ISOLATION AND CHARACTERIZATION OF *Proteus mirabilis* AND *Escherichia coli* BACTERIOPHAGES

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ISOLATION AND CHARACTERIZATION OF *Proteus mirabilis* AND *Escherichia coli* BACTERIOPHAGES

by

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

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LIST OF ABBREVIATIONS

APS	Ammonium persulfate
ATCC	American Type Culture Collection
BLASTx	Basic Local Alignment Search Tool-standard
BLASTn	Basic Local Alignment Search Tool-nucleotide
BSA	Bovine serum albumin
bp	Base pair
CDC	Centers for Disease Control and Prevention
Cfu	Colony forming unit
ddH_2O	Deionized 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
EHEC	Enterohaemorrhagic *E. coli*
FDA	Food and Drug Administration
HC	Hemorrhagic colitis
HCl	Hydrochloride acid
HUS	Hemorrhagic uremic syndrome
ICTV	International Committee on Taxonomy of Viruses
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb/kbp	Kilobase pair
kDa	Kilodalton
LB	Luria-Bertani
LPS	Lipopolysaccharide
$MgCl_2$	Magnesium chloride

MOI	Multiple of infection
mRNA	messenger RNA
NaCl	Sodium chloride
NaOH	Sodium hydroxide
$NaOAc.3H_2O$	Sodium acetate trihydrate
NCBI	National Center for Biotechnological Information
NGS	Next Generation Sequencing
OD	Optical density
ORFs	Open reading frames
Pfu	Plaque forming unit
PCR	Polymerase Chain Reaction
P. mirabilis	*Proteus mirabilis*
RAST	Rapid Annotations using Subsystems Technology
RNA	Ribonucleic Acid
RNase A	Ribonuclease A
Rpm	Rotations/revolutions per minute
SAP	Shrimp alkaline phosphatase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
ssDNA	Single stranded deoxyribonucleic acid
STEC	Shiga toxin-producing *E. coli*
Stx	Shiga toxins
Taq	*Thermus aquaticus*
TBE	Tris-Borate-EDTA
TEM	Transmission electron microscope
TEMED	Tetramethylethylenediamine
TMS	Tris-Magnesium-Sodium
Tris base	Tris (hydroxymethyl)-aminomethane
Tris-HCl	Tris hydrochloric acid
tRNA	Transfer ribonucleic acid
UTIs	Urinary tract infections

UV	Ultraviolet
VTEC	Verotoxin-producing *E. coli*
Vt	Verocytoxin
WHO	World Health Organization
w/v	Weight/volume
v/v	Volume/volume

LIST OF SYMBOLS

B	Beta
Φ	Phi
g	Gravity acceleration
L	Litre
µg	microgram
µl	microliter
ml	milliliter
mm	millimeter
mM	millimolar
ng	nanogram
nm	nanometer
M	molar
u	unit
V	volt

PEMENCILAN DAN PENCIRIAN BAKTERIOFAJ *Proteus mirabilis* dan BAKTERIOFAJ *Escherichia coli*

ABSTRAK

Malaysia mempunyai kepelbagaian faj yang luas yang masih belum diterokai daripada segi aplikasi dan ini termasuk terapi faj untuk merawat bakteria yang merintang kesan antibiotik. Dalam kajian ini, tiga faj iaitu pPM_01, pEC_02 dan pEC_03 masing-masing khusus kepada *Proteus mirabilis*, *Escherichia coli* O157:H7 dan *Escherichia coli* ATCC 13706 telah berjaya dipencilkan dari loji rawatan kumbahan mentah di Batu Maung, Pulau Pinang, Malaysia. Mikroskopi pancaran elektron mencadangkan bahawa faj pPM_01 tergolong dalam keluarga *Siphoviridae*, manakala faj pEC_02 dan pEC_03 tergolong dalam keluarga *Myoviridae*. Pencilan mempunyai julat perumah yang sempit dan DNA bebenang ganda dua sebagai bahan genetik. Selain itu, analisis enzim pembatasan DNA dan profil protein separa menunjukkan faj jelas berbeza antara satu sama lain dan juga berbeza daripada faj yang umum seperti T4, T7 dan λ. Faj pPM_01 dan faj pEC_02 dikelaskan sebagai faj lisis manakala pEC_03 dipercayai sebagai faj lisogenik. Untuk langkah pencirian seterusnya, hanya faj lisis pPM_01 dan pEC_02 dipilih. Kejangkitan tertinggi faj pPM_01 dicatatkan pada pH 6, 37°C dan kepekatan garam 0.17M, manakala untuk faj pEC_02, kejangkitan tertinggi dicatatkan pada pH 7, julat suhu daripada 10°C sehingga 45°C dan kepekatan garam 0.17M. Analisis keluk pertumbuhan selangkah faj pPM_01 mendedahkan tempoh gerhana, tempoh pendam dan saiz letusan masing-masing adalah 20 min, 25 min, dan 32 partikel faj bagi setiap sel yang dijangkiti. Sebaliknya, analisis ke atas faj pEC_02 menunjukkan tempoh gerhana, tempoh pendam dan saiz letusan masing-masing adalah 25 min, 30 min, dan 18 partikel faj bagi setiap sel yang dijangkiti. Dalam kajian genomik separa, analisis jujukan DNA menunjukkan faj pEC_02 berkongsi identiti dengan faj *Escherichia* vB_EcoM_PhAPEC2. Anotasi dan analisis fungsian jujukan genom penuh faj pPM_01 mendedahkan bahawa faj tersebut berkait rapat secara evolusi kepada faj

Enterobacter Enc34 dengan 67% identiti dalam 14% liputan pertanyaan. Identiti yang rendah dengan jujukan dalam pangkalan data mencadangkan bahawa faj pPM_01 berpotensi sebagai pencilan yang baru. Panjang jujukan genom penuh faj pPM_01 adalah 58,546 bp dan keseluruhan komposisi bes guanina-sitosina (GC) adalah 46.9%. Sebanyak tujuh puluh rangka bacaan terbuka (ORFs) dianotasi berdasarkan pangkalan data NCBI untuk protein fungsian tersebut. Sebanyak 20 ORFs telah ditetapkan fungsi berdasarkan jujukan protein homolog yang diketahui; ORFs yang dianotasi ini kebanyakannya adalah komponen berstruktur dan protein pemasangan kepala dan ekor, serta enzim yang terlibat dalam replikasi, pemulihan, pembungkusan dan pengubahsuaian DNA. ORFs yang selebihnya adalah protein yang ditetapkan sebagai protein hipotetikal, yang mana menunjukkan lanjutan kajian genomik faj pPM_01 diperlukan untuk menjelaskan lebih banyak lagi penemuan yang novel. Ketiadaan gen virulen atau toksik dalam jujukan genom penuh membuktikan faj lisis pPM_01 adalah selamat untuk digunakan dalam kajian lanjutan melibatkan terapi faj. Ujian *in vitro* menunjukkan faj pPM_01 adalah sangat lisis dan virulen untuk mengurangkan pertumbuhan *P. mirabilis* di dalam kultur kaldu daripada ~0.51 kepada ~0.08 OD_{600} dalam masa 250 minit.

