IDENTIFICATION AND CHARACTERISATION OF COLIPHAGES FROM SEAWATER IN BATU FERRINGHI, PENANG

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IDENTIFICATION AND CHARACTERISATION OF COLIPHAGES FROM SEAWATER IN BATU FERRINGHI, PENANG

by

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LIST OF ABBREVIATONS

bp	Base pair
cfu	Colony forming unit
conc.	Concentration
dH_2O	Distilled water
ddH_2O	Double distilled water / ultrapure water
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
EDTA	Ethylene-diamine-tetra-acetic acid
F^+	Fertility factor
HCl	Hydrochloric acid
kb	kilo-base pair
kDa	kilo-dalton
LB	Lysogeny broth
M	Molarity
MOI	Multiplicity of infection
MPN	Most probable number
MraY	Phospho-MurNAc-pentapeptide translocase
mRNA	Messenger ribonucleic acid
N	Count
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NEB	New England Biolabs
PCR	Polymerase chain reaction
pfu	Plaque forming unit
RNA	Ribonucleic acid
rpm	Revolutions per minute

SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
ssDNA	Single-stranded deoxyribonucleic acid
TBE	Tris-borate-EDTA
TEM	Transmission electron microscope
TEMED	Tetramethylenediamine
Tris	tris(hydroxymethyl)aminomethane
UV	Ultra violet
v/v	Volume per volume
w/v	Weight per volume

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LIST OF SYMBOLS

Ø	Phi
ß	beta
TM	Trademark
®	Registered trademark
µm	Micrometer
µM	Micro-molarity

PENGECAMAN DAN PENCIRIAN KOLIFAJ DARIPADA AIR LAUT DI BATU FERRINGHI, PULAU PINANG

ABSTRAK

Patogenik mikroorganisma dalam air laut di pantai rekreasi yang tercemar boleh membawa ancaman kepada pengunjung pantai dan hidupan laut. Dalam sistem pemantauan kualiti air, kolifaj telah dikenalpasti sebagai petunjuk biologi untuk mengesan kehadiran virus yang menjangkiti manusia. Oleh itu, tujuan penyelidikan ini adalah untuk mengkaji kepelbagaian faj dalam air laut yang tercemar di Batu Ferringhi serta mengkaji ciri-ciri faj yang telah dipencilkan. Morfologi bagi lima pencilan faj telah ditentukan melalui mikroskop transmisi elektron. Tiga pencilan kolifaj yang menjangkiti *E. coli* ATCC 11303 dicadangkan tergolong sebagai *Myoviridae*, manakala dua kolifaj yang spesifik terhadap *E. coli* ATCC 13706 tergolong sebagai *Microviridae*. Faj-faj *Microviridae* yang dinamakan sebagai K3 dan K5 telah dicirikan melalui analisis spektrum perumah dan protein. Berdasarkan penemuan analisis protein, faj K3 dan faj K5 dianggap sebagai virus daripada strain yang sama. Oleh itu, tumpuan penyelidikan diberi kepada faj K5, dimana pencirian fizikokimia dan genomik serta kitaran hidup faj telah diuji. Faj K3 dan faj K5 menunjukkan spektrum perumah yag amat khusus terhadap *E coli* ATCC 13706. Profil-profil protein faj K3 dan faj K5 memaparkan pola yang serupa. Profil-profil protein menunjukkan bahawa kedua-dua faj tersebut tergolong sebagai *Microviridae*. Namun, mereka tidak menyerupai faj ØX174. Faj K5 berupaya menjangkiti bakteria dalam persekitaran yang mengandungi pH antara pH 6 dan 10. Ia kehilangan keupayaan berjangkit dalam persekitaran yang terlalu berasid (pH 1 hingga 3) dan terlalu beralkali (pH 12). Selain itu, ia dapat bertoleransi dengan kepekatan garam

yang tinggi, dimana bilangan faj menunjukkan turun-naik yang sedikit tetapi berkurang secara perlahan dalam kepekatan garam 0% (g/L) hingga 20% (g/L). Kejangkitan optima berlaku pada kepekatan garam 1% (g/L). Walau bagaimanapun, bilangan faj menurun sebanyak 3% semasa ketiadaan garam. Sementara itu. faj K5 berupaya menjangkiti bakteria apabila terdedah kepada suhu antara 10°C dan 44.5°C. Kejangkitan menurun secara mendadak pada 50°C dan ia dilemahkan pada 60°C. Kitaran replikasi tunggal bagi faj K5 adalah 45 minit, dengan tempoh pendam selama lima minit dan purata saiz letusan sebanyak 35 faj partikel bagi setiap jangkitan sel. Kajian genomik telah mengenalpasti bahawa faj K5 memiliki DNA bebenang tunggal berlingkar. Berdasarkan analisis jujukan separa genom dan perbandingan profil genom yang dipotong oleh *Hae*III, genom faj K5 mempamerkan 99% persamaan dengan genom faj ID11. Kesimpulannya, penyelidikan ini menunjukkan bahawa faj K5 berasal daripada air laut. Penemuan penyelidikan juga menunjukkan bahawa faj K5 berpotensi sebagai bio-penunjuk untuk mengesan kehadiran virus yang menjangkiti manusia dalam sistem pemantauan kualiti air laut.

