Master's Thesis

Characterization of *Acinetobacter baumannii* bacteriophages and determination of phage-resistance costs in bacterial hosts

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There has been a worldwide recession on effective antibiotics against increasing number of multi-antibiotic resistant bacteria across multiple species. *Acinetobacter baumannii* has arisen as most notable multi-drug resistant species in clinical care associated infections. This is due to *A. baumannii*'s efficient genetic machinery and upregulation of innate resistance mechanisms, which has allowed the accumulated resistance towards multiple classes of antibiotics. Furthermore, it has remarkable capability to survive harsh environments, which makes it possible for the bacteria to endure a long time and transmit to a possible host. New strains of *A. baumannii* have emerged, which have acquired resistance towards almost all known classes of antibiotics. With increasing number of reports about multi-drug resistant *A. baumannii* infections and outbreaks, new therapeutic approaches are needed to be pursued. Using viruses that target bacteria (phages) to combat against bacterial drug resistance is a potential therapy option. The aims of the study were to isolate phages from sewage water against 4 different antibiotic resistant *A. baumannii* strains, assess the phage resistance mechanisms of development and determine their viability of phage therapy. Phage isolations were attempted from wastewater without success. Thus, 7 previously isolated phages from the same wastewater source, were characterized via plaque assays, growth tests, genome sequencing and electron microscopy. All phages seem to be novel and have potential for usage in phage therapy, but more information is still needed for proper therapy assessment.

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Antibioottien teho on heikennyt maailmanlaajuisesti ja resistenssisyys monia eri antibiootteja vastaan on yleistynyt lähes kaikissa eri bakteerilajeissa. *Acinetobacter baumannii* on noussut esiin merkittävimpänä monen antibioottiresistanssin omaavana patogeenina, varsinkin sairaalaympäristöissä. Tämä johtuu pääosin *A. baumanni*in tehokkaista geneettisistä ominaisuuksista, kuten sisäisen puolustuksen uudelleenjärjestelystä, jonka avulla bakteeri on kyennyt muun muassa keräämään resistenssisyyksiä monia antibiootteja vastaan. Se myös kykenee selviämään erilaisissa olosuhteissa, täten lisäten sen leviäväisyyttä. *A. baumanni*ista on jo löytynyt kantoja, jotka ovat resistenttejä kaikille tunnetuille antibiooteille. Antibioottiresistenttien *A. baumannii* -kantojen infektiomäärien kasvaessa, uudet hoitokeinot ovat tarpeen. Faagiterapia eli bakteerispesifisten virusten (bakteriofaagien) käyttö on mahdollinen vaihtoehto antibioottiresistenttien bakteeri-infektioiden hoitoon. Tutkimuksen tavoitteena oli eristää faageja jätevedestä neljää eri antibioottiresistenttiä *A. baumannii* kantaa vastaan, tutkia faagiresistenssin kehitystä ja määrittää resistenssiyden vaikutukset bakteerin kasvuominaisuuksiin. Faagieristys jätevedestä ei onnistunut useista yrityksistä huolimatta. Täten, seitsemän aikaisemmin eristettyä faagia karakterisoitiin tutkimuksessa plakkianalyyseillä, kasvukokeilla, DNA sekvensoinnilla ja

elektronimikroskopoinnilla. Kaikki faagit olivat aikaisemmin tuntemattomia ja ovat mahdollisesti potentiaalisia faagiterapiaa varten.

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TERMS AND ABBREVIATIONS

TERMS

Bacteriophage	A virus that infects and replicates in bacteria and archaea
Capsid	A coiled or polyhedral protein structure, that encloses and protects the nucleic acid of a virus
Lytic	Relating to lysis or the destruction of the cells; producing lysis
Lysogenic	Harbouring a temperate virus as a prophage or plasmid, which has the capability to undergo lysis
Nosocomial	Acquired or occurring in a hospital
Plaque	Area devoid of bacterial growth due to destruction of the cells by a bacteriophage
Prophage	Genetic material of a temperate bacteriophage, which has integrated into bacterial host genome in a stable manner, capable of being replicated and expressed
Temperate phage	Bacteriophage capable of integrating into bacterial host genome, rarely causing lysis
Titer	The strength of a solution or the concentration of a substance in an solution determined by titration

ABBREVIATIONS

abs.	Absorbance
LB	Lysogeny broth
LB-Plate	100 x 15 mm petri dish covered with a LB-agar layer
OD595	Optical density at 595 nm
PFU	Plaque forming unit
RT	Room temperature

1 INTRODUCTION

1.1 *Acinetobacter baumannii*

Acinetobacter baumannii has emerged throughout the world as one of the most successful pathogens plaguing the modern healthcare system (Lin and Lan 2014). It is an opportunistic pathogen and is most commonly associated with hospital-derived (nosocomial) infections (Montefour *et al.* 2008). It infects most prevalently immunocompromised individuals, especially in prolonged hospital care (Lin and Lan 2014). It has shown broad resistance to most first-line antibiotics and it is one of the so-called ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogens, which cause most of the antibiotic resistant nosocomial infections in the world (Rice 2008). Furthermore, *A. baumannii* has been declared by WHO (World Health Organization) as number 1 priority pathogen for new research and development of new antibiotics (WHO 2017).

Acinetobacter spp. are non-motile, non-fastidious, oxidase-negative, non-fermenting, catalase-positive, aerobic Gram-negative coccobacilli (Baumann *et al.* 1968, Lin and Lan 2014). *Acinetobacter* genus includes 26 named species and 9 genomic species (Nocera *et al.* 2011). They can be found practically from all soil and surface water samples and are thus deemed ubiquitous in nature (Peleg *et al.* 2008). However, no systematic study has been conducted to examine the different *Acinetobacter* species natural occurrence in nature and some of them do not have their natural habitat in the environment (Peleg *et al.* 2008). They can be difficult to identify due to de-staining difficulties, which can lead to mistaken classification as Gram-positive (Peleg *et al.* 2008). Furthermore, four of the species (*A. baumannii*, *A. calcoaceticus*, Acinetobacter genomic species 3 and Acinetobacter genomic species 13TU) are

difficult to differentiate from each other due to their comparable phenotypes and are referred in some instances as *A. calcoaceticus*-complex (Gerner-Smidt *et al.* 1991).

A. baumannii is considered the most significant among the *Acinetobacter* species regarding infections in clinical surroundings and reported outbreaks (Lin and Lan 2014). Before the 1970's, it was considered as a low priority pathogen due to its susceptibility to range of antibiotics, such as tetracyclines, aminoglycosides and β-lactams (Bergogne-Bérézin & Towner, 1996). Currently, strains of *A. baumannii* have arisen with resistance to almost all know antibiotics (Falagas and Bliziotis 2007, Ahmed *et al.* 2016, Nowak and Paluchowska 2016). This is credited to its incredible capability to acquire antimicrobial resistance mechanisms (Lee *et al.* 2017) and upregulation of innate resistance mechanisms (Howard *et al.* 2012). Multiple different resistance mechanisms, such as efflux pumps, permeability defects, modification of target sites, aminoglycoside-modifying enzymes and β-lactamases, have been detected in *A. baumannii* strains (Lee *et al.* 2017). These different mechanisms can target singular antibiotic class or work in tandem (Lin and Lan 2014) and the accumulation of these mechanisms have progressively diminished the range of available antibiotics against *A. baumannii* (Lee *et al.* 2017). Furthermore, *A. baumannii* has remarkable resistance towards desiccation and disinfectants (Jawad *et al.* 1998, Wisplinghoff *et al.* 2007). These attributes contribute towards clinical outbreaks and enable *A. baumannii* 's prolonged survival in clinical environment. For example, bed rails have been discovered to be possible secondary reservoirs for infection (Catalano 1999). The ever-diminishing repertoire of treatment options against *A. baumannii*, together with its natural survival tenacity in clinical surroundings, raises the need for new therapeutic tools.

1.3 Bacteriophages

Phages, short for bacteriophages, are bacteria specific viruses and are considered one of the most abundant genetically replicating entities on earth (Fuhrman 1999, Comeau *et al.* 2007, Suttle 2005, 2007). They were first discovered by William Twort

in 1915, but their antimicrobial ability was first realised in 1917 by Felix d'Herelle (Duckworth 1976). However, most of the bacteriophage research as possible therapeutic agents were quickly abandoned after the discovery of antibiotics (Chanishvili 2012, Altamirano & Barr 2019). The research continued in few places, such as in Eliava Institute in Georgia and Ludwik Hirszfeld Institute of Immunology and Experimental Therapy in Poland, which are still up and running to this day (Chanishvili *et al.* 2009, Górski *et al.* 2017, Międzybrodzki *et al.* 2017).

Bacteriophages have either DNA or RNA genome, enveloped in a protective protein coat (capsid) and they reproduce in Bacteria or Archea, causing cell lysis. They can infect hosts through a variety of host surface molecules, such as protein receptors, lipopolysaacharide receptors and flagella, each of which are specific to different phages (Grove & Marsh 2011). Most of the phages discovered so far are tailed phages (*Caudovirales*), which have a tail-like protein structure that is involved in host receptor targeting, cell wall penetration and genome delivery. This was the original basis for their taxonomy, where they were divided into three different morphotypes, with contractile tail (*Myoviridae)*, long non-contractile tail (*Siphoviridae*) and short non-contractile tail (*Podoviridae*) (Bradley 1967, Ackermann & Eisenstark 1974). Over the years the taxonomy has been refined and expanded, but with the advancement in molecular technology, the genomic diversity of phages became more apparent. This led into the creation of multiple new subfamilies inside the order of *Caudovirales*, which in turn resulted the ICTV's Bacterial and Archeal Viruses Subcommittee transitioning into more genome-based classification for phages (Lavigne *et al.* 2008, 2009, Krupovic *et al.* 2016, Adriaenssens *et al.* 2018, 2020).

Phages can exhibit three different life cycles once they have been adsorbed by the host cell: lytic, lysogenic and chronic. In lytic cycle, the phages infect and swiftly hijack the hosts cellular machinery and redirect them to reproduce the phage progeny, which ultimate leads to the cell bursting and thus releasing new virions (complete virus particle) into the surrounding environment (Weinbauer & Rassoulzadegan 2004, Abedon *et al.* 2008**)**. The lysogenic life cycle oppositely, is

where a temperate phage integrates into the hosts genome as a prophage, without immediately killing the host, where it can remain stable for thousands of generations, affecting the host gene expression in a process called lysogenic conversion (Little *et al.* 2014, Łoś *et al.* 2014)**.** These temperate phages can also switch from lysogenic to lytic life cycle, depending on different environmental stressors, such as the amount of UV light and nutrient availability **(**Campbell 1988**,** Wilson *et al.* 1996, Munson-McGee 2018). The chronic life cycle occurs mostly in archaeal phages, but also in few temperate and filamentous phages. Here, the phage does not cause any cell disruption or cell lysis, but instead virions are constantly being formed and released, only slowing the host cells growth (Munson-MacGee *et al.* 2018). With these processes, phages influence microbe populations through the manipulation of their biology and thus in turn, affect the entire microbiome (Weinbauer & Rassoulzadegan 2004, Abedon *et al.* 2008**).**

Bacteria have evolved multiple different immune systems in the response to the pressure exerted by phages. Phages in turn, have developed countless ways to circumvent and overcome these defences, which has resulted in a continuous arms race between them, and contributed greatly to the bacterial immune diversity (Samson *et al*. 2013, Dy *et al.* 2014, Houte *et al.* 2016). Since phages are reliant on receptors for infection, bacteria have developed methods to disguise, alter, mask, or lower the expression of receptors through modifications on the surface of the bacteria (Høyland-Kroghsbo *et al.* 2013, Seed *et al.* 2014). Furthermore, temperate phages have also been seen to transfer phage resistance and prevent subsequent infections. This is achieved by affecting the configuration membrane receptors and by brining genes with it that encode for beneficial factors, such as membrane permeability and defence systems (Cumby *et al.* 2012, Dy *et al.* 2014). Bacteria can also bud out outer membrane vesicles, containing phage-targeted receptors, which act as a decoy for phages and reduces the number of possible infections in the surrounding population (Reyes-Robles *et al.* 2018). Phages have developed around this due to the selective pressure towards recognizing these altered receptors, which

has led to variety of receptor-binding protein configurations (Meyer *et al.* 2012). Furthermore, this also often increases the range of the receptors that the phage can bind on to, effectively increasing the host range for the phage and its progeny (Habusha *et al.* 2019).

A well-studied bacterial defence mechanism, restriction-modification (RM) system, differentiates foreign DNA and dismantles phage DNA after its injection. They are found in 90% of all known bacteria and have many different known mechanisms of action, many of which are dependent on DNA methylation targeting and restriction endonuclease cleavage (Oliviera *et al.* 2014, Loenen *et al.* 2014,). However, phages have evolved many strategies to overcome RM and RM-like systems, which are mainly DNA modifications by methylation, acetamidation, hydroxymethylation, glucosylation and glycosylation, to prevent endonuclease targeting and cleavage (Vasu & Nagaraja 2013, Pleška *et al.* 2017). Bacteria have also developed another defence, which specifically targets and cleaves foreign DNA and RNA sequences: clustered regularly interspaced short palindromic repeats (CRISPR)–associated protein (Cas) system. In contrast to RM, CRISPR-Cas needs previous encounter with foreign genetical material to recognize and dismantle it, providing the bacteria essentially with an adaptive immunity (Jackson *et al.* 2017). To circumvent this defence, phages have been found to mutate the sites that are targeted, encode for proteins that inactive CRISPR-Cas systems and modify their DNA to reduce Cas complex binding and cleavage (Bondy-Denomy *et al.* 2013, Strotskaya *et al.* 2017, B. N. J. Watson *et al.* 2019).

In comparison to most other bacterial defensive mechanisms, abortive infection (Abi) anti-phage systems protect the whole bacterial population by self-destruction of infected bacteria (Houte *et al.* 2016). Abi is essentially self-induced cell death through different metabolic routes, such as phosphorylation pathway, toxin-antitoxin system or formation of membrane leakage channels (Parma *et al.* 1992, Fineran *et al.* 2009, Depardieu *et al.* 2016). These systems in bacteria, and how phages have developed to avoid them are still not completely understood yet, but certain

T4-phages have been detected to bypass toxin-antitoxin and Rex membrane leakage channels Abi systems (Snyder 1995, Otsuka & Yonesaki 2012).

