

**ISOLATION AND CHARACTERIZATION OF  
BACTERIOPHAGE FOR VIBRIOSIS THERAPY IN  
FISH**

**MOHAMMAD TAMRIN BIN MOHAMAD LAL**



PERPUSTAKAAN  
UNIVERSITI MALAYSIA SABAH  
**UMS**  
UNIVERSITI MALAYSIA SABAH

**THESIS SUBMITTED IN PARTIAL FULFILLMENT  
FOR THE DEGREE OF DOCTOR OF PHYLOSOPHY**

**BORNEO MARINE RESEARCH INSTITUTE  
UNIVERSITI MALAYSIA SABAH  
2016**

# UNIVERSITI MALAYSIA SABAH

## BORANG PENGESAHAN STATUS TESIS

JUDUL: **ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGE FOR VIBRIOSIS THERAPY IN FISH**

IJAZAH: **DOCTOR OF PHYLOSOPHY (MARINE BIOTECHNOLOGY)**

Saya **MOHAMMAD TAMRIN BIN MOHAMAD LAL**, Sesi Pengajian **2012-2016**, mengaku membenarkan tesis Doktor Falsafah ini disimpan di Perpustakaan Universiti Malaysia Sabah dengan syarat-syarat kegunaan seperti berikut:-

1. Tesis ini adalah hak milik Universiti Malaysia Sabah.
2. Perpustakaan Universiti Malaysia Sabah dibenarkan membuat salinan untuk tujuan pengajian sahaja.
3. Perpustakaan dibenarkan membuat salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. Sila tandakan ( / )


SULIT

(Mengandungi maklumat yang berdarjah keselamatan atau kepentingan Malaysia seperti yang termaktub di dalam AKTA RAHSIA RASMI 1972)

TERHAD

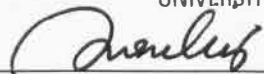
(mengandungi maklumat TERHAD yang telah ditentukan oleh organisasi/badan di mana penyelidikan dijalankan)

TIDAK TERHAD



**MOHAMMAD TAMRIN BIN MOHAMAD LAL**

Disahkan oleh  
**NURULAIN BINTI ISMAIL**  
LIBRARIAN  
UNIVERSITI MALAYSIA SABAH



(Tandatangan Pustakawan)

Tarikh: 20 Mei 2016



(Prof Madya Dr. Julian Ransangan)  
Penyelia

# CERTIFICATION

NAME : **MOHAMMAD TAMRIN BIN MOHAMAD LAL**  
MATRIC NO. : **PY1211001T**  
TITLE : **ISOLATION AND CHARACTERIZATION OF  
BACTERIOPHAGE FOR VIBRIOSIS THERAPY IN FISH**  
DEGREE : **DOCTOR OF PHYLOSOPHY  
(MARINE BIOTECHNOLOGY)**  
VIVA DATE : **18 DECEMBER 2015**

## CERTIFIED BY:

1.

**SUPERVISOR**

Assoc. Prof. Dr. Julian Ransangan

Signature




UMS  
UNIVERSITI MALAYSIA SABAH

A handwritten signature in black ink, written over a horizontal line, representing the signature of the supervisor.

# DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, excerpts, equations, summaries and references, which have been duly acknowledged.

20 May 2016



.....  
Mohammad Tamrin Bin Mohamad Lal  
(PY1211001T)



UMS  
UNIVERSITI MALAYSIA SABAH

PERPUSTAKAAN  
UNIVERSITI MALAYSIA SABAH

## ACKNOWLEDGEMENTS

I owe my innermost gratitude to Allah *s.w.t.* for allowing me to finish my doctoral PhD study. I would thank the first and foremost, my family for their moral supports from the beginning until the end of this thesis. They are always on my side and encourage me during good and hard time.

I would like to express my appreciation to my supervisor, Associate Professor Dr. Julian Ransangan, Deputy Director of Borneo Marine Research Institute, Universiti Malaysia Sabah, for his continuous guidance, critics, moral supports, financial support and advices throughout the completion of this thesis. Thank you for having me as your student as well as your team.

I am also indebted to many colleagues who contribute their times, ideas and advices. Special thanks to Prof. Motohiko Sano from Tokyo University of Marine Science and Technology, Tokyo, Japan for his contribution in taking transmission electron microscopy images of the bacteriophage samples.

This thesis would have remained incomplete had it not been for the Borneo Marine Research Institute (BMRI), Universiti Malaysia Sabah, which provided the facilities during my postgraduate research. I would also like to thank the lecturers and staffs of BMRI for their critics, ideas, advices and guidance.

I wish to thank the UMS Postgraduate Center and the Ministry of Education Malaysia for providing both administrative and financial supports, respectively, throughout my PhD candidature. My deepest appreciation should also be dedicated to agencies and individuals who directly and indirectly contributed toward the completion of this thesis.