IDENTIFICATION AND CHARACTERISATION OF COLIPHAGES FROM SEAWATER IN BATU FERRINGHI, PENANG

ABSTRACT

Pathogenic microorganisms in polluted recreational seawater would be harmful to beachgoers and marine lives. Coliphages are recognised as bioindicator for the presence of human viruses in water quality monitoring. Thus, the purpose of this study was to survey the phage diversity in the polluted seawater in Batu Ferringhi and to study the characteristics of the isolated phages. The morphology of five isolated phages was determined through transmission electron microscopy visualisation. Three isolated coliphages that infected *E. coli* ATCC 11303 were suggested belonged to *Myoviridae*, whereas two coliphages which specific to *E. coli* ATCC 13706 were of *Microviridae*. The *Microviridae* phages namely K3 and K5 were characterised by host range and protein analysis. Based on the findings of protein analysis, phages K3 and K5 were assumed the same strain of viruses. Thus, the study was focused on phage K5 for physicochemical and genomic characterisation as well as phage life cycle. Phages K3 and K5 showed strictly specific to the host, *E coli* ATCC 13706. The protein profiles of phages K3 and K5 showed similar patterns. The profiles indicated that the phages were belonged to *Microviridae* but they were not identical to phage ØX174. Phage K5 was infectious in pH condition between pH 6 and 10. It lost infectious ability in extremely acidic (pH 1 to 3) and alkaline (pH 12) conditions. In addition, it could tolerate at high concentration of saline, where the phage titres demonstrated slight fluctuations but decreased slowly throughout 0% (w/v) to 20% (w/v) of NaCl. The optimum infectivity occurred at 1% of NaCl. However, phage titre decreased 3% in the

absence of NaCl. Meanwhile, phage K5 was infectious upon exposure to temperature between 10°C and 44.5°C. The infectivity decreased drastically at 50°C and it was inactivated at 60°C. The single replication cycle for phage K5 was 45 minutes, with the latent period of five minutes and average burst size of 35 pfu per infected cell. The genomic study identified that phage K5 possessed supercoiled circular single-stranded DNA. Based on the partial genome sequences analysis and genome profiles comparison cleaved by *Hae*III, the genome of phage K5 demonstrated 99% similarity to the genome of phage ID11. In conclusion, the study showed that phage K5 was originated from seawater. The findings also showed that phage K5 could be used as a potential bioindicator for the presence of human viruses in seawater quality monitoring.

CHAPTER 1

INTRODUCTION

1.1 PROBLEM STATEMENT

In early February 2014, the incidence of black discharge covered the seawater at Batu Ferringhi beach alarmed our nation about the unsafe of our coastal recreational water. According to New Straits Times dated 10 February 2014, the source of the pollution was the vicinity river, Sungai Batu Ferringhi. High level of *Escherichia coli* counts at 16,000 MPN per 100 ml had been reported. Microbial pollution in coastal waters is often impacted by the runoff discharges consisted of sewage and domestic discharges that contains human and animal excreta as well as organic matters (Boehm *et al*., 2009). Faecal bacteria that released from the excreta will replicate well in the presence of organic matters in the polluted water (Ishi and Sadowsky, 2008). *Escherichia coli* are widely used as faecal indicator bacteria (FIB) for faecal pollution monitoring in environments. This is because *E. coli* are originated from the intestinal of human and warm-blooded animals. In addition, the occurrence of *E. coli* also indicates the presence of potential pathogens in the environments (Ishi and Sadowsky, 2008). In spite of this, the presence of human enteric viruses was reported to be uncorrelated with FIB (Jiang *et al*., 2001; Jiang *et al*., 2007). Therefore the problem statements for this study are described as below:

(i) The current water quality monitoring system in Malaysia is based on FIB. However, the absence of FIB is inadequately to reflect the absence of human

viral pathogens since the viability of viruses is more persistent than bacteria (Jiang *et al*., 2001; Jiang and Chu, 2004).

(ii) Waterborne diseases related to recreational seawater caused by viral pathogens should have raised great concern from government and public. Polluted recreational seawater in Batu Ferringhi can cause waterborne diseases to beachgoers and harmful effect to marine lives. In addition, oceans are known as the largest reservoirs for viruses, with the viral abundance approximately 10^{29} (Wilhelm and Suttle, 1999; Breitbart, 2012). For these reasons, virology assessment is important for the seawater quality monitoring.

1.2 OBJECTIVES

Bacteriophages or phages are viruses that attack bacteria, while coliphages are viruses that infect *E. coli*. The occurence of coliphages was reported to be correlated with the presence of *E. coli* and coliforms (Burbano-Rosero *et al*., 2011). Bacteriophages are also recognised as indicators for the presence of human viruses in water quality assessment, due to the similar features and properties shared by phages and human enteric viruses (Grabow, 2001). Furthermore, the presence of phages was reported to be correlated with the existence of human viral pathogens in water (Jiang *et al*., 2001; Wade *et al*., 2003).

The purposes of this study are to survey the phage diversity in the polluted seawater and to study the characteristics of the isolated phage. In the preliminary stage, the isolated coliphages were identified belonged to the family of *Myoviridae* and *Microviridae*. Then, the further investigation was focused on the *Microviridae*

phage namely K5. Tailed phages are ubiquitous in the biosphere and were the majority isolated for studies (Ackermann, 2005; 2007). *Myoviruses* are tailed phages, while *Microviruses* are non-tailed phages. Therefore, the isolated *Microviruses* were of the interested in this study. Specifically, the objectives of this research are described as below:

(i) To isolate and identify coliphages from recreational seawater in Batu Ferringhi.

(ii) To characterise the isolated coliphages based on their morphological, host range, proteins, physicochemical and genetic properties as well as phage life cycle.

Chapter 2

LITERATURE REVIEW

2.1 VIRUS

2.1.1 General overview

Viruses are the simplest biological entities composed of protein capsid and either DNA or RNA as genetic material. The genome can be either single-stranded or double-stranded and either linear or circular (Tortora *et al*., 2004). Virus must enter a cell to replicate but it brings destructive to the cell. A cell that a virus can infect is called as host. Viruses can be divided into two categories: extracellular state and intracellular state. In extracellular state, viral particles are also called as virions. A virion is metabolically inert and does not have biosynthetic function. Nevertheless, virion is an infectious particle (Acheson, 2007). It is a complete structure composed of genome packaged in protein coat and is readily to transmit its genome into the host (Tortora *et al*., 2004). The intracellular state is initiated when a virion attaches to a cell and viral replication occurs in the cell (Madigan *et al*., 2003). This process is also called as viral infection. In the infected cell, replication and synthesis of viral components occur and end up with releasing of new virions. The new virions will then infect the adjacent hosts and the process of infection could be repeated (Paul, 2008).

