CHARACTERIZATION OF A NEW BACTERIAL VIRUS FROM ORANGUTAN (*Pongo pygmaeus*)

USWATUN BAHIRAH BT AHMAD HISHAM

UNIVERSITI SAINS MALAYSIA

2015

CHARACTERIZATION OF A NEW BACTERIAL VIRUS FROM ORANGUTAN (*Pongo pygmaeus*)

by

USWATUN BAHIRAH BT AHMAD HISHAM

Thesis submitted in fulfillment of the requirements for the degree of Master of Science

March 2015

ACKNOWLEDGEMENT

In the name of Allah, The Most Gracious and The Most Merciful

Prayers and peace be upon His Prophet Muhammad S.A.W.

Foremost, I would like to express the deepest appreciation to my supervisor, Assoc. Prof. Dr. Yahya Mat Arip for his exemplary guidance and constant motivation throughout this project. His continuous support, patience and knowledge will always be remembered. I am grateful for his effort and time despite of his extraordinarily busy schedule.

I express warm thanks to all postgraduate students from Lab 218 for all guidance and advices. It is through their kindness and encouragement that I have gained and grown. To all lab assistants and all members of the Electron Microscope Unit staffs, I will always appreciate your helps and your cooperation upon completion of this project.

To the Ministry of Higher Education and USM, I would like to take this opportunity to thank you for your generosity in funding this project. I sincerely appreciate the Institute of Postgraduate Studies USM for granted me the USM Fellowship.

I am highly indebted to my beloved parents and family for their endless love, blessings and encouragement. I will always love and treasure them forever. A special thanks to my beloved sister, Hanis Shaheera Bt. Ahmad Hisham for being there when I need it the most. To my fiancé, Mohd Ashraf B. Abdul Rahman, thank you for your prayers. For all who has contributed to this project, I owe you a heartfelt appreciation. Thank you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

APS	Ammonium persulfate
ATCC	American Type Culture Collection
BLASTn	Basic Local Alignment Search Tool-nucleotide
bp	Base pair
dH_2O	Distilled water
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
dNTP	Deoxynucleotide triphosphates
dsDNA	Double stranded deoxyribonucleic acid
E. coli	*Escherichia coli*

EB	Elution buffer
EDTA	Ethylenediaminetetraacetic acid
ICTV	International Committee on Taxonomy of Viruses
kb	Kilobase pair
kDa	Kilodalton
LB	Luria-Bertani
$MgCl_2$	Magnesium chloride
MOI	Multiplicity of infection
NaCl	Sodium chloride
$NaOAc.3H_2O$	Sodium acetate trihydrate
NCBI	National Center for Biotechnological Information
NEB	New England BioLabs

nm	Nanometer
OD	Optical density
PCR	Polymerase chain reaction
pfu	Plaque forming unit
RNA	Ribonucleic acid
RNase A	Ribonuclease A
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
ssDNA	Single stranded deoxyribonucleic acid
TBE	Tris-Borate-EDTA
TEM	Transmission electron microscope

TEMED	Tetramethylethylenediamine
TMS	Tris-Magnesium-Sodium
Tris base	Tris (hydroxymethyl)-aminomethane
Tris-HCl	Tris hydrochloric acid
w/v	Weight/volume

LIST OF APPENDICES

LIST OF SYMBOLS

Φ	Phi
®	Registered trademark
™	Trademark
β	Beta

PENCIRIAN SATU VIRUS BAKTERIA BAHARU DARIPADA

ORANGUTAN (*Pongo pygmaeus*)

ABSTRAK

Bakteriofaj atau faj ialah virus yang menjangkiti bakteria. Dalam kajian ini, faj UB1 yang telah dipencilkan sebelum ini yang menjangkiti *Escherichia coli* O157:H7 (*E. coli* O157:H7) telah berjaya dicirikan. Faj UB1 menghasilkan plak jelas dengan anggaran diameter 0.3 mm. Mikrograf electron transmisi menunjukkan bahawa faj UB1 mempunyai panjang keseluruhan ~250 nm dengan diameter ~105 nm. Faj tersebut mempunyai kepala ikosahedral dan ekor kontraktil menjadikannya berkemungkinan berasal daripada keluarga *Myoviridae*. Faj UB1 mempunyai spektrum perumah yang sempit memandangkan ia hanya menjangkiti tiga daripada dua belas bakteria yang diuji. Viabiliti faj UB1 ini mencatatkan nilai tertinggi dalam persekitaran neutral (pH 7), suhu rendah dan medium (10°C ke 50°C) serta kepekatan garam sodium klorida daripada 0.5% g/L ke 3% g/L. Kitaran replikasi faj UB1 mengambil masa selama 50 minit dengan tempoh terpendam selama 5 minit dan saiz pecahan sebanyak 44 partikel faj bagi setiap sel yang dijangkiti. Analisis protein separa mendedahkan bahawa jalur protein utama dengan saiz kira-kira ~40 kDa ke ~46 kDa yang mungkin merupakan protein kapsid. Perbandingan profil protein faj UB1 dengan faj T4 dan T7 menunjukkan bahawa kesemua faj berbeza antara satu sama lain. Penghadaman enzim pembatasan untuk faj UB1, faj T4, faj T7 serta faj *Myoviridae* lain yang menjangkiti *E. coli* O157:H7 menjana profil yang berbeza. Analisis genom DNA faj UB1 menunjukkan bahawa ia berkongsi

persamaan jujukan genom yang tinggi dengan faj PBECO 4 yang dipencilkan dari sisa kumbahan di Korea dan turut menjangkiti hos yang sama. Sebagai tambahan, kedua-dua faj UB1 dan PBECO 4 mempunyai morfologi yang hampir sama dan berkemungkian kedua-duanya adalah spesies yang sama. Namun, profil enzim pembatasan untuk kedua-dua faj menunjukkan sedikit perbezaan jadi berkemungkinan bahawa faj terpencil adalah strain yang berbeza. Secara ringkasnya, kajian ini telah mendedahkan pengetahuan tentang biologi faj UB1 dan juga ciri-cirinya pada peringkat genom.

