PURIFICATION AND CHARACTERISATION OF VIBRIOPHAGE ISOLATED FROM MANGALAVANAM MANGROVE

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by

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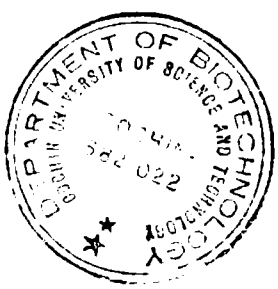
CERTIFICATE

This is to certify that the research work presented in the thesis entitled " **Purification and characterisation of Vibriophage isolated from Mangalavanam mangrove**" is based on the original research work carried out by Miss. Archana Kishore under my guidance and supervision at the Department of Biotechnology, Cochin University of Science and Technology, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, and that no part thereof has been presented for the award of any other degree.



SARITA G. BHAT

DECLARATION

I hereby declare that the work presented in this thesis entitled "**Purification and characterisation Of Vibriophage isolated from Mangalavanam mangrove**" is based on the original research carried out by me at the Department of Biotechnology, Cochin University of Science and Technology, Cochin under the guidance of Dr. Sarita G. Bhat, Senior Lecturer, Cochin University of Science and Technology and the thesis or no part thereof has been presented for the award of any degree, diploma, associate ship or other similar titles or recognition.

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Archana Kishore

Dedicated to My Parents &
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ABBREVIATIONS

%	-	Percentage
°C	-	Degree Celsius
A	-	Adenine
A°	-	Armstrong
A_{280}	-	Absorbance at 280nm
APS	-	Ammonium per sulphate
BLAST	-	Basic Local Alignment Search Tool
bp	-	Base pairs
BSA	-	Bovine serum albumin
C	-	Cytosine
cfu	-	Colony forming units
DNA	-	Deoxy ribo nucleic acid
dNTP	-	deoxy nucleotide tri phosphate
dsDNA	-	double stranded DNA
DW	-	Distilled water
EDTA	-	Ethylene diamine tetra acetic acid
g	-	grams
G	-	Guanine
Hrs	-	Hours
ICTV	-	International Committee on Taxonomy of Viruses
kDa	-	Kilo Dalton
kV	-	Kilo Volt
LB	-	Luria Bertani

M	-	Molar
mg	-	milligram
ml	-	milliliter
mm	-	millimeter
MOI	-	Multiplicity of Infection
MSA	-	Multiple sequence alignment
NJ	-	Neighbour Joining
nm	-	Nanometer
OD	-	Optical density
ORF	-	Open reading frame
PAGE	-	Polyacrylamide gel electrophoresis
PCR	-	Polymerase chain reaction
PEG	-	Polyethylene glycol
Pfu	-	plaques forming units
RNA	-	Ribonucleic acid
rpm	-	Rotations per minute
rRNA	-	ribosomal RNA
RT	-	Room temperature
SDS	-	Sodium dodecyl sulphate
sp.	-	Species
ssDNA	-	single stranded DNA
T	-	Thymine
TE	-	Tris EDTA
TEM	-	Transmission Electron Microscope
TEMED	-	N-N-N'-N'-tetramethyl ethylene Diamine
UV	-	Ultra violet

v/v	-	volume/volume
µg	-	microgram
µl	-	microlitre
µM	-	micromole

CONTENTS

Chapter 1

INTRODUCTION

Phages are viruses that infect prokaryotes and were first described in the early 1900s (d'Herelle, 1917; Twort, 1915). They can be classified into two groups, virulent and temperate phages. Virulent phages induce lytic infection, resulting in the lysis of the host cells and producing clear plaques on lawns of susceptible bacteria. Temperate phages integrate their DNA within the genome of the host bacteria, resulting in a lysogenic infection and the phage genome is passed to all daughter cells at cell division. Lysogenic phages generally develop hazy or turbid plaques on lawns of susceptible host cells. Another class of phages known as filamentous phages have a filamentous capsid with mostly a circular ssDNA molecule. Eg. M13, fd etc. The earliest reports of bacteriophage activity in marine ecosystems date back to the first quarter of this century (ZoBell, 1946), although the existence of indigenous marine bacteriophages was not confirmed until 1955 (Spencer, 1955).

The phage is probably the most widely distributed biological entity in the biosphere, with an estimated population of $>10^{30}$ or ~10 million per cubic centimeter of any environmental niche where bacteria or archaea reside (Wommack and Colwell, 2000). There are an estimated $\sim 10^{10}$ phage per liter of surface seawater (Bergh *et al.*, 1989) and about 10^7 to 10^9 per g of sediment or topsoil (Danovaro and Serresi, 2000; Danovaro *et al.*, 2001; Hodgson, 2000). In the ocean, phages are major predators of bacteria and significant sinks of essential nutrients (e.g., nitrogen and phosphorus) (Wilhelm and Suttle, 1999). It is now known that viral abundances typically exceed those of bacteria and routinely occur at $>10^7$ particles ml^{-1} (Mann, 2005).

There is diversity at one level, in the types of phages that infect individual or interrelated bacterial species and at another level, among genomically related phages that do not share the same bacterial hosts. One example is the lytic Enterobacterial dsDNA phage T4, which has relatives that are specific to *Aeromonas*, *Vibrio*, *Acinetobacter*, and other bacterial species. The genome sequences of few T4-like phages were sequenced and found not only to share homologies with T4, but also to differ from one another in size, organization of the T4-like genes, content of other putative genes, and DNA mobile elements. The T4-related phages are known to cross bacterial species barriers and possess plastic genomes that can acquire and lose genetic cassettes through their travels in the microbial world (Petrov *et al.*, 2006). In addition to evolving by serving as traffickers of microbial genes, phage genomes evolve through the accumulation of mutations in both acquired and core genes. Sequence divergence among homologues of the essential genes for phage propagation within a phage family can be used as a source of information about the determinants of specificity of the protein-protein and protein-nucleic-acid interactions that underlie biological function (Karam, 2005).

Phages are known to be major conduits of genetic exchange, transducing an estimated 10^{25} to 10^{28} bp of DNA per year in the world's oceans (Jiang and Paul, 1998; Paul, 1999). Currently, bacteriophages are considered to be dynamic partners in microbial food webs (Abedon, 2006), and it is thought that, on average, approximately, 20% of the standing stock of bacteria are lysed by phages on a daily basis (Chibani-Chennoufi *et al.*, 2004; Suttle, 1994). Until recently, the enormous influences of the phages on the various ecosystems were not realized (Fuhrman, 1999; Wommack and Colwell, 2000). Hence it is important to study the representative viruses from the marine environment and examine their relationship to other bacteriophages.

Marine phages might offer new approaches to lysogeny, including novel integration mechanisms and unique control of the lysogenic switch. For instance, studies with natural populations have indicated that environmental cues on various scales of time and space dictate the lysogenic decision in the marine environment (McDaniel *et al.*, 2002; Weinbauer *et al.*, 2003), yet the genomic underpinnings of these processes are completely unknown. Finally, studies on marine phage isolates, including mutant, expression and protein analysis, will be vital for solving the mysteries of many of the unidentified ORFs in these genomes. Together, efforts in these areas will augment our understanding of the functioning of oceanic phages in nature (Paul and Sullivan, 2005).

Vibrios are the hosts for a variety of bacteriophages, generally called vibriophages, which include lytic as well as temperate phages. Frequently, *Vibrio* sp. and vibriophage, which are common in seawater (Borsheim, 1993; Thompson *et al.*, 2004) and easily culturable (Eilers *et al.*, 2000), have served as model systems in studies of host-virus interactions in the water column. First report of vibriophages dates back to 1965 (Monsur *et al.*, 1965) and since then a large number of them are being used for phage typing. A number of filamentous phages have been reported in *Vibrios* some of which have been implicated in virulence gene transfer among the strains. Egs. CTX Φ encoding Cholera Toxin, KSF-1 Φ etc (Faruque *et al.*, 2005; Waldor and Mekalanos, 1996).

The evolutionary events responsible for the emergence of epidemic cholera are as fascinating and important as they are enigmatic. It has been shown that multiple horizontal gene transfer events lie at the core of this evolutionary potential. It is clear, however, that the ToxRS regulatory system and the gene clusters encoding cholera toxin and the toxin-coregulated pilus (TCP) are nearly always present in pathogenic strains (Faruque *et al.*, 1998). The discovery of the fact that the genes encoding cholera toxin are carried on the genome of a

filamentous phage (CTXΦ) represents the clearest example of horizontal transfer of virulence genes between *V. cholerae* strains and the phage (Waldor and Mekalanos, 1996). This observation implied a temporal pathway for the evolution of pathogenesis. Acquisition of the TCP cluster followed by infection with CTXΦ could represent sequential steps in the conversion of a nonpathogenic environmental isolate into a highly virulent strain. Although filamentous phage had not previously been recognized as being responsible for the lysogenic conversion of bacterial pathogens, they seem quite well suited for this purpose (Waldor and Mekalanos, 1996).

The significant role played by the phages in changing the dynamics of various ecosystems and the role they play in maintaining the genomic diversity of the bacterial communities have been studied. The importance of phages in the lateral transfer of genes among the different host systems has also increased the interest in them.

Phages are no longer just tools of molecular biology. They are now recognized to play a critical role in bacterial pathogenesis (Boyd *et al.*, 2001; Wagner and Waldor, 2002) and bacterial population dynamics (Burroughs *et al.*, 2000; Hendrix, 2003). In Western medicine today, there is renewed interest in phage therapy (Thacker, 2003). Phage-derived proteins are currently being used as molecular machines (Smith *et al.*, 2001), diagnostic agents (Schuch *et al.*, 2002), and therapeutic agents (Loeffler *et al.*, 2001; Nelson *et al.*, 2001; Schuch *et al.*, 2002) and for drug discovery (Liu *et al.*, 2004).

Phages are excellent sources of many enzymes and biochemical transactions that are broadly represented in all divisions of life (Karam, 2005). The large number of phylogenetic variants of biologically interesting proteins and nucleic acids that one can derive from sequenced phage genomes are treasure

troves for studies of biological structure in relation to function. Interest in phage and phage gene products as potential therapeutic agents is also rapidly increasing and is likely to have profound impact on the pharmaceutical industry and biotechnology in general over the coming years. With the widespread development of antibiotic resistance in pathogenic bacteria, the need for new antibiotics and alternative strategies to control microbial infections is of increasing urgency. Bacteriophages may yet have a role to play in the treatment of infection both independently and in combination with antibiotic therapy. There is a general sense that the best is yet to come out of phage research.

OBJECTIVES OF THE STUDY

The aquatic environment often plays the role of incubator and that the phages specific for *Vibrios* harbor the virulent genes and attribute pathogenicity to *Vibrio*. Thus often there is emergence of new strains with new virulent genes. Whereas, the literature available to substantiate horizontal gene transfer through phages are rather limited and consequently there is a need to understand this phenomenon. Hence, the present study was undertaken to isolate vibriophages from the aquatic environments of Kerala, purify, and characterize them for presence of virulence genes and host specificity.

Specific objectives towards this aim included the following:

1. To isolate host vibrios from aquatic environment
2. To isolate Vibriophage from aquatic environment.
3. To purify the Vibriophage.
4. To identify and characterize the Vibriophage with reference to their viability and cell adsorption, host specificity and the partial characterization of the phage genome.

Chapter 2

REVIEW OF LITERATURE

Bacteriophages are viral cellular parasites that depend on bacterial host processes to produce viral proteins and viral particles (Boyd *et al.*,2001). Bacterial viruses were discovered independently by Twort in 1915 and by d'Herelle in 1917. Today, phages are universally recognized as a group of bacteria-specific viruses, i.e. ultramicrobes of diverse character exhibiting all the signs of a long history of manifold variation, adaptation and specialization. In the 1940s and 1950s, pioneering studies into the structure and physiology of host/phage interactions (Delbruck, 1940; Delbruck, 1945; Doermann, 1952; Ellis and Delbruck, 1939; Luria and Delbruck, 1943) laid the basis for the development of molecular biology, which in turn became the foundation for a spectrum of new biotechnology-based industries (Marks and Sharp, 2000).

Bacteriophages which infect most prokaryotic groups of organisms have been isolated from diverse ecological habitats. Ecologically, phages are as varied and as versatile as their hosts with some able to survive extremes of temperature up to 95°C and extremes of pH, as low as pH1 (Raven *et al.*,1986; Sharp *et al.*,1985; Zillig *et al.*,1988).

2.1 General characteristics of bacteriophages

The phage is probably the most widely distributed biological entity in the biosphere, with an estimated population of $>10^{30}$ or ~10 million per cubic centimeter of any environmental niche where bacteria or archaea reside (Wommack and Colwell, 2000).

Bacteriophages, like other viruses, carry their genetic information in the form of either DNA or RNA. Most bacteriophages have tails, the tips of which bind to specific receptor molecules, which may be carbohydrate, protein or lipo-polysaccharide molecules on the surface of the host bacterium. The bacteriophage injects its nucleic acid into the host, thereafter utilizing the genetic machinery of the host to replicate its genetic material and to transcribe this to form new phage capsule material for the formation of new phage particles. The number of phages produced during a single cycle of infection (the burst size) varies between 50 and 200 new phage particles.

Bacteriophages can be classifed into two groups- as virulent and temperate phages. Virulent phages induce lytic infection, resulting in the lysis of host cells, thereby producing clear plaques in lawns of susceptible bacteria. On the other hand, temperate phages integrate their DNA within the genome of the host bacteria, resulting in a lysogenic infection and the phage genome is passed to all daughter cells at cell division.

Some phage genomes are spontaneously induced, resulting in transcription and production of new phage particles, which then infect and lysogenise other uninfected hosts. Induction of a lysogenic microorganism (lysogenic induction) can occur through the action of mutagenic agents such as ultraviolet light and chemical mutagens. Lysogenic phages generally develop hazy or turbid plaques on lawns of susceptible host cells. The lysogenisation of a strain with a particular phage confers immunity to infection by other related phage sharing the same immunity group profile. Phages with different immunity profiles are able to infect normally and cells can become lysogenised by a number of different phages belonging to different immunity groups (Campbell, 1996).

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2.2 Bacteriophage classification

Classification of viruses is not nearly as well developed as the classification of cellular organisms. The phages are classified in to different species based on a number of factors. Distinguishing features used as criterions for phage classification include host range, virion morphology, genetic material, genome structure, genome sequence etc.

The existing system of classification of phages is based primarily on the nature of the genomic material. Bacteriophage genomes consist of either single or double stranded DNA or RNA and may vary in size from 4 to 725 kb and may be circular or linear (Ackerman, 1998). In some cases the DNA may consist of separate discrete segments and in the case of phage ΦCH1 from *Natronobacterium magadii,* the phage head contains both DNA and RNA (Whitte *et al.*,1997). Temperate phages are generally double stranded DNA >20 kb. Tailed phages contain 50:50 DNA/protein although it appears that large parts of the genome are non-essential. Phage P22 can lose 40% of its genome without affecting lytic growth or lysogeny (Poteete, 1988).

Bacteriophages have been classified into 20 families and they are listed in Table 2.1.

Table 2.1 Classification of bacteriophages (http://www.thebacteriophages.org)

Group	Features	Genome	Type
Corticoviridae	Icosahedral capsid with lipid layer	Circular, supercoiled,double stranded DNA	Pseudoalteromonas phage PM2
Cystoviridae	Enveloped, icosahedral capsid, lipids	Three molecules of linear double stranded RNA	Pseudomonas phage Φ6
Fuselloviridae	Pleomorphic,envelope,lipids, no capsid,	circular supercoiled dsDNA	Acidianus phage DAV1, Haloarcula phage His 1
Inoviridae genus inovirus	Long filaments with helical symmetry	Circular single stranded DNA	Vibriophage CTXΦ, fs2, VSK, M13
Inoviridae genus plectrovirus	Short rod with helical symmetry	Circular single stranded DNA	Acholeplasma phage MS2,Qβ
Leviviridae	Quasi-Icosahedral capsid	One molecule of linear single stranded DNA	Acinetobacter phage 142, MI2 PRD1
Lipothrixviridae	Enveloped filaments, lipids	Double stranded DNA	Acidianus LDAΦ, Sulfolobus phage SIFE
Microviridae	Icosahedral capsid		Bdellovibrio phage MAC1, Enterobacteria phage G13, G14
Myoviridae, A1	Tail contractile, head isometric		Vibriophage CP-T1, K139, VP10, VP17
Myoviridae, A2	Tail contractile, head elongated (length/width ratio= 1.3-1.8)		Vibriophage KVP20, KVP40
Myoviridae, A3	Tail contractile, head elongated (length/width ratio= 2 or more)		Salmonella phage 11, 12, 16-19
Plasmaviridae	Pleomorphic envelope, lipids, no capsid	Circular super coiled double stranded DNA	Acholeplasma phage L2
Podoviridae C1	Tail short and non contractile, head isometric		Vibriophage e1,e5, FK, N5
Podoviridae C2	Tail short and non contractile, head elongated (length/width ratio=1.4)		Vibriophage PA1, 7
Podoviridae C3	Tail short and non contractile, head elongated (length/width ratio=2.5 or more)		Vibriophage 7-8, 70A-2
Rudiviridae	Helical rods	Linear double stranded DNA	Sulfolobus phage SIRV
Siphoviridae B1	Tail long and non contractile, head isometric		Vibriophage P13, P38, VP3, VP6
Siphoviridae B2	Tail long and non contractile, head elongated (length/width ratio=1.2-2)		Vibriophage VP5, VP16, VP15
Siphoviridae B3	Tail long and non contractile, head elongated (length/width ratio=2.5 or more)		E-coli phage H19-J, 933H
Tectiviridae	Icosahedral capsid with inner lipoprotein vesicle	Linear double stranded DNA	Bacillus phage AP50, Pseudomonas phage PRD1, PR3

2.3 Marine phages

Marine viruses (principally bacteriophages) are the most abundant biological entities on the planet (Hendrix *et al.*,1999). The earliest reports of bacteriophage activity in marine ecosystems date back to the first quarter of this century (ZoBell, 1946). After three decades, minimum estimates of viral abundances of $>10^4$ particles ml^{-1} was reported (Torrella and Morita, 1979). Later it was reported that viral abundances typically exceed those of bacteria and routinely occur at $>10^7$ particles ml^{-1} (Bergh *et al.*,1989; Proctor and Fuhrman, 1990). Bacteriophages are considered to be dynamic partners in microbial food webs (Bratbak *et al.*,1990; Bratbak *et al.*,1994; Fuhrman and Suttle, 1993), and on average, approximately, 20% of the standing stock of bacteria are lysed by phages on a daily basis (Suttle, 1994).

Lytic marine phages can divert carbon flow in grazing food chains towards the dissolved organic matter (DOM) pool, thus fueling heterotrophic activity and potentially augmenting atmospheric carbon dioxide levels (Fuhrman, 1999). In contrast, marine temperate phages can replicate lytically (also potentially increasing DOM levels during prophage induction events), but are also capable of altering host phenotype (Paul and Jiang, 2001). Both types of phages can participate in gene transfer and alter microbial genotypes (Jiang and Paul, 1998). Yet, despite their biogeochemical and genetic importance, to date, only 17 marine phage genomes appear in the GenBank database (Table 2.2) (Paul and Sullivan, 2005).