Viruses are referred as obligate intracellular parasites. Since they are small, they carry genome that only encodes the functions which they cannot adapt from their hosts (Madigan *et al*., 2003; Acheson, 2007; Ryan and Ray, 2010). Some

viruses have fewer than 10 genes while some have 30 to 100 genes. The species that carry few genes will depend heavily on the hosts and use almost all of the cellular function including host genes for the progeny virions reproduction (Grabow, 2001). In contrast, the species that possess more genes have less dependent on the host since they have their own nucleic acid polymerases in the genomes for viral genome transcription (Grabow, 2001; Madigan *et al*., 2003).

Viral capsids are composed of many copies of one or different kinds of protein subunits. The proteins are arranged precisely, repetitively and symmetry surrounding the genome. Nevertheless, the number of different protein subunits is restricted by the size of the genome. Some viruses possess only one kind of protein in the capsids. However, some viruses with smaller genome encounter this limitation by self-assembling several structural subunits into a large assembly, which is known as capsomers (Grabow, 2001; Madigan *et al.,* 2003).

Some viruses possess enveloped capsids and they are known as ónenveloped virusesô. The envelope constituted of lipid and glycoprotein. The lipid membranes are derived from the cellular membranes when releasing from the infected hosts, while the proteins are encoded by the viruses. In contrast, viruses that do not possess membranes at their outer shells are called as ónaked capsid virusesô. On the other hand, some viruses possess spikes, which composed of glycoprotein that protrude from the surface of capsids. The spikes are the particular structures that bind to the receptors on the host cells (Madigan *et al*., 2003). Capsids or envelopes are the outer shells that protect the genome and as the aid to entry the hosts (Ryan and Ray, 2010). Basically, capsids are categorized as helical (cylindrical) and icosahedral (spherical) based on the shapes. For example, tobacco mosaic viruses are helical whereas

bacteriophages phi-X174 are icosahedral. However, some bacteriophages possess complex structures, such as phage T4, where the structure of phage T4 is consisted of icosahedral head and helical tail (Madigan *et al.,* 2003).

Virus taxonomy is established by the International Committee for Taxonomy of Viruses (ICTV) and the first publication was released in 1971. The hierarchical taxon levels are based on the virion and genome structures. The general taxonomy structure with the taxon suffixes in italics is shown as follows:

Order (-*virales*)

Family (-*viridae*)

Subfamily (-*virinae*)

Genus (-*virus*)

Species

The number of orders, families, subfamilies, genus and species increased with the continual emergence of new viruses. The latest ICTV report was released in August 2016, with a total of 8 orders, 122 families, 35 subfamilies, 735 genera and 4,404 species of viruses were defined in the taxonomy (https://talk.ictvonline.org/taxonomy/p/taxonomy_releases).

2.1.2 Host range

Viruses can infect humans, animals, invertebrates, vertebrates, plants, fungi and bacteria (Tortora *et al*., 2004). However, viruses are specific to the hosts, and frequently referred as narrow host range (Ackermann and Dubow, 1987). Viruses

that infect humans are usually harmless to animals and plants, vice-versa. Likewise, viruses that infect prokaryote do not infect humans, animals and plants. The host range is determined by the availability of the specific receptors on the hosts for viral recognition and attachment (Madigan *et al*., 2003). The receptor sites are located at different parts of the hosts, such as cell walls, plasma membranes and fertility (sex) fimbriae (Grabow, 2001; Tortora *et al*., 2004). Typically, viruses can be categorized according to the hosts they infect. Therefore, we have human viruses, animal viruses, plant viruses and bacterial viruses (bacteriophages). In some cases, some viruses are broad host spectrum. For instance, rabies viruses can infect plants, insects and mammals (Ryan and Ray, 2010).

2.2 BACTERIOPHAGE

2.2.1 History

The existence of bacteriophage was first reported by Ernest Hanbury Hankin in 1896, when he found an antibacterial agent against *Vibrio cholera* in the river waters of Ganges and Jumma in India (Sharp, 2001). Later in 1910, Felix dõHerelle observed clear spots on the culture of the genus *Coccobacilli* in Mexico. Again in 1915, he found clear spots within cultures of dysentery bacilli when he was in France. Eventually in 1917, dõHerelle declared that the phenomenon of clear spots was caused by filterable viruses that killed bacteria. (Duckworth, 1976; Keen, 2012). Hence, he named this virus as ótbacteriophageô, which means ótaters of bacteriaô (Duckworth, 1987; Keen, 2012). In the meantime, Frederick Twort, who also

discovered this lytic agent in 1915 but the further discovery had discontinued (Duckworth, 1976).

With the concept of bacterial killer, phage therapy was developed and used therapeutically to combat bacterial diseases between 1920 and 1940 (Duckworth, 1976). However, a controversy began when phage therapy was not effective to some diseases (Adams, 1959). Thus, many researchers disputed that the clear spots phenomenon was attributed by enzyme (Duckworth, 1976). In 1940s, chemotherapy was developed against bacterial diseases. The application of phage therapy in clinical treatment was then replaced by antibiotics (Keen, 2012). Nevertheless, the presence of bacteriophage was rediscovered and the phagesô structural as well as their physiology were studied intensively as model system between 1940s and 1950s (Sharp, 2001).

