the virus particle (Abrescia *et al.*, 2004; Rydman *et al.*, 2001). At least 11 of the 12 fivefold vertices are occupied by a receptor-recognizing complex formed by monomeric receptor-binding protein P2 (64 kDa), trimeric spike protein P5 (34 kDa), pentameric penton protein P31 (14 kDa) and internal membrane protein P16 (13 kDa) (Rydman *et al.*, 1999; Jaatinen *et al.*, 2004). One of the fivefold vertices is different (special vertex), where the DNA-packaging machinery exists (Gowen *et al.*, 2003; Strömsten *et al.*, 2003). This special vertex complex consists of the integral membrane proteins P20 (5 kDa) and P22 (6 kDa) as well as packaging efficiency factor P6 (18 kDa) and packaging ATPase P9 (26 kDa) (Karhu *et al.*, 2007; Strömsten *et al.*,

Three supplementary figures are available with the online Supplementary Material.

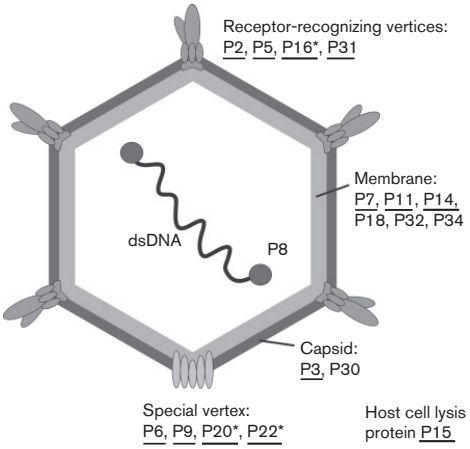

Fig. 1. Schematic representation of the PRD1 virion. Proteins involved in this study are underlined. Membrane proteins at the vertices are indicated with an asterisk.

2003, 2005). Although an *in vitro* DNA-packaging system is available (Žiedaitė *et al.*, 2009), the underlying molecular details of the DNA-packaging machinery and its structure remain to be solved.

Initial recognition of the host cell occurs when protein P2 interacts with the PRD1 receptor (Grahn *et al.*, 1999; Mindich *et al.*, 1982a). This is followed by irreversible binding, which eventually leads to DNA injection. The internal membrane of PRD1 forms a tube structured by membrane-associated proteins that serves as a DNA injection apparatus (Bamford & Mindich, 1982; Lundström *et al.*, 1979; Peralta *et al.*, 2013). Several virus proteins taking part in this process have been recognized: P18 is essential for tube formation and the major membrane-associated protein P11 is necessary for the initiation of DNA delivery (Grahn *et al.*, 2002a). Other virus genome injection-related membrane proteins are P14 and P32. The PRD1 virion contains two lytic enzymes, P7 and P15, of which the latter is responsible for host cell lysis (Caldentey *et al.*, 1994; Mindich *et al.*, 1982b; Pakula *et al.*, 1989). The function of P15 is intriguing, because it is associated with the virion, which is an unusual feature in virus–host cell lysis systems. Protein P7 is a transglycosylase (the conserved motif locates in its N-terminal domain) and takes part in DNA delivery; however, it is not an absolute requirement for the process (Rydman & Bamford, 2000). As phages generally rely on different muralytic enzymes for cell wall penetration and lysis, the exact roles of P7 and P15 have remained obscure.

PRD1 virus structure and functions have been analysed by complementing not only structural approaches, but also techniques relying on biochemical and/or genetic manipulation of the virus (Bamford & Mindich, 1982; Mindich *et al.*, 1982a, b; Strömsten *et al.*, 2003). In PRD1, the numerous suppressor-sensitive mutants have been invaluable in studying protein interactions and assigning functions to the corresponding proteins, e.g. revealing mechanisms for virus genome packaging (Karhu *et al.*, 2007; Strömsten *et al.*, 2005) or entry (Bamford & Bamford, 2000; Grahn *et al.*, 2002a, b). PRD1 is the first and the only virus with an internal membrane so far solved by X-ray crystallography at the atomic level (~4 Å resolution; Abrescia *et al.*, 2004; Cockburn *et al.*, 2004). Other approaches, such as cryo-electron microscopy, cryo-electron tomography, X-ray crystallography of structural proteins, antibody labelling and biochemical virion dissociation experiments, have also provided insights into virion organization and subunit interactions (Benson *et al.*, 1999; Caldentey *et al.*, 1993; Gowen *et al.*, 2003; Peralta *et al.*, 2013; San Martín *et al.*, 2002). However, these approaches have only provided information about interactions of soluble proteins and their possible membrane association; they could not be used to study the inner parts of the virion. Thus, the interactions of the majority of the membrane proteins and membrane-associated proteins still remain unresolved.

In this study, we applied blue native (BN)-PAGE for the analysis of protein complexes and membrane proteins and their interactions in bacteriophage PRD1. The presence of lytic enzyme P15 in the packaging-deficient mutant virus particles was also analysed by zymogram assay in order to unravel its localization and interactions in the virion.

RESULTS AND DISCUSSION

BN-PAGE as a tool to separate complexes of dissociated virus particles

BN-PAGE, combined with the solubilization of the membrane in mild conditions, is a method capable of preserving weak protein–protein interactions. Originally, it was used in the separation of mitochondrial protein supercomplexes (Schägger & von Jagow, 1991), but was later also employed in a variety of applications, such as determination of oligomeric states and native masses and identification of protein–protein interactions (Wittig *et al.*, 2006). For viruses, BN-PAGE has been used for studying protein interactions in extracts of hepatitis C virus-infected cells as well as in measles virus particles (Brindley & Plemper, 2010; Stapleford & Lindenbach, 2011). Prior to BN-PAGE analysis, samples are usually treated with non-ionic detergents to obtain solubilized membrane protein complexes. One widely used mild detergent, digitonin, even preserves some intact supercomplexes (Wittig *et al.*, 2006). *N*-dodecyl-β-maltoside (DDM) is a stronger detergent and it delipidates proteins more efficiently than digitonin, leading to more disrupted samples. Although SDS is an anionic detergent, it has been used in low concentrations prior to BN-PAGE (Klodmann *et al.*, 2011). BN-PAGE is based on the ability of Coomassie brilliant blue

(CBB) dye to bind hydrophobic proteins and provide them with a negative net charge, but not to interfere with most of the protein–protein interactions. However, some weaker interactions might be disrupted, which can be occasionally overcome using lower concentrations of CBB (Neff & Dencher, 1999). In BN-PAGE, protein samples covered with CBB are run in non-denaturing gel electrophoresis, leading to the separation of proteins and protein complexes according to their mass. The separated protein complexes can be resolved into their subunits in second-dimension denaturing SDS-PAGE and identified using Western blotting or MS.

Here, the purified PRD1 particles were treated with mild detergents, i.e. digitonin or DDM, to obtain protein complexes. Preliminary screenings were carried out using detergent concentrations between 0.01 and 10 % (w/v) as recommended previously (Reisinger & Eichacker, 2007). The PRD1 membrane is very resistant to various conditions, such as heating and basic pH (Caldentey *et al.*, 1993). Also, e.g. Triton X-100 (1 %) is not able to solubilize the membrane entirely at room temperature (Luo *et al.*, 1993a). Consequently, we combined the heat treatment with the detergent treatments when using digitonin or DDM. We obtained the largest amount of visible protein complexes in the BN-PAGE gel when 70 °C was used (data not shown for other temperatures). This is consistent with the notion that the virus capsid consisting of the trimeric major capsid protein P3 is very stable and dissociates completely only at 75 °C (Caldentey *et al.*, 1993). In addition to mild detergents, we used the strong ionic detergent SDS, as it is known that the PRD1 membrane can be dissolved by SDS treatment even at room temperature, leaving the P3 protein capsid intact (Luo *et al.*, 1993a).

The final experimental conditions were chosen based on the repeatability of the dissociations and maximizing the amount of protein complexes. The treated samples were analysed using a 5–16 % BN-PAGE gradient gel (the first-dimension gel) to separate detached protein complexes (Fig. 2a). Treatment with 0.1 % SDS (20 min, on ice) resulted in six visible complexes having masses of ~100–700 kDa (Fig. 2b). Treatment with 1 % digitonin or 2 % DDM at 70 °C produced nine (~70–680 kDa) and 16 (~30–900 kDa) protein complexes, respectively (Fig. 2b). We are aware of the mass estimation problems with soluble protein markers in BN-PAGE (Wittig *et al.*, 2010), and therefore we used the estimations here only to differentiate between complexes and to have some rough values of their masses.

Production of new polyclonal antisera against lytic enzyme P15

To identify the protein species in different complexes, the analysis was performed using denaturing second-dimension SDS-PAGE gels combined with Western blotting. We utilized monoclonal and polyclonal antibodies recognizing PRD1 structural proteins (P2, P3, P5, P6, P7/P14, P9, P11, P16, P22, P31), and produced a new polyclonal antibody against P15 to be able to detect it in the formed subviral complexes. For this, gene *XV* was cloned, and the protein was expressed and purified for antibody production. Sequencing of the cloned gene revealed a point mutation (Trp6 to Leu), which was assessed not to affect antibody production, because polyclonal antibodies recognize several epitopes of the protein. The new antibody recognized specifically the viral protein P15 by Western blotting (Figs

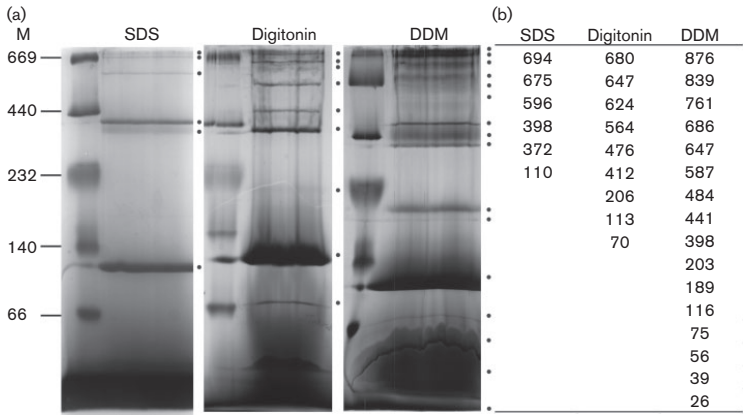

Fig. 2. (a) Complexes of the PRD1 analysed by BN-PAGE using 5–16 % acrylamide gradient gels (the first-dimension gel analysis). The disruption conditions were SDS (0.1 %, on ice, 20 min), digitonin (1 %, 70 °C, 10 min) and DDM (2 %, 70 °C, 10 min). M, HMW Native Marker (kDa; GE Healthcare). The complexes detected are marked with dots on the right. (b) Estimated masses (kDa) of the complexes obtained using different disruption conditions.

S1–S3, available in the online Supplementary Material). In the sequential labelling, antibody detecting P15 was used first. The specificity was also tested with highly purified PRD1 Sus232 mutant (amber mutation in gene *XV*) particles. No signal was obtained by Western blotting (data not shown). Antibodies detecting P8, P18, P20, P30, P32 and P34 were not available for Western blotting, and so these protein species were not included in the analysis.

Receptor-recognizing vertex complex can be resolved by BN-PAGE after SDS disruption

Interpretation of the second-dimension results was occasionally quite challenging, because the complexes were not always separated clearly, which is typical for native gels. However, we were able to determine the composition of most of the complexes. The second-dimension data are shown in Figs S1–S3 and summarized in Table 1. Some of the structural proteins were detected in several complexes. Many proteins were not found in any of the multiprotein complexes obtained, but found as monomers or homomultimers (see below).

Table 1. Proteins identified in the complexes by Western blotting in the second-dimension SDS-PAGE gels after different disruptions: SDS (treatment with 0.1 % SDS, on ice, 20 min), digitonin (treatment with 1 % digitonin, 70 °C, 10 min) and DDM (treatment with 2 % DDM, 70 °C, 10 min)

Complexes of similar protein composition are grouped (see Figs 2 and S1–S3).

