CHARACTERIZATION OF BACTERIOPHAGES SPECIFIC TO DRUG RESISTANT *PSEUDOMONAS AERUGINOSA* ISOLATED FROM WASTEWATER

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(Miss Rungnapha Wannasutta)

Researcher

บทคัดย่อ

ชื่อเรื่อง : การศึกษาคุณลักษณะของแบคเทอริโอเฟจจำเพาะต่อ *Pseudomonas aeruginosa* ที่ดื้อยา ซึ่งแยกได้จากน้ำเสีย

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ศัพท์สำคัญ : แบคเทอริโอเฟจ แบคเทอริโอเฟจบำบัด *P. aeruginosa* ที่ดื้อยา

การอุบัติขึ้นของ *Pseudomonas aeruginosa* ดื้อยาก่อให้เกิดความกังวลไปทั่วโลก ทั้งนี้เนื่องจากการติดเชื้อแบคทีเรียดังกล่าว ไม่สามารถรักษาได้ด้วยยาปฏิชีวนะส่วนใหญ่ที่ใช้กันในปัจจุบัน ในช่วงเวลาที่มีการค้นหาวิธีการใหม่ในการควบคุมแบคทีเรียดื้อยา การรักษาโรคด้วยแบคเทอริโอเฟจถือเป็นวิธีการหนึ่งที่มีความเป็นไปได้สูง การศึกษานี้มีวัตถุประสงค์เพื่อแยกและจัดจำแนกแบคเทอริโอเฟจที่จำเพาะต่อ *P. aeruginosa* ดื้อยาสายพันธุ์ SH01 และเพื่อทดสอบความสามารถในการยับยั้งแบคทีเรียดังกล่าวในหลอดทดลองเมื่อใช้แบคเทอริโอเฟจเดี่ยว ๆ หรือเมื่อใช้แบคเทอริโอเฟจมากกว่า 1 ชนิดร่วมกัน ในการศึกษานี้สามารถแยกแบคเทอริโอเฟจได้ 3 ชนิด ได้แก่ WS-23 WS-40 และ WS-45 จากน้ำทิ้งต่างแหล่งกัน โดยแบคเทอริโอเฟจเหล่านี้สามารถทำให้เกิดพลาค (plaques) ใสขนาดเล็ก ซึ่งมีเส้นผ่านศูนย์กลางประมาณ 1 ถึง 2 มิลลิเมตร และยับยั้งได้เฉพาะ *P. aeruginosa* ดื้อยาสายพันธุ์ SH01 เท่านั้น แต่ไม่ยับยั้งแบคทีเรียสายพันธุ์อื่น ๆ ที่ใช้ในการศึกษานี้ จีโนมของแบคเทอริโอเฟจดังกล่าวถูกย่อยสลายได้โดยเอนไซม์ตัดจำเพาะหลายชนิด ได้แก่ *Eco*RI *Hin*dIII *Kpn*I และ *Pst*I จากการศึกษาด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่าน พบว่า แบคเทอริโอเฟจทุกชนิดมีหัวแบบ isometric head และมีหางแบบยืดหดได้ จากลักษณะของจีโนมและรูปร่างของแบคเทอริโอเฟจทำให้สามารถจัดจำแนกแบคเทอริโอเฟจเหล่านี้ไว้ใน สกุล *Myoviridae* จากกราฟแสดงการเจริญของแบคเทอริโอเฟจ พบว่าแบคเทอริโอเฟจ WS-23, WS-40 และ WS-45 มีระยะแฝง (latent period) เท่ากับ 30, 40 และ 40 นาที ตามลำดับ มีระยะปลดปล่อยอนุภาค (burst period) เท่ากับ 180, 150 และ 180 นาที ตามลำดับ และมี burst size เท่ากับ 45, 76 และ 30 PFU/infected cell ตามลำดับ เมื่อนำแบคเทอริโอเฟจแต่ละชนิดไปทดสอบความสามารถในการยับยั้ง *P. aeruginosa* ดื้อยาสายพันธุ์ SH01 ในหลอดทดลอง พบว่า แบคเทอริโอเฟจ WS-23,

WS-40 และ WS-45 สามารถลดจำนวนของเซลล์แบคทีเรียลงประมาณ 3, 6 และ 6 log CFU/ml ตามลำดับเมื่อเปรียบเทียบกับชุดการทดลองควบคุม ซึ่งไม่มีการเติม แบคเทอริโอเฟจ อย่างไรก็ตาม กลับพบว่าความสามารถในการยับยั้งการเจริญของแบคทีเรีย เมื่อใช้แบคเทอริโอเฟจ 2 ชนิดร่วมกัน ได้แก่ WS-23 ร่วมกับ WS-40, WS-23 ร่วมกับ WS-45 และ WS-40 ร่วมกับ WS-45 และเมื่อใช้แบคเทอริโอเฟจ 3 ชนิดร่วมกัน (WS-23 ร่วมกับ WS-40 และ WS-45) ลดลงเมื่อเทียบกับความสามารถในการยับยั้งการเจริญของแบคทีเรีย เมื่อใช้ แบคเทอริโอเฟจเพียงชนิดเดียว เพื่อจะอธิบายผลที่เกิดขึ้นในลักษณะเช่นนี้จำเป็นต้องมีการทดลองต่อไป โดยสรุปแล้ว แบคเทอริโอเฟจที่ได้จากการศึกษานี้มีความสามารถในการยับยั้ง *P. aeruginosa* ดื้อยาสายพันธุ์ SH01 ดังนั้นจึงอาจจะเป็นประโยชน์ในการนำไปใช้ในการรักษาโรคที่เกิดจากแบคทีเรียดังกล่าว

ABSTRACT

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KEYWORDS : BACTERIOPHAGE/ BACTERIOPHAGE THERAPY /DRUG RESISTANT *PSEUDOMONAS AERUGINOSA*

The emergence of drug resistant *Pseudomonas aeruginosa* has become a global concern because its infections cannot be cured by most of presently used antibiotics. In the midst of finding alternative therapeutic approaches to control drug resistant bacteria, bacteriophage therapy is considered as a most promising one. The aims of this study are to isolate and classify bacteriophages specific to drug resistant *P. aeruginosa* SH01 and to examine their ability to inhibit its specific bacterial host *in vitro* when used individually or as mixtures of more than one bacteriophage. In this study 3 bacteriophages, WS-23, WS-40 and WS-45, were isolated from different wastewater sources and found to produce small clear plaques of 1-2 mm in diameter. All of them inhibited only drug resistant *P. aeruginosa* SH01, but not other bacteria used in this study. Their genomes were digested by various restriction enzymes including *Eco*RI, *Hin*dIII, *Kpn*I and *Pst*I. As revealed by transmission electron microscopy, each of the bacteriophage had an isometric head with a contractile tail. Based on their genomic and morphological characteristics, all of the 3 bacteriophages were classified as members in the family *Myoviridae*. From one-step growth curves, the bacteriophages WS-23, WS-40 and WS-45 had latent periods of 30, 40 and 40 min, respectively, burst periods of 180, 150 and 180 min, respectively, and burst sizes of 45, 76 and 30 PFU/infected cell, respectively. When each bacteriophage was used to inhibit *P. aeruginosa* SH01 *in vitro*, it was found that the bacteriophages WS-23, WS-40 and

WS-45 reduced the bacterial cells by 3, 6 and 6 log CFU/ml, respectively as compared to the control treatment (no bacteriophage added). However, inhibitory activity of the mixtures of 2 bacteriophages (WS-23 plus WS-40, WS-23 plus WS-45 and WS-40 and WS-45) and 3 bacteriophages (WS-23, WS-40 and WS-45) was weaker than that of single bacteriophage. Further investigation is required to explain the decrease of inhibitory activity when these bacteriophages are used together. In conclusion, bacteriophages obtained from this study were able to inhibit drug resistant *P. aeruginosa* SH01; hence, they may be useful as therapeutic agents to treat diseases caused by the bacterium.

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CHAPTER 1

INTRODUCTION

1.1 Background and rational of the study

Pseudomonas aeruginosa is a gram-negative bacillus. It is motile by a single polar flagellum. This organism is an important opportunistic human pathogen. It causes several diseases such as urinary tract infection, respiratory system infection, dermatitis, soft tissue infection, bacteremia, bone and joint infection, gastrointestinal infection, ventilator associated pneumonia and a variety of systemic infections particularly in patients with compromised immune system. The most common approach for treating *P. aeruginosa* infections is antibiotic therapy. Antibiotics normally used to treat *P. aeruginosa* infections are antipseudomonal penicillin (e.g., piperacillin), antipseudomonal cephalosporin (e.g., ceftazidime), fluoroquinolones (e.g., ciprofloxacin), carbapenems (imipenem, meropenem and doripenem) and aminoglycosides (gentamicin, tobramycin and amikacin). However, this therapeutic approach has been found not to be effective for many strains of *P. aeruginosa* due to the emergence of drug resistant *P. aeruginosa.* Its ability to resist to antibiotics can be intrinsic or occurred through the adoption of genes encoding resistance determinants. Multiple mechanisms have been reported to be attributed to drug resistance of *P. aeruginosa* including production of enzymes modifying or inactivating antibiotics, upregulation of multidrug efflux pumps and cell wall mutation leading to the low permeability of the cell wall to antibiotics.

The emergence of multidrug resistant pathogenic bacteria has urged scientists to search for alternative therapeutic approaches to combat infectious diseases caused by the problematic bacteria. One of the potential candidates is phage therapy. This therapeutic approach uses bacteriophages, bacterial viruses, as antimicrobial agents to inhibit bacterial growth or to kill bacteria. Since the discovery by Twort in 1915 and by d'Hérelle in 1917 (Kutter and Sulakvelidze, 2005), bacteriophages have been used for treatment and prophylaxis of various bacterial infectious diseases. Therapeutic phages have been reported to have advantages over

antibiotics. They are very specific to their hosts; therefore, dysbiosis and chances of developing secondary infections are avoided. They replicate at the site of infection and are thus available where they are most needed. No serious side effects have been described for therapeutic phages. A few minor side effects of the phage may have been due to the liberation of endotoxins from bacteria lysed in vivo by the phage. Such effects may also be observed when antibiotics are used.

In this study, bacteriophages specific to drug resistant *P. aeruginosa* were isolated from various sources such as hospital, agricultural, industrial and household wastewater. Their genomes and morphological characteristics were studied for bacteriophage classification. Their bacterial host ranges and one-step growth kinetics were also examined in this study. Moreover, the effectiveness of using the isolated bacteriophages as individual or in combinations to inhibit drug resistant *P. aeruginosa* were assessed. The bacteriophages obtained from this study may be useful as alternative therapeutic agents against drug resistant *P. aeruginosa.*

1.2 Objectives of the study

1.2.1 To isolate bacteriophages specific to drug resistant *P. aeruginosa* from wastewater.

1.2.2 To identify the isolated bacteriophages based on their morphological characteristics and genomes.

1.2.3 To study host ranges of the isolated bacteriophages.

1.2.4 To study one-step growth kinetics of the isolated bacteriophages.

1.2.5 To examine the effectiveness of the isolated bacteriophages as individual or in combinations to inhibit drug resistant *P. aeruginosa.*

CHAPTER 2

LITERATURE REVIEW

2.1 Characteristics of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative asporogenous rod shaped organism. Cells are approximately 0.2 to 0.6 μm in length and 1.2 μm in width and can occur as single or in pair. Single cell is motile by a single polar flagellum. The bacterium can grow at temperature ranging from 7 to 44 °C but the optimized temperature for growth is 37 °C. It can grow in pH raging from 4.5-9.0 but cannot grow at pH 4.0. *P. aeruginosa* is sometimes described as an obligate aerobe, but this description is misleading because most strains can grow under anoxic conditions with nitrate as a terminal electron receptor. *P. aeruginosa* cannot grow fermentatively and for this reason it is referred to in the clinical literature as a "nonfermenter" (Colwell, 1964). *P. aeruginosa* strains produce a variety of pigments including pyocyanin (blue-green), fluorescein (yellow-green and fluorescent, now also known as pyoverdin), and pyorubin (red-brown).
The production of these pigments can be enhanced by growing the bacterial cell on appropriate agar. For examples Pseudomonas agar P (aka King A media) is used for enhancing pyocyanin and pyorubin production and Pseudomonas agar F (aka king B medium) is used for enhancing fluorescein production (King et al., 1954).

2.2 Pathogenesis of *P. aeruginosa*

P. aeruginosa is an important opportunistic human pathogen. In hospitals, *P. aeruginosa* survive well in moist environments such as skin, respirators, humidifiers etc (Pitt, 1986). *P. aeruginosa* is becoming more significant in the medical bacteriology because of the increased frequency of hospitalized patients infected by the bacterium. It causes several diseases such as urinary tract infection (Nickel et al., 1985), respiratory system infection, dermatitis, soft tissue infection, bacteremia, bone and joint infection (Miskew et al., 1983), gastrointestinal infection, ventilator-associated pneumonia (Brewer et al., 1996) and a variety of systemic

infection particularly in patients with severe burns and in cancer (Chatzinikolaou et al., 2000) and AIDS patients (Shepp et al., 1994). The emergence of multidrug resistant strains of *P. aeruginosa* has increased over the past decade (Obritsch et al., 2004), leading to the difficultly in treating *P. aeruginosa* infection by conventional antibiotics.

The pathogenesis of *P. aeruginosa* is largely caused by multiple bacterial virulence factor including adhesins, toxins secreted via type III secretion system and a number of secreted virulence factors (Waite et al., 2005). The pathogenic mechanism of *P. aeruginosa* was well studied in its respiratory infection. *P. aeruginosa* is an opportunistic pathogen that, after being acquired from the environment, colonizes the respiratory epithelium in patients with predisposing conditions such as cystic fibrosis, mechanical ventilation, immunodeficiency or preexisting respiratory disease. The motile surface appendages of *P. aeruginosa*, flagella and pili, are responsible for bacterial motility. These appendages also act as initial tethers in facilitating the bacterium to adhere to epithelial cells by binding to the epithelial surface glycolipid asialo-GM1. Additionally, lipopolysaccharides (LPSs) also play a similar role in bacterial adhesion through asialo-GM1 binding. The irreversible adhesion of the bacterium to epithelial cells is the initial critical step in colonization of the respiratory epithelium. Upon bacterial cell adhesion, the type III secretion system, a major virulence determinant, is activated. The type III secretion system allows *P. aeruginosa* to inject secreted toxins through a syringe-like apparatus directly into the eukaryotic cytoplasm. Four effector proteins are known: ExoY, ExoS, ExoT, and ExoU and all participate, at varying levels, in the cytotoxicity of *P. aeruginosa* leading to invasion and dissemination of *P. aeruginosa*. Other virulence factors secreted via type II secretion system into the extracellular space such as elastase, alkaline phosphatase, exotoxin A, and phospholipase C participate in invasion by destroying the protective glycocalix of the respiratory epithelium and by exposing epithelial ligands to *P. aeruginosa*. These secretins also participate in cytotoxicity.

In acute infections, invasion, dissemination and extensive tissue damage predominate. However, in chronic infection, particularly in cystic fibrosis patients, *P. aeruginosa* may adapt, by losing its most immunogenic features such as pili and flagella to avoid clearance, and by isolating itself from host defenses and adhering to the respiratory epithelium by forming biofilms.
In chronic infections, persistent inflammatory state is maintained by extracellular secreted virulence factors.

Several virulence factors have been found to play important roles in pathogenesis of *P. aeruginosa.* They includes adhesins, toxins secreted via type III secretion system and secreted virulence factors (Kipnis et al., 2006).