ISOLATION AND CHARACTERIZATION OF *Proteus mirabilis* AND *Escherichia coli* BACTERIOPHAGES

ABSTRACT

Malaysia has a huge untapped phage diversity with potential applications, including a phage therapy that treats antibiotic-resistant bacteria. In this study, three phages namely, pPM_01, pEC_02 and pEC_03 specific to *Proteus mirabilis*, *Escherichia coli* O157:H7 and *Escherichia coli* ATCC 13706 respectively, were successfully isolated from a raw sewage treatment facility in Batu Maung, Penang, Malaysia. Transmission electron microscopy (TEM) analysis suggested that phage pPM_01 belongs to the *Siphoviridae* family while phages pEC_02 and pEC_03 belong to the *Myoviridae* family. The isolates had narrow host range and double-stranded DNA as genetic material. Besides that, DNA restriction analysis and partial protein profile analysis showed that they were significantly different from one another as well as from the common phages T4, T7 and λ. Phages pPM_01 and pEC_02 were classified as lytic phages whereas pEC_03 was allegedly suspected to be a lysogenic phage. For the next characterization steps, only lytic phages pPM_01 and pEC_02 were selected. The highest infectivity of phage pPM_01 was recorded at pH 6, 37°C and 0.17M of salt concentration, meanwhile for phage pEC_02, the highest infectivity was recorded at pH 7, temperature range from 10°C to 45°C and 0.17M of salt concentration. Single-step growth curve analysis of phage pPM_01 revealed that the eclipse period, latent period and burst size were 20 min, 25 min, and 32 pfu/cell respectively. On the other hand, analysis on phage pEC_02 showed that the eclipse period, latent period and burst size were 25 min, 30 min and 18 pfu/cell, respectively. In the partial genomic study, DNA sequence analysis demonstrated that phage pEC_02 shared identity with *Escherichia* phage vB_EcoM_PhAPEC2. Whole genome sequence annotation and functional analysis of phage pPM_01 revealed that the phage is evolutionarily closely related to *Enterobacter* phage Enc34 with 67% identity of 14% query coverage. Low identity with the sequences in the database suggested phage

pPM_01 as a potentially new isolate. The whole genome sequence of phage pPM_01 was 58,546 bp in length and the overall guanine-cytosine (GC) base composition was 46.9%. Seventy open reading frames (ORFs) were annotated based on the NCBI database for their functional proteins. Twenty ORFs were designated functions according to their known homologous protein sequences; these annotated ORFs were mainly head and tail structural components and assembly proteins, as well as enzymes that were involved in DNA replication, repairing, packaging, and modification. The remaining ORFs were proteins assigned as hypothetical proteins, which indicate further genomic study of phage pPM_01 is required to elucidate more novel discoveries. The absence of virulent or toxic genes in the whole genome sequence indicated that the lytic phage pPM_01 is safe to proceed with further research involving phage therapy. *In vitro* testing showed that phage pPM_01 was highly lytic and virulent in reducing the growth of *P. mirabilis* in broth culture from ~0.51 to ~0.08 OD_{600} within 250 minutes.

CHAPTER 1

1 GENERAL INTRODUCTION

1.1 Problem statement

Malaysia, ranked 14th in the top seventeen mega-diverse countries list, has more than 70% of the biosphere's biodiversity. Currently, this mega-diversity concept focuses only on flora and fauna (Greenpeace International, 2004), where microbes, also included as part of the mega-diversity in Malaysia, lack attention and were involved in few studies. Microbial communities are interconnected to flora and fauna through various relationships (Nihorimbere *et al*., 2011); therefore, the biodiversity in Malaysia could support a vast range of microbial communities. However, microbes, particularly phages are less explored and uncovered (Ackermann, 2001; Ackermann, 2011; Rohwer, 2003). Malaysia, as one of the mega-diverse countries, could harbor a novel and putatively potential phage applicable in numerous sectors.

Over the past decades, phages were applied as entities able to fight disease-causing bacteria (Verbeken *et al*., 2014). Even though phage therapy has been practiced in Europe and the former Soviet Union, clinical settings for therapeutic use of phages were largely abandoned in Western Europe due to the introduction of conventional antibiotics, such as penicillin (Kutateladze and Adamia, 2010; Verbeken *et al*., 2014). Due to the worldwide crisis associated with the continuous emergence of multidrug resistance in bacteria, modern medicine has once again started showing interest in phage therapy, particularly among countries that used antibiotics as antimicrobial agents: Western Europe and the United States (Deresinski, 2009; Inal, 2003). In the European Union (EU), the annual number of infections caused by multidrug-resistant bacteria was more than 400 000 cases, resulting in 25 000 deaths with losses greater than 1.5 billion Euros (Verbeken *et al*., 2014).

In this study, three common bacteria that resisted several antibiotics and caused severe diseases in humans were studied: *Proteus mirabilis*, *Escherichia coli* O157:H7 and *Escherichia coli* ATCC 13706 (Chen *et al*., 2012; Nguyen and Sperandio, 2012; Srivastava

and Vasudev, 2011; Wendel *et al*., 2009). *P. mirabilis* and a number of *E. coli* strains (including serotypes) have been well documented in scientific literature as etiologic agents of severe diseases associated with urinary tract infections (UTIs) and other various complications. Moreover, these bacteria contributed to unacceptably high rates of nosocomial infections in hospitals and clinic due to their resistance towards present antibiotics (Luzzaro *et al*., 2011). Furthermore, biofilm formation by these pathogens render them too difficult to be controlled or eradicated by antibiotics (Carson *et al*., 2010). The more virulent *E. coli* serotype, *E. coli* O157:H7 also causes bloody diarrhea, haemorrhagic colitis (HC) and haemorrhagic uremic syndrome (HUS) in humans (Mount and Pollak, 2007). Annually, approximately eight out of 100 000 humans were infected by this pathogenic bacterium in Washington, North America and the cases were sporadic (Guandalini, 2004; MacDonald *et al*., 1988; Mount and Pollak, 2007)

Access to phage therapy remains problematic due to the fact that published reports of well-established and commercialized phages were restricted to the Soviet Union (Chibani-Chennoufi *et al*., 2004c). This issue proved difficult for scientific communities to approach phage therapy. In order to develop new isolated phages into phage therapy, phage background studies need to be considered. Background studies are crucial since various factors, such as safety analysis, medical parameters and phage stability, would affect phage therapy in clinical settings (Cairns *et al*., 2009; Jończyk *et al*., 2011).

