Escherichia coli bacteriophages as candidates for phage therapy and their involvement in membrane vesicle biogenesis

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Certificate of Authorship/Originality

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Content page

List of Figures

List of Tables

Abbreviations

Abbreviation	Meaning
3D-SIM	Three-dimensional structured illumination microscopy
^{o}C	Degree Celsius
aa	Amino acid/s
Abi	Abortive infection
ATP	Adenosine triphosphate
att	Attachment site
BLAST	Basic Local Alignment Search Tool
bp	Base pair/s
CDS	Coding sequence
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CsCl	Caesium chloride
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EOP	Efficiency of plating
ESBL	Extended spectrum β-lactamase
ESCs	Extended spectrum cephalosporins
EU	Endotoxin unit
ExPEC	Extra intestinal pathogenic *E. coli*
FQR	Fluoroquinolone-resistance
g	Relative centrifugal force
G+C	Guanine + Cytosine
h	Hour/hours
ICTV	International Committee on Taxonomy of Viruses
Kb	Kilo base
Kda	Kilo dalton
Kv	Kilo volt
L	Litre
LAL	Limulus amebocyte lysate
LB	Lysogeny broth
LPS	Lipopolysaccharide
M	Molar
Mb	Mega base
MDR	Multi drug resistant
Min	Minute/minutes
µl	Microlitre
mL	Mililitre
MLST	Multi locus sequence typing
mM	Miliomolar
mRNA	Messanger Ribonucleic acid
MVs	Membrane vesicles
MWCO	Molecular weight cut-off value
NCBI	National Centre for Biotechnology information
ng	Nanogram
nm	Nanometer
nr	Non-reductant nucleotide
nt	Nucleotides

Abbreviation	Meaning
NTP	Nucleotide triphosphate
PB	Phage buffer
pfu	Plaque forming unit
R-M	Restriction modification
RNA	Ribonucleic acid
rpm	Revolutions per minute
SAR	Sequence anchored release
SDS	Sodium dodecyl sulphate
Sec	Second/s
ST	Sequence type
Syd1	vB_EcoS_Syd1
Syd6	vB_EcoP_Syd6
Syd8	vB_EcoP_Syd8
T4	Bacteriophage T4
T7	Bacteriophage T7
TA	Toxin-antitoxin
TEM	Transmission electron microscopy
tRNA	Transfer ribonucleic acid
US FDA	The United States Food and Drug Administration
UV	Ultraviolet
v	Version

Research outputs

Publications

Mandal P. K., Sim E. M., Mansfield D., Haggerty L., Venturini C., Iredell J. R., Whitchurch C. B., & Petty N. K., (2020) Three virulent bacteriophages to combat the multidrug resistant *Escherichia coli* clone ST131 (Manuscript in preparation).

Mandal P. K., Ballerin G., Petty N. K., & Whitchurch C. B., (2020) Membrane Vesicles biogenesis in *Escherichia coli* through Bacteriophage infection (Manuscript in preparation).

Mandal P. K., Sim E. M., Whitchurch C. B., & Petty N. K., (2020) Phage purification and endotoxin removal utilising Triton X-114 and ultrafiltration (Manuscript in preparation).

Conference proceedings

Mandal P. K., Ballerin G., Petty N. K., & Whitchurch C. B., (July 2019) **ORAL PRESENTATION**. Membrane vesicles (MVs) as an outcome of bacteriophage mediated bacterial lysis. *Australian Society of Microbiology Annual Scientific Meeting*, Adelaide, Australia.

Abstract

The emergence of multidrug-resistant (MDR) bacterial pathogens is one of the biggest threats to global health. The WHO has recently prioritized the carbapenem-resistant Gram-negative bacteria, including *Escherichia coli*, as critical pathogens for which new therapeutic treatments are urgently required. However, there have been no new antibiotics approved in the past few decades and there is a trend of rapid development of bacterial resistance against the available antibiotics. Thus, non-conventional approaches are essential to control such MDR bacteria and therapy-utilising bacteriophages currently experiencing revival as an effective strategy. Bacteriophages are bacterial viruses that infect and multiply in bacteria thereby lysing them. Bacteriophages can only be cultivated in a susceptible bacterial host and may contain potential contaminants from bacterial cell. Membrane vesicles (MVs) are spherical nanostructures produced by bacteria and are also observed within bacteriophage preparations. However, there is no direct evidence linking bacteriophage activity and MVs formation. This Thesis established a collection of three new bacteriophages Syd1, Syd6, and Syd8 infecting one of the high risks MDR *Escherichia coli* Sequence Type 131 (ST131) and determined the source of MVs within bacteriophage preparations.

The genomic analysis determined that Syd1, Syd6, and Syd8 each lacked genes encoding for lysogeny, bacterial virulence, or antibiotic resistance. Electron microscopy and comparative genomic analyses revealed that Syd1, Syd6, and Syd8 were both morphologically and genetically distinct from each other and other previously characterized ST131 infecting bacteriophages. These three bacteriophages each possessed a completely distinct host recognition gene from each other and other closely related bacteriophages, indicating that each bacteriophage targeted a different surface receptor. Host range analysis on a panel of *E. coli* strains showed that all three bacteriophages can infect multiple ST131 strains efficiently and had different host range infectivity patterns, suggesting them as appropriate candidates for therapy. Live-cell super-resolution microscopy of *E. coli* infected with bacteriophages T4 and T7 showed that during bacteriophage infection, bacterial cells lyse explosively and MVs were formed from shattered membrane fragments. Electron microscopy revealed the presence of different forms of MVs within phage lysates, consistent with MVs formation through phage mediated bacterial lysis. A bacteriophage purification method utilising

ultrafiltration and Triton X-114 phase separation was optimised to remove endotoxins and MVs from bacteriophage preparations. Validation of the method on a panel of *E. coli* bacteriophages including Syd1, Syd6, and Syd8 showed the possibility of up to 5-log reduction in endotoxin and MVs were not detected from electron microscopy of any of these purified bacteriophages.

Chapter overview

This Thesis is composed of six chapters. Chapter one provides a general introduction to the *Escherichia coli* specifically a high-risk strain *E. coli* ST131, bacteriophage specifically tailed phages, phage therapy and preparation and purification of phages. Chapter two describes the materials and methodologies used to achieve the aims of this thesis. Chapter three details the isolation, morphology, genomic and host range characterization of three distinct bacteriophages Syd1, Syd6 and Syd8, which can infect and kill *E. coli* ST131 strains. Chapter four explores the membrane vesicles (MVs) biogenesis in *E. coli* through virulent bacteriophage infection and demonstrates the source of MVs within phage preparation. Chapter five describes a phage purification method, which is proposed for separating MVs from phages and getting pure phages with minimal endotoxin level. Chapter six discuss the main conclusions of the studies presented in this thesis and outlines recommendations for future work.

1 Introduction

1.1 *Escherichia coli*: a critical bacterial pathogen

The World Health Organisation recently published a list of priority bacterial pathogens, that are resistant to most of the currently available antibiotics. The different bacterial pathogens were categorised into three priority levels based on the threat level: critical; high; and, medium. The most critical priority was given to Gram-negative multi-drug resistant (MDR) bacteria that have evolved resistance to the last line of antibiotics. These pathogens pose a significant threat especially in clinical settings due to the paucity of any other effective treatments. Specifically, the critical group is composed of carbapenem-resistant *Acinetobacter baumannii,* carbapenem-resistant *Pseudomonas aeruginosa,* and carbapenem-resistant and third-generation cephalosporin-resistant Enterobacteriaceae, which includes *Escherichia coli* (Tacconelli et al., 2018).

Escherichia coli is a Gram-negative, facultative anaerobic, nonsporulating, rod-shaped bacteria which encompasses an enormous population of bacteria with a high degree of phenotypic and genetic diversity. Due to its diversity, various typing methods were developed and used to classify *E. coli* strains. Serotyping based on bacterial cell surface antigens: O antigen (part of the lipopolysaccharide layer); H antigen (flagellar antigen); and, K antigen (capsular antigen), differentiated *E. coli* into 190 different serogroups (Stenutz *et al.*, 2006). Later, a multi-locus enzyme electrophoresis (MLEE) technique was developed that divided *E. coli* into five major phylogenetic groups (A, B1, B2, D and E) (Herzer *et al.*, 1990) based on the electrophoretic mobility of enzymes in *E. coli* strains. More recently, multi-locus sequence typing (MLST) utilised the sequences of seven house-keeping genes to assign an allelic profile or sequence type (ST) to any *E. coli* isolate (Urwin & Maiden, 2003, Wirth *et al.*, 2006). Indeed, as of 26/06/2019 there are 9,029 STs of *E. coli* on the PubMLST database (https://pubmlst.org) with the number increasing as more isolates are sequenced.

Most *E. coli* strains are harmless, occurring as commensal organisms of the gastrointestinal tracts of warm-blooded animals and humans and either pose no threat or are beneficial to the host. However, various *E. coli* strains cause intestinal as well as extra-intestinal infections and are one of the most common bacterial pathogens in humans

(Kaper *et al.*, 2004, Leimbach *et al.*, 2013). Intestinal pathogenic *E. coli* are mostly responsible for diarrheal diseases in human and animals whereas extra-intestinal pathogenic *E. coli* (ExPEC) are the most common cause of urinary tract infection, bacteraemia, neonatal meningitis and sepsis. Based on the pathogenesis and the associated human disease, *E. coli* are grouped into various pathotypes (Table 1.1) (Kaper *et al.*, 2004). Pathogenic *E. coli* strains can be identified by the presence of genes encoding virulence factors including genes associated with adherence, colonization, cellular invasion, cell surface molecules, secretion, transport, and siderophore formation (Finlay & Falkow, 1997, Kaper *et al.*, 2004).

Table 1. 1: Pathotypes of *E. coli* and associated human diseases (Kaper et al., 2004).

Pathogen	Pathotype	Responsible for
Intestinal pathogenic *E. coli*	Enterohemorrhagic *E. coli* (EHEC)/ Shiga-toxin-producing *E. coli* (STEC)/Verocytotoxin-producing *E. coli* (VTEC)	Haemorrhagic colitis and haemolytic-uremic syndrome
	Enterotoxigenic E. *coli* (ETEC)	Traveller's diarrhoea
	Enteropathogenic *E. coli* (EPEC)	Diarrhoea in children
	Enteroaggregative *E. coli* (EAEC)	Persistent diarrhoea
	Diffusely adherent *E. coli* (DAEC)	Diarrhoea in children
	Enteroinvasive *E. coli* (EIEC)	Diarrhoea and dysentery
Extra-intestinal pathogenic *E. coli* (ExPEC)	Uropathogenic *E. coli* (UPEC)	Urinary tract infections
	Neonatal meningitis *E. coli* (NMEC)	Meningitis and sepsis

Pathogenic *E. coli,* particularly ExPEC strains, have developed resistances to multiple classes of antibiotics used to treat human and animal infections. The most common first-line oral antibiotics amoxicillin, trimethoprim, sulfamethoxazole, and amoxicillin plus clavulanic acid have steadily shown to be ineffective against community acquired *E. coli* infection (Canton & Coque, 2006). Furthermore, most pathogenic *E. coli* have also become resistant to fluoroquinolones and extended-spectrum cephalosporins (ESCs) due to the expression of extended-spectrum β-lactamases (ESBLs) (Paterson & Bonomo, 2005). ESBLs are enzymes produced from bacteria, which hydrolyse the β-lactam ring of β-lactam antibiotics rendering them ineffective. Six major families of ESBLs have been

characterised based on grouping the enzymatic properties: SHV type; TEM type; CTX-M type; OXA type; PER type; and, GES type, encoded by *bla*$_{SHV}$ *bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{OXA}$, *bla*$_{PER}$ and *bla*$_{GES}$ respectively (Bush *et al.*, 1995, Shaikh *et al.*, 2015).

The first ESBLs were detected in 1985. These were the plasmid-borne β-lactamases *bla*$_{TEM-1}$, *bla*$_{TEM-2}$ and *bla*$_{SHV-1}$ derivatives which do not confer resistance to ESC (known as wild type ESBL) and were largely a hospital-acquired problem. However, since 2000, plasmid-borne CTX-M (*bla*$_{CTX-M}$) which hydrolyses ESCs has emerged as a major ESBL and appears as a major clinical problem. Moreover, in many geographic regions, clinically isolated *E. coli* are found to be CTX-M positive, including CTX-M-15 (one of the most dominant variants of this enzyme group), which is highly proficient at hydrolysing the third generation cephalosporins cefotaxime and ceftazidime (Bonnet, 2004, Canton & Coque, 2006).

Initially, it was unclear whether the widespread occurrence of these CTX-M-15-positive *E. coli* was due to strain-to-strain transfer of the corresponding plasmids or other mobile genetic elements surrounding the plasmid-mediated *bla*$_{CTX-M-15}$ gene (Pallecchi *et al.*, 2004, Gangoue-Pieboji *et al.*, 2005, Lavollay *et al.*, 2006) or whether it was due to the global clonal spread of the multi-drug resistant strains themselves (Prats *et al.*, 2000, Manges *et al.*, 2001, Oliveira *et al.*, 2002). The second mechanism was found to be more apparent and the increased resistance observed among *E. coli* strains, since 2008, has therefore been linked with the worldwide dissemination of a particular clone of *E. coli* designated as *E. coli* sequence type 131 (ST131) (Coque *et al.*, 2008, Nicolas-Chanoine *et al.*, 2008, Johnson *et al.*, 2010).

1.2 *Escherichia coli* ST131: a multi drug resistant clonal complex

E. coli ST131 is an extra intestinal pathogenic *E. coli* belonging to the phylogenetic group B2 of *E. coli.* It is mainly responsible for a high proportion of urinary tract and bloodstream infections (Totsika *et al.*, 2011, Nicolas-Chanoine *et al.*, 2014, Petty *et al.*, 2014) but has also been the cause of other extra-intestinal infections such as intra-abdominal infections, wound infections, meningitis, osteomyelitis, myositis, epididymo-

orchitis, peritonitis and pneumonia (Bert *et al.*, 2010, Johnson *et al.*, 2010, Assimacopoulos *et al.*, 2012, Banerjee & Johnson, 2014).

The *E. coli* ST131 isolates commonly harbour a large number of virulence-associated genes encoding various virulence factors. The most common virulence factor genes possessed by *E. coli* ST131 are: *fimH* (type 1 fimbriae), *sat* (secreted auto transporter toxin), *kpsMII* (group II capsule synthesis), *usp* (uropathogen- specific protein), *iucD* (aerobactin), *iutA* (aerobactin receptor), *iha* (adhesion siderophore receptor), *ompT* (outer membrane receptor), *malX* (pathogenicity island marker) and *traT* (serum resistance) (Karisik *et al.*, 2008, Van der Bij *et al.*, 2012, Blanco *et al.*, 2013, Banerjee & Johnson, 2014). The underlying mechanism of virulence includes attachment to epithelial cells by bacterial surface components (including type I fimbriae and flagella), circumvention of the host defences via an impenetrable capsule, and invasion and destruction of the host cell by the secretion of toxic factors (such as cytotoxic necrotizing factors and haemolysin) (Johnson, 1991, Kaper *et al.*, 2004, Kakkanat *et al.*, 2015). The high virulence of *E. coli* ST131 clone in comparison with other ExPEC strains together with the strain's worldwide dissemination, has made it a high-risk bacterial pathogen (Banerjee & Johnson, 2014, Nicolas-Chanoine *et al.*, 2014). Indeed, this clonal group of *E. coli* was first detected in 2000 and has since been identified in multiple countries of three continents (North America, Europe and Asia) simultaneously in 2008 (Nicolas-Chanoine *et al.*, 2014). Now, it is known to be the most prevalent *E. coli* lineage among ExPEC isolates worldwide.

Phylogenetically, *E. coli* ST131 consists of three lineages, namely clade A (which consist of strains from O16:H5 serogroup encoding *fimH*41), clade B (O25b:H4 serogroup encoding *fimH*22) and clade C also known as H30 clade (O25b:H4 serogroup encoding *fimH*30) (Petty *et al.*, 2014). However, ST131 strains having O25b:H4 serogroup with other *fimH* variants (*fimH*24, *fimH*27, *fimH*32, *fimH*34, *fimH*35 *fimH*54, *fimH*65 and *fimH*191) have also been reported (Banerjee & Johnson, 2014, Nicolas-Chanoine *et al.*, 2014). Of these lineages, clade C, which is associated with fluoroquinolone-resistance (FQR) due to point mutations within the *parC* and *gyrA* genes (*parC-1aAB* and *gyrA-1AB* allele), has become the most dominant lineage detected globally since the 2000s (Nicolas-Chanoine *et al.*, 2014, Petty *et al.*, 2014). The clade C strains were further divided into two sub clades C1/H30R and C2/H30-Rx based on their association with FQR and

carriage of a plasmid encoded *bla*$_{CTX\text{-}M\text{-}15}$ (Figure 1.1). The majority of the clade C strains are FQR with *bla*$_{CTX\text{-}M\text{-}15}$ and are resistant to extended spectrum cephalosporins (Johnson *et al.*, 2013, Price *et al.*, 2013, Petty *et al.*, 2014). Phylogenetic studies revealed that clade C evolved from the clade B in a stepwise evolutionary process, sequentially acquiring mutations, genomic islands and antibiotic resistance genes carried on plasmids (Ben Zakour *et al.*, 2016, Stoesser *et al.*, 2016).

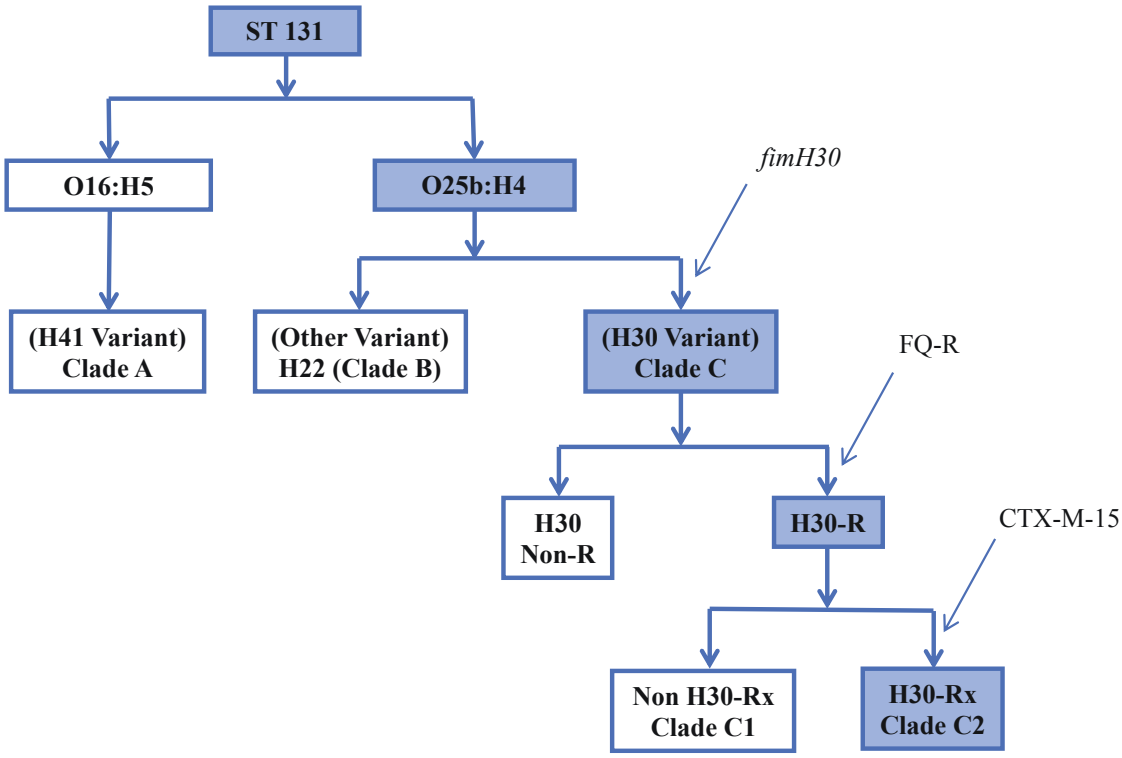

Figure 1. 1: Evolutionary development of multi-drug resistant *E. coli* ST131. Figure adapted from Banerjee & Johnson (2014).

The rapid emergence and successful spread of *E. coli* ST131 clone is strongly associated with development of antibiotic resistance (Figure 1.1) (Platell *et al.*, 2011, Rogers *et al.*, 2011). The current highly occurring ST131 strains are resistant to most of the antibiotics used for treatment including fluoroquinolones and ESCs. They harbour a plasmid encoding the *bla*$_{CTX\text{-}M\text{-}15}$ gene together with other antibiotic resistance genes for various antibiotics including aminoglycosides and ciprofloxacin (*aac (6)-Ibcr*), chloramphenicol (*catB4*), macrolides (*mphA*), sulphonamides (*sul1*), trimethoprim (*dhfr*$_{XVII}$), and tetracycline (*tetA,*) (Woodford *et al.*, 2009, Nicolas-Chanoine *et al.*, 2014).

1.3 Alternative approaches to combat multi drug resistant bacterial infections

The development of resistance against multiple antibiotics such as occurs in *E. coli* ST131 has triggered governments and health organizations around the world to call for the development of new antibiotics. The need for novel antibacterial agents is crucial in order to control global multi-drug resistant bacterial strains as they are rapidly becoming resistant to the last line antibiotics (Peirano *et al.*, 2014). However, there have been no new antibiotics approved in the past few decades (Conly & Johnston, 2005, Fernandes, 2006). In addition, it has also been reported that bacteria could develop resistance to the antibiotics within a very short period of time after their initial usage. Indeed, bacterial resistance to almost all of antibiotics has already been observed (CDC, 2013, Ventola, 2015). This trend of bacterial resistance and the antibiotic development crisis could cause severe public health consequences globally. Thus, alternative non-conventional approaches instead of the classical antibacterial agents have been investigated to help control multi-drug resistant bacterial infections.

So far, various possible alternative-to-antibiotics approaches have been recognized as potentially useful clinically (Gill *et al.*, 2015, Czaplewski *et al.*, 2016). Based on the level of development and available research findings, these approaches have been broadly categorized into three major groups (Table 1.2): Group One includes approaches that are focused on clinical development; Group Two includes approaches currently in preclinical development; and Group Three includes all the other approaches that are at their early stage of research and require proof of concept studies to validate the approach. Of all the identified approaches, three of the Group One approaches utilize either natural bacteriophages, engineered bacteriophages or a bacteriophage lysin (Czaplewski *et al.*, 2016) indicating that therapy-utilizing bacteriophages might be an effective way to combat MDR infections including *E. coli* ST131 .

Table 1. 2: Development stages of alternative to antibiotic treatments for MDR Infections (Czaplewski *et al.*, 2016).

Group	Level of Development	Approaches
1	Clinical	Natural bacteriophages, Engineered bacteriophages, Bacteriophage lysins, Antibodies, Probiotics, Immune stimulation, and Vaccines.
2	Pre-clinical	Antimicrobial peptides, Natural/synthetic peptides, and Antibiofilm peptides
3	Other	Immune suppression, Anti-resistance nucleic acids, Antibacterial nucleic acids, Toxin sequestration using liposomes, Antibiotic-degrading enzymes, Metal chelation, Alphamers, Apheresis of protective antibodies, Immune stimulation by P4 peptide.

1.4 Bacteriophages

Bacteriophages (also called phages) are obligate intracellular bacterial viruses, which infect and multiply only within bacterial cells. They are natural, ubiquitous viruses, equipped with the useful property of being able to kill bacteria. Structurally, they are acellular entities composed of nucleic acid and protein (Joanne *et al.*, 2008). Bacteriophages are a very diverse group of viruses, being the most numerically abundant form of life on Earth (Hendrix, 2002). It is estimated that there is approximately 10 times more bacteriophages than bacteria and archaea with an approximate number of 10^{32} bacteriophages on Earth and densities of $2.5x10^{8}$ particles per millilitre in water and $1.5x10^{7}$ particles per gram in soil (Ashelford *et al.*, 2003). They are ubiquitous and inhabit everywhere including different adverse niches such as the deep sea (Danovaro & Serresi, 2000), polar inland waters (Sawstrom *et al.*, 2008), solar salterns (Guixa-Boixereu *et al.*, 1996), surface sands of the Sahara (Prigent *et al.*, 2005) hot springs (Lin *et al.*, 2010) acidic hot springs (Rice *et al.*, 2001), alkaline lakes (Jiang *et al.*, 2004), and Antarctic lakes (Kepner *et al.*, 1998).

1.4.1 Morphology and classification of bacteriophages

Based on the accumulated evidence from transmission electron micrographs of more than 6,196 phages, diverse types of phage morphology have been described (Figure 1.2) (Ackermann & Prangishvili, 2012). Besides the morphology, the molecular composition and organization of the phage genome is also variable (DNA or RNA, double stranded or single stranded, linear or circular) (Ackermann, 2006). Hence, bacteriophages are classified on different criteria including their morphology (tailed, polyhedral, filamentous, pleomorphic), capsid symmetry (binary, cubical/polyhedral, filamentous/helical, pleomorphic); genomic structure / organization (Ackermann, 2006).

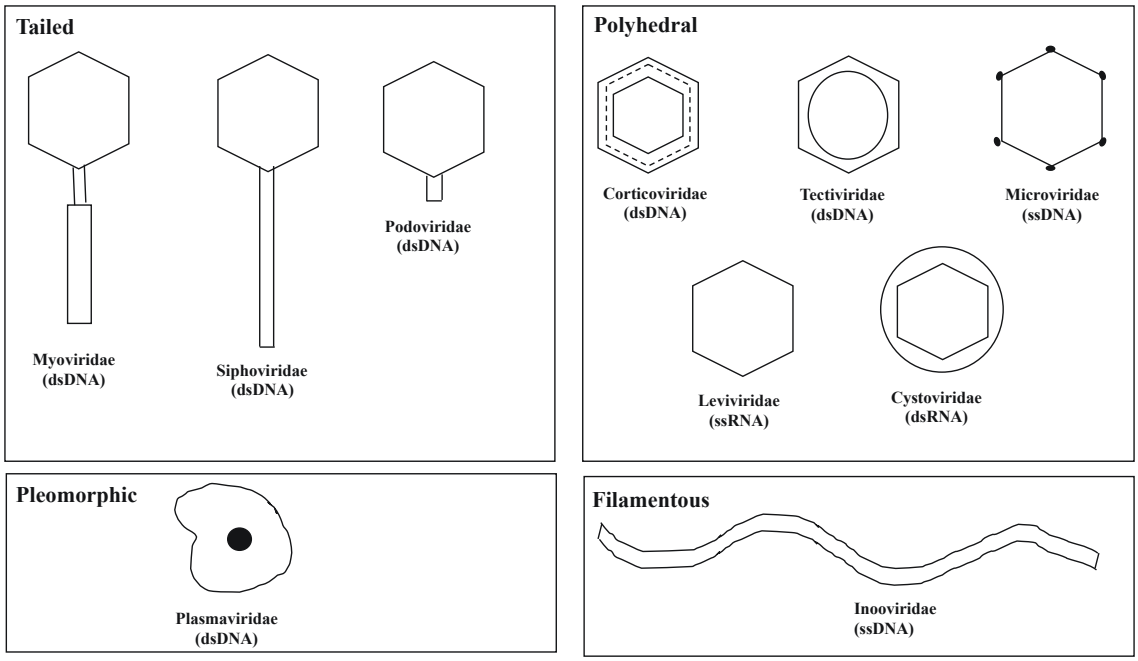

Figure 1. 2: Diversity of bacteriophage morphology. Schematic representative diagrams of each of the 10 known phage families, which are broadly divided into tailed, polyhedral, pleomorphic and filamentous morphotypes. Figure adapted from Ackermann & Prangishvili (2012).

The taxonomic classification of bacteriophages is maintained by the International Committee on Taxonomy of Viruses (ICTV; http://www.ictvonline.org). The ICTV classifies phages based on the evaluation of various properties including the morphological structure of the phage, the molecular composition of the phages, genomic structure / organization and sequence similarity. A recent report of ICTV classified phages into 14 families with 204 genera (Adriaenssens & Brister, 2017). Amongst these,

the most abundant (96%) are dsDNA tailed phages belonging to the order *Caudovirales* (Ackermann & Prangishvili, 2012).

1.4.2 *Caudovirales*: the tailed phages

Caudovirales (also referred to as the "tailed phages") have a structural organization with binary symmetry. They contain a double stranded DNA genome enclosed within a protein cage called a "head" or "capsid". The head is made up of many copies of one or several different proteins with a very stable organization. The shape of the head may be filamentous, spherical or pleomorphic with the most common geometry being icosahedral symmetry. The head is connected to the tail, a single hollow tube-like structure composed of many repeating proteins, via a connector. The connector is a hetero-oligomer comprised of many proteins subunits which serves as an adaptor between the head and tail (Lurz *et al.*, 2001). The tail may vary in its length and flexibility. Based on the tail architecture, tailed phages are categorized into three major families namely, *Myoviridae, Siphoviridae* and *Podoviridae* (Figure 1.3) (Ackermann, 2006).

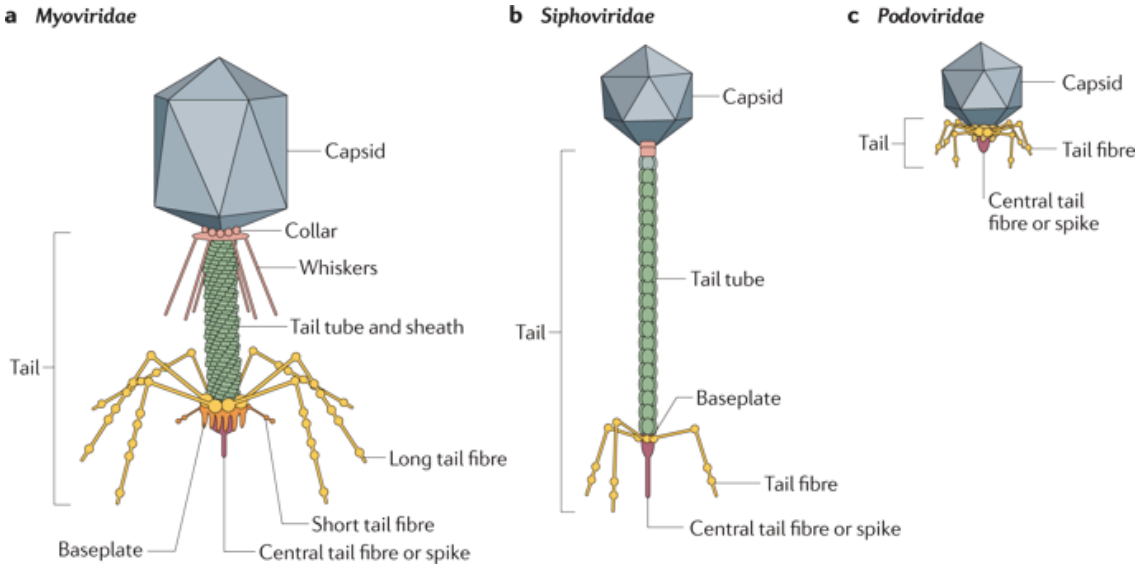

Figure 1. 3: Tailed phages structure. A representative structure of each of the three tailed phage families. Figure copied from Nobrega *et al*. (2018) with permission.

Myoviridae phages, such as T4 consist of a long contractile tail with a rigid internal tube surrounded by a contractile sheath. The distal end of the tail tube possesses a complex structure comprised of a baseplate from which tail fibres and tail spikes emanate (Leiman & Shneider, 2012). Tail fibres are thin protrusions which can be long or short, whereas

tail spikes are short and thick trimeric intertwined protrusions (Ackermann & DuBow, 1987, Nobrega *et al.*, 2018). The tail of *Siphoviridae* bacteriophages, T5, λ for example, consist of a long flexible non-contractile tail tube without any sheath. Like *Myoviridae*, they also have a base plate with tail fibres and tail spikes, although *Siphoviridae* bacteriophages without a baseplate-like structure have also reported (Fokine & Rossmann, 2014, Nobrega *et al.*, 2018). In contrast to both *Myoviridae* and *Siphoviridae*, the *Podoviridae* bacteriophages, such as T7, P22, possess short tails without any sheath or baseplate-like structure. In addition, their tails consist of an upper tail adaptor protein that connects the tail to the capsid and a lower nozzle, which is surrounded by six or twelve tail fibres or tail spikes (Cuervo *et al.*, 2013)

The tail and its accessory structures play a major role in the entry of the bacteriophage genome into the host bacterium during the infectivity process. It serves as a signal transmitter as well as a pipeline through which the genome is delivered into the host cell during infection (Leiman *et al.*, 2010). The initial reversible binding of tailed bacteriophages onto the bacterial surface receptor is mediated by the peripheral tail fibres (Casjens & Molineux, 2012). Bacteriophages can recognise different surface receptors on the bacterial envelope, including lipopolysaccharide (Prehm *et al.*, 1976, Heller & Braun, 1979), capsular polysaccharides (Pickard *et al.*, 2010), fimbriae, flagella (Raimondo *et al.*, 1968, Pate *et al.*, 1979), outer membrane proteins (Wang *et al.*, 2000, German & Misra, 2001) and teichoic acid (Xia *et al.*, 2011). After the establishment of initial attachment, the irreversible binding of the central tail fibre or spike occurs which results in subsequent injection of the bacteriophage genome into the bacterial host (Casjens & Molineux, 2012).

Due to the structural diversity of the tail architecture between the three families of tailed bacteriophages, they utilize different mechanisms to inject their DNA into the host cells. *Myoviridae* bacteriophage, after attaching to their host cell, undergoes a change in the base plate conformation resulting in sheath contraction, which facilitates the tail tube puncturing the bacterial outer membrane and delivery of the viral DNA (Leiman & Shneider, 2012, Hu *et al.*, 2015). In *Siphoviridae*, the fibre or spike that projects from the centre of the base plate, also referred as tail tip protein, directly penetrates the bacterial cell envelope after attachment (Davidson *et al.*, 2012). *Podoviridae* bacteriophages

release a needle-like extension from their tail in order to penetrate the host membrane following attachment (Casjens & Molineux, 2012).

1.4.3 Genomes of bacteriophages

The tailed bacteriophages all contain dsDNA genomes but the genome size varies enormously ranging from 11.6 kb (*Mycoplasma* phage P1) (Tu *et al.*, 2001) to 497 kb (*Bacillus megaterium* phage G) (Pedulla *et al.*, 2003). Despite the genome size variability, the genome of dsDNA bacteriophages has modular organization with genes performing similar functions, such as head morphogenesis, tail morphogenesis, DNA replication, bacterial lysis etc., clustered together in functional modules (Campbell, 1994, Hatfull, 2008). Additionally, there is also a general order in the organization of the structural module genes where the head morphogenesis genes are located upstream of the tail morphogenesis genes (Casjens, 2005). Another notable feature of bacteriophage genomes is the genome mosaicism where each individual bacteriophage genome is considered as a combination of functional modules that can be exchanged among the population (Hendrix *et al.*, 2003). Genomic mosaicism in bacteriophages can be observed either at the nucleotide level by comparative genomic analysis or at the amino acid sequence level, if nucleotide sequence similarity is no longer recognizable. At the nucleotide level, regions of genomic similarity flanked by regions of genomic differences are observed, which correspond to the boundaries of DNA segments with different evolutionary origins (Juhala *et al.*, 2000).

1.4.4 Bacteriophage lifecycles

Bacteriophage multiplication depends upon the fate of their life cycle within the bacterial host. Based on the individual bacteriophages' life cycle, bacteriophages can be divided into two major types: virulent and temperate bacteriophages.

1.4.4.1 Virulent bacteriophages

Virulent bacteriophages (strictly lytic bacteriophages) infect bacteria, hijack their machinery, multiply intracellularly and finally lyse the bacterial cell to release them from the host bacteria thereby killing them (Deresinski, 2009) (Figure 1.4). Virulent bacteriophages reproduce using the metabolism and machinery of the host bacterium.

Once the bacteriophage irreversibly binds to the specific target bacterial cell, the bacteriophage genetic material is immediately injected, and the host metabolism is redirected to synthesize bacteriophage structural components by replication, transcription and translation. Bacteriophage DNA is replicated by *theta* or a rolling circle mechanism (Weigel & Seitz, 2006) forming long linear concatemers (genome units linked head-to-tail) which is then cleaved and packaged into the assembled heads by the phage terminase enzyme by one of two known packaging mechanisms: *cos* packaging or headful packaging.

In *cos* packaging, the phage terminase recognizes either the '*cos* site' or the '*pac* site' (packaging site) and makes a specific cut initiating DNA packaging and proceeds until the next *cos* or *pac* site is recognized by the terminase and cut identically. The *cos* site is a complementary protruding identical length single strand of 7 to 19 nucleotide at 5' or 3' end whereas the '*pac*' site is a specific repeat sequence in concatemeric DNA. Due to the specific recognition of initial and final cleavage sites, the length of packaged genomic DNA is 100% accurate among bacteriophage progeny (Catalano *et al.*, 1995, Fujisawa & Morita, 1997) and produce bacteriophage genomes with either 5'/3' cohesive ends or terminal repeats.

In the headful packaging mechanism, the initial terminase recognition of concatemeric DNA occurs at a specific *pac* site but, the second cut is nonspecific after the complete filling of the bacteriophage head. Headful packaging results in packaging over 100% of the genome length with direct terminal repeats that vary from 2% to 10% of the total bacteriophage genome length. Due to this non-specificity, this mechanism ensures terminal redundancy with circular permutations of the original genome configuration among bacteriophage progeny (Oliveira *et al.*, 2005, Rao & Black, 2005).

After DNA packaging, assembly of the bacteriophage tails to bacteriophage heads occurs, followed by the lysis of the host cell envelope, resulting in the release of progeny bacteriophages from the bacterium and the death of the host cell.

Bacteriophage mediated bacterial cell lysis is a carefully regulated and temporally scheduled process, which requires expression of lysis module genes, especially lytic enzymes (Young, 1992). Bacteriophage lytic enzymes, also known as phage lysins/endolysins, have muralytic activity, which degrade the peptidoglycan layer (or

murein) of the bacteria and thus dictate the process of bacteriophage mediated bacterial cell lysis (Young, 1992). Based on the type of muralytic activity, phage endolysins can be broadly divided into three functional groups namely (i) glucosaminidase, (ii) amidase, and (iii) endopeptidase. Glucosaminidase include N-acetylmuramidase (lysozyme), N-acetylglucosamidase and lytic transglycosylase, which act on the glyosidic bonds that link the amino sugars N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) in the peptidoglycan layer. Amidases cleave amide bonds between NAM and L-alanine of the cross-linking oligopeptide stems whereas endopeptidases cleave peptide bonds of the interpeptide bridge (Young, 1992, Fischetti, 2010, Oliveira *et al.*, 2013).

The bacteriophage endolysin is synthesized during bacteriophage replication and accumulates harmlessly within the host cell until access to the cell wall murein guided by a holin protein occurs (Young, 1992). Holins are bacteriophage encoded membrane proteins that accumulate harmlessly in the membrane. There are two mechanisms documented for the translocation of phage lysin to the peptidoglycan layer (Young, 2014) which depend upon the type of holin -lysin system of a particular bacteriophage. In a canonical holin-lysin system, the canonical holin is triggered at a specific time to polymerize in the membrane to form holes, which allow the endolysin to gain access to the peptidoglycan layer (Dewey *et al.*, 2010, White *et al.*, 2011). In the non-canonical SAR (sequence anchored release) endolysin system, endolysin is anchored within the membrane in an inactive form and distributed throughout the periplasmic space. Pinholin, a membrane-tethered enzyme accumulates in the periplasm in an inactive form (Xu *et al.*, 2004, Pang *et al.*, 2009). Pinholin triggers activation of SAR endolysin by forming small heptameric channels in the membrane that serve to collapse the proton motive force thereby depolymerizing the membrane. The membrane depolymerization allows the SAR endolysins to refold into an active form and hydrolyse the peptidoglycan (Park *et al.*, 2007).

The endolysin mediated murein hydrolysis is sufficient to lyse Gram-positive bacteria. However, due to the presence of the outer membrane, lysis of Gram-negative bacteria requires an additional protein complex termed spanin (Summer *et al.*, 2007, Berry *et al.*, 2012). Spanin is a two-component membrane spanning protein complex that consists of a small outer membrane lipoprotein and an integral cytoplasmic membrane protein, designated as o-spanin and i-spanin respectively (Summer *et al.*, 2007, Young, 2014).

Some bacteriophage utilises a single component spanin, designated as u-spanin, that possess an N-terminal outer-membrane lipoprotein signal and a C-terminal transmembrane domain (Summer *et al.*, 2007, Young, 2014). Spanins catalyse the fusion of the inner and outer membranes after degradation of the peptidoglycan layer and thus disrupt the membranes. In the absence of spanin function, the lytic cycle in Gram-negative bacteria terminates in a spherical cell form, apparently capable of withstanding the internal osmotic pressure of the cell and resisting lysis, whereas in the presence of spanin function, the cycle completes the bursting of the cell releasing the bacteriophage progeny (Berry *et al.*, 2012, Rajaure *et al.*, 2015).

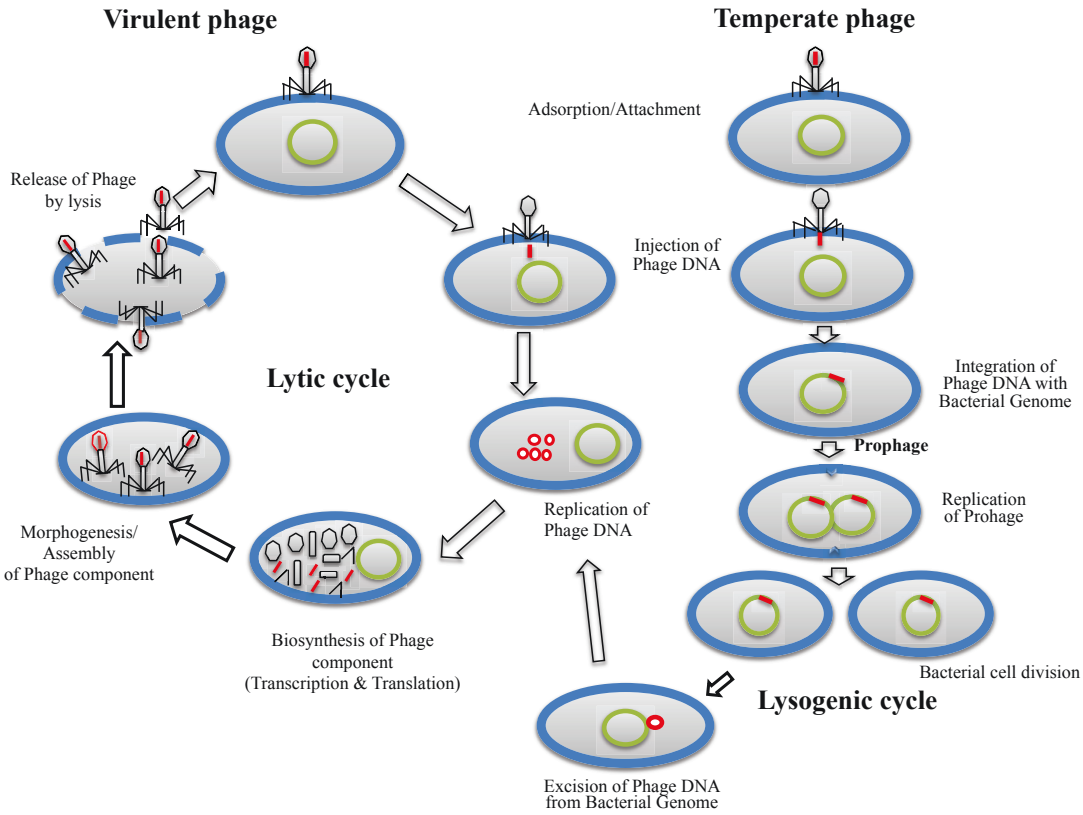

Figure 1. 4 : Life cycle of bacteriophages. The lytic life cycle begins with replication of injected bacteriophage DNA in bacteria followed with synthesis of bacteriophage proteins, which assemble into bacteriophage progeny and release from host by lysing the bacterial cell envelope. In the lysogenic cycle, bacteriophage DNA integrates into the bacterial chromosome and replicate as part of its genome as prophage. Prophage may also excise from host bacteria and enter into lytic life cycle. Figure adapted from Campbell (2003).

1.4.4.2 Temperate bacteriophage

Temperate bacteriophage may also follow the lytic lifecycle upon infection of a host bacterium, or alternatively, after injection, the bacteriophage genetic material is

incorporated it into the host genome and resides as a stable element called a “prophage” in the host chromosomal DNA or as a free plasmid molecule without integration. The prophage or free plasmid molecule thus generated in the bacterial cell are propagated in bacterial progeny through bacterial replication during the lysogenic cycle (Figure 1.4) (Lwoff, 1953, Ikeda & Tomizawa, 1968).

The integration of bacteriophage DNA into the bacterial chromosome is mediated by a bacteriophage encoded integrase, which catalyzes site-specific recombination between similar genomic sequences present in the bacteriophage (*attP*) and the bacterial genome (*attB*) (Nash, 1981, Campbell *et al.*, 1992). Based on the mode of catalysis, bacteriophage integrases are broadly divided into two groups: tyrosine integrases and serine integrases. Tyrosine integrases, such as lambda integrase, consists of a N-terminal DNA binding domain and a C-terminal catalytic domain. The DNA binding domain recognizes a longer *attP* to bind and the catalytic domain utilizes a catalytic tyrosine to mediate strand cleavage. These also require other accessory proteins encoded by the bacteriophage itself or from the host bacteria for recombination (Groth & Calos, 2004, Van Duyne, 2005). Serine integrases consist of a N-terminal catalytic domain followed by a DNA binding domain and multiple structural C-terminal domains. They recognize shorter *attP* to bind, utilize a catalytic serine for strand cleavage and do not require any bacterial host factors (Groth & Calos, 2004, Rutherford *et al.*, 2013).

Generally, a temperate bacteriophage establishes a long-term stable relationship with the target bacterial cell replicating together with the host, but they may excise from host genome, a process known as prophage induction resulting in the initiation of the lytic cycle (Figure 1.4). This process is mediated by bacteriophage-encoded excisionase together with integrase and other host proteins, and is regulated by bacteriophage-encoded repressor proteins (Miller *et al.*, 1981). Excisionase are DNA binding enzymatic proteins that possesses an unusual winged-helix DNA binding structure and interact with integrase and/or DNA to inhibit bacteriophage integration (Sam *et al.*, 2002). Phage repressors such as lambda CI repressor consist of a N-terminal DNA binding domain and a C-terminal dimerization domain, which is required for cooperative binding repression. In the well-studied temperate bacteriophage λ, the decision of a lysogenic vs lytic cycle is controlled by CI repressor and Cro (a DNA binding regulatory protein) through CI–Cro double negative feedback loop. The CI repressor expresses from the lysogenic

promoter (P_{RM}) and functions as anti-lytic regulators. CI protein specifically binds to the operators overlapping with Cro and other lytic genes promoter (P_R) and prevents the RNA polymerase from binding to it thus no Cro or lytic pathway genes produce. In contrast, Cro antagonizes the production of CI by specifically binding to the P_{RM} and thus promotes expression of lytic module genes (Campbell, 2003, Schubert *et al.*, 2007). The conversion of lysogenic to lytic life cycle of temperate bacteriophage depends on various factors such as chemical stress, environmental factors and the host metabolic state.

The lack of the phage integrase, excisionase and repressor genes in the bacteriophage genome are genomic indicators of obligatory virulent bacteriophages that can kill bacteria specifically by lysis (Małgorzata Łobocka *et al.*, 2014).

1.4.5 Co-evolution of bacteriophages and bacteria

As bacteriophage-bacteria interactions are one of the most common interactions on earth whereby bacteriophages can multiply themselves at the expense of bacterial death, bacteria have evolved various mechanisms to evade bacteriophage infection and killing. However, bacteriophages being the most abundant organism, evolve rapidly to counter bacterial defence mechanisms. Hence, there is an evolutionary arms-race between the bacteriophage and the bacteria that results in extensive co-evolution of both.

1.4.5.1 Adsorption Resistance

Adsorption resistance is a mechanism by which bacteria can prevent the bacteriophage from accessing its receptor and thus become resistant to bacteriophage infection. Bacteria can exhibit adsorption resistance to bacteriophages either by modifying their cell surface receptors (i.e. receptor modification) or producing extracellular masking polymers that hide the bacteriophage receptor (Holst Sorensen *et al.*, 2012). For instance, the outer-membrane protein TraT in *E. coli* F+ strain masks or modifies the conformation of the outer-membrane protein A (OmpA), which is a receptor for many T-even-like *E. coli* bacteriophages (Riede & Eschbach, 1986).

However, bacteriophages can overcome the receptor modification by either evolving to recognise new surface receptors (Meyer *et al.*, 2012), or changing the specificity of their host recognition proteins through tail fibre modification. Bacteriophages can also produce

enzymes to degrade extracellular polymers masking the surface receptor and thus allow the bacteriophage to bind its receptor.

1.4.5.2 Restriction–Modification (R-M) systems

Restriction-modification (R-M) is a defence system possessed by bacteria that utilize a restriction endonuclease enzyme to degrade the foreign DNA and a methyltransferase to protect the host DNA (Bickle & Kruger, 1993, Vasu & Nagaraja, 2013). When bacteriophage DNA enters into a bacterial cell harbouring a R-M system, it is identified by the bacterial restriction enzyme that then cuts the bacteriophage DNA at specific restriction sites (Pingoud *et al.*, 2005) whereas the host DNA is protected from cleavage through modification of the restriction sites by the methyltransferase (Tock & Dryden, 2005).

Bacteriophages have evolved various anti-restriction strategies to cope with these bacterial R-M systems. These include modification of restriction sites by incorporation of modified bases (Warren, 1980), methylation of DNA, lacking endonuclease recognition sites (Kruger & Bickle, 1983, McGrath *et al.*, 1999), masking its restriction site (Iida *et al.*, 1987), enhancing host methyltransferase to methylate bacteriophage DNA (Loenen & Murray, 1986), and producing an enzyme to degrade a co-factor required by the R-M system (Studier & Movva, 1976).

1.4.5.3 The CRISPR/Cas system

The CRISPR/Cas system is considered to be a bacterial immunity process that confers resistance to foreign genetic elements like bacteriophage DNA (Barrangou *et al.*, 2007). CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) are loci present within the bacterial genome, composed of 21–48 bp direct repeats interspaced by non-repetitive DNA of similar length (26–72 bp) called “spacers”. The CRISPR loci usually flanked by a varying number of *cas* genes, which encodes CRISPR associated proteins responsible for degradation of foreign DNA (Barrangou & Horvath, 2009). The CRISPR/Cas system begins with a step called “adaptation”, where a new spacer acquired from foreign DNA (protospacer) are incorporated into the CRISPR loci. The updated CRISPR loci is transcribed to produce short crRNA which contains segments of a CRISPR repeat and a protospacer and subsequently complexes with the Cas proteins to

form the CRISPR- Cas complex. The CRISPR- Cas complex then seeks out foreign DNA with nucleotide sequence identical to the protospacer and cleaves the foreign DNA (Sorek *et al.*, 2008).

The CRISPR locus of bacteriophage-resistant bacterial strains contain a spacer acquired from the bacteriophage genome and thus the bacterial cell become resistant towards bacteriophages carrying a genome with a similar sequence. However, bacteriophages can bypass the bacterial CRISPR-Cas system by having a single point mutation or a deletion in their protospacers to prevent detection by the CRISPR-Cas complex (Deveau *et al.*, 2008). Bacteriophages can also evade the bacterial CRISPR-Cas system by encoding either an anti-CRISPR protein that interferes with the CRISPR-Cas systems (Bondy-Denomy *et al.*, 2013) or a bacteriophage borne CRISPR-Cas system to inactivate the host antiviral defence (Seed *et al.*, 2013).

