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Characterization of *Pseudomonas* lytic phages and their application as a cocktail with antibiotics in controlling *Pseudomonas aeruginosa*

Doctoral Thesis

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2020

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List of abbreviations

CDS	Coding sequence
CF	Cystic fibrosis
CFTR	CF transmembrane conductance regulator
COG	Cluster of Orthologous Group
PAI	*Pseudomonas* autoinducer
T4P	Type IV pili
TLR	Toll-like receptor
LPS	Lipopolysaccharide
ECDC	European Centre for Disease Prevention and Control
CDC	Centers for Disease Control and Prevention
MDR	Multi-drug resistant
XDR	Extensively drug-resistant
PDR	Pandrug-resistant
HGT	Horizontal gene transfer
MHB	Muller Hinton broth
CIP	Ciprofloxacin
MEM	Meropenem
MIC	Minimal inhibitory concentration
CLSI	Clinical Laboratory Standard institute
CFU	Colony forming unit
SNP	Single nucleotide polymorphism
ESBL	Extended spectrum beta-lactamase
MBL	Metallo-beta-lactamase
TEM	Transmission electron microscopy
PFU	Plaque forming unit

Chapter 1 General Introduction

1.1 *Pseudomonas aeruginosa*- the opportunistic pathogen

Pseudomonas aeruginosa, a Gram-negative and rod-shaped bacterium that can be widely found in natural environment such as water and soil (1). Complete genome sequence from *P. aeruginosa* PAO1, a laboratory standard strain was reported in year 2000 (2). It was isolated from human wound in Melbourne, Australia. The size of genome was found to be 6,264,404 base pair (bp) and there are 5586 coding sequence (CDS) according to the latest record (year 2019) from Pseudomonas Genome Database (http://www.pseudomonas.com/). Genome of *P. aeruginosa* is GC-rich (GC content: 66.6%). Genes were classified in different categories according to their function by Cluster of Orthologous Groups (COGs) as shown in Table 1-1.

Table 1-1. Classification of genes encoded in PAO1 based on COGs

Categories	Number of CDS
General function (prediction only)	709
Amino acid transport and metabolism	558
Function unknown	548
Transcription	526
Signal transduction mechanisms	455
Energy production and conversion	366
Inorganic ion transport and metabolism	352
Cell wall/membrane/envelope biogenesis	282
Lipid transport and metabolism	270
Carbohydrate transport and metabolism	261
Coenzyme transport and metabolism	218
Translation, ribosomal structure and biogenesis	207
Secondary metabolites biosynthesis, transport and catabolism	206
Posttranslational modification, protein turnover, chaperones	201
Intracellular trafficking, secretion, and vesicular transport	182
Replication, recombination and repair	160
Cell motility	160
Nucleotide transport and metabolism	107
Defense mechanisms	74
Cell cycle control, cell division, chromosome partitioning	39
Chromatin structure and dynamics	3
RNA processing and modification	2

Reference: (http://www.pseudomonas.com/)

According to Table 1-1, genome of *P. aeruginosa* encodes versatile of genes related to transportation and metabolism of nutrients. This might be related to its ability to survive in

various kinds of environment. There were 455 genes related to signal transduction, showing that genes in *P. aeruginosa* are highly regulated (2).

Whole genome sequencing of *P. aeruginosa* PAO1 also provided insights to the reason why *P. aeruginosa*'s infection is difficult to be treated. *P. aeruginosa* has various kinds of efflux system that export compounds such as antibiotics from the cell (3). This will be further discussed in section 1.3.

P. aeruginosa is infamous as opportunity pathogen that caused various kind of infection inside hospital setting (nosocomial infection) or outside hospital setting (community-acquired infection) (1). Example of infection are summarized in Table 1-2.

Table 1-2. Infections caused by *P. aeruginosa*

Community acquired	**Description**
Keratitis	Eye's infection commonly found in contact lens wearer, or people with eye injuries
Otitis externa	Inflammation of outer ear canal
Bones and soft tissue infection	Occurs in punctured wound of the foot
Endocarditis	Inflammation of inner lining of heart chamber and heart valve. Bacteria can travel to hear via blood stream.
Folliculitis	Inflammation of hair follicles
Nosocomial	
Respiratory tract infection	Ventilator-associated pneumonia
Urinary tract infection	Catheter- associated urinary tract infection (CAUTI)
Surgical infection	Wound infection after surgery

Reference: (1)

The other serious clinical problem caused by *P. aeruginosa* which draws public's attention is chronic lung infection in cystic fibrosis (CF) patients (4). Cystic fibrosis is caused by mutation in gene encoding CF transmembrane conductance regulator (CFTR). It is a type of autosomal recessive disease which is prevalent in the United States and Europe. There were more than 2,000 types of mutations found to contribute to this disease and mutations can be genetically inherited (5). Mutations in CFTR can affect multiple organs such as lungs, pancreas, gastrointestinal tracks and etc. Defective CFTR failed to secrete chloride and bicarbonate ions, causing accumulation of sodium ion and hypersecretion of mucus. As a result, mucociliary clearance is impaired in lungs and leads to chronic lung inflammation, which is the main cause of mortality in CF patients (5). The most dominant bacteria that caused chronic lung infection in CF patients is *P. aeruginosa* (4). Sixty-eight mutations were found in the genome of *P. aeruginosa* that persisted in CF patient for 8 years. Those mutations resulted in a hypermutable strain with increased antibiotic resistance (6). Similar phenotypic characteristics were observed

in comparative study between CF strains and non CF strain, whereby CF strains were found to be hypermutable and were highly resistant to antibiotics compared to non CF strains (7).

Human immune system is activated when entry of foreign materials, such as bacteria to our body is detected and the first line of immune system that respond to such invasion is innate immunity (8). Toll-like receptors (TLR) recognize bacterial molecules such as lipopolysaccharide (LPS), flagellin, lipoteichoic acid and etc. and in turn activate the production of proinflammatory cytokine. Virulence factors of *P. aeruginosa* stimulate host cell immune system to produce proinflammatory cytokine and chemokine and resulted in phagocytosis (4). Virulence factors produced by *P. aeruginosa* were summarized in Table 1-3 below.

1.2 Emergence of multidrug-resistant bacteria

The discovery of Penicillin by Sir Alexander Fleming in year 1928 marked the important victory in human history for western medicine in treating bacterial infection (9). However, the first penicillin-resistant bacteria were soon discovered (10). Different varieties of antibiotics were continued to be discovered but bacteria could become resistant to newly introduced drugs again in a short time as shown in Fig.1-1. The emergence of antibiotic resistance are caused by several factors: overuse of antibiotics, inappropriate prescription and extensive use in agriculture as growth supplement (10).

There are various classes of antibiotics which target different metabolism and physiological function of bacteria, as summarized in Fig.1-2 (11). However, bacteria can easily become resistant to the drug used against it through several ways. For example, bacteria can acquire resistant genes by plasmid, through phage transduction, conjugation or by uptake free DNA from the environment (12). Not only that, bacteria is able to modify antibiotic's target by mutation and thus decrease the affinity of antibiotics to its target whereby fluoroquinolone resistance can be archived by point mutations in DNA gyrase (13). On the other hand, over express of efflux transporter system helps to export the antimicrobial agents inside the cell. Meanwhile, antibiotics can also be degraded by enzyme and thus inactivate the antimicrobial agents (14,15). Antibiotic resistance mechanisms in bacteria are summarized in Fig.1-3.

Antibiotic resistant mechanism specific to *P. aeruginosa* will be discussed in the next section.

Table 1- 3. Virulence factors of *P. aeruginosa.*

Component	Mechanism	Roles in pathogenesis
Type IV pilus (T4P)	Adhesin: recognizes GalNAcbeta1-4Gal receptor in asiaglycolipids	Mediates attachment to epithelial surfaces, provides twitching motility essential for biofilm formation, activates inflammation
Flagella	Motility, chemotaxis, mucin-binding, major immunostimulant through toll-like receptor 5 recognition	Facilitates tissue invasion, activates inflammation
Alginate mucoexopolysaccharide	Antiphagocytic	Characterized chronic infections in cystic fibrosis
Elastase	Cleaves elastin, proteins including immunoglobulin A	Destroys extracellular matrix components
Alkaline protease	Cleaves proteins	Causes tissue destruction
Phospholipase C (hemolytic and nonhemolytic)	Cleaves phosphatidylcholine sphingomyelin	Degrades pulmonary surfactant, facilitates infection in the lung
Phospholipase D (PldA and PldB; type IV secretion)	Activates PI3K/Akt pathway	Facilitates intracellular invasion through actin rearrangement
Neuraminidase	Releases sialic acid from glycoconjugates	Facilitates colonization
Cytotoxin	Forms pores in membranes	Causes tissue destruction
Pseudomonas autoinducer (PAI)	Homoserine lactone derivative, a secreted cofactor necessary for the expression of elastase, alkaline protease, neuraminidase and biofilm formation	Coordinates gene expression within a population of organisms
Exotoxin A	ADP ribosylating enzyme, which inactivates EF-2, inhibiting protein synthesis.	Causes tissue destruction
Exoenzyme S, T, U (type III secreted toxins)	ADP ribosylating enzymes (S and T) and toxins with specific eukaryotic targets, including GTPases; phospholipase A_2	Facilitates invasion, cytotoxicity, interferes with cytoskeletal integrity
Siderophores (pyochelin, pyoverdine)	High-affinity iron binding capacity	Facilitates iron acquisition for bacterial metabolism
Phenazines (pyocyanin)	Blue-green pigment; oxidant activity	Destroys ciliary activity, toxic to airway cells, oxidative stress

Reference: (1)

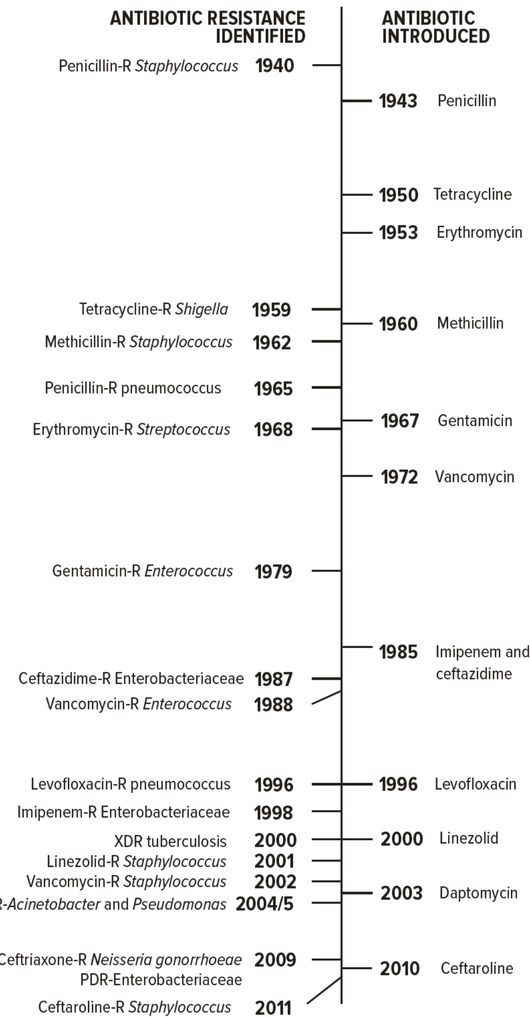


Figure 1- 1. Timeline and major event in the discovery of new antibiotic and emergence of resistance. Reference: (16).

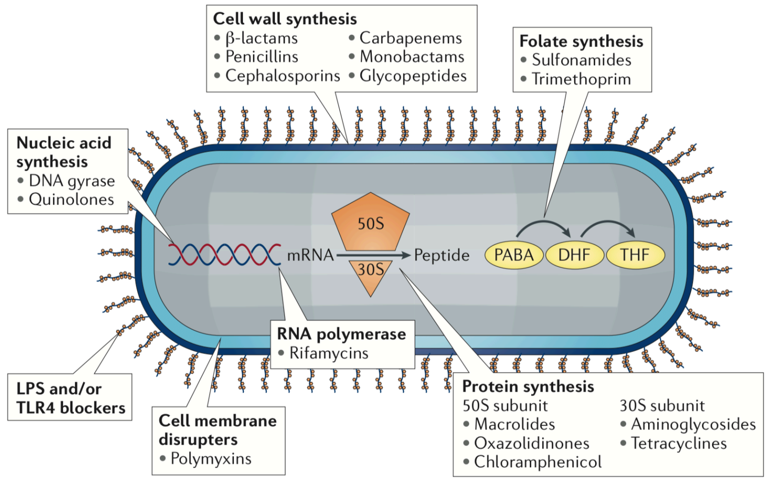


Figure 1- 2. Target Sites of Different Classes of Antibiotics. Reference (11).

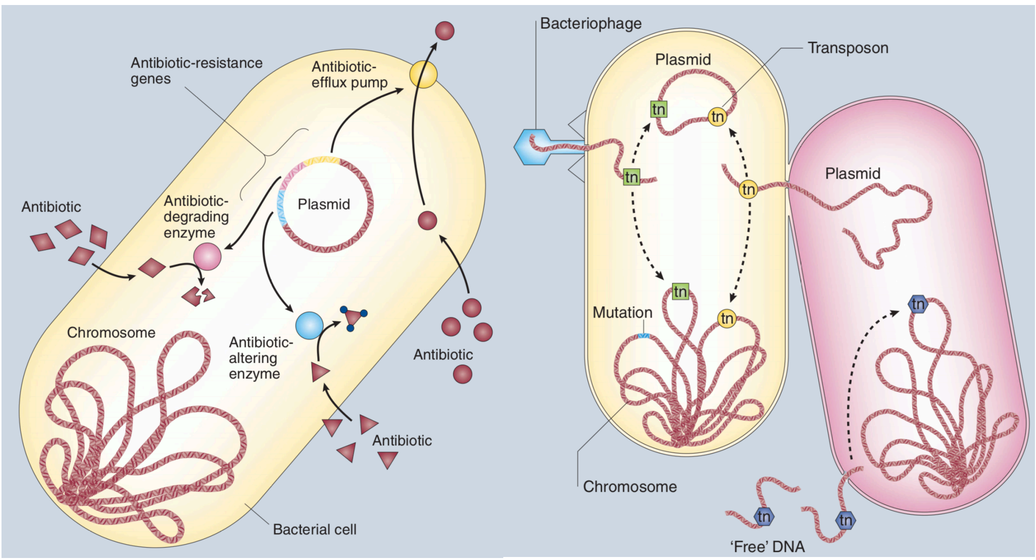


Figure 1-3. Mechanisms that contribute to antibiotic resistance. Reference: (15).

1.3 *Pseudomonas aeruginosa*'s resistant mechanism to antibiotics

A study in Spain showed that it costed 3 times in treating antibiotic resistant *P. aeruginosa* compared to non-resistant strains (17). It is very hard to be eradicated once it is established especially among CF and immunocompromised patients (4,18).

According to the latest international standard definitions proposed by a group of experts including those from the European Centre for Disease Prevention and Control (ECDC), the Centers for Disease Control and Prevention (CDC) and etc., antibiotic resistant bacteria can be divided into three categories: multi-drug resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) bacteria (19). Antibiotic resistant *P. aeruginosa* can be classified base on the following definition:

1. MDR: strain resistant to at least one type of antibiotic in more than 3 antimicrobial classes.
2. XDR: strain resistant to at least one type of antibiotic in all or sensitive to less than 2 antimicrobial classes.
3. PDR: strains resistant to all antibiotic in all antimicrobial classes.

Classes of antibiotic specially used against *P. aeruginosa* are summarized in table 1-4 below. Meanwhile, antibiotic resistant mechanisms possessed by *P. aeruginosa* will be discussed in the following sections and Fig.1-4 summarized the resistant mechanisms of *P. aeruginosa*.

1.3.1 Intrinsic resistant mechanism

Intrinsic resistant mechanism can be defined as natural resistant mechanism possessed by bacteria due to its natural physiological characteristics, such as structure of outer

Table 1- 4. Classes of antimicrobial and their antibiotics.

Antimicrobial category	Antibiotic
Aminoglycosides	Gentamicin
	Tobramycin
	Amikacin
	Netilmicin
Antipseudomonal carbapenems	Imipenem
	Meropenem
	Doripenem
Antipseudomonal cephalosporins	Ceftazidime
	Cefepime
Antipseudomonal fluoroquinolones	Ciprofloxacin
	Levofloxacin
Antipseudomonal Penicillin + ß- lactamase inhibitors	Ticarcillin-clavulanic acid
	Piperacillin-tazobactam
Monobactams	Aztreonam
Phosphonic acids	Fosfomycin
Polymyxins	Colistin
	Polymyxin B

Reference: (19)

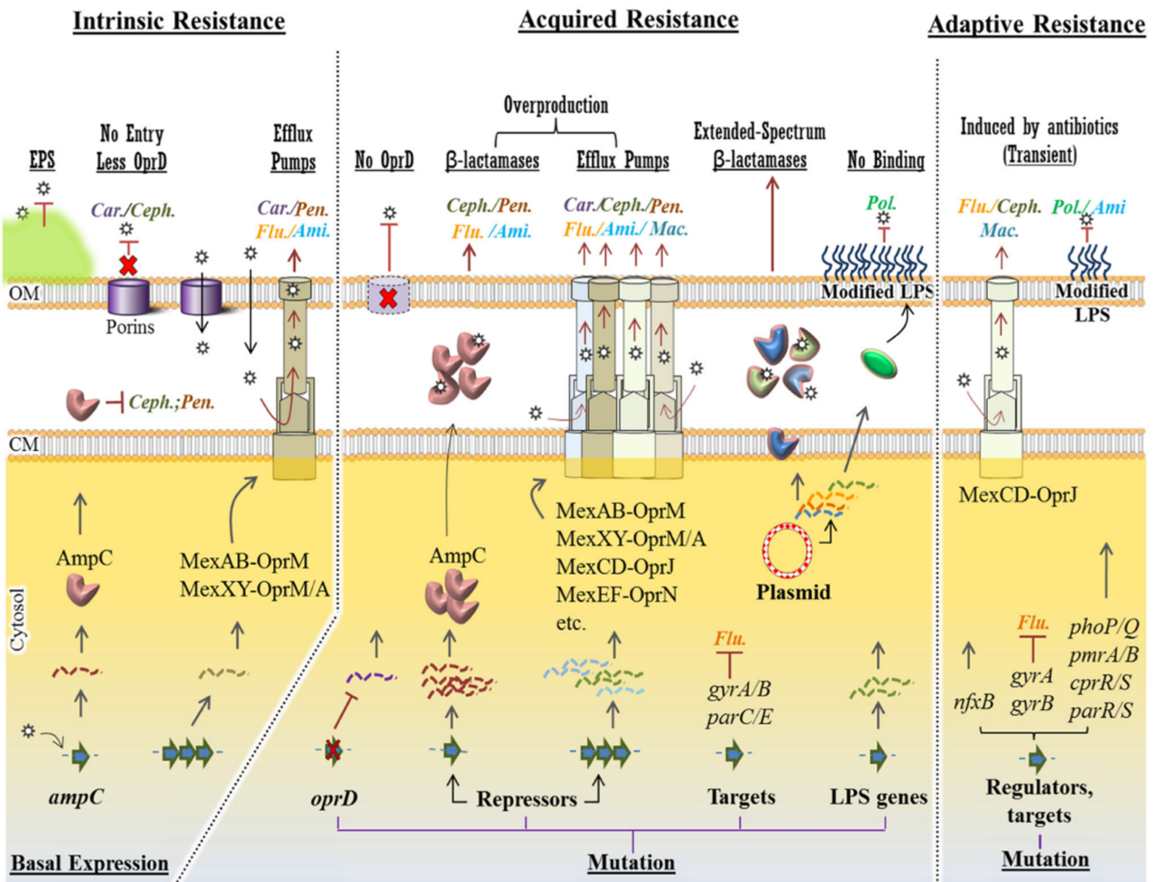


Figure 1- 4. Antibiotic Resistant Mechanisms of *P. aeruginosa*. (Car., Carbapenems; Ceph., Cephalosporins; Pen., Penicillins; Ami., Aminoglycosides; Flu., Fluoroquinolones; Mac., Macrolides and Pol., Polymyxins), EPS (extracellular polymeric substances), LPS (lipopolysaccharide) CM, cytoplasmic membrane; OM, outer membrane. Reference: (20).

membrane (21), especially *P. aeruginosa*. Naturally, the outer membrane permeability of *P. aeruginosa* is 12-100 times lower compared to *Escherichia coli* (22). Its low outer membrane permeability is contributed by limited number of major porin, OprF with large channels (23). There are more OprF with narrow channel present in the outer membrane of *P. aeruginosa* compared with OprF with large channel and thus limiting the entry of certain antibiotic (24). Not only that, *P. aeruginosa* can express less OprD, a porin that facilitate transportation of amino acids and Imipenem, thus limiting the influx of Imipenem to the cell (25).