SDS	Digitonin	DDM
372–694 kDa	**412–680 kDa**	**398–876 kDa**
P2	P2	P2
P5	P5	P5
P16	P6	**116–203 kDa**
P31	P9	P2
110 kDa	**113–206 kDa**	P5
P2	P2	P7
P5	P5	P16
P11	P16	P31
		75 kDa
		P7/P14
Monomers (<66 kDa)		
P3	P2	P2
P6	P6	P6
P9	P9	P9
P7/P14	P7/P14	P11
P11	P11	P16
P15	P16	P22
P16	P22	P31
P22	P31	
P31		
Spread through the whole gel		
P3	P3	P3
	P11	P11

After treatment with SDS (0.1 %), the complexes with estimated masses of 372 and 398 kDa in the first-dimension gel included at least proteins P2, P5, P31 and P16 (Fig. 3). The receptor-recognizing vertex of PRD1 contains a P2 monomer (64 kDa), P5 trimer (3×34 kDa) and P31 pentamer (5×14 kDa). In addition, there are five copies of protein P16 (13 kDa) linking the P2–P5–P31 complex to the underlying membrane (Abrescia *et al.*, 2004). The P2–P5–P31–P16 complex has a theoretical molecular mass of 301 kDa, which is approximately the same as of those obtained by 0.1 % SDS treatment (estimated as 372 and 398 kDa complexes; Fig. 2).

In addition to those complexes, using 0.1 % SDS, we obtained several other protein complexes with higher molecular masses (~600 kDa and over), but with similar protein composition (Table 1, Figs 3 and S1). Major capsid protein P3 was detected in each of these complexes. It is possible that the peripentonal P3 trimers surrounding the vertex structures were present in these complexes in varying amounts, which would explain why complexes with different sizes were detected. It is known that SDS treatment of the PRD1 procapsid (Sus1 mutant particles missing protein P9) detaches the peripentonal trimers from the receptor-recognizing vertex structure leaving the rest of the P3 shell intact (Butcher *et al.*, 1995; Luo *et al.*, 1993a). This released complex is the same as that observed here by BN-PAGE. In addition, the transformation of the membrane to a tube for DNA delivery is accompanied by decapping of some of the vertex structures, including peripentonal P3 trimers (Peralta *et al.*, 2013).

We also obtained smaller complexes containing P2, P5 and P16 by treatment with digitonin or DDM, which may represent further dissociated versions of the vertex structure (Table 1, Figs S2 and S3). Following DDM treatment, P31 seemed to be present in some amounts in these complexes. After SDS treatment, some minor amounts of P2 appeared at ~140 kDa in the second dimension, as well as significant amounts of P5 (Table 1, Figs 3 and S1). These results are in accordance with the model suggesting that P5 and P2 proteins interact with each other as separate spikes, and that P5 attaches to the virus particle via P31 (Huiskonen *et al.*, 2007). Under most conditions, P16 and P31 appeared partly as monomers (Table 1, Figs S1–S3). Interestingly, no monomeric spike protein P5 was detected in any of the disruptions.

Major capsid protein P3 and major membrane protein P11 were detected in almost every protein complex

In certain cases, one protein species could be found in all of the complexes. The major capsid protein P3 was detected in several complexes in every disruption condition tested (Table 1, Figs S1–S3). It is the major protein species in the virion, most probably having a wide interaction network with several other structural proteins. Notably, the major capsid protein P3 was never seen as a monomer in the

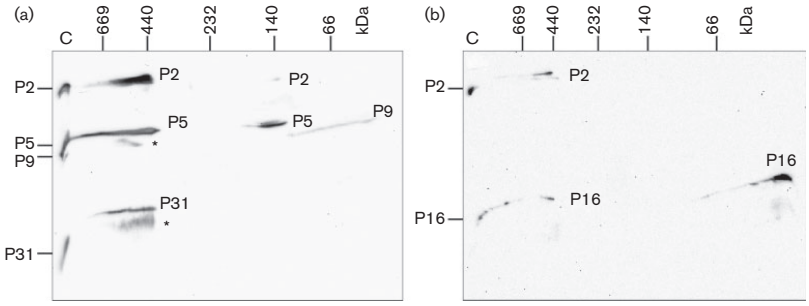

Fig. 3. PRD1 dissociated with 0.1 % SDS (20 min, on ice) and analysed by 5–16 % BN-PAGE followed by second-dimension SDS-PAGE (16 %) and Western blotting. Proteins were sequentially labelled by antibodies recognizing (a) P31, P5, P2 or P9 and (b) P16 or P2 (indicated next to the bands). The bands marked with asterisks are unspecific labelling or degradation products of the nearest band above. C, PRD1 WT used as a standard in the second-dimension gel. Molecular masses (kDa; on top) refer to the marker bands in the first-dimension BN-PAGE gel (see Fig. 2).

milder disruption conditions (Table 1, Figs S2 and S3). The smallest units were >100 kDa, indicating that P3 always appeared in some bigger complexes or at least as a trimer. It has previously been shown that the P3 trimer is very stable (Caldentey *et al.*, 1993; Mindich *et al.*, 1982a). A fraction of major capsid protein P3 (43 kDa; Stewart *et al.*, 1993) was found to be monomeric after treatment with SDS, known to be a strong detergent.

Aggregation factor P11 is the major membrane protein and also appeared over a wide mass range in the BN-PAGE gels (Table 1, Figs S1– S3). It seemed to exist at least partly in some of the complexes with P2 and P5, although we cannot draw any specific conclusions. However, it has been proposed that P11 covers the membrane vesicle, making the vesicular particles insoluble without the capsid shell (Bamford & Mindich, 1982). This might explain why it was found associated with so many different protein species. In addition, the membrane protein P16 was associated with several protein complexes after digitonin and DDM disruption (Table 1, Figs S2 and S3). This can be broadly explained by its association with vertex structures and major capsid protein P3.

Components of the packaging vertex dissociate readily

We interpreted proteins smaller than the 66 kDa marker detected in second-dimension SDS-PAGE gels as monomers. Certain proteins, such as P6 and P9, we found mostly in that area, indicating that they detached as monomers in all of the tested conditions. Proteins P6 and P9 are part of the DNA-packaging machinery located at the special vertex, and the packaging enzyme P9 is the last protein to be incorporated to the forming virion (Strömsten *et al.*, 2003, 2005). In addition, the proposed locations of P6 and P9 indicate localization on the surface of the virion (Gowen *et al.*, 2003; Strömsten *et al.*, 2003), which might explain their release in the presence of detergents. In digitonin disruption, P6 and P9 were occasionally seen in the complexes of ⩾412 kDa (Table 1, Fig. S2). However, the special vertex protein P22 was always monomeric. Thus, no conclusions about packaging vertex protein interactions could be drawn.

Proteins P7 and P14 appear to form a heteromultimer attached to the virus membrane

P7 and P14 were found as monomers in SDS and digitonin treatment (Table 1, Figs S1 and S2). However, a specific protein complex obtained by 2 % DDM treatment at 70 °C had an estimated mass of ~75 kDa (Figs 2 and S3, Table 1). Protein P7 has previously been detected in cross-linking experiments in a complex having a mass of similar range (Luo *et al.*, 1993b). Here, the obtained complex included at least proteins P7 (27 kDa) and P14 (15 kDa) (Fig. 4a). In addition, protein P11 might take part in this complex, but it was detected through the whole gel, so specific conclusions could not be drawn (Fig. S3, Table 1). The mass of the complex indicated that P7 and P14 formed a heteromultimeric complex, as predicted previously (Fig. 4b) (Rydman & Bamford, 2000). Taking into account that mass estimations based on BN-PAGE analyses are only approximates, it is possible that the 75 kDa complex consisted of more than one P7 or P14 unit.

Both proteins P7 and P14 are encoded by the gene *VII* (Fig. 4c) (Hänninen *et al.*, 1997). The full-length gene product P7 includes the transglycosylase domain at its N terminus and the predicted transmembrane helix at the C terminus (Rydman & Bamford, 2000). The 3′ end of the gene encodes protein P14, and thus most probably both P7 and P14 are membrane proteins. According to Davis *et al.* (1982), the copy number of protein P7 is ~20 copies per virion. Based on Western blots of purified virus using the mAb detecting both P7 and P14, the copy number of P14

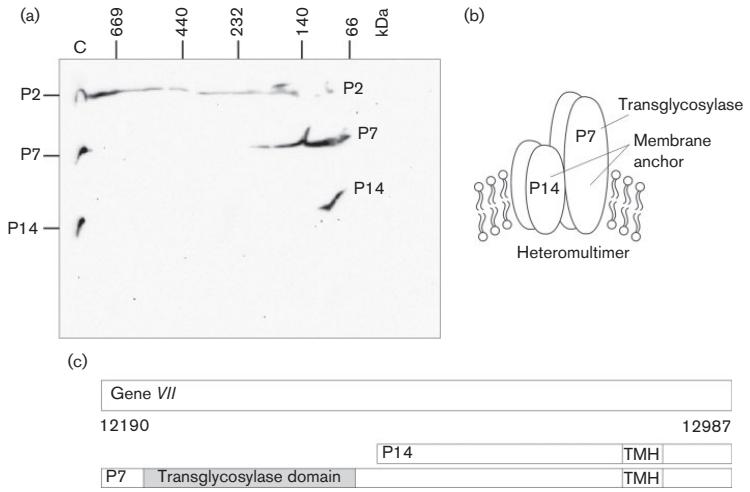

Fig. 4. PRD1 proteins P7 and P14. (a) PRD1 dissociation with 2 % DDM (70 °C, 10 min). Proteins were resolved by BN-PAGE (5–16 %) followed by second-dimension SDS-PAGE (16 %) and Western blotting. Proteins were identified by sequential labelling using antibodies recognizing P7/P14 or P2 (indicated next to the bands). C, PRD1 WT used as a standard in the second-dimension gel. Molecular masses (kDa; on top) refer to the marker bands in the first-dimension BN-PAGE gel (see Fig. 2). (b, c) Schematic representations of the heteromultimeric P7/P14 complex (b), and gene *VII* encoding P7 and P14 (c). Transglycosylase motif and predicted transmembrane helix (TMH) are indicated. The numbers refer to PRD1 genome coordinates (nt) (GenBank accession number AY848689).

may be very similar (Fig. 4a, the control on the left). There could be two copies of both proteins for each of the vertices, as suggested by Rydman & Bamford (2000). Our data are in accordance with this estimation, because two P7 and two P14 proteins would form a complex of ~84 kDa.

Zymogram analysis suggests that P15 is not part of the special vertex

Using BN-PAGE, P15 could be detected only after 0.1 % SDS treatment and only as a monomer (Table 1, Fig. S1). It is possible, that the interaction of P15 with other structural proteins in PRD1 is so weak that it cannot be detected by BN-PAGE. No conclusions about its interactions or location could be drawn. We utilized zymogram analysis to test whether P15 could be part of the special vertex of the virion, as suggested previously (Rydman & Bamford, 2002). Zymogram analysis relies on protein PAGE, where the peptidoglycan cast in the separation gel is used as a substrate for proteins with peptidoglycan-hydrolysing activity (Bernadsky *et al.*, 1994). The proteins first separated in the denaturing gel are renaturated and their lytic activity can be then observed as clear zones after staining the gel. We conducted experiments using purified WT particles and two P20-deficient mutant particles, Sus526 and Sus400 (amber mutation in gene *XX*; Strömsten *et al.*, 2003). In addition, the ATPase P9 and the packaging-complex-associated protein P6 were missing from the mutant particles. As reported previously (Rydman & Bamford, 2000), the zymogram assay revealed that the PRD1 virion contained two lytic proteins, P7 and P15 (Fig. 5). Both mutant particles had similar lytic peptidoglycan-degrading activities as the WT particles (Fig. 5). This contradicts previously published results (Rydman & Bamford, 2002) and questions the localization of P15 as a part of the special vertex. The absence of P7 causes delayed DNA synthesis and virion liberation (Rydman & Bamford, 2000), but does not prevent virus entry. Rydman & Bamford (2002) speculated that P15 might replace P7. If this is the case, they would probably localize similarly in the virus capsid, and P15 might function in both cell lysis and cell penetration.