1.4.5.4 Abortive infection (Abi) systems

Bacterial abortive infection systems are programmed cell death systems that can be initiated by bacteriophage infection. These are post-bacteriophage infection resistance mechanisms of bacteria which limit the bacteriophage's propagation and lead to the death of the infected bacterial cell (i.e. suicide) (Chopin *et al.*, 2005), thereby providing protection to the remaining bacterial population. The genes encoding Abi systems are usually found in prophages and plasmids (Samson *et al.*, 2013). There are more than 20 Abi systems so far documented that disrupt a crucial step of bacteriophage multiplication such as bacteriophage DNA replication (Parma *et al.*, 1992), transcription or translation (Snyder, 1995). Some Abi systems may also function as a toxin-antitoxin (TA) system under bacteriophage infection, leading to the toxin induced death of the bacterial cell and the abortion of bacteriophage infection (Fineran *et al.*, 2009, Dy *et al.*, 2014). Bacteriophages could escape TA mediated abortive infection by encoding their own antitoxin or a pseudo-antitoxin that mimics the antitoxin thereby neutralizing the toxins effects (Fineran *et al.*, 2009, Otsuka & Yonesaki, 2012). Bacteriophages could also bypass other Abi systems by producing a molecule that can hijack the host Abi system (Samson *et al.*, 2013).

1.4.6 Cultivation and purification of bacteriophages

Being an obligatory bacterial virus, bacteriophages can only be cultivated in a susceptible bacterial host. Therefore, growth for both the bacterial host and bacteriophages should be optimized prior to mass production. The removal of viable bacteria including endotoxins and pyrogens, which are released after bacterial lysis, is another essential step to obtain a clinical grade bacteriophage preparation (Merabishvili *et al.*, 2009). The traditional techniques to yield bacteriophage preparation typically consist of four phases: (i) bacteriophage cultivation, (ii) bacteriophage harvesting, (iii) bacteriophage concentration, and (iv) bacteriophage purification (Yamamoto *et al.*, 1970, Sambrook & Russell, 2001, Bonilla *et al.*, 2016).

The propagation of bacteriophages in the appropriate bacterial host is the first step of bacteriophage preparation, which is done either on solid media or in liquid media. The solid media utilizes the double agar plate assay in which an agar plate with appropriate nutrient media (bottom agar) is seeded with a layer of soft agar (top agar) containing a bacterial culture and bacteriophage mixture. After incubation at optimal conditions for bacterial growth and thus bacteriophage infection, bacteriophages produce a visible plaque with confluent lysis on the top layer of agar. The bacteriophages cultivated on the agar plate are then harvested by either scrapping off the entire top agar layer or diffusing the bacteriophages into buffer (Su *et al.*, 1998, Mattila *et al.*, 2015, Bonilla *et al.*, 2016). In liquid media, host bacteria grow to mid-exponential phase and are then infected with a suitable number of bacteriophages for bacteriophage multiplication (Su *et al.*, 1998, Bonilla *et al.*, 2016). Whatever the method of bacteriophage propagation, the next step is to remove bacterial contaminants by preparing an initial bacteriophage lysate by low speed centrifugation and subsequent filter-sterilization of the supernatant to remove the bacterial cellular debris. Additionally, DNase and RNase treatment may also use for removing host DNA and RNA contaminants.

To get a high bacteriophage titre, the bacteriophage lysate is concentrated by using various techniques with the most common methods being precipitation, ultracentrifugation and ultrafiltration. Precipitation of bacteriophages from the lysates is done by using polyethylene glycol (PEG) in the presence of high salt and the precipitated bacteriophage particles pelleted by centrifugation (Sambrook & Russell, 2006).

Ultracentrifugation (110,000 – 160,000 g) can directly be used to pellet the bacteriophage particles which are then carefully separated from the supernatant (Winkler *et al.*, 1976, Sambrook & Russell, 2006). Ultrafiltration by using low molecular weight cut off (100 KDa) ultra-centrifugal filter device can filter bacteriophages from the lysates (Bonilla *et al.*, 2016). However, all of these processes result in the extraction of other impurities from a large-scale lysate and thus require further processing.

The final step in the traditional approach of bacteriophage preparation is the purification of the concentrated bacteriophage using cesium chloride (CsCl) density gradient centrifugation. This method uses three different concentration of CsCl solution to prepare three different density gradients which separates the bacterial proteins from the bacteriophage particles through ultracentrifugation (Sambrook & Russell, 2006). Although the traditional CsCl method of bacteriophage purification removes most of the impurities of the media and bacterial constituents, bacterial endotoxin in the purified bacteriophage is still a problem. Another notable issue of the traditional bacteriophage preparation method is that the core steps used for bacteriophage preparation are exactly the same as the method used to purify bacterial membrane vesicles (Figure 1.5) (Klimentová & Stulík, 2015). Additionally, bacterial membrane vesicles range in size from 20 to 300 nm, which in terms of physical size, have a clear overlap with the size of a bacteriophages (24 to 200 nm). As such, it is highly likely that this method to purify bacteriophages will result in the co-purification of bacterial membrane vesicles. In fact, membrane vesicles (MVs) have been seen in transmission electron microscopy of various bacteriophage preparations (Sullivan *et al.*, 2005, Guang-Han *et al.*, 2016, Schiettekatte *et al.*, 2018). However, there has been little attempt to remove MVs from bacteriophage preparation for therapeutic use.

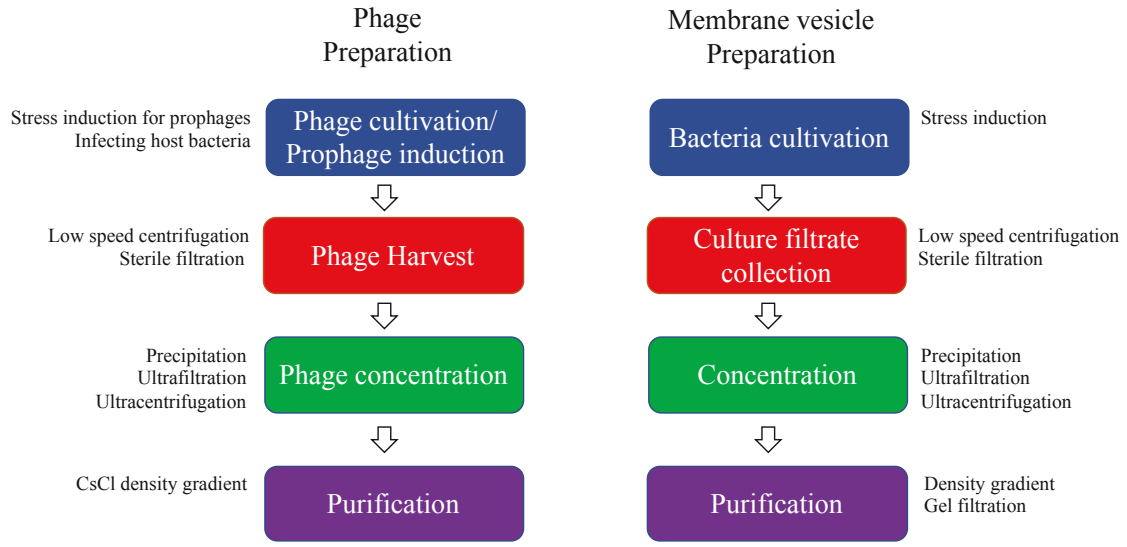

Figure 1. 5: Core steps for bacteriophage and MVs preparation. Each colour box represents a core step and the same colour represents the similar step between bacteriophage and MVs preparation method. Figure adapted from Klimentova & Stulik (2015).

1.4.7 Membrane vesicles in bacteriophage preparations

Membrane vesicles are non-replicating, spherical nanostructures that prevail in all three domains of life but are most common in bacteria (Deatherage & Cookson, 2012). Both Gram-positive and Gram-negative bacteria produce MVs, though the process is mostly studied in Gram-negative bacteria. So far, there are two principal processes in Gram-negative bacteria that result in the production of MVs, namely membrane blebbing and explosive cell lysis (Moller *et al.*, 2005, Turnbull *et al.*, 2016, Toyofuku *et al.*, 2019).

Membrane blebbing is a process of protrusion of the outer membrane from bacteria resulting into the formation of MVs often referred to as outer membrane vesicles (OMVs). Generally, membrane blebbing occurs as a result of cell envelope disturbances that can be caused by either an imbalance of peptidoglycan biosynthesis or an intercalation of hydrophobic molecules into the outer membrane, either of which is likely induced by various cell-associated stress factors (Volgers *et al.*, 2018, Toyofuku *et al.*, 2019). The factors that has been reported for the membrane blebbing include antimicrobial peptides (Urashima *et al.*, 2017), antibiotics (Bauwens *et al.*, 2017), lysozymes (Metruccio *et al.*, 2016), nutrient deprivation (Roier *et al.*, 2016), temperature stress (McBroom & Kuehn, 2007), oxidative stress and oxygen tension (Sabra *et al.*, 2003). MVs formed by membrane blebbing are produced by viable bacteria and have thus been reported as a

protective mechanism to various stresses (McBroom & Kuehn, 2007, Baumgarten *et al.*, 2012).

In contrast, explosive cell lysis results in formation of MVs from shattered membrane fragments that self-assemble following the bursting of the bacterial cell following degradation of bacterial cell envelope (Turnbull *et al.*, 2016). In *Pseudomonas aeruginosa*, the biogenesis of MVs from explosive cell lysis has been shown to occur due to the expression of a prophage based endolysin (Turnbull *et al.*, 2016). As mentioned in section 1.4.4.1, bacteriophage lysin is a major player responsible for the bacteriophage mediated bacterial lysis. Indeed, it has been previously reported that bacteriophage lyse its host bacteria through a bursting phenomenon similar to that of explosive cell lysis (Young, 1992, Berry *et al.*, 2012). As such it is likely that bacteriophage mediated bacterial lysis during bacteriophage cultivation would also produce MVs, though to-date this has not been conclusively demonstrated.

MVs are thought to have various roles in microbial communities, specifically in intercellular molecular exchange that includes delivery of a variety of molecular cargoes including proteins and nucleic acids (Mashburn & Whiteley, 2005, Bitto *et al.*, 2017, Jan, 2017). They are involved in diverse biological processes such as pathogenicity (Ellis & Kuehn, 2010), biofilm formation (Schooling & Beveridge, 2006), horizontal gene transfer (Domingues & Nielsen, 2017) and decoys to defend bacteria from antibiotics (Kulkarni *et al.*, 2015), antimicrobial peptides (Urashima *et al.*, 2017) and most importantly from bacteriophage predation (Manning & Kuehn, 2011, Reyes-Robles *et al.*, 2018). Additionally, MVs have been reported to cause immunomodulatory effects in *in vivo* studies (Alaniz *et al.*, 2007, Codemo *et al.*, 2018). Thus, MV contamination in bacteriophage preparation could be problematic for the optimal efficacy of any potential phage therapy.

1.4.8 Endotoxin in bacteriophage preparation

Endotoxins are lipopolysaccharides associated with the outer membrane of Gram-negative bacteria, which is shed after bacterial cell lysis. They are complex amphipathic molecules consisting of a lipid component linked to a polysaccharide core (Raetz, 1990, Holst *et al.*, 1996). Released endotoxins can exist as either monomers (molecular weight of 4-20 KDa), micelles (molecular weight of 300-1000 KDa) or vesicles with a molecular

weight greater than 1000 KDa (Petsch & Anspach, 2000, Mueller *et al.*, 2004, Gorbet & Sefton, 2005). Endotoxins may elicit different types of pathophysiological effects if the body is exposed to them systemically (Bertok, 1998) and can cause toxic shock, tissue injury and even death depending upon the amount of endotoxin exposure (Morrison & Ulevitch, 1978, Mattern *et al.*, 1994, Rietschel *et al.*, 1994).

As a standard, quantification of endotoxin is expressed as an Endotoxin Unit (EU), which corresponds to the activity of 0.1 ng of *E. coli* lipopolysaccharide. The maximal level of endotoxin in pharmaceutical and biological products used for intravenous applications is set at 5 EU per kilogram of body weight per hour (Malyala & Singh, 2008, Dawson, 2017). With regards to phage therapy, the current consensus within experts in the field of phage therapy is that the limit of endotoxin is dependent on dose and method of administration and the route of administration (Pirnay *et al.*, 2015). However, the limit of toxicity for intravenous application was also similarly agreed upon at 5 EU per kilogram of body weight per hour (Pirnay *et al.*, 2015). The standard procedure to detect endotoxin is via a limulus amebocyte lysate test (LAL test).

There are currently multiple methods that can be used to remove endotoxin from concentrated and CsCl purified bacteriophage preparations including proprietary commercial kits (Merabishvili *et al.*, 2009, Morello *et al.*, 2011), ion exchange chromatography (Hou & Zaniewski, 1990), affinity chromatography (Issekutz, 1983, Hanora *et al.*, 2005) and organic solvent extraction (Szermer-Olearnik & Boratyński, 2015). However, most of these procedures are tailored for specific bio-products that lack generality. A study was previously conducted to compare the strategies for the removal of endotoxin (Van Belleghem *et al.*, 2017) by the different methods. Specifically, either a phage lysate or a CsCl purified bacteriophage preparation were first subjected to endotoxin removal with a commercial kit and then subsequently treated with various reagents including organic solvents, detergents and enzymes. This study reported that endotoxin removal efficiency is bacteriophage specific and that complete removal of endotoxin is not a trivial task (Van Belleghem *et al.*, 2017). It has been reported that diluting the bacteriophage preparations to levels below the limit of endotoxin for intravenous use following endotoxin removal can be effective in managing residual endotoxin. However, this is predicated on the bacteriophage preparation being

sufficiently concentrated so that it can be further diluted (Merabishvili *et al.*, 2009, Morello *et al.*, 2011, Schooley *et al.*, 2017).

1.5 Phage therapy: a potential therapeutic approach against multidrug resistant bacteria

1.5.1 Introduction to phage therapy

Phage therapy is the treatment of bacterial infectious disease by using bacteriophages as an antibacterial agent. It is an alternative, non-compound-based approach in which natural virulent phages that infect and kill pathogenic bacteria are used for therapeutic purposes (Weber-Dabrowska *et al.*, 2000, Miedzybrodzki *et al.*, 2012). Phage therapy may be applied as either monophage therapy, which involves the use of only a single phage type, or polyphage therapy, where a mixture of two or more phage types possessing a diversity of host ranges are combined into a phage cocktail (Chan & Abedon, 2012). Monophage therapy involves either a single, specific narrow host range phage or a broader host range polyvalent monophage. Clinically, it can be used after careful matching between the bacterial isolate and an individual phage isolate. Matching phages are either isolated from natural environments or obtained from phage banks (collection of previously characterized phage isolates) (Miedzybrodzki *et al.*, 2012, Chan *et al.*, 2013). This approach is applied as targeted therapy for a critically ill patient with recalcitrant bacterial infections that are resistant to all antibiotics. Polyphage therapy (phage cocktail), on the other hand, is more appropriate for widespread use as a phage therapy due to the broader host range (Carlton, 1999).

1.5.2 The history of phage therapy

The concept of phage therapy was first put forward by Felix d'Herelle (French-Canadian microbiologist) in 1917, during the pre-antibiotic era (Duckworth, 1976). The first use of phage therapy was in 1919 by d'Herelle on patients with bacillary dysentery (Dublanchet & Fruciano, 2008), however it was not until 1921 when the first report of human trial of phages was published by Richard Bruynoghe and Joseph Maisin, who successfully used bacteriophages to treat staphylococcal skin disease (Bruynoghe & Maisin, 1921). Since then, various studies were carried out by different scientists and scholars exploring the

efficacy of phage therapy on different types of bacterial diseases such as bubonic plague (d'Herelle, 1925), chronic furunculosis, staphylococcal septicaemia (Schultz, 1932), typhoid fever (Knouf *et al.*, 1946), skin infections (Cisło *et al.*, 1987), and cholera (Summers, 1993),. It was first applied commercially as antibacterial therapeutics in Russia and Eastern Europe (Kutateladze & Adamia, 2008). However, by the 1940s, after the discovery of antibiotics, the concept of phage therapy was marginalized due to the success of antibiotics to treat bacterial infections, poor understanding of the biology of bacteriophages, low-quality control of phage preparations and a lack of scientifically approved clinical trials.

In the antibiotic era, the use of phages as therapeutic agents was overshadowed by the worldwide use of antibiotics due to the broad-spectrum activity, effectivity and affordability of these newly discovered compounds (Sulakvelidze *et al.*, 2001, Kutateladze & Adamia, 2008). Most of the phage therapy research and clinical applications were abandoned by the Western world but the therapeutic approaches of phages persisted in the former Soviet countries, most notably in Georgia and Poland where phages are still used as treatment of choice for bacterial diseases (Abedon *et al.*, 2011). However, in the past few decades due to the rapid emergence of multi-drug resistant bacteria and slow development of newer antibiotics, phage therapy has re-emerged as an alternative to antibiotics (Alisky *et al.*, 1998, Matsuzaki *et al.*, 2005, Vandamme, 2014).

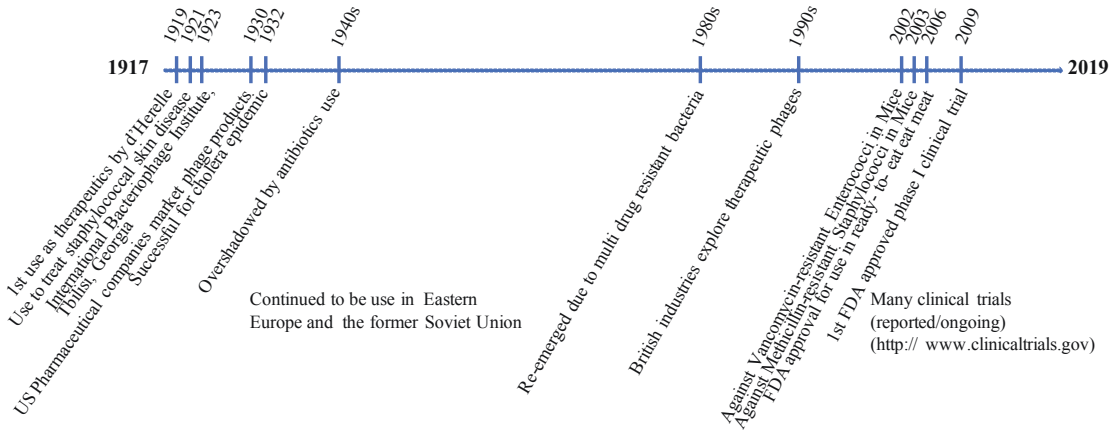

Figure 1. 6: Historical milestones in phage therapy

Major interest in the phage therapy approach was rekindled after 1980 following the publication of experiments in animals supporting the effectiveness of phage therapy

(Matsuzaki *et al.*, 2005). Smith *et al.* (1982) compared the effectiveness of anti-K1capsule specific phages with multiple antibiotics for treating mice challenged intramuscularly or intracerebrally with *E. coli* and revealed that a single intramuscular dose of an anti-K1 phage is more effective than multiple intramuscular doses of tetracycline, ampicillin, chloramphenicol, or trimethoprim plus sulfafurazole (Smith & Huggins, 1982). Since this report, many other reports on the effectiveness of phage therapy have been published which utilised various animal model studies to evaluate the efficacy and safety of phage therapy (Smith & Huggins, 1983, Bogovazova *et al.*, 1991, Soothill, 1992, Barrow *et al.*, 1998, Biswas *et al.*, 2002, Chibani-Chennoufi *et al.*, 2004). Despite the narrow host range and the potential for development of phage resistant mutants, these studies supported the view that bacteriophages could be useful in the treatment of human infections and have paved the way for well-designed human clinical trials for phage therapy in the 21st century.

1.5.3 Significance of phage therapy

As phages are specific with a narrow host range, they can selectively kill the target bacteria without showing any negative effect on the normal human microbiota and human cells (i.e. selective toxicity) (Alisky *et al.*, 1998). Phages have the potential for self-replicating and multiply in the target bacterial population lysing them significantly. Hence, a single dose of appropriate phages is sufficient to treat the infection (Carlton, 1999). Furthermore, once the targeted bacterial pathogen is lysed completely, the phages stop their multiplication automatically due to the lack of suitable host cells and will disperse harmlessly without any accumulation of side effects on the kidney, liver or any other organs. Phages are therefore safer than many antibiotics (Kutter *et al.*, 2010).

More importantly, bacteria that have resistance to particular antibiotics do not confer any cross-resistance with therapeutic phages because the mode of action of phages is entirely different from those of antibiotics. So, phages can kill antibiotic resistant bacteria effectively (Carlton, 1999, Kutter *et al.*, 2010, Loc-Carrillo & Abedon, 2011). Although, bacteria may mutate and develop resistance to specific phages, phages also have the ability to evolve, which implies that there is theoretically no limit to phage discovery. New phages effective against multi-drug resistant strains of particular bacteria can be discovered rapidly with low cost in comparison with the discovery and development of

antibiotics. Moreover, phage preparations can be formulated in a variety of ways to overcome administration issues (Kutateladze & Adamia, 2010, Loc-Carrillo & Abedon, 2011).

1.5.4 Quality of phages for therapy

In 2015, the European Medical Agencies (EMA) reviewed many aspects of the quality of phages for therapy and recommended that only phages that are unable to transfer any harmful host bacterial DNA into non-targeted bacteria were fit to use (Pelfrene *et al.*, 2016). Thus, therapeutic phages should not only be free from lysogeny module genes but should also not possess any gene sequence with homology to genes for antibiotic resistance, virulence or toxin production (Céline *et al.*, 2010). Only the pure phage preparations, free from host-cell proteins, DNA, pyrogenic exotoxins, endotoxins, residual agents such as solvents, and haemolysins are recommended to use for therapy. However, the limit of endotoxin is dependent on the dose and method of administration and the route of administration (Pirnay *et al.*, 2015). Assessment of phage potency through titre determination and negative-staining transmission electron microscopy (as an orthogonal test) is also recommendable for the purpose of quality control.

1.5.5 Patenting and regulation of phage therapy

In view of the fact that phages are naturally occurring biological agents and have been used as therapeutic agents since 1920, the core concept of phage therapy is not patentable (Clark & March, 2006). However, the individual characterized phages can be patented by using the Budapest Treaty (http://www.wipo.int). Though, there is also the possibility that other phages can be isolated from the environment that are competitive with the existing phage preparations (Parracho *et al.*, 2012). Thus, broad-spectrum phage cocktails containing multiple characterized phage strains can be licensed and patented to avoid such problems.

The major medicinal regulatory authorities, the US FDA and the EMA consider phages as biological agents. According to the European Human Code for Medicines, therapeutic phages are kept under biological medicinal products (Directive 2001/83/EC) and in the USA, it is kept under the guidelines of the Division of Vaccine and Related products, but these directives do not specifically apply to phages, highlighting the need for a new

regulatory framework particular for therapeutic phages (Parracho *et al.*, 2012, Verbeken *et al.*, 2012). In accordance with the current regulatory act, it is difficult to register a new phage immediately after its discovery (Vandamme, 2014). However, it has been proposed that therapeutic changes using replacement of phages should follow a shorter assessment time frame like that of influenza vaccine which is the model currently being used to obtain approval (Pelfrene *et al.*, 2016).

1.6 Scope and objective of this study

E. coli, and specifically the multi-drug resistant strain *E. coli* ST131, continue to evolve as high-risk human pathogens. As antibiotic resistance spreads, it is imperative to identify and investigate possible alternative strategies to combat such high-risk multi-drug resistant bacterial strains. Bacteriophages are natural, ubiquitous particles able to kill bacteria specifically, and thereby represent a potential therapeutic as a natural antimicrobial agent. Indeed, natural bacteriophages infecting *E. coli* ST131 strains have been already reported, which can kill *E. coli* ST131 strains *in vitro* and *in vivo* (Dufour *et al.*, 2016, Green *et al.*, 2017). This study was conducted to further understand the biology of different phages against ST131 strains and determine the potential of using such bacteriophages as therapeutics. The overall aim of this thesis is to investigate bacteriophages to control high risk bacterial pathogens, with an emphasis on the preparation and purification of bacteriophages for therapy. To achieve this, following three major objectives were set for this thesis:

1. To characterize virulent bacteriophages with potential for use in phage therapy against *E. coli* ST131.
2. To determine the source of MVs within bacteriophage preparations.
3. To develop a cost effective and simple bacteriophage purification method accounting for MVs and endotoxin removal.

2 Materials and Methods

2.1 Media supplements and solutions

All the components of the media and solutions used in this study are listed in Table 2.1. All were made with ultrapure de-ionized water (dH2O), unless otherwise stated. All the media and solutions were sterilised by autoclaving at 121 °C for 20 minutes if not specified differently.

Table 2. 1: Media and solutions

Media/solutions	Constituents
Lysogeny Broth (LB)	1% (w/v) NaCl (Oxoid: LP0005), 1% (w/v) tryptone (Oxoid: LP0042) and 0.5% (w/v) yeast extract (Oxoid: LP0021) in dH_2O.
LB agar	1% (w/v) NaCl (Oxoid: LP0005), 1% (w/v) tryptone (Oxoid: LP0042), 0.5% (w/v) yeast extract (Oxoid: LP0021) and 1.5% (w/v) agar (Oxoid: LP0011) in dH_2O.
Overlay LB agar	1% (w/v) NaCl (Oxoid: LP0005), 1% (w/v) tryptone (Oxoid: LP0042), 0.5% (w/v) yeast extract (Oxoid: LP0021) and 0.35% (w/v) agar (Oxoid: LP0011) in dH_2O.
Overlay LB agarose	1% (w/v) NaCl (Oxoid: LP0005), 1% (w/v) tryptone (Oxoid: LP0042), 0.5% (w/v) yeast extract (Oxoid: LP0021) and 0.6% (w/v) agarose (Pierce:PIE17852) in dH_2O.
LB gellan gum (LBGG)	1% (w/v) NaCl (Oxoid: LP0005), 1% (w/v) tryptone (Oxoid: LP0042), 0.5% (w/v) yeast extract (Oxoid: LP0021) and 0.8% (w/v) gellan gum (MP Biomedicals:180106) in dH_2O.
Phage buffer	10mM Tris (pH 7.4), 10mM $MgSO_4$ (Sigma:83266) and 0.01% (w/v) Gelatin (Sigma: G9391) in dH_2O.
Lambda diluent	10 mM Tris (pH 7.4) and 8 mM $MgSO_4$ (Sigma:83266) in dH_2O.
1X TBE	0.89 M Tris, 0.89 M Boric acid and 0.02 M Ethylenediaminetetraacetic acid (Medicago: 12-9112-10)

Media/solutions	Constituents
Agarose gel mix (Gel Red)	0.7% (w/v) agarose (Pierce:PIE17852), 0.125X Gel Red (Biotium:41003), 1X TBE
Gel loading buffer	18%(v/v) gel loading buffer (Sigma: G2526) in dH2O.

2.2 Bacterial strains and bacteriophages

E. coli strains used in this study are listed in Table 2.2. All bacterial strains were mixed with 15% (v/v) glycerol for cryopreservation at -80 °C and were revived on LB agar at 37 °C overnight. Overnight cultures were routinely grown in 5 mL of LB in loosely capped 15 mL tubes in an orbital shaker (200 rpm) at 37 °C.

Table 2. 2.: Bacterial strains used in this study

***E. coli* strains**	**Relevant information**	**Reference/Source**
EC958	Human pathogen (UPEC) ST131 Clade C strain	(Totsika *et al.*, 2011, Forde *et al.*, 2014)
SE15	ST131 Clade A	(Toh *et al.*, 2010)
S120EC	ST131 Clade A	(Petty *et al.*, 2014)
S128EC	ST131 Clade B	(Petty *et al.*, 2014)
S135EC	ST131 Clade C	(Petty *et al.*, 2014)
CFT073	Human pathogen (UPEC)	(Welch *et al.*, 2002)
UTI89	Human pathogen (UPEC)	(Chen *et al.*, 2006)
E2348/69	Human pathogen (EPEC)	(Iguchi *et al.*, 2009)
H10407	Human pathogen (ETEC)	(Crossman *et al.*, 2010)
MG1655	K12 laboratory strain	(Blattner *et al.*, 1997)
SE11	Commensal strain	(Oshima *et al.*, 2008)
JIE97	ST131 isolate	Westmead hospital, Sydney, NSW
JIE100	ST131 isolate	Westmead hospital, Sydney, NSW
JIE101	ST131 isolate	Westmead hospital, Sydney, NSW
JIE110	ST131 isolate	Westmead hospital, Sydney, NSW
JIE118	ST131 isolate	Westmead hospital, Sydney, NSW
JIE143	ST131 isolate	Westmead hospital, Sydney, NSW
JIE154	ST131 isolate	Westmead hospital, Sydney, NSW
JIE157	ST131 isolate	Westmead hospital, Sydney, NSW
JIE186	ST131 isolate	Westmead hospital, Sydney, NSW
JIE188	ST131 isolate	Westmead hospital, Sydney, NSW
JIE224	ST131 isolate	Westmead hospital, Sydney, NSW
JIE286	ST131 isolate	Westmead hospital, Sydney, NSW
JIE289	ST131 isolate	Westmead hospital, Sydney, NSW
JIE295	ST131 isolate	Westmead hospital, Sydney, NSW
JIE461	ST131 isolate	Westmead hospital, Sydney, NSW
JIE494	ST131 isolate	Westmead hospital, Sydney, NSW
JIE804	ST131 isolate	Westmead hospital, Sydney, NSW

E. coli strains	Relevant information	Reference/Source
JIE1296	ST131 isolate	Westmead hospital, Sydney, NSW
JIE1301	ST131 isolate	Westmead hospital, Sydney, NSW
JIE1302	ST131 isolate	Westmead hospital, Sydney, NSW
JIE1308	ST131 isolate	Westmead hospital, Sydney, NSW
JIE1311	ST131 isolate	Westmead hospital, Sydney, NSW
JIE1315	ST131 isolate	Westmead hospital, Sydney, NSW
JIE1323	ST131 isolate	Westmead hospital, Sydney, NSW
JIE1324	ST131 isolate	Westmead hospital, Sydney, NSW
JIE1325	ST131 isolate	Westmead hospital, Sydney, NSW
JIE1326	ST131 isolate	Westmead hospital, Sydney, NSW
JIE3172	ST131 isolate	Westmead hospital, Sydney, NSW
JIE3173	ST131 isolate	Westmead hospital, Sydney, NSW
JIE3184	ST131 isolate	Westmead hospital, Sydney, NSW
JIE3195	ST131 isolate	Westmead hospital, Sydney, NSW
JIE3208	ST131 isolate	Westmead hospital, Sydney, NSW
JIE3235	ST131 isolate	Westmead hospital, Sydney, NSW
JIE3247	ST131 isolate	Westmead hospital, Sydney, NSW
JIE3266	ST131 isolate	Westmead hospital, Sydney, NSW
JIE3307	ST131 isolate	Westmead hospital, Sydney, NSW
JIE3320	ST131 isolate	Westmead hospital, Sydney, NSW
JIE3348	ST69 isolate	Westmead hospital, Sydney, NSW
JIE3365	ST131 isolate	Westmead hospital, Sydney, NSW
JIE3411	ST131 isolate	Westmead hospital, Sydney, NSW
JIE3418	ST131 isolate	Westmead hospital, Sydney, NSW
JIE3424	ST131 isolate	Westmead hospital, Sydney, NSW
JIE3430	ST131 isolate	Westmead hospital, Sydney, NSW
JIE3431	ST131 isolate	Westmead hospital, Sydney, NSW
JIE3432	ST131 isolate	Westmead hospital, Sydney, NSW
JIE3454	ST131 isolate	Westmead hospital, Sydney, NSW
JIE3459	ST131 isolate	Westmead hospital, Sydney, NSW
JIE3465	ST131 isolate	Westmead hospital, Sydney, NSW
JIE3684	ST131 isolate	Westmead hospital, Sydney, NSW
JIE3772	ST131 isolate	Westmead hospital, Sydney, NSW
JIE3905	ST131 isolate	Westmead hospital, Sydney, NSW
JIE3996	ST131 isolate	Westmead hospital, Sydney, NSW

All the bacteriophages used in this study are listed in Table 2.3. All bacteriophage stocks were stored in phage buffer (PB) at 4 °C, unless otherwise stated. Bacteriophages were suspended in either PB or lambda diluent.

Table 2. 3: Bacteriophages used in this study

Bacteriophages	**Relevant information**	**Reference**
T4	Virulent Myovirus that infects *E. coli*	(DSM4505)
T7	Virulent Podovirus that infects *E. coli*	(DSM4623)
Syd1	Virulent Siphovirus that infects *E. coli* EC958	This study
Syd6	Virulent Podovirus that infects *E. coli* EC958	This study
Syd8	Virulent Podovirus that infects *E. coli* EC958	This study

2.3 Bacteriophage methods

2.3.1 Isolation of bacteriophages

Bacteriophages were isolated from sewage effluent collected from a wastewater treatment plant in Sydney, Australia, using a protocol adapted from a standard enrichment method for bacteriophage isolation as detailed by Van Twest and Kropinski (Van Twest & Kropinski, 2009). Briefly, 5 mL of effluent sample were chloroform (Sigma: C2432) treated and the resulting supernatant was filtered through 0.45µm pore size membrane filter (FilterBio®, FBS25CA045S) to remove any large sized impurities. The filtrate was then either directly plated onto *E. coli* ST131 strain EC958 or enriched in a liquid culture containing *E. coli* ST131 strain EC958.

The filtrate or enriched eluent was serially diluted (up to 10^{-10} fold) in phage buffer and titrated using double agar overlay method. Briefly, 10 µL of the diluted bacteriophage was mixed with 100 µL of an overnight culture of *E. coli* strain EC958 in 4 mL overlay top medium. The *E. coli*/phage mix was then poured over LB agar. Plates were then incubated at 37°C until plaques were visible (approximately 20 hours. For both methods, single plaques of different morphologies were cored and transferred into separate tubes containing 500 µL of PB to allow the bacteriophage to diffuse into the liquid media. The bacteriophage suspension was then subsequently chloroform (Sigma: C2432) treated and filtered through 0.45µm pore size membrane filter (FilterBio®, FBS25CA045S). The filtrate thus obtained was serially diluted (up to 10^{-5} fold) and titrated using

aforementioned double agar overlay method and single plaque of different morphologies were harvested separately as mentioned above. This process was repeated until only a single plaque morphotype is observed.

Note: This section of work was performed by Dylan Mansfield, Honours student, The ithree institute, University of Technology Sydney.

2.3.2 Propagation and harvesting of bacteriophages

Phage lysates of pure plaque suspensions were prepared from a purified single plaque suspension using the protocol described by Sambrook and Russell (Sambrook & Russell, 2006) with modifications. Briefly, bacteriophages were serially diluted in phage buffer. For each dilution, 100 µL of overnight bacterial host (*E. coli* EC958) culture and 10 µL of bacteriophage suspension was added to 2.5 mL of molten overlay LB agarose and incubated overnight at 37°C. The plate with a mosaic pattern (defined as plate where single plaques are starting to merge into one another) was set to chill at 4 °C for 15 minutes before 3 mL of lambda diluent was added. The flooded plate was then kept rocking overnight at 4 °C. The lambda diluent was subsequently aspirated then centrifuged at 4000 x *g* for 10 min at 4 °C. The resulting supernatant was filtered using a 0.45 µm syringe filter (Sartorius: 16555K) to remove bacterial debris. The filtrate was re-filtered using a 0.22 µm syringe filter (Sartorius: 16534K) to obtain phage lysate.

2.3.3 Concentration and purification of bacteriophages

Bacteriophage concentration and purification was performed by using an optimized bacteriophage purification method, which is detailed in Chapter V (section 5.2.6). The major methods used in the optimized protocol are outlined below.

2.3.3.1 Ultrafiltration of bacteriophage

Ultrafiltration of bacteriophage samples was performed using 100 KDa Amicon® Ultra-4 centrifugal filter device (Sigma: 100K MWCO) according to manufacturer's instructions with modifications. Briefly, the entire phage lysate volume was filtered through an Amicon® 100K MWCO Ultra centrifugal filter at room temperature by centrifugation at 4000 x *g*, until the minimum volume that can be retained by the

Amicon® Ultra centrifugal filter remained. Once all lysate was passed through the filter, bacteriophage concentrates were filled with lambda diluent and treated with DNaseI (Roche: 4536282001) and RNaseA (Roche: 10109142001) to a final concentration of 100 units mL^{-1} and 55 µg mL^{-1} respectively. Three rounds of buffer exchange/buffer washing with lambda diluent was subsequently performed according to manufacturer's instructions at 4 °C. The final retentate was diluted up to the required volume after the third buffer exchange/ buffer washing.

2.3.3.2 Endotoxin removal using Triton X -114

Endotoxin removal from bacteriophage lysate was performed according to the protocol by Aida and Pabst (Aida & Pabst, 1990) with modifications. Briefly, Triton X-114 (Sigma: X114) was added to phage lysate at a final assay concentration of 1% (v/v), vortexed and incubated on ice for 10 minutes. The Triton X-114 treated phage lysate was then subsequently incubated at 37 °C for 10 minutes and centrifuged at 16,000 x *g* for 5 minutes to obtain a phase separation with aqueous (top) and detergent (bottom) layer. The aqueous layer was then aspirated and transferred to a new tube. The Triton X-114 treatment and phase separation were subsequently repeated for a total of ten rounds (Figure 2.1).

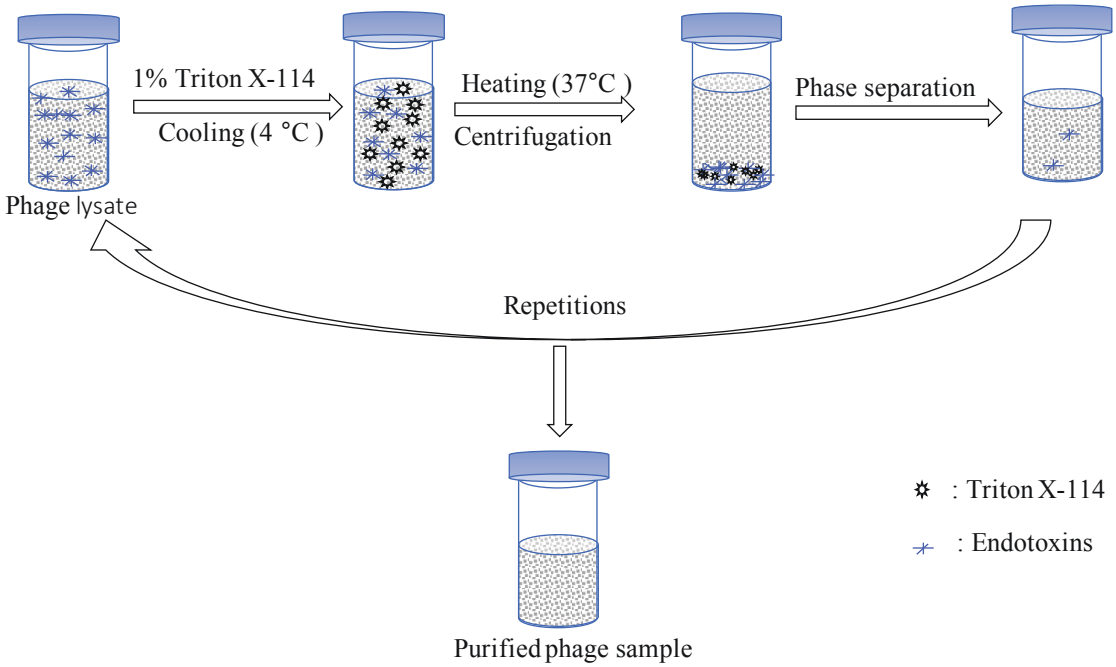

Figure 2. 1: Endotoxin removal by Triton X-114 phase separation method. Figure adapted from London *et al.* (2016).

2.3.3.3 Triton X-114 quantification

Triton X-114 within purified bacteriophage lysates was determined according to the protocol as detailed in a study by Teodrowicz *et al*. (Teodorowicz *et al.*, 2017) with modifications. The control tests for residual triton X-114 determination was performed as with the endotoxin removal steps detailed in section 2.4.3.2 except instead of phage lysates, lambda diluent was used. The residual triton X-114 within control tests were determined from a standard curve generated from Triton X-114 standard solutions. Briefly, 0.1 % (v/v), 0.0125 % (v/v), 0.025 % (v/v), 0.05 % (v/v), 0.005 % (v/v), 0.0025 % (v/v) and 0.00125 % (v/v) standard concentration of Triton X-114 in lambda diluent was prepared. The 280 nm absorbance of these standards (in triplicate) were determined using a Nanodrop and a standard curve was generated. The coefficient of determination (r^2) was ≥ 0.98 for all test performed.

2.3.3.4 Endotoxin quantification

Endotoxin in bacteriophage samples was quantified using Pierce™ LAL Chromogenic Endotoxin Quantification Kit (ThermoFisher Scientific: 88282) according to manufacturer's instructions in a 96 well microtiter plate (Falcon: 353072). Optical density of the LAL test was obtained at 405 nm using a plate reader (TECAN SPARK 10M) and endotoxin level was calculated using the standard curve prepared as part of the LAL test. The coefficient of determination (r^2) was ≥ 0.98 for all tests performed.

2.3.4 Bacteriophage quantification

2.3.4.1 Quantitative plaque assay

Double agar overlay plaque assays were performed according to the Kropinski protocol (Kropinski *et al.*, 2009) with some modifications. Briefly, phage lysates were serially diluted (up to 10^{-10} fold) in phage buffer. For each dilution, 100 µL of overnight bacterial culture and 10 µL of bacteriophage suspension was added to 4 mL of molten LB soft overlay media, mixed gently and poured onto a LB agar plate. The overlay media was left to set at room temperature for 30 minutes before incubating at 37 °C overnight. Bacteriophages were counted on plates with between 30 to 300 plaques, and bacteriophage titres were reported as plaque forming units (pfu mL^{-1}).

2.3.4.2 Semi-quantitative Spot test

Spot tests were performed according to protocol described previously by Kutter (Kutter, 2009) with modifications. Briefly, 100 µL of overnight culture of a bacterial strain of interest was added to 4 mL of molten LB soft overlay media, mixed gently and poured onto a LB agar plate. The overlay media was left to set at room temperature for 30 minutes. Log-fold serial dilutions (up to 10^{-10} fold) of each bacteriophage were prepared in phage buffer and 10 µL of each phage dilution and a phage diluent (as a negative control on each plate) were subsequently spotted onto the surface of the set overlay media (example of which is in Figure 2.2). The plate was incubated at room temperature for 30 minutes and then overnight at 37 °C. After incubation, plaques were counted in spots with between 3 to 30 plaques, and bacteriophage titres were reported as pfu mL^{-1}.

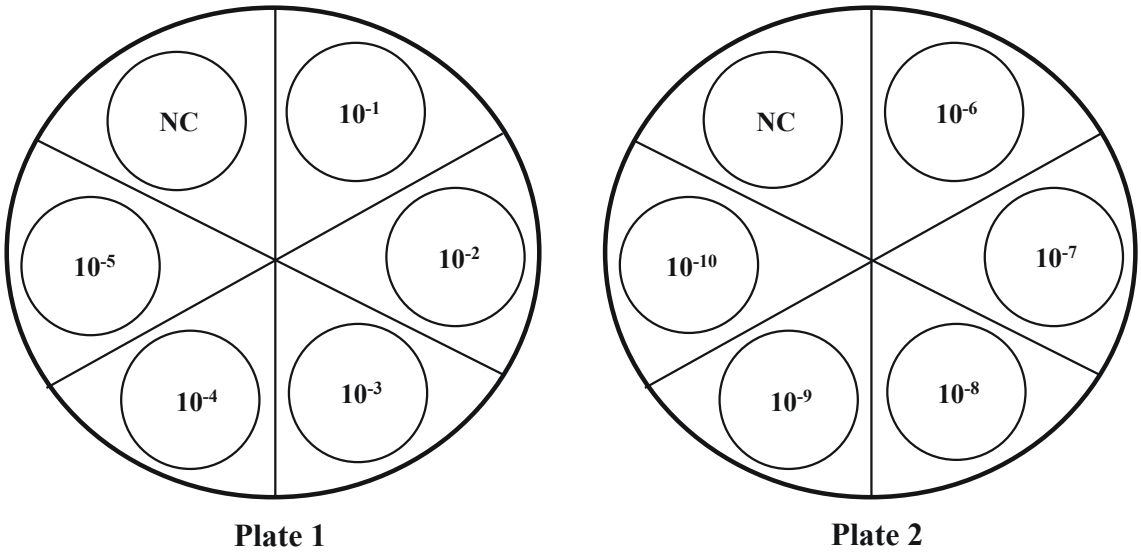

Figure 2. 2: Arrangement of log-fold dilutions of bacteriophage upon spot plate assays. Values on spots refer to the dilution factor of the bacteriophage preparations and NC implies negative control.

2.3.5 Bacteriophage host range test

The host range of bacteriophage isolates was assessed using the semi- quantitative spot test method as mentioned in section 2.3.4.2 (Mazzocco *et al.*, 2008, Kutter, 2009). On spot tests, plates that showed inhibition of bacterial growth at lower dilutions of bacteriophage spots and formation of single plaques at higher dilutions were scored as "bacteriophage infection" (Figure 2.3A) while plates with visibly decreasing inhibition at

higher dilutions with no plaque formations were scored as “lysis from without” (Figure 2.3B). Absence of growth inhibition on all the spots was recorded as “non-infective”.

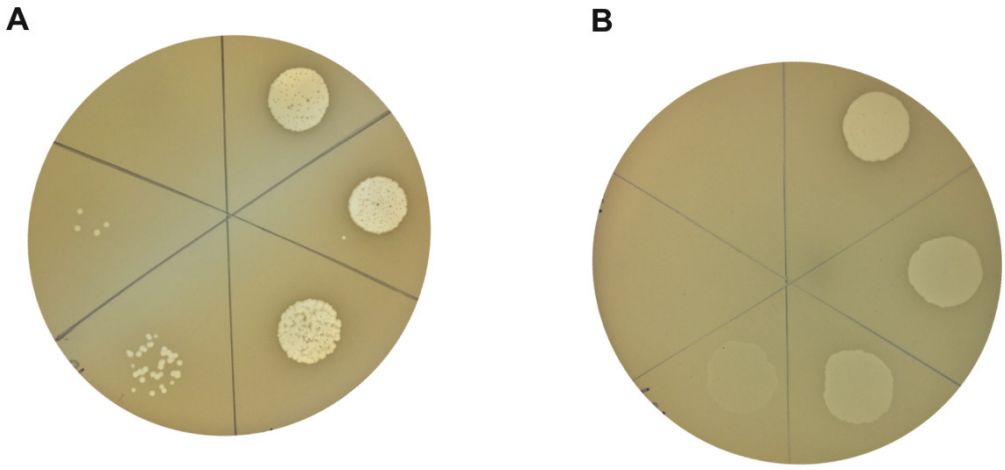

Figure 2. 3: A representative visual assessment of spot tests. (A) bacteriophage infection and (B) lysis from without.

2.3.6 Bacteriophage Efficiency of Plating (EOP)

All bacterial isolates exhibiting the ability to form bacteriophage plaques on a particular bacterial isolate in spot plate assays were taken forward for analysis by efficiency of plating (EOP). For all the infective strains, three independent aforementioned quantitative spot tests were performed, dilutions with 3 to 30 plaques were counted and bacteriophage titre was reported as plaque forming units (pfu mL^{-1}). Efficiency of plating (EOP), was calculated as the ratio of bacteriophage titre obtained from infection of test strain to the bacteriophage titre obtained from infection with the reference strain *E. coli* EC958.

2.3.7 Isolation of spontaneous bacteriophage resistant bacterial mutants

To isolate spontaneous bacteriophage resistant mutants, a high-titre spot assay was performed. Briefly, 100 µL of overnight culture of a bacterial strain was added to 4 mL of molten LB soft overlay media, mixed gently and poured onto a LB agar plate. The overlay media was left to set at room temperature for 30 minutes and 20 µL of bacteriophage suspension with titre ≥ 10^{10} pfu mL^{-1} was subsequently spotted onto the

surface of the set overlay media. The plate was dried at room temperature for 30 minutes and then incubated at 37 °C overnight. After incubation, three bacterial colonies from within the spot were picked with sterilized pipette tips. The isolated colonies were streaked onto a fresh LB agar plate and incubated at 37 °C overnight. The isolated bacteria were tested for bacteriophage resistance using the spot assay (as mentioned in section 2.4.4.2) and the resistant bacteria were passaged thrice serially on LB agar plates. The isolated single bacterial colony after three serial passages was then re tested for bacteriophage resistance using the spot test.

2.4 Genome sequencing and bioinformatics analysis

2.4.1 Bacteriophage DNA extraction

Bacteriophage DNA was extracted using a standard phenol: chloroform extraction method (Pickard, 2009) with modifications. Phase separation was facilitated by using 1.5 mL Phase Lock Gel tubes (5-prime: 2900306) according to manufacturer's instructions. Briefly, bacteriophage DNA was extracted from 450 µL of phage lysate with a titre more than 10^8 pfu mL^{-1}. To remove any possible nucleic acid contamination, the phage lysate was first treated with DNaseI (Roche: 4536282001), RNaseA (Roche: 10109142001) and lysozyme (Thermofisher: 90082) to a final concentration of 100 units mL^{-1}, 55 µg mL^{-1} and 1 mg mL^{-1} respectively, and incubated for 30 minutes at 37 °C. Bacteriophage lysis was performed by adding 20% SDS (Sigma: 05030-F) and proteinase K (Roche: 3115828001) to a final concentration of 0.5% and 100 µg mL^{-1} respectively and incubated for another 30 minutes at 37 °C. DNA was then extracted with phenol: chloroform: isoamyl alcohol 25:24:1 (Sigma:77617) three times followed by chloroform: isoamyl alcohol 24:1 (Sigma:25666) once. Bacteriophage DNA was precipitated by adding 45 µL of 3 M sodium acetate (Sigma: S7899) and 500 µL of 100% isopropanol (Sigma: I9516) and kept at room temperature for 15 minutes. Precipitated DNA sample was then pelleted by centrifugation at 12000 x *g* for 20 minutes and the pellet was washed twice with 70% ethanol (Sigma: E7023) and resuspended in 50 µL of elution buffer (10mM Tris.Cl, pH 8). Extracted DNA was evaluated for purity (A260/A280 ratio = 1.8-2.0 and only a A260 peak in the nanodrop spectra) and quantity (> 4 ng/ul) using a Nanodrop One. When required (i.e. when the A260/A280 ratio was less than 1.8), DNA was further purified using SPRIselect (Beckman Coulter: B23317)) according to manufacturers' instruction.

An aliquot of DNA was run on a 0.7% (w/v) agarose gel in 1x TBE buffer for 1 hour at 75V and visualising on a UV transilluminator to assess DNA integrity.

Note: This section of work was performed by Dylan Mansfield, Honours student, The ithree institute, University of Technology Sydney.

2.4.2 Bacteriophage DNA sequencing

The extracted bacteriophage DNA was sequenced at the ithree institute, University of Technology Sydney sequencing facility using the Illumina MiSeq platform generating 300 bp-paired end reads. Low quality and short reads of less than 30 bp were removed and low quality trailing ends were trimmed using PRINSEQ (Schmieder & Edwards, 2011).

2.4.3 Bacteriophage genome assembly and verification

Bacteriophage genomes were assembled from the filtered reads using SPAdes (Bankevich *et al.*, 2012) v3.5 using a K-mer size of 125 bp. The filtered reads were then mapped onto the respective assembly using BWA-MEM (Li, 2013) v0.7.12 with default settings and visualised using Tablet v1.15.09.01 (Milne *et al.*, 2010), IGV v2.3.77 (Thorvaldsdottir *et al.*, 2013), QualiMap v2.2 (Garcia-Alcalde *et al.*, 2012) and Artemis (Rutherford *et al.*, 2000) to check for misassembles. BLASTn of each assembly was subsequently performed against the genome of *E. coli* ST131 strain EC958 (accession number: HG941718) (Forde *et al.*, 2014) to check for any prophage region from the host and against the genome of PhiX (accession number: J02482) (Sanger *et al.*, 1978) to check for any PhiX contamination in assembly.