1.3 Phage Therapy

Phage therapy, where phages are utilized to eradicate and weaken bacterial infections, is a potential way to combat against ever-rising tide of multidrug-resistant bacteria species (d'Herelle 1931, Chanishvili 2012). Phages have been in clinical use almost since early 1920, but most of the trials were poorly organized and the lacked consistency, which lead into safety and efficacy concerns towards phage therapy (Altamirano & Barr 2019). Thus, phage therapy research remained active only in few locations in Eastern Europe (Chanishvili *et al.* 2009, Rohde *et al.* 2018). Since then, phage therapy has been studied and used in preclinical and clinical human trials at Eliava Institute of Bacteriophage and the Institute of Immunology and Experimental Therapy, against multiple different common bacterial pathogens (Kutateladze and Adamia 2008). In the face of post -antibiotic era heralded by WHO, phage therapy research has been revitalized all over the world (WHO 2014). Multiple studies have been done using animal models to examine the viability of phage therapy against multiple different clinically significant pathogens (Soothill 1992, Wang *et al.* 2006, Watanabe *et al.* 2007, Chan *et al.* 2016). In recent years, numerous case studies have been performed with humans using phage therapy, successfully treating variety of infections caused by a multitude of different bacterial pathogens (Zhvania *et al.* 2017, Fish *et al.* 2018, Hoyle *et al.* 2018, LaVergne *et al.* 2018, Law *et al.* 2019, Nir-Paz. *et al.* 2019). Furthermore, antibiotics combined with phages have yielded effective results as well against different pathogens and have even been seen to re-sensitize them to previously resisted antibiotics (Comeau 2007, Ryan *et al.* 2012, Waqas *et al.* 2017, Altamirano 2021). Overall, there have been great leaps in the field of phage therapy, but there are still many aspects that needs to be figured out in phage therapy, such as different regulatory hurdles, before safe and proper clinical use (Lee *et al.* 2017, Pelfrene *et al.* 2016).

1.4 Aims of the study

So far, there are relatively few studies and limited data available on *A. baumannii* phages (Lee *et al.* 2017). The aims of this study were to find out if therapeutically potential phages could be isolated against multidrug-resistant *A. baumannii* strains, and see how the resistance against phages emerge in bacteria, and how does it affects the bacterial phenotype. These will be achieved by plaque (area devoid of bacteria due to phages) assays, growth tests with bacterial hosts, stability assessments, genome sequencing and electron microscopy. For the bacteria, the relative cost of adapting against multiple different phages will most likely be higher, than against one, and this will affect the fitness negatively. The outcome of phage isolation cannot be estimated since it is highly dependent on the isolation time, and optimal isolation timepoints have not yet been determined.

2 MATERIALS AND METHODS

2.1 *Acinetobacter baumannii* strains

The 4 strains of *A. baumannii* (AB2, AB3, AB5, AB6) used in the research, were acquired for previous study (see Mattila *et al.*, 2015) from Turku University Hospital. The bacteria were cultured using Lysogeny Broth (LB) -medium (Sambrook *et al.*, 1989) shaken at 230 rpm in + 37◦C.

2.2 Phage isolation, stock preparation and phage stability

Phage isolation was attempted from 11 sewage samples, taken from wastewater treatment plant in Jyväskylä, Finland (Nenäinniemi), 7 soil samples, collected around University of Jyväskylä's Department of Biological and Environmental sciences campus, and 1 horse manure sample from Joutsa, Central Finland. Three of the sewage samples were taken at an earlier timepoint compared to the others and all the other solid samples were taken across three months (Table 1). All the sewage samples were filtered using 0.2 µm filter with 0.8 µm prefilter before usage to remove possible unwanted bacteria. Soil samples and the horse manure sample were centrifuged at 5000 RPM for 10 min, in room temperature (RT), to separate any solid material, before filtering with 0.2 µm filter with 0.8 µm prefilter. The samples were enriched by using 20 µl sample together with 20 µl of LB and 100 µl of *A. baumannii* strain 6 (AB6) overnight in +37 ◦C on a shaker at 210 RPM. The enriched samples were afterwards plated to 1% LB-agar containing 55 cm² petri dishes (LB-plate) with three different volumes; 50 µl, 100 µl and a streak with 1 µl loop, alongside with 100 µl of AB6 and 3 ml of LB with 0.7 % soft-agar (LB-soft). The LB-plates were incubated in +37 ◦C overnight and subsequently checked for plaques. Plaques were picked from the LB-plates using 100 µl pipette tips and placed into Eppendorf tubes with 500 µl of LB. The solution was then vortexed briefly (~3 min) and plated as previously, using 50 and 100 µl volumes and a 1 µl loop. This plaque picking process was repeated, after which if semi-confluence was observed, the

plate was used for stock preparation. The stock was prepared by collecting the LB soft-agar layer, mixing it with 5 ml of LB (per plate) and by incubating it in +37 °C on a shaker at 210 RPM for 4 hours. After incubation, the mixture was centrifuged at 5000 RPM for 10 min, in RT and filtered using 0.2 µm with 0.8 µm prefilter. Phage concentration (titer) of the resulting phage stocks were tested by using 100 µl of undiluted and three different dilutions, 10^{-3}-10^{-6}, of the made stock on LB-plates with 100 µl of AB6 and 3 ml of LB-soft (LB with 0.7 % soft-agar). The plates were incubated overnight in +37 °C, after which the resulting plaques were counted. The titer in PFU (plaque forming units)/ml was calculated using the equation:

$$\frac{PFU}{ml} = \frac{number\ of\ plaques}{used\ lysate\ volume \times dilution\ factor}$$

Plates with 25-250 PFU (Breed and Dotterrer 1916) range were used and averaged to calculate the final titers. The stocks were stored in +4 °C and were retitered after ~3 (1P1, 1P2 and 3P1) or ~10 (3P1, 6P1 and 6P2) months, to determine the phage stability.

2.3 Phage host range determination

Host range for the phages was determined by drop test. All the AB strains were plated to LB-plates, using 100 µl of o/n culture and 3 ml of LB-soft (LB with 0.7 % soft-agar). After left for ~15 min to solidify in RT, 5 µl droplets of each phage stock were put on top of the agar lawn and the plates were incubated in +37 °C o/n. Infective phages were determined by clear spot, area devoid of bacterial growth, at the droplet site.

2.4 Host and Bacteriophage DNA extraction and genome analysis

The phage DNA from 1P1 (10^8 PFU/ml), 1P2 (10^8 PFU/ml), 3P1 (10^8 PFU/ml), 6P1 (10^8 PFU/ml) and 6P2 (10^9 PFU/ml) were extracted using Norgen Phage DNA Isolation Kit (Cat. 46800, 46850), with a minor modification to 1a. step to utilize

unbranded DNase I: 1 µl of DNase I (1 mg/ml) were added to the 1 ml phage samples, which were incubated for 30 min in +37 °C for activation and 5 min in +75 °C for inactivation. DNA extraction for 3P2 (10^8 PFU/ml) and AB6 (o/n culture) was done with the following modified protocol from Santos (Santos *et al*. 2009), together with Qiaqen DNeasy Blood & Tissue Kit (Cat. 69504). The samples were DNase and RNase treated, to remove unwanted genomic contamination, by adding 1 µl of DNase I (1 mg/ml) and 10 µl of RNase to 1 ml samples, which were incubated at +37 °C for 30 min. Afterwards, 20 µl of 0.2 µm filtered 2 M $ZnCl_2$ was added (total concentration of 40 µM) and the samples were incubated at +37 °C for 5 min and centrifuged at 10000 RPM for 1 min. The supernatants were discarded, and the resulting pellets were resuspended in 0.5 ml of 0.2 µm filtered TES buffer (0.1 M Tris-HCL pH 8, 0.1 M EDTA, 0.3% SDS) and incubated at 60 °C for 15 min. 20 µl of protease K (20 mg/ml) was added to the samples to remove any protein contamination and the samples were incubated at 37 °C for 2h. The subsequent purification was done using DNeasy Blood & Tissue Kit's protocol with half volumes. 500 µl of AL buffer was added to samples, together with 500 µl of 96% ethanol, and were mixed by vortexing. The sample mixtures were transferred to the elution column and were centrifuged at 6000 x g for 1 min. 500 µl of AW1 buffer were added and the columns were centrifuged at 6000 x g for 1 min. The columns were washed twice by adding 500 µl of AW2 buffer and centrifuging at 20 000 x g for 3 minutes. The DNA was eluted by adding 50 µl of PCR grade water to the spin column membranes and incubating them for 1 min in RT, before centrifuging at 6000 x g for 1 min. The elution step was repeated for additional DNA yield. All the flowthroughs were discarded after each wash. DNA extraction for 2P1 was unsuccessful with both methods.

The AB6 was sequenced using PacBio RSII and assembled with HGAP3 -pipeline. The 6 phages were sequenced using Illumina MiSeq PE3000 and libraries were prepared using Nextera Flex (Illumina).

The sequenced genomes were assembled into contig sequences, after they were quality checked, trimmed and possible host sequences were removed, using CLC Genomics Workbench 12 (Qiagen). The quality check was performed after each step, using CLC's quality check using QC for sequencing reads -function. At the start, majority of the sequences from all phages had PHRED score between 30-40 and thus their quality was deemed acceptable. The sequences were trimmed, removing 5 bases from 5' and 3' ends and filtering lengths below 50 bp and above 500 bp. The trim quality score was set as 0.01 for 1P1, 3P2, 6P1 and 6P2, while 0005 was used for 1P2 and 3P1. These settings led to the best results, the lowest length distribution, least amount of ambiguous bases, PHRED scores of >90 and good quality distribution.

The assembled genomes were inspected for host native sequences and annotated using Rapid Annotation using Subsystem Technology, RAST (Aziz *et al.* 2008, Overbeek *et al.* 2014, Brettin *et al.* 2015). Phage conservative structure proteins, such as tail or capsid proteins, were chosen from the results and used with RAST's Psi-Blast to find close relatives, from which discern information about the phages themselves. The whole genomes were ran through NCBI's Microbial Nucleotide BLAST using discountinous megablast and megablast settings, to verify the novelty of the phages. The phage genomes were compared to each other with NCBI Aliqn Sequences Nucleotide Blast, using megablast setting.

2.5 Cultivation of phage-resistant hosts

Phage resistance hosts were cultivated with singular-, multi- and sequential phage exposures, using phages the strains were deemed to be susceptible against in the previous host range determination. Singular- and multi exposures were done for each AB-strain for 2 days, using either one or all infective phages, respectively. AB2 and 5 were exposed in order towards 3P2, 6P2, 1P1 and 1P2. AB3 and 6 were exposed in order towards 3P1, 6P1, 1P1, 1P2, 3P2 and 6P2. Exposures were started by adding 1 ml of o/n grown host, 4 ml of LB and 10 µl of phage stock(s) and

incubating overnight in +37 °C on a shaker at 210 RPM. On the second day, 50 µl of the previous exposure was transferred to 5 ml of LB, containing 50 µl of the phage stock(s), and the new exposure was incubated overnight in +37 °C on a shaker at 210 RPM. The second day exposure was plated using 1 µl loop and the LB-plate was incubated o/n in +37 °C. Afterwards, one resistant colony was picked and placed into 5 ml of LB and incubated in +37 °C. The phage susceptibility of the arisen resistant strain was tested with 5 µl drop test. The sequential phage exposures were done as the singular ones, but after each exposure the resulting resistant strain was subjected to another phage, until all the available phages the strain was originally susceptible against, were used. AB6 -strain was also exposed towards 1P1 and 1P2 individually, and towards AB3 and AB6 sequentially, due to emerged vulnerability after other exposures. Additionally, AB3 and 6 were exposed to 3P2 and 6P2 due to same reason.

2.6 Growth Characteristics Measurement

The growth of all the original and the cultivated resistant strains (except 1P1 and 1P2 exposed AB3 and 6) was measured using Thermo Fisher Multiskan™ FC Microplate Photometer with Skanit software. The strains were inoculated to 3 ml of LB and grown o/n in +37◦C at 210 RPM. Two parallel runs were done for each strain, by diluting the o/n grown bacteria with 1:100 ratio to either LB or 10% LB, and 200 µl of each diluted strain were pipetted to 96-plate with three replicates. 200 µl of LB or 10% LB was pipetted to three wells for background measurement. The wells on the plate edge were filled with H_2O to prevent excess evaporation. The plate's absorbance (abs.) was measured in 595nm wavelength 5 min intervals for 20 h in +37◦C, from which average rate, max rate and max peak values were collected.

2.7 Bacteriophage morphology

The morphology of the phages was studied using JEM-1400 transmission electron microscope (TEM) and their plaque morphology via visual examination. Phage lysates of 1P1 (10^8 PFU/ml), 1P2 (10^8 PFU/ml), 3P1 (10^8 PFU/ml), 6P1 (10^8 PFU/ml) and 6P2 (10^9 PFU/ml) were purified and concentrated for imaging via ultracentrifugation. 5 ml of lysates were centrifuged at 25 000 x g (14 500 RPM) for 2 hours at 4°C. The resulting opaque pellets were resuspended in 5 ml of filtered (0.2 µm filter with 0.8 prefilter) 0.1 M ammonium acetate (pH=7). The centrifugations and resuspensions were repeated twice, before suspending the pellets in 70 µl of sterile 0,02M potassium phosphate (pH=7,5).

The phage concentrates were negatively stained using uranyl acetate (UA) or phosphotungstic acid (PTA). 5 µl of phage concentrate was transferred to Formvar carbon-coated 200-mesh grid and were left to bind for 30 seconds or 2 minutes. Afterwards, the excess concentrate was dried using filter paper. The grids were then stained using 5 µl of PTA (phosphotungstic acid) (pH=7) or 2 µl of UA (2%) for 30 seconds or 2 minutes, and the excess was removed using filter paper.