2.2.2 Overview of bacteriophage

Bacteriophages or phages are ubiquitous, abundant and morphologically diverse in the biosphere. Phages are prevalent in waters, soils, foods, sewages and sludge. Besides, they are also detected in biological fluids of humans and animals, such as, urine, faeces, saliva, rumen and serum (JoŒzyk *et al*., 2011). Numerous phages in the oceans, sewages and fertile soils had been reported, as shown in Table 2.1 (Sharp, 2001; Ackermann, 2011). High phage titres ($>10^4$ ml^{-1}) in the waters of Yaquina Bay (Oregon, United States) was reported for the first time by Torrella and Morita in 1979 using transmission electron microscope. Subsequently, a total of 10^5 to 10^7 ml^{-1} of phages was detected from sewages in 1980 (Bitton, 1987). According to the study on decay rates of viral infectivity, more than 50% of viruses were

infectious (Wilhem *et al*., 1998b). Thus, this indicates that viral infection brings significant mortality to prokaryotes in aquatic systems (Weinbauer, 2004).

Phages possess only one type of genome, either single-stranded or double-stranded DNA or RNA. The virions are found with tailed, cubic (icosahedral), filamentous or pleomorphic (Weinbauer, 2004; Ackermann, 2011). In addition, some have facultative structures which composed of spikes or head appendages, collar and tail fibres (Weinbauer 2004). Phages with double-stranded DNA and tailed are numerous and ubiquitous in the biosphere (Ackermann, 2005).

After the advent of electron microscope, at least 5,360 tailed and 208 cubic, filamentous and pleomorphic phages were viewed in 1959. The 5,360 tailed phages comprised of 96.2% of the total phages. The tailed phages were dsDNA from the order of *Caudovirales* which constituted of 60% of *Siphoviridae,* 25% of *Myoviridae* and 15% of *Podoviridae* (Sharp, 2001; Ackermann, 2007; Ackermann, 2011). *Myoviruses* are the phages with contractile sheath, neck and central tails. *Siphoviruses* have simple and long flexible tails, while *Podoviruses* possess very short and non-contractile tails (Ackermann, 1998; Ackermann, 2009). On the other hand, the study on the bacteriophage diversity in Lake Plußsee (Germany) revealed that the total of 39 isolates constituted of 50% of *Siphoviridae*, 18% of *Myoviridae* and 19% of *Podoviridae* (Demuth *et al*., 1993). This implied that *Siphoviridae* were the most populated in the environments. The classification and overview of phage families are illustrated in Figure 2.1 and 2.2.

Phage life cycle consisted of sequential steps: (a) attachment or adsorption of phage to a susceptible bacterium, (b) penetration of hostôs cell wall and injection of genome into the cytoplasm, (c) biosynthesis of viral genomes and proteins,

(d) maturation and assembly of progeny phages, and (e) release and transmission of new phages (Duckworth 1987; Tortora *et al.*, 2004). Basically, there are two types of phage replication, i.e. lytic cycle and lysogenic cycle. Phages that carry out lytic infection are referred as virulent phage, whereas phages that undergo lysogenic cycle are known as temperate phages. Lytic infection results in cell lysis and production of progeny phage, followed by continual infection to new hosts. In contrast, lysogenic infection involves integration of phage-host genome which provides genetic diversity, and the viral production occurs during an induction event (Ortmann *et al.,* 2002, Ackermann, 2003). The processes of lytic and lysogenic cycles of bacteriophage are illustrated in Figure 2.3.

Table 2.1: Distribution of phages in the environment (Sharp, 2001)

Medium	**Phage density**
Seawater	10^6 ï 10^7 tailed phage cm^{-3}
Soil	10^7 $pfug^{-1}$
Sewage	10^5 ï 10^7 $pfucm^{-3}$
Sewage (by electron microscopy)	10^8 ï 10^{10} total phage particles
Human faeces	10^5 $pfucm^{-3}$

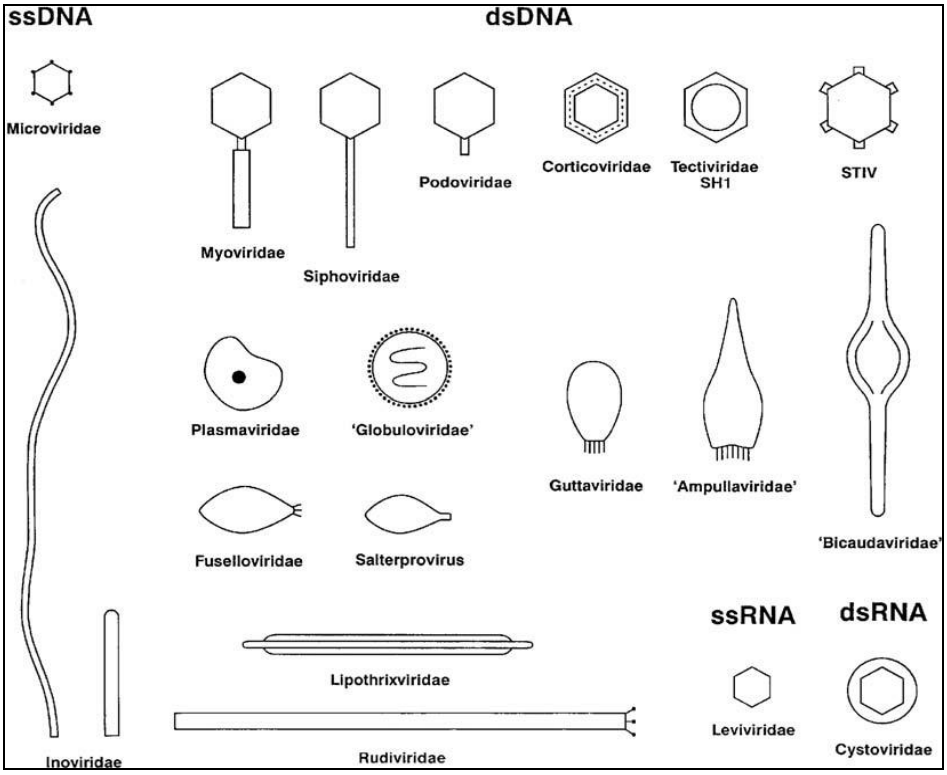
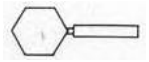
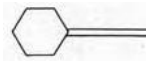


Figure 2.1: Phage classification and characterisation (Ackermann, 2005)

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, 70ï 80 nm	ϕ6	3
	*Inoviridae*	ssDNA (C), filamentous or rods, 85ï 1950 x 7 nm	fd	66
	*Plasmaviridae*	dsDNA (C), lipidic envelope, no capsid, 80 nm	MVL2	5

[a] From reference 1. (Ackermann, 2007) C, circular; L, linear.