CHARACTERIZATION OF A NEW BACTERIAL VIRUS FROM ORANGUTAN (*Pongo pygmaeus*)

ABSTRACT

Bacteriophages or phages are viruses that infect bacteria. Previously isolated phage, UB1 that infects *Escherichia coli* O157:H7 (*E. coli* O157:H7) was successfully characterized. Phage UB1 produced clear plaques with a diameter of 0.3 mm. Transmission electron micrograph revealed that phage UB1 was ~250 nm long with a diameter of ~105 nm. The phage had an icosahedral head and a contractile tail where it probably belongs to the *Myoviridae* family. Phage UB1 had a narrow host range where it only infected three out of twelve bacteria tested. The viability of phage UB1 was highest in neutral condition (pH 7), lower to medium temperature (10°C to 50°C) and in 0.5% w/v to 3% w/v salinity. Replication cycle of phage UB1 took around 50 minutes with short latent period of 5 minutes and burst size of 44 phage particles per infected cell. Partial protein profile analysis revealed major protein band of about ~40 kDa to ~46 kDa which could be the capsid protein. Comparison of the protein profile of phage UB1 with phage T4 and T7 showed that they were distinct from each other. Restriction enzyme digestion of phage UB1, phage T4, phage T7 and other *Myoviridae* phages infecting *E. coli* O157:H7 generated a different profile. Genome sequence analysis of phage UB1 DNA revealed that it shared high sequence similarity with phage PBECO 4 that was isolated from raw sewage in Korea which infects the same host. In addition, both phages shared almost similar morphology and could probably be the same species. Nevertheless,

restriction enzyme digestion profile of both phages generated a slight different profile which suggests that phage UB1 could be another strain. In summary, this study has provided the knowledge regarding phage UB1 biology as well as its characteristics at genomic level.

CHAPTER 1

INTRODUCTION

Since many phages are yet to be discovered and due to the increasing frequency of antibiotic resistance bacteria as well as the decreasing frequency of new antibiotics discovered has rekindled interest in the use of phage against pathogenic bacteria (Sulakvelidze *et al*., 2001; Sulakvelidze, 2011). Realizing once again the importance of phage as a bio-control agent, there is an urgent need to search for a potential new phage to treat bacterial infections (Anany *et al*., 2011). Recently, Hisham (2012) had successfully isolated a phage that is specific to *Escherichia coli* O157:H7 from the feces of orangutan (*Pongo pygmaeus*).

E. coli O157:H7 was selected as the phage host due to its pathogenicity and resistance to antibiotics (Aslam *et al*., 2010). There are many outbreaks of *E. coli* O157:H7 associated with food-borne diseases reported which are increasing worldwide after its first major outbreak in 1982 in the United States (Trueba *et al*., 2013; Verma *et al*., 2013). In addition, this pathogen resists antibiotic treatment making it difficult to be killed which opens the opportunity for phage to control this pathogen (Nordmann *et al*., 2011).

The reason why orangutan was chosen to isolate phage was due to the bacteriophage specificity. Since orangutan shared 95% of genetic similarities with human (Wildman *et al*., 2003), sample from orangutan might have the specific phage that could infect *E. coli* O157:H7, in human. In addition, the microbiota of orangutan could be the same as human since Enterobacteriaceae species were successfully isolated from the fecal sample of Sumatran orangutan (*Pongo abelii*) by Darmawi *et*

al., (2014) where the same species were also isolated from human as well (Guentzel, 1996; Voets *et al*., 2012; Soraas *et al*., 2014). Thus, if phage UB1 was able to infect *E. coli* O157:H7 and live inside orangutan without interfering with their microbiota, it would also means that phage UB1 would less likely to interfere with human microbiota.

The main purpose of this project is to further characterize the isolated phage, UB1, as the continuation from the previous work by Hisham (2012). The characterization of phage UB1 would lead to better understanding with regards to phage biology and its bio-control potentials. Specifically, the objectives of this project are:

1. To characterize phage UB1 based on:
    - morphology
    - physicochemical attributes and
    - molecular profiles
2. To determine host specificity of phage UB1.
3. To study phage UB1's replication cycle by single-step growth curve.

CHAPTER 2

LITERATURE REVIEW

2.1 Bacteriophage

Biosphere contains many types of organisms and the most abundant organism is bacteriophage or phage. Originated from the Greek word, *phagein* which means to eat, phage is defined as a virus of prokaryotic cells (Cescon, 2013). It was termed as bacteriophage since virulent bacteriophage would lyse bacterial cells they infect (Wommack & Colwell, 2000). This ubiquitous microorganism was found wherever bacteria thrive and certainly the concentration of bacteriophage is related to the concentration of bacteria present. It was estimated that there is about 10^{31} of bacteriophages exists on the earth (Fernandez-Gacio *et al*., 2003; Clokie & Millard, 2011).

As an obligate intracellular parasite, bacteriophages could only live and replicate once inside bacterial cells (Fischetti, 2008). Inside the host cells, the bacteriophages would become parasites by utilizing the host's machineries to produce new phage progenies (Lopez & Arias, 2010). Bacteriophage is composed of a genetic material enclosed in a capsid or protein coat. Their genomes could be either single stranded or double stranded DNA (deoxyribonucleic acid) or RNA (ribonucleic acid) which may exists as linear or circular (Sharp, 2001; Ackermann, 1998). Bacteriophages with double stranded DNA dominate the population. The morphologies of the bacteriophage could only be observed under the transmission electron microscope (TEM) since they are too small (20-250 nm) to be seen under the light microscope (Guttman *et al*., 2005; Gillen, 2007).

2.2 History of bacteriophages

The discovery of bacteriophages started over a century ago in 1896, where a British bacteriologist, Ernest Hanbury Hankin found something which had antibacterial activity against *Vibrio cholera* in the waters of Ganges and Jumna rivers in India (Nigam *et al*., 2014). The antibacterial activity from the unknown source was able to pass through a very fine porcelain filter and retain its lytic activity. However, he did not study this phenomenon further (Goodridge & Bisha, 2011). About 20 years later in 1915 after Hankin's finding, Frederick Twort who was also a British bacteriologist proposed the existence of viruses that infect bacteria by the secretion of bacteria that could lyse the neighboring cells. He observed that *Micrococcus* colonies had a clear transformation instead of their opaque creamy-white appearance (Twort, 1915; Summers, 2011). When he added some of the clear material to the bacteria lawn, similar appearances formed although they have been passed through a very fine filter. He called this phenomenon as 'transmissible lysis'. This findings has lead to the understanding of lytic infections of viruses (Bronfenbrenner & Korb, 1924; Summers, 2011).