2.2.1 Adhesins

The adherence ability to host cells appears to be quite important for bacterial colonization. *P. aeruginosa* produces at least two types of proteins adhesins, pili and adhesive cell surface components (nonpilus adhesins). The pili of *P. aeruginosa* are type 4 pili, which are characterized by having a methylated phenylalanine as the first amino acid of the processed pilin subunit. The *P. aeruginosa* pili bind preferentially to asialoG_{M1} ganglioside on host cell. The pili are located at one pole of the cell and, when attached to a host cell, cause a twitching motility that allows the bacteria to move along the cell surface. The pili of *P. aeruginosa* mediate binding to epithelial cell, but not to mucin. However, *P. aeruginosa* is capable of binding to mucin.
This binding is mediated by the nonpilus adhesins. There appear to be two of these.
One mediates binding to both mucin and epithelial cells and the other one mediates binding to mucin only.

Another nonpilus adhesin is lipopolysaccharide (LPS). It is responsible to the adhesion of *P. aeruginosa* to a chloride channel protein called CFRT (cystic fibrosis transmembrane conductance regulator). The gene encoding this protein is mutated in many cystic fibrosis patients (Salyers and Whitt, 2002).

2.2.2 Toxins secreted via type III secretion system

P. aeruginosa has a type III secretion system that facilitates the injection of toxic proteins (toxins) directly from the bacterial cytoplasm into the cytoplasm of host cell. The type III secretion system (TTSS) of *P. aeruginosa* is a complex pilus-like structure allowing the translocation of toxic proteins form the bacteria across the bacterial membranes and in to the eukaryotic cytoplasm through a needle-like appendage forming a pore in the eukaryotic membrane (Kipnis et al., 2006). There are four known toxins, ExoY, ExoS, ExoT and ExoU, variably expressed in different strains and isolates, injected into host cells by *P. aeruginosa* through the TTSS (Frank, 1997). ExoS, ExoT and ExoY have clearly defined enzymatic activities and in general, are responsible for altering cellular cytoskeletal components. Intoxication with ExoS,

ExoT and ExoY causes cell rounding and detachment, and may contribute to infection by inhibiting or preventing bacterial uptake and phagocytosis (Barbieri and Sun, 2004).

ExoU, another toxic protein delivered by the TTSS, possesses a unique cytotoxic effect. It is capable of destroying cellular monolayers during short infection periods (Finck-Barbancon et al., 1997).

2.2.2.1 ExoS

ExoS is a bi-functional cytotoxin with two active domains, a C-terminal ADP-ribosyltransferase domain and N-terminal Rho GTPase-activiting protein (GAP) domain (Fu et al., 1993). ExoS is toxic for polymorphonuclear luekocytes (PMNs) (Salyers and Whitt, 2002). The ADP-ribosyltransferase activity necessitates a eukaryotic cell cofactor: 14-3-3 protein (Fu et al., 1993). The pathogenic role of ExoS is mainly attributable to the ADP-ribosyltransferase activity leading to disruption of normal cytoskeletal organization (Shaver and Hauser, 2004; Maresso and Barbieri, 2002), although GAP activity also plays a similar role (Shaver and Hauser, 2004; Krall et al., 2002).

2.2.2.2 ExoT

ExoT is similar to ExoS, with dual ADP-ribosyltransferase and GAP activities. However, it is considered to be a minor cytotoxin because its ADP-ribosyltransferase is thought to be deficient compared to that of ExoS (Sundin et al., 2004).

2.2.2.3 ExoY

ExoY is an adenylate cyclase (Yahr et al., 1998) injected directly into the host cytosol by the TTSS and increases cytosolic cAMP. The increased cytosolic cAMP causes the increase of pulmonary microvascular intercellular gap formation and lung permeability (Sayner et al., 2004).

2.2.2.4 ExoU

P. aeruginosa delivers the toxin ExoU to eukaryotic cells via a type III secretion system. Intoxication with ExoU is associated with lung injury, bacterial dissemination and sepsis in animal model and human infection. ExoU is produced and secreted as a 74 kDa protein (687 amino acids) and predicted to be mainly hydrophilic (Sato et al., 2003). It possesses a unique cytotoxic effect, which is rapid and potent. This toxin is toxic for macrophage but not for PMNs, which seem to be the main targets of ExoS. How ExoU kills macrophages remains to be

determined (Salyers and Whitt, 2002). ExoU is widely recognized as the major cytotoxin secreted via the TTSS playing an important role in the pathogenesis of *P. aeruginosa* infection (Ader et al., 2005). It is 100 times more cytotoxic than ExoS (Hauser et al., 2002).

2.2.3 Secreted virulence factors

2.2.3.1 Pyocyanin

Pyocyanin is a blue pigment metabolite of *P. aeruginosa* that has been shown to have numerous pathogenic effects such as increasing interleukin-8 (IL-8), depressing host-response, and inducing apoptosis in neutrophils (Leidal et al., 2001; Denning et al., 1998; Allen et al., 2005).

2.2.3.2 Pyoverdine

Pyoverdine is a siderophore, a small molecule chelating iron from the environment for use in *P. aeruginosa* metabolism. It has been shown to play a role in *P. aeruginosa* virulence (Meyer et al., 1996; Takase et al., 2000) by regulating the secretion of other *P. aeruginosa* virulence factors, exotoxin A, endoprotease and its own secretion (Lamont et al., 2002).

2.2.3.3 Alkaline protease

Alkaline protease is a fibrin lysing protease secreted by *P. aeruginosa,* through a type I secretion system (Guzzo et al., 1991). Although its pathogenic role is only clear in corneal infection as is the case for most *P. aeruginosa* proteases, it may participate in the pathogenesis of acute lung injury (Kipnis et al., 2004).

2.2.3.4 Elastase

Elastase is a metalloproteinase secreted by *P. aeruginosa* into the extracellular space through a type II secretion system. Elastase has been shown to have a role in the pathogenesis of *P. aeruginosa* respiratory infection by rupturing the respiratory epithelium through tight-junction destruction, thus increasing epithelial permeability (Azghani et al., 1993).

2.2.3.5 Protease IV

Protease IV also has a role in pathogenesis of *P. aeruginosa.* It is involved in the pathogenesis of lung infection through degradation of surfactant proteins A, D and B (Malloy et al., 2005).

2.2.3.6 Exotoxin A

Exotoxin A (ExoA), secreted into the extracellular space through a type II secretion system, is a ADP-ribosyltransferase inhibiting elongation factor-2 (EF-2) thereby inhibiting protein synthesis and leading to cell death (Pavlovskis et al., 1978). Among extracellular toxin, ExoA has a major role in *P. aeruginosa* virulence (Miyazaki et al., 1995). ExoA has also been shown to depress host response to infection (Schultz et al., 2000).

2.2.3.7 Phospholipase C

Phospholipase C, more specifically hemolytic phospholipase C, is a phospholipase secreted by *P. aeruginosa* into the extracellular space through a type II secretion system. Hemolytic phospholipase C targets eukaryotic membrane phospholipids and has been shown to participate in the pathogenesis of *P. aeruginosa* acute lung injury and in inflammation (Konig et al., 1997).

2.3 Antibiotic treatment of *P. aeruginosa*

P. aeruginosa is naturally resistant to a large range of antibiotic and may demonstrate additional resistance after unsuccessful treatment. Therefore, the most commonly used strategy to treat *P. aeruginosa* infection has been the combination of two antibiotics. Traditional therapy for *P. aeruginosa* infection is the administration of ß-lactam antibiotic together with aminoglycosides. Today, several antibiotics are still found to be active against *P. aeruginosa* (Table 1) (Pitt, 1986).

Table 1 Major antibiotics active against *P. aeruginosa*

Antibiotic	MIC_{50}* (mg/ml)	MIC_{90}* (mg/ml)
Amikcin	4	32
Azlocillin	16	64
Carbenicillin	64	128
Cefsulodin	4	16
Ceftazidime	2	8
Ciprofloxacin	0.25	1
Gentamicin	2	8
Piperacillin	16	64
Ticarcillin	32	128
Tobramycin	1	4

* Minimum inhibitory concentration for 50% and 90% of clinical isolates (Pitt, 1986)

However, it is not unusual to find *P. aeruginosa* developing drug resistance during therapy, leading potentially to treatment failures (Bonomo and Szabo, 2006). This phenomenon makes *P. aeruginosa* infection difficult to treat and may life-threatening, particularly if the causative strain is multi-resistant.

2.4 Drug resistant *P. aeruginosa*

P. aeruginosa is noted for its intrinsic resistance to antibiotics and for its ability to acquire genes encoding resistance determinants (Bonomo and Szabo, 2006). The resistance to multiple classes of antibiotics seriously compromises our ability to treat patients who are infected with the pathogen. In many instance, there are perilously few antibiotic choices. Its intrinsic and acquire resistance can occur via multiple mechanisms, including production of ß-lactamases and carbapenemases, upregulation of multidrug efflux pumps, and finally cell wall mutation leading to low permeability of its cell wall to antibiotic (Lambert, 2002).

2.4.1 Low permeability of cell wall to antibiotics

All of the major classes of antibiotics used to treat *P. aeruginosa* infections have to cross the cell wall to reach their targets. The innate resistance of *P. aeruginosa* to all classes of antibiotics has generally been attributed to the low permeability of its cell wall. Failure of antibiotics to accumulate within the organism is due to a combination of restricted permeability of the outer membrane and the efficient removal of antibiotic molecules that do penetrate by the action of efflux pump as described below (Lambert, 2002).

2.4.2 Multidrug efflux systems

The multidrug efflux systems are composed of three protein components, an energy-dependent pump located in the cytoplasmic membrane, an outer membrane porin and a linker protein which couples the two membrane components together (Nikaido, 1998). This tripartite arrangement forms an efficient extrusion system for toxic molecules present in the cytoplasm, the cytoplasmic membrane or the periplasm, the region between the outer and cytoplasmic membranes. Four different antibiotic efflux systems have been described in *P. aeruginosa*: mexAB-oprM, mexXY-oprM, mexCD-oprJ and mexEF-oprN10. All classes of antibiotics except the polymyxins are susceptible to extrusion by one or more of the efflux systems (Poole, 2001). Figure 1 shows how *P. aeruginosa* uses the efflux systems to expel antibiotics from the cell.

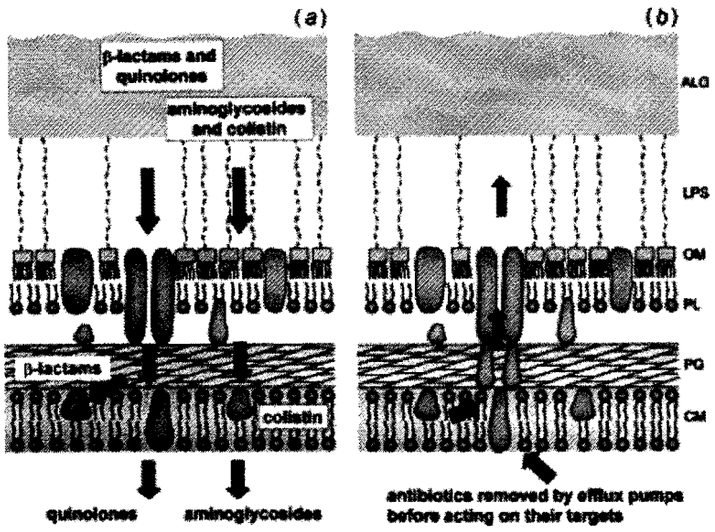


Figure 1 Schematic representation of the arrangement of components in the cell wall of *P. aeruginosa* efflux systems

CM=cytoplasmic membrane; OM=outer membrane; PG=peptidoglycan; LPS=lipopoly-saccharide; ALG=alginate. (a) The pathways for penetration of β-lactams and quinolones through porin channels in the OM. Aminoglycosides and colistin promote their own uptake by interacting with the LPS on the outer face of the OM. (b) How efflux systems reverse the diffusion of antibiotics across the OM. The efflux pumps comprise three components: an energy-dependent pump in the CM, a porin in the OM and an adapter protein joining the two membrane components. Antibiotics which have entered the cell are collected from the cytoplasm, the cytoplasmic membrane or the periplasm and expelled from the cells through the porin (Lambert, 2002).

2.4.3 Inactivation and modification of antibiotics

All *P. aeruginosa* strains possess the *amp*C gene for the inducible chromosomal ß -lactamase. However, induction alone probably does not account for resistance in *P. aeruginosa* strains. Instead, over-expression of the enzyme results from spontaneous mutation in the regulatory gene, *amp*R. This has occurred particularly where heavy reliance has been placed on ceftazidime therapy (Giwercman et al., 1990). Over-production of the *amp*C ß-lactamase poses a particular threat to cephalosporins. Inactivation of aminoglycosides occurs through production of enzymes which transfer acetyl, phosphate or adenylyl groups to amino and hydroxyl substituents

on the antibiotics. Prior to the recognition that aminoglycosides are susceptible to efflux, inactivation was regarded as the major mechanism of resistance for this group of antibiotics.

2.4.4 Changes in targets

This mechanism of resistance results from mutational changes in target enzymes which result in maintenance of their vital role in cell metabolism but resistance to the action of selective inhibition by antibiotics. Changes in the structure of the ribosome 30S subunit (the aminoglycoside target) influence streptomycin sensitivity but not that of the anti-pseudomonal aminoglycosides. Alteration in the penicillin-binding proteins of *P. aeruginosa* resulting in resistance to ß -lactams has been reported but is not currently a major problem in *P. aeruginosa* strains (Srikumar et al., 1999).

2.4.5 Cell aggregation

In infection, *P. aeruginosa* grows as aggregate of cell (microcolonies) encased in a protective alginate polysaccharide. This mode of growth also occurs on surfaces, where it is referred to as a biofilm. The characteristic property of all biofilm is their remarkable resistance to eradication by physical and biochemical treatments, including antibiotics (Stewart and William, 2001).

2.5 The nature of bacteriophages

Bacteriophages (also called "phages") are bacterial viruses that can be robusted antibacterial agents *in vitro*. However, they were used as therapeutic agents, during a number of trials from the 1920s to the 1950s. Phages were discovered in 1915 by British microbiologist Felix Twort, and independently in 1917, by French-Canadian microbiologist Felix d'Hérelle. Twort didn't pursue his discovery, whereas d'Hérelle systematically investigated the nature of bacteriophages and explored their ability to function as therapeutic agents (Kutter and Sulakvelidze, 2005).

Bacteriophages are viruses that only infect bacteria. They are like complex spaceships (Fig. 2). Each phage particle (virion) contains its nucleic genome (DNA or RNA) enclosed in a protein or lipoprotein coat, or capsid; the combined nucleic acid and capsid form the nucleocapsid. Phages, like all viruses, are absolute parasites. Although they carry all the information to direct

their own reproduction in an appropriate host, they have no machinery of generating energy and no ribosomes for making proteins.

Phages are extremely heterogeneous in their structural, physicochemical, and biological properties. Virions are tailed, polyhedral, filamentous, and pleomorphic (Fig. 2), and phage families may be listed in this order for convenience. The vast majority contain dsDNA, however, ssDNA, ssRNA, or dsRNA are found in small groups of phages. All DNA phages contain a single molecule of DNA. Several types of phages have lipid containing envelopes or internal vesicles.