2.4.4 Bacteriophage genome annotation and curation

Automated genome annotation was performed using Prokka v1.1 (Seemann, 2014) and Artemis (Rutherford *et al.*, 2000) was used to consolidate the data for manual curation of the automated annotation. BLASTp was performed on all predicted coding sequences (CDS) against the non-redundant protein sequences (nr) database of NCBI (Altschul *et al.*, 1990) and start codons were verified manually. Conserved protein motifs were predicted using the Conserved Domain Database (CDD) and InterPro

(https://www.ebi.ac.uk/interpro/) (Finn *et al.*, 2017) v.61.0 searches. Presence of either antibiotic resistance or virulence genes was detected using the antibiotic resistance gene database ARDB (http://ardb.cbcb.umd.edu/) (Liu & Pop, 2009) and the virulence factor database VFDB,(http://www.mgc.ac.cn/VFs/) (Chen *et al.*, 2005). Signal peptide and transmembrane domain were predicted using PHOBIUS (Kall *et al.*, 2004) while theoretical isoelectric point (pI) and molecular weight of protein product were calculated by using ExPASy ProtParam tool (Wilkins *et al.*, 1999). The online analysis tools PePPER (http://genome2d.molgenrug.nl) (de Jong *et al.*, 2012) and ARNold, (http://rna.igmors.u-psud.fr/toolbox/arnold/) (Lesnik *et al.*, 2001) were used to predict regulatory promoters and putative rho-independent terminators respectively. The predicted promoters were manually checked against promoter sequence TTGACA(N_{15-18}) TATAAT and promoters with up to 2 base difference were identified as possible regulatory promoters.

2.4.5 Comparative genomic analysis

Closely related members for each of the bacteriophage genomes in the public database was performed by using BLASTn against the non-redundant /nucleotide (nr/nt) database of NCBI for viral entries (taxid: 10239) (Altschul *et al.*, 1990). All the closely related bacteriophage genomes used for comparative genomic analysis were downloaded from GenBank and are listed together with accession numbers in Table 2.4. Whole genome nucleotide alignments for each bacteriophages with their closest related bacteriophages were generated using BLASTn and visualized using either Easyfig version 2.1 (Sullivan *et al.*, 2011), ACT v13.0.0 (Carver *et al.*, 2005) and BRIG 0.95 (Alikhan *et al.*, 2011). For pairwise comparison, bacteriophage genomes from the database were reordered as per the respective reference bacteriophage sequence and high-quality diagrams were generated using Easyfig version 2.1 (Sullivan *et al.*, 2011) and BRIG 0.95 (Alikhan *et al.*, 2011).

Table 2. 4: Genomes used in this study

Genomes	Accession	Host Genus	Reference
Bacterial genome			
Escherichia coli EC958	HG941718	N/A	(Forde *et al.*, 2014)
Bacteriophage genome that infect *E. coli* ST131			
LM33_P1	LT594300	*Escherichia*	(Dufour *et al.*, 2016)

Genomes	Accession	Host Genus	Reference
HP3	KY608965	*Escherichia*	(Green *et al.*, 2017)
phiEC1	KY608966	*Escherichia*	(Green *et al.*, 2017)
CF2	KY608967	*Escherichia*	(Green *et al.*, 2017)
Bacteriophage genome similar to Syd1			
vB_EcoS_Golestan	MG099933	*Escherichia*	No associated manuscript
K1ind2	GU196280	*Escherichia*	(Bull *et al.*, 2010, Anany *et al.*, 2015)
K1ind3	GU196281	*Escherichia*	(Bull *et al.*, 2010, Anany *et al.*, 2015)
K1G	GU196277	*Escherichia*	(Bull *et al.*, 2010, Anany *et al.*, 2015)
ST2	MF153391	*Escherichia*	No associated manuscript
K1ind1	GU196279	*Escherichia*	(Bull *et al.*, 2010, Anany *et al.*, 2015)
vB_EcoS_L	KY295896	*Escherichia*	(Baig *et al.*, 2017)
vB_EcoS_G	KY295895	*Escherichia*	(Baig *et al.*, 2017)
K1H	GU196278	*Escherichia*	(Bull *et al.*, 2010, Anany *et al.*, 2015)
vB_EcoS_P	KY295898	*Escherichia*	(Baig *et al.*, 2017)
BPS11T2	MG646668	*Salmonella*	No associated manuscript
SETP13	KF562864	*Salmonella*	(De Lappe *et al.*, 2009)
BPS11Q3	KX405002	*Salmonella*	No associated manuscript
LSPA1	KM272358	*Salmonella*	(Zeng *et al.*, 2015)
vB_SenS_PVP-SE2	MF431252	*Salmonella*	No associated manuscript
FSLSP-101	KC139511	*Salmonella*	(Moreno Switt *et al.*, 2013)
STP03	KY176369	*Salmonella*	No associated manuscript
SS3e	AY730274	*Salmonella*	(Kim *et al.*, 2012)
Jersey	KF148055	*Salmonella*	(Anany *et al.*, 2015)
SE2	JQ007353	*Salmonella*	(Tiwari *et al.*, 2012)
LPSE1	KY379853	*Salmonella*	No associated manuscript
vB_SenS_Ent1	HE775250	*Salmonella*	(Turner *et al.*, 2012)
f18SE	KR270151	*Salmonella*	(Segovia *et al.*, 2015)
fSE1C	KT962832	*Salmonella*	(Santander *et al.*, 2017)
vB_SenS_Ent3	HG934470	*Salmonella*	(Anany *et al.*, 2015)
vB_SenS_Ent2	HG934469	*Salmonella*	(Anany *et al.*, 2015)
wksl3	JX202565	*Salmonella*	(Kang *et al.*, 2013)
SETP7	KF562865	*Salmonella*	(De Lappe *et al.*, 2009)
fSE4S	KT881477	*Salmonella*	(Santander *et al.*, 2017)
f2SE	KU951146	*Salmonella*	No associated manuscript
f3SE	KU951147	*Salmonella*	No associated manuscript
ST1	MF001366	*Salmonella*	No associated manuscript
ST3	MF001364	*Salmonella*	No associated manuscript
MA12	KX245013	*Salmonella*	(Lee *et al.*, 2016)
vB_SenS_AG11	JX297445	*Salmonella*	No associated manuscript
SETP3	EF177456	*Salmonella*	(De Lappe *et al.*, 2009)
Bacteriophage genome similar to Syd6			

Genomes	Accession	Host Genus	Reference
PhiEco32	EU330206	*Escherichia*	(Savalia *et al.*, 2008, Pavlova *et al.*, 2012)
vB_EcoP_SU10	KM044272	*Escherichia*	(Khan Mirzaei *et al.*, 2014)
NJ01	JX867715	*Escherichia*	(Li *et al.*, 2012)
172-1	KP308307	*Escherichia*	No associated manuscript
LAMP	MG673519	*Escherichia*	No associated manuscript
KBNP1711	KF981730	*Escherichia*	No associated manuscript
EP335	MG748548	*Escherichia*	No associated manuscript
ECBP2	JX415536	*Escherichia*	No associated manuscript
Bacteriophage genome similar to Syd8			
vB_CroP_CrRp3	MG775042	*Citrobacter*	(Carolina M. Mizuno, 2018)
vB_EcoP_R	KY295899	*Escherichia*	(Baig *et al.*, 2017)
vB_EcoP_C	KY295892	*Escherichia*	(Baig *et al.*, 2017)
VEc3	MG251390	*Escherichia*	No associated manuscript
AAPEc6	KX279892	*Escherichia*	(Nonis *et al.*, 2017)
vB_EcoP_D	KY295893	*Escherichia*	(Baig *et al.*, 2017)
vB_EcoP_B	KY295891	*Escherichia*	(Baig *et al.*, 2017)
vB_EcoP_ACG-C91	JN986844	*Escherichia*	No associated manuscript
K1E	AM084415	*Escherichia*	(Stummeyer *et al.*, 2006)
K1-5	AY370674	*Escherichia*	(Scholl *et al.*, 2004)
mutPK1A2	MG004687	*Escherichia*	(Lehti *et al.*, 2017)
SP6	AY288927	*Salmonella*	(Dobbins *et al.*, 2004, Scholl *et al.*, 2004)
BP12B	KM366097	*Salmonella*	No associated manuscript
UAB_Phi78	GU595417	*Salmonella*	(Bardina *et al.*, 2016)
vB_EcoP_K	KY295897	*Proteus*	(Baig *et al.*, 2017)
PM85	KM819695	*Proteus*	No associated manuscript
vB_PmiP_Pm5460	KP890822	*Proteus*	(Melo *et al.*, 2016)
PM93	KM819696	*Proteus*	No associated manuscript

2.5 Microscopy methods

2.5.1 Bacterial lysis assay

A liquid culture assay used for bacteriophage propagation (Bonilla *et al.*, 2016) was adapted to demonstrate bacteriophage mediated bacterial lysis (bacterial lysis assay). Briefly, 1 mL of *E. coli* overnight culture was diluted in 10 mL of fresh LB and incubated at 37 °C for 1 hour until early log phase. 10 µL of purified bacteriophage sample with a titre more than 10^8 pfu mL^{-1} was then added to the bacterial suspension. 1 µL of the mixture just after bacteriophage addition was used for microscopic examination to

visualize bacterial lysis and the mixture was incubated further up to 5 hours at 37 °C with shaking at 100 rpm for bacteriophage propagation. A control test with bacteriophage diluent (lambda diluent) instead of bacteriophage sample in the protocol was used to validate the assay. Three independent tests for each of the bacteriophage and control were performed.

2.5.2 Phase contrast and Super-resolution microscopy

Both phase contrast and super resolution microscopic analysis was performed according to the methods described by Turnbull and Whitchurch (Turnbull & Whitchurch, 2014) with some modification. Briefly, cells from a liquid culture were spotted on LB solidified gellan gum (LB gellan gum/LBGG). The LBGG nutrient media was supplemented with the fluorescent dye FM1-43FX (Life Technologies) to a final concentration of 5 μg mL^{-1} to visualise the cell membrane for super-resolution microscopy.

Phase contrast microscopy was performed using an Olympus IX71 inverted microscope fitted with an environmental chamber (Solent Scientific, Segensworth, UK) and AnalySIS Research acquisition software (Olympus Australia, Notting Hill, VIC, Australia).

Super resolution 3D-structured illumination microscopy (3D-SIM) was performed using the DeltaVision OMX SR microscope. Live images were captured using a 1.42 numerical aperture 60x oil objective, standard filter set, a scientific CMOS 512x512 pixel 15-bit camera (PCO AG, Kelheim, Germany) and AquireSR software. Raw 3D-SIM images were obtained through section using a 125-nm Z-step size, which were then reconstructed using SoftWorX software (Applied Precision, GE Healthcare).

2.5.3 Electron microscopy

A drop (5 μl) of bacteriophage suspension was deposited onto a glow-discharged formvar carbon-coated 200-mesh copper grids (ProSciTech: GSCu200CH-100) with an adherence time for 1 minute and stained with 2 % (w/v) uranyl acetate solution (pH 4.5) for 1 minute. Grids were examined with a Transmission electron microscope (FEI Tecnai, T_{20} TEM) operated at 120 kV acceleration voltages. Images were captured using a Gatan camera. Dimensions of MVs and each of the bacteriophage components were measured using FIJI

(Schindelin *et al.*, 2012). Dimension of bacteriophages was established by measurement of 10 different images of each bacteriophage. Each bacteriophage isolate was assigned to a respective family in accordance with the recommended guidelines of the International Committee on Taxonomy of Viruses (ICTV), based upon examination of virion particle morphology.

2.5.4 Image analysis

Phase-contrast microscopy images were analysed, and movies prepared using FIJI (v. 2.0.0) (Schindelin *et al.*, 2012). Reconstructed 3D-SIM images were rendered and presented using IMARIS (v. 9.2.1, Bitplane Scientific). Linear adjustments to signal contrast and brightness were made in the images presented but no gamma settings were changed. Transmission electron microscopy images were analysed using FIJI (v. 2.0.0) (Schindelin *et al.*, 2012) and dimension of images were measured with their respective image scale. The frequency of cell lysis with respect to time was determined using FIJI (v. 2.0.0) (Schindelin *et al.*, 2012).

2.6 Statistical analysis

Results from three independent tests (n =3) were expressed as mean ± SD if not specified differently. Statistical analyses were carried out using GraphPad Prism 8 software. Unpaired t-tests were used to evaluate the significance, unless otherwise specified.

3 Characterisation of three distinct bacteriophages infecting *E. coli* ST131

3.1 Introduction

Escherichia coli sequence type 131 (ST131) has become the most dominant lineage of multi-drug resistant extra intestinal pathogenic clonal *E. coli* complex globally (Johnson *et al.*, 2013, Nicolas-Chanoine *et al.*, 2014, Petty *et al.*, 2014). ST131 lineages are rapidly becoming resistant to the last line antibiotics, encouraging searches for alternative control therapies. Phage therapy is currently experiencing a revival as a strategy to control such multidrug resistant bacteria. Four bacteriophages so far, namely LM33_P1, HP3, CF2 and EC1 were found to infect *E. coli* ST131 strains (Dufour *et al.*, 2016, Green *et al.*, 2017). However, none of these could kill all the tested ST131 strains, suggesting the need for finding more virulent bacteriophages with different host range patterns. Previously, a set of 16 bacteriophages infecting *E. coli* ST131 reference strain EC958 were isolated from effluent water and sequenced in the Petty lab (Dylan Mansfield honors thesis, 2016). These bacteriophages are referred to in this thesis as Syd1-Syd16. Comparative nucleotide analysis revealed that all 16 bacteriophages could belong to three distinct types based on nucleotide identity. This chapter briefly describes the genomes of these 16 bacteriophage genome assemblies, and details the morphological, genomic and host range characterization of three representative bacteriophages that could be useful as constituents in a phage therapy cocktail to combat *E. coli* ST131 infections.

3.2 Results

3.2.1 Verification of bacteriophage assembly revealed that each of the bacteriophage assembled in a single contig

The genomes of each of the 16 bacteriophages were previously assembled into multiple contigs, with each assembly consisting of a single large contig (> 44 kb) and multiple smaller contigs (427 bp to 5511 bp) (Table 3.1). The only exception in the data set is the assembly of Syd8, Syd13 and Syd14, which had additional large contigs of length 24,389 bp, 22,120 bp and 41,000 bp respectively.

Table 3. 1: Assembly statistics of bacteriophage genomes

Bacteriophages	No. of filtered reads	Mean read length (bp)	Assembly length (bp)	No. of contigs	Largest contig		Second largest contig		Other contigs	
					Length (bp)	Read depth	Length (bp)	Read depth	Mean read length (bp)	Mean read depth (X)
Syd1	98,792	242	58,612	24	44,305	546X	1,756	4X	570	1.8X
Syd2	373,494	274	93,579	91	44,305	2193X	1405	3X	538	2X
Syd3	161,219	241	71,560	46	44,305	781X	5,511	458X	494	2X
Syd4	271,242	244	56,978	28	44,305	1428X	1135	2X	463	1.6X
Syd5	41,877	281	55,305	25	44,305	232X	941	2X	455	1.3X
Syd6	205,396	268	141,551	76	76,200	709X	5,511	174X	809	3X
Syd7	55,700	231	86,117	39	44,305	269X	4354	9X	1012	3.7X
Syd8	406,302	279	157,299	74	45,393	2479X	24,389	10X	1215	3.4X
Syd9	85,635	287	158,872	164	45,393	322X	5,511	1392X	667	2X
Syd10	300,562	259	76,523	70	44,305	1661X	2451	4X	464	2X
Syd11	565,209	267	136,895	127	44,305	3230X	5422	6X	697	5.5X
Syd12	54,420	283	101,995	87	45,393	290X	5,511	26X	601	2.2X
Syd13	285,802	252	148,718	200	44,305	1543X	22120	6X	508	2X
Syd14	351,702	260	112,033	52	44,305	1951X	41263	13X	529	2X
Syd15	25,521	281	51,432	15	44,305	138X	567	2X	504	2X
Syd16	351,234	259	73,459	62	44,305	1949X	981	2X	469	2X

To check for potential misassembled, filtered reads (reads with minimum length of 30 bp, and quality of 20) were mapped onto their respective assemblies. It was observed from the mapping profile that in each assembly, except Syd3, Syd6, Syd9 and Syd12, the largest contig had a significant higher read depth than the rest of the contigs (Table 3.1; Figure 3.1). In Syd3, Syd6, Syd9 and Syd12, the second largest contig also had a high read depth. More importantly, of those contigs, only two in Syd3, Syd6 and Syd9 and one largest contig in each of the other bacteriophage assemblies had a read depth of more than 50X (Table 3.1), which is an optimum sequencing depth required for *de novo* assembly of small genomes (Desai *et al.*, 2013). This degree of discrepancy of contig read depth coverage in genome assemblies (Figure 3.1) is unusual and indicates possible contamination (Douglass *et al.*, 2019). Sequencing contamination in various draft genomes has been reported previously, and can stem from different sources such as human DNA , Phi X-174 DNA used for quality control in the sequencing, or cross contamination from other DNA samples sequenced in the same multiplex experiments (Mukherjee *et al.*, 2015, Kryukov & Imanishi, 2016, Lu & Salzberg, 2018, Douglass *et al.*, 2019). Therefore, each of the assemblies were examined further to identify possible contamination sources.

To check for the presence of PhiX-174, a sequencing control for the MiSeq platform and a possible source of contamination (Mukherjee *et al.*, 2015), each bacteriophage assembly was compared using BLASTn (Altschul *et al.*, 1990) to the PhiX-174 genome (GenBank accession number: J02482) (Sanger *et al.*, 1978). Pairwise comparison revealed that in Syd3, Syd6, Syd9 and Syd12, a complete genome of PhiX-174 could be assembled into a single contig with 99% nucleotide identity across the entire length (Figure 3.2 C, F, I and L). Fractions of the PhiX-174 genome could also be assembled from other contigs in each of these assemblies. Similarly, PhiX-174 genomes could be assembled with high nucleotide identity from different contigs in all the other assemblies, except Syd2 and Syd4 (Figure 3.2). All BLASTn matches to PhiX-174 were localised to the smaller contigs in each of the assemblies, and there were no matches to the first, high coverage contigs in any assemblies (Figure 3.2 A-P). Taken together, these results indicate likely PhiX-174 contamination during sequencing and confirmed that PhiX-174-derived sequence reads were not filtered from the dataset. Thus, regions with coverage less than or equal to the mean read depth of the associated PhiX-174 contigs should be treated as possible contaminants due to low level of misassigned reads.

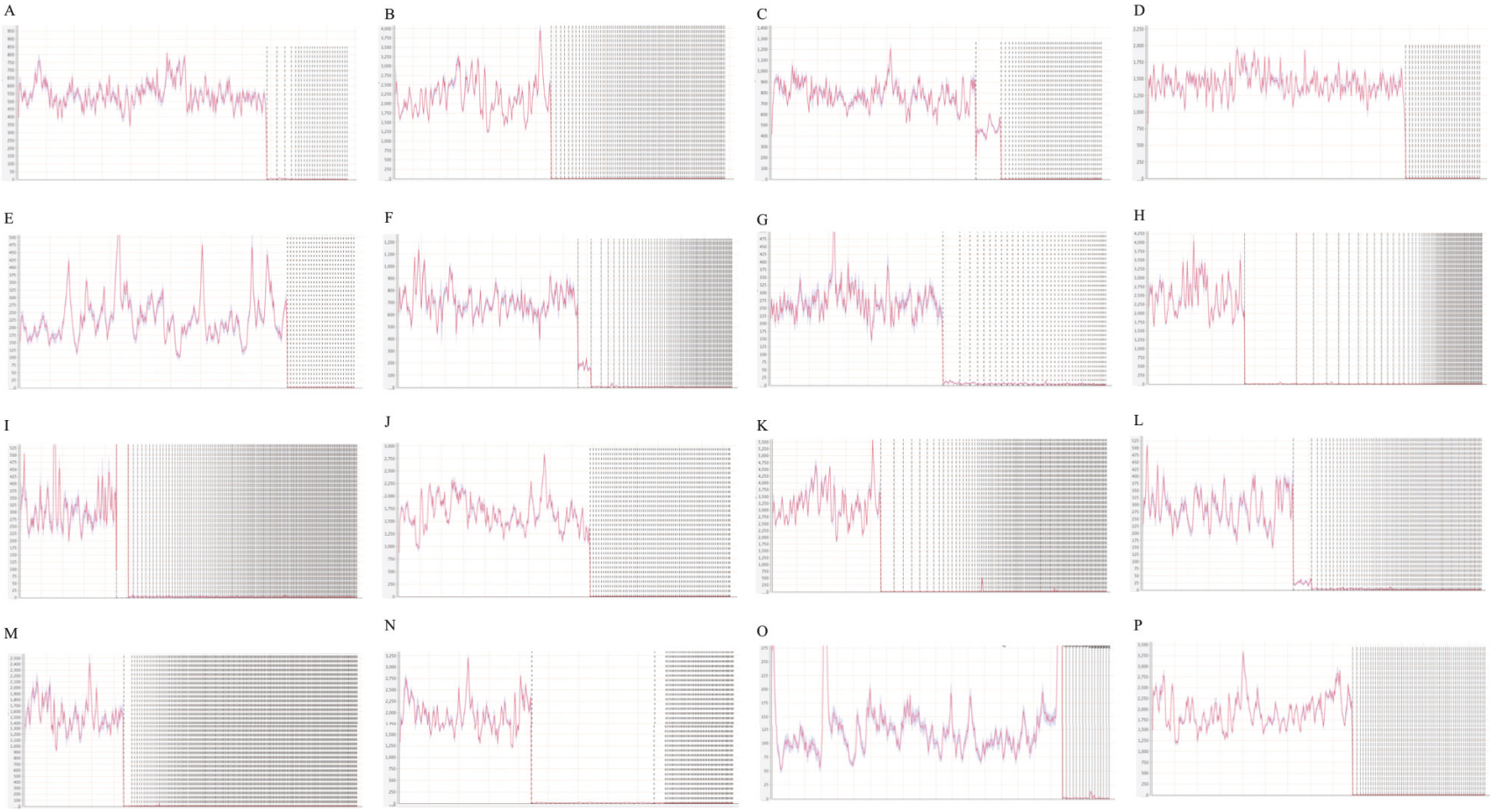

Figure 3. 1: Read coverage over assembly. (A) Syd1, (B) Syd2, (C) Syd3, (D) Syd4, (E) Syd5, (F) Syd6, (G) Syd7, (H) Syd8, (I) Syd9, (J) Syd10, (K) Syd11, (L) Syd12, (M) Syd13, (N) Syd14, (O) Syd15, and (P) Syd16. The X-axis represents the genome length of assembly while the Y-axis represents the depth of read coverage over a single nucleotide position and dotted vertical lines separate individual contigs. Images were generated using QualiMap v2.2 (Garcia-Alcalde *et al.*, 2012)

Figure 3. 2: Pairwise BLASTn comparison of PhiX-174 genome with bacteriophage assembly. (A) Syd1, (B) Syd2, (C) Syd3, (D) Syd4, (E) Syd5, (F) Syd6, (G) Syd7, (H) Syd8, (I) Syd9, (J) Syd10, (K) Syd11, (L) Syd12, (M) Syd13, (N) Syd14, (O) Syd15, and (P) Syd16. In each comparison, the complete genome of PhiX-174 is represented as a horizontal black line. Alternating orange and brown rectangles represent contigs and grey shading between genomes represents nucleotide identity scaled according to the grey bar. Images were generated using Easyfig v2.1 (Sullivan *et al.*, 2011)

Moreover, the length of the PhiX-174 assembled contig in the assemblies of Syd3, Syd6, Syd9 and Syd12 had the same size of 5,511 bp, which is 125 bases longer than the reported genome of PhiX-174 (5,386 bp) (Sanger *et al.*, 1978). When self-to-self BLASTn of the PhiX-174 contig of Syd3, Syd6, Syd9 and Syd12 was performed, it was revealed that each of these contigs was flanked by a 125 bp repeat on either end of the contig, a characteristic of a circular genome assembly (Hunt *et al.*, 2015). Notably, the deletion of one of the repeat sequences from the PhiX-174 contigs in each of these assemblies yields an intact PhiX-174 genome of 5,386 bp, which was reported to be a circular genome (Sanger *et al.*, 1978).

To see if other contigs in our set of bacteriophage assemblies also had similar features, self-to-self BLASTn was performed on each of the assemblies. It was noted that- each of the assemblies also had a 125 bp repeat sequence at both ends of the high coverage, largest first contig. Apart from the largest first contigs of each of the assemblies and the PhiX-174 contigs of Syd3, Syd6, Syd9 and Syd12 assembly, none of the other contigs in any assemblies showed such terminal repeat sequences. In addition, the 125 bp repeat sequence of each of the largest first contigs and the PhiX-174 contigs in Syd3, Syd6, Syd9 and Syd12 were completely different from each other. Since a K-mer size of 125 bp was the parameter used in the assembly process, the presence of these 125 bp repeats in all of the largest first contigs, together with the PhiX contigs of Syd3, Syd6, Syd9 and Syd12, suggested that this could be an assembly artefact due to a circular genome (Hunt *et al.*, 2015).

3.2.2 Comparative nucleotide analysis revealed three types of bacteriophages

The largest contig of Syd1, Syd2, Syd3, Syd4, Syd5, Syd7, Syd10, Syd11, Syd13, Syd14, Syd15 and Syd16 assemblies were exactly the same size (44,305 bp), and the largest contig of Syd8, Syd9 and Syd12 were also the same size (45,393 bp) (Table 3.1). To determine if similar sized bacteriophage contigs were identical to each other, BLASTn was performed. The 44.3 kb contigs of Syd1, Syd2, Syd3, Syd4, Syd5, Syd7, Syd10, Syd11, Syd13, Syd14, Syd15 and Syd16 were highly similar to each other (Figure 3.3). In fact, Syd2 shared 100% nucleotide identity with entirety of Syd4, and Syd5, Syd7 and Syd11 also had identical nucleotide sequences. Likewise, the 45.4kb contig of Syd8, Syd9

and Syd12 were also very similar (Figure 3.3). In contrast, the assembly of Syd6, which has a large contig of 76.2 kb, was distinct from both the 44.3 kb and 45.4 kb bacteriophages (Figure 3.3).

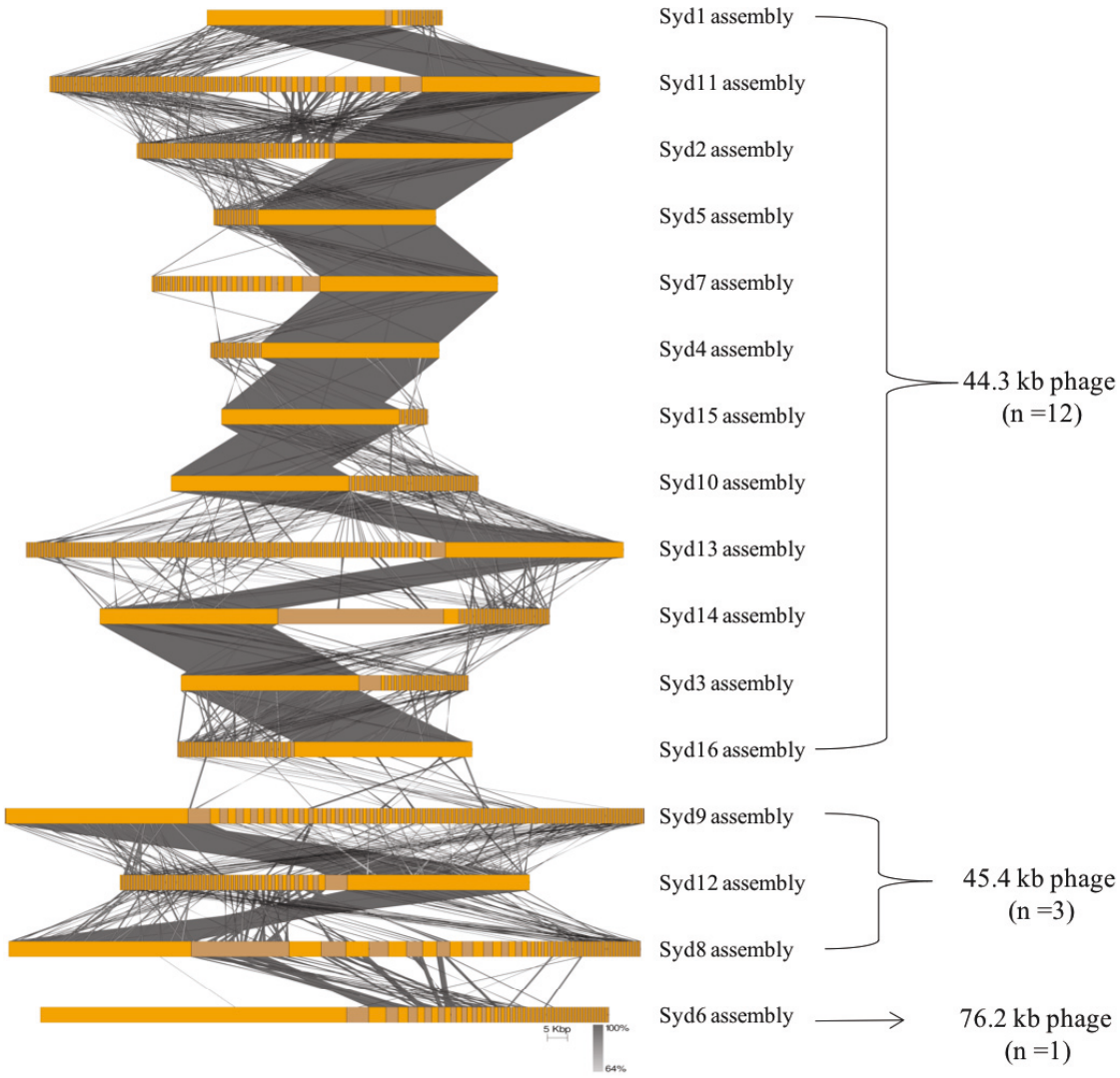

Figure 3. 3: BLASTn comparison between all the assemblies. Alternating orange and brown represent contigs of each assembly. The homology in nucleotide sequence of largest orange colour bacteriophage contig is represented by grey shading between them and scaled according to the grey bar. Image was generated using Easyfig 2.1 (Sullivan et al., 2011).

Notably, the differences among the similar sized bacteriophages were detected only in the 125 bp terminal repeat sequences. Indeed, the 125 bp sequence that was repeated was identical in bacteriophages that were similar across the entire nucleotide sequence from first to last base but different among others. Each of these different 125 bp sequences were still present in all the other similar size bacteriophages, elsewhere in a single copy in the genome if not in terminal repeats. This difference in 125 bp terminal repeat

sequences in highly similar assemblies suggested that this could also be an assembly artefact.

To confirm if these were truly assembly artefacts and not the bacteriophage direct terminal repeat ends, one representative bacteriophage from each type of bacteriophage was selected to check for assembly artefacts and downstream analysis. Specifically, Syd1 and Syd8 as the first isolates from their respective sets (indicated by the bacteriophage number) were used as representatives of the 44.3 kb and 45.4 kb bacteriophages respectively, and Syd6 was used as the third type. Each of these three bacteriophage contigs possessing the 125 bp repeats were reordered into pseudomolecules. In each of the bacteriophage pseudomolecules, the 5' and 3' 125 bp repeats were positioned next to each other, and filtered reads were subsequently mapped back onto their respective pseudmolecules.

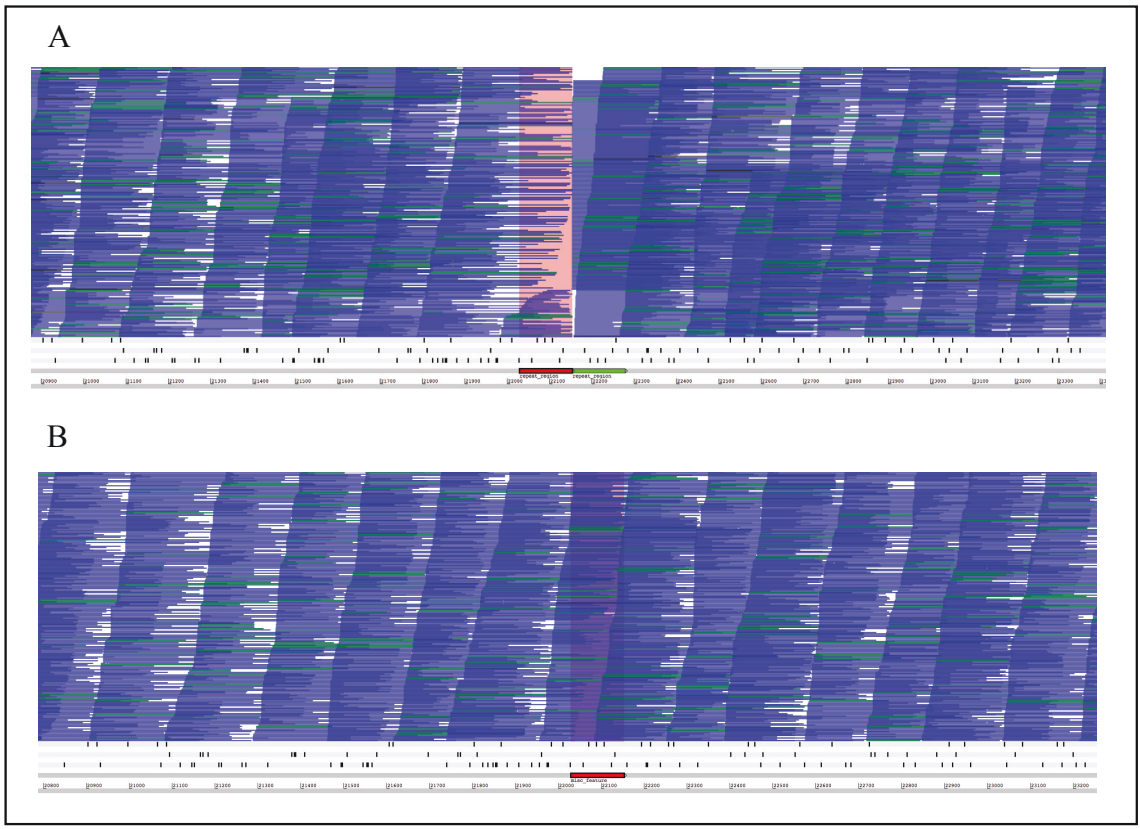

Figure 3. 4 A mapping profile over pseudomolecule. Pseudomolecules of Syd1 assembly was used as a representative and all the other bacteriophage pseudomolecules showed the same result (A) Mapping of psuedomolecule with 5' (in green colour) and 3' (in red colour) 125 bp sequences located next to each other; no reads crossing both 5' 125 and 3' 125 region (B) Mapping of psuedomolecule with only one 125 bp sequence; reads passing through 125 repeat regions. Images were generated using Artemis 16.0.0 (Rutherford *et al.*, 2000)

In all three bacteriophages assemblies, the re-mapping results revealed no reads spanning across either 125 bp terminal repeat (Figure 3.4A), which indicated that this was either an assembly artefact or a linear molecule with bacteriophage direct terminal repeats. To distinguish these possibilities, one of the 125 bp repeats from each of the pseudomolecules was deleted and the reads mapped back onto their respective pseudomolecule. In all three representative bacteriophages, the re-mapping revealed a similar result showing a high percentage of reads mapping to the 125 bp repeat region (Figure 3.4B). Of all the reads that covered the first base of the 125 bp repeats, more than 30% of reads mapped across the entire 125 bp sequence inclusive of flanking sequence (Table 3.2). This confirmed there was only one copy of the 125 bp repeat sequence in each of the representative bacteriophage genomes, indicating that the genomes had circular assembly. This suggested that these genomes were either circularly permuted or have terminal redundancy, in which exact termini sequence was not sequenced. It has been reported that Illumina sequencing techniques, which used Nextera based DNA sample preparation (i.e. transposon mediated adapter ligation), led to the loss of phage termini sequence and thus should not be able to sequence phage terminal sequence (Rihtman *et al.*, 2016, Garneau *et al.*, 2017). Hence, It was concluded that the 125 bp repeat in the assembled bacteriophages were assembly artefacts, and thus one of the 125 bp repeat sequences was removed from all bacteriophage contigs to correct the genome assembly. This process also confirmed that bacteriophages with similar genome size were undeniably the same bacteriophage, showing that the same bacteriophage was isolated and sequenced multiple times. Bacteriophages Syd1, Syd6 and Syd8 were investigated further as the representative of these three different types of bacteriophages.

Table 3. 2: Mapping profiles of the 125 bp repeat sequence in representative pseudomolecules

Mapping description	Syd1	Syd6	Syd8
Total number of reads mapped at 125 bp region	845	1217	3809
No. of mapped reads passing through entire 125 bp region inclusive of flanking	259	378	1148
% of mapped read passing through entire 125 bp region inclusive of flanking	30.65%	31.05%	30.13%

3.2.3 Bacteriophage Syd1, Syd6 and Syd8 each produce different types of plaque morphology

The bacteriophages Syd1, Syd6 and Syd8 propagated efficiently on *E. coli* ST131 strain EC958 and formed clear plaques, typical for virulent bacteriophages. However, the plaques produced by each differed in both their appearance and size (Figure 3.5A-C). Plaques formed from Syd1 infections were 1 - 1.5 mm in size with a halo surrounding the plaque, which showed variability in size (Figure 3.5A). This variation in halo size from Syd1 plaques could be due to diffusion of the bacteriophage in the agar, inactivation of bacteriophage particles by bacterial debris, diffusion of bacteriophage unrelated soluble enzymes, and/or aging of the bacterial lawn (Abedon & Yin, 2009). Infection of *E. coli* ST131 strain EC958 with bacteriophage Syd6 resulted in small pinpoint (0.5 mm) plaques (Figure 3.5B) while infection with Syd8 resulted in large clear plaques of 2 - 2.5 mm in diameter (Figure 3.5C).

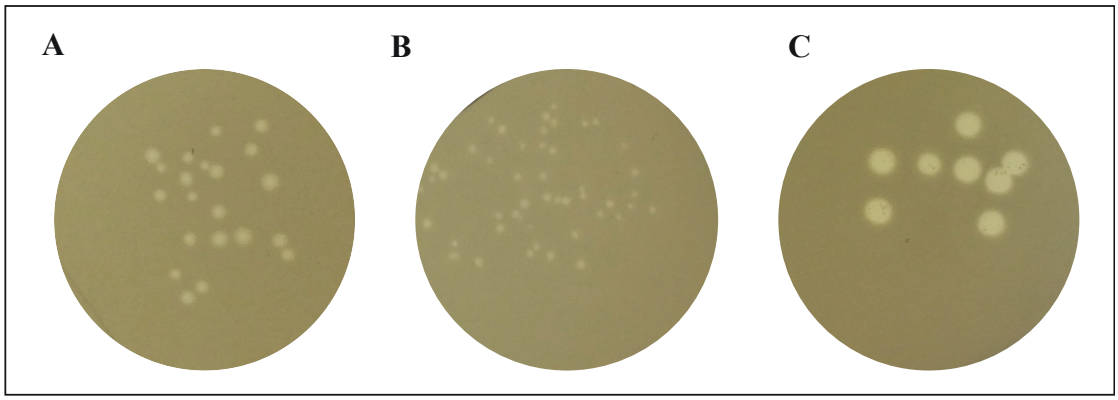

Figure 3. 5: Plaque morphologies of bacteriophages. (A) Syd1, (B) Syd6, and (C) Syd8 as grown on *E. coli* ST131 strain EC958.

3.2.4 Bacteriophage Syd1, Syd6 and Syd8 are tailed bacteriophages with distinct morphologies

Examination by transmission electron microscopy (TEM) revealed that bacteriophages Syd1, Syd6 and Syd8 were each morphologically different, but that each belonged to the order *Caudovirales* (i.e. the tailed phages) (Figure 3.6A-C). Using TEM, multiple images (n =10) for each of the bacteriophages were taken and analysed for their morphotype. Bacteriophage Syd1 was identified as a member of the *Siphoviridae* family. The bacteriophage particle consisted of an icosahedral head of 63 (±1.2) nm and a non-

contractile tail of 116 (±0.5) nm in length. The bacteriophage tail showed transverse striations and possessed a 20 (±0.35) nm wide baseplate with tail spikes at the end of its tail (Figure 3.6A). Bacteriophages Syd6 and Syd8 were both observed to be members of the *Podoviridae* family but with differences in head and tail features. Bacteriophage Syd6 possessed a prolate head of 134 (±2) nm × 60 (±2) nm in dimension with a short tail of 19.32 (±0.2) × 9.5 (±0.3) nm (Figure 3.6B). Bacteriophage Syd8 had an icosahedral head of 57.7 (±1.6) nm and a very short stubby tail of 13.8 (±0.67) nm with appendages, of approximately the same width emanating from the central tail tube (Figure 3.6C). These appendages are likely to be tail spikes, a structure that some Podoviruses possess (Casjens & Molineux, 2012, Nobrega *et al.*, 2018).

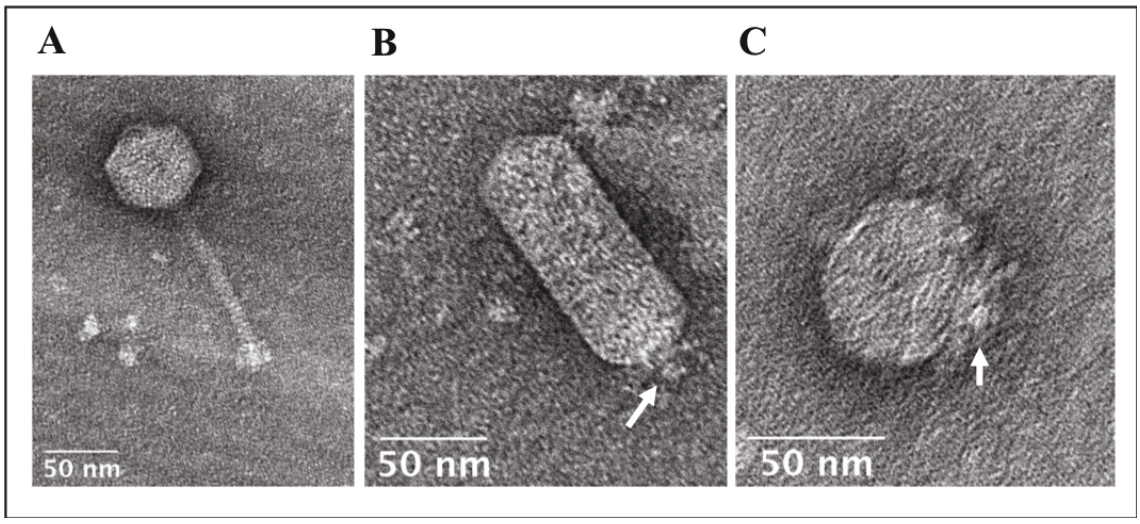

Figure 3. 6: Representative transmission electron micrographs of bacteriophages (A) Syd1, (B) Syd6 and (C) Syd8. Scale bar is 50 nm. White arrow points to the tail of the respective bacteriophages.

Notably, the observed morphologies of each of these three bacteriophages were distinct from bacteriophages previously reported to infect ST131 strains. Three out of four previously reported ST131 infecting bacteriophages, namely HP3, CF2 and EC1 were all Myoviruses, of which HP3 and EC1 consisted of a T4-like head and tail morphology whereas CF2 had a long contractile tail with prolate head (Green *et al.*, 2017). Bacteriophage LM33_P1 on the other hand was predicted to be T7-like Podoviruses by the authors based on genomic analysis (Dufour *et al.*, 2016). These morphological differences indicated that bacteriophage Syd1, Syd6 and Syd8 could represent new groups of ST131-infecting bacteriophages. Based on the morphological data, Syd1, Syd6 and Syd8 were renamed vB_EcoS_Syd1, vB_EcoP_Syd6 and vB_EcoP_Syd8

respectively according to the bacterial virus nomenclature standard (Kropinski *et al.*, 2009) (i.e. vB for the virus of bacteria; Eco for *E. coli*, the host species; S and P for *Siphoviridae* and *Podoviridae*, the virus family; followed by the laboratory name). However, the shorthand of Syd1, Syd6 and Syd8 is used throughout this thesis for simplicity.

3.2.5 The three bacteriophage genomes are distinct from each other and from other *E. coli* ST131 infecting bacteriophages.

BLASTn comparisons between the Syd1, Syd6 and Syd8 genomes revealed that all three bacteriophages were genomically distinct with no significant nucleotide similarities shared between them (Figure 3.7). These genomic comparisons also correlated with the different morphologies observed under the electron microscope (Figure 3.6), confirming that Syd1, Syd6 and Syd8 were each morphologically and genomically distinct from each other. The genomes of the three bacteriophages were also compared to the genomes of published bacteriophages infecting *E. coli* ST131 namely LM33_P1 (GenBank accession number LT594300), HP3 (GenBank accession number KY608965), phiEC1 (GenBank accession number KY608966) and CF2 (GenBank accession number KY608967) (Dufour *et al.*, 2016, Green *et al.*, 2017). BLASTn comparison showed that none of the query bacteriophages shared any significant nucleotide identity to the genomes of bacteriophage Syd1, Syd6 and Syd8 (Figure 3.7), with the exception of a small region (1.5 Kb) of Syd1 with 87% nucleotide identity to LM33_P1. Collectively, all these results implied that Syd1, Syd6 and Syd8 are completely distinct and novel bacteriophages that could infect *E. coli* ST131.

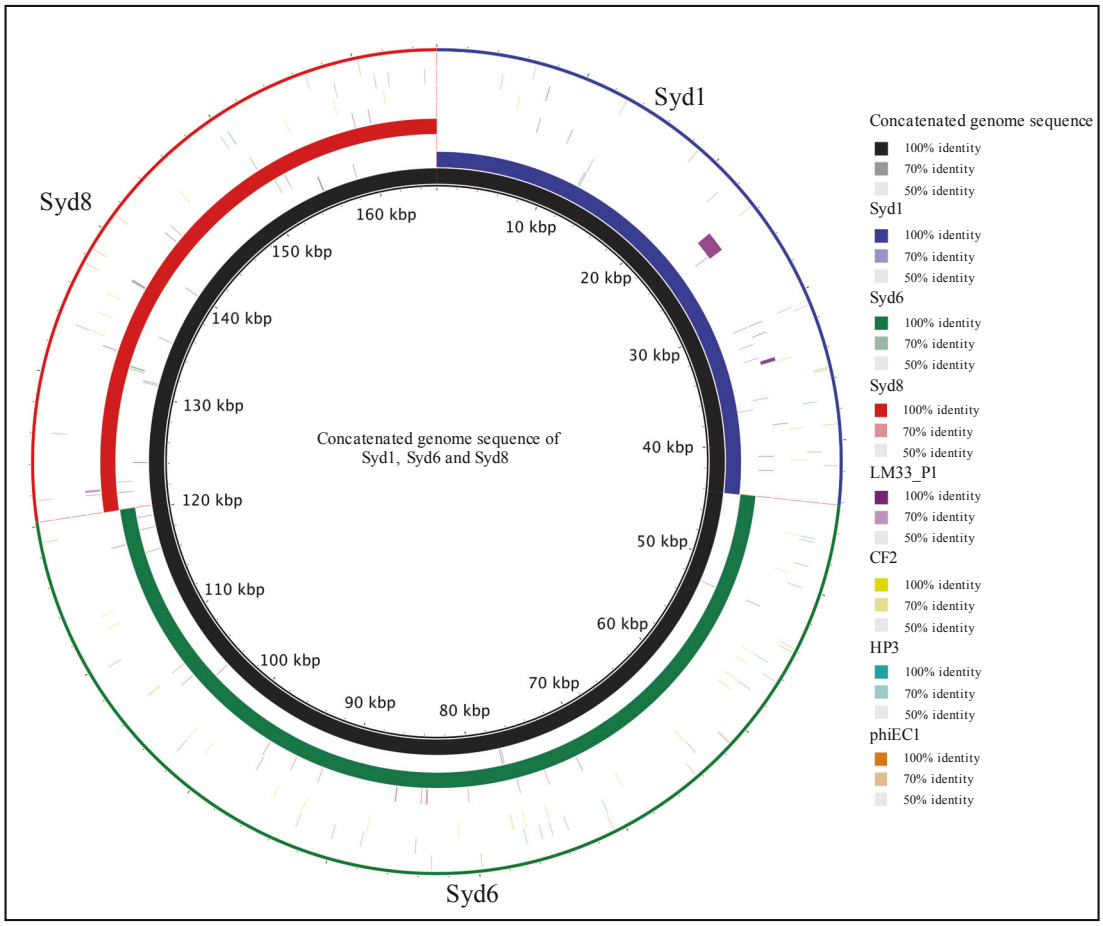

Figure 3. 7: BLASTn comparison of a concatenated genome sequence of Syd1, Syd6 and Syd8 against each other and other bacteriophages which infect *E. coli* ST131 strains. The innermost ring represents the reference sequence, a Multi-FASTA file consisting of Syd1, Syd6 and Syd8. Each ring represents a query genome with coloured region representing BLASTn matches to the reference. Nucleotide identity of BLASTn matches is scaled according to the figure key. Image was generated using BRIG (Alikhan *et al.*, 2011).

3.2.6 Genomic properties of Syd1, Syd6 and Syd8

The genome of bacteriophage Syd1 is 44,180 bp in size with a G+C content of 50.64%, which is close to that of its host *E. coli* (50.8%) (Forde *et al.*, 2014) whereas the Syd6 and Syd8 have genomes of 76,075 bp and 45,268 bp with an overall G+C content of 42.08% and 44.8 % respectively (Table 3.2), lower than the host *E. coli* G+C content. The genome of Syd1 contained 76 predicted coding sequences (CDSs), 52 of which are on the direct strand and 24 on the complementary strand (Figure 3.8A). Bacteriophage Syd6 genome had 125 CDSs and a single tRNA gene, 25 of which are on the direct strand and 100, including the tRNA, on the complementary strand (Figure 3.8B). The genome of Syd8 consisted of 52 CDSs, all of which are on the direct strand (Figure 3.8C). The majority of the CDSs in each of the three bacteriophages utilized ATG as the start codon (89.47%,

90.4% and 94.23% in Syd1, Syd6 and Syd8 respectively) with a small proportion of CDSs starting with either GTG or TTG. Only one CDS in Syd6 utilised TTG whereas Syd8 did not utilise TTG as a start site. All the three stop codons were utilised by each of the bacteriophages, with the majority being TAA (44.73%, 60.8% and 65.38% in Syd1, Syd6 and Syd8 respectively), followed by TGA (42.10%, 28.8 % and 21.15% in Syd1, Syd6 and Syd8 respectively).

Searching potential *E. coli* (host) promoter sequence within these genomes revealed each bacteriophage possessed promoter sequences similar to *E. coli*. Five, six and two potential *E. coli* promoters were identified within the genome of Syd1, Syd6 and Syd8 respectively (a list of all the predicted promoters and terminators in the genomes of Syd1, Syd6 and Syd8 is provided in Appendix 1, 2 and 3 respectively). Identifying *E. coli*-like promoters in each of these bacteriophages suggested that these could be bacteriophage early promoters, potentially used by *E. coli* to transcribe and translate the early genes of the bacteriophages. A summary of the features of each of the bacteriophage genomes is listed in Table 3.3, and the predicted functional annotations describing the properties of individual CDSs are provided in Appendix 4, 5, and 6 respectively.

Table 3. 3: General features of the Syd1, Syd6 and Syd8 genomes.