Mohammad Tamrin Bin Mohamad Lal  
20 May 2016

## ABSTRACT

Bacterial infections in aquaculture are commonly treated using antibiotics. However, due to health concern and environmental issues, new control strategies for bacterial diseases are needed. Therefore, this study was conducted to isolate and characterize bacteriophage that are potentially be used as therapy for fish bacterial diseases. Four species of bacterial pathogens (*Vibrio alginolyticus*, *V. harveyi*, *V. parahaemolyticus* and *Photobacterium damsela*) were targeted for bacteriophage isolation. Each bacteriophage isolate was spotted onto different bacterial pathogens (*V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus* and *Ph. damsela*) lawns. The bacteriophage morphology was determined using TEM and the whole genome sequence of bacteriophage was achieved using Illumina sequencing and *de novo* assembly. The stability of the bacteriophage was evaluated on different levels of pH, temperatures and bile concentrations. The bactericidal effect of the bacteriophage was evaluated using the *in vitro* co-culture method. In addition, the toxicity of the bacteriophage was evaluated against brine shrimp (*Artemia* sp.) and Asian seabass (*Lates calcarifer*) juveniles. This study has successfully isolated bacteriophage which were effective against *V. alginolyticus*, *V. harveyi* and *V. parahaemolyticus*. The bacteriophage isolates exhibited high specificity to its host with exception to *V. harveyi* phage that was also capable of infecting *V. parahaemolyticus* ATCC 17802. All phage isolates were classified under the double stranded DNA phage. The TEM analysis revealed that the *V. alginolyticus* phage, *V. harveyi* phage and *V. parahaemolyticus* phage were belong to the Family of *Myoviridae*, *Myoviridae* and *Siphoviridae*, respectively. The complete genome of *V. alginolyticus* phage was estimated at 248,088 bp and has high homology to *Vibrio* phage VH7D. Meanwhile, *V. parahaemolyticus* phage genome was 56,637 bp and hypothetically novel. Interestingly, all the phages possess methylated genome. The bioinformatics analyses revealed that the phage genomes have low significant homologies to vibrio virulent genes and toxin related proteins. All phage isolates were stable at 50 °C but completely deactivated at temperatures higher than 60 °C. The phage also stable at wide range of pH (4-9?) and high bile concentrations. Further analysis showed that the *V. parahaemolyticus* phage required high level of multiplicity of infection (MOI 100) to suppress the growth of its host but *V. harveyi* and *V. alginolyticus* phages required low MOI (0.01) to achieve similar effect. The findings of this study showed that the characteristics of the bacteriophage complied with the phage therapy requirement whereby all phages exhibited bactericidal effect and highly specific. The methylated genome allows the bacteriophage to survive from the defence mechanisms of the host bacteria. Lack of virulence genes prohibits the phage from contributing virulence to host bacteria through horizontal gene transfer. Furthermore, the phages were stable in both acidic and alkaline conditions which make them withstand the extreme condition of the gastrointestinal environment during therapy through oral administration. Most importantly, the bacteriophage were not toxic to the target animals. With these characteristics, the isolated phages seem beneficial for therapeutic use against vibriosis in aquaculture.

## **ABSTRAK**

### **PEMENCILAN DAN PENGENALPASTIAN BAKTERIOFAJ UNTUK TERAPI VIBRIOSIS IKAN**

Jangkitan bakteria di akuakultur pada umumnya dirawat menggunakan antibiotik. Namun, penggunaannya yang boleh menyebabkan masalah kesihatan dan menjejaskan alam sekitar memerlukan strategi kawalan jangkitan bakteria yang baru. Oleh itu, kajian ini dilakukan untuk memencil dan mengenalpasti bakteriofaj yang berpotensi untuk digunakan bagi tujuan terapi. Empat spesies bakteria patogen (*Vibrio alginolyticus*, *V. harveyi*, *V. parahaemolyticus* dan *Photobacterium damsela*) digunakan untuk tujuan pemencilan bakteriofaj. Setiap isolat bakteriofaj diuji ke atas hamparan bakteria patogen. Morfologi bakteriofaj tersebut ditentukan menggunakan TEM dan penjujukan keseluruhan genom bakteriofaj dihasilkan menggunakan penjujukan Illumina dan pemasangan genome *de novo*. Tahap kestabilan bakteriofaj dikaji pada tahap pH, suhu dan kepekatan hempedu yang berbeza. Kesan bakterisidal bakteriofaj ditentukan menggunakan ujian ko-kultur secara *in vitro*. Kesan toksik bakteriofaj pula ditentukan menggunakan ujian toksik terhadap anak udang (*Artemia sp.*) dan ikan siakap (*Lates calcarifer*). Kajian ini berjaya memencilkan bakteriofaj yang berkesan melawan *V. alginolyticus*, *V. harveyi* dan *V. parahaemolyticus*. Isolat bakteriofaj tersebut amat spesifik terhadap perumahnya kecuali pada isolat bakteriofaj *V. harveyi* yang boleh menjangkiti *V. parahaemolyticus* ATCC 17802. Semua isolate faj (*V. alginolyticus*, *V. harveyi* and *V. parahaemolyticus*) adalah faj DNA dwibebeban. Analisis TEM menunjukkan faj *V. alginolyticus*, faj *V. harveyi* and faj *V. parahaemolyticus* masing-masing berada pada Famili Myoviridae, Myoviridae and Siphoviridae. Jujukan genom lengkap bagi faj *V. alginolyticus* dianggarkan pada 248,088 bp dan homolog kepada *Vibrio phage VH7D*. Manakala, genom *V. parahaemolyticus* adalah 56,637 bp dan berkemungkinan novel. Menariknya, kebanyakan faj tersebut memiliki genom bermetil. Analisa bioinformatik menunjukkan genom-genom tersebut memiliki homolog yang rendah terhadap gen virulen dan protein toksin vibrio. Semua faj adalah stabil pada suhu 50 °C tetapi tidak aktif pada suhu lebih tinggi dari 60 °C. Faj tersebut stabil pada julat pH yang besar (4-9) dan boleh bertoleransi pada tahap kepekatan hempedu yang tinggi. Analisis lanjut menunjukkan bahawa Faj *V. parahaemolyticus* memerlukan MOI yang tinggi (MOI 100) untuk membantutkan pertumbuhan perumahannya, namun, faj *V. harveyi* dan *V. alginolyticus* boleh membantutkan pertumbuhan perumahannya pada MOI yang rendah (MOI 0.01). Dapatan kajian ini menunjukkan bakteriofaj tersebut menepati kriteria-kriteria untuk calon terapi dimana ia menunjukkan aktiviti bakterisidal yang tinggi dan amat spesifik. Genom bermetil juga membolehkan bakteriofaj bermandiri dari mekanisme pertahanan perumah. Gen virulen yang tidak dikesan menghadkan peningkatan tahap virulen bakteria melalui perpindahan gen. Selain itu, faj tersebut stabil dalam keadaan berasid dan beralkali membolehkan mereka bertoleransi dengan keadaan ekstrem gastrousus ikan selepas pemberian secara oral. Seterusnya, faj tersebut tidak toksik pada haiwan sasaran. Kesimpulan dari sifat-sifat yang dinyatakan, faj yang dipencilkan dalam kajian ini mungkin berfaedah untuk kegunaan terapeutik menentang vibriosis di akuakultur.