Phages can also be divided into two classes based on lifestyle: virulent or temperate. Virulent phages can only multiply by means of a lytic cycle; the phage virion adsorb to the surface of a host cell and inject its genome, which takes over much of host metabolism and sets up molecular machinery for new phages. The host cell then lyses minute or hour later leading the liberation of many new phages. Temperate phages, in contrast, have a choice for productive modes when they infect a new host cell. Sometimes the infecting phage initiates a lytic cycle, resulting in lysis of the cell and release of new phages, previously shown. The infecting phage may alternatively initiate a lysogenic cycle. In this cycle, the phage genome is in quiescent state called a prophage, often integrated into host genome but sometimes maintaines as a plasmid.
It remains in this condition indefinitely, being replicated as its host cell reproduces to make a clone of cell all containing prophages; these cells are said to be lysogenized or lysogenic (i.e., capable for producing lysis) because one of these prophages occasionally come out of its quiescent condition and enters the lytic cycle. Temperate phages can help protect their host from infection by other phages and can lead to significant changes in the properties of their hosts, including restriction system and resistance to antibiotics and the other environmental insults (Lenski and Levin, 1985).

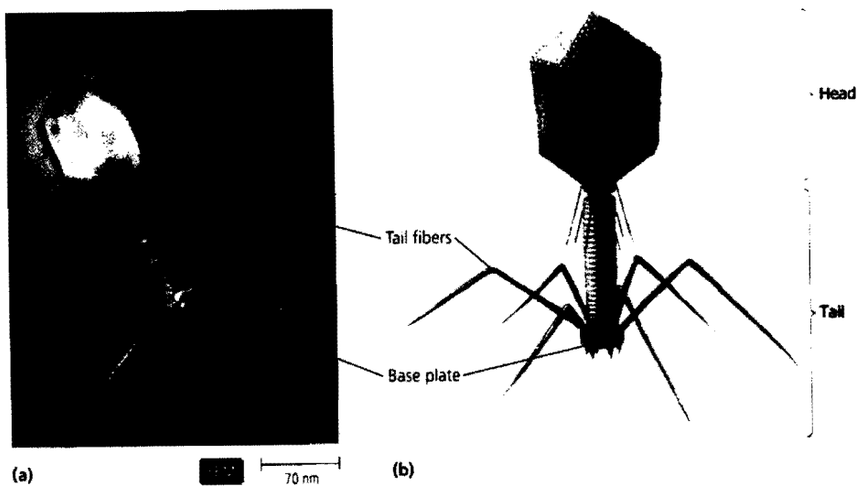


Figure 2 The complex shape of bacteriophage T4

It includes an icosahedral head and ornate tail that enables viral attachment and penetration (Bauman, 2009).

2.6 Bacteriophage classification

The International Committee for Taxonomy of viruses (ICTV) classifies viruses into three orders, 61 families, and 241 genera. Bacteriophage virions are tailed, filamentous, polyhedral, or pleomorphic (Fig. 3). Bacteriophages constitute one order, 13 families, and 30 genera (Table 1). A few types have lipid containing envelopes or contain lipid as part of the particle wall. Most phages contain dsDNA, but there are small group with ssDNA, ssRNA, or dsRNA (Ackermann, 2003). Taxonomic names of order, families, and genera are typically constructed from Latin and Greek root and end in *–virales*, *-viridae*, and *–virus*, respectively. Most genera of "cubic", filamentous and pleomorphic phages have Latinized names. So far, tailed phage genera have vernacular names only (e.g., "T-4 like viruses").

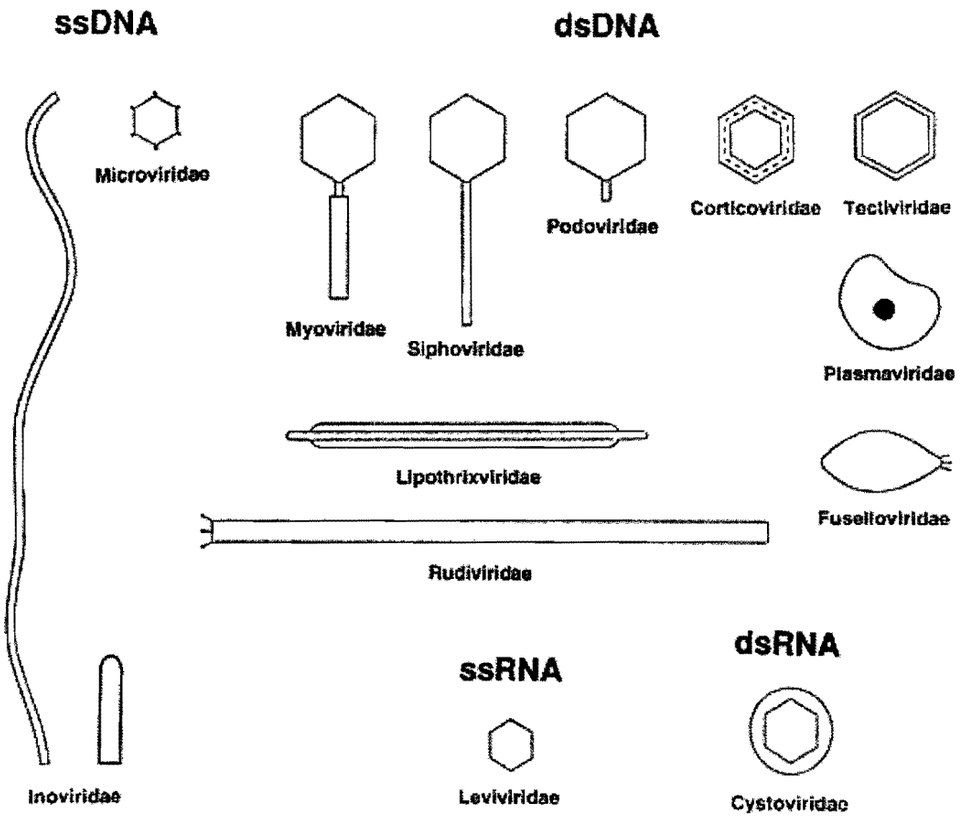


Figure 3 Schematic representation of major phage groups (Ackermann, 2003)

Table 2 Classification and basic properties of bacteriophages

Shape	Nucleic acid	Order and families	Genera	Example	Member	Characteristics
Tailed	**DNA, ds, L**	***Caudovirales***	**15**		**4950**	
		Myoviridae	6	T4	1243	Tail contractile
		Siphoviridae	**6**	**λ**	**3011**	**Tail long, noncontractile**
		Podoviridae	3	T7	696	Tail short
Polyhedral	**DNA, ss, C**	***Microviridae***	**4**	**ΦX174**	**40**	
	ds, C, T	*Corticoviridae*	1	PM2	3?	Complex capsid, lipid
	ds, L	***Tectiviridae***	**1**	**PRD1**	**18**	**Internal lipoprotein vesicle**
	RNA, ss, L	*Leviviridae*	2	MS2	39	
	ds, L, S	***Cystoviridae***	**1**	**Φ6**	**1**	**Envelope, lipid**
Filamentous	DNA, ss, C	*Inoviridae*	2	fd	57	Filaments or rod
	ds, L	***Lypothrixviridae***	**1**	**TTV1**	**6?**	**Envelope, lipid**
	ds, L	*Rudiviridae*	1	SIRV1	2	Resembles TMV
Pleomorphic	**DNA, ds, C, T**	***Plasmaviridae***	**1**	**L2**	**6**	**Envelope, lipid, no capsid**
	ds, C, T	*Fuselloviridae*	1	SSV1	8?	Spindle-shaped, on capsid

C, circular; L, linear; S, segmented; T, superhelical; ss, single-stranded; ds, double-stranded (Ackermann, 2001)

2.6.1 Tailed phages (Order Caudovirales)

Tailed phages (Ackermann, 1998) constitute the largest and most widespread group of bacterial viruses. Virions consist of a protein shell and linear dsDNA only. Phage particles are not enveloped, since their heads (capsids) have cubic symmetry and their tails are helical. Their heads are icosahedra, either regular (85% of tailed phages) or prolate. Capsids are composed of capsomers, but capsomers are difficult to observe and capsids usually appear smooth. Phage tails are true helices or consist of stacked disks, and they usually possess terminal structures such as base plates, spikes, or fibers (Ackermann, 1998). The DNA of tailed phages are a single linear, double stranded filament. Some DNAs contain unusual base such as 5-hydroxy-methylcytosine. DNA sizes vary between 17 and over 700 kb and tail lengths range from 10 to 800 nm (Ackermann, 2003). Tailed phages are divided into three families including *Myoviridae*, with contractile tails consisting of a sheath and a central tube (25% of tailed phages) (Ackermann, 2001) *Siphoviridae*, with long, noncontractile tails (61% of tailed phages) and *Podoviridae*, with short, noncontractile tails (14% of tailed phages) (Table 3). Three families show considerable overlap in their physicochemical properties. However, the capsids of *Myoviridae* tend to be larger and to contain more DNA than those of the other two families (Ackermann, 1998).

Table 3 Tailed phages genera (Ackermann, 2001)

Family	Genus	Type species	Species	Member	Principal hosts
Myoviridae	T4-like viruses	T4	7	47(+100)	Euterobacteria
	P1-like viruses	P1	3	12	Euterobacteria
	P2-like viruses	P2	2	16	Euterobacteria
	Mu-like viruses	Mu	1	2	Euterobacteria
	SPO1-like viruses	SPO1	1	13	*Bacillus*
	ΦH-like viruses	ΦH	1	2	*Halobacterium*
Siphoviridae	λ-like viruses	λ	1	7	Euterobacteria
	T1-like viruses	T1	1	11(+50)2	Euterobacteria
	T5-like viruses	T5	1	5(+20)	Euterobacteria
	L5-like viruses	L5	1	4(+15)	*Mycobacterium*
	c2-like viruses	c2	1	5(+500)	*Lactococcus*
	ΨM1-like viruses	ΨM1	1	3	*Methanobacterium*
Podoviridae	T7-like viruses	T7	3	26	Euterobacteria
	P22-like viruses	P22	1	11	Euterobacteria
	Ø29-like viruses	Ø29	4	12	*Bacillus*

2.6.2 Polyhedral phages

2.6.2.1 Polyhedral DNA phages

Microviridae (ssDNA)

Virions are unenveloped icosahedra (27 nm in diameter) with 12 capsomers and contain circular ssDNA. Virions are quite small such as ØX174. Phages infect different hosts (*Euterobacteria*, *Bdellovibrio*, *Chlamydia*, and *Spiroplasma*) and are classified into four genera. Their DNA replicates, via the rolling-cycle model, as a double strand replicative form. Genome sequence data have been used to divide the family into two subfamilies, one for phage propagating in proteobacteria and the other for *Chlamydia*, and *Spiroplasma* phages (Brentlinger et al., 2002).

Corticoviridae (dsDNA)

The family has a single member, phage PM2, was isolated from seawater and the first bacteriophage in which the presence of lipids was demonstrated. Like tectiviruses, it has a protein capsid with an internal phospholipoprotein vesicle, but is little known (Ackermann, 2001).

Tectiviridae (dsDNA)

The viral particle has a rigid protein capsid containing a thick, flexible lipoprotein vesicle. In addition, *bacillus* tectiviruses have apical spikes. After the phage adsorb to their host bacteria, or chloroform, the vesicle transforms itself into a tail-like tube of about 60 nm in length, which serves as a nucleic acid injection device. Despite the family's small size, tectiviruses infect apparently unrelated bacteria.

2.6.2.2 Polyhedral RNA phages

Leviviridae (ssRNA)

The leviviruses are unenveloped and morphologically similar to polioviruses. Their genome consists of four partially overlapping genes, and their RNA acts as mRNA and therefore positive-stranded. Most known leviviruses are plasmid-specific coliphages that adsorb to F or sex pili. They have been divided, by serology and genome structure, into two genera.

Cystoviridae (dsRNA)

Cystoviruses have icosahedral capsids surrounded by lipid-containing envelopes, and they are unique among bacteriophage because they containing a dodecahedral RNA polymerase complex (Bamford et al., 1993) and three molecules of dsRNA. The capsids of infecting Cystoviruses enter the space between the bacterial cell wall and the cytoplasmic membrane. This family has a single "official" member. They are specific for phytopathogenic bacterium *Pseudomonas syringae*.

2.6.3 Filamentous phages

2.6.3.1 *Inoviridae* (ssDNA)

The family consists of two genera with different particle morphologies and host ranges. The phage DNA replicates as a double-stranded form via a rolling circle mechanism. This similarity in replication seems to derive from the nature of phage rather than

from a common origin of the two genera. The 42 phages of the genus Inovirus, which have been classified into 29 species, are long rigid of flexible filaments whose length reflects the size of their genomes. They infect enterobacteria and their relatives, the genus *Thermus*, clostridia and propionibacteria. Virions are sensitive to chloroform and sonication and they are very resistant to heat. The genus *Plectrovirus*, whose virions are short, straight rods, includes 15 members that infect only mycoplasmas. Progeny inoviruses are extruded from host cells, which survive and may produce phages indefinitely.

2.6.3.2 *Lipothrixviridae* (dsDNA)

This family includes six viruses of extremely thermophilic archaebacteria. Viral particles are characterized by the combination of a rod-like shape, a lipoprotein envelope, and a nucleosome-like core. In contrast to Inoviruses, the progeny are released by lysis. Lipothrixviruses may be taxonomically heterogeneous and form two subfamilies (Arnold et al., 2000).

2.6.3.3 *Rudiviridae* (dsDNA)

This family includes two viruses of different lengths, which were isolated from the thermophilic archaeon *Sulfolobus*. Particles are straight, rigid rods without envelopes, have conspicuous fixation structures at one end, and resemble the tobacco mosaic virus (Peng et al., 2001).

2.6.4 Pleomorphic phages

2.6.4.1 *Plasmaviridae* (dsDNA)

The family has only one member, *Acholeplasma* virus MVL2 or L2. Virions do not have capsids, but they possess an envelope and a dense nucleoprotein granule. Like enveloped vertebrate viruses, plamaviruses infect their host by fusion of the viral envelope with the mycoplasmal cell membrane, and progeny viruses are released by budding.

2.6.4.2 *Fuselloviridae* (dsDNA)

Fuselloviruses are spindle-shaped with short spikes at one end. The best-studied member of the family, SSV1, is harbored in the archaeon *Sulfolobus shibatae* as a plasmid and as an integrated prophage. It has not been propagated because of the absence of a suitable host, but it is inducible by UV light and mitomycin C. The coat consists of two hydrophobic

proteins and host lipids, and is disrupted by chloroform. Fuselloviruses are liberated by extrusion from the host cell (Ackermann, 2003).

2.7 Application of bacteriophages

2.7.1 Phage for the detection of pathogenic bacteria (phage typing)

The specificity of interaction of a virus with its host cell immediately lends itself to methods for identification of bacteria, in particular the pathogens. One of the first uses of phage was in typing schemes, where a panel of phages with different lytic spectra is used to discriminate between different isolates of a bacterial species or genus, according to their ability to infect the isolate and form plaques. When using an identical set of phages, typing results are highly reproducible in different laboratories, although it is important that the phages used are propagated under specified conditions. The ease of use and inexpensiveness of phage typing also contribute to the fact that it is still among the most widely used methods for strain identification of many bacteria, in particular, *Salmonella*, *Listeria*, and *Staphylococcus*.

2.7.2 Phage displays

The development of phage display (Smith, 1985) has revolutionized the isolation of peptides with binding affinity for particular substrates. In this system, libraries of peptide variants (often produced using mutational PCR methods) are cloned into filamentous phage vectors as gene fusion to coat proteins. The cloned sequences are expressed along with the phage coat proteins and incorporated into the mature phage particle and are thus "displayed" on the surface of the mature virions (Smith and Petrenko, 1997). For instance, clone expressing with a particular affinity can be recovered from libraries of phage particles by bionpanning experiments, where the target ligand is bound to a solid surface and then incubated with phage particles. Phages that do not bind tightly to the ligand are washed away, and the remaining bound fraction of the library, which is enriched for clones expressing peptides that bind to the target ligand, is recovered. Repeated rounds of bionpanning lead to rapid identification of specific clones expressing peptides with the highest binding affinity (Hayhurst and Georgiou, 2001).