Properties	Syd1	Syd6	Syd8
Genome	ds DNA	ds DNA	ds DNA
Size of genome	44,180 bp	76,075 bp	45,268 bp
G+C content	50.64%	42.08%	44.8%
Number of CDSs	76	125	52
Number of tRNA	-	1	-
Number of Functionally assigned proteins	30	32	24
Number of regulatory promoters	5	6	2
Number of Rho-independent terminators	13	15	7

The BLASTn comparison of each of the bacteriophage genomes to the virus genomes (taxid: 10239) from NCBI non-reductant nucleotide (nr/nt) database revealed that each bacteriophage was similar to groups of bacteriophages previously studied by others. An in-depth genomic analysis of each of the bacteriophages with comparisons to these closely related bacteriophages is described further in the sections below.

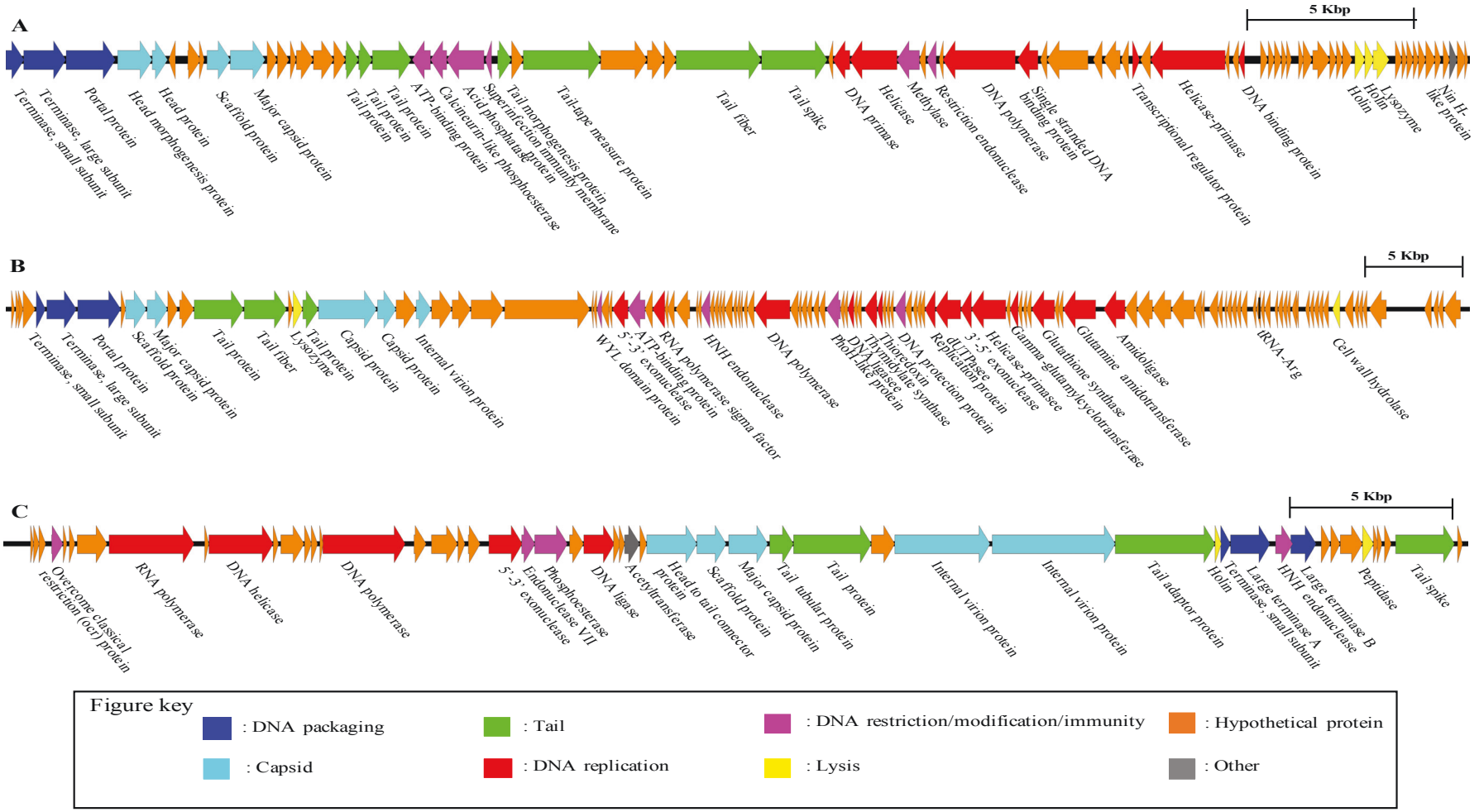

Figure 3. 8: Linear representation of (A) Syd1, (B)Syd6, and (C) Syd8 genome. CDSs are colour-coded according to function listed within the figure key. Direction of arrows represents transcriptional direction. Images were generated using Easyfig 2.1(Sullivan *et al.*, 2011).

3.2.7 Bacteriophage Syd1 is genetically similar to members of "Jerseyvirinae"

BLASTn of the genome of bacteriophage Syd1 against the non-redundant/nucleotide (nr/nt) database of NCBI revealed that it had high levels of nucleotide identity with the bacteriophage subfamily Jerseyvirinae. The closest match resembled K1ind2 and K1ind3, with 86 % nucleotide identity over 73 % of query length (the list of all other closely related bacteriophages with nucleotide identity over Syd1 genome is in Appendix 7). Jerseyvirinae is a subfamily of *Siphoviridae*, which includes strictly lytic bacteriophages with Jersey-type morphotype (a distinct Siphoviruses morphotype that consist of a tail-terminal disk-like structure with six club-shaped spikes) and which have similar genomic organization and a high degree of synteny (Anany *et al.*, 2015). However, based on whole genome sequence alignments, genome organization, protein homology, and phylogenetic analysis, this subfamily formed three distinct genera: Jerseylikevirus, K1glikevirus and Sp3unalikevirus (Anany *et al.*, 2015). Members of this subfamily have been documented to infect either *Salmonella species* or *E coli*. Bacteriophage Syd1 was identified as a member of K1glikevirus, which consists of *Escherichia* bacteriophages K1G, KIH, K1ind1, K1ind2 and K1ind3, that share more than 79 % nucleotide identity with each other over their entire length (Anany *et al.*, 2015).

The comparative genomic analysis of Syd1 with the closely related Jerseyvirinae bacteriophages revealed that, like all the other Jerseyvirinae bacteriophages, the Syd1 genome had a syntenic gene order and a similar modular organization with four distinct functional gene clusters (2 on the direct strand and 2 on the complementary strand) (Figure 3.8A). All CDSs on the complementary strand except Syd1_060 encoded replication, regulation and immunity functions, whereas all CDSs on the direct strand, except Syd1_0460, encoded structural (packaging, morphogenesis, head and tail); and lysis module functions (Figure 3.8A).

The arrangement of structural module genes in bacteriophage Syd1 is in accordance with other Siphoviruses with packaging genes followed by the head genes 5' to the tail genes (Hatfull, 2008). However, like all the other Jerseyvirinae bacteriophages, the structural module is interrupted with a cluster of CDSs oriented in the opposite direction, which comprise a regulation/immunity module (Turner *et al.*, 2012, Anany *et al.*, 2015). All the

structural genes except genes encoding a hypothetical protein (Syd1_0270), a tail fibre (Syd1_0290) and the tail spike (Syd1_0310) showed high similarity (≥ 70 % nucleotide identity over entire gene) with their respective counterparts from other Jerseyvirinae bacteriophages (Figure 3.10). These similarities within structural module genes of Syd1 with other Jerseyvirinae bacteriophages also correlated with its similar Jersey-type morphotype observed under the electron microscope (Figure 3.2A), confirming Syd1 as a Jerseyvirinae bacteriophage.

The replication module in Syd1 is comprised of 21 CDSs with nine functionally characterized genes that play an important role in the bacteriophage replication mechanism. The major genes identified in the genome of Syd1 were genes encoding a primase (Syd1_0330), a helicase (Syd1_0340), a DNA cytosine methylase (Syd1_0350), a restriction endonuclease (Syd1_0370), a DNA polymerase (Syd1_0390) and a helicase-primase (Syd1_0480). All of these genes were well conserved within members of Jerseyvirinae bacteriophages except the genes encoding the DNA primase and the genes encoding the DNA cytosine methylase (Figure 3.10). It has been previously reported that within the Jerseyvirinae subfamily, the bacteriophages belonging to the *E. coli* infecting K1glikevirus each consisted of an additional gene encoding a C-5 cytosine-specific methylase within their core replication genes (Anany *et al.*, 2015). Notably, this was not present in other bacteriophages from this subfamily with the exception of *Salmonella* bacteriophage Jersey (GenBank accession number KF148055) and FSLSP-101 (GenBank accession number KC139511) (Anany *et al.*, 2015). Bacteriophage borne methylases have been previously shown to protect the bacteriophage genome post infection from destruction by host restriction enzymes enabling its successful multiplication within the targeted host bacteria (Hattman, 1977, Murphy *et al.*, 2014), and it is plausible that this is a defence strategy employed by the K1glikevirues to evade host defences.

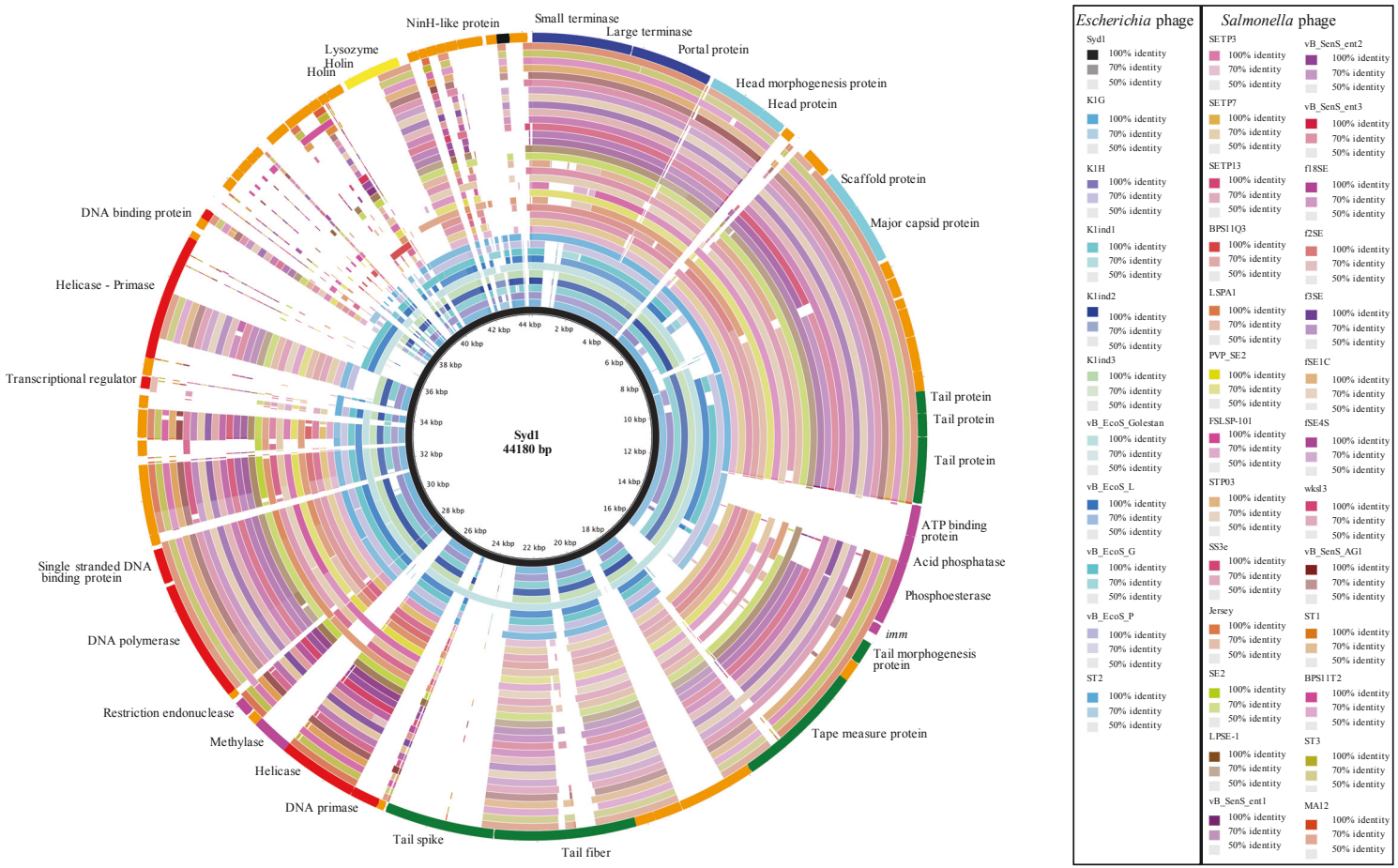

Figure 3. 9: BLASTn comparison of Syd1 against the Jerseyvirinae subfamily. The genome of Syd1 is represented in a circular form in the centre. Each coloured ring represents a different query Jerseyvirinae genome. Coloured regions represent BLASTn matches to the reference and percentage identity is scored according to the figure key. The outermost ring is consisted of the CDSs encoded by Syd1 and colour coded as per figure 3.8. Image was generated using BRIG (Alikhan *et al.*, 2011).

The gene encoding DNA cytosine methylase of Syd1 was located at the analogous location as in other K1glikevirus but did not show any nucleotide identity with them (Figure 3.9). Instead it showed high nucleotide identity (≥90%) with the gene encoding a methylase from *Salmonella* phage Jersey (Locus tag: N271_gp37) and FSL SP-101 (Locus tag: FD193_gp32). As expected, the encoded protein has a high homology with the methylase of these two bacteriophages (93% amino acid identity over 100%). The DNA primase, which was identified due to the presence of a conserved TOPRIM domain (cd01029) and its distant relation to DNA primase of T5 (Locus tag: AAX12008) (Wang *et al.*, 2005) (48% amino acid identity over 94%), was not present within any of the closely related bacteriophages except vB_EcoS_Golestan (GenBank accession number MG099933). The presence of a primase and helicase in the replication unit of Syd1 suggests that Syd1 probably has a T4 and T5 like DNA replication mechanism. Bacteriophage T4 and T5 follow a coordinated leading and lagging strand DNA replication mechanism that utilizes a primosome complex comprised of a primase and helicase to synthesize primers on the lagging strand (Ilyina *et al.*, 1992, Jing *et al.*, 1999, Noble *et al.*, 2015).

In addition to the aforementioned core replication genes, one single stranded DNA binding protein (SSB) encoding gene (Syd1_0400) and two helix-turn-helix sequence-specific DNA binding proteins encoding genes (Syd1_0460 and Syd1_0510) were also identified within the replication module genes. Syd1_0460 has a XRE-family like Helix-turn-Helix domain (PF01381) with a transcriptional direction opposite to that of replication module and is flanked by a regulatory promoter upstream and a putative rho-independent terminator downstream. This suggests that the Syd1_0460 encoded protein could be a bacteriophage transcriptional regulator. In bacteriophages, transcriptional regulators have been reported to regulate expression of the bacteriophages immediate early genes, with delayed early and late gene expression (Yang *et al.*, 2014)

Like the other Jerseyvirinae bacteriophages, Syd1 also contains a cluster of early genes (Syd1_0200 to Syd1_0230) responsible for regulation and immunity functions (Anany *et al.*, 2015). However, among these four genes, only the genes encoding a calcineurin-like phosphoesterase (syd1_0220) and a superinfection immunity membrane protein encoding *imm* gene (syd1_0230) were well conserved among most of the Jerseyvirinae bacteriophages (Figure 3.9). The gene encoding a predicted superinfection immunity

membrane protein consists of a conserved domain from the PF14373 family and exhibits distant relationship with the T4 superinfection immunity membrane protein (Locus tag: T4045) (28% amino acid identity over 84%) (Miller *et al.*, 2003). Like the T4 *imm* protein, the Syd1 superinfection immunity protein was also predicted to localize to the cytoplasmic membrane, as it consists of two transmembrane domains with both N and C terminal non-cytoplasmic domains. The superinfection immunity protein in bacteriophageT4 protects the host from further infection of the same bacteriophage type and thus prevent bacteriophages from superinfecting a single cell (Lu & Henning, 1989).

Strikingly, Syd1_0200 and Syd1_0210 were only found within K1glikevirus that infect *E. coli* (Figure 3.10). Syd1_0200 and Syd1_0210 encode an ATP-binding protein and an acid phosphatase respectively and show distant relationship to DNA metabolism genes from *E. coli* phage T1 (GenBank accession number: AY216660) (Roberts *et al.*, 2004). The Syd1_0200 encoded ATP-binding protein has 38% amino acid identity over 97% of T1 putative Kinase (T1p62) and the Syd1_0210 encoded acid phosphatase showed 47% amino acid identity over 98% of the T1 polynucleotide kinase/phosphatase (T1p64). Additionally, the Syd1_0200 encoded ATP-binding protein contains a protein motif for a phosphate binding NTPase fold (IPR027417) that could function as a protein kinase. As such the protein kinase (Syd1_0200) together with the phosphatase (Syd1_0210) in the Syd1 genome would function as regulatory proteins. Indeed, the process of phosphorylation and dephosphorylation carried out by protein kinases and protein phosphatases is the most common regulatory system controlling the regulation of protein interactions in prokaryotes and eukaryotes (Cheng *et al.*, 2011). Protein kinases modify the target protein by phosphorylation, whereas protein phosphatases remove a phosphate group from the target protein, resetting protein function. Conservation of these tandem genes within the regulatory/immunity module gene cluster of Jerseyvirinae bacteriophages that infects *E. coli,* suggested that these could regulate the metabolism of the infected *E. coli* host by modifying their proteins to optimise bacteriophage production (Gone & Nicholson, 2012).

The host lysis module of Syd1 encodes a holin-lysin system comprised of two holins (Syd1_0630 and Syd1_0640) followed by phage lysozyme (Syd1_0650). Syd1_0630 encodes a predicted 102 amino acid sized protein with 3 transmembrane domains, which includes a N-terminal periplasmic domain and a C-terminal cytoplasmic domain, a

feature similar to the S holin of bacteriophage lambda (Grundling *et al.*, 2000). Syd1_0640 encodes a predicted 90 amino acid sized protein that contains 2 transmembrane domains. Both holin-encoding genes show significant nucleotide identity (≥ 80% identity over 100%) with their counterparts from all the *Escherichia* phages from Jerseyvirinae, whereas none of the *Salmonella* phage from the group except LSPA1 (GenBank accession number KM272358) had any nucleotide sequence identity with these holins (Figure 3.10). In contrast to these significant differences between holins of *Escherichia* and *Salmonella* phages from this subfamily, the lysin (i.e. phage lysozyme) encoding gene showed high levels of nucleotide identity (≥ 70% nucleotide identity), and the predicted protein had more than 75% amino acid identity over 98% of its length. More importantly, the lysin of all these bacteriophages have a similar glycoside hydrolase (PF00959) functional domain that can act on the glycosidic bond (between N-acetylmuramic acid and N-acetylglucosamine) linking the amino sugar in the bacterial peptidoglycan (Tsugita & Inouye, 1968). This suggests that all of the Jerseyvirinae bacteriophages, including Syd1, could utilise a common mechanism of murein lysis of the host cells.

3.2.8 Bacteriophage Syd6 is genetically similar to phiEco32-like bacteriophages

Bacteriophage Syd6 showed high homology and is genetically similar to phiEco32-like bacteriophages, with 97% nucleotide identity over 93% of the reference bacteriophage phiEco32 genome (GenBank accession number: EU330206) (Savalia *et al.*, 2008) (a list of all other closely related phiEco32 like bacteriophages with nucleotide identity over Syd6 genome is in Appendix 8). BLASTn pairwise alignment of Syd6 with phiEco32 revealed a similar genomic organization with the three temporal classes of genes (i.e. early genes, middle gene and late genes) (Pavlova *et al.*, 2012) (Figure 3.11). The distinct early, middle and late temporal groups of genes are common in bacteriophage genomes, which typically expresses sequentially over the bacteriophage infection cycle (Yang *et al.*, 2014). In phiEco32, the middle gene cluster encode proteins involved in bacteriophage DNA replication and modification, and the late gene products code for structural and packaging proteins. However, most of the early gene products are hypothetical proteins that have not been functionally identified yet (Savalia *et al.*, 2008, Pavlova *et al.*, 2012).

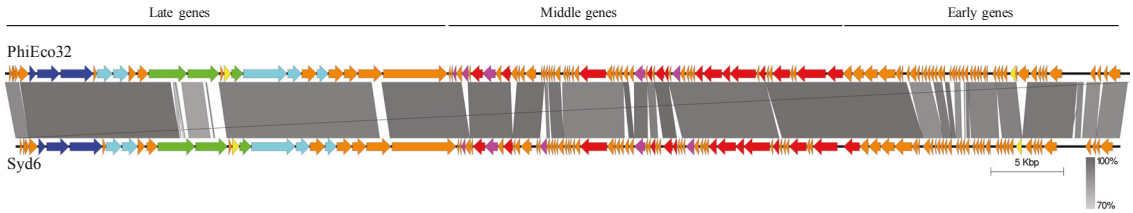

Figure 3. 10: Pairwise BLASTn comparison of Syd6 with reference bacteriophage phiEco32. Each arrow represents the CDS of each bacteriophage and their transcriptional direction and colour coded as per figure 3.8. Nucleotide identity between genomes is represented by grey shading between them and scaled according to the grey bar. Image was created using Easyfig 2.1(Sullivan *et al.*, 2011)

Based on the comparison with the genome of bacteriophage phiEco32, modules for bacteriophage structure and packaging, nucleotide metabolism and replication, and host cell lysis were identified within Syd6 genome. The structural gene module of Syd6 consisted of 25 CDSs, all of which showed more than 90% amino acid identity to respective phiEco32 proteins except the Syd6_0140 encoded tail fibre and Syd_0240 encoded hypothetical bacteriophage protein (Appendix 9). Indeed, all of these structural genes were well conserved among all members of the phiEco32 like bacteriophages and shared more than 90 % of nucleotide identity (Figure 3.11). In addition, the gene which encoded the bacteriophage lysozyme (Syd6_0160) was also present within the cluster of structural module genes. This high level of nucleotide identity across the entire structural module genes among these bacteriophages was further corelated with the similar bacteriophage morphology observed for Syd6, phiEco32 and vB_EcoP_SU10 (Savalia *et al.*, 2008, Khan Mirzaei *et al.*, 2014). This result clearly implies that all of these bacteriophages are morphologically and genomically similar to each other and thus could belong to an interbreeding bacteriophage group.

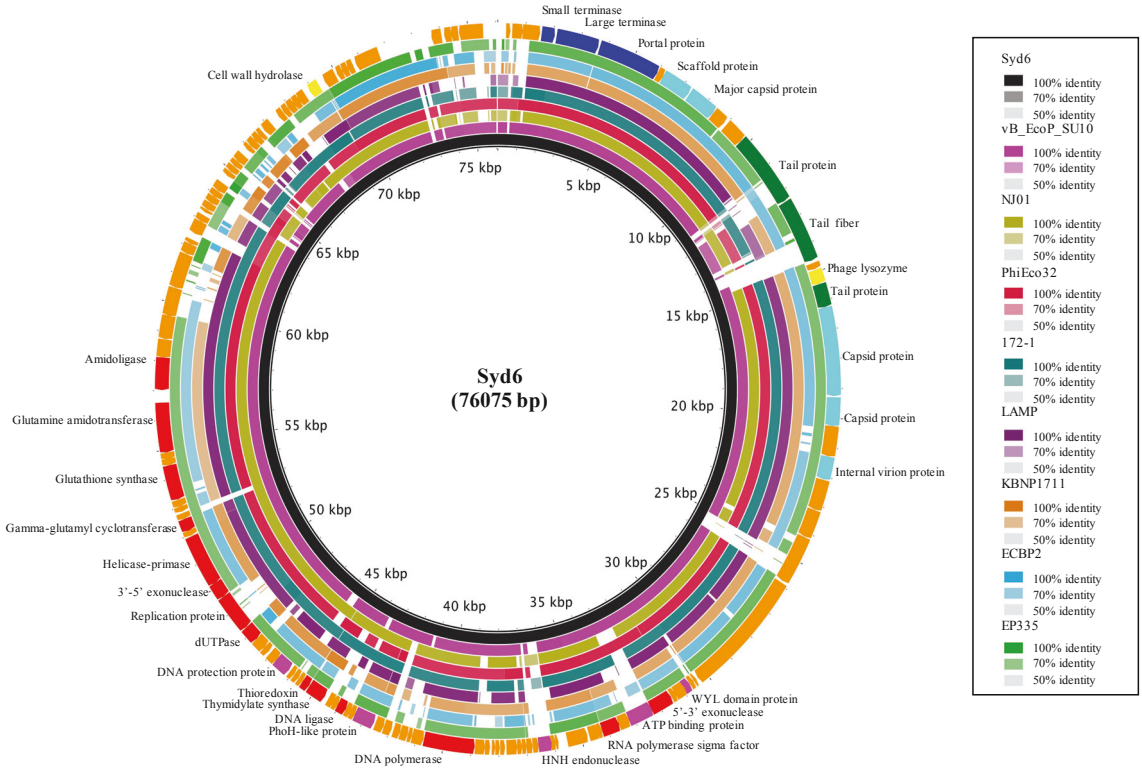

Figure 3. 11: BLASTn comparison of vB_EcoS_Syd6 against the genomes of PhiEco32-like bacteriophages. The innermost ring represents the reference sequence, vB_EcoS_Syd6 genome and the outermost ring represents the CDSs of it. Other coloured rings represent each query genome, and coloured region represent BLASTn matches with the reference. Percentage identity of the coloured regions is listed in Figure key. Image was generated using BRIG (Alikhan *et al.*, 2011).

Genes that were functionally predicted to be involved in DNA replication are genes that encode a 5'-3' exonuclease (Syd6_0310), a RNA polymerase sigma factor (Syd6_0340), a DNA polymerase (Syd6_0510), a DNA ligase (Syd6_0610), a 3'-5' exonuclease (Syd6_0760) and a helicase-primase (Syd6_0770), all of which were well conserved among all the phiEco32 like bacteriophages (Figure 3.11). Notably, the RNA polymerase sigma factor (sigma70-ECF) of Syd6 exhibited 100% amino acid identity with phiEco32 RNA polymerase ECF sigma factor, suggesting that both Syd6 and phieco32 utilized similar bacteriophage promoters. Furthermore, with the aid of a conserved consensus element tAATGTAtA of bacteriophage phiEco32 promoters (Pavlova *et al.*, 2012), six similar bacteriophage promoters were identified within the Syd6 genome (Appendix 2).

Additionally, seven CDSs encoding enzymes for nucleotide metabolism, thymidylate synthase (Syd6_0640), thioredoxin (Syd6_0650), dUTPase (Syd6_0740), gamma glutamyl cyclotransferase (Syd6_0790), glutathione synthase (Syd6_0830), glutamine amidotransferase (Syd6_0860) and amidoligase (Syd6_0870), were predicted within the

Syd6 genome. All of these genes were also well conserved within all the phiEco32 like bacteriophages (Figure 3.11), suggesting that they are core genes for bacteriophage replication mechanisms. Thioredoxin and dUTPase are well known for their role in DNA replication. Thioredoxin plays a role in stabilizing the DNA polymerase that increase its processivity (Huber *et al.*, 1987, Tabor *et al.*, 1987) whereas dUTPase removes dUTP from the deoxynucleotide pool, and reduces incorporation of dUTP into DNA during replication (Chen *et al.*, 2002). Additionally, dUTPase also produces the dTTP precursor dUMP via the thymidylate synthase pathway (Hirmondo *et al.*, 2017). Hence, it is likely that Syd6_0640 encoded thymidylate synthase could be responsible for the conversion of dUMP to dTMP. Moreover, Syd6_0790 encoded gamma glutamyl cyclotransferase and Syd6_0830 encoded glutathione synthetase could play roles in the synthesis and metabolism of glutathione, which would act as a ubiquitous reducing agent in reductive mechanisms involved in DNA and protein synthesis. Likewise, the Syd6_0860 encoded glutamine amidotransferase and the Syd6_0870 encoded amidoligase enzymes could aid *de novo* synthesis of purine nucleotides and play a role in nucleotide metabolism.

Five genes for DNA modification/restriction were also located within the early and middle gene cluster of DNA replication and metabolism genes. These include genes encoding for a WYL domain (PF13280) containing DNA binding protein (Syd6_0280), ATP-binding protein (Syd6_0320), HNH homing endonuclease (Syd6_0400), phoH-like protein (Syd6_0580) and a DNA protection protein (Syd6_0690) consisting of a ferritin like DNA binding protein motif from the DNA protecting protein family (cd01043). All of these genes were conserved among all the closely related bacteriophages, with the exception of the absence of HNH endonuclease in KBNP1711 (GenBank accession number: KF981730). Notably, the HNH endonucleases did not share any nucleotide identity with each other and were located at different positions among these bacteriophages, with the exception of *Escherichia* phage 172-1 (GenBank accession number: KP308307) and NJ01 (GenBank accession number: JX867715). In bacteriophage 172-1 and bacteriophage NJ01 the HNH endonuclease were located at the same position as to Syd6 and also showed more than 70 % nucleotide identity with it. Homing endonucleases are selfish freestanding mobile elements, common in bacteriophage genomes but do not provide any obvious selective advantage to their host genome and can move independently (Belle *et al.*, 2002).

The pairwise comparative genomic analysis of the gene content and positioning of genes from Syd6 in reference to bacteriophage phiEco32 showed the insertion and deletions of 16 coding sequences within these two bacteriophage genomes (Figure 3.10; Appendix 9). Seven CDSs present in Syd6, predicted to encode hypothetical proteins (Syd6_0560, Syd6_0600, Syd6_0630, Syd6_0660, Syd6_0670, Syd6_0800 and Syd6_01120), were not present in the phiEco32 genome (Figure 3.11). However, all of these genes were found to be highly similar (more than 97% nucleotide identity over entire length) to genes from different phiEco32 like bacteriophages. Likewise, nine genes of phiEco32 (Phi32_37, Phi32_59, Phi32_91, Phi32_97, Phi32_100, Phi32_101, Phi32_102, Phi32_118 and Phi32_119) were not present in the Syd6 genome (Appendix 9) but were found within other bacteriophages from this group. A similar phenomenon of insertions and deletions of multiple hypothetical bacteriophage genes was also observed among other phiEco32 like bacteriophages. All these results suggest the occurrence of horizontal gene transfer within this bacteriophage group. Horizontal gene transfer has been reported among bacteriophage populations and bacteriophages may evolve through exchange of a given gene or set of genes for other functionally equivalent gene/s from bacteriophages in the same interbreeding group (Botstein, 1980, Brussow & Desiere, 2001).

3.2.9 Bacteriophage Syd8 is genetically similar to SP6-like bacteriophages.

Bacteriophage Syd8 showed high homology and is genetically similar to the Sp6-like bacteriophages from the *Podoviridae* subfamily "Autographivirinae". When the genome of Syd8 was compared to the genome of the reference bacteriophage SP6 (GenBank accession number: AY288927) (Dobbins *et al.*, 2004), Syd8 shared 74 % nucleotide identity over 79 % of the reference bacteriophage. When compared to the rest of the SP6-like bacteriophages, the genome of Syd8 most closely resembled vB_CroP_Crp3 with 87% nucleotide identity over 86% of query length (the list of all other closely related bacteriophages with nucleotide identity over Syd8 genome is in Appendix 10). The pairwise nucleotide sequence alignment of Syd8 with all the other closely related bacteriophages showed the same modular organization with similar gene order among all of these bacteriophages. A representative of the similar gene order in Syd8 with respect to the reference *Salmonella* phage SP6 and *Escherichia* phage K1E (GenBank accession number AM084415) is shown in Figure 3.13.

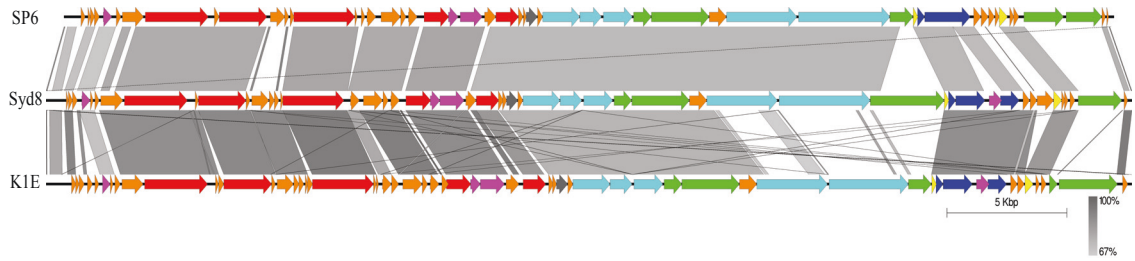

Figure 3. 12: Pairwise BLASTn comparison of Syd8 with reference bacteriophage SP6 and K1E. Each arrow represents the CDS of each bacteriophage and their transcriptional direction, and colour coded as per figure 3.8. Nucleotide identity between genomes is represented by grey shading between them and scaled according to the grey bar. Figure was created using Easyfig 2.1(Sullivan *et al.*, 2011)

Based on comparison with the genome of reference bacteriophage SP6, modules for bacteriophage nucleotide metabolism and replication, structure and packaging and host cell lysis were identified within Syd8 genome. Genes that were functionally predicted to be involved in DNA replication are the DNA-dependent RNA polymerase (Syd8_080), DNA helicase (Syd8_0100), DNA polymerase (Syd8_0160), 5'-3' exonuclease (Syd8_0210), endonuclease VII (Syd8_0220), phosphoesterase (Syd8_0230) and DNA ligase (Syd8_0250). All of these replication genes encode proteins that show more than 80% amino acid identity to their counterparts from SP6 (Appendix 11) and are well conserved among all the closely related bacteriophage (Figure 3.14). Likewise, all the identified structural module genes encoded proteins except the tail adaptor protein (Syd8_0380) and the tail spike (Syd8_0510) show high homology having 70 to 85% amino acid identity with their respective counterparts from SP6 (Appendix 11) and are also conserved among all the closely related bacteriophages (Figure 3.14). However, the internal virion proteins encoding genes (Syd8_0360 and Syd8_0370) of Syd8 show higher nucleotide identity with the respective genes from *Salmonella*, *Citrobacter* and *Proteus* phage than the closely related *Escherichia* phages (Figure 3.14), suggesting that Syd8 may have evolved from bacteriophages that might not have initially infected *E. coli*.

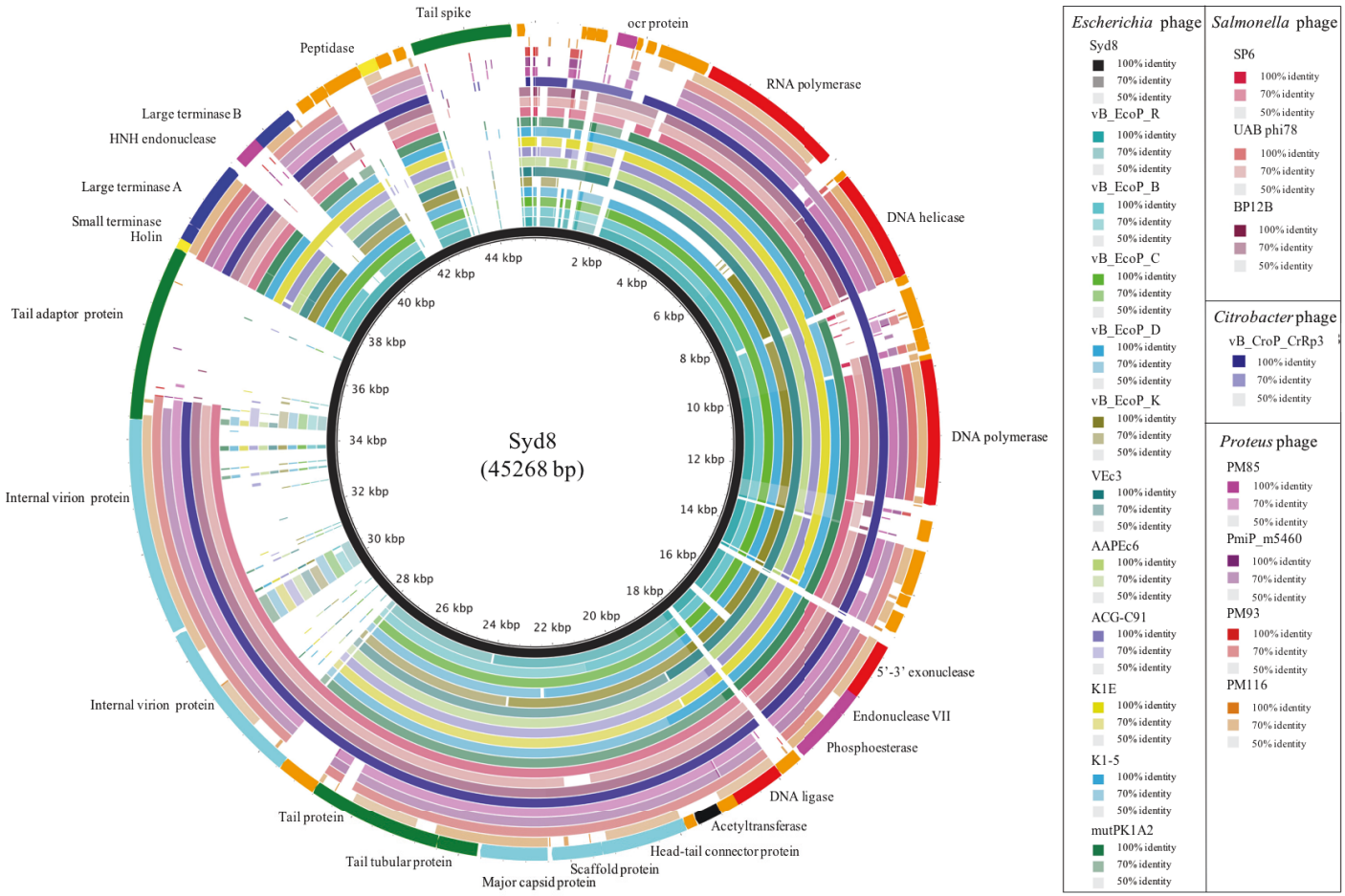

Figure 3. 13: BLASTn comparison of Syd8 against all the closely related bacteriophages. The innermost ring represents the reference sequence, vB_EcoS_Syd8 genome and the outermost ring represents the CDSs of it. Other coloured rings represent each query genome, and coloured region represent BLASTn matches with the reference. Percentage identity of the coloured regions is listed in Figure key. Image was generated using BRIG (Alikhan *et al.*, 2011)

In contrast to SP6-like bacteriophages, where the genomes encode two tail spike protein genes and a tail adaptor protein for binding of these two sets of tail spike proteins (Stummeyer *et al.*, 2006), Syd8 has only one predicted tail spike protein and a homologous tail adapter protein (Figure 3.15).

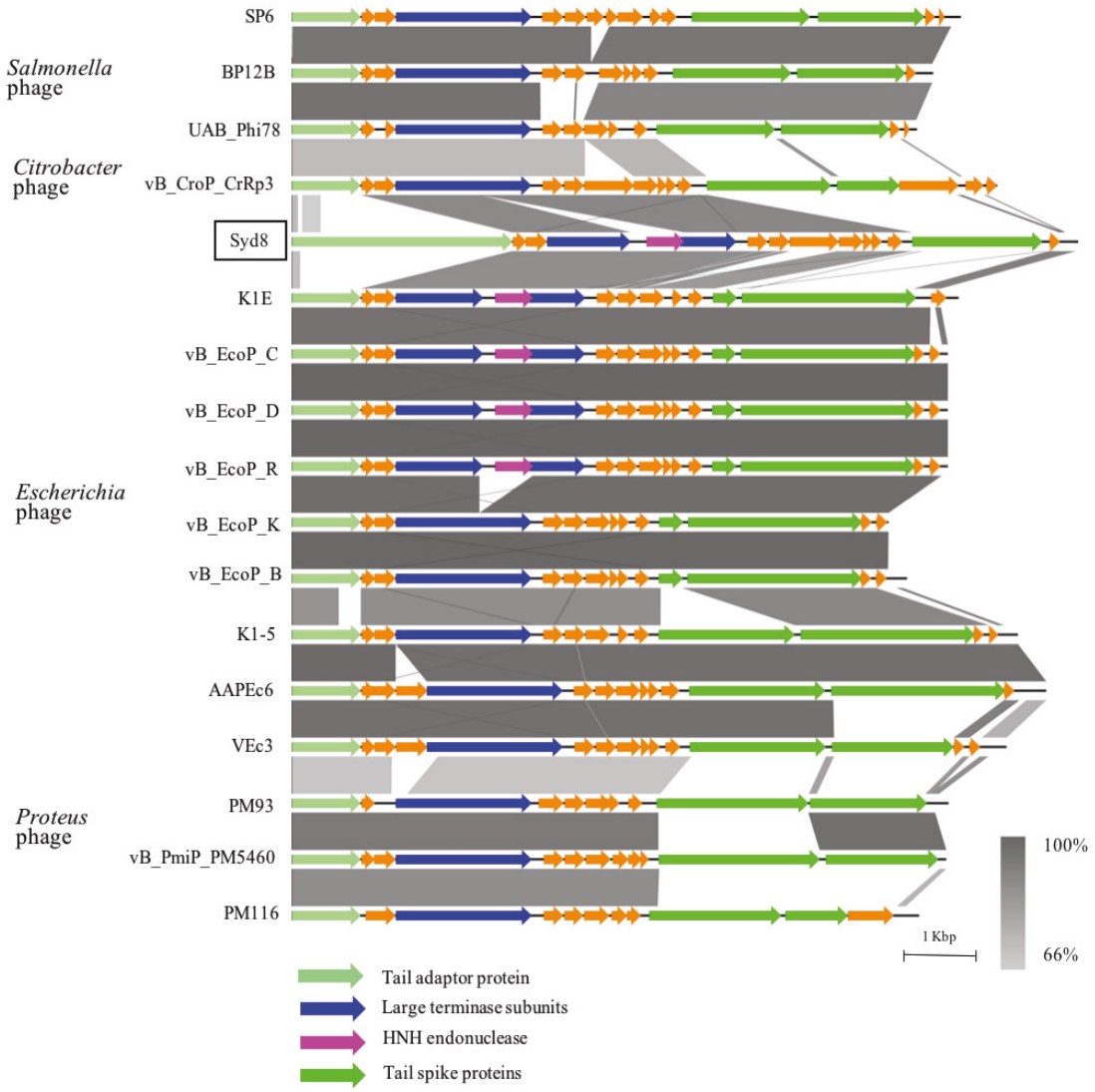

Figure 3. 14: Pairwise BLASTn comparison of genomic region from tail adaptor to the end of Syd8 with SP6-like bacteriophages. Each arrow represents individual CDS and direction indicate transcriptional direction. Light green colour shows the tail adaptor protein, Dark green colour indicates the tail spike protein, blue colour indicates large terminase subunits of the respective bacteriophages and the pink colour shows HNH endonuclease. The nucleotide identity between genomes is represented by grey shading between them and scaled according to the grey bar. Figure was created using Easyfig 2.1(Sullivan *et al.*, 2011).

The homology of the Syd8 tail adaptor protein with the SP6 like tail adaptor protein is however limited to the N-terminal region that contains a protein motif for a bacteriophage T7 tail fibre protein (PF03906). Notably, the Syd8 tail adaptor gene (3081 bp) is larger than its counterpart from SP6-like bacteriophages (960 bp) and has a C-terminal

intramolecular chaperone auto-processing domain (IPR030392). The intramolecular chaperone auto-processing domain in the C-terminal of the endosialidase tail spikes of K1 and K1-5 catalyses trimerization-dependent auto-proteolysis, and thus self-release after folding and assembly of the mature protein, which is essential to stabilize the final structure of the protein (Schwarzer *et al.*, 2007). Hence, it is likely that the tail adaptor protein in Syd8 is processed after cleavage of the chaperone auto-processing domain, which allows the binding of the tail spike to it.

The packaging module genes of bacteriophage Syd8 show an insertion of 746 bp segment within a large terminase gene splitting it into two subunits (Syd8_0410 and Syd8_0430), when compared to reference bacteriophage SP6. However, BLASTn comparison with bacteriophage K1E showed that this insertion was also present in the genome of bacteriophage K1E (Figure 3.13). When this 746 bp insertion sequence was further examined, an intron region followed by a CDS encoding homing endonuclease (Syd8_0420) containing a C–terminal DNA binding protein motif (IPR011991) and N - terminal catalytic domains of GIY-YIG family (cd10448) was identified. This suggested that this fragment is likely to be a self-splicing group I intron. To determine if the removal of this 746 bp region occurred via splicing, filtered reads were mapped onto the genome of Syd8 and mapping over the large terminase was investigated. No reads were observed to be truncated at both the 5’ and 3’ end of the insertion (Figure 3.15), which indicated that there was no subpopulation of bacteriophages that had possessed an excision of this insertion. This in turn suggested that it was likely that the region was spliced out at the mRNA level. Notably, the same self-splicing group I intron was observed in packaging module of a set of SP6 like *Escherichia* phages namely vB_EcoP_C, vB_EcoP_D and vB_EcoP_R including K1E and Syd8 but not in any of other bacteriophages in the comparison group (Figure 3.14).

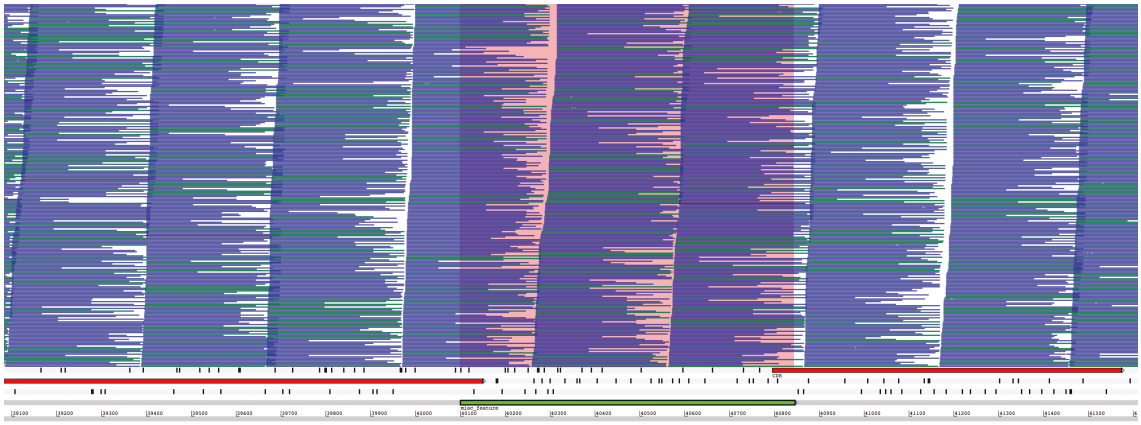

Figure 3. 15: A mapping profile over Syd8 terminase region. Mapping profile is localised to the region consisting of the terminase (in red frame) and the intron (in green) Image was generated by using Artemis 16.0.0 (Rutherford *et al.*, 2000)

3.2.10 The three representative bacteriophages each possess distinct host recognition proteins

The genomic analyses above revealed that all three representative bacteriophages were strictly virulent bacteriophages, devoid of any lysogeny module genes or their homologues. In addition, none of these bacteriophages possessed any bacterial virulence genes or antibiotic resistance genes, suggesting that they could be suitable for therapeutic purposes. More importantly, the aforementioned results from the genomic comparisons showed that each of the three bacteriophages possesses a distinct host recognition gene (Figure 3.9), and also had nucleotide dissimilarity from their closely related bacteriophages (Figures 3.9, 3.11 and 3.13). On closer inspection of the respective host recognition genes of each of these bacteriophages, it was revealed that in the genome of Syd1(Syd1_0310) and in the genome of Syd6 (Syd6_0140) the difference was limited to the C-terminal end in their respective related bacteriophage groups. Indeed, both bacteriophages had highly conserved N-terminal amino acid sequence motifs in their host recognition proteins as compared within their respective related bacteriophage groups. The tail spike encoding gene of Syd8 (Syd8_0510), which did not show any significant nucleotide identity with the tail spike of any of the closely related bacteriophages, showed 50% amino acid identity, limited to the N-terminal 61 amino acid residue of the tail spike of both K1E (Locus tag: CAJ29458) and K1-5 (Locus tag: AF322019_2). Notably, the respective amino acid residues from the N-terminal region in K1E and K1-5 tail spikes have been reported to interact with the adaptor protein aiding their attachment to the tail protein (Leiman *et al.*, 2007).

It has been previously reported that the host recognition protein in tailed bacteriophages consists of a N-terminal domain for binding to the tail and a C-terminal domain for interacting with its receptor present on the bacterial surface, which determines its specificity (Wang *et al.*, 2000, Dupont *et al.*, 2004). The conservation of N-terminus amino acid sequences and the differences observed on the C-terminus within the host recognition proteins of all these three bacteriophages suggested that they could specifically target separate receptors on ST131. Interestingly, the C-terminal region of the host recognition protein of Syd1 showed high amino acid sequence identity (90% identity over 100% query cover) with the C-terminal host recognition protein of LM33_P1 (Locus tag: SBT28132). This high sequence identity between two completely different bacteriophages (Figure 3.16) suggested that both Syd1 and LM33_P1 could utilize similar mechanisms for host recognition. The presence of an enzymatic functional domain belonging to the pectin lyase superfamily (IPR011050) within the host recognition protein further suggested that these bacteriophages could modify the bacterial surface by their enzymatic activity, exposing the bacteriophage receptors (Latka *et al.*, 2017). In fact, both Syd1 and LM33_P1 (Dufour *et al.*, 2016) formed clear plaques with a visible halo, which lends weight to the possible enzymatic activity of their host recognition protein and thus a similar mechanism for host recognition. However, the utilization of the same receptor of both Syd1 and LM33_P1 on *E. coli* ST131 is unlikely due to the C terminal regions not being identical. Differences in amino acid sequence have been previously shown to alter host specificity (Heineman *et al.*, 2008, Le *et al.*, 2013).

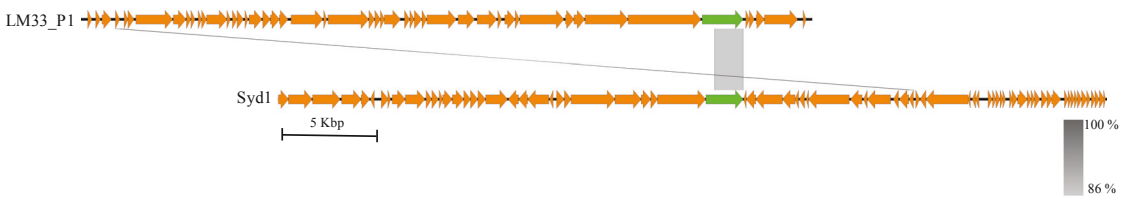

Figure 3. 16: Pairwise BLASTn comparison of Syd1 with LM33_P1. Each arrow represents the CDS of each bacteriophage and their transcriptional direction. Nucleotide identity between genomes is represented by grey shading between them and scaled according to the grey bar. Green colour arrow shows the host recognition protein of the bacteriophages. Figure was created using Easyfig 2.1(Sullivan *et al.*, 2011)

The host recognition protein in bacteriophage Syd6 contains a bacteriophage tail collar domain (PF07484) in the N-terminal region and an intramolecular chaperone auto-processing domain (PF13884, IPR030392) in the C-terminal region. Interestingly, all the closely related bacteriophages of Syd6, despite having nucleotide dissimilarity on their

host recognition proteins, possessed a similar C-terminal functional domain. C-terminal chaperone auto-processing domains in bacteriophage host recognition proteins assist folding and assembly of the protein (Schwarzer *et al.*, 2007). Conservation of host recognition protein N-terminal regions among highly similar bacteriophages, which have similar functional domains in their C-terminal regions, suggests that the host recognition proteins of each of these bacteriophages may undergo a maturation process that could determine their final structure. These structural differences may confer different host recognition abilities and thus distinct receptor specificities.