# LIST OF CONTENT

TITLE	Page
CERTIFICATION	i
DECLARATION	ii
ACKNOWLEDGEMENT	iii
ABSTRACT	iv
<b>ABSTRAK</b>	v
LIST OF CONTENTS	vi
LIST OF TABLES	vii
LIST OF FIGURES	xi
LIST OF ABBREVIATIONS	xii
LIST OF SYMBOLS	xx
LIST OF APPENDICES	xxi
<b>CHAPTER 1: GENERAL INTRODUCTION</b>	xxii
1.1 Common Bacterial Pathogens in Marine Fish Aquaculture	1
1.2 Treatments Option for Bacterial Diseases	1
1.3 Bacteriophage	2
1.3.1 Taxonomy of Bacteriophages	2
1.3.2 Structure of Caudovirales	3
1.3.3 Bacteriophage Lifecycle	4
a. Attachment	5
b. Penetration	6
c. Replication	7
1.4 Marine Bacteriophage	7
1.5 Bacteriophage for Aquaculture Pathogens	8
1.6 Bacteriophage Therapy in Aquaculture	9
1.7 Advantages and Disadvantages of Bacteriophage Therapy in Aquaculture	12
1.8 Objectives	13
<b>CHAPTER 2: ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGE AS POTENTIAL BIOCONTROL OF <i>Photobacterium damsela</i>, <i>Vibrio alginolyticus</i>, <i>V. harveyi</i> and <i>V. parahaemolyticus</i></b>	16
2.1 Introduction	16
2.2 Materials and Methods	17
2.2.1 Sampling	17
a. Water	17
b. Sand	17
c. Biofilm	17



	d.	Fish and Bivalve Organs	17
2.2.2		Bacterial Host for Bacteriophage Isolation	18
2.2.3		Screening of Bacteriophage	19
2.2.4		Isolation and Confirmation of Bacteriophage Plaque	20
2.2.5		Purification of Bacteriophage	20
2.2.6		Propagation of Bacteriophage	20
2.2.7		Bacteriophage Host Range Test	21
2.2.8		Bacteriophage Molecular Characterization	22
	a.	Precipitation of Bacteriophage	22
	b.	Genomic DNA Extraction	23
	c.	Standardization of Genomic DNA	24
	d.	Nature of Nucleic Acid	24
	e.	Restriction Fragment Length Polymorphism	24
	f.	Random Amplified Polymorphic DNA	25
	g.	Protein Profiling	26
2.3		Results	27
2.3.1		Screening of Bacteriophage	27
2.3.2		Isolation and Confirmation of Bacteriophage	29
2.3.3		Purification of Bacteriophage	29
2.3.4		Purified Bacteriophage Isolates	30
2.3.5		Bacteriophage Host Range	31
	a.	Host Specificity of Bacteriophage Isolates	31
	b.	Host Range of <i>Vibrio harveyi</i> and <i>V. parahaemolyticus</i> Bacteriophages	33
2.3.6		Nature of Nucleic Acid	35
2.3.7		Restriction Fragment Length Polymorphism	39
2.3.8		Random Amplified Polymorphic DNA	43
2.3.9		Protein Profiles	45
2.4		Discussion	49
2.5		Conclusion	55
<b>CHAPTER 3: MORPHOLOGICAL AND GENOME ANALYSES OF THREE BACTERIOPHAGE INFECTING THREE VIBRIO SPECIES (<i>Vibrio alginolyticus</i>, <i>Vibrio harveyi</i> AND <i>Vibrio parahaemolyticus</i>)</b>			<b>56</b>
3.1		Introduction	56
3.2		Materials and Methods	57
3.2.1		Bacteriophage Isolates	57
3.2.2		Bacteriophage Enrichment	57
3.2.3		Morphological Examination of Bacteriophage Isolates	57
3.2.4		Extraction of Genomic DNA	58
	a.	Extraction and Purification of VALLPKK3 and VPLPKK5 Genomic DNA	59
	b.	Extraction and Purification of VHLPKM4 Genomic DNA	59
	c.	Evaluation of Purified Bacteriophage DNA	60
3.2.5		Bacteriophage DNA Library Construction, Sequencing and Sequence Assembly	60
3.2.6		Genome Sequence Analyses	61
	a.	Homology Search	61

	b.	Genome Annotation	61
	c.	Genome Map	62
	d.	Nucleotide Sequence Accession Number	63
	e.	Virulence Factor	63
	f.	Single Gene Analysis	63
3.3		Results	63
	3.3.1	Morphology of VALLPKK3, VHLPKM4 and VPLPKK5	63
	3.3.2	Bacteriophage Genomic DNA	65
	3.3.3	Homology Search	66
	3.3.4	Genome Annotation	67
	a.	VALLPKK3 Genome Overview	67
		i. General Features of VALLPKK3 Genome	67
		ii. Putative Functional Protein of VALLPKK3 Genome	73
		iii. Genome Map of Bacteriophage VALLPKK3	86
	b.	VPLPKK5 Genome Overview	88
		i. General Features of VPLPKK5 Genome	88
		ii. Putative Functional Protein of VPLPKK5 Genome	90
		iii. Genome Map of Bacteriophage VPLPKK5	93
	c.	Nucleotide Sequence Accession Number	95
	3.3.5	Lysogeny and Virulence Factors	95
	3.3.6	Single Gene Analysis	100
3.4		Discussion	101
	3.4.1	<i>Vibrio alginolyticus</i> Phage VALLPKK3	101
	3.4.2	<i>Vibrio harveyi</i> Phage VHLPKM4	101
	3.4.3	<i>Vibrio parahaemolyticus</i> Phage VPLPKK5	102
	3.4.4	Putative Functions of the CDSs in VALLPKK3 Genome	102
	3.4.5	Putative Functions of the CDSs in VPLPKK5 Genome	108
	3.4.6	Lysogeny and Toxin Genes in VALLPKK3 and VPLPKK5 genomes	110
	3.4.7	Phylogenetic Analysis of VPLPKK5 Using DNA Polymerase Gene	111
3.5		Conclusion	112
	<b>CHAPTER 4:</b>	<b>GROWTH CHARACTERISTICS OF BACTERIOPHAGE INFECTING AQUACULTURE BACTERIAL PATHOGENS</b>	<b>113</b>
4.1		Introduction	113
4.2		Materials and Methods	114
	4.2.1	Bacteriophage Isolates	114
	4.2.2	Bacteriophage Adsorption Assay	114
	4.2.3	Bacteriophage One Step Growth	114
	4.2.4	Bacteriophage Tolerance Test	115
		a. Temperature Tolerance Test	115
		b. pH Tolerance Test	115
		c. Bile Salt Tolerance Test	116
4.3		Results	116
	4.3.1	Bacteriophage Adsorption Assay	116