CONCLUSIONS

Viral membranes, for which the lipids are acquired from the host cell cytoplasmic membrane, can reside either outside or inside a proteinaceous capsid, or be the only protecting layer for the genome, as seen in the pleomorphic archaeal and bacterial viruses (Dybvig *et al.*, 1985; Pietilä *et al.*, 2012). Common for all virus morphotypes is that the viral lipid bilayer is supplemented with virus-specific transmembrane or membrane-associated proteins. In addition, the membrane organization and protein–protein interactions in the membranes are not typically symmetrically organized. Although the structure of the icosahedral

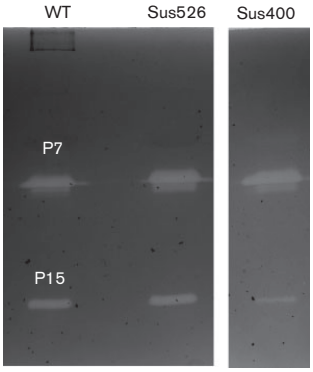

Fig. 5. Zymogram analysis of the purified PRD1 WT and special vertex mutant particles Sus526 and Sus400 (missing proteins P6, P9 and P20). Viruses were highly purified by rate zonal and equilibrium centrifugation in sucrose gradients. Positions of P7 and P15 protein lytic activities are indicated.

PRD1 virus with an internal membrane has been solved at 4 Å resolution (Abrescia *et al.*, 2004; Cockburn *et al.*, 2004), only one viral transmembrane protein was visualized in the X-ray electron density map. Various methods are needed to probe the complex structures of membrane-containing viruses, especially those techniques that could solve asymmetrical protein–protein interactions, including interactions with the membranes. One approach is BN-PAGE combined with screening different dissociation conditions. We have illustrated here that this technique is suitable for detecting membrane protein interactions in a rigid virus with strong protein–protein interactions. The feasibility of BN-PAGE was demonstrated by showing that we could identify the whole PRD1 vertex structure formed of four protein species: P2, P5, P31 and P16 (Fig. 3). P16 is the transmembrane protein linking the vertex structure to the virus membrane (Abrescia *et al.*, 2004; Jaatinen *et al.*, 2004). Although different mild detergents were tested, only SDS treatment was able to detach the vertices from the virus particle, allowing the analysis of its subunits by electrophoresis. The mass estimation of the complex formed of P2, P5, P31 and P16 obtained by BN-PAGE is in agreement with the theoretical mass of the vertex structure. Using DDM treatment of PRD1 particles, we could also demonstrate that transmembrane protein P7 with lytic activity and transmembrane protein P14 possibly interact together in a complex, in which they might have equal copy numbers (Fig. 4). These proteins function during virus entry at the peptidoglycan digestion and DNA translocation step (Grahn *et al.*, 2002a). To further probe the location of lytic enzymes in the virion, we utilized zymogram analysis of P20-deficient mutant virus particles (Fig. 5) and analysed whether the lytic enzyme P15 is absent in the particles without the special vertex, as suggested previously (Rydman & Bamford, 2002). Our analysis proposes that the special vertex does not include protein P15; thus, it is still unclear where the lytic enzymes of PRD1 reside in the virion.

METHODS

Production and purification of PRD1 particles. PRD1 WT was grown on *Salmonella enterica* sv. Typhimurium LT2 DS88, and PRD1 mutants Sus232 (amber mutation in gene *XV*), Sus400 (amber mutation in gene *XX*) and Sus526 (amber mutation in gene *XX*) were grown on suppressor strains DB7156, PSA and DB7154, respectively (Mindich *et al.*, 1976, 1982b; Strömsten *et al.*, 2003; Winston *et al.*, 1979). Bacteria were grown in Luria–Bertani (LB) medium (Sambrook *et al.*, 1989) with appropriate antibiotics at 37 °C. For production of WT and mutant PRD1 particles, DS88 cells grown to a cell density of 1×10^9 c.f.u. ml^{-1} were infected at m.o.i. 7. Cells containing Sus232 mutant particles missing lytic enzyme P15 were collected by centrifugation (Sorvall SLA-3000 rotor, 5000 r.p.m., 15 min, 4 °C) 2 h after infection and disrupted with a French press (Thermo; 1000 p.s.i., twice). For disruption, cells were resuspended in 20 mM potassium phosphate buffer (pH 7.4, 1/80 of the original volume). Before polyethylene glycol (PEG) precipitation, buffer was added to restore the volume. Phage particles were precipitated by 10 % (w/v) PEG-6000/0.5 M NaCl and purified by 5–20 % (w/v) rate zonal sucrose gradient centrifugation (defined as 1 × virus; Beckman SW28 rotor, 24 000 r.p.m., 1 h, 15 °C). For zymogram analysis and P15 antibody specificity tests, material was further purified by 20–70 % equilibrium sucrose gradient centrifugation (defined as 2 × virus; Beckman SW28 rotor, 22 000 r.p.m., 17–19 h, 15 °C) (Bamford & Bamford, 1990, 1991). Purified particles were collected by differential centrifugation (Beckmann 45Ti rotor, 33 000 r.p.m., 3 h, 5 °C; Beckmann 70Ti rotor, 33 000 r.p.m., 3 h or 40 000 r.p.m., 1 h 45 min, 5 °C) and suspended in 50 mM Bistris/HCl buffer (pH 7.0, 1 × virus) or 20 mM potassium phosphate buffer (pH 7.2, 2 × virus). Protein concentration was determined by the CBB method using BSA as a standard (Bradford, 1976).

Detergent treatment of virus samples. Purified WT 1 × virus (1 mg ml^{-1}) was incubated in 50 mM Bistris/HCl, 500 mM 6-aminohexanoic acid and 1 mM EDTA, pH 7.0 (Schägger & von Jagow, 1991) containing 0.01–1.0 % (w/v) SDS for 20 min on ice. Treatments with DDM (0.5–2.5 %, w/v) or digitonin (0.1–2.5 %, w/v) were carried out by incubation at 70 °C for 10 min. Optimized detergent concentrations were 0.1 % SDS, 2 % DDM and 1 % digitonin.

Gel electrophoresis. After detergent treatments, glycerol and CBB G-250 were added to a final concentration of 5 and 0.5 % (w/v), respectively. Samples were loaded onto a linear 5–16 % (w/v) acrylamide gradient gel prepared as described previously (Schägger & von Jagow, 1991). The first-dimension gels for complex separation were run at 4 °C for at least 16 h at 100–500 V (SE400 vertical unit gel electrophoresis system; GE Healthcare). Current was limited to 15 mA. Molecular masses of the protein complexes were determined using Quantity One 1-D analysis software (Bio-Rad) and a HMW Native Marker (GE Healthcare). One complex (680 kDa, 1 % digitonin treatment) was added manually because the program did not recognize it. Vertical gel slices from the first-dimension gel were boiled in 5 % (v/v) β-mercaptoethanol/5 % (w/v) SDS for 10 min and placed on the second-dimension gel (SE400 vertical unit gel electrophoresis system; GE Healthcare). Second-dimension SDS-PAGE was performed using 16 % (w/v) acrylamide gels as described previously (Olkkonen & Bamford, 1989). Agarose (0.5 %, w/v) in the gel running buffer was laid on top of the gel slice to support the control sample (20–30 μg purified 1 × PRD1 absorbed in a slice of

Whatman paper) and to seal the gel cassette. Gels were run at 50–200 V for 16–24 h. Current was limited to 15 mA. To test P15 antibody specificity, SDS-PAGE was performed as described except that the current was limited to 25 mA. PRD1 WT and Sus232 sample series of 40, 20, 10 and 5 μg were used in the analysis.

Zymogram analysis. Peptidoglycan sacculus was obtained from *Escherichia coli* DH5α cells (Hanahan, 1983) as described previously (Rydman & Bamford, 2002). Zymogram analysis was used to detect muralytic activity of proteins as described by Bernadsky *et al.* (1994). Peptidoglycan preparation was added at a final concentration of 15 % (v/v) to the separation gel of the SDS-16 % (w/v) polyacrylamide gel (Olkkonen & Bamford, 1989). After electrophoresis, the gels were rinsed and incubated for 30–60 min in distilled water at 4 °C. The gels were incubated for 60 min in renaturation buffer (25 mM potassium phosphate buffer, pH 7.2, 0.2 % Triton X-100) with gentle agitation at 4 °C and incubated in fresh renaturation buffer for 48–72 h at 4 °C. Zymograms were stained with 0.1 % (w/v) methylene blue /0.01 % (w/v) KOH at room temperature for 1 h and destained with distilled water. Virus concentrations were optimized with Quantity One 1-D analysis software (Bio-Rad) using P3 protein on gels as a standard. An aliquot of 5 μg 2× virus or lysozyme control was used in zymogram analysis.

Glutathione *S*-transferase (GST)-P15 cloning and recombinant P15 protein purification. Standard molecular biology techniques were used in DNA manipulations (Sambrook *et al.*, 1989). A derivative of expression plasmid pGEX-4T-3 (GE Healthcare), in which a TEV cutting site was included, was used for cloning. PCR was used to amplify the gene *XV* sequence using the PRD1 genome as a template. Primers hybridizing the target area were designed: 5′ primer PRD1_P15F (ATATATGAAGACACCATGGGACAATATACACTTT-GGGATATTATCAGCCG) containing a *Nco*I site and 3′ primer PRD1_P15R (ATATATATGCGGCCGCTTATTTCACCCCGTAAAA-TTGGCAGGCT) containing a *Not*I site. Amplified fragments were inserted between the *Nco*I and *Not*I sites of plasmid pGEX-4T-3 TEV and transformed into *E. coli* HB101 (Bolívar, 1979; Boyer & Roulland-Dussoix, 1969). The sequence of the insert was determined (ABI Prism 3130xl; University of Jyväskylä). Plasmid pSPM1 was transformed into *E. coli* BL21-Gold (Carstens & Waesche, 1999), resulting in the GST-P15-producing strain BL21-Gold (pSPM1).

For production of GST-P15, an overnight culture of BL21-Gold (pSPM1) cells was diluted 1/20 and grown at 28 °C until the OD_{550} reached 0.75 (Clormic; Selecta). Protein production was induced with 1 mM IPTG and the temperature was reduced to 18 °C. After overnight growth the cells were collected (Sorvall SLA-3000 rotor, 5000 r.p.m., 10 min, 4 °C) and suspended in 1/100 of the original volume using PBS (pH 7.3) containing 1 mM DTT/5 % (v/v) glycerol. Cells were stored at −80 °C.

Concentrated cell suspension (100×) of BL21-Gold (pSPM1) (18 ml) was thawed and buffer (PBS/1 mM DTT/5 % glycerol) was added to a final volume of 40 ml to purify recombinant protein P15. After disruption of cells with a French press (Thermo) at 2000 p.s.i. (twice), Pefabloc SC (Roche) was added to a concentration of 1 mM. The cell debris were removed by centrifugation (Sorvall SS-34 rotor, 20 000 r.p.m., 30 min, 4 °C) after which protein solution was filtered (Sartorius Minisart; 0.45 μm). For purification, GST-P15 was bound to an affinity column at 20 °C (Glutathione Sepharose 4 Fast Flow matrix; GE Healthcare). After washing the column with PBS containing 1 mM DTT, GST-P15 was eluted with 40 mM reduced glutathione in PBS containing 1 mM DTT, filtered and the buffer was changed (Amicon Ultra-15 Millipore; Ultracel-10K) to 20 mM HEPES/1 mM EDTA/150 mM NaCl/1 mM DTT (pH 7.9). The GST-tag was cleaved with ProTEV protease (Promega; 0.3 U μl^{-1}) at 8 °C overnight. P15 was purified by gel filtration (GE Healthcare; HiLoad 26/60; Superdex; 1 ml min^{-1}; 20 mM HEPES/1 mM EDTA/150 mM NaCl/1 mM DTT, pH 7.9). P15-containing fractions were pooled and concentrated (Millipore; Amicon Ultra-15, Ultracel-10K). The protein purity (~90 %) was estimated by SDS-PAGE using 15 % (w/v) acrylamide gel (Olkkonen & Bamford, 1989) and the concentration was determined by the Bradford assay using BSA as a standard (Bradford, 1976). The identity of the purified protein was confirmed by MS analysis with a (high-performance nano) LC-(H)ESI Orbitrap Elite Hybrid Mass Spectrometer (Thermo Scientific; University of Helsinki) and its activity was tested by zymogram analysis. Polyclonal sera against protein P15 were raised in rabbit using purified P15 as antigen (Inbiolabs).