In 1917, Felix d'Herelle discovered the same phenomenon in dysentery bacilli. He observed a transparent spots on lawns of dysentery bacilli. When he mixed the filtered faeces emulsion with broth cultures of pure dysentery bacilli, he noticed the lysis of broth cultures (Matsuzaki *et al*., 2005; Keen, 2012). He also found that this virus needs a living bacteria to multiply itself and he termed his virus as bacteriophage or phage (Dimmock, 1994; Keen, 2012). Realizing the potential and the significant of phages, an institute was opened in 1923 which specialized in the study of bacteriophage and named as the Eliava Institute located in Tbilisi, Georgia.

Between 1920's and 1930's, bacteriophage properties have been studied widely. Since the development of the Eliava Institute, much research has been conducted to study the biology of phages and its application in various fields (Lobocka & Szybalski, 2012).

2.3 Structure of bacteriophages

The invention of electron microscope seventy years ago has lead a breakthrough for researchers to analyse the bacteriophage structure (Urban, 2008). The use of electron helps to increase the resolution power and magnification to visualize microorganisms which is smaller than bacteria (Wurtz, 1992). Using the electron microscope, the general architecture of bacteriophages was revealed and further studies were conducted to explore its structure and organizations.

2.3.1 Basic architecture of bacteriophages

The underlying feature of bacteriophages is that all of them have nucleic acid surrounded by a protein coat or a capsid (Parija, 2009). The nucleic acid may exist in the form of single stranded or double stranded DNA (deoxyribonucleic acid) or RNA (ribonucleic acid). Capsid consists of a molecular complex or oligomer which is made of protein subunits called protomers which are then linked to form larger subunits called capsomers. Capsomers are the morphological subunits which form the capsid (Bradley, 1967, Mehrotra, 2009). Some bacteriophage particles may be enclosed in an envelope or may have a tail attached to the capsid. The tail structure may be short or long. The tail is divided into non-contractile or contractile tails which composed of many subunits arranged in helical form. Tailed bacteriophages mostly have an extra appendage attached at the tip of the tail while some of them may have a base plate attached with tail fibres (Leiman *et al*., 2003).

2.3.2 Basic morphological types of bacteriophages

Electron microscope has enabled the visualization of bacteriophages and its classification in accordance to its morphology. All of the bacteriophages observed under the electron microscope were grouped into six groups as shown in Figure 2.1 (Abedon, 2005). Group A is made up of complex phages with hexagonal head and a contractile tail. The rigid tail is usually composed of various appendages such as tail fibres. Bacteriophage T4 falls into this group since it is a bacteriophage with complex structure (Bradley, 1967).

Group B consists of phages with long flexible and non-contractile tails. The head of bacteriophages in this group is also in hexagonal shape as in group A. Bacteriophage T5 is classified into this group. Bacteriophages with a very short tail and with six-sided head are grouped into group C. The tail is usually non-contractile and often attached with extra appendages. Phages from *Podoviridae* family such as phage T7 made up group C (Clokie *et al*., 2011; Bradley, 1967).

In group D, it differs slightly where the tailless phages have no large apical subunits. As in groups A, B and C the head of bacteriophages in group D is also six-sided except that at the tip of the hexagon has a node-like structure or large capsomer attached to it which is present on phage ΦX174. In group E, the bacteriophages structure are made up of the head only without the present of tail or apical subunits and the virion just consists of a simple hexagonal head as observed in phage MS2. Bacteriophages in group F are comprised of filamentous phages which are long and flexible such as phage M13 (Bradley, 1967; Maramorosch, 1971; Clokie *et al*., 2011).

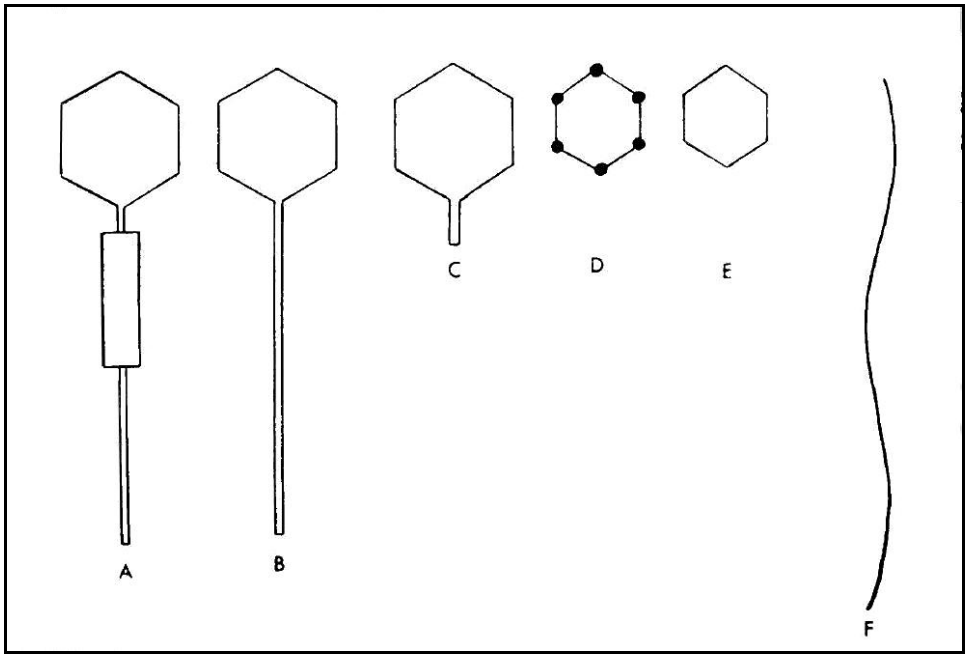

Figure 2.1 Six fundamental morphotypes of bacteriophages (Bradley, 1967).

