CHARACTERIZATION OF BACTERIOPHAGE KA ISOLATED FROM HOT SPRING AT KUALA WOH, PERAK

By

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ABSTRACT

CHARACTERIZATION OF BACTERIOPHAGE KA ISOLATED FROM HOT SPRING AT KUALA WOH, PERAK

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Bacteriophages are obligate intracellular parasites targeting bacteria. There are 13 families of bacteriophages which have been classified based on morphology, nucleic acid, and presence of envelope. The potential of bacteriophage as an alternative biocontrol agent has recently been revisited due to the occurrence of antibiotic-resistant bacteria. Bacteriophage therapy can be very effective in certain conditions and has advantages over antibiotics. Characterization is a preliminary step towards designing a bacteriophage for biocontrol. Hence, this study focused on characterization of bacteriophage KA isolated from a hot spring at Kuala Woh, Perak based on physiological properties, partial genomic properties and morphology. Bacteriophage KA isolates were grown against *E. coli* BL21 (DE3) and precipitated using polyethylene glycol (PEG). Physiological properties such as host range, temperature and pH stability were tested. Nucleic acid properties of bacteriophage KA were determined by DNase and RNase digestion of the extracted nucleic acid, followed by restriction enzymes digestion. PEG-precipitated bacteriophage was purified using density gradient caesium

chloride ultracentrifugation prior to transmission electron microscopy. Results showed that the buoyant density of bacteriophage KA is approximately 1.4 g/mL. Bacteriophage KA which is specific towards *E. coli* BL21 (DE3) and *Shigella boydii* could not tolerate 60°C and above, and it favours a neutral environment around pH 6 to pH 8. Bacteriophage KA contains a DNA genome with size larger than 10,000 base pairs. However, it was shown that its genome does not have restriction sites for *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, *Sac*I and *Xba*I. As a result, the bacteriophage genome could not be deduced as double-stranded DNA. Transmission electron microscopy showed that the bacteriophage has an icosahedral head and a long non-contractile tail, which are characteristics of the family *Siphoviridae*. Therefore, further characterization of bacteriophage KA is necessary as it may harbour potential enzymes that are useful in medical and biopharmaceutical industries.

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DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at Universiti Tunku Abdul Rahman or other institutions.

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CHU SAY YING

APPROVAL SHEET

This project report entitled "**CHARACTERIZATION OF BACTERIOPHAGE KA ISOLATED FROM HOT SPRING AT KUALA WOH, PERAK**" was prepared by CHU SAY YING and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

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PERMISSION SHEET

It is hereby certified that **CHU SAY YING** (ID Number: **13ADB03315**) has completed this final year project entitled "**CHARACTERIZATION OF BACTERIOPHAGE KA ISOLATED FROM HOT SPRING AT KUALA WOH, PERAK**" under the supervision of Dr. Michelle Ng Yeen Tan (supervisor) from the Department of Biomedical Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project dissertation in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

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(CHU SAY YING)

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LIST OF ABBREVIATIONS

×*g*	Times gravity (acceleration due to gravity)
°C	Degree Celcius
%	Percentage
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
bp	Base pair
CsCl	Caesium chloride
dsDNA	Double-stranded deoxyribonucleic acid
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
g	Gram
g/mL	Gram per millilitre
h	Hour
HCl	Hydrochloric acid
ICTV	International Committee on Taxonomy of Viruses
Kb	Kilo base pair
L	Litre
LB	Luria Bertani
LPS	Lipopolysaccharide
mL	Millilitres
mM	Millimolar
min	Minutes

MWCO	Molecular weight cut off
NaCl	Sodium chloride
nm	Nanometer
oc	Open circular
OD_{600}	Optical density measure at wavelength 600 nm
PAGE	Polyacrylamide gel electrophoresis
pfu/mL	Plaque forming units per millilitre
PEG	Polyethylene glycol
pH	Power of hydrogen
RNA	Ribonucleic acid
rpm	Revolution per minute
s	seconds
SDS	Sodium dodecyl sulphate
S. boydii	*Shigella boydii*
S. dystenteriae	*Shigella dysenteriae*
S. flexneri	*Shigella flexneri*
S. sonnei	*Shigella sonnei*
spp.	All species in a given genus
ssDNA	Single-stranded deoxyribonucleic acid
TBS	Tris-buffered saline
µg	Microgram
µL	Microlitre
V	Volts
w/v	Weight per volume
w/w	Weight per weight

CHAPTER 1

INTRODUCTION

Bacteriophages, commonly known as phages, are viruses, which are obligate intracellular parasites of bacteria. They cannot survive on their own, and therefore they are not considered a living organism by biologists. However, they can reproduce within host cells by hijacking the host's protein synthesis machinery, often with devastating results to the host organism (Rakhuba, et al., 2010).

Bacteriophages are commonly found in the environment and they can be easily isolated from sea water, soils, sewage, fresh water and river (Sundar, et al., 2009). Bacteriophages are the most abundant form of life, there are around 1,031 bacteriophages present on Earth. However, out of these phages, only a few hundreds of phages have been sequenced (Harper, et al., 2014). Meanwhile, most bacteriophages genes remained unknown as an estimation of less than 0.0002% of the all the phage metagenome has been sampled. (Rohwer, 2003). Many of the genes identified are ORFs (open reading frames) among the genomes that have been sequenced, which exhibit no similarity with other organisms. This could be resulted from the high diversity nature of bacteriophages. As a result, it is tremendously challenging in terms of mapping the functional elements of bacteriophage (Kumar, et al., 2002). Due

to the massive and readily available diversity of bacteriophages, sampling from the environment is useful to isolate and characterize naturally evolved bacteriophages for almost any applications in the industries (Kelly, et al., 2011).

Recently, there has been an increased concern in the application of bacteriophage as an alternative antimicrobial therapy in various fields such as treating human infections, veterinary usage, agricultural and food safety. In certain conditions, phage therapy are highly effective and has several special advantages as compared to antibiotics (Tan, Chan and Lee, 2014). For instance, phages have special benefit for localized application, as they can penetrate deeper into a localized area as long as the infection is still there, instead of reduced quickly in concentration of the phage which occur in antibiotics. Once the specific bacteria host they targeting are killed, the phages stop reproducing. Moreover, secondary resistance is less likely to develop in phages, which is relatively common in antibiotics. Therefore, due to the increasing development of antibiotic resistant bacteria and challenges in designing new classes of antibiotics to act on antibiotic resistant bacteria, it is essential to apply phages to target the bacterial infections (Phage Therapy Centre, 2016).

According to Ackermann (2011), classification is important to facilitate comparisons and understanding of all the possible phages found on Earth. Classification is also necessary for identification of novel and therapeutic phages, as well as those, which are harmful in biotechnology and industrial

fermentations. In addition, classification also helps to identify industrially important phages particularly in patent application purposes.

Therefore, the objective of this study was to characterize an unknown phage KA isolated from a hot spring located at Kuala Woh, Perak. The phage was characterized based on its host range, physiological properties, partial genomic properties and morphology for its potential application in medical field as well as in other industries.

CHAPTER 2

LITERATURE REVIEW

2.1 General Overview of Bacteriophage (Phage)

2.1.1 Biology of Phage

Viruses are tremendously tiny particles that are infectious. They are not noticeable under a light microscope. They occur in a many different forms and target nearly all living systems such as bacteria, insects, plants and animals. The viruses that target and hijack resources of bacteria are known as bacteriophages, commonly referred as “phages”. The word “bacteriophage” indicates “to eat bacteria”, because virulent bacteriophages can lyse susceptible bacterial cells. Bacteriophages are obligate intracellular parasites, meaning that they could not survive outside a host. They can only survive and multiply inside bacteria by hijacking the host biosynthetic machinery (Orlova, 2012).

Bacteriophages, similar to bacteria, are very commonly found in all natural environments such as soil, sediments, water (both river and seawater), and in living or dead plants or animals. Essentially, phages can be isolated from almost any environment that can support bacterial growth, thus bacteriophages are directly related to presence or the bacteria population. On Earth, they are

considered the most abundant 'life' forms as there are an estimated of 10^{32} bacteriophages that can be found on the planet (Elbreki, et al., 2014). For instance, it is estimated that aquatic environments have a total phage population above 10^{31}. Many terrestrial ecosystems have been shown to harbour 10^{7} phages per gram of soil; and sewage is known to contain 10^{8} to 10^{10} phage per millilitre (Danovaro, et al., 2011).

2.1.2 Basic Architecture of Phage

The most general structure of bacteriophages are the head or capsids that act as a protective covering for the genome hidden inside the capsid and some supportive structures that provide interface with membrane of a bacterium in order to release the phage genome (Negash and Ejo, 2016). Figure 2.1 shows the structure of a T4 bacteriophage.

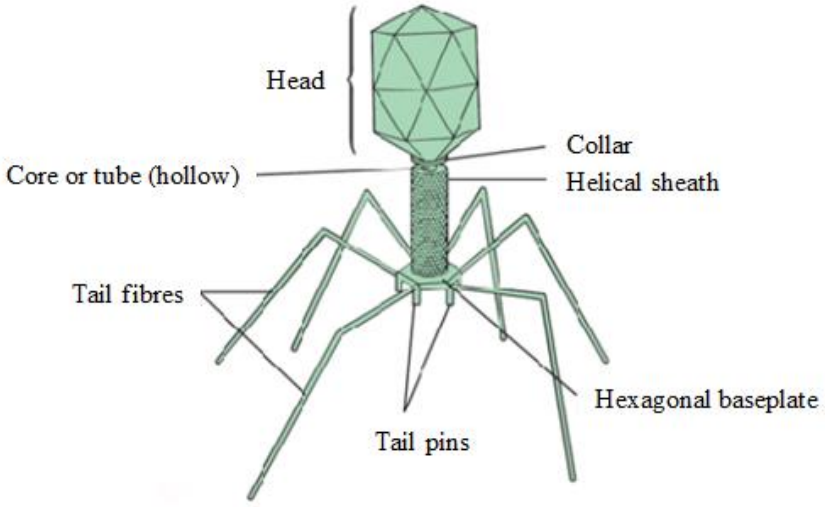

Figure 2.1: Structure of a T4 bacteriophage.

Bacteriophages range significantly in terms of their complexity, from simple viruses to complicated or complex viruses. Size of most phages range from 24 to 200 nm in length. Moreover, phage genome size can range from 5 kbp such as bacteriophage *f*X174 (Sanger, et al., 2007) to more than 280 kbp such as bacteriophage *f*KZ (Mesyanzhinov, et al., 2002). T4 is among the largest phages, with about 200 nm in length and 80 to 100 nm in width (Leiman, et al., 2003).

All phages consist of a capsid structure which varies in size and shape. Shape of some phages can be icosahedral, helical or filamentous. The head or capsid protecting its nucleic acid can be composed of DNA or RNA. Moreover, the capsid is made of numerous copies of one or even more distinct proteins (Mayer, 2016). The nucleic acid and capsid together are known as the nucleocapsid. Some viruses consists of an envelope that covers around the protein capsid. This envelope is shaped from the host cell's lipid membrane when the virus is released out from the host cell (Hans, 1996).

Most phages consist of tails that is bound to the head structure. The tail is a hollow tube for the passage of nucleic acid during infection process. The tail size may varies and there are minority of phages which are not tailed. For example, in the T4 phage which is a complex phage, the tail is wrapped by a contractile sheath which would contracts during the infection process of a bacterium (VanMeter, Hubert and William, 2010). There is a base plate as

well as tail fibres attached to the end of the tail of complex phages like T4. The tail penetrates through the bacterium cell envelope during infection, subsequently, nucleic acids translocate from the phage into the host cell. In addition, there are host cell receptor recognition molecules which are attached to the tail, which facilitates host recognition as well as nucleic acid delivery (Yap and Rossmann, 2014).

2.2 Classifications of Phage

2.2.1 Morphology

There are approximately 5,100 phages which had been reported. They are categorized into 13 families as shown in Figure 2.2 according to the morphology, nucleic acid type, and presence of a lipid or envelope (Matsuzaki, et al., 2005).

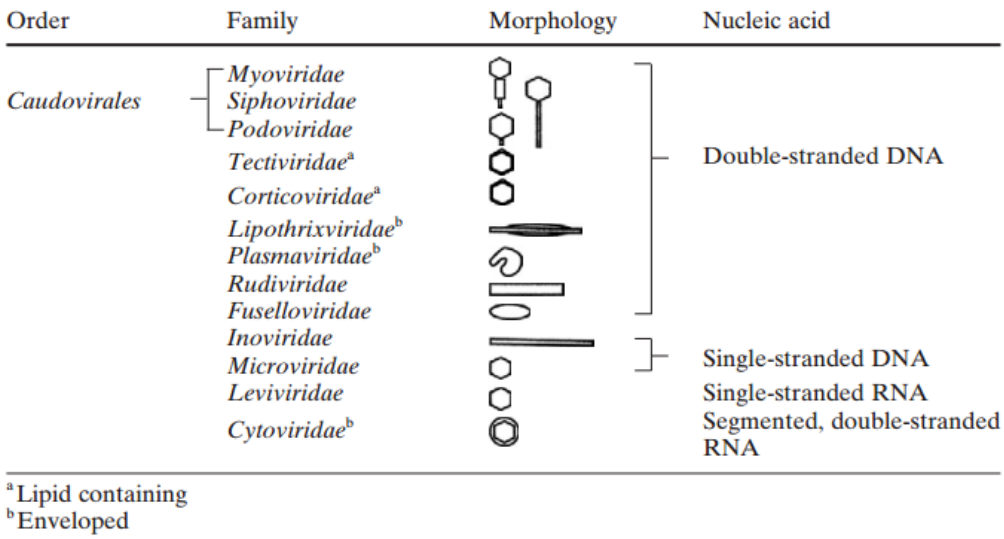

Figure 2.2: Classification of bacteriophage based on morphology, nucleic acid type, and presence of a lipid or envelope (Matsuzaki, et al., 2005).