In this study, an assessment on isolated phages infecting *P. mirabilis* and *E. coli* O157:H7 were extensively analyzed, including *in vitro* testing of phage pPM_01 infecting *P. mirabilis*. Work regarding these isolated phages would help scientific communities worldwide in developing phage applications particularly phage therapy. This study could be the starting point of a sustainable phage research program in Malaysia.

1.2 Objectives

Realizing the potential of Malaysian biodiversity in harboring an assorted phage community and contributing to the issues of multidrug resistant bacteria, research on phages was conducted. The overall objectives of this study are summarized as follows:

i. To isolate potentially new phages infecting *P. mirabilis*, *E. coli* O157:H7 and *E. coli* ATCC 13706 from raw sewage.
ii. To perform physicochemical characterization including the effects of pH, temperature, and salt concentration on the infectivity of the selected phages.
iii. To conduct biological characterization including morphological structure, single-step growth curve, host range analysis, proteomic and genomic of selected phages.
iv. To analyze the effects of phage pPM_01 on the growth of *P. mirabilis* during *in vitro* study.

1.3 Outline of thesis

This thesis consists of seven chapters. Following the general introductory chapter, Chapter 2 outlines the literature review that includes the introduction of virus, overview of phages, interaction between phage and host, and significance of *P. mirabilis* and *E. coli* O157:H7. The final part is the potential application of phages and the advantages of phage therapy over conventional medicine.

Chapter 3 describes the general materials and methods, which includes experimental materials and procedures adopted from previous studies.

Isolation of phages specific to *P. mirabilis*, *E. coli* O157:H7 and *E. coli* ATCC 13706 from Malaysian environment are described in Chapter 4. The whole process of sample collection, phage isolation and phage morphological structure determination are clearly illustrated.

Chapter 5 entitled “Characterization of the isolated phages” presents data analysis of the phage isolates. The focus points are the physicochemical (the effect of pH, temperature, NaCl concentration on the infectivity of the selected phages) as well as biological (host range analysis, single-step growth curve, proteomic and partial genomic analysis) features of selected phages.

Following characterization of the isolated phages chapter, Chapter 6 describes the genomic and *in vitro* analysis of phage pPM_01 infecting *P. mirabilis*. For genomic study, this includes primer walking sequencing method and the Illumina sequencing technology.

Finally, Chapter 7 of the thesis summarizes the work done and suggests future work in the light of the findings from the present work.

CHAPTER 2

2 LITERATURE REVIEW

2.1 Virus definition

Viruses are the smallest and simplest life forms particles (when growing inside their hosts) on the Earth (Acheson, 2007) and at the borderline of living and nonliving organism (Pelczar *et al*., 2010). Viruses are natural obligate intracellular parasites that possess their own genetic information; however, they rely on the host metabolic machinery to carry out replication cycle (Madigan *et al*., 2008; Voyles, 2002; Willey, 2008).

Viral replication is not the only process that would involve host cell for vital metabolic function (Pelczar *et al*., 2010). In fact, viruses are unable to generate usable chemical energy (ATP) (Cann, 2001) and it is incapable to directly synthesize their own proteins since they do not encode a complete translation system (ribosomes, tRNAs and associated enzymatic machinery). Moreover, they lack enzyme systems that could generate molecular building blocks of life, such as nucleotides, amino acids, carbohydrates and lipids (Acheson, 2007).

Basically, they could exist in two different forms: extracellular and intracellular (Madigan *et al*., 2008). Once inside a host cell, viruses exist as replicating nucleic acids that usurp the host's metabolism to synthesize their own protein components in order to produce matured virus particles (Willey, 2008). Whereas, in the extracellular form, a complete virus entity comprises of one or more DNA or RNA molecules enclosed in a capsid (coat protein) (Pelczar *et al*., 2010). In this infectious form of viruses,a nucleic acid that wrapped in the capsid is called as a virion (Acheson, 2007).

Some of them might have complex additional layers comprise of carbohydrate, lipids and additional proteins (Figure 2.1) (Acheson, 2007; Cornelissen *et al*., 2012; Willey, 2008). These infectious viruses are microscopic particles carrying nucleic acids, which encode proteins that enable replication as well as their transmission from one host cell to

another host cell (Pelczar *et al*., 2010; Willey, 2008). The capsid would function as a protection for the viral genome outside the host cell and used as a vehicle for virus entry since the capsid contains various binding proteins that could adsorb to the host receptors (Pelczar *et al*., 2010). Outside the host, this virion has completely no metabolic activities (Harper, 2012).

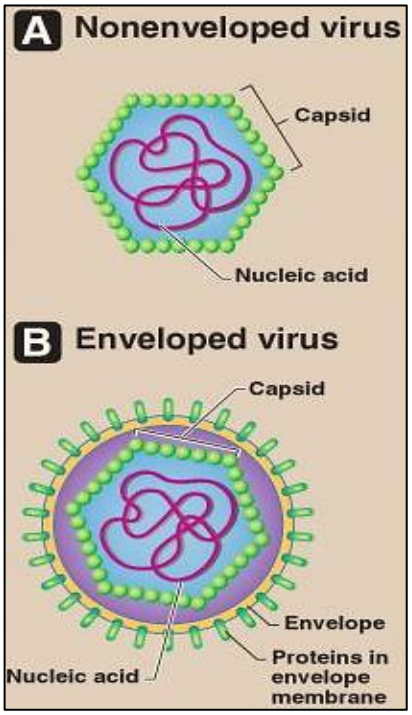


Figure 2.1: The general structure of a virus in the infectious form. The virus could be classified as (A) non-enveloped and (B) enveloped viruses (Cornelissen *et al*., 2012).

2.2 Nature of the virion

Viruses are found everywhere in nature and tend to appear wherever cellular life occurs (Voyles, 2002). They are well known to cause various diseases in humans via infection of the human cells as well as infecting all the kingdom of *Animalia*, including both vertebrates and invertebrates (Pelczar *et al*., 2010; Voyles, 2002). Viruses have also been described by their ability to infect plant (*Plantae*), filamentous fungi and yeast, all types of algae, protozoans, phytoplankton and zooplankton as well as bacteria (Acheson, 2007; Voyles, 2002).

Disease outbreaks in domesticated animals and plants by viruses could lead to the destruction of thousands or millions of animals and plants for avoidance even more widespread epidemics (Acheson, 2007). Bacterial viruses are the viruses that have been investigated in details and produced wealth of information in the fundamental process of molecular genetics (Acheson, 2007; Cann, 2001; Voyles, 2002).