In contrast to Syd1 and Syd6, the host recognition protein in bacteriophage Syd8 did not have any predicted functional domain at its C terminus and was not only novel in the comparison group but also in the database. This novel Syd8 host recognition protein suggests that it could target a specific receptor on *E. coli* EC958. In conclusion, the differences observed among host recognition proteins of Syd1, Syd6 and Syd8 suggests that these three bacteriophages are utilizing different receptors on *E. coli* ST131 strains and could have distinct host specificities.

3.2.11 Different patterns of host range infectivity in *E. coli* ST131 strains

Host range infectivity of the three representative bacteriophages was assessed by infecting a panel of 65 *E. coli* strains, including 56 ST131 strains (Table 3.4). Infections were scored based on phenotypic presentation on agar plates. Infections that showed inhibition of bacterial growth at lower dilutions and formation of plaques at higher dilutions were scored as “bacteriophage infection”. Infections that showed inhibition of growth at lower dilutions with visibly decreasing inhibition at higher dilutions without plaque formation were scored as “lysis from without”. (Abedon, 2011). Lysis from without is a non-productive bacteriophage infection in which either bacteriophage or bacteriophage-based lysin induces lysis of bacteria from outside or the bacteria undergo bacteriophage-mediated suicidal death (Delbruck, 1940, Chopin *et al.*, 2005, Molineux, 2006, Abedon, 2011). The phenomenon of bacteriophage mediated suicidal death of bacteria is also known as abortive infection.

Host range analysis revealed that bacteriophage Syd6 is a more effective killer of *E. coli* ST131 strains. Syd6 showed zones of lysis on 52 of the tested *E. coli* ST131 strains, but productive bacteriophage infection was only confirmed in 45 strains. As such, killing of 7 strains was likely due to lysis from without (Table 3.4). Bacteriophage Syd1 killed 48 of the tested *E. coli* ST131 strains but as seen in Syd6, not all of these killings were attributable to bacteriophage infection, with 29 *E. coli* ST131 strains killed by lysis from without (Table 3.4). In contrast, Syd8 could infect only six of the tested ST131 strains. All three bacteriophages were unable to kill commensal, laboratory and other pathogenic *E. coli* strains from the test panel, with the exception of a clinical multidrug resistant ST69 strain JIE3348, which was infected by Syd6 and had lysis from without with Syd1. The infectivity patterns of each of the three bacteriophages were completely different, which indicated that each of the three bacteriophages likely utilised distinct surface receptors. More importantly, 52 out of 56 of the tested *E. coli* ST131 strains from the clinical sample set were killed by these three bacteriophages, though killing of six were attributed due to lysis from without. Notably, four strains were infected with all the three bacteriophages, 15 were infected with any two of them and 27 were infected with any one bacteriophage.

Infective strains for each of the bacteriophages were taken forward to ascertain the relative efficiency of plating (EOP) in reference to the host strain EC958. EOP is a quantification of the relative efficiencies with which different bacteria can be infected by a particular bacteriophage (Kutter, 2009). Indeed, EOP is a relative estimation of plaquing on susceptible strains to its host strain, which may depend on various factors (Kutzner, 1961). Hence, the efficiency of bacteriophage based on the EOP value can be classified as “high” if the ratio is more than 0.5, which represent 50% of the pfu found for the host bacteria. An EOP between 0.5 and 0.1 can be considered as “moderate” whereas anything less than 0.1 would be the “low” efficiency (Viazis *et al.*, 2011, Khan Mirzaei & Nilsson, 2015).

On EOP assays, bacteriophage Syd1 showed high efficiency on 12/19 (63.2%) infective strains, moderate efficiency on 2/19 (10.5%) and low on 5/19 (26.3%) infective strains. Likewise, Syd6 had high efficiency on 39/45 (86.7%), moderate on 4/45 (8.9%) and low on 2/45 (4.4%) infective strains. In contrast, Syd8 showed high efficiency on all the infective strains (6/6; 100%) notably with EOP greater than 1.0. Hence, in light of this EOP data bacteriophage Syd8 appears to be the most efficient in killing the ST131 strains

that are susceptible to it. However, as it could only kill a small proportion (6/52; 11.5%) of tested ST131 strains, it would not be recommended as effective alone for use as a therapeutic bacteriophage against ST131 strains. On the other hand, both Syd1 and Syd6 showed variability with their EOPs, both with a high percentage scored as “high” efficiency. These results indicate that bacteriophage Syd1 and Syd6 would be the most efficient therapeutic bacteriophages for killing ST131 strains. However, Syd6 could be the best among them as it not only killed relatively more ST131 strains but also the majority of killing was due to bacteriophage infectivity with a high efficiency.

Table 3. 4: Host range infectivity pattern of Syd1, Syd6 and Syd8 and Efficiency of Plating (EOP). Host range infectivity results were reported as "+" for bacteriophage infection, "-" for no infection and "LO" for lysis from without. EOP values represents the ratio of plaques formed upon a bacterial isolate relative to the propagating host strain EC958. N/A implies not applicable/not tested. All data were determined from three independent tests.

***E. coli* strains**	**Type**	**Syd1**		**Syd6**		**Syd8**	
		Infectivity	**EOP**	**Infectivity**	**EOP**	**Infectivity**	**EOP**
EC958	ST131	+	1.0	+	1.0	+	1.0
S135EC	ST131	LO	N/A	LO	N/A	-	N/A
S128EC	ST131	-	N/A	-	N/A	-	N/A
S120EC	ST131	-	N/A	+	0.94	+	1.2
SE15	ST131	-	N/A	+	1.42	-	N/A
JIE97	ST131	+	0.89	+	0.99	+	1.43
JIE100	ST131	LO	N/A	LO	N/A	-	N/A
JIE101	ST131	+	0.46	+	0.90	-	N/A
JIE110	ST131	-	N/A	+	0.61	-	N/A
JIE118	ST131	+	0.02	+	0.50	-	N/A
JIE143	ST131	LO	N/A	+	1.58	-	N/A
JIE154	ST131	+	0.86	+	1.29	+	1.08
JIE157	ST131	+	0.02	+	0.53	-	N/A
JIE186	ST131	LO	N/A	LO	N/A	-	N/A
JIE188	ST131	LO	N/A	+	0.14	-	N/A
JIE224	ST131	LO	N/A	+	0.08	-	N/A
JIE286	ST131	+	0.99	+	1.3	-	N/A
JIE289	ST131	LO	N/A	+	0.084	-	N/A
JIE295	ST131	LO	N/A	LO	N/A	-	N/A
JIE461	ST131	+	0.0003	-	N/A	-	N/A
JIE494	ST131	-	N/A	+	1.21	+	1.52
JIE804	ST131	-	N/A	+	0.247	-	N/A
JIE1296	ST131	+	1.06	+	1.34	-	N/A
JIE1301	ST131	LO	N/A	+	0.61	-	N/A
JIE1302	ST131	LO	N/A	+	0.57	-	N/A

E. coli strains	Type	Syd1		Syd6		Syd8	
		Infectivity	EOP	Infectivity	EOP	Infectivity	EOP
JIE1308	ST131	LO	N/A	+	1.02	-	N/A
JIE1311	ST131	+	0.85	+	0.68	-	N/A
JIE1315	ST131	+	0.93	+	0.9	-	N/A
JIE1323	ST131	+	0.85	+	1.07	+	1.33
JIE1324	ST131	+	0.59	+	0.94	-	N/A
JIE1325	ST131	+	0.63	+	0.93	-	N/A
JIE1326	ST131	LO	N/A	LO	N/A	-	N/A
JIE3172	ST131	LO	N/A	+	0.82	-	N/A
JIE3173	ST131	LO	N/A	+	1.15	-	N/A
JIE3184	ST131	LO	N/A	LO	N/A	-	N/A
JIE3195	ST131	LO	N/A	+	1.38	-	N/A
JIE3208	ST131	LO	N/A	+	1.17	-	N/A
JIE3235	ST131	-	N/A	-	N/A	-	N/A
JIE3247	ST131	+	.0003	+	0.99	-	N/A
JIE3266	ST131	LO	N/A	+	0.97	-	N/A
JIE3307	ST131	LO	N/A	+	1.33	-	N/A
JIE3320	ST131	+	0.10	LO	N/A	-	N/A
JIE3348	ST69	LO	N/A	+	0.38	-	N/A
JIE3365	ST131	LO	N/A	+	0.3	-	N/A
JIE3411	ST131	LO	N/A	+	1.28	-	N/A
JIE3418	ST131	LO	N/A	+	1.43	-	N/A
JIE3424	ST131	+	1.32	+	0.97	-	N/A
JIE3430	ST131	LO	N/A	+	1.18	-	N/A
JIE3431	ST131	-	N/A	-	N/A	-	N/A
JIE3432	ST131	LO	N/A	+	1.51	-	N/A
JIE3454	ST131	+	1.38	+	0.94	-	N/A
JIE3459	ST131	LO	N/A	+	1.06	-	N/A
JIE3465	ST131	-	N/A	-	N/A	-	N/A
JIE3684	ST131	LO	N/A	+	1.03	-	N/A
JIE3772	ST131	LO	N/A	+	0.94	-	N/A
JIE3905	ST131	LO	N/A	+	0.91	-	N/A

E. coli strains	Type	Syd1		Syd6		Syd8	
		Infectivity	EOP	Infectivity	EOP	Infectivity	EOP
JIE3996	ST131	+	0.0004	+	1.07	-	N/A
CFT073	UPEC	-	N/A	-	N/A	-	N/A
UTI89	UPEC	-	N/A	-	N/A	-	N/A
H10407	ETEC	-	N/A	-	N/A	-	N/A
2348/69	EPEC	-	N/A	-	N/A	-	N/A
SE11	Commensal	-	N/A	-	N/A	-	N/A
MG1655	Commensal	-	N/A	-	N/A	-	N/A

3.2.12 Bacteriophage resistant host mutants to each of the three representative bacteriophages and their cross infectivity

As bacterial resistance against therapeutic phages has been reported in various animal model studies (Oechslin, 2018), an *in vitro* assay was performed to isolate spontaneous host mutants resistant to each of the three representative bacteriophages. Briefly, the host strain was spotted with a high titre of each of the bacteriophages, and a bacterial colony from within the bacteriophage spot was isolated and tested for bacteriophage resistance. Specifically, three *E. coli* EC958 mutants were isolated, namely EC958R1, EC958R6, EC958R8, which were resistant to Syd1, Syd6 and Syd8 respectively. Each of these three mutants stably retained the respective bacteriophage resistance through subsequent generations, as checked with bacteriophage infectivity testing. The host range test of each of the three bacteriophages on those resistant host mutants was performed to see whether one bacteriophage could infect the resistant bacterial variants of another bacteriophage. Bacteriophages Syd1 and Syd6 infected EC958R8 and bacteriophage Syd8 infected EC958R1 and EC958R6. However, bacteriophage Syd1 was unable to infect *E. coli* EC958R6 and bacteriophage Syd6 did not infect EC958R1. In addition, Syd1 and Syd6 made clearer and larger plaques on *E. coli* EC958R8 strains than those on wild-type (*E. coli* EC958) (Figure 3.17) and the EOP of both Syd1 and Syd6 on this mutant was also higher than that of the wild type (Table 3.5). In contrast, no such changes of plaque morphology by Syd8 on either EC958R1 or EC958R6 was observed, while the EOP on both of these mutants was lower than that of wild type.

Table 3. 5: Host range infectivity and EOP of Syd1, Syd6 and Syd8 on bacteriophage resistant host mutants. Host range infectivity results were reported as "+" for bacteriophage infection and "-" for no infection. EOP values represents the ratio of plaques formed upon a bacterial isolate relative to the propagating host strain EC958. N/A implies not applicable/not tested. All data were determined as mean from three independent tests.

E. coli Strain	Syd1		Syd6		Syd8	
	Infectivity	EOP	Infectivity	EOP	Infectivity	EOP
EC958	+	1.0	+	1.0	+	1.0
EC958R1	-	N/A	-	N/A	+	0.54
EC958R6	-	N/A	-	N/A	+	0.62
EC958R8	+	1.04	+	1.16	-	N/A

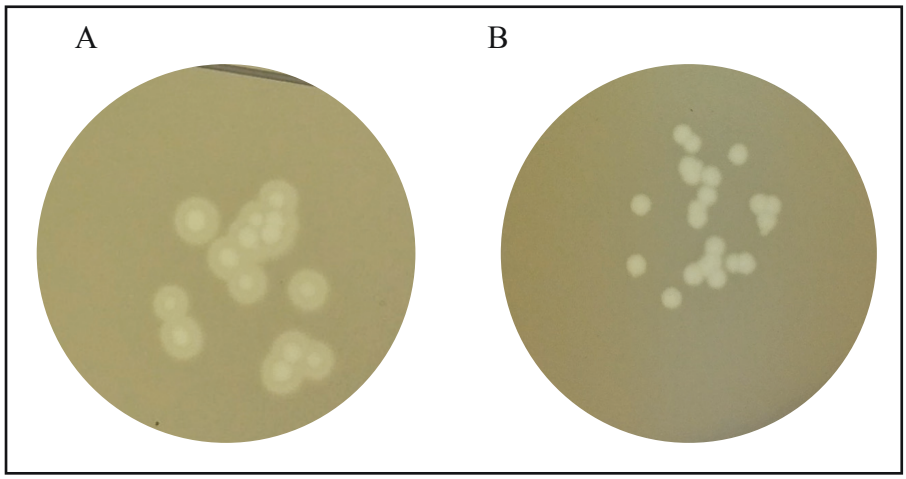

Figure 3. 17: Plaque morphologies of bacteriophage Syd1 (A) and Syd6 (B) on Syd8 resistant *E. coli* EC958 mutant. Plaque morphologies of bacteriophage Syd1 and Syd6 on wild type E. *coli* EC958 is as in Figure 3.1.

As described in earlier sections, these three bacteriophages were likely to be able to utilize different surface receptors on *E. coli* EC958. So, if a mutation occurred within a gene encoding the bacteriophage receptor for a single bacteriophage, it could still be infected by the other two bacteriophages. Hence, it is likely that the mutations that occurred in the Syd8 resistant mutant could be in the gene encoding the putative bacteriophage receptor but that might not be the case for the Syd1 and Syd6 resistant mutants. Bacteriophages can also develop resistance to bacteria through mechanisms other than receptor modification, which will make them resistant to more than one type of bacteriophage. It is therefore possible that Syd1 and Syd6 resistant mutants could have a common mechanism for host resistance that made them resistant to both of the bacteriophages. Although further work needs to be done to analyse the mechanism for resistance for each of the bacteriophages, the data presented here clearly indicated that even if mutation of the host arises, another bacteriophage could still be effective, which suggests that cocktails of bacteriophages would be effective therapeutics against ST131 lineages.

3.3 Discussion

In this chapter, three distinct bacteriophages, namely Syd1, Syd6 and Syd8, were characterised, all of which can kill multiple *E. coli* ST131 strains. These three bacteriophages were not only morphologically and genomically distinct from each other

but also from other previously published bacteriophages that infect *E. coli* ST131. Most importantly, the functional analysis of all identified genes within each bacteriophage reveals that all three were devoid of any lysogeny module genes or their homologues. No gene products with homology to characterised integrases, resolvases or excisionase and bacteriophage encoded repressors were found. In addition, none of these bacteriophages possessed any bacterial virulence genes or antibiotic resistance genes. The closely related bacteriophage groups for Syd1, Syd6 and Syd8 were, respectively, Jerseyvirinae, phiEco32 like and Sp6-like bacteriophages reported to be strictly lytic (Dobbins *et al.*, 2004, Savalia *et al.*, 2008, Anany *et al.*, 2015). Thus, bacteriophages Syd1, Syd6 and Syd8 could be appropriate candidates for therapeutic agents according to the current consensus on bacteriophage therapy (Pirnay *et al.*, 2015, Schooley *et al.*, 2017).

Bacteriophage Syd1, Syd6 and Syd8 are not novel and have high sequence identity with three different groups of known bacteriophage groups. However, it is noteworthy that these bacteriophages are differentiated from their closely related bacteriophages almost exclusively by differences in the putative host recognition gene. Since all of these bacteriophages were isolated selectively on *E. coli* ST131 strain EC958, differences in the gene encoding the host recognition proteins of all of these bacteriophages is likely due to their affinity to specific receptors present on the surface of *E. coli* ST131 strains. This specificity was further highlighted when none of these bacteriophages showed any kind of infectivity on commensal *E. coli* strains, laboratory and other pathogenic *E. coli* strains tested except a multidrug resistant ST69 strain JIE3348. This ST69 strain was infected by Syd6 and had lysis from without with Syd1. Importantly, within the five most common lineages of *E. coli,* ST69 is the only other lineage that also includes multidrug resistant strains (Kallonen *et al.*, 2017). Hence, it is likely that bacteriophage Syd6 targets a surface receptor on *E. coli* ST131 that could also be present on the surface of multidrug resistant ST69 strains. As such it could be possible that Syd6 could specifically kill the multi drug resistant *E. coli* strains and thus it would be worth testing the infectivity of Syd6 on other ST69 strains.

The differences observed among host recognition proteins of Syd1, Syd6 and Syd8, which also consist of distinct functional domains, reveals that these three bacteriophages could utilize different receptors on *E. coli* ST131 strains. This was further confirmed by distinct host range infection profiles of each bacteriophage over a panel of clinical ST131

strains. Similar findings were also reported by Green *et al*. (2017), who showed that all of their bacteriophages able to infect *E. coli* ST131 strains have different host range patterns, and were unable to kill all the ST131 strains tested (Green *et al.*, 2017). *E. coli* ST131 comprises different clades that have differences in O-antigen (Johnson *et al.*, 2013, Petty *et al.*, 2014), capsule loci (Alqasim *et al.*, 2014, Petty *et al.*, 2014), and other surface proteins due to these genes encoded in mobile genomic elements (genomic islands, prophages and plasmids) (Ben Zakour *et al.*, 2016). Hence, it is possible that the receptors that the bacteriophage recognises are either only conserved within a particular subset of the ST131 strains or are masked by the different types of capsule produced by different strains (Dufour *et al.*, 2016). Additionally, it could also be possible that some bacterial strains escaped the bacteriophage infectivity because of their anti-bacteriophage defence mechanisms.

Bacteria may prevent bacteriophage infectivity if they are equipped with either the bacteriophage adsorption blocking system or a system to prevent bacteriophage multiplication such as Restriction-modification (R-M) system, CRISPR/Cas system or Abortive infection system (Labrie *et al.*, 2010). No functional CRISPR/Cas system has been reported in *E. coli* ST131 strain but various types of R-M system, including orphan methylases on different mobile genomic elements has been reported within ST131 strains (Forde *et al.*, 2015, Shaik *et al.*, 2017). This variability among the R-M systems within ST131 clones suggests that this could be the one of the major mechanisms affecting bacteriophage infectivity of these clones. Abortive infection, which prevents bacteriophage multiplication but allows suicidal death of infected bacteria, may also provide a lysis from without type reaction (Chopin *et al.*, 2005, Abedon, 2011). As lysis from without reaction was observed with bacteriophage Syd1 and Syd6 specifically on some *E. coli* ST131 strains only, these could be speculated as the result of abortive infection. However, a role of bacteriophage based lysin could not be excluded completely. Since lysis from without especially by Syd1 was found to be equally effective for bacterial killing as bacteriophage infection, deciphering the biological mechanism is an important future aspect for designing phage therapy against *E. coli* ST131 strains.

The finding of *in vitro* spontaneous host resistant mutants to each of the three phages and their ability to retain the bacteriophage resistance through subsequent generations suggested that bacterial variants resistant to these bacteriophages could also develop *in*

vivo. Occurrence of bacterial resistance against therapeutic bacteriophages have been reported in various animal model studies as well as in human clinical trials (Oechslin, 2018), which could affect favourable outcomes of the therapy. As so, the problem of emergence of bacteriophage resistant bacterial mutants against therapeutic bacteriophages could only be tackled if resistant variants to one bacteriophage can be infected with another bacteriophage in a phage cocktail. The cross-infectivity data of Syd1, Syd6 and Syd8 on its resistant host mutants however clearly indicated that it would depend on the type of resistance acquired by the bacteria. Thus, it is possible that bacterial mutants that acquire resistance to one bacteriophage can also become resistant to another bacteriophage within a cocktail. Interestingly, it has also been reported in various animal model studies that bacteriophage-resistant bacterial mutants may become less virulent while conferring bacteriophage resistance (Smith & Huggins, 1982, Capparelli *et al.*, 2010, Pouillot *et al.*, 2012). Hence, it is likely that spontaneous mutation of bacteria conferring resistance to Syd1 and Syd6 may pose a reduced threat to health or may even become avirulent. However, this needs to be further investigated by characterising the bacteriophage resistant mutants genomically and phenotypically for their virulence.

Whilst much information still remains to be elucidated for each of these bacteriophages and their resistant bacterial mutants, specifically *in vivo* efficiency, the work reported here indicates that these bacteriophages are possible candidates for phage therapy targeting *E. coli* ST131 strains. It would therefore be worth investigating the *in vivo* efficacy of each of these bacteriophages in animal model studies. To study the in *vivo* effectivity of bacteriophages in an animal model requires pure bacteriophages free from any host bacterial contaminants, especially those which could involve an immunomodulatory effect. The next chapters in this thesis focus on identifying possible contaminants in bacteriophage preparations, and the purification process needed to obtain therapeutic grade bacteriophage preparations suitable for further studies.

3.4 Conclusion

Phage therapy is currently experiencing a revival as a promising strategy to control bacterial infections, especially against MDR bacteria. This study presents morphological, genomic and host range characterization of three different bacteriophages that could kill *E. coli* ST131 strains *in vitro*. Genomic analysis confirmed that not only are these three

virulent bacteriophages genetically distinct, they also possessed different host recognition genes, which indicated that they are likely to utilise different bacterial receptors. This was further highlighted when each bacteriophage showed distinct infection profiles over the panel of clinical ST131 strains. Moreover, none of these bacteriophages were able to infect commensal *E. coli* strains, laboratory or other pathogenic *E. coli* strains tested, except for a multidrug resistant ST69, confirming their potential as therapeutic bacteriophages against multidrug resistant *E. coli*. While each bacteriophage individually was not able to kill all tested ST131 strains, they are useful candidates for a combined therapeutic phage cocktail to combat *E. coli* ST131 infections.

4 Membrane vesicle biogenesis in *E. coli* through virulent bacteriophage infection.

4.1 Introduction

Membrane vesicles (MVs) are nanostructures enclosed by a lipid bilayer, which may contain cellular components. MVs prevail in all three domains of life and are common in bacteria (Deatherage & Cookson, 2012). In Gram-negative bacteria, MVs are produced either through membrane blebbing (Li *et al.*, 1998, Moller *et al.*, 2005) or through explosive cell lysis (Turnbull *et al.*, 2016). Membrane blebbing occurs due to cell envelope disturbances induced from various stress response whereas explosive cell lysis occurs due to degradation of peptidoglycan by a bacteriophage-based endolysin (Turnbull *et al.*, 2016, Volgers *et al.*, 2018, Toyofuku *et al.*, 2019). Bacteriophage mediated lysis of Gram-negative bacteria has been reported as a cell bursting phenomenon similar to that of explosive cell lysis (Young, 1992, Berry *et al.*, 2012) and images of MV-like structures have been seen in transmission electron microscopy of various bacteriophage preparations (Sullivan *et al.*, 2005, Guang-Han *et al.*, 2016, Schiettekatte *et al.*, 2018). However, there is currently a lack of knowledge on the source of MVs in phage lysates and also no evidence of bacteriophage mediated MVs formation. Thus, this chapter aimed to examine MVs biogenesis associated with virulent bacteriophage infection in *E. coli.* This chapter explores MVs biogenesis in a model organism *E. coli* K-12 strain MG1655 through virulent bacteriophages T4 and T7 infection and also confirms MVs within lysates of *E. coli* ST131 infecting bacteriophages Syd1, Syd6, and Syd8. This chapter demonstrates for the first time that bacteriophage mediated bacterial lysis is the source of MVs within phage lysates.

4.2 Results

4.2.1 Live cell imaging of bacterial lysis during bacteriophage infection

An assay using the usual condition for bacteriophage propagation in liquid medium (Bonilla *et al.*, 2016) was utilized to examine bacteriophage mediated bacterial lysis using live-cell imaging. Specifically, the liquid culture of *E. coli* was infected with

bacteriophages and time-series captured with a phase-contrast microscope. To validate the assay results, three independent tests of *E. coli* infected with bacteriophages T4 and T7 (suspended in lambda diluent) and negative control (lambda diluent) were performed. Time-lapse imaging at one frame /min was performed continuously for an hour at 37°C to observe changes within the bacterial cell population due to bacteriophage infection. Under the established optimum conditions for *E. coli* growth, the addition of either bacteriophage T4 or T7 showed lysis of many bacterial cells whereas no lysis was observed in the control samples (Figure 4.1, Appendix 12; Movies 1A, 1B and 1C). Indeed, in the negative control, bacteria cells were observed to be multiplying, increasing their number within the field of view (Appendix 12; Movie 1C). These observations indicated that the lysis was due to bacteriophage infection and not due to any extraneous condition or any experimental artefacts. This further indicated that the assay was suitable for the investigation of bacteriophage mediated bacterial lysis under live cell imaging conditions. The assay was also performed on *E. coli* ST131 strain EC958 with bacteriophages Syd1 and Syd8 (n =1). Similar to what was observed on *E. coli* K-12 strain MG1655 with bacteriophages T4 and T7, bacterial lysis was observed under live cell imaging on cultures that were infected with both bacteriophage Syd1 and Syd8 but not with the lambda diluent (Figure 4.2).

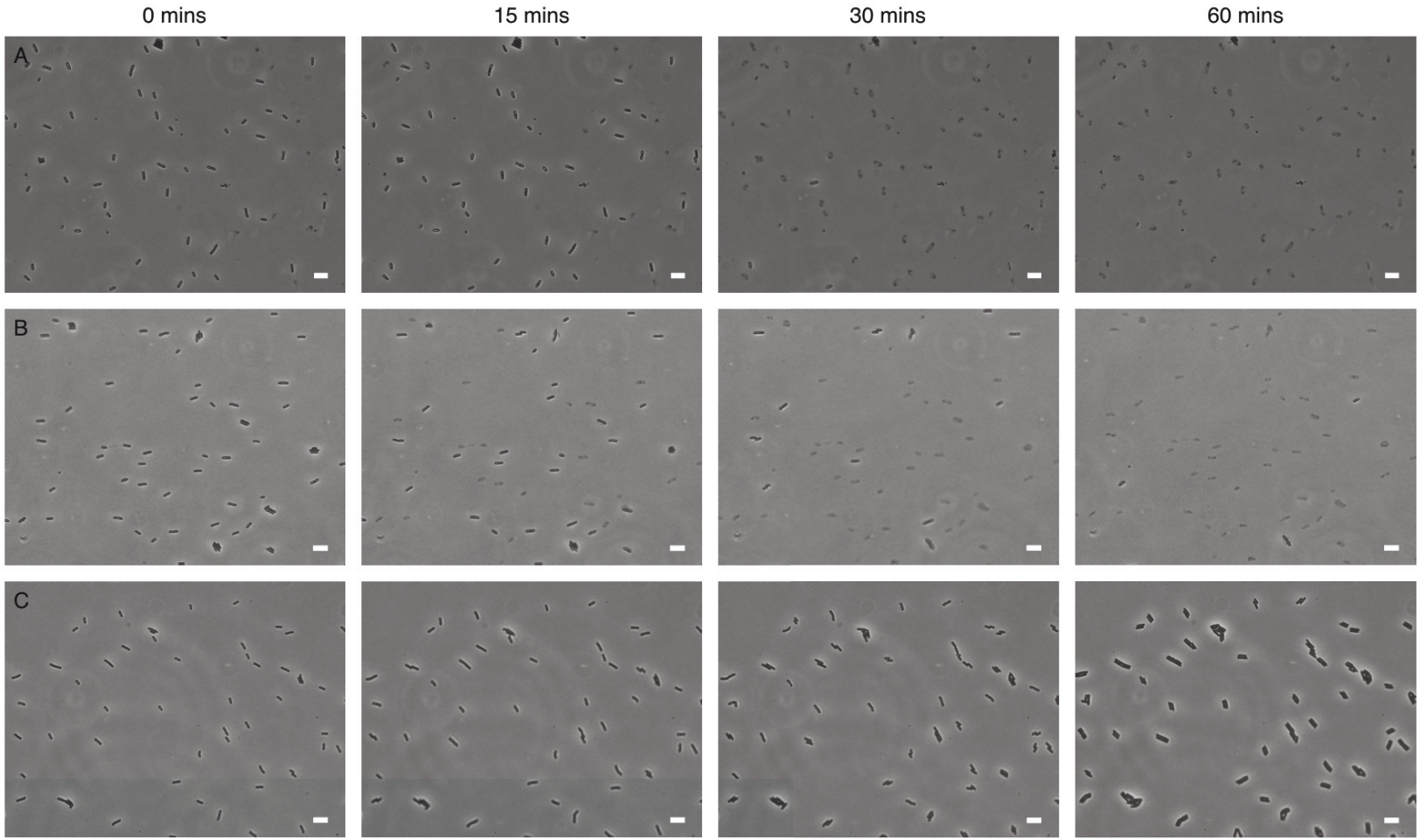

Figure 4. 1: Time lapse image sequence of *E. coli* MG1655 treated with (A) bacteriophage T4, (B) bacteriophage T7 and (C) lambda diluent. The black thin rods in each frame represent a live bacterium. Images were taken using phase contrast (Olympus IX71). Scale bar is 2 μm. Figure was generated using Omerofig.

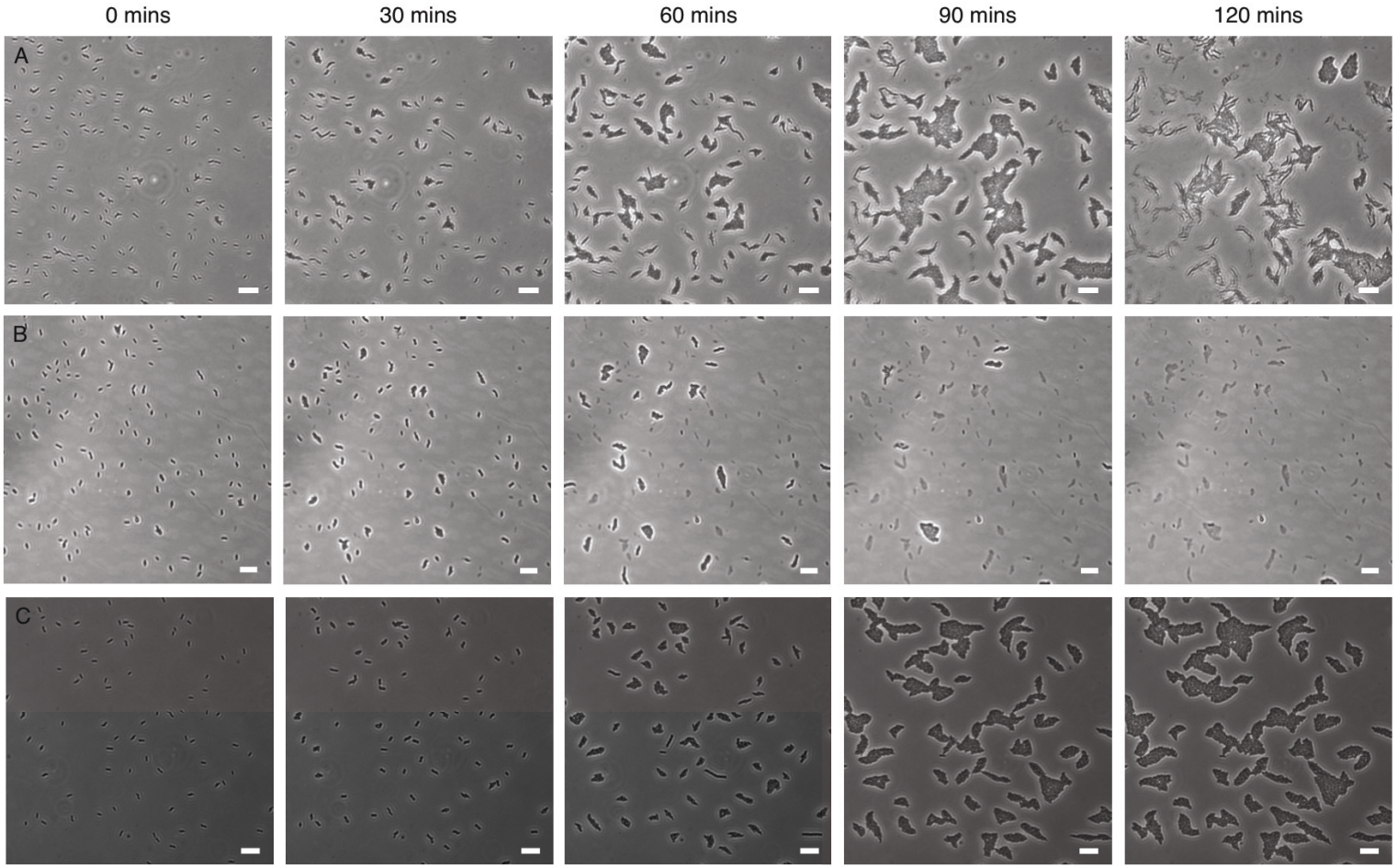

Figure 4. 2: Time lapse image sequence of *E. coli* ST131 strain EC958 treated with (A) bacteriophage Syd1, (B) bacteriophage Syd8, and (C) lambda diluent. The black thin rods in each frame represent a live bacterium. Images were taken using phase contrast (Olympus IX71). Scale bar is 2 μm. Figure was generated using Omerofig.

To establish the appropriate time-frame for capturing of lysis events from T4 and T7 infection, the time at which most of the lysis event occur with each of the bacteriophages was determined. The time-lapse data obtained from image sequences of each bacteriophage revealed that under the experimental condition, 50 % of the cell lysis event occurred within 17-32 minutes with bacteriophage T4 and within 8-24 minutes for infections with bacteriophage T7 (Figure 4.3). Thus, the determined time was utilized to demonstrate the cell lysis event in detail at higher magnification (section 4.2.2).

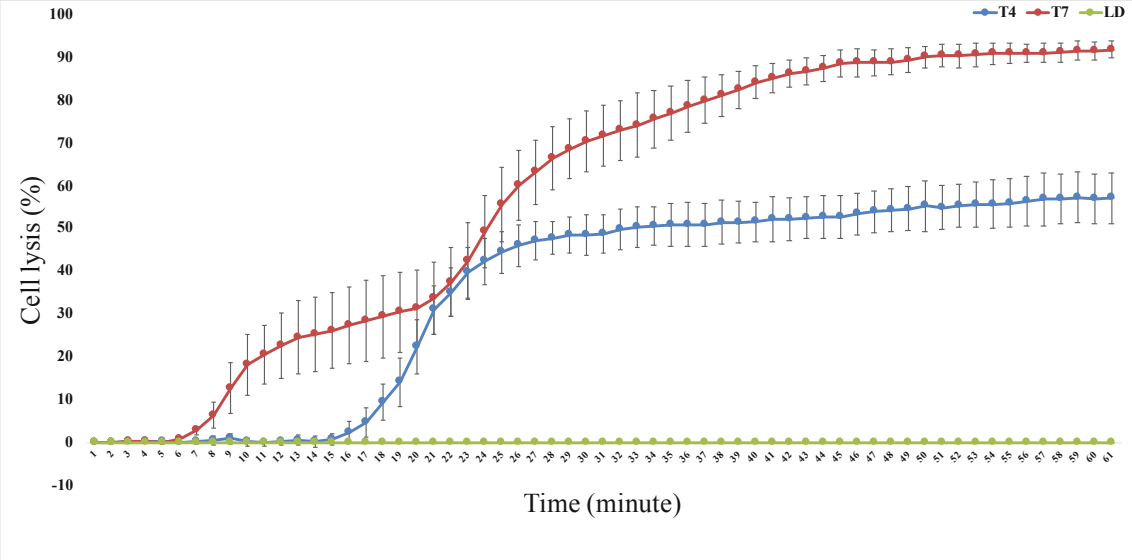

Figure 4. 3: Cumulative percentage of bacterial lysis under bacteriophage treatments. Graphs showing percentage of lysed *E. coli* K-12 strain MG1655 treated with bacteriophage T4 (in blue), T7 (in red) and Lambda diluent (LD) control (in green), over an hour period of time. The error bar represents standard deviation from mean of three different fields of view (n =3). More than 1000 cells (1,170 for T4; 1,669 for T7 and 1,470 for LD) were followed for lysis.

4.2.2 Bacteriophage mediated explosive cell lysis in *E. coli*

Time-lapse series at higher magnification of single-cell lysis events with both the T4 and T7 bacteriophages were acquired. The observation of multiple independent experiments (n=5 for T4; n=4 for T7) resulted in the acquisition of 103 and 118 cell lysis events for bacteriophage T4 and T7 respectively, three of which are shown in Figure 4.4A-C as time-lapse series. Time-lapse microscopy revealed that lysis occurred as an explosion of the cell with the expulsion of cellular content (Appendix: 12; Movie 2). Lysis resulted either in a direct blowout without any gross changes in cellular morphology or in progressive changes in the morphology to a spherical form before bursting (Figure 4.4A-C).

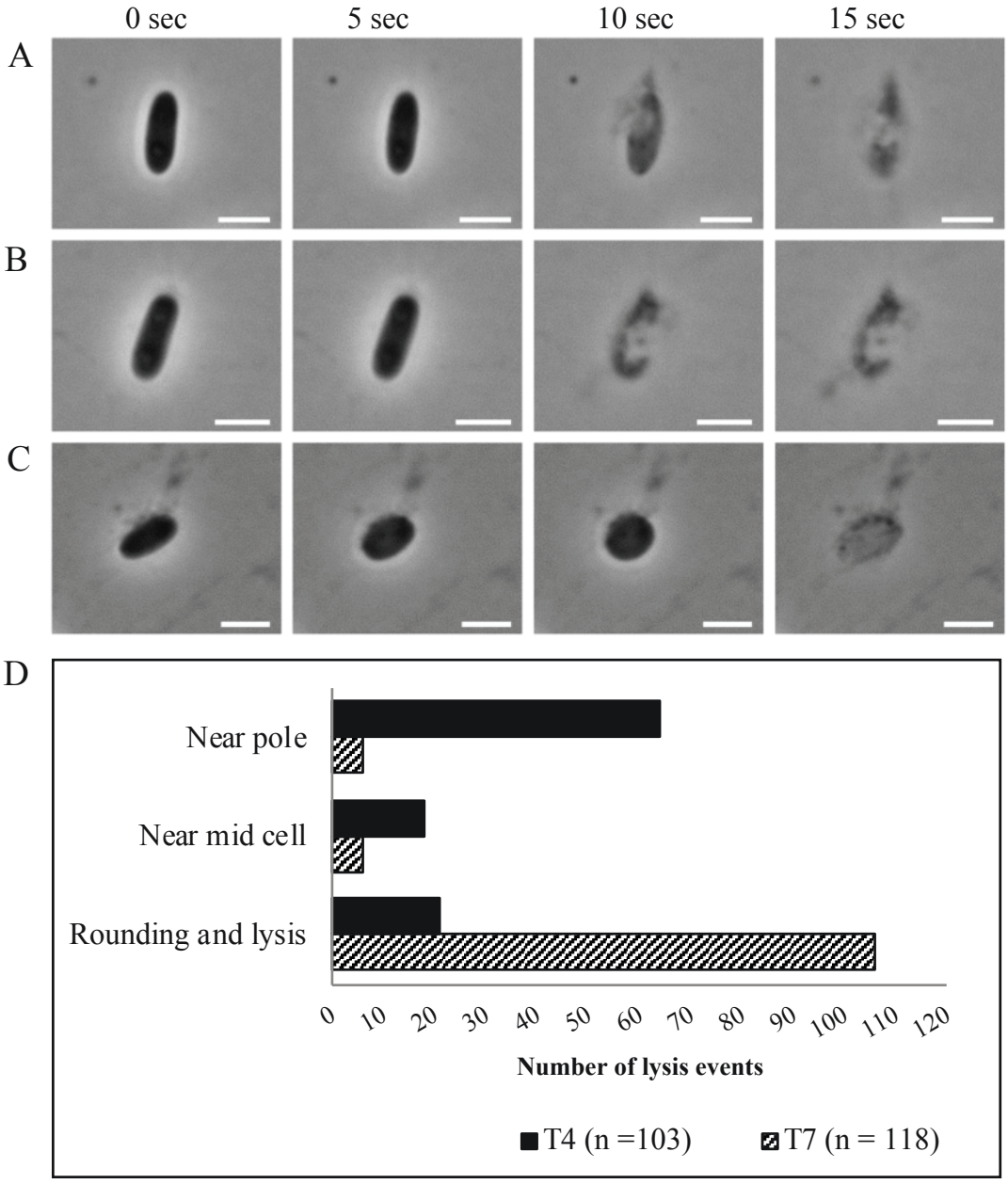

Figure 4. 4: Different patterns of explosive cell lysis in *E. coli*. Time lapse representative images of *E. coli* MG1655 treated with bacteriophage T4 showing (A) lysis as direct blowout near pole without any gross change in cell morphology, (B) lysis as direct blowout near mid cell without any gross change in cell morphology, (C) gross change in morphology before lysis (i.e. rounding and lysis), and (D) a bar chart on the number of different types of lysis pattern observed with bacteriophage T4 and T7. Images were taken using phase contrast (Olympus IX71). Scale bar is 2 μm.

For lysis due to bacteriophage T4 infection, the majority of lysis events (64/103; 62.13%) showed a pattern whereby the cell abruptly ruptured near a pole (Figure 4.4D). This was followed by lysis events (21/103; 20.39%) which showed the rounding of rod-shaped cells into spheres (Figure 4.4D) and lysis events (18/103; 17.47%) that showed abrupt lysis near the mid cell (Figure 4.4D). For lysis events that showed the

rounding of cells, the most common morphology observed before lysing was a prolate sphere. The same three different lysis patterns were also observed when MG1655 cells were infected with bacteriophage T7. Unlike the infection with bacteriophage T4, the majority of lysis events (106/118; 89.83%) showed the pattern of the rounding of rod-shaped cells into spheres before lysis (Figure 4.4D). Such a phenomenon of the rounding of a cell before bursting has been termed as explosive cell lysis (Turnbull *et al.*, 2016). On account of the data that revealed the cells were lysed explosively regardless of either the rounding up before lysing or the direct blowout, all the observations recorded here are collectively interpreted as explosive cell lysis.

4.2.3 Bacteriophage mediated explosive cell lysis resulted MV biogenesis in *E. coli*

As explosive cell lysis has been previously showed to generate MVs (Turnbull *et al.*, 2016), 3D- structured illumination microscopy (3D-SIM) of *E. coli* infected with bacteriophages was performed in the presence of the lipophilic dye FM1-43 to visualize cell membranes and MVs. This live-cell super-resolution microscopy technique showed that bacteriophage infection is associated with cell lysis and the formation of numerous MVs (Figure 4.5A). No lysis or MVs were observed in control samples (Figure 4.5B).

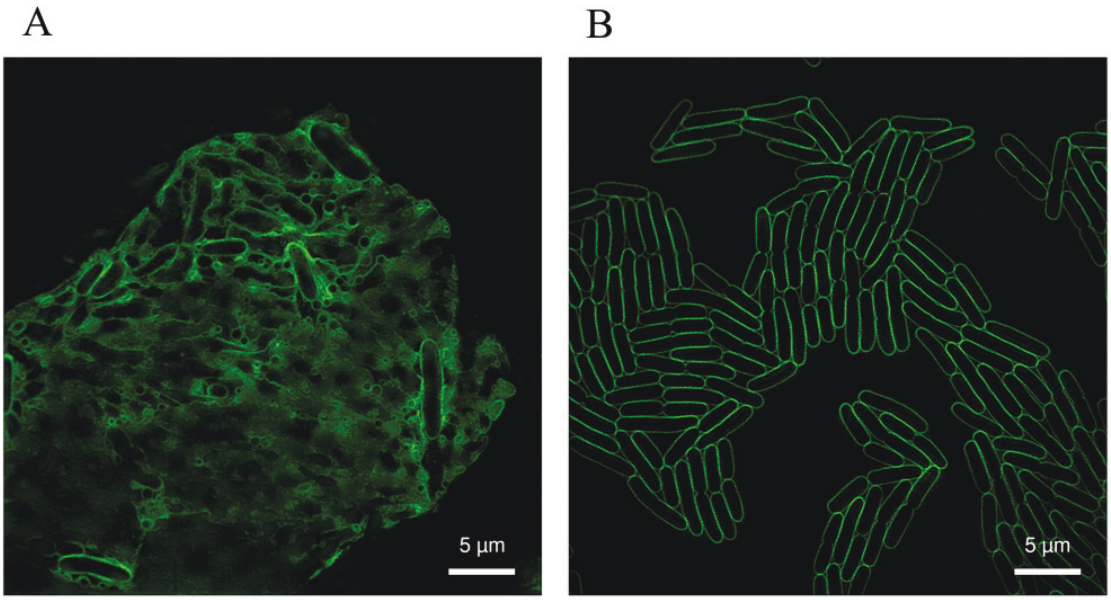

Figure 4. 5: Cell lysis results in MVs formation. Image of *E. coli* MG1655 (A) treated with bacteriophage T7 showing MVs within lysed cell population and (B) treated with lambda diluent

does not show any lysis or MVs. The membrane dye FM1-43X (Green colour) was used for membrane staining. Images were acquired using 3D-SIM (DeltaVision OMX SR). Scale bar is 5 μm.

Time-lapse imaging using 3D-SIM live-cell super-resolution microscopy further demonstrated that irrespective of the lysis pattern, the exploding bacteria produced MVs from shattered membrane fragments that quickly vesicularized after the explosion (Figure 4.6A-B; Appendix 12: Movies 3A and 3B). Analysis of individual cells using 3D-SIM revealed that after lysis, the membrane curled inward and then rounded up forming spherical MVs, which persisted in the same topology thereafter (Figure 4.6 A-B; Appendix 12: Movies 3A and 3B). There were no differences in steps of MVs formation through explosive cell lysis, whether it was due to infection with bacteriophage T4 or bacteriophage T7. Similarly, the process was also found to be independent of individual cell lysis pattern with the process being the same whether the lysis resulted in a direct blowout without any gross changes in cellular morphology or in progressive changes in the morphology before rupturing. Indeed, the findings on this process of MVs formation through bacteriophage mediated explosive cell lysis agreed with the observations described by Turnbull *et. al.,* (2016), who showed that the MV biogenesis from explosive cell lysis was due to the curling and self-annealing of the shattered membrane fragments from the lysed cell (Turnbull *et al.*, 2016). The process of MVs formation from the lysed cell was very dynamic and it was observed that a single explosive cell could produce multiple MVs, which had variable dimensions. The reproducibility of the results from the multiple cell lysis events under both the T4 and T7 bacteriophage infection suggest that MV formation through bacteriophage-mediated lysis is a common route for MVs formation during bacteriophage cultivation.

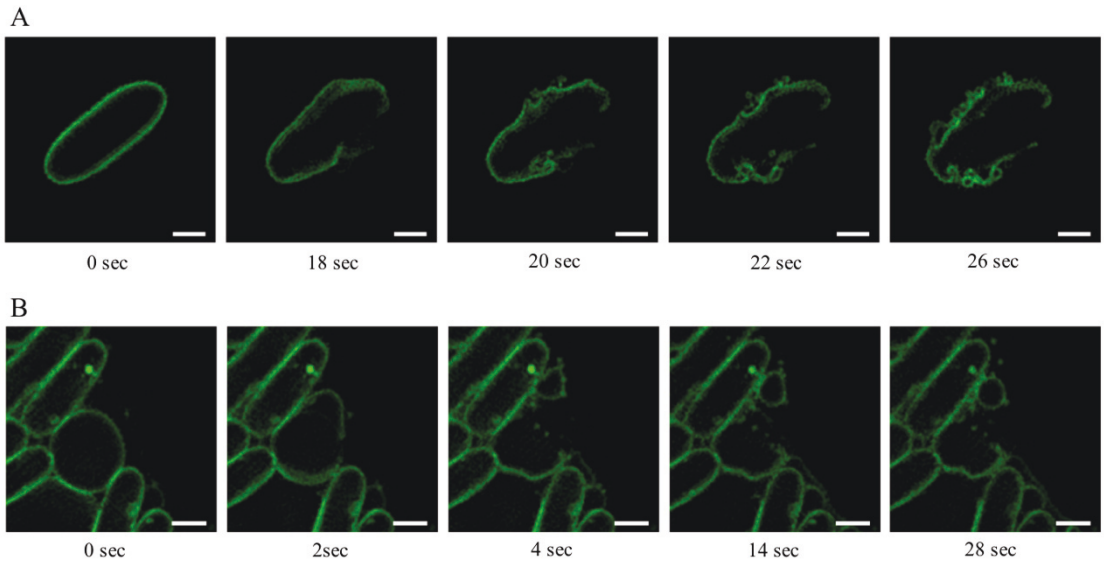

Figure 4. 6: MVs formation through explosive cell lysis in *E. coli*. Time lapse image sequence of a lysis event in *E. coli* MG1655 treated with (A) T4 and (B) T7 showing MVs formation from shattered membrane fragments produce after explosive cell lysis. A membrane dye FM1-43X (Green colour) was used for membrane staining. Images were acquired using 3D-SIM (DeltaVision OMX SR). Scale bar is 1 μm. Figure was generated using Omerofig.

4.2.4 Membrane blebbing in *E. coli* under bacteriophage infection

In addition to the MVs biogenesis through cell lysis, cells with membrane blebs were also observed in the bacterial populations that were not lysed by bacteriophage infection (Figure 4.7). The observation from multiple independent experiments (n= 2 for T4 and n= 4 for T7) resulted in the acquisition of 28 and 99 cells with membrane blebs under bacteriophage T4 and T7 infection condition respectively. Indeed, the formation of membrane blebs was captured on MG1655 under both the T4 and T7 bacteriophage infected conditions by time-lapse imaging with 3D-SIM which demonstrated that the cell began to protrude its outer membrane and produced different sized membrane blebs (Fig 4.6A-B; Appendix Appendix 12: Movies 4A and 4B). It was observed that a single cell formed multiple membrane blebs, which were variable in size and which could also merge forming a bigger bleb (Appendix 12: Movies 4B).

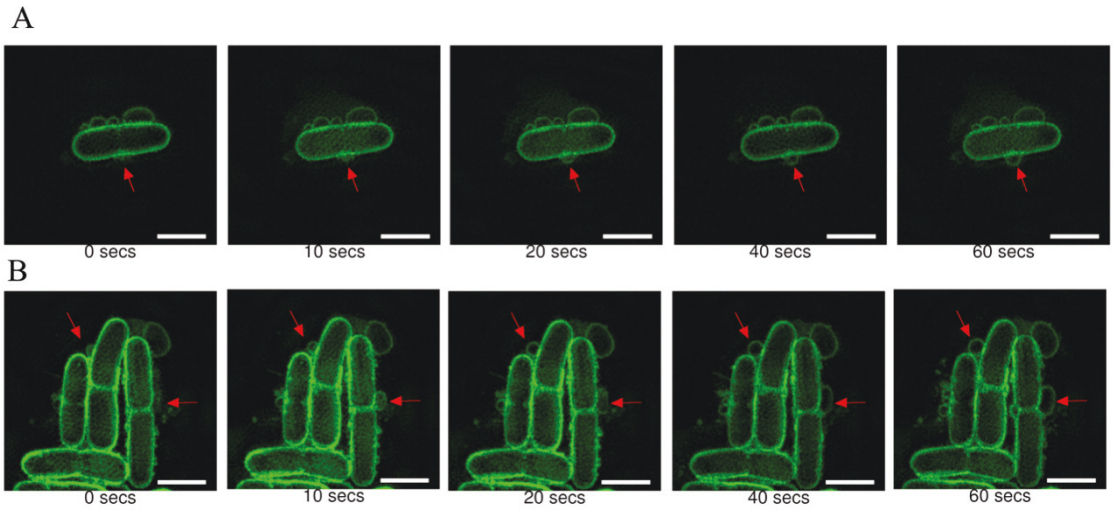

Figure 4. 7: Membrane blebbing in *E. coli* under bacteriophage infection. Time-lapse image sequence of membrane blebbing in *E. coli* MG1655 treated with (A) T4 and (B) T7. Red arrow indicates site of membrane blebs on cell. A membrane dye FM1-43X (Green colour) was used for membrane staining. Images were acquired using 3D-SIM (DeltaVision OMX SR) Scale bar is 2 μm. Figure was generated using Omerofig.