Tailed and double-stranded DNA bacteriophages account for 96% of all identified bacteriophages which contributes to the majority of phages on the planet. They consist of either an isometric or a prolate head with an attached tail (Xiang and Rossmann, 2011). According to Fokine and Rossmann (2014), tailed phages belong to the order *Caudovirales* and are categorized into three families based on their morphological features of the tail which are *Myoviridae* with long rigid contractile tails, *Podoviridae* with short contractile tails and *Siphoviridae* with long flexible non-contractile tails. The genome of *Caudovirales* is has a special set of modules with distinctive history of evolution that have been swapped among phages (Swanson, et al., 2012). Figure 2.3 shows the electron micrographs of some examples of tailed phages. KVP20, KVP40 and KVP241 belong to the *Myoviridae* family whereas ϕMR11 is a *Staphylococccus* phage which belongs to the *Siphoviridae* family (Matsuzaki, et al., 2005). Since tailed phages are the biggest population of bacteriophages, they are easily isolated and they are also the most studied phages in terms of their biochemical and structural aspects (Swanson, et al., 2012).

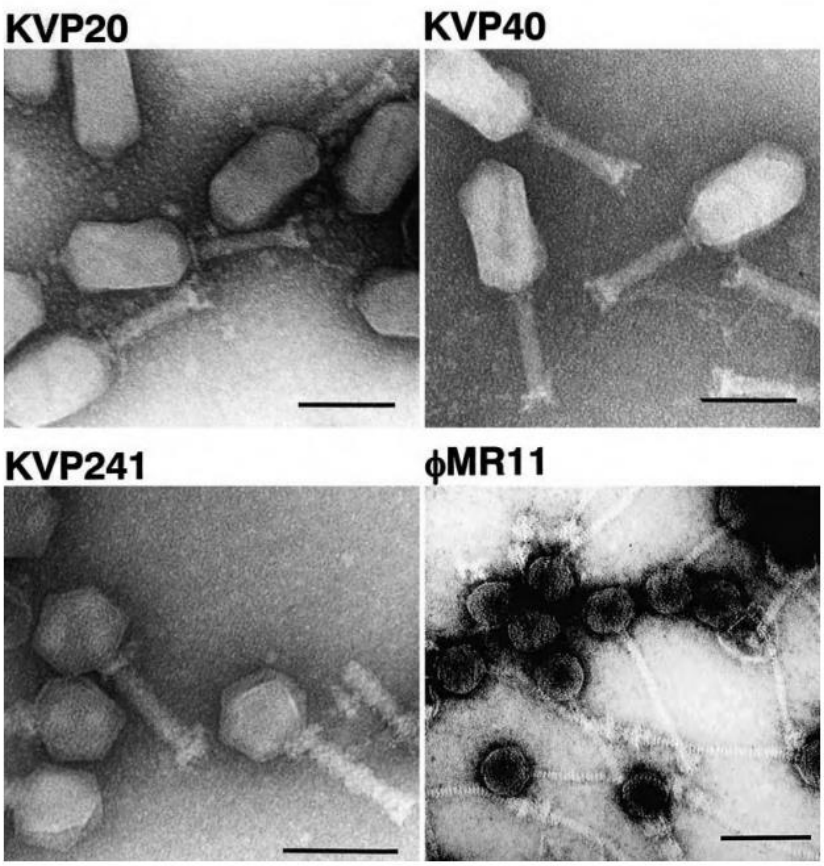

Figure 2.3: Electron micrographs of four tailed phages which are KVP20, KVP40, KVP241 and ϕMR11. They were negatively stained using 2% uranyl acetate at pH 4.0.Bars are 100 nm (Matsuzaki, et al., 2005).

According to Arnold, Ziese and Zilig (2000), filamentous and pleomorphic phages account about 3.6% of all known bacteriophages. They have been classified into ten minor families (Ackermann, 2009). These phages have significant difference in terms of their features and characteristics. Pleomorphic phages are classified into a low number of known members under three families that require further characterization (Orlova, 2012). *Plasmaviridae* are inclusive of phages composed of double-stranded DNA that are protected by a lipoprotein envelope. On the other hand, members of the *Fuselloviridae* family have double-stranded DNA enclosed in a lemon-shaped

capsid, in addition to some short spikes at one end. Lastly, *Guttaviridae* family with double-stranded DNA appear as droplet-shaped virus-like particles under transmission electron microscope (Orlova, 2012).

There are also phages with either helical or filamentous structure. The *Inoviridae* family is inclusive of phages that have long and rigid, or flexible filaments with a variety of lengths have been characterized by virion length, structure of head as well as DNA content. Moreover, the *Lipothrixviridae* phages are classified by a lipoprotein envelope as well as a rod-like shape. Furthermore, the *Rudiviridae* phages have straight rigid rods without envelopes with is highly similar to tobacco mosaic virus. All of the phages mentioned above are composed of double-stranded DNA (Arnold, Ziese and Zilig, 2000).

Next, there are phages with an icosahedral shape of head. For instance, *Leviviridae* phages have single-stranded RNA genome that is packed in small capsids. Phages with icosahedral head and a DNA genome are classified into three families which are *Microviridae, Corticoviridae* and *Tectiviridae*. The *Microviridae* includes small virions and single circular single-stranded DNA. The *Microviridae* family is currently only represented by PM2, which is a maritime phage. It has a capsid composed by proteins' outer layer as well as an inner lipid bilayer (Huiskonen et al., 2004). The last family, *Tectiviridae,* is classified by a lipoprotein vesicle which covers the protein capsid with

double-stranded DNA. These phages consist of spikes on their envelope. In the current International Committee on Taxonomy of Viruses (ICTV) release, the family *Microviridae* contains the Microvirus genus and the subfamily *Gokushovirinae*, which contains three genera such as Bdellomicrovirus, Chlamydiamicrovirus and Spiromicrovirus (Adams, Hayden and Casjens, 1983).

2.2.2 Genome

Most of the ICTV- classified dsDNA tailed phage to the order of *Caudovirales*. Despite the importance of ssDNA phages in the development of molecular biology and their heavy exploitation for biotechnological purposes, ssDNA phages only represent 11% of the phages in the ICTV database. The majority of these are filamentous phages belonging to the family *Inoviridae* and *Microviridae*, which only represents 2% of the database (Szekely and Breitbart, 2016). Figure 2.4 shows the classification of phage genome and the respective families.

DNA genome						RNA genome	
dsDNA				ssDNA		dsRNA lipid containing *Cystoviridae*	ssRNA *Leviviridae*
Caudovirales (dsDNA tailed phages)			dsDNA lipid containing *Tectiviridae*	Filamentous *Inoviridae*	Icosahedral *Microviridae*		
Podoviridae	*Myoviridae*	*Siphoviridae*	**PRD1**	**M13, fd, f1**	**phiX174**	**phi6, phi8, phi12**	**MS2, Qβ, fr, GA, R17, f2, phiCb5**
P22, T7, phi29, N4	**T4, Mu, G, P2/P4**	**Lambda, SPP1, HK97, T5, p2**					

Figure 2.4: Classification of bacteriophages based on properties of genome (Aksyuk and Rossmann, 2011).

Single-stranded DNA phage is classified into two general classes: the filamentous or rod-shapes phages, and icosaheral phages. Most of the single-stranded DNA phages belong to the family *Microviridae* (Streips and Yasbin, 2002). In 1963, three new single-stranded DNA filamentous phages have been reported. For instance, f1 phage (Zinder, et al., 1963), fd phage (Marvin and Hoffmann-Berling, 1963), and M13 phage (Hofschneider, 1963), which have been isolated from sewers in New York, Heidelberg, and Munich, respectively. These isolates have been shown to be closely related and they may be considered mutants of the same phage (Salivar, Henry and Pratt, 1964).

According to Dikjeng, Kuzmickas and Anderson (2009), only a few RNA phage isolates exist and RNA phages appear to be very rare even in metagenomic surveys specifically targeting viral RNA. For example, some of RNA phage which could only survive and multiply in *Escherichia coli* K-12 males (Hfr and F^+) have been isolated (Loeb, 1960). There is one serological group of these phages, designated as f2, which was known for its small virion size with spherical shape. It has a diameter of about 20 mμ, and it contains solely of RNA (Loeb and Zinder, 1961). In another study, an isolated phage similar to f2, known as MS2 phage has been isolated which was then classified as an RNA phage (Davis, Strauss, and Sinsheimer, 1961).

2.2.3 Life Cycle

According to Orlova (2012), apart from the morphological and genomic classifications, phages can also be divided into two groups based on their life cycles such as lytic cycle and lysogenic cycle as shown in Figure 2.5.

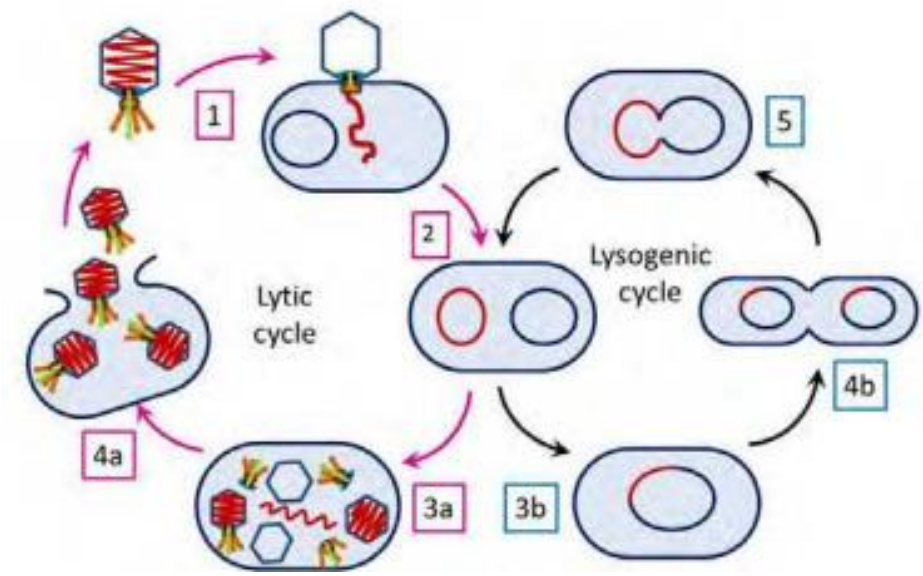

Figure 2.5: Lytic cycle and lysogenic cycle of bacteriophage. **1**: Phage first attach to the host cell and injects its DNA; **2**: Injected DNA initiates lytic or lysogenic cycle; **3a**: Synthesis of new phage DNA and proteins and assembly of virions; **4a**: Virions are released upon cell lysis; **3b** and **4b**: In lysogenic cycle, phage genome integrates into the bacterial chromosome, resulting in a prophage which exists in every daughter cells as bacteria reproduces; **5**: Prophage excises from the bacterial chromosome and lytic cycle is initiated (Orlova, 2012).

Absorption to a suitable host is a multistep process that can be divided into reversible and irreversible events and represents the starting point of the phage life cycle (Abedon, 2006). Initial binding to the target cell is a reversible process in which the tail fibers of the phage attach to a surface receptor of a bacterium. However, the first interaction for some phages such as *Bacillus*

subtilis phage ϕ29 and *Caulobacter crescentus* phage ϕCb13 with the host is via their head appendages (Guerrero-Ferreira, et al., 2011). The initial association of the phage head to the bacterial host provides additional time for the tail fibres to acquire the primary receptor, usually outer membrane proteins, pili, capsule, lipopolysaccharides and peptidoglycan of gram-positive bacteria (Rakhuba, et al., 2010). Upon successful attachment to the host cell, the phages migrate to secondary receptor sites on the cell surface, a process which is enzymatically controlled in some phages such as P22, a Podoviridae phage.

Once irreversibly bound to the secondary receptors DNA delivery is triggered, it is followed by ejection of DNA into the host cells. Although there is no common mechanism among phages for DNA ejection, bacteriophage such as phage λ is believed to deliver its genome through diffusion while T4 phage employs a cell puncturing method (Gonzalez-Huici, Salas and Hermoso, 2004). Following the successful penetration of the bacterial membrane, the internal pressure of the phage's genome (produced as a by-product of DNA packaging in the head) is released (Casjens and Molineux, 2012). This internal pressure is used to eject the phage's DNA into the cell at between 60,000 to 75,000 bps (reported for phage λ and T5). In tailed phages such as P22 and T7, some proteins are also released into the bacterial cell along with DNA injection. These proteins are important in the inhibition of host defence mechanisms in DNA delivery, or in the replication of phage DNA (Casjens and Molineux, 2012). An ejected phage genome can result in one of three main replication cycles which include temperate, virulent or lytic, and chronic life cycles.