Table 2.2 Marine phage genomes sequenced to date

Heterotrophic bacterial phages	Size (bp)	Group	Accession No.	Reference
Published phage genomes				
Pseudoalteromonas espejianana BAL-31 PM2	10 079	Corticoviridae	NC_000867	(Mannisto *et al.*,1999)
Roseobacter SIO1	39 906	Podoviridae	NC_002519	(Rohwer *et al.*,2000)
Vibrio parahaemolyticus VP16T	46 012	Myoviridae	AY328852	(Seguritan *et al.*,2003)
Vibrio parahaemolyticus VP16C	49 575	Myoviridae	AY328853	(Seguritan *et al.*,2003)
Vibrio parahaemolyticus VpV262	46 012	Podoviridae	AY095314	(Hardies *et al.*,2003)
Vibrio cholerae K139	33 106	Myoviridae	AF125163	(Kapfhammer *et al.*,2002)
Vibrio harveyi VHML	43 193	Myoviridae	AY133112	(Oakey *et al.*,2002)
Listonella pelagia f HSIC	39 766	Siphoviridae	AY772740	(Paul *et al.*,2005)
Vibrio parahaemolyticus KVP40	244 835	Myoviridae	AY283928	(Miller *et al.*,2003)
Picocyanophages				
*Synechococcus*WH7803 P60	47 872	Podoviridae	AF338467	(Chen and Lu, 2002)
Prochlorococcus MED4 P-SSP7	44 970	Podoviridae	AY939843	(Sullivan *et al.*,2005)
Synechococcus WH7803 S-PM2	~194 000	Myoviridae	AJ630128	(Mann *et al.*,2005)
Prochlorococcus NATL1A P-SSM2	252 401	Myoviridae	AY939844	(Sullivan *et al.*,2005)
Prochlorococcus NATL2A P-SSM4	178 249	Myoviridae	AY940168	(Sullivan *et al.*,2005)
Unpublished phage genomes				
Vibrio parahaemolyticus Vp2	39 853	Podoviridae	NC_005879	(D Wang *et al.*,unpublished)
Vibrio parahaemolyticus Vp2	39 786	Podoviridae	NC_005891	(D Wang *et al.*,unpublished)
Flavobacterium sp.	36 107	Unclassified	NC_006356	(M Borriss *et al.*,unpublished)

As prophages, marine phages may also confer a wide range of traits to their hosts including: immunity to superinfection (Hershey, 1971); toxin production (Waldor and Mekalanos, 1996) and the capability to transfer modular blocks of genes (Jiang and Paul, 1998; Paul, 1999). Although there are technical challenges associated with isolating, purifying and cloning marine phages for genomic sequencing (Paul *et al.*,2002), nearly all new marine phage genomes have led to novel observations. Each sequence has also increased the number of known unique open reading frames (ORFs), because most of the marine phage ORFs (60–80%) lacks similarity to anything already in GenBank. This high percentage of unique ORFs contrasts greatly with more well studied phages (e.g. coliphages and streptococcal phages), which may have >80% homology to previously cultivated phages and, often, even gene order is highly conserved (Chibani-Chennoufi *et al.*,2004).

2.4 Filamentous phages

The filamentous phages constitute a large family of bacterial viruses that infect a variety of Gram negative bacteria, using pili as receptors (Russel *et al.*,1997). They are long deoxyribonucleoproteins about 5.5 nm in diameter. Their deoxyribonucleic acid (DNA) is a single-stranded ring containing fewer than 10,000 nucleotides (Marvin and Hohn, 1969).

The best characterized filamentous phages are M13, fd and f1, which are similar in nature that infect *Escherichia coli* via F pili and, to a lesser extent, IKe, which uses N or P pili. The phage particles are rods about 65 Å in diameter and 9000 Å in length, and they contain a circular ss DNA which is extended along the particle axis with an imperfectly base-paired segment that constitutes the packaging signal at one end. The particle is a tube formed by many copies of the major coat protein, with a few copies each of several minor coat proteins located at the ends (Marvin and Hohn, 1969). The current understanding of the infection

process of filamentous bacteriophages comes mainly from studies of Ff phages (M13, fd, f1) infecting *Escherichia coli* carrying the F episome, coding for the F-pilus, which is the primary receptor of the host cell (Jacobson, 1972). The infection is specifically mediated by coat protein 3 (g3p) of the phage, which is located at one end of the phage particle (Karlsson *et al.*,2003).

Upon entry into the cytoplasm, host enzymes convert the phage ssDNA to a super-coiled ds form which is the template for rolling-circle replication and for phage gene expression (Model and Russel, 1988). The phage genome encodes eleven proteins, of which five are part of the virion, three are required for phage DNA synthesis and three serve assembly functions (Model and Russel, 1988).

Filamentous phages do not kill their host, and infected cells continue to grow and divide indefinitely while producing phage. Phage particles do not form in the cytoplasm; rather they are continually extruded or secreted across the bacterial membranes as they are assembled, without causing cell lysis. These properties and their filamentous morphology distinguish them from most other bacterial viruses.

A few characteristics of the filamentous phages are as follows.

(i) These are among the smallest viruses known, so that it is feasible to define all of the virus functions at the molecular level and to consider the interaction of the various functions: the "systems" aspect of molecular biology.

(ii) In particular, the small genome size makes it relatively easy to study the mechanics, enzymology, and regulation of DNA replication and recombination of filamentous phages.

(iii) The purified virions themselves are a plentiful source of homogeneous stable deoxyribonucleoprotein for physical studies. This is the only bacterial virus which has been crystallized in a form suitable for structure

analysis with X-ray diffraction techniques. Study of the unusual method of virus penetration and release may lead to useful knowledge about the nature of the bacterial cell envelope. In fact, the cell membrane itself may be an important factor in the assembly of the virion.

(iv) Infection by filamentous phages alters the metabolism of the bacterial cell without killing the cell. One may hope to learn more about the bacterial cell by studying the nature of these perturbations. Since useful information has been obtained by measuring parameters of bacterial growth after shift of temperature or medium, so useful information may be obtained by measuring such parameters after filamentous phages infection.

2.5 Vibriophages

Vibriophages are a group of bacteriophages infecting the members of the family Vibrionaceae and this family contains the greatest number of reported phage-host systems for the marine environment (Moebus, 1987), with the genus *Vibrio* comprising most of the hosts (Moebus and Nattkemper, 1981). Since the first report of the isolation of a bacteriophage specific for *Vibrio parahaemolyticus* (Nakanishi *et al.*, 1966), these phage host systems have received much attention (Baross and Liston, 1968; Baross *et al.*, 1978; Hidaka and Tokushige, 1978; Kaneko and Colwell, 1973; Koga and Kawata, 1981; Koga *et al.*, 1982; Sklarow *et al.*, 1973). These studies, however, were largely concerned with the isolation, morphology, sensitivity to heat and chemicals, burst size, and host range of such phages. The study of the genetic diversity of marine Vibriophages from different regions have also been attempted by several workers (Comeau *et al.*, 2006; Kellogg *et al.*, 1995).

Bacterial viruses (phages) are known to play a critical role in the evolution of pathogenic bacterial species, and *V. cholerae* in particular. For example, cholera toxin genes are transferred to nontoxigenic strains by means of a lysogenic

filamentous phage, CTXΦ (Waldor and Mekalanos, 1996). An alternative mechanism of cholera toxin acquisition by *Vibrio cholerae* have also been studied using a generalized transducing bacteriophage CP-T1 (Boyd and Waldor, 1999). Temperate bacteriophage K139 was isolated from a *Vibrio cholerae* O139 isolate and characterized. The phage genome consisted of a 35 kbp, double-stranded, linear DNA molecule that circularizes and integrates into the chromosome in a site specific manner (Reidl and Mekalanos, 1995). KSF-1Φ, a novel filamentous phage of *Vibrio cholerae*, supported morphogenesis of the RS1 satellite phage by heterologous DNA packaging and facilitated horizontal gene transfer (Faruque *et al.*,2005a).

The relationship among the local incidence of cholera, the prevalence in the aquatic environment of *Vibrio cholerae*, and bacterial viruses that attack potentially virulent O1 and O139 serogroup strains of this organism (cholera phages) was studied in Dhaka, Bangladesh (Faruque *et al.*,2005a). Significantly more environmental water samples contained either a phage or a phage-susceptible *V. cholerae* strain rather than both. The number of cholera patients varied seasonally during the period of study and frequently coincided with the presence of pathogenic *V. cholerae* strains in water samples that otherwise lacked detectable cholera phages. Interepidemic periods were characterized by water samples containing cholera phages but no viable bacteria. The conclusion was that cholera phages can influence cholera seasonality and may also play a role in emergence of new *V. cholerae* pandemic serogroups or clones (Faruque *et al.*,2005b).

A novel filamentous phage VGJΦ, isolated from the strain SG25-1 of *Vibrio cholerae* O139 infected all the O1 and O139 strains tested (Campos *et al.*,2003a). The genome of this has been almost fully characterized and found to integrate in the same site as CTXΦ. Phage 493 is known to use mannose-sensitive hemagglutinin (MSHA) as a receptor, and it has been suggested that this phage

played some role in the emergence of the O139 epidemic serotype of *V. cholerae* (Jouravleva *et al.*,1998a; Jouravleva *et al.*,1998b); however, its sequence and gene structure have not been described. Phage VSK is able to integrate into the *V. cholerae* chromosome (Kar *et al.*,1996), but the integration site has not been determined. Phages fs1 and fs2 have been sequenced, and their genomic structures have been described (Honma *et al.*,1997; Ikema and Honma, 1998).

A number of *V. parahaemolyticus* viruses (VpVs) have been isolated and characterized from the coastal waters of Japan, Laos, Hawaii, Florida, and the Pacific and Atlantic coasts of North America (Baross *et al.*,1974; Comeau *et al.*,2005; Hardies *et al.*,2003; Kellogg *et al.*,1995; Nakanishi *et al.*,1966; Nakasone *et al.*,1999; Seguritan *et al.*,2003; Sklarow *et al.*,1973). All isolates, except a few filamentous phages (*Inoviridae* family), belong to the three families of tailed phages, *Myoviridae*, *Siphoviridae* and *Podoviridae* (Ackermann *et al.*,1984). The viruses are usually species-specific and sometimes strain-specific, although a few demonstrate broad host ranges against *Vibrio* and the related genus *Photobacterium* (Baross *et al.*,1974; Comeau *et al.*,2005; Matsuzaki *et al.*,1998; Matsuzaki *et al.*,1992; Nakanishi *et al.*,1966). Temperate phage VpVs have also been isolated using mitomycin C and ultraviolet light as inducing agents (Koga and Kawata, 1991; Ohnishi and Nozu, 1986) with one study finding that <10% of *V. parahaemolyticus* isolates harboured lysogens (Muramatsu and Matsumoto, 1991). Filamentous phages Vf12 and Vf33 of *Vibrio parahaemolyticus* have been isolated and the gene organizations of their conserved regions were found to be similar to that of CTX phage of *Vibrio cholerae* and coliphage Ff of *Escherichia coli* (Chang *et al.*,1998).

Seven bacteriophages specific to *Vibrio harveyi*, the causative agent of luminous vibriosis in shrimp, were isolated from coastal aquaculture systems like shrimp farms, hatcheries and tidal creeks along the east and west coasts of India.

All the seven phages were found to have the typical head and tail morphology with double-stranded DNA as genetic material. Morphologically, six phages were grouped under family Siphoviridae and one under Myoviridae (Shivu *et al.*,2007).

The complete genome sequence of a few vibriophages were determined like Phage KVP40 and KVP20 (Miller *et al.*,2003), VHML infecting *V. harveyi* (Oakey *et al.*,2002), VpV262 infecting *V. parahaemolyticus* (Hardies *et al.*,2003), VP16T and VP16C infecting *V. parahaemolyticus* (Seguritan *et al.*,2003). Temperate phages showing no evidence of lysis was observed in *V. vulnificus* (Marco-Noales *et al.*,2004).

2.6 Role of vibriophages in the pathogenecity of the hosts

Toxigenic strains of *Vibrio cholerae* belonging to the O1 and O139 serogroups cause cholera, a devastating diarrhea disease that occurs frequently as epidemics in many developing countries (Faruque *et al.*,1998). Epidemics of cholera occur regularly in the Ganges Delta region of Bangladesh and India. The occurrence of epidemics are known to coincide with increased prevalence of the causative *V. cholerae* strain in the aquatic environment (Khan *et al.*,1984). But , bacterial viruses in the environment have recently been found to inversely correlate with the abundance of toxigenic *V. cholerae* in water samples and the incidence rates of cholera (Faruque *et al.*,2005b). These data strongly suggest that phage predation in the environment likely influences the temporal dynamics of cholera epidemics. Phages also play a role in the emergence of pathogenic clones and may also be involved in territorialism between different strains of *V. cholerae* (Faruque and Mekalanos, 2003; Faruque *et al.*,2005b; Jouravleva *et al.*,1998a). For example, cholera toxin genes were transferred to nontoxigenic strains through a lysogenic filamentous phage (Waldor and Mekalanos, 1996), and the emergence and dominance of *V. cholerae* O139 in Bangladesh and India during 1992-1993 may

have involved phages both as a means of horizontal gene transfer as well as a bacteriocidal selective mechanism (Faruque *et al.*,2005b; Jouravleva *et al.*,1998a). Recently, it has also been recognized that some bacteriophages carry powerful diversity-generating retroelements (DGR) that are used to alter host range of the phages (Doulatov *et al.*,2004; Liu *et al.*,2002). Thus, possible DGR-driven changes in the host range of vibriophages may lead to the emergence of new phage types that could impact the epidemiology of cholera or the emergence of new serogroups of *V. cholerae* or related *Vibrio* sp (Faruque *et al.*,2005a).

It is becoming increasingly clear that filamentous phages can play critical roles in horizontal gene transfer among *V. cholerae*. This is not only because of some virulence gene clusters are actually phage genomes, but also their flexible capsid structure that allows packaging of heterologous DNA. The CTXΦ genome comprises a 'core region' that carries the ctxAB operon as well as genes essential for the formation of CTXΦ particles, and an RS2 region carrying genes that encode products required for the integration, replication and regulatory functions of CTXΦ (Waldor *et al.*,1997). Although the lysogeny of CTX prophage is maintained by the phage repressor protein RstR, the anti-repressor protein RstC, which influences the replication and transmission of CTXΦ, is encoded by another adjacently located satellite phage genome RS1 (Davis *et al.*,2002). Conversely, RS1 uses CTXΦ morphogenesis genes to produce RS1Φ particles, demonstrating both symbiotic and parasitic interactions between a phage and a satellite-phage in *V. cholerae* (Davis *et al.*,2002; Faruque *et al.*,2002). Production of RS1Φ particles can also occur using functions encoded by another filamentous phage called KSF-1Φ (Faruque *et al.*,2003). RS1Φ produced by this process are capable of infecting recipient strains in a TCP-independent manner. Therefore, the interplay between the CTX prophage, RS1 and KSF-1Φ promotes efficient dissemination of CT genes and simultaneously enhances the virulence and evolutionary fitness of *V. cholerae* strains (Faruque and Mekalanos, 2003). Because the CTXΦ uses TCP

pili as its receptor for infecting recipient cells, it appears that acquisition of the TCP island is possibly the initial genetic event required for the evolution of epidemic strains. Moreover, colonization is a prerequisite for establishing a productive infection by *V. cholerae*, and given the role of TCP in colonization it appears that acquisition of the TCP island would provide a strong selective advantage to any *V. cholerae* recipient (Faruque and Mekalanos, 2003).

2.7 Vibriophage research in India

A phage typing system was developed at National Institute of Cholera and Enteric Diseases, India for the typing of *V. cholerae* for epidemiologic importance (Basu and Mukerjee, 1968; Chakrabarti *et al.*,2000; Chattopadhyay *et al.*,1993; Mukerjee *et al.*,1957; Sarkar, 1993; Sarkar *et al.*,1999). Two phages were characterized as *V. cholerae* O1 biotype ELTor and named as ΦIVI-1 and ΦIVI-2. Both phages belonged to the family *Podoviridae* according to the International Committee on Taxonomy of Viruses (Sur *et al.*,2006). Three new O1 ElTor vibriophages named AS1, AS2 and AS3, were isolated from the sewage and pond waters of the outskirts of Kolkata (Sen and Ghosh, 2005a). A few phages, named AS4, with hexagonal heads and abnormally long tails with typical curly projections were also found in the water samples. Physicochemical characterization of phage N5 was done (Sen and Ghosh, 2005b). An unusual filamentous bacteriophage VSK, containing single stranded, circular DNA as its genome was isolated from *Vibrio cholerae* O139 strains P07 and B04 (Kar *et al.*,1996). Unlike other single stranded DNA phages, VSK can integrate its genome into the chromosome of the host and enter into a lysogenic state. Another phage e5 which can lyse only the ElTor strains of *Vibrio cholerae* was also partially characterized (Basu *et al.*,1993).

2.8 Phages as gene-transfer particles

Tailed phages are the most efficient gene-transfer particles developed in evolution. They represent densely compacted phage DNA (Zhang *et al.*,2000) encased in a protective protein shell (the phage head) (Conway *et al.*,2001). To this remarkable DNA storage device is added an equally efficient DNA transfer device, the phage tail and its associated fibres. This structure assures both the specific recognition of the appropriate host cell and the guided injection of the phage DNA into the bacterial cell (Kanamaru *et al.*,2002; Molineux, 2001). Some bacteria have learned to use phages for their own purposes. In *Pseudomonas aeruginosa*, two phage-tail gene clusters have developed into bacteriocins (Nakayama *et al.*,2000). The defective *Bacillus subtilis* prophage PBSX has maintained the capacity to build a size-reduced phage head into which 13 kb fragments of random bacterial DNA are packaged. A prophage remnant of *Rhodobacter capsulatus* acts as a gene-transfer agent for random 4.5 kb fragments of bacterial DNA in bacteria-controlled DNA exchange between cells in the stationary phase (Lang and Beatty, 2000). Prophage-like elements from *Mycobacterium tuberculosis* encode active integration/excision systems (Bibb and Hatfull, 2002).

A particularly interesting case is the 15 kb-long pathogenicity island SaPI1 from *Staphylococcus aureus* encoding the toxin Tst involved in toxic shock. In cells infected with *S. aureus* phage 80α, SaPI1 is excised from the chromosome; it replicates autonomously and interferes with phage growth by directing the encapsidation of its own DNA into specially tailored small phage 80α heads commensurate with its size. Upon phage-mediated transfer to a recipient organism, SaPI1 integrates by means of its own integrase (Ruzin *et al.*,2001).