Figure 2.2: Overview of phage families (Ackermann, 2011)

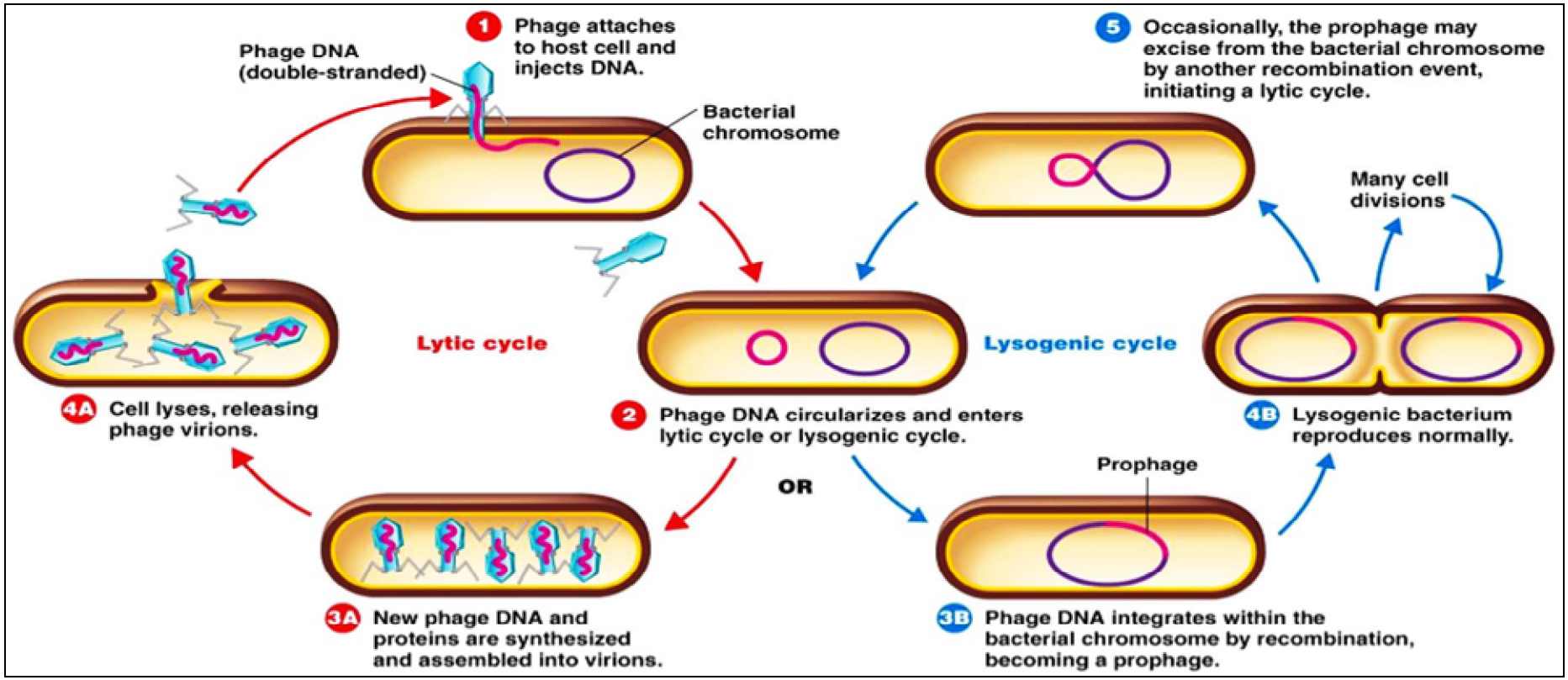


Figure 2.3: The lytic and lysogenic cycle of bacteriophage

(Assessed on 10.12.2016; link address: https://www.google.com/imgres?imgurl=http%3A%2F%2F1.bp.blogspot.com%2F-cfFDaYYRXcc%2FUJEqjCiJt6I%2FAAAAAAAAABlw%2FMouX5JrGVEE%2Fs640%2Flytic%2Band%2Blysogenic%2Bcycle.jpg&imgrefurl=http%3A%2F%2Fwww.biologyexams4u.com%2F2012%2F11%2Fbacteriophage-lytic-and-lysogenic-cycle.html&docid=DYjTHSHxPRTDcM&tbnid=K9MLPg5q_OXTYM%3A&vet=10ahUKEwjPj927jeLSAhVBLY8KHeHDDQwQMwg7KBYwFg..i&w=448&h=273&bih=639&biw=1280&q=phage%20life%20cycle&ved=0ahUKEwjPj927jeLSAhVBLY8KHeHDDQwQMwg7KBYwFg&iact=mrc&uact=8)

2.3 MARINE PHAGE

Bacteriophages are tremendously abundance, with approximately 10^{29} in the ocean (Wilhelm and Suttle, 1999; Breitbart, 2012). In addition, viral abundance was reported exceeding the bacterial density by five to ten-folds in marine waters (Hara *et al*., 1996; Wilhelm and Suttle, 1999; Wommack and Colwell, 2000). Although many studies about phage density in aquatic communities had been done, the variable data on the viral counts did not offer a meaningful comparison for the actual phage density (Grabow, 2001). This is because various factors are contributed to the survival of phages in different aquatic environments. Furthermore, the inconsistency of techniques used for the detection and enumeration of phages might not reflect the overall dynamics of phage populations (Green *et al*., 2000). For example, higher phage titres were reported through the enumeration via direct electron microscopy, instead of plaque assays technique (Ewert and Paynter, 1980; Rosario *et al*., 2009).