2.2.1 Virion size

Viruses are smaller than bacteria and eukaryote cells as illustrated in Figure 2.2. Even though viruses are known to be predators, they are smaller than their host cells (Voyles, 2002). *Pandoravirus salinus*, a giant virus that has been isolated from the mouth of the Tunquen river (coast of central Chile) was discovered as the largest virus with size of ovoid particles 1 µm in length and 0.5 mm in diameter and known to infect *Acanthamoeba castellanii* which belongs to the amoeba's group (Philippe *et al*., 2013). Meanwhile, the smallest (only16-18 nm) virus is known as *Porcine circoviruses* (PCVs) and it belongs to the genus *Circovirus* (Ellis, 2014; Mettenleiter and Sobrino, 2008). Nanometer is a common unit used for measuring viruses and it must be viewed under scanning or transmission of electron

microscopy to examine their basic morphological structures (Acheson, 2007; Madigan *et al.*, 2008; Willey, 2008).

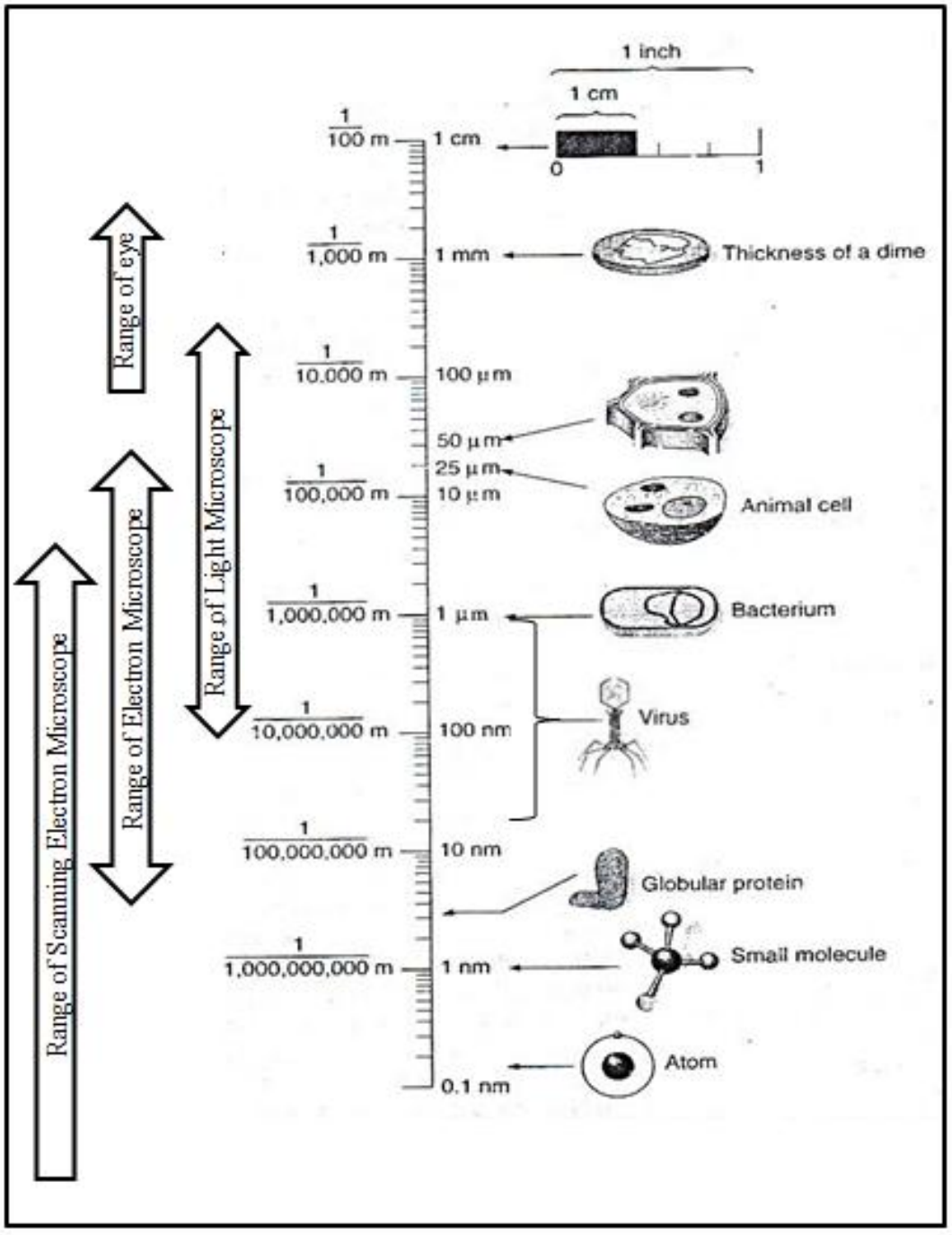


Figure 2.2: Relative sizes of various biological cells and other components. Viruses are far smaller than their host cells. Note that the vertical scale of this figure is logarithmic to accommodate a vast range of sizes depicted. The diagram is an adaptation from Voyles (2002).

2.2.2 Virion genome

Nucleic acid type of virus particle is the most fundamental of viral components (Cornelissen *et al*., 2012). The genome of a virus particle could either be DNA or RNA, of which could either be single-stranded or double-stranded. Single-stranded RNA and double-stranded DNA are the most common types of virus genomes that are found in nature (Cornelissen *et al*., 2012; Willey, 2008). Mostly, plant viruses contain single-stranded RNA, whereas majority of the bacterial viruses consist of double-stranded DNA (Willey, 2008).

Single-stranded RNA compose of a base sequence identical to mRNA sense in which it serves as a template for direct protein synthesis and is called a plus strand or positive strand. The negative strand or antisense (complementary strand) however, could not be used as a template for protein synthesis (Cornelissen *et al*., 2012; Willey, 2008). The largest size of the virus genome 2.77 Mb belongs to the DNA of *Pandoravirus salinus* and correspond to its size as the largest virus among all known viruses (Philippe *et al*., 2013).

In addition, the smallest size of the viral genome is less than 1800 nucleotides that possessed by the smallest virus known as *Porcine circoviruses* (PCVs) (Mettenleiter and Sobrino, 2008; Wen *et al*., 2014). Generally, all the host cells genomes are in the form of double-stranded DNA (Voyles, 2002).

2.2.3 Virion architecture

Structures of virion are relatively diverse since they have different size, shape as well as chemical composition (Madigan *et al*., 2008). At the most basic structure, a virion contains a central core of nucleic acid genome. It is surrounded by a capsid protein and the resulting structure is called as a nucleocapsid (Harper, 2012). The virion surface often possesses complex proteins and sugars known as glycoproteins and sometimes might be embedded in the envelope (Parasion *et al*., 2014). The capsid protein that enclosed the viral

genome is built from repeating protein subunits called capsomers (Pelczar *et al*., 2010). These small protein subunits actually reduce the amount of genetic coding capacity of the viral genome that is used to encode the capsid proteins (Acheson, 2007).