This approach has led to the development of phage antibody technology where the peptide displayed is the antigen-binding domain of an antibody molecule. A library of such phage includes billions of clone expressing antibody-derived peptides with different binding

affinities that can then domain of an affinity selection process (Rader and Barbas, 1997). This approach has been successfully used to develop a range of detection assays, including the identification of a variety of viruses (Petrenko and Vodyanoy, 2003), the differentiation of *Candida* species in clinical specimens (Bliss et al., 2003) and the species-specific detection of *Bacillus* spores (Zhou et al., 2002).

2.8 Phage therapy

2.8.1 Definition of phage therapy

Phage therapy refers to the treatment of bacterial infection with intact phage (Petty et al., 2007). Currently emergence of pathogenic bacteria resistant to most, if on all, currently available antibiotics has become a critical problem in modern medicine (Sulakvelidze et al., 2001). Phage therapy is an alternative therapy against multidrug resistant pathogens because phages and antibiotics inhibit bacteria with different mechanisms.

2.8.2 History of phage therapy

From the time that the first bacteriophage was discovered, the role of bacteriophages in the course of bacterial infectious diseases was of crucial interest. The first report of the therapeutic use of phage was a note by Bruynoghe and Maisin (1921) from Louvain in which they injected a preparation of staphylococcal phage in the local region of cutaneous boils. They noted both reductions in swelling and in pain as well as some reduction in fever.

In the summer of 1919, d'Hérelle carried out extensive tests of phage as prophylaxis against the natural infection of chicken by *Bacillus gallinarun*, the bacterium that causes avian typhosis.

He reported these results in 1921. Phage therapy was also evaluated in field trail against bovine hemorrhagic septicemia (*barbone*) in Indochina. With evidence of therapeutic effectiveness of phage in both gastrointestinal disease (avian typhosis) and septicemic disease (*barbone*), d'Hérelle extended his trials to human beings. He first determined the safety of his phage preparation by self-administration. He also injected his coworkers as well as his family.

This procedure was considered sufficient to evaluate the safety of this material. After being assured that no harmful effects attended the ingestion of the Shiga-bacteriophage, this treatment was applied for therapeutic purposes to patients afflicted with bacillay dysentery.

The work that attracted the most attention for phage therapy was probably d'Hérelle's report of four cases of bubonic plaque which he treated with antiplaque phage. While he was stationed at the League of Nations Quarantine Station in Alexandria Egypt, d'Hérelle observed four patients on a ship passing through the Suez Canal, all of whom had laboratory diagnosed bubonic plaque. d'Hérelle treated all four with antiplaque phage preparations by direct injection of phage into buboes. All of four patients remarkably recovered and this result was reported in the widely read French medical periodical, *La presse médical.* On the basics of this work, d'Hérelle was invited by the British government to go to India to work on phage therapy of the plaque at the Haffkine Institute in Bombay. This short visit led to the later establishment of the Bacteriophage Inquiry in India, under the patronage of the Indian Research Fund Association. This project studied the application of phage therapy in India, especially for cholera epidemics that regularly occurred in association with religious festivals and pilgrimages.

From the initial reports from India in the 1920s and 1930s, it seems consistently observed that the severity and duration of cholera symptoms and the overall mortality from the disease were reduced in patients given cholera-specific phage by mouth. In several WHO-sponsored studies in Pakistan in the 1970s, in which phage was compared with antibiotics (tetracycline), high-dose phage therapy seemed about equivalent to tetracycline in certain aspects of the clinical control of cholera.

Interest in phage therapy has waxed and waned since its inception: first early enthusiasm, followed by critical skepticism and abandonment, and currently renewed interest and reappraisal. The changing attitudes toward phage therapy reflect both scientific and cultural influences.

While many early phage therapy trials were reported to be successful, and many of the major pharmaceutical firms sold phage preparations, there were also failures. The Council on pharmacy and chemistry, established in 1950 by the American Medical Association to set standard for drugs and lead the battle against quack remedies, undertook the evaluation of phage therapy in the mid-1930s. The voluminous report of the Council concluded with an ambiguous assessment of the literature on phage therapy, acknowledging that there were both positive and negative results in the literature. The Council was concerned that the biological nature of bacteriophage was poorly understood and that the lack of standards for purity and potency of

phage made it impossible to compare most of the published studies. Such a report might have generated more research and new and better understanding, but in the World War II and the discovery of antibiotic effectively diverted efforts away from further study of phage therapy in the United States. Some very good research was conducting during the early 1940s by scientist like Rene Dubos at Harvard and physicians fighting typhoid in Los Angeles, but it largely disappeared from view very recently. d'Hérelle had returned to France and was held under virtual house arrest in Vichy during the war, and thus the most vigorous advocate for phage therapy was silenced.
In Europe, however, there are two major efforts in phage therapy continuing in a decidedly military context: the Soviet Union waged war against the Finns, phage therapy was extensively used to treat these war-wounded. Phage therapy continued to be researched extensively with military support after the war and become part of the general standard of care there, particularly in the Republic of Georgia. The German military was also engaged in use of phage therapy (Kutter and Sulakvelidze, 2005).

Over recent decades, a growing body of literature has validated the use of bacteriophage for therapy and prophylaxis in the war against drug-resistant bacteria. After the antibiotic era, increasing of antibiotic-resistant bacterial strains has become a major health problem in hospital. One solution to overcoming the multi-resistance problem might be finding alternative remedies against drug-resistant pathogens. This is an important challenge to modern medicine. Scientists and clinicians alike are looking retrospectively to find an alternative treatment in the form of phage therapy (Kutateladze and Adamia, 2010).

2.8.3 Comparison of phages and antibiotics: advantages and limitations of phage-based therapy

One of the areas that had made the idea of phage therapy attractive is the increasing prevalence of antibiotic-resistant bacteria. The wide-spread use of antibiotics in modern medical practice is related to their rapid antibacterial action and broad spectrum of activity. Although both phages and antibiotics can be used to treat bacterial infection diseases, they have advantages and disadvantages. Some of the key differences between therapeutic phages and antibiotics are summarized in Table 4.

Table 4 Comparison of bacteriophage and antibiotic therapy

Advantages	
Bacteriophage	**Antibiotic**
1) High specificity for particular bacterium, therapy reducing the possibilities of secondary infections developing. 2) Repeated administration is unlike because as the target bacterium is present, the phage will be able to reproduce. 3) Cheap to produce and to date without any observed side-effects. 4) The receptors to which phages are targeted on the bacterial cell surface are virulence factors, so when bacteria develop phage-resistance, they are usually altered, which results in an attenuation of virulence. 5) Finding a phage which will be active against bacteria which has developed phage resistance is rapid, taking only a matter of day.	1) Active against wide range of bacteria, thereby avoiding the need to characterize the infective bacterium.

Table 4 Comparison of bacteriophage and antibiotic therapy (Continued) (Inal, 2003)

Disadvantages	
Bacteriophage	**Antibiotic**
1) Causative agent may need to be identified in order to use appropriate phage, unless a phage cocktail is used.	1) Repeated administration is needed and antibiotic therapy is often associated with side-effects such as intestinal problems, secondary infection, e.g. with yeast. 2) Once the bacteria develop antibiotic resistance, as opposed to phage resistance, it remains pathogenic. 3) The development of novel antibiotics (need for example when bacteria develop resistance) takes on the order of years. 4) Because of a non-specific mode of action, antibiotic also destroy the commensal microflora especially in the intestine, which may lead to intestinal disorders. 5) Expensive to produce.

2.8.4 Phage therapy of drug-resistant bacteria

Several studies have supported the use of phage therapy for treating disease caused by drug resistant bacteria.

Phage therapy was successfully used as therapeutic and prophylactic treatment for multidrug resistant *P. aeruginosa* infection in immunocompetent mice. A curative dose of phage intranasally administrated to the mice 2 hour after the onset of the infection allowed more than 95% survival. Furthermore, a four-day preventive treatment resulted in a 100% survival (Morello et al., 2011).

Jikia (2005) used bacteriophage in combination with ciprofloxacin to treat wound infection caused by a strain of *Staphylococcus aureus* resistant to many antibiotics (including ciprofloxacin). The treatment resulted in clinical improvement associated with rapid elimination of the aetiologic bacterial strain (Jikia et al., 2005).

Phage K, a lytic phage, isolated by O'Flaherty et al. (2005) was shown to have in vitro inhibition ability against a wide range of drug resistant *Staphylococcus aureus* strains including methicillin resistant *S. aureus* (MRSA), *S. aureus* strains with vancomycin resistance and those with teicoplanin resistance. Their study also showed that phage K had potential in the prevention and/or treatment of infections caused by antibiotic resistant staphylococci (O'Flaherty et al., 2005).

In December 2001, three Georgian lumberjacks form the village of Lia were exposed to a strontium-90 source. Two of them developed severe local radiation injuries which subsequently became infected with multidrug-resistant *Staphylococcus aureus*. Approximately 1 month after hospitalization, they were treated with PhagoBioDerm (a wound-healing preparation consisting of a biodegradable polymer impregnated with ciprofloxacin and bacteriophages). Purulent drainage stopped within 2-7 days. Clinical improvement was associated with rapid elimination of the aetiologic agent, a strain of *S. aureus* resistant to many antibiotics (including ciprofloxacin), but susceptible to the bacteriophages contained in the PhagoBioDerm preparation (Jikia et al., 2005).

2.8.5 Phage cocktail and the combined phage and antibiotic therapy

The major concern regarding the use of phage therapy in the treatment of infection is the development of phage-resistant bacteria. In earlier phage resistance work, Luria and Delbruck in 1943 made the general observation that a decrease in turbidity occurred within several hours upon attack of a pure bacteria culture by phage. However, continued incubation of the culture resulted in the growth of a bacterial variant that was resistant to the attacking phage. Several groups have developed phage cocktails alleviate the phage resistance problem.

Tanji et al. (2004) described a method used to develop a phage cocktail to control the foodborne pathogen *E. coli* O157:H7. These cocktails were shown to delay the development of phage-resistant cells, when compared with the use of a single phage (Tanji et al., 2004).

O'Flynn et al. (2004) evaluated the cocktail of three bacteriophages for biocontrol of *E. coli* O157:H7 both in vitro and in vivo. This study concerns the exploitation of bacteriophages as biocontrol agents to eliminate the pathogen *E. coli* O157:H7. Two distinct lytic phages (e11/2 and e4/1c) isolated against a human strain of *E. coli* O157:H7, a previously isolated lytic phage (pp01), and a cocktail of all three phages were evaluated for their ability to lyse the bacterium in vivo and in vitro. Phage e11/2, pp01, and the cocktail of all three virulent phages resulted in a 5-log-unit reduction of pathogen numbers in 1 h at 37 °C. While using phage e4/1c resulted in the high frequency of bacteriophage-insensitive mutant. In a meat trial experiment, the phage cocktail containing all of the three phages completely eliminated *E. coli* O157:H7 from the beef meat surface in seven of nine cases (O'Flynn et al., 2004).

Fu et al. (2010) investigated the effect of pretreating hydrogel-coated catheters with *P. aeruginosa* bacteriophage on biofilm formation by *P. aeruginosa* in a in vitro model. They found that the application of phages, especially phage cocktails, to the surface of indwelling medical devices could mitigate biofilm formation by the bacteria (Fu et al., 2010).

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table 5. All of bacterial cultures were grown aerobically in Brian heart infusion (BHI) broth at 37°C except *P. aeruginosa* which was grown in Luria-Bertani (LB) broth at 37°C. Stock cultures of all bacteria containing 20% glycerol (v/v) were maintained at -20°C.

3.2 Sewage water collection and preparation

P. aeruginosa clinical strain was used as a host organism for enriching and isolating bacteriophages from sewage water samples. The sewage water samples for phage isolation were collected from various sources such as hospitals, animal farms, industries and houses in Ubon Ratchathani, Thailand. In brief, 10 ml of each sewage water sample was centrifuged at 3,000 rpm for 15 min. The supernatant was filtered through a 0.22 μm membrane filter (SartoriousAG, Goettingen, Germany). Five ml of the filtrate was added to an equal volume of double strength LB broth. The 100 μl of an overnight culture of *P. aeruginosa* was added to the mixture and incubated at 37°C for 24 h. After incubation, the culture was centrifuged at 3,000 rpm for 15 min and the supernatant was filtered through a 0.22 μm membrane filter. The resulting filtrate was used for examining the presence of phage by spot test method (Lu et al, 2003).

Table 5 Bacterial strains used in this study

Bacterial strains	Source[a]
Escherichia coli O157:H7 (clinical strain)	SH
Escherichia coli EBSL^{+}(clinical strain)	SH
Staphylococcus epidermidis ATCC12228	ATCC
Shigella dysenteriae DMS2173	DMS
Serratia marcescens ATCC8100	ATCC
Pseudomonas aeruginosa SH01	SH
Pseudomonas aeruginosa ATCC27853	ATCC
Salmonella typhimurium ATCC1331	ATCC
Bacillus cereus ATCC11778	ATCC
Bacillus subtilis ATCC6633	ATCC
Enterobacter aeruginosa ATCC13048	ATCC
Proteus vulgaris ATCC29905	ATCC
Salmonella typhi DMS5784	DMS
Klebsiella pneumonia ATCC27736	ATCC

[a] ATCC, American Type Culture Collection; DMS, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; SH, Sapprasitiprasong hospital, Ubon Ratchathani, Thailand

3.3 Phage detection and host range determination

Spot test method (Lu et al, 2003) was performed for detection of phage in the prepared filtrate samples. This method was used as preliminary screening of phage by observing the occurrence of a clear zone at the area where the tested sample was spotted. In brief, 5 ml of soft agar (0.7% agar) was added to 100 µl of a log-phase host culture, gently mixed, and overlaid onto solidified basal LB agar plate. After allowing the soft agar to solidify for 15 to 20 min, 10 µl of the prepared filtrate sample was spotted onto top agar layer. The spotted sample was allowed to dry and the plate was incubated at 37°C overnight before observing the presence of a clear zone. A clear zone at the spot area, representing the lysis of host cells, indicated the activity of phage. The sample giving a positive result was subjected to plaque assay to confirm the presence of phage.

The spot test method was also used to determine phage host range. All bacterial strains used as hosts in phage host range determination experiments are presented in Table 5.

3.4 Plaque assay

Plaque assay was conducted using the double layer agar plate method of Adams (1959) with some modifications. A phage containing sample was subjected to ten-fold serial dilution using normal saline as a diluent. One hundred µl of the suitable dilution was added to 0.1 ml of log phase *P. aeruginosa* culture. The mixture was incubated for 15 min at 37°C, mixed with 5 ml of soft LB agar (0.7% agar, prewarmed to 60°C) and poured onto the surface of a LB agar plate. The plate was incubated at 37°C overnight to allow plaques to form.

3.5 Plaque purification

P. aeruginosa phage was as follows. After plaque assay, a single plaque was picked from the bacterial host lawn and transferred into a tube containing 10 ml of early log phase *P. aeruginosa* culture in LB broth. The tube was then incubated at 37°C overnight to allow bacterial cell lysis to occur. The phage lysate was centrifuged at 3,000 rpm for 15 min. The supernatant was filtrated through 0.22 µm membrane filter. The resulting filtrate or phage suspension was kept as a phage stock at 4°C.

3.6 Plaque titer determination

Phage titer determination was performed by plaque assay using only the dilution giving 30 to 300 plaques per plate. Plaques formed on bacterial lawns were counted and the numbers were used to calculate the phage titer in plaque forming units per ml (PFU/ml).