2.3.3 Bacteriophage T4

One of the most complex viruses, bacteriophage T4 is the most studied phage since it has become a model for scientists to understand the biology of phages. Moreover, 168,903 base pairs (bp) of full genome sequence of phage T4 has allowed the understanding of bacteriophages at the molecular level (Miller *et al.*, 2003). Bacteriophage T4 is classified into the *Myoviridae* family which is susceptible to bacteria *E. coli* (Lobocka & Szybalski, 2012). The structure of bacteriophage T4 is shown in Figure 2.2. The total length of bacteriophage T4 is about 200 nm long and the diameter of its elongated icosahedron capsid is between 80 to 100 nm wide. It has double stranded DNA as its genetic material and it has 274 open reading frames. More than 40 out of the total open reading frames encode for its structural proteins (Leiman *et al*., 2003).

The elongated icosahedron capsid of bacteriophage T4 structure is made up of more than 3000 polypeptide chains with at least 12 different types of proteins. The hexagonal shape of the capsid is made up of three proteins which are gp23, gp24 and gp20 (Kanamaru *et al*., 2002). The capsid and the tail are separated by a neck and collar. The rigid tail of bacteriophage T4 is made up of two predominant layers which are the tail tube and contractile sheath. The contractile sheath surrounds the tail tube and during contraction, the diameter expands from 24 nm to 33 nm. At the end of the tail, tail plate is connected to the tail pins and tail fibres aids in the attachment of bacteriophage particle to the host cell receptors (Kostyuchenko *et al*., 2005; Lobocka & Szybalski, 2012).

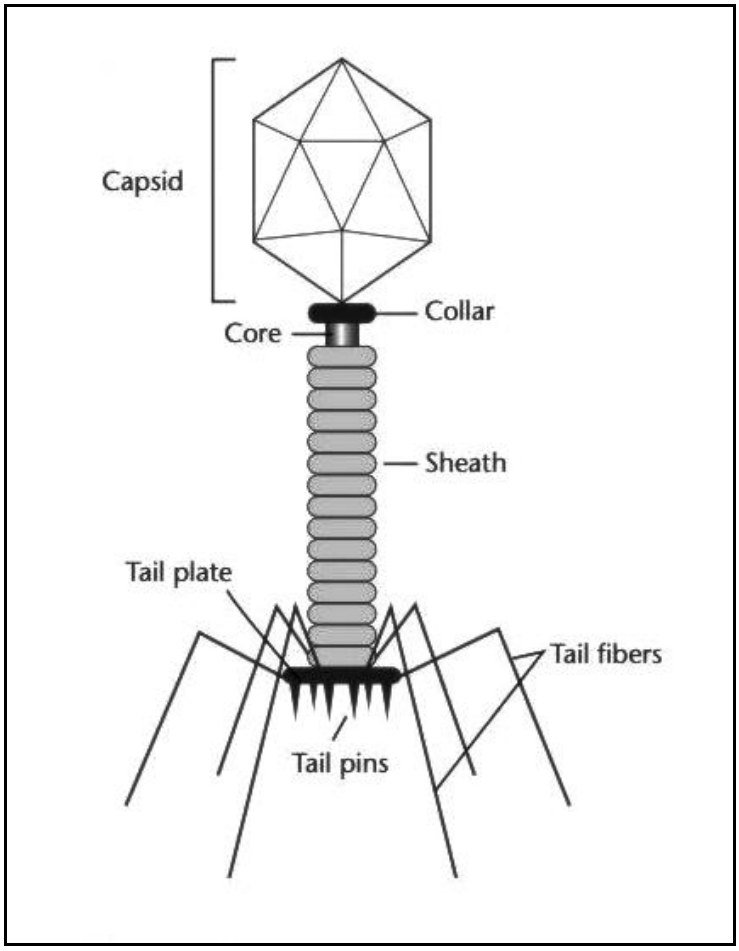

Figure 2.2 Morphology of bacteriophage T4 (Lobocka & Szybalski, 2012).

2.4 Evolution of bacteriophages

The advance in molecular biology has enabled scientists to study the evolution of bacteriophages. DNA sequence of phage genomes was used as tool to understand the differences between bacteriophages species. DNA sequence analysis of phage genomes revealed that the evolution of phage occurred through horizontal gene transfer (Canchaya *et al*., 2003). In horizontal exchange of sequences, the process of evolution takes place by first, random non-homologous recombination and second, recombination of variant gene generated from homologous gene transfer (Clokie *et al*., 2011).

The most rapid evolving group of organisms in this earth is the tailed, dsDNA bacteriophages. This ancient and most abundant microorganism has become a model to address questions regarding phage evolution which could be applied to the whole virus population (Hendrix, 2002). Lambdoid phages are bacteriophages that have lysogenic life cycle or termed as temperate phages. Lambdoid phages were used as a model in this study since it exchange genetic segment actively with lambda phage. It integrates its DNA into bacterial genome by site-specific recombination catalysed by the integrase enzyme. The different mechanism of DNA recombination that takes place has lead to many types of recombinant genes in the process of evolution such as the non-homologous and homologous recombination (Groth & Calos, 2004).

2.4.1 Mosaicsm of phage DNA and non-homologous recombination

The architecture of phage DNA is made up of mosaic structure. It denotes that in a single species of phage, its genome is composed of variant combination of DNA sequences which are formed through horizontal genetic exchange among the population (Hendrix, 2002 and 2003). Mosaicism of phage DNA may arise from

extensive non-homologous recombination between lambdoid phages, which is the exchange of genetic elements between two different DNA molecule (Clark, 2009; Hendrix, 2002). During non-homologous gene transfer, genomes of two lambdoid phages would exchange DNA segments with each other. When the sequence of both lambdoid phage is compared, certain regions would have high degree of similarity or homologous while the other regions may show slight or no similarity. This phenomenon suggests that the non-homologous recombination may originate between different ancestors of one of the phage genome. As an example, for phage HK97 and HK022, when both genomic sequences are compared, it showed about 99% of sequence similarity for major capsid genes. Meanwhile, both phages are only 31% identical in protein gp55 which is required in DNA replication (Hendrix, 2002). This percentage of similarity showed the difference in phage genome that arose from non-homologous recombination which then leads to different phage species. Mosaicism of phage DNA has lead to the evolution of phage species which vary greatly in terms of its head morphology, tail structure, size of phage particle as well as its host range (Hendrix *et al*., 1999).