4.3.2	Bacteriophage One Step Growth	118
4.3.3	Bacteriophage Tolerance Test	121
4.4	Discussion	124
4.5	Conclusion	126
<b>CHAPTER 5: BACTERICIDAL AND TOXICITY ASSESSMENTS OF BACTERIOPHAGE</b>		127
5.1	Introduction	127
5.2	Materials and Methods	128
5.2.1	<i>In Vitro</i> Co-culture	128
5.2.2	<i>In Vitro</i> Toxicity to <i>Artemia</i> Spp.	128
5.2.3	<i>In Vivo</i> Toxicity Study in Fish	129
5.3	Results	129
5.3.1	<i>In Vitro</i> Co-culture	129
5.3.2	<i>In Vitro</i> Toxicity to <i>Artemia</i> Spp.	133
5.3.3	<i>In Vivo</i> Toxicity Study Asian Seabass, <i>Lates calcarifer</i>	134
5.4	Discussion	135
5.5	Conclusion	136
<b>CHAPTER 6: GENERAL DISCUSSION</b>		137
<b>CHAPTER 7: GENERAL CONCLUSION</b>		144
<b>REFERENCE</b>		145
<b>APPENDICES</b>		174



UMS  
UNIVERSITI MALAYSIA SABAH

## LIST OF TABLES

	Page
Table 1.1	Taxonomy of bacteriophage <span style="float: right;">4</span>
Table 1.2	List of studies on the isolation of bacteriophage against fish pathogenic bacteria <span style="float: right;">10</span>
Table 1.3	Advantages and disadvantages of bacteriophage therapy in aquaculture <span style="float: right;">14</span>
Table 2.1	List of bacterial strains used for bacteriophage isolation <span style="float: right;">18</span>
Table 2.2	List of bacterial strains used for bacteriophage host range <span style="float: right;">22</span>
Table 2.3	Restriction endonucleases used in RFLP analysis of the bacteriophage genomic DNA <span style="float: right;">23</span>
Table 2.4	Purified bacteriophage isolates for respective bacterial host <span style="float: right;">30</span>
Table 2.5	Susceptibility of bacterial species to the bacteriophages <span style="float: right;">32</span>
Table 2.6	Lytic spectra of <i>V. harveyi</i> phages against <i>V. harveyi</i> isolates <span style="float: right;">34</span>
Table 2.7	Lytic spectra of <i>V. parahaemolyticus</i> phages against <i>V. parahaemolyticus</i> isolates <span style="float: right;">34</span>
Table 2.8	Molecular weight of structural proteins from the representative <i>V. alginolyticus</i> , <i>V. harveyi</i> and <i>V. parahaemolyticus</i> bacteriophages <span style="float: right;">49</span>
Table 3.1	Free on-line protein search tools <span style="float: right;">62</span>
Table 3.2	Morphological dimensions of VALLPKK3, VHLPKM4 and VPLPKK5 <span style="float: right;">65</span>
Table 3.3	Concentration and purity of the bacteriophage genomic DNA <span style="float: right;">66</span>
Table 3.4	Nucleotide BLAST search for VALLPKK3, VHLPKM4 and VPLPKK5 genome sequence <span style="float: right;">67</span>
Table 3.5	Predicted ORFs of VALLPKK3 <span style="float: right;">68</span>
Table 3.6	Putative function of CDSs in Bacteriophage VALLPKK3 <span style="float: right;">74</span>
Table 3.7	Predicted ORFs of VPLPKK5 <span style="float: right;">89</span>
Table 3.8	Putative function of CDSs in Bacteriophage VPLPKK5 <span style="float: right;">91</span>
Table 3.9	Result of the virulence factors screening of VALLPKK3 against VFDB and MvirDB databases <span style="float: right;">96</span>
Table 3.10	Result of the virulence factors screening of VPLPKK5 against VFDB and MvirDB databases <span style="float: right;">99</span>

## LIST OF FIGURES

	Page
Figure 1.1	Schematic diagram of T4 bacteriophage. 5
Figure 1.2	Schematic diagram of the lytic and lysogenic lifecycle of bacteriophage. → = lytic cycle, → = enter lysogenic cycle, → = switch to lytic cycle. 28
Figure 2.1	Screening of bacteriophage. The bacteriophage plaque formed on the bacterial lawn (red arrow). A: <i>Vibrio alginolyticus</i> bacteriophage plaques, B: <i>V. harveyi</i> bacteriophage plaques, C: <i>V. parahaemolyticus</i> bacteriophage plaques. 29
Figure 2.2	Confirmation of bacteriophage from individual plaque isolated from bacterial lawn. A: <i>Vibrio alginolyticus</i> bacteriophages, B: <i>V. harveyi</i> bacteriophages, C: <i>V. parahaemolyticus</i> bacteriophages. 30
Figure 2.3	Purification of bacteriophage isolates from different bacterial lawn. Red arrow shows a well-isolated single plaque. A: Representative of <i>Vibrio alginolyticus</i> bacteriophage isolates, VALLPKK19, B: Representative of <i>V. harveyi</i> bacteriophage isolates, VHLPKM3, C: Representative of <i>V. parahaemolyticus</i> bacteriophage isolates, VPLPKK13. 31
Figure 2.4	Susceptibility of bacteria to bacteriophage: Susceptible (clear lysis), intermediate susceptible (turbid lysis) and resistant (no lysis). 36
Figure 2.5	Gel electrophoresis analysis of <i>V. alginolyticus</i> , <i>V. harveyi</i> and <i>V. parahaemolyticus</i> bacteriophages genomic nucleic acid digested with DNase I enzyme. A) Total genomic nucleic acid of <i>V. alginolyticus</i> bacteriophage isolates; Lane 1: VALLPKK1, Lane 2: VALLPKK2, Lane 3: VALLPKK3, Lane 4: VALLPKK4, Lane 5: VALLPKK5, Lane 6: VALLPKK6, Lane 7: VALLPKK7, 8: Lane VALLPKK8, 9: Lane VALLPKK9, Lane 10: VALLPKK10, Lane 11: VALLPKK11, Lane 12: VALLPKK12, Lane 13: VALLPKK13, Lane 14: VALLPKK14, Lane 15: VALLPKK15, 36