On the other hand, there are four types of active multidrug efflux pumps in *P. aeruginosa.* They are MexAB-OprM, MexXY/OprM (OprA), MexCD-OprJ, and MexEF-OprN as shown in Fig.1-4 These efflux system is highly regulated and their expression level varies under different conditions (20). The MexAB-OprM is consecutively expressed and thus constantly repelling toxic molecules and antibiotics. Besides, MexXY- (OprA) is expressed at

basal level unless induced by protein synthesis inhibitors (20). There are more resistant nodulation-cell division (RND) system predicted to encode in the genome of *P. aeruginosa* compared to *E. coli*, *B. subtilis* and *M. tuberculosis* (Fig.1-5).

Other than efflux pump, gene encoding ß-lactamase can be found in the chromosome of *P. aeruginosa* and this enzyme is basally expressed (26). Expression of this enzyme enable *P. aeruginosa* to become resistant to aminopenicillins and most of the cephalosporin (20).

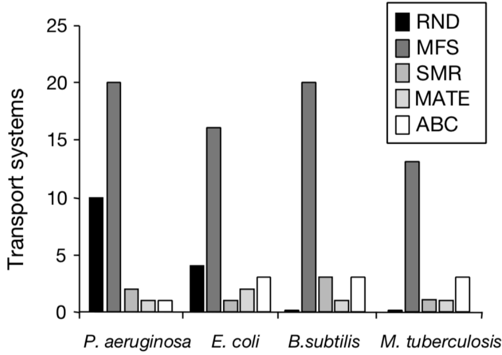


Figure 1- 5. Comparison of the number of predicted drug efflux systems in *P. aeruginosa*, *E. coli, B. subtilis and M. tuberculosis.* resistance/nodulation/cell division family (RND); major facilitator superfamily (MFS); small multidrug resistance family (SMR); multidrug and toxic compound extrusion family (MATE); ATP-binding cassette family (ABC). Reference: (27).

1.3.2 Adaptive resistant mechanism

Expressions of resistance gene can be induced under prolonged presence of antibiotics (22). The presence of Imipenem induced the expression of chromosomal ß-lactamase (AmpC) in *P. aeruginosa.* Regulation of AmpC is controlled by complex regulatory systems. The AmpR, positively regulates the expression of AmpC depending on the presence of N-acetylglucosamine-1,6-anhydro-N-acetylmuramic acid oligopeptide. This molecule is produced when peptidoglycan is breakdown by ß-lactam antibiotics (28). The other genes involved in the regulation of AmpC includes AmpD, AmpG and AmpE (29).

Another adaptive resistant mechanism of *P. aeruginosa* included the overexpression of MexCD-OprJ efflux pump which confers resistance to fluoroquinolone antibiotics. This could be regulated by AlgU, a stress response sigma factor. (20).

On the other hand, *P. aeruginosa* can become resistant to certain polycationic antimicrobials such as aminoglycosides, polymyxins and cationic antimicrobial peptides through alteration of lipid A structure in lipopolysaccharide (LPS). Multiple mutations in

cognate regulatory proteins such as the two-component systems PhoP-PhoQ, PmrA-PmrB, CprR- CprS, and ParR-ParS contributed to the alternation of LPS (20).

1.3.3 Acquired resistant mechanism

Acquired resistance mechanism can be obtained through gene mutation or by acquisition of plasmids harboring resistant gene from other bacteria (20). These traits are irreversible and can be inherited (22).

Mutation in AmpR and AmpD leads to overproduction of AmpC. As a result, *P. aeruginosa* become resistant to wider range of antibiotics, including most ß-lactams, monobactams, third and fourth generation of cephalosporins (20). The mexAB-oprM operon in *P. aeruginosa* is negatively controlled by mexR, nalD, nalB, and nalC. Mutations in these loci resulted in the overproduction of MexAB-OprM complex and conferring a higher resistance to carbapenem antibiotics. Overexpression of other multidrug efflux pumps such as MexXY and MexCD-OprJ can occur through mutations in regulatory loci, causing over expression and a higher resistance to a various kinds of antimicrobial agents (20).

Another clinically prevalent resistance mechanism is attributed to OprD porin channel. This porin channel is localized in the outer membrane of *P. aeruginosa*. It is a carbapenem-specific porin (Fig. 1-4) and thus loss or reduction of OprD can reduce permeability of carbapenems (24).

Horizontal gene transfer (HGT) can occur among bacteria, either intraspecies or interspecies and it is the culprit of the widespread of resistant mechanisms (30). *P. aeruginosa* carrying plasmid encoding metallo-ß-lactamase was isolated from Japanese hospital in 1996 (31). *P. aeruginosa* carrying other metallo-ß-lactamase encoding plasmid were reported in France, England and Brazil in the next decade (32–34).

Multidrug resistant *P. aeruginosa* is an alarming situation in clinical setting due to its sophisticated antibiotic resistant mechanisms. Thus, alternative treatment is crucial in order to overcome this problem. Treating *P. aeruginosa* infection using bacteriophage is a potential alternative method.

1.4 Bacteriophage

Bacteriophage was first discovered by William Twort in 1915. Felix d'Herelle discovered phage's ability to kill bacteria (35). Other than its ability to kill bacteria that causes infections, the study of molecular biology of phages has enable scientist to discover that deoxyribonucleotide (DNA) as the inheritance materials in cell, which was demonstrated by

the famous Hershey and Chase experiment (36). Phages can be classified based on their taxonomy and genetic composition as shown in Fig. 1-6B.

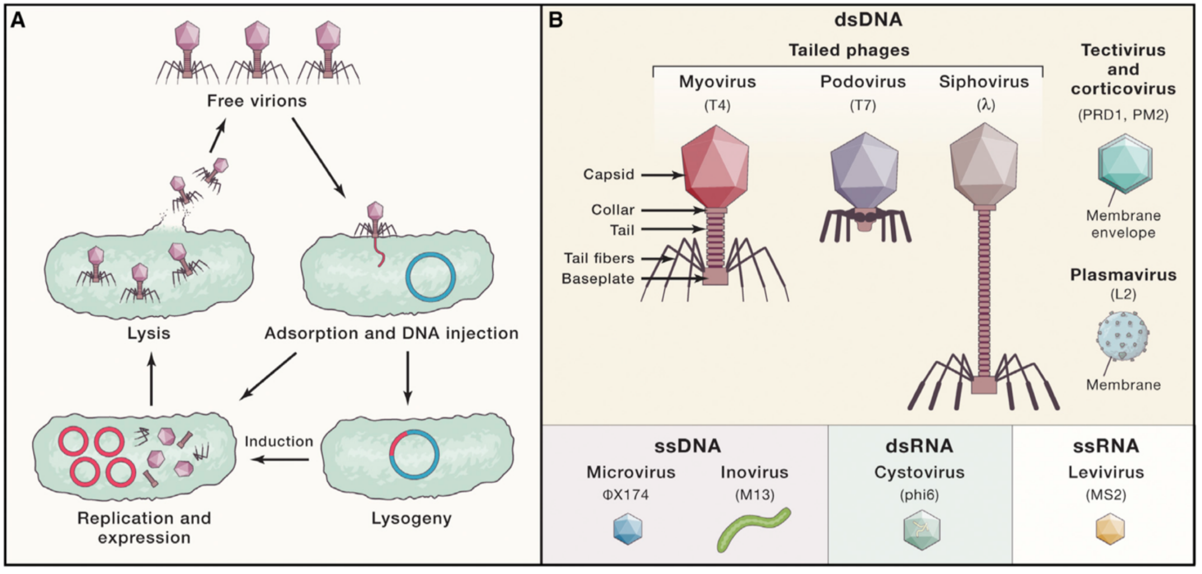


Figure 1- 6. (A)The life cycle of phage. (B) Phage taxonomy based on morphology and genome composition. Reference: (37).

Bacteriophages are obligatory parasite and they could not propagate without infecting a host cell (37) . As shown in Fig. 1-6A, phage infection starts with the adsorption to host cell, through recognition of specific host cell receptor present on the surface of host cell (38). Next, DNA carried inside the capsid of phage is injected into host cell. At this point, phage can enter into lytic or lysogenic lifecycle. After that, phage undergoes lytic life cycle and hijacks the cellular machinery of host cell for DNA replication, translation and protein synthesis for phage particles. Synthesized phage DNA and phage component are assembled before released from the host cells (39). Temperate phage can lysogenize their DNA into host genome and propagate together with host cell without killing them, or enter into lytic life cycle by certain inducing factor (37). The ability of bacteriophage to lyse targeting cell is the main key point to be used as antimicrobial in phage therapy.

1.5 Phage therapy: the alternative

The escalation of multidrug resistant bacteria has diverted public's attention toward phage therapy (40). During pre-antibiotic era, phage was used to treat infection caused by bacteria such as dysentery and *Staphylococcal* skin disease. Phage was being produced to be commercialized by d'Herelle's laboratory in Paris and The Oswaldo Cruz Institute in Rio de Janeiro, Brazil (41). However, phage therapy was soon be abandoned by the western world after the discovery of antibiotics due to several short comings compared to antibiotics. Host

range of bacteriophage is narrow and the results of phage therapy could not be reproduced due to variation in phages used for therapy (40). Use of phage therapy was continued in Eastern Europe, such as the Eliava Institute of Bacteriophage, Microbiology and Virology in Tbilisi, Georgia and the Institute of Immunology and Experimental Therapy in Wroclaw, Poland (40).

Other than its short comings, phage therapy showed several advantages over antibiotics. First, specificity of phage host range minimized the disruption of normal flora. Second, new phages can readily be isolated from the environment due to their abundance in nature (35). Third, some phages were able to degrade and penetrate through biofilm (42).

The most successful story of phage therapy could be of the Petterson case. Tom Petterson was infected with *Acinetobacter baumannii* during his trip to Egypt. Treatment with a combination of antibiotics later resulted in the appearance of *A. baumannii* that become resistant to all available drugs (43). A personalized phage cocktail was prepared for the treatment and this cocktail successfully eradicated *A. baumannii* revived patient from critical condition, in addition of sublethal concentration of antibiotic (44).

1.6 Aim of this research

This research has the following aims:

1. To elucidate the spontaneous mutations in *P. aeruginosa* that leads to antibiotic resistance

2. To Isolate lytic phages of *P. aeruginosa*

3. To investigate effective alternative treatment against *P. aeruginosa* by using phage cocktail complemented with antibiotic which has potential to be applied in the treatment of infection caused by *P. aeruginosa*.

1.7 Thesis structure

Figure 1-7 below shows the structure of this thesis

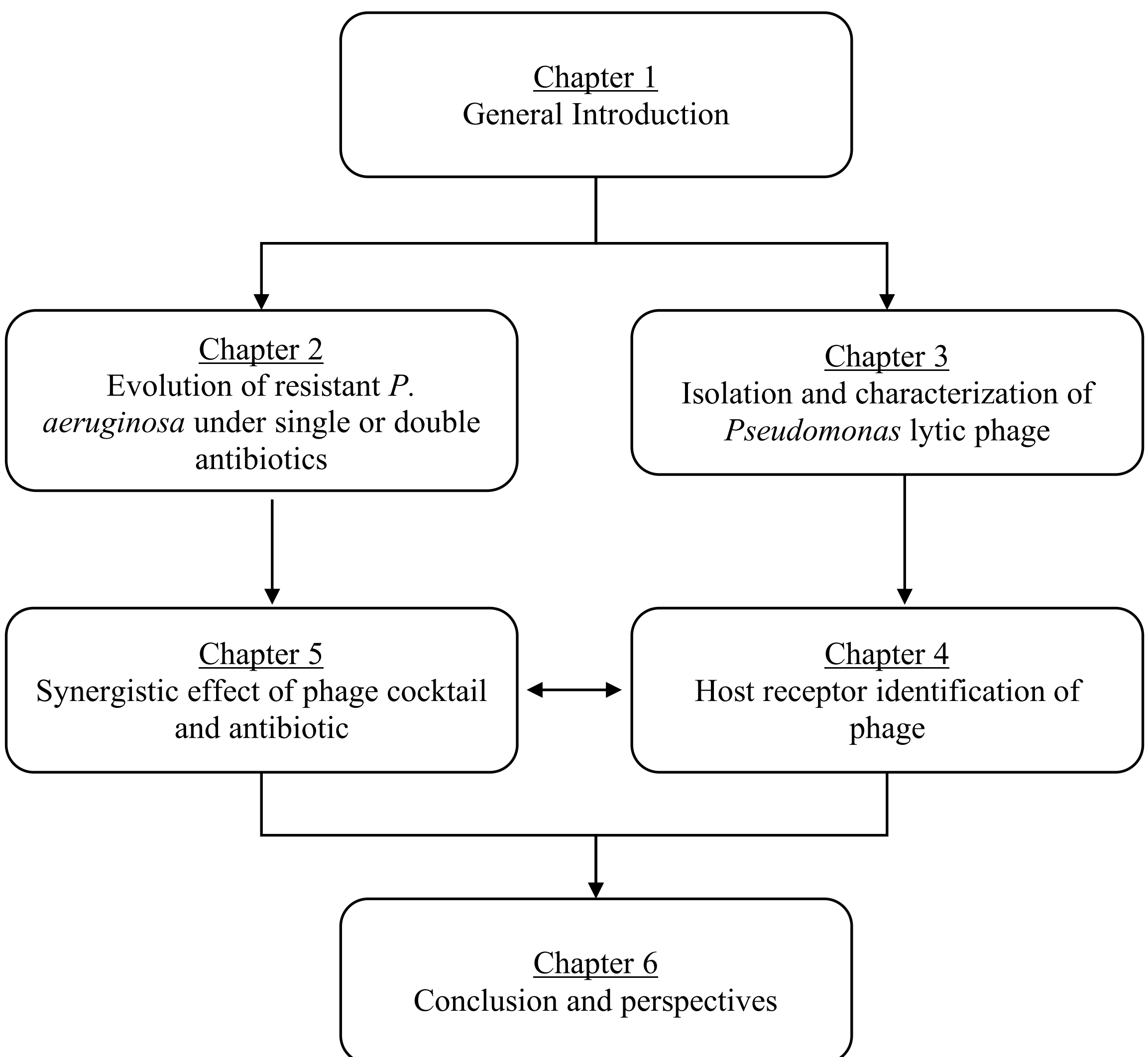


Figure 1-7. Thesis structure. The arrows indicate relationship between each chapter.

Chapter 2 Evolution of resistant *P. aeruginosa* under single or double antibiotics

2.1 Introduction

Acronym ESKAPE refers to a group of antibiotic bacteria that became problematic issue in clinical setting. The ESKAPE pathogens, include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter spp*. has posed new threats to the hospital setting (45). The ESKAPE pathogens are predicted to be more prevalent in becoming resistant to antimicrobial agents in coming years (45). Thus, it is important to study the mechanism that contribute to their resistance.

P. aeruginosa is intrinsically resistant to antibiotics commonly used for treating bacterial infection. Thus, special groups of antibiotics are needed for the treatment, such as carbapenem, fluoroquinolone and polymyxin (46). It is common to use combination of antibiotic for treatment in hospital while treating infection caused by *P. aeruginosa*. However, the major drawbacks of combination treatment are toxicity caused by antibiotics and the appearance of multidrug resistant bacteria (47). Treatment failures were often caused by superbug that become resistant to all available antibiotics after prolonged treatment, especially in citric fibrosis (CF) patients with chronic lungs infection (48).

Carbapenem and fluoroquinolone are common antibiotic used to treat infection caused by *P. aeruginosa* (49) and the representative antibiotic in each class is shown in Fig 2-1.

Structure	Meropenem (carbapenem)	Ciprofloxacin (fluoroquinolone)
Target	Penicillin binding protein	DNA gyrase
Mechanism	Interfere with cell wall synthesis	Interfere with DNA replication

Figure 2-1. Structure, target and inhibitory mechanism of meropenem (MEM) and ciprofloxacin (CIP).

2.2 Materials and methods

2.2.1 Bacterial strains, antibiotic and medium

Pseudomonas aeruginosa PAO1, a standard lab strain was used throughout the experiment, otherwise stated. Muller Hinton broth (MHB) was used in the stepwise batch culturing of *P. aeruginosa* PAO1. Each liter of MHB contains 2 g of beef infusions solids, 17.5 g of casein hydrolysate, and 1.5 g of starch. Ciprofloxacin (CIP) and Meropenem (MEM) which belong to fluoroquinolone and carbapenem, respectively were used in stepwise batch culturing of *P. aeruginosa*.

2.2.2 Stepwise batch culturing of *P. aeruginosa*

Ciprofloxacin-resistant, meropenem-resistant, and PAO1 resistant to both antibiotics were generated via batch culturing by increasing the concentration of antibiotic in stepwise manner. The cultivation was conducted in TVS062CA compact rocking incubator (TVS062CA, ADVANTEC, Tokyo, Japan) at 40 rpm. Briefly, *P. aeruginosa* PAO1 wildtype was challenged with sub-lethal concentration of antibiotic, which was half of its minimal inhibitory concentration (MIC). After culturing for 24-48h at 37°C, bacterial culture in stationary phase was transferred to new medium with increased concentration (Fig. 2-2). Stepwise batch culturing was carried out for 4-6 rounds. Each round was duplicated. Optical density value at wavelength of 660 (OD_{660}) of cultivation was automatically recorded in every 15 min. One

culture without oxacillin was used as a control. Antibiotic resistant bacteria were isolated from each batch by plating the bacteria culture on LB agar plate. Single colony was picked up and streaked for three times before storing it as glycerol stock. Each strain was named 00X, with X referring to the type of antibiotic it was originated from, and 00 stands for the concentration of antibiotic. The acronym is as follow: CIP (C) and MEM (M). For example, strain isolated from MHB added with 16 µg/mL of ciprofloxacin would be named as 16C.

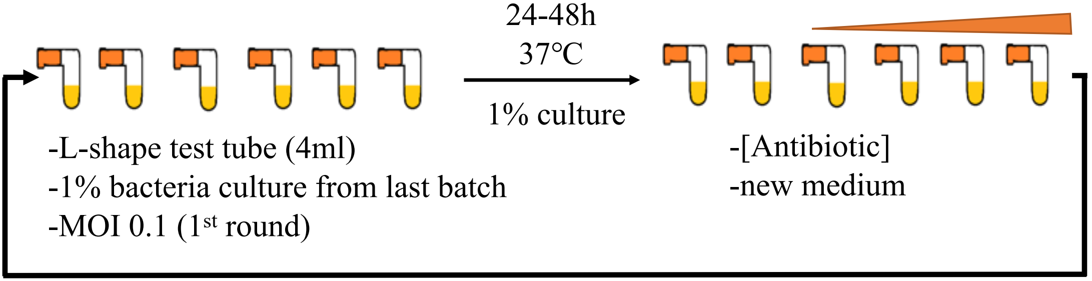


Figure 2-2. Batch wise culturing of *P. aeruginosa* PAO1 challenged with antibiotics

2.2.3 Minimal Inhibitory Concentration (MIC)

Minimal inhibitory concentration (MIC) of wild type and evolved *P. aeruginosa* PAO1 were determined according to standard protocol from Clinical Laboratory Standard institute (9th Edition) (CLSI) with modification. Briefly, an overnight culture was diluted with MHB to 10^6 colony forming unit (CFU) per ml. Antibiotic was added to MHB and were serially diluted for 2 times in 96-wells plate (from lane 1-10). Each well contained 100 µL of MHB with antibiotic. One hundred microliter of adjusted bacterial culture was added to the wells. All tests were done in triplicates. Positive control was included in lane 11, only adding bacteria culture to MHB without antibiotic. Sterile MHB was used as a negative control.