3 RESULTS

3.1 Phage isolation

The objective was to isolate phages against *A.baumannii* from soil and sewage samples. However, after 19 isolation attempts (Table 1) with AB6, 16 potential plaque picks and two rounds of plaque purification, no phages were found. Thus, seven *A. baumannii* -phages, 1P1, 1P2, 2P1, 3P1, 3P2, 6P1 and 6P2 isolated in a previous study (Mattila *et al.* 2015) were used in further experiments.

Table 1. Phage isolation attempts chronologically.

Sample	Date	Sample	Date
Waste Water Sample 1	05.06.18 – 11.06.18	Waste Water Sample 7	02.07.18 – 11.07.18
Waste Water Sample 2	12.06.18 – 15.06.18	Waste Water Sample 8	02.07.18 – 11.07.18
Soil sample 1	18.06.18 – 21.06.18	Waste Water Sample 9	02.07.18 – 11.07.18
Horse Manure Sample	19.06.18 – 21.06.18	Waste Water Sample 10	02.07.18 – 11.07.18
Soil sample 2	25.06.18 – 29.06.18	Waste Water Sample 11	02.07.18 – 11.07.18
Soil sample 3	25.06.18 – 29.06.18	Waste Water Sample 12	02.07.18 – 11.07.18
Soil sample 4	25.06.18 – 29.06.18	Soil sample 5	10.07.18 – 17.07.18
Waste Water Sample 4	26.06.18 – 29.06.18	Soil sample 6	10.07.18 – 17.07.18
Waste Water Sample 5	26.06.18 – 29.06.18	Soil sample 7	10.07.18 – 17.07.18
Waste Water Sample 6	26.06.18 – 29.06.18		

3.2 Phage morphology

Imaging of phages 1P1 and 1P2 with transmission electron microscope identified them as *Myoviridae* (Figure 1 and Appendix 1) by the characteristic elongated icosahedral head and contractile long tail, both of which were ~100 µm in length. The 3P1 and 6P1 phages (Figure 2 and Appendix 2) were classified to be *Podoviridae* or *Autographiviridae* due to their short and small stature, ~60 nm diameter icosahedral head and short tail. The 3P2 and 6P2 (Figure 3 and Appendix 3) shapes and dimensions were similar to that of 1P1 and 1P2, which classifies them as *Myoviridae,* but the exact shape of the head was unidentifiable. Furthermore, as the particles were empty, they appeared darker; their capsids ununiform and tail shape shorter and thicker.

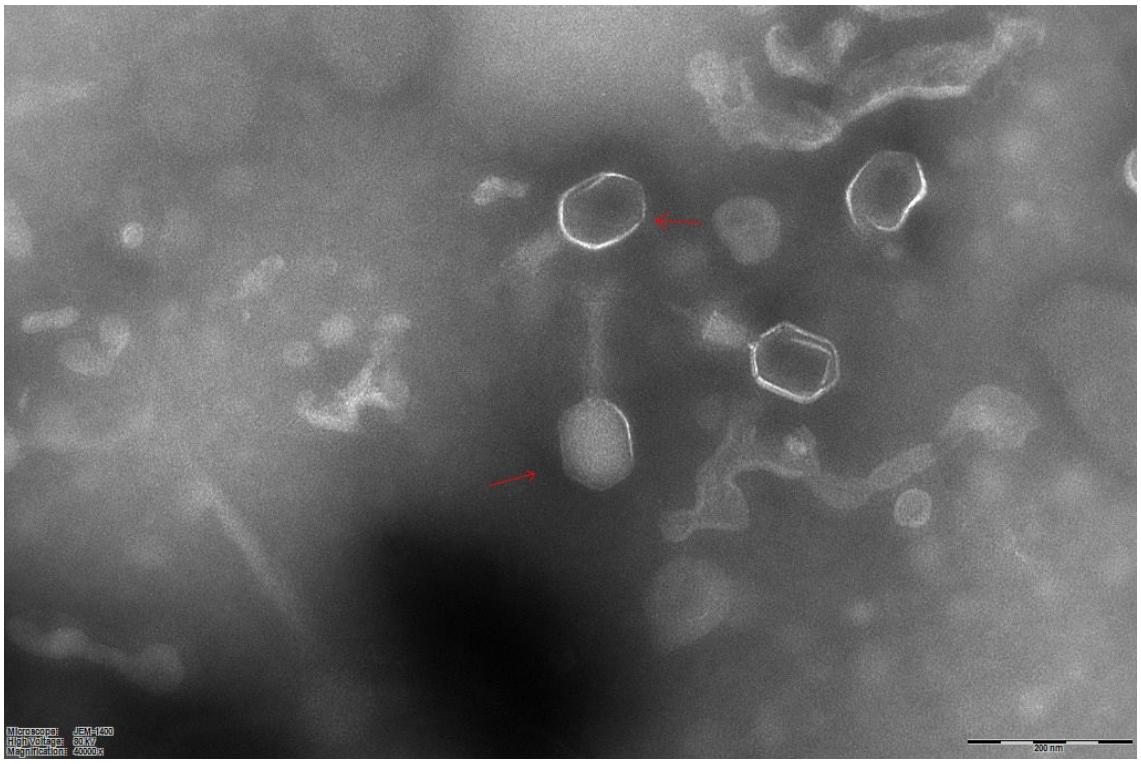

Figure 1. Concentrated 1P1 phage stained with PTA and imaged with JEM-1400 Transmission electron microscope at 40000 magnification. Time used for sample binding and staining was 30 s.

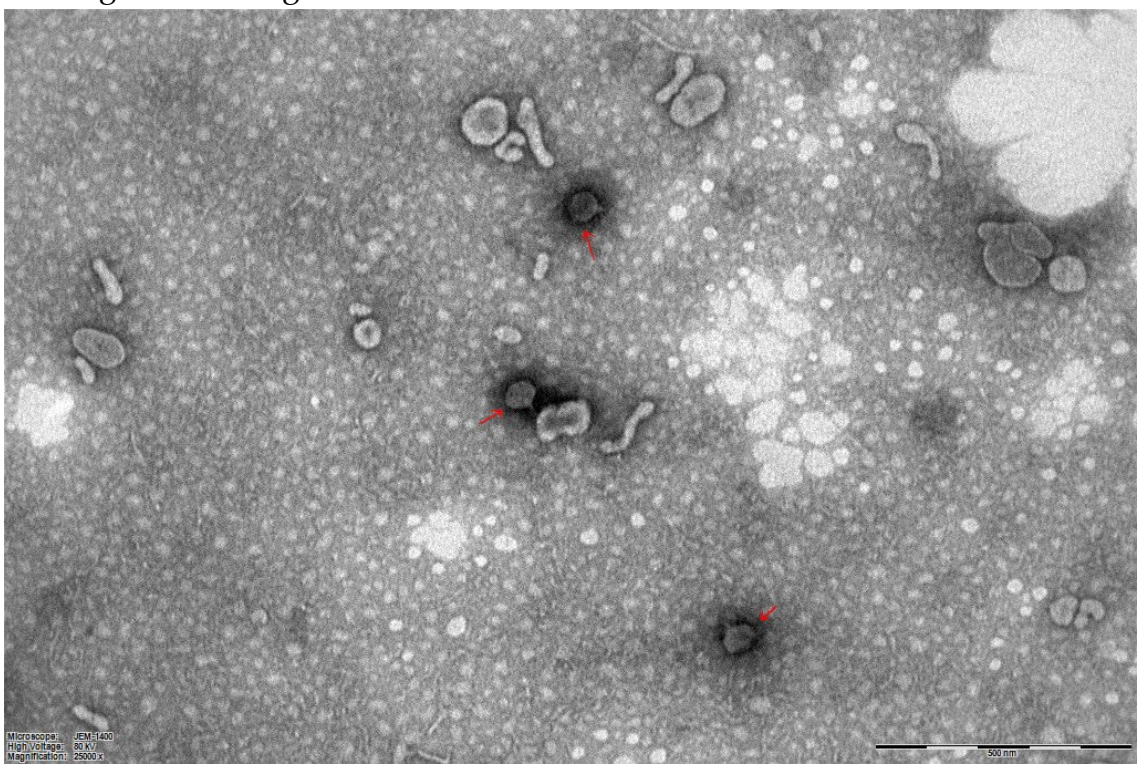

Figure 2. Concentrated 6P1 phage stained with PTA and imaged with JEM-1400 Transmission electron microscope at 25000 magnification. Time used for sample binding was 2m and for staining 30 s.

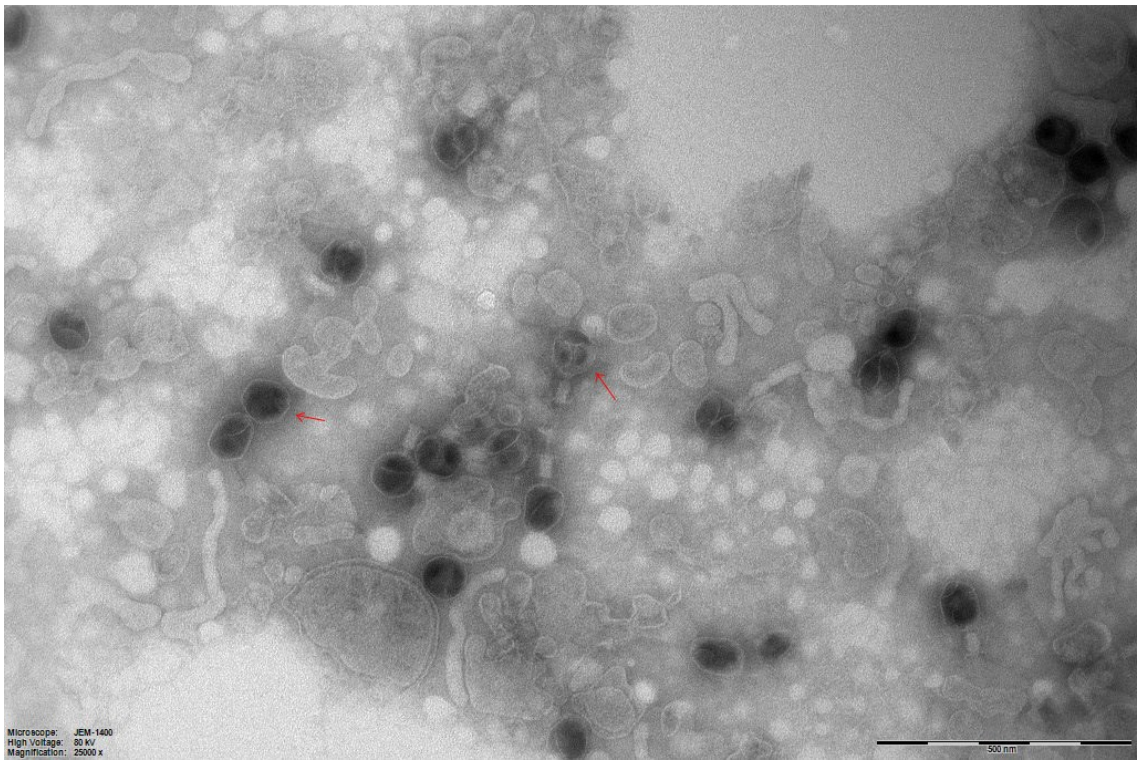

Figure 3. Concentrated 6P2 phage stained with PTA and imaged with JEM-1400 Transmission electron microscope at 25000 magnification. Time used for sample binding was 2m and for staining 30 s.

3.3 Genome analysis

The genome assembly for 1P1 resulted in one consensus sequence with the length of 165 kb. Phage tail sheath monomer, Phage tail completion and Phage major capsid proteins were chosen from 1P1's RAST annotation results to be used for Psi-Blast. These produced multiple hits with high query cover and high sequence identities, from which, notable matches were vB_ApiM_fHyAci03 (Pulkkinen *et al.* 2019), KARL-1 (Jansen *et al.* 2018), ZZ1 (Jin *et al.* 2012) and AbTZA1 (Nir-Paz *et al.* 2019) (Table 2). NCBI's Microbial Nucleotide BLAST (Table 5) produced 0% query covers with both discontiguous and normal megablast settings, but highest sequence identities were for *A.baumannii* complete genome, *Solemya elarraichensis* whole genome shotgun sequence and *Sedimenticola selenatireducens* whole genome shotgun sequence.

1P2 assembly created nine consensus sequences, from which one was ~165 kb in length and was chosen for further analyses, while others ranged from 275-345 bp. From the 1P2's RAST annotation results, Phage capsid/scaffold and Phage head completion proteins were chosen for comparison with Psi-Blast. These produced similar results to 1P1, with high query cover and sequence identities towards vB_ApiM_fHyAci03, KARL-1, ZZ1 and AbTZA1 (Table 2). RAST annotated and identified DNA topoisomerase 1 from contig 3, Phenylacetate-coenzyme A ligase from contig 4 and VgrG protein from contig 5, which all were closely related to either *A. baumannii* or *A. pittii*. The NCBI Microbial nucleotide BLAST results for 1P2 were nearly identical to that of 1P1, with minor deviation in sequence identity percentage. The NCBI Microbial Nucleotide BLAST (Table 5) had identical results to that of 1P1.

Table 2. Six highest scoring results from RAST-Psi BLAST for chosen 1P1 and 1P2 proteins. 1* indicates the phage, 2* the chosen protein and 3* description of results.