VPLPKK6, Lane 7: VPLPKK7, Lane 8: VPLPKK8, Lane 9: VPLPKK9, Lane 10: VPLPKK10, Lane 11: VPLPKK11, Lane 12: VPLPKK12, Lane 13: VPLPKK13, Lane 14: VPLPKK14, Lane 15: VPLPKK15, Lane 16: VPLPKK16, Lane 17; VPLPKK17, Lane 18: VPLPKK18, Lane 19: VPLPKK19, Lane 20: VPLPKK20, Lane M: 1 kb DNA Ladder (Promega).

Figure 2.7

Gel electrophoresis analysis of *V. alginolyticus*, *V. harveyi* and *V. parahaemolyticus* bacteriophages genomic nucleic acid digested with S1 Nuclease enzyme. A) Total genomic nucleic acid of *V. alginolyticus* bacteriophage isolates; Lane 1: VALLPKK1, Lane 2: VALLPKK2, Lane 3: VALLPKK3, Lane 4: VALLPKK4, Lane 5: VALLPKK5, Lane 6: VALLPKK6, Lane 7: VALLPKK7, 8: Lane VALLPKK8, 9: Lane VALLPKK9, Lane 10: VALLPKK10, Lane 11: VALLPKK11, Lane 12: VALLPKK12, Lane 13: VALLPKK13, Lane 14: VALLPKK14, Lane 15: VALLPKK15, Lane 16: VALLPKK16, Lane 17; VALLPKK17, Lane 18: VALLPKK18, Lane 19: VALLPKK19, Lane 20: VALLPKK20. B) Total genomic nucleic acid of *V. harveyi* bacteriophage isolates; Lane 1: VHLPKM1, Lane 2: VHLPKM2, Lane 3: VHLPKM3, Lane 4: VHLPKM4, Lane 5: VHLPKM5, Lane 6: VHLPKM6, Lane 7: VHLPKM7, 8: Lane VHLPKM8. C) Total genomic nucleic acid of *V. parahaemolyticus* bacteriophage isolates; Lane 1: VPLPKK1, Lane 2: VPLPKK2, Lane 3: VPLPKK3, Lane 4: VPLPKK4, Lane 5: VPLPKK5, Lane 6: VPLPKK6, Lane 7: VPLPKK7, 8: Lane VPLPKK8, 9: Lane VPLPKK9, Lane 10: VPLPKK10, Lane 11: VPLPKK11, Lane 12: VPLPKK12, Lane 13: VPLPKK13, Lane 14: VPLPKK14, Lane 15: VPLPKK15, Lane 16: VPLPKK16, Lane 17; VPLPKK17, Lane 18: VPLPKK18, Lane 19: VPLPKK19, Lane 20: VPLPKK20, Lane M: 1 kb DNA Ladder (Promega).

38

Figure 2.8

RFLP analysis of *V. alginolyticus* phage DNA using *Bam*HI (A), *Alu*I (B), *Hind*III (C), *Hae*III (D) and *Eco*RI (E). Lane 1: VALLPKK1, Lane 2: VALLPKK2, Lane 3: VALLPKK3, Lane 4:

40

VALLPKK4, Lane 5: VALLPKK5, Lane 6: VALLPKK6, Lane 7: VALLPKK7, 8: Lane VALLPKK8, 9: Lane VALLPKK9, Lane 10: VALLPKK10, Lane 11: VALLPKK11, Lane 12: VALLPKK12, Lane 13: VALLPKK13, Lane 14: VALLPKK14, Lane 15: VALLPKK15, Lane 16: VALLPKK16, Lane 17; VALLPKK17, Lane 18: VALLPKK18, Lane 19: VALLPKK19, Lane 20: VALLPKK20, Lane M: 1 kb DNA Ladder (Promega).

Figure 2.9 RFLP analysis of *V. harveyi* phage DNA using *Bam*HI (A), *Alu*I (B), *Hind*III (C), *Hae*III (D) and *Eco*RI (E). Lane 1: 41

VHLPKM1, Lane 2: VHLPKM2, Lane 3: VHLPKM3, Lane 4: VHLPKM4, Lane 5: VHLPKM5, Lane 6: VHLPKM6, Lane 7: VHLPKM7, 8: Lane VHLPKM8, Lane M: 1 kb DNA Ladder (Promega).

Figure 2.10 RFLP analysis of *V. parahaemolyticus* phage DNA using 42

*Bam*HI (A), *Alu*I (B), *Hind*III (C), *Hae*III (D) and *Eco*RI (E).

Lane 1: VPLPKK1, Lane 2: VPLPKK2, Lane 3: VPLPKK3, Lane 4: VPLPKK4, Lane 5: VPLPKK5, Lane 6: VPLPKK6, Lane 7: VPLPKK7, Lane 8: VPLPKK8, Lane 9: VPLPKK9, Lane 10: VPLPKK10, Lane 11: VPLPKK11, Lane 12: VPLPKK12, Lane 13: VPLPKK13, Lane 14: VPLPKK14, Lane 15: VPLPKK15, Lane 16: VPLPKK16, Lane 17; VPLPKK17, Lane 18: VPLPKK18, Lane 19: VPLPKK19, Lane 20: VPLPKK20, Lane M/M1: 1 kb DNA Ladder (Promega), Lane M2: 100 bp DNA Ladder (promega), Lane M3: Lambda/HindIII Marker (Promega).