3.7 One-step growth experiment

The one-step growth experiment was performed according to the method of Leuschner et al. (1993) with some modifications. A mid-log phase culture of *P. aeruginosa* was centrifuged at 12,000 rpm for 2 min (WiseSpin®CF-10 Personal centrifuge, DAIHAN Scientific).
The bacterial cell pellet was resuspended in one-fifth of the initial volume of fresh LB broth. Phage was added to the bacterial suspension at a multiplicity of infection (MOI) of 0.1 and then allowed to adsorb to the bacterial host cells at 37°C for 30 min. Phage-embedded bacterial cells were harvested by centrifugation at 12,000 rpm for 2 min, and gently resuspended in fresh LB broth. Test sample was taken at 0, 10, 20, 30, 40, 60, 80, 100, 120, 150, 180, 210 and 240 min for phage titer determination.

3.8 Phage genome analysis

The small scale preparation of bacteriophage DNA based on phage precipitation by zinc chloride was performed following the method of Santos (Santos, 1991) with some modifications. Briefly, 60 µl of filter sterilized 2 M $ZnCl_2$ solution was added to 2.94 ml of phage suspension to obtain the final concentration of $ZnCl_2$ of 40 mM. The mixture was incubated at 37°C for 2 min and centrifuged at 10,000 rpm for 5 min. The pellet was resuspended with 500 µl TES buffer (0.1 M Tris-HCl, pH 8; 0.1 M EDTA and 0.3% SDS), mixed by pipetting and incubated at 60°C for 15 min. After incubation, 180 µl of a 3 M potassium acetate solution (pH 5.2) was added, mixed thoroughly and left on ice for 15 min to allow a white, dense precipitate to form. The precipitate was removed by centrifugation for 2 min at 12,000 rpm.
The supernatant was collected to a new tube and mixed with an equal volume of isopropanol. After incubation on ice for 5 min, the solution was centrifuged for 15 min at 12,000 rpm to precipitate phage genome. The pellet was washed with 500 µl of 70% ethanol and dried at room

temperature. The phage genome was resuspended in 50 µl TE buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA) before checking by agarose gel electrophoresis.

For restriction analysis, phage genome was digested with *Kpn*I restriction enzyme and then analyzed by agarose gel electrophoresis.

Agarose gel electrophoresis was performed using 1% w/v of agarose at constant voltage of 100V for 30 min. Gel was stained with 0.5 µg/ml of ethidium bromide for 5 min and visualized under UV transilluminator (Benchtop UV Transilluminators, Ultra-Violet Products Ltd.).

3.9 Phage preparation for electron microscopy

Bacteriophage was concentrated by precipitation with polyethylene glycol (PEG) following the method of Yamamoto et al. (1970). In brief, NaCl was added to the phage suspension to a final concentration of 0.5 M. After continuous mixing by using magnetic stirrer at 4 °C for 1 h, polyethylene glycol 8000 (PEG 8000) was gradually added to the phage susupension, with constant magnetic stirring, to a final concentration of 10% w/v. The mixture was continuously stirred 4°C overnight to allow the phage particles to form a precipitate. Precipitated phage particles were collected by centrifugation at 4 °C for 20 min at 12,000 rpm. Phage pellet was resuspended in sterilized phosphate buffer. To remove PEG 8000 from the phage pellet, 1/5 volume of chloroform was added to the phage pellet and intermittently mixed by inversion for 5 min. The mixture was centrifuged at 10,000 rpm for 5 min to precipitate PEG 8000. The supernatant which was phage suspension was collected into a new sterilized tube for transmission electron microscopy (TEM).

3.10 Transmission electron microscopy (TEM)

For TEM, purified phage was deposited on a carbon-coated Formvar film on copper grids, stained with 2% (w/v) uranyl acetate and examined in a JEOL JEM-1230 Electron microscope (JEOL, Tokyo, Japan) at 80 kV accelerating voltage.

3.11 Bacterial inactivation by single bacteriophage

Bacterial host was cultured in 2 ml of LB broth to obtain a cell concentration of 10^8 CFU/ml. One hundred µl of the cultured bacterial host was diluted in 9 ml of fresh LB broth, resulting in the bacterial culture with a cell concentration of 10^6 CFU/ml which was used as a starting bacterial host in this experiment. Phage was added at a MOI of 0.01, 0.1 and 1. Samples from each MOI were taken at 0, 2, 4, 6, 8, 10, and 12 h for bacterial cell counting. One hundred µl of each sample was subjected to ten-fold serial dilution using normal saline (0.85 % w/v NaCl) as a diluent. One hundred µl of each dilution was spread onto the surface of LB agar. After incubation at room temperature for 15 min to allow the applied solution to absorb to the LB agar, the plate was incubated at 37°C overnight. Bacterial cell number was calculated from the plates containing 30 to 300 bacterial colonies. For the control, fresh LB broth was used instead of phage to add to the bacterial host culture.

3.12 Bacterial inactivation by multiple bacteriophages

Bacterial host was cultured in 2 ml of LB broth to obtain a cell concentration of 10^8 CFU/ml. One hundred µl of the cultured bacterial host was diluted in 9 ml of fresh LB broth, resulting in the bacterial culture with a cell concentration of 10^6 CFU/ml which was used as a starting bacterial host in this experiment. Three ml of bacterial host culture was infected with various combinations of three phages (WS-23, WS-40 and WS-45) as presented in Table 6. Samples were taken at 0, 2, 4, 6, 8, 10, and 12 h for bacterial cell counting using the method described above. For the control, fresh LB broth was used instead of phage combinations to add to the bacterial host culture.

Table 6 Phage combinations used in this study

Combination of phage	Phage (10^4 PFU/ml)	Volume of phage (ml)	Volume of bacteria (10^6 CFU/ml)	Total volume (ml)
1.	WS-23	1.5	3.0	6.0
	WS-40	1.5		6.0
2.	WS-23	1.5	3.0	6.0
	WS-45	1.5		6.0
3.	WS-40	1.5	3.0	6.0
	WS-45	1.5		6.0
4.	WS-23	1.0	3.0	6.0
	WS-40	1.0		6.0
	WS-45	1.0		6.0

CHAPTER 4

RESULTS

4.1 Bacteriophage isolation

Forty six wastewater samples were used to screen for bacteriophages specific to the drug resistant *P. aeruginosa* SH01. By using spot test method, filtrates prepared from only 3 wastewater samples (samples 23, 40 and 45) produced inhibition zones on the lawn of *P. aeruginosa* SH01 (Table 7, Fig. 4). When all of the three filtrates were subjected to plaque assay using *P. aeruginosa* SH01 as a host, they produced clear plaques of 0.1-0.2 mm in diameter, indicating the presence of bacteriophages in the filtrates (Fig. 5). The bacteriophages were designated WS-23, WS-40 and WS-45.

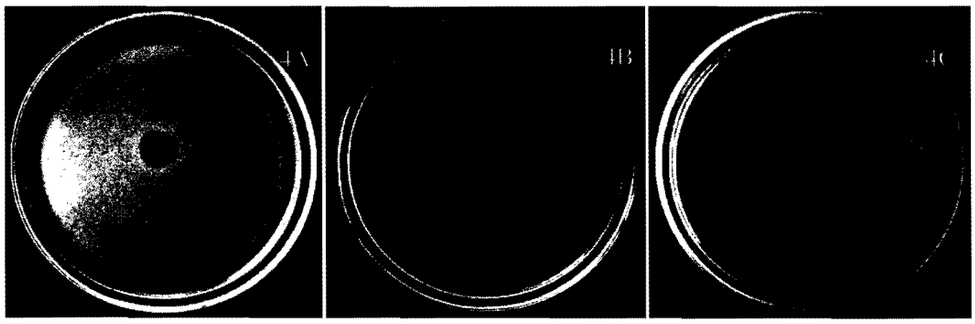


Figure 4 Inhibition zones on the lawn of *P. aeruginosa* SH01 produced by filtrates prepared from wastewater samples 23, 40 and 45 using spot test method

Table 7 Presence of phage in wastewater samples

Wastewater sample	Source	Spot test[a]	Phage	Wastewater sample	Source	Spot test[a]	Phage
01	Hospital	-		24	Animal farm	-	
02	Hospital	-		25	House	-	
03	House	-		26	House	-	
04	House	-		27	House	-	
05	House	-		28	House	-	
06	Hospital	-		29	House	-	
07	Hospital	-		30	House	-	
08	Hospital	-		31	Hospital	-	
09	Hospital	-		32	Hospital	-	
10	Hospital	-		33	House	-	
11	Animal farm	-		34	House	-	
12	Animal farm	-		35	House	-	
13	Hospital	-		36	Animal farm	-	
14	Hospital	-		37	Animal farm	-	
15	Animal farm	-		38	Animal farm	-	
16	Animal farm	-		39	House	-	
17	House	-		40	House	+	WS-40
18	House	-		41	House	-	
19	Animal farm	-		42	House	-	
20	House	-		43	Hospital	-	
21	House	-		44	House	-	
22	House	-		45	House	+	WS-45
23	Animal farm	+	WS-23	46	House	-	

[a] -, No inhibition zone produced; +, inhibition zone produced

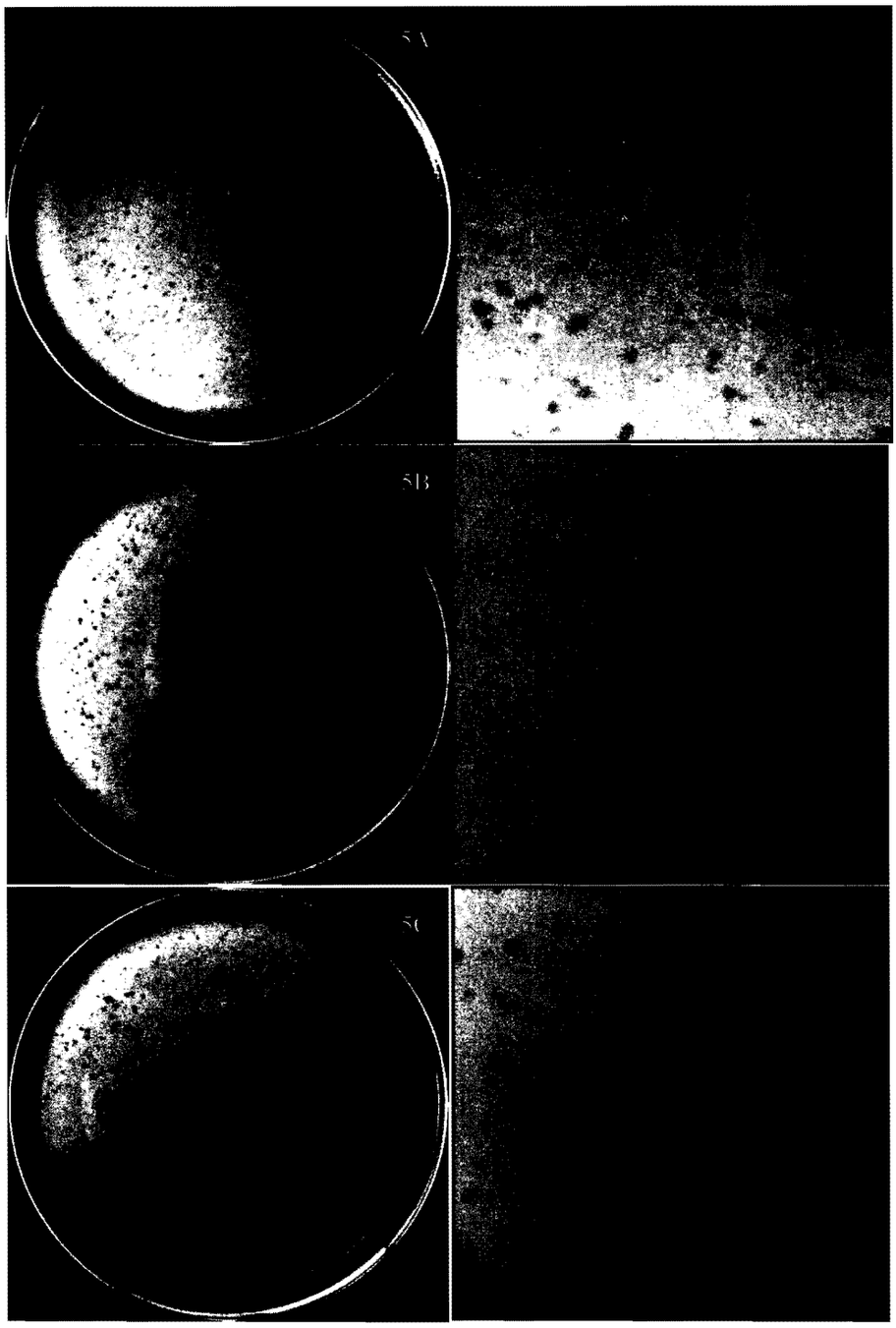


Figure 5 Clear plaque produced by filtrates prepared from wastewater samples 23, 40 and 45 using plaque assay

4.2 Host range determination

All of three isolated bacteriophages were examined for their ability to lyse various species of bacteria listed in Table 8. It was found that all of the bacteriophages were be able to inhibit only *P. aeruginosa* SH01 but not the other bacterial strains used in this study (Table 8). These results suggested that all of the bacteriophages had a very narrow host range.

Table 8 Ability of phages WS-23, WS40 and WS-45 to lyse various bacterial hosts

Hosts	Lysis by bacteriophages[a]		
	WS-23	**WS-40**	**WS-45**
Escherichia coli O157:H7	-	-	-
Escherichia coli EBSL^{+}	-	-	-
Staphylococcus epidermidis ATCC12228	-	-	-
Shigella dysenteriae DMS2173	-	-	-
Serratia marcescens ATCC8100	-	-	-
Pseudomonas aeruginosa SH01	+	+	+
Pseudomonas aeruginosa ATCC27853	-	-	-
Salmonella typhimurium ATCC1331	-	-	-
Bacillus cereus ATCC11778	-	-	-
Bacillus subtilis ATCC6633	-	-	-
Enterobacter aeruginosa ATCC13048	-	-	-
Proteus vulgaris ATCC29905	-	-	-
Salmonella typhi DMS5784	-	-	-
Klebsiella pneumoniae ATCC27736	-	-	-

[a] +, Lysis; -, No lysis

4.3 Bacteriophage genome analysis

Sensitivity to restriction enzymes of genomes extracted from the isolated bacteriophages was studied. It was found that they were digested by all of the restriction enzymes used in this study including *Eco*RI, *Hin*dIII, *Kpn*I (Fig. 6) and *Pst*I. These resulted indicated that the genomes of all isolated bacteriophages were double stranded DNA. Furthermore, the digestion patterns of all 3 genomes by *Kpn*I were different (Fig. 6), suggesting that all of the isolated bacteriophages were not the same type of bacteriophages.

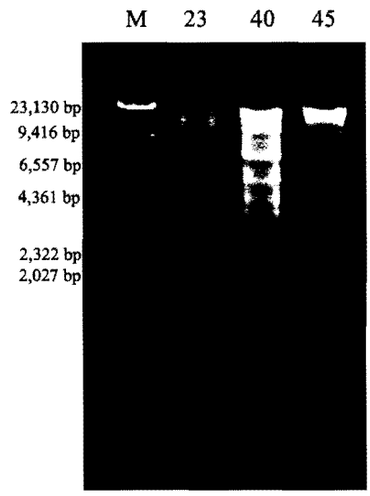


Figure 6 Agarose gel electrophoresis of bacteriophage genomes digested with *Kpn*I (Lane 1 = λ DNA digested with *Hind*III, Lane 2 = phage WS-23, Lane 3 = phage WS-40 and Lane 4 = phage WS-45)

4.4 Transmission electron microscopy of bacteriophage

Morphology analysis by TEM revealed that the bacteriophages WS-23, WS-40 and WS-45 had similar morphological characteristics (Fig.7). All of them had a isometric head and a contractile tail. The isometric heads of the bacteriophages WS-23, WS-40 and WS-45 were 130, 110 and 110 nm in diameter, respectively (Table 9). The contractile tails of the bacteriophages WS-23, WS-40 and WS-45 were 150, 180 and 100 nm in length, respectively (Table 9).
The different dimension of the heads and tails of the isolated bacteriophages emphasized that these bacteriophages were not the same bacteriophages.