Many bacterial virulence determinants are encoded by accessory element-borne genes, rather than those that are chromosomally located. In the 1950s, the β prophage of *Corynebacterium diptheriae* was implicated in the increased virulence of strains harbouring this phage (Freeman, 1951). Many such virulence determinants are now believed to be phage-encoded, for example, the genes coding for shiga-like toxins (SLT) in strains of *E coli* 0157 are located on bacteriophage genomes (Rietra *et al.*,1989; Strockbine *et al.*,1986). The conversion of non-toxigenic strains of *E coli* to toxigenic strains has also been demonstrated. A phage, H19A, isolated from *E. coli* serotype O26:H19, encoding SLTI, has been used to convert the enteroadherent *E. coli* RDEC-1, which is a rabbit pathogen, into a significantly more virulent strain, RDEC-H19A (Sjogren *et al.*,1994). It has been proposed that the new strain, RDEC-H19A, may form the basis of an animal model for shiga-like toxin production (Tarr, 1994). More recently, VT genes have been identified in other bacterial strains including *Citrobacter freundii* (TschaÈpe *et al.*,1995) and *Aeromonas* sp. (Haque *et al.*,1996). Similarly, in *Vibrio cholerae*, the structural genes for cholera enterotoxin are located on the genome of a bacteriophage, CTXΦ, which is related to the *E coli* phage M13. For full virulence, *V cholerae* requires two factors for virulence, cholera toxin and toxin-coregulated pili, which are required for colonisation. The phage CTXΦ uses the pili as its receptor. Infection by CTXΦ was reported to occur more efficiently in the gastrointestinal tract of mice than in vitro (Waldor and Mekalanos, 1996).

Other bacterial toxins that are encoded on bacteriophage genomes include type A exotoxin in *Streptococcus pyogenes* (Yu and Ferretti, 1991) coded on bacteriophage T12, staphylokinase and enterotoxin A (Betley and Mekalanos, 1985) in *S aureus* (Coleman *et al.*,1989) and cytotoxins in *P. aeruginosa* (Hayashi *et al.*,1993) The transfer of neurotoxin type E from strains of *Clostridium butyricum* to non-toxigenic strains of *Cl botulinum* has been shown to be mediated by bacteriophage (Zhou *et al.*,1993). Other virulence determinants coded on

bacteriophages include capsule production by *Streptococcus pnuemoniae* (Russell, 1991) and lipopolysaccharide acetylase (Clark *et al.*,1991). Transfer of bacterial virulence determinants by bacteriophages is extensively reviewed (Cheetham and Katz, 1995). Phages do encode such virulence factors, the repercussions of the possibility of horizontal transfer of these factors cannot be ignored. Whilst the factors alone generally do not confer pathogenicity to the recipient strain, if the recipient already has a number of virulence determinants, phage-mediated transfer of additional factors can only increase the subsequent virulence of the strain. This transfer may play a role in the emergence of seemingly new pathogens (Levin and Tauxe, 1996). Careful consideration should be given to the potential for increased pathogenicity following phage transfer of virulence components when designing regimes for phage-related microbial control techniques (such as phage therapy). Widespread, indiscriminate use of bacteriophages may exacerbate problems with bacterial pathogens rather than control them.

2.9 Lateral Gene Transfer

With about 100 sequenced genomes of bacteria in the public database and many more to come, genomics has changed our understanding of microbiology. In fact, the genomes of bacteria are remarkably fluid. A substantial part of the bacterial DNA is not transferred from the parental cell to its descendent ('vertical' transfer), but is acquired horizontally by transformation, conjugation or transduction ('lateral' transfer) (Bushman, 2002).

Virulence genes were apparently transferred between phages belonging to different phage groups (Mirold *et al.*,2001) or infecting different bacterial species (Desiere *et al.*,2001) thereby increasing the lateral spread of these genes in bacteria. Sequencing data from coliphages and dairy phages have demonstrated that large phage gene clusters were transferred between distinct groups of phages

(Ravin *et al.*,2000) confirming tenets of the classical modular theory of phage evolution. The strikingly different GC-content of the left and right arm of phage lambda suggests the heterologous origin of this reference phage. The mosaic character of phages was greater in Gram-negative than in Gram-positive bacteria (Bru¨ssow and Desiere, 2001). In some lambdoid coliphages short conserved sequences were identified at the boundaries of functional modules. This suggested homologous recombination as the driving force for lateral gene transfer between phages (Clark *et al.*,2001). However, the comparison of other lambdoid coliphage genomes suggested that non-homologous recombination occurs everywhere and the observed order in phage genome organisation is the consequence of selection forces eliminating all non-viable recombinants (Juhala *et al.*,2000). Recent sequencing data has identified hybrids between phage genera, phage families (Allison *et al.*,2002; Recktenwald and Schmidt, 2002) and even temperate and virulent phages (Canchaya *et al.*,2003).

2.10 Specialised transduction

Resolvase-type integrases from phages of Gram-positive bacteria have no requirements for cofactors facilitating their integration into heterologous hosts (Groth *et al.*,2000). If a prophage is imprecisely excised from the heterologous host, small segments of flanking bacterial DNA can be copackaged with the phage DNA and transferred to the original host ('specialized transduction'). In accordance with this model, prophages from low GC content Gram positive bacteria frequently contain extra genes in the vicinity of attR, the right attachment site. Sometimes these genes differ in GC-content from the surrounding DNA and suggest a phage-mediated gene transfer from a rare heterologous host differing in GC content (Ferretti *et al.*,2001). In the case of pathogenic bacteria, these extra genes frequently encoded important virulence factors like bacterial toxins (Baba *et al.*,2002; Beres *et al.*,2002; Smoot *et al.*,2002). These extra genes were also

observed in commensals and free-living bacteria and belonged to the few prophage genes expressed in the lysogenic state (Ventura *et al.*,2002); only in a few cases did database matches suggest a physiological role for these extra genes. In fact, when bacteria were grown under conditions that mimicked pathological conditions (Smoot *et al.*,2001), or when they were grown in infected animals (Dozois *et al.*,2003), prophage genes belonged to the most prominent genes of the entire bacterial chromosome that changed the expression level.

2.11 Generalised transduction

Phages such as *Salmonella* phage P22 or coliphage Mu occasionally commit the error to package even a headfull of bacterial DNA instead of phage DNA. Upon infection of the next host, this bacterial DNA can be incorporated into the bacterial chromosome ('generalised transduction'). Despite the interest in gene flux in the environment, sparked by the discussion of the risks associated with the release of genetically modified microorganisms, only a few recent reports have investigated generalised transducing phages in terrestrial habitats (in *Streptomyces* and *Listeria*) (Burke *et al.*,2001; Hodgson, 2000). One technical report addressed the problem of PCR-detection of phage-encapsidated bacterial DNA when working with uncultivatable bacteria and their phages (Sander and Schmieger, 2001).

By contrast, phage ecology and phage-mediated DNA transfer has become a focus of interest in marine microbiology (Williamson *et al.*,2002). Researchers realised that viruses (most of them probably phages) outnumber bacteria in the open ocean by a factor of ten (Wommack and Colwell, 2000). In view of the large volume of the world's oceans and the high titre of phage particles of 10^7/ml of seawater, phages particles are the most abundant biological entities on earth (Bru¨ssow and Hendrix, 2002). If one anticipates a transduction frequency of 10^{-8} per plaque forming unit for marine phages (Jiang and Paul, 1998), it was calculated

that phage mediated gene transfer takes place at the incredible rate of about 20 million billion times per second in the oceans (Bushman, 2002). However, the genomics of the predominant marine bacteria and their phages is still in its infancy. Only a handful of marine phages have been sequenced (Paul *et al.*,2002) from the 400–7000 viral types estimated in 100 litre water samples (Breitbart *et al.*,2002).

Since their discovery at the start of the 20th century, bacteriophages have helped shape the development of modern biotechnology. From the early hopes of developing methods for the control of microbial infections, later eclipsed by the discovery of antibiotics, to the latest developments in phage display technology, bacteriophages have played key roles in biotechnological advancements. Their usefulness is underlined by the diversity of their uses in biotechnology. Phages have been used as tracers in the environment, as challenge agents for monitoring and validation, and as bacterial typing systems. In the field of molecular biology they have been used as sources of enzymes and have been invaluable as cloning vectors. There is a re-awakened interest in phage therapy, sparked by concerns over the emergence of drug resistant micro-organisms, and the re-emergence of pathogens believed to have been eradicated from the developed world. The continuing struggle between phages and their hosts resulting in the infection of microbial fermentations will continue to present a challenge to the biotechnology industry. Increased concerns over the ability of phages to transfer genes, especially virulence factors, amongst microbial populations, potentially resulting in the creation of pathogens with enhanced virulence, have ensured that fundamental research on the biology of phages is likely to continue unabated. At the end of the 20th century, the emergence of the powerful technique of phage display, with its potential to revolutionize screening in the biotechnology and pharmaceutical industries, provides ample testament to the pioneering work and beliefs of d'Herelle, Twort and other workers, who achieved so much at its beginning.

Prophages contribute a substantial share of the mobile DNA of their bacterial hosts and seem to influence the short-term evolution of pathogenic bacteria. Automated methods for systematic investigation of prophages and other mobile DNA elements in the available bacterial genome sequences will be necessary to understand their role in bacterial genome evolution. In the past, phages were mainly investigated as the simplest model systems in molecular biology. Now it is increasingly realised that phage research will be instrumental in the understanding of bacterial abundance in the environment. One can predict that phage research will impact diverse areas such as geochemistry and medicine. Success will largely depend on integrative multidisciplinary approaches in a field that has, until recently, been dominated by reductionist thinking (Canchaya et al., 2003).

Chapter 3

MATERIALS AND METHODS

3.1 Screening of *Vibrio*

Environmental *Vibrios* were isolated from the samples, which were to be used later for the isolation of the marine vibriophages, since the phages required a suitable host for their survival.

3.1.1 Sample

Water samples were collected from different locations in the marine and mangrove environments off the coast of Kerala. They were collected aseptically in sterile containers and transported under chilled condition to the laboratory.

3.1.2 Plating procedures

The samples were serially diluted and 100µl of the diluted water sample was used as inoculum. Pour plating technique was employed using thiosulphate-citrate bile salts-sucrose (TCBS) agar medium (Himedia) for the isolation of the environmental strains of *Vibrios*. The inoculated plates were then incubated at 37°C for 18 to 24 hours and subsequently examined for growth. Isolated single cell colonies were picked, repeatedly purified on Nutrient Agar (NA) plates, sub-cultured, and stocked and used for further characterization.

3.1.3 Identification of *Vibrios*

All the isolates obtained from TCBS medium were identified as *Vibrios* based on their morphological and biochemical characteristics, as outlined in Bergey's Manual of Systematic Bacteriology (Buchanan and Gibbons, 1974).

3.1.4 Maintenance of culture

After purification, the cultures were maintained on NA slants with paraffin overlay, on semisolid agar slants and as glycerol stock.

3.2 Phage isolation

1. Water samples were collected from the same locations, previously used for isolation of environmental *Vibrios,* for the screening of vibriophages. The samples were collected in sterile containers and transported under refrigerated condition to the laboratory.
2. One litre of the water sample was centrifuged at 10,000 x g for 10 minutes at 4°C in order to separate the suspended solid particles from the water.
3. The supernatant was then collected and filtered through 0.22μ nitrocellulose membrane (Sartorius).
4. 50 ml of this filtrate was then added to equal volume of mid-log phase cultures of *Vibrio* species previously isolated, and was incubated overnight at 150rpm towards enrichment of the phages in water and to ease their detection.
5. The culture broth was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was filtered through 0.22μ nitrocellulose membrane (Sartorius) and the filtrate was plated employing soft agar overlay

technique (modified from Gratia, 1936) as detailed below, using the corresponding host *Vibrios* sp. used previously for enrichment.

The hosts were inoculated into Luria Broth (HiMedia) and incubated at 28±2°C, with shaking at 120 rpm for 3.5 hrs until the $O.D_{600}$=1 (~10^8 cfu/ml). One ml of this host culture was mixed with one ml of the enriched phage culture and incubated at 37°C for 30 min. The Luria Agar plates were then overlaid by soft agar (Luria Broth having 0.8% agar) containing this mixture of host and the phage. The plates were allowed to set for 10 minutes followed by incubation at 37°C and checked for plaques every 2 hrs. Appropriate controls were also maintained.

The host infected by the phage could be segregated by the appearance of visible plaques. The host *Vibrio* for which phage could be isolated was designated as *Vibrio* sp. MV-5 and was then further identified using 16S rRNA sequencing for confirmation of its identity as described under section 3.6.3.

3.3 Phage Purification

Phage purification was done by the single plaque transfer method, whereby a single plaque was picked using a sterile toothpick and transferred to 3 ml mid log phase culture of host *Vibrio* sp. MV-5 in LB and incubated overnight at 150rpm. The culture was centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant was filtered through 0.22µ nitrocellulose membrane (Sartorius). The filtrate was plated as described under section 3.2. The procedure was repeated 6 times in order to ensure uniform plaque size.

3.4 Large scale production of phage lysate

The plates prepared as described in the previous section and showing infective centers at the rate 10^8 PFU/ml, were washed with SM buffer using the following method.

1. The plates with well developed plaques were overlaid with 10 ml of SM buffer as described below.

   SM buffer

NaCl	-	5.8 g
$MgSO_4.7H_2O$	-	2.0 g
1 M Tris HCl (pH 7.5)	-	50 ml
2% gelatin	-	5.0 ml

   Volume was made up to 1 litre with distilled water and autoclaved. This was then stored at 4°C.
2. The plates were incubated at 4°C overnight with gentle rocking so that the phages could diffuse easily into the SM buffer.
3. The phage suspension from each plate was recovered and pooled into a sterile polypropylene container. The plates were rinsed once again with 2 ml of SM buffer and pooled.
4. Chloroform was added to this pooled mixture to a final concentration of 5% (v/v). This suspension was mixed well by vortexing and incubated at room temperature for 15 min.
5. The cell debris was then removed by centrifugation at 5,000x g for 10 min.
6. The supernatant was transferred to a sterile polypropylene tube. The steps 4 and 5 were repeated whenever the supernatant appeared cloudy or when there was a high amount of cell debris, until the supernatant became clear.
7. To this clear supernatant, chloroform was added to a final concentration of 0.3% (v/v) and stored at 4°C.
8. The titer of this lysate was checked after serial dilution.

3.5 Concentration of phage particle

Phage concentration was carried out using PEG 6000 as detailed below (Sambrook *et al.*, 2000).

1. Overnight culture of *Vibrio* sp. MV-5 was transferred to 200 ml LB at 1% (v/v) level and incubated at room temperature (RT) at 150 rpm for 3.5 hrs.
2. Phage was added at a multiplicity of infection (MOI) of 0.2 and incubation at room temperature was continued at 150 rpm for 12-15 hours.
3. The broth was centrifuged at 10,000 rpm for 20 min.
4. The supernatant was collected and filtered through 0.22μ nitrocellulose membrane (Sartorius).
5. To the filtrate, DNase I and RNase were added, to get a final concentration of 1μg/ml each, and incubated at RT for 30 min.
6. Solid NaCl was added to a final concentration of 1M, dissolved by stirring with a sterile glass rod and kept in ice for 1 hr.
7. Centrifuged at 11,000 x g for 10 min at 4°C.
8. Solid PEG 6000 was added to the supernatant at a final concentration of 10% (w/v), dissolved by slow stirring on a magnetic stirrer at RT, and kept in ice overnight.
9. Centrifuged at 11,000 x g for 10 min at 4°C.
10. The supernatant obtained was discarded completely and the pellet was resuspended in 5 ml of PBS as described below.

Phosphate Buffered Saline (PBS)

NaCl	-	8.0 g
KCl	-	0.2 g
Na_2HPO_4	-	1.44 g
KH_2PO_4	-	0.24 g

The above salts were dissolved in 800 ml of distilled water, pH was adjusted to 7.4 with 1N HCl and the volume was made up to 1 litre, sterilized by autoclaving for 20 min and stored at RT.

11. PEG and cell debris were extracted from the phage suspension by adding an equal volume of chloroform, vortexing for 30 seconds, and centrifugation at 3,000 x g for 15 min at 4°C.
12. The aqueous phase containing the phage particles were recovered and stored at –20°C.

3.6 Confirmation of identity of host by ribotyping

The *Vibrio* sp. MV–5, was the only host that was successfully infected during the phage isolation process and hence its identity was confirmed by 16S rRNA sequence analysis.

3.6.1 Template preparation for PCR (Sambrook *et al.*, 2000)

1. Log phase culture of *Vibrio* sp. MV –5 in LB (40 ml) was taken in a sterile Oakridge tube and centrifuged at 5000 rpm for 10 min at 4°C.
2. The supernatant was discarded and the pellet was blot dried.
3. The cell pellet was dissolved in 8.75 ml of TE buffer, and 50 µl of Proteinase K (10mg/ml) and 10% SDS (1 ml) was added, mixed gently and incubated at 37°C for 1 hr.
4. To this equal volume of phenol-chloroform mixture (1:1) was added, mixed gently and kept for 10 min.

5. This was centrifuged at 10,000 rpm for 10 min at 4°C, the supernatant was transferred to another sterile tube using sterile cut tip and the steps 4 and 5 were repeated three times.
6. To the aqueous phase taken in a sterile 50 ml beaker, 0.1 volume of 5M sodium acetate (pH 5.2) and 20 ml of isopropanol were added. The DNA was collected by spooling on a glass rod and then washed with 70% ethanol.
7. The DNA dissolved in 1 ml TE buffer and electrophoresis performed on a 0.8% agarose gel.

3.6.2 Agarose gel electrophoresis (Sambrook *et al.*, 2000)

The agarose gel electrophoresis was carried out in order to check the quality of the DNA obtained.

(i) Agarose gel with a concentration of 0.8% was prepared for electrophoresis.

(ii) 10 µl of the DNA was loaded on to the gel and electrophoresis was carried out at 80 volts for 1 h or until the migrating dye (Bromophenol blue) had traversed two-thirds distance of the gel. λ DNA/EcoR1 Hind III double digest (Bangalore Genei) was used as the marker.

(iii) The gel was stained in a freshly prepared 0.5mg/ml ethidium bromide solution for 20 min.

(iv) The gel was viewed on a UV transilluminator, and image captured with the help of Gel Doc system (Bio Rad).

3.6.3 Ribotyping using partial 16S rRNA gene

Identification of the *Vibrio* sp. MV-5 was done by ribotyping using a primer pair for 16S rDNA. A portion of the 16S rRNA gene (1.5kb) was amplified from the genomic DNA (Reddy *et al.*, 2000; Reddy *et al.*, 2002a; Reddy *et al.*,

2002b; Shivaji *et al.*, 2000). Products after PCR amplification was subjected to sequencing, followed by homology analysis.