Figure 2.11 RAPD analysis of all bacteriophage DNA using (GTG)<sub>5</sub>. A) *V.* 44

*alginolyticus* bacteriophage, Lane 1: VALLPKK1, Lane 2: VALLPKK2, Lane 3: VALLPKK3, Lane 4: VALLPKK4, Lane 5: VALLPKK5, Lane 6: VALLPKK6, Lane 7: VALLPKK7, 8: Lane VALLPKK8, 9: Lane VALLPKK9, Lane 10: VALLPKK10, Lane 11: VALLPKK11, Lane 12: VALLPKK12, Lane 13: VALLPKK13, Lane 14: VALLPKK14, Lane 15: VALLPKK15, Lane 16: VALLPKK16, Lane 17; VALLPKK17, Lane 18: VALLPKK18,

Lane 19: VALLPKK19, Lane 20: VALLPKK20, Lane VAL: *V. alginolyticus* DNA, Lane - : Sterile distilled water. B) *V. harveyi* bacteriophage, Lane 1: VHLPKM1, Lane 2: VHLPKM2, Lane 3: VHLPKM3, Lane 4: VHLPKM4, Lane 5: VHLPKM5, Lane 6: VHLPKM6, Lane 7: VHLPKM7, 8: Lane VHLPKM8, Lane VH: *V. harveyi* DNA, Lane - : Sterile distilled water. C) *V. parahaemolyticus* bacteriophage. Lane 1: VPLPKK1, Lane 2: VPLPKK2, Lane 3: VPLPKK3, Lane 4: VPLPKK4, Lane 5: VPLPKK5, Lane 6: VPLPKK6, Lane 7: VPLPKK7, 8: Lane VPLPKK8, 9: Lane VPLPKK9, Lane 10: VPLPKK10, Lane 11: VPLPKK11, Lane 12: VPLPKK12, Lane 13: VPLPKK13, Lane 14: VPLPKK14, Lane 15: VPLPKK15, Lane 16: VPLPKK16, Lane 17; VPLPKK17, Lane 18: VPLPKK18, Lane 19: VPLPKK19, Lane 20: VPLPKK20, Lane VP: *V. parahaemolyticus* DNA, Lane - : Sterile distilled water. Lane M: 1 kb DNA Ladder (Promega).

Figure 2.12

SDS-PAGE analysis of structural proteins of *V. alginolyticus* bacteriophages. Protein bands are indicated by yellow numbers. The most predominant protein bands are indicated by red arrows. Lane 1: VALLPKK1, Lane 2: VALLPKK2, Lane 3: VALLPKK3, Lane 4: VALLPKK4, Lane 5: VALLPKK5, Lane 6: VALLPKK6, Lane 7: VALLPKK7, 8: Lane VALLPKK8, 9: Lane VALLPKK9, Lane 10: VALLPKK10, Lane 11: VALLPKK11, Lane 12: VALLPKK12, Lane 13: VALLPKK13, Lane 14: VALLPKK14, Lane 15: VALLPKK15, Lane 16: VALLPKK16, Lane 17; VALLPKK17, Lane 18: VALLPKK18, Lane 19: VALLPKK19, Lane 20: VALLPKK20, Lane M: Broad Range Protein Molecular Weight Marker (Promega).

46

Figure 2.13

SDS-PAGE analysis of structural proteins of *V. harveyi* bacteriophages. Protein bands are indicated by yellow numbers. The most predominant protein bands are indicated by red arrows. Lane 1: VHLPKM1, Lane 2: VHLPKM2, Lane 3: VHLPKM3, Lane 4: VHLPKM4, Lane 5: VHLPKM5, Lane 6:

47



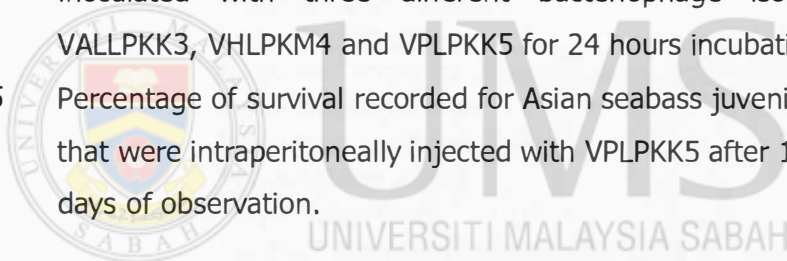
	VHLPKM6, Lane 7: VHLPKM7, 8: Lane VHLPKM8, Lane M: Broad Range Protein Molecular Weight Marker (Promega).	
Figure 2.14	SDS-PAGE analysis of structural proteins of <i>V. parahemolyticus</i> bacteriophages. Protein bands are indicated by yellow numbers. The most predominant protein bands are indicated by red arrows. Lane 1: VPLPKK1, Lane 2: VPLPKK2, Lane 3: VPLPKK3, Lane 4: VPLPKK4, Lane 5: VPLPKK5, Lane 6: VPLPKK6, Lane 7: VPLPKK7, 8: Lane VPLPKK8, 9: Lane VPLPKK9, Lane 10: VPLPKK10, Lane 11: VPLPKK11, Lane 12: VPLPKK12, Lane 13: VPLPKK13, Lane 14: VPLPKK14, Lane 15: VPLPKK15, Lane 16: VPLPKK16, Lane 17; VPLPKK17, Lane 18: VPLPKK18, Lane 19: VPLPKK19, Lane 20: VPLPKK20, Lane M: Broad Range Protein Molecular Weight Marker (Promega).	48
Figure 3.1	Electron micrograph of VALLPKK3. Bars = 100 nm (A), 200 nm (B).	64
Figure 3.2	Electron micrograph of VHLPKM4. Bars = 200 nm	64
Figure 3.3	Electron micrograph of VPLPKK5. Bars = 100 nm	65
Figure 3.4	Purified bacteriophage genomic DNA. 1: DNA of VALLPKK3; 2: DNA of VHLPKM4; 3: DNA of VPLPKK5; M: 1 kb DNA Ladder (Promega).	66
Figure 3.5	A circular genome map illustrating the genes in bacteriophage VALLPKK3. nt = nucleotide.	87
Figure 3.6	A circular genome map illustrating the genes in bacteriophage VPLPKK5. nt = nucleotide.	94
Figure 3.7	The phylogenetic tree of DNA polymerases from <i>Siphoviridae</i> was constructed using Neighbor-Joining Method. Bootstrap values based on 1000 resampling. Only values greater than 50 are shown.	100
Figure 4.1	Adsorption of VALLPKK3 to <i>V. alginolyticus</i> ATCC® 17749™.	117
Figure 4.2	Adsorption of VHLPKM4 to <i>V. harveyi</i> VHJR7.	117
Figure 4.3	Adsorption of VPLPKK5 to <i>V. parahaemolyticus</i> KPHGV1.	118
Figure 4.4	One step growth curve of VALLPKK3 infected with <i>Vibrio</i>	119