2.4.2 Homologous recombination

Homologous recombination is the exchange of same DNA sequence between the same or closely related DNA (Clark, 2009). In phage genome, homologous recombination actually occurs at higher frequency than non-homologous recombination. Homologous recombination usually does not result in the change of DNA sequence, however, in certain events, it may result in notable changes of the phage genome. For example, such recombination may cause the formation of mosaic joints and reassortment of flanking gene (Hendrix, 2002). When the DNA sequence of lambdoid phage is observed, it is found that the mosaic joints may be actually the sites

of phage ancestral recombination (Susskind & Botstein, 1978). The position of mosaic joints in the DNA sequence is not arbitrary located, but is present at fixed locations across the phage genomes. Through mosaic joints, it is presumably that phage evolution occurred by homologous recombination or site-specific mechanisms in the conserved clusters of genes or between genes (Hendrix, 2002; Juhala *et al*., 2000, Susskind & Botstein, 1978). Finally after mosaic joints are successfully formed, reassortment of flanking genes would take place by homologous recombination (Campbell & Botstein, 1983). These events play an important role in novel recombination of genes and deliver variant novel genes in the population of phages. For example, genomic profile of phage P22 and λ has shown that both phages have a similar genetic organization which means that both DNA sequences contain some homologs. These homologs could exchange genetic segments and generate new viable hybrids. Through homologous recombination, it has revealed the reason why phage P22 and λ shared many same characteristics such as the physiology and gene organizations (Cowie & Szafranski, 1967; Botstein & Kleckner, 1977; Gemski *et al*., 1972, Skalka & Hanson, 1972; Hilliker & Botstein, 1976).

2.5 Diversity of bacteriophages

Bacteriophages exist in a staggering number and ubiquitous in the biosphere as its estimated total population size reached about 10^{31} phage particles (Chibani-Chennoufi *et al*., 2004). The estimation of phage density and its diversity is basically based on the existence of its host since phage is an obligate intracellular parasite of bacteria (Clokie e*t al*., 2011). Thus, estimation of phage population should be related to the host concentrations since phage only exists wherever bacteria thrive (Whitman *et al*., 1998). The ratio of phage to its host is estimated to be ten-to-one. Numerically if prokaryotic organisms concentration is 10^{30} individual cells and thus, the population

size of phage is supposed to be 10^{31} phage particles. The concentrations of phage are able to be presented in numerical data with the help of electron or epifluorescence microscopy as well as DNA staining (Abedon, 2008).

Bacteriophages are found in almost all geographical location on earth as long as bacterial host is present. It was reported that phage concentration was surprisingly very high in the ocean and coastal water and even more prominent in freshwater such as the lakes (Bergh *et al*., 1989). Since phage-to-host ratio is ten-to-one, the concentrations of phage in the eutrophic estuarine water are 10^7 particles per ml while bacterial density is 10^6 cells per ml. Meanwhile in the marine sediments, phage titers are counted to be around 10^9 phage particles per gram (Danovaro *et al*., 2002). On the other hand, in the terrestrial ecosystem, extrapolations by electron microscopy total counts have unveiled about 10^7 phages in one gram of soil (Ashelford *et al*., 2003).

Interestingly, phage does not only exist in the environments but are also found inside the human guts, ruminants and dairy products such as cheese. A survey was conducted to study the presence of phages in human. Stool sample was taken from about 600 healthy adults and the results shown that 34% of the samples are positive for the presence of coliphages (Furuse, 1987). In ruminants, their inabilities to digest cellulose cause them to rely completely on bacteria for that function. Since bacteria is present, phage concentrations in ruminants are as high as 10^7 phage particle for each gram of faeces. In dairy products like cheese which is fermented by lactic acid bacteria, *Lactococcus lactis*, phage often contaminates the food with titers up to 10^5 phage per m^3 in the air and 10^9 phage in one ml of whey. This findings showed that phage is disperse everywhere which reflects its high diversity (Neve *et al*., 1994).

2.6 Classification of bacteriophages

Every year, at least 100 novel phages are being characterized. Scientists estimated that the total number of phage species is about 100 millions which make bacteriophages constitute the largest virus group known (Rohwer, 2003). Bacteriophage classification is significant since it allows differentiation between phages and aids in understanding of phages. On the other hand, this classification helps in identification of newly isolated as well as its potential in various sectors.

2.6.1 History of bacteriophages classification

The notion for phage classification was pioneered in 1937 by an Australian microbiologist, Sir Macfarlane Burnet. He demonstrated that bacteriophages have a wide range of size and different resistance and sensitivity against physicochemical agents (Burnet, 1933; Sankaran, 2006). In 1943 after the invention of electron microscope, Ernst Ruska was the first to classify bacteriophages based on morphology (Ruska, 1943; Ackermann, 2009). Five years later in 1948, Holmes classified bacteriophages according to its host range where he proposed one genus with 46 species under the order *Virales* and family *Phagineae* (Holmes, 1948). However, since host range was not the criteria implemented to classify bacteriophages, his work was never been recognized (Karthik *et al*., 2014; Holmes, 1948). After that in 1962, Lwoff, Horne and Tournier suggested the criteria for virus classification which should be based on type of genetic materials (DNA or RNA), shape of capsid, number of capsomers and presence or absence of viral envelope. Based on these criteria, they classified tailed phages into order *Urovirales*, filamentous phage into family *Inoviridae* and ϕX-type such as ϕX174 phage under *Microviridae* family (Ackermann, 2006). In 1967, Bradley proposed six basic morphotypes of

bacteriophages which has become the fundamental in phage classification until today (Bradley, 1967). Since bacteriophage classification is very significant and more phage groups are added over time, the International Committee on Taxonomy of Viruses (ICTV) previously known as the Provisional Committee on Nomenclature of Viruses (PCNV) was established in 1966 and become the organization responsible for the classification of viruses (Ackermann, 2009).