1*	**1P1**					
2*	Phage tail sheath monomer					
3* Description		Max Score	Total Score	Query Cover	E value	Seq iden.
Phage tail seath protein	[*AB* phage vB_ApiM_fHyAci03]	1296	1296	100%	0.00E+00	99.24%
Phage tail seath protein	[AB phage KARL-1]	1295	1295	100%	0.00E+00	99.09%
Phage tail seath protein	[AB baumannii]	1225	1225	100%	0.00E+00	99.40%
Phage tail seath protein	[AB phage AbTZA1]	1225	1225	100%	0.00E+00	92.40%
Phage tail seath protein	[AB phage ZZ1]	1119	1119	99%	0.00E+00	83.76%
Phage tail completion protein						
Phage tail completion and sheath stabilizer protein	[AB phage vB_ApiM_fHyAci03]	387	387	100%	3.00E-136	100.00%
Phage tail completion and sheath stabilizer protein	[AB phage KARL-1]	385	385	100%	2.00E-135	99.47%
T4-like virus tail tube protein gp19	[AB baumannii]	347	347	100%	2.00E-120	86.24%
Phage tail completion and sheath stabilizer protein	[AB phage ZZ1]	289	289	97%	2.00E-97	71.35%
Phage gp3 tail completion and seath stabilizer protein	[Acinotebacter phage Acj9]	260	260	95%	5.00E-86	69.61%
Phage major capsid protein						
Phage major capsid protein	[AB phage vB_ApiM_fHyAci03]	1037	1037	100%	0.00E+00	99.62%
Phage major head protein	[AB phage KARL-1]	1032	1032	100%	0.00E+00	99.24%
Hypothetical protein	[AB phage AbTZA1]	997	997	100%	0.00E+00	94.87%
Mahor capsid protein Gp23	[AB baumannii]	994	994	100%	0.00E+00	94.86%
Phage gp23 major head subunit precursor	[AB phage Acj9]	922	922	98%	0.00E+00	88.20%
1P2						
Phage capsid and scaffold protein						
Phage prohead core protein	[AB phage vB_ApiM_fHyAcio3]	271	271	100%	6.00E-92	99.30%
Uncharacterized protein	[AB baumannii]	249	249	100%	6.00E-83	91.55%
Phage prohead core protein	[AB phage ZZ1]	199	199	98%	3.00E-63	70.71%
Phage gp68 procore core protein	[AB phage Acj9]	183	183	99%	5.00E-57	69.50%
Phage gp68 procore core protein	[AB phage Acj61]	181	181	95%	3.00E-56	66.18%
Phage head completion protein						
Phage head completion protein	[AB phage vB_ApiM-fHyAci03]	320	320	100%	9.00E-111	100%
Phage head completion protein	[AB phage KARL-1]	313	313	100%	3.00E-108	97%
Uncharacterized protein	[AB baumannii]	296	296	100%	3.00E-101	91%
Phage gp4 head completion protein	[AB phage Acj9]	223	223	100%	1.00E-72	68%
Phage head completion protein	[AB phage ZZ1]	223	223	99%	2.00E-72	67%

3P1 generated two consensus sequences, with the lengths of 275 bp and 42 kb. The 42 kb length contig was chosen for further analysis, but it did not generate any subsystem information through RAST, and only available usable information was from closest neighbor section, where *Staphylococcus Epidermis* gave a score of 50. Only recognized proteins by RAST were putative RNA-polymerase, DNA ligase phage-associated protein, two different Phage HNH homing endonucleases (ACLAME 27 and 312) and Phage endonuclease. Using these proteins with the compare region function in this section of RAST, the RNA-polymerase was found to be similar within various *Autographiviridae* phages, such as *Pseudomonas* phage LKA1 (Ceyssens *et al.* 2006) and *Klebsiella* phage KP34 (Drulis-Kawa *et al.* 2011), and T7-like phages, such as *Yersinia* phage Yepe2 (Genbank accession no. EU734170) and *Enterobacteria* phage BA14 (Mertens *et al.* 1982). The ACLAME 27 HNH homing endonuclease was similar with *Myoviridae* phage Felix 01 (Felix & Callow *et al.* 1943), while towards ACLAME 312 nothing was found to compare with. The Phage endonuclease sequence could be found from many *Pseudomonas* phages, such as LKA1, LKD16 (Ceyssens *et al.* 2006) and PT5 (Genbank accession no. EU056923), all of which belonged to *Autographiviridae*. Furthermore, DNA ligase phage-associated protein was found from multiple different *Autographiviridae* –phages as well. When ran through NCBI's Microbial Nucleotide BLAST (Table 5), megablast setting gave no results. Discontiguous megablast setting resulted in 3% query cover and ~71% sequence identity towards *A.baumannii* complete genome, but the rest of the results were with 0% query cover and rather high E-values.

3P2 assembled into 18 different consensus sequences, from which three were 69 kb (contig 2), 38 kb (contig 1) and 23 kb (contig 3) in length, while the rest varied between 204-8900 bp. RAST annotation for contig 1 produced two comparable proteins, Topoisomerase IV subunit B and T4-like phage DexA exonuclease A. These proteins were ran through RAST's Psi-Blast, which resulted in multiple high percentage query covers and sequence identities (Table 3). For Topoisomerase IV subunit B, most notable hits were towards AbTZA1, and ACj9 (Petrov *et al.* 2010)

and for T4-like phage DexA exonuclease A –protein, towards AbTZA1, KARL-1, and vB_ApiM_fHyAci03. Both proteins matched highly against *AB* itself.

Table 3. Four to five highest scoring results from Rast-Psi BLAST for chosen 3P2 proteins. 1* indicates the phage, 2* the chosen protein and 3* description of results.

1*	**3P2**					
2*	Topoisomerase IV subunit B					
3* Description		Max Score	Total Score	Query Cover	E value	Seq iden.
DNA gyrase, subunit B (type II topoisomerase)	[AB baumannii]	1221	1221	100%	0.00E+00	100%
topoisomerase IV subunit B	[AB phage AbTZA1]	1219	1219	100%	0.00E+00	99.67%
gp60plus39 DNA topoisomerase subunit	[AB phage Acj9]	1013	1013	99%	0.00E+00	81.56%
gp60plus39 DNA topoisomerase subunit	[AB phage Acj61]	963	963	100%	0.00E+00	75.08%
DNA topoisomerase subunit	[AB phage ZZ1]	958	958	100%	0.00E+00	75.66%
T4-like phage DexA exonuclease A						
Uncharacterized protein	[AB baumannii]	447	447	100%	6.00E-159	100%
exonuclease A	[AB phage AbTZA1]	446	446	100%	2.00E-158	99.54%
exonuclease A	[AB phage KARL-1]	400	400	99%	4.00E-140	85.78%
exonuclease A	[AB phage vB_ApiM_fHyAci03]	397	397	99%	4.00E-139	85.78%
Phage tail completion protein						
Phage T4-like virus tail tube protein gp19	[AB baumannii]	388	388	100%	1.00E-136	100%
Phage tail completion and seath stabliizer protein	[AB phage KARL-1]	350	350	100%	2.00E-121	86.77%
Phage tail completion and seath stabliizer protein	[AB phage vB_ApiM_fHyAci03]	347	347	100%	2.00E-120	86.24%
Phage tail completion and seath stabliizer protein	[AB phage ZZ1]	285	285	97%	1.00E-95	71.20%
Phage head completion protein						
Uncharacterized protein	[AB baumannii]	321	321	100%	2.00E-111	100%
Phage head completion protein	[AB phage vB_ApiM-fHyAci03]	296	296	100%	3.00E-101	90.60%
Phage head completion protein	[AB phage KARL-1]	295	295	100%	4.00E-101	88.59%
Phage head completion protein	[E.coli phage vB_EcoM_PhAPEC2]	227	227	99%	5.00E-74	68.24%
Phage capsid and scaffold protein						
Uncharacterized protein	[AB baumannii]	274	274	100%	8.00E-93	100%
Phage prohead core protein	[AB phage vB_ApiM_fHyAcio3]	248	248	100%	2.00E-82	90.85%
Phage prohead core protein	[AB phage ZZ1]	198	198	98%	8.00E-63	71.43%
Phage gp68 procore core protein	[AB phage Acj9]	178	178	99%	4.00E-55	66.67%

Annotation of contig 2 lead into three commensurable proteins: Phage tail completion, Phage head completion and Phage capsid/scaffold protein. Psi-Blast of the Phage tail completion protein resulted in 100% match to T4-Like virus tail tube protein gp19 from *A.baumannii* and other numerous high percentage query covers and sequence identities, most eminent being ZZ1, KARL-1 and vB_ApiM_fHyAci03 (Table 3). Phage head completion protein's Psi-Blast showed high similarity to KARL-1, vB_ApiM_fHyAci03 and *AB*. The Phage capsid/scaffold protein's result was nearly identical to the Phage tail protein, where *AB*, ZZ1, ACj9 and vB_ApiM_fHyAci03 were the most noteworthy. The NCBI Microbial Nucleotide BLAST (Table 5) had only one result with megablast setting towards *Heliobacter bilis*, with 3% query cover and ~71% sequence identity and discontinuous megablast had

low scoring matches towards *Luteibacter rhizovicinus* and *A. baumannii* complete genome.

6P1 assembly produced two consensus sequences with 238 bp and 42 kb lengths. These sequences did not give any subsystem information when ran through RAST and the longer one gave similar, if not identical results to that of 3P1, such as 50 score towards *Staphylococcus epidermis* from closest neighbor section and the same recognized proteins with same comparative results. The NCBI's Microbial Nucleotide Blast (Table 5) even produced identical results to that of 3P1.

6P2 produced 2 consensus sequences in assembly, 134 bp and 134 kb in lengths. Three proteins were selected for comparison from the RAST annotation results: Phage tail completion-, Phage head completion- and Phage capsid/scaffold protein. When used with RAST's Psi-Blast, all the proteins produced high query covers and sequence identities with ZZ-1, KARL-1 and vB_ApiM_fhyAci03 (Table 4). Phage head completion protein and Phage capsid/scaffold protein produced high query covers and sequence identities with *E. coli* phage vB_EcoM_PhAPEC2 (Tsonos *et al.* 2014) and thus far unidentified from *A. baumannii*. 6P2's NCBI Microbial Nucleotide BLAST results were the same as with 3P2.

Table 4. Four highest scoring results from Rast-Psi BLAST for chosen 6P2 proteins. 1* indicates the phage, 2* the chosen protein and 3* description of results.

1*	6P2					
2*	Phage tail completion protein					
3* Description		Max Score	Total Score	Query Cover	E value	Seq iden.
Phage T4-like virus tail tube protein gp19	[AB baumannii]	388	388	100%	1.00E-136	100%
Phage tail completion and seath stabliizer protein	[AB phage KARL-1]	350	350	100%	2.00E-121	86.77%
Phage tail completion and seath stabliizer protein	[AB phage vB_ApiM_fHyAci03]	347	347	100%	2.00E-120	86.24%
Phage tail completion and seath stabliizer protein	[AB phage ZZ1]	285	285	97%	1.00E-95	71.20%
Phage head completion protein						
Uncharacterized protein	[AB baumannii]	321	321	100%	2.00E-111	100%
Phage head completion protein	[AB phage vB_ApiM-fHyAci03]	296	296	100%	3.00E-101	90.60%
Phage head completion protein	[AB phage KARL-1]	295	295	100%	4.00E-101	88.59%
Phage head completion protein	[E.coli phage vB_EcoM_PhAPEC2]	227	227	99%	5.00E-74	68.24%
Phage capsid and scaffold protein						
Uncharacterized protein	[AB baumannii]	274	274	100%	8.00E-93	100%
Phage prohead core protein	[AB phage vB_ApiM_fHyAcio3]	248	248	100%	2.00E-82	90.85%
Phage prohead core protein	[AB phage ZZ1]	198	198	98%	8.00E-63	71.43%
Phage gp68 procore core protein	[AB phage Acj9]	178	178	99%	4.00E-55	66.67%

The NCBI's Align Sequences Nucleotide BLAST with megablast setting, showed 100% query cover between 1P1 and 1P2 with 98.86% sequence identity, and 98% query cover between 3P1 and 6P1 with 100% sequence identity. 3P2 and 6P2 had 2 contigs to compare, from which the first contig produced 100% query cover and sequence identity, and the second 0% query cover but 100% sequence identity. No significant similarities were found between 1P1/1P2 and 3P1/6P1, 1P2/1P2 and 3P2/6P2, nor 3P1/6P1 and 3P2/6P2.

Table 5. Four highest scoring NCBI's Microbial Nucleotide Blast results for 1P1/1P2, 3P1/6P1 and 3P2/6P2. 1* indicates the phage, 2* the used blast setting and 3* description of results.

1*	**1P1 and 1P2**				
2*	Megablast				
3* Description	Max Score	Total Score	Query Cover	E value	Seq iden.
A.baumannii strain AB030, complete genome	161	161	0%	8.00E-34	100.00%
S. elarraichensis gill symbiont isolate Se-Cadez Svelesiana sym scaffold 14, whole genome shotgun sequencing	135	135	0%	5.00E-26	97.47%
S. selenatireducens DSM 17993 A3GODRAFT scaffold 1.2 C, whole genome shotgun sequencing	134	134	0%	2.00E-25	97.44%
M. rhizosphaerae strain Ca-68, whole genome shotgun sequencing	134	134	0%	2.00E-25	95.29%
Discontinious Megablast					
L. rhizovicinus DSM 16549 Contig 235, whole genome shotgun sequence	248	248	0%	7.00E-60	73.51%
C. mytili strain KCTC 52417 KCTC52417, whole genome shotgun sequence	205	293	0%	7.00E-47	66.88%
R. pneumotropicus DSM 21403 A3GEDRAFT scaffold 5.6, whole genome shotgun sequence	177	232	0%	3.00E-38	66.68%
T. oleivorans MIL-1 complete genome	168	168	0%	5.00E-36	68.12%
3P1 and 6P1					
Discontinuous Megablast					
Description	Max Score	Total Score	Query Cover	E value	Seq iden.
A.baumannii strain AB030, complete genome	766	766	3%	0.00	70.89%
B. fibrisolvens DSM 3071, whole genome shotgun sequence	50,9	50,9	0%	0,88	91.43%
W. bombi strain R-53094, whole genome shotgun sequence	49,1	49,1	0%	3,1	84.09%
B. ligniniphilus strain L1 contig120, whole genome shotgun sequence	48,2	48,2	0%	3,1	72.62%
3P2 and 6P2					
Megablast					
Description	Max Score	Total Score	Query Cover	E value	Seq iden.
Helicobacter bilis strain AAQJH, complete genome	52,8	52,8	0%	0,11	100.00%
Discontinious Megablast					
L. rhizovicinus DSM 16549 Contig 235, whole genome shotgun sequence	205	505	0%	2.00E-47	73.47%
A.baumannii strain AB030, complete genome	161	435	1%	2.00E-34	67.92%
A. gyllenbergii NIPH 230 adfcg-supercont1.2, whole genome sequence	125	125	1%	1.00E-23	55.53%
L. rhizovicinus DSM 16549 Contig 236, whole genome shotgun sequence	104	104	0%	4.00E-17	71.01%

3.4 Host range and Resistance emergence

Strains AB2, AB3, AB5 and AB6 susceptibility against phages was studied through different phage exposures, using singular phage, simultaneous multi phage or sequential serial phage exposure setups. AB2 and AB5 serial phage exposure order was 3P2, 6P2, 1P1 and 1P2 (Serial 1-4), and for AB3 and AB6 it was 3P1, 6P1, 1P1, 1P2, 3P2 and 6P2 (Serial 1-6). Only originally infective phages were used against each strain (Table 6).