Antibodies and Western blotting. Western blotting was performed by transferring the proteins from the SDS-PAGE gels onto PVDF membranes (Millipore), followed by visualization with peroxidase-conjugated secondary antibodies (Dako) and chemiluminescent detection using a Super Signal West Pico (Thermo) system. Specific rabbit antisera recognizing PRD1 proteins P2 (Grahn *et al.*, 1999), P6 (Karhu *et al.*, 2007), P3 (Rydman *et al.*, 2001), P5 (Hänninen *et al.*, 1997), P9, P22 (Strömsten *et al.*, 2003), P15 (this study) and P31 (Rydman *et al.*, 1999) as well as mAbs 7N41 (for P7 and P14), 11T25 (for P11) and 16A201 (for P16) (Hänninen *et al.*, 1997) were used as primary antibodies for sequential labelling.

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Supplemental Information

Probing of protein interactions in the membrane-containing virus PRD1
Sari Mattila, Hanna M. Oksanen and Jaana K. H. Bamford
Journal of General Virology
jaana.bamford@jyu.fi, University of Jyväskylä.

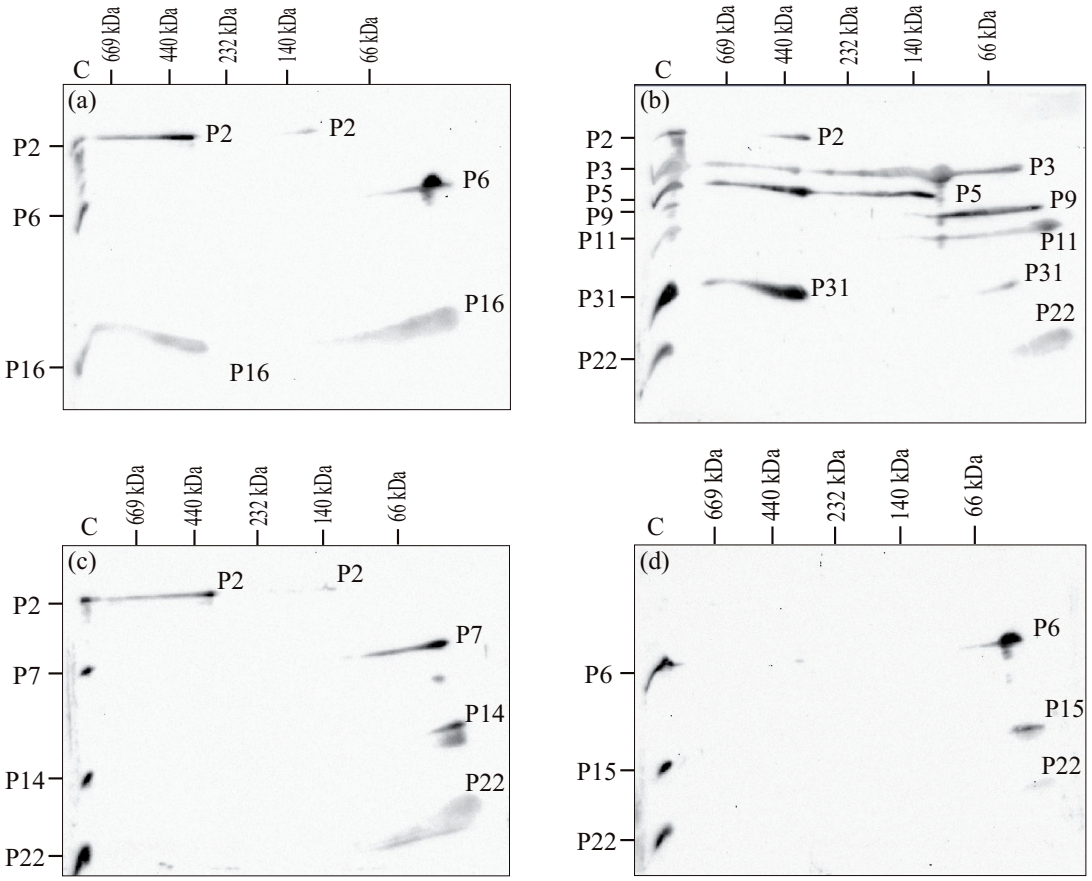

Fig. S1. Protein complexes of PRD1 after 0.1 % SDS-treatment (20 min on ice). Proteins were resolved by BN-PAGE (5 - 16 %) followed by SDS-PAGE (16 %). Western blotting was used for the detection of proteins. Proteins were identified by sequentially labeling using the (a) antibodies recognizing P2, P6 or P16, (b) antibodies recognizing P2, P3, P5, P9, P11, P22 or P31, (c) antibodies recognizing P2, P7/P14 or P22, and (d) antibodies recognizing P6, P15 or P22. PRD1 wt (C) was used as a standard in the second dimension gel. PRD1 structural proteins are indicated on the left. Molecular masses (on top) refer to the marker bands in the first dimension BN-PAGE gel (see Fig. 2).

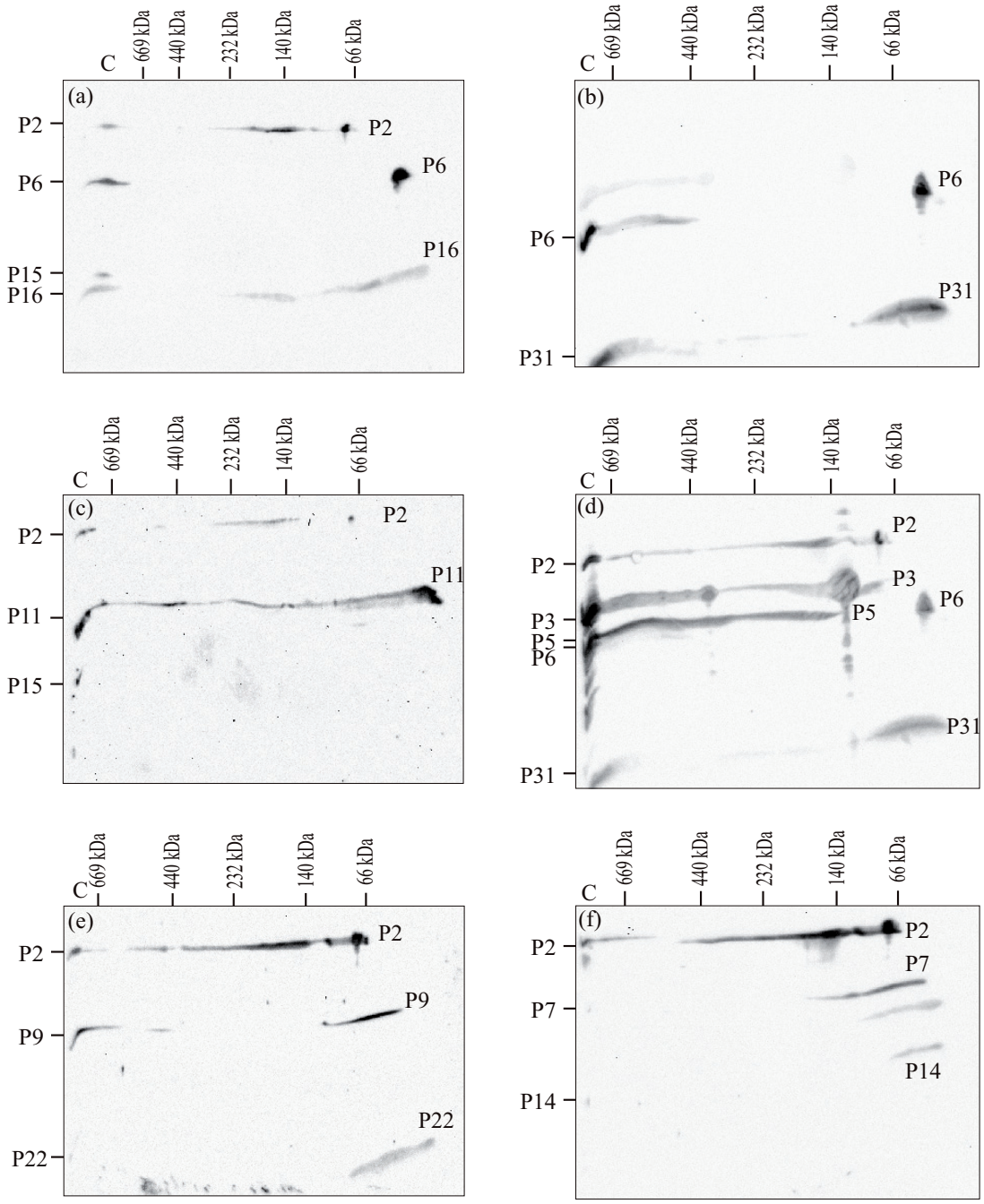

Fig. S2. PRD1 dissociation with 1 % Digitonin (70 °C, 10 min). Proteins were resolved by BN-PAGE (5-16 %) followed by the second dimension SDS-PAGE (16 % acrylamide) and Western blotting. Proteins were identified by sequentially labeling using the (a) antibodies recognizing P2, P6, P15 or P16, (b) antibodies recognizing P6 or P31, (c) antibodies recognizing P2, P11 or P15, (d) antibodies recognizing P2, P3, P5, P6 or P31 (P6 appears above P5 on the right of the gel due to a migration artefact), (e) antibodies recognizing P2, P9 or P22, and (f) antibodies recognizing P2 or P7/P14. PRD1 wt (C) was used as a standard in the second dimension gel. PRD1 structural proteins are indicated on the left. Molecular masses (on top) refer to the marker bands in the first dimension BN-PAGE gel (see Fig. 2).

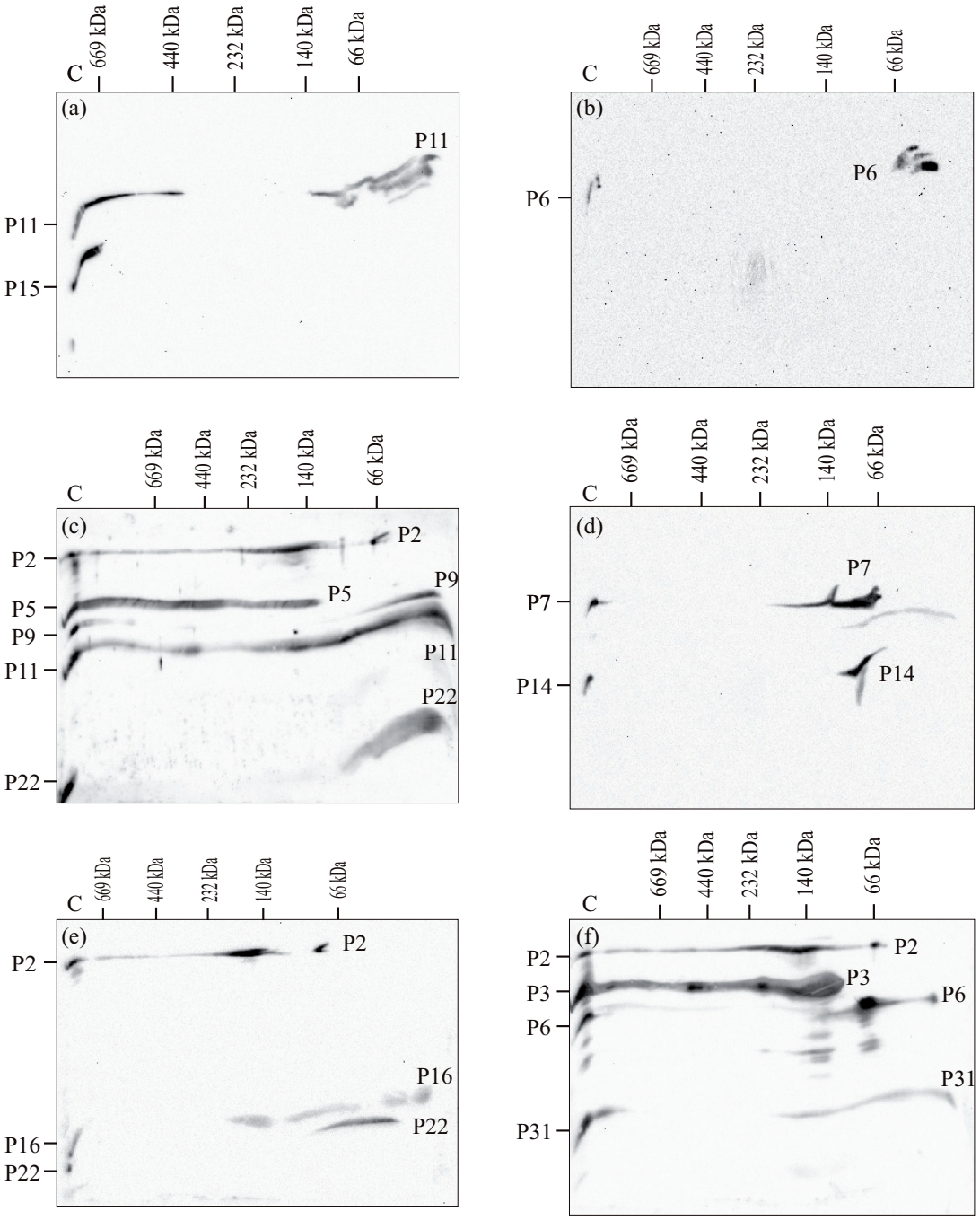

Fig. S3. PRD1 dissociation with 2 % DDM (70 °C, 10 min). Proteins were resolved by BN-PAGE (5-16 %) followed by the second dimension SDS-PAGE (16 % acrylamide) and Western blotting. Proteins were identified by sequentially labeling using the (a) antibodies recognizing P11 or P15, (b) antibody recognizing P6, (c) antibodies recognizing P2, P5, P9, P11 or P22, (d) antibody recognizing P7/P14, (e) antibodies recognizing P2, P16 or P22, and (f) antibodies recognizing P2, P3, P6 or P31. PRD1 wt (C) was used as a standard in the second dimension gel. PRD1 structural proteins are indicated on the left. Molecular masses (on top) refer to the marker bands in the first dimension BN-PAGE gel (see Fig. 2).