Sequence	Amplicon	Reference
16SF 5' AGTTTGATCCTGGCTCA 3'	**1500** bp	(Shivaji *et al.*, 2000)
16SR 5' ACGGCTACCTTGTTACGACTT 3'	**1500** bp	(Reddy *et al.*, 2002a; Reddy *et al.*, 2002b)

3.6.3.1 DNA sequencing

Nucleotide sequences of the PCR amplicon was determined by the ABI Prism 310 genetic analyzer, using the big dye Terminator kit (Applied Biosystems) at BioServe India Ltd., Hyderabad. The identity of the sequences determined were established by comparing the sequences obtained with the gene sequences in the database using BLAST software (Altschul *et al.*, 1980).

3.6.3.2 Phylogenetic tree construction

Phylogenetic trees were constructed using the neighbour joining methods implemented in CLUSTAL W. Trees were constructed using nucleotide evolutionary model for estimating genetic distances based on synonymous and non-synonymous nucleotide substitutions. Trees were visualized using the CLUSTAL W N-J tree.

3.7 Characterization of phage

The vibriophage was characterized for its morphology using Transmission Electron Microscopy (TEM) and the protein profile was studied using SDS-PAGE.

The various psychochemical parameters for propagation as well as for the adsorption of the phage to the host were also analyzed.

3.7.1 Electron microscopy

The morphology of the phage isolates was determined by TEM at CCMB, Hyderabad (modified method of Suttle and Chan, 1993). Phage lysates were filtered through 0.2µm pore size filters and concentrated by PEG precipitation as described in section 3.5. The phages were transferred to 400-mesh carbon-coated copper grids by floating the grids on drops of filtered lysate for ~30 min. The grids were stained with 1% uranyl acetate and photographed at 80 kV using TEM.

3.7.2 Electrophoresis

The phage lysate prepared by concentration (as stated in section 3.5) was subjected to electrophoresis analysis by non-denaturing Native–PAGE and denaturing SDS-PAGE in a vertical slab electrophoresis system (Hoefer mini vertical electrophoresis unit). Electrophoresis was carried out in a 10% polyacrylamide gel (Laemelli, 1970). SDS–PAGE was carried out under both reducing and non-reducing conditions, i.e., with and without β-mercaptoethanol respectively.

3.7.2.1 Polyacrylamide Gel Electrophoresis Reagents

1) **Stock acrylamide solution (30:0.8)**

Acrylamide (30%)	-	60.0 g
Bis-acrylamide (0.8%)	-	1.6 g
Distilled water (DW)	-	200.0 ml

Stored in amber colored bottle at 4°C.

2) Stacking gel buffer stock (0.5M Tris-HCl, pH 6.8)

Tris buffer - 6 g in 40 ml DW
Titrated to pH 6.8 with 1MHCl (~ 48 ml) and made up to 100 ml with DW.
Filtered with Whatman No. 1 filter paper and stored at 4°C.

3) Resolving gel buffer stock (3M Tris-HCl, pH 8.8)

Tris buffer - 36.3 g
Titrated to pH 8.8 with 1M HCl (~48 ml) and made up to 100 ml with DW.
Filtered with Whatman No. 1 filter paper and stored at 4°C.

4) Reservoir buffer for Native-PAGE (pH 8.3)

Tris buffer - 3.0 g
Glycine - 14.4 g
Dissolved and made up to 1L with DW.
Prepared as 10X concentration and stored at 4° C.

5) Reservoir buffer for SDS-PAGE (pH 8.3)

Tris buffer - 3.0 g
Glycine - 14.4 g
SDS - 1.0 g
Dissolved and made up to 1L with DW.
Prepared as 10X concentration and stored at 4° C

6) Sample buffer for Native-PAGE

Tris-HCl (pH 6.8) - 0.0625 M
Glycerol - 10% (v/v)
Bromophenol blue - 0.01%
Prepared as 2X concentrations and stored at 4°C.

7) Sample buffer for Reductive SDS-PAGE

Tris-HCl (pH 6.8) - 0.0625 M
Glycerol - 10% (v/v)
SDS - 2%
Dithiothreitol - 0.1M
Bromophenol blue - 0.01%
Prepared as 2X concentrations and stored at 4°C

8) Sample buffer for Non-reductive SDS-PAGE

Tris-HCl (pH 6.8)	-	0.0625 M
Glycerol	-	10% (v/v)
SDS	-	2%
Bromophenol blue	-	0.01%

Prepared as 2X concentrations and stored at 4°C

9) SDS (10%) - 1 g in 10 ml DW

10) Sucrose (50%) - 5 g in 10 ml DW

(autoclaved at 121° C for 15 minutes and stored at 4°C.)

11) Protein staining solution

Coomassie brilliant blue (0.1%)	-	100 mg
Methanol (40%)	-	40 ml
Glacial acetic acid (10%)	-	10 ml
DW	-	50 ml

12) Destaining solution

Methanol (40%)	-	40 ml
Glacial acetic acid (10%)	-	10 ml
DW	-	50 ml

13) Protein Markers for Native-PAGE

Separate markers from Sigma-Aldrich were used

Components		**Volume**	**MW (M_r)**
Bovine Serum Albumin	-	10 µl	66,000
Chick albumin	-	10 µl	45,000
Carbonic anhydrase	-	5 µl	29,000
Lactalbumin	-	10 µl	14,200

Markers were with 65µl of native 1X sample buffer, and 30 µl of marker mix was loaded to the gel.

14) Protein Markers for SDS-PAGE

Low molecular weight marker mix (Amersham Pharmacia) was used. Lyophilized marker mix was reconstituted in 1X sample buffer for reductive SDS-PAGE, boiled for 5 minutes, and 5µl of marker was loaded on to the gel. The composition of the marker mix is as given below.

Components		MW (M_r)
Phosphorylase b	-	97,000
Bovine Serum Albumin	-	66,000
Ovalbumin	-	45,000
Carbonic anhydrase	-	29,000
Trypsin inhibitor	-	20,100
α-Lactalbumin	-	14,400

3.7.2.2 Native Polyacrylamide Gel Electrophoresis

3.7.2.2.1 Gel preparation

Resolving gel (10%)

Acrylamide: bis-acrylamide (30:0.8)	-	10.0 ml
Resolving gel buffer stock	-	3.75 ml
Ammonium persulphate (APS 10%)	-	0.15 ml
Water	-	16.25 ml
TEMED	-	15.0 µl

Stacking Gel (2.5%)

Acrylamide: bis-acrylamide (30:0.8)	-	2.5 ml
Stacking gel buffer stock	-	5.0 ml
Ammonium persulphate (APS 10%)	-	0.1 ml
Water	-	12.5 ml
TEMED	-	15.0 µl

Sample buffer(1X)

Native-PAGE sample buffer (2X)	-	1.0 ml
50% Sucrose	-	0.4 ml
DW	-	0.6 ml

Sample preparation

Ten µl of 1X sample buffer was added to 30 µl sample, mixed well and 25 µl sample and 5 µl marker mix was loaded to the gel.

3.7.2.2.2 Procedure

a. The gel plates were cleaned and assembled.

b. **Resolving gel** –All the components except APS were added in to a beaker, mixed gently, and finally added APS. The mixture was immediately poured into the gel assembly and a layer of butanol was added over the gel and allowed to polymerize at least for one hour. Removed the butanol and washed with water to remove all the traces of butanol.

c. **Stacking gel** – All the components of stacking gel except APS were added into a beaker, mixed gently. Finally APS was added and the contents were poured into the gel assembly, above the resolving gel and the comb was immediately inserted between the glass plates. This was allowed to polymerize at least for 30 minutes.

d. Gel was placed in the electrophoresis apparatus, and upper and lower reservoirs were filled with reservoir buffer for Native-PAGE.

e. The gel was pre run for 1 hr at 80 V.

f. The protein sample was loaded to the gel and was run at 80V until the sample entered the resolving gel.

g. When the dye front entered the resolving gel, the current was increased to 100V.

h. The run was stopped when the dye front reached 1 cm above the lower end of the glass plate.

i. The gel was removed from the cast and stained for at least one hr in the staining solution.

j. The gel was destained till the bands became clear and were then observed using the transilluminator.

3.7.2.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The purified phage protein was subjected to SDS-PAGE, under reductive and non-reductive conditions for evaluating the nature of phage polypeptide. Low molecular weight marker (Amersham Pharmacia) was used as standard and molecular weight was determined using Quantity One Software (Biorad).

3.7.2.3.1 Reductive SDS –PAGE

3.7.2.3.1.1 Gel preparation

Resolving gel (10%)

Acrylamide: bis-acrylamide (30:0.8)	-	10.00 ml
Resolving gel buffer stock	-	3.75 ml
10% SDS	-	0.30 ml
Ammonium persulphate (APS)	-	0.15 ml
Water	-	15.95 ml
TEMED	-	15.00 µl

Stacking Gel (2.5%)

Acrylamide: bis-acrylamide (30:0.8)	-	2.5 ml
Stacking gel buffer stock	-	5.0 ml
10% SDS	-	0.2 ml
Ammonium persulphate (APS)	-	0.1 ml
Water	-	12.3 ml
TEMED	-	15.0 µl

Sample buffer(1X)

SDS-PAGE sample buffer (2X)	-	1.0 ml
50% Sucrose	-	0.4 ml
DW	-	0.6 ml

3.7.2.3.1.2 Sample preparation

Hundred µl of 1X sample buffer was added to the concentrated phage lysate, mixed well, boiled for 5 minutes in a water bath, cooled to room temperature and 25 µl of this sample and 5 µl of low molecular weight marker mix was loaded onto the gel.

3.7.2.3.1.3 Procedure

Procedure followed for SDS poly acrylamide gel electrophoresis was essentially the same as that of Native-PAGE which is described in section 3.7.2.2 with the exception that the reservoir buffer used was that of SDS-PAGE.

3.7.2.3.2 Non-reductive SDS –PAGE

3.7.2.3.2.1 Gel preparation

Resolving and stacking gel was prepared as described in section 3.7.2.3.1.1

Sample buffer (1X)

Sample buffer for Non-reductive SDS-PAGE (2X)	-	1.0 ml
50% Sucrose	-	0.4 ml
DW	-	0.6 ml

3.7.2.3.2.2 Sample preparation

Twenty µl of 1X sample buffer was added to concentrated phage lysate (30µl), mixed well and 25 µl of this sample and 5µl low molecular weight marker mix was loaded on to the gel.

3.7.2.3.2.3 Procedure

SDS poly acrylamide gel electrophoresis was performed following the same procedure as that of Native-PAGE which is as described in section 3.7.2.2.2 with the exception that the reservoir buffer used was that of SDS-PAGE.

3.7.2.4 Silver staining

3.7.2.4.1 Solutions and reagents for silver staining

1. Wash solution - 20% ethanol in distilled water
2. Sensitizer - 12.5% gluteraldehyde in distilled water
3. Stain - 2 ml of 20% aqueous solution of silver nitrate
   1 ml $(NH_4)_2OH$
   5 ml of 5 N NaOH
   Made up to 100 ml with Wash solution. Mixed and used freshly.
4. Developer - 100 µl of 37% formaldehyde solution
   25 µl 2.3 M citric acid
   Made up to 100 ml with Wash solution. Mixed and used freshly.
5. Fixing solution - 100 ml acetic acid and 400 ml methanol made up to 1 litre with DW
6. Preserving solution - 50 ml glycerol and 50 ml acetic acid made up to 500 ml with DW

3.7.2.4.2 Procedure

1. The gel was run till the dye front reached 1 cm above the lower end of the glass plate, and was transferred to the fixing solution and kept for 10 minutes on a Gel rocker.

2. This was followed by washing with distilled water for 10 minutes.
3. The sensitizer was added to the gel and allowed to react for 10 minutes.
4. The gel was washed twice with distilled water for 10 minutes each, and followed by Wash solution for 15 min.
5. The gel was stained for 15 minutes, followed by washing twice with Wash solution for 10 minutes each.
6. The bands were then allowed to develop by rinsing in the developer solution for 2-10 minutes, after which the gel was transferred to the preserving solution.

3.8 Effect of various parameters on phage propagation

Many physical and chemical factors like temperature, pH, Ca^{2-}, NaCl, and sugars that affect the propagation, viability and adsorption of phages, the effect of multiplicity of infection (MOI) and one-step growth of the phage, and the effect of various other factors like inducers, and sugars were also studied.

3.8.1 Determination of optimal multiplicity of infection (MOI) (Lu *et al.*, 2003)

The multiplicity of infection (MOI) is the average number of phage per bacterium infected. The MOI is determined by dividing the number of phage added (Volume in ml x pfu/ml) by the number of bacteria added (Volume in ml x cell/ml).

Vibrio sp. MV –5 was inoculated into 100 ml of Luria broth and incubated at room temperature for 3.5 hrs on a shaker at 120 rpm until the $O.D_{600}$=1 (1 x 10^8 cfu/ ml). This host culture was infected with the phage lysate at a final concentration of 0.01, 0.1, 0.5, 1, 5 and 10 pfu/ml and the mixture was incubated at

R. T for 3.5 hrs. This mixture was centrifuged at 9,000 x g for 3 min. The supernatant was collected, filtered through 0.22μ nitrocellulose membrane (Sartorius) and assayed to determine the phage titer after proper dilution. Phage-free cultures (containing only *Vibrio* sp MV–5) and host-free cultures (containing only phage) were used as controls. All assays were performed in triplicates. The MOI resulting in the highest phage titer within 3.5 hours was considered as optimal and was used in all subsequent large scale production of the phage and other characterization studies.

3.8.1.1 One-step growth curve (Capra *et al.*, 2006)

The One-step growth curve helps in the study of the latent period, the rise period and the burst size of the phage.

Latent period is the elapsed time between adsorption of a phage particle to its host cell and lysis of that cell with the release of phage progeny.

The **rise period** is the interval from the end of the latent period until all phages are extra cellular.

Burst size is the mean number of phage particles liberated per infected bacterium. The burst size is influenced by the length of the latent period. Generally, longer the time before the lytic burst, the larger is the number of bacteriophages released by the infected bacterium.

$$\text{Burst size} = \frac{\text{Final PFU} - \text{Initial PFU}}{\text{Number of infected bacterial cells}}$$

Vibrio sp. MV–5 was inoculated to 200ml of Luria broth and incubated for 3.5 hrs at 120 rpm so that $O.D_{600}$ =1 (1 x 10^8 cfu/ ml). The cells were harvested by centrifugation at 10,000 rpm for 10 min and resuspended in 1/5 of the initial volume (40 ml) of Luria Broth. Phage was added at an MOI of 5 and allowed to

adsorb for 5 min at 37° C. The cells were harvested again by centrifugation at 10,000 x g for 5 min and resuspended in 200 ml Luria Broth. This was incubated at 37°C. At 10 min intervals; phage counts was made using sample aliquots of 4ml after serial dilution. The soft agar overlay assay method was used. All studies were continued till 4 hrs of growth and were carried out in triplicates. The latent period, the rise period and the burst size of the phage were calculated.

3.8.1.2 Phage adsorption

The first step in the growth of bacteriophage is its attachment to susceptible bacteria. This process is called adsorption.

The adsorption studies were carried out as described below(Ellis and Delbruck, 1939; Foschino *et al.*, 1995), except that the unadsorbed phages were obtained by filtration instead of centrifugation. *Vibrio* sp. MV–5 was inoculated in to 100 ml of Luria broth and incubated for 3.5 hrs at 120 rpm so that OD_{600}=1 (1 x 10^8 cfu/ ml). This mid log phase culture was infected with phage lysate at an MOI of 5. This was considered as time zero. Sample aliquot of 5ml was drawn and the rest was incubated at 37°C at 150 rpm in a water bath shaker (SciGenics India Limited). Aliquots of 5 ml were sampled at 3, 6, 10, 15, 20, 25 and 30 min time intervals after infection. All the samples drawn were immediately filtered using 0.22μ nitrocellulose membrane (Sartorius) syringe filter. The samples were assayed for phage titer using double layer agar plate method after appropriate dilutions. All plating was done in triplicates. The percentage of phage adsorption was calculated as follows:

$$\text{Percentage of adsorption} = \frac{\text{(Mean of control titer) – (Mean of residual titer)}}{\text{(Mean of control titer)}} \times 100$$

The phage titer observed at time zero was considered as the control titer.

3.8.1.3 Effect of various parameters on phage adsorption

3.8.1.3.1 Effect of temperature on adsorption (Quiberoni *et al.*, 2004)

One ml of exponentially growing *Vibrio* sp. MV-5 was added to each of the 8 flasks containing 50 ml Luria broth and incubated at Room Temperature for 3.5 hrs at 150 rpm. Phage was added at an MOI of 5 and incubated at 0, 10, 20, 30, 37, 40, 45and 50° C for 30 min. After centrifugation at 12,000 x g for 4 min, the supernatants with appropriate dilutions were assayed using soft agar overlay method for determining the unadsorbed phages. All platings were done in triplicates. The phage titer was compared with the control titer.

3.8.1.3.2 Effect of pH on adsorption (Capra *et al.*, 2006)

Exponentially growing *Vibrio* sp. MV-5 culture was centrifuged at 10,000 x g for 20 min and the cells were resuspended in Luria broth, previously adjusted to the desired pH. Phage was added at an MOI of 5 and incubated at 37°C for 30 min. The mixtures were centrifuged at 10,000 x g for 5 min. The supernatants were assayed with exponentially growing *Vibrio* sp. MV – 5 culture using soft agar overlay method to determine surviving pfu. All platings were done in triplicates. The percentage of adsorption was calculated as described in section 3.8.1.2.

The pfu of phage grown in Luria Broth without pH adjustment was considered as control.

3.8.1.3.3 Effect of NaCl on adsorption

Exponentially growing host culture was added to Luria Broth with 0.1, 0.25, 0.5, 0.75 and 1M NaCl. Phages were added at an MOI of 5 and the mixture

incubated for 30min at 37°C. After centrifugation at 10,000 x g for 5min, the supernatants were assayed for unadsorbed phages and the counts were compared with the titre of a control which contain no NaCl in L B. All platings were done in triplicates.

3.8.1.4 Effect of Calcium ions on phage propagation (Lu *et al.*, 2003)

The effect of Calcium ions on the propagation of phages was studied. Ca^{2+} requirement not only stabilizes the coiled DNA inside the phage capsid and greatly improves the adsorption rate, but also controls the penetration efficiency of phage DNA into the bacterial cells. (Se´chaud *et al.*, 1988)

Vibrio sp. MV–5 was inoculated into 100 ml of sterile Luria broth and incubated for 3.5 hrs at 120 rpm so that $O.D_{600}$=1 (1 x 10^8 cfu/ ml). 10 ml each of this mid log phase host culture was added to five, 15 ml Mc Cartney bottle. 1M $CaCl_2$ solution was prepared and filter sterilized. Appropriate volumes of $CaCl_2$ were added to the host aliquots to make 0, 1, 10, 20 and 30 mM concentrations. After the final volume was adjusted to 15 ml with sterile distilled water, each tube was infected with the phage at an MOI of 5. All tubes were incubated at 37°C for 30 min. 1ml aliquots were drawn and centrifuged at 10,000 x g for 10 min. The supernatants were serial diluted and assayed using soft agar overlay method for determining the phage titer. All platings were done in triplicates.