The original strains AB2 and AB5 were vulnerable to phages 1P1, 1P2, 2P1, 3P2 and 6P2, whereas AB3 and AB6 were vulnerable to 2P1, 3P1, 3P2, 6P1 and 6P2. After 2 weeks of +4 °C storage, AB2 and AB5 lost their sensitivity towards 2P1. Furthermore, AB3 became slightly resistant against 2P1 and AB6 slightly vulnerable against 1P1, which were seen as more faded plaques at the droplet sites.

AB2's exposure to phages 1P1, 2P1, 3P1 or 6P2 did not change phage susceptibility against other phages. Exposure to 1P2, 3P2 or 6P1 emerged a resistance in AB2 towards 2P1. Multi phage exposure left AB2 resistant against all but 1P1 and 1P2. The first serial exposure induced a resistance to 2P1, 3P1 and 6P1. In the second serial exposure, AB2 developed resistance towards all but 1P1 and 1P2, the third and fourth one removed vulnerability to all phages.

Interestingly, after exposing AB3 towards 3P1, it became susceptible towards 1P1 and 1P2, but also developed resistance against 3P1 and 6P1. AB3 generated resistance towards 6P1 after exposure, but it became susceptible to 1P1 and 1P2. 3P2 exposure yielded resistance towards all phages, except 3P1 and 6P1. AB3's exposure to 6P2 left it susceptible against 3P1, 3P2 and 6P1. Multi exposure left AB3 vulnerable against 1P1, 3P1 and 6P1. The first and second serial exposures generated same results as the 3P1 exposure. Third serial exposure eliminated all vulnerabilities, except towards 1P1. Fourth serial exposure left AB3 resistant to 1P2, 2P1, 3P1, 6P1 and 6P2. Fifth serial exposure added resistance to 3P2 and the final sixth exposure removed susceptibility towards all but 3P1 and 6P1.

AB5 became resistant to all but 3P2 and 6P2 phages, after 1P1, 1P2 or multi exposure. Exposing AB5 to 2P1, 3P2 or 6P2, resulted in susceptibility towards 1P1, 1P2, 3P2 and 6P2. The first serial exposure for AB5 induced same vulnerabilities as with 2P1, 3P2 or 6P2 exposure. The second serial exposure generated additional resistance towards 6P2 and the third to 1P1 and 1P2. AB5's host range did not alter after the fourth serial exposure.

AB6 became susceptible against all phages, after exposure to 1P1, but the 2P1 infection was weak. 1P2 exposure of AB6 induced resistance towards 1P1, 1P2 and 2P1. 3P1 or 6P1 left AB6 resistant to 2P1, 3P1 and 6P1, while 3P2 or 6P2 exposure generated resistance towards all but 3P1 and 6P1. The multi exposure eliminated susceptibility towards all phages. The first three serial exposures left AB6 vulnerable against 1P1, 1P2, 3P2 and 6P2. The fourth and the fifth generated resistance for AB6 towards 1P1 and 1P2, but induced vulnerability towards 3P1 and 6P1. The sixth exposure left AB6 susceptible against 1P1 and 1P2, but resistant to all other phages.

Table 6. *A. baumannii* phage susceptibility chart, showing all the strains used in this study and their phage vulnerabilities. Susceptibility is shown as +, where additional brackets indicate weak infection, and resistance as –. The designation after strain, marks the phage(s) which the strain has been exposed towards.

	Phage								Phage						
Strain	1P1	1P2	2P1	3P1	3P2	6P1	6P2	Strain	1P1	1P2	2P1	3P1	3P2	6P1	6P2
AB2	+	+	+	-	+	-	+	AB5	+	+	+	-	+	-	+
AB2*	+	+	-	-	+	-	+	AB5*	+	+	-	-	+	-	+
AB2 1P1	+	+	+	-	+	-	+	AB5 1P1	-	-	-	-	+	-	+
AB2 1P2	+	+	-	-	+	-	+	AB5 1P2	-	-	-	-	+	-	+
AB2 2P1	+	+	+	-	+	-	+	AB5 2P1	+	+	-	-	+	-	+
AB2 3P1	+	+	+	-	+	-	+	AB5 3P2	+	+	-	-	+	-	+
AB2 6P1	+	+	-	-	+	-	+	AB5 6P2	+	+	-	-	+	-	+
AB2 3P2	+	+	-	-	+	-	+	AB5 Multi	-	-	-	-	+	-	+
AB2 6P2	+	+	+	-	+	-	+	AB5 Serial 1	+	+	-	-	+	-	+
AB2 Multi	+	+	-	-	-	-	-	AB5 Serial 2	+	+	-	-	+	-	-
AB2 Serial 1	+	+	-	-	+	-	+	AB5 Serial 3	-	-	-	-	+	-	-
AB2 Serial 2	+	+	-	-	-	-	-	AB5 Serial 4	-	-	-	-	+	-	-
AB2 Serial 3	-	-	-	-	-	-	-	AB6	-	-	+	+	+	+	+
AB2 Serial 4	-	-	-	(+)	-	-	-	AB6*	(+)	-	-	+	+	+	+
AB3	-	-	+	+	+	+	+	AB6 1P1	+	+	(+)	+	+	+	+
AB3*	-	-	(+)	+	+	+	+	AB6 1P2	-	-	-	+	+	+	+
AB3 2P1	-	-	-	+	-	+	-	AB6 2P1	-	-	-	+	-	+	-
AB3 3P1	+	+	+	-	+	-	+	AB6 3P1	+	+	-	-	+	-	+
AB3 6P1	+	+	+	+	+	-	+	AB6 6P1	+	+	-	-	+	-	+
AB3 3P2	-	-	-	+	-	+	-	AB6 3P2	-	-	-	+	-	+	-
AB3 6P2	-	-	-	+	+	+	-	AB6 6P2	-	-	-	+	-	+	+
AB3 Multi	+	-	-	+	-	+	-	AB6 Multi	-	-	-	-	-	-	-
AB3 Serial 1	+	+	+	+	+	-	+	AB6 Serial 1	+	+	-	-	+	-	+
AB3 Serial 2	+	+	+	-	+	-	+	AB6 Serial 2	+	+	-	-	+	-	+
AB3 Serial 3	+	-	-	-	-	-	-	AB6 Serial 3	+	+	-	-	+	-	+
AB3 Serial 4	+	+	-	-	+	-	-	AB6 Serial 4	-	-	-	+	+	+	+
AB3 Serial 5	+	+	-	-	-	-	-	AB6 Serial 5	-	-	-	+	+	+	+
AB3 Serial 6	-	-	-	+	-	+	-	AB6 Serial 6	+	+	-	-	-	-	-

3.5 Growth Characteristic Measurements

The changes in bacterial growth characteristics max peak, max rate and average rate, were studied with spectrophotometer. They were measured using *A. baumannii* strains, which were exposed against originally effective phages with native original strains as control.

AB2's max peak at Optical density of 595 nm (OD595) (Figure 4) lowered after exposure towards either 2P1, 3P2, Serial 1, Serial 2, Serial 3 or Serial 4. This could be also seen in 10% LB (Figure 5) with 2P1, Serial 1, Serial 3 and Serial 4 exposed strains. 6P2 exposure increased max peak in normal LB and in 10% LB, while 1P2 exposure increased max peak only in 10% LB. Max rate decreased in 2P1, 3P2, Serial 2, Serial 3 and Serial 4 exposed strains in normal LB, which was only shared by Serial 3 across both measurement conditions. However, slight increase in max rate is noticeable with 1P1, 6P2, Multi and Serial 1 exposed strains, but this was only observed in both medias with 1P1. Average rate lowered slightly with 2P1, 3P2, 6P2, Serial 1, Serial 2 and Serial 3 exposed strains. This could be only seen with Serial 3 and 4 exposed strains in 10% LB.

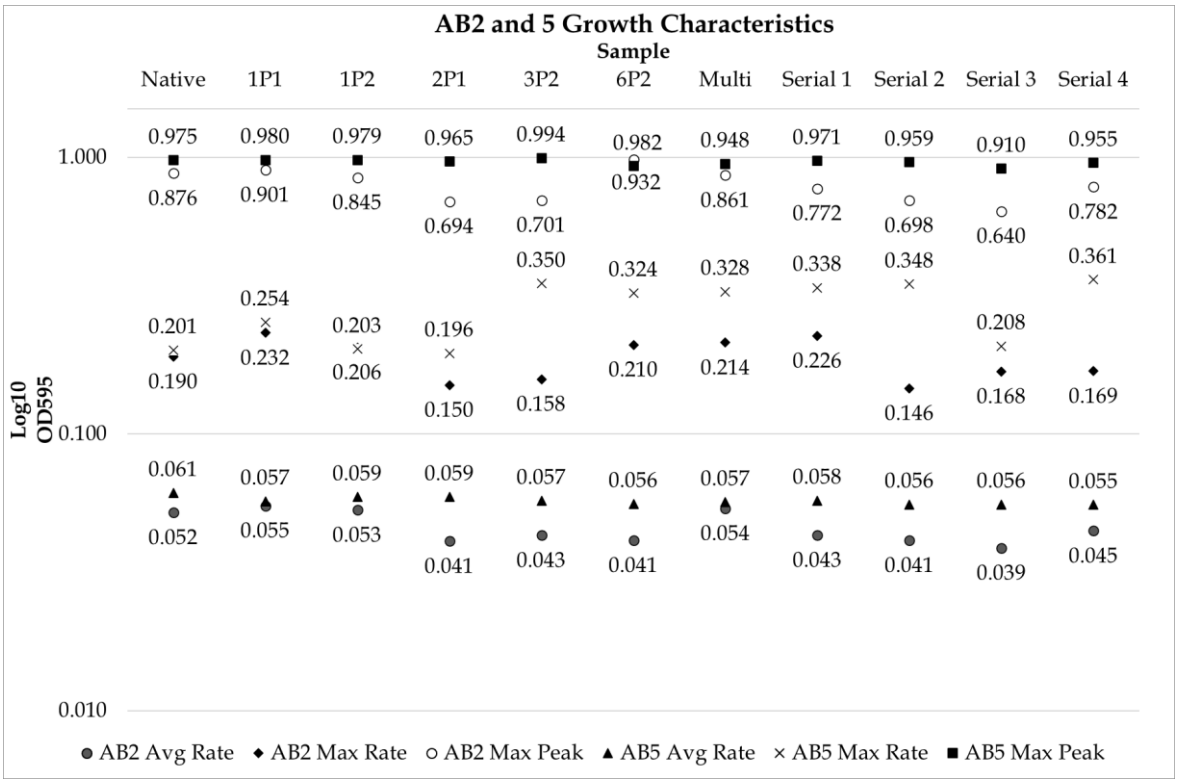

Figure 4. Growth characteristics consisting of average rate, max rate and max peak, measured from growth curve for AB2 and AB5 strains. The different strains tested are shown on the X-axis, on top of the figure and the designation after strain marks the phage(s), which the strain has been exposed towards. AB2 and AB5 Serial - strains were exposed in order, to 3P2, 6P2, 1P1 and 1P2. Y-axis shows Optical Density at 595 nm in Log10 -scale and the exact values of each data point are shown above or below them.

AB5's max peak at OD595 (Figure 4) after each exposure had little to no divergence compared to the native strain in normal LB. Comparatively in 10% LB (Figure 5), minor OD595 decreases could be seen with 1P2, 3P2, 6P2, Multi, Serial 1, Serial 2 and Serial 3 exposed strains. Significant increase in max rate was observed with 3P2, 6P2, Multi, Serial 1, Serial 2 and Serial 4 exposed strains in normal LB, but decreased in 10% LB with 1P2, 3P2, 6P2, Serial 1 and Serial 2 exposed strains. The average rate did not change meaningfully in normal LB, but slight decrease could be seen in 10 % LB with 1P2, 3P2 and Serial 2 exposed strains.

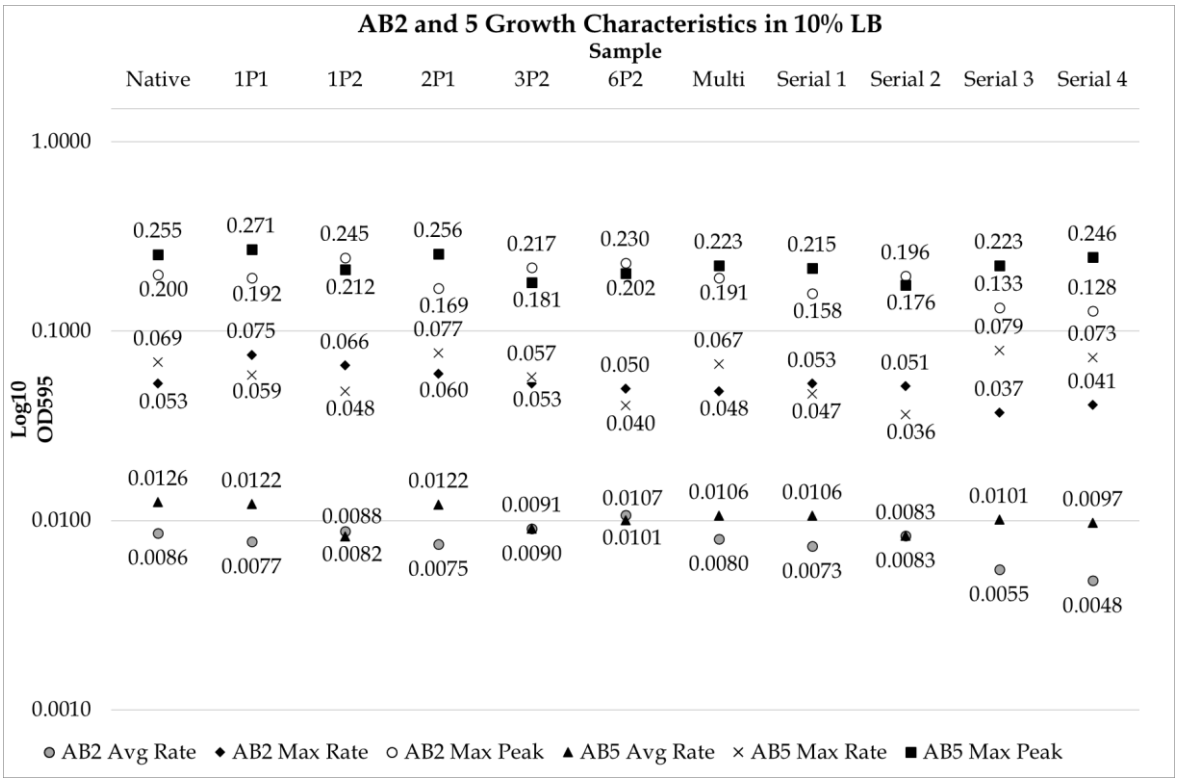

Figure 5. Growth characteristics consisting of average rate, max rate and max peak, measured from growth curve for AB2 and AB5 strains in 10% LB. The different strains tested are shown on the X-axis, on top of the figure and the designation after strain marks the phage(s), which the strain has been exposed towards. AB2 and AB5 Serial -strains were exposed in order, to 3P2, 6P2, 1P1 and 1P2. Y-axis shows Optical Density at 595 nm in Log10 -scale and the exact values of each data point are shown above or below them.