II

CONJUGATIVE ESBL-PLASMIDS DIFFER IN THEIR POTENTIAL TO RESCUE SUSCEPTIBLE BACTERIA VIA HORIZONTAL GENE TRANSFER

by

Sari Mattila, Pilvi Ruotsalainen, Ville Ojala, Timo Tuononen, Teppo Hiltunen & Matti Jalasvuori 2016.

Submitted manuscript

III

SCOPING THE EFFECTIVENESS AND EVOLUTIONARY OBSTACLES IN USING PLASMID-DEPENDENT PHAGES TO FIGHT ANTIBIOTIC RESISTANCE

by

Ville Ojala, Sari Mattila, Ville Hoikkala, Jaana K. H. Bamford, Teppo Hiltunen & Matti Jalasvuori 2016.

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IV

ON-DEMAND ISOLATION OF BACTERIOPHAGES AGAINST DRUG-RESISTANT BACTERIA FOR PERSONALIZED PHAGE THERAPY

by

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On-Demand Isolation of Bacteriophages Against Drug-Resistant Bacteria for Personalized Phage Therapy

*Sari Mattila, Pilvi Ruotsalainen and Matti Jalasvuori**

Department of Biological and Environmental Science, Centre of Excellence in Biological Interactions, University of Jyväskylä, Jyväskylä, Finland

Bacteriophages are bacterial viruses, capable of killing even multi-drug resistant bacterial cells. For this reason, therapeutic use of phages is considered as a possible alternative to conventional antibiotics. However, phages are very host specific in comparison to wide-spectrum antibiotics and thus preparation of phage-cocktails beforehand against pathogens can be difficult. In this study, we evaluate whether it may be possible to isolate phages on-demand from environmental reservoir. We attempted to enrich infectious bacteriophages from sewage against nosocomial drug-resistant bacterial strains of different medically important species in order to evaluate the probability of discovering novel therapeutic phages. Stability and host-range were determined for the acquired phages. Our results suggest that on-demand isolation of phages is possible against *Pseudomonas aeruginosa, Salmonella* and extended spectrum beta-lactamase *Escherichia coli* and *Klebsiella pneumoniae*. The probability of finding suitable phages was less than 40% against vancomycin resistant *Enterococcus* and *Acinetobacter baumannii* strains. Furthermore, isolation of new phages against methicillin resistant *Staphylococcus aureus* strains was found to be very difficult.

Keywords: antibiotic resistance, ESBL, MRSA, phage therapy, phage cocktails, bacteriophages

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Benjamin K. Chan, Yale University, USA

****Correspondence:***
Matti Jalasvuori
matti.jalasvuori@jyu.fi

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INTRODUCTION

Antibiotic resistance is an emerging global health crisis, resulting from the continuous use (and misuse) of antibiotics in healthcare, farming industry, and elsewhere (Cantas et al., 2013; World Health Organization [WHO], 2014). Phage therapy refers to the utilization of bacteriophages (or just phages, viruses infecting bacteria) to treat bacterial diseases (Abedon et al., 2011). Given the increasing number of drug-resistant bacterial infections, especially within hospital settings, the exploration of alternatives to conventional antibiotics has become an important research objective (Finch, 2011; Sommer and Dantas, 2011). Bacteriophages are very abundant (Hendrix et al., 1999) and every bacterium is likely to have their own specific viruses that could be utilized as antibacterial agents (Clokie et al., 2011; Flores et al., 2011; Örmälä and Jalasvuori, 2013). Historically, phages were used therapeutically already in the early 20th century (Sulakvelidze et al., 2001). Yet, the discovery of broadly effective antibiotics led to the demise of the development of phage therapy in western countries and only as the antibiotics are starting to fail there has been a serious attempt to restore the old tool. However, the second coming of phage therapy faces challenges regarding

to the strict regulatory guidelines and the development of effective therapeutic practices (Gill and Hyman, 2010; Lu and Koeris, 2011; Keen, 2012). Yet, phage therapy can provide an evolutionarily sustainable alternative to conventional antibiotics, should we be able to adjust our regulations and procedures to meet the special requirements of phage based medicine (Keen, 2012; Örmälä and Jalasvuori, 2013).

It is important to note that phages infect bacterial hosts very selectively. Often, the narrow host-range is considered as an advantage over traditional antibiotics since phage treatment can focus accurately on the pathogen without harming commensal bacterial flora (Loc-Carrillo and Abedon, 2011). On the other hand, bacteria develop resistance also to phages rapidly, and thus the achieved antibacterial effect may be transient (Hyman and Abedon, 2010; Labrie et al., 2010). When multiple different phages are used simultaneously in a phage cocktail, development of resistance is less likely (Skurnik et al., 2007; Chan et al., 2013). However, it is challenging to obtain a set of phages that is effective against all variants of a given pathogen (Pirnay et al., 2011; Chan et al., 2013). There can be a tradeoff between the host range and the therapeutic efficacy of a cocktail for a specific species of bacteria: when the number of phages in a cocktail increases in an effort to increase the host range of the cocktail, the number of phages against a specific strain of bacteria may decrease. Therefore, the host specificity of phages, while in theory beneficial, poses a practical problem when combined with the rapidly emerging resistant phenotypes.

In principle, it is possible to acquire bacteriophages on-demand to treat, for example, infections that are resistant to all known antibiotics and off-the-shelf (standardized) phage-therapy products (Keen, 2012; Örmälä and Jalasvuori, 2013). Tailoring a therapeutic cocktail personally for each patient would allow the cocktails to comprise phages that are effective against the bacterial strains responsible of the infection (Pirnay et al., 2011; Chan et al., 2013). Therefore and in comparison to premade cocktails, a personalized phage therapy does not carry a surplus of ineffective phages. Indeed, there are older studies suggesting that tailored phage treatments are several times more effective compared to standardized cocktails (Zhukov-Verezhnikov et al., 1978), and thus effective phage-therapy practices to treat constantly changing bacterial pathogens may depend on the adjustment of the treatment to the causative agent (Keen, 2012).

Generating a personal set of phages requires that the pathogen is isolated and, then, effective bacteriophages obtained against it. One possible way for identifying suitable viruses is to have a variety of bacteriophages isolated and prepared beforehand and then the causative pathogen screened through the phage-library (Chan et al., 2013). Alternatively, phages may be isolated as needed from environmental reservoirs. In some cases, the latter option may be inevitable due to the lack of infectious phages in the premade libraries against all possible bacterial variants. Ultimately, environment serves as the only source of practically endless phage variety and thus exploitation of the environmental resources forms the basis for personalized phage medicine.

While phages are known to be abundant, it is obvious that all environments cannot contain infective phages against all different bacterial hosts (see e.g., Flores et al., 2011; Atanasova et al., 2012). To the best of our knowledge, the probability of finding therapeutically useful phages against different resistant pathogens on-demand has not been studied *per se* despite the fact that it is likely to be the limiting factor in attempts to update premade cocktails or to generate on-demand personalized therapies (Chan et al., 2013). As an example, hospital acquired wound infections have been suggested to be especially suitable target for phage therapy as the causative agents are generally resistant to various antibiotics (Loc-Carrillo et al., 2012). Yet, there might be multiple different bacterial species present in these infections, including, e.g., *Staphylococcus aureus*, *Enterococcus faecium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa,* and *Acinetobacter baumannii* (Agnihotri et al., 2004). Therefore, a successful phage-based treatment can be dependent on the practicality of being able to simultaneously and rapidly isolate new durable phages against very different pathogens.

In this study, we provide an evaluation of the on-demand isolation of phages against the most common hospital borne resistant pathogens: methicillin resistant *S. aureus* (MRSA), extended spectrum beta-lactamase (ESBL) *E. coli* and *K. pneumoniae*, multi-drug resistant (MDR) *P. aeruginosa*, vancomycin resistant *Enterococcus* (VRE), *A. baumannii* and different *Salmonella* species. All aforementioned species are also listed in CDC's report on the top 18 drug-resistant threats to the United States in 2013 (CDC, 2013). These bacteria commonly cause infections of skin, lung and urinary tract, as well as foodborne infections among others and affect people all around the world disregarding their background (CDC/FDA/NIH, 2011).

Sewage is known to be an optimal resource of phages (Lobocka et al., 2014), thus a wastewater treatment plant in Jyväskylä, Finland (Nenäinniemi) was used as the environmental reservoir for phage hunt. The stability of the acquired viruses and their cross-infectivity on other potential host strains were determined.

We demonstrate vast differences in probabilities of finding novel phages against different hosts by using enrichment method for isolation. There appears to be severe constraints in isolating phages on-demand against pathogens like MRSA. On the other hand, it seems feasible to obtain phages against ESBL positive *E. coli* and *K. pneumoniae* as well as *P. aeruginosa*.

MATERIALS AND METHODS

Bacteria Strains and Culturing Conditions

Bacterial strains used in this study were mostly purchased from Medix Laboratories or acquired from Turku University Hospital (Supplementary Table S1). One *Klebsiella* strain and four *Enterococccus* strains were obtained from commercial culture collections. Aside from six bacterial strains, all had caused (antibiotic resistant) human infections and thus they represent pathogens that could have been treated with phages. Overall, we obtained 12 MRSA strains, 16 *E. coli* ESBL strains, 6 *K. pneumonia* (ESBL) strains, 17 *P. aeruginosa* MDR strains, 9 *A. baumannii* strains, 10 *E. faecium* (VRE) strains, 4 *Enterococcus faecalis* (VRE) strains, and 9 different *Salmonella* strains. Detailed

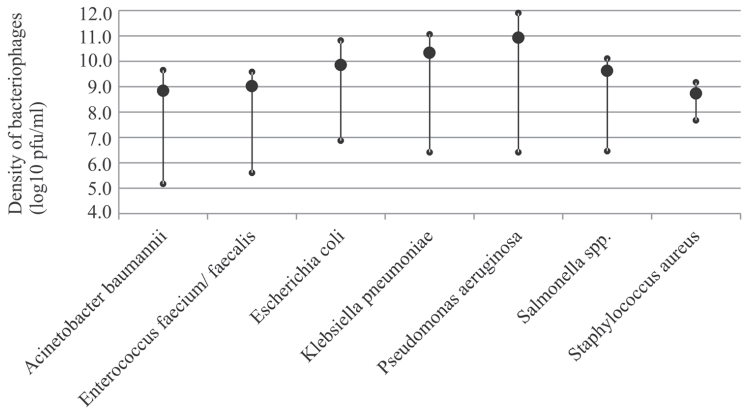

FIGURE 1 | Average density of infectious bacteriophage particles in the prepared stocks of each host species (large circle). Small circles indicate the maximum and minimum values observed.