2.2.4 Whole genome sequencing

Overnight culture was prepared by inoculating single colony into 2 mL LB broth (10 g polypeptone, 10 g NaCl and 5 g yeast extract per liter). Culture was incubated for 16 h, 37°C, 120 rpm. Five hundred microliter of overnight culture was palleted at 5,000 g for 1 min. Next, palleted bacteria was washed with distilled water for 2 times. Washed cell was used for DNA extraction using GenElute Bacterial Genomic DNA kits (Sigma, Germany), following instruction from manufacturer. Extracted genomes were submitted to BGI (Hongkong) for whole genome sequencing by Illumina HiSeq platform with genome coverage (sequencing depth) = 100-fold with 100-bp paired end. Ciprofloxacin-resistant (16C), meropenem-resistant (128M), and strain resistant to both antibiotic (4C32M) were sequenced. The sequence was

mapped against genome sequence of *P. aeruginosa* wildtype PAO1 (Accession number: NC_002516) and Single Nucleotide Polymorphisms (SNPs) were identified by SAMtools (version 1.10), Pilon or manually checked by visualizing mapped file on Tablet (version 1.19).

2.3 Results

2.3.1 Stepwise batch culturing of *P. aeruginosa*

The MIC of CIP and MEM of *P. aeruginosa* PAO1 wildtype were determined to be 0.25 µg/mL and 2 µg/mL, respectively. The starting concentration of antibiotic was fixed at half of MIC (sub-lethal concentration). In the first stepwise batch culturing with CIP, resistant mutants appeared around 19 h and 21 h in the concentration of 0.125 µg/mL and 0.25 µg/mL, respectively (Fig. 2-3, left). At round 2, bacteria growth was similar regardless of the presence of antibiotic. Due to machine error, the OD_{660} from 24 h to 48 h was not recorded. In round 3, the concentration of antibiotic was doubled, growth of CIP resistant bacteria was observed in between 18-20 h. At round 4, the growth of bacteria was not so much different from the control (Fig. 2-3). On the other hand, bacteria could grow under 16 µg/mL of CIP and turbidity of bacteria culture remained lower than the control and the culture grew under 8 µg/mL of CIP.

In stepwise batch culturing with MEM, cells resistant to 1 µg/mL of MEM appeared around 15 h (Fig. 2-4) in round 1. Meanwhile, cells resistant to 2 µg/mL appeared around 5 h later at 20 h. Bacteria resistant to 16 µg/mL of MEM appeared around 15 h and its growth become stagnant from 20-28 h before start growing again. At round 4, cells resistant to 64 µg/mL of MEM appeared at about 18 h and 20 h. Meanwhile, growth of cells resistant to 128 µg/mL of MEM were observed after 40 h (Fig. 2-4). Fluctuation in OD value was caused by cell aggregation in the culture (Fig. 2-6).

During the treatment with both CIP and MEM at sub-lethal level, turbidity of cells increased for first 3 h before plummeted to less than OD_{660} 1 (Fig. 2-5). Resistant cells grew at 18 h and 30 h. In around 2, resistant mutant appeared around 20 h and 35 h in each replicate. At round 6, no resistant cells were observed until 65 h in replicate 2. While cell in replicate 1 remained at OD_{660} 0 up to 92 h (Fig. 2-5).

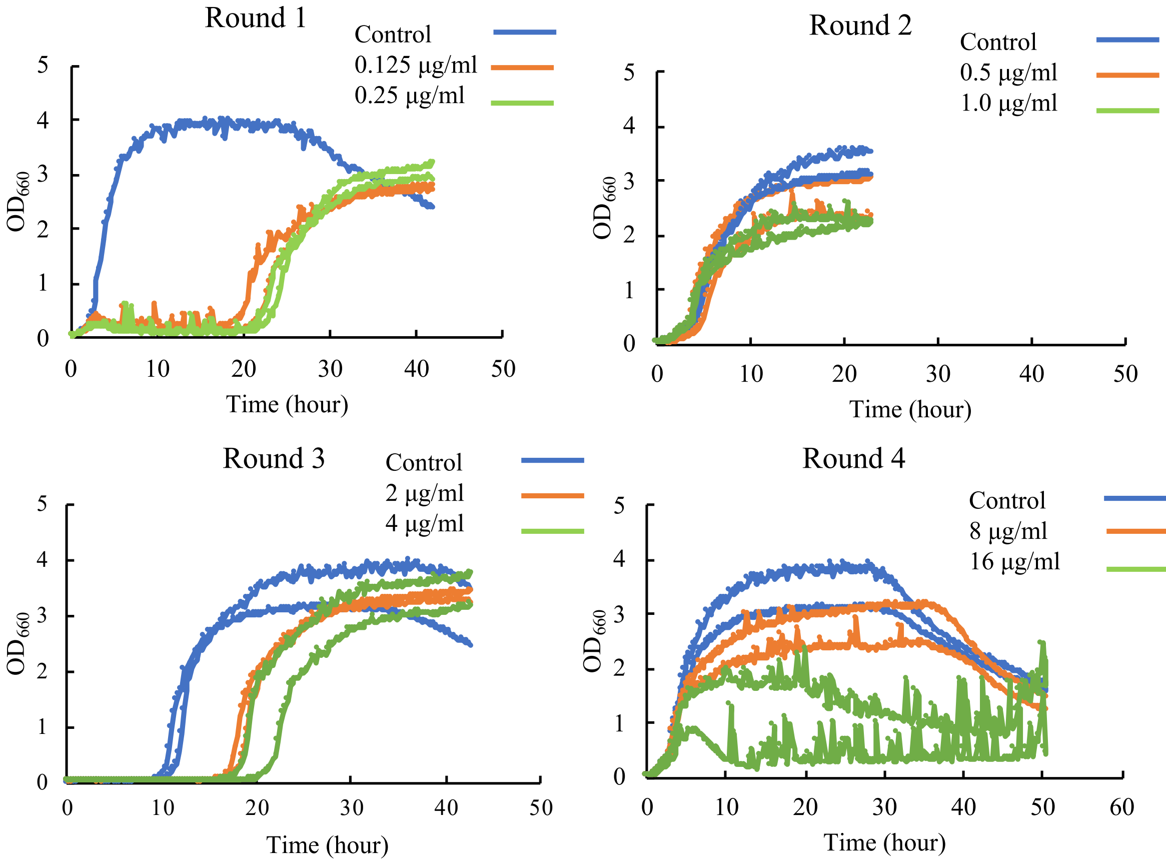


Figure 2-3. Growth curves of *P. aeruginosa* in stepwise batch culturing with ciprofloxacin (CIP). Control in round 1 was *P. aeruginos*a PAO1 wildtype. Control in round 2 onwards were the resistant mutant culture appeared in previous batch. For each concentration, 2 replicates were conducted.

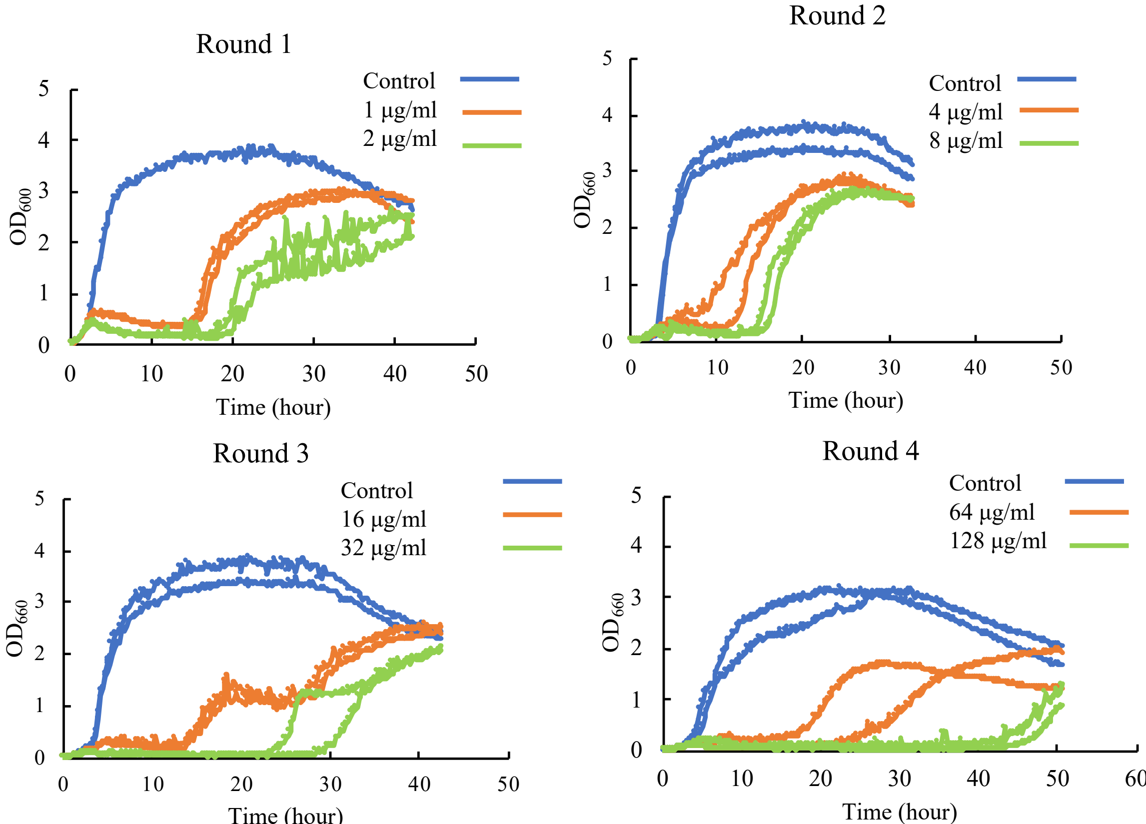


Figure 2-4. Growth curves of *P. aeruginosa* in stepwise batch culturing with Meropenem (MEM). Control in round 1 was *P. aeruginos*a PAO1 wildtype. Control in round 2 onwards were the resistant mutant culture appeared in previous batch. For each concentration, 2 replicates were conducted.

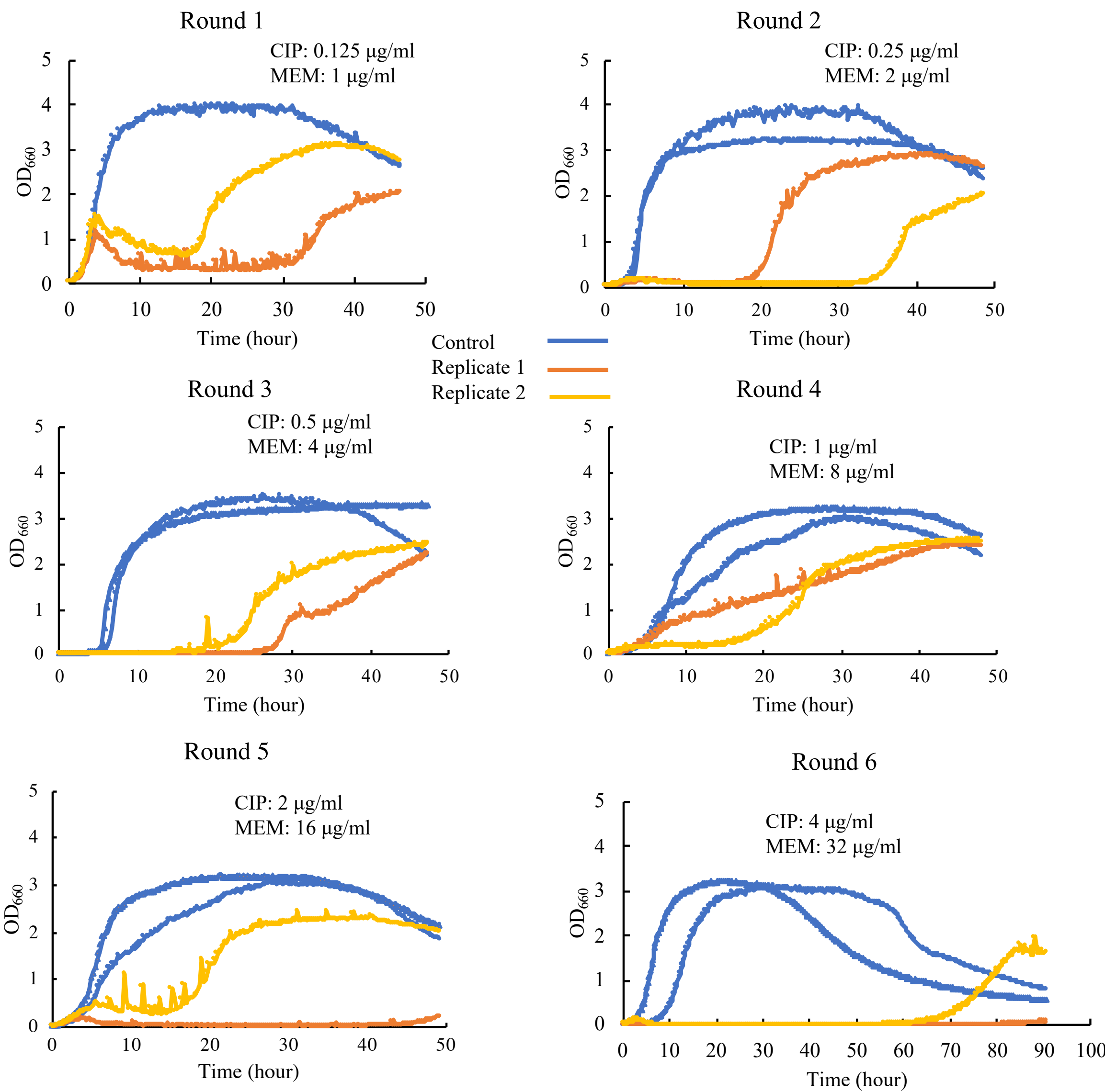


Figure 2-5. Growth curves of *P. aeruginosa* in stepwise batch culturing with CIP and MEM. Control in round 1 was *P. aeruginos*a PAO1 wildtype. Control in round 2 onwards were the resistant mutant culture appeared in previous batch. For each concentration, 2 replicates were conducted.

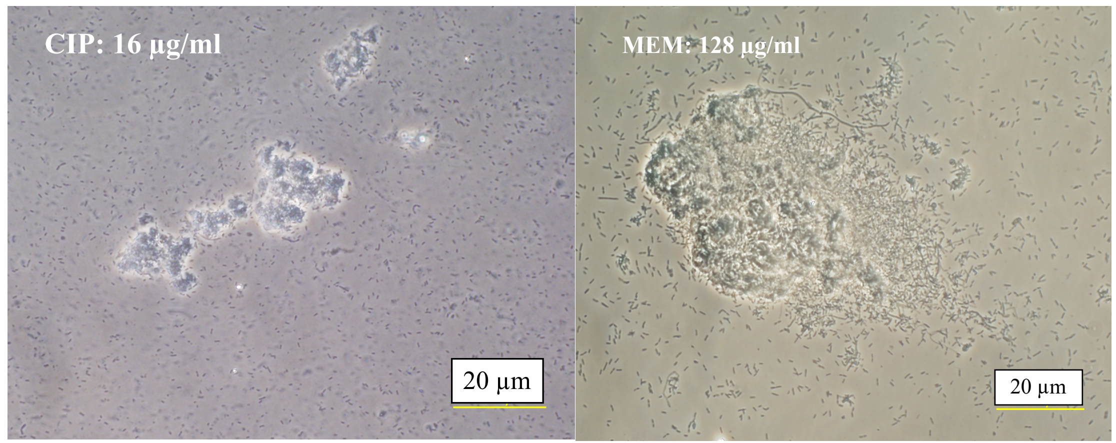


Figure 2-6. Cell aggregation observed by light microscope.

2.3.2 MIC of resistant strain isolated at the end of batch culture

Resistance to antibiotic is defined by CLSI reference breakpoint (table 2-1), which is fixed at specific concentration (µg/mL). According to this standard, bacteria can be divided into 3 categories as sensitive (S), intermediate (I) and resistant (R) to an antibiotic where I and R are classified as resistant strain. Mutant strains isolated in this experiment were classified into non-resistant or resistant strain is based on their MIC value.

Table 2-1. Breakpoint reference of ciprofloxacin and meropenem (CLSI)

	Concentration (µg/mL)		
Antibiotics	Sensitive (S)	Intermediate (I)	Resistant (R)
Ciprofloxacin	≤ 1	2	≥ 4
Meropenem	≤ 4	8	≥ 16

The MIC of MEM mutant strains isolated from each round showed higher MIC than the concentration of MEM it was cultured with, except strain 8M whereby its MIC value remained at 8 µg/mL. All isolated resistant mutants had become resistant to MEM (table 2-2). Meanwhile, strain 2M and strain 8 M remained sensitive to CIP. Cross resistance was observed in strain 32M and 128M, which showed intermediate resistance to CIP.

The mutant strain 0.25C remained sensitive to CIP, while strains isolated from the consecutive rounds became resistant to CIP. Contrary to MEM resistant strains, all CIP resistant strains did not gain resistance to MEM and all the MIC value remained less than 1 µg/mL.