3.8.1.5 Effect of temperature on phage propagation (Lu *et al.*, 2003)

A 1.5 ml centrifuge tube containing 900 µl sterile distilled water was pre-heated to temperature ranging from 50 to 100°C. 100 µl of phage sample was added (MOI 5) to the pre-heated tube. The tubes were maintained at these temperatures for varying intervals, i.e., 15 sec, 30 sec, 1 min, 2 min and 3 min. The

tubes after incubation were immediately placed in ice. Samples were assayed using soft agar overlay method to determine the number surviving pfu. All plate assays were done in triplicates.

3.8.1.6 Effect of pH on phage propagation (Capra *et al.*, 2006)

Influence of pH on phage viability was evaluated by incubating the phage in suitable buffers of different pH, ranging from 2-11. Hydrochloric acid-potassium chloride buffer was used for the studies at pH 2, while the Citrate-phosphate buffer system was used for pH 3-6; Phosphate buffer for pH 7, Tris (hydroxymethyl) amino methane buffer system for pH 8 and 9 and Carbonate-bicarbonate buffer for pH 10 and 11. The phage was added at an MOI of 5 to 15 ml of sterilized buffer solutions and incubated at 37°C for 30 min. These were assayed with the mid log phase *Vibrio* sp. MV–5 using soft agar overlay method to determine the surviving pfu. The plate assay was done in triplicates.

3.8.1.7 Effect of sugars on phage propagation (Quiberoni *et al.*, 2004)

Sugars play a very important role in phage propagation. Sugars are known to be a part of bacterial cell wall. In most of the phages, these sugars present on the host cell wall form an essential component of phage receptor structures or that their conformation is recognized by phages.

Hence the effect of various sugars like arabinose, dextrose, galactose, fructose, maltose, mannitol, mannose, lactose, rhamnose, ribose, xylose and glucosamine hydrochloride was studied. The sugars were added to a final concentration of 500 mM/l to each phage sample (10^6 pfu/ml) respectively (MOI 5). The mixture was incubated at 37°C for 30min. The mixtures were diluted adequately and assayed with the mid log phase *Vibrio* sp. MV–5 for plaques by the

soft agar overlay method to determine the surviving pfu. The results were compared with titres of control samples without the saccharides and then expressed as a percentage of phage inactivation.

3.8.1.8 Effect of NaCl on phage propagation

In order to study the effect of NaCl on phage viability, NaCl solutions of varying concentrations such as 0.1M, 0.25M, 0.5M, 0.75M, 1M, 2M and 3M were prepared in deionised water. Phage sample was added at an MOI of 5 and incubated for 30 min at 37°C. The surviving phages were immediately diluted and assayed for plaques by the soft agar overlay method. The results were expressed as percentage of initial phage counts.

3.9 Effect of inducers

The phage lysate at an MOI of 5 was added to a log phase culture of the *Vibrio* sp. MV-5 in sterile Luria broth. Mitomycin C (Sigma-Aldrich) was added at a concentration of 20 µg/ml and incubated at 37°C for 30 min. The mixture was plated and the counts were compared with the titre of a control without Mitomycin C.

3.10 Concerted effect of optimized parameters on vibriophage ΦMV-5 propagation and adsorption

The cumulative effect of all the parameters optimized in earlier studies such as temperature, pH, Calcium ions and which improved and increased phage adsorption were studied. Phage lysate was added at an MOI of 5 to Phosphate buffer (pH 7) with 10 mM $CaCl_2$ and incubated at 37°C. Aliquots were sampled at

10 min intervals, mixed with mid log *Vibrio* sp. MV-5 also at pH 7, followed by incubation for 30min and assayed using soft agar overlay method.

3.11 Broth clearance experiment

The broth clearance experiment was carried out to study the clearance of the *Vibrio* sp. MV-5 in broth. The host was inoculated into LB and incubated at 120 rpm at 37°C until the O D_{600} =1. This was followed by addition of phage lysate at an MOI of 5. This experiment was carried out under two conditions, shake culture at 120 rpm at 37°C and the other static culture at 37°C. A control was also maintained without phage lysate. Readings were taken at definite intervals from all the sets at 600 nm to check for clearance.

3.12 Host range analysis

Experimental evidence that phage exert a strong selective pressure on microbial populations comes from host-range analysis of phage isolates and the observation that very closely related bacterial species and even strains of the same species are infected by different phages (Moebus, 1991; Suttle and Chan, 1993; Waterbury and Valois, 1993).

Host range analysis was done using 225 different strains of environmental *Vibrios* and 7 clinical strains. The cultures were grown upto exponential phase and the phage lysate was added at an MOI of 5. The mixture was incubated for 30min and plated using the soft agar overlay technique. All platings were done in triplicates. The plates were incubated at appropriate temperatures and were observed for plaques.

3.13 Isolation of phage DNA

The phage lysate prepared as described in section 3.8 was used for DNA isolation. 11 ml of the phage lysate was clarified by centrifugation at 8,000 rpm at 4°C for 10 minutes. Solid PEG 6000 (10% w/v) and NaCl (1M) was added and incubated for 40 min at 0°C. The phage particles were recovered by centrifugation at 10,000 rpm at 4°C for 10 min. The pellet was resuspended in 11 ml TE buffer (10 mM Tris HCl (pH 8.0), 0.5 mM EDTA, 10 mM $MgCl_2$). The suspension was treated with equal volume of chloroform followed by centrifugation at 6,000 rpm at 16°C for 5-10 min. The aqueous phase was collected and centrifuged at 25,000 rpm at 4°C for 30 minutes. The pellet was suspended in 0.6 ml TE + $MgCl_2$ and clarified from bacterial debris by centrifugation for 2 min. Added 3 µl 20% SDS, 8 µl 0.5M EDTA, 20 µl RNase A (20 mg/ml) and incubated for 15 min at 70°C. The solution extracted with phenol-chloroform-isoamyl alcohol mixture (25:24:1) followed by centrifugation at 10,000 rpm at 4°C for 10 min. This extraction with chloroform–isoamyl alcohol mixture was repeated twice. DNA was precipitated by adding 1-2 volume of ethanol in presence of 0.15-0.5 M NaCl. The DNA was pelleted by centrifugation at 10,000 rpm at 4°C for 10 min. The pellet was immediately dissolved in 30 µl TE buffer without drying. The purity of DNA was checked by running on a 1% agarose gel.

3.13.1 Acridine orange staining (McMaster and Carmichael, 1977)

Acridine orange is a nucleic acid selective metachromatic stain used for cell cycle determination. It interacts with DNA and RNA by intercalation or electrostatic attraction respectively. Acridine orange intercalated with double stranded DNA fluoresces green (525 nm); while single stranded DNA or RNA bound electrostatically with acridine orange fluoresces red (>630 nm).

DNA run on 1% agarose gel was stained in acridine orange (Sigma-Aldrich) at 30 µg/ml for 30 min in 10 mM phosphate buffer (pH 7.0) followed by destaining in the same buffer for 30 min. The gel was viewed in UV-transilluminator (BioRad).

3.13.2 Restriction analysis

The restriction pattern of the phage DNA was studied using the enzymes *Eco* RI, *Bam* HI, *Bgl* II, *Hind* III, *Not* I, *Pst* I and *Sau* 3AI. The digestions were performed according to the instructions of the manufacturer. The generated fragments were separated by agarose gel electrophoresis as described in section 3.6.2.

3.13.3 Polymerase Chain Reaction for major coat protein of ΦMV-5

3.13.3.1 Preparation of template

The template DNA was prepared as described in section 3.13.

3.13.3.2 Designing of primers

Major coat proteins (MCP) contribute a bulk of the phage genome and can be used for comparing the phylogenetic relationship of the phage to other reported ones. The primers used in this study were designed on the basis of nucleotide sequences of MCPs of reported vibriophages - KVP 40 and KVP 20 and a coliphage T4. The sequences were aligned and three pairs of forward and reverse homologous stretches were identified and the selected primers were as follows:

Forward primers

1. 5'-AAATGGAAAGAATTGCTTGAA-3'
2. 5'-ATGGGTATGGTACGTCGTGCT-3'
3. 5'-TTCCGCATCGACAAACAAGTT-3'

Reverse primers

4. 5'-ACCAGACTTACCGATTTGAGC-3'
5. 5'-GCGTGATGCAATGATGAAGTT-3'
6. 5'-ACCGTAACGAGTTTTGAACCC-3'

The primers were synthesized by Integrated DNA Technologies, Inc. USA.

3.13.3.3 PCR Mix composition

Deionised water	-	12.5 µl
Taq Buffer	-	2.5 µl
dNTP	-	10 mM
Forward primer	-	10 pico mol
Reverse primer	-	10 pico mol
DNA	-	50 ng
Taq (1/10)	-	0.6 U

3.13.3.4 PCR conditions

Initial denaturation	-	94°C	(2 min)
Denaturation	-	94°C	(45 sec)
Annealing	-	varying temperatures based on the primers (1min)	
Primer extension	-	72°C	(2 min)
Go to step 2 and Repeat 29 times			
Final extension	-	72°C	(10 min)
	-	4°C	(5 min)

PCR was performed in a thermal cycler (Eppendorf master cycler personal).

3.13.4 Sequencing

Nucleotide sequences were determined by the ABI Prism 310 genetic analyzer by using the big dye Terminator kit (Applied Biosystems) at BioServe India Ltd., Hyderabad. The identity of the sequence was established using BLAST software (Altschul *et al.*, 1980).

3.13.5 Multiple sequence alignment (MSA) and analysis

The MSA sequence analysis was done using CLUSTALW and the relationship of the mcp sequence with other reported phage coat proteins was checked.

3.14 PCR screening for virulence /virulence related genes

PCR based screening was carried out to check for the presence of virulence genes on the phage DNA and on the host *Vibrio* sp.MV-5 genome. Screening was done for six virulence genes -*tcpA, toxR, ace, zot, ctxA* and *sxt* employing PCR.

3.14.1 PCR with *tcpA* F and *tcpA* R (Keasler and Hall, 1993)

tcpA denote Toxin co-regulated pili, and is the key virulence factor in *Vibrio cholerae*. The presence of *tcpA* is indicated by a positive amplification.

Primer sequence

Forward primer: 5' – CACGATAAGAAAACCGGTCAAGAG – 3'

Reverse primer: 5' – ACCAAATGCAACGCCGAATGGAGC – 3'

Amplicon size: 618 bp

PCR Mix composition

10X PCR buffer	-	2.5µl
2.5mM each dNTPs	-	2.0µl
Forward primer (10 picomoles)	-	1.0µl
Reverse primer (10 picomoles)	-	1.0µl
*Taq*DNA polymerase	-	1U
Template DNA (as prepared above)	-	3.0µl
Sterile Distilled water	-	to a final volume of 25µl

PCR conditions

Annealing at 60°C, 1 min.

Extension at 68°C, 1.5min.

PCR was performed in a thermal cycler (Eppendorf master cycler personal).

3.14.2 PCR with *toxR* F and *toxR* R (Singh *et al.*, 2001)

ToxR is a direct virulence factor. A positive amplification indicates its presence.

Primer sequence

Forward primer: 5' – CCTTCGATCCCCTAAGCAATAC – 3'

Reverse primer: 5' – AGGGTTAGCAACGATGCGTAAG – 3'

Amplicon size: 779 bp

PCR Mix composition

10X PCR buffer	-	2.5µl
2.5mM each dNTPs	-	2.0µl
Forward primer (10 picomoles)	-	1.0µl
Reverse primer (10 picomoles)	-	1.0µl
*Taq*DNA polymerase	-	1U
Template DNA (As prepared above)	-	3.0µl
Sterile Distilled water	-	to a final volume of 25µl

PCR conditions

Annealing at 60°C, 1 min.

Extension at 68°C, 1.5min.

PCR was performed in a thermal cycler (Eppendorf master cycler personal).

3.14.3 PCR with *zot* F and *zot* R (Singh *et al.*, 2001)

Zonula occludens toxin is known as *zot*. Positive amplification will denote its presence. It can be called a direct virulence factor.

Primer sequence

Forward primer: 5' – TCGCTTAACGATGGCGCGTTTT – 3'

Reverse primer: 5' – AACCCCGTTTCACTTCTACCCA – 3'

Amplicon size: 947 bp

PCR Mix composition

10X PCR buffer	-	2.5µl
2.5mM each dNTPs	-	2.0µl
Forward primer (10 picomoles)	-	1.0µl
Reverse primer (10 picomoles)	-	1.0µl
*Taq*DNA polymerase	-	1U
Template DNA (As prepared above)	-	3.0µl
Sterile Distilled water	-	to a final volume of 25µl

PCR conditions

Annealing at 60°C, 1 min.

Extension at 68°C, 1.5min.

PCR was performed in a thermal cycler (Eppendorf master cycler personal).

3.14.4 PCR with *ctxA* F and *ctxA* R (Singh *et al.*, 2001)

ctxA is the most important virulence factor of *Vibrio cholerae*. It codes for cholera toxin A subunit.

Primer sequence

Forward primer: 5' – CTCAGACGGGATTTGTTAGGCACG – 3'

Reverse primer: 5' – TCTATCTCTGTAGCCCCTATTACG – 3'

Amplicon size: 302 bp

PCR Mix composition

10X PCR buffer	-	2.5µl
2.5mM each dNTPs	-	2.0µl
Forward primer (10 picomoles)	-	1.0µl
Reverse primer (10 picomoles)	-	1.0µl
*Taq*DNA polymerase	-	1U
Template DNA (As prepared above)	-	3.0µl
Sterile Distilled water	-	to a final volume of 25µl

PCR conditions

Annealing at 60°C, 1 min.

Extension at 68°C, 1.5min.

PCR was performed in a thermal cycler (Eppendorf master cycler personal).

3.14.5 PCR with *ace* F and *ace* R (Singh *et al.*, 2001)

Accessory cholera enterotoxin is called as *ace*. A positive amplification will indicate its presence. This is also a direct virulence factor.

Primer sequence

Forward primer: 5'– TAAGGATGTGCTTATGATGGACACCC – 3'

Reverse primer: 5'– CGTGATGAATAAAGATACTCATAGG – 3'

Amplicon size: 289 bp

PCR Mix composition

10X PCR buffer	-	2.5µl
2.5mM each dNTPs	-	2.0µl
Forward primer (10 picomoles)	-	1.0µl
Reverse primer (10 picomoles)	-	1.0µl
*Taq*DNA polymerase	-	1U
Template DNA (As prepared above)	-	3.0µl
Sterile Distilled water	-	to a final volume of 25µl

PCR conditions

Annealing at 62°C, 1 min.

Extension at 68°C, 1.5min.

PCR was performed in a thermal cycler (Eppendorf master cycler personal).

3.14.6 PCR with *sxt* F and *sxt* R (Thungapathra *et al.*, 2002)

sxt is an integrative conjugative element, which is around 100kb in size. A part of ***sxt***, the partial integrase gene will be amplified if the element is present. This is a virulence associated gene.

Primer sequence

Forward primer: 5' – TTATCGTTTCGATGGC – 3'

Reverse primer: 5' – GCTCTTCTTGTCCGTTC – 3'

Amplicon size: 803 bp

PCR Mix composition

10X PCR buffer	-	2.5µl
2.5mM each dNTPs	-	2.0µl
Forward primer (10 picomoles)	-	1.0µl
Reverse primer (10 picomoles)	-	1.0µl
*Taq*DNA polymerase	-	1U
Template DNA (As prepared above)	-	3.0µl
Sterile Distilled water	-	to a final volume of 25µl

PCR conditions

Annealing at 54°C, 1.5min.

Extension at 68°C, 1.5min.

PCR was performed in a thermal cycler (Eppendorf master cycler personal).

3.14.7 Agarose gel electrophoresis (Sambrook *et al.*, 2000)

(i) Agarose gel with a concentration of 1.5% or 2% (depending on the expected amplicon size) was prepared for electrophoresis of the PCR products.

(ii) Ten µl of the PCR products was loaded on to the gel and electrophoresed at 80V for 1hr or until the migrating dye (Bromophenol blue) had traversed two-thirds distance of the gel. λ DNA/EcoR1 Hind III double digest (Bangalore Genei) was used as the marker.

(iii) The gel was stained in freshly prepared 0.5mg/ml ethidium bromide solution for 20 min.

(iv) The gel was viewed on a UV transilluminator, and the image was captured with the help of Gel Doc system (Bio Rad).

Chapter 4

RESULTS

4.1 Isolation and identification of *Vibrios*

In order to isolate Vibriophages, initially suitable host bacteria were isolated from different locations in the marine and mangrove environments off the Kerala coast. One hundred and twenty eight strains of environmental *Vibrios* were isolated from these different marine sources, and identified based on their morphological and biochemical characteristics.

The isolates, which were Gram negative, oxidase positive, fermentative with/without gas on MOF media and which showed yellow/green colored colonies on TCBS (Thiosulphate Citrate Bile salt Sucrose) agar were segregated as *Vibrios*. These isolates were later used as hosts for the screening of phages.

4.2 Phage isolation

Several attempts were made for the isolation of phages from the marine and mangrove water samples using the host *Vibrios* isolated from the same sample. The phage was isolated from water sample collected from Mangalavanam mangrove, Kochi (Fig. 4.1), using the *Vibrio* sp. MV-5 isolated from the same location. This strain was then further subjected to species level identification by ribotyping using partial 16S rRNA gene and also screened to check for the presence of virulence genes.

Fig. 4.1 The study area – Mangalavanam mangrove

The phage was purified using repeated plating and picking of single isolated plaques on the lawns of *Vibrio* plates. The plaques obtained were small, pinhead sized (Fig. 4.2), and were turbid indicating a filamentous nature of the phages. For convenience, the phage was named ΦMV-5. From the plates, phage lysates were prepared in large quantities for further analyses.

Fig. 4.2 Plate showing pinhead sized turbid plaques of ΦMV-5 phage

4.3 CHARACTERIZATION OF HOST *VIBRIO*

4.3.1 Morphological and biochemical characters

The host bacterium *Vibrio* sp. MV-5 was Gram negative, oxidase positive and fermentative on MOF media. On TCBS agar it grew as pin- head sized, round, smooth colonies, which were sucrose non-fermenting and therefore green in color.

4.3.2 16S rRNA ribotyping of *Vibrio* sp. MV-5

The host *Vibrio* sp. MV-5 was further subjected to species level identification based on ribotyping using partial 16S rRNA gene, which could be amplified and sequenced. The sequence is presented as below (Fig.4.3). The identity was confirmed by comparing the sequences with Genbank entries, by BLAST programme.