The architecture of virion is usually made up of two simple forms: helix and icosahedral (Figure 2.3) (Harper, 2012). The capsid with helical symmetry form contains proteins that are aligned in a helix, which surrounds the viral genome and appears as a rod-like. One of the best studied plant virus was tobacco mosaic virus that possesses a capsid in helical shape orientation (Cann, 2001). Meanwhile, for capsid protein with icosahedral symmetry, capsomers proteins form an outer shell that enclose the viral genome in the center (Harper, 2012). Three-dimensional (3D) structure of a virus composes of 20 triangular facets and 12 vertices. Each triangular facet is made up of an equilateral triangle and each of the 12 vertices is the intersection of five triangular facets (Voyles, 2002). Some of the bacterial viruses have icosahedral heads that are greater in length than in width, resulting in the icosahedral shape distortion (Harper, 2012; Pelczar *et al*., 2010).

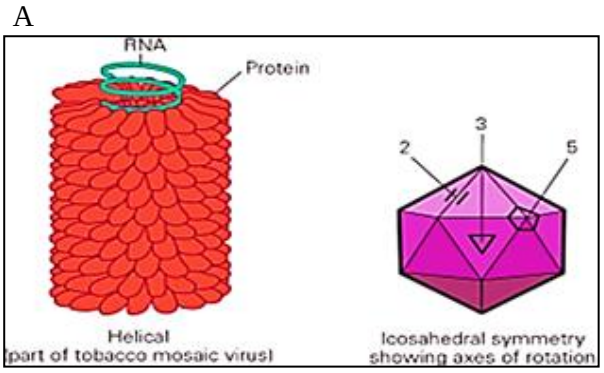


Figure 2.3: Schematic diagram of virus particles. Illustrated are two types of the most common capsid morphologies: (A) A capsid with helical symmetry, proteins aligned in a helix around the nucleic acid and appears as rod-like. (B) 3D of symmetry icosahedron structure that show axes of rotation; the proteins form an outer shell around the nucleic acids in the center. The diagram is an adaptation from Harper (2012).

2.3 Overview of phages

2.3.1 The discovery of phages

Bacterial virus or bacteriophage (phages for short) was discovered independently by a British pathologist Frederick William Twort in England in 1915 (Adams, 1959; Parasion *et al*., 2014). Twort described an acute infectious disease of staphylococci that showed marked changes in the colonial morphology. Sometimes, bacterial colonies dissolve and disappear due to the cells being lysed by an unknown agent (Pelczar *et al*., 2010).

Twort found that the lytic agent was a filterable infectious agent and indefinitely transmitted in series from colonies to other colonies (Ackermann, 2003; Adams, 1959; Sulakvelidze *et al*., 2001). The highly diluted form of this lytic agent could lyse other bacteria; however, heating the filtrates destruct the lytic property of the infectious agent (Pelczar *et al*., 2010). Twort, then described the concept of phage nature in a remarkable paper (Inal, 2003); yet, the paper remained unnoticed by scientists at that time and he failed to continue his discovery since he had wartime missions in the British Army (Adams, 1959).

In 1917, a French Canadian bacteriologist, Felix Hubert d'Herelle who was working at the Pasteur Institute in Paris observed the lysed *Shigella* cultures in broth (Ackermann, 2003). He filtered the lysed cultures and inoculated again the fresh cultures of *Shigella* with the filtrates. The bacterial culture was grown separately as a control. He found that the control culture was turbid as predicted, whilst, the bacterial culture mixed with the filtrate was completely cleared (Adams, 1959).

He summarized the filterable lytic agent as a virus with invisible entities that are parasitic to bacteria (Sulakvelidze *et al*., 2001). Upon the discovery of this phenomenon, also called as *Twort-d'Herelle phenomenon*, he coined the term of "bacteriophage" which means bacteria eater and introduced the phages to treat bacterial infectious diseases (Abedon, 2005; Gravitz, 2012).

2.3.2 Phage diversity

Phage is a virus of prokaryote (Ackermann, 2011). Phages are known to be the largest group of viruses and they use most of the bacteria and archae species as hosts for propagation (Brovko *et al*., 2012; Parasion *et al*., 2014). They are obligate parasites that could be found in all habitats in the world where bacteria or archae proliferate (Grath and van Sinderen, 2007). In the size range from 20 to 200 nm (Sulcius *et al*., 2011), phages are believed to be the most diverse life form on the planet (Hatfull, 2008, Rohwer, 2003) and occupying the biosphere with an estimated of 1×10^8 species. The predicted global viral population is 1×10^{30} to 1×10^{31} phage particles or approximately 10 million per cubic centimeter of every environmental niche (Breitbart and Rohwer, 2005; Brovko *et al*., 2012; Deresinski, 2009; Grath and van Sinderen, 2007; Hendrix, 2003; McAuliffe *et al*., 2007).

Sea water is the biggest and densest natural source for phages, which up to 10^7 phages particles per milliliter could be found in the coastal seawater (Hendrix, 2003). However, the phage concentrations fluctuate according to the seasons and the geographical locations (Chibani-Chennoufi *et al*., 2004a). They exert substantial control on communities of marine bacteria and phytoplankton in both species composition and biological production. These phages directly affect the matter pathways as well as energy transfer in this system (McAuliffe *et al*., 2007).

In fact, there is an estimation that 70% marine bacteria might be killed by the phages predator (Prescott, 1993). For every second, it was predicted that approximately 10^{25} phages might start an infection (Yamamoto *et al*., 2014). Hence, it is not surprising that phages actually contribute to the ecological balance of microbial life in the biosphere (McAuliffe *et al*., 2007). Their ubiquitous could be illustrated as following examples: per drop of seawater, it is estimated there are 10^6 phage particles and per gram of soil, 10^8 phage particles are predicted (Deresinski, 2009).

The comparison of nucleotide sequences of phage genomes actually discovers them to be enormously diverse entities in this biosphere (Hendrix, 2003). It is impossible to find highly similar nucleotide sequence to other database entries except the genome of the phage has a known close relative phage and infect the same host or is closely related temperate phage. This observation suggest that the host preferences as a substantial barrier for genetic exchange to occur (Brüssow and Hendrix, 2002; Hatfull and Hendrix, 2011).