TABLE 1 | Summary of the decrease in phage titers as observed after 1-month storage at +4°C.

Host bacterium	Average decrease in titer (log10)
Acinetobacter baumannii	0.973
Enterococcus faecium/ faecalis	0.222
Escherichia coli	0.496
Klebsiella pneumoniae	0.594
Pseudomonas aeruginosa	0.437
Salmonella sp.	0.529
Staphylococcus aureus	0.491

characterization of the bacterial strains was beyond the scope of this paper.

All bacteria were cultured in Lysogeny Broth (LB) -medium (Sambrook et al., 1989) at +37°C shaken 230 rpm (*Enterococcus* strains were cultivated without shaking).

Isolation Protocol

The following isolation protocol with slight modification in individual experiments was used throughout the study. Either unprocessed sewage samples or supernatants of turbid samples (centrifuged 3000–6000 *g* in Megafuge 1.0R, Heraeus, or in Eppendorf centrifuge 5702 R, 10–15 min at +4°C) were used in the enrichment steps. In cases where previous isolation attempts had failed to yield phages, the supernatant was also filtrated through a 0.45 μm filter to remove all remaining bacterial cells. The first enrichment step was conducted using 20–30 ml of sewage water filled up to 30–40 ml with LB-broth, depending on the volume of collected sewage samples. The target bacterial strain was added (50–200 μl o/n culture grown in LB-broth, 300 μl in case of *E. faecium* and *E. faecalis*) to enrich (potential) phages in the sample. These enrichments were cultivated overnight at +37°C, shaken 230 rpm. Bacteria from this enrichment culture were removed by centrifugation (3000–6000 *g* in Megafuge 1.0R, Heraeus, or in Eppendorf centrifuge 5702 R, 15–20 min, +4°C) and filtration (0.2 or 0.45 μm filter). The amount of potential phages in a 2.5 ml sample of the bacteria-free enrichment were further amplified by adding 2.5 ml of LB-broth and 50–100 μl of the target host bacterium and were grown overnight as above. The sample from this second enrichment step was centrifuged at 13 000 *g* for 15 min at room temperature and at least 10 μl the supernatant was plated on a LB-agar containing petri dish along with 100–300 μl of the host strain and 3 ml of melted 0.7% soft-agar. The plates were incubated overnight at +37°C. If plaques were observed on the bacterial lawn, a separate plaque was picked and transferred into 500 μl of LB-broth. A sample from this plaque-stock was further plated on the same host strain. Plaque-purification was performed three times for all discovered phages in order to isolate a single homogenous phage from the potentially heterogeneous phage mix that may have been present in the initial enrichment.

Due to poor isolation success for *S. aureus*, different modifications of the above-described method were used for enriching phages. The volume of the first enrichment step as well as the number of enrichment steps was increased (120 ml sewage sample + 70 ml L broth + 1 ml host overnight cultures in the first step). Rotation speed during shaken cultivation steps was varied between 100, 120, 180, or 360 rpm. In addition, samples from different sources were used for phage enrichment (River in Ljubljana, Slovenia, a water-lock sample from the Helsinki university hospital and soil samples from a livestock farm). These samples were not included in analysis of isolation success from sewage.

Preparation of Phage Stock

Semi-confluent plates (i.e., plates of which about half of the area is covered by phage induced plaques and the rest is bacterial lawn) were prepared by plating 100 μl of host strain (300 μl

TABLE 2 | Probability for discovering a bacteriophage from a sewage sample against different pathogens.

Bacterial pathogen	Mean hit %*	Isolation attempts	Number of strains hit
Acinetobacter baumannii	38.9	34	5/9
Enterococcus faecium/faecalis	33.9	27	5/14
Escherichia coli	90.6	35	15/16
Klebsiella pneumoniae	83.3	15	6/6
Pseudomonas aeruginosa	79.4	44	15/17
Salmonella sp.	88.9	11	8/9
Staphylococcus aureus	6.1	117	1/12

*As calculated over the bacterial strains of the given species.

of *Enterococcus* strains) and 3 ml of melted soft-agar with appropriate dilution of the phage stock. Plates were incubated overnight at +37°C. The soft-agar layers of semi-confluent plates were combined with 2.5–5 ml of LB-broth/plate. The combination was incubated for 4 h at +37°C, 230 rpm, and centrifuged at 6000 *g* for 15 min at +4°C (Megafuge 1.0R, Heraeus). If we were unable to get semi-confluent plates, we used as a combination "over-infected" plates supplied with 100–700 μl of the overnight-cultivated host strain. The supernatant was filtered (0.2 μm filter) and stored at +4°C.

Cross Infection Tests

All phages were used to cross-infect all different bacterial strains of its original host species (excluding *P. aeruginosa* phages as only half of them were used) for preliminary evaluation of their host range. Cross-infection tests were done by spotting 8 μl of phage stock dilution (1:10 or 1:100) on 100 μl bacterial overnight culture in soft-agar (0.7%). Plates were incubated at +37°C overnight. Formation of less opaque spots on the bacterial lawn was scored as a successful infection.

Phage Stock Stability

The titer of each phage stock was determined by standard double agar overlay method by plating a dilution series (10^{-2}–10^{-8}) immediately after preparation of the stock. Titer of the stock was determined again after 1-month storage (+4°C) to estimate the stability of the stock in LB-medium.

RESULTS

We evaluated the feasibility for generating a personalized phage-product on-demand against different bacterial pathogens. We chose bacterial species from seven different genuses that are responsible for the majority of hospital acquired bacterial infections, namely *Escherichia*, *Salmonella*, *Klebsiella*, *Pseudomonas*, *Staphylococcus*, *Enterococcus,* and *Acinetobacer*. Total of 283 phage isolation attempts were conducted for 83 different host strains. Overall 108 bacteriophages were discovered. All of these viruses were characterized for their plaque morphology and stability (described individually for each virus in Supplementary Table S2).

Phages were isolated via three consecutive plaque-picking steps to avoid mixed-culture stocks. Due to different plaque morphologies and titers, the preparation of phage stocks was adjusted for each phage. However, no actual optimization of phage production was carried out. The density of viable phage particles was measured immediately after the preparation of the stock (**Figure 1**). In order to determine their viability for acute use, the number of viable particles was re-measured 1 month later (see summary in **Table 1**). On average, the titers of the stocks decreased around 0.5 log10 during the 1-month storage in L-broth in 4°C. However, for some phages of *Enterococcus*, the titers could no longer be resolved. Phage-specific titers and plaque morphologies are listed in Supplementary Table S2.

The probability for finding an infectious bacteriophage from sewage for different host bacterium varied substantially (**Table 2**). Namely, phages for only a single *S. aureus* strain, SA10, were discovered in total of 117 enrichment attempts (the phages specific to the one *S. aureus* strain were obtained at the same time and they produced visually identical plaques, thus we selected only one of these phages for subsequent analyses). Conversely, almost every isolation attempt yielded a bacteriophage for *E. coli*, *K. pneumoniae*, *P. aeruginosa,* and *Salmonella* strains. Phage isolation for *Acinetobacter* and *Enterococcus* had success rates between 30 and 40%. Given the medical importance of MRSA, we decided to investigate whether alternative source materials would be more suitable for discovering phages. We obtained water samples from a water lock situated in a room used to treat MRSA-patients in Helsinki University Hospital. Two phages for a single strain (SA10) were found from these samples. A single MRSA-specific bacteriophage was isolated from a set of soil samples acquired from a livestock farm. Also, a water sample from river Ljubljana, Slovenia, produced a single bacteriophage for strain SA10. Yet, we failed to find a single phage for any of the ten other MRSA-strains used in the isolation attempts.

As presented in **Figure 2**, we studied the host-range of the obtained phages in order to determine their cross-infectivity and thus the potential to combine previously isolated phages into phage-cocktails. Aside from a couple of exceptions, almost all phages isolated for any given *P. aeruginosa* strain could also infect majority of the other strains. However, we neither found any phages for strain PA15 nor did any of the other phages infect this strain. In addition, only 4 out of 20 tested *Pseudomonas* phages infected strain PA6. Detailed characterization of these particular strains was beyond the scope of this paper.

Along with *Pseudomonas* phages, some of the *Salmonella* phages had a wide host range. *E. coli* phages tended to infect more than one strain, except EC1P1, EC11P2, EC15P2, and EC16P1. For other bacterial species, isolated phages generally had less

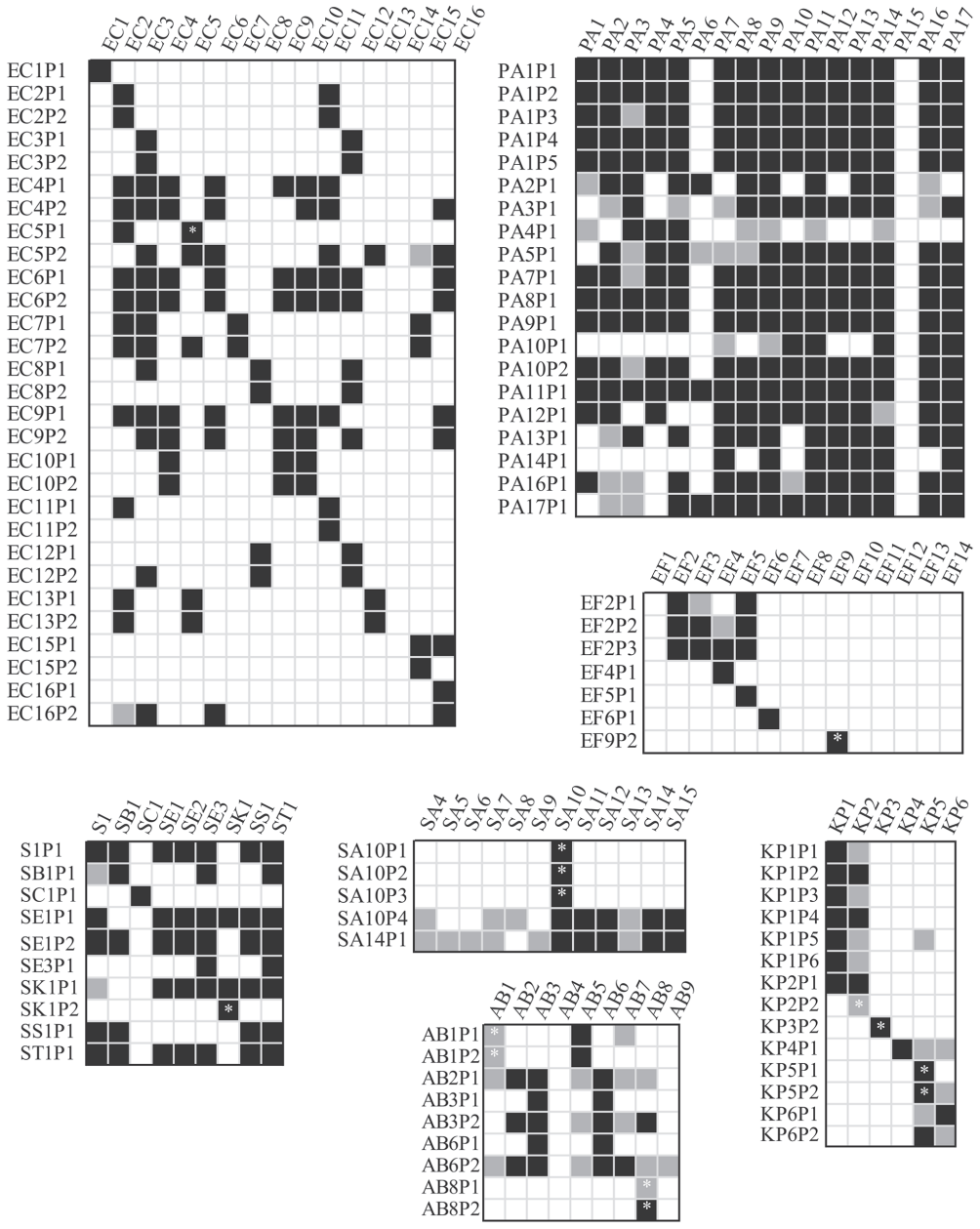

FIGURE 2 | Cross-infectivity of the isolated bacteriophages. Measurements where less than 10^5 pfu/ml were used are indicated with an asterisk. Only half of the isolated *Pseudomonas* phages were used in the experiments. White background indicates no lysis area, black marks clearly detected lysis area and light gray indicates very dim lysis area in the spot test. AB, *Acinetobacter baumannii*; EC, *Escherichia coli*; EF, *Enterococcus faecium* or *faecalis*; KB, *Klebsiella pneumoniae*; PA, *Pseudomonas aeruginosa*; S, *Salmonella* sp., and SA, *Staphylococcus aureus*.

alternative hosts, if any, indicating that a rapid preparation of a personalized phage-cocktail is likely to require multiple separate but simultaneous phage enrichments using a single bacterial strain as a host. Especially, *Klebsiella* and *Enterococcus* phages are very host specific. Sometimes phage stocks produced only a dim inhibition area on alternative hosts (presented as light gray coloring in **Figure 2**). This suggests that something, but not necessarily the phage in the prepared stocks was restricting the growth of the bacterium. Furthermore, phages isolated for any particular host often had similar infection patterns. This suggests that additional isolation attempts using the same isolation source for enrichment may not be the best choice for improving the host-range of the cocktail.