As it has been reported that bacteria can produce MVs through blebbing under various physiological stressors (Sabra *et al.*, 2003, McBroom & Kuehn, 2007, Roier *et al.*, 2016), further investigation on MG1655 treated with lambda diluent was performed to see whether membrane blebs were produced under this experimental control condition. The 3D-SIM live imaging of multiple cells from three independent experiments (n = 3), did not show a single cell with any visible membrane blebs. These observations indicate that the blebbing observed under the experimental conditions was due to bacteriophage infection. Strikingly, it was noteworthy to observe that cells with membrane blebs did not lyse explosively. It has been previously proposed that under various stressors that causes internal damage to the bacteria, membrane blebbing contribute to bacterial survival by enabling the removal of misfolded proteins (McBroom & Kuehn, 2007, MacDonald & Kuehn, 2012). Hence, it is plausible that membrane blebbing under bacteriophage infection condition could be the protective mechanism utilized by bacteria against bacteriophage infection.

4.2.5 Membrane vesicles within phage lysates

Transmission electron microscopy (TEM) of the phage lysates that were prepared from the bacteriophage infection assay experiments (n =3) was performed to see if MVs were present within them. Under the microscope, MVs were observed within all of the lysates

of both bacteriophage T4 and T7. Indeed, TEM micrographs of MG1655 cell lysates resulting from bacteriophage T4 and bacteriophage T7 infection showed MVs together with the respective bacteriophages and the cell debris (Figure 4.8 A and B). These results were consistent with the results of live-cell imaging. Moreover, the lysates prepared with bacteriophage Syd1, Syd6 and Syd8 were also subjected to TEM analysis for the reproducibility of the results. As expected, TEM images of EC958 cell lysates resulting from bacteriophage Syd1, Syd6, and Syd8 infection also revealed similar results showing MVs within each of them (Figure 4.8 C-E). Hence, it was interpreted that the occurrence of bacteriophage mediated bacterial lysis during bacteriophage cultivation could be the source of MVs within phage lysate.

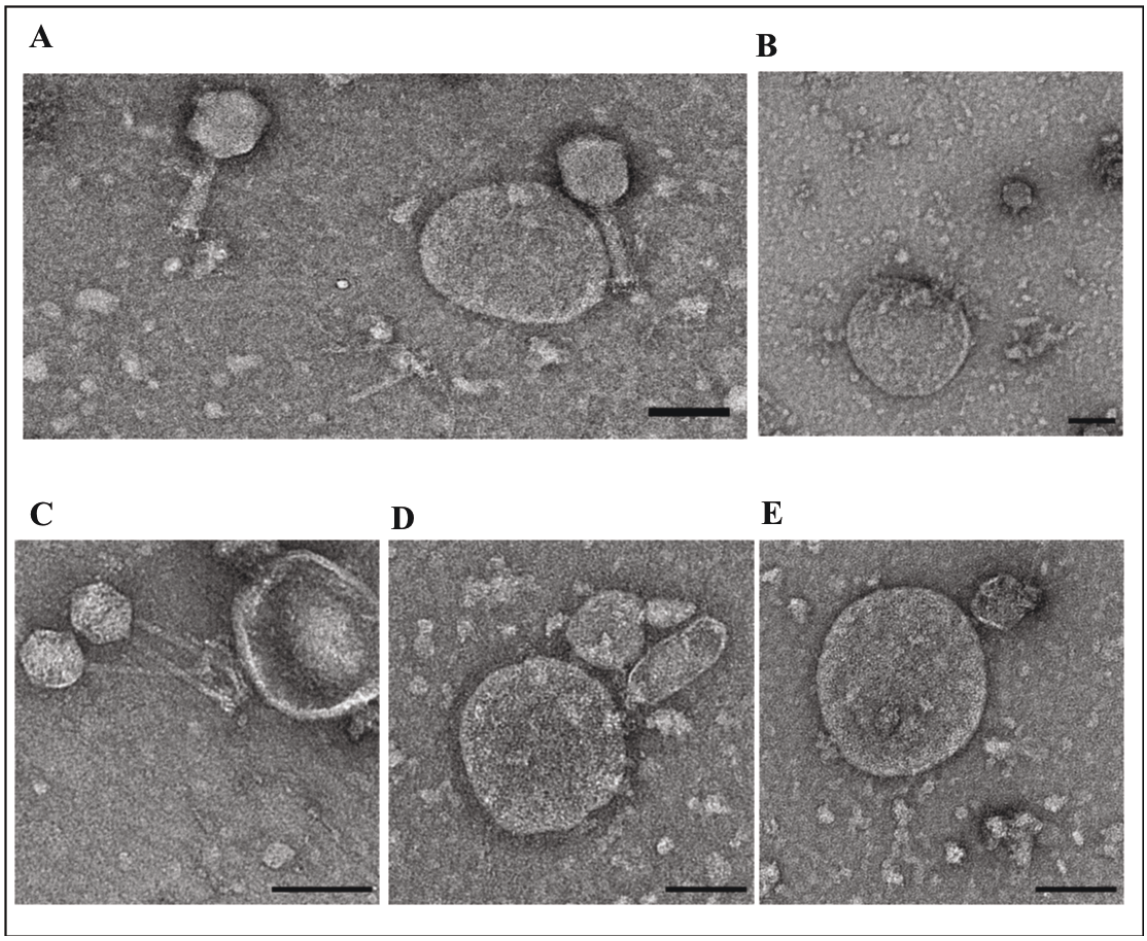

Figure 4. 8: MVs within phage lysates. TEM image of (A) T4 with MV, (B) T7 with MV, (C) Syd1 with MV, (D) Syd6 with MV, and (E) Syd8 with MV. Scale bar is 100 nm.

Using TEM, 50, 62, 27, 73 and 15 random images of MVs in lysates prepared from bacteriophages T4, T7, Syd1, Syd6, and Syd8 respectively were acquired to study the morphological features of the MVs. Analysis of TEM images of MVs revealed that each of the bacteriophages resulted in MVs with variable sizes (Figure 4.9).

Specifically, MVs from T4 were 45-266 nm with an average of 126.8 nm (SD ± 53.9) whereas from T7 were 63-283 nm with an average of 180.9 nm (SD ± 48.0). MVs observed within bacteriophage Syd1, Syd6 and Syd8 were 141 nm (SD ± 45.6), 151.2 nm (SD ± 53.4) and 173.5 nm (SD ± 52.3) respectively. Collectively, the observed sizes of the MVs from all of these bacteriophages was within the range of 50 – 300 nm, which is indeed correlated with the sizes of bacterial MVs mentioned in various literature (Beveridge, 1999, Schwechheimer & Kuehn, 2015, Turnbull *et al.*, 2016, Toyofuku *et al.*, 2019).

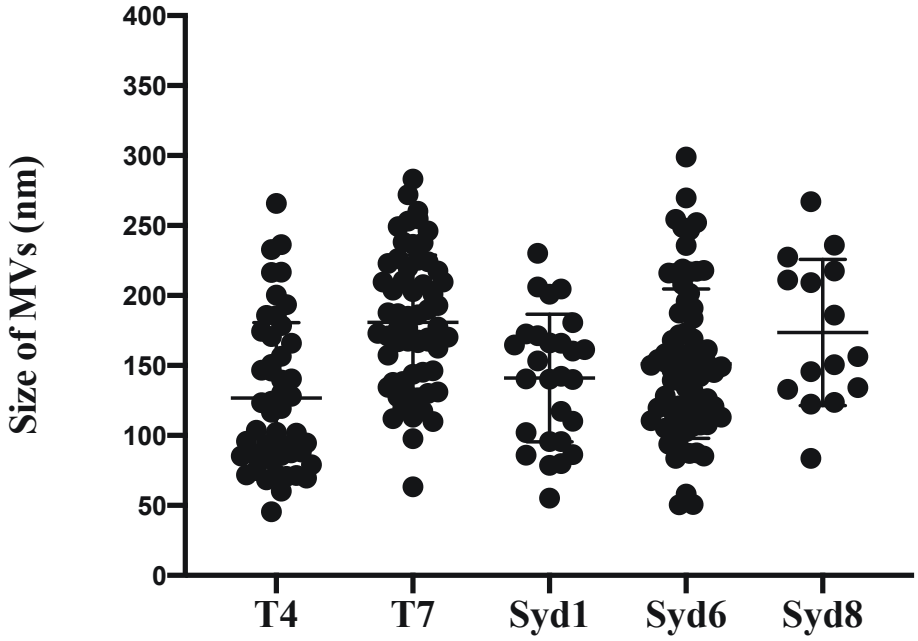

Figure 4. 9: Sizes of MVs observed within different phage lysates. Each black dot represents an individual MV within each of the phage lysates.

The morphological analysis of individual MV within each of the phages further revealed that MVs were not only variable on their size but also differ in their morphological feature. Both the classic outer-membrane vesicles which consist of a single membrane layer and outer-inner membrane vesicles which consist of both inner and outer membrane layers were observed within each of the phage lysates (Figure 4.10). Additionally, some irregular MVs with multiple membrane layers were also observed within each of the phage lysates.

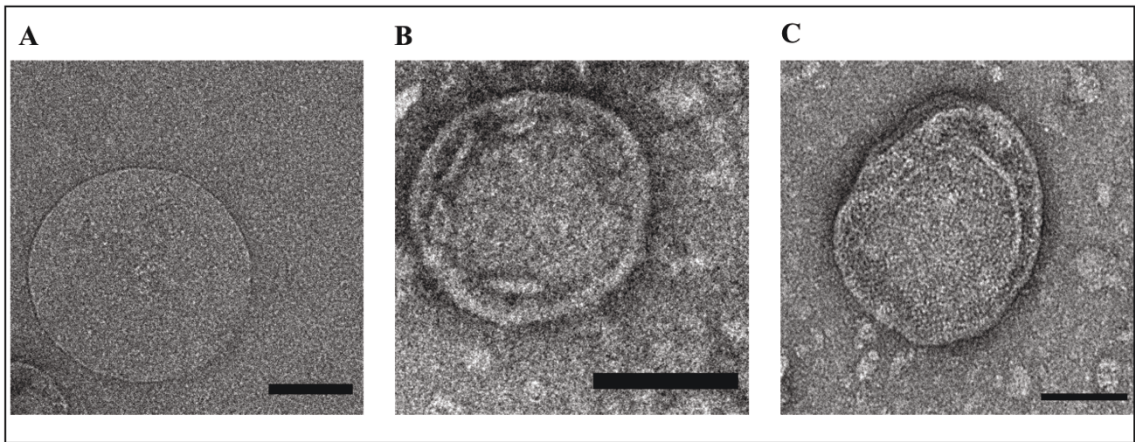

Figure 4. 10: MVs morphotypes. Representative TEM image of (A) Classic OMV (B) OIMV and (C) irregular MV observed within phage lysates. Scale bar is 100 nm.

It is currently thought that blebbing of membrane material of living cells can give rise to classic OMVs whereas explosive cell lysis leads to the formation of outer-inner membrane vesicles (OIMVs) as well as explosive outer membrane vesicles (EOMVs) (Toyofuku *et al.*, 2019). Thus, observations of different morphotypes of MVs within each of the phage lysates revealed that these MVs were the outcome of bacteriophage mediated bacterial lysis. These results presented in this chapter show for the first time that under bacteriophage infection, bacteria can produce MVs through explosive cell lysis as well as membrane blebbing. Together these data indicate that bacterial populations under bacteriophage infection, conditions that are perhaps closer to those found in nature, can result in the formation of different types of MVs.

4.3 Discussion

The results presented in this chapter demonstrated that both T4 and T7 bacteriophages mediate explosive cell lysis of their host *E. coli* MG1655 with a different pattern of lysis. Generally, bacterial cell lysed either at a particular site without any gross morphological changes or become round before lysing. Irrespective of differences in lysis patterns, the lysis event was explosive. Live imaging showed that majority of lysis with T4 occurred at or near polar region without any gross morphological change, whereas with T7 was rounding before bursting. The aforesaid two distinct patterns of bacteriophage-mediated lysis were reported to be associated with bacteriophage holin-lysin system by Berry *et. al.*, (2012), who showed that with lambda bacteriophage consisting of canonical holin-lysin system, the majority of lysis events were of the former type whereas its isogenic hybrid consisting of Pinholin-SAR endolysin caused majority of lysis events of the latter

type. Though at low frequencies, rounding before bursting was also shown with canonical holin lysin system and the direct blowout near the pole or mid cell shown with Pinholin-SAR endolysin system (Berry *et al.*, 2012).

Strikingly, despite having similar lysis systems (canonical holin-lysin system) in both bacteriophage T4 and bacteriophage T7 (Young, 1992, Young, 2014), the results presented here show that these bacteriophages have two distinct lysis patterns. These findings suggested that bacteriophage mediated cell lysis pattern may not solely be associated with the bacteriophage holin-lysin system but could also depend on other factors. Notably, the bacteriophage T4 encoded lysin has muramidase activity (PF00959) that can act on glycosidic bond (between N-acetylmuramic acid and N-acetylglucosamine) linking the amino sugar in the peptidoglycan (Tsugita & Inouye, 1968), whereas the T7 encoded lysin has zinc amidase activity (PF01510) that act on the amide bonds between N-acetylmuramic acid and L-alanine of the cross-linking oligopeptide stems and interpeptide bridge in the peptidoglycan (Inouye *et al.*, 1973). Hence, it can be inferred that this difference within the enzymatic activity of individual phage lysin could be responsible for their dominant type of lysis patterns. Since both of these bacteriophages showed all the different types of lysis pattern, it could also be due to a non-specific accumulation of phage lysin within the cell wall.

Notwithstanding the lysis pattern, the explosive cell lysis of *E. coli* produced MVs from shattered membrane fragments. In concordance with Turnbull *et. al*., (2016) MVs biogenesis after explosive cell lysis was due to inward curling followed by rounding up of the membrane fragments, which ultimately formed spherical MVs, and persisted in the same topology thereafter (Turnbull *et al.*, 2016). Indeed, the process of MV biogenesis through bacteriophage mediated explosive cell lysis in *E. coli* was the same as previously observed in *Pseudomonas aeruginosa.* As the process of MV biogenesis through explosive cell lysis was similar in two different types of Gram-negative bacteria in two different conditions, it is inferred that the process could be common in all Gram-negative bacteria. More importantly, under 3D-SIM live imaging different sizes of MVs producing from lysis of a single cell was observed and the TEM data also correlated with showing variable sizes of MVs within phage lysates.

Moreover, the dynamic nature of explosive cell lysis could also result in the transfer of MVs produced by explosive cell onto the surface of the nearby cells and may thus cause

intercellular molecular exchange including bacteriophage receptors among cells. The observations that shattered membrane fragments generated immediately after explosive lysis transferred onto the surface of the nearby cells where it rounded up producing MVs suggested that this could transfer bacteriophage receptors from one cell to another. Indeed, Tzipilevich *et. al*. (2017) has shown that bacteriophage resistant cells can be lysed by the bacteriophage if co-cultured with the bacteriophage sensitive cells and reported that bacteriophage invasion to resistant cells occurs after lysis of the majority of sensitive cells. They interpreted their finding as the acquisition of bacteriophage sensitivity by resistant bacteria occurred due to exchange of bacteriophage receptors through MVs produced from sensitive cells (Tzipilevich *et al.*, 2017). Since the explosive cell lysis resulted into the binding of the membrane from the lysed cell to the membrane of the non-lysed cell, it can be inferred that the acquisition of membrane-based bacteriophage receptors could occur through the MVs produced from explosive cell lysis.

The observations presented in this chapter also demonstrated that bacteriophage infection not only produces MVs after cell lysis but also trigger MV formation from the cell through a membrane blebbing mechanism. The super-resolution live imaging analyses comprehensively showed that the cell protrudes outer membrane blebs under bacteriophage-infected condition. As membrane blebbing was reported to be as a cell protective mechanism under different kinds of stress (McBroom & Kuehn, 2007, Baumgarten *et al.*, 2012), these observations suggest that this could be the same for the bacteriophage infection. Since the cells with membrane blebs did not lyse, it can be inferred that this could be the bacterial survival mechanism against bacteriophage infection. Whilst much of this information remains to be elucidated at the molecular level, the study reported here advances the knowledge on sources of MVs within bacterial populations and phage lysates.

4.4 Conclusion

Virulent bacteriophages of *E. coli* mediate a lytic burst of the host cell that results in an explosion with the expulsion of the cellular content at a particular site in the cell. Generally, cell lysis may occur either as direct blowout without any gross changes in cellular morphology or in progressive changes in the round morphology before rupturing. Regardless of the differences in lysis pattern, bacteriophage mediated lysis is explosive

and results in shattered membrane fragments that form MVs through self-assemblies of the fragments. Additionally, bacteriophage infected cells that do not lyse can also produce MVs through membrane blebbing. This chapter not only confirmed the observations of MVs within bacteriophage lysates but also provided direct evidence for bacteriophage mediated MVs formation, which underpin the presence of MVs in phage lysates.

5 Development of an optimized bacteriophage purification method.

5.1 Introduction

As bacteriophages can only be propagated from bacteria, this limitation mandated the need for isolating and purifying bacteriophage samples from their bacterial host. There is large range of potential contaminants that can result from propagating bacteriophages, which include media contaminants, bacterial debris, extracellular DNA, and most importantly endotoxins and the MVs that can result from bacterial lysis during bacteriophage propagation process. Both endotoxins and MVs can interfere with the bacteriophage biology studies especially when investigating therapeutic bacteriophage candidates *in vivo*. In terms of physical size, there is a clear overlap with the size of MVs and bacteriophages. In addition to this size overlap, the core steps used for MVs preparation (Klimentová & Stulík, 2015) and for bacteriophage preparation (Yamamoto *et al.*, 1970, Sambrook & Russell, 2001, Bonilla *et al.*, 2016) are the same and as such, bacteriophages and MVs can be co-purified. Several methods have been documented for endotoxin removal from bacteriophages, however most of those procedures lacked generality and also does not account for MVs (Boratynski *et al.*, 2004, Oslizlo *et al.*, 2011, Cooper *et al.*, 2014, Szermer-Olearnik & Boratyński, 2015, Van Belleghem *et al.*, 2017, Hietala *et al.*, 2019). Additionally, it has also been reported that endotoxin removal efficiency is bacteriophage specific and the complete removal of endotoxin is not a trivial task (Van Belleghem *et al.*, 2017).

Previously Triton X-114 was trialled as a method of endotoxin removal for the purpose of investigating the bacteriophage metagenome from pathogenic *E. coli* (Sim and Petty unpublished data). Triton X-114 ($(C_2H_4O)_n$ $C_{14}H_{22}O$, n = 7 or 8) or (1,1,3,3-tetramethylbuityl) phenyl-polyethylene glycol is a non-ionic detergent that is very good for solubilizing membranes and has reversible phase-separation ability. Due to this, it has been utilized for the separation of hydrophilic proteins from hydrophobic lipid membranes (Bordier, 1981, Brusca & Radolf, 1994). Removal of endotoxin from protein samples by extraction with Triton X-114 is based on the principle of two-phase partitioning, in which the TritonX-114-water system is maintained outside the miscibility

area by adjusting the temperature to above 20°C and then separated by centrifugation. Endotoxins partition favorably into the Triton X-114 phase, while the desired hydrophilic protein molecules like bacteriophages remained in the aqueous phase, which can be easily separated (Aida & Pabst, 1990, Adam *et al.*, 1995). Hence, the subsequent rounds of the treatment could potentially remove MVs and endotoxins from bacteriophage samples. Thus, the aim of the work presented in this chapter was to adapt and optimize the method of bacteriophage purification utilizing Triton X-114. This chapter describes the usage of Triton X-114 and ultrafiltration in the bacteriophage purification method for separating MVs from bacteriophages and getting pure bacteriophages with minimal endotoxin level, suitable for therapeutics.

5.2 Results

5.2.1 Total retention of bacteriophage through 100 KDa ultrafiltration

Technically, ultrafiltration with molecular weight cut off 100 KDa (100K MWCO) would allow the capture of all particles of approximately 30 nm in size and thus, is able to capture all bacteriophages. Indeed, ultrafiltration using Amicon® Ultra-15 centrifugal filter device has been used previously for bacteriophage concentration (Bonilla *et al.*, 2016). Thus, 100 KDa ultrafiltration was considered as a key component for bacteriophage concentration in the bacteriophage purification method. As such, the efficiency of 100 KDa Amicon® Ultra-4 centrifugal filter device to retain bacteriophages were first ensured by direct filtering of the lysate of bacteriophage T4, Syd1, Syd6 and Syd8. Ten millilitres each of T4, Syd1, Syd6 and Syd8 was concentrated to 1.5 mL through the Amicon® Ultra-4 centrifugal filter device and the total number of bacteriophages in both the initial lysate and the concentrate was calculated from bacteriophage titre. There was no significant loss observed in total number of bacteriophages after concentration, though there was a slightly less observed in Syd1 and Syd6 (Table 5.1).

Table 5. 1: Bacteriophage quantity in pre and post 100 KDa ultrafiltration.

Bacteriophage sample	Total number of bacteriophages (pfu)	
	In initial lysate	In concentrate
T4	1.35E+12	1.35E+12
Syd1	1.95E+12	1.05E+12
Syd6	2.05E+11	1.05E+11
Syd8	5.0E+11	6.0E+11

In addition, the filtrate of each of the bacteriophage samples was also titrated to determine bacteriophage titre within them. On titration, no bacteriophages were detected in any of the filtrate with any of these bacteriophages. This finding suggested that total number of bacteriophages present within each of the lysates were retained on the filter device. Since only one test was performed with each of the bacteriophage, the low values observed within Syd1 and Syd6 and more with Syd8 could be the user error. Each of these bacteriophages has different dimension and completely distinct morphology, which also represents all three families of the tailed phages. Thus, this result indicated that 100 KDa Amicon® Ultra-4 centrifugal could be effectively used for bacteriophage concentration in the bacteriophage purification protocol.

5.2.2 Adaptation of bacteriophage cultivation process to minimize buffer and media constituents in bacteriophage lysate

To ensure that media constituents that were used for bacteriophage propagation do not leach into the final product, media and buffer used for bacteriophage propagation, namely LB, phage buffer and lambda diluent, had their media constituents examined. For all buffers, the constituents can be filtered through 100 KDa Amicon® Ultra-4 centrifugal filter device, due to its size/and or molecular weight but gelatin (225 bloom, corresponding to between 50 KDa to 100 KDa) might be a problem. To determine if gelatin could affect ultracentrifugation, T4 bacteriophage suspended in a buffer containing gelatin (phage buffer) and a buffer not containing gelatin (lambda diluent) were ultrafiltered and the time required to reach a 50 μL concentrate from 4 mL was measured. Bacteriophage T4 suspended in a buffer with gelatin took 53 minutes 20

Seconds (± 11minutes 33 seconds) to reach 50 µL while T4 suspended in gelatin-free buffer took 46 minutes 40 seconds (± 5 minutes 46 seconds) to reach the same volume (Figure 5.2). There was no statistically significant ($p = 0.4216$; unpaired t-test) difference between both conditions but as the molecular weight of gelatin ranges, this was too unpredictable and thus buffer without gelatin (lambda diluent) should be used for bacteriophage harvesting.

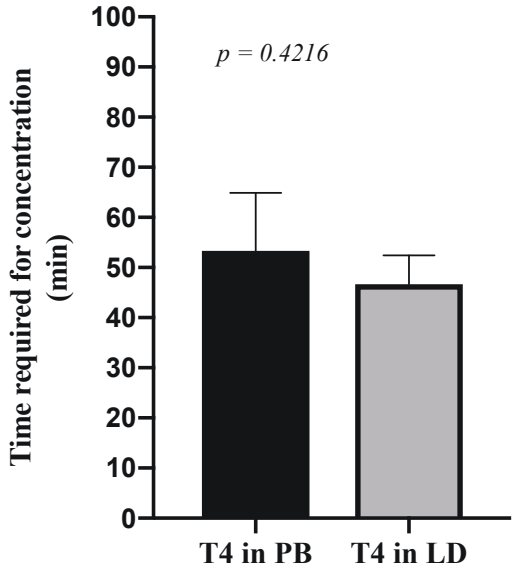

Figure 5. 1: Bacteriophage harvesting buffer and bacteriophage concentration time on 100 KDa ultrafiltration. A bar diagram showing total time required for concentration of 4 mL T4 bacteriophage in phage buffer (PB) and lambda diluent (LD) through 100 KDa ultrafiltration device (n =3).

Since gelatin in phage buffer has been reported as bacteriophage stabilizing agent required for long term storage (Engel *et al.*, 1974, Fortier & Moineau, 2009), the next thing investigated was the bacteriophage stability in lambda diluent. For this, a plaque purified T4 bacteriophage suspended separately in phage buffer and lambda diluent was stored at 4°C and titrated at different days up to 2 weeks to observe for any drop-in bacteriophage titre. On comparison, there were no drop-in bacteriophage titre in both the phage buffer and lambda diluent, indicating that bacteriophage T4 is stable in lambda diluent. The observed bacteriophage stability coupled with the relatively efficient filtering through 100 KDa ultrafiltration suggested that lambda diluent could be the buffer of choice.

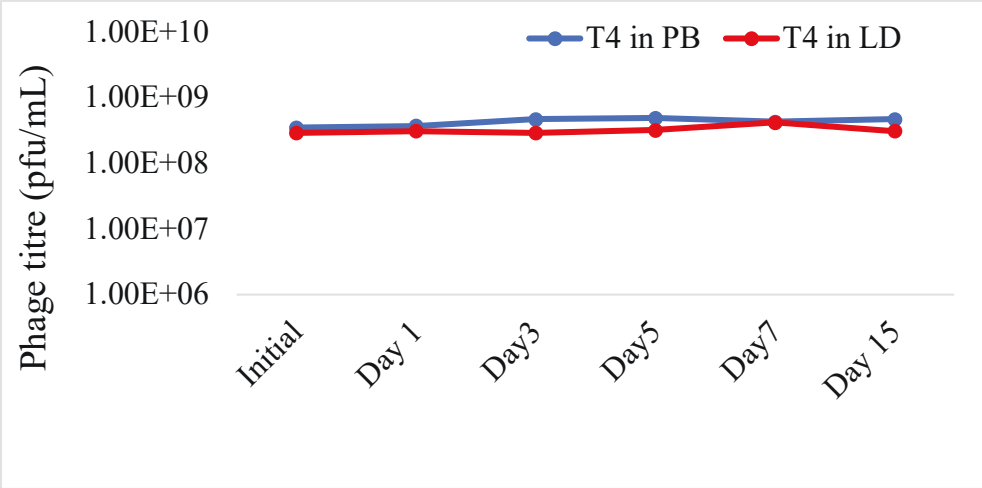

Figure 5. 2: Stability of T4 in phage buffer and lambda diluent. A graph showing T4 phage titre harvested in phage buffer (PB) and lambda diluent (LD) over a time period.

Two different harvesting methods were compared to determine if there was an impact on ultrafiltration. The first method was a top agar scrape method while the second was a slow diffusion method (Sambrook & Russell, 2006). Both methods required the propagation of bacteriophages and the formation of confluent lysis on dual layered agar, but the main difference was in harvesting of bacteriophages from the agar plate. Agar scraping required the scraping of the top overlay agar while diffusion required the flooding of the agar plate with lambda diluent followed by an overnight incubation at 4°C to allow diffusion of bacteriophages into the liquid media (Sambrook & Russell, 2006). From a comparison of both these processes, it was likely that the agar scraping will introduce additional contaminants and residual agar into the system and affect ultrafiltration. To determine the effect of these methods on 100 KDa ultrafiltration, bacteriophage T4 was propagated using both methods and the bacteriophage lysate were each ultra-filtrated. In addition, bacteriophage T4 was also propagated using the liquid media (LB) where entire LB containing bacteriophages was harvested and the lysate was subjected to ultrafiltration. On ultrafiltration, it took 50 minutes (± 10 minutes) for scraping method, 26 minutes 40 seconds (± 5 minutes 46 seconds) for diffusion method, and 26 minutes 40 seconds (± 5 minutes 46 seconds) for liquid method (Figure 5.4).

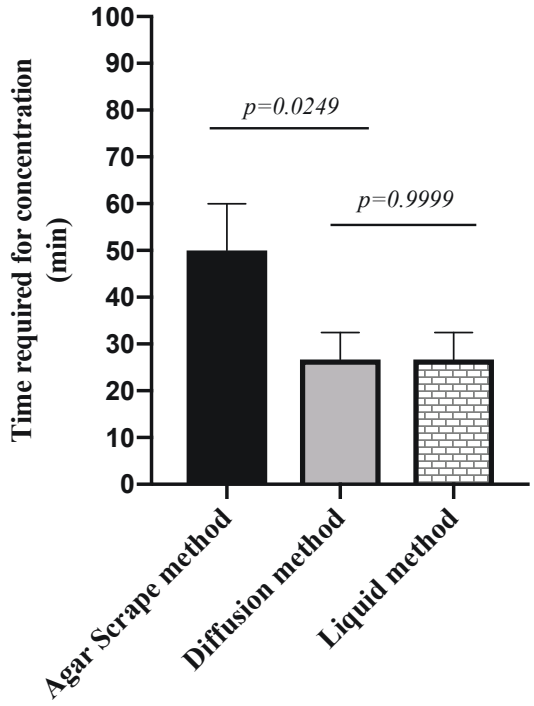

Figure 5. 3: Bacteriophage harvesting method and bacteriophage concentration time on 100 KDa ultrafiltration. A bar diagram showing total time required for concentration of 4 mL T4 bacteriophage harvested by agar scrape method, diffusion method and liquid cultivation method through 100 KDa ultrafiltration device (n =3).

When the timings were compared, there was a significant difference ($p = 0.0249$; unpaired t-test) between the agar scrape and diffusion methods with diffusion being faster, whereas there was no significant difference ($p = 0.9999$; unpaired t-test) between the diffusion method and the liquid cultivation method. While diffusion will add an additional day for the protocol when propagating bacteriophages on solid media, the likelihood of not introducing additional contaminants that could affect ultrafiltration was determined to be more beneficial. Thus, a slow diffusion bacteriophage harvesting method was adopted for the protocol. The liquid cultivation method could also be used if a large volume of bacteriophage is required or bacteriophages can not cultivate well on solid media. However, the liquid cultivation method required a buffer exchange following bacteriophage harvest before endotoxin removal. This is because, in liquid cultivation method bacteriophages were in LB, which would not be suitable for the Triton X-114 treatment and thus need to be exchanged with lambda diluent using an additional ultrafiltration step.

5.2.3 Optimization of Triton X-114 treatment for maximal removal of endotoxins from phage lysate

Endotoxin removal using 1% (v/v) Triton X-114, a method previously suggested for protein purification (Aida & Pabst, 1990) was adapted and evaluated for endotoxin removal from phage lysate. Phage lysate of T4 were prepared and then phase separated with 1% (v/v) Triton X-114 up to five rounds and endotoxin level was quantified after each round by the standard Limulus Amoebocyte Lysate (LAL) test. The initial endotoxin level was 5.56 x 10^4 EU mL^{-1} and after five rounds of Triton X-114 treatment, endotoxin levels were reduced to 96 EU mL^{-1}. However, this reduction was not constant through the five rounds as the majority of the endotoxin was removed by the second round of phase separation with minimal removal from rounds three to five (Table 5.2). To eliminate all traces of endotoxin from phage lysate, endotoxin removal was performed for another five rounds and this brought the endotoxin level down to 6.38 EU mL^{-1} (Table 5.2). Taking the results together, it appeared that endotoxin removal was more efficient in the presence of higher levels of endotoxin, which was in agreement with observations previously made by others (Van Belleghem *et al.*, 2017).

Table 5. 2: Residual endotoxin level and rate of endotoxin removal from successive rounds of 1% Triton X-114 treatment of T4 lysate. Initial endotoxin level = 5.55 x 10^4 EU mL^{-1}.

Triton X-114 treatment rounds	Residual endotoxin (EU mL^{-1})	Rate of endotoxin removal (%)	
		Cumulative	Per round
Round 1	8090	85.45	85.45
Round 2	479	99.13	94.07
Round 3	378	99.32	21.08
Round 4	240	99.57	36.50
Round 5	96	99.82	60
Round 10	6.38	99.988	N/A

Higher final assay concentrations of Triton X-114 including 1% (v/v), 2% (v/v), 3% (v/v), 5% (v/v) and 7% (v/v) were further tested (Table 5.3) to investigate if higher final assay concentrations of Triton X-114 could lead to more efficient removal of endotoxin. As 10 rounds could not remove all traces of endotoxin, the residual endotoxin level was tested after 20 rounds of treatment. It was observed that only 5% (v/v) and 7% (v/v) Triton X-114 could reduce endotoxin levels to below 5 EU mL^{-1} (a maximal level of endotoxin in

pharmaceutical and biological products that is used for intravenous application), indicative that higher concentrations of Triton X-114 can remove endotoxin more efficiently. However, this came with a trade-off as bacteriophage titres for both conditions were lower at 5.4 x 10^6 pfu mL^{-1} and 4.0 x 10^5 pfu mL^{-1} for 5% (v/v) and 7% (v/v) Trion X-114 treatments respectively. After 20 rounds of endotoxin removal, lysates treated with 1% (v/v), 2% (v/v) and 3% (v/v) Triton X-114 each had bacteriophage titres at 10^7 pfu mL^{-1} and residual endotoxin levels ranging from 5.09 EU mL^{-1} to 7.79 EU mL^{-1}.

Table 5. 3: Residual endotoxin levels and bacteriophage titres from successive rounds of endotoxin removal from a phage lysate with varying concentration of Triton X-114.

Number of rounds	Residual endotoxin (EU mL^{-1})	Phage titre (pfu mL^{-1})
1% (v/v) Triton X-114		
20 rounds	7.79	3.3 x 10^7
30 rounds	5.98	1.0 x 10^6
2% (v/v) Triton X-114		
20 rounds	6.99	3.10 x 10^7
30 rounds	5.54	1.6 x 10^6
3% (v/v) Triton X-114		
20 rounds	5.09	3.3 x 10^7
30 rounds	0.61	5.0 x 10^5
5% (v/v) Triton X-114		
20 rounds	2.69	5.4 x 10^6
7% (v/v) Triton X-114		
20 rounds	0.51	4.0 x 10^5

To get an endotoxin level below 5 EU mL^{-1}, lysates treated with 1% (v/v), 2% (v/v) and 3% (v/v) Triton X-114 were further processed for another 10 rounds. At round 30, all three conditions showed reductions in endotoxin levels with the largest drop being the lysate treated with 3 % (v/v) Triton X-114. However, this also had a bacteriophage titre lower (Table 5.3). Taken together, these results indicated that higher final assay concentrations of Triton X-114 could remove endotoxin more efficiently but bacteriophage titre would be negatively impacted. Similarly, increase in number of rounds would also remove more endotoxin but that could also result in reduced bacteriophage titre. As the majority of endotoxins were removed within 10 rounds of

treatment with 1% (v/v) Triton X-114 and increasing final assay concentrations of Triton X-114 and/or number of rounds negatively impacted on bacteriophage titre, the protocol adopted a 1% (v/v) Triton X-114 treatment for 10 rounds for endotoxin removal.

5.2.4 Residual Triton could be removed through ultrafiltration

The presence of residual Triton within Triton X-114 treated protein samples has been reported previously (Teodorowicz *et al.*, 2017, Van Belleghem *et al.*, 2017). This could be toxic to cells if the level is above its critical micelle concentration (0.01%). Hence, the ability to remove Triton X-114 was another major consideration for the protocol. Since Triton X-114 has maximum absorbance at 280 nm similar to that of protein, protein free samples are required for Triton quantification. Thus, residual Triton within Triton X-114 treated bacteriophage samples was measured by quantifying Triton concentrations in protein free solutions using spectroscopic absorbance at 280 nm (Teodorowicz *et al.*, 2017). Lambda diluent was treated with 1% (v/v) Triton X-114 up to five rounds (n =3) and after each round the aqueous layer was quantified for residual Triton. Quantification of Triton X-114 showed a residual level of 0.014 % (± 0.0008 %) after the first round of Triton X-114 treatment, whereas from round two onwards the level was only slightly decreased after each round (Figure 5.5). Residual Triton X-114 after five rounds was 0.010 % (± 0.0002 %). However, treatment of the samples for another five rounds (in total 10 rounds as per requirement of the protocol), showed a residual Triton level of 0.011 % (± 0.0013 %). This indicated that there could be residual Triton X-114 left within the bacteriophage preparation post endotoxin removal and the level would be around its critical micelle concentration.

As Triton X-114 has an average molecular weight of 534 Da, it was likely that it could be filtered out by ultrafiltration with molecular weight cut off 100 KDa. To investigate if residual Triton X-114 could be removed by ultrafiltration the same samples treated with 10 rounds of 1% Triton X-114 (n =3) were subjected to ultrafiltration followed with washing with lambda diluent. The 100 µl concentrate thus obtained was quantified (n=3) for residual Triton and it was found to be 0.0004 % (± 0.0001 %), which was way below the critical micelle concentration.

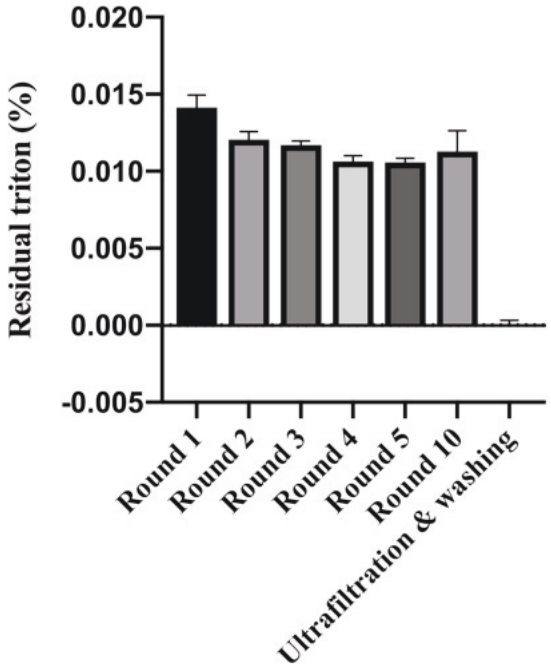

Figure 5. 4: Residual Triton X-114 in bacteriophage sample. A bar diagram showing level of residual Triton X-114 quantified in control sample from successive rounds of 1% Triton X-114 treatment and ultrafiltration.

5.2.5 Residual endotoxin removal through ultrafiltration

Since, ultrafiltration using 100 KDa filters has been used for endotoxin removal from different solutions (Sweadner *et al.*, 1977, Jang *et al.*, 2009), the ability for the 100 KDa Amicon® Ultra-4 centrifugal filter device to remove residual endotoxin in bacteriophage samples after Triton treatment rounds was investigated. Briefly, a pooled sample consisting of previous endotoxin treated bacteriophage T4 samples, was put through a single round of ultrafiltration. Similarly, an endotoxin spiked lambda diluent was also put through a single round of ultrafiltration. When quantified by LAL test, the initial pooled T4 sample possessed an endotoxin level of 4.97 EU mL^{-1} while the spiked lambda diluent possessed an endotoxin level of 2.36 EU mL^{-1}. After one round of ultrafiltration, the endotoxin level of both samples dropped to 0.20 EU $mL^{-1}$and 0.16 EU mL^{-1} for the pooled T4 sample (95.85% decrease) and spiked lambda diluent (93.05% decrease) respectively. This result suggested that ultrafiltration could also reduce residual endotoxin levels if performed after endotoxin removal with Triton treatments. Taken together, these results indicate that ultrafiltration with the 100 KDA MWCO filter should be performed after endotoxin removal.

5.2.6 Designing an optimized protocol for bacteriophage purification

Based on the results of all the aforementioned parameter testing, a bacteriophage purification protocol was designed centring on a previously established method of removing endotoxin by Triton X-114 as well as ultrafiltration. This process will take between five to six days and there are two workflows: one for bacteriophages that propagate well in solid media and one for bacteriophages that propagate in liquid culture (Figure 5.6). Propagation in liquid media, would require two rounds of ultrafiltration with the first ultrafiltration occurring post-harvest and the second ultrafiltration occurring post endotoxin removal by Triton X-114. This was because phase separation cannot properly occur in LB which necessitated a buffer exchange. Also based on the parameter testing, the concentration of Triton X-114 used for validation was 1% (v/v) over 10 rounds of phase separation. The general outline of the developed bacteriophage propagation and purification method is illustrated in Figure 5.6 and the detail protocol is described in Appendix 13.

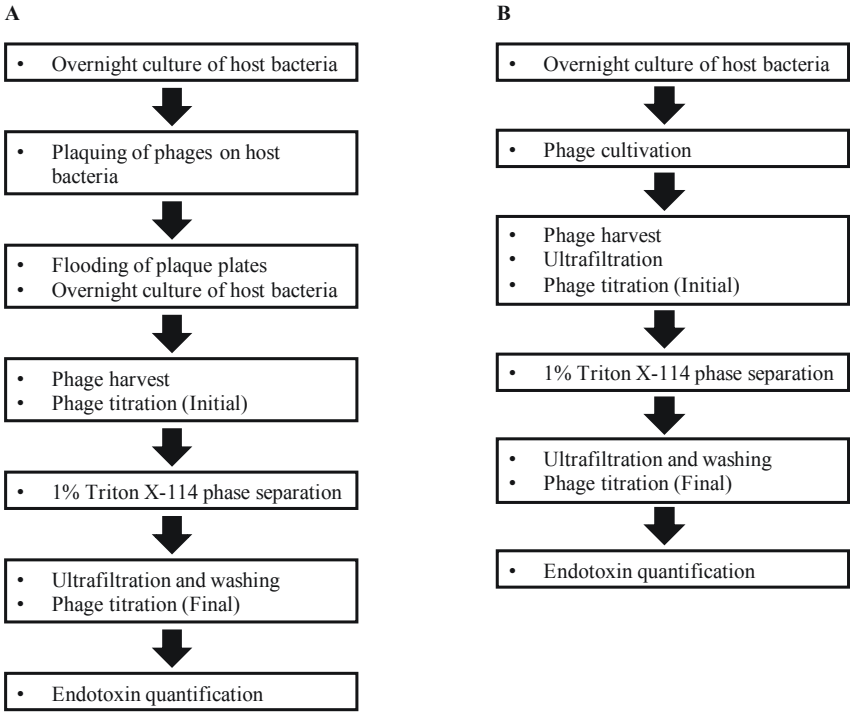

Figure 5. 5: A schematic representation of the optimized bacteriophage propagation and purification method. (A) for bacteriophages that propagate in solid media and (B) for bacteriophages that propagate in liquid media.

5.2.7 Validation that the optimized bacteriophage purification protocol reduces levels of Triton, endotoxin and membrane vesicles

To validate the optimized bacteriophage purification protocol (Appendix 13), the standard *E. coli* bacteriophage T4 and *E. coli* ST131 bacteriophages Syd1, Syd6 and Syd8 were harvested and purified using the optimized protocol and assayed for residual Triton, endotoxin and membrane vesicles. No residual Triton was detected within any of the control samples (n =4). The endotoxin levels within each of the bacteriophage harvests before purification was more than 10^5 EU mL^{-1} except the bacteriophage T4 (Figure 5.7A) and the highest level was detected within Syd8. The endotoxin level within each of the purified samples was more than 10 EU mL^{-1} except for bacteriophage T4 and the highest level detected was with bacteriophage Syd6 (Figure 5.7A). The titration of each of the bacteriophage samples showed the expected reduction in bacteriophage titre after purification and the amount of reduction in titre varied among the different bacteriophages. However, the reduction in titre was within 1 to 2-log scale and the titre of each of the purified bacteriophage samples was around 10^{10} pfu mL^{-1} (Figure 5.7B).

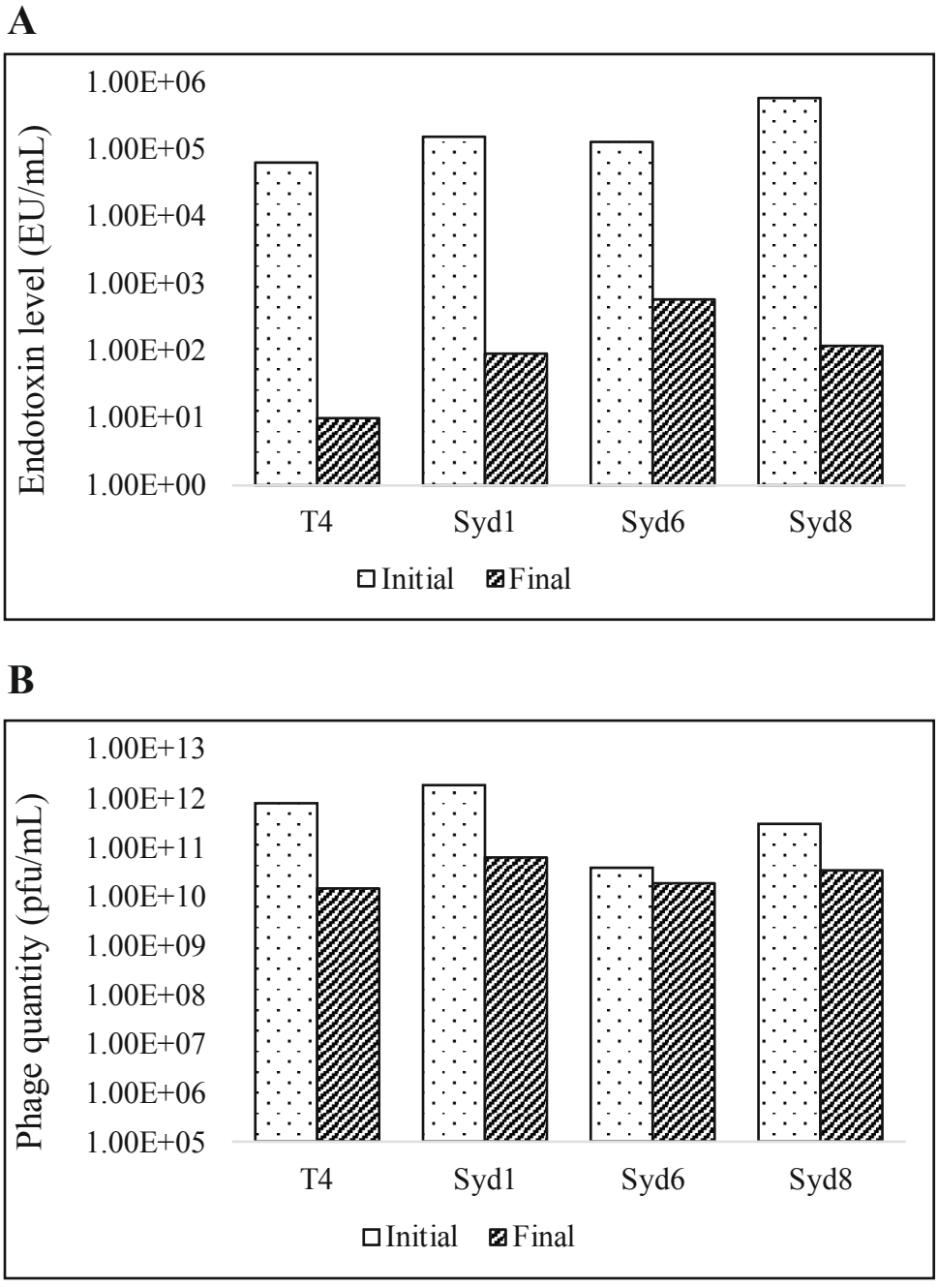

Figure 5. 6: Endotoxin level and bacteriophage quantity pre and post purification. A bar diagram showing (A) the endotoxin concentration (EU mL^{-1}), and (B) the bacteriophage quantity (pfu mL^{-1}) within each of the bacteriophage sample before and after purification.

The data obtained from the validation of the optimized method of bacteriophage purification with different bacteriophages revealed that the removal of endotoxin below 10 EU mL^{-1} was not achievable by this protocol, though it could remove 4 to 5-log endotoxin from the initial bacteriophage harvest with an expected 1 to 2 logs reduction in titre to a final titre of around 10^{10} pfu mL^{-1} . Since, a titre around 10^{8} pfu mL^{-1} is usually used as a therapeutic bacteriophage titre (Cerveny *et al.*, 2002, Huff *et al.*, 2006,

Merabishvili *et al.*, 2009), the endotoxin concentration within each of the sample was further normalized in the form of endotoxin per plaque forming unit by dividing the endotoxin concentration by the bacteriophage titre. Normalization of the endotoxin level within purified samples in the form of EU per 10^8 pfu mL^{-1} revealed the endotoxin level ≤ 5 EU mL^{-1} in all the purified bacteriophage samples (Figure 5.8). Thus, dilution of the purified sample will be recommended for further lowering of the endotoxin to desirable levels.

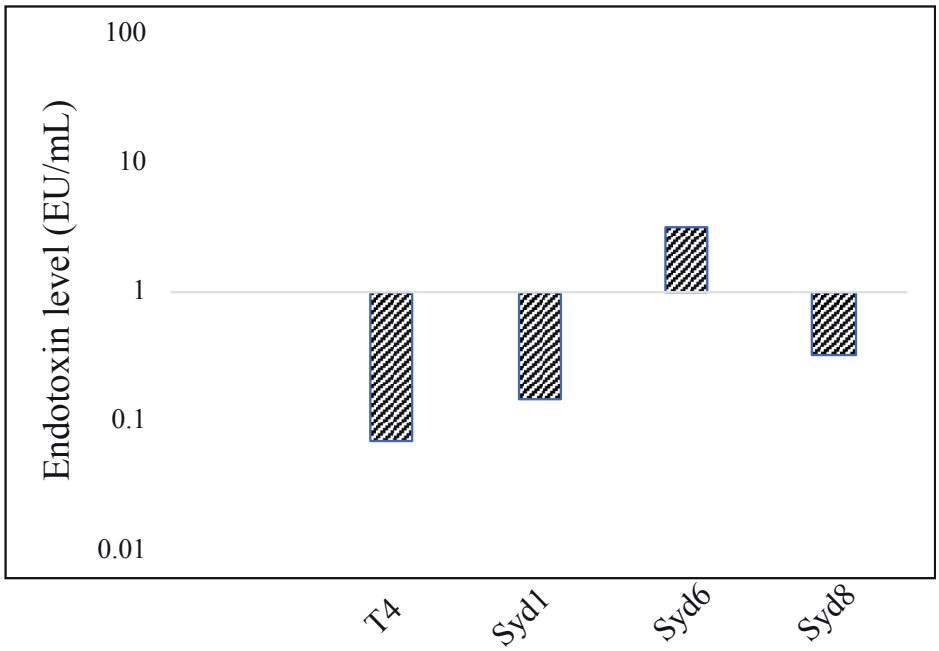

Figure 5. 7: Normalized endotoxin level within purified bacteriophage preparation. A bar diagram showing the endotoxin concentration (EU mL^{-1}) within purified bacteriophages, normalized against a phage therapeutic titre 10^8 pfu mL^{-1}.