2.6.2 Criteria of bacteriophages classification

The ICTV is the only body responsible in developing, improving and sustaining universal virus taxonomy. The most significant criteria to classify phages are the nature of nucleic acid whether it has single stranded or double stranded DNA or RNA, its morphology, physicochemical characteristics and the genomic profile (Ackermann, 2011).

2.6.3 The families of bacteriophages

Since the negative staining method was invented in 1959, about 5568 bacteriophages have been visualized by the electron microscope until the year 2007. There are more than 5500 phages examined and tailed phages constitute 96% of the total while others are in the form of cubic (cubic symmetry and icosahedral shape), filamentous and pleomorphic (Ackermann, 2007). Until now, the classification of phages is based on the Bradley's six basic morphological types of bacteriophages (Bradley, 1967).

In recent classification, ICTV has recognizes one order, 14 families with another five families anticipating official classification (Ackermann, 2009). Figure 2.3 shows the morphotypes of bacteriophage families. *Caudovirales* is the order made up

of tailed phages. In this order, tailed phages with double-stranded DNA which constitute the largest number of viruses are categorized into three families, *Myoviridae* for phages with contractile tails, *Siphoviridae* for long and non-contractile and *Podoviridae* for short tails. Cubic phages are grouped into seven small families which are *Microviridae* (ssDNA), *Corticoviridae* (dsDNA), *Tectiviridae* (dsDNA), *SH1* or *Serpentine-Lake-Hispanica* (dsDNA), *SITV* or *Sulfolobus-Icosahedral-Turreted-Virus* (dsDNA), *Leviviridae* (ssRNA) and *Cystoviridae* (dsRNA). Three small groups are made up of filamentous phages which are *Inoviridae* (ssDNA), *Lipothrixviridae* (dsDNA) and *Rudiviridae* (dsDNA). Pleomorphic phages which has no fixed shape are having dsDNA as genetic material and are classified into *Plasmaviridae, Fusseloviridae, Guttaviridae, Ampullaviridae, Bicaudaviridae* and *Globuloviridae* (Ackermann, 2009). Table 2.1 shows the overview of phage families with general descriptions of their characteristics.

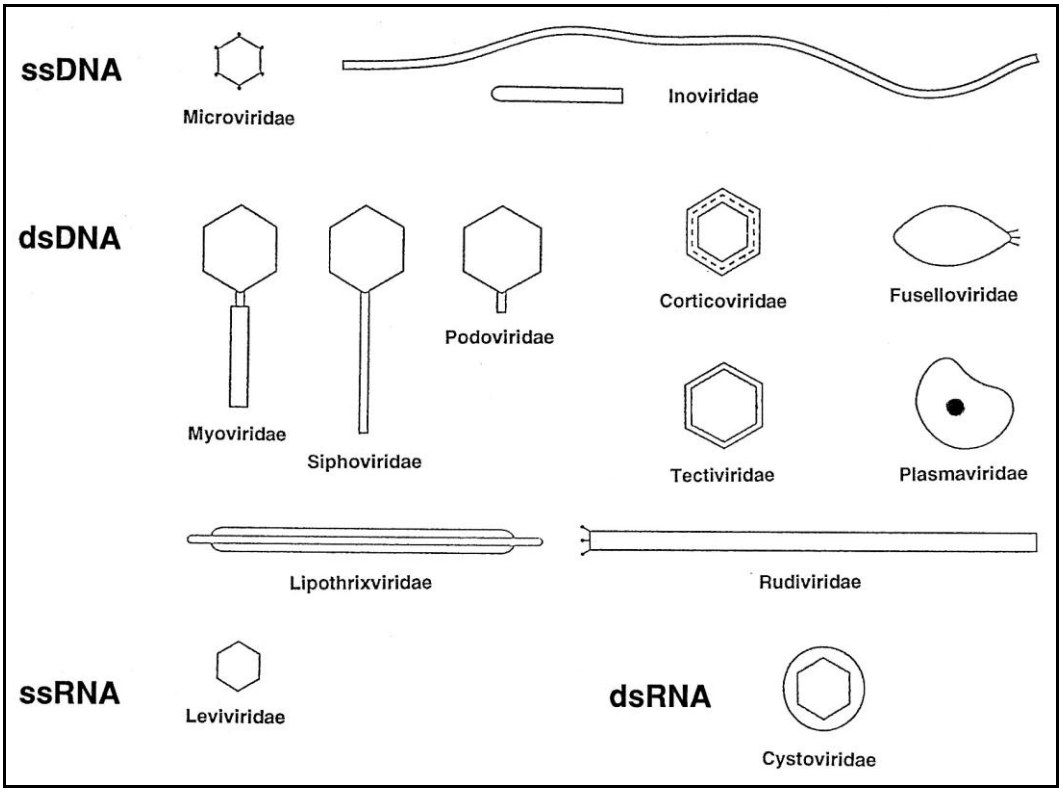

Figure 2.3 The morphology of 13 families of phages (Ackermann, 2009).

Table 2.1 Overview of phage families (Ackermann, 2009).

Shape	Nucleic acid	Family	Genera	Particulars	Example	Total Members
Tailed	dsDNA (L)	*Myoviridae*	6	Tail contractile	T4	1,320
		Siphoviridae	7	Tail long, noncontractile	λ	3,229
		Podoviridae	4	Tail short	T7	771
Polyhedral	ssDNA (C)	*Microviridae*	4	Conspicuous capsomers	ϕX174	40
	dsDNA (C,S)	*Corticoviridae*	1	Complex capsid, lipids	PM2	3?
	dsDNA (L)	*Tectiviridae*	1	Double capsid, lipids, pseudo-tail	PRD1	19
	dsDNA (L)	SH1*		Double capsid,	SH1	1
	dsDNA (C)	STIV*		Turret-shaped	STIV	1
	ssRNA (L)	*Leviviridae*	2	Poliovirus-like	MS2	39
	dsRNA (L, M)	*Cystoviridae*	1	Envelope,	*φ*6	3
Filamentous	ssDNA (C)	*Inoviridae*	2	Long filaments,	M13	67
	dsDNA (L)	*Lipothrixviridae*	4	Envelope, lipids	TTV1	7
	dsDNA (L)	*Rudiviridae*	1	Stiff rods, TMV-like	SIRV-1	3
Pleomorphic	dsDNA (C, S)	*Plasmaviridae*	1	Envelope, no capsid, lipids	L2	5
	dsDNA (C, S)	*Fuselloviridae*	1	Lemon-shaped, envelope, lipids?	SSV1	11
	dsDNA (L, S)	—	1**	Lemon-shaped, envelope	His1	1
	dsDNA (C, S)	*Guttaviridae*	1	Droplet-shaped	SNDV	1