2.3.1 Diversity of marine phages

Marine phages are genetically diverse, but they are not morphologically diverse (Zhang *et al*., 2011). Their sizes are approximately 25 nm to 300 nm with the genomesô sizes between 4 kb and 630 kb (Garza and Suttle, 1995; Takao *et al*., 2005). It is estimated 5000 viral genotypes in 200 litres of seawater and a million viral genotypes in one kilogram of marine sediments (Breitbart and Rohwer, 2005).

Among the 75 bacteriophages isolated from the seawater in North Sea and northern Atlantic in 1987, only tailed DNA phages were observed through electron microscopy (Frank and Moebus, 1987). Subsequently, studies concluded that tailed

phages with dsDNA were prevalent in aquatic environments (Wommack and Colwell, 2000; Breitbart *et al*., 2002). On one hand, Sandaa (2008) reported that approximately 40% of dsDNA phages in marine possessed genomes between 20 kb and 40 kb. Nevertheless, single-stranded DNA phages (Angly *et al*., 2006; Reyes and Jiang, 2010) and RNA phages (Børsheim, 1993; Proctor, 1997; Cully *et al*., 2003) were also detected from seawaters.

In 21st century, metagenomics approach is used widely to study the diversity of phages in the environments, especially marine waters (Clokie *et al*., 2011). The assessment on the coastal waters of Scripps Pier and Mission Bay in California, U.S.A. demonstrated that 75% to 90% of the isolated viruses were of phages. In addition, there were between 374 and 7,114 types of phages in the two marine communities (Breitbart *et al*., 2002). This indicates the enormous diversity of phage genetics in marine ecosystem. Despite the great diversity, the dominance of the most abundance phages was reported low, which comprised of only 2% to 3% of the population (Breitbart *et al*., 2002).

In fact, local viral diversity of seawater is higher than global viral diversity (Breitbart and Rohwer, 2005; Angly *et al*., 2006). This could be explained by the observation of "long-distance" phage-host systems, especially between *Vibriophages* and *Vibrio sp*. (Hidaka, 1980; Kellogg *et al*., 1995). Hidaka (1980) discovered that the phages against the *Vibrio* (bacteria that he isolated near Hawaii) were found in the seawater of south Japan. Likewise, Kellogg and co-workers (1995) reported that 60 *Vibriophages* with similar morphology, genetically related and shared the same host were detected in marine over a geographic distance of more than 4,500 miles. This phenomena were suggested as related to the movement of phages between

environments (Breitbart and Rohwer, 2005), where phages could be distributed widely through natural currents (sea waves) and cargo ships (Kellogg *et al*., 1995). For this reason, global diversity of phages is relatively limited compared to the local viral diversity.

2.3.2 Distribution of bacteriophage in seawater

Phage abundance decreases along transects from coastal to deep sea. The viral density in coastal waters is 10-fold higher than oligotrophic open sea (Sharp, 2001). According to Paul and Kellogg (2000), the highest viral density of 10^6 to 10^7 ml^{-1} was detected in coastal water, intermediate rate of 10^5 to 10^6 ml^{-1} was found in offshore surface water, while the lowest viral density of 10^4 to 10^5 ml^{-1} was discovered in the deep sea. In addition, phage abundance varies along the depth of marine waters. For instance, a study on the viral density in Baltic Sea (Europe) demonstrated that viral abundance decreased with depth in the oxic (oxygen) layer, but increased again in the anoxic layer (Weinbauer, 2004). However, viral abundance was unaffected in the bottom anoxic water level of Chesapeake Bay (Maryland and Virginia of United States) (Wommack *et al*., 1992). On the other hand, phage density of 10^8 to 10^9 cm^{-3} in the sea floor sediment had been reported by Suttle (2005).

The distribution of bacteriophages is typically influenced by the environmental conditions, such as physical forces, temperature, solar radiation, salinity, organic matter and pH value. In addition, host availability is also the main factor that affects the distribution of phages (Kirchman, 2000), as elaborated below:

2.3.2(a) Physical forces

Water is the medium of transmission for phages in aquatic environments (Kirchmann, 2000). Marine phages can be distributed by natural sea waves or freighters (Kellogg *et al*., 1995). The ballast water-exchange system in the ships causes the transmission of microorganisms from coastal waters to mid-ocean seas, vice-versa (Leichsenring and Lawrence, 2011). An investigation on the invasion of viruses into Chesapeake Bay in Maryland and Virginia of United States revealed that there was estimated 7.4×10^{6} ml^{-1} of viruses invaded through ballast waters and the number was one order of magnitude more than bacterial density (Ruiz *et al*., 2000). Moreover, Drake and co-workers (2007) speculated that 6.8×10^{19} viruses would be imported into Chesapeake Bay by ballast ships annually. Thus, the distribution of marine phages between biomes is believed could trigger ecological risk to the marine ecosystems in terms of biogeochemical cycling, bacterial diversity and marine organisms (Leinschenring and Lawrence, 2011).