*alginolyticus* ATCC® 17749™ at MOI of 0.001. The number of PFU per infected cell in untreated culture (○) and chloroform-treated culture (Δ) are also shown. The burst size, latent period and eclipse are indicated as B, L and E, respectively.

- Figure 4.5 One step growth curve of VALLPKK3 infected with *Vibrio harveyi* VHJR7 at MOI of 0.001. The number of PFU per infected cell in untreated culture (○) and chloroform-treated culture (Δ) are also shown. The burst size, latent period and eclipse are indicated as B, L and E, respectively. 120
- Figure 4.6 One step growth curve of VPLPKK5 infected with *V. parahaemolyticus* KPHGV1 at MOI of 0.001. The number of PFU per infected cell in untreated culture (○) and chloroform-treated culture (Δ) are also shown. The burst size, latent period and eclipse are indicated as B, L and E, respectively. 121
- Figure 4.7 The temperature stability of VALLPKK3 (■), VHLPKM4 (■) and VPLPKK5 (■). All isolates were incubated at various range of temperature (40 °C, 50 °C, 60 °C, 70 °C, 80 °C, 90 °C and 100 °C) for 1 hour. Data are the means from three independent experiments (Mean ± Standard Deviation). 122
- Figure 4.8 The temperature stability of VALLPKK3 (■), VHLPKM4 (■) and VPLPKK5 (■). All isolates were incubated at various range of pH (2, 3, 4, 5, 6, 7, 8 and 9) for 24 hours. Data are the means from three independent experiments (Mean ± Standard Deviation). 123
- Figure 4.9 The bile salt stability of VALLPKK3 (■), VHLPKM4 (■) and VPLPKK5 (■). All isolates were incubated at various range of bile salt concentration (5000, 6000, 7000, 8000 and 9000 ppm) for 24 hours. Data are the means from three independent experiments (Mean ± Standard Deviation). 123
- Figure 5.1 Co-culture of VALLPKK3 phage and *Vibrio alginolyticus* ATCC® 17749™ at MOI 0 (●), 0.01 (●), 1 (●) and 100 131

(●). The absorbance at OD<sub>600</sub> was measured per hour basis. The results are shown as the mean ± standard deviations from triplicate experiments.

- Figure 5.2 Co-culture of VHLPKM4 phage and *Vibrio harveyi* VHJR7 at MOI 0 (○), 0.01 (●), 1 (◐) and 100 (◑). The absorbance at OD<sub>600</sub> was measured per hour basis. The results are shown as the mean ± standard deviations from triplicate experiments. 131
- Figure 5.3 Co-culture of VPLPKK5 phage and *Vibrio parahaemolyticus* KPHGV1 at MOI 0 (○), 0.01 (●), 1 (◐) and 100 (◑). The absorbance at OD<sub>600</sub> was measured on hour basis. The results are shown as the mean ± standard deviations from triplicate experiments. 132
- Figure 5.4 Percentage of survival of *Artemia* nauplii (instar 2 – 3) inoculated with three different bacteriophage isolates: VALLPKK3, VHLPKM4 and VPLPKK5 for 24 hours incubation. 132
- Figure 5.5 Percentage of survival recorded for Asian seabass juveniles that were intraperitoneally injected with VPLPKK5 after 10 days of observation. 133



## LIST OF SYMBOLS

$^{\circ}\text{C}$	Degree Celcius
$\mu\text{l}$	microliter
$\text{OD}_{600}$	Optical Density at 600 nm
%	Percent
$\text{mM}$	Milimolar
$\text{ml}$	Mililiter
$\text{l}$	Liter
$\text{xg}$	Times gravity
$\text{M}$	Molar
$\text{ng } \mu\text{l}^{-1}$	Nanoram per microliter
$\text{nm}$	Nanometer
$\Phi$	Bacteriophage
$\Phi$	Bacteriophage
$\Phi$	Bacteriophage
$\Psi$	Bacteriophage
$\text{cfu ml}^{-1}$	Colony forming unit per mililiter
$\text{pfu ml}^{-1}$	Plaque forming unit per mililiter



UMS  
UNIVERSITI MALAYSIA SABAH

## LIST OF APPENDICES

		Page
Appendix 1	Location of field samplings	174
Appendix 2	Samples used in screening of bacteriophage	175
Appendix 3	Concentration of Bacteriophage Genomic DNA	179
Appendix 4	BLAST result using VFDB database of ORF231 from VALLPKK3 genome	180
Appendix 5	BLAST result using VFDB database of orf64 from VPLPKK5 genome	181
Appendix 6	Adsorption test	182
Appendix 7	One step growth test	183
Appendix 8	Temperature tolerance test	185
Appendix 9	pH tolerance test	186
Appendix 10	Bile salt tolerance test	187
Appendix 11	<i>In vitro</i> co-culture test <i>v. alginolyticus</i> bacteriophage	188
Appendix 12	<i>In vitro</i> co-culture test <i>v. harveyi</i> bacteriophage	190
Appendix 13	<i>In vitro</i> co-culture test <i>v. parahaemolyticus</i> bacteriophage	192
Appendix 14	<i>In vitro</i> toxicity test	194
Appendix 15	<i>In vivo</i> toxicity test	196
Appendix 16	List of Publications	197