AB3's max peak at OD595 (Figure 6) in normal LB had a slight decrease with 3P1 exposed strain and noticeable decline in all Serial exposures, highest being with Serial 3 at ~0.2 abs. All AB3's max peak values in 10% LB (Figure 7) were slightly lower than native strain, with 3P1 and Serial exposed strains showing a decrease of 0.08-0.2 in absorbance. No significant differences were found in max rate between all exposed strains when measured in normal LB. However, noticeable decline in max rate were seen with 3P1, 6P1, Multi, Serial 3 and Serial 5 exposed strains in 10% LB. Average rate between strains showed little variance with both media, but slight decrease could be seen in 10% LB with Serial exposed strains, especially with Serial 1, Serial 2 and Serial 4.

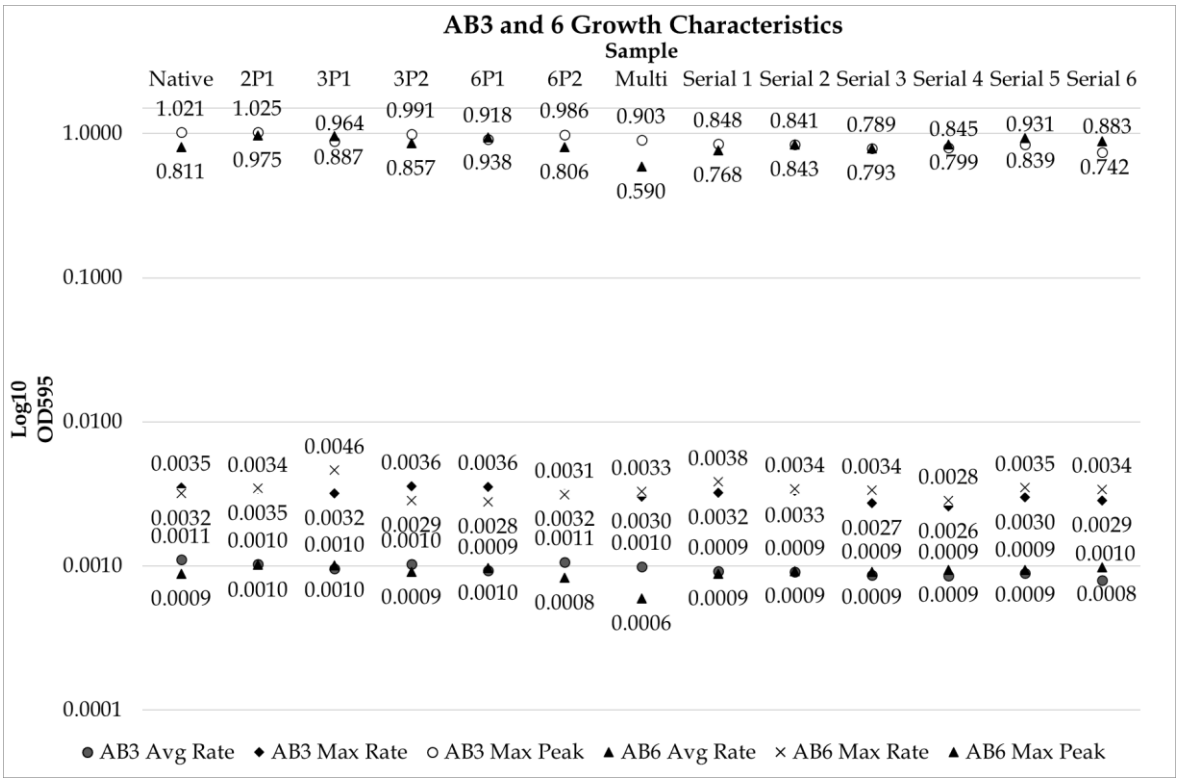

Figure 6. Growth characteristics consisting of average rate, max rate and max peak, measured from growth curve for AB3 and AB6 strains. The different strains tested are shown on the X-axis, on top of the figure and the designation after strain marks the phage(s), which the strain has been exposed towards. AB3 and AB6 Serial -strains were exposed in order, to 3P1, 6P1, 1P1, 1P2, 3P2 and 6P2. Y-axis shows Optical Density at 595 nm in Log10 -scale and the exact values of each data point are shown above or below them.

AB6's max peak in normal LB at OD595 (Figure 6) decreased ~0.2 abs. with multi exposed strain and increased by ~0.17 abs. with 2P1 and 3P1 exposed strains, while others had a slight increase or stayed relatively close to the native strain. Max peak declined 0.05-0.14 abs. in all exposed strains using 10% LB, multi exposure having the biggest decline to native strain (Figure 7). Max and average rates had minimal deviation compared to the native strain, but slight increase could be seen in max rate with 3P1 exposed strain and slight decrease in average rate with multi exposed strain. Comparatively in 10% LB, average and max rates declined slightly with all exposures, between 0.0001-0.0006 with max peak and 0.00001-0.00009 with average rate.

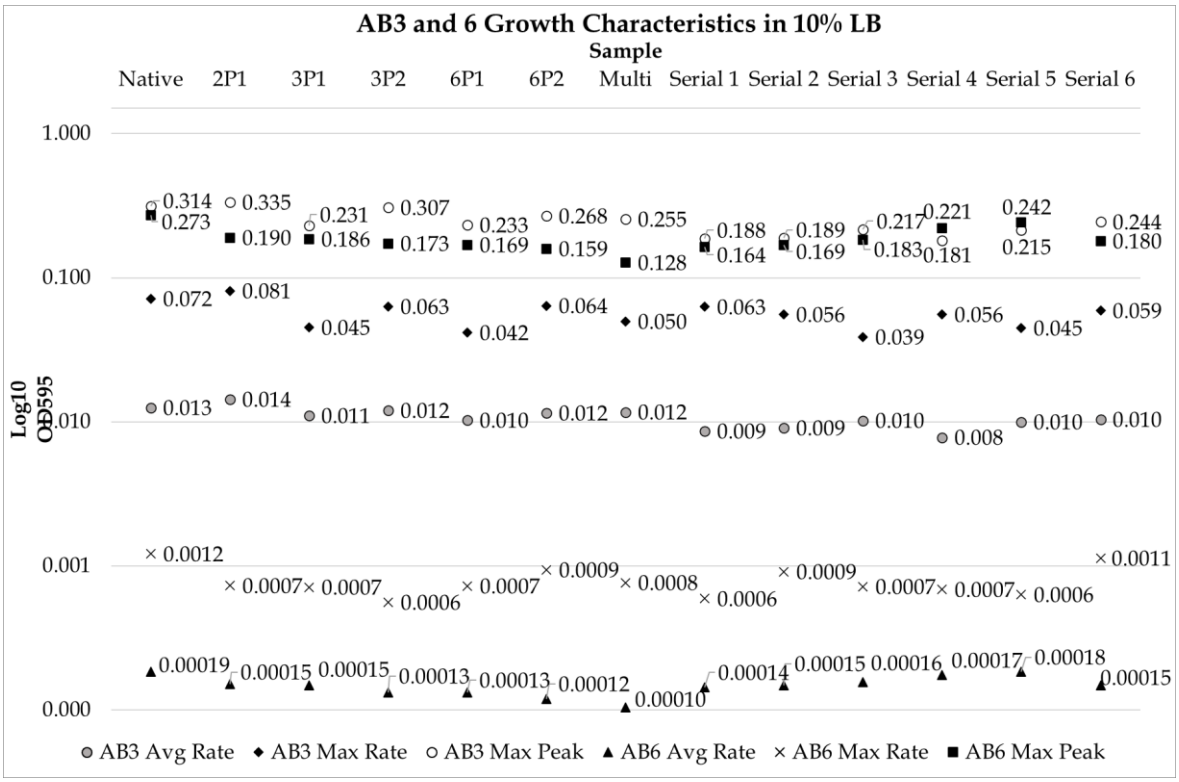

Figure 7. Growth characteristics consisting of average rate, max rate and max peak, measured from growth curve for AB3 and AB6 strains in 10% LB. The different strains tested are shown on the X-axis, on top of the figure and the designation after strain marks the phage(s), which the strain has been exposed towards. AB3 and AB6 Serial -strains were exposed in order, to 3P1, 6P1, 1P1, 1P2, 3P2 and 6P2. Y-axis shows Optical Density at 595 nm in Log10 -scale and the exact values of each data point are shown above or below them.

3.6 Phage stability titrations

The initial titers for 1P1, 1P2 and 3P2 were 1.77E+07 PFU/ml, 7.97E+07 PFU/ml and 3.29E+07 PFU/ml, respectively. After 3-month storage in +4 °C, 1P1, 1P2 and 3P2 titers increased to 8.5E+07 PFU/ml, 4.37E+08 PFU/ml and 3.6E+08 PFU/ml, respectively.

3P1's initial titer after production was 2.9x10^8 PFU/ml and increased after 10-month storage in +4 °C to 9.8E+08 PFU/ml. 6P1's titer in turn, increased after the same period from 1.2x10^8 PFU/ml to 2.57E+09 PFU/ml. Contrarily, 6P2's titer dropped from 2.3x10^8 PFU/ml to 1.00E+06 PFU/ml in 10 months.

4 DISCUSSION

4.1 Phage isolation

Phages can be found almost anywhere on Earth, but the isolation of new phages against certain species is most likely from a location, that is natural to the bacterial species. Phages against bacteria, known to infect and colonize in humans, should be searched from sources with notable human influence. Thus, wastewater is considered to be good source for phage isolation, due to its human origin and high bacterial concentrations (Lobocka *et al.* 2014).

In the study by Mattila *et al*., phage isolation success rate against *A. baumannii* was calculated as 38.9%. With this rate out of the 19 attempted isolations in this study, 7 phages would have been found on average. The low isolation success rate could be due to the wastewater reservoirs not being completely static or closed off ecosystems, which causes shifts and changes in the phage populations. Thus, the success rates and probabilities for successful phage isolation can vary greatly over time, even if the location is the same. The plaque morphology is also determining factor for isolation. Most of the phages isolated in Mattila's study, exhibited pinprick-like plaque morphology. This makes plaque identification from the agar lawn difficult, since one can easily mistake a plaque as just an agar lawn imperfection and vice versa. Furthermore, only one strain of AB was used for phage isolation in this study, which greatly diminishes the possibility for successful phage isolation and has been criticized to yield phages with narrower host infectivity (Ross *et al.* 2016).

As *A. baumannii* has emerged as a clinically relevant pathogen and is most often linked to hospital obtained infections (Montefour *et al.* 2008), the source for phage isolation should also reflect this. In multiple studies (Lin *et al.* 2010, Ghajavand *et al.* 2017, Shen *et al.* 2012, Xu *et al.* 2020) hospital derived wastewater has been used successfully as a source for lytic phages against *A.baumannii*. Thus, in future phage

isolation attempts, hospital derived wastewater should be considered as a source when targeting nosocomially relevant bacteria.

4.2 Host-Phage interactions

The exposure towards 1P1 generated resistance for AB5, but not for AB2. The resistance did not seem to have a high fitness cost, when comparing the growth properties of 1P1 exposed AB5 to the native strain, not even in low nutrient conditions. AB5 even managed to generate and retain the resistance after multi phage exposure. AB2 generated resistance towards 1P1 only after third serial exposure, i.e., after it had already been exposed and had generated resistance to 3P2 and 6P2. At this point, all AB2's growth properties decreased across the board, but it could be the result of the accumulated fitness cost, rather than just 1P1 resistance. Although, since the receptors used by these phage's are still unknown, it is difficult to evaluate if the fitness cost is additive across the sequential exposures (Wright *et al.* 2019). Overall, it can be said that 1P1 resistance seems to require higher adaptive cost for AB2 compared to AB5.

1P1's effectiveness and resistance cost for AB3 and AB6 is hard to estimate without growth measurements or any further information, such as the aforementioned phage receptors, from which concretely to see the effects of phage resistance (Scanlan *et al.* 2015). Based on the sensitivity tests, both strains had resistance towards 1P1 after different exposure conditions approximately half of the time. Each individual phage exposed strain of AB3 and AB6, that had resistance towards 1P1, had no significant decrease in measured growth. This implies that 1P1 resistance does not have high fitness cost to generate for these strains. Although, AB6 strains did have decrease in growth parameters in low nutrient conditions, which implies that the resistance towards 1P1 does have some detriment attached to it. Furthermore, AB6's weak susceptibility towards 1P1 after being stored on solid media in +4 ^{o}C, suggests that the resistance is linked to adaptations to these conditions. The sixth serial exposure for AB3 and the fourth for AB6, generated

resistance towards 1P1, which also lowered their growth properties in normal LB and in low nutrient conditions. In this case, the resistance load could be accumulative, since AB3 had additional resistance towards 2P1, 3P2 and 6P2 after the sixth serial exposure and AB6 had additional resistance towards 2P1 after fourth exposure. Thus, the growth measurements for these strains would not be solely suitable for evaluating solely 1P1's resistance cost to fitness.