Lytic, or virulent, phages hijack the host transcription-translation apparatus to produce the viral particles (Rakhuba, et al., 2010). leading to the destruction of the bacterial host during early and late infection stages. During the early infection stage (the time between DNA delivery and replication initiation), early and middle genes are expressed in which the products are responsible for DNA replication. In the late stage of infection (time between replication initiation and burst), genes encoding for structural and assembly components for the production of phage particles are transcribed. Cell lysis begins with the assembly of phage particles, followed by bacterial cell membrane lysis, and lastly the releasing of phage progeny to search for a new host (Abedon, 2006). The activity of two groups of enzymes, endolysins and holins, mediate lysis of the bacterial cell membrane. Endolysins are peptidoglycan degradators and holins (hole-formers) accelerate the endolysins access to the peptidoglycan layer (Gonzalez-Huici, Salas and Hermoso, 2004).

Upon successful infection, the lifecycle of a temperate phage can progress in one of two alternative pathways; the lytic or the lysogenic cycle which are dependent on the prevailing conditions. The lytic pathway is similar to that of lytic phages, in which the phage genome remains in the cytoplasm, and then replicates and expresses the phage genes which lead to cell death and release of progeny phages. In the lysogenic cycle, the phage DNA in most cases becomes integrated in the host chromosome at a specific site via site-specific recombination, in which it stays dormant and replicates as part of the host chromosome (Cho, Gumport and Gardner, 2002). A genetic switch containing

at least two promoters and two repressor controls the choice between lytic growth and establishment and maintenance of lysogenic growth (Renberg-Eriksson, et al., 2001). Although lysogenic growth is often a stable state, external factors can induce the conversion from lysogenic to lytic growth as seen in phage λ where lysis can be induced by activation of the SOS response pathways (Krebs et al., 2013).

However, only minority of phages can reproduce as part of a chronic infection or in a pseudolysogenic manner. The chronic infection (life cycle) is used by members of the Inoviridae (filamentous phages). These phages contain circular ssDNA that is packaged into long protein filaments, similar to bacterial pili, which are then released without causing lysis of the bacterial host (Yamada, 2013). In pseudolysogeny, an ejected genome remains in the host cytoplasm which neither integrate into the host genome nor proceed to cell lysis. This phenomenon was initially observed in bacteria from nutrient poor environments, in which pseudolysogenic phages lysed the bacteria when additional nutrients were provided to the culture. A pseudolysogenic state can be induced by the production of a holin inhibitor in *E. coli* phage T4 which delays the onset of cell lysis (Cenens et al., 2013).

2.2.4 Physiological Properties

2.2.4.1 Host Range

An alternative approach has been used to group phages according to the types (strains or species) of bacteria that a bacteriophage is able to infect. It is generally believed that most bacteriophages are only capable of infecting a narrow range of bacteria that are closely related (Cho, Gumport and Gardner, 2002). This is due to a combination of factors including specificity of phages' host binding proteins, biochemical interactions during infection, presence of related prophages or particular plasmids (especially for phages adsorbing to pili) and bacterial phage-resistance mechanisms (Roossinck, 2005).

2.2.4.2 Temperature and pH

Thermophilic phages play extraordinarily important roles in the processes of evolution, biogeochemistry, genetic exchange as well as ecology in extreme environments (Prangishvili, Forterre and Garrett, 2006). According to Szekely and Breitbart (2016), the phage particles which are resistant to temperature shifts suggests that phages are able to transfer DNA laterally from these extreme conditions. In a study conducted by Roossinck (2005), twelve *Thermus* phages were isolated from a hot spring located in California. It was found that temperature and pH stability of *Thermus* phages are not dependent of their geographic position. The twelve phages were studied for their heat resistance and it was found to be generally high and resembled myovirus ϕYS40 of *Thermus thermophilus*, which resisted heat treatment at 80°C for 30

min. Meanwhile, in another study conducted by (Yamada, 2013), a tectivirus ϕNS11 isolated from a hot spring in Beppu was found to have an optimum growth at temperature from 60°C to 65°C. Therefore, the heat resistance of majority of the *Thermus* phages shows adaptation to their environment. According to Ackermann (2007), PH65 which is a filamentous phage is known as a naturally heat-resistant as inoviruses are still stable at temperature of 80°C even if their habitats or hosts are mesophilic.

On the other hand, in terms of pH resistance, the twelve phages studied by Roossinck (2005) are stable between pH 4 to pH 10, thus they show slight resistance to acidic and alkaline environments. According to Achermann (1987), the usual pH resistance of tailed phages is between pH 5 to pH 9, pH 5 to pH 8 for tectiviruses, and pH 4 to pH 10 for inoviruses. On the other hand, Sakaki and Oshima (1976) reported the optimum pH for *B. acidocaldarius* phage ϕNS11 was at pH 3.5. Its activity shows significant declination at both pH 2 and pH 6. Therefore, these findings showed that specific phage exhibits different tolerance level against different pH.

2.3 Sources of Phage Isolation

As mentioned previously, phages are common in all natural environments on earth, such as soil, sediments, river and seawater. Phages can also be found in humans and living animals. For instance, isolation can be done from faeces, urine, saliva, spit, rumen and serum. Phages and their bacterial hosts

contribute to the intestinal flora. They can penetrate deep into different organs and tissues, and they can pass through the central nervous system (Frenkel and Solomon 2002).

A study conducted by Müller, et al. (2011) reported phages against *Erwinia amylovora* isolated from orchards in North America and Germany have been characterized for their possible control of fire blight which affects pear and apple trees. Based on their morphology, ϕEa1h and ϕEa100 were assigned to *Podoviridae* family. Phages are the only known microbial predators in extreme environments like hot springs. For instance, a novel phage ϕOH3 isolated from Obama hot spring in Japan against a hyperthermophilic bacterium *Thermus thermophiles* has been characterized (Nagayoshi, et al., 2016). Phage ϕOH3 was classified into the *Inoviridae* family and consists of a flexible filamentous particle of 830 nm in length and 8 nm in width. ϕOH3 was found stable at temperatures ranging from 70℃ to 90℃ and at pH ranging from pH 6 to pH 9.

In another study, a *Thermus Siphoviridae* phage, TSP4, has been isolated from Tengchong hot spring in China (Lin, et al., 2010). TSP4 phage was used to study its morphology, thermal stability, pH and organic solvent stability, the restriction enzyme digestion pattern and its composition of protein. The TSP4 phage was classified as the *Siphoviridae* family as it has a hexagonal head with diameter of 73 nm, a very long and flexible tail which is 785 nm in length and 10 nm in width. TSP4 was found very stable at temperature of 65℃ and

pH 7.6**.** Apart from that, a total of 115 bacteriophage strains have been isolated from alkaline hot springs in the U.S.A, Iceland, Russia and New Zealand (Nagayoshi, et al., 2016). These phages were then characterized into the family of *Myoviridae*, *Siphoviridae*, *Tectiviridae*, and *Inoviridae.*

2.4 Applications of Phage

Recently, the usage of phage or its products in food industries has become an alternative method for biocontrol against pathogens to enhance the safety of various types of food (Hagens and Loessner, 2010). Phages have also shown effectiveness in the elimination of pathogens which cause food poisoning such as *Listeria monocytogenes* (Leverentz, et al., 2003), *Salmonella* spp. (Leverentz, et al., 2001) and *Campylobacter jejuni* (Atterbury, et al., 2003). Another study demonstrated the problem of *Enterobacter sakazakii* growth in infant formula milk (Kim, et al. 2007). It was shown that the newly isolated T4-like phage ESP 732-1 against this infectious pathogen shown effectiveness in suppressing the growth of the organisms present in infant formula milk, at temperatures of 24℃ and 37℃. It was also found that the effectiveness of killing was dose-dependent, as the most effective dose was found to be phage with concentration of 10^9 pfu/mL in order to eliminate the target organisms completely.

In addition, administration of phage has shown effectiveness for the control of fish diseases and for disinfectant of food purposes have also been reported.

For example, Nagayoshi, et al. (2016) succeeded in eliminating fish pathogens such as *Pseudomonas plecoglossicida* and *Lactococcus garvieae* from cultured fish. Furthermore, animal products contamination can occur during the process of slaughter or milking. For instance, two studies reported an experimental treatment of sheep using phage (Atterbury, et al., 2003). In the studies, administration of phage DC22 in an synthetic rumen system that was made similar to sheep showed bacteria clearance. On the other hand, Sheng, et al. (2006) administrated phage KH1 and SHI to treat infections in mice, sheep, and cattle. It was found that oral administration of KH1 to sheep did not decrease numbers of the target intestinal bacteria. However, oral administration of either SH1 alone or a combination of KHI and SH1 in mice showed successful complete eradication of the targeted bacteria. Therefore, combination of different phages is shown to be more effective than a phage alone.

According to Sulakvelidze, Zemphira and Glenn-Morris (2001), phages have shown to be more effective in therapeutic purposes in human and animals compared to antibiotics for the treatment of infectious diseases although the antimicrobial activities of lytic phages give identical effects as antibiotics. For example, in one study conducted by Kaźmierczak, Górski and Dąbrowska, (2014), patients suffering from infectious lethal diseases of lungs which caused by *Staphylococcus aureus* have been treated with either phage therapy or antibiotic. The bacteriophage therapy showed no side effect and 82% of the treated patients have completely recovered as compared to the antibiotics treated patients with 64% recovery.

A study conducted by Matsuzaki, et al. (2005) reported an effective way of eradicating *Helicobacter pylori* by phage display technique. In the study, M13, a modified filamentous phage, which has a coat protein attached with an antibody specific to an antigen, was created. The modified M13 was not able to multiply in *Helicobacter pylori*, however, it showed suppression on its growth *in vitro*. Furthermore, it was found that administration of the phage orally could decrease the number of bacteria in mice's stomachs.

Proteins produced by bacteriophage have also been investigated as alternatives to the usage of complete and intact phage in crop infections. Phage lysozyme is a protein that can cause fragmentation of the bacterial cell wall. It has also been investigated as a treatment for plant infections. In a study conducted by Doss, et al. (2017), phage lysozyme was isolated from phage *f*Xo411 against *Xanthomonas oryzae*. The isolated lysozyme (Lyse411) was tested against some other bacterial strains and the results were shown to effectively reduce the bacterial concentration in both *Xanthomonas* and *Stenotrophomonas*. In another study by Wittmann, Eichenlaubet and Dreiseikelmann (2010), endolysins from phage CMP1 was transformed into tomato seeds. When the transformed plants were infected with *Clavibacter michiganensis*, the transgenic plants which contain the endolysins showed less severe infection as compared to the infected wild-type tomato plants. Therefore, based on these findings, it was shown that phage endolysin protein is not toxic to plants and it can be very beneficial in warding off infection.

Nowadays, on the market, there is availability of several commercial phages. For instance, Listex P100 is one of the most famous commercially available phage established by a Dutch company known as Micreos which has been proved safe to eradicate *Listeria monocytogenes* in food products of either raw or canned food (Żaczek, Weber-Dąbrowska and Górski, 2015). Another three commercially available phages which are ListShield, EcoShield and SalmoFresh which can target some well-known food pathogens, namely *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella* serotypes, respectively.

In brief, these convincing findings have proved that phages show high effectiveness in eradicating bacterial infections. The specificity of phages towards particular bacteria, the capability of phages to multiply in infected animal hosts, and the low-risk of phages make them very effective as antibacterial agents. Since multiple drug-resistant bacteria has been a global concern, phage therapy is very promising as an alternative treatment. Apart from that, potential products produced by phage such as endolysin can be further explored for both medical and industrial applications. Therefore characterization of newly isolated bacteriophages is very important in order to fully understand them and discover their potential applications.

CHAPTER 3

MATERIALS AND METHODS

3.1 Phage Sample and Bacterial Strain

Phage KA isolate used in this study was previously isolated by a postgraduate student from a 55°C, pH 7.6 hot spring in Kuala Woh, Perak. *Escherichia coli* BL21 (DE3) which was used as bacteria host for phage KA was obtained from a postgraduate student. Another seven bacterial strains: *Escherichia coli* TOP10 cells enterotoxigenic *Escherichia coli* (ETEC), enteropathogenic *Escherichia coli* (EPEC), *Shigella boydii*, *Shigella dysenteriae*, *Shigella flexneri* and *Shigella sonnei* were obtained from Dr. Tan Gim Cheong, Department of Biomedical Science, Faculty of Science, Universiti Tunku Abdul Rahman.

3.2 Reagents, Chemicals and Equipment

The reagents and chemicals used throughout this study with their respective manufacturers are listed in Table 3.1.

Table 3.1: List of reagents and chemicals.

Reagents/ Chemicals	**Manufacturers, Country**
1 Kb DNA ladder	Gene DireX Inc., USA
10X Buffer Tango (with BSA)	Thermo Scientific, USA
Acetic acid	QRec, Malaysia
Agar-agar powder	HmbG Chemicals, Germany
Caesium chloride	Bio Basic Canada Inc., Canada
Chloroform:isoamyl alcohol (24:1)	Sigma-Aldrich, USA
DNase I	Vivantis Sdn. Bhd., Malaysia
Ethanol	Merck, Germany
Ethylenediaminetetraacetic acid	QReC, Malaysia
Hydrochloric acid	VWR Prolabo, Singapore
Luria Bertani agar	Laboratorios Conda, Spain
Luria Bertani broth	Laboratorios Conda, Spain
Novel juice	Gene DireX Inc., USA
Phenol saturated with TE buffer	Nacalai Tesque, Inc., Japan
Polyethylene glycol 8000 (PEG 8000)	Promega Corporation, USA
Proteinase K	Bio Basic Inc., Malaysia

Table 3.1: List of reagents and chemicals (continued).