GTGGGGTCAGGGGCTGAGCAGCCACGGTATAGATGGTGTTGTTGT
NCNTTGGGGTAACGTGGGGGGTGTGTGTGCTGGGAGTGCCAGTGA
TGGGGATAACGTTGGATTGCTGTATACCGTATACGCCTACGGGGGA
GGGTGGGCCTTCGGGCTNGCNGGTTGGTATGCCCGGGTGGGTTTA
GCTTGTTTNGTGTGGGGTAAGTGCNCTCACCACGNGCACCATNCCC
TAGGCTGNTTCTGAGCAGGGATGATCAGCCCACANGTGGAAACTG
AACACACGTCCCCAAACTCCCTACGGGGAGGACACCACGGGGGGA
AATATTGCANAATGGNGCAAACCTTATGCACCCAATANCGGGGGTG
GTGTAAAGCGAAAGGGCCTTTCGGGGGTTGTAAAACCCACTTTNTC
AAGCGAGAGCGAAGGGAAAAGGTCAGAAACCCTAAATAATCTTGC
TTGGGGTTTTTTACCATGTTTCCNTCGCAAGAACACAAACACGCNC
CCGGCTTAAACCTTCCGTTCCCCAAGCCAAGCCTCGGGCGGGGAA
AATAACGGGAGGGTGCCAANCCGTTTTAATTCGGGAAAATTAACAT
TGGNCCGTTAAAAACGCCNACNCCGCGGGCGGGNTGTGGGCATCA
CATTAAAAATTTTNAAAACCCCCCGGCCTCAAACCTGGGANATTTN
CACTTTATAAAAAACTTTNTCCGGTAGAGTTTTTTGTAAAAGGGGG
GGGGAAA

Fig. 4.3. The sequence obtained from ribotyping using partial 16 S rRNA gene of *Vibrio* sp. MV-5

Partial sequence of the 16S rRNA gene was submitted to GenBank (accession number EF506946) through BankIt programme, at NCBI site (http://www.ncbi.nlm.nih.gov/BankIt).

4.3.2.1 Phylogenetic tree construction

The identity of the *Vibrio* sp. MV-5 could be confirmed by comparing the sequences with Genbank entries, by BLAST programme (Altschul *et al.*, 1980), and the interrelation with other *Vibrios* based on the phylogram constructed using CLUSTALW N-J programme (Fig. 4.4).

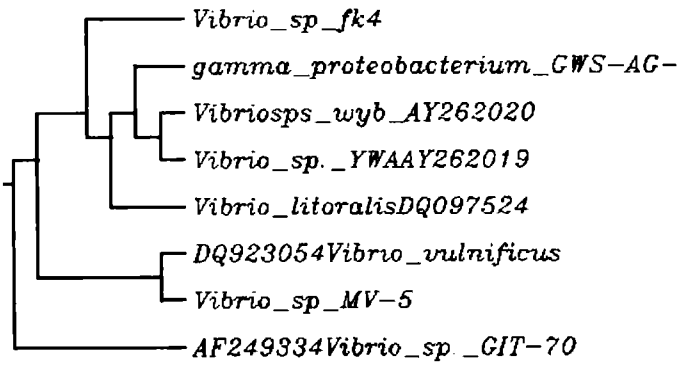


Fig. 4.4 Phylogram of host *Vibrio* sp. MV-5

From the phylogram, it is inferred that the host *Vibrio* sp. MV-5 has close similarity to *Vibrio vulnificus*.

4.4 Concentration of ΦMV-5 phage

The ΦMV-5 phages were concentrated using polyethylene glycol (PEG) precipitation method. From the results (Fig. 4.5), it was observed that 100% precipitation of phage was obtained using >40% PEG saturation. It was also noted that although the precipitation of phages was higher at >20% PEG concentration, other proteins from the medium also got precipitated. This was also clear when the precipitates were observed from the SDS-PAGE analysis pattern. In order to avoid this protein contamination, 20% PEG saturation level was used in all further studies.

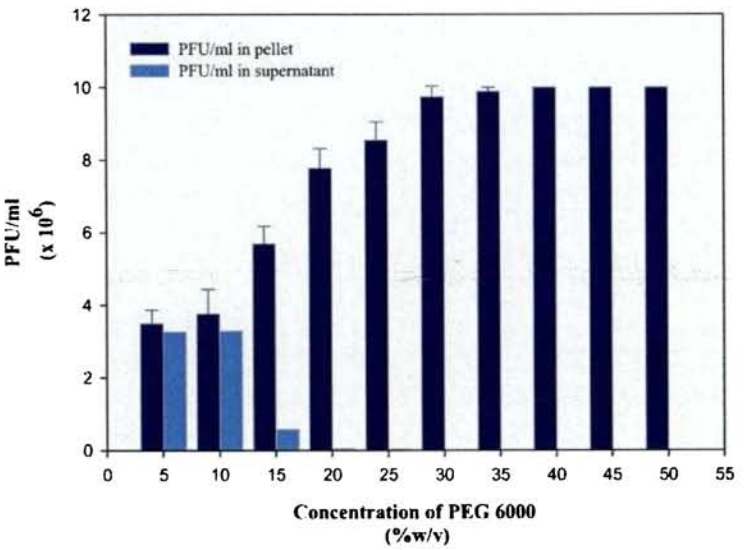


Fig 4.5 Precipitation of ΦMV-5 phage using different concentrations of PEG

4.5 CHARACTERISATION OF PHAGE

4.5.1 Electron microscopy

Transmission electron microscopic studies conducted towards elucidation of the structure of the vibriophage ΦMV-5 indicated that it is a filamentous phage, (Fig. 4.6), and from the structure, it is considered to be Inovirus-like bacteriophage. Phages were observed to be long filamentous structures that were approximately 1.1 μm in length and 0.03 μm in diameter. The phage particles also showed a pointed head and a blunt tail, which are the characteristics of all filamentous phages.

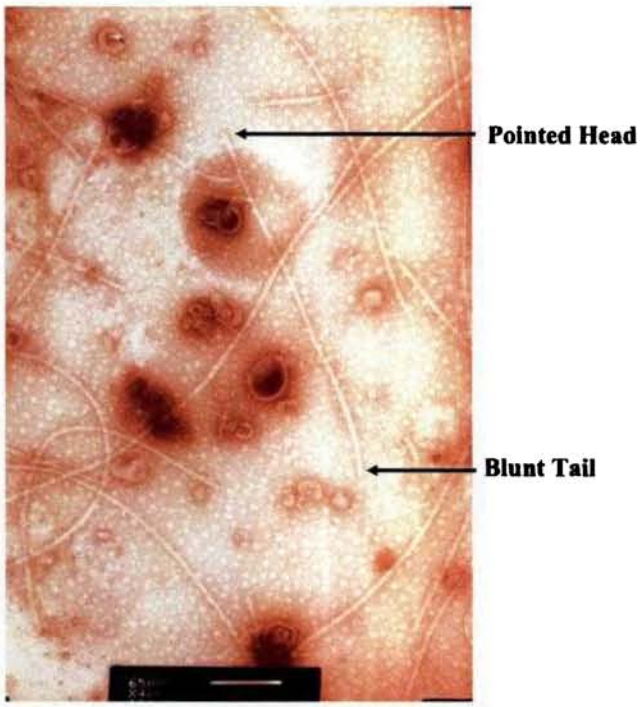


Fig. 4.6 The morphology of ΦMV-5 phage as obtained in Transmission Electron Microscopy

4.5.2 Electrophoresis

4.5.2.1 Native Polyacrylamide Gel Electrophoresis

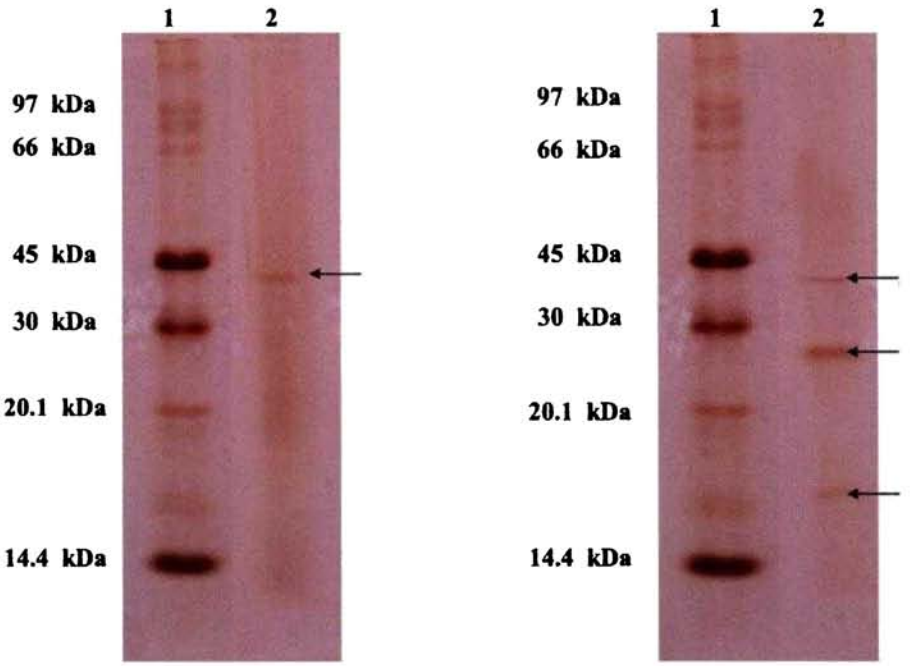
The ΦMV-5 phage precipitated using 20% PEG, was analyzed by native polyacrylamide gel electrophoresis (PAGE). The sample was visualized as a single protein band on the gel, thereby confirming its homogeneity and purity (Fig. 4.7).

Fig. 4.7 Native PAGE analysis of ΦMV-5 phage after PEG precipitation

4.5.2.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE pattern of the purified ΦMV-5 under reducing and non-reducing conditions is presented in the Fig 4.8. A single polypeptide band with a molecular weight of ~ 45 kDa was observed in non-reductive SDS-PAGE (Fig 4.8 A). The molecular weight was confirmed to be 42.95 kDA using Quantity One® software (BioRad).

Under reducing conditions, (in the presence of β-mercaptoethanol), in addition to the ~45 kDa molecular weight protein band, two other protein bands with molecular weights i.e. ~ 29 and 18 kDa respectively, were also observed (Fig 4.8 B).



Lane 1 – Marker
Lane 2 – Φ MV-5 phage concentrated with PEG 6000

Lane 1 - Marker
Lane 2 - Φ MV-5 phage concentrated with PEG 6000 and treated with β-mercaptoethanol

Fig. 4.8 SDS PAGE pattern of ΦMV-5 phage protein under reducing (A) and non reducing (B) conditions

4.5.3 Effect of various parameters on ΦMV-5 phage propagation

4.5.3.1 Determination of optimal multiplicity of infection (MOI)

Multiplicity of infection is defined as the ratio of virus particles infected to that of the potential host cells. The MOI resulting in highest phage titer under standard conditions was considered as the optimal MOI and used in all subsequent

large-scale phage production. From the data obtained (Fig. 4.9), the optimal multiplicity of infection was observed to be 5 PFU/ml.

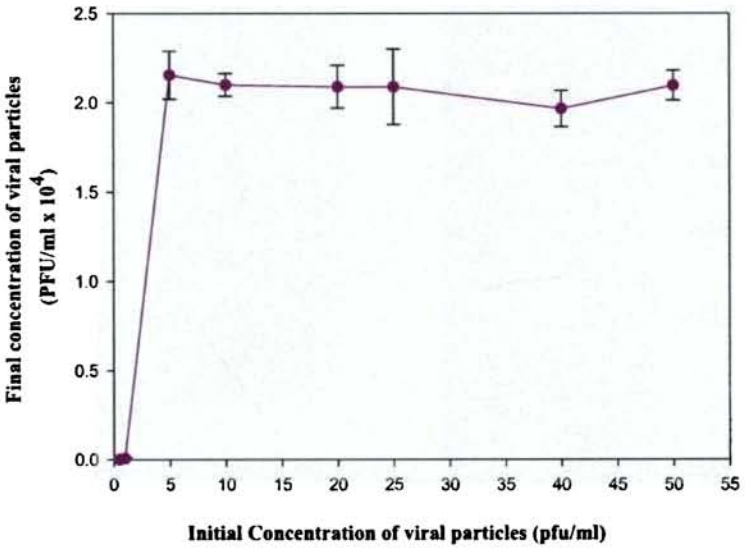


Fig 4.9 Determination of optimal multiplicity of infection (MOI) for Vibriophage ΦMV-5

4.5.3.2 One-step growth curve

The latent period, rise period and the burst size were calculated from the one-step growth curve (Fig. 4.10). Here, a one step growth curve was determined for vibriophage ΦMV-5 at an MOI of 5 and at 37°C. From this, the latent period for the phage was found to be approximately up to 30 minutes and the rise period was 50 minutes. The burst size was calculated to be 60 phages per cell.

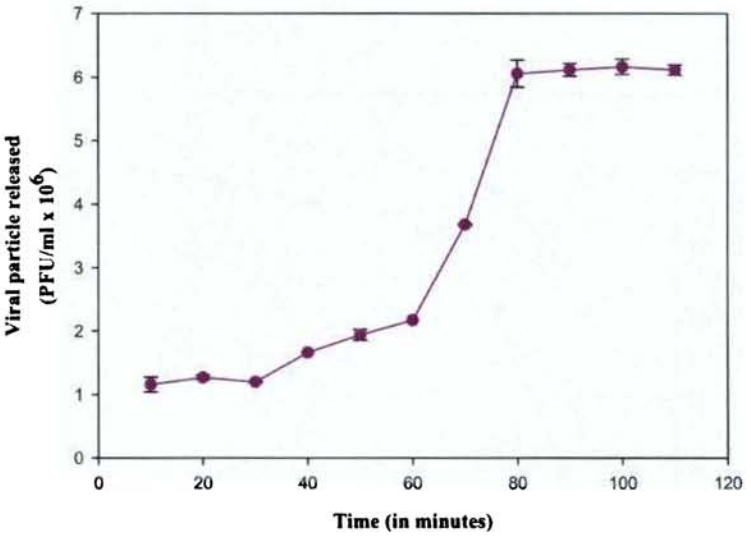


Fig 4.10 One step growth curve of vibriophage ΦMV-5 at 37°C

4.5.3.3 Phage adsorption

The adsorption rates of vibriophage ΦMV-5 are shown in Fig 4.11. From the results, it is observed that although about 90% of the phage particles got adsorbed on to the host cells within 15 min of incubation at 37°C, about 94% of the phage particles could get adsorbed in 25 min, and almost 100% adsorption took place in 30 min.

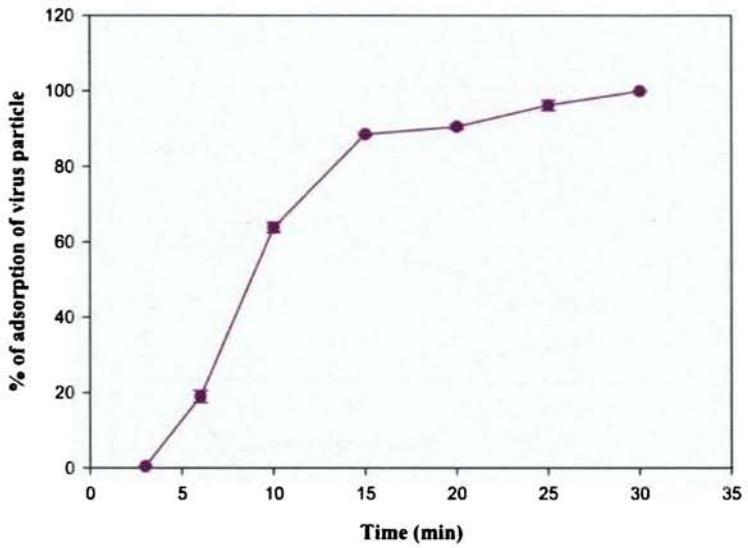


Fig. 4.11 Adsorption kinetics of vibriophage ΦMV-5

4.5.3.4 Effect of various parameters on phage adsorption

4.5.3.4.1 Effect of temperature on adsorption

The effect of temperature on adsorption of the ΦMV-5 to the host cells was investigated at temperatures ranging between 0 to 50°C, after incubation for 30 minutes at these test temperatures. The results obtained are as shown in Fig. 4.12. The maximum percentage of adsorption was obtained at temperatures ranging between 30°C and 40°C, and the highest was observed at 37°C. Approximately 95% adsorption was obtained at 37°C. There was no adsorption at 0°C, 10°C and 50°C.

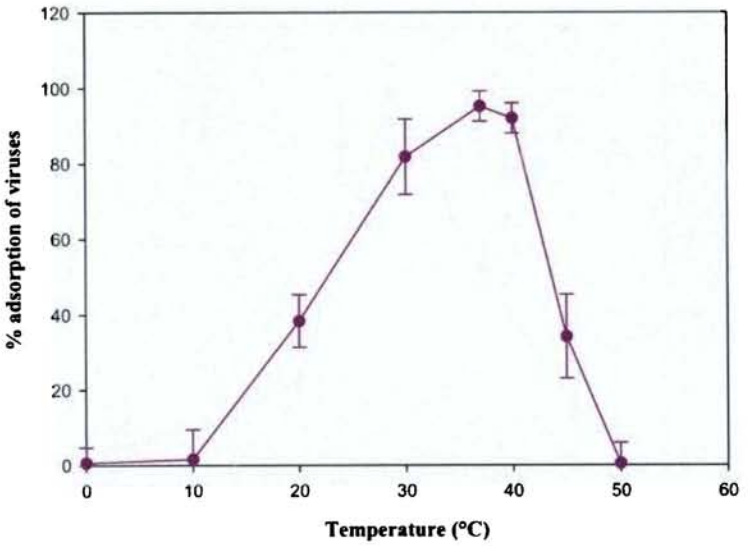


Fig. 4. 12 The effect of temperature on adsorption of phage ΦMV-5

4.5.3.4.2 Effect of pH on adsorption

The influence of pH on the adsorption of phage ΦMV-5 to host cells was studied in the pH range of 5 to 10. There was 16% adsorption at pH 5 followed by 52%, 100%, 21%, 10% and 4% at pH 6, 7, 8, 9 and 10 respectively (Fig. 4.13). The adsorption was found to increase from pH5 and reached an optimum at pH7, the phage titer decreased beyond pH7.