Interestingly, 1P2 exposures had some deviating results compared to 1P1, even though they seem highly similar genetically and morphologically. AB5 exposure towards 1P2 lowered all measured growth properties in low nutrient conditions, which was not seen after 1P1 exposure. Additionally, AB2's sensitivity towards 2P1 disappeared after 1P2 exposure, which was not case after 1P1 exposure. AB6 sensitivity also differed between 1P1 and 1P2 exposures and AB3 was only sensitive towards 1P1 after multi exposure and fourth serial exposure, but not towards 1P2. These suggest minor, but possibly critical differences between 1P1 and 1P2.

2P1 exposure had minimal effect on growth with any other strain besides AB2, although all the strains were initially sensitive to it. Intriguingly, even though 2P1 seemed to hinder AB2's growth, it did not generate resistance, nor did its sensitivity profile change after exposure. One reason could be, that the changes needed for resistance overall, were very costly or even too detrimental. Thus, AB2 did not have the capacity or enough time to complete the changes necessary for resistance, or that changes were reverted as soon as the selective pressure subsided (Blockhurst *et al.* 2005). Another possibility is, that no actual resistance generation was needed because of downregulation of 2P1 targeted receptors due the pressure exerted by the phages or the population density (Høyland-Kroghsbo *et al.* 2013). AB5 and 6 did generate resistance and were mainly resistant to 2P1 across all the exposures. This could be due to minimal negative effects, brought on by the resistance generation for these strains, which also explains the minimal effect of 2P1 resistance on their growth. Although, AB6's Max Peak value decreased after 2P1 exposure in 10% LB, but this happened with all AB6 strains. In this case, AB6 could have had some

interaction with all the phages, or that AB6 is just sensitive towards variations in nutrient availability, resulting in slow growth.

Interestingly, all the strains except AB3, generated resistance towards 2P1 passively after 2 weeks in +4 °C on LB agar covered petri dishes. It is known that *in vivo* and *in vitro* conditions differ greatly (Oechslin *et al.* 2017) and cause bacteria to adapt and change to its new environment (Dalhoff 1985). In this case, these induced changes are most likely linked to the passive 2P1 resistance development, which could be a downregulated receptor not needed for these specific conditions. For AB3, this receptor might be critical for these specific adaptations, thus it was not able to completely remove all the 2P1 targeted receptors, leading into only partial resistance and a weak infection by 2P1.

Based on the sensitivity tests, 3P1 seems to be equally effective against AB3 and AB6. However, the growth measurements show 3P1 resistance only affecting AB3 negatively. AB3's max peak and somewhat max rate in normal LB and all measured parameters in 10% LB decreased, while AB6 max rate and peak showed an increase in normal LB. AB6 growth increase could be due to changes induced by 3P1, which are favourable and are seen as increased max peak and rate values. Furthermore, AB3's resistance generation towards 3P1 after exposure seems not to be absolute, since AB3 was resistant after initial single exposure, but not after the first sequential exposure, where the exposure conditions were identical. 3P1 did not infect AB2 or 5 initially, nor after any exposure.

3P2 seems to be more effective against AB2 and AB5 based on the sensitivity tests. AB5 did not generate resistance towards 3P2 after any exposure and AB2 did only after multi phage exposure and onwards from the third sequential exposure. Additionally, 3P2 exposure decreased AB2's growth in normal LB, but not in low nutrient conditions and vice versa for AB5. In this case, the receptor might be involved in nutrient intake, which could cause the targeted nutrient to become a limiting factor for growth (Luckey & Nikaido 1980). For AB2, this could be the

reason for limited growth in normal conditions, but in low nutrient conditions another limiting factors could arise, which explain the minimal effects. For AB5, the nutrient might be especially crucial, which causes growth to be negatively affected in 10% LB. Due to the abundance of nutrients in normal LB, AB5 could possibly compensate for this by simply increasing the intake through other metabolic routes, even though they might be more contrived or costly (Parter *et al.* 2007).

AB3 and AB6 were sensitive towards 3P2 after over half of the exposures, but no noticeable changes were seen in the growth measurements when compared to the native strain after 3P2 exposure. This indicates that 3P2 resistance does not carry high fitness cost for AB3 or AB6.

AB2 and AB5 were both resistant towards 6P1 initially and after every exposure, whereas 6P1 was effective against AB3 and 6 after half of the exposures. 6P1 did not affect AB3's or AB6's growth in normal LB, but all the measured growth parameters decreased notably in low nutrient conditions. This implies a cost to the resistance, which is not apparent without the presence of other stress factors. Intriguingly, AB6 did not even generate resistance towards 6P1 after exposure, but the lowered growth could be result of partial resistance development. Based these results, the sole effect of 6P1 resistance to AB6 is hard to evaluate. Even more so due to AB6's decreased growth in 10% LB across all the exposed strain measurements. Furthermore, 3P1 and 3P2 exposures made AB6 sensitive towards 6P1 and 6P2, and vice versa. This suggests that these four phages use the same receptor, or that the receptors are closely linked.

6P2 is more effective against AB2, AB5 and AB6 based on the sensitivity tests, as none of them managed to develop resistance after exposure and were susceptible against 6P2 after most of the exposures. AB3 was sensitive towards 6P2 initially and after 3P1, 6P1, Serial 1 and 2 exposures. However, no effect on growth could be seen after exposure and resistance development in normal LB and only slight decrease in all measured values in low nutrient conditions. Furthermore, since none of the

other strains became resistant towards 6P2, it is hard to evaluate the sole effect of 6P2 resistance on growth. Interestingly, 6P2 did alter the sensitivity profiles for all strains after exposure, but this is only reflected in the growth measurements for 6P2 exposed AB2 and AB5. This could be due to same kind of partial changes towards resistance as with 2P1, where the resistance for 6P2 must carry a high fitness cost for these three strains, or that it is mechanically or metabolically challenging to develop.

All these sensitivity tests, growth measurements and their results can only be used to determine cursory interactions and effectiveness of the phages against the tested bacterial strains. Overall, 1P1 and 1P2 seemed to be most effective against AB2 and AB5, with higher resistance cost to AB2 than AB5. 2P1 affected only AB2 and AB3, while AB5 and 6 were resistant towards it after every exposure. 3P1 and 6P1 were only effective against AB3 and AB6, since AB2 and AB5 were resistant to them. 3P2 and 6P2 was more effective against AB2 and AB5 since neither of them managed to develop resistance towards it.

Furthermore, it can be said that the effects of phage exposure or resistance cannot be straight away reflected to growth properties on these bacteria. Each phage, bacterial strain and their pairing are unique and can behave in unexpected ways. Thus, more information is needed to figure and filter out more broadly infecting phages, and to make solid conclusions on each host/phage pairing (Ross *et al.* 2016). Ideally, information needs to be gathered about the phage receptors themselves (Wright *et al.* 2019). Such as their sequence and their part in other bigger complexes, to figure out all the possible intricacies and interactions behind them. This detailed information about the phage receptors would be ideal in figuring out phage/host pairings, which would help in selecting a proper phage against different bacterial strains.

4.3 Phage characterization

High similarity between 1P1 and 1P2, 3P1 and 6P1, 3P2 and 6P2 seen from the genetic and morphologic analyses, strongly suggest that they are the same phages between them. This is also supported by the phage sensitivity results to an extent, in which the phage susceptibility and resistance emerge mostly at the same time for these phage pairs. The sensitivity profiles differed between 1P1 and 1P2 exposures towards AB2 and AB6, 3P1 and 6P1 exposures towards AB2 and AB3, and between 3P2 and 6P2 exposures towards AB2, AB3 and AB6. Even between two same phage single exposures differed after 3P2 exposure for AB3. Even though, these phages are similar between them, the slight differences might be just in the right areas to cause critical changes, i.e., in the receptor binding proteins themselves. This would explain the high similarity between them and the slightly differing sensitivity results. Furthermore, the differences between 3P2 and 6P2 might be even bigger, since 6P2 final assembly size was almost double of 3P2's. Still, the compared regions were almost identical, as were the RAST annotation and the blast results derived from it, which firmly suggests them being similar, if not identical.

The structural proteins in phages are fairly conserved, so these were good reference points to be used for NCBI cross-referencing. Proteins identified through RAST annotation for 1P1, 1P2, 3P2 and 6P2 BLAST comparison results produced high percentage matches against vB_ApiM_fHyAci03 (Pulkkinen *et al.* 2019), KARL-1 (Jansen *et al.* 2018), and ZZ1 (Jin *et al.* 2012), which are myoviruses or t4-like viruses. Morphology gleamed from TEM-imagery also supports the conclusion that the 1P1, 1P2, 3P2 and 6P2 phages are *Myoviridae*. 1P1 and 1P2 can be even further categorized to *Tevenvirinade* or t4-like phages, by the elongated head visible in EM-images. As the head morphology is unclear in the 6P2 EM-image, further classification cannot be derived from it. The contractile tail can be seen in the images, but it provides no further taxonomization for 3P2 and 6P2. The phages matched with 1P1, 1P2, 3P2 and 6P2 however, all belong to *Tevenvirinae* subfamily. This strongly implies that 3P2 and 6P2 are indeed part of the *Tevenvirinae* as well.

All the compared proteins from 3P1 and 6P1, beside ACLAME HNH homing endonucleases, were found from many distinct *Autographiviridae* (Ceyssens *et al.* 2006, Drulis-Kawa *et al.* 2011) and few T7-like phages (Mertens *et al.* 1982, Savalia *et al.* 2020). Furthermore, the TEM-imagery for 6P1 strongly indicate it and 3P1 being *Podoviridae* or *Autographiviridae.* Therefore, these two phages most likely belong to *Autographiviridae* family.

Previously discovered and sequenced *A. baumannii* phages have mainly belonged to *Caudovirales*, especially to *Myoviridae* or *Autographiviridae* family, where the size of the genomes for *A.baumannii Tevenvirinae* and *Autographiviride* has been recorded to be 159-168 Kb and 40-42 Kb, respectively (Turner *et al.* 2017). The size of 1P1 and 1P2 (165 Kb) matches the previously recorded genome range for *Tevenvirinae*, as do the 3P1 and 6P1 genome for *Autographiviride*. 3P2's and 6P2's genome size differs notably between each other and from recorded size for *Tevenvirinae*. This is most likely due to errors in the genome sequencing or the assembly. They both are highly similar between each other in the whole genome comparison and the RAST annotation provided the same proteins for both phages, which matched with only *Tevenvirinae*.

Because no exact matches, nor matches with any markable query covers were found with full genome cross-referencing, it can be assumed that these phages are previously undiscovered and novel. Furthermore, phages 1P1, 1P2, 3P2 and 6P2 have great potential as future phage therapy candidates, due to high similarity towards multiple phages, that were deemed good for phage therapy. This is crucial due to urgent need for new treatment options towards *A.baumannii* infections (WHO 2017), especially since the chance for successful phage isolation towards *A. baumannii* seems to be even smaller than previously thought.

4.4 Phage therapy assessment

Not considering the related regulatory framework involving phage therapy, the most important characteristics for a phage to be considered for phage therapy are stability, producibility, host range and life cycle (Parracho *et al.* 2012, Pelfrene *et al.* 2016, Pirnay *et al.* 2015, Cooper *et al.* 2016, Ross *et al.* 2016). The phages stability was tested in the isolation study by Mattila *et al.* (2014), after 1-month +4 °C storage. This showed the *A.baumannii* phage stock titers to drop by log 0.973, which does not match with the more recent tests. The titer measurement for phages is highly dependent on the exact methods that are employed in the lab, such as how and how long were the phages stored, how well the used samples were vortexed, which dilutions were chosen for plaque counting, how freshly plated the host was and how many replicates were made**.** These could explain the differences in stability results between these studies and the 0.5-1 log titer increase between the measured time points.

Overall, the phages seemed to be quite stable in LB at +4 °C and no significant titer drop was observed by phage therapy standpoint. Furthermore, the phages could be even revived from 4-year-old, +4 °C in LB stored phage stocks (unpublished data). 3-month measurement is more than enough to determine phages rudimentary suitability for phage therapy application, when the concern is that will the phage stay active during its journey from the production into the targeted treatment area. Although, the phage stability issue is not so one dimensional and one must consider the application route and the targeted area of treatment as well. Different phages are more stable in different conditions. For example, where one phage might stay active for long periods in human plasma, another might inactivate almost instantly (Schooley *et al.* 2017). To this end, the used phage should be tested in a medium reflecting its treatment environment. There are also options for different storage mediums and preservation strategies, which can increase the stability and longevity of phages (Malik *et al.* 2017). These methods however, also suffer from the same issue that stability varies between different phages types.

The only virulence factor found across all the characterized phages was a Copper-translocating P-type ATPase (CopA) in 3P2 and 6P2, which is involved in copper related homeostasis and defence (Wu *et al.* 2008). This could be host derived sequence, but when comparing the sequence through RAST, the only comparable regions came from other phages. However, this gene is non-relevant to most treatment situations and thus, it can be concluded that these phages are devoid of transmissible virulence factors and are safe for phage therapy use.

The production of these phages using agar plate collection method, yielded phage concentrations between 1E+05 to 1E+09, settling mostly in the range of 1E+06-1E+08. The relatively low titers and the variability of them between different productions, infers questionable suitability for phage applications. Phages used for therapy applications, must be readily producible in sufficient quantities for multiple possible applications. This is to ensure that the whole treatment proceeds promptly, which might be even critical for treating patients with far progressed infections. The appliance method also determines how much, how concentrated and how purified the end phage product needs to be. Furthermore, all phage therapy treatment cases are unique, and as such, no universal treatment doses have been determined. For example, Schooley *et al.* (2017) used a cocktail containing 4 different phages with 1E+09 PFU per dose through intracavitary installations and intravenously, with 2h – 12h appliance intervals for 59 days. In this case, the amount of phages that was needed for treatment is roughly estimated around 1E+11-1E+12. Moreover, due to intravenous appliance, the phage product needed to be purified to meet the 5 EU/kg per h limitation set by FDA and European Pharmacopeia, which is a potential step for phage loss. In another study treating burn wound infections, the used treatment dose was 20 ml of ~1E+06 PFU/ml per day for a week, using an alginate-based dressing (Algosteril, Les Laboratoires Brothier, Paris, France) on the wound to spread the phages. However, as with the stability, the optimal production conditions between different phages can also vary greatly. Only one production method has been used for these phages in this and the earlier study (Mattila *et al.*

2015). Other methods, such as the traditional liquid culture production or more recently surfaced culture bottle-based production (Rubalskii *et al.* 2020), should be tried to get the bigger picture of the producibility of these phages.