Figure 7 Electron micrographs of drug resistant *P. aeruginosa* phages (A, WS-23; B, WS-40 and C, WS-45 phages)

Table 9 Morphological characteristics of bacteriophages WS-23, WS-40 and WS-45 as revealed by TEM

phage	Head		Tail	
	Type	Diameter	Type	Length
WS-23	Isometric	130 nm	Contractile tail	150 nm
WS-40	Isometric	110 nm	Contractile tail	180 nm
WS-45	Isometric	110 nm	Contractile tail	100 nm

4.5 One-step growth kinetics of bacteriophage

One-step growth experiments were performed to verify the latent periods, burst periods and burst sizes of isolated *P. aeruginosa* bacteriophages. As shown in Fig. 8, for each bacteriophage, a triphasic curve, including the latent period, rise period and plateau period, was obtained from the dynamic change in the number of bacteriophage during one replicative cycle. From one-step growth curve depicted in Fig. 8, the bacteriophage WS-23, WS-40 and WS-45 had latent periods of about 30, 40 and 40 min, respectively. The burst periods of the bacteriophages WS-23, WS-40 and WS-45 were approximately 180, 150 and 180 min respectively.

The estimated burst sizes of the bacteriophages WS-23, WS-40 and WS-45 were 45, 76 and 30 PFU/infected cell, respectively.

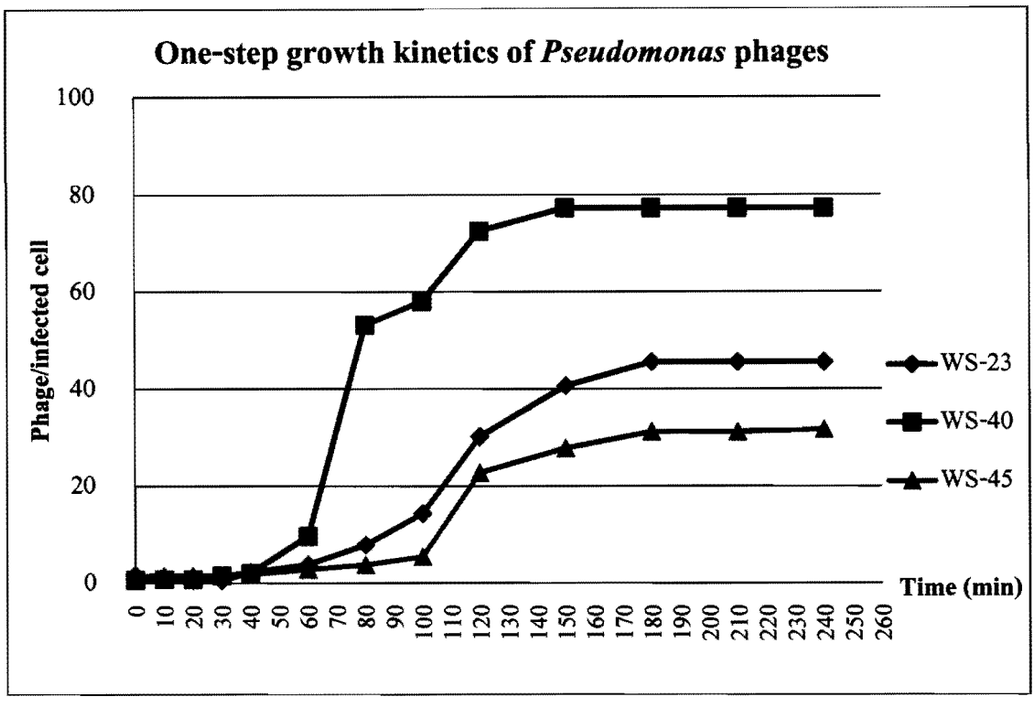
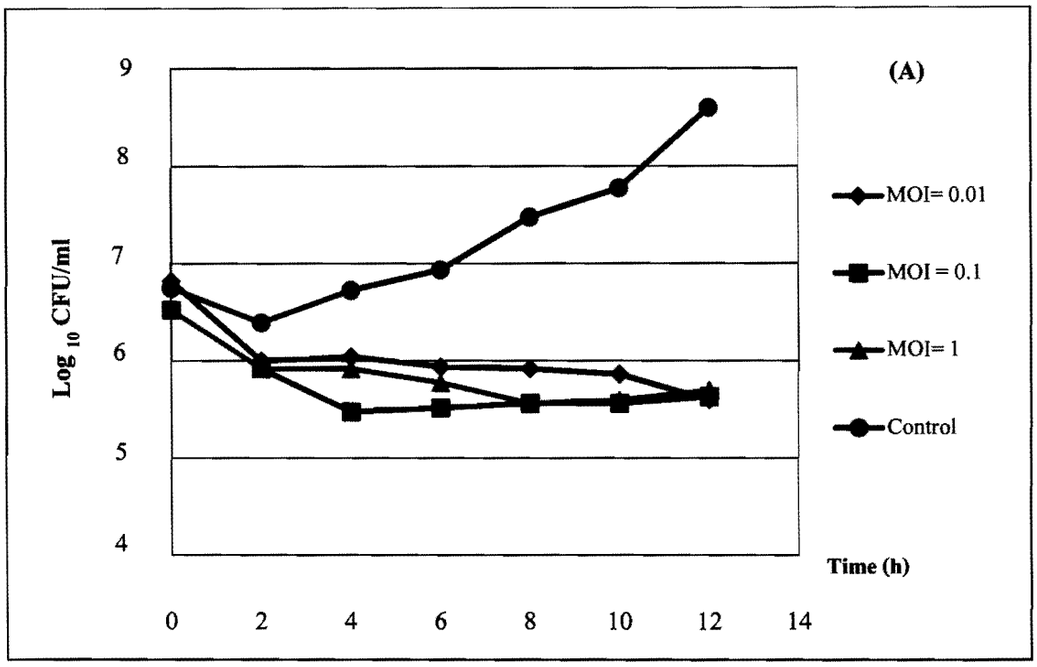
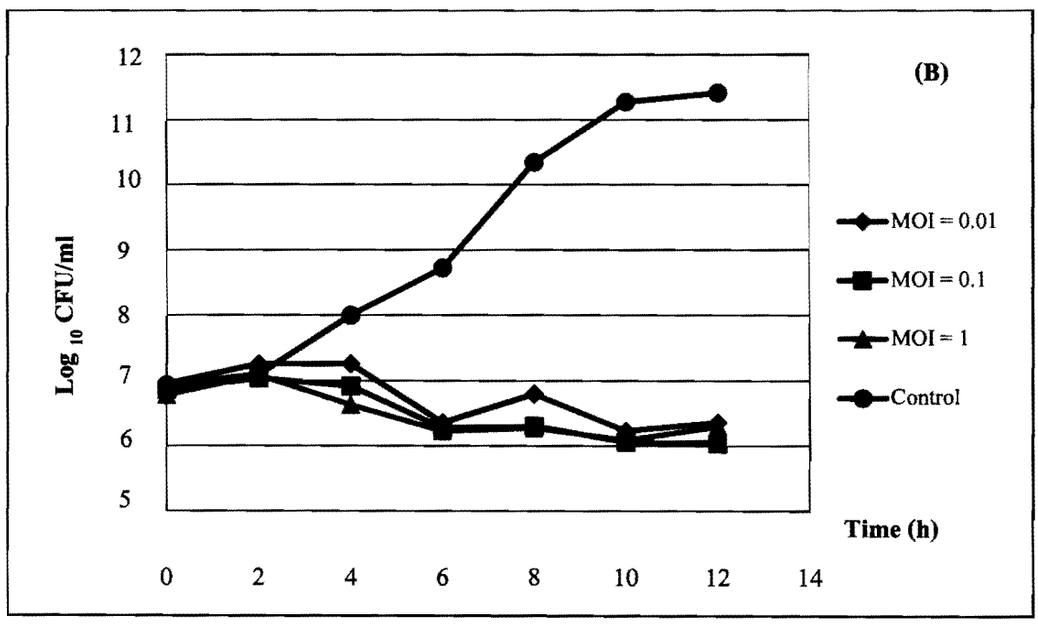


Figure 8 One-step growth curves of bacteriophages WS-23, WS-40 and WS-45

4.6 Bacterial inactivation by single bacteriophage

Examination of inhibitory ability of the bacteriophages WS-23, WS-40 and WS-45 against *P. aeruginosa* SH01 was performed *in vitro* by using individual bacteriophages. When single bacteriophage treatments were conducted, *P. aeruginosa* SH01 at the final concentration of 10^6 CFU/ml was treated either with WS-23, WS-40 and WS-45 at the MOI of 0.01, 0.1 and 1. After 12 h of incubation, MOI independent reduction of *P. aeruginosa* SH01 was obtained from all single bacteriophage treatments. During the course of treatment, the bacteriophage WS-23 caused the reduction of *P. aeruginosa* SH01 by 3 log CFU/ml, compared to the control (no bacteriophage) (Fig. 9A) while the other 2 bacteriophages (WS-40 and WS-45) lowered the number of *P. aeruginosa* SH01 by 6 log CFU/ml, compared to control (Fig. 9B, 9C). From the results of single bacteriophage treatment, the optimal MOI of all bacteriophages used in this study to inhibit *P. aeruginosa* SH01 was 0.01. Therefore, for the double bacteriophage treatments and triple bacteriophage treatments, each bacteriophage was used at the MOI of 0.01.


(A)
Log 10 CFU/ml
9
8
7
6
5
4
0
2
4
6
8
10
12
14
Time (h)
MOI= 0.01
MOI = 0.1
MOI= 1
Control


(B)
Log 10 CFU/ml
12
11
10
9
8
7
6
5
0
2
4
6
8
10
12
14
Time (h)
MOI = 0.01
MOI = 0.1
MOI = 1
Control

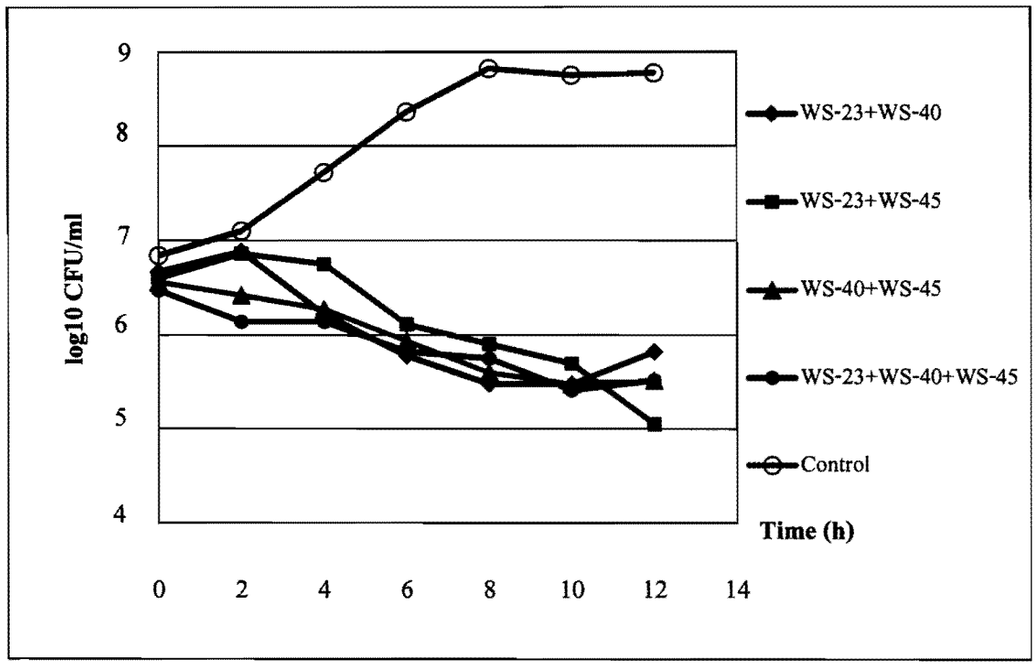


Figure 10 Inhibition of *P. aeruginosa* SH01 by multiple bacteriophages

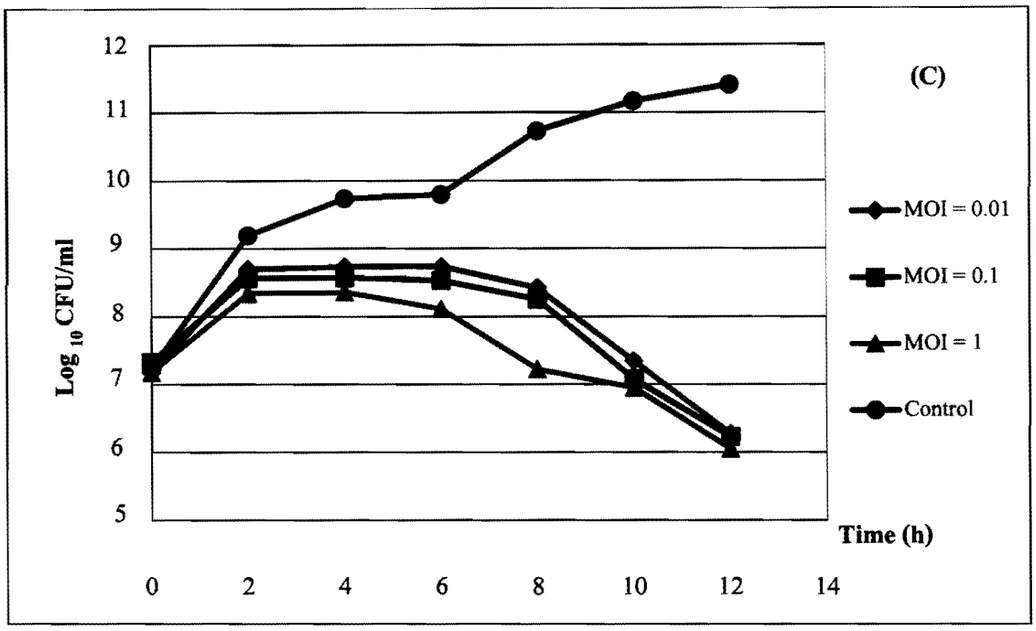


Figure 9 Inhibition of *P. aeruginosa* SH01 by single bacteriophage (A=WS-23, B=WS-40 and C=WS-45)

4.7 Bacterial inactivation by multiple bacteriophages

Inhibition of *P. aeruginosa* SH01 with multiple bacteriophages was performed using double bacteriophages and triple bacteriophages. For double bacteriophage treatment, 3 separate experiments were prepared using WS-23 plus WS-40, WS-23 plus WS-45 and WS-40 plus WS-45 while triple bacteriophage treatment used all of the bacteriophages at the same time. During the 12 h incubation period, all of the four treatments exhibited similar inhibition patterns and by the end of the incubation time, the bacterial cell number was reduced by 3 log CFU/ml, compared to the control for all of the treatments (Fig. 10).

CHAPTER 5

DISCUSSION

Treatment of bacterial infection with antibiotics is considered as a non-surgical technique to prevent or fight against pathogens in the human body. This therapeutic approach has become the most popular treatment, especially for disease caused by bacterial infections because many antibiotics have a broad spectrum of inhibition, each of which can kill a wide variety of bacterial pathogens. Furthermore, most antibiotics are cost effective and readily available. However, antibiotics have some very undersirable properties, such as:

(1) They have side effects that can sometimes prove to be more difficult to manage than the ailment they are meant to cure.