Table 2-2. Minimal Inhibitory Concentration (MIC) of *P. aeruginosa* PAO1 and antibiotic resistant strains

Strain	**MIC (µg/mL)**	
	Ciprofloxacin	**Meropenem**
PAO1	0.25 (S)	2 (S)
2M (MEM resistant 2 µg/ml)	0.125 (S)	64(R)
8M	1 (S)	8 (I)
32M	2 (I)	512 (R)
128M	2 (I)	256 (R)
0.25C (CIP resistant 0.25µg/ml)	1 (S)	0.5 (S)
1C	2 (I)	1 (S)
4C	8 (R)	1 (S)
16C	128 (R)	<0.125 (S)
0.25C 2M	4 (R)	128 (R)
1C 8M	4 (R)	128 (R)
4C 32M	8 (R)	64 (R)

2.3.3 Spontaneous mutation found in resistant strains

Table 2- 3. Mutations found in ciprofloxacin resistant PAO1 (16C)

Deletion

Gene	Reference	Deletion (bp)	Type of mutation
Glycyl-tRNA synthetase beta chain, *glyS*	PA0008	15	Deletion of 5 aa (in frame)
Energy transducer protein, *tonB2*	PA0197	18	Deletion of 6 aa (in frame)
Probable multidrug resistant efflux pump	PA1237	87	Frameshift and deletion of 29 aa
Probable outer membrane component of multidrug efflux pump	PA1238	38	Frameshift →no stop codon
Sigma factor regulator, *femR*	PA1911	5	Nonsense → premature stop codon
Probable esterase/deacetylase	PA2098	44	Nonsense → premature stop codon
Branched-chain alpha-keto acid dehydrogenase, *bkdB*	PA2249	15	Deletion of 5 aa (in frame)
Anti-sigma factor for Haem uptake, *hasS*	PA3409	6	Framshift→2aa deletion
Regulator of ethanolamine catabolism, e*atR*	PA4021	31	Nonsense→premature stop codon
Dihydroaerugionic acid synthase, *pchE*	PA4226	4	Nonsense→premature stop codon
Probable ferredoxin	PA4772	6	Deletion of 2 aa
Probable transmembrane sensor	PA4895	28	Nonsense→premature stop codon
Probable transcriptional regulator	PA5431	100	Nonsense→premature stop codon

Nonsense point mutation

Gene	Reference	Mutation	Amino acid changes
Probable transmembrane sensor	PA2094	G→T	GAA (glu)→ TAA (stop codon)
DNA gyrase subunit A, *gyrA*	PA3168	C→A	GAC (Asp)→TAC (Tyr)
Transcriptional regulator, *nfxB*	PA4600	A→C	ACC (Thr)→CCC (Pro)

Table 2-4. Mutations found in PAO1 (128M)

Deletion

Gene	Reference	Deletion (bp)	Type of mutation
Outer membrane protein, *oprD*	PA0958	1	Nonsense→premature stop codon
Probable multidrug resistance efflux pump	PA1237	33	Deletion of 11 AA (in frame) (macrolide related)
Dehydroxyproline dehydrogenase alpha-subunit, *lphE*	PA1226	43	Frameshift→no stop codon
GspCD subunit for type II secretion, *xphA*	PA1867	39	Deletion of 13 aa
Non-ribosomal peptide synthetase, *ambB*	PA2305	1	Nonsense → premature stop codon
Anti-σ factor, *sbrR*	PA2985	122	Nonsense → premature stop codon
Precorrin-3-mythlase *cobJ*	PA2903	15	Nonsense → premature stop codon
Anti-sigma factor for Haem uptake, *hasS*	PA3409	48	Deletion of 16 aa
Phosphomannomutase/ phosphoglucomutase, *algC*	PA5322	6	Deletion of 2 aa (at magnesium binding site)
Guanosine-3',5'-big(diphosphate)-3'-pyrophohydrolase, *spoT*	PA5338	6	Deletion of 2aa
Glycolate oxidase, *glcF*	PA5353	12	Deletion of 4aa
Probable transcriptional regulator	PA5431	20	Nonsense→premature stop codon

Table 2-5. Mutations found in PAO1 (4C32M)

Deletion

Gene	Reference	Deletion (bp)	Type of mutation
D-hydroproline dehydrogenase alpha-subunit *lphE*	PA1266	6	Deletion of 2 aa
Flagella biosynthetic protein *fliP*	PA1446	A	Nonsense→premature stop codon (54aa)
Threonyl-tRNA-synthetase *thrS*	PA2744	3	1 aa deletion
Anti-σ factor *sbrR*	PA2895	4	Nonsense → premature stop codon
Transport protein *hasD*	PA3406	8	Frameshift → no stop codon
Anti-sigma factor for Haem uptake, *hasS*	PA3409	10	Nonsense→ premature stop codon
protein-PII uridylyltransferase, *glnD*	PA3658	1	Nonsense → premature stop codon

Beta-lactamases induction permease, *ampO*	PA4219	7	Nonsense→ premature stop codon
Two component sensors, *cbrA*	PA4725	14	Nonsense→ premature stop codon
glycolate oxidase subunit, *glcF*	PA5353	9	Deletion of 3 aa

Nonsense point mutation

Gene	Reference	Mutation	Amino acid changes
DNA gyrase subunit, *gyrB*	PA0004	C→T	TCC (glu)→ TTC Ser→Phe
Outer membrane protein, *oprD*	PA0958	C→A	GAA→TAA Glu→stop codon
DNA gyrase subunit, *gyrA*	PA3168	T→C	GAC→GGC Asp→Gly

For all antibiotic resistant strains, several deletions and point mutation (except 128M) were found which conferred to nonsense mutations. All the mutations were summarized in table 2-3, 2-4 and 2-5 for respective resistant strain. In strains 16C and 128M, mutations were found in probable efflux pumps which could be related to antibiotic resistance. However, no mutation related to efflux pump was found in 4C32M.

One base pair deletion was found in *oprD* and resulted in frameshift mutation that yielded premature stop codon in 128M, which was isolated from round 4, cultured with 128 μg/mL. Whereas, no mutation in *oprD* was found in CIP-resistant stain (16C). One-point mutation was found in *gyrA* in strain 16C. Whereas, one-point mutation was found in *gyrA* and *gyrB* 4C32M. One-point mutation in *oprD* that yield stop codon was found to accumulate in 4C32M which is resistant to both antibiotic (table 2-6). Mutations commonly related to CIP and MEM resistance were summarized in table 2-6.

Table 2-6. Mutation found commonly in genes related to CIP and MEM resistance.

Gene	Reference no.	CIP-resistant (16C)	MEM-resistant (128M)	CIP-MEM resistant (4C32M)
oprD	PA0958	X	O	O
gyrA	PA3168	O	X	O
gyrB	PA0004	X	X	O

X-absent; O-present

2.4 Discussion

2.4.1 Stepwise batch culturing of *P. aeruginosa*

P. aeruginosa tends to grow in micro aggregates in liquid culture and will disperse during starvation (50). In the presence of antibiotic, *P. aeruginosa* can form aggregate encapsulated by alginate to protect from antibiotics (51). The aggregation of cells was observed during the co-culture with gradually increased antibiotic.

2.4.2 MIC of resistant strain isolated at the end of batch culture

Sublethal antibiotic treatment was found to promote the emergence of antibiotic-resistant bacteria, as shown in the co-culture experiment. Culture of *P. aeruginosa* by 0.25 µg/mL of CIP or 2 µg/mL of MEM leads to the emergence of mutant that was highly resistant to CIP and MEM, respectively. The same was observed by Toprak and colleagues when they challenged the *E. coli* with concentration increased gradually (52).

2.4.3 Spontaneous mutation found in resistant strains

Carbapenem is the last resort for treating infection caused by gram negative bacteria. Among the beta-lactams, carbapenem is stable against most beta-lactamases, enzymes that inactivate beta- lactams) such as AmpC and the extended spectrum beta-lactamases (ESBLs) (53). However, the emergence of carbapenem resistant bacteria in recent years has become a critical issue in clinical setting and is usually associated with metallo-beta-lactamases (MBLs) (34).

In the case of *P. aeruginosa*, resistant strains without the production of MBLs were isolated from the clinical strains (54), showing that *P. aeruginosa* can become resistant to carbapenem via other mechanism. Thus, it is important to study about the mutation that confer resistant to antibiotics via spontaneous chromosomal mutations. The OprD was inactivated in strain 128M whereby OprD mutations is often related to carbapenem resistance in clinical strains as OprD is a transport proteins which facilitate the entrance of meropenem into the cell (55,56).

Ciprofloxacin is another common antibiotic used for the treatment of. *P. aeruginosa*'s infection (49). Ciprofloxacin binds to DNA gyrase and caused double stranded break in chromosomal DNA (57). Resistance to ciprofloxacin is often achieved by point mutation that decrease the binding affinity to *P. aeruginosa* by single point mutation (58).

In strain 128M and 4C32M, mutation was found in gene *sbrR* which encode for protein involved in the regulation of swarming motility and biofilm formation in *P. aeruginosa* (59). Gene *tonB2* which involved in quorum sensing of *P. aeruginosa* was mutated in 16C (60).

Besides, mutations were also found in genes involved in the production of structural gene. In 128M, mutation was found in *algC*, a gene encoding for phosphomannomutase and phosphoglucomutase that are involves in the biosynthesis of core lipopolysaccharide (LPS), alginate and rhamnolipids (61,62). In strain 4C32M, gene involving in synthesis of flagella

(*fliP*) was mutated. Flagella is important for *P. aeruginosa* for mobility and attachment of bacteria to host cells (1).

Other than having mutation in gene commonly confers to antibiotic resistance, there were random mutation found in genes involved in gene regulation and structural genes, which could reduce the virulence or fitness of the antibiotic strain. Bacteria that gained antibiotic resistance were found to be less fit if compared to wildtype (63).

Chapter 3 Isolation and characterization of *Pseudomonas* lytic phage

3.1 Introduction

Phages are bacterial viruses and they occupy habitats where bacteria thrive. They are classified into 13 families according to morphology, type of nucleic acid, and presence or absence of an envelope or lipid. The majority of phages are "tailed phages," composed of an icosahedral head and tail. All tailed phages have double-stranded DNA. According to the morphologic features of the tail, they are classified into 3 families, *Myoviridae*, *Siphoviridae*, and *Podoviridae* (37).

P. aeruginosa is ubiquitous in the environment, and thus phage infectious to *P. aeruginosa* can be found in wide range of geographical areas (64). Due to the emergence of antibiotic resistant bacteria, phage therapy has gained its attention again. New phages are continuously being isolated from the environment and the understand of these phages will contribute to the efficacy of phage therapy.

3.2 Materials and methods

3.2.1 Bacteria, culture media and growth condition

Standard strain *P. aeruginosa* PAO1 was used in all experiments, unless otherwise stated. All experiments were conducted in LB Broth at 37°C, with shaking at 120 rpm, unless otherwise stated.

3.2.2 Isolation and preparation of phage stock

Phages were isolated from sewage influent obtained from the municipal wastewater treatment plant in Tokyo using *P. aeruginosa* PAO1 as the host by double layer agar plating method. Phages were propagated and purified by the method described elsewhere. Briefly, purified phage was propagated by mixing 1% of overnight culture of PAO1 in liquid LB and incubated overnight. Host cells were removed by centrifugation (11,000 g, 20 min, 4°C) before concentration by polyethylene glycol 6000-NaCl (PEG-NaCl) method and filtered with a 0.45 μm Millex-GP filter (Merck, Millipore, Germany). Workflow of phage isolation was summarized in Fig. 3-1.

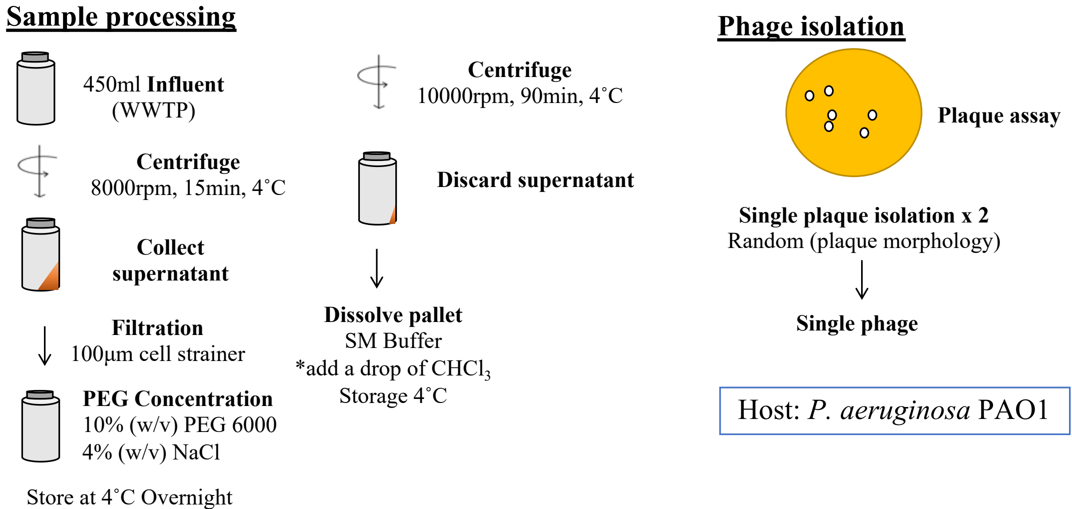


Figure 3-1. Workflow of phage isolation by influent from wastewater treatment plant.

3.2.3 TEM imaging of phages

Phages were observed by transmission electron microscope (TEM) as described previously (65). Briefly, PEG-NaCl concentrated phage lysate was purified by cesium chloride (CsCl) step centrifugation (step densities, 1.46, 1.55 and 1.63 g/ml) and concentrated phage suspension (10^9 plaque forming unit (PFU)/ml) was spotted on top of a hydrophilic plastic-carbon-coated copper grid (Nissin EM Corporation). Phages were allowed to adsorb for 1 minute before removing excess sample. Next, 10 µl of distilled water was spotted on the grid and removed after a short time. Phages were stained by 2% uranyl acetate or EM Stainer (Nissin EM Corporation). Excess stain was removed after 1 minute, and the grid was allowed to air-dry for 30 minutes before observing with the JEOL JEM-1400Plus (TEM) operating at 80 kV.

3.2.4 Characterization of phage growth and determination of phage host range

A one-step growth curve was constructed to determine the phage's burst size and latent period as previously described, with modification (66). Briefly, the phage was added to refreshed overnight culture of *P. aeruginosa* PAO1 (OD_{660nm} =1) at multiplicity of infection (MOI) 0.01, and incubated at 37°C for 10 minutes, with shaking at 120 rpm. Unbound phage was removed by centrifugation and washed with chilled LB medium five times. Cells infected with phage were incubated at 37°C for one hour. The phage's number was tittered by double layer agar method. A set of lab collection of *P. aeruginosa* were used for preliminary host range test for selecting phages. Host range of phage that was selected was determined using 58 strains of clinical *P. aeruginosa* collected from Jichi Medical University Hospital (Tochigi, Japan), by

dropping 1 μL of phage lysate of 10^7 PFU/ml on *P. aeruginosa* mixed with 0.5% top agar (w/v). All the clinical strains were isolated from different sporadic cases, originated from patients admitted in different wards. Motility of clinical strains were examined (3.2.5 swarming test and 3.2.6 twitching test) as one of the phenotypic characteristics. Results were scored as clear (sensitive), turbid (sensitive) or no plaque (resistant). Spot test was also carried out using mutant strains (16C, 32M, 128M and 4C32M) from Chapter 2 in order to examine phage's efficacy towards antibiotic resistant strains.

3.2.5 Swarming test

Swarming agar was prepared as followed based on developed protocol (67). First, 6 g of agar was autoclaved in 800ml of water. All the solutions with mentioned final concentration below were added to melted agar: 200 ml 5X M8 solution (30 g Na_2HPO_4, 15 g KH_2PO_4, 2.5 g NaCl/1L), 10 ml of 20% glucose (final concentration:0.2%), 25 ml casamino acids (final concentration: 0.5%) and 1M $MgSO_4$ (final concentration: 1mM). The solution was gently stirred until mixed well, and 25 mL of agar solution was poured in each plate. Agar were left in room temperature for overnight. Overnight culture was prepared by inoculating bacteria into 96 wells plate with 100 μL LB. 1μL of bacteria was dropped on top of the agar (3 strains in each plate). The plates were incubated upright in 37°C for 20 hours (inside humidified polystyrene box) before measuring their longest and shortest swarming diameter (Fig.3-2).

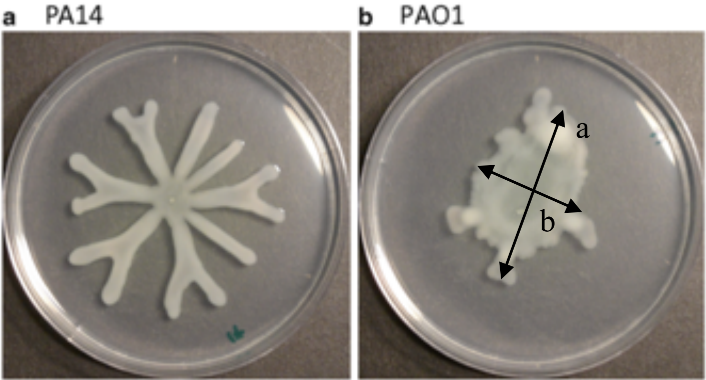


Figure 3- 2. Example of swarming test shown by different strains of *P. aeruginosa*. a: longest diameter; b: shortest diameter. Reference: (67)

3.2.6 Twitching test

Twitching test was carried out based on developed protocol (67). 10 mL 1% LB agar were poured for each plate. Single colony were picked up by toothpick and stabbed through the agar and touched the bottom of plate for 5 times. Plates were incubated upside down in 37°C

for 20 hours (inside humidified polystyrene box). About 5mL TM developer solution (400mL water, 100mL glacial acetic acid, 500mL methanol; stored in 4°C) were flooded on the surface and incubated for ~30min before washing away with water and air-dried. Twitching area could be visualized clearer. The surface bacterial colony was scraped off before longest (a) and shortest (b) twitching diameter were measured (Fig.3-3B). Twitching area were calculated as: $\pi \times \frac{1}{2} a \times \frac{1}{2} b$

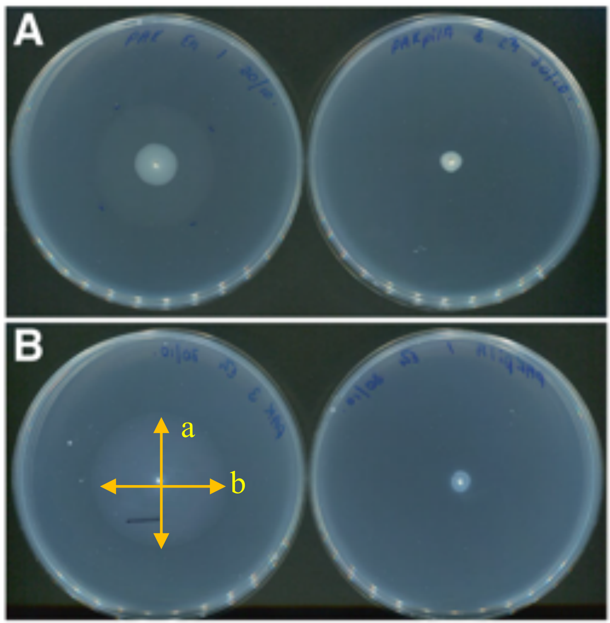


Figure 3-3. Example of twitching test result. Plates showed twitching of wildtype *P. aeruginosa* PAK (left) and pili mutant (right). (A) Before staining. (B) After staining and removal of surface colony. Reference: (67).

3.2.7 DNA extraction, sequencing, genome analysis, phage growth characterization

Genomic DNAs of phages were extracted by phage DNA isolation kit (Norgen Biotek Corp., Thorold, ON, Canada) and were submitted to BGI (Hongkong) for whole genome sequencing by Illumina HiSeq platform with genome coverage (sequencing depth) = 100-fold with 100-bp paired end. The sequence was assembled using Velvet De Novo Assembler v1.2.10 (EMBL-EBI). Open reading frames (ORFs) were predicted and annotated using the RAST server (http://rast.nmpdr.org/) and phage-carried tRNA genes were identified using tRNA Scan SE ver. 1.21 software. A phylogenetic tree was constructed using VICTOR Virus Classification and Tree Building Online Resource based on settings recommended for prokaryotic viruses and was visualized with FigTree based on a method described previously (68).

3.3 Results

3.3.1 Phage selection

A total of 46 strains of phages was picked up. Then, seven phages were selected and sent for sequencing based on preliminary host range test using lab strains (table 3-1). All of the selected phages could infect more than half of the strains.

Table 3- 1. Host range of isolated phages against *P. aeruginosa* lab collection.

	P. aeruginosa									
Phage	PAO1	28	29	30	31	32	33	34	35	36
ΦPA01	C	C	N	C	T	N	C	N	C	C
ΦPA02	C	C	N	T	T	T	C	N	T	N
ΦPA05	C	C	N	C	T	N	C	N	T	N
ΦPA13	C	C	N	T	T	N	T	N	C	N
ΦPA21	C	C	T	C	T	N	C	N	C	C
ΦPA27	T	T	C	C	C	N	C	N	T	T
ΦPA35	C	C	N	C	T	T	C	N	C	C

*28: IAM 1504; 29: IAM 1271; 30: ATCC 7700; 31: ATCC 10145; 32: NCTC 6750; 33: NBRC 3455; 34: ATCC 9027; 35: ATCC 15442; 36: ATCC 27853

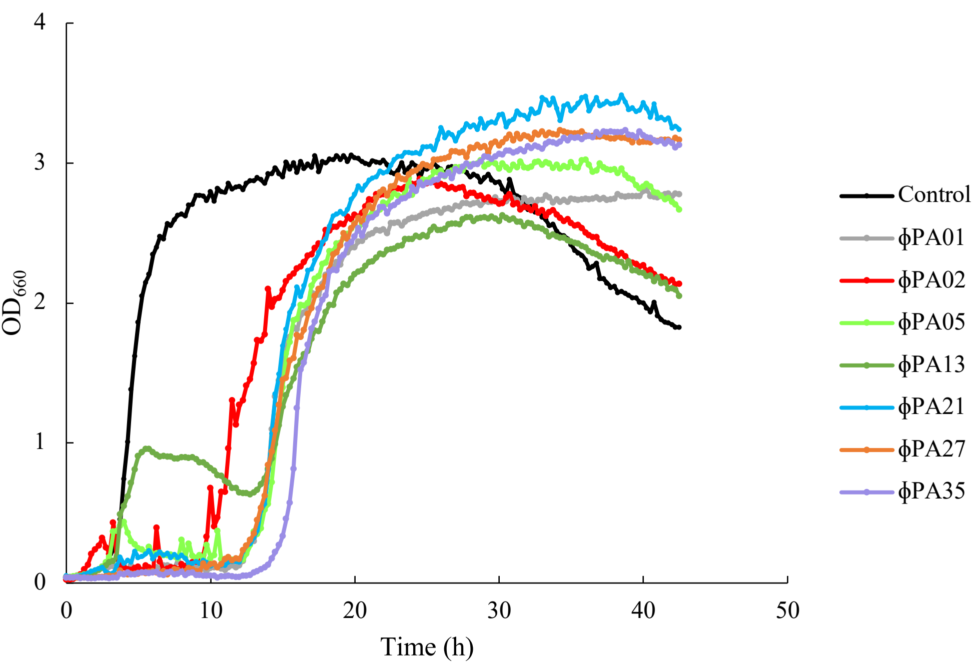


Figure 3-4. Growth curve of *P. aeruginosa* infected with different phages at MOI 0.1.