Reagents/ Chemicals	**Manufacturers, Country**
Restriction enzymes (*Bam*HI, *Eco*RI, *Eco*RV, *Xba*I)	BIORON, Germany
Restriction enzyme (*Hin*dIII)	New England Biolabs, UK
Restriction enzyme (*Sac*I)	Thermo Scientific, USA
RNase A	Bio Basic Inc., Malaysia
Sodium acetate	Bio Basic Canada Inc., Canada
Sodium chloride	Bio Basic Canada Inc., Canada
Sodium dodecyl sulphate (SDS)	Bio Basic Canada Inc., Canada
Tris	Bio Basic Inc., Malaysia
Tryptone	Oxoid Ltd., England
Yeast extract	Laboratorios Conda, Spain

The equipment and laboratory wares used throughout this study with their respective manufacturers are listed in Table 3.2.

Table 3.2: List of equipment and laboratory wares.

Equipment/Laboratory Wares	**Manufacturers, Country**
Autoclave machine	Hirayama, Japan
Centrifuge tubes (15 mL and 50 mL)	TRP, Europe
Gel electrophoresis set	Major Science, Taiwan
High speed centrifuge	Sigma 2-16PK Sartorius, Germany
Incubator	Memmert, Germany
Laminar flow cabinet	Esco Micro Pte. Ltd., Singapore
MP-300V power supply	Medigene Sdn. Bhd., Malaysia
Microcentrifuge machine	Thermo Scientific, USA
Microcentrifuge tubes (0.2 mL and 1.5 mL)	Greiner Bio-One, Austria
Microwave oven	Sharp, Japan
Nano-spectrophotometer	Implen, USA
Needle (0.70 × 38mm)	Terumo Corporation, Europe
Petri dishes	Nest Biotechnology Co, Ltd, China
pH meter	Sartorius, Germany
Media bottles	Kimax, Germany
Shaking incubator	Yihder Co, Ltd, Taiwan

Table 3.2: List of equipment and laboratory wares (continued).

Equipment/Laboratory Wares	**Manufacturers, Country**
SnakeSkin Pleated Dialysis Tubing	Thermo Scientific, USA
Spectrophotometer and cuvettes	Biochrom, USA
Syringe (1 mL/cc)	Muzamal Sdn. Bhd., Malaysia
Table-top microcentrifuge machine	Sigma 2-16PK Sartorius, Germany
TH-641 polyallomer tube	Thermo Scientific, USA
Transmission electron microscope (JEM-2100F)	Jeol USA Inc., USA
Ultracentrifuge machine	Thermo Scientific, USA
UV transilluminator	UVP, Canada
Vortex mixer	Gemmy Industrial Corp., Taiwan
Water bath	Copens Scientific, Malaysia
Weighing balance	Sartorius, Germany

3.3 Amplification and Growth of Phage

3.3.1 Preparation of Luria Bertani (LB) Agar Plates

In a 1 L media bottle, 17.5 g LB agar powder was dissolved in 500 mL deionized water and autoclaved. The autoclaved warm LB agar was poured into sterile Petri dishes and allowed to solidify. Lastly, the LB agar plates were stored at 4°C until use.

3.3.2 Preparation of Top Agar

In a 100 mL media bottle, 0.6 g tryptone, 0.42 g agar powder, 0.3 g sodium chloride and 0.3 g yeast extract were dissolved in 60 mL deionized water. The mixture was then autoclaved. The top agar was melted using a microwave oven and 4 mL of the melted top agar was aliquoted into each test tube. Next, the test tubes were covered with aluminium foil and kept in a 55°C water bath until use.

3.3.3 Preparation of Luria Bertani (LB) Broth

In a 1 L media bottle, 16 g of LB broth powder was dissolved in 800 mL deionized water. Then, the mixture was mixed well and autoclaved.

3.3.4 Preparation of 50 mM Tris-HCl, 150 mM NaCl Tris-Buffered Saline (TBS), pH 7.6

In a 1 L media bottle, 4.84 g Tris and 7.01 g sodium chloride were dissolved in 600 mL of deionized water. Then, the pH of the mixture was adjusted to pH 7.6 with 37% hydrochloric acid. The solution was topped up to 1 L and autoclaved.

3.3.5 Preparation of Log-phase *Escherichia coli* Culture

Escherichia coli BL21 (DE3) bacterial stock was streaked onto a LB agar plate and incubated at 37°C overnight. The next day, a single colony of the bacteria was inoculated into 40 mL of LB broth. The culture was then incubated overnight at 37°C in a shaking incubator with agitation at 200 rpm. The next day, the overnight culture obtained was inoculated into fresh LB broth at a ratio of 1:20. The culture was grown in a shaking incubator at 37°C, with agitation speed of 200 rpm until the absorbance of the culture at OD_{600} reached between 0.6 to 0.8 before subsequent phage infection was carried out.

3.3.6 Plaque Assay

Plaque assay was carried out to obtain an isolated plaque for subsequent amplification. Firstly, the phage stock was serially diluted with TBS buffer. Then, 4 mL of top agar was aliquoted into several test tubes and covered with aluminium foil before incubating them in a 55°C water bath until use. Next, 50

µL of diluted phage and 200 µL of log-phase *E. coli* culture were added into the 4 mL of top agar and vortexed. The mixture was then poured onto the LB agar plate and swirled gently to completely cover the surface of LB base agar. The agar plate was allowed to solidify and incubated overnight at 37°C.

3.3.7 Phage Amplification

An isolated plaque was picked up with a sterile yellow pipette tip. The plaque was then mixed with 1 mL of TBS buffer and left overnight at 4°C to allow phage diffusion from the plaque into the buffer. The next day, the phage-containing TBS buffer was inoculated into 150 µL of log-phase *E. coli* culture and incubated in a shaking incubator at 37°C with agitation at 200 rpm for 30 min. After that, the approximately 1.15 mL culture was added into 4 mL melted top agar and vortexed. Then, the mixture was poured onto a LB agar plate and allowed to solidify. The plate was then incubated overnight at 37°C.

The next day, confluent phage growth on the plate would be observed. Then, 5 mL of TBS buffer was poured onto the plate and stored in the fridge overnight to allow diffusion of phage into the buffer. On the following day, the TBS buffer containing the diffused phage was transferred into several 1.5 mL microcentrifuge tubes, then centrifuged at 10,000 ×*g* for 5 min at 4°C. Next, 2 mL of the supernatant obtained was added into 20 mL of log-phase *E. coli* culture and incubated in a shaking incubator at 37°C with agitation at 200 rpm

until total lysis was observed. Another tube with only 20 mL of log-phase *E. coli* culture was prepared to serve as a negative control.

The 20 mL phage-infected culture was centrifuged at 12, 000 ×*g* for 30 min at 4°C to pellet down bacterial debris. The supernatant was then used to infect the 120 mL of log-phase *E. coli* culture and incubated in a 37°C shaking incubator with agitation at 200 rpm until total lysis was observed. Then, approximately 140 mL phage lysate was transferred into 50 mL centrifuge tubes and centrifuged at 12, 000 ×*g* for 30 min at 4°C to pellet down bacterial debris. The supernatant was collected for phage precipitation using 20% PEG-NaCl as described in Section 3.4.2. The PEG-precipitated phage was used for subsequent large scale phage amplification.

3.4 Phage Precipitation

3.4.1 Preparation of 20% (w/v) Polyethylene Glycol 8000 (PEG 8000) Containing 2.5 M Sodium Chloride (NaCl)

In a 500 mL media bottle, 100 g of PEG 8000 and 73.05 g of NaCl were added to 300 mL of deionized water. The mixture was then stirred and heated gently until the powder dissolved completely. The dissolved PEG-NaCl solution was then topped up to 500 mL using deionized water and autoclaved.

3.4.2 Phage Precipitation with Double 20% (w/v) PEG-NaCl

After centrifugation, the supernatant containing phage was transferred into a sterile beaker with a magnetic stirrer bar. Next, 20% (w/v) PEG-NaCl solution was added into the supernatant to precipitate the phage. The volume of PEG-NaCl solution added was 25% (v/v) of the total volume of the supernatant obtained. The mixture was then constantly stirred overnight at 4°C. The next day, the mixture was centrifuged at 12, 000 ×*g* for 30 min at 4°C. The supernatant was then carefully discarded and the pellet was washed with 10 mL TBS buffer. The PEG-precipitated phage was then collected into several 1.5 mL microcentrifuge tubes and centrifuged at 15, 000 ×*g* for 10 min at 4°C. Next, the supernatant was transferred into another sterile breaker with a magnetic stirrer bar and 20% (w/v) PEG-NaCl solution was added. The volume of PEG-NaCl solution added was 25% (v/v) of the total volume of the supernatant obtained. The mixture was then constantly stirred at 4°C for approximately 2 h. The double PEG-precipitated phage was then collected into several 1.5 mL microcentrifuge tubes and centrifuged at 12, 000 ×*g* for 30 min at 4°C. Then, the supernatant was discarded and the pellet was resuspended in 1 mL of TBS buffer. The PEG-precipitated phage was then stored in a 1.5 mL microcentrifuge tube and kept at 4°C until use. Plaque assay (Section 3.3.6) was carried out to determine the titre of the PEG-precipitated phage. The phage titre (pfu/mL) was calculated using the formula below:

$$\frac{\text{pfu}}{\text{mL}} = \frac{\text{Number of plaques}}{\text{Dilution} \times \text{volume of diluted virus added (mL)}}$$

3.5 Caesium Chloride Ultracentrifugation

3.5.1 Preparation of Caesium Chloride Gradients

Thirty millilitres of TBS buffer in a 50 mL centrifuge tube was placed on an electronic weighing scale and tared. For the preparation of caesium chloride solution with a density of 1.3 g/mL, caesium chloride crystals were added into the TBS buffer gradually and mixed well. An empty 1.5 mL microcentrifuge tube was placed on another electronic weighing scale and tared. Then, 1 mL of the caesium chloride solution was aliquoted into the 1.5 mL microcentrifuge tube and the weight was measured. The caesium chloride solution was then transferred back into the 50 mL centrifuge tube. Caesium chloride crystals were added until the desired densities were obtained. For the preparation of caesium chloride with densities of 1.3 g/mL, 1.4 g/mL, 1.5 g/mL and 1.7 g/mL, the amount of caesium chloride crystals added were 13.213 g, 18.135 g, 24.256 g and 39.4594 g, respectively. The four caesium chloride gradients were then stored at 4°C until use.

3.5.2 Preparation of Phage Sample

Before ultracentrifugation, 1 mL PEG-precipitated phage was diluted to 3 mL using TBS buffer. Then, 0.228 g of caesium chloride crystals were added to the 3 mL of phage sample and mixed well to obtain a density of 1.15 g/mL.

3.5.3 Layering of the Densities and Phage Sample

A needle attached with a 1 mL syringe was used to layer the caesium chloride solution. Firstly, 1.5 mL of 1.7 g/mL caesium chloride gradient was placed into the TH-641 polyallomer tube. Next, 2 mL of 1.5 g/mL caesium chloride gradient was layered on top of the 1.7 g/mL gradient slowly. Then, 2.5 mL of 1.4 g/mL gradient was layered, followed by 2.5 mL of 1.3 g/mL caesium chloride gradient. Lastly, the 1.15 g/mL phage sample was layered on top of the 1.3 g/mL caesium chloride until the tube was about 95% filled. The tube was then slowly lowered into the bucket using a pair of forceps and screwed shut. A total of two samples were prepared and the weight of the two buckets with phage sample were balanced prior to ultracentrifugation.

3.5.4 Ultracentrifugation

The ultracentrifuge machine together with a swing-out rotor (TH-641) and empty buckets were first pre-cooled to 4°C. Then, the TH-641 rotor was removed from the machine and all the six buckets with or without the samples were hung onto the rotor. Then, the rotor was placed back into the machine and vacuumed. The samples were then centrifuged at 280, 000 $\times g$ for 4 h.

After ultracentrifugation has completed, the machine was de-vacuumed. The rotor was then removed from the machine and placed onto the holder carefully. The polyallomer tubes were removed from the buckets using a pair of forceps.

Next, the distinct bands containing the purified phage were collected separately into 1.5 mL microcentrifuge tubes and stored at 4 °C.

3.5.5 Dialysis

In order to remove caesium chloride from the purified sample, 1 L of TBS buffer was dispensed into a sterile 1 L beaker with a magnetic stirrer and pre-cooled at 4 °C until use. A SnakeSkin Pleated Dialysis Tubing with 10,000 MWCO was first clamped on one side. TBS buffer was then used to run through the clamped end to ensure no leakage. Then, the dialysis tubing was filled with the purified phage sample up to 1/3 of the total length of the tube. Then, the other end of the dialysis tubing was clamped. The sample was then dialyzed in the TBS buffer with constant stirring overnight at 4°C. On the next day, the dialyzed sample was collected and plaque assay was carried out to determine the titre of the purified phage.

3.6 Physiological Characterizations of Phage

3.6.1 Host Range

Seven different bacterial strains: *E. coli* TOP10 cells, enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), *Shigella boydii*, *Shigella dysenteriae*, *Shigella flexneri* and *Shigella sonnei* were streaked onto LB agar plates and incubated at 37°C overnight. Another positive control plate was also prepared using *E. coli* BL21 (DE3). The next day, a single colony from each bacteria

were inoculated into 15 mL centrifuge tubes containing 3 mL of LB broth separately. The tubes were then incubated in a shaking incubator at 37°C, 200 rpm for 3 to 4 h.

Then, 50 µL of PEG-precipitated phage with similar dilution factor and 200 µL of each log-phase bacteria were added into different 4 mL melted top agar and vortexed. The mixtures were then poured onto respective LB agar plates. After the agar has solidified, the plates were incubated at 37°C overnight. Plaque formation was observed and recorded the next day.