Table 2.1 Continued

	dsDNA (L)	Ampullaviridae*		Bottle-shaped, helical NC	ABV	1
	dsDNA (C)	Bicaudaviridae*		Two-tailed, development cycle, helical NC	ATV	1
	dsDNA (L)	Globuloviridae*		Envelope, spherical, lipids, helical NC	PSV	1

C, circular; L, linear; M, multipartite; NC, nucleocapsid; S, supercoiled; -, no name; *, non-classified; **, genus *Salterprovirus*.

2.7 Mechanisms of phage infections

In 1939, Ellis and Delbruck had performed an experiment to illustrate the nature of bacteriophages infection which is referred to as ‘single-burst experiment’ or ‘one step growth curve’. This experiment showed that virus has three fundamental phases during replication as shown in the Figure 2.4. The three phases are first, the initiation of infection, second, the replication and expression of the viral genome and lastly, the release of mature virus progeny from the infected cell (Uldis, 2001). The process of bacteriophage infections starts when it encounters a susceptible host cell. The cell wall of the bacterium is the easy target for bacteriophage adsorption instead of other cell receptor units such as pili, flagella or capsule (Dimmock *et al*., 2007). After the bacteriophage has adhered to the cell wall using their tail fibres and tail pins, the tail sheath tighten to allow the tail core to penetrate the cell wall. Phage lysozyme helps to digest the proteins beneath the phage to allow penetration of the tail core. The nucleic acid would pass through from the head into the core canal of the tail and then injected into the cytoplasm of the cell (Douglas, 1975). This phase represents the latent period in which there are no visible effect on the host caused by virus infection because only viral genome replication takes place (Cann, 1993).

During attachment, phage head that appear dark under the electron microscope indicates the presence of genome while phage heads that seems white means that it has lost its nucleic acid and appear ghostly since it has injected its genome into its host (Douglas, 1975). After bacteriophage has successfully injected its genome, it would either enter lytic or lysogenic state before producing new phage progeny (Dimmock *et al*., 2007).

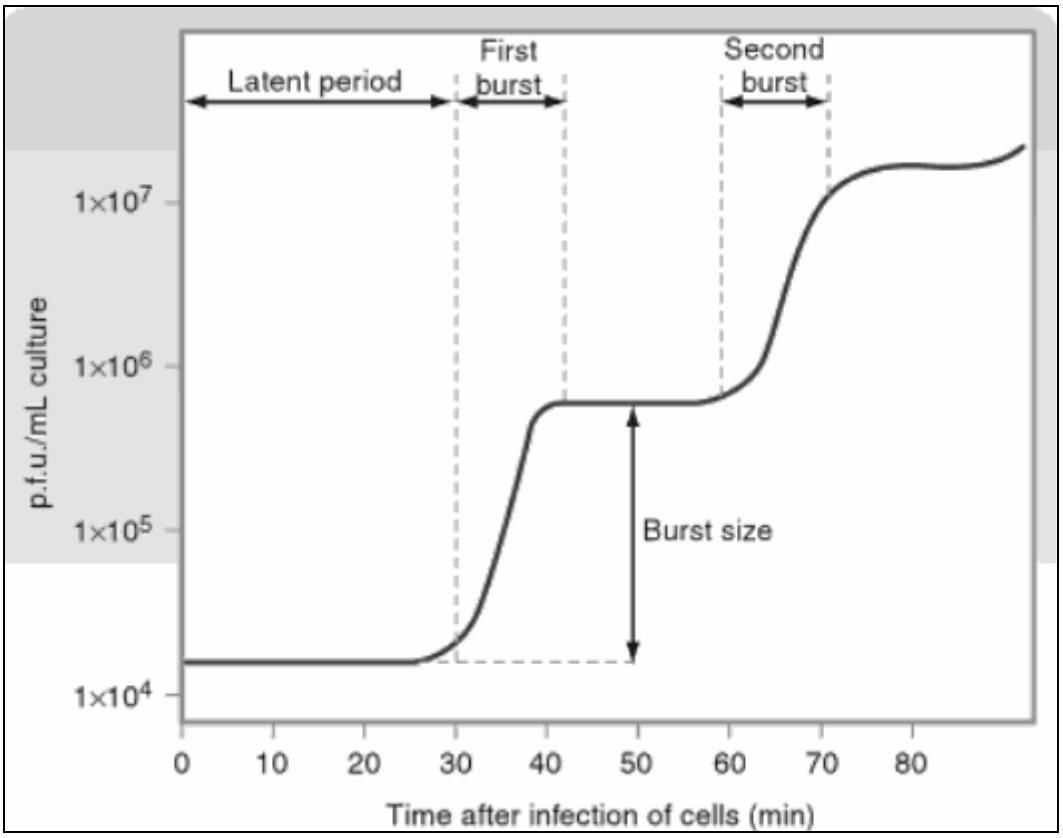

Figure 2.4 The single step growth curve of bacteriophage replication (Cann, 1993).

2.7.1 The lytic cycle

Lytic cycle infection is where the host cells has lysed and new phage progeny was released (Singleton, 2004). Lytic cycle begins when the phage nucleic acid are replicated. Since most bacteriophages contain DNA as their nucleic acid, they replicate and transcribe their DNA in the host nucleus. Viral DNA is translated using host RNA polymerase. There are two phases of viral transcription that is the early and late phase. The early phase produces early proteins that have catalytic and regulatory nature which aids in viral genome replication. Late genes helps in the production of structural proteins which made up viral capsid or envelope (Hurst, 2000).