Hence, based on the phage diversity, they might play an important role in the bacterial evolution in the biosphere (Hendrix, 2003; McAuliffe *et al*., 2007). Phage, which kills almost one-half of the bacterial population worldwide every 48 hours, has contributed to the acceleration of bacterial mutation rates. This leads to the constant emergence of bacteria resistance to the phages (Pal *et al*., 2007). Nevertheless, antagonistic coevolution evolved by this parasite would able to overcome bacteria resistance since phages mutate at higher rates (Buckling and Rainey, 2002; Comeau and Krisch, 2005). The evolution that takes place for billions of years derived from the relationship of prey-predator reveals the phages as rich potential candidates for antimicrobial compounds (Deresinski, 2009; Sau *et al*., 2008).

2.3.3 Classification of phages

Like other viruses, phage nucleic acid is surrounded by a capsid in order to protect the core from nucleases and other harmful materials (Voyles, 2002). Mostly, phage genome comprises of a single copy of a nucleic acid molecule, and it could be either single- or double-stranded linear or circular DNA, or single-stranded linear RNA (Madigan *et al*., 2008). The genome size of phages varies starting from approximately 3300 nucleotides single-stranded RNA viruses of *Escherichia coli* to almost 500 kb of *Bacillus megaterium* phage G genome. The smallest genome size for the double-stranded DNA tailed phages were *Mycoplasma* phage P1 (*Podoviridae*) with ~11.5 kb, *Lactococcus* phage c2 (*Siphoviridae*)

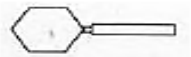
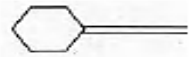
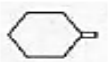
with ~21 kb as well as *Pasteurella* phage F108 (*Myoviridae*) with ~30 kb (Hatfull and Hendrix, 2011).

Phages structures might be physically differentiated by shape: tailed, polyhedral (icosahedral or quasi-icosahedral bodies), filamentous as well as pleomorphic. Of the 6000 viruses examined by transmission electron microscopy, 5360 of them are tailed phages and only 179 are cubic, filamentous or pleomorphic phages (Ackermann, 2011; Parasion *et al*., 2014). Currently, the International Committee on Taxonomy of Viruses (ICTV) classifies phages in a hierarchical and holistic system into one order with 10 families (Table 2.1) (Ackermann, 2011).

The double-stranded DNA tailed phages account for more than 96% reported phages in the scientific literature and presumably constitute the majority of phages on the Earth (Ackermann, 2011; Grath and van Sinderen, 2007). Tailed phages that belong to order *Caudovirales* consist of three families: *Myoviridae*, *Siphoviridae* and *Podoviridae*. They could be characterized as contractile, long and non-contractile, or short tails, respectively (Ackermann, 2011). In both families *Myoviridae* (e. g., T4-like phages) and *Siphoviridae* (e. g., λ-like phages), six genera have been created, separately as well as three genera in the family *Podoviridae* (e. g., T7-like phages) (Ackermann, 1998).

Members of *Caudovirales* comprise a cubic symmetry head and a helical tail that form binary symmetry. They contain no envelope and often possess capsid protein and the DNA only. Sometimes, icosahedral head of phages might elongate; however, 85% of them were found to be isometric heads (Abedon, 2005; Ackermann, 2003). Uniquely, tailed phages consist of tail with a hollow tube that allows the phage nucleic acid to pass through into the host cytoplasm (Brovko *et al*., 2012; Lobocka and Szybalski, 2012). The tail sizes are diverse and some of the phages do not possess this structure (Brovko *et al*., 2012).

Table 2.1: Overview of phage families (Ackermann, 2011)

Shape	Order or family	Nucleic acid, particulars, size	Member	Number[a]
	Caudovirales	dsDNA (L), no envelope		
	*Myoviridae*	Tail contractile	T4	1312
	*Siphoviridae*	Tail long, noncontractile	λ	3262
	*Podoviridae*	Tail short	T7	771
	*Microviridae*	ssDNA (C), 27 nm, 12 knoblike capsomers	ϕX174	38
	*Corticoviridae*	dsDNA (C), complex capsid, lipids, 63 nm	PM2	3?
	*Tectiviridae*	dsDNA (L), inner lipid vesicle, pseudo-tail, 60 nm	PRD1	19
	*Leviviridae*	ssRNA (L), 23 nm, like poliovirus	MS2	38
	*Cystoviridae*	dsRNA (L), segmented, lipidic envelope, 70–80 nm	ϕ6	3
	*Inoviridae*	ssDNA (C), filaments or rods, 85–1950 x 7 nm	fd	66
	*Plasmaviridae*	dsDNA (C), lipidic envelope, no capsid, 80 nm	MVL2	5

[a] From reference 1. C, circular; L, linear.

Besides, in certain phages, a contractile sheath surrounds the tail and the tail would contract upon infection. T4-like phages tend to be more complex by having a baseplate as well as tail fibers that are attached to the baseplate. The tail fiber often contains proteins that would recognize the receptor molecules on the surface of its host cell (Brovko *et al*., 2012). Figure 2.4 (A) shows the overview structure of T-even phage specific to *E. coli* that contains double-stranded DNA in their heads. The DNA length of the phage is only 6% of the DNA length of its host (Heritage *et al*., 1996). Meanwhile, Figure 2.4 (B) clearly shows the electron micrograph of T4-like phage morphological structure infecting *E. coli* O157:H7.

The rest of seven families that possess polyhedral, filamentous and pleomorphic shapes are classified based on the profound differences of their nucleic acid structure and content (Ackermann, 2011). The members of these families are comparatively small (Lobocka and Szybalski, 2012). Of seven families, four have lipids and two of them possess lipoprotein envelopes (Ackermann, 2011). Phage classification often encounters problems when dealing with tailed phages since they have extraordinary numbers and enormous of data reported so far (Ackermann, 1998; Ackermann, 2003).

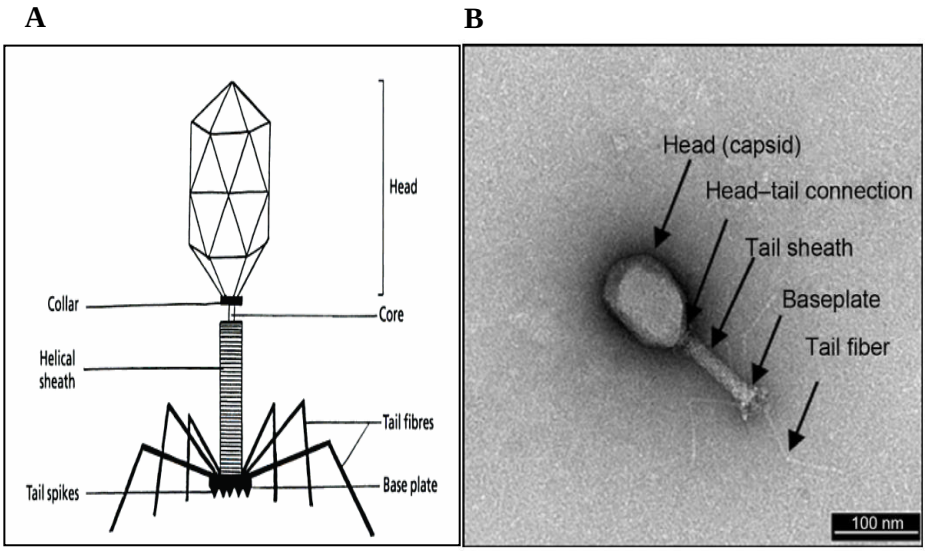


Figure 2.4: The basic structure of *Myoviridae* members. The images illustrate the structures of *E. coli* phages: (A) Schematic diagram of T-even phage (Heritage *et al*., 1996) and (B) electron micrograph of T4-like phage, EcoM-AG2 (Brovko *et al*., 2012). Both of the images show the phages structures with icosahedral head, tail sheath, baseplate, and tail fiber.