3.6.2 Effect of Temperature on Phage Infectivity

Fifty microlitres of phage with similar dilution factor were aliquoted into ten 0.2 mL microcentrifuge tubes labelled as 50°C, 52°C, 54°C, 56°C, 58°C, 60°C and 24°C (control). These tubes except the control tube were then heat treated in a water bath accordingly for 10 min. The control sample was kept at room temperature.

After heat treatment, 50 µL of the heat-treated phage and 200 µL of log-phase *E. coli* culture were added into 4 mL melted top agar in different tubes and vortexed. The mixtures were then poured onto a LB agar plate. After the agar has solidified, the plates were incubated at 37°C overnight. Plaque formation was observed and recorded the next day.

3.6.3 Effect of pH on Phage Infectivity

Ten millilitres of LB broth was prepared in five universal bottles with pH ranging from pH 5 to 9. Positive control in this study was LB broth at pH 7. The pH of the LB broths were adjusted accordingly and autoclaved. Then, 200 µL of overnight *E. coli* culture was added into each universal bottle and incubated in a shaking incubator at 37°C with agitation at 200 rpm for 3 to 4 h. Next, 100 µL of phage with the same dilution factor was used to infect the log-phase *E. coli* culture and incubation was continued until complete lysis was observed. The phage-infected cultures were then centrifuged at 12,000 ×*g* for 30 min at 4°C. The supernatants obtained were serially diluted using TBS buffer prior to plaque assay.

3.7 Partial Genomic Characterization

3.7.1 Preparation of 10 mM Tris, pH 7.4

In a 100 mL media bottle, 0.061g Tris base powder was dissolved in 40 mL deionized water and mixed well. Then, the pH of the solution was adjusted to pH 7.4 using HCl. The solution was then topped up to 50 mL with deionized water.

3.7.2 Preparation of 20% Sodium Dodecyl Sulphate (SDS)

In a 15 mL centrifuge tube, 2 g SDS powder was dissolved in 10 mL deionized water.

3.7.3 Preparation of 3 M Sodium Acetate

In a 500 mL media bottle, 73.83 g sodium acetate powder was dissolved in 200 mL deionized water. The solution was then topped up to 300 mL with deionized water and autoclaved.

3.7.4 Preparation of 70% Alcohol

In a 15 mL centrifuge tube, 7 mL absolute ethanol was added to 3 mL of deionized water.

3.7.5 Preparation of 0.5 M Ethylenediaminetetraacetic Acid (EDTA)

In a 100 mL media bottle, 9.306 g of EDTA powder was dissolved in 30 mL of deionized water. The solution was then adjusted to pH 8 and topped up to 50 mL with deionized water.

3.7.6 Preparation of 10X Tris-Acetate-EDTA (TAE) Buffer

In a 1 L media bottle, 48.4 g of Tris base powder and 11.4 mL glacial acetic acid were added. Next, 20 mL of 0.5 M EDTA with pH 8 and 800 mL of deionized water were added into the mixture. The mixture was mixed well and topped up to 1 L with deionized water. The buffer was then autoclaved prior to use.

3.7.7 Preparation of 1X Tris-Acetate-EDTA (TAE) Buffer

In a 1 L media bottle, 10X TAE buffer was mixed with 900 mL of sterile deionized water to obtain 1 L of 1X TAE buffer.

3.7.8 Phage Nucleic Acid Extraction Using Phenol:Chloroform:Isoamyl Alcohol (PCI)

Two hundred microlitres of PEG-precipitated phage was aliquoted into a 1.5 mL microcentrifuge tube. Then, 200 µL of 10 mM Tris with pH 7.4 was added into the tube followed by 2 µL of 20% SDS. Next, about 0.80 µL of proteinase K (10 mg/mL) was added to a final concentration of 20 µg/mL. The mixture was then incubated in a 37°C water bath overnight. The next day, the mixture was heat inactivated in a heat block at 65°C for 20 min. An equal volume of ready-to-use phenol saturated with TE buffer was added into the tube and agitated vigorously for 3 min. The mixture was then centrifuged at 14,000 ×*g* for 5 min at room temperature. The upper layer was carefully removed and discarded. An equal volume of chloroform:isoamyl alcohol (24:1 ratio) was added to the tube and mixed vigorously, followed by centrifugation at 14, 000 ×*g* for 5 min at room temperature. The upper layer was carefully removed and discarded. Then, 3 M sodium acetate and 100% ethanol were added to the tube at a ratio of 1:10 and 2:1, respectively. Next, the mixture was agitated for a few second and placed in a -20°C freezer for 30 min. The mixture was then centrifuged at 14,000 ×*g* at room temperature. The supernatant was discarded and 1 mL of 70% ethanol was used to wash the pellet. The mixture was then centrifuged again at 14,000 ×*g* for 5 min at room temperature. The supernatant

was discarded and the pellet was air dried. Then, 200 µL of sterile deionized water was used to resuspend the pellet and stored at -20°C.

3.7.9 Quantification of Extracted Phage Nucleic Acid

The extracted phage nucleic acid was quantified by measuring the concentration and absorbance of the nucleic acid. The absorbance was measured at wavelengths of 260 nm and 280 nm. Firstly, the nano-spectrophotometer was calibrated and a submicroliter cell was properly placed into the cell holder. Secondly, 1 µL of sterile deionized water was used to blank the machine. Then, the measurement window was cleaned and 1 µL of the phage nucleic acid was placed on it. Next, a lid with 1 mL pathlength was cleaned before placing on the submicroliter cell. Measurements were recorded.

3.7.10 DNase and RNase Digestion of Phage Nucleic Acid

In order to determine the type of phage nucleic acid, the nucleic acid was treated with DNase and RNase separately. The reaction mixtures were prepared according to Table 3.3 and incubated for approximately 1 h at room temperature.

Table 3.3: DNase and RNase digestion reaction mixtures.

Components	**DNase Treatment**	**RNase Treatment**	**Negative Control**
Bacteriophage Nucleic Acid (µL)	2.0	2.0	2.0
10X Buffer Tango With BSA (µL)	0.5	-	-
DNase (µL)	0.2	-	-
RNase (µL)	-	0.7	-
Deionized Water (µL)	2.3	2.3	3.0
Total (µL)	5.0	5.0	5.0

After digestion, agarose gel electrophoresis was carried out. In a 100 mL conical flask, 20 mL of 1X TAE buffer was added to 0.2 g of agarose powder and heated in a microwave oven until the agarose powder was completely dissolved. The mixture was allowed to cool down slightly before pouring the gel mixture into the casting tray of electrophoresis set. The comb was then inserted slowly into the gel and the gel was allowed to solidify.

Once the 1% (w/v) agarose gel has solidified, the comb was carefully removed and the gel was placed in a gel tank filled with 1X TAE buffer. Then, 5 µL of 1 Kb DNA ladder was mixed with 1 µL of novel juice and 2 µL of the pre-stained ladder was then loaded into the first well. Next, 5 µL of the treated samples were mixed separately with 1 µL of novel juice and loaded into their respective wells. Then, the gel was subjected to electrophoresis at 80 V for 45 min or until the dye front reached the bottom of the gel. The gel was then visualized using a UV transilluminator.

3.7.11 Restriction Enzyme Digestion of Phage Nucleic Acid

Six restriction enzymes; *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, *Sac*I and *Xba*I were used to digest the phage nucleic acid. The positive control (plasmid DNA) for *Bam*HI, *Eco*RI, *Eco*RV and *Hin*dIII were pBR322 while the positive control for *Sac*I and *Xba*I were pUC19. The reaction mixtures were prepared based on Table 3.4 and incubated overnight at 37°C.

Table 3.4: Restriction enzyme digestion mixtures.

Components	**Volume (µL)**
Phage nucleic acid/Plasmid DNA	5.0
Restriction enzyme	0.5
Restriction enzyme buffer	1.0
PCR water	3.5
Total	10.0

The next day, the reaction mixtures were heat inactivated by incubating them in a water bath as described in Table 3.5.

Table 3.5: Heat inactivation of restriction enzyme mixtures.

Restriction Enzyme	**Temperature (°C)**	**Duration (min)**
*Bam*HI	80	20
*Eco*RI	65	20
*Eco*RV	80	20
*Hin*dIII	80	20
*Sac*I	65	20
*Xba*I	65	20

After heat inactivation, agarose gel electrophoresis was carried out. The prepared agarose gel was placed in a gel tank with 1X TAE buffer. The first well was loaded with 2 μL of pre-stained 1 Kb DNA ladder. Next, 1 μL of novel juice was mixed separately with each of the heat inactivated samples and loaded into their respective wells. The gel was then subjected to electrophoresis at 80 V for 45 min or until the dye front reached the bottom of the gel. The gel was then visualized using the UV transilluminator.

3.8 Morphological Characterization

3.8.1 Transmission Electron Microscopy

One droplet of purified sample and another droplet of 2% uranyl acetate were placed on a parafilm with a sterile Pasteur pipette. A carbon-coated grid was placed on the sample for 3 min. Then, the grid was dried using a filter paper. Next, the shiny side of the grid was placed on top of the uranyl acetate droplet for one minute. Again, the grid was dried using a filter paper.

The dried grid was then viewed under a JEM-2100F transmission electron microscope located at Institute of Bioscience, Universiti Putra Malaysia, Serdang, Selangor. The magnification used was 10,000×.

CHAPTER 4

RESULTS

4.1 Purification of Bacteriophage with Caesium Chloride Ultracentrifugation

PEG-precipitated phage was subjected to caesium chloride (CsCl) density gradient ultracentrifugation to purify the phage particles prior to phage observation under transmission electron microscope (TEM). Ultracentrifugation also allowed the determination of buoyant density of the phage KA by measuring the density of the CsCl solution in the corresponding phage-containing bands (Bachrach and Friedmann, 1971).

Based on Figure 4.1, two bands were formed after ultracentrifugation. The first upper band formed at a density of about 1.3 g/mL showed no infectivity as there was no plaque formation observed in plaque assay. Meanwhile, the second lower band which appeared much thicker compared to the first upper band was estimated to have a density of 1.4 g/mL with average phage titre of 5.24×10^{13} pfu/mL. Therefore, the buoyant density of phage KA is approximately 1.4 g/mL.

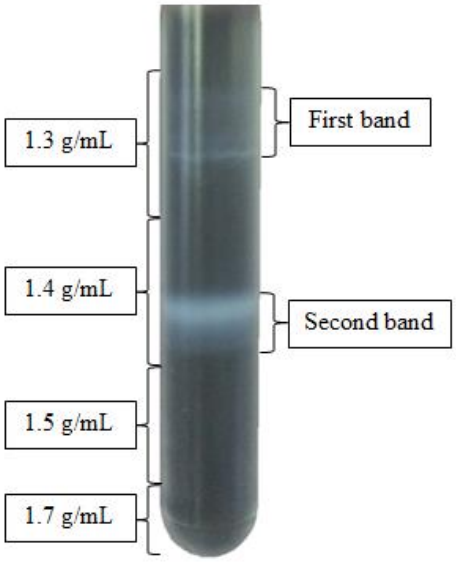

Figure 4.1: Purification of phage KA using density gradient caesium chloride ultracentrifugation. Two distinct bands with different densities were observed at 1.3 g/mL and 1.4 g/mL.

4.2 Physiological Characterizations of Phage

4.2.1 Host Range

In order to determine different types of bacteria susceptibility to phage KA, seven different gram-negative bacterial strains which include *E. coli* TOP10 cells, enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), *Shigella boydii*, *Shigella dysenteriae, Shigella flexneri* and *Shigella sonnei* were used. The infectivity was determined by plaque assay. *E. coli* BL21 (DE3) served as the positive control Based on Table 4.1, phage KA specifically infects *E. coli* BL21 and *S. boydii*, but does not infect and lyse other types of *E. coli* or *Shigella* species including *E. coli* TOP10 cells, enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), *Shigella dysenteriae*, *Shigella flexneri* and *Shigella sonnei*, therefore phage KA has

high host specificity towards *E. coli* BL21 and *S. boydii* out of the seven tested bacteria.

Table 4.1: Infectivity of phage KA against different bacterial hosts.

Bacteria Host	**Infectivity**
E. coli TOP10 cells	−
Enteropathogenic *E. coli* (EPEC)	−
Enterotoxigenic *E. coli* (ETEC)	−
Shigella boydii	+
Shigella dysenteriae	−
Shigella flexneri	−
Shigella sonnei	−
E. coli BL21 (DE3) (Positive control)	+

(+) there was infectivity

(−) no infectivity

4.2.2 Effect of Temperature on Phage Infectivity

The PEG-precipitated stock was subjected to heat stability test at 50°C, 52°C, 54°C, 56°C, 58°C and 60°C for 10 min. Infectivity of phage was determined by plaque assay. Based on Figure 4.2, the phage infectivity decreased gradually as the temperature increased from 50°C to 60°C. In addition, a significant drop of phage titre was observed when the temperature was increased beyond 55°C. At 60°C, the phage completely lost its infectivity.