DISCUSSION

Due to the enormous variety of bacteriophages in environmental reservoirs, on-demand isolation of novel phage-antibacterials is a potential way to generate a personalized medicine for treating bacterial infections that are resistant to conventional drugs. In this study, we evaluated the feasibility of isolating phages for such therapeutic cocktails.

The efforts required to find phages differs substantially between bacterial species. Phages can be readily discovered for *E. coli*, *K. pneumoniae*, *P. aeruginosa,* and *Salmonella* species. Although virus production was neither optimized nor standardized in this study, phages of these hosts also readily generated high-density virus stocks (**Figure 1**). In contrast, we found it very challenging to isolate phages against *Staphylococcus* strains despite of several attempts that were conducted at different times of year and from multiple sources (sewage, river, hospital water lock, and livestock farm soil samples). It was also more laborious to isolate phages for *E. faecium* and *faecalis* and *A. baumannii,* although it must be noted that we had only handful of these strains and we performed only few isolation attempts for them. Nevertheless, based on the results, the on-demand discovery of phages appears to be feasible for some but not all bacteria. This highlights the importance of premade wide-host range cocktails or the existence of other antimicrobial solutions against species such as *S. aureus* (such as the one developed by Kelly et al., 2011). Also, teixobactin, the first new potential antibiotic to be discovered in 30 years is very effective against bacteria lacking the outer membrane (such as *S. aureus* and *Enterococcus*; Ling et al., 2015). Yet, gram-negative pathogens with the impermeable outer membrane (e.g., *E. coli*, *Salmonella*, *K. pneumonia,* and *P. aeruginosa*) are inherently resistant to antibiotics like teixobactin, but contrastingly appear to be suitable targets for obtaining a cocktail from environmental reservoir (sewage) as needed. Also, better preservability and wider host-range of these phages supports the on-demand isolation approach. While conventional tools for antibiotic development may still remain relevant, in the face of worsening world-wide antibiotic resistance crisis we should be actively exploring these promising alternatives in order to retain the upper hand against all pathogens.

Generalization of the obtained results must be done while acknowledging the potential sources of error. First, while we collected our sewage samples at different times (over the timespan of almost 2 years), only a single wastewater management plant was used. Although the biological material in these plants changes constantly, the phage populations may still be substantially different in different plants, thus possibly skewing the chances for finding phages against certain species. Moreover, the host ranges of some phages appear identical, suggesting that the hosts themselves may be genetically very close to one another. Second, albeit we performed several hundred isolation attempts, just a few isolations were performed for any particular strain and thus the achieved probabilities should be treated as a case study rather than an exhaustive evaluation. Third, we did not perform an in-detail characterization for the isolated phages. Such characterization, at least to some extent, will be necessary during actual therapy practices (Skurnik et al., 2007; Merabishvili et al., 2009; Keen, 2012), as bacteriophages are known to carry undesirable genes coding for toxins and antibiotic resistances (Loc-Carrillo and Abedon, 2011). However, separating lytic phages from temperate phages (possibly when accompanied with genome sequencing and analysis) should be enough and feasible for the rapid assessment of safety (Chan et al., 2013). Also, phage stocks have to be purified from (host-bacterium generated) endotoxins before therapeutic use (Keen, 2012). These steps were not performed or their effects on phages evaluated in this study.

CONCLUSION

The success of on-demand isolation of phages appears to be critically dependent on the bacterial host. Promisingly, against pathogens for which conventional antibiotics are becoming the least useful, such as ESBL *E. coli* and *K. pneumoniae*, personalized phage therapy could be considered as a potential alternative.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01271

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Conflict of Interest Statement: 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.

Table S1. Bacterial strains used in this study.

Bacterial strains	Short name	Source
Acinetobacter baumannii 57163	AB1	Turku University Hospital
Acinetobacter baumannii 59999	AB2	Turku University Hospital
Acinetobacter baumannii 62060	AB3	Turku University Hospital
Acinetobacter baumannii 20692	AB4	Turku University Hospital
Acinetobacter baumannii 57163	AB5	Turku University Hospital
Acinetobacter baumannii 18243	AB6	Turku University Hospital
Acinetobacter baumannii 26006	AB7	Turku University Hospital
Acinetobacter baumannii 18510	AB8	Turku University Hospital
Acinetobacter species 18560	AB9	Turku University Hospital
Enterococcus faecium 61285	EF1	Turku University Hospital
Enterococcus faecalis 58897	EF2	Turku University Hospital
Enterococcus faecalis	EF3	ATCC 19433
Enterococcus faecalis	EF4	ATCC 29212
Enterococcus faecalis	EF5	ATCC 33186
Enterococcus faecium	EF6	ATCC 9790
Enterococcus faecium 59776	EF7	Turku University Hospital
Enterococcus faecium 61244	EF8	Turku University Hospital
Enterococcus faecium 61027	EF9	Turku University Hospital
Enterococcus faecium 60803	EF10	Turku University Hospital
Enterococcus faecium 60734	EF11	Turku University Hospital
Enterococcus faecium 60703	EF12	Turku University Hospital
Enterococcus faecium 60457	EF13	Turku University Hospital
Enterococcus faecium 60145	EF14	Turku University Hospital
Escherichia coli 10AE5909	EC1	Medix
Escherichia coli 12UM05186	EC2	Medix
Escherichia coli 10UU11258	EC3	Medix
Escherichia coli 11UT10019	EC4	Medix
Escherichia coli 11AN03027	EC5	Medix
Escherichia coli 11UT12639	EC6	Medix
Escherichia coli 11UO03492	EC7	Medix
Escherichia coli 11UU07697	EC8	Medix
Escherichia coli 11UM05271	EC9	Medix
Escherichia coli 57262	EC10	Turku University Hospital
Escherichia coli 57294	EC11	Turku University Hospital
Escherichia coli 57189	EC12	Turku University Hospital
Escherichia coli 57253	EC13	Turku University Hospital
Escherichia coli 55027	EC14	Turku University Hospital
Escherichia coli 56895	EC15	Turku University Hospital
Escherichia coli 57361	EC16	Turku University Hospital
Klebsiella pneumoniae	KP1	DSM681
Klebsiella pneumoniae 10UO03898	KP2	Medix
Klebsiella pneumoniae 61705	KP3	Turku University Hospital
Klebsiella pneumoniae 61784	KP4	Turku University Hospital
Klebsiella pneumoniae 61837	KP5	Turku University Hospital
Klebsiella pneumoniae 61794	KP6	Turku University Hospital
Pseudomonas aeruginosa 61841	PA1	Turku University Hospital
Pseudomonas aeruginosa 61823	PA2	Turku University Hospital
Pseudomonas aeruginosa 61790	PA3	Turku University Hospital
Pseudomonas aeruginosa 61432	PA4	Turku University Hospital
Pseudomonas aeruginosa 11AN03663	PA5	Medix
Pseudomonas aeruginosa 26153	PA6	Turku University Hospital
Pseudomonas aeruginosa 62314	PA7	Turku University Hospital
Pseudomonas aeruginosa 62263	PA8	Turku University Hospital
Pseudomonas aeruginosa 62224	PA9	Turku University Hospital
Pseudomonas aeruginosa 62206	PA10	Turku University Hospital
Pseudomonas aeruginosa 62180	PA11	Turku University Hospital
Pseudomonas aeruginosa 62181	PA12	Turku University Hospital
Pseudomonas aeruginosa 62172	PA13	Turku University Hospital
Pseudomonas aeruginosa 62109	PA14	Turku University Hospital
Pseudomonas aeruginosa 62090	PA15	Turku University Hospital
Pseudomonas aeruginosa 62069	PA16	Turku University Hospital
Pseudomonas aeruginosa 61932	PA17	Turku University Hospital
Salmonella, Group B, ESBL strain 12FB01687	SB1	Turku University Hospital
Salmonella, Group C, 13FB10784	SC1	Turku University Hospital
Salmonella enteritis (36.)	SE1	Turku University Hospital
Salmonella enteritidis 13FB9205	SE2	Turku University Hospital
Salmonella enteritidis FB11214	SE3	Turku University Hospital
Salmonella Kreber (37.)	SK1	Turku University Hospital
Salmonella stanley FB3820	SS1	Turku University Hospital
Salmonella typhimurium FB7595	ST1	Turku University Hospital
Salmonella, quality control strain 18048	S1	Turku University Hospital
Staphylococcus aureus 11AN02972	SA4	Medix
Staphylococcus aureus 10MR01905	SA5	Medix
Staphylococcus aureus 11AE06590	SA6	Medix
Staphylococcus aureus 11AE07165	SA7	Medix
Staphylococcus aureus 11AN03312	SA8	Medix
Staphylococcus aureus 10AN02929	SA9	Medix
Staphylococcus aureus 10AE05905	SA10	Medix
Staphylococcus aureus 10AE06192	SA11	Medix
Staphylococcus aureus 60820	SA12	Turku University Hospital
Staphylococcus aureus 61765	SA13	Turku University Hospital
Staphylococcus aureus 60881	SA14	Turku University Hospital
Staphylococcus aureus 60689	SA15	Turku University Hospital

Table S2. Isolated phages.