Besides, growth curve of *P. aeruginosa* PAO1 infected with isolated phages was examined (Fig.3-4). In Fig. 3-4, all phages, except ϕPA13, suppressed the growth of P. *aeruginosa* PAO1 up to 9-13 h. Among all, mutant resistant to ϕPA02 appeared earlier at approximately 9.5 h. When infected by ϕPA13, PAO1 regrow at 3 h before OD_{660} decreased again at around 6 h until 12.5 h. The OD_{660} increased again from 13 h.

Next, antibiotic resistant PAO1 described in Chapter 2 were also used to examine phage host range of each phage (Figure 3-5). All phage could not infect 128M (PAO1 strain isolated from 128 µg/mL) and ϕPA27 showed turbid plaque even though it could suppress the growth of *P. aeruginosa* PAO1. Antibiotic resistant strain 16C, 32M and 4C32 M is sensitive to all phages except ϕPA27. ϕPA27 formed turbid plaque on wildtype, 16C and 32M.

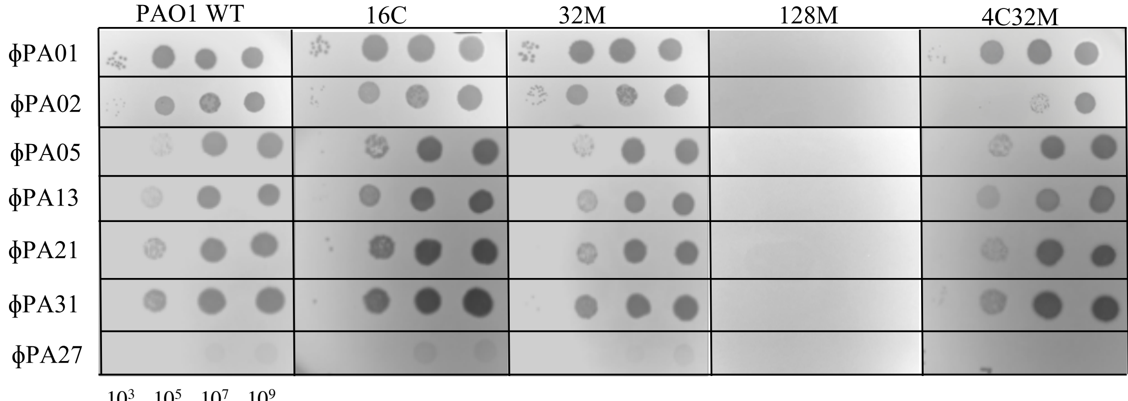


Figure 3-5. Result of spot test of isolated phages against *P. aeruginosa* PAO1 antibiotic resistant mutants described in chapter 2.

Table 3- 2. Genomic characteristics of isolated phages

Phage	Length (bp)	GC content (%)	Identity (%)	Similar phage species (genus)
ΦPA01	66220	55.4	95	vB_PaeM_LBL3 (*Pbunalikevirus*)
ΦPA35	66135	55.4	92	vB_PaeM_E217 (*Pbunalikevirus*)
ΦPA05	65477	55.5	99	Pseudomonas phage LMA2 (*Pbunalikevirus*)
ΦPA11	66260	55.7	98	Pseudomonas phage LMA2 (*Pbunalikevirus*)
ΦPA13	64518	55.0	98	Pseudomonas virus F8 (*Pbunalikevirus*)
ΦPA02	279267	36.8	97	Pseudomonas PhiKZ (*Phikzlikevirus*)
ΦPA27	279269	36.8	97	Pseudomonas PhiKZ (*Phikzlikevirus*)

Furthermore, based on whole genome sequencing result, these phages could be classified into two genera, *Pbunalikevirus* and *Phikzlikevirus* (table 3-2). For further study, one phage from each genus was chosen. Phage ΦPA01 and ΦPA02 were chosen based on their host range and the growth curve of PAO1 when infected with phages. Phage ΦPA27 was not chosen due to the possibility of being a lysogenic phage.

3.3.2 Phage morphology

Based on the morphology of ϕPA01 and ϕPA02 observed (Fig. 3-6A-D), they were classified to the *Myoviridae* family. Both phages displayed a capsid head connected to a long contractile tail (Fig. 3-6B and 3-6D, shown in black arrows). ϕPA02 showed a distinct morphology with a large capsid with the diameter and height of 121±3 nm and 134±4 nm, respectively. This feature is similar to reported giant phages. The relaxed tail fiber of ϕPA02 was 206±7 nm long and 25±1 nm wide. Meanwhile, the diameter and height of ϕPA01's capsid was 73±6 nm and 73±2 nm respectively, with a 148±3 nm long and 21±1 nm wide tail sheath, measured in extended state. Its features are similar to phages from *Pbunalikevirus.*

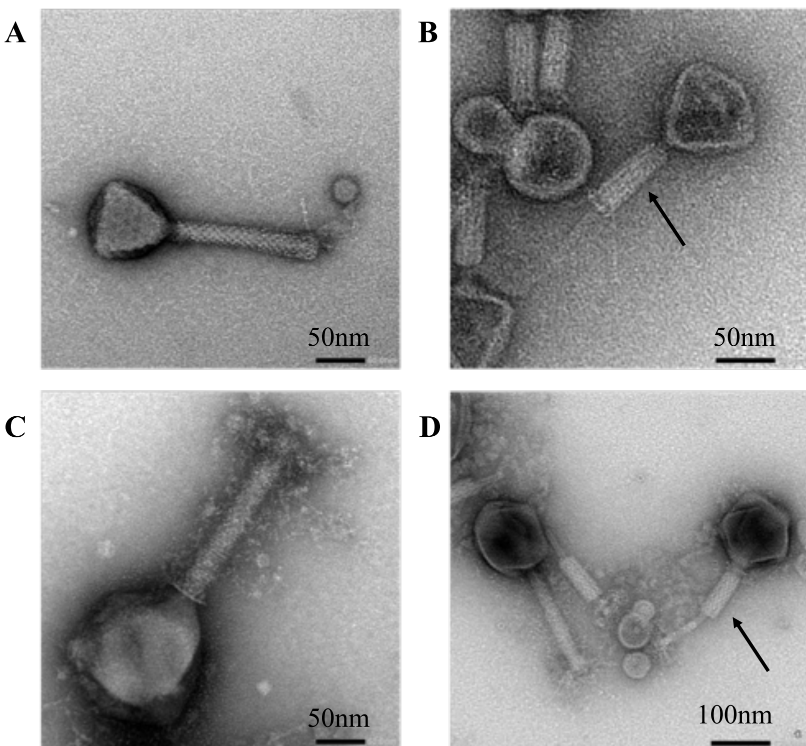


Figure 3-6. TEM image of (A,B) ϕPA01 and (C,D) ϕPA02. Arrows show the contracted tail. Sizes of scale bar are indicated. (Staining: A and C -uranyl acetate; C and D- EM Stainer) One-step growth curve of (E) ϕPA01 and (F) ϕPA02.

3.3.3 Phage growth and host range

The one-step growth curve (Fig. 3-7A, B) showed that latent periods of ϕPA01 and ϕPA02 were 30 min and 35 min (in addition to 10 min of adsorption time), each with a burst size of 32 and 49 phage particles per cell. Phage ϕPA01 and ϕPA02 were able to lyse 36% and 47% of clinical *P. aeruginosa*, respectively, and showed different host spectra to 58 clinical isolates (Table 3-3). In addition, all antibiotic resistant mutant, except 128M remained sensitive to both phages, as shown in Fig. 3-5.

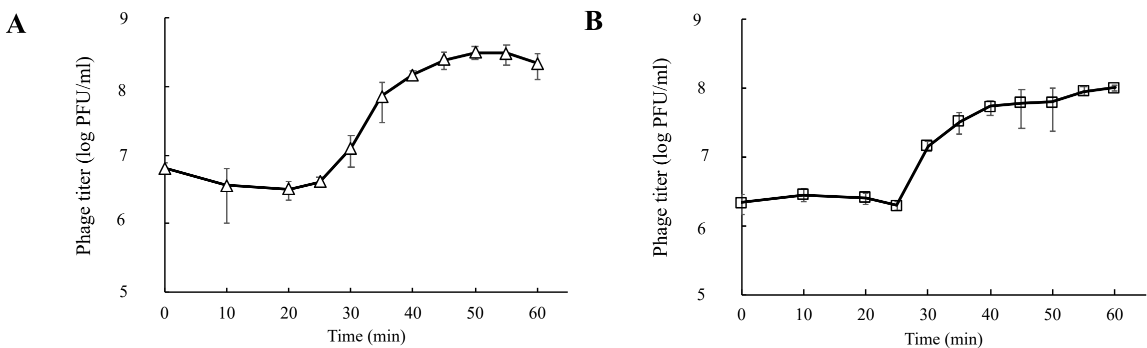
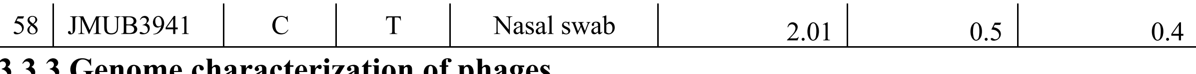


Figure 3-7. One step growth curve of (A) ϕPA01 and (B) ϕPA02.

Table 3-3. Results of host range on clinical *P. aeruginosa*'s collection (Jichi Medical University Hospital) isolated from different sporadic cases from patients admitted to different wards. (C-clear plaque; T: Turbid plaque; -:no plaque)

	Strain	ϕPA01	ϕPA02	source	Twitching area (cm^2)	Swarming diameter-longest (cm)	Swarming diameter-shortest (cm)
1	JMUB3508	-	C	Wound	0.33	0.7	0.6
2	JMUB3581	C	T		6.15	1.7	1
3	JMUB3585	-	-		3.30	1.2	1.2
4	JMUB3588	-	-		4.34	2.3	1.8
5	JMUB3594	C	C		1.02	0.8	0.8
6	JMUB3876	C	T		0.33	0.5	0.5
7	JMUB3944	-	C		6.83	6.5	5
8	JMUB4022	-	C		0.19	3.5	1.7
9	JMUB4024	C	T		0.16	1.1	1.1
10	JMUB3509	-	C	Urine	0.38	0.6	0.6
11	JMUB3511	-	-		4.12	0.7	0.7
12	JMUB3576	-	T		5.11	0.4	0.4
13	JMUB3579	T	-		5.73	0.6	0.6
14	JMUB3580	C	-		5.31	1.7	1.5
15	JMUB3582	C	-		5.31	1	0.9

16	JMUB3584	-	-		0.28	3.1	2
17	JMUB3586	-	-		5.72	1.2	1
18	JMUB3590	T	T		4.32	1.2	0.7
19	JMUB3592	C	-		5.11	0.7	0.6
20	JMUB3593	-	C		4.34	1	0.8
21	JMUB3600	C	-		5.89	0.5	0.5
22	JMUB3871	-	-		4.91	1.6	1.3
23	JMUB3872	-	-		4.15	1	0.9
24	JMUB3874	T	-		6.83	1	0.9
25	JMUB3878	-	T		0.19	1.6	0.9
26	JMUB3879	-	T		0.71	0.4	0.4
27	JMUB3885	-	C		4.70	6	5.5
28	JMUB3886	-	C		0.57	0.5	0.5
29	JMUB3940	-	-		0.07	0.5	0.5
30	JMUB3942	-	-		0.09	1.4	1.1
31	JMUB3943	-	T		0.86	0.6	0.5
32	JMUB4021	C	C		6.13	4	2.5
33	JMUB4023	C	C		6.38	1	0.9
34	JMUB3578	-	T	Tracheal secretion	6.15	0.9	0.9
35	JMUB3596	-	-		0.28	2	1.5
36	JMUB3598	-	T		2.40	1.5	0.8
37	JMUB3873	C	-		0.33	0.6	0.5
38	JMUB3881	C	-		0.33	1	1
39	JMUB3457	-	-	Sputum	0.33	3	2.4
40	JMUB3574	-	-		5.51	0.9	0.7
41	JMUB3577	C	T		5.11	0.6	0.6
42	JMUB3583	-	-		0.20	0.4	0.3
43	JMUB3589	-	-		0.28	0.5	0.5
44	JMUB3591	T	T		3.80	2	1.7
45	JMUB3595	-	-		4.52	0.9	0.9
46	JMUB3597	-	-		0.24	0.4	0.4
47	JMUB3877	-	C		0.33	1.9	1.1
48	JMUB3880	C	-		5.51	1	1
49	JMUB3882	-	-		0.63	0.4	0.4
50	JMUB3883	-	T		0.22	0.6	0.5
51	JMUB3938	T	-		4.91	1.5	1.3
52	JMUB3939	-	-		0.13	0.5	0.4
53	JMUB3458	-	-	Pus	0.50	1	1
54	JMUB3459	-	-		3.61	1.6	1.3
55	JMUB3460	-	C		4.90	0.9	0.9
56	JMUB3587	-	-		0.20	0.5	0.5
57	JMUB3887	C	C		0.49	0.6	0.6



58	JMUB3941	C	T	Nasal swab	2.01	0.5	0.4

3.3.3 Genome characterization of phages

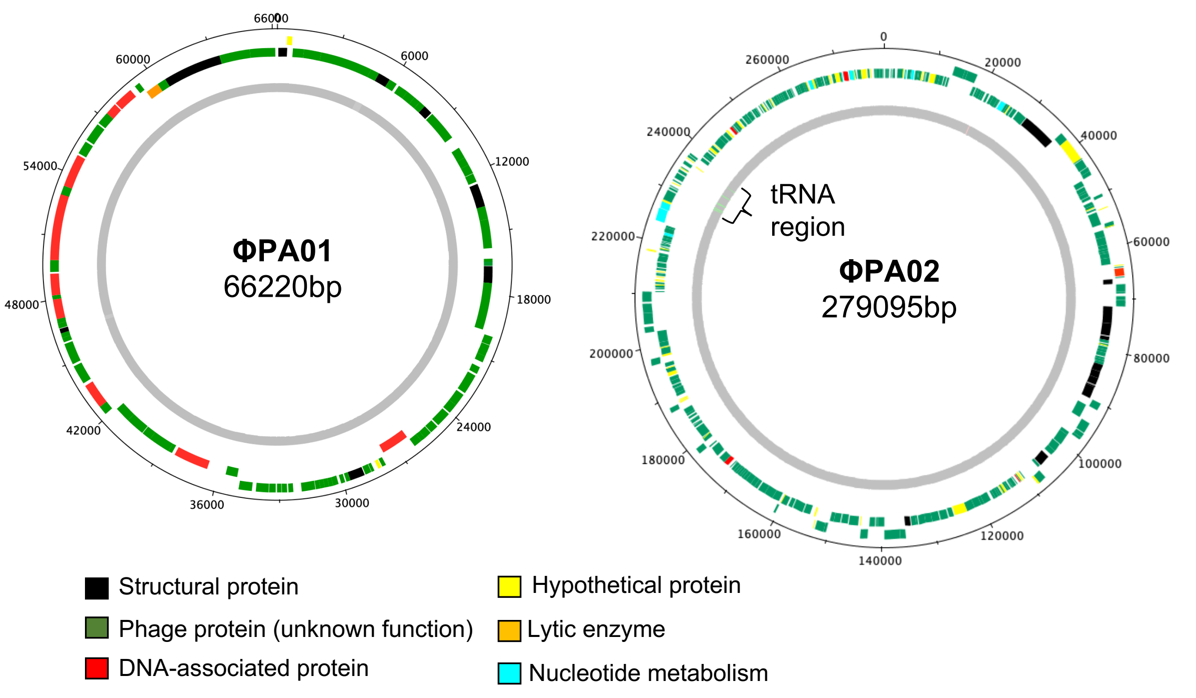


Figure 3-8 Genomic map of phage ϕPA01 and ϕPA02.

It was revealed that ϕPA01 (66,220 bp) and ϕPA02 (279, 095 bp) belonged to the genus *Pbunalikevirus* and *Phikzlikevirus*, respectively. Function of more than half of the genes in ϕPA02 were unknown (Fig. 3-8). Analysis via BLASTn showed that the genome sequence of ϕPA01 had highest similarities to phage LBL3 and PB1 (95%). Meanwhile, ϕPA02 had highest similarities to PhiKZ and KTN4 (99%). Phylogenetic tree showed that these two phages have far evolutionary relationship to each other (Fig. 3-9).

The genome of ϕPA02 encodes 6 tRNAs specific for Leucine (TAA), Proline (TGG), Isoleucine (CAT), Aspartic acid (GTC), Asparagine (GTT) and Threonine (TGT). All the tRNA encoding gene were found to concentrate in the same region. However, no tRNA encoding gene was found in ϕPA01. Table 3-4 summarized the genomic characteristics of 4 similar phages to each phage. Phage KZ, KTN4, PA7 and SLS carry genes encoding for tRNA. Meanwhile similar phages to ϕPA01 does not carry gene encoding for tRNA.

Virion associated RNA polymerase (RNAP) (gp48, gp48, gp82 and gp156) and non-virion associated RNAP (gp109, gp164, gp165, gp166 and gp185) were encoded in ϕPA02's genome. However, such kind of transcriptional machinery was not found in ϕPA01. Besides, no integrase and virulence genes were found in the genome of either phage. GC contents of

ϕPA01 and ϕPA02 were 55.4% and 36.8% respectively. ϕPA02's genome encodes putative 343 ORFs, which is about four times larger than ϕPA01 (92 ORFs).

Table 3- 4. Genomic characterization of ΦPA01 and ΦPA02 and comparison with similar phages.