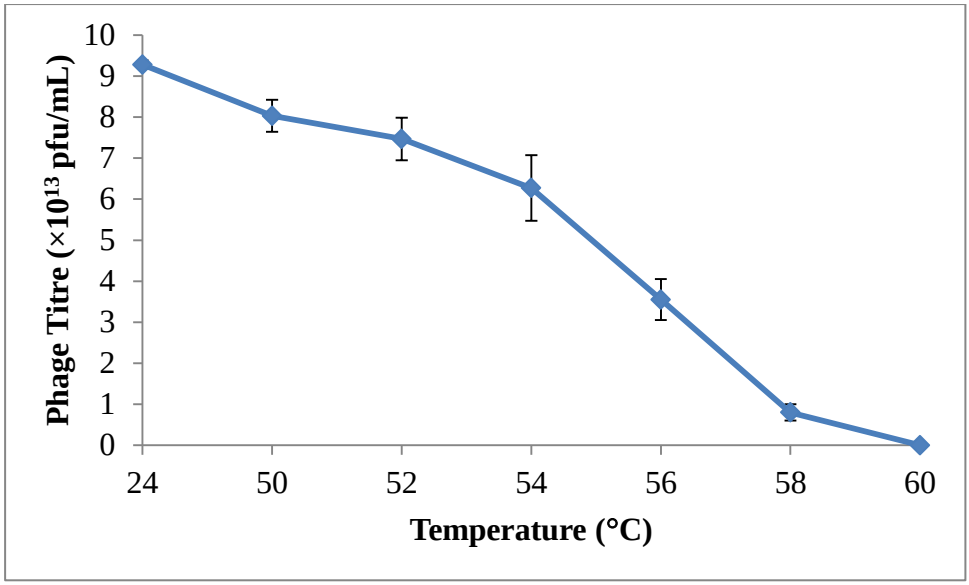

Figure 4.2: Effect of temperature on phage infectivity. Error bars represent distribution of data.

4.2.3 Effect of pH on Phage Infectivity

In this study, phage KA was grown in LB broth with pH ranging from pH 5 to pH 9. Based on Figure 4.3, phage KA could tolerate pH 5 and pH 9 and grew optimally at pH 7. Therefore, phage KA favoured a neutral environment, around pH 6 to pH 8.

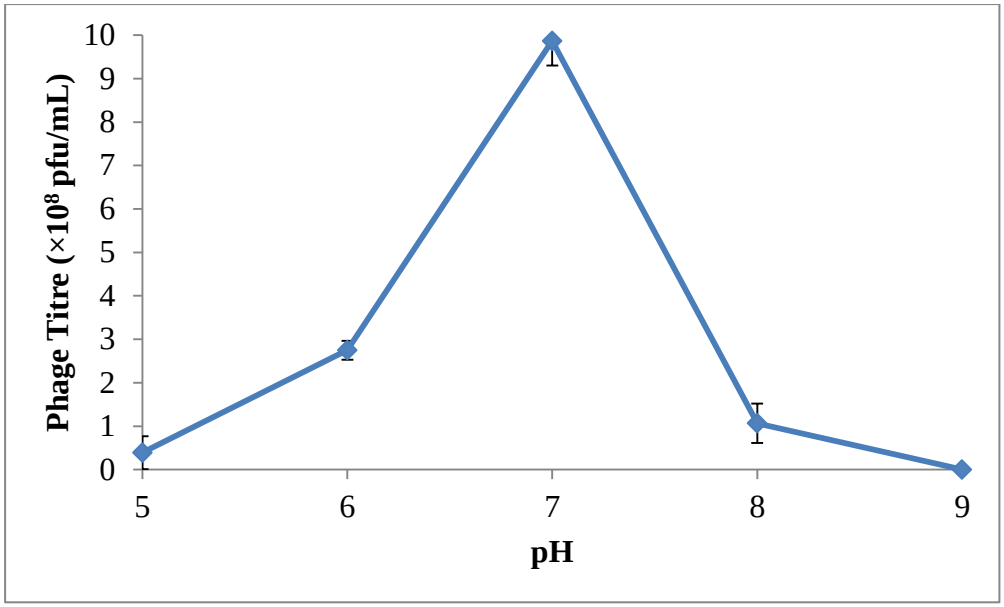

Figure 4.3: Effect of pH of LB medium on phage infectivity. Error bars represent distribution of data.

4.3 Partial Genomic Characterization

4.3.1 Phage Nucleic Acid Extraction

PEG-precipitated phage was subjected to nucleic acid extraction using phenol:chloroform:isoamyl alcohol method and quantified using a nano-spectrophotometer. The absorbance of the extracted nucleic acid measured at wavelengths of 260 nm and 280 nm (A_{260}/A_{280}) as well as 260 nm and 230 nm (A_{260}/A_{230}) was 1.791 and 2.037, respectively. Meanwhile, the yield of the extracted nucleic acid was 81.5 ng/µL.

4.3.2 DNase and RNase Digestion of Phage Nucleic Acid

The extracted phage nucleic acid was subjected to DNase and RNase digestion to determine the nature of the nucleic acid. If the phage nucleic acid is composed of DNA, it would be digested by DNase, thus no band would be observed on the agarose gel electrophoresis. Conversely, if the phage nucleic acid was made up of RNA, it would be digested by RNase and no band would be observed. The gel image of the DNase or RNase digested phage nucleic acid after gel electrophoresis is shown in Figure 4.4.

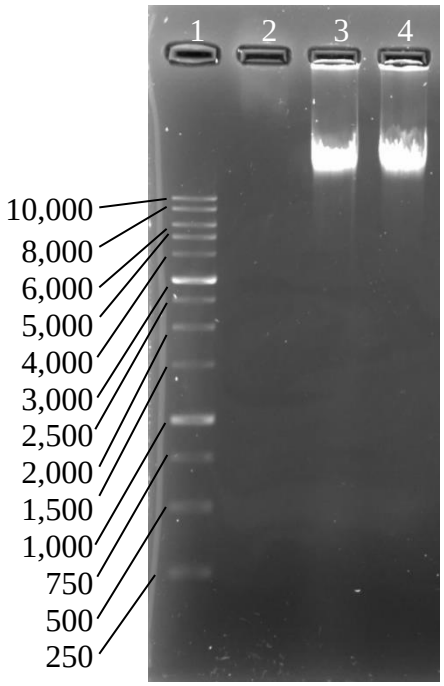

Figure 4.4: Gel image of the digested nucleic acid using DNase and RNase. Lane 1: 1 kb DNA ladder; Lane 2: DNase-treated phage nucleic acid; Lane 3: RNase-treated phage nucleic acid; Lane 4: Phage nucleic acid without any treatment (negative control).

Based on Figure 4.4, phage nucleic acid treated with DNase did not show any band, whereas Lane 3 and Lane 4 that were loaded with RNase treated nucleic acid and nucleic acid without treatment, respectively, showed presence of one single band. Therefore, the genome of phage KA is made up of DNA. The phage genome size was estimated to be larger than 10,000 bp.

4.3.3 Restriction Enzyme Digestion of Phage Nucleic Acid

The phage nucleic acid was then subjected to digestion using six different types of restriction enzymes such as *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, *Sac*I and *Xba*I. Figure 4.5 shows the gel image of the phage nucleic acid after treatment with six different restriction enzymes. All the six restriction enzymes were not able to cut the phage nucleic acid loaded in Lane 2 to Lane 7 as the bands remained at the same level as the untreated phage nucleic acid in Lane 8 which served as a negative control.

Plasmid DNA which is a double stranded circle can exist in three conformations, which are supercoiled, open-circular (oc), and linear. Lane 15 and 16 showed the uncut plasmid DNA pBR322 and pUC19, respectively, which served as positive controls. Plasmid DNA pBR322 and pUC19 are 4,362 bp and 2,686 bp in length, respectively (Watson, 1988). Bands observed in Lane 9 to Lane 14 were the linearized plasmid DNA digested with restriction enzymes.

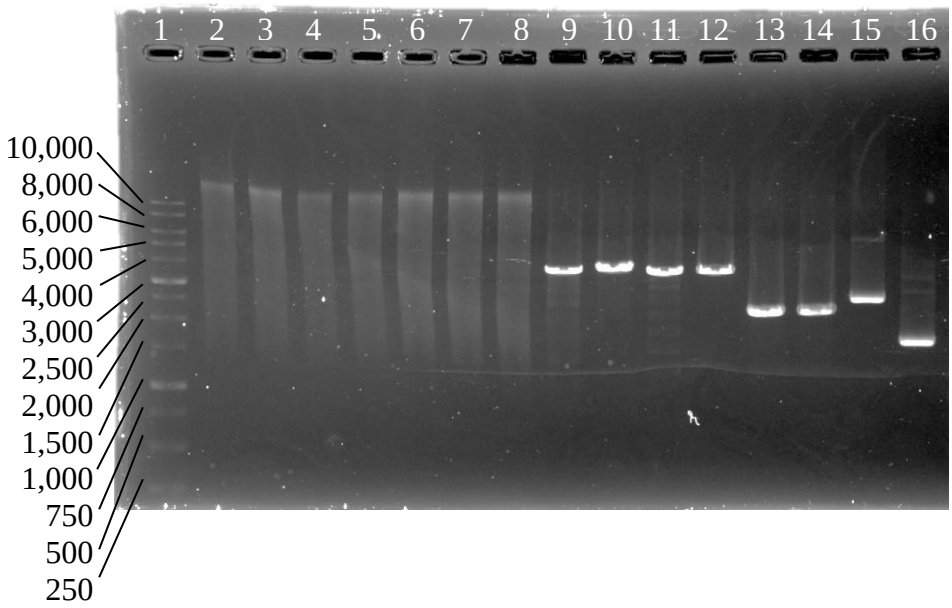

Figure 4.5: Gel image of phage DNA digested with different restriction enzymes. Lane 1: 1 kb DNA ladder; Lane 2: *BamHI*-treated phage DNA; Lane 3: *EcoRI*-treated phage DNA; Lane 4: *EcoRV*-treated phage DNA; Lane 5: *HindIII*-treated phage DNA; Lane 6: *SacI*-treated phage DNA; Lane 7: *XbaI*-treated phage DNA; Lane 8: Undigested phage DNA (negative control); Lane 9: *BamHI*-treated pBR322 DNA; Lane 10: *EcoRI*-treated pBR322 DNA; Lane 11: *EcoRV*-treated pBR322 DNA; Lane 12: *HindIII*-treated pBR322 DNA; Lane 13: *SacI*-treated pUC19 DNA; Lane 14: *XbaI*-treated pUC19 DNA; Lane 15: Undigested pBR322 DNA (negative control); Lane 16: Undigested pUC19 DNA (negative control).

4.4 Morphological Characterization

4.4.1 Transmission Electron Microscopy

Dialyzed caesium chloride purified phage was then observed on a negatively-stained carbon-coated grid using a transmission electron microscope (TEM) under magnification of 10,000×. Figure 4.6 shows the TEM micrograph of phage KA. The virion of phage KA is approximately 140 nm in length. Phage KA has a long tail and an icosahedral head. No tail fibres were observed.

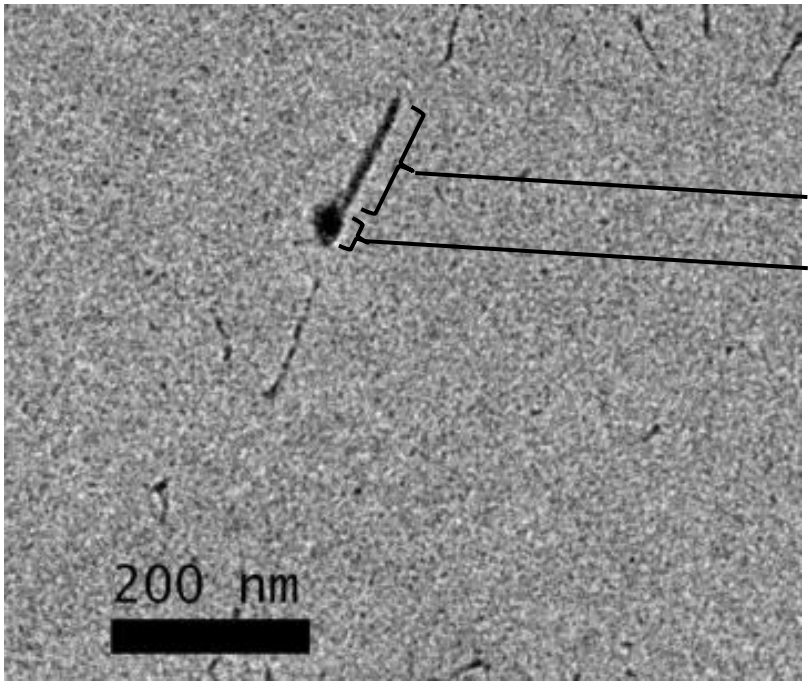

Figure 4.6: TEM micrograph of negatively-stained phage KA under magnification of 10,000×. The bar represents 200 nm in length.

CHAPTER 5

DISCUSSION

5.1 Purification of Phage with Caesium Chloride Ultracentrifugation

According to Majekodunmi (2015), particles move through the density gradient until a point at which their density is equivalent to the density of the surrounding is reached in isopycnic or equilibrium buoyant density separations. Based on Figure 4.1, ultracentrifugation of phage KA resulted in the formation of first upper band at a density of 1.3 g/mL which are composed of unwanted bacterial debris, phage ghosts and internal proteins as no infectivity was detected by doing plaque assay. Meanwhile, the second lower band which appeared thicker was formed at a density of 1.4 g/mL with an average phage titre of 5.24×10^{13} pfu/mL. Therefore, phage KA has a buoyant density of approximately 1.4 g/mL.