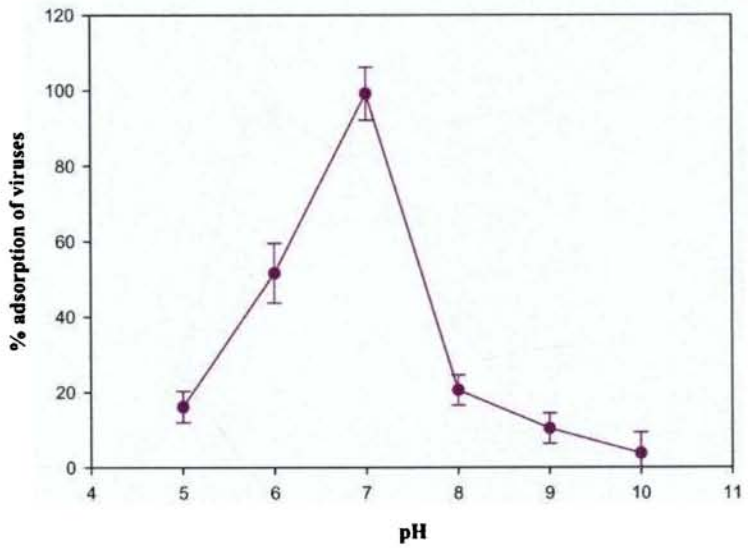


Fig. 4.13 The effect of pH on adsorption of ΦMV-5 phage to host cells

4.5.3.4.3 Effect of NaCl on adsorption

Results obtained for the study on the effect of NaCl on phage adsorption are shown in Fig. 4.14. The maximum adsorption of 65% was obtained at 0.2M NaCl. There were no significant decrease in the titer at the higher concentrations tested.

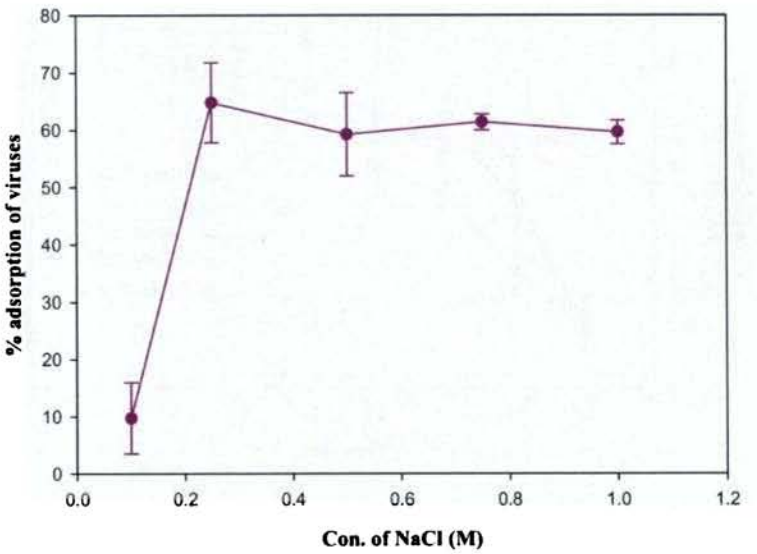


Fig. 4.14 The effect of NaCl on adsorption of ΦMV-5 phage to host cells

4.5.3.5 Effect of Calcium ions on phage propagation

The effect of $CaCl_2$ on propagation of vibriophage ΦMV-5 was investigated using varying concentrations of the same in the phage lysate (Fig. 4.15). A concentration of 10 mM $CaCl_2$ was optimum for the propagation of the phage. There was no observed increase in the effect on propagation beyond this concentration.

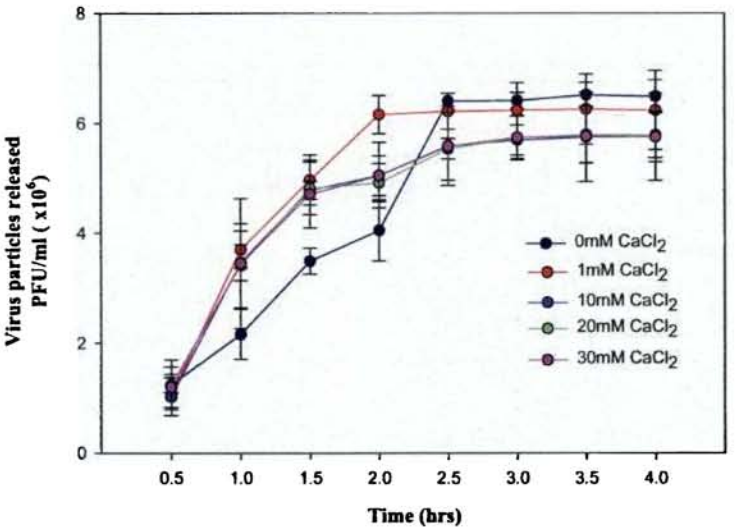


Fig. 4.15 The effect of $CaCl_2$ on propagation of ΦMV-5 phage

4.5.3.6 Effect of temperature on phage propagation

Effect of temperature on vibriophage ΦMV-5 viability was investigated by heat treatment at 50°C, 60°C, 70°C, 80°C, 90°C and 100°C each, for different time intervals ranging from 15 sec, 30 sec, 1 min, 2 min and 3 min. Survivor curves of the phage at 50°C, 60°C and 70°C are shown in Fig. 4.16. There was an observed decrease in the PFU at 50°C, 60°C and 70°C corresponding to time of temperature treatment. But it also showed that temperatures up to 70°C could be tolerated by the vibriophage ΦMV-5. Phage titers decreased below the detection limit when the phage was subjected to heat treatment at 80°C, 90°C and 100°C even for 15 sec.

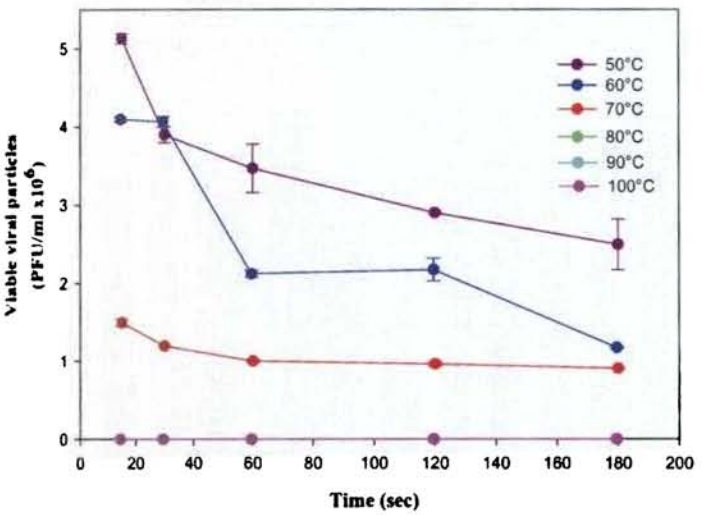


Fig 4.16 The survival curves of vibriophage ΦMV-5 at different temperatures

4.5.3.7 Effect of pH on phage propagation

Results obtained for the study on the viability of the vibriophage ΦMV-5 over a pH range of 2-11 are shown in Fig. 4.17. From the results, it is obvious that there is a gradual effect on the viability of the phage particles in the pH range from 5 to 11 at 37°C, with viability highest at pH 7 and gradually decreasing on either side of this pH. While on one hand the phage is able to tolerate higher alkaline pH, being viable even at pH 11, the viral suspension was completely inactivated at pH 2-4 after 30 min, indicating higher sensitivity to very low pH.

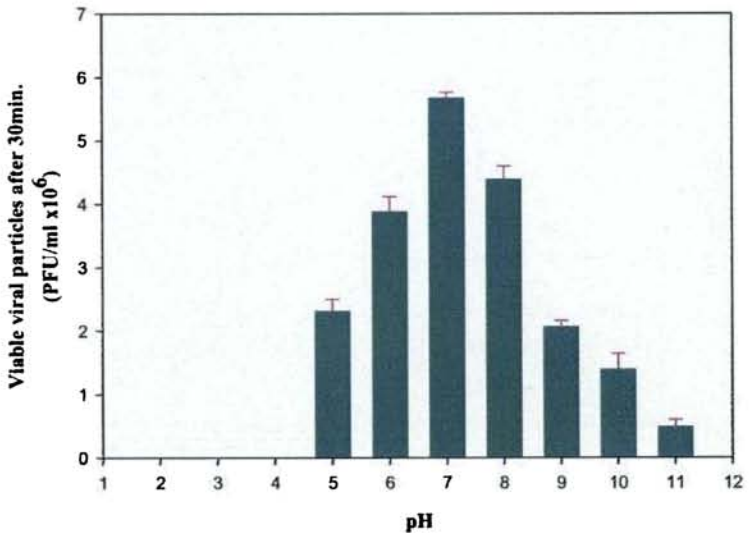


Fig. 4.17 Effect of pH on viability of vibriophage ΦMV-5 after 30 min at 37°C

4.5.3.8 Effect of sugars on phage propagation

The effect of sugars on the viability of phage ΦMV-5 was tested using arabinose, dextrose, galactose, fructose, maltose, mannitol, mannose, lactose, rhamnose, ribose, xylose and glucosamine hydrochloride, each at a final concentration of 500 mM/l, and the result is shown in Fig 4.18. It is evident that all the sugars tested, affected the viability of the phage; rate of inhibition ranging between 80 to 100 %. Glucose amine caused 100% inhibition of phage viability.

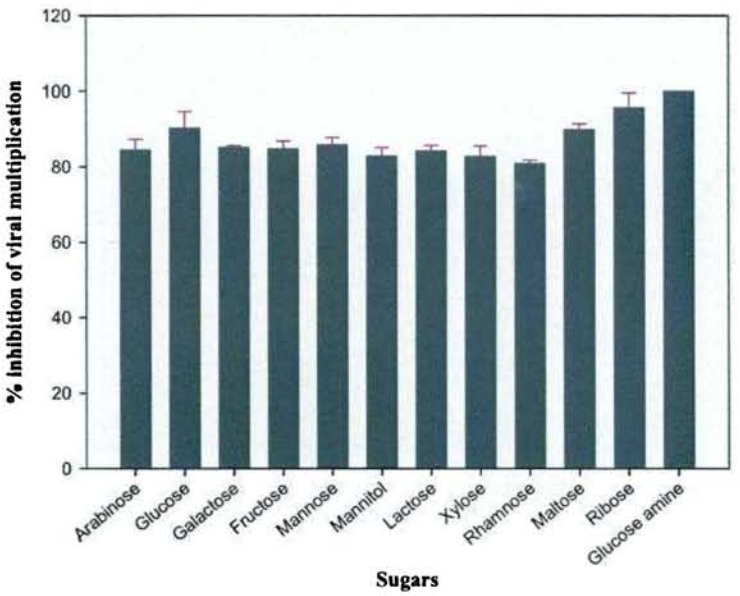


Fig. 4.18 Effect of sugars on the viability of vibriophage ΦMV-5

4.5.3.9 Effect of NaCl on phage propagation

From the data presented in Fig. 4.19 on the studies on the effect of different concentrations of NaCl on the viability of phage ΦMV-5, it is inferred that maximum PFU/ml was observed at 0.25 M NaCl concentration and beyond this, though the titer values decreased, the phage was able to tolerate up to 3M NaCl.

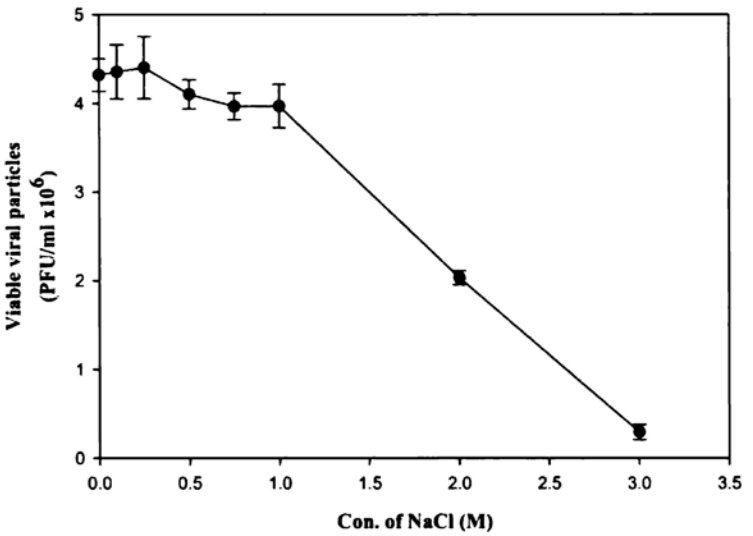


Fig. 4. 19 Effect of NaCl on the viability of vibriophage ΦMV-5

4.6 Effect of inducers

The effect of Mitomycin C, a DNA damaging agent (conc. of 20 μg/ml) on the induction of phage ΦMV-5 was studied. The titer increased significantly and was found to be ~ 250% higher in the case of the phage induced with Mitomycin C compared to the uninduced control (Fig. 4.20). This also indicates that the phage is lysogenic and can be induced.

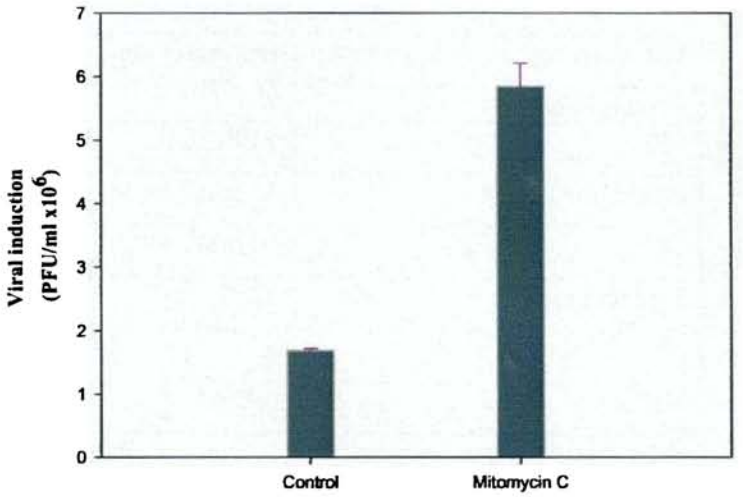


Fig. 4.20 The effect of Mitomycin C on induction of vibriophage ΦMV-5

4.7 Concerted effect of optimized parameters on vibriophage ΦMV-5 propagation and adsorption

All the parameters optimized for vibriophage ΦMV-5 propagation and adsorption to the host cells (Table 4.1), were integrated and the results obtained is presented in Fig.4.21.

Table 4.1 Optimal conditions for propagation and adsorption of phage ΦMV- 5

Parameters	**Optimal value**
Propagation	
MOI	5 PFU/ml
Incubation period	30 min
$CaCl_2$	10 mM
Temperature	37°C
pH	7
NaCl	0.25 M
Adsorption to host	
Temperature	37°C
pH	7
NaCl	0.25 M

It was observed that the burst size had almost doubled compared to the control, increasing from 60 to 121.

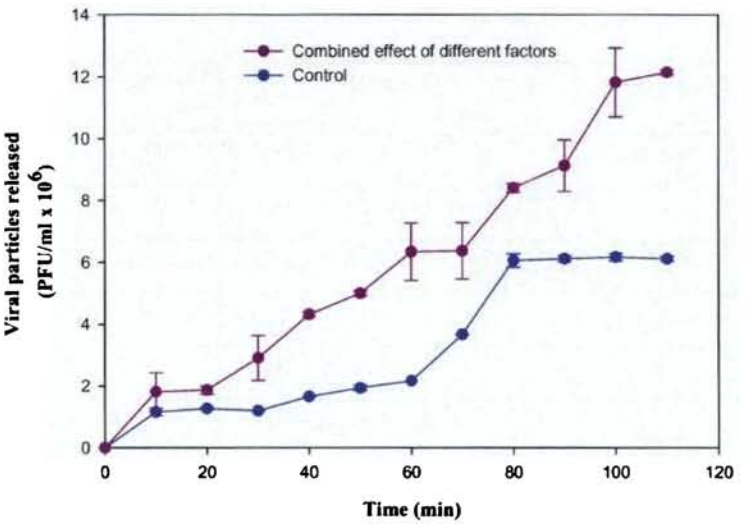


Fig. 4.21 Concerted effect of optimized parameters on propagation and adsorption of vibriophage ΦMV-5

4.8 Broth clearance experiment

The results obtained for the broth clearance experiment are depicted in Fig. 4.22. There was no pronounced clearing of the broth under both shaking condition as well as static conditions, even after 48 hrs of incubation. The rate of growth was comparatively slow under static condition. This substantiates the filamentous nature of the phage ΦMV-5.

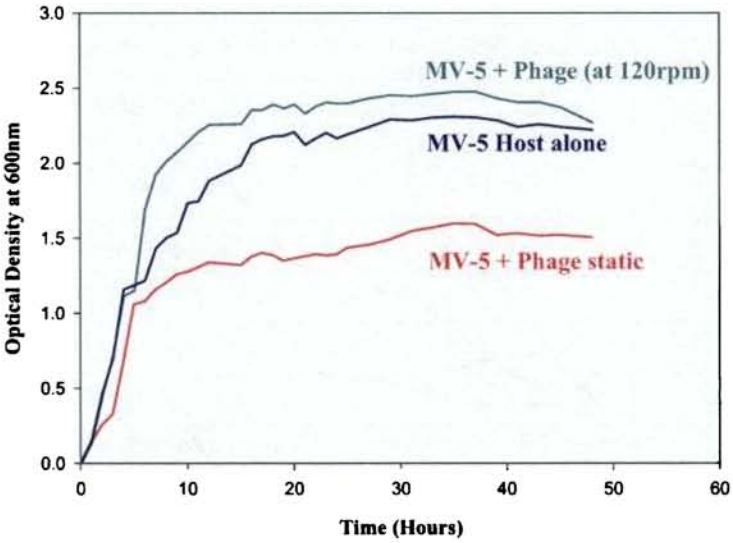


Fig. 4.22 Broth clearance experiment

4.9 Host range analysis

The host specificity of ΦMV-5 phage was studied using 225 strains of environmental *Vibrios* and 7 clinical strains.

Of the 225 strains of environmental *Vibrios* tested, ΦMV-5 phage showed specificity to only 22 strains. ΦMV-5 showed no specificity towards the other clinical (pathogenic) strains like *E. coli, Vibrio cholerae O139, V. parahaemolyticus O3:K6, Vibrio cholerae O1 Inaba Classical, Vibrio cholerae O1 Ogawa ElTor CO 366, Vibrio cholerae C26 and V. cholerae 01VC 20* . The list of positive strains is given in Table 4.2.

Table 4.2 List of strains sensitive to ΦMV-5 phage

Sl. No.	Culture	Identity	Reference
1	ALPVC 4	*Vibrio cholerae*	(Bernard, 2006)
2	CHAVA 3	*Vibrio parahaemolyticus*	,,
3	CHV22	*Vibrio alginolyticus*	,,
4	EKM 10	*Vibrio* sp.	,,
5	P3	*Vibrio harveyi*	,,
6	P4	*Vibrio alginolyticus*	,,
7	P6	*Vibrio harveyi*	,,
8	P9	*Mucus bacterium*	,,
9	P10	*Vibrio* sp.	,,
10	PV8 1	Bacterium JB 8	,,
11	C10	*Vibrio vulnificus*	,,
12	KG 5	*Vibrio parahaemolyticus*	,,
13	AF6	*Vibrio* sp.	,,
14	MUS 6	*Vibrio* sp.	,,
15	MUS 12	*Vibrio* sp.	,,
16	MUS 13	*Vibrio furnissii*	,,
17	MUS 14	*Vibrio* sp.	,,
18	MUS 15	*Vibrio harveyi*	,,
19	MV 3	*Vibrio* sp.	,,
20	MV 7	*Vibrio* sp.	,,
21	MV 8	*Vibrio* sp.	,,
22	MV 15	*Vibrio* sp.	,,

4.10 Isolation of phage DNA

The phage DNA was isolated and observed as single band on agarose gel electrophoresis (Fig. 4.23).