(2) They destroy friendly bacteria along with disease-causing bacteria. The body needs the friendly bacteria for a number of processes like detoxification, easy elimination of wastes and cleansing of the blood and the liver.

(3) Over-use of antibiotics can lead to their therapeutic incompetence due to bacterial mutation. Simply stated, it means that certain bacteria can undergo mutation to tolerate or resist to a particular antibiotic. Penicillin and tetracycline are classic examples of how antibiotics become ineffective in killing some bacteria when they are abused.

Emergence of a large number of antibiotic resistant bacteria in recent years has urged scientists to look for alternative approaches to fight against bacterial infectious diseases. One of the promising approaches is phage therapy.

Phage therapy is the therapeutic and prophylactic use of lytic bacteriophages to treat pathogenic bacterial infection. An important benefit of phage therapy is derived from the observation that bacteriophages are much more specific than most antibiotics that are in clinical use. Theoretically, phage therapy is harmless to the eukaryotic host undergoing therapy, and it should not affect the benefit normal flora of the host.

Phage therapy also has few, if any, side effects, as opposed to drugs, and does not stress the liver. Since phages are self-replicating in their target bacterial cell, a single, small dose is theoretically efficacious. On the other hand, this specificity may also be disadvantageous because a specific phage will only kill a bacterium if it is a match to the specific subspecies. Thus, phage mixtures may be applied to improve the chance of success. Phages are currently being used therapeutically to treat bacterial infections that do not respond to conventional antibiotics. Moreover, they are reported to be especially successful where bacteria have constructed a biofilm composed of a polysaccharide matrix that antibiotics cannot penetrate.

Currently, there are a large number of research works focusing on finding bacteriophages virulent to drug resistant bacteria and this number tends to increase in the future. From previous works, many bacteriophages specific to drug resistant bacteria have been found. Thamniamton et al. (2010) isolated phage Φkpdr1 specific to drug resistant strain of *Klebsiella pneumoniae* DR1 from hospital sewage water. Hsieh et al. (2011) isolated lytic phage Stau2 that strong lytic activity to multi-drug resistant *Staphylococcus aureus* from endotracheal tube that was collected from hospital. In the other hand, Ahiwale et al. (2011) isolated lytic phage BVPaP-3 against biofilm forming multi-drug resistant *P. aeruginosa* from the Pavana river water.

In this study, 3 bacteriophages specific to *P. aeruginosa* SH01 were isolated from 3 different sources. Although *P. aeruginosa* SH01 is a clinical strain isolated from a patient admitted in the hospital, it is not unusual to find its specific bacteriophages in wastewater collected from places other than hospitals. This is because it has been known for a long time that bacteriophages are wide spread in the environment. They can be found in air, soils, water reservoirs, foods, household and hospital wastewater and so on. Several previous works reported the discovery of bacteriophages from places outside from where their specific hosts exist. Bacteriophage JG024 specific to *P. aeruginosa* originally from patient was isolated from sewage (Garbe et al., 2010). Bacteriophage KPO1K2 specific to *Klebsiella pneumoniae* originally from clinical isolation was isolated from sewage (Verma et al., 2009).

In order to classify the isolated bacteriophages, information on their genome and morphology are required. Digestion of the genomes of the bacteriophages WS-23, WS-40 and WS-45 by all of the restriction enzymes used in this study suggested that their genomes were double stranded DNA. By using TEM, all of the bacteriophages were found to have an isometric

with a contractile tail. According to the International Committee on Taxonomy of Viruses, tailed bacteriophages with double stranded DNA are classified in the *Caudovirales* order. This order contains three families, namely, the *Myoviridae* (with long, contractile tail), the *Siphoviridae* (with long, noncontratile tail), and the *Podoviridae* (with short tail). Based on their genomic and morphological characteristics, the bacteriophages WS-23, WS-40 and WS-45 were tentatively classified as a member of *Myoviridae* family. Besides our bacteriophages, several *P. aeruginosa* bacteriophages have been found to be members of the family *Myoviridae* such as *P. aeruginosa* phage PAK-P3 (Merello et al., 2011), *P. aeruginosa* phage JG024 phage (Garbe et al., 2010) and *P. aeruginosa* phage KPP10 (Watanabe et al., 2007) However, bacteriophages specific to *P. aeruginosa* is not restricted to the family *Myoviridae*, many of them were classified as members in the family *Siphoviridae* and *Podoviridae* such as *P. aeruginosa* phage D3 (*Siphoviridae*) (Uchiyama et al., 2008), *P. aeruginosa* phage [φ] KMV(*Podoviridae*) (Lavigne et al., 2003) and *P. aeruginosa* phage O4 (*Podoviridae*) (Li et al., 2010).

Bacteriophages host range is one of the parameters needed to be considered when a bacteriophage is selected to be used as a therapeutic agent. A bacteriophage with a broad host range may not be suitable for therapeutic use because it has a tendency to be virulent to beneficial normal flora residing in human body. However, a bacteriophage with a narrow inhibitory spectrum sometime causes limitation in its therapeutic use. This problem can be overcome by using a cocktail or a combination of several bacteriophages. All of the bacteriophages isolated in this study had a very narrow host range. They inhibited only its specific host, *P. aeruginosa* SH01, but the rest of the tested bacteria. Several bacteriophages were found to possess the same characteristics. Examples of these bacteriophages are bacteriophage PHL4 specific to only *Salmonella enteritidis* (Higgins et al., 2005), bacteriophage KPP10 specific to only *P. aeruginosa* (Watanabe et al., 2007), bacteriophage HP1 specific to only *Helicobacter pylori* (Heinegg et al., 1993).

Use of combination of bacteriophages to treat bacterial infections is not only broadens bacteriophage host range but sometime also increase inhibitory activity against a specific bacterial host. In this study, we designed to test whether the inhibitory activity against *P. aeruginosa* SH01 was increased when double and triple bacteriophages were used to inhibit the bacterial strain,

compared to single bacteriophages treatment. From our results, single bacteriophage treatment with the bacteriophages WS-23, WS-40 and WS-45 exhibited higher inhibitory activity against *P. aeruginosa* SH01 than all of three double bacteriophage treatments and the triple bacteriophage treatment. The decrease in the inhibitory activity against *P. aeruginosa* SH01 when the bacteriophages were used in combination may be due to the incompatibility of these bacteriophages or antagonistic interaction among these bacteriophages. However, more experiments are required to explain this finding. Similar result was found by O'Flynn et al. (2004). They used 3 bacteriophages, e11/2, e4/1c and pp01, to treat *Escherichia coli* O157:H7 as individually and as a mixture of 3 bacteriophages. It was found that all of the treatments produced similar numbers of bacteriophage insensitive mutants. However, the advantage of using bacteriophage mixture over the use of single bacteriophage has also found. Tanji et al. (2005) reported that use of single bacteriophage (SP15, SP21 or SP22) and double bacteriophages caused the regrowth of the specific host, *Escherichia coli* O157:H7, while use of a cocktail of all three bacteriophages did not cause the regrowth of the host. Finding from our study and previous research work indicate that it is not necessary that use of a mixture of bacteriophage will exhibit the better inhibitory activity than use of single bacteriophage. Therefore, it is mandatory to always check before making a decision to use single bacteriophage or multiple bacteriophages for therapeutic purpose.

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APPENDIX

POSTER CONTRIBUTION TO CONFERENCE

NATIONAL CONFERENCE

Rungnapha Wannasutta, Roongtip Iyara, Chayanee Chimpalee, Tranuk Somnate, Parichat Phumkhachorn, Pongsak Rattanachaikunsopon. Characterization of bacteriophages specific to drug resistant *Pseudomnas aeruginosa* isolated from wastewater. The 37th Congress on Science and Technology of Thailand. 231, 2011.

Roongtip Iyara, Rungnapha Wannasutta, Chayanee Chimpalee, Tranuk Somnate, Parichat Phumkhachorn, Pongsak Rattanachaikunsopon. "Isolation of dichlorvos degradging bacteria from agricultural siol", The 37th Congress on Science and Technology of Thailand. 348, 2011.

CHARACTERIZATION OF BACTERIOPHAGES SPECIFIC TO DRUG RESISTANT *PSEUDOMNAS AERUGINOSA* ISOLATED FROM WASTEWATER

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Abstract: Drug resistant bacteria have been a global public health threat because they cannot be controlled by most of antibiotics. However, bacteriophage therapy has recently become a potential approach to control such bacteria. In this study, we intended to isolate and partially characterize lytic bacteriophages specific to drug resistant *Pseudomonas aeruginosa*. Three filtrates prepared from wastewater samples were found to contain lytic bacteriophages as examined by the spot test method and plaque assay. The bacteriophages were designated PPA1, PPA2 and PPA3. They were highly specific to drug resistant *P. aeruginosa* as shown by host range study. When genomes of all three bacteriophages were digested with restriction enzyme *Kpn*I, it was found that their digestion patterns were different, indicating that they were likely to be different bacteriophages. The bacteriophages obtained from this study may be useful for controlling drug resistant *P. aeruginosa* infection.

Introduction: *Pseudomonas aeruginosa* is a Gram-negative bacillus with a single polar flagellum. It is an important opportunistic human pathogen causing several diseases such as urinary tract infection, respiratory system infection, dermatitis, soft tissue infection, bacteremia, bone and joint infection, gastrointestinal infection, ventilator associated pneumonia and a variety of systemic infection particularly in patients with compromised immune system. The most common approach for treating *P. aeruginosa* infections is antibiotic therapy. However, this therapeutic approach has been found not to be effective for many strains of *P. aeruginosa* due to the emergence of drug resistant *P. aeruginosa*. This problem has urged scientists to search for alternative therapeutic approaches to combat infectious diseases caused by the problematic bacteria. One of the potential candidates is bacteriophage therapy. This therapeutic approach uses bacteriophages, bacterial viruses, as antimicrobial agents to inhibit growth or kill bacteria. There have been several reports showing the ability of bacteriophages to kill drug resistant bacteria.[1,2,3] Therefore, it is of interest to find bacteriophages for use as therapeutic agent to control drug resistant *P. aeruginosa*.

This study aims to isolate bacteriophages specific to drug resistant *P. aeruginosa* from wastewater and to partially characterize them in some aspects such as host range and genome. The bacteriophages from this study may be useful as potential therapeutic agents for controlling drug resistant *P. aeruginosa* infection.

Methodology: Wastewater samples for bacteriophage isolation were collected from various sources such as hospitals, animal farms, industries and houses in Ubon Ratchathani, Thailand. Each sample was subjected to bacteriophage enrichment by mixing the sample with an equal volume of double strength BHI broth containing drug resistant *P. aeruginosa*, kindly donated from Sappasitiprasong Hospital, Ubon Ratchathani, Thailand. After incubation at 37°C overnight, the culture was centrifuged at 4,500 xg for 10 min and the supernatant was filtered through a membrane with a pore size of 0.22 μm. The presence of a lytic bacteriophage in the filtrate was examined by using the spot test method. Overnight culture of host bacterial strain was smeared thoroughly on a BHI agar plate by a sterile swab. Five μl of the filtrate was spotted on the bacterial lawn. The plate was incubated at 37°C overnight. A clear zone in the plate indicated the presence of bacteriophage. The clear zone producing filtrates were confirmed for the presence of bacteriophage by plaque assay which were performed as described by Lu *et al.*[4] Each purified bacteriophage was obtained from single plaque. Spot test method was also used for host range study using a variety of host bacterial strains as listed in Table 1.

Bacteriophage nucleic acid was extracted by using PureLink Viral RNA/DNA Mini Kit (Invitrogen, USA). For restriction analysis, bacteriophage nucleic acid was digested with *Kpn*I and then analyzed by agarose (0.8% w/v) gel electrophoresis at constant voltage of 100V.

Results, Discussion and Conclusion: Filtrates prepared from 3 wastewater samples were found to produce clear zones on lawns of drug resistant *P. aeruginosa* by spot test (Figure 1 and Table 2). By using plaque assay, clear plaques were obtained from all filtrates indicating that bacteriophages in the filtrates responsible of antibacterial activity were lytic bacteriophages (Figure 2). The bacteriophages were designated as PPA1 to PPA3. All of them were highly specific to the drug resistant *P. aeruginosa* as shown by host range study (Table 1). By using agarose gel electrophoresis, it was found that *Kpn*I could digest nucleic acid isolated from all of the bacteriophages (Figure 3) indicating that their genomes were double stranded DNA. Since all of the bacteriophages had different restriction digestion patterns (Figure 3), they were likely to be different bacteriophages although they had the same host range. However, to confirm whether the bacteriophages are different, morphological study of the

bacteriophages by transmission electron microscope (TEM) and determination of their genome size are required. These studies are underway in our laboratory. The bacteriophages obtained from this study will be used individually an in combination with each other to test for the ability to control the drug resistant *P. aeruginosa* both *in vitro* and in animal models.

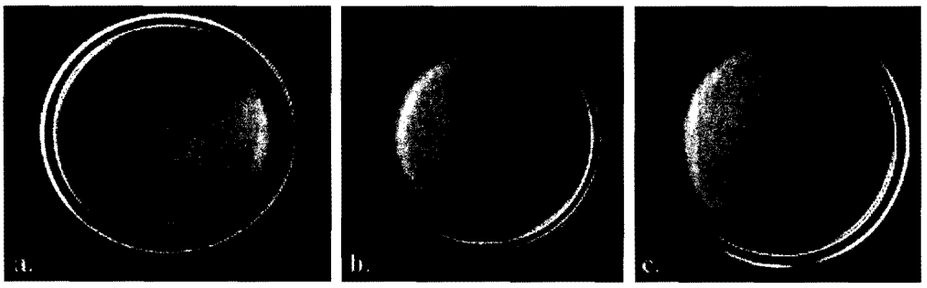

Figure 1. A clear zone on a lawn of drug resistant *P. aeruginosa* as examined by the spot test method. a, b and c = filtrates containing bacteriophages PPA1, PPA2 and PPA 3, respectively

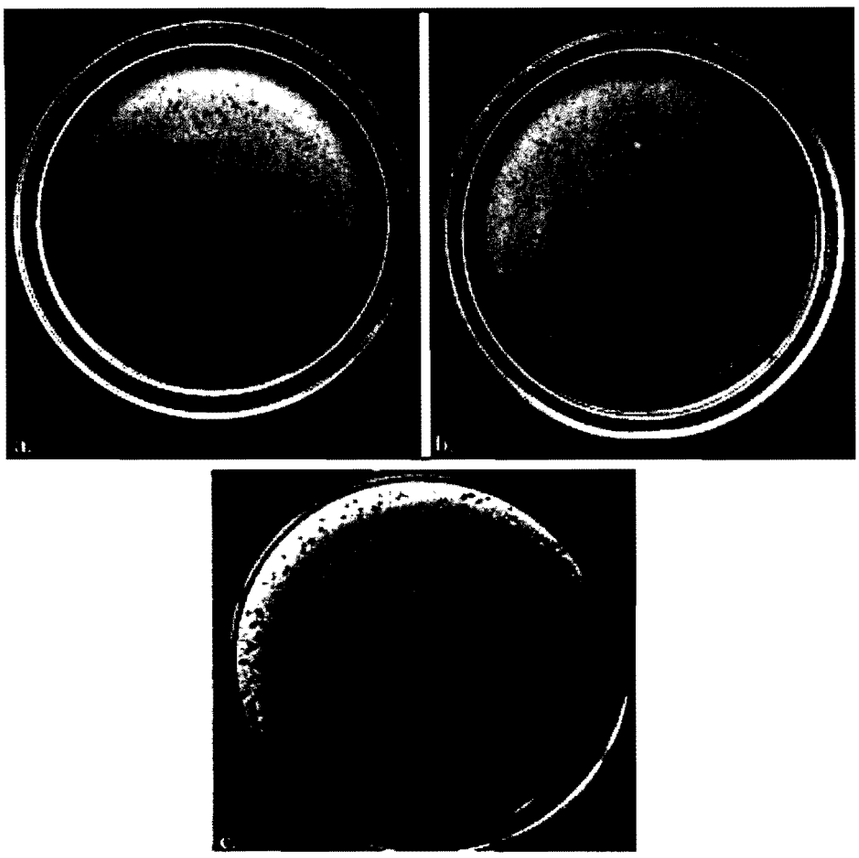

Figure 2. Clear plaques on a lawn of drug resistant *P. aeruginosa* as examined by plaque assay. a, b and c = filtrates containing bacteriophages PPA1, PPA2 and PPA 3, respectively

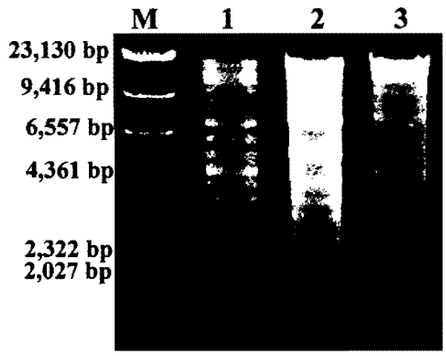

Figure 3 Restriction digestion patterns of bacteriophage DNA analyzed by agarose gel electrophoresis. M = λ DNA digested with *Hin*dIII; 1, 2 and 3 = *Kpn*I digested DNA of bacteriophages PPA1, PPA2 and PPA3, respectively.