In a study conducted by Ghei, et al (1968), in caesium chloride solution, the buoyant density of T5 that infects *E. coli* is 1.508 g/mL. However, another phage XP5 targeting *E. coli*, was revealed to be less dense than T5 as its buoyant density is 1.45 g/mL. Ghei and colleagues (1968) indicated the difference in buoyant density between T5 and XP5 was due to some difference in overall chemical composition. In addition, difference in DNA fraction in the intact phage or fraction of guanine and cytosine to adenine to thymine in the

DNA of phage could lead to different buoyant density. These findings also showed close similarity to phage KA with buoyant density of 1.4 g/mL. Therefore, it is suggested that phage targeting similar host may exhibit similar buoyant densities, ranging from approximately 1.4 g/mL to 1.5 g/mL. The slight difference in density could be due to different chemical composition.

In another study conducted by Kaiser (1986), ultracentrifugation of a mixture of phage lambda particles and ghost particles which had expelled their DNA resulted in two bands formation. One of the bands was formed at density 1.5 g/mL and was composed almost solely of phage particles. The other band was obtained at a density of 1.3 g/mL with only ghost particles. In the present study, phage KA particles were found at density of 1.4 g/mL and ghost particles were formed at 1.3 g/mL. Therefore, phage particles are shown to be denser than ghost particles of which DNA is absent.

According to Osborn, Weiner and Weber (1970), bacterial debris, internal proteins and phage ghost particles can be recovered from caesium chloride zones with different densities. High caesium chloride salt aids in removing any protein contaminants binding closely to the phage particles. As a result, protein contaminants are separated from phage to give pure phage particles. However, due to the high ionic strength of salt solutions, protein-protein interaction and nucleic acid-protein bonds could be disrupted (Steward and Culley, 2010). In this study, the titre of phage KA before ultracentrifugation was 9.28×10^{13} pfu/mL. However, after ultracentrifugation, the phage titre

decreased slightly to 5.24×10^{13} pfu/mL. This could be due to intolerance of phage KA towards the high ionic strengths of caesium chloride and its toxicity towards virus particles (Lorenz, et al., 2009).

5.2 Physiological Characterizations of Phage

The virulence of a phage depends on the presence of particular specific receptors on the surface of host cell and environmental conditions, such as temperature, pH and certain cations in the media (Lau, et al., 2012). These environmental factors can affect the ability to adsorb and efficiency of phages to cause lysis (Fischetti, 2008). Therefore, investigations of these conditions are required to determine the suitable route of administration of the phage targeting certain pathogen.

5.2.1 Host Range

Most phages can target or inactivate the growth of only a single specific host strain (Huang, et al., 2013). Based on Table 4.1, among the eight gram-negative bacteria, phage KA could only infect and lyse *E. coli* BL 21 (DE3) and *Shigella boydii*, which showed that phage KA has a narrow host range. A specific phage strain is only able to infect a narrow host range or a specific bacteria and majority of phage are only able to lyse different strains within a species (Clokie, et al., 2011). *E. coli* and *Shigella* spp. belong to the Enterobacteriaceae family and classified as one distinctive species in the genus *Escherichia* (Pupo, Lan and Reeves, 2000).

Sugar polymers in gram-negative bacteria can protect the underlying surface structures by forming thick coats in order to protect the bacterium from host immune system. In this way, a barrier against bacteriophage infection is also created (Taylor and Roberts, 2005). However, attachment sites can also be found on the protective sugar coats specific for certain phages. Only phages with polysaccharide depolymerases as tail spike proteins can penetrate into the protective coat and to access to the receptors on the cell surface (Buettner, et al., 2012). This specificity in the phage-bacteria interaction is also determined by the specificity for adsorption, which is highly dependent on the nature and structural of receptors found on bacterial cell surface (Rakhuba, et al., 2010). Various components of the bacterial outermost cell layer, such as flagella, O antigen of lipopolysaccharides, outer membrane porin protein C, and cobalamin transporter BtuB, are receptors found on bacterial cell surface for phage attachment (Hong, et al., 2008).

According to Edwards, et al. (2016), most coliphages can infect *E. coli*'s close relative *Shigella*. Accordong to Ud-Din and Wahid (2014), *Shigella* and *E. coli* share a common ancestor and they are 80-90% similar at nucleotide level, therefore the proteins or gene encoded for their surface receptor may be similar, therefore enabling subsequent infection process. This is in accordance to phage KA as it can also infect both *E. coli* BL21 and *Shigella boydii*. *Shigella* species can cause diarrheal disease (Kim and Ravinder, 2010). *S. boydii* is quite rare in developed countries as compared to other *Shigella* species and is usually related with individuals who have gone to endemic areas

before (Kim and Ravinder, 2010). In this study, phage KA could cause lysis to *S. boydii*, therefore phage KA can be used to combat *S. boydii* infections.

Besides, BL21 (DE3) is the most commonly used *E. coli* strain for high expression of recombinant proteins and highest yield of target protein can be produced as compared to endogenous proteins (Robichon, et al., 2011). As phage KA could cause lysis to *E. coli* BL 21 (DE3), therefore phage KA can be potentially used to disrupt cells for the release of recombinant protein. In addition, the endolysin produced by phage KA can be further explored to determine its potential applications in medical field and other industries.

Narrow host range was also demonstrated in a previous study conducted by Zhou, et al., 2015, whereby an *E. coli* specific phage JS09 isolated from sewage samples of a swine farm in China was only able to lyse 16 *E. coli* B and K strains among 46 *E. coli* and *Salmonella* spp. strains. None of the *Salmonella* spp. strains were lysed. Another study showed that coliphages MER01 and MER02 isolated from sludge has a narrow host range, as they infect only two of the four tested *E. coli* strains, which are *E. coli* NDM-1, *E. coli* J53, *E. coli* K-12 and *E. coli* C3000 (Yu, et al., 2017). In comparison to the antibiotics usage, phage therapy can be more beneficial due to its narrow host range, therefore it is less harmful to the normal body microbiota. Antibiotics, on the other hand, can cause disruption to the normal gastrointestinal microbiota. As a result, opportunistic secondary infections

caused by organisms such as *Clostridium difficile* are more likely to occur (Merril, Scholl and Adhya, 2003).

Based on previous findings, there are also broad-host range phages that can infect distinct genera or species strains. In a previous study, phage HY01 isolated from a swine faecal sample was shown to infect both *E. coli* O157:H7 and *S. flexneri*. The study revealed that it could be due to two different host specificity-associated genes that are present in the phage genome, as a result, phage HY01 can lyse both different host strains (Park, et al., 2012). The ability of phage to infect broad-host range is advantageous in the biocontrol of different pathogens. For instance, by cloning a variety of host specificity-associated genes into a phage via genetic engineering, a novel phage with broad-host range could be produced for biocontrol of different pathogens (Lee, et al., 2016). Therefore, further investigations should be done to determine host specificity-associated genes in genome of phage KA.

In another study, phage SFP10 isolated from soil sample was shown to target *Salmonella Typhimurium* and *E. coli* O157:H7. Sequencing and analysis of the genome of phage SFP10 was performed. It was found that a tail fibre protein and two tail spike proteins is present in the phage SFP10, which are encoded by the host specificity decision gene cluster consisting of three genes. Subsequently, the tail fibre protein is shown to be highly specific to *E. coli* O157:H7 by performing comparative genomic analysis and BLASTp analysis.

However, the two tail spike proteins show higher specific towards *Salmonella Typhimurium* (Park, et al., 2012).

5.2.2 Effect of Temperature on Phage Infectivity

Temperature is an important factor that determines phage survival. It can affect the extent of attachment, penetration and multiplication of phages (Jończyk, et al., 2011). Based on Figure 4.1, it was shown that the effect of temperature is inversely related to the infectivity of phage KA. Phage infectivity decreased gradually as the temperature increased from 50°C to 60°C and showed highest stability at room temperature. According to Yamada (2013), heat resistance of majority of the *Thermus* phages shows adaptation to their environment.

In the present study, although phage KA was isolated from a 55 ℃ hot spring, it was shown that this phage could not tolerate temperatures higher than 55 ℃. This is in accordance with a study conducted by Lin, et al. (2010), whereby a *Thermus* bacteriophage TSP4 isolated from a neutral hot spring at 65 ℃ at Tengchong, China was most stable at 60 ℃ and its survival decreased significantly after 1 hr of heat incubation at 70 ℃ and 80 ℃. In addition, another study conducted by Mishra, Choi and Kang (2012) demonstrated that thermostable phages from *Siphoviridae* family isolated from a hot spring in China are shown to be exhibit stability at 60 ℃ but their phage titres were significantly decreased by half at temperature of 70 ℃.

Since phage KA was isolated from a 55°C hot spring, theoretically the phage infectivity should be the highest at 55°C among other temperatures tested as that is the optimum temperature for phage KA. However, the phage without any heat treatment showed the highest infectivity. According to Beata, et al. (2016), the way of storing phage should be determined by the phage biology and properties. This is to retain the survival of phage particles in its original living conditions or environment. However, storing phage in a cooling condition could also maintain their survival.

A study conducted by Cooper, Denyer and Maillard (2014), three phages which are GL-1, GL-$1_{2.5}$, and LP-M_{10} were challenged against *Pseudomonas aeruginosa* by storing them at different temperatures. It was found that storage of these phages at both 4℃ and room temperature was able to maintain their survival even after 180 days with minimal decrease of survival. However, after 300 days, 40% of the phage was killed. Therefore, it is suggested that temperature of phage storage should be similar to the environment of the phage where it resides in. Moreover, the duration of storage can also influence phage stability. Since phage KA was not stored at 55℃ which is the temperature of the hot spring that it resides in, and phage KA has been stored at 4℃ for quite some time, its stability may have been affected.

5.2.3 Effect of pH on Phage Infectivity

According to Taj, et al. (2014), pH plays an important role to determine the survival of phage in an environment. In this study, optimal pH was determined by testing the stability of phage KA under pH ranging from pH 5 to pH 9 of growth medium. Based on Figure 4.3, phage KA could not tolerate pH 5 and pH 9. However, phage KA could tolerate a neutral environment, around pH 6 to pH 8. These results suggested that extreme pHs might affect phage stability (Yang, et al., 2010).

Previous findings have shown that the survival of phage can be indirectly affected by pH of media by influencing the extent of phage adsorption to other bacterial cells (Taj, et al., 2014). Moreover, unfavourable environment can damage phages' structural elements (head, tail, envelope) as well as causing loss of lipid and disruption in DNA structural changes, these can lead to inactivation of a phage (Ackermann, Tremblay and Moineau, 2004). In another study, stability of MS2 phage was tested in different salt solutions, it was found that the phage titre is unaffected by the presence of monovalent salts (Mylon et al., 2010). Moreover, according to Langlet et al. (2008), high ionic strength in low pH of media can increase the aggregation of phage.

However, according to Ly-Chatain (2014), at pH lower than pH 4.5, the proliferation of some phages is limited. It was shown that in food contamination cases, the pathogenicity of some pathogenic bacteria is also

generally reduced at pH below pH 4.5. For example, the phage T4 from *Myoviridae* family is found to be unstable at pH less than pH 5. Phage PM2 from *Corticoviridae* family also completely loses its lytic activity at pH 5.0 and a temperature of 37℃ after 1 hr (Ly-Chatain, 2014). Moreover, it was revealed that stomach acid can have a negative effect on phage survival if it is injected orally. This could result in failure in treatment (Watanabe, et al., 2007). Since phage KA favoured neutral environment but not acidic environment, therefore, it may not be able to survive in the highly acidic environment of stomach.

In a previous study, Jończyk, et al (2011) had demonstrated the stability of five different marine phages in media. Different inorganic salts were added into the media, such as distilled water, 0.5% and 3% of NaCl solution, artificial seawater, seawater broth and artificial seawater. It was observed that all phages were most inhibited in a medium containing 0.5% NaCl than in the other media. It was also found that the phages were most active in salt concentrations similar to seawater. Similarly, in the present study, phage KA isolated from the pH 7.6 hot spring at Kuala Woh with unknown salt concentrations favoured a neutral environment around pH 6 to pH 8, and it showed the highest stability at pH 7 which is near to the pH of the hot spring. Therefore, it is convinced that phages are most stable at the environment where they reside at. Moreover, different environments can have different pH.

Several studies have shown that phage exhibits stability in a wide range of pH regimes (Langlet, et al., 2008). For instance, T4 phage isolated against *E. coli* BL21 strain was shown to be stable at different pH regimes ranging from pH 4 to pH 10 (Taj, et al., 2014). However, there are also phages with narrow pH stability. In a study conducted by Krasowska, et al. (2015), two *Bacillus subtilis*-specific bacteriophages which are SPOσ and SIOΦ isolated from soil. It was found that SIOΦ and SPOσ phages are the most resistant to acid (pH 4) and alkaline (pH 9 and pH 10). The optimum pH values for both phages were 7.0 and 8.0. Lasobras, et al. (2007) have shown that phage Arπ isolated from soil, which was morphologically classified as a member of *Siphoviridae*, is stable in a narrow pH range (6.0 to 8.0). These findings are in line with the results obtained in this study as phage KA also showed narrow pH stability and grows optimally around a neutral environment.

5.3 Partial Genomic Characterization

5.3.1 Phage Nucleic Acid Extraction

Based on the results described in Section 4.3.1, the absorbance of the extracted nucleic acid measured at (A_{260}/A_{280}) and (A_{260}/A_{230}) was 1.791 and 2.037, respectively. In addition, the yield for the extracted nucleic acid was 81.5 ng/µL. According to Wang, et al. (2011), the A_{260}/A_{280} of pure nucleic acid is within the range of 1.8 to 2.0 whereas the optimum A_{260}/A_{230} values are typically in the range of 2.0 to 2.2 (Paul, 2001). Therefore, the extracted nucleic acid in this study was considered pure as the values obtained were close or within the range. The A_{260}/A_{280} value obtained in this study was

slightly lower than the range and this could be due to presence of phenol residual during the extraction procedure.