Phage	Host	Plaque morphology	Titer	Titer after 1 month
AB1P1	*Acinetobacter baumannii* 57163	small, bright and some dim	4.2×10^{6}	3.0×10^{3}
AB1P2	*Acinetobacter baumannii* 57163	small, bright and some dim	2.0×10^{6}	7.2×10^{3}
AB2P1	*Acinetobacter baumannii* 59999	small, dim, rough edges, roundish	6.9×10^{7}	3.9×10^{7}
AB3P1	*Acinetobacter baumannii* 62060	medium-size, bright, big halo	4.5×10^{9}	9.1×10^{8}
AB3P2	*Acinetobacter baumannii* 62060	small, ,bright, round	1.8×10^{8}	3.5×10^{7}
AB6P1	*Acinetobacter baumannii* 18243	small and medium-size, dim, big halo	3.5×10^{8}	5.7×10^{8}
AB6P2	*Acinetobacter baumannii* 18243	small, dim, rough edges, roundish	1.1×10^{9}	1.5×10^{8}
AB8P1	*Acinetobacter baumanni* 18510	extremely small, slightly dim	1.5×10^{5}	2.6×10^{5}
AB8P2	*Acinetobacter baumannii* 18510	small, dim, rough edges, roundish	3.4×10^{5}	7.2×10^{4}
EC1P1	*Escherichia coli* 10AE5909	small, bright center surrounded by misty ring	6.0×10^{7}	5.6×10^{7}
EC2P1	*Escherichia coli* 12UM05186	big, bright	1.2×10^{10}	6.7×10^{9}
EC2P2	*Escherichia coli* 12UM05186	big, bright	7.6×10^{8}	1.3×10^{9}
EC3P1	*Escherichia coli* 10UU11258	medium-size, bright	1.7×10^{8}	8.4×10^{6}
EC3P2	*Escherichia coli* 10UU11258	medium-size, bright	4.6×10^{8}	1.3×10^{7}
EC4P1	*Escherichia coli* 11UT10019	small, bright	2.1×10^{7}	8.9×10^{6}
EC4P2	*Escherichia coli* 11UT10019	small, bright	1.6×10^{8}	2.6×10^{8}
EC5P1	*Escherichia coli* 11AN03027	small, bright	7.4×10^{8}	8.8×10^{5}
EC5P2	*Escherichia coli* 11AN03027	extremely small, bright	3.5×10^{8}	N/A
EC6P1	*Escherichia coli* 11UT12639	small, bright	1.6×10^{9}	2.9×10^{9}
EC6P2	*Escherichia coli* 11UT12639	small, bright	1.9×10^{9}	3.1×10^{9}
EC7P1	*Escherichia coli* 11UO03492	small, slightly dim	3.9×10^{8}	2.8×10^{8}
EC7P2	*Escherichia coli* 11UO03492	small, slightly dim	2.1×10^{10}	3.6×10^{10}
EC8P1	*Escherichia coli* 11UU07697	extremely small, slightly dim	2.4×10^{7}	7.3×10^{7}
EC8P2	*Escherichia coli* 11UU07697	extremely small, slightly dim	1.2×10^{7}	4.0×10^{7}
EC9P1	*Escherichia coli* 11UM05271	extremely small, bright	1.3×10^{9}	1.5×10^{9}
EC9P2	*Escherichia coli* 11UM05271	extremely small, bright	1.5×10^{10}	7.3×10^{9}
EC10P1	*Escherichia coli* 57262	small, bright	6.6×10^{10}	6.7×10^{10}
EC10P2	*Escherichia coli* 57262	small, bright	6.3×10^{10}	5.4×10^{10}
EC11P1	*Escherichia coli* 57294	big, bacterial growth in the middle	2.5×10^{9}	2.4×10^{8}
EC11P2	*Escherichia coli* 57294	small, some bright and some dim	1.6×10^{9}	4.0×10^{8}
EC12P1	*Escherichia coli* 57189	small, dim	1.2×10^{8}	8.5×10^{7}
EC12P2	*Escherichia coli* 57189	small, slightly dim	7.0×10^{7}	2.6×10^{7}
EC13P1	*Escherichia coli* 57253	small, dim	2.0×10^{8}	1.1×10^{8}
EC13P2	*Escherichia coli* 57253	small, dim	4.2×10^{8}	8.0×10^{7}
EC15P1	*Escherichia coli* 56895	small, bright, halo	9.0×10^{9}	9.6×10^{8}
EC15P2	*Escherichia coli* 56895	medium-size, dim, vague halo	2.3×10^{9}	4.0×10^{8}
EC16P1	*Escherichia coli* 57361	big and medium-size, bright, vague ring	3.7×10^{9}	6.3×10^{9}
EC16P2	*Escherichia coli* 57361	small/medium-size, bright	6.0×10^{8}	6.0×10^{8}
EF2P1	*Enterococcus faecalis* 58897	small, bright	3.8×10^{9}	1.7×10^{9}
EF2P2	*Enterococcus faecalis* 58897	small, bright	2.3×10^{9}	1.3×10^{9}
EF2P3	*Enterococcus faecalis* 58897	medium-size, brigth	8.8×10^{8}	7.3×10^{8}
EF4P1	*Enterococcus faecalis* ATCC 29212	big, bright, difficult to determine titer	3.5×10^{8}	N/A
EF5P1	*Enterococcus faecalis* ATCC 33186	big, bright, difficult to determine titer	7.0×10^{7}	N/A
EF6P1	*Enterococcus faecium* ATCC 9790	medium-size, bright, difficult to determine titer	8.0×10^{5}	N/A
EF9P2	*Enterococcus faecium* 61027	medium-size, round, dim, difficult to determine titer	4.0×10^{5}	N/A
KP1P1	*Klebsiella pneumoniae* DSM681	big, bacterial growth in the middle of plaque	6.8×10^{10}	1.7×10^{10}
KP1P2	*Klebsiella pneumoniae* DSM681	small, bright center surrounded by misty ring, some big	4.9×10^{8}	2.3×10^{7}
KP1P3	*Klebsiella pneumoniae* DSM681	medium-size, slightly turbid center	3.0×10^{10}	3.2×10^{9}
KP1P4	*Klebsiella pneumoniae* DSM681	medium-size, slightly turbid center	4.7×10^{10}	1.8×10^{10}
KP1P5	*Klebsiella pneumoniae* DSM681	big, turbid, bacterial growth in the middle of plaque	1.2×10^{11}	1.1×10^{10}
KP1P6	*Klebsiella pneumoniae* DSM681	variable size, some bright, some had bacterial growth in the middle	3.0×10^{10}	6.1×10^{9}
KP2P1	*Klebsiella pneumoniae* 10UO03898	medium-size, bright	1.5×10^{8}	2.5×10^{8}
KP2P2	*Klebsiella pneumoniae* 10UO03898	small, bright	2.6×10^{8}	2.1×10^{5}
KP3P2	*Klebsiella pneumoniae* 61705	medium-size, dim	1.0×10^{7}	1.5×10^{7}
KP4P1	*Klebsiella pneumoniae* 61784	small, bright, halo	1.4×10^{9}	4.4×10^{8}
KP5P1	*Klebsiella pneumoniae* 61837	small, bright	1.7×10^{9}	7.0×10^{8}
KP5P2	*Klebsiella pneumoniae* 61837	small, bright	5.0×10^{8}	2.9×10^{8}
KP6P1	*Klebsiella pneumoniae* 61794	small, slightly dim	4.0×10^{9}	1.8×10^{9}
KP6P2	*Klebsiella pneumoniae* 61794	small, dim	4.1×10^{8}	8.0×10^{7}
PA1P1	*Pseudomonas aeruginosa* 61841	small, bright, vague thin ring around	2.0×10^{11}	1.0×10^{11}
PA1P2	*Pseudomonas aeruginosa* 61841	small, bright, vague thin ring around	9.3×10^{10}	6.5×10^{10}
PA1P3	*Pseudomonas aeruginosa* 61841	small, bright, vague thin ring around	8.1×10^{10}	3.6×10^{10}
PA1P4	*Pseudomonas aeruginosa* 61841	small, bright, vague thin ring around	1.1×10^{11}	6.8×10^{10}
PA1P5	*Pseudomonas aeruginosa* 61841	small, bright, vague thin ring around	1.6×10^{11}	1.1×10^{11}
PA2P1	*Pseudomonas aeruginosa* 61823	small, dim	9.5×10^{8}	1.8×10^{11}
PA2P2	*Pseudomonas aeruginosa* 61823	small, round, turbid center surrounded by bright ring	5.0×10^{8}	5.0×10^{7}
PA3P1	*Pseudomonas aeruginosa* 61790	small/medium-size, dim	3.3×10^{10}	2.9×10^{8}
PA3P2	*Pseudomonas aeruginosa* 61790	small/medium-size, very dim, disfigured plaques	3.4×10^{8}	1.9×10^{8}
PA4P1	*Pseudomonas aeruginosa* 61432	small, dim	5.8×10^{8}	1.3×10^{7}
PA4P2	*Pseudomonas aeruginosa* 61432	round, bright	3.7×10^{9}	2.2×10^{9}
PA5P1	*Pseudomonas aeruginosa* 11AN03663	medium-size, bright	6.0×10^{9}	7.0×10^{9}
PA5P2	*Pseudomonas aeruginosa* 11AN03663	medium-size, bright, round, some small and dim	6.7×10^{8}	1.7×10^{6}
PA7P1	*Pseudomonas aeruginosa* 62314	small, dim, rough edges	2.1×10^{10}	3.9×10^{10}
PA7P2	*Pseudomonas aeruginosa* 62314	medium-size, slightly dim	1.5×10^{11}	5.5×10^{10}
PA8P1	*Pseudomonas aeruginosa* 62263	small, halo	1.4×10^{11}	1.3×10^{11}
PA8P2	*Pseudomonas aeruginosa* 62263	small, bright	6.1×10^{8}	2.9×10^{6}
PA9P1	*Pseudomonas aeruginosa* 62224	small, bright, halo	1.4×10^{11}	1.9×10^{11}
PA9P2	*Pseudomonas aeruginosa* 62224	big, bright	1.2×10^{9}	3.4×10^{7}
PA10P1	*Pseudomonas aeruginosa* 62206	big, dim	7.0×10^{9}	6.0×10^{9}
PA10P2	*Pseudomonas aeruginosa* 62206	small, bright, halo	1.0×10^{11}	1.1×10^{11}
PA10P3	*Pseudomonas aeruginosa* 62206	small, dim, vague halo	6.7×10^{10}	6.8×10^{9}
PA11P1	*Pseudomonas aeruginosa* 62180	small, dim, rough edges	2.0×10^{11}	1.4×10^{11}
PA11P2	*Pseudomonas aeruginosa* 62180	small, round, dim	8.0×10^{11}	5.4×10^{11}
PA12P1	*Pseudomonas aeruginosa* 62181	medium-size, slightly dim, rough edges	1.7×10^{8}	2.5×10^{8}
PA12P2	*Pseudomonas aeruginosa* 62181	small/medium-size, slightly dim, round	1.4×10^{11}	1.2×10^{11}
PA13P1	*Pseudomonas aeruginosa* 62172	medium-size, bright	1.2×10^{11}	1.1×10^{11}
PA13P2	*Pseudomonas aeruginosa* 62172	small, dim, some had halo	2.7×10^{11}	4.2×10^{11}
PA14P1	*Pseudomonas aeruginosa* 62109	extremely small, slightly dim	2.6×10^{8}	2.1×10^{4}
PA14P2	*Pseudomonas aeruginosa* 62109	big, slightly dim, round	4.1×10^{9}	4.2×10^{9}
PA16P1	*Pseudomonas aeruginosa* 62069	extremely small, dim, disfigured plaques, difficult to determine titer	1.4×10^{8}	6.7×10^{7}
PA16P2	*Pseudomonas aeruginosa* 62069	small, round (slightly rough edges), dim	9.5×10^{7}	6.6×10^{7}
PA17P1	*Pseudomonas aeruginosa* 61932	small/medium-size, slightly dim,	3.0×10^{9}	3.9×10^{8}
PA17P2	*Pseudomonas aeruginosa* 61932	small/medium-size, slightly dim, round, halo	2.4×10^{9}	1.9×10^{9}
SA10P1	*Staphylococcus aureus* 10AE05905	small, slightly dim	7.4×10^{8}	2.3×10^{8}
SA10P2	*Staphylococcus aureus* 10AE05905	small, slightly dim	1.5×10^{9}	7.0×10^{8}
SA10P3	*Staphylococcus aureus* 10AE05905	small, slightly dim, roundish	4.0×10^{8}	1.0×10^{8}
SA10P4	*Staphylococcus aureus* 10AE05905	small, dim, round	5.0×10^{7}	3.3×10^{7}
SA14P1	*Staphylococcus aureus* 60881	small, slightly dim	4.7×10^{7}	1.0×10^{6}
S1P1	*Salmonella* , quality control strain 18048	small, dim, round	1.2×10^{10}	2.1×10^{10}
SB1P1	*Salmonella* , Group B, ESBL-strain 12FB01687	small and medium-size, dim, roundish	3.5×10^{8}	1.8×10^{8}
SC1P1	*Salmonella* , Group C, 13FB10784	extremely small, bright, round	1.3×10^{10}	3.0×10^{6}
SE1P1	*Salmonella enteritis* (36.)	small, dim	1.1×10^{8}	9.2×10^{7}
SE1P2	*Salmonella enteritis* (36.)	in high density: small, dim, round / in lower density: big, dim halo, bright center, round	1.3×10^{7}	1.0×10^{8}
SE3P1	*Salmonella enteritidis* FB11214	big, turbid center, bright halo, round	7.8×10^{7}	5.0×10^{7}
SK1P1	*Salmonella Kreber* (37.)	extremely small, bright	1.9×10^{7}	9.8×10^{6}
SK1P2	*Salmonella Kreber* (37.)	extremely small, slightly dim, round	2.9×10^{6}	1.6×10^{6}
SS1P1	*Salmonella stanley* FB3820	small, extremely dim, roundish	N/A	N/A
ST1P1	*Salmonella typhimurium* FB7595	big, turbid center, bright halo, round	1.2×10^{10}	2.4×10^{10}