2.3.2(b) Temperature

Phages are prevalent in all types of environments where bacteria grow (JoCEzyk *et al*., 2011). They can sustain in broad range of temperatures, even in hot springs and Sahara as well as freeze waters of polar inland waters and North Sea (Wichels *et al*., 1998; Breitbart *et al*., 2004; Prigent *et al*,. 2005; SäwstrR,m *et al*., 2008; Lin *et al*., 2010). In spite of this, marine phages are more sensitive to heat (Spencer, 1955). High temperature affects phages infectivity, which involving attachment, penetration, replication and latent period (JoCEzyk *et al*., 2011; White *et al*., 2012). This was corroborated with the findings of Wiebe and Liston (1968),

where the isolated marine phages were found infectious only at temperature below 23°C, even though the hosts could survive at 33°C and above. In addition, Reyes and Jiang (2010) also reported that less frequency of coliphages were detected in coastal waters during warm temperature and long period of solar radiation exposure.

On the contrary, too low temperature also affects phages density in the seawater. Cochran and Paul (1998) found greatest numbers of viruses (1.32×10^7 viruses ml^{-1}) during summer but smallest number of viruses (0.57×10^7 viruses ml^{-1}) in the winter. This phenomenon was suggested due to the prophage induction at temperature above 19°C. It could also be evidenced by the findings that more than 50% of lysogeny was detected at temperature below 15°C in the Baltic Sea (Europe) and deep waters of Mediterranean Sea (Weinbauer *et al*., 2003). In addition, the greatest emergence of lysogeny could be correlated with the low reproduction of bacteria during winter (Paul, 2008).

2.3.2(c) Solar radiation

Visible light can penetrate until 300 metres in open oceans (Madigan *et al*., 2003). In surface seawaters, the solar radiation causes damage to the capsids and/or genome of the phages (Sinton *et al*., 1999; Fisher *et al*., 2011). The UV-B component of sunlight causes damage to viral genome (Kirchmann, 2000), whereas the photochemical mechanism destroys phagesô capsids (Sinton *et al*., 1999; Fisher *et al*., 2011). A study using laboratory strain phages demonstrated that F-RNA coliphages MS2 were more resistant to sunlight, compared to somatic coliphages T7 and ØX174 (Kapuscinski and Mitchell, 1983). Conversely, the studies on the naturally occurring phages in seawater showed that somatic coliphages were more resistant to sunlight

inactivation than F-RNA phages (Water Research Centre. 1991; Sinton *et al*., 1999). Even so, the three studies concluded that coliphages were more resistant to sunlight than thermotolerant faecal coliforms and *Escherichia coli* (Kapuscinski and Mitchell, 1983; Water Research Centre. 1991; Sinton *et al*., 1999). On the other hand, Love and co-workers (2010) reported that sunlight inactivation rate of F^+ RNA coliphages was slower than F^+ DNA and somatic coliphages.

Compared to F-RNA phages, somatic coliphages were highly susceptible (with 58% of inactivation) to UV-B wavelengths below 318 nm (Sinton *et al*., 1999). In contrast, F-RNA phages were less likely susceptible (with only 17% of inactivation) to wavelengths below 318 nm and were inactivated in longer wavelengths due to photooxidative damage to the host-binding proteins (Davies-Colley *et al*., 1999, Sinton *et al*., 1999). In spite of this, hosts may repair damaged viruses by using UV-A light (Wilhelm and Suttle, 1999; Wommack and Colwell, 2000). It had been reported that about 40% to 80% of the sunlight-damaged phages were repaired daily by their hosts (Wilhem *et al*., 1998a; Weinbauer, 2004). The viral repair mechanism involves enzymatic excision of damaged oligonucleotides at one DNA strand, followed by nucleotide re-synthesis by using the complementary strand. (Harm, 1980; Weinbauer, 2004).

2.3.2(d) Salinity

Phages are able to sustain in broad range of salinity. Despite sauerkraut and brines, phages also had been detected in high salinity environments, such as coastal salt marshes (Zachary, 1974), salt pond (Kauri *et al*., 1991) and Great Salt Plains (Seaman and Day, 2007). Phages that require certain concentration of salt for their

optimum growth are categorised as halophiles. Whilst, phages that do not require high salinity but can tolerate it are defined as halotolerant phages (Oren, 2008). Phages that isolated from salty environments were usually dsDNA tailed phages, which constituted of *Myoviridae*, *Siphoviridae* and *Podoviridae* (Wichels *et al*., 1998). Zachary (1974) reported wide distribution and abundance of marine phages in coastal marshes contained salinity of greater than 8%. In addition, the morphology of the isolated phages were highly diverse and in the category of dsDNA phages (Zachary, 1974). On the other hand, halophages namely ū gspC isolated from Great Salt Plains (Oklahoma, U.S.A.) were found contained large genome (340 kb) in the capsids. Since the morphology of phage ū gspC was similar with the other *Myoviridae* phages, it was predicted that the large genome might encode environmentally relevant genes, which enable the phages to adapt in the high salinity environment (Seaman and Day, 2007).

2.3.2(e) Nutrient and pH

Phage proliferation is influenced by the host metabolism. Eutrophic environment that contained high organic matters and nutrients sustains higher metabolism and growth rate of bacteria. Consequently, it increases the production rate of phages (Weinbauer and Suttle, 1999; Bongiorni *et al*., 2005). On the contrary, depletion of nutrient elements such as phosphorous, carbon, nitrogen, iron or sulphur in the environment reduces viral density (Wilson *et al*., 1996; Clokie *et al*., 2011). In addition, the presence of suspended organic matter such as humic acid in aquatic environments was reported had protection effect towards phages (Mitchell and Jannasch, 1969; Yang and Griffiths, 2013). On the other hand, marine phages are

affected by pH values which deviating from the seawaters. Nevertheless, it had been reported that some isolated marine phages were stable for months in a broad range of pH values (Børsheim, 1993; Weinbauer, 2004).