The transmission electron microscopy of purified *E. coli* ST131 bacteriophages Syd1, Syd6 and Syd8 including Bacteriophage T4 confirmed the level of purity of these bacteriophages. As can be seen in the Figure 5.8, purified bacteriophages had a clean background. In contrary, the bacteriophage from crude lysates (an initial filtrate of the bacterial cell lysates obtained from bacteriophage infection) had a lots of cell debris and possible endotoxins. More importantly, as mentioned in Chapter 4, MVs were also observed within each of the crude bacteriophage lysates but no such structures were observed within any of the purified bacteriophage samples. These results clearly indicated that the method not only lowers the endotoxin from bacteriophage preparation but also remove MVs successfully.

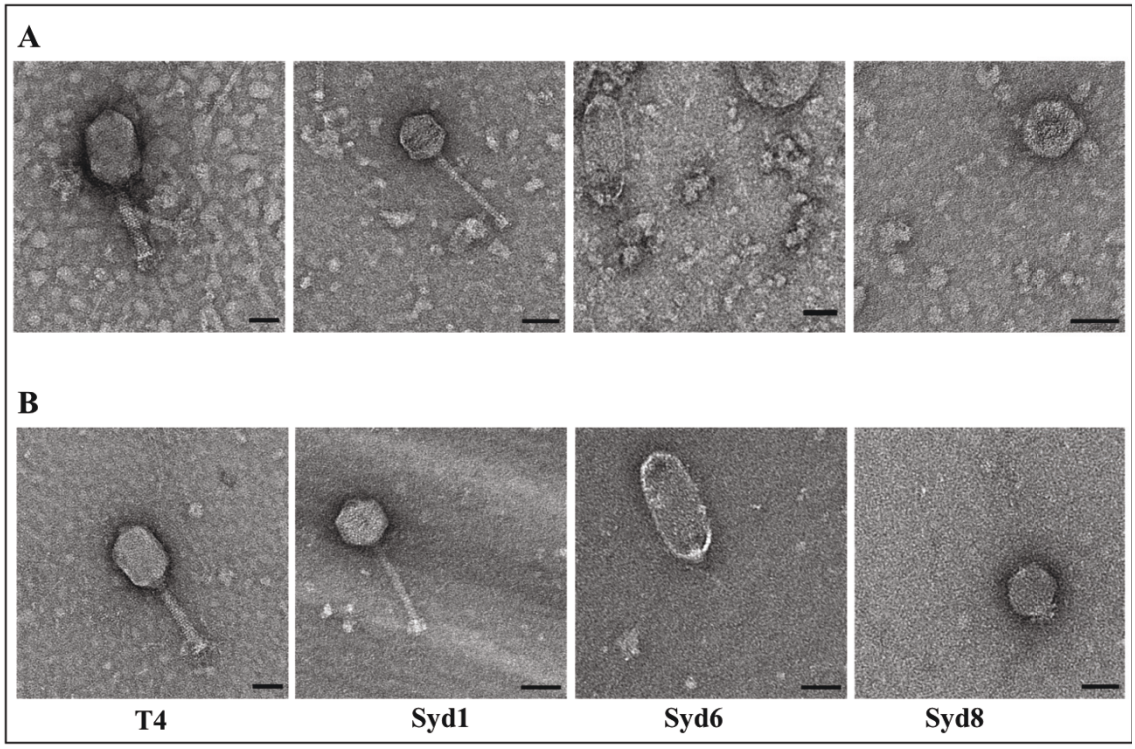

Figure 5. 8: Electron micrograph of crude and purified bacteriophages. Transmission electron microscopic images of various bacteriophages in (A) crude phage lysates, and (B) purified phage preparation. Image labelled with respective bacteriophage name and scale bar is 50 nm.

5.3 Discussion

This chapter described on the optimisation of a bacteriophage purification protocol that could be utilised to remove endotoxins together with MVs from bacteriophage preparation. The pipeline approach of combining different established methods with adaptation was used to achieve pure bacteriophage free from MVs. Combination of the modified bacteriophage cultivation method, ultrafiltration for bacteriophage concentration and utilization of a Triton X-114 phase separation protocol for endotoxin removal is a unique approach for bacteriophage preparation in the laboratory. Specifically, as Triton X-114 is a non-ionic detergent, it is very good for solubilizing membranes and thus not only remove endotoxins but also the membrane vesicles from the phage lysates through phase separation (Bordier, 1981, Brusca & Radolf, 1994).

This bacteriophage purification methodology presents various significant advantages in comparison to traditional methods. Firstly, bacteriophages that are sensitive to components of traditional methods such as caesium chloride (Sillankorva *et al.*, 2010)

and chloroform (Olsen *et al.*, 1974, Taniguchi *et al.*, 1984) can be purified by this method. However, as with all reagents, it would be prudent to test the viability of the bacteriophage of interest in Triton X-114 before proceeding. In addition, this protocol does not make use of ultracentrifugation, an instrument not readily available to all laboratories, and thus allowing this method to be simple and easily adopted by research groups.

Moreover, the traditional method for bacteriophage purification and the method for MVs preparation are virtually similar to each other. As such it is hard to separate MVs from bacteriophages using the standard traditional methods. This problem is further highlighted when MVs-like structures can be seen in some bacteriophage electron micrographs indicating that bacteriophage purification methods are likely to co-purify bacteriophage and MVs (Sullivan *et al.*, 2005, Guang-Han *et al.*, 2016, Schiettekatte *et al.*, 2018). Presence of MVs within bacteriophage preparation not only decoy the bacteriophage activity (Manning & Kuehn, 2011, Reyes-Robles *et al.*, 2018) but also can induce immunomodulatory effects if used for *in vivo* studies (Alaniz *et al.*, 2007, Codemo *et al.*, 2018). It is therefore mandatory to remove MVs together with the endotoxins from the bacteriophage preparation prior to performing any *in vivo* studies for phage therapy.

The method presented here utilized Triton X-114, which indeed solubilizes membranes and extracts the fragments through phase separation and thus can efficiently remove the MVs. Indeed, the TEM based qualitative analysis of the purified bacteriophages T4, Syd1, Syd6 and Syd8 did not show any MVs within any of them. Each of the purified bacteriophages had a clear background free from the debris. These results suggested that MVs were extracted from each of the bacteriophage samples during the rounds of Triton X-114 phase separation. Thus, it can be interpreted that the optimized method developed here removes the MVs from the bacteriophage preparation. However, complete removal of MVs can not be claimed only from the TEM data, and further quantitative analyses of MVs within purified bacteriophage preparations is required. Whilst more work needs to be done for direct quantification of MVs within purified bacteriophage samples, the results presented here strongly suggest that this method could be very useful for separation of MVs from bacteriophages.

The other advantage of this method is the removal of endotoxin, which will provide assurance that bacteriophage preparations to be used for downstream therapy applications are free of the toxic effects of endotoxin. It has been previously noted that complete

removal of endotoxin is not only a trivial task but efficiency of endotoxin removal is also bacteriophage specific (Van Belleghem *et al.*, 2017). The results presented here showed that this also held true for this protocol as not all bacteriophages showed similar efficiency in removal of endotoxin. In addition, the method also showed that there will be some bacteriophage lost during processing which can be up to a two-log reduction in titre. Barring the possibility of bacteriophage loss due to Triton X-114 sensitivity, a possible explanation for bacteriophage loss post successive rounds of endotoxin removal lies at the removal of the aqueous phase during Triton X-114 phase separation. As with all methods that require the removal of the aqueous phase, there is a trade-off as to how much of the aqueous phase to aspirate as the closer aspiration occurs towards the interphase the more sample is recovered but there will also be a risk of aspirating contaminants. Hence it is prudent to sacrifice some aqueous phase to avoid contamination.

Importantly, there are ways to modify the protocol to suit the needs of the end-user. The number of rounds of Triton X-114 treatment can be modified and end-users can include as many rounds as required. However as reported in the parameter testing (Section 5.2.3), bacteriophage titres will be negatively affected as the number of rounds of phase separation increases. A way to counteract this loss in titre is to have a larger amount of starting bacteriophages, keeping in mind that titre lost can be up to three logs drop for 30 rounds of phase separation.

5.4 Conclusion

The optimized bacteriophage preparation and purification protocol proposed here is simple and user friendly. It does not require specialised instruments and reduces levels of contaminants (triton, endotoxin, MVs) to levels acceptable for therapeutic applications. Whilst more work needs to be done for direct quantification of MVs within purified bacteriophages, the endotoxin quantification together with the TEM based qualitative analysis of purified bacteriophages suggest that the method could remove MVs completely. Thus, this protocol will be useful not only for bacteriophage biologists or microbiologist but also researchers interested in metagenomics or microbiome studies which involves bacteriophages. The implementation of this method in genomic/metagenomic studies could be useful to infer conclusions on bacteriophages and/or possible DNA of interest carried by bacteriophages.

6 General discussion

6.1 Overview

The emergence of the multidrug-resistant (MDR) bacterial pathogens that are resistant to most of the available antibiotics is an urgent threat in the clinical setting. The WHO has published a list of high-risk pathogens that can pose a significant threat in both the clinical and community settings. Notably, the Gram-negative bacteria *Acinetobacter baumannii, Pseudomonas aeruginosa,* and Enterobacteriaceae including *Escherichia coli* are prioritized as critical pathogens because of their resistance to the last line antibiotic carbapenems (Tacconelli *et al.*, 2018). The need for novel antibacterial agents is essential to control these superbugs but there have been no new antibiotics approved in the past few decades (Conly & Johnston, 2005, Fernandes, 2006). Furthermore, there has been a trend of development of bacterial resistance against the used antibiotics within a very short period (CDC, 2013, Ventola, 2015). It is therefore essential to have alternative non-conventional approaches instead of the classical antibacterial agents to control such multidrug-resistant bacteria. Various non-conventional approaches have been identified and one of the potential options is phage therapy that uses bacteriophages to combat MDR bacterial pathogens (Czaplewski *et al.*, 2016, Rohde *et al.*, 2018, Kortright *et al.*, 2019).

Phage therapy relies on the isolation and use of naturally occurring bacteriophages from environmental sources. As the newly isolated bacteriophages should be strictly virulent for phage therapy (Weber-Dabrowska *et al.*, 2016), bacteriophage candidates for phage therapy must be screened genetically for any genes that could be responsible for the temperate lifestyle. Also, they should be free from high homology gene sequences from the pathogenic bacteria and should not be responsible for any horizontal gene transfer including transduction (Pelfrene *et al.*, 2016). Furthermore, it is also necessary to check the *in vitro* host ranges among various bacterial pathogens as well as commensal strains and to evaluate *in vivo* in animal models for their efficacy and safety (Loc-Carrillo & Abedon, 2011, Elbreki *et al.*, 2014). In general, natural therapeutic phages should meet basic criteria regarding pharmacodynamics and pharmacokinetics, i.e., they should have a perfect antibacterial effect, less or no potential side effects in the patient and good ability to reach the target bacterial pathogen *in situ* (Abedon & Thomas-Abedon, 2010).

The main focus of this thesis was to investigate natural bacteriophages effective against one of the high-risk multidrug-resistant globally disseminated bacterial pathogens, *E. coli* ST131, and to explore their possible usage in phage therapy. This included an investigation that illustrated the morphological, genomic and host range characterization of three bacteriophages, namely Syd1, Syd6, and Syd8, to examine their ability to kill *E. coli* ST131 strains. This work also investigated the sources of possible MVs contaminants in these bacteriophage preparations, and the purification process needed to obtain therapeutic grade bacteriophage preparations suitable for further studies. The following sections of this chapter briefly discuss the significance of the findings in this study and provide insights on the selection and preparation of bacteriophages for phage therapy.

6.2 New bacteriophages Syd1, Syd6 and Syd8 are potential candidates for phage therapy against *E. coli* ST131

Chapter 3 of this Thesis focused on characterization of the bacteriophage Syd1, Syd6, and Syd8. Genomic analyses revealed that each of these bacteriophages did not possesses any lysogeny module genes nor any toxin-encoding or antibiotic resistance mediating genes. Thus, based on the available guidelines on bacteriophage selection for phage therapy (Lobocka *et al.*, 2014, Pirnay *et al.*, 2015), all three bacteriophages are potential candidates to be used in phage therapy against *E. coli* ST131 strains. More importantly, the comparative whole-genome analysis and morphological features of each of these bacteriophages confirmed them to be completely distinct from each other and also from other previously identified *E. coli* ST131 infecting bacteriophage (Dufour *et al.*, 2016, Green *et al.*, 2017). Though each of these bacteriophages is similar to a particular group of previously published bacteriophage, they each possessed a completely different host recognition gene from their closely related bacteriophage groups. Similar results have been reported with another ST131 infecting bacteriophage LM33_P1, which is also highly similar to a group of bacteriophages from the database but has a distinct host recognition protein (Dufour *et al.*, 2016). It is known that bacteriophage utilize its host recognition protein (tail spike and/or tail fibre) to bind specifically to the bacterial surface receptor (Wang *et al.*, 2000, Walter *et al.*, 2008, Garcia-Doval & van Raaij, 2012). Thus, the differences in the gene encoding the host recognition proteins of all of these

bacteriophages are suggestive of their affinity for receptors present on the surface of ST131 strains.

The *in vitro* host range and efficiency of plating tests showed that each of the three-bacteriophage demonstrated different host range infectivity patterns within E. *coli* ST131 strains. These findings are similar to the results that have been reported previously with the other ST131 infecting bacteriophage HP3, CF2, and EC1, all of which showed differences in their host range infectivity patterns (Green *et al.*, 2017). The host range infectivity pattern of a bacteriophage reveals its specificity, which depends on the specific interaction between the bacterial cell surface receptor and the bacteriophage receptor binding proteins (Casjens & Molineux, 2012, Chatterjee & Rothenberg, 2012). Generally, bacteriophage utilize bacterial cell surface structural component as their specific receptor which include (i) Major structural components such as capsule, lipopolysaccharides, pili and flagella, and (ii) Outer membrane proteins such as structural proteins, transport proteins, and porins (Rakhuba *et al.*, 2010, Bertozzi Silva *et al.*, 2016). So, the most parsimonious explanation of the differences in the observed host range infectivity patterns of these three bacteriophages is that each bacteriophage displays a host range limited to those *E. coli* ST131 strains that possess the particular surface receptor specific for each bacteriophage.

Notably, no single bacteriophage alone exhibited lysis of all ST131 strains tested. This result agrees with previous findings on other ST131 infecting bacteriophages reported by Dufour *et al.* (2016) and Green *et al.* (2017). All these findings together indicate that it is not trivial to find a single bacteriophage which could infect all ST131 strains. The most plausible reason for this is the existing genotypic and phenotypic variations within the ST131 strains. There are various clades and subclades of ST131 that have differences in their structural components such as O- antigen (Price *et al.*, 2013, Petty *et al.*, 2014), capsule loci (Alqasim *et al.*, 2014, Petty *et al.*, 2014), variants in type I fimbriae (*fimH* variants) (Nicolas-Chanoine *et al.*, 2014, Petty *et al.*, 2014), and other surface proteins due to these genes encoded in mobile genomic elements (genomic islands, prophages and plasmids) (Ben Zakour *et al.*, 2016). The *E. coli* EC958 used for isolation of bacteriophage Syd1, Syd6, and Syd8 contains various mobile genomic elements, most of which are not well conserved in all the three clades of ST131 (Petty *et al.*, 2014, Shaik *et al.*, 2017). Specifically, there has been 7 genomic islands (GI-*SelC*, GI-*leuX*, GI-*thrW*,

GI-*pheV*, ROD-1, ROD-2, and ROD-3) and 8 prophages including one cryptic prophage reported in the genome of EC958 (Totsika *et al.*, 2011, Forde *et al.*, 2014). Though these genomic islands and prophages are conserved in the ST131 clade C strains (Petty *et al.*, 2014), GI-*SelC,* prophage 6 and prophage 8 region are present only in some C2/H30-Rx and are absent in C1/H30R subclades (Matsumura *et al.*, 2016). Furthermore, genomic islands may contain genes encoding various bacteriophage defence systems like the toxin-antitoxin system, and R-M system, which could enable different strains to escape bacteriophage infection (Makarova *et al.*, 2011). Likewise, prophages may also contain various membrane proteins and superinfection immunity proteins that could protect the bacteria from infection by various bacteriophage and thus also affect the bacteriophage host infectivity pattern (McGrath *et al.*, 2002). It is therefore plausible that the differences in genomic islands and prophages within ST131 strains could be responsible for preventing infection by a single bacteriophage type on all ST131 strains.

Interestingly, the bacteriophage Syd6 was also able to infect an *E. coli* ST69 strain indicating that it could target a receptor common on both ST131 and ST69 strains. Importantly, within the five most common lineages of *E. coli,* ST69 is the only other lineage that also includes multidrug-resistant strains (Kallonen *et al.*, 2017). Hence, it is possible that the bacteriophage Syd6 may target a surface receptor that could also be present on the surface of multidrug-resistant ST69 strains. As the assignment of a Sequence Type in *E. coli* via the MLST scheme is based on the sequence of seven house-keeping genes, it does not represent the entire bacterial genome (Urwin & Maiden, 2003, Wirth *et al.*, 2006). Thus, it is likely that difference ST may consist of multiple similar genes and the strain from the same ST may have different gene contents especially due to the mobile genomic elements. The strains susceptible to infection by a particular bacteriophage must possess similar target bacteriophage receptors and thus must have similar genes for the respective bacteriophage target. Absence or mutation within these genes will lead to the abolishment of susceptibility to infection by that bacteriophage. Therefore, identifying the bacterial genes involved in a particular bacteriophage infection is useful for the selection of bacteriophages for therapeutics cocktails against targeted bacterial pathogens. Phage cocktails consisting of bacteriophages targeting different types of receptors not only broaden the host range but also are more effective in delaying the emergence of bacteriophage resistant bacteria (Chan *et al.*, 2013, Bai *et al.*, 2019).

As bacteriophages Syd1, Syd6 and Syd8 are likely to target different receptors, it will be worth identifying the receptors of these bacteriophages to design a phage cocktail against ST131. Bacteriophage receptors can be identified through the traditional approach by constructing a series of deletion mutants in host bacteria through site-directed mutagenesis and studying bacteriophage infection in the mutant (Hong *et al.*, 2008, Filippov *et al.*, 2011) or using a high-throughput method like random transposon mutagenesis (Shin *et al.*, 2012, Kim *et al.*, 2019) or transposon-directed insertion site sequencing (TraDIS) (Cowley *et al.*, 2018). In addition to these approaches, comparative genomic analysis of all the strains tested for bacteriophage infectivity could be another crucial way to see if a common target could be predicted within a set of bacteriophages sensitive bacteria. This predicted bacteriophage receptor could then be confirmed by selective gene knockout and complementation experimentation. Thus, comparative genomic analysis of all the *E. coli* ST131 strains tested for bacteriophage infectivity of Syd1, Syd6 and Syd8 will be an important future work to identify receptor of each of these three bacteriophages.

The spontaneous bacterial mutants isolated against each of these three bacteriophages showed two distinct patterns on cross infectivity by those bacteriophages. In the first type, the mutant resistant to one bacteriophage was susceptible to infection with another bacteriophage whereas in the second type the mutant resistant to one bacteriophage also showed resistance to other and vice versa. The first type of cross-infectivity pattern suggests that the bacteriophage resistant mutant could likely have the mutation in gene encoding the bacteriophage receptor whereas the second type suggests resistance might be due to acquired anti-phage immunity. The EC958 strain does not contain any complete CRISPR/Cas loci and immunity due to abortive infection would lead to suicidal death providing a lysis from without type reaction. It is therefore unlikely that either of these could be the mechanism of bacteriophage resistance for any of resistant mutant strains identified in this study. Furthermore, all three bacteriophages infected the wild type EC958 strains successfully despite the presence of various types of R-M system, including orphan methylases on different mobile genomic elements in EC958 (Forde *et al.*, 2015). So, it is also unlikely that any of these systems could have any role in the spontaneous bacteriophage resistance mechanism.

Therefore, comparative genomic analysis of the wild type and spontaneous bacteriophage resistant bacterial mutant using whole-genome sequencing will be an important future step to determine if the mutation occurs in bacteriophage receptor gene/s. Identifying the mechanism responsible for individual bacteriophage resistance could be then further investigated on all the other clinical ST131 strains tested for bacteriophage infectivity. This could determine if strains resistant to infection by a particular bacteriophage have a similar variation in their respective bacteriophage receptor gene/s. These investigations would provide an insight into these three bacteriophage's infectivity and resistance patterns in general and could help to design phage cocktails against *E. coli* ST131.

Overall, each of these three virulent bacteriophages gave a promising outcome by killing multiple ST131 strains *in vitro* with high efficiency of plating. However, an *in vivo* study on the performance of these bacteriophages is a crucial next step to confirm their applicability for phage therapy (Małgorzata Łobocka *et al.*, 2014, Weber-Dabrowska *et al.*, 2016, Harada *et al.*, 2018). Specifically, the understanding of the ability of these bacteriophages to reach the targeted tissue and knowledge of the immune response against the bacteriophages *in vivo* are equally important. Likewise, the information on the role of these bacteriophages on gene transfer through transduction is also a prerequisite to select them as therapeutic phages. In order to test these parameters with bacteriophage Syd1, Syd6, and Syd8, it is mandatory to use pure bacteriophage preparations without any bacterial contaminants. Thus, the following sections discuss identifying possible contaminants in the bacteriophage preparations, and also the purification processes to obtain therapeutic-grade bacteriophage preparations suitable for further studies.

6.3 Generation of MVs during bacteriophage cultivation is problematic for bacteriophage preparation

In view of the fact that bacteriophages can only be propagated from a bacterial host, separation of bacteriophages from their bacterial host and other bacterial cell components including endotoxins is necessary to obtain pure bacteriophages. Purified bacteriophages with an acceptable level of endotoxin are important for bacteriophage biology studies, especially when studying or applying bacteriophages in the context of the eukaryotic system such as when examining the interactions of bacteriophages with the immune

system or characterizing the *in vivo* activity of the bacteriophages in an animal model of infection.

Chapter 4 of this Thesis examined the hypothesis that MVs are produced as a consequence of phage-mediated cell lysis. Through the use of live microscopic observation of *E. coli* under virulent bacteriophage infection it was demonstrated that the bacteriophage mediated explosive cell lysis resulted in the production of MVs. The TEM analysis of bacteriophage lysates further confirmed the presence of MVs within bacteriophage sample resulted from bacteriophage infection and bacterial lysis. These findings determined for the first time that the source of MVs within bacteriophage preparation is due to the phage infection itself and confirmed the occurrence of MVs within bacteriophage preparations, which has been previously observed in electron micrographs of various bacteriophage preparations.

Notably, MVs have been shown to act as a decoy to defend bacteria from bacteriophage predation (Manning & Kuehn, 2011, Reyes-Robles *et al.*, 2018). Therefore, MVs in bacteriophage preparations can likely bind to bacteriophages and reduce the bacteriophage titre and lower the efficiency of infectivity of bacteriophages. MVs could also induce immunomodulatory effects if used for *in vivo* studies (Codemo *et al.*, 2018). In addition, MVs play various roles in microbial communities, specifically in interactions between bacterial cells and intercellular molecular exchange that includes delivery of a variety of molecular cargoes including nucleic acids (Mashburn & Whiteley, 2005, Bitto *et al.*, 2017, Jan, 2017) and thus may also mediate horizontal gene transfer (Domingues & Nielsen, 2017).

The significance of horizontal gene transfer has recognised to be an important issue in the perspective of phage therapy (Pirnay *et al.*, 2015, Rodriguez-Rubio *et al.*, 2017, Schneider, 2017). In addition to the killing of susceptible bacterial hosts and formation of viral progeny via the lytic life cycle, bacteriophages can also lead to the mispackaging of bacterial DNA, which can be transferred into a susceptible host via a process known as transduction (Fineran *et al.*, 2009). Virulent bacteriophages have been reported to do low-level transduction, however none of those studies accounted for the presence of MVs within their bacteriophage preparations, which is likely to occur. As membrane vesicles (MVs) can also encapsulate bacterial DNA (Mashburn & Whiteley, 2005, Bitto *et al.*, 2017, Jan, 2017), it will be difficult to know if bacterial DNA that appears in a

bacteriophage preparation is from bacteriophage mediated transduction and not from MVs. Rates of transduction are lower than that of the packaging of bacteriophage DNA, particularly generalised transduction where random bits of DNA are mispackaged (Fineran *et al.*, 2009). Hence, the correct level of transduction through virulent bacteriophages can only be determined if there are no MVs within the bacteriophage preparation.

Moreover, it is likely that MVs produced through explosive cell lysis could also package bacterial toxin molecules as well as bacteriophage lysins that are produced within the bacterial cell during bacteriophage multiplication (Toyofuku *et al.*, 2019). As such, transfer of toxin or lysin to the bacterial cell through bacteriophage derived MVs could also lead to lysis from without type reactions. Likewise, MVs mediated acquisition of bacteriophage receptor could also lead to the killing of non-targeted bacteria (Tzipilevich *et al.*, 2017); effectively negating the advantage that bacteriophage-mediated killing is specific. Considering all these possibilities, the removal of MVs from bacteriophage preparations is crucial. So, preparation of bacteriophage with an acceptable level of endotoxin and free from MVs is important before starting any molecular, metagenomic or *in vivo* bacteriophage studies.

It was unknown prior to this study that MVs are produced during cultivation of virulent bacteriophages through explosive cell lysis and thus would be one of the major contaminants in bacteriophage preparation. Though MVs-like structures have been observed in electron micrographs of different bacteriophages (Sullivan *et al.*, 2005, Guang-Han *et al.*, 2016, Schiettekatte *et al.*, 2018), none of the bacteriophage preparation methods so far explicitly mentioned its removal. The traditional methods used for bacteriophage purification (Yamamoto *et al.*, 1970, Sambrook & Russell, 2001, Bonilla *et al.*, 2016) utilize similar core steps that are also used for MVs purification (Klimentová & Stulík, 2015), and thus are highly likely to prepare bacteriophages with MVs. As the traditional method could not remove endotoxins from bacteriophage preparations, most of the reported bacteriophage purification methods were specifically focused on endotoxin removal from bacteriophage preparations (Boratynski *et al.*, 2004, Merabishvili *et al.*, 2009, Oslizlo *et al.*, 2011, Szermer-Olearnik & Boratyński, 2015, Van Belleghem *et al.*, 2017, Hietala *et al.*, 2019). It is therefore necessary to have a method, which can remove endotoxins together with the MVs from bacteriophage preparation to

get a pure bacteriophage sample for various *in vivo* studies in the laboratory and for clinical use.

6.4 Use of Triton X-114 and ultrafiltration can remove endotoxin and MVs from bacteriophage preparation

Chapter 5 of this Thesis describes the development of a purification method to remove contaminating endotoxin and MVs from phage preparations. Removal of endotoxin from protein samples by extraction with Triton X-114 is based on the principle of two-phase partitioning, in which the TritonX114-water system is maintained outside the miscibility area by adjusting the temperature. Endotoxin, being hydrophobic, is partitioned favourably into the Triton X-114 phase, while the desired protein molecules remain in the aqueous phase (Liu *et al.*, 1998), which can be easily separated (Aida & Pabst, 1990, Teodorowicz *et al.*, 2017). Since Triton X-114 is very good for solubilizing membranes and has the reversible phase-separation ability, it can not only extract the endotoxin but also the MVs. Concentration of the bacteriophage sample by ultrafiltration trapped bacteriophages on the filter due to their larger size whereas bacterial macromolecules and media components can pass through due to their smaller size (Bonilla *et al.*, 2016). Since Triton X-114 has a molecular size that can be pass through 100 KDa ultrafiltration device, residual Triton X-114 within the Triton treated bacteriophage sample is easily removed while also concentrating the bacteriophages. Subsequent rounds of washing remove the traces of Triton resulting in bacteriophage samples free from any residual Triton X-114, which could otherwise be harmful for eukaryotic cells.

Proof of principle studies performed on range of *E. coli* bacteriophages demonstrated that the method has the potential for 4 to 5-log reduction of initial endotoxin levels from the bacteriophage sample. However, there was not complete removal of endotoxin from any of the bacteriophage preparations and not all bacteriophages showed similar efficiency in loss of endotoxin. Thus, regarding the removal of endotoxin, the anticipated results from the method were in an agreement with other methods, which reported that the complete removal of endotoxin is not only a trivial task but efficiency of endotoxin removal is also bacteriophage specific (Van Belleghem *et al.*, 2017). Similarly, in agreement with observations previously made by others, it appeared that endotoxin removal was more efficient in the presence of higher levels of endotoxin (Van Belleghem *et al.*, 2017). As

endotoxins are complex amphipathic molecules and exist in different forms (Petsch & Anspach, 2000, Mueller *et al.*, 2004, Gorbet & Sefton, 2005), it is possible that at the lower concentrations endotoxin is present as highly dispersed small molecules that would be difficult to extract and thus remove. A method reported in the literature to manage residual endotoxin levels in bacteriophage preparation post endotoxin removal, describes diluting the bacteriophage preparations to levels below the limit of endotoxin acceptable for intravenous use (Merabishvili *et al.*, 2009, Morello *et al.*, 2011, Schooley *et al.*, 2017).

Although the efficiency of the method described in Chapter 5 seems to be similar to other methods regarding endotoxin removal, the method significantly accounts for MVs removal. The TEM imaging of phage samples purified by this method did not reveal any MVs, which suggests that the method removed MVs successfully. However, TEM is only a qualitative and does not provide sufficient quantitative analysis. Thus, further work is required for the direct quantification of MVs within purified bacteriophages. Additionally, cytotoxicity assays with the purified bacteriophage samples on different cell lines to check the cytotoxic effect of residual Triton X-114 should also be a requirement before studying or applying those bacteriophages in the context of the eukaryotic system. Since the purification methods were tested only on a set of *E. coli* bacteriophages, it will also be imperative to test the methods on other bacteriophages to evaluate its performance for universal use. Due to the time limitation for this thesis, all of these tests are considered as the future direction to improve the efficiency and performance of this method.

6.5 Conclusions and Final remarks

Whilst more issues still need to be resolved to develop bacteriophages as a successful alternative to antibiotics to control the emerging high-risk bacterial pathogens, the results and their analysis presented in this thesis provides insight for the selection and preparation of bacteriophages for phage therapy. Also, this thesis provides clear evidence of bacteriophage mediated MVs formation and explores an unappreciated route for MVs formation that could be the major process responsible for the abundance of MVs in nature.

Specifically, the work presented in this thesis has provided fundamental understanding of three new bacteriophages against multidrug-resistant bacteria *E. coli* ST131 and also identified MVs as one of the possible contaminants in bacteriophage preparation. As the use of bacteriophages has been commended as one of the potential approaches to combat multidrug-resistant bacteria, understanding the *in vivo* dynamics of bacteriophages is crucial. The *in vivo* studies of bacteriophages require pure bacteriophages free from any toxic or immunomodulatory agents such as endotoxins, cytotoxins or MVs containing these. Thus, the finding of MVs within bacteriophage preparations as a result of bacteriophage mediated bacterial lysis revealed that these should also be removed from bacteriophage preparation before doing any *in vivo* studies. Lack of accountability of MVs removal by any of currently available bacteriophage purification methods suggested exploring a method of bacteriophage purification which is suitable for removal of both MVs and endotoxins from bacteriophage preparation. Use of the modified bacteriophage cultivation process and utilization of Triton X-114 for bacteriophage purification and 100 KDa ultrafiltration for bacteriophage concentration showed lowering endotoxin levels to an acceptable level and successful removal of MVs from bacteriophage preparation.

Overall, the work presented in this thesis adds important new knowledge to our current understanding of bacteriophage-mediated killing of bacterial pathogens and the formation of MVs from bacteria under bacteriophage infection. This also provided a notion that bacteriophage purification for phage therapy is a very important step to avoid any notorious effects caused by contaminants present within the bacteriophage sample. Based on all the findings and data from this thesis, the following recommendations can be made for the use of bacteriophages in phage therapy:

1. Though *E. coli* ST131 strains all belong to the same clonal complex, it is hard to obtain a single bacteriophage capable of infecting all ST131 strains. Thus, multiple bacteriophages targeting different receptors should be useful to design a phage cocktail to combat multidrug resistant *E. coli*.
2. Determination of bacteriophage receptors and analysis of *in vitro* bacteriophage resistant mutants should be a prerequisite for *in vivo* studies of bacteriophages for phage therapy.
3. MVs could be present within the bacteriophage preparations and would thus be problematic for bacteriophage mediated horizontal gene transfer and also for

immunomodulatory effects. Thus, MVs should be removed from bacteriophage preparation before doing any *in vivo* studies.

4. The optimized method of Triton X-114 and ultrafiltration presented here could successfully remove the MVs from bacteriophage preparations, however this should be investigated further to obtain therapeutic grade bacteriophages for animal model studies.

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Appendices

Appendix 1: Promoters and terminator sequences in bacteriophage Syd1 genome

S. N.	Position	Sequence (5' → 3')
Regulatory promoters		
1	27776..27804c	TTGACGACCTAATAGCAAACTATTATAGT
2	31185..31213c	TTGACACCTGAATAGTTAGCTATTATAGT
3	33889..33917c	TTGACATGTGAATAGTTCGCTATTATAGT
4	33906..33933	TTCACATGTCAATGGGCTTTTCTAAAAT
5	41843..41871	TTGACTATTATCCTCGACCCTATTATATT
Predicted terminators		
1	3305..3341	GTTCACCTATAGCCCCGAAAGGGGCTTTCTTATAGTA
2	4871..4911c	AATAAAACTAAGGCCCCATAACGGGGCCTTTTGTCATTCTG
3	4873..4912	GAATGACAAAAGGCCCCGTTATGGGGCCTTAGTTTTATTT
4	5435..5477	TAAGGACTAGCGCCCTGCTTTAAGGCAGGGCTTTACTTATCGA
5	7828..7868	GCTATAATCGAGAGGGACTTCGGTCCCTCTTTTCATTTACT
6	12220..12258c	TGTAAGAAAAAGGCCCCGAAAGGGGCCTTAGTTTTATGC
7	12221..12259	CATAAAACTAAGGCCCCTTTCGGGGCCTTTTTCTTACAA
8	24774..24812c	AGTAGAGAAAAGGCCCCGTTTGGGGCCTTAGTTTTAAGC
9	24774..24815	GCTTAAAACTAAGGCCCCAAACGGGGCCTTTTCTCTACTCTT
10	30454..30492	AATTTAACTAAGGCCCTTCATTGGGCCTTTTTACTAGGG
11	34178..34215	CGTTAGGTGAAGGGGCTTAAAGCCCCTTTCTTTTTATA
12	41778..41823	CTCCAGTTTTATCCCGGCCCTGACCCAGCCGGGATTTTTTTTTATC
13	44093..44131	AACTGATACAAGCCCTCTACGGAGGGCTTTTCTGTACAT

Appendix 2: Promoters and terminator sequences in bacteriophage Syd6 genome

S. N.	Position	Sequence (5' → 3')
Regulatory promoters		
1	37707..37735 c	TTGACATGAATATTGGTTTAGATTACGAT
2	40662..40690 c	TTGACAGAGATGACTGTCGCTGATATAAT
3	49599..49628 c	TTGACCCTTACGACCCACCTTCATTATATT
4	72619..72647 c	TTGACAGCGAAAACAACATGATTCATAAT
5	73252..73280 c	TTGACACCGAAAACAGCCTGATATATAGT
6	75588..75615 c	TTGACAGCCTTTTCCTTTATTATATAGT
PhiEco32 like promoters		
1	1558..1583	TGTTGTTGATAATGTAGAAGGAGTAT
2	8973..8998	GCTAGTGTGTAATGTATATTCTTTGT
3	25855..25880	TGCCTTCTATAATGTATATAGGGGGT
4	35576..35601 c	GTATAAGCGTAATGTATACTATACAT
5	42577..42603 c	AGTAACTTATAATGTATACTCCTTAG
6	47074..47099 c	GTAACTCTCAAATGTATATAGAGTAC
Predicted terminators		
1	1525..1563	AAGTAATATTTGCCTCCGTTGGGAGGCTTTTTGTGTTGT

S. N.	Position	Sequence (5' → 3')
2	3668..3707	CTAAAGTAAGGGGGAGGTTTGTCCTCCCTTCTTTTATTTA
3	8918..8961	CTTAAAATCAGACCCTGCCTTCGGGCGGGGTTTTTTCGTTTTAA
4	19251..19293	GAGATAACAACAAGGGGCTTCGTGCCCCTTTTATTGTTTTGGA
5	30329..30380 c	GAAACAAATAAAGCCCTCTTGGATTTCTCCTTGAGGGCTTTTCTTTTATCAC
6	30331..30382	GATAAAAGAAAAGCCCTCAAGGAGAAATCCAAGAGGGCTTTATTTGTTTCTA
7	34726..34766 c	GTTATAGCTTAGCCACCTTCGGGTGGCTTTTTTCGTTGGAG
8	37741..37780 c	TTAAATAAGAAGCTGCCTTCGGGCGGCTTTTCTTGTCTAA
9	41890..41935 c	CTCTAGAACAAAGGCTGCCTAATCAGGTGGCCTTTTCTTTTATATA
10	62268..62309 c	ATTTAAAGCTTGACTCTTTTCGGAGAGTCCTTTTTTAAATTC
11	64507..64551 c	TAATAGGATAAGGGAGGATTCGTCCCCTCCCCCTATTTTATTGAA
12	66753..66796 c	TCAATAAAGAAAGGCTGCCTTCGGGCGGCCTTTGTTGTTTTAAT
13	69327..69370 c	CTTTCAGGTTTGCCCCGGCTACGGCTGGGGCTTTTTTATGCCCG
14	70735..70782 c	AATAAACATAAGGCGCTATCTTAACGGGTAGCGCCTTTTCTTTTATCA
15	73560..73600 c	TCCTTCCTATAAGCCTCATAGCGAGGCTTTTTTCTTTTCTA

Appendix 3: Promoters and terminator sequences in bacteriophage Syd8 genome

S. N.	Position	Sequence (5' → 3')
Regulatory promoters		
1	671..699	TTGACAACGCCATCCCAATGTAATACTAT
2	1321..1349	TTGACAGCCACGGCATACAAGGTTACATT
Sp6 like promoters		
1	1958..1975	ATTTAGCTGACACTATAA
2	5989..6006	ATTTACCGGACACTATAG
3	8289..8306	ATTTACCGGACACTATAG
4	9176..9193	ATTTTGCCGACACTATAG
5	12604..12621	ATTTACTGGACACTATAG
6	13067..13084	AATTACTGGACACTATAG
7	14298..14315	ATTTACTGGACACTATAG
8	17424..17441	ATTTAGTTGACACTATAG
9	22344..22361	ATTTACTGGACACTATAG
10	37714..37730	CTATACTGGACACTATAG
11	40567..40584	ATTTACTGGACACTATAG
12	42920..42937	ATTTACTGGACACTATAG
Predicted terminators		
1	8505..8551	TATTTATCAGGGCTTGTCTCACATGTGAGACAGGCTCTTATTAAGTA
2	14733..14785	TTTTAATTGATACCCTGTCTGCCTTAGTGTAGGCAGGGTCTTTTGCGTAATAG
3	17437..17479	TATAGAACAAAGGGTAGGTATTAGCTTGCCCTTGATTGTATAG
4	23623..23670	AATAACCTATGCCCTATCTACCTTGCGTAGGTAGGGTTCTTTTGTTTA
5	37984..38027	TTACAGCAGACCTTTCCGTATACGGCGGAAGGTTTGCTTCTCTT
6	42609..42650	TAGAAGTGCTGGCAGGTCTTATTGGCCTGCTTGTCTCTGCTA
7	44839..44882	TCTAATATCAAGGCAGTGCGTAAGCACTGCTTTTCTCGCAACTT

Appendix 4: Functional annotation of bacteriophage Syd1

Locus_tag	Position	CDS size (bp)	Properties of encoded protein					
			Size (aa)	Mol. Wt. (KDa)	pI	Signal peptide	Conserved domain	Function
Syd1_010	1-549	549	182	20.2	6.38	No		Small terminase subunit
Syd1_020	539-1810	1272	423	47.4	6.51	No	PF03237, IPR004921	Large terminase subunit
Syd1_030	1823-3304	1482	493	54.7	4.79	No		Portal protein
Syd1_040	3374-4417	1044	347	38.6	5.2	Yes	PF04233, IPR006528	Head morphogenesis protein
Syd1_050	4417-4878	462	153	16.3	4.41	No	IPR007110	Head protein
Syd1_060	4912-5115	204	67	8.0	4.5	No		Hypothetical protein
Syd1_070	5490-5876	387	128	13.9	9.12	Yes		Hypothetical protein
Syd1_080	5839-5985	147	48	5.5	4.46	No		Hypothetical protein
Syd1_090	6068-6772	705	234	25.6	5.66	No		Scaffold protein
Syd1_0100	6772-7821	1050	349	37.8	4.72	No		Major capsid protein
Syd1_0110	7881-8180	300	99	10.0	9.1	No		Hypothetical protein
Syd1_0120	8193-8543	351	116	12.0	4.39	No	IPR008983	Hypothetical protein
Syd1_0130	8580-8759	180	59	6.7	5.23	No		Hypothetical protein
Syd1_0140	8763-9275	513	170	17.7	4.57	No		Hypothetical protein
Syd1_0150	9278-9892	615	204	20.9	9.05	No	IPR007110	Hypothetical protein
Syd1_0160	9892-10251	360	119	13.0	7.93	No		Hypothetical protein
Syd1_0170	10248-10643	396	131	14.6	9.85	No	PF04883	Tail protein
Syd1_0180	10643-11056	414	137	15.0	4.85	No	PF13554	Tail protein
Syd1_0190	11059-12225	1167	388	40.8	4.46	No		Tail protein
Syd1_0200	12254-12808	555	184	20.0	5.03	No	IPR027417	ATP-binding protein
Syd1_0210	12808-13296	489	162	18.6	5.52	No	PF03767	Acid phosphatase
Syd1_0220	13293-14423	1131	376	42.4	8.44	No	PF12850, IPR029052	Calcineurin-like phosphoesterase
Syd1_0230	14462-14653	192	63	7.4	9.86	No	PF14373, 2 transmembrane domains	Superinfection immunity membrane protein

Locus_tag	Position	CDS size (bp)	Properties of encoded protein					
			Size (aa)	Mol. Wt. (KDa)	pI	Signal peptide	Conserved domain	Function
Syd1_0240	14832-15248	417	138	15.9	4.9	No	PF08748	Tail morphogenesis protein
Syd1_0250	15254-15613	360	119	13.6	5.13	No		Hypothetical protein
Syd1_0260	15606-17945	2340	779	83.1	4.83	No	COG3941	Tail-tape measure protein
Syd1_0270	17945-19342	1398	465	50.0	4.54	No		Hypothetical protein
Syd1_0280	19346-19861	516	171	19.1	4.39	No	PF08875	Hypothetical protein
Syd1_0290	19858-20223	366	121	14.1	6.5	No		Hypothetical protein
Syd1_0300	20214-22781	2568	855	93.8	4.55	No	COG4733	Tail fibre protein
Syd1_0310	22794-24779	1986	661	69.2	5.4	No	IPR011050	Tail spike protein
Syd1_0320	24808-24945	138	45	5.2	5.26	No		Hypothetical protein
Syd1_0330	24942-25454	513	170	19.4	6.82	No	cd01029	DNA primase
Syd1_0340	25454-26878	1425	474	53.5	8.48	No	IPR1400, PF00176, cd00079, IPR027417	Helicase
Syd1_0350	26871-27554	684	227	25.1	6.15	No	COG0270	DNA-cytosine methylase
Syd1_0360	27551-27742	192	63	7.1	9.22	No		Hypothetical protein
Syd1_0370	27773-28060	288	95	10.9	9.26	No	PF08774, IPR0148830	Restriction endonuclease
Syd1_0380	28142-28273	132	43	4.6	6.5	Yes	1 transmembrane domain	Hypothetical protein
Syd1_0390	28263-30443	2181	726	8.2	7	No	cd08642, PF00476, PF01612, IPR001098, IPR012337	DNA polymerase I
Syd1_0400	30503-31129	627	208	23.4	4.94	No	PF10991, IPR012340	Single stranded DNA binding protein
Syd1_0410	31199-31402	204	67	8.1	4.16	No		Hypothetical protein
Syd1_0420	31399-32640	1242	413	45.9	6.31	No	PF10926	Hypothetical protein
Syd1_0430	32792-33064	273	90	10.8	9.17	No		Hypothetical protein
Syd1_0440	33108-33608	501	166	18.7	4.41	No		Hypothetical protein
Syd1_0450	33633-33860	228	75	8.1	4.39	No		Hypothetical protein
Syd1_0460	33982-34197	216	71	7.7	8.25	No	cd00093, PF01381, IPR010982	Transcriptional regulator protein

Locus_tag	Position	CDS size (bp)	Properties of encoded protein					
			Size (aa)	Mol. Wt. (KDa)	pI	Signal peptide	Conserved domain	Function
Syd1_0470	34211-34525	315	104	12.0	5.65	No	2 transmembrane domains	Hypothetical protein
Syd1_0480	34522-36774	2253	750	83.8	5.28	No	IPR027417 PF13481	Helicase-primase
Syd1_0490	36771-36899	129	42	5.0	11.41	No		Hypothetical protein
Syd1_0500	36995-37180	186	61	7.2	8.76	No		Hypothetical protein
Syd1_0510	37177-37359	183	60	7.1	9.78	No	IPR009061 PF12728 IPR010982	DNA binding protein
Syd1_0520	37839-38057	219	72	8.2	9.05	Yes		Hypothetical protein
Syd1_0530	38076-38261	186	61	7.0	10	No		Hypothetical protein
Syd1_0540	38261-38476	216	71	8.0	7.88	No		Hypothetical protein
Syd1_0550	38476-38631	156	51	6.0	5.59	No		Hypothetical protein
Syd1_0560	38628-38777	150	49	5.5	10.61	No		Hypothetical protein
Syd1_0570	38986-39135	150	49	5.7	4.41	No		Hypothetical protein
Syd1_0580	39125-39397	273	90	10.4	8.09	No		Hypothetical protein
Syd1_0590	39415-39930	516	171	19.5	5.59	No		Hypothetical protein
Syd1_0600	39927-40130	204	67	7.4	6.54	Yes		Hypothetical protein
Syd1_0610	40133-40315	183	60	6.9	4.25	No	PF10930	Hypothetical protein
Syd1_0620	40315-40608	294	97	11.3	11.04	No		Hypothetical protein
Syd1_0630	40684-40992	309	102	10.6	6.77	No	3 transmembrane domains	Putative holin class I
Syd1_0640	40985-41257	273	90	10.0	9.24	No	2 transmembrane domains	Putative holin class II
Syd1_0650	41235-41723	489	162	17.0	9.81	No	PF00959, IPR023346	Lysozyme
Syd1_0660	41906-42106	201	66	7.3	7.89	No		Hypothetical protein
Syd1_0670	42106-42255	150	49	5.3	8.1	No		Hypothetical protein
Syd1_0680	42252-42449	198	65	7.3	4.13	No		Hypothetical protein
Syd1_0690	42446-42604	159	52	6.1	10.59	No		Hypothetical protein
Syd1_0700	42601-42825	225	74	8.7	4.67	No		Hypothetical protein
Syd1_0710	42825-43115	291	96	10.4	4.28	No		Hypothetical protein
Syd1_0720	43102-43284	183	60	6.7	5.07	No		Hypothetical protein

Locus_tag	Position	CDS size (bp)	Properties of encoded protein					
			Size (aa)	Mol. Wt. (KDa)	pI	Signal peptide	Conserved domain	Function
Syd1_0730	43347-43541	195	64	7.4	6.76	No		Hypothetical protein
Syd1_0740	43538-43774	237	78	9.0	9.39	No	PF06322	NinH-like protein
Syd1_0750	43771-44043	273	90	10.4	4.56	No		Hypothetical protein
Syd1_07600	43997-44098c	102	33	3.6	8.29	No		Hypothetical protein

Appendix 5: Functional annotation of bacteriophage Syd6

Locus_tag	Position	CDS size (bp)	Properties of encoded protein					
			Size (aa)	Mol. Wt. (KDa)	pI	Signal peptide	Conserved domain	Function
Syd6_010	297-458	162	53	5.777	7.94	No		Hypothetical protein
Syd6_020	518-729	213	70	8.02	9.89	No		Hypothetical protein
Syd6_030	651-905	255	84	9.861	4.24	No		Hypothetical protein
Syd6_040	898-1530	633	210	24.113	4.57	No		Hypothetical protein
Syd6_050	1587-2060	474	157	17.095	4.48	No		Terminase small subunit
Syd6_060	2130-3671	1542	513	58.141	6.26	No	PF03237	Terminase large subunit
Syd6_070	3733-5976	2244	747	84.948	4.93	No	PF11498, COG4372	Portal protein
Syd6_080	5986-6222	237	78	8.696	6.83	No		Hypothetical protein
Syd6_090	6222-7298	1077	358	41.2	4.21	No		Scaffolding protein
Syd6_0100	7340-8398	1059	352	38.659	6.98	No		Major capsid protein
Syd6_0110	8410-8922	513	170	16.544	4.01	No		Hypothetical protein
Syd6_0120	9024-9776	753	250	28.716	4.71	No		Hypothetical protein
Syd6_0130	9786-12344	2559	852	90.197	4.58	No		Tail protein
Syd6_0140	12385-14583	2199	732	79.256	5.58	No	PF07484, PF13884, IPR030392	Tail fibre
Syd6_0150	14676-14894	219	72	7.571	9.3	Yes		Hypothetical protein
Syd6_0160	14923-15414	492	163	18.214	8.85	No	PF00959, IPR023346	Phage lysozyme
Syd6_0170	15427-16230	804	267	29.226	5.87	No	PHA02582	Tail protein
Syd6_0180	16240-19257	3018	1005	110.254	4.73	No	IPR003343, PF02368	Capsid protein
Syd6_0190	19301-20269	1029	342	35.326	4.63	No	PF04582	Capsid protein

Locus_tag	Position	CDS size (bp)	Properties of encoded protein					
			Size (aa)	Mol. Wt. (KDa)	pI	Signal peptide	Conserved domain	Function
Syd6_0200	20271-21305	1035	344	37.377	6.4	No		Hypothetical protein
Syd6_0210	21315-22097	783	260	27.529	9.26	No	IPR023346, PF01464	Internal virion protein
Syd6_0220	22117-23169	1053	350	35.845	9.87	No		Hypothetical protein
Syd6_0230	23182-24150	969	322	33.254	4.33	No		Hypothetical protein
Syd6_0240	24164-25843	1680	559	60.897	5.38	No	TIGR02168	Hypothetical protein
Syd6_0250	25911-30332	4422	1473	162.977	5.75	No	PTZ00121	Structural protein
Syd6_0260	30406-30513 c	108	35	3.981	4.94	No		Hypothetical protein
Syd6_0270	30526-30687 c	162	53	6.062	4.13	No		Hypothetical protein
Syd6_0280	30696-30950 c	255	84	9.998	7.87	No	PF13280	WYL domain protein
Syd6_0290	30916-31317 c	402	133	15.283	8.89	No	cd00085	Hypothetical protein
Syd6_0300	31304-31483 c	180	59	7.157	9.45	No		Hypothetical protein
Syd6_0310	31497-32315 c	819	272	31.949	5.42	No	IPR020045, PF01367, PF02739	5'-3' exonuclease
Syd6_0320	32302-33195 c	894	297	33.466	5.06	No	2 IPR027417	ATP-binding protein
Syd6_0330	33192-33584 c	393	130	14.555	6.07	No	PF10686/ IPR019627	Hypothetical protein
Syd6_0340	33581-34225 c	645	214	25.82	5.63	No	IPR013325, TIGR02937	RNA polymerase sigma factor
Syd6_0350	34225-34398 c	174	57	6.392	4.68	No		Hypothetical protein
Syd6_0360	34370-34720 c	351	116	13.478	5.86	No		Hypothetical protein
Syd6_0370	34778-35524 c	747	248	27.414	5.25	No		Hypothetical protein
Syd6_0380	35840-35947 c	108	35	4.028	8.14	No	1 transmembrane domain	Hypothetical protein
Syd6_0390	35941-36123 c	183	60	7.252	9.3	No		Hypothetical protein
Syd6_0400	36095-36568 c	474	157	18.257	9.17	No	PF00847, PF13392	HNH endonuclease
Syd6_0410	36565-36780 c	216	71	8.089	4.04	No		Hypothetical protein
Syd6_0420	36783-37004 c	222	73	7.963	9.26	No		Hypothetical protein
Syd6_0430	37001-37168 c	168	55	6.662	4.13	No		Hypothetical protein
Syd6_0440	37165-37341 c	177	58	6.766	8.71	Yes		Hypothetical protein
Syd6_0450	37338-37730 c	393	130	14.923	4.72	No		Hypothetical protein