Phage (Accession number)	Genome size (bp)	Identity (%)	Query coverage (%)	tRNA no.	ORFs no.	Reference
ϕPA01 (AP19535)	66220	-	-	0	92	This study
LBL3 (NC_011165.1)	64427	95	96	0	92	(69)
PB1 (NC_011810.1)	65764	95	96	0	93	(69)
KTN6 (NC_041865.1)	65994	95	97	0	91	(70)
KPP12 (NC_019935.1)	64144	94	94	0	88	(71)
ϕPA02 (AP019418)	279095	-	-	6	343	This study
KZ (NC_004629.1)	280334	99	97	6	343	(72)
KTN4 (KU521356.1)	279593	99	97	6	368	(73)
PA7 (NC_042060.1)	266743	99	93	6	337	unpublished
SL2 (NC_042081.1)	279696	98	95	4	355	(74)

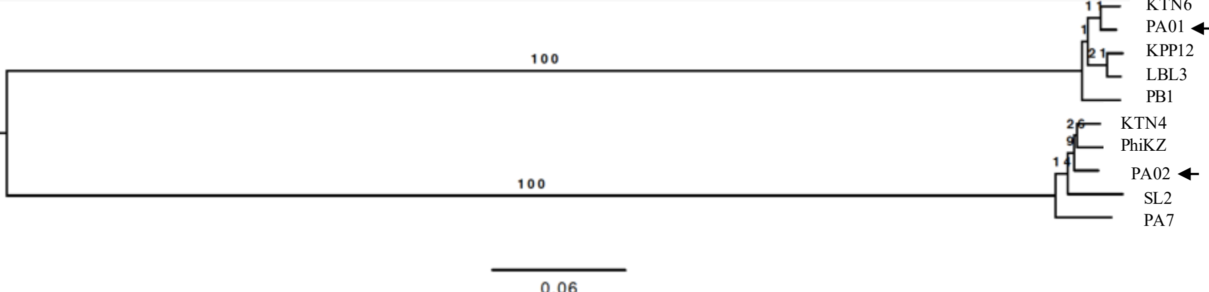


Figure 3-9. Phylogenetic relationship of ϕPA01 and ϕPA02 generated by VICTOR (Virus Classification and Tree Building Online Resource) with settings recommended for prokaryotic viruses. The tree was visualized with FigTree. The numbers above the branches are Genome-BLAST Distance Phylogeny pseudo-bootstrap support values from 100 replications. The analysis yielded 8 clusters at species and two at genus and family level. Phage ϕPA01 and ϕPA02 are shown with arrows.

3.4 Discussion

3.4.1 Infectivity of phage to clinical strains

Clinical strains were isolated from wound, urine, tracheal secretion, sputum, pus and nasal swab from different patients admitted in different wards in 2017, further showing the potential to apply ϕPA01 and ϕPA02 across different patients with different local infection.

Previous study showed that phages belonged to *Pbunalikevirus* and *Phikzlikevirus* have board host range (70,73).

Not only that, antibiotic resistant mutant strains (32M, 16C, 4C32M) generated from chapter 2 were sensitive to ϕPA01 and ϕPA02. Phage-antibiotic synergism were demonstrated in other studies (68,75,76) and this indicated the potential of combining ϕPA01 and ϕPA02 to be applied to antibiotic resistant bacteria. A diabetic patient infected with multidrug resistant *A. baunmannii* were successfully treated with cocktail phages (77). On the other hand, application of phage OMKO1 to *P. aeruginosa* multidrug resistant strain had restored their sensitivity to antibiotics (78).

3.4.2 Genomic characterization of phage

A well characterized phage is one of the key factors determining the success of a phage therapy. Phages from the genera *Pbunalikevirus* and *Phikzlikevirus* were able to control the growth and biofilm formation of *P. aeruginosa* in *vitro* (70,73) and in *vivo* (71,79) in previous studies. Genomic analysis has revealed the absence of integrase, genes associated with toxin and virulence in ϕPA01 and ϕPA02, suggesting that they are potentially safe candidates for phage therapy.

Previous study showed that ϕKZ protein transcription was independent on its host. Five predicted ORFs which encode for virion associated RNAP and non-virion associated RNAP were found in ϕPA02 but not in ϕPA01 (80). This showed that ϕPA02 is independent to host transcription mechanism compared to ϕPA01. Meanwhile, gene encoding for tRNA were commonly found in giant phages infecting *P. aeruginosa* (81,82) and other bacteria (83). Due to difference in GC content of ϕPA02 (36.8%) with its host (66.6%), the presence of tRNA encoding genes could aid this phage in optimizing codons usage that were rare in the host cell (84). Phage T4 without tRNA showed lower burst size and slower rate of protein synthesis compared with phage T4 with tRNA (85). Presence of tRNA encoding gene in ϕPA02 could contribute in its fitness. The absent of tRNA encoding gene in ϕPA01 showed that its transcription mechanism is different from ϕPA01.

Chapter 4 Host receptor identification of phage

4.1 Introduction

4.1.1 Host receptor of phage

Life cycle of phage begins with contacting with host bacteria by recognizing host receptor present on bacterial cell surface. Various kinds of host receptors were reported including outer membrane proteins, lipopolysaccharide (LPS), pili or flagella (38).

The LPS of *P. aeruginosa* is made up of three domains. They are lipid A, core oligosaccharide and O polysaccharides which can be further divided into two: common polysaccharide antigen (A-band) and O-specific antigen (B-band) as shown in Fig. 4-1A and B. The A-band consists of repeated unit of D-rhamnose (average ~70 monosaccharide), while B-band consists of different sugar in one repeating unit (e.g: MAN-MAN-FUC). Meanwhile, some mutant appeared as rough colony that is devoid of A-band, B-band or has incomplete core LPS (Fig. 4-1C).

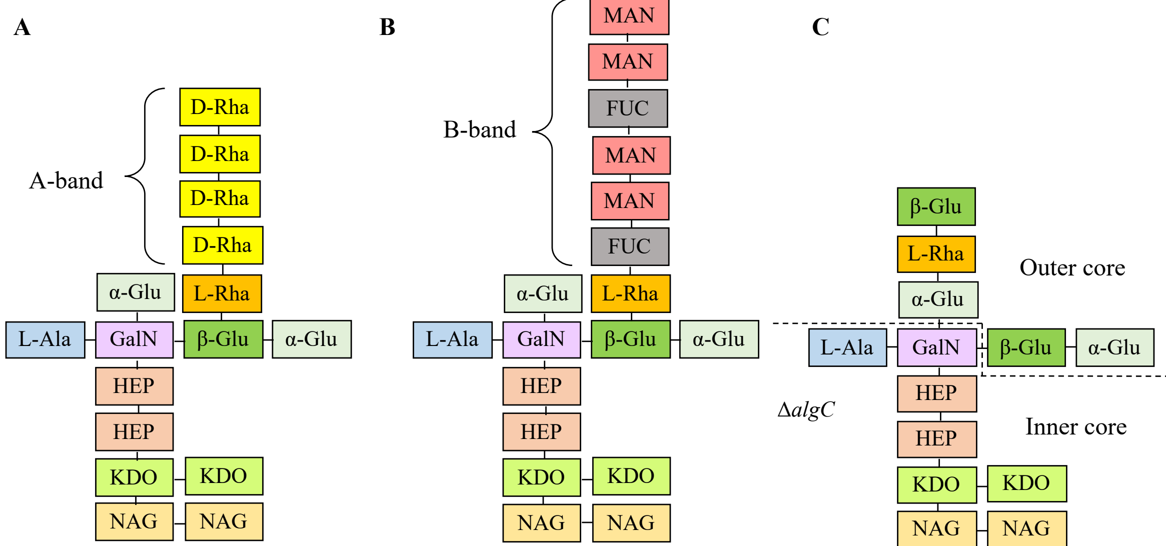


Figure 4-1. Schematic diagram of lipopolysaccharide (LPS) of *P. aeruginosa*, lipid A not shown. MAN, mannose; FUC, fucose; GalN, N-galactosamine; HEP, heptose; NAG, N-acetylglucosamine; KDO, 2-keto-3-deoxyoctulosonic acid. (Derived from Kohler et al, 2010).

Treatment with phage cocktail were proven to be more effective compared to treatment with single phage (86). A potential phage cocktail is made up with phages that

target different host receptor (87) or from distinct linage (74) as phages from the same lineage tend to adapt cross resistance (70).

In this chapter, the cross resistance of phages described in Chapter 3 was examined by generating phage resistant strain of respective phage in order to confirm their potential to be used as cocktail.

4.1.2 Bacterial immunity: CRISPR/Cas9 system as useful molecular cloning tool

A bacterial adaptive immune systems, regularly interspaced short palindromic repeats (CRISPR)/Cas9 (88) has been developed into genome editing tool widely used (89). In this system, the Cas9 DNA nuclease forms a complex with a single guide RNA (sgRNA). By reprogramming 20-nucleotide (nt) sequence of sgRNA, this complex can be precisely guided to genomic locus via base pairing of designed sgRNA with the genomic DNA adjacent to protospacer-adjacent motif (PAM). The Cas9 DNA nuclease generates a double-stranded DNA break in the genome by cleaving the target locus. Bacteria do not possess the non-homologous end-joining repair pathway, and thus the double stranded break will lead to cell death. Therefore, cells that have undergone homologous recombination could be selected. A genetic editing system based on this system had been developed by Chen and colleague, with the incorporation of λ-Red system to enhance homologous recombination in *P. aeruginosa* (90). A two-plasmid system was utilized in this method. Plasmid pCasPA carries locus that express Cas9 and λ-Red proteins. Meanwhile, pACRISPR carries sgRNA expression cassettes and repair template can be inserted in this plasmid.

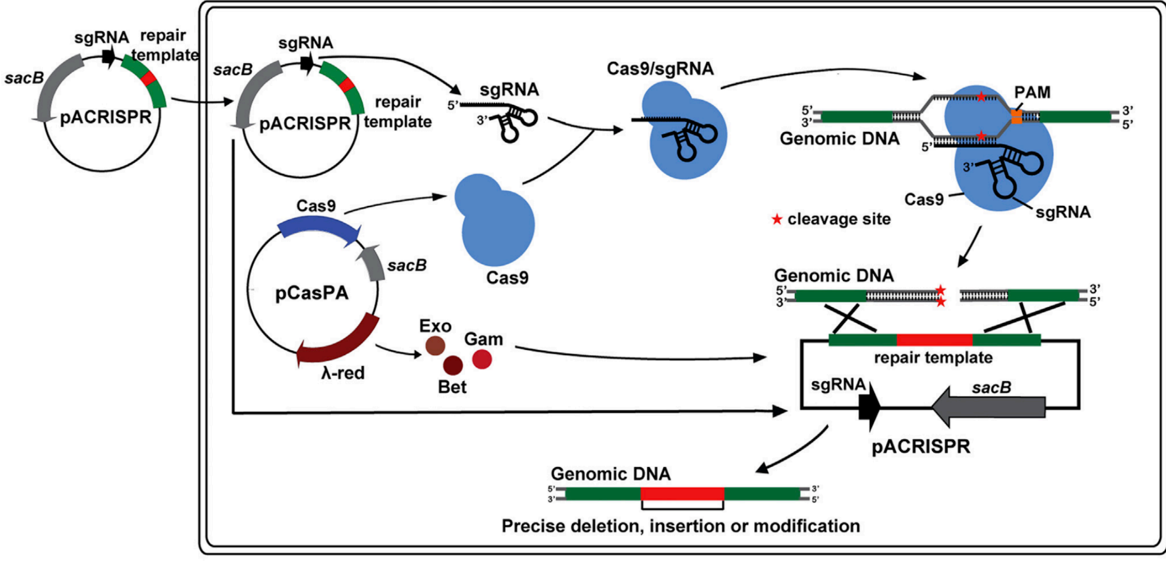


Figure 4- 2 Genetic editing of *P. aeruginosa* based on CRISPR/Cas9, combining λ-Red system. This system contains two plasmids, whereby λ-Red system was supplied by one of the plasmids. Reference: (90).

4.2 Methods and materials

4.2.1 Generation and isolation of phage resistant strains

Phage resistant strains were generated by repeated co-culturing of *P. aeruginosa* PAO1 and phage based on a previous study (66). The co-culture was started by infecting PAO1 with phage at MOI 0.1 and cultured for 48 h before transferring 1% (v/v) cells at stationary phase to the new medium. Serial transfer was continued 4-5 rounds and phage-resistant colonies were isolated from each round by plating the stationary bacterial cell culture on LB plates. Each isolated resistant strain was assigned as PA0X-RY, where (0X) indicates the type of phage to which the bacteria is resistant (01- ϕPA01 and 02- ϕPA02) while (Y) indicates the batch number (R1-R5).

4.2.2 Spot test and determination of phage adsorption rate

Spot test was carried out by dropping 1.5 μL of phage lysate of 10^7 PFU/ml on *P. aeruginosa* mixed with 0.5% top agar and incubated overnight, supplemented with 150 μg/mL of Kanamycin (KAN) when it is necessary. Adsorption efficiency of phages on *P. aeruginosa* phage resistant strain was measured by titrating free phages present in the supernatant after 20 minutes of cell-phage contact at MOI of 0.01. One hundred microliters of cell-phage solution was sampled and immediately added to 9.9 ml of chilled SM buffer. The solution was gently vortexed before taking 1 ml for centrifugation (10,000 g, 5 minutes, 4°C) in order to remove the bacterial cells before titration of phage concentration. Adsorption efficiency was calculated by dividing the number of adsorbed phages by the initial number of phages. Statistical analysis was carried out using two-tailed student's t-test.

4.2.3 Whole genome sequencing of phage resistant strain

Overnight culture of phage resistant strain was prepared by inoculating single colony into 2 mL LB broth. Culture was incubated for 16 h, 37°C, 120 rpm. Five hundred microliter of overnight culture was palleted at 5,000 g for 1 min. Next, palleted bacteria was washed with distilled water for 2 times. Washed cell was used for DNA extraction using GenElute Bacterial Genomic DNA kits (Sigma Aldrich), following instruction from manufacturer. Extracted

genomes were submitted to BGI (Hongkong) for whole genome sequencing by Illumina HiSeq platform with genome coverage (sequencing depth) = 100-fold with 100-bp paired end. The sequence was mapped against genome sequence of *P. aeruginosa* wildtype PAO1 (Accession number: NC_002516) and Single Nucleotide Polymorphisms (SNPs) were identified by SAMtools, Pilon or manually checked by visualizing mapped file on Tablet.

4.2.4 Molecular cloning, plasmids constructions and genome editing

Knockout of *algC* (Δ*algC*) was generated by the method reported (90) based on CRIPSR/Cas9 system with modification. Primers and plasmids used in this study are listed in table 4-1. Plasmid pCasPA and pACRISPR were provided by Quanjiang Ji (ShanghaiTech University).

First, spacers for target gene were searched by online software CHOPCHOP (91). The double stranded spacer was generated by phosphorylation using T4 polynucleotide kinase and was inserted to plasmid pACRISPR by Golden Gate assembly. Homologous arms for gene deletion with flanking region around 300 base pair (bp) was generated from overlap PCR. The purified DNA product was digested by restriction enzyme and inserted to pACRISPR-spacer by ligation with T4 ligase. All enzymes were purchased from New England Biolabs. Propagation of plasmids were done using *E. coli* JM109. Plasmid was extracted using NucleoSpin® Plasmid (MACHEREY-NAGEL GmbH), following the instructions from manufacturer.

P. aeruginosa PAO1 was transformed with pCasPA (PAO1-pCasPA) and the transformant was selected by 100 µg/mL of tetracycline (TET). Single colony of PAO1-pCasPA was inoculate into 100 mL of LB medium added with 100 µg/mL TET and cultured for approximately 5 hours to reach OD_{600} 1. After that, arabinose with 2 µg/mL final concentration was added into the culture and further cultured for 2 hours for the induction of λ-Red proteins. Induced cells were collected by centrifugation at 3,500 g, 5 min, 4°C. Cells were washed 2 times with chilled 10% glycerol. Finally, cells were dissolved in 1 mL of 10% glycerol. Plasmid pCRISPR carrying designed gRNA and homologous arms for recombination was electroporated into PAO1-pCasPA by Gene Pulser® (Bio-Rad) with the setting of 2,100 V, 100 Ω, 25 µF, using cuvette with 1 mm gap (Bio-Rad). After 1-2 h of recovery in SOC (Tryptone 20 g, yeast extract 5 g, NaCl 0.5 g and 20 mM glucose per liter) medium, recombinant strain was selected on LB plate supplemented with 100 µg/mL TET and 150 µg/mL carbenicillin (CAR). Knockout mutant was screened by PCR and plasmids were cured by plating transformant on LB plate supplemented with 5% sucrose.

All the primers and plasmids used in this study were summarized in table 4-1 and table 4-2.

Table 4-1 Primers used in this study.

Primer	Description	Sequence 5’→3’	Restriction site
AlgC_spacer oligo1	Oligo for making spacer (forward)	GTGGGGCCGCGTAGTACAGCACCG	
AlgC_spacer oligo2	Oligo for making spacer (reverse)	AAACCGGTGCTGTACTACGCGGCC	
AlgC_arm1_Fw	Homologous arm at upstream	ATACTCGAGCCGGCCTGTCCATCTACATCG	XhoI
AlgC_arm1_Rv	of *algC*	AGCTCCGACATGTCAGTGGCTGCCGGAATG	
AlgC_arm2_Fw	Homologous arm at	GGCAGCCACTGACATGTCGGAGCTCCCCATGAC	
AlgC_arm2_Rv	downstream of *algC*	ATAAAGCTTCGTCGATGAAGTGGCTCTCGA	HindIII
AlgC_com_Fw	Cloning for *algC* from wild	ATATCTAGACCCCGAACACAGGACGAGACGC	XbaI
AlgC_com_Rv	type PAO1	ATAGGATCCTCAGAAGGGCACGGGCAGCG	BamHI

*The restriction site is underlined.

Table 4- 2 Plasmids used in this study.

Plasmid	Description	reference
pCasPA	Plasmid for genome editing based on CRISPR/Cas9 system, harboring Cas9 protein and the λ-Red system, tetracycline resistant marker, *tetA* and counter selectable marker, *sacB*	69
pACRISPR	Plasmid for genome editing based on CRISPR/Cas9 system, gRNA expression, carbenicillin resistant marker, *bla* and counter selectable marker, *sacB*	69
pACRISPR_AlgC	pACRISPR plasmid containing spacer and editing template for *algC*	This study
pBBR1-MCS2	Plasmid with kanamycin selection marker, *kanR* and multiple cloning site for blue white selection~	71
pBBR1_AlgC	Plasmid with inserted *algC* from *P. aeruginosa* PAO1 wildtype	This study

Complementation of *algC* was done by cloning *algC* gene from wildtype PAO1 using Ex *Taq* polymerase (TAKARA, Japan) and ligated into plasmid pBBR1-MCS2 (92), which was kindly provided by Kenneth Peterson (Louisiana State University Medical Center,

Shreveport, LA, USA). Competent cell was prepared base on the method developed by Choi et al. (93). Briefly, 6 mL of overnight culture of *P. aeruginosa* was pelleted at 16,000 g, 2 min at room temperature (25°C) and washed twice with 300 mM of sucrose solution. Plasmid was electroporated into the cell by same method above, by using 2 mm gap cuvette and with the adjusted setting of 2,500 V, 200 Ω, 25 μF. Transformant was screened on LB plate supplemented with 150 μg/mL of KAN.

4.3 Results

4.3.1 Generation of phage resistant strains

In the co-culture of *P. aeruginosa* with Phage-resistant strains were isolated from each batch of co-culture conducted up to four or five rounds. In Fig. 4-3, lysis of bacterial cell was observed at the beginning of round 1 co-culture. Bacteria regrow again around 10 h.