2.3.2(f) Host availability

The distribution and abundance of bacteriophages are likely depending on the existence of the bacterial hosts due to the fact that viruses are obligate intracellular parasites (Mei and Danovaro, 2004; Clokie *et al*., 2011). Benthic create favourable conditions for viral infections since bacterial production rate in the sediments of the Mediterranean Sea was reported 50 to 400 times greater than in the water column (Mei and Danovaro, 2004). This could be evidenced by the findings that high viral abundance in the aquatic sediments of the Mediterranean Sea, with the value of 40 to 80 times higher than in the water column (Mei and Danovaro, 2004).

2.3.3 Occurrence of lysogeny in marine bacteria

Lytic phages are prevalent and frequently isolated from marine (Goyal *et al*., 1987; Weinbauer, 2004). It had been reported that 65% of the isolated phages from Atlantic Ocean were of lytic (Moebus and Nattkemper, 1981; Moebus, 1983; Weinbauer, 2004). Nevertheless, lysogenic phages also had been detected in marine. Some investigations reported that approximately 40% of bacteria isolated from marine and estuaries were of lysogens (Ackermann and DuBow, 1987; Jiang and Paul, 1994; Jiang and Paul, 1998a). However, the highest incidence of lysogeny was

reported by Stopar and co-workers (2004), where 71% of the bacterial isolates from the Gulf of Trieste (northern Adriatic Sea) were found contained lysogenic phages.

Generally, the percentage of lysogens in offshore is higher than turbid coastal and estuarine water (Jiang and Paul, 1996; Weinbauer and Suttle, 1999). It was suggested that solar radiation penetrates deeper into the water column at offshore area compared to turbid coastal water and causes rapid destruction of infectivity in free phages (Wilhem *et al*., 1998b). Hence, phages survive in pristine offshore water by staying temperate in their host (Weinbauer and Suttle, 1999). On the contrary, low percentage of lysogens in coastal and estuaries is due to prophage induction, which resulted from natural environmental factors and anthropogenic impacts (Paul, 2008). The environmental pollutants such as polynuclear aromatic hydrocarbons, polychlorinated biphenyls and pesticides could trigger the induction activity in prophages (Jiang and Paul, 1996; Cochran and Paul, 1998). The study on viral production in the Mediterranean Sea could be an evident, where only lytic phages and no lysogens were detected in the Port of Ancona (Italy) and the Gulf of Thermaikos (Greece), due to the two regions contained high levels of polynuclear aromatic hydrocarbon and heavy metals (Mei and Davorano, 2004).

By harbouring in the hosts, phages gain protection from proteolytic digestion, grazing by protists and UV inactivation (Weinbauer, 2004). In fact, lysogenic seems beneficial to prophages but harmful to the hosts. Not only the host itself, the closely related adjacent strains will also be lysed by the phages. Hence, prophages are described as ódangerous molecular time bombô, which might affect the entire host population in marine (Paul, 2008). Before being lysed, the infected hosts still can continue with their normal activities such as transforming and oxidizing organic

matters in the environment (Weinbauer, 2004). The energy and material of the hosts are used to synthesise viral macromolecules, while portions of bacterial genome are used to synthesise viral genome (Wikner *et al*., 1993). This actually causes metabolic burdens to the hosts because the host cells served as bioreactors or factories for the production of phage progeny (Weinbauer, 2004).

2.3.4 Significance of phages in marine ecosystem

Viruses are abundant, ubiquitous and ecologically important in the marine ecosystems (Breitbart and Rohwer, 2005). Phages are recognised as potential controlling agents of the microbial community due to their obligate parasitic behaviour (Thomas *et al*., 2011). They play important roles in the microbial food webs, controlling the host diversity in a community and coding for genes that is beneficial to the hosts to sustain in a community (Sandaa, 2008; Zhang *et al.*, 2011).

2.3.4(a) The role of phages in microbial food webs

Bacteriophages are crucial components of oceanic microbial food webs due to their lytic capabilities toward microbial communities (Paul, 2008; Thomas *et al*., 2011). Viral lysis results in mortality of prokaryotes. It had been postulated that 10^{23} of prokaryotes were infected by phages every second (Hendrix, 2003). Upon bacterial mortality, the releasing of nucleic acids and proteins into environments affects carbon, nitrogen and phosphorus cycling as well as nutrient flow in the marine food web (Noble and Fuhrman, 1997; Middelboe and Lyck, 2002; Sekar and Kandasamy, 2013). Although viral lysis reduces the transfer of carbon to higher

trophic levels, the activity indeed increases the recycling of nutrients in the food webs (Noble and Fuhrman, 1997; Sekar and Kandasamy, 2013).

Significant sources of dissolved organic carbon (DOC) pool in marine are derived from bacterial components, such as capsular polysaccharides, cell wall components, porins, proteins and nucleic acids (Stoderegger and Herndl, 1998; Ogawa *et al*., 2001). Phages play important roles by converting these cellular components into dissolved organic matter (DOM) (Fenchel, 1994). In marine pelagic system, phage lysis generates 6% to 26% of carbon fixed by photosynthesis channelled to the DOM pool (Wilhelm and Suttle, 1999; Sekar and Kandasamy, 2013). At least 50% of carbon from the oceanic DOC pool has been used for microbial respiration and reproduction (Breitbart and Rohwar, 2005).

In Lake Plußsee of Germany, high abundance of bacteria was detected in the oxic epilimnion and thermocline layer of metalimnion due to the highest proportion of DOM in these regions (Weinbauer and HØfle, 1998; Weinbauer *et al*., 2003). On the contrary, DOM in the hypolimnion is a refractory carbon skeleton with depletion of nitrogen and phosphorus which could not support bacterial growth (MÎnster and Albrecht, 1994). Therefore, this implies that the growth of bacteria is influenced by the amount and composition of DOM (Weinbauer and HØfle, 1998; Weinbauer *et al*., 2003).

2.3.4(b) Viral impact on prokaryotic diversity

The role of phages in sustaining and equilibrating the diversity of prokaryote species could be explained through the concept of ôkilling the winnerô (Tingstad,