After phage has synthesized its genome, viral assembly and maturation would take place. During assembly, bacteriophage head is the first to be produced as shown in Figure 2.5. The assembled head matures into prohead without any DNA inside. Then, it develops into a mature head when DNA was inserted into head. Collar is then attached after a mature head is produced (Dimmock *et al*., 2007). Then, the tail begins to assemble. The tail consists of plug, wedge, core, base plate, tube, sheath and tail fibres. The length of tail is determined by 'Vernier' principle in which as the sheath and core develop by the addition of monomers, this polymerization halts when unequal lengths is reached until there are twelve sheath monomers to seven core monomers (Douglas, 1975; Katsura & Hendrix, 1984). After tail has assembled, mature head would then join the tails and lastly the tail fibres would attach to base plate. Assembly stage has created a complete bacteriophage progeny (Dimmock *et al*., 2007).

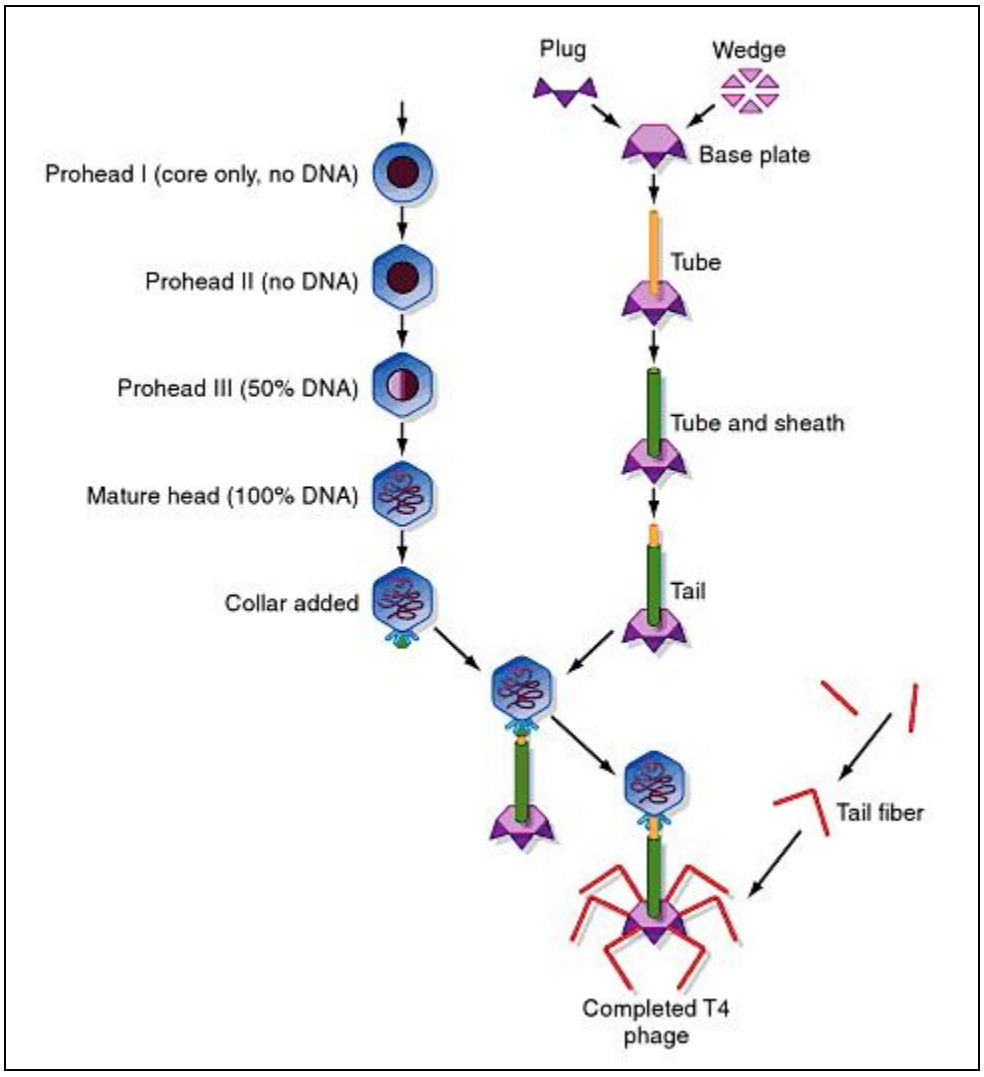

Figure 2.5 The assembly of bacteriophage during lytic cycle (Cann, 1993).

The production of new phage progeny could be seen with naked eye through plaque formation. The number of plaques increases sharply during this time based from the one step growth curve shown by Figure 2.4. It is due to the assembly and release of new virions from the infected cells. To release themselves, these bacteriophage then secretes endolysins that will degrade the peptidoglycan layer in host cell wall so that they are free to infect another host (Campbell & Campillo-Campbell, 1963; Fischetti, 2005). From the Figure 2.4, this step represents the first burst phase since bateriophage was released and waiting to infect new host (Cann, 2011).

2.7.2 The lysogenic cycle

Lysogenic bacterium is a bacterium that have spread the power to produce bacteriophage (Lwoff, 1953). Lysogeny occur when a bacteriophage integrate its genome into bacteria chromosome after infecting its host cells. The integrated genome is passed from one generation to another each time the cell divides. Such bacteriophage is termed as temperate bacteriophage. Prophage is the viral gene that are not expressed and integrated into the host nucleic acid (Madigan *et al*., 2011).

Coliphages, which is the bacteriophages infecting *E. coli* such as lambda (λ) phages is the most notable temperate viruses. It could either enter lysogenic or lytic cycle depending on the environmental condition. Lambda phages contains linear double stranded DNA as its genetic material enclosed within a capsid and attached to it is a long tail (Madigan *et al*., 2011). When a lambda DNA enters host cytoplasm, it would be transformed into prophage by joining the end, forming circular molecule. As in Figure 2.6, crossing over between lambda phage DNA and host genomes causing the integration of the whole phage DNA into host chromosome. This creates a stable