In this study, proteinase K was used in nucleic acid extraction. Proteinase K is useful for protein digestion as it cleaves glycoproteins and inactivates RNase and DNase in the presence of 0.5% to 1% SDS solution to release genomic DNA (Hebron, Yu and Jun, 2009). A study conducted by Fatma, et al. (2011) has compared the DNA yield with and without the addition of SDS with proteinase K for DNA extraction. It was found that the DNA yield for the extraction protocol with the usage of SDS and proteinase K was higher than the other protocol without proteinase K.

5.3.2 DNase and RNase Digestion of Bacteriophage Nucleic Acid

Phage genome can be composed of either DNA or RNA. Based on results in Figure 4.4, the genome of phage KA was found to be composed of DNA as it was digested by DNase but not RNase. In addition, the phage genome size was estimated to be larger than 10,000 base pairs. Since phage KA is a lytic phage, its genome was compared with other lytic phages' genomes that were made up of DNA.

Several studies have shown that some DNA phage isolated consists of really large genome size. For instance, a lytic SPC35 phage that can target *Salmonella enterica serovar Typhimurium* and *Escherichia coli* revealed a

genome size of 118,351 bp (Kim and Ryu, 2011). Similarly, an isolated lytic phage phiE142 from animal faecal samples against *Salmonella* and multidrug-resistant *Escherichia coli* O157:H7 strains consist of a DNA genome of 121,442 bp in size (Amarillas, et al., 2016). Based on these findings, both the reported phages were able to infect *E. coli* strains, but the genome size was significantly larger than phage KA genome.

On the other hand, there are also lytic DNA phages that have relatively smaller genome size as compared to the lytic phages described previously. For example, a lytic phage P8625 targeting *Verrucomicrobia* that was classified under the family *Siphoviridae*, isolated from surface seawater consists of genome with a size of 32,894 bp (Choi, et al., 2015). Another study conducted by Turner, et al. (2017) demonstrated that a new lytic *Acinetobacter baumannii* phage, vB_AbaS_Loki isolated from activated sewage sludge. Genome sequencing revealed that Loki that is a siphovirus encapsulates a 41,308 bp genome. Therefore, it can be concluded that lytic phage is usually a DNA phage. However, the genome size of lytic DNA phage may vary. In general, these genomes are packaged into their capsids at similar densities. Moreover, the capsid size may vary as it is dependent on the size of genome (Hatfull and Hendrix, 2011).

5.3.3 Restriction Enzyme Digestion of Bacteriophage Nucleic Acid

According to Pingoud, Wilson and Wende (2014), type II restriction enzymes as well as other types of restriction enzymes are present exclusively in unicellular microorganisms such bacteria and prokaryotes or archaea. Their primary function is to protect host genome against foreign infectious DNA molecules, such as phage DNA. Each restriction enzyme can recognize a specific sequence in double-stranded DNA with high accuracy and cleaves at a specific position, therefore producing fragments.

Based on Figure 4.5, the phage DNA could not be digested by the six different type II restriction enzymes, namely *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, *Sac*I and *Xba*I. This shows that phage KA genome does not have the restriction sites for the enzymes. According to Pingoud and Jeltsch (2001), in the presence of magnesium ions, type II restriction enzymes recognize short sequences of 4 to 8 bp which are usually palindromic and cleave the DNA within or near to the recognition site.

There are also cases whereby phage genome could not be digested. For example, the DNA of coliphage PR772 isolated from Africa was not digested by four restriction endonucleases, namely *Bam*HI, *Bgl*II, *Eco*RI and *Hin*dII. Previously, it has been found that phages without restriction enzyme sites, is probably due to an evolutionary response to pressures exerted from their host restriction enzymes, mainly members of the *Tectiviridae* family (Lute, et al.,

2004). The genomes of PR772 and another phage PRD1 were compared and it was found that their DNA sequences showed 97.2% similarity. However, transmission electron microscopy of PR772 confirmed that it is classified to the *Tectiviridae* family, which is similar to PRD1 in terms of structure. It was also found that there were unique restriction enzyme sites present in PR772 but not in PRD1 (Lute, et al., 2004). In another study conducted by Kulikov, et al. (2014), DNA of phage 9g isolated from horse faeces against *Escherichia coli* C600 showed resistance to *Eco*RV, *Hae*III, *Dra*I, *Eco*RI and *Mbo*I. The resistance of phage DNA to several restriction enzymes shows the possibility that one or more of the DNA bases could have been modified, therefore providing protection of the genomes against degradation by restriction enzymes. For example, chromosomes of T-even phages consist of glucosylated hydroxymethylcytosine residues, therefore unable to be recognised by its specific restriction enzymes (Lehman and Pratt, 2008).

Since type II restriction enzymes are only able to cleave double-stranded DNA, the genome of phage KA cannot be deduced as double-stranded DNA. However, there is still possibility that it is made up of double-stranded DNA since only six restriction enzymes used in this study. It could have restriction sites for some enzymes such as *Xho*I, *Kpn*I, *Sal*I and *Pst*I which could cut the phage T4 genome against *E. coli* (Patrick, Kutter and Nakanishi, 2010). Therefore, further investigations need to be carried out on phage KA genome to determine its genomic properties.

However, since no bands or restriction fragments observed in the gel image, the genome of phage KA could be made up of single-stranded DNA. As a result, the type II restriction enzymes could not cleave the genome and generate restriction fragments. According to Szekely and Breitbart (2016), ssDNA phages only represent 11% of the phages in the ICTV database. The majority of these are filamentous phages belonging to the family *Inoviridae*, including Ff phages; while the family *Microviridae*, which includes φX174, only represents 2% of the database. Furthermore, most of the ssDNA phages are related to members of the family *Microviridae*, which is typically regarded as a group of specialized phages that only infect a narrow range of hosts, namely *Enterobacteria* and intracellular parasites such as Chlamydia (Cherwa and Fane, 2011).

5.4 Morphological Characterization

5.4.1 Transmission Electron Microscopy

Approximately 96% of known phages in the order *Caudovirales* are tailed viruses of that comprise three families. They are *Podoviridae* with short tail, *Myoviridae* with long and contractile tail, and *Siphoviridae* with long and non-contractile tail, which account for 14%, 61% and 25%, respectively (Fokine and Rossmann, 2014). According to Aprea, et al. (2015), bacteriophages are differentiated based on their head shape of either icosahedral or elongated, as well as presence or absence of a tail structure. By comparing the tail to the diameter of head, the tail can be estimated as long or short tail. It can also be

contractile or non-contractile Appendices or tail-fibres are present in some of the phages.

Based on Figure 4.6, phage KA has an icosahedral head and a tail. The tail is a long and non-contractile tail as the tail is longer than the head diameter, and contractile sheath was not detected. The length of the virion of phage KA is approximately 140 nm. According to (Bradley, 1967), the length of phage's tail provides background information of its stability and resistance in its living environment. In fact short (tail shorter than head diameter) and phages without tail are usually more resistant, whereas phages with long tails are more prone to damage, therefore causing them to lose its infectious activity. On the other hand, tailed phages are composed of double-stranded DNA whereas phages without tail show completely dissimilar genomic properties of either single-stranded DNA or RNA which are relatively less common. This is in accordance to phage KA as it has long tail and it could not tolerate extreme pH or temperature. Moreover, phage KA is composed of DNA. However, phage KA could be not confirmed as double-stranded DNA.

According to Ackermann (2007), bacteriophages may have different morphological structures even if they are residing in similar environments. For instance, phages residing in hot springs have been classified into five different archea viruses which are *Ampullaviridae*, *Bicaudaviridae*, *Globuloviridae*, SH1 and STIV due to their dissimilar morphological forms. Firstly, *Ampullaviridae* isolated from Italian volcanic springs consists of a cone-

shaped body, a nucleocapsid which is helical in shape, and polar tail fibres. *Bicaudaviridae*, consists of a helical nucleocapsid as well as grow tail-like appendages at two ends, was isolated from acidic hot springs in China. In the same Italian hot spring, another phage of *Globuloviridae* family poccesses a helical nucleocapsid lipid with a spherical envelope.

On the other hand, SH1 isolated from an alkaline lake in Australia have polyhedral head and a lipid nucleocapsid which was classified as *Tectiviridae*. Lastly, STIV isolated from a hot spring in Yellowstone National Park are phages with polyhedral head targeting hyperthermophilic bacteria (Ackermann, 2007). Meanwhile, phage KA has an icosahedral head and long non-contractile tail which are morphological characteristics of a *Siphoviridae* family. However, its morphology is different from the other phages isolated from hot springs or similar environments as mentioned above. Therefore, it can be concluded that different phages from similar environments could have different morphology.

Since phage KA has a long tail, comparisons with *Myoviridae* and *Siphoviridae* which also have long tail was done. In a study done by Oliveira, et al. (2017), two lytic *Morganella morganii* bacteriophages (vB_MmoM_MP1, vB_MmoP_MP2) isolated from wastewater treatment plant sewage samples located in Braga (Portugal) shown that MP1 is a myovirus with an elongated icosahedral head (85 nm in length and 65 nm in width) and a contractile tail of 111 nm in length and 17 nm in width. In

another study conducted by Amarillas, et al. (2016) stated that phage phiE142 which is infectious to *Salmonella* and multidrug-resistant *E. coli* O157:H7 strains is classified to the order *Caudovirale*s under *Myoviridae* family due to an isometric head with average diameter of approximately 58 nm and a long non-flexible contractile tail of approximately 120 nm in length.

According to Tamakoshi, et al. (2011), a lytic phage ϕTMA isolated from a hot spring in Japan against *Thermus thermophilus* HB27 consists of an icosahedral head as well as a contractile tail. Meanwhile, another study conducted by Kim, et al. (2010) claimed that a novel bacteriophage SP18 found from the Gap River in Korea that targets *Shigella sonnei* showed morphological characteristics of the family *Myoviridae*, which is an icosahedral head and a contractile tail with fibres. However, since phage KA does not have contractile tail and tail fibres were not observed, phage KA could not be classified under *Myoviridae* family.

Since phage KA could not be classified under *Myoviridae* family, further comparisons with *Siphoviridae* phage were done. In a study conducted by Li, et al. (2010), phage EEP isolated during an electroporation experiment in which engineered *E. coli* strain BL21 (DE3) was used as competent cell. BL21 (DE3) cells were subsequently used as host cells for EEP. It was found that phage EEP which has an icosahedral head and a long flexible non-contractile tail, was classified as a member of the *Siphoviridae* family.

Another study conducted by Othman, Askora and Abo-Senna (2015) isolated phage ϕBM from a religious structure (Kaaba) located in Makkah, Saudi Arabia. It was revealed that phage ϕBM has an icosahedral head with diameter of about 65 ±5 nm and a long non-contractile tail of about 300 ±10 nm in length, about 17 ±2 nm in width. These findings are in accordance to the morphology of phage KA as it also has an icosahedral head and a long non-contractile tail. Therefore, it is convinced that phage KA can be classified under the *Siphoviridae* family of *Caudovirales* order.

5.5 Limitations of Study

The exact genome size of the phage KA could not be determined. Moreover, the electron micrograph of phage KA provides limited details in terms of structure as the magnification used for transmission electron microscopy was not high enough to visualize the details of phage KA.

5.6 Future Studies

Firstly, the temperature stability test should be further investigated by using different incubation period. In addition, whole genome mapping of phage KA could be done to determine its genomic sequence. Pulse field gel electrophoresis could also be performed to determine the size of phage KA genome. Moreover, the magnification used for transmission electron microscopy could be increased to have a better visualization of the virion.

CHAPTER 6

CONCLUSION

Characterization of a novel phage is very important for the discovery of it potential applications in medical field or other industries. In this study, phage KA isolated from a hot spring at Kuala Woh, Perak was characterized based on its physiological properties, genomic properties and morphological properties. Caesium chloride density gradient ultracentrifugation showed that phage KA has buoyant density of approximately 1.4 g/mL which is quite similar to other phage infecting *E. coli* as well. In terms of physiological properties, phage KA was found to be specific towards *E. coli* BL21 (DE3) and *S. boydii*. Therfore, phage KA can be used to disrupt cells for the release of recombinant protein and to combat *S. boydii* infections.

Moreover, phage KA was most stable at room temperature and it could not tolerate temperature of 60°C and above. Phage KA favours a neutral environment around pH 6 to pH 8. Therefore, phage KA probably can survive in human with body temperature of around 37°C. However, it could survive in acidic condition of the stomach. In terms of genomic properties, the genome of phage KA is composed of DNA with a size of more than 10,000 bp. In addition, phage KA does not have recognition sites for the six restriction enzymes which are *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, *Sac*I and *Xba*I.

Therefore, phage KA genome was unable to be confirmed as double-stranded DNA. Further investigations should be carried out by using other uncommon restriction enzymes that may cut the phage genome. Morphologically, phage KA has an icosahedral head and a long non-contractile tail, with a virion size of about 140 nm in length. Therefore, phage KA may be classified as *Siphoviridae* family of the *Caudovirales* order.

In conclusion, further investigations should be done on phage KA as it may harbour potential enzymes that can be used in medical and biopharmaceutical industry.

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