Table 1. Host range of bacteriophages PPA1, PPA2 and PPA3

Bacterial host	Bacteriophage		
	PPA1	PPA2	PPA3
Bacillus cereus ATCC 11778	-	-	-
Bacillus subtilis ATCC 6633	-	-	-
Enterobacter aeruginosa ATCC 13048	-	-	-
Escherichia coli EBSL+[a]	-	-	-
Escherichia coli O157:H7 ATCC 35150	-	-	-
Klebsiella pneumoniae ATCC 27736	-	-	-
Proteus vulgaris ATCC 29905	-	-	-
Pseudomonas aeruginosa (drug resistant)[a]	+	+	+
Pseudomonas aeruginosa ATCC 27853	-	-	-
Salmonella typhi DMS 5784	-	-	-
Salmonella typhimurium ATCC 1331	-	-	-
Serratia marcescens ATCC 8100	-	-	-
Shigella dysenteriae DMS 2173	-	-	-
Staphylococcus epidermidis ATCC 12228	-	-	-

[a] Clinical strain donated from Sappasitiprasong Hospital, Ubon Ratchathani, Thailand

Table 2. Source of isolated bacteriophages PPA1, PPA2 and PPA3

Wastewater sample	Source	Isolated bacteriophage
1	Sappasitiprasong Hospital	PPA1
2	Animal farm	-
3	Animal farm	PPA2
4	Textile factory	-
5	Meatball factory	-
6	Fermented rice noodle factory	PPA3
7	Household	-
8	Household	-
9	Household	-

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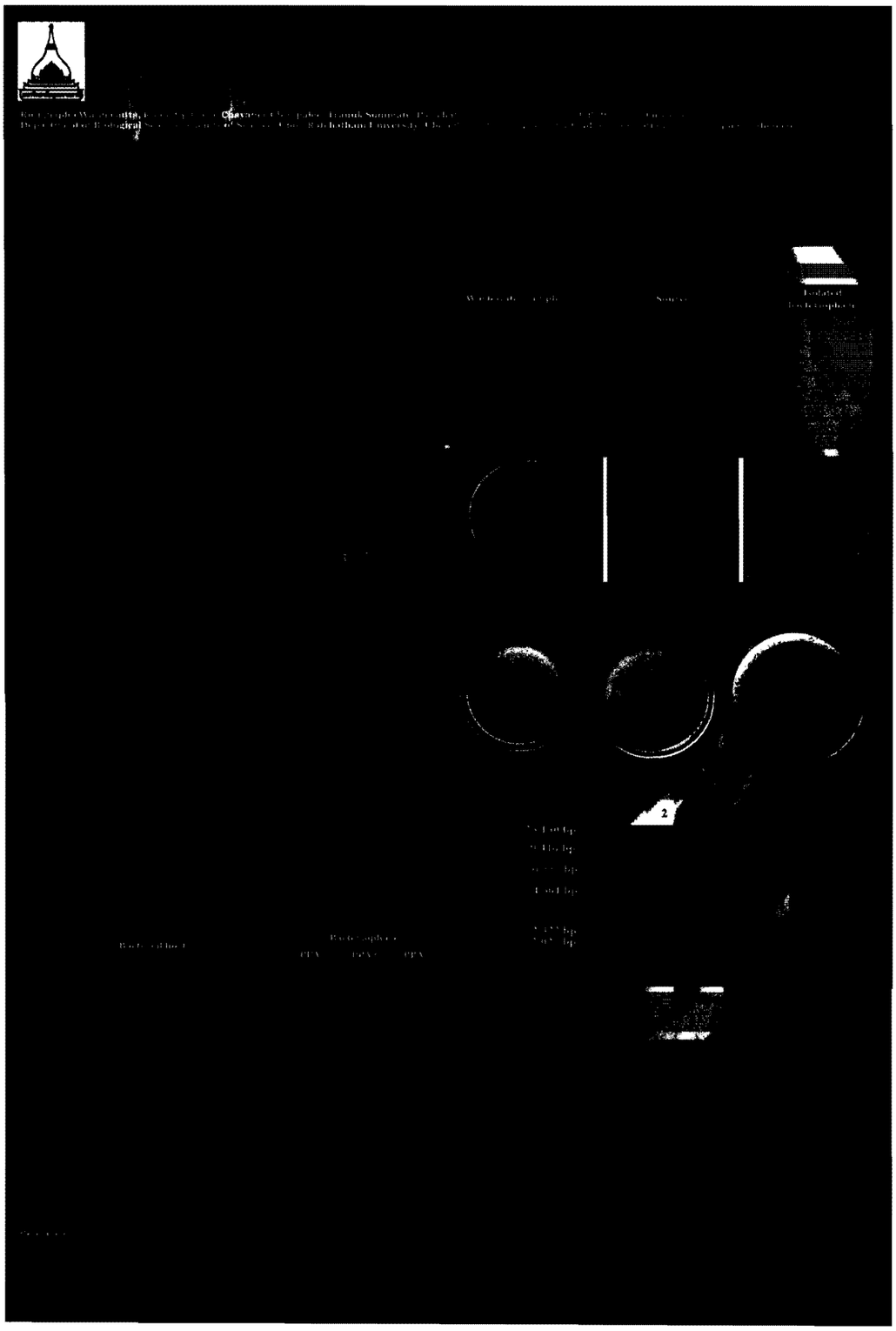
Keywords: Bacteriophage, Bacteriophage therapy, drug resistant *Pseudomonas aeruginosa*



ISOLATION OF DICHLORVOS DEGRADING BACTERIA FROM AGRICULTURAL SOIL

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Abstract: The objective of this study is to isolate bacteria with ability to degrade dichlorvos, an insecticide commonly used in agriculture. From the selection of bacteria isolated from agricultural soil samples on the mineral salt agar containing only dichlorvos as sole carbon source, six isolates of bacteria (DD1 to DD6) were found to be able to grow on the agar. Three of the bacteria (DD1, DD2 and DD3) were Gram-negative bacilli while the rest of them (DD4, DD5 and DD6) were Gram-positive cocci. DD1 and DD3 were found to be more tolerant to dichlorvos than the other isolated bacteria. Ability to grow in the mineral salt medium supplemented with 1,000 ppm of dichlorvos was observed in DD1 and DD3 but not in the rest of the bacteria. From plasmid isolation, all of the bacteria, except DD3, contained no plasmid. The bacterium DD3 harbored a plasmid with a size of approximately 2.3 Kb. Plasmid curing analysis revealed that the plasmid free derivative of DD3 still maintained its ability to grow on the mineral salt agar containing only dichlorvos as sole carbon source. These results suggested that genes responsible for dichlorvos degrading ability of all bacteria were localized on their chromosomes. The bacteria obtained from this study may be useful as bioremediation agents to obliterate dichlorvos from environment.

Introduction: Dichlorvos is one of the most commonly used organophosphate insecticides in agriculture in Thailand. However, the use of dichlorvos has become a public concern because it can pose potential threat to environment and to non-target organisms. The application of dichlorvos in agricultural areas not only results in its contamination of soil at the target sites, but also leads to the contamination of aquatic environment via soil percolation, air drift, and surface run-off.[1, 2] The excessive and frequent application of dichlorvos may result in high levels of its residues accumulated on agricultural crops, posing a potential health hazard to consumers.

Bioremediation of soil and water contaminated with organophosphate insecticides by using bacteria to degrade the insecticides has become of great interest because the approach has considered to be safe, inexpensive and effective. Since many strains of bacteria have been found to be able to degrade organophosphate insecticides, it is of

interest to find a bacterial strain capable of degrading dichlorvos for bioremediation. Genes responsible for degradation of organophosphate insecticides have been found in both bacterial chromosomes and plasmids.[3] This study aims to isolate dichlorvos degrading bacteria from agricultural soil, to partially identify the isolated bacteria and to determine the location of genes responsible for dichlorvos degradation. The bacteria obtained from this study may be useful for bioremediation of soil and water contaminated with dichlorvos.

Methodology: Agricultural soil samples were collected from paddy fields treated with dichlorvos annually for the last 5 years in Ubon Ratchathani province, Thailand. The soil samples were subjected for the isolation of dichlorvos degrading bacteria on mineral salt agar [KH_2PO_4 (0.2 g), K_2HPO_4 (0.8 g), $MgSO_4.7H_2O$(0.2 g), $CaSO_4$(0.1 g), $(NH_4)_6Mo_7O_{24}.4H_2O$ (0.001 g), $(NH_4)_2SO_4$ (5.0 g) and agar (1.5%)] supplemented with 100 ppm of dichlorvos as a carbon source.

The isolated bacteria were evaluated for their survival ability in mineral salt medium containing different concentrations of dichlorvos ranging from 100 to 1,000 ppm. The minimal inhibitory concentration (MIC) of dichlorvos for each bacterial isolate was taken as the lowest concentration of dichlorvos that resulted in the absence of detectable growth.

The morphology of the dichlorvos degrading bacteria was investigated by light microscopy after Gram staining.

Plasmid isolation was performed using the method of Anderson and McKay.[4] Plasmid DNA was analyzed by agarose gel elctrophoresis on 1.0% (w/v) agarose gel in Tris-acetate buffer at 60 V. The DNA was visualized on a UV transilluminator after staining with 0.5 μg/ml of ethidium bromide solution.

Plasmid containing bacterium (DD3) was subjected to plasmid curing using ethidium bromide as a plasmid curing agent. The plasmid cured derivative (plasmid free derivative) of DD3 was tested for its ability to degrade dichlorvos on mineral salt medium supplemented with 100 ppm of dichlorvos.

Results, Discussion and Conclusion: Six bacterial colonies were found to be able to grow on the mineral salt agar supplemented with 100 ppm of dichlorvos as a carbon source. Because all of the bacteria grew on the medium having only dichlorvos as sole carbon source, they were considered as dichlorvos degrading bacteria and designated DD1 to DD6. Three of the bacteria (DD1 to DD3) were Gram-negative bacilli while the rest of them (DD4 to DD6) were Gram-positive cocci (Table 1 and Figure 1). DD1

and DD3 were more tolerant to dichlorvos than the other isolated bacteria as indicated by their MIC values (Table 1). To determine the location of dichlorvos degrading genes in all of the bacteria, plasmid isolation and plasmid curing were performed. Plasmid isolation revealed that DD1, DD2, DD4, DD5 and DD6 did not carry a plasmid indicating that genes responsible for their dichlorvos degrading ability are localized on chromosome. However DD3 was found to have a plasmid with a size of approximately 2.3 kb (Figure 2). The plasmid cured derivative (plasmid free derivative) of DD3 was able to grow on mineral salt medium supplemented with 100 ppm of dichlorvos, indicating that the gene responsible for their dichlorvos degrading ability was likely localized on chromosome.

Table 1. Some characteristics of dichlorvos degrading bacteria isolated from agricultural soil

Bacterial isolates	Gram staining	Morphology	MIC (ppm)
DD1	Gram-negative	Bacillus	1,000
DD2	Gram-negative	Bacillus	500
DD3	Gram-negative	Bacillus	1,000
DD4	Gram-positive	Coccus	500
DD5	Gram-positive	Coccus	500
DD6	Gram-positive	Coccus	500

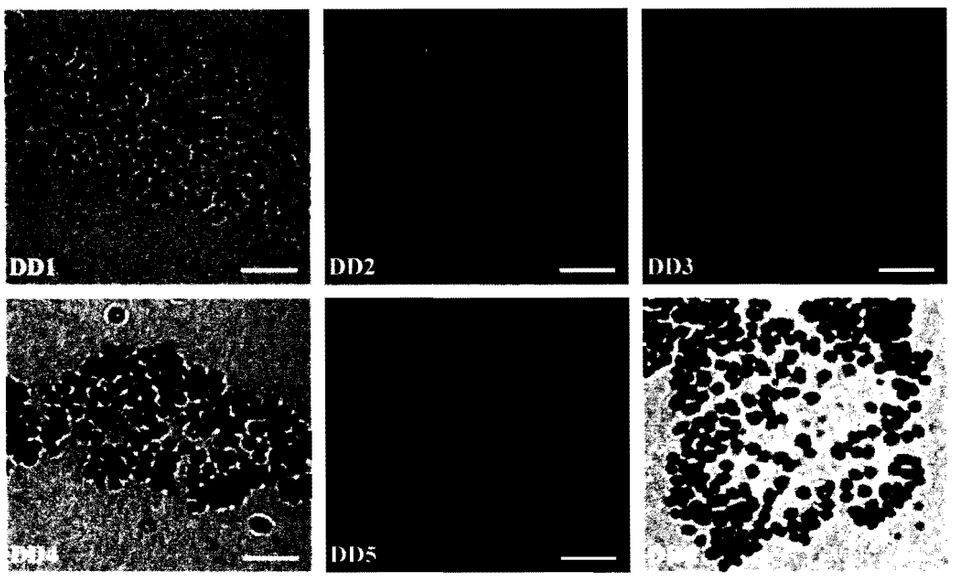

Figure 1. Gram staining and morphology of dichlorvos degrading bacteria DD1 to DD6 as studied by light microscope (Bar = 5 μm)

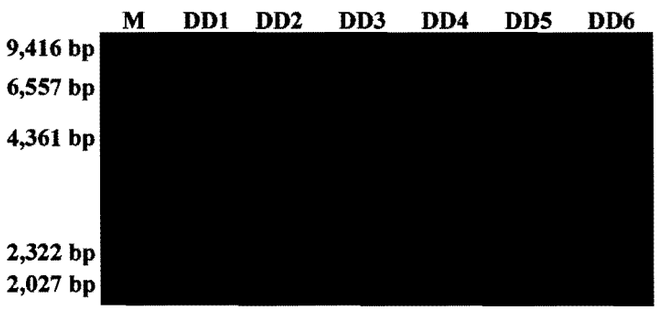

Figure 2. Plasmid isolated from dichlorvos degrading bacteria DD1 to DD6 (M = lambda DNA digested with *Hin*dIII)

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