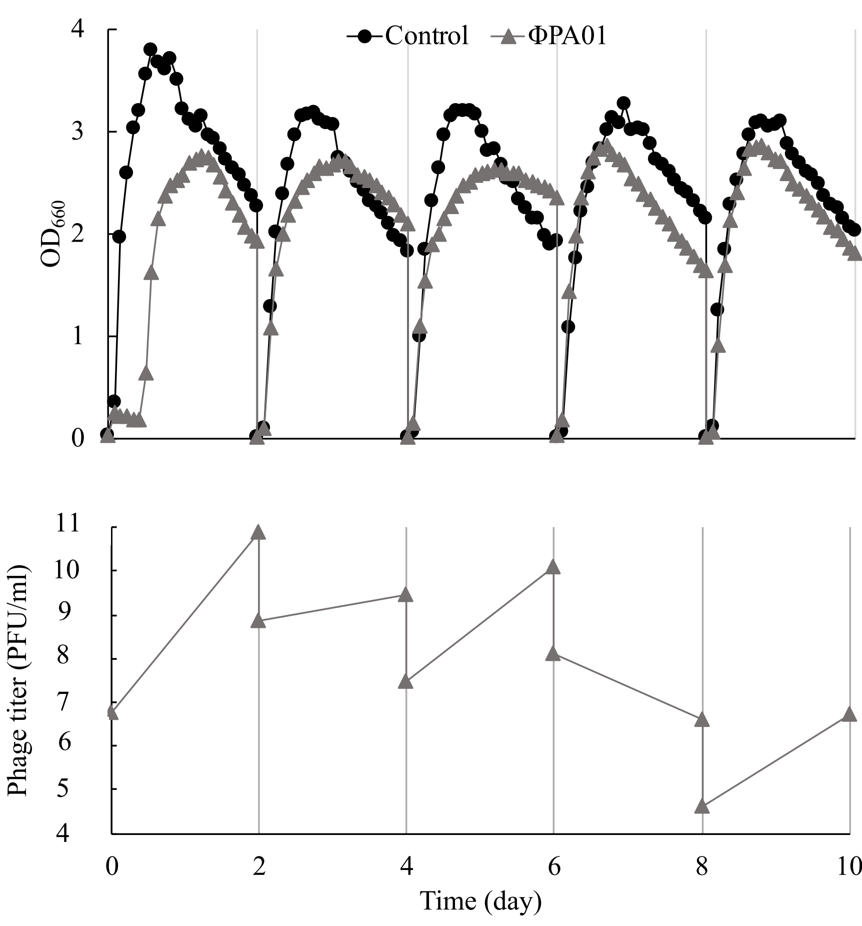


Figure 4-3. Co-culture of *P.aeruginosa* PAO1 with ϕPA01. Bacteria growth (top) and phage titer in the co-culture (bottom).

Concentration of phage increased from 10^6 to 10^{11} PFU/mL. From second round onward, no decrease of OD_{660} was observed. However, growth of phage was observed in round 2 and round 3. Phage was not propagated in round 4. However, propagation of phage was observed again in round 5.

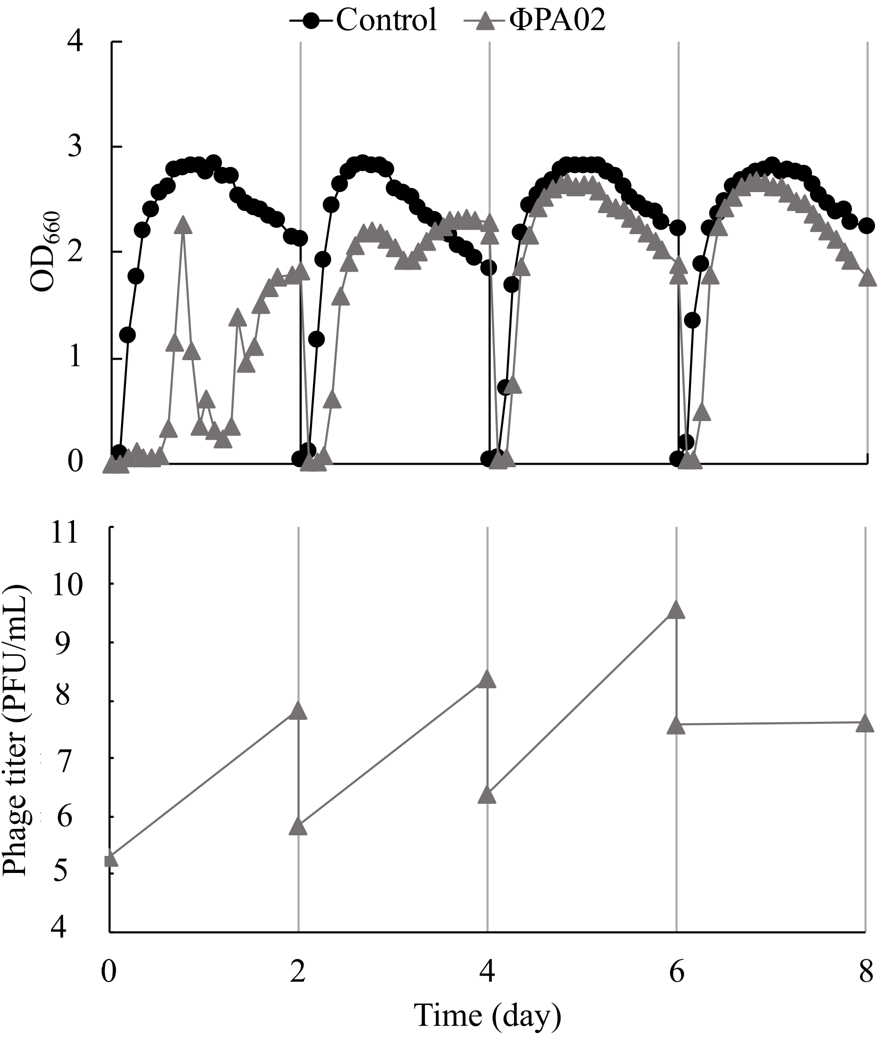


Figure 4-4. Co-culture of *P. aeruginosa* PAO1 with ϕPA02. Bacteria growth (top) and phage titer in the co-culture (bottom).

In the first round of co-culture with ϕPA02, slight decrease in OD_{660} was observed between 6-8h. Second lysis curve 19-22.5 h, whereby OD_{660} plummeted from 2.3 to 0.3 and increased again. In between 33-35 h, lysis occurred with a small decreased in OD_{660}. In round 2, lysis curve was observed in between18-26 h, with a decrease of OD_{660} from 2.2 to 1.9. No lysis curve occurs in round 3 and 4. Phage concentration increased for 10^5 to 10^7 (PFU/mL) in

round 1 and 2. In round 3, phage increased from 10^6 to 10^9 PFU/mL. Number of phages maintained at the same level in round 4.

According to Fig. 4-5, phage-resistant mutants isolated from co-culture were resistant to their respective phages used for infection. Besides, adsorption efficiency of ϕPA01 to its resistant strains decreased drastically compared to adsorption to wildtype PAO1. Respective phage resistant strain occurred in the first round of co-culture. ϕPA01-resistant mutants remained slightly sensitive to ϕPA02, except R5-PA01R, which became resistant to both phages.

Meanwhile, ϕPA02-resistant mutants remained sensitive to ϕPA01. Adsorption efficiency of ϕPA01 toward ϕPA02-resistant mutants reduced significantly, except R1-PA02, compared with wildtype. Whereas, ϕPA02's adsorption efficiency remained at around 73-98% towards ϕPA01-resistant mutants (R1-R4), with decreased infectivity. Adsorption of ϕPA02 to R5-PA01R dropped to 39%.

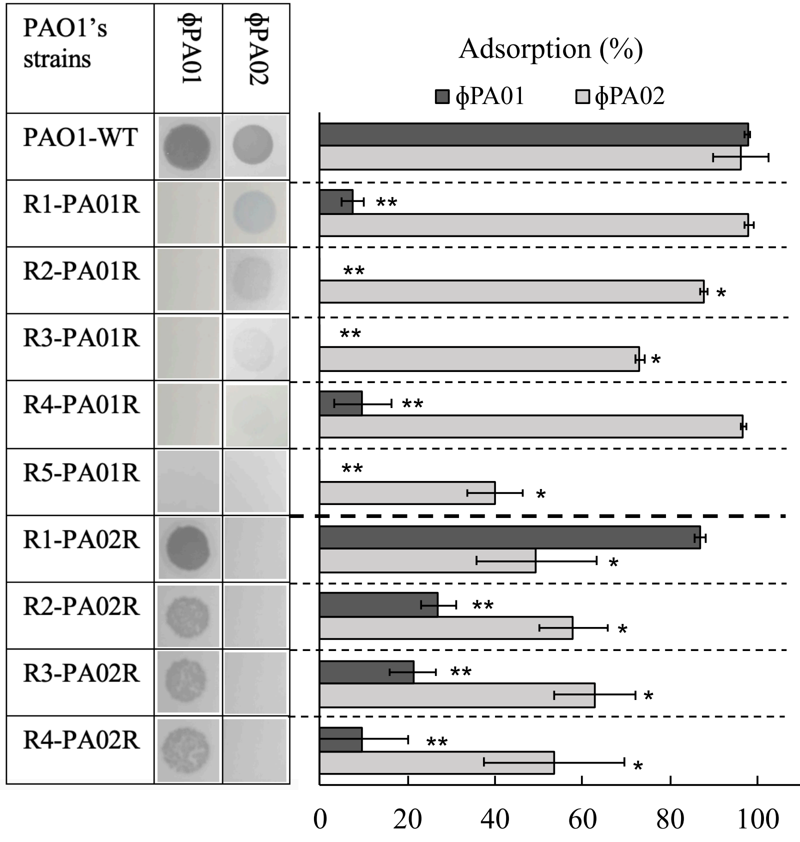


Figure 4-5. Cross-resistant analysis of ϕPA01 and ϕPA02 by spot test (left) and adsorption efficiencies (right) to phage resistant strains. Single asterisk and double asterisks indicate statistical difference ($P<0.01$) of ϕPA01 and ϕPA02, respectively. All adsorption test was carried out in triplicates.

4.3.2 Mutation in phage resistant strains

Strain R5-PA01R was sequenced to reveal spontaneous mutation that confer resistance to both phages. Adsorption of both phages to R5-PA01R was significantly decreased (Fig. 4-5). Nucleotide deletion of base pair A at position 1284 was found in *algC* (PA5322) as shown in Fig. 4-6A. Deletion of one base pair caused frameshift mutation and resulted in premature stop codon at position of 473 amino acid (AA). Truncated protein with 472 amino acids (aa) was produced compared to wildtype, 868 aa (Fig 4-6).

Phage resistant strain of R4-PA01R and R4-PA02R were sequenced to elucidate mutations that confer resistance to respective phage. Partial deletion spanning across two genes: a putative glycosyltransferase, *ssg* (PA5001) and hypothetical protein (PA5002) were found in in R4-PA01R (Fig. 4-7A).

Meanwhile, strain R4-PA02R showed one-point mutations in the following genes: *fliF* (PA1101), *opmB* (PA2525), *rocS1* (PA3946) and a hypothetical protein (PA5148). Detailed mutations are summarized in Fig. 4-7B. These mutations found were not reflected in R5-PA01R strain.

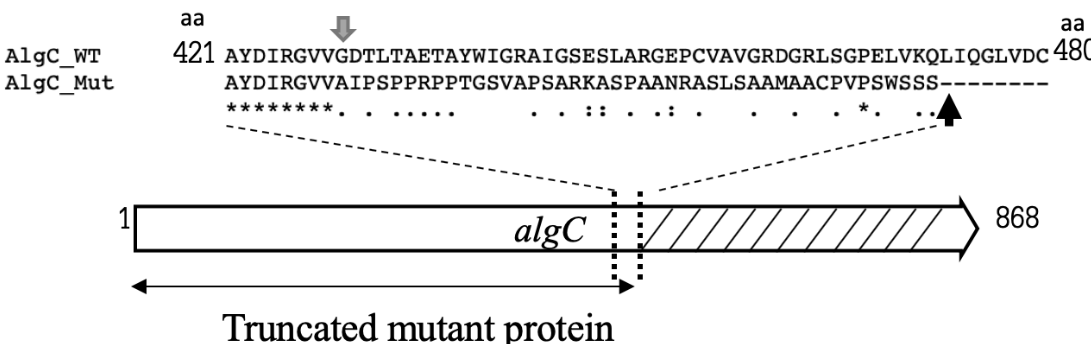
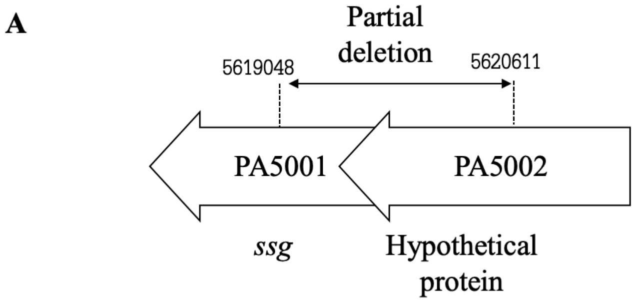


Figure 4-6. Mutation of *algC* found in R5-PA01. Frameshift mutation occurred from 429 aa (shown in grey arrow) and stop codon was introduced at 473 aa (black arrow).



B

Gene	Nucleotide alternation	Amino acid alternation (position)
fliF (PA1101)	CGG→<u>G</u>GG	Arg→Gly (318)
opmB (PA2525)	TAC→<u>C</u>AC	Tyr→His (251)
rocS1 (PA3946)	CCG→ C<u>T</u>G	Pro→Leu (724)
Hypothetical protein (PA5148)	ACC→<u>T</u>CC	Thr→Ser (47)

Figure 4-7. (A) Partial deletion found in R4-PA01R. (B) One-point mutation found in R4-PA02R.

4.3.3 Mutation in *algC* blocked adsorption of of ϕPA01 and ϕPA02 to host

Deletion mutant of *algC* (Δ*algC*) became resistant to phage ϕPA01 and ϕPA02. Adsorption of phage ϕPA01 was completely blocked while adsorption of ϕPA02 was drastically decreased to 39 % compared to wildtype. This indicated that adsorption of phage to host cell was hindered. Complementation of *algC* restored the sensitivity of Δ*algC* to ϕPA01 and ϕPA02. Besides, adsorptions of both phages to Δ*algC* were restored back to the level comparable to wildtype.

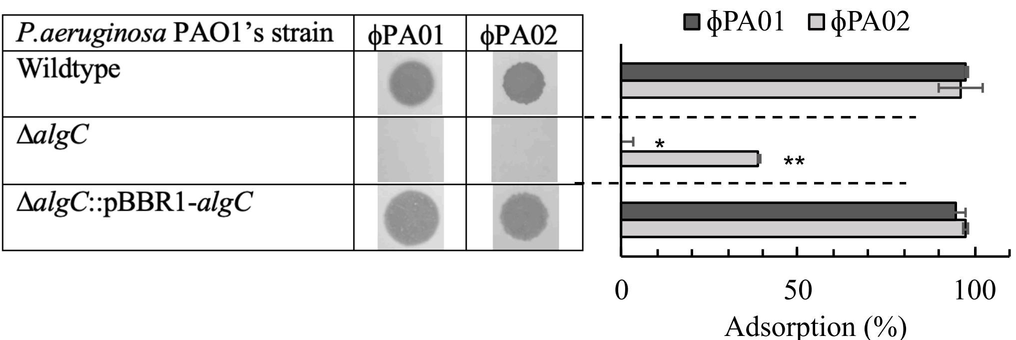


Figure 4-8. Spot test and adsorption of phage to PAO1 wildtype, *algC* knockout and *algC* knockout complemented with wildtype *algC*.

4.4 Discussion

4.4.1 Generation of phage resistant strains

The low adsorption of ϕPA01 and ϕPA02 to its resistant mutants might be due to the masking or loss of host receptor. Infection of ϕPA02 might be blocked in the post adsorption process since it remained highly adsorbed to all ϕPA01 and ϕPA02-resistant mutants (73-98% and 49-63%, respectively), except R5-PA01R. In previous study, resistant strains of closely related PB1-phages that utilized the same host receptor for infection showed cross-resistance (70). Thus, our results suggested that phages from distance lineage are potential phage cocktail candidates, whereby host resistant to both phages only appeared after 5th batch of continuous co-culture.

4.4.2 Mutations found in phage resistant strain

Truncated AlgC was produced in R5-PA01R due to the introduction of premature stop codon. Gene *algC* encodes enzyme with dual functions: phosphoglucomutase and phosphomannomutase (94).

Gene *algC* acts as phosphoglucomutase in the production of precursors for LPS core precursors and rhamnolipids such as UDP-D-Glucose, UDP-D-Mannose and dTP-L-Ramnose residues (62,94). Mutant of *ΔalgC* was devoid of complete core oligosaccharide, A-band and B-band (O-specific antigen) in outer membrane (Fig. 4-9).

Meanwhile, AlgC also act as phosphomannomutase in converting mannose-6-phosphate into mannose-1-phosphate in the initial step of alginate synthesis (61).

Function of gene *ssg* is not well studied in *P. aeruginosa.* However, transposon mutant of *P. alkylphenolia* (95) in gene homologous to *ssg* had incomplete LPS without B-band, further supporting that host receptor of ϕPA01 was LPS, similar to its nearest phages KTN6 and KPP22 (70,76).

Point mutations found in R4-PA02 mutant were probably related to the latter steps in life cycle of phage after phage adsorption, since adsorption of ϕPA02 to this strain remained relatively high (Fig.4-5). Bacteria can employ different strategy to block phage infection at different step of phage cycle: adsorption, phage DNA injection and protein translation.

Gene *fliF* encodes the precursor forming M-ring in the base of flagella (96). Product of gene *opmB* was found to be part of component in efflux pump (97) while *rocS1* regulates the expression of adhesins important for biofilm formation (98).

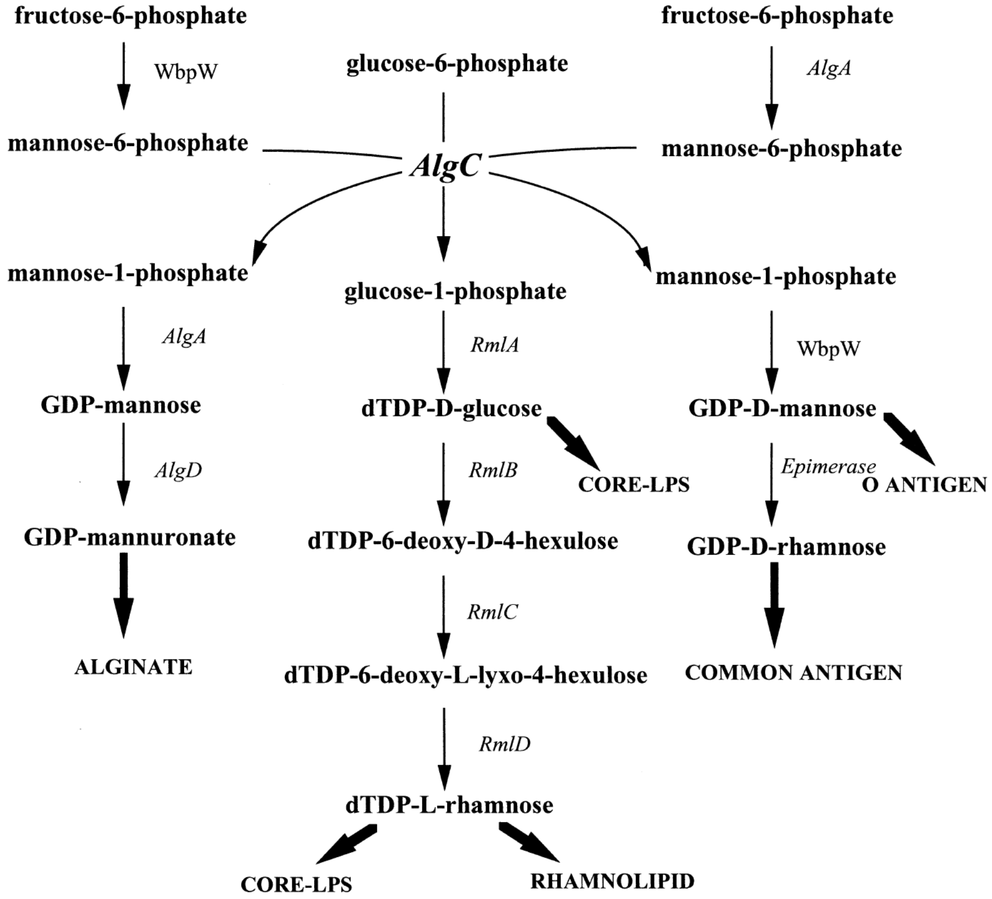


Figure 4-9. Gene *algC* involved in different biosynthesis pathway. Reference: (94).

Distinct mutations found in each phage resistant mutant suggested that ϕPA01 and ϕPA02 have different phage infection mechanism. Closely related phages of *S. aureus* were found to specifically recognize different components in wall teichoic acid (99). Even though both phages used LPS for host recognition, phage specifically recognized certain component in LPS as host receptor since LPS is a complex structure made up of different components (100).