The phages did not exhibit lysogeny during any of the experiments and no prophages were detected in the AB6 genome. Nonetheless, the life cycle of all the phages still needs to be confirmed by going through all the sequences for any lysogenic related genes. Furthermore, all the used host strains need to be sequenced to affirm the absence of any prophage integration.

The host range for these phages is still undetermined, due to lack of available *A.baumannii* strains. Only 4 strains were used for host range determination, whereas in other studies, 20-127 different strains were used (Lin *et al.* 2010, Jin *et al.* 2012, Jansen *et al.* 2018, Pulkkinen *et al.* 2019). For phage therapy, it is good to have broad ranged phages that can be used against most oncoming infections. However, there is no scientific consensus yet, what exactly defines what a broad host range for a phage is. For example, across 4 different phage -characterization & therapy studies, a broad host range varied between 3 and 39 (Lin *et al.* 2010, Jin *et al.* 2012, Jansen *et al.* 2018, Pulkkinen *et al.* 2019). Nevertheless, these phages still need more extensive and defined host range analysis before proper therapy use.

It is not detrimental for a phage from phage therapy perspective if the target host becomes resistant towards it. There always will be some form of resistance development in host-phage interactions, but the point of phage therapy is not necessarily to clear out the infection by itself. Phage resistance always carries some sort of fitness cost, which can interrupt the balance of the infection and possibly help the immune system to clear it out. Furthermore, phage resistance can also suscept the bacteria towards previously non-working antibiotics (Altamirano *et al.* 2021) or even other phages (Regeimbal *et al.* 2016) and these treatment strategies have already been tested successfully on mouse models. Loss of virulence have also been observed due to phage resistance, such as in Regeimbal's study, where the

phages shifted the bacteria population towards uncapsulated state (Regeimbal *et al.* 2016). In fact, this could also be the case with AB6 when infecting with either 1P1/1P2, 3P1/6P1 or 3P2/6P2, since AB6 seemed to become susceptible towards 3P1/6P1 after either 1P1/1P2 or 3P2/6P2 infection and vice versa.

The sensitivity and growth measurements suggest that the optimal personalized phage therapy approach is highly dependent on the bacterial strain. For AB6, using simultaneous multi-phage cocktail seemed to have the biggest impact on its growth properties. As for AB2, the biggest impact on growth happened gradually over the course of the serial exposures. For AB3, no apparent effects were seen in normal LB measurements, but in low nutrient conditions the serial exposures lowered the growth noticeably more, compared to multi-phage exposure. AB5, no other apparent effects on growth were seen in normal LB, besides an increase in max rate after multi-phage exposure and 3P2/6P2 exposure. In low nutrient conditions however, biggest impact was seen after 3P2 and Serial 2. These results emphasize the necessity of phage cocktail customization for each patient strain and question the traditional simultaneous multi-phage cocktail appliance.

5 CONCLUSIONS

When isolating phages, one should consider the location, time and the bacterial species. Many phages exhibit plaque morphologies, which are hard to distinguish from other debris and imperfections. Thus, multiple possible plaques should be collected to increase the chance for isolation.

The 1P1/1P2 and 3P2/6P2 results indicated close relations to previously published *A. baumannii* phages, some of which have already been deemed as good potential candidates for phage therapy applications. Furthermore, the phages stayed active and stable for sufficient amount of time for them to be useful in phage therapy. Further studies are still needed, to confirm their producibility, host range and that they are indeed lytic.

Phage resistance does not necessarily carry detrimental fitness cost and in most cases the cost is not even apparent. Furthermore, phage resistance does not exclude phages from phage therapy use, since it might render the bacteria susceptible to other phages, previously resistant antibiotics and the immune system.

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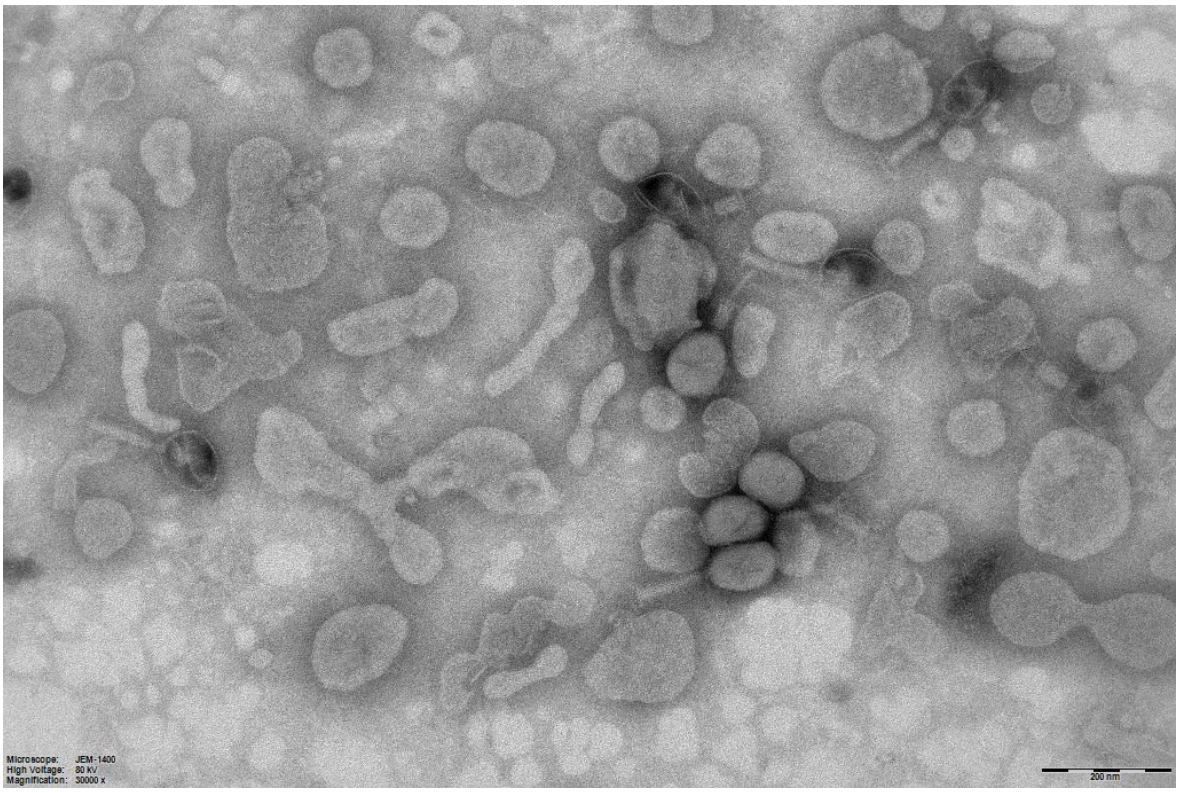
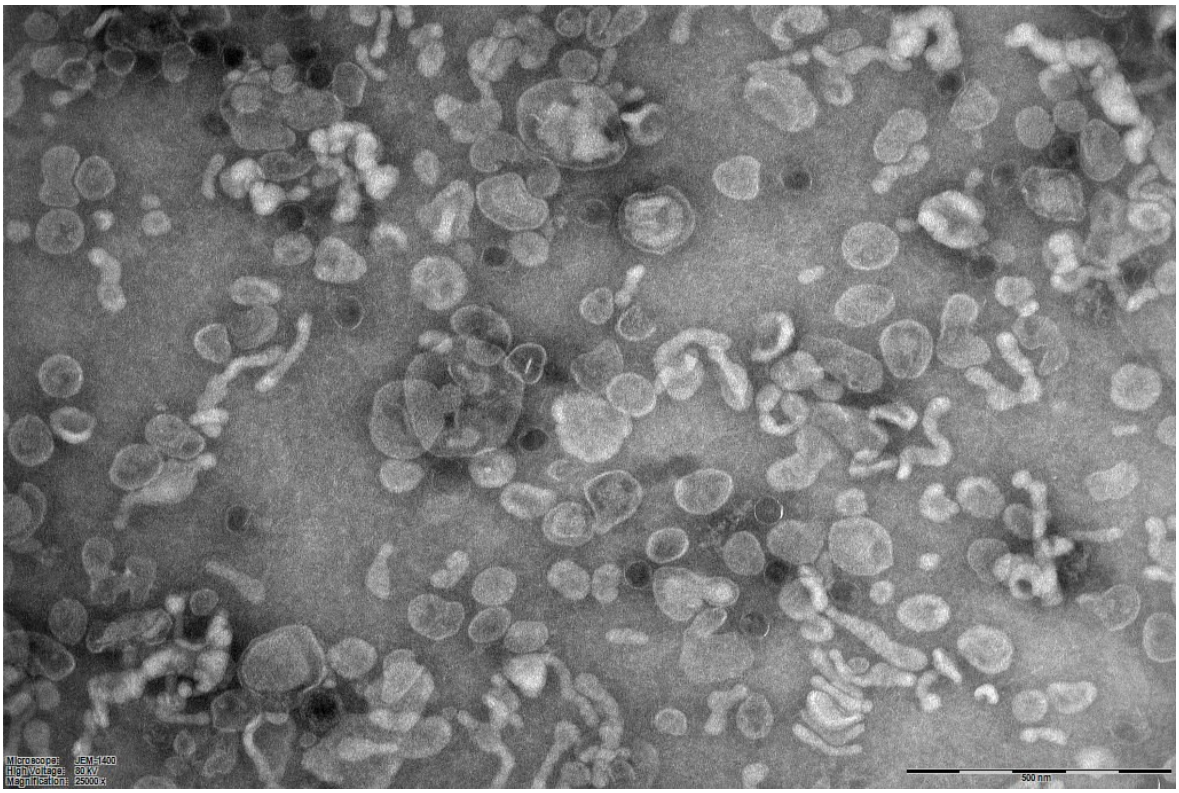
APPENDIX 1. 1P2 TEM-image



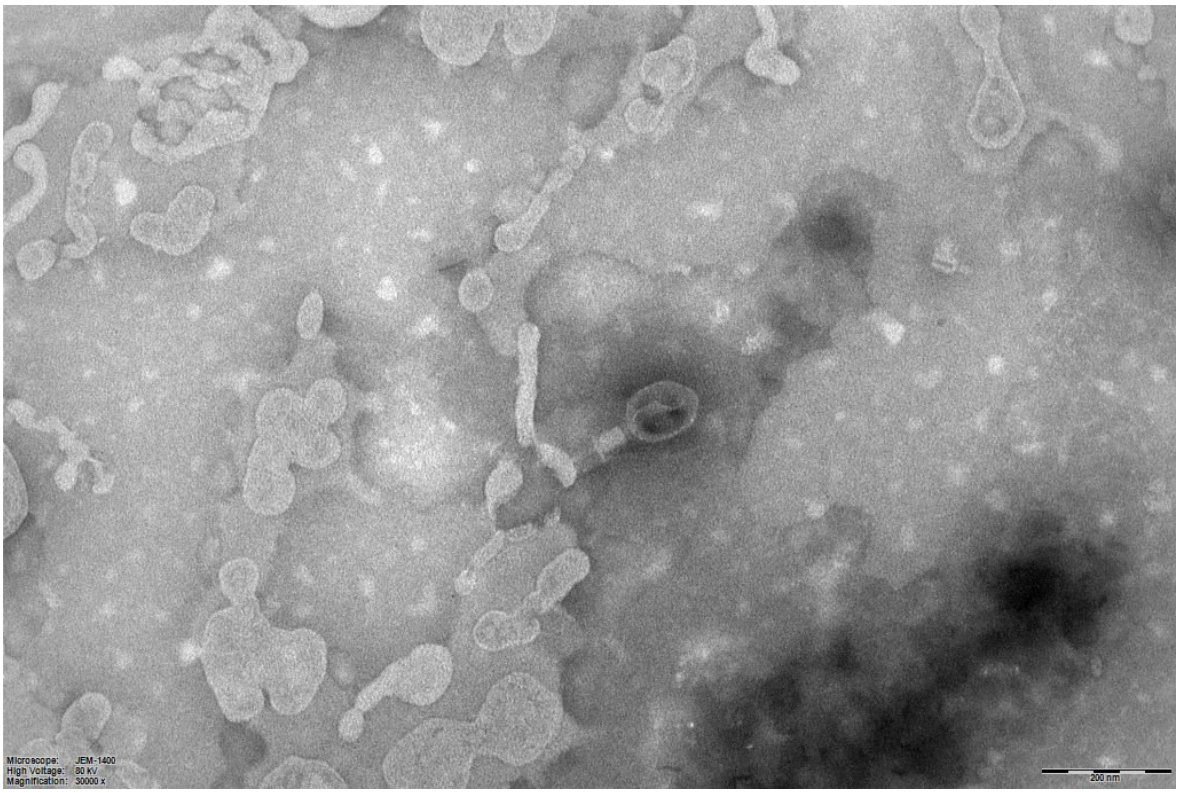
Concentrated 1P2 phage stained with PTA and imaged with JEM-1400 Transmission electron microscope at 30000 magnification. Time used for sample binding and staining was 30 s.

APPENDIX 2. 3P1 TEM-image



Concentrated 3P1 phage stained with PTA and imaged with JEM-1400 Transmission electron microscope at 25000 magnification. Time used for sample binding and staining was 30 s.

APPENDIX 3. 3P2 TEM-image



Concentrated 3P2 phage stained with PTA and imaged with JEM-1400 Transmission electron microscope at 30000 magnification. Time used for sample binding and staining was 30 s.