Locus_tag	Position	CDS size (bp)	Properties of encoded protein					
			Size (aa)	Mol. Wt. (KDa)	pI	Signal peptide	Conserved domain	Function
Syd6_0460	37777-37953 c	177	58	6.296	4.56	No		Hypothetical protein
Syd6_0470	37963-38130 c	168	55	6.315	5.14	No		Hypothetical protein
Syd6_0480	38130-38258 c	129	42	5.169	4.21	No		Hypothetical protein
Syd6_0490	38314-38505 c	192	63	6.967	7.87	No		Hypothetical protein
Syd6_0500	38502-38864 c	363	120	14.85	7.66	No		Hypothetical protein
Syd6_0510	38873-40717 c	1845	614	69.123	9.15	No	PF00476/ IPR001098	DNA polymerase I
Syd6_0520	40734-41156 c	423	140	16.422	5.2	No		Hypothetical protein
Syd6_0530	41158-41316 c	159	52	6.181	4.42	No		Hypothetical protein
Syd6_0540	41320-41604 c	285	94	10.868	10.11	No		Hypothetical protein
Syd6_0550	41606-41860 c	255	84	9.606	10.6	No		Hypothetical protein
Syd6_0560	41930-42223 c	294	97	11.594	9.28	No		Hypothetical protein
Syd6_0570	42234-42548 c	315	104	10.827	11.46	No		Hypothetical protein
Syd6_0580	42605-43342 c	738	245	27.616	6.85	No	PF02562, IPR027417	Phosphate starvation-inducible phoH-like protein
Syd6_0590	43339-43536 c	198	65	7.388	9.1	No		Hypothetical protein
Syd6_0600	43521-43721 c	201	66	7.702	4.5	No		Hypothetical protein
Syd6_0610	43718-44041 c	324	107	12.393	4.84	No	PF01653/ IPR013839	DNA ligase
Syd6_0620	44038-44223 c	186	61	7.266	9.74	No		Hypothetical protein
Syd6_0630	44204-44437 c	234	77	9.242	9.13	No		Hypothetical protein
Syd6_0640	44584-45240 c	657	218	24.862	6.24	No	PF02511	Thymidylate synthase
Syd6_0650	45256-45531 c	276	91	10.06	5.33	No	PF00085, IPR012336	Thioredoxin
Syd6_0660	45533-45709 c	177	58	6.395	9.36	No		Hypothetical protein
Syd6_0670	45722-45907 c	186	61	6.73	7.92	Yes	1 transmembrane domain	Hypothetical protein
Syd6_0680	45917-46096 c	180	59	6.383	6.52	No	2 transmembrane domains	Hypothetical protein
Syd6_0690	46157-46720 c	564	187	21.21	4.71	No	IPR009078, PF00210	DNA protection protein
Syd6_0700	46713-47042 c	330	109	12.237	8.79	No		Hypothetical protein
Syd6_0710	47115-47324 c	210	69	8.339	9.79	No		Hypothetical protein

Locus_tag	Position	CDS size (bp)	Properties of encoded protein					
			Size (aa)	Mol. Wt. (KDa)	pI	Signal peptide	Conserved domain	Function
Syd6_0720	47324-47533 c	210	69	7.906	10.56	No		Hypothetical protein
Syd6_0730	47490-47729 c	240	79	9.148	9.89	No		Hypothetical protein
Syd6_0740	47730-48263 c	534	177	19.618	5.59	No	IPR029054, PF00692, cd07557	dUTPase
Syd6_0750	48273-49586 c	1314	437	49.184	4.89	No	COG0617, cd05398	Replication protein
Syd6_0760	49588-50145 c	558	185	21.689	4.96	No	PF01612	3'-5' exonuclease
Syd6_0770	50139-51929 c	1791	596	66.645	5.09	No	PF03796, PF13155	Helicase-primase
Syd6_0780	51956-52159 c	204	67	8.347	9.51	No		Hypothetical protein
Syd6_0790	52149-52568 c	420	139	15.766	4.65	No	IPR013024, PF06094	Gamma-glutamyl cyclotransferase (GGCT)
Syd6_0800	52570-52779 c	210	69	7.971	5.81	No		Hypothetical protein
Syd6_0810	52833-53018 c	186	61	6.702	4.38	No		Hypothetical protein
Syd6_0820	53015-53257 c	243	80	9.303	5.41	No		Hypothetical protein
Syd6_0830	53270-54463 c	1194	397	44.171	5.51	No	TIGR00768	Glutathione synthase
Syd6_0840	54446-54690 c	225	74	8.599	5.26	No		Hypothetical protein
Syd6_0850	54671-54904 c	234	77	8.626	9.36	No		Hypothetical protein
Syd6_0860	54907-56595 c	1689	562	63.686	5.25	No	IPR029055	Glutamine amidotransferase
Syd6_0870	57018-58121 c	1104	367	42.161	5.27	No	IPR022025/ PF12224	Putative amidoligase enzyme
Syd6_0880	58118-58735 c	618	205	23.249	5.96	No		Hypothetical protein
Syd6_0890	58745-59551 c	807	268	30.07	5.45	No	IPR025681/ PF14395	Hypothetical protein
Syd6_0900	59544-60515 c	972	323	36.85	4.98	No		Hypothetical protein
Syd6_0910	60526-61725 c	1200	399	46.59	4.84	No		Hypothetical protein
Syd6_0920	61737-62099 c	363	120	13.889	9.94	No		Hypothetical protein
Syd6_0930	62102-62254 c	153	50	5.583	5.96	Yes		Hypothetical protein
Syd6_0940	62481-62945 c	465	154	17.814	9.93	No	2 transmembrane domains	Hypothetical protein
Syd6_0950	62945-63178 c	234	77	8.45	10.35	No		Hypothetical protein
Syd6_0960	63285-63401 c	117	38	4.462	10.12	No		Hypothetical protein

Locus_tag	Position	CDS size (bp)	Properties of encoded protein					
			Size (aa)	Mol. Wt. (KDa)	pI	Signal peptide	Conserved domain	Function
Syd6_0970	63410-63658 c	249	82	9.148	8.64	No		Hypothetical protein
Syd6_0980	63664-63909 c	246	81	8.915	9.22	No		Hypothetical protein
Syd6_0990	63911-64141 c	231	76	8.625	6.55	No		Hypothetical protein
Syd6_01000	64150-64461 c	312	103	12.201	10.08	No		Hypothetical protein
Syd6_01010	64757-64930 c	174	57	6.039	3.37	No		Hypothetical protein
Syd6_01020	64940-65161 c	222	73	8.169	7.86	No		Hypothetical protein
Syd6_01030	65171-65368 c	198	65	7.525	9.82	No		Hypothetical protein
Syd6_01040	65370-65669 c	300	99	10.592	4.91	Yes		Hypothetical protein
Syd6_01050	65894-66058 c	165	54	6.756	9.14	No		Hypothetical protein
Syd6_01060	66066-66317 c	252	83	9.326	7.75	No		Hypothetical protein
Syd6_01070	66314-66538 c	225	74	8.572	9.23	No		Hypothetical protein
Syd6_01080	66535-66711 c	177	58	6.333	9.4	No		Hypothetical protein
Syd6_01090	66790-67041 c	252	83	9.461	7.77	No		Hypothetical protein
Syd6_01100	66989-67129 c	141	46	5.237	10.53	No		Hypothetical protein
Syd6_01110	67488-67706 c	219	72	8.006	4.89	No		Hypothetical protein
Syd6_01120	67717-67929 c	213	70	8.038	6.24	No		Hypothetical protein
Syd6_01130	67932-68183 c	252	83	9.313	7.71	No		Hypothetical protein
Syd6_01140	68173-68409 c	237	78	9.092	6.75	No		Hypothetical protein
Syd6_01150	68399-68695 c	297	98	11.531	8.82	No	1 transmembrane domain	Hypothetical protein
Syd6_01160	68895-69284 c	390	129	15.015	9.67	No	PF07486/ IPR011105	Cell wall hydrolase
Syd6_01170	69526-69969 c	444	147	16.41	10.1	No		Hypothetical protein
Syd6_01180	70039-70248 c	210	69	7.739	10.18	No		Hypothetical protein
Syd6_01190	70245-70469 c	225	74	8.825	10.3	No		Hypothetical protein
Syd6_01200	70466-70684 c	219	72	8.457	9.64	No		Hypothetical protein
Syd6_01210	70796-71695 c	900	299	33.208	5.84	No		Hypothetical protein
Syd6_01220	73667-74032 c	366	121	13.264	5.17	Yes		Hypothetical protein
Syd6_01230	74130-74390 c	261	86	9.549	4.11	No		Hypothetical protein

Locus_tag	Position	CDS size (bp)	Properties of encoded protein					
			Size (aa)	Mol. Wt. (KDa)	pI	Signal peptide	Conserved domain	Function
Syd6_01240	74380-74646 c	267	88	9.834	4.47	No		Hypothetical protein
Syd6_01250	74671-75543 c	873	290	31.399	5.78	No		Hypothetical protein

Appendix 6: Functional annotation of bacteriophage Syd8

Locus_tag	Position	CDS size (bp)	Properties of encoded protein					
			Size (aa)	Mol. Wt. (KDa)	pI	Signal peptide	Conserved domain	Function
Syd8_010	842-937	96	31	3.705	3.21	No		Hypothetical protein
Syd8_020	934-1110	177	58	6.959	4.83	No		Hypothetical protein
Syd8_030	1103-1312	210	69	8.179	5.84	No		Hypothetical protein
Syd8_040	1492-1851	360	119	13.635	3.89	No	PF08684; IPR014798	ocr (Overcome classical restriction) protein
Syd8_050	1854-1967	114	37	4.364	7.9	No		Hypothetical protein
Syd8_060	2041-2217	177	58	6.68	7.75	Yes		Hypothetical protein
Syd8_070	2282-3205	924	307	33.327	4.96	No		Hypothetical protein
Syd8_080	3269-5896	2628	875	98.581	6.05	No	IPR029262; PF14700	DNA-dependent RNA polymerase
Syd8_090	6221-6349	129	42	5.115	10.71	No	IPR013232/ PF08200	Hypothetical protein
Syd8_0100	6353-8341	1989	662	74.598	5.77	No	IPR0341544; IPR007694	DNA helicase
Syd8_0110	8341-8502	162	53	5.976	9.4	No	2Transmembranedomain	Hypothetical protein
Syd8_0120	8575-9297	723	240	27.285	9.36	No		Hypothetical protein
Syd8_0130	9308-9526	219	72	8.031	9.46	No		Hypothetical protein
Syd8_0140	9516-9713	198	65	7.427	10.59	No		Hypothetical protein
Syd8_0150	9778-9885	108	35	4.455	3.82	No		Hypothetical protein
Syd8_0160	9872-12418	2547	848	96.533	6.6	No	IPR012337; IPR001098	DNA polymerase I
Syd8_0170	12691-13068	378	125	14.754	5.8	Yes		Hypothetical protein
Syd8_0180	13231-14037	807	268	29.556	4.71	No		Hypothetical protein
Syd8_0190	14047-14265	219	72	79.17	7.01	No		Hypothetical protein
Syd8_0200	14370-14738	369	122	13.152	4.45	No		Hypothetical protein

Locus_tag	Position	CDS size (bp)	Properties of encoded protein					
			Size (aa)	Mol. Wt. (KDa)	pI	Signal peptide	Conserved domain	Function
Syd8_0210	15006-16034	1029	342	38.823	6.47	No	IPR029060; IPR020045	5'-3' exonuclease
Syd8_0220	16019-16429	411	136	15.473	9.77	No	IPR004211/ PF0294	Endonuclease VII
Syd8_0230	16422-17429	1008	335	38.208	7.87	No	PF00149; IPR029052	Phosphoesterase
Syd8_0240	17497-17934	438	145	17.329	6.24	No	IPR021739	Hypothetical protein
Syd8_0250	17936-18883	948	315	35.633	8.19	No	IPR012310; PF14743	DNA ligase
Syd8_0260	18855-19070	216	71	7.926	3.82	No		Hypothetical protein
Syd8_0270	19033-19203	171	56	6.479	7.87	No		Hypothetical protein
Syd8_0280	19200-19661	222	73	8.475	9.25	No	IPR016181; PF00583	Acetyltransferase
Syd8_0290	19671-19880	210	69	7.1	9.3	No		Hypothetical protein
Syd8_0300	19882-21432	1548	515	57.647	5.89	No	IPR020991/ PF12236	Head-to-tail connector protein
Syd8_0310	21432-22337	906	301	32.553	4.27	No		Scaffolding protein
Syd8_0320	22416-23624	1209	402	43.49	5.21	No	PHA02004	Major capsid protein
Syd8_0330	23680-24420	741	246	27.727	5.83	No	PF17212	Tail tubular protein
Syd8_0340	24420-26822	2403	800	89.663	5.25	No		Tail protein
Syd8_0350	26822-27547	726	241	24.459	9.47	Yes		Hypothetical protein
Syd8_0360	27548-30490	2943	980	107.71	5.48	No	IPR023346	Internal virion protein
Syd8_0370	30558-34361	3804	1267	138.26	6.18	No	PHA03415; PHA03413	Internal virion protein
Syd8_0380	34361-37444	3081	1026	114.68	5.44	No	PF03906; IPR030392	Tail adaptor protein
Syd8_0390	37444-37638	195	64	7.421	6.52	No	PF10746; 1Transmembrane domain	Holin
Syd8_0400	37622-37924	303	100	10.904	6.05	No		Small terminase subunit
Syd8_0410	37924-39141	1218	405	45.974	6.11	No		DNA packaging A
Syd8_0420	39311-39844	534	177	20.239	9.48	No	IPR011991	DNA binding protein
Syd8_0430	39786-40565	780	259	29.856	9.21	No		DNA packaging B
Syd8_0440	40724-41005	282	93	9.898	8.79	No		Hypothetical protein
Syd8_0450	41023-41310	288	95	10.021	6.71	No		Hypothetical protein
Syd8_0460	41312-42001	690	229	25.293	4.7	No		Hypothetical protein
Syd8_0470	42001-42345	345	114	12.78	9.3	No	PF08291; IPR009045	Peptidase

Locus_tag	Position	CDS size (bp)	Properties of encoded protein					
			Size (aa)	Mol. Wt. (KDa)	pI	Signal peptide	Conserved domain	Function
Syd8_0480	42339-42476	138	45	5.182	5.11	No		Hypothetical protein
Syd8_0490	42454-42603	150	49	4.965	7.78	Yes		Hypothetical protein
Syd8_0500	42681-42890	210	69	7.481	8.68	Yes		Hypothetical protein
Syd8_0510	43023-44843	1821	606	66.013	5.76	No	PF12708; IPR011050	Tail spike
Syd8_0520	44941-45090	150	49	5.348	10.12	No		Hypothetical protein

Appendix 7: Nucleotide identity of bacteriophages that genomically resemble with Syd1

S. N.	Bacteriophage	Accession	Size (bp)	GC%	BLASTn Identity %	Query cover
1	*Escherichia* phage vB_EcoS-Golestan	MG099933	44829	50.6	93	88
2	*Escherichia* phage ST2	MF153391	44517	50.8	92	73
3	*Escherichia* phage K1ind2	GU196280	42765	51.34	86	73
4	*Escherichia* phage K1ind3	GU196281	43461	51.15	86	73
5	*Escherichia* phage K1G	GU196277	43587	51.06	87	72
6	*Escherichia* phage K1ind1	GU196279	42292	51.27	82	72
7	*Escherichia* phage vB_EcoS_L	KY295896	41039	51.12	88	67
8	*Escherichia* phage vB_EcoS_G	KY295895	41519	50.78	88	66
9	*Escherichia* phage K1H	GU196278	41632	51.16	88	65
10	Escherichia phage vB_EcoS_P	KY295898	41184	51.28	86	65
11	*Salmonella* phage BPS11T2	MG646668	43797	49.71	74	67
12	*Salmonella* phage LSPA1	KM272358	41880	50.48	83	67
13	*Salmonella* phage BPS11Q3	KX405002	43788	49.7	76	67
14	*Salmonella* phage SETP13	KF562864	42665	49.78	74	66
15	*Salmonella* phage MA12	KX245013	41224	49.87	73	66
16	*Salmonella* phage ST1	MF001366	42285	49.92	73	66
17	*Salmonella* phage ST3	MF001364	42266	49.93	73	66

S. N.	Bacteriophage	Accession	Size (bp)	GC%	BLASTn Identity %	Query cover
18	*Salmonella* phage FSLSP-101	KC139511	41873	50.26	75	65
19	*Salmonella* phage LPSE1	KY379853	41854	49.82	74	65
20	*Salmonella* phage SE2	JQ007353	43221	49.64	73	65
21	*Salmonella* phage f18SE	KR270151	41868	49.79	73	65
22	*Salmonella* phage fSE1C	KT962832	41720	49.73	73	65
23	*Salmonella* phage SETP7	KF562865	42749	49.9	73	65
24	*Salmonella* phage fSE4S	KT881477	41768	49.78	73	65
25	*Salmonella* phage f2SE	KU951146	41865	49.79	73	65
26	*Salmonella* phage f3SE	KU951147	41867	49.79	73	65
27	*Salmonella* phage vB_SenS PVP-SE2	MF431252	42425	49.98	73	65
28	*Salmonella* phage vB_SpuS_Sp4		43614		77	64
29	*Salmonella* phage wksl3	JX202565	42633	49.79	73	64
30	*Salmonella* phage VB_SenE_Ent3	HG934470	42764	49.79	77	63
31	*Salmonella* phage vB_SenS_Ent1	HE775250	42391	49.78	73	63
32	*Salmonella* phage SS3e	AY730274	40793	50.07	73	63
33	*Salmonella* phage VB_SenE_Ent2	HG934469	42093	49.91	76	62
34	*Salmonella* phage STP03	KY176369	43428	49.57	74	60
35	*Salmonella* phage Jersey	KF148055	43447	49.97	89	59
36	*Salmonella* phage SETP3	EF177456	42572	49.85	73	59
37	*Salmonella* phage vB_SenS_AG11	JX297445	41546	49.9	73	59

Appendix 8: Nucleotide identity of bacteriophages that genomically resemble with Syd6

S. N.	Bacteriophage	Accession	Size (bp)	GC%	BLASTn Identity %	Query cover
1	Phage vB_EcoP_SU10	KM044272	77327	42.1	97	91
2	Enterobacteria phage phiEco32	EU330206	77554	42.05	97	90
3	Enterobacteria phage NJ01	JX867715	77448	42.27	94	90
4	*Escherichia* phage 172-1	KP308307	77266	41.98	93	90
5	*Escherichia* phage LAMP	MG673519	68521	42.23	94	79
6	*Escherichia* phage KBNP1711	KF981730	76184	42.36	83	68
7	*Escherichia* phage EP335	MG748548	76622	42.53	85	67
8	*Escherichia* phage ECBP2	JX415536	77315	42.42	83	59

Appendix 9: Identity of Syd6 encoded proteins with reference bacteriophage PhiEco32 proteins.

Syd6		PhiEco32			Syd6 identity to PhiEco32	
Locus_tag	CDS size (aa)	Locus_tag	CDS size (aa)	Encoded protein	aa identity (%)	Coverage (%)
Syd6_010	53	phi32_1	66	Hypothetical protein	100	100
		phi32_2	46	Hypothetical protein		
Syd6_020	70	phi32_3	59	Hypothetical protein	97	84
Syd6_030	84	phi32_4	84	Hypothetical protein	93	100
Syd6_040	210	phi32_5	231	Hypothetical protein	98	97
Syd6_050	157	phi32_6	157	Hypothetical protein	98	100
Syd6_060	513	phi32_7	513	Terminase large subunit	99	100
Syd6_070	747	phi32_8	747	Portal protein	99	99

Syd6		PhiEco32			Syd6 identity to PhiEco32	
Locus_tag	CDS size (aa)	Locus_tag	CDS size (aa)	Encoded protein	aa identity (%)	Coverage (%)
Syd6_080	78	phi32_9	78	Hypothetical protein	100	100
Syd6_090	358	phi32_10	361	Scaffolding protein	95	100
Syd6_0100	352	phi32_11	352	Major head protein	100	100
Syd6_0110	170	phi32_12	170	Bacterial Ig-like domain	98	100
Syd6_0120	250	phi32_13	250	Conserved hypothetical protein	99	100
Syd6_0130	852	phi32_14	880	Putative tail fibre	73	100
Syd6_0140	732	phi32_15	722	Tail fibre	78	63
Syd6_0150	72	phi32_16	72	Putative holin	97	100
Syd6_0160	163	phi32_17	163	Lysis protein	98	100
Syd6_0170	267	phi32_18	267	Putative structural protein	99	100
Syd6_0180	1005	phi32_19	1005	Bacterial surface protein	99	100
Syd6_0190	342	phi32_20	322	Putative tail tip fibre protein	99	100
Syd6_0200	344	phi32_21	343	Conserved hypothetical protein	61	99
Syd6_0210	260	phi32_22	260	Internal virion protein	97	100
Syd6_0220	350	phi32_23	350	Hypothetical protein	99	100
Syd6_0230	322	phi32_24	322	Putative DNA injection protein	98	100
Syd6_0240	559	phi32_25	551	Hypothetical protein	88	59
Syd6_0250	1473	phi32_26	1473	Hypothetical protein	98	100
Syd6_0260	35	phi32_27	35	Hypothetical protein	91	100
Syd6_0270	53	phi32_28	53	Conserved hypotsxhetical protein	94	100
Syd6_0280	84	phi32_29	75	Hypothetical protein	97	89
Syd6_0290	133	phi32_30	132	Hypothetical protein	95	98

Syd6		PhiEco32			Syd6 identity to PhiEco32	
Locus_tag	CDS size (aa)	Locus_tag	CDS size (aa)	Encoded protein	aa identity (%)	Coverage (%)
		phi32_31	150	HNH endonuclease		
Syd6_0300	59	phi32_32	59	Hypothetical protein	100	100
Syd6_0310	272	phi32_33	272	5'-3' exonuclease	98	100
Syd6_0320	297	phi32_34	297	ATP-binding protein	98	99
Syd6_0330	130	phi32_35	130	Putative GTP-binding protein	99	100
Syd6_0340	214	phi32_36	214	RNA polymerase ECF sigma factor	100	100
		phi32_37	146	Appr-1-p processing enzyme family		
Syd6_0350	57	phi32_38	57	Hypothetical protein	98	100
Syd6_0360	116	phi32_39	116	Conserved hypothetical protein	93	100
Syd6_0370	248	phi32_40	248	Hypothetical protein	100	100
Syd6_0380	35	phi32_41	35	Hypothetical protein	91	100
Syd6_0390	60	phi32_42	59	Hypothetical protein	100	88
Syd6_0400	157					
Syd6_0410	71	phi32_43	71	Hypothetical protein	99	100
Syd6_0420	73	phi32_44	47	Hypothetical protein	64	53
Syd6_0430	55	phi32_45	55	Hypothetical protein	96	100
Syd6_0440	58	phi32_46	58	Hypothetical protein	100	100
Syd6_0450	130	phi32_47	130	Hypothetical protein	100	100
Syd6_0460	58	phi32_48	58	Hypothetical protein	100	98
Syd6_0470	55	phi32_49	55	Hypothetical protein	100	100
Syd6_0480	42	phi32_50	42	Hypothetical protein	100	100

Syd6		PhiEco32			Syd6 identity to PhiEco32	
Locus_tag	CDS size (aa)	Locus_tag	CDS size (aa)	Encoded protein	aa identity (%)	Coverage (%)
Syd6_0490	63	phi32_51	63	Conserved hypothetical protein	98	100
Syd6_0500	120	phi32_52	121	Hypothetical protein	71	97
Syd6_0510	614	phi32_53	614	DNA polymerase-like protein	99	100
Syd6_0520	140	phi32_54	137	Hypothetical protein	84	100
Syd6_0530	52	phi32_55	52	Hypothetical protein	100	100
Syd6_0540	94	phi32_56	94	Hypothetical protein	91	100
Syd6_0550	84	phi32_57	85	Hypothetical protein	67	100
Syd6_0560	97					
Syd6_0570	104	phi32_58	105	Hyopothetical protein	100	100
		phi32_59	110	Hyopothetical protein		
Syd6_0580	245	phi32_60	247	Phosphate starvation-inducible phoH-like protein	99	99
Syd6_0590	65	phi32_61	61	Hyopothetical protein	97	93
Syd6_0600	66					
Syd6_0610	107	phi32_62	109	NAD-dependent DNA ligase	96	98
Syd6_0620	61	phi32_63	61	Hypothetical protein	93	100
Syd6_0630	77					
Syd6_0640	218	phi32_64	218	Thymidylate synthase thyX/thy1	99	99
Syd6_0650	91	phi32_65	91	Thiol-disulphide isomerase and thioredoxin	100	100
Syd6_0660	58					
Syd6_0670	61					
Syd6_0680	59	phi32_66	59	Hypothetical protein	98	100
Syd6_0690	187	phi32_67	187	DNA-binding protein	99	100

Syd6		PhiEco32			Syd6 identity to PhiEco32	
Locus_tag	CDS size (aa)	Locus_tag	CDS size (aa)	Encoded protein	aa identity (%)	Coverage (%)
Syd6_0700	109	phi32_68	109	Conserved hypothetical protein	100	100
Syd6_0710	69	phi32_69	69	Hypothetical protein	99	100
Syd6_0720	69	phi32_70	69	Hypothetical protein	88	100
Syd6_0730	79	phi32_71	65	Hypothetical protein	90	74
Syd6_0740	177	phi32_72	177	dCTP deaminase	99	100
Syd6_0750	437	phi32_73	437	Hypothetical protein	86	100
Syd6_0760	185	phi32_74	185	Type I DNA polymerase	99	100
Syd6_0770	596	phi32_75	596	primase/helicase	99	100
Syd6_0780	67	phi32_76	67	Hypothetical protein	100	100
Syd6_0790	139	phi32_77	139	Conserved YtfP/UPF0131 protein	99	100
Syd6_0800	69					
Syd6_0810	61	phi32_78	61	Hypothetical protein	95	100
Syd6_0820	80	phi32_79	49	Hypothetical protein	100	100
Syd6_0830	397	phi32_80	397	ATP_grasp enzyme	99	100
Syd6_0840	74	phi32_81	74	Hypothetical protein	100	100
Syd6_0850	77	phi32_82	77	Hypothetical protein	100	100
Syd6_0860	562	phi32_83	676	Glutamine amidotransferase	99	100
Syd6_0870	367	phi32_84	367	Hypothetical protein	99	100
Syd6_0880	205	phi32_85	205	Hypothetical protein	100	100
Syd6_0890	268	phi32_86	268	Conserved hypothetical protein	99	100
Syd6_0900	323	phi32_87	323	Hypothetical protein	97	100

Syd6		PhiEco32			Syd6 identity to PhiEco32	
Locus_tag	CDS size (aa)	Locus_tag	CDS size (aa)	Encoded protein	aa identity (%)	Coverage (%)
Syd6_0910	399	phi32_88	399	Hypothetical protein	95	100
Syd6_0920	120	phi32_89	120	Hypothetical protein	99	100
Syd6_0930	50	phi32_90	50	Hypothetical protein	100	100
		phi32_91	71	Hypothetical protein		
Syd6_0940	154	phi32_92	154	Hypothetical protein	63	100
Syd6_0950	77	phi32_93	77	Hypothetical protein	100	100
Syd6_0960	38	phi32_94	38	Hypothetical protein	100	100
Syd6_0970	82	phi32_95	82	Hypothetical protein	99	100
Syd6_0980	81	phi32_96	81	Hypothetical protein	98	100
		phi32_97	82	Hypothetical protein		
Syd6_0990	76	phi32_98	76	Hypothetical protein	99	100
Syd6_01000	103	phi32_99	107	Hypothetical protein	95	100
		phi32_100	73	Hypothetical protein		
		phi32_101	43	Hypothetical protein		
		phi32_102	81	Hypothetical protein		
Syd6_01010	57	phi32_103	57	Hypothetical protein	91	100
Syd6_01020	73	phi32_104	73	Hypothetical protein	92	100
Syd6_01030	65	phi32_105	82	Hypothetical protein	24	95
Syd6_01040	99	phi32_106	93	Putative phage lipoprotein	75	100
Syd6_01050	54	phi32_107	59	Hypothetical protein	59	100
Syd6_01060	83	phi32_108	83	Conserved hypothetical protein	96	100
Syd6_01070	74	phi32_109	74	Hypothetical protein	97	100

Syd6		PhiEco32			Syd6 identity to PhiEco32	
Locus_tag	CDS size (aa)	Locus_tag	CDS size (aa)	Encoded protein	aa identity (%)	Coverage (%)
Syd6_01080	58	phi32_110	58	Hypothetical protein	97	100
Syd6_01090	83	phi32_111	69	Conserved hypothetical protein	96	83
Syd6_01100	46	phi32_112	46	Hypothetical protein	100	100
Syd6_01110	72	phi32_113	72	Hypothetical protein	96	100
Syd6_01120	70					
Syd6_01130	83	phi32_114	85	Hypothetical protein	70	98
Syd6_01140	78	phi32_115	78	Hypothetical protein	95	100
Syd6_01150	98	phi32_116	98	Hypothetical protein	96	100
Syd6_01160	129	phi32_117	129	Hypothetical protein	100	100
		phi32_118	43	Hypothetical protein		
		phi32_119	265	Agglutinating adhesin		
Syd6_01170	147	phi32_120	147	Hypothetical protein	98	100
Syd6_01180	69	phi32_121	69	Hypothetical protein	83	100
Syd6_01190	74	phi32_122	74	Hypothetical protein	99	100
Syd6_01200	72	phi32_123	72	Hypothetical protein	100	100
Syd6_01210	299	phi32_124	299	Hypothetical protein	98	100
Syd6_01220	121	phi32_125	121	Hypothetical protein	86	100
Syd6_01230	86	phi32_126	93	Hypothetical protein	93	100
Syd6_01240	88	phi32_127	290	Hypothetical protein	64	98

Appendix 10: Nucleotide identity of bacteriophages that genomically resemble with Syd8

S. N.	Bacteriophage	Accession	Size (bp)	GC%	BLASTn Identity %	Query cover
1	*Citrobacter* phage vB_CroP_Crp3	MG775042	44349	45.15	87	86
2	*Salmonella* phage UAB Phi78	GU595417	43931	47.4	74	80
3	*Salmonella* phage SP6	AY288927	43769	47.22	74	79
4	*Salmonella* phage BP12B	KM366097	43602	47.55	74	79
5	*Escherichia* phage vB_EcoP_C	KY295892	44970	44.96	88	73
6	*Escherichia* phage vB_EcoP_R	KY295899	44941	44.95	87	73
7	Enterobacteria phage K1E	AM084415	45251	45.05	75	73
8	*Escherichia* phage VEc3	MG251390	44895	44.97	90	72
9	*Escherichia* phage vB_EcoP ACG-C91	JN986844	43731	45.28	90	72
10	*Escherichia* phage vB_EcoP_D	KY295893	44931	44.97	88	72
11	*Escherichia* phage K1-5	AY370674	44385	45.24	87	72
12	*Escherichia* phage AAPEc6	KX279892	44560	45.17	91	71
13	*Escherichia* phage mutPK12	MG004687	44784	45.06	89	71
14	*Escherichia* phage vB_EcoP_B	KY295891	44018	45.03	88	71
15	*Escherichia* phage vB_EcoP_K	KY295897	37775	45.11	81	59
16	*Proteus* phage vB_PmiP Pm5460	KP890822	44573	39.57	70	71
17	*Proteus* phage PM85	KM819695	43642	39.32	70	70
18	*Proteus* phage PM93	KM819696	45169	39.36	70	70
19	*Proteus* phage PM116	KU946962	44601	39.21	70	67
20	*Lelliottia* phage phD2B	KM370384	44366	51	74	55
21	*Pectobacterium* phage POP72	KY744566	44760	49.7	68	55
22	*Pectobacterium* phage PP1	JQ83790	44400	49.73	68	54

Appendix 11: Identity of Syd8 encoded proteins with reference bacteriophage SP6 proteins.

Syd8		SP6			Syd8 identity to Sp6	
Locus_tag	**CDS size (aa)**	**Locus_tag**	**CDS size (aa)**	**Encoded protein**	**aa identity (%)**	**Coverage (%)**
		gp1	57	Hypothetical protein		
Syd8_010	31	gp2	33	Hypothetical protein	78	87
Syd8_020	58	gp3	58	Hypothetical protein	69	100
Syd8_030	69	gp4	69	Hypothetical protein	29	100
Syd8_040	119	gp5	119	inhibits EcoB and EcoK host restriction	63	85
Syd8_050	37					
Syd8_060	58	gp6	63	Hypothetical protein		
Syd8_070	307	gp7	294	Hypothetical protein	64	100
Syd8_080	875	gp8	874	SP6 DNA-directed RNA polymerase	85	100
Syd8_090	42	gp9	65	Hypothetical protein	74	100
Syd8_0100	662	gp10	661	SP6 primase	91	100
Syd8_0110	53					
Syd8_0120	240	gp11	203	Hypothetical protein	45	99
Syd8_0130	72			Hypothetical protein		
Syd8_0140	65	gp12	56	Hypothetical protein	73	75
Syd8_0150	35	gp13	53	Hypothetical protein	80	28
Syd8_0160	848	gp14	849	Phage putative DNA polymerase	90	100
		gp15	32	Hypothetical protein		
		gp16	48	Hypothetical protein		
Syd8_0170	125	gp17	125	Hypothetical protein	70	100
Syd8_0180	268	gp18	268	Hypothetical protein	84	100

Syd8		SP6			Syd8 identity to Sp6	
Locus_tag	CDS size (aa)	Locus_tag	CDS size (aa)	Encoded protein	aa identity (%)	Coverage (%)
Syd8_0190	72	gp19	72	Hypothetical protein	68	100
Syd8_0200	122	gp20	122	Hypothetical protein	82	100
Syd8_0210	342	gp21	342	Exonuclease	83	99
Syd8_0220	136	gp22	136	Endonuclease VII	88	100
Syd8_0230	335	gp23	304	Hypothetical protein	93	90
Syd8_0240	145	gp24	156	Hypothetical protein	77	67
Syd8_0250	315	gp25	315	Hypothetical protein	80	100
Syd8_0260	71	gp26	64	Hypothetical protein	83	83
Syd8_0270	56	gp27	42	Hypothetical protein	61	71
Syd8_0280	73	gp28	153	Hypothetical protein	74	100
Syd8_0290	69	gp29	69	Hypothetical protein	70	100
Syd8_0300	515	gp30	515	Head portal protein	87	99
Syd8_0310	301	gp31	300	Scaffolding protein	68	100
Syd8_0320	402	gp32	401	Major capsid protein	85	100
Syd8_0330	246	gp33	246	Tail protein	85	100
Syd8_0340	800	gp34	803	Tail protein	78	100
Syd8_0350	241	gp35	239	Internal virion protein	64	97
Syd8_0360	980	gp36	978	Hypothetical protein	81	99
Syd8_0370	1267	gp37	1270	Internal virion protein	88	100
Syd8_0380	1026	gp38	319	Tail adaptor protein	48	23
Syd8_0390	64	gp39	65	putative holin	79	95
Syd8_0400	100	gp40	99	Small terminase subunit	77	98
Syd8_0410	405	gp41	631	Large terminase subunit	81	96
Syd8_0420	177					
Syd8_0430	259	gp41	631	Large terminase subunit	75	93
		gp42	99	Hypothetical protein		
Syd8_0440	93	gp43	99	Hypothetical protein	38	89

Syd8		SP6			Syd8 identity to Sp6	
Locus_tag	CDS size (aa)	Locus_tag	CDS size (aa)	Encoded protein	aa identity (%)	Coverage (%)
Syd8_0450	95	gp43	99	Hypothetical protein	52	95
Syd8_0460	229	gp44	81	Hypothetical protein	34	24
		gp45	55	Hypothetical protein		
Syd8_0470	114	gp46	118	Hypothetical protein	76	100
Syd8_0480	45			Hypothetical protein		
Syd8_0490	49	gp47	56	Hypothetical protein	88	100
Syd8_0500	69	gp48	72	Hypothetical protein		
		gp49	550	P22 like tail spike		
Syd8_0510	606	gp50	495	Tail spike		
Syd8_0520	49	gp51	47	Hypothetical protein	85	95
		gp52	25	Hypothetical protein		

Appendix 12: List of movies

Movie 1A: Lysis of *E. coli* MG1655 treated with bacteriophage T4.

Live imaging of *E. coli* MG1655 infected with bacteriophage T4. The black thin rods are live bacteria, which lyse due to bacteriophage infection during period of imaging.

Time-lapse images were taken using phase contrast microscope (Olympus IX71) under 20X objective using 1 frame per minute up to 1 hour and video was made with 10 frame per second. Time is shown in minutes.

Movie 1B: Lysis of *E. coli* MG1655 treated with bacteriophage T7.

Live imaging of *E. coli* MG1655 infected with bacteriophage T7. The black thin rods are live bacteria, which lyse due to bacteriophage infection during period of imaging

Time-lapse images were taken using phase contrast microscope (Olympus IX71) under 20X objective using 1 frame per minute up to 1 hour and video was made with 10 frame per second. Time is shown in minutes.

Movie 1C: Multiplication of *E. coli* MG1655 treated with lambda diluent.

Live imaging of *E. coli* MG1655 treated with lambda diluent. The black thin rods are live bacteria, which show multiplication during period of imaging.

Time-lapse images were taken using phase contrast microscope (Olympus IX71) under 20X objective using 1 frame per minute up to 1 hour and video was made with 10 frame per second. Time is shown in minutes.

Movie 2: Explosive lysis of *E. coli* MG1655 under bacteriophage infection.

Live imaging of *E. coli* MG1655 treated with bacteriophage T4. Bacterial cells lyse explosively and show different pattern of lysis. Bacteria lyse either as direct blowout without any gross change in cell morphology or rounding before lysis.

Time-lapse images were taken using phase contrast microscope (Olympus IX71) under 100X oil immersion objective using 1 frame per 5 seconds and video was made with 10 frame per second. Time is shown in seconds.

Movie 3A: MVs formation through bacteriophage T4 mediated explosive lysis of *E. coli* MG1655.

Live imaging of *E. coli* MG1655 lysis event under bacteriophage T4 infected condition. The cell lyses explosively without any gross morphological changes near pole. Exploding bacterial cell produce shattered membrane fragments, which curl up inward and then round up forming spherical Membrane vesicle.

A membrane dye FM1-43X (Green colour) was used for membrane staining and Time-lapse images were taken using 3D-SIM (DeltaVision OMX SR) under 60X objective using 1 frame per 2 seconds and video was made with 10 frame per second.

Movie 3B: MVs formation through bacteriophage T7 mediated explosive lysis of *E. coli* MG1655.

Live imaging of *E. coli* MG1655 lysis event under bacteriophage T7 infected condition. The bacteria with a gross morphological change to round lyses explosively. Exploding

bacterial cell produce shattered membrane fragments, which round up and bind to the nearby cell forming spherical Membrane vesicle.

A membrane dye FM1-43X (Green colour) was used for membrane staining and Time-lapse images were taken using 3D-SIM (DeltaVision OMX SR) under 60X objective using 1 frame per 2 seconds and video was made with 10 frame per second.

Movie 4A: Membrane blebbing under bacteriophage T4 infected condition.

Live imaging of *E. coli* MG1655 blebbing under bacteriophage T4 infected condition. The bacteria show formation of a membrane bleb. The membrane bleb forms by protrusion of outer membrane of cell.

A membrane dye FM1-43X (Green colour) was used for membrane staining and Time-lapse images were taken using 3D-SIM (DeltaVision OMX SR) under 60X objective using 1 frame per 2 seconds and video was made with 10 frame per second.

Movie 4B: Membrane blebbing under bacteriophage T7 infected condition.

Live imaging of *E. coli* MG1655 blebbing under bacteriophage T7 infected condition. The cells show multiple membrane blebs. The membrane blebs form by protrusion of outer membrane of cell.

A membrane dye FM1-43X (Green colour) was used for membrane staining and Time-lapse images were taken using 3D-SIM (DeltaVision OMX SR) under 60X objective using 1 frame per 2 seconds and video was made with 10 frame per second

To view these movies, use the URL address below:

https://code.research.uts.edu.au/MIF/Workflows/mandal-phd

Note: Use either Chrome or Firefox. Safari on OSX does not display in-browser previews of the movies. Though, they can be downloaded.

Appendix 13: Optimized bacteriophage propagation and purification protocol

Bacteriophage propagation and harvesting

Note:

(1) Only one out of the two harvesting methods presented here is required to proceed with the protocol.
(2) Prior to performing bacteriophage harvest, the user will be required to already have a bacteriophage suspension ready.

Method I: Plate harvest

1 Inoculate 5 ml of LB broth with a single colony of host bacteria of interest and incubate over night at 37°C with shaking at 200 rpm.
2 Aspirate and dispense 3 ml of molten harvest agarose into the 5 sterile tubes and keep in oven set at 55°C.
3 Prepare 1:10 serial dilutions of each phage lysate in lambda diluent
Note: user has to know beforehand which dilution will yield a mosaic plate, i.e. the dilution that will result in a plate whereby plaques are starting to merge with one another.
4 Take a tube containing molten harvest agarose. Aspirate and dispense 10 ul of phage dilution of interest and 100 µl of overnight indicator bacterial strain into it. Mix gently by inverting tubes, taking care not to introduce air bubbles and pour mixture over the surface of the LB agar. Swirl the petri dish to ensure that the harvest agarose overlays the entirety of the LB agar and allow the plate to set on the bench with uniform flat surface. <TROUBLESHOOTING>
5 Repeat the step 4 for additional three plates with same phage dilution and one plate with lambda diluent (instead of phage diluent) as a control.
6 Incubate the petri dishes overnight facing upwards, in a 37°C incubator.
7 After an overnight incubation, remove plates from incubator and chill all the plates for 15 minutes at 4°C.

8 Add 3 ml lambda diluent to flood each of the plate, seal the plates with parafilm and incubate overnight at 4°C with gentle rocking

9 The next day, collect phage-containing lambda diluent into a sterile centrifuge tube.

10 Centrifuge the tubes at 4000 xg for 10 minutes at 4°C.

11 Collect the supernatant (phage suspension) from the centrifuged tubes taking care not to disturb the pellet.

12 Filter the phage suspension once through a 0.45 µm pore sized syringe filter into a sterile tube.

13 Filter the 0.45 µm filtrate through a 0.22 µm pore sized syringe filter into a sterile tube.

14 Perform phage quantification to get titre of the bacteria cleared phage lysate.
Note: Bacteria cleared phage lysate can be stored at 4°C *until endotoxin removal. This is the sample to use for testing of initial endotoxin level.*

Method II: Liquid harvest

15 Inoculate 5 ml of LB broth with a single colony of host bacteria of interest and incubate over night at 37°C with shaking at 200 rpm.

16 Add 1 ml of overnight culture into 9 ml of LB in an Erlenmeyer flask and incubate for one hour at 37°C with shaking at 200 rpm.

17 Add 10 µL of phage sample with a titre more than 10^8 pfu mL^{-1} and place the flask back in the 37°C incubator with shaking at 200 rpm for 5 hours.

18 Centrifuge the bacterial culture at 4000 xg for 10 minutes at 4°C.

19 Collect the supernatant (phage suspension) from the centrifuged tubes taking care not to disturb the pellet.

20 Filter the phage suspension once through a 0.45 µm pore sized syringe filter into a sterile tube.

21 Filter the 0.45 µm filtrate through a 0.22 µm pore sized syringe filter a syringe filter into a sterile tube.

22 Put the entire volume of phage lysate through Amicon® Ultra-4 filter device and centrifuge at 4,000 x *g* for 30 minutes at room temperature. <TROUBLESHOOTING>
Note: this is a buffer exchange step, phage suspension in LB does not phase properly.

23 Once all phage lysates have been put through the Amicon® Ultra-4 filter device, top up upper reservoir of Amicon® Ultra-4 filter device to maximum volume with lambda diluent for buffer exchange and centrifuge 4,000 x *g* for 30 minutes.

24 Discard filtrate and dilute retentate to maximum tube volume with lambda diluent and centrifuge at 4,000 x *g* for 20 minutes at room temperature.

25 Transfer retentate to a sterile tube and perform phage quantification to get titre of the bacteria cleared phage lysate

Note: Bacteria cleared phage lysate can be stored at 4°C *until endotoxin removal. This is the sample to use for testing of initial endotoxin level.*

Endotoxin removal

Note: Triton X-114 residual test (steps 39 - 44) has to be done in tandem with this part of the procedure.

26 To the phage sample of interest, add Triton X-114 to a final concentration of 1 % (v/v). Vortex the tube to ensure proper mixing of Triton X-114 with the phage sample.

27 Cool the tubes on ice for 10 minutes and thereafter, incubate the tubes at 37°C for 15 minutes.

Note: After cooling on ice, the mixture will appear clear and after incubation at 37°C*, the mixture will appear cloudy.*

28 Centrifuge the tubes for 7 minutes at 16,000x*g* to sediment detergent and endotoxin.

29 Remove aqueous phase, without aspirating detergent phase, and dispense into fresh tubes.

Note: The phase separation will most likely not yield a horizontal interphase, so care has to be observed while removing the aqueous phase.

30 Repeat steps 26 to 29 for another 9 times, making a total of 10 rounds

Bacteriophage concentration and residual Triton X-114 removal

31 Add Triton X-114 treated phage lysate into the upper reservoir of Amicon® Ultra-4 filter device and centrifuge at 4,000 x *g* for 30 minutes. <TROUBLESHOOTING>

32 Discard filtrate and top up retentate with Triton X-114 treated phage lysate to maximum tube volume and centrifuge at 4,000 x *g* for 30 minutes. Repeat until all phage lysate has been put through the Amicon® Ultra-4 filter device.

33 Top up the upper reservoir of Amicon® Ultra-4 filter device to maximum volume with lambda diluent for buffer exchange and centrifuge 4,000 x g for 30 minutes.

34 Discard filtrate and to the retentate, add DNaseI to a final concentration of 100 units mL^{-1} and incubate at 37°C for 30 minutes.

35 Dilute DNaseI treated retentate to maximum tube volume with lambda diluent chilled at 4°C and centrifuge at 4,000 x *g* for 20 minutes at 4°C.

36 Discard the filtrate and top up the upper reservoir of Amicon® Ultra-4 filter device to maximum volume with lambda diluent chilled at 4°C. Centrifuge at 4,000 x *g* for 20 minutes at 4°C and discard filtrate. Repeat this step one addition time making it a total of three washes with lambda diluent at 4°C.

37 Transfer retentate into a sterile tube after the third wash.

38 Perform phage quantification to get titre of the Triton X-114 treated phage lysate <TROUBLESHOOTING>.

Note: Triton X-114 treated phage lysate should be stored at 4°C *until endotoxin quantification.*

Residual Triton X-114 test

39 To the Lambda diluent buffer, add Triton X-114 to a final concentration of 1 % (v/v). Vortex the tube to ensure proper mixing of Triton X-114 with the buffer.

40 Perform the steps 28 to 31.

41 Add Triton X-114 treated sample into the upper reservoir of Amicon® Ultra-4 filter device and centrifuge at 4,000 x *g* for 30 minutes. <TROUBLESHOOTING>

42 Discard filtrate and top up retentate with Triton X-114 treated phage lysate to maximum tube volume and centrifuge at 4,000 x *g* for 30 minutes. Repeat until all sample has been put through the Amicon® Ultra-4 filter device.

43 Discard the filtrate and top up the upper reservoir of Amicon® Ultra-4 filter device e to maximum volume with lambda diluent chilled at 4°C. Centrifuge at 4,000 x *g* for 20 minutes at 4°C and discard filtrate. Repeat this step twice making it a total of three washes with lambda diluent at 4°C.

44 Transfer retentate into a sterile tube after the third wash.

45 Prepare a solution containing 1% of Triton X-114 standard by adding10 µl of the Triton X-114 Stock with 990 µl of lambda diluent in 1.5 ml sterile tube (standard Vial A) and vigorously vortex the solution for 10 minutes before proceeding.

Note: Take care not to introduce air bubbles.

46 Prepare different concentration of Triton X-114 Standard solution as per volume designated in Table 1 and vortex each of the solution gently for 1 minute before use.

Table 1: Dilutions and procedures for preparing Triton X-114 Standard solutions.

Vial	Volume of Triton X-114 stock (µl)	Volume of Vial A (µl)	Lambda diluent buffer (µl)	Final Triton X-114 concentration (%)
A	10	-	990	1
1	-	100	900	0.1
2	-	50	950	0.05
3	-	25	975	0.025
4	-	12.5	988.5	0.0125
5	-	5	995	0.005
6	-	2.5	997.5	0.0025
7	-	1.25	998.75	0.00125

47 Keep the Triton X-114 standard tubes (1 to 7) and triton treated Lambda diluent tubes on ice.

48 Measure the absorbance of all the standards and samples in duplicates at 280 nm using NanoDrop.

49 Determine the average absorbance of all individual standard and prepare a standard curve by plotting the absorbance for each standard versus its concentration in %.

Note: The coefficient of determination, r^2 must be ≥ 0.98.

50 Use the formulated standard curve to determine the residual triton concentration in Triton treated samples and record it.

Quantification of residual endotoxin level

51 Perform endotoxin quantification test on both the bacteria cleared phage lysate and the Triton X-114 treated lysate according to instructions listed on the Pierce™ LAL Chromogenic Endotoxin Quantitation Kit manual. Manual also contains troubleshooting steps pertaining to this assay. For samples with endotoxin levels exceeding requirements see table 5.5. <TROUBLESHOOTING>

Troubleshooting

Potential problems encountered, and its troubleshooting steps are listed in Table 2.

Table 2: Troubleshooting steps on phage purification protocol

Step	Problem	Solution
5	Harvest agarose solidifying rapidly due to low volume which can lead to molten agarose not covering the entire plate.	1) Pre-warm empty tubes in an oven before use. 2) Dispense harvest agarose into pre-warm tubes and keep in oven until use.
22, 32	Ultrafiltration units compromised, and lysates drain into collection tube independent of centrifuging	This problem is hard to spot (only noticed at DNase treatment stage) and by the time it is noticed it can be too late. Instead of pooling filtrates into a single waste receptacle, consider collecting filtrates in sterile tubes until just after the DNase treatment step.
30	Accidentally aspirating detergent layer- which can be visible as swirls when dispensing aqueous layer into a new clean tube.	Mencius of the detergent layer is clear and may not be partitioned horizontally throughout the entire circumference of the tube. Make sure to check around the tube and mark out the highest point and leave enough of the aqueous layer behind.
39	Low phage titre after process	Plan for up to 2 logs drop in phage titre. If sensitivity to Triton X-114 is suspected, users should consider other methods. If phages are not sensitive to Triton X-114, starting with a larger volume during phage harvest can help counteract titre lost.
43	Remaining endotoxin level higher than what user requires.	If phage titre allows, user can dilute Triton X-114 treated sample in lambda diluent to achieve required endotoxin levels.

Step	Problem	Solution
		Another solution will be to increase the rounds of Triton X-114 treatment. However, users will have to keep in mind the possibility to losing phage titre. See troubleshooting of step 39 to find a balance between required phage titre and endotoxin levels.