4.4.3 Mutation of *algC* blocked adsorption and infection of ϕPA01 and ϕPA02 to host

Noteworthy, sequencing of R5-PA01R, which blocked adsorption of ϕPA01 and ϕPA02 showed frameshift mutation that resulted in premature stop codon in *algC* gene. By completely deleting *algC*, it was confirmed that both phages required LPS as their receptor. Previous study showed that mutant of *algC* could not produce rhamnolipids and alginates which are important in biofilm formation (101). This mutant was less virulent compared to

wildtype in burned mouse modal (102), showing that bacteria that become phage resistant would have trade off in its virulence.

Chapter 5 Synergistic effect of phage cocktail and antibiotic

5.1 Introduction

Resistant bacteria can emerge in the treatment via antibiotic or phage (45,66). Bacteria can become resistant to phage by blocking phage at different stage of life cycle (103). Phage cocktail was more effective in controlling the growth of *E.coli* compared to single phage (86). Combination of phage and antibiotic showed synergism effect in killing the host (75,104). This is due to the different type of selection pressures asserted onto the host (78).

Study about combination of phage cocktail and antibiotic is scarce and thus it is important to conduct study using phage cocktail and antibiotic. Since not all antibiotic will produce synergy effect with phage (76).

5.2 Methods and materials

Treatment effect of phage cocktail and antibiotics combination

In order to examine the treatment effects of ϕPA01, ϕPA02, the phage cocktail and antibiotics combination, two sets of experiments were set up. In the first experiment, ϕPA01, ϕPA02 or phage cocktail were added at 1 h (early logarithmic growth phase) after 1% (v/v) of stationary overnight culture was inoculated in 4 mL fresh LB broth and incubated at 37°C, with shaking at 40 rpm. Phages were added at MOI of 1.

In the second experiment, ciprofloxacin (CIP, 0.25 μg/ml), meropenem (MEM, 2 μg/ml), or combination of CIP (0.25 μg/ml) and MEM (2 μg/ml) were added at the same condition. The antibiotics' concentrations were decided based on MIC of *P. aeruginosa* PAO1 determined in our lab.

Growth of *P. aeruginosa* in each condition was monitored at 15-minute intervals, for a minimum of 48 h based on optical OD_{660} using TVS062CA BioPhoto recorder (Advantec, Tokyo, Japan). The value was recorded every 15 min.

To observe propagation of each phage and number of surviving PAO1 cells in phage cocktail-antibiotic's condition, the third experiment was carried out in 20 ml LB in shake flask (due to volume limitation for sampling) at 37°C with shaking at 100 rpm. One milliliter of medium was sampled each time. The sample was centrifuged (10,000 g, 5 minutes, 4°C) and pelleted cells were washed three times with Phosphate Buffer Saline (PBS) before plating for cell count. Phage titer was determined using the supernatant from centrifugation. Titration of ϕPA01 and ϕPA02 in the cocktail experiment was done using ϕPA02-resistant PAO1 generated

in our lab and *P. aeruginosa* NBRC 3080 strain (resistant to ϕPA01 but sensitive to ϕPA02), respectively.

5.3 Results

Phage ϕPA01 and ϕPA02 suppressed the growth of *P. aeruginosa* PAO1 to 10 h and 8 h, respectively (Fig. 5-1A). Since cross-resistance to both phages was not observed, we combined ϕPA01 and ϕPA02 as a phage cocktail to treat *P. aeruginosa*. The phage cocktail was able to suppress the growth of bacteria up to 20 h. Based on Fig. 5-1B, an antibiotic resistant mutant appeared around 20-22 h when treated with CIP (0.25 μg/ml) or MEM (2.0 μg/ml).

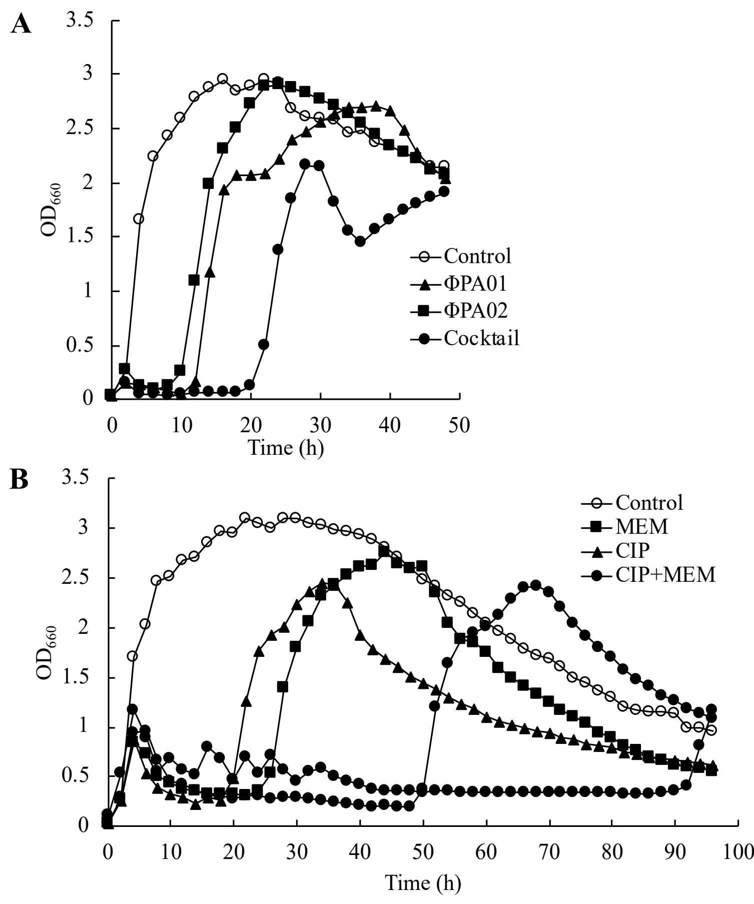


Figure 5-1. Treatment of *P. aeruginosa* PAO1 with (A) single phage and phage cocktail; (B) with CIP (0.25 μg/ml), MEM (2 μg/ml) or the combination of both antibiotics at the same concentration with single antibiotic.

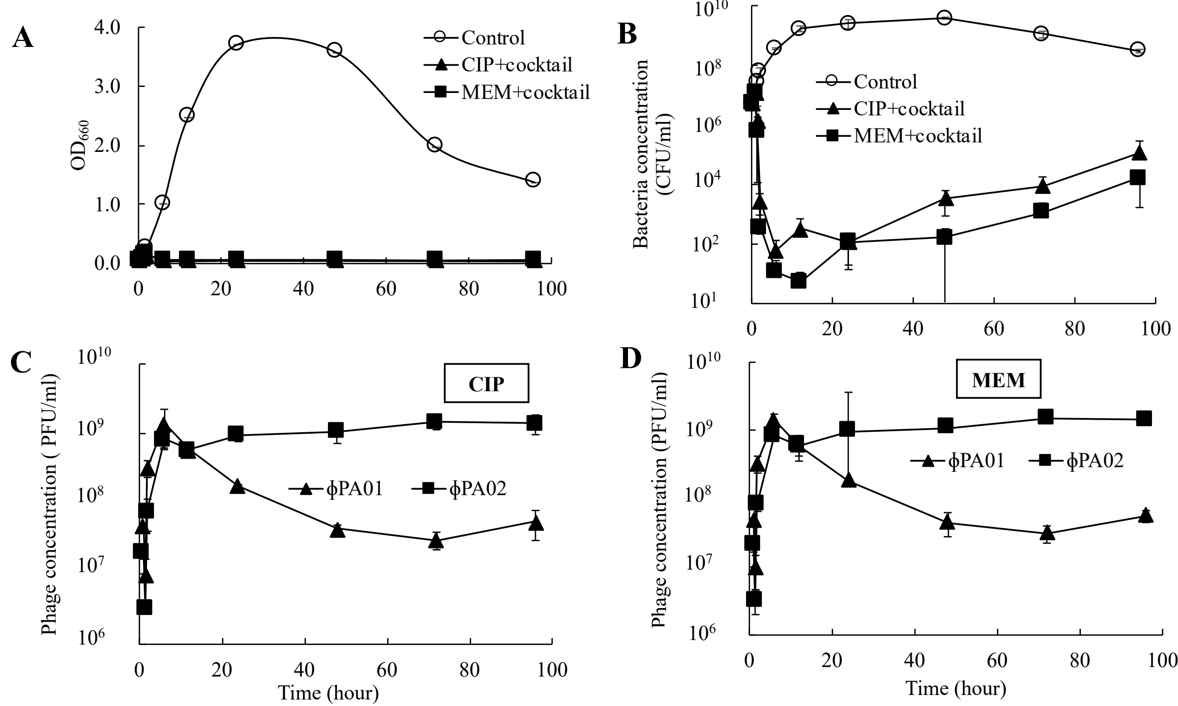


Figure 5- 2. Phage propagation and bacteria growth in phage cocktail and antibiotic treatment. (A) Killing curve of *P. aeruginosa* PAO1 with different treatments (MOI:1.0; CIP: 0.25 μg/ml; MEM: 2 μg/ml). (B) Growth curve of PAO1 treated with phage cocktail and CIP or MEM measured by OD_{660}. (C, D) Phage titer of ϕPA01 and ϕPA02 with addition of CIP or MEM, respectively. All phages and antibiotics were added at 1 h. Each condition was performed in triplicate and the means ± standard errors are indicated.

The resistant mutant appeared around 50 h and 94 h when treated with a combination of CIP and MEM. Furthermore, when infected with the phage cocktail, together with CIP (0.25 μg/ml) or MEM (2.0 μg/ml), OD_{660} of bacteria culture remained low at 0.1 up to 96 h (Fig. 5A) and about 99.9% of viable cells were reduced within 2 h (Fig. 5-2A). Treatment using the phage cocktail together with CIP reduced cells to 10^2 CFU/ml at around 6 h and bacteria regrew around 12 h. Based on Fig. 5-2B, approximately 10^5 CFU/ml of viable cells were detected at the end of the experiment (96 h). Meanwhile, the phage cocktail treatment together with MEM further reduced viable cells up to 12 h, to less than 10^2 CFU/ml, while approximately 10^4 CFU/ml of viable cells were detected at 96 h. Production of ϕPA01 and ϕPA02 progeny phages were observed in both treatments with CIP and MEM, respectively (Fig. 5-2C and 5-2D). Phage titer of ϕPA01 and ϕPA02 peaked at 6 h after addition of phage and antibiotics at 1 h, while the number of both phages decreased at 1.5 h (0.5 h after phage addition). Titer of ϕPA01

decreased to about 5.0 x10^7 PFU/ml at 96 h, while titer of ϕPA02 remained stable until the end of the experiment in both conditions. Phages' propagation was not inhibited by either CIP or MEM. These results clearly showed that applications of the phage cocktail together with CIP or MEM were more effective in controlling *P. aeruginosa* compared with the phage cocktail alone.

5.4 Discussion

Studies have shown that phage cocktails are more potent compared with a single phage in vitro (74,86) or as a preventive treatment in medical devices (105). due to the different selection pressures asserted by each phage (78).

The results show that a cocktail of ϕPA01 and ϕPA02 was able to suppress the growth of *P. aeruginosa* for longer than a single phage, due to different mechanisms in phage infection. This was supported by the absence of cross-resistance in phage resistant strains in chapter 4 (Fig.4-5), except R5-PA01R which only appeared in the 5th round of co-culture. Host that gained resistance to both phages might pay higher fitness cost compared to resistance to single phage.

P. aeruginosa often becomes resistant during treatment with antibiotic (47). even with a combination of antibiotics targeting different components (CIP: DNA gyrase; MEM: peptidoglycan) as shown in our results (Fig. 5-1B). The inconsistency in emergence times for mutants in the combined antibiotic treatment is possibly due to random mutation (58) or the presence of a variant subpopulation which is more resistant to antibiotics, as found in *Enterobacter cloacae* (106). In this study, the result showed that a phage cocktail of ϕPA01 and ϕPA02 together with either CIP or MEM is more effective than a phage cocktail or antibiotic alone in suppressing the growth of resistant *P. aeruginosa*, showing the potential of ϕPA01 and ϕPA02 to be used as a cocktail for phage treatment together with CIP or MEM. This study showed that propagation of both phages was not impeded by CIP and MEM, as shown by the increment of phage titer (Fig. 5-2C, 5-2D). Selection pressure by different phages in the cocktail in addition to antibiotic might assert high selective pressure on *P. aeruginosa* (78). Even though resistant cells emerged at the end of the treatment, these cells might pay a high fitness cost in order to become resistant to two phages and antibiotic (78).

Resistance to CIP and MEM was found in OprD membrane protein and DNA gyrase respectively (Chapter 2), which were different from resistant mechanism towards ϕPA01 and ϕPA02 (Chapter 4). Thus, our treatment strategy using lytic phages from different linage as a

cocktail complemented with CIP or MEM might have exerted a high selection pressure against *P. aeruginosa* and thus managed to delay the regrow of resistant bacteria.

Chapter 6 Conclusion and prospective

In chapter 2, *P. aeruginosa* gained resistance toward 2 antibiotics through accumulation of mutations that confer resistance to ciprofloxacin and meropenem, respectively. The mutations found was located in *oprD* and DNA gyrase. Besides, mutations were found in genes involve in gene regulation and in the production of virulence factor.

In chapter 3, two bacteriophages were isolated from sewage influent. Phage ϕPA01 and ϕPA02 were found to belong to *Pbunalikevirus* and *Phikzlikevirus*, respectively. These phages are unique to each other at genomic level and have far evolutionary relationship.

In chapter 4, cross-resistant to phage was investigated to determine the potential of ϕPA01 and ϕPA02 to be used as phage cocktail. Resistant strains of ϕPA01 showed slight cross-resistance to ϕPA02. Meanwhile, resistant strain of ϕPA02 remained sensitive to ϕPA01. Host receptor was identified as lipopolysaccharide (LPS). Each phage resistant strain showed unique mutations, indicating that *P. aeruginosa* employ different mechanisms to block the infection of ϕPA01 and ϕPA02.

In chapter 5, we showed the possibility to use ϕPA01 and ϕPA02 with distinct features as a phage cocktail together with antibiotic as a strategy in treating Pseudomonal infection. Growth of *P. aeruginosa* was not observed during the treatment with phage cocktail complemented with either ciprofloxacin or meropenem. In relation to Chapter 2 and Chapter 3, distinct mutations were found in antibiotic resistant and phage resistant strains, supporting the efficacy of using ϕPA01 and ϕPA02 as phage cocktail together with antibiotic for treatment.

In vitro study of the therapeutic effect of a phage cocktail complemented with antibiotics, which is an important initial step before applying it in clinical setting also provides an important insight for future clinical application. Further examination *in vivo* using animal models is needed before application to patients in order to improve the success rate of treatment.

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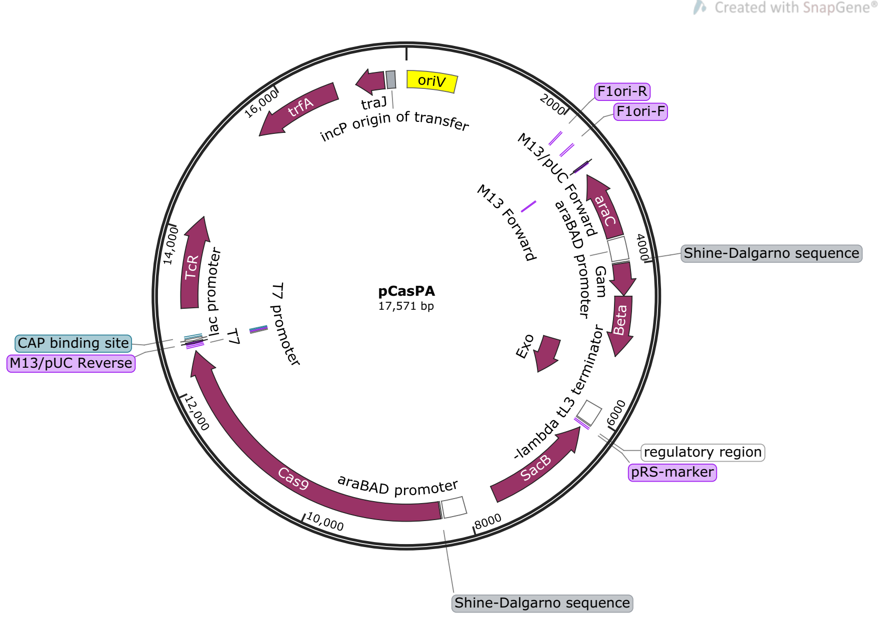
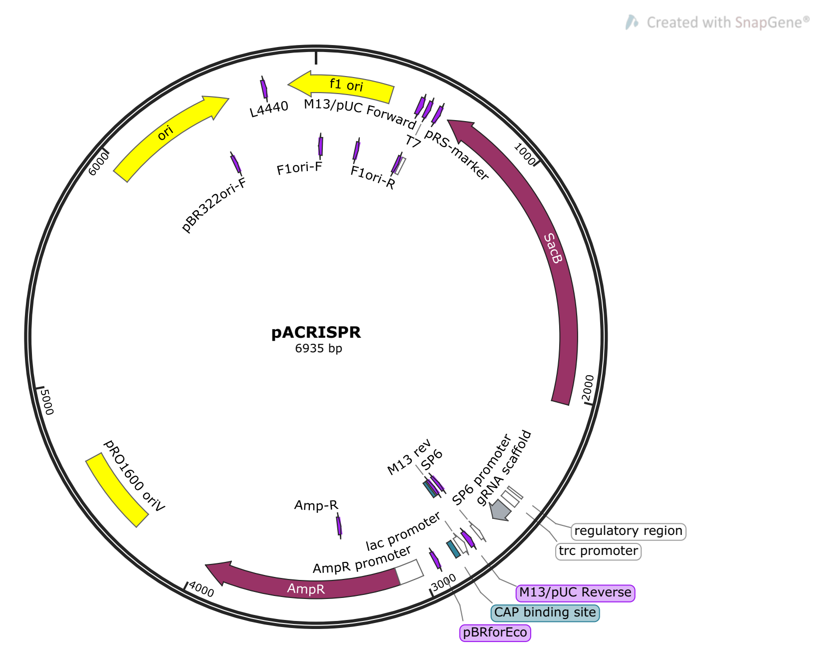
Appendix

Genomic map of plasmids used in this study

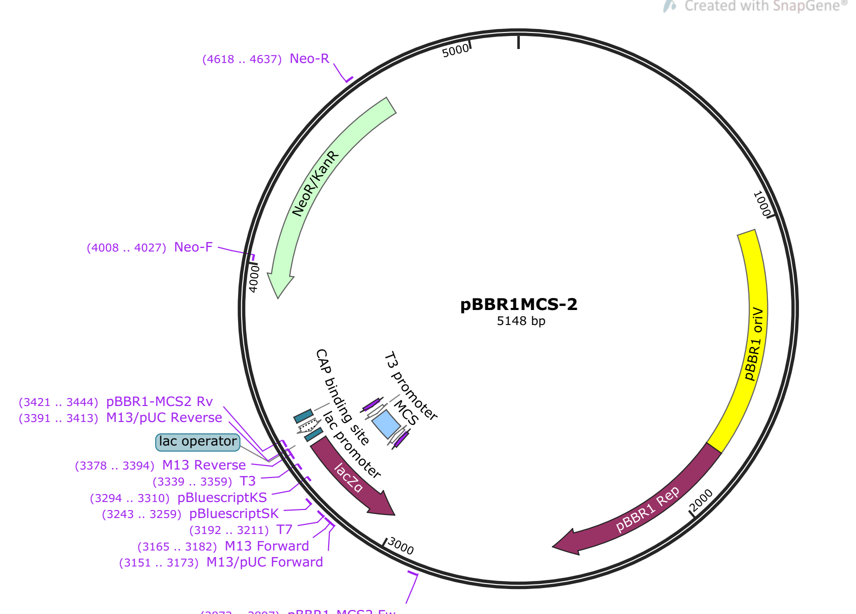
Plasmid pCasPA:



Plasmid pACRISPR:



Plasmid pBBR1MCS-2



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