2.3.4 Phage life cycle

Two types of phages are found in nature: lytic (or virulent) and lysogenic (or temperate) (Madigan *et al*., 2008; Maramorosch and Shatkin, 2006; Parasion *et al*., 2014; Willey, 2008). Typical life cycle of a phage is illustrated in Figure 2.5 (Brovko *et al*., 2012). By simple definition, lytic phages would destroy or kill their host cells. In the lytic infective process, lytic phage infects host cells and replicates abundantly. They cause disruption to the host cell metabolism by subverting it to form progeny of the phage (Pelczar *et al*., 2010). Host cells then burst or lyses, releasing new phages progeny that eventually spreads to infect other bacterial host cells (Brovko *et al*., 2012; Pelczar *et al*., 2010). The time taken for a whole phage life cycle to complete usually within one to two hours and the number of phages released depend on the type of phage (Brovko *et al*., 2012).

A basic lytic cycle of phages contains the following five steps: adsorption (attachment), penetration, latent period, maturation and lysis (Brovko *et al*., 2012; Madigan *et al*., 2008; Willey, 2008). The initial step of the lytic cycle for dsDNA tailed phage begins with adsorption of the phage towards specific bacterial host cell (Cann, 2001; Parasion *et al*., 2014). The infection starts when the specialized adsorption structures that present at the tip of the phage such as tail fibers or spikes bind to the specific receptor on the surface of the host cell (Brovko *et al*., 2012). Bacterial mutants that do not have the ability to synthesize specific receptors for phage binding or attachment would eventually become resistant to infections by specific phages (Pelczar *et al*., 2010).

The second step of the lytic cycle is penetration. Phages with different morphological structures might have different mechanisms of nucleic acid injection towards the bacteria (Willey, 2008). Phage produce an enzyme called lysozyme to puncture the cell wall of the bacteria (Madigan *et al*., 2008) and the DNA genome located in the phage head is then injected through the tube of the tail into the host cytoplasm upon tail sheath contractions (e. g., T4 phage) (Brovko *et al*., 2012; Pelczar *et al*., 2010).

Latent period or viral components synthesis is the third stage of viral infection. After DNA is immediately passed to the cytoplasm, the early proteins are produced for DNA replication as well as to modify the host metabolic machinery (Brovko *et al*., 2012). DNA is transcribed to mRNA and is often initiated by the host cell RNA polymerase that directs the transcription of the genes encoding phage protein synthesis (Willey, 2008). A number of phage DNA copies are synthesized for transcription and translation of late proteins, which build up the capsomeres subunits and other components of the tail assembly (Brovko *et al*., 2012; Willey, 2008).

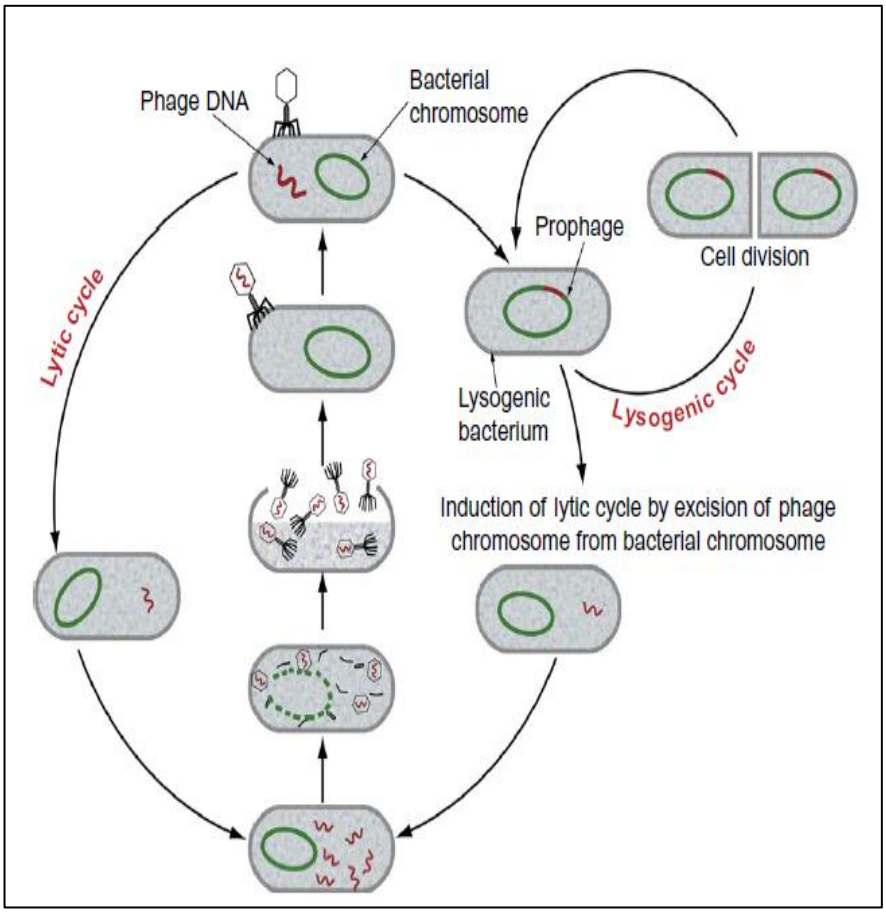


Figure 2.5: Typical life cycle of a phage. Life cycle depicting the lytic and lysogenic pathways of phage when it infects bacterial cells. The diagram is an adaptation from Brovko *et al*. (2012).

The next step of phage replication is maturation. Within this period, new phage components are assembled to form complete virion (Cann, 2001). DNA copy is packaged into procapsid (preassembled protein capsid) and most of the phages would assemble via complex interactions between major head proteins and scaffolding protein (Brovko *et al*., 2012; Willey, 2008). The head and tails of member *Caudovirales* are assembled using different pathways and are eventually combined after the encapsidation of DNA (Brovko *et al*., 2012; Pelczar *et al*., 2010).