# CHAPTER 1

## GENERAL INTRODUCTION

### 1.1 Common Bacterial Pathogens in Marine Fish Aquaculture

Fish in captivity as well as in the natural habitat are exposed to many kinds of bacterial diseases including as vibriosis, streptococcosis and bacterial kidney disease (BKD) (Toranzo *et al.*, 2005). Vibriosis is one of the bacterial diseases which often occurs in aquaculture. Vibriosis may cause by various *Vibrios* such as *V. anguillarum*, *V. ordalii*, *V. salmonicida*, *V. vulnificus*, *V. harveyi*, *V. alginolyticus*, *V. cholerae*, *V. fischeri*, *V. furnisii*, *V. ichthyenteri*, *V. logei*, *V. pelagius*, *V. splendidus*, *V. tapetis* or *V. wodanis* (Toranzo *et al.*, 2005; Won and Park, 2008; Austin and Austin, 2007). The outbreak of vibriosis has been reported to occur worldwide involving many marine organisms (Austin and Austin, 2007) and also freshwater fishes (Geng *et al.*, 2014). Fish affected by this disease generally shows typical signs of haemorrhage on the base of fins, exophthalmia, corneal opacity and skin lesions. Meanwhile, the moribund fish will experience severe anemia which manifested by pale gills (Toranzo *et al.*, 2005). Study by Ransangan and Mustafa (2009) showed that *V. harveyi* is responsible for mortality in Asian seabass (*Lates calcarifer*) cultured in Sabah, Malaysia.

Streptococcosis had been reported both in freshwater and marine fish aquaculture. Although it can be caused by many *Streptococcus* species, most of the infections in marine aquaculture are due to *Streptococcus iniae* (Musa *et al.*, 2007). Infected fish normally showed meningoencephalitis, panophthalmitis, skin lesion, necrosis, corneal opacity and hemorrhage (Musa *et al.*, 2007). Streptococcosis can easily be transmitted through contact with infected fish or contaminated feeds (Musa *et al.*, 2007).

*Photobacterium damsela* is a marine bacterium that causes infection in a variety of marine fish (Rivas *et al.*, 2013). Fish species which are reported to be affected by this pathogen include rainbow trout (Pedersen *et al.*, 2009), seabass

(Labella *et al.*, 2006) and turbot (Fouz *et al.*, 1992). This pathogen is reported to causing wound infections and haemorrhagic septicemia in fish. (Rivas *et al.*, 2013).

## **1.2 Treatments Option for Bacterial Diseases**

Fish diseases caused by bacteria are commonly treated with antibiotics. However, due to health concern and environmental issues, the use of antibiotics is no longer accepted in many countries including Malaysia (Musa *et al.*, 2008). Studies also showed that rampant use of antibiotic can promote the development of antibiotic resistant bacteria in net cage aquaculture environment (Tendencia and de la Pena, 2001). Hence, there is a need for development of noble strategies which are harmless to both consumers and environment, in fighting for bacterial pathogens in aquaculture.

The use of vaccines in aquaculture has been shown to successfully protect fish against bacterial diseases, such as vibriosis (Sun *et al.*, 2009), edwardsiellosis (Liu *et al.*, 2005), furunculosis (Gudmundsdóttir and Björnsdóttir, 2007), streptococcosis (Heath and Feldman, 2005) and pasteurellosis (Andreoni and Magnani, 2014). According to Collado *et al.* (2000), vaccine application was better solution against vibriosis. However, vaccine is only promoting the fish immune system to resist the bacterial infection without controlling the proliferation of the target bacteria itself. Therefore, other strategy to control the target bacteria is necessary.

The increasing interest in the application of bacteriophages in aquaculture is something worthy to investigate (Nakai and Park, 2002). Due to its host specificity, bacteriophages normally do not disturb the natural bacterial flora inside the fish. Therefore, isolation of bacteriophages which have the ability to kill fish bacterial pathogens may provide new avenue for diseases control in aquaculture.

## **1.3 Bacteriophage**

Bacteriophage are viruses which prey on bacteria (Gillis and Mahillon, 2014). Similar to other viruses, they are absolute parasitic to bacteria (Kutter and Sulakvelidze, 2005). Bacteriophage were first discovered by Federick Twort and

Felix d'Herelle in 1915 and 1917, respectively (Duckworth, 1976). Independently, Felix d'Herelle characterized this virus and named as bacteriophage, meaning "bacterial eater" (D'Herelle, 1917). The subsequent decades, researchers continue to examine the nature of the bacteriophage. In fact, the bacteriophage have been used as model microorganism to investigate the various aspect of viruses (Keen, 2015), such as virion structure, genetics and viral replication system. Hershey and Chase (1952) reported that the DNA was the carrier of genetic information in bacteriophage. The T4 bacteriophage was also used as a tool to study the discontinuous replication of DNA by Okazaki *et al.* (1968). The bacteriophage lambda has been extensively used for a range of studies including understanding of gene regulation (Ptashne *et al.*, 2004) and vector for gene analysis (Chauthaiwale, 1992). In addition, the extensive study on bacteriophage genome has provide the insight into the identification of novel biochemical mechanisms (Miller *et al.*, 2003a).

The intensive study on therapeutic use of bacteriophage began in 1920 (Carlton, 1999). After the discovery of the first antibiotic, Penicillin in 1928 (Garrod, 1947), the study of therapeutic possibilities of bacteriophage was abandoned in favour of the wider usage of antibiotics (Gill and Hyman, 2010). However, the research on bacteriophage continued in the Eastern Europe and former Soviet Union (Sulakvelidze *et al.*, 2001). The lack of international peer review and limited number of English articles have somehow contributed to unavailability of the progress of these works to the international scientific communities. The interest in bacteriophage therapy was only revived in recent years following the rampant occurrence of antibiotic resistant bacteria (Keary *et al.*, 2013).

### **1.3.1 Taxonomy of Bacteriophages**

The initial classification of bacteriophage was based on the different in host specificities (Nelson, 2004). With the advent of electron microscope, the bacteriophage was classified using morphology. To date, approximately 96% of the bacteriophage belong to the order Caudovirales have been successfully examined via electron microscopy (Ackermann, 2003). The current report on the taxonomy of bacteriophage is listed in Table 1.1.