The final step of the phage life cycle is a lysis. Phages are released from the bacterial host by lysis of the host cell (Cann, 2001). Specific enzymes (endolysin and holin) that are synthesized during late proteins expressions would hydrolyze the host cell wall from the inside to liberate the mature phages. These phages are capable of initiating a new life cycle over and over again by infecting new susceptible bacterial host cells (Pelczar *et al*., 2010; Willey, 2008).

Lysogenic is an alternative phage life cycle that is exhibited by a certain phage known as the temperate phage. It infects the host cell and directly incorporates its genome into the host genome (prophage) or it might exist as an element of episomal in the bacteria (Brovko *et al*., 2012). This eventually leads to the permanent association between prophage and the host cell as well as all the progenies (Madigan *et al*., 2008). The host cells that harbor these prophages are termed as lysogenic bacteria. When the host cells become lysogens, the phages could not lyse the bacteria and liberate their progenies (Willey, 2008).

In fact, the lysogenic relationship of temperate phages with its respective host cell form a secure home for prophage genome (Madigan *et al*., 2008), disrupt the replication of non-virulent homologous phages as well as might change the host cell phenotype (Brovko *et al*., 2012). The lysogens might harbor the temperate phages for many generations until the phages are induced by adverse environmental effects (e. g., UV or other chemicals) that finally produce hundred copies of phages and released by host cell lysis (Parasion *et al*., 2014; Pelczar *et al*., 2010).

2.3.5 Genomics and evolution of phages

The elucidation of the first reported genome sequence of phage ΦX174 has left a significant impact in the genomic era in 1977 (McAuliffe *et al*., 2007). The number of isolated phages is more than 5000 but only approximately 750 of these have been completely published in the public database entries and the numbers keep increasing every day (Hatfull and Hendrix, 2011). Apparently, the available information obtained would be useful to understand the contribution of phages towards evolution, ecological balance, virulence as well as therapeutic aspects (McAuliffe *et al*., 2007).

However, these available database only reflect a minor proportion of the phages diversity in the biosphere (McAuliffe *et al*., 2007). The introduction of metagenomic analysis to study viral population reveals an amazing diversity of phages, primarily from seawater, soil and gut communities (Breitbart and Rohwer, 2005; Hatfull and Hendrix, 2011). Abundance of novel genes has been identified and it is proposed that uncultured phage communities are the largest untapped source of genetic information present in the biosphere (McAuliffe *et al*., 2007; Rohwer, 2003).

Phage genomics are always the best tracks to explore fundamental genomics. All the problems that have been encountered in the bacterial genomics are always discussed based on the phage genomics perspectives: origin unity and diversity, vertical and horizontal (lateral) genes transfer, non-orthologous gene displacement, synteny versus instability of the genes order, tree versus web-like phylogeny as well as gene-splitting against domain accretion (McAuliffe *et al*., 2007).

As mentioned before, double-stranded DNA tailed phages are believed to be the largest group of entities in the biosphere and perhaps they are a very ancient group of virus (Ackermann, 2011; Brüssow and Hendrix, 2002). Comparative genomic analysis of phage groups has discovered that the mechanisms of phage evolution include lateral gene transfer, non-homologous recombination of different genomes, and reassortment of variant sequences

created by homologous recombination. Tailed phages which belong to *Caudovirales* that have been sequenced so far allow an access of large common genetic pool (Grath and van Sinderen, 2007; Hendrix *et al*., 1999).

Presumably, high frequency of horizontal gene exchange is accountable for genetic mosaicism and evidently emerge from non-homologous recombination of ancestral genes sequences. Modules of mosaicism are varied, which are often individual genes, but could be large blocks of genes corresponding to protein domains (Hendrix, 2003). By means of temperate phages, the probabilities for a genetic exchange to occur might exist among phages and prophages as well as bacterial genomes that they proliferate (Brüssow and Kutter, 2005).

Vertical gene transfer might play an important role for evolution in the phage families, especially those of virulent phages. Gene recombination could only recombine among virulent phages with other phages upon simultaneous infection, or via homologs sequences of resident prophages on the bacterial genomes or with complementary sequences on the plasmid (Brüssow and Kutter, 2005). For example, the members of T4-like phages has a few notably genetic exchange and they form quite a distinct group, but still there is an evidence of lateral exchange reported for those of non-essential genes (Grath and van Sinderen, 2007). Short conserved sequences have been revealed at the functional units boundaries in some lambdoid phages proposing that the homologous or site-specific recombination leads to lateral gene exchange among these phages (Grath and van Sinderen, 2007; Hendrix, 2003).

However, mycobacteriophages comparative genomic analysis proposed that lateral gene exchange through non-homologous recombination might cause the evolution of this group (Pedulla *et al*., 2003). Non-homologous recombination happens randomly throughout the genome and the appearance of this event might be due to natural selection, which eliminates the progeny that possesses the DNA inserted at the middle sequence of functional relevant genes (Hendrix *et al*., 2000). Thus, this leads to the elucidation of new module

junctions as well as generation of coding sequences in which it reveals the creativity of the process and a dominant force in the evolution of phage genomes (Brüssow and Hendrix, 2002; Grath and van Sinderen, 2007).

2.4 Phage-host interactions

2.4.1 Bacterial hosts evolution: the role of phages

Bacterial evolution is often associated with the continuous generation of genetic variants and the major contributions of this constantly evolving processes are point mutations, genetic rearrangements as well as lateral gene transfer. Extensive dynamic genomes produced by lateral gene exchange results in huge amounts of DNA are being inserted and deleted from the bacterial chromosomes (McAuliffe *et al*., 2007). Besides, this kind of genetic exchange is responsible for the majority of intraspecies genetic variation (Hambly and Suttle, 2005) and is found to leave a substantial impact on the evolution of the bacterial host genomes (McAuliffe *et al*., 2007).

In some species of bacteria, prophages account for a major contribution of bacterial genomic diversity. Meanwhile, transposons, pathogenicity islands (PAIs) as well as integrative plasmids might involve in other species (McAuliffe *et al*., 2007). Undoubtedly, the integration of the phage DNA into the bacterial genome (prophage) has become an extremely common phenomenon; prophage increasing the metabolic burden of the host as well as causing the bacteria to lyse when induction occurs (Pelczar *et al*., 2010).

Lysogenic conversion genes might happen in certain conditions, in which the prophage tends to express some of their genes in the lysogen, thus alter the phenotype of the host by providing selective benefit (Brüssow and Hendrix, 2002; Madigan *et al*., 2008). These genes might increase cell fitness, or protect the hosts from other phage infection as well as increasing the host's virulence (Waldor and Mekalanos, 1996).