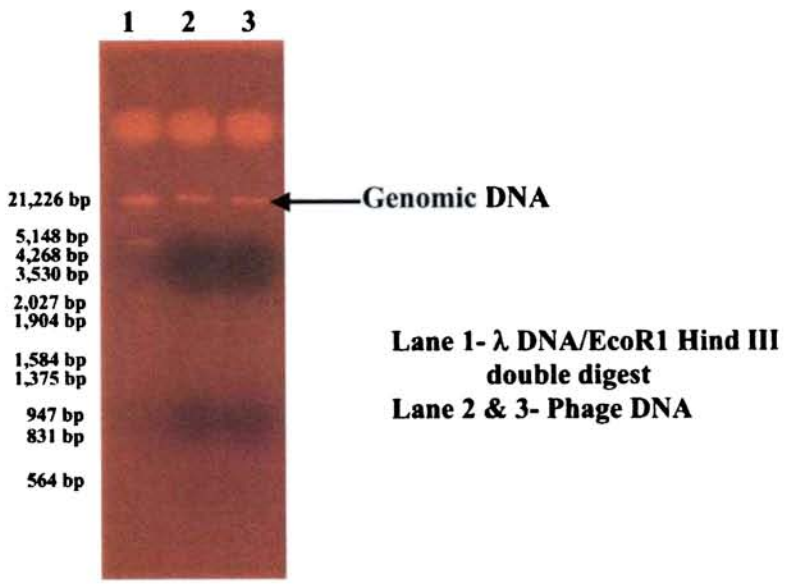


Fig. 4.23 Agarose gel electrophoresis of vibriophage ΦMV-5 DNA

4.10.1 Acridine orange staining

The phage ΦMV-5 DNA was stained using acridine orange to identify the nature of the DNA molecule. Acridine orange differentially stains the single and double stranded DNAs. As shown in Fig. 4.24, the DNA fluoresces green, indicating the double stranded nature of the DNA of phage ΦMV-5.

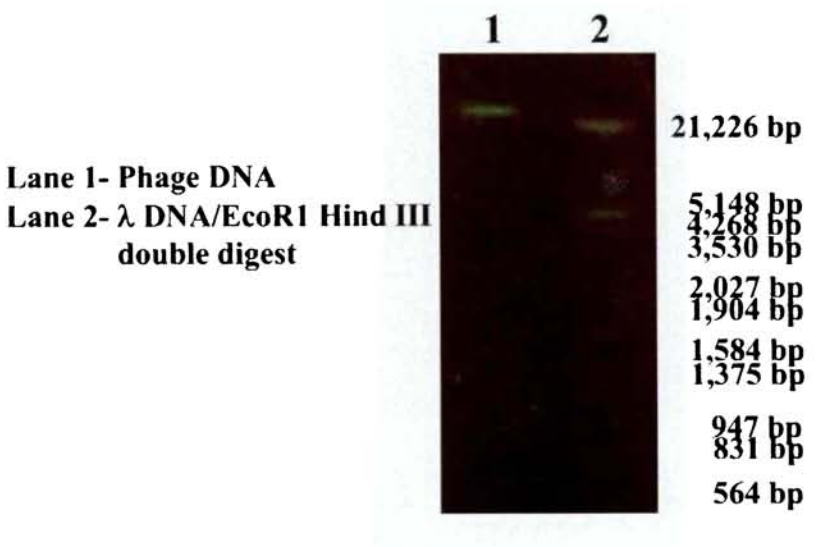


Fig. 4.24 Agarose gel electrophoresis of phage ΦMV-5 DNA stained with acridine orange

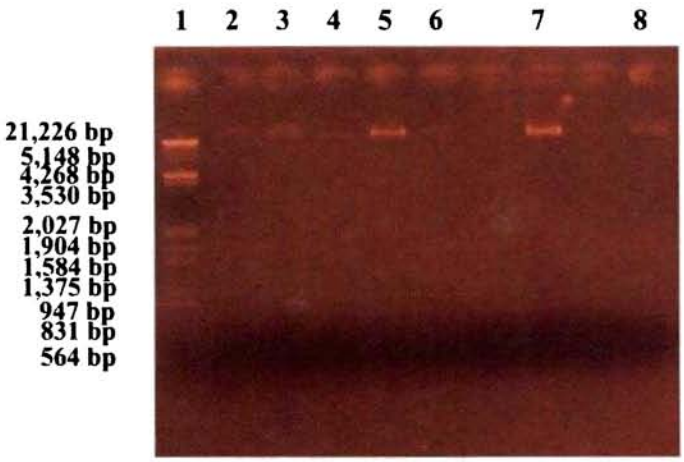
4.10.2 Restriction analysis

The restriction pattern of the phage ΦMV-5 DNA was studied using the restriction enzymes listed in Table 4.3.

Table 4.3 Restriction enzymes and their corresponding restriction sites

Restriction enzyme	Restriction site
Eco RI	G↓AATTC
Bam HI	G↓GATCC
Bgl II	A↓GATCT
Hind III	A↓AGCTT
Not I	GC↓GGCCGC
Pst I	CTGCA↓G
Sau 3AI	↓GATC

From the results presented in Fig. 4.25, it was observed that the phage ΦMV-5 DNA was resistant to all the restriction enzymes tested.



Lane 1	**λ DNA/EcoR1 Hind III double digest**
Lane 2	**EcoRI**
Lane 3	**Bam HI**
Lane 4	**Bgl II**
Lane 5	**Hind III**
Lane 6	**Not I**
Lane 7	**Pst I**
Lane 8	**Sau 3AI**

Fig. 4.25 Restriction pattern of ΦMV- 5 phage DNA

4.10.3 Polymerase Chain Reaction for major coat protein of ΦMV- 5

The major coat protein (mcp) was amplified using the degenerate primers synthesized. An amplicon size of ~1kb (Fig. 4.26) was generated.

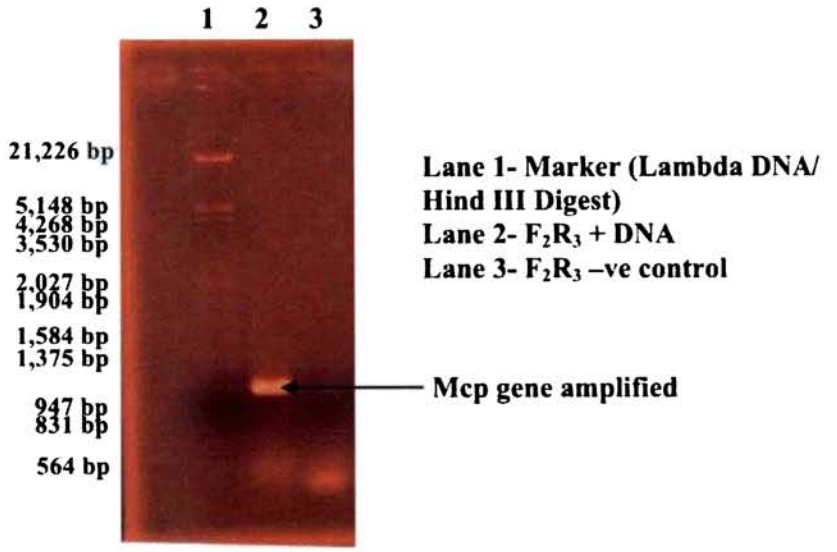


Fig. 4.26 PCR amplification of mcp gene of ΦMV-5 DNA

4.10.4 Sequencing and analysis

The amplified fragment thus obtained was sequenced and the sequence is as shown in Fig. 4.27. The sequence of the partial mcp gene was submitted to GenBank (accession number EF506947) through BankIt programme, at NCBI site (http://www.ncbi.nlm.nih.gov/BankIt). The sequence was compared with other phage sequences in the database using BLAST programme and the result is presented in Fig. 4.28.

AACGGCAGCGACGCCGTCCGTGCTAAGGGTTAGTACCTTCGTGCTGAAGG
GTGTGCCATTCTCTCGTCGAGCGCGCGACTTCCCTTTGTCGGCTCGCTTTG
GGATGTCCGGGCTAGCTTATGACGCTCCCGTCCAAACCATATCCACGGGA
TTCTTGACCTGTGCTTGGCTGACCCTGCGTTCACGTTCCGTAACCGTAGGT
CTTCGGGAGATGCACCCCTCCTCTCCTTACGAGGAAAACCGACCTCTGCG
GGTTCCATCCACCCCCAGGCCTGTGCACTGGGTCTGTTCCACGCTTTCCTA
CAAGAGGATTTCACCACTGCCCCGCAGGTCCCTTT

Fig. 4.27. The sequence obtained from partial mcp gene of *Vibriophage* ΦMV-5

Caudovirales	5 hits	5 orgs [root; Viruses; dsDNA viruses, no RNA stage] .
Podoviridae	3 hits	3 orgs
. . Vibriophage VpV262	1 hits	1 orgs [T7-like viruses; unclassified T7-like viruses]
. . unclassified Podoviridae ...	2 hits	2 orgs
. . . Vibrio phage VP2	1 hits	1 orgs
. . . Vibrio phage VP5	1 hits	1 orgs
. Phage phiJL001	1 hits	1 orgs [Siphoviridae; unclassified Siphoviridae]
. Vibrio phage KVP40	1 hits	1 orgs [Myoviridae; T4-like viruses; Vibrio phage nt-1 sensu lato]

Fig. 4.28 Taxonomic position of Vibriophage ΦMV-5 based on partial sequence of mcp gene

Results of the BLAST analysis, indicate that vibriophage ΦMV-5 has a similarity to double stranded DNA viruses and to other vibriophages VpV262, VP2, VP5, ΦJL001 and KVP40.

4.11 PCR screening for virulence/ virulence related genes

Results of the study on screening of six virulence genes associated with *Vibrios* are presented below. *tcp A, tox R, ace, zot, ctx A* and *sxt* are the virulence genes looked for in the DNA of phage ΦMV-5 DNA and the host *Vibrio* sp. MV-5.

4.11.1 PCR with *tcpA* F and *tcpA* R

tcpA codes for the A subunit of a cluster of proteins making toxin-co-regulated pili. This is a gene which is directly responsible for virulence in *Vibrio cholerae*. PCR screening for checking the presence of this gene yielded positive result for both phage ΦMV-5 DNA and the host *Vibrio* sp. MV-5 (Fig. 4.28).

4.11.2 PCR with *toxR* F and *toxR* R

toxR codes for transmembrane regulatory protein. ToxR is required for expression of virulence factors in the human diarrhoeal pathogen *Vibrio cholerae,* including the cholera toxin (CT) and the toxin co-regulated pilus (TCP). From the results of PCR assay for detecting *toxR* (Fig. 4.28), the positive amplicon ~779 bp was observed for both phage ΦMV-5 DNA and the host *Vibrio* sp. MV-5.

4.11.3 PCR with *zot* F and *zot* R

Zonula occludence toxin (ZOT) is a toxin affecting tight junctions of cells. Even though originally reported from *Vibrio cholerae,* as part of this study the

presence of this gene was checked in both phage ΦMV-5 DNA and the host *Vibrio* sp. MV-5 and was found to be present in both genomes (Fig. 4.28).

4.11.4 PCR with *ctxA* F and *ctxA* R

The key virulence factor in *Vibrio cholerae,* the Cholera toxin gene has two subunits linked together. The presence of either one subunit may indicate the presence of the other. Here, the presence of 'A' subunit gene of cholera toxin was checked by PCR, and it was observed that the phage ΦMV-5 DNA and *Vibrio* sp MV-5 was devoid of the virulence factor (Fig. 4.28).

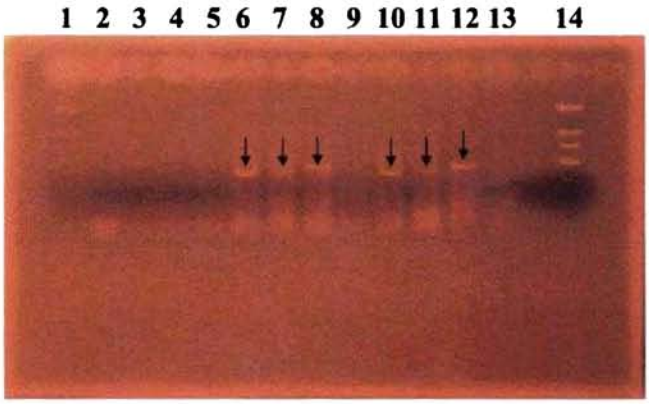


Fig. 4.28 PCR amplification for screening of virulence genes in ΦMV-5 DNA and host *Vibrio* sp. MV-5

1 & 14- Marker (Lambda DNA/Hind III Digest)
2- -ve control for *tcpA* amplified fragment
3- -ve control for *toxR* amplified fragment
4--ve control for *zot* amplified fragment
5--ve control for *ctxA* amplified fragment
6- *tcpA* amplified fragment of ΦMV-5 DNA
7- *toxR* amplified fragment of ΦMV-5 DNA
8- *zot* amplified fragment of ΦMV-5 DNA
9- *ctxA* amplified fragment of ΦMV-5 DNA
10- *tcpA* amplified fragment of host *Vibrio* sp. MV-5
11- *toxR* amplified fragment of host *Vibrio* sp. MV-5
12-*zot* amplified fragment of host *Vibrio* sp. MV-5
13-*ctxA* amplified fragment of host *Vibrio* sp. MV-5

4.11.5 PCR with *ace* F and *ace* R

ACE stands for accessory cholera enterotoxin. This is a newly found toxin reported in *V. cholerae* itself. Presence of *ace* was observed only in the phage ΦMV-5 DNA and not in the host *Vibrio* sp. MV-5 as shown in Fig. 4.29.

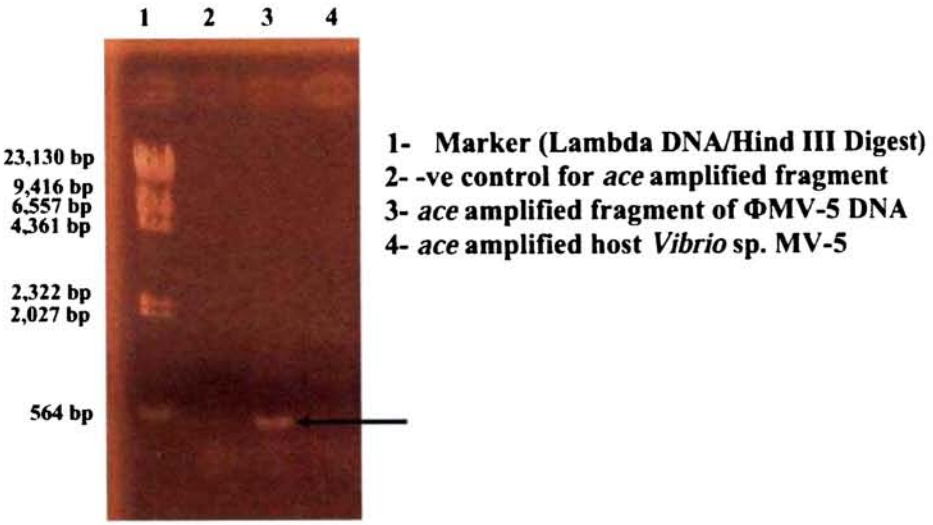


Fig. 4.29 PCR amplification of *ace* gene of ΦMV-5 DNA and host *Vibrio* sp. MV-5

4.11.6 PCR with *sxt* F and *sxt* R

sxt is around 100 kb, integrative conjugative element, which is indicated by the presence of sulfamethoxazole, trimethoprim, streptomycin, and chloramphenicol resistance genes clustered together near the 5' end of the element. The result of PCR amplification of this element of the phage ΦMV-5 DNA and the host *Vibrio* sp. MV-5 turned out to be positive (Fig. 4.30).

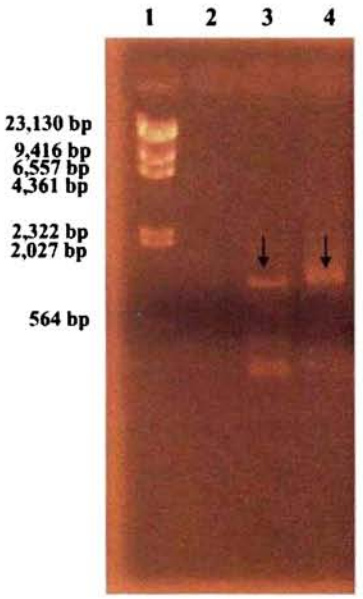


Fig. 4.30 PCR amplification of *sxt* gene of ΦMV-5 DNA and host *Vibrio* sp. MV-5

4.12 Virulence genes associated with phage ΦMV-5 and sensitive host *Vibrios*

The virulence pattern associated with the phage ΦMV-5 and the host *Vibrios* which are infected by the phage were analysed and the result is shown in Table 4.4. From the data presented in this table it is evident that the isolated vibriophage is capable of infecting different species of Vibrios including *Vibrio cholerae, V. parahaemolyticus, V. alginolyticus, V. harveyi, V. vulnificus,* and *V. furnissi* which did not harbor *tcpA, toxR, ace, ctxA, zot* and *sxt* except *V. cholerae* which harbored *toxR* and *sxt.* The specific host, *V. vulnificus* MV-5, of phage ΦMV-5 showed the presence of *tcpA, toxR, zot* and *sxt.* Results indicate probability of *V. vulnificus* MV-5 acquiring these toxin genes from the phage.

Table 4.4 Virulence genes associated with phage ΦMV-5 and sensitive host *Vibrios*

Organism	Identity based on 16S rRNA gene	tcpA	toxR	ace	ctxA	zot	sxt
ALPVC 4	*Vibrio cholerae*	-	+	-	-	-	+
CHAVA 3	*Vibrio parahaemolyticus*	-	-	-	-	-	-
CHV22	*Vibrio alginolyticus*	-	-	-	-	-	-
EKM 10	*Vibrio* sp.	-	-	-	-	-	-
P3	*Vibrio harveyi*	-	-	-	-	-	-
P4	*Vibrio alginolyticus*	-	-	-	-	-	-
P6	*Vibrio harveyi*	-	-	-	-	-	-
P9	*Mucus bacterium*	-	-	-	-	-	-
P10	*Vibrio* sp.	-	-	-	-	-	-
PV8 1	Bacterium JB 8	-	-	-	-	-	-
C10	*Vibrio vulnificus*	-	-	-	-	-	-
KG 5	*Vibrio parahaemolyticus*	-	-	-	-	-	-
AF6	*Vibrio* sp.	-	-	-	-	-	-
MUS 6	*Vibrio* sp.	-	-	-	-	-	-
MUS 12	*Vibrio* sp.	-	-	-	-	-	-
MUS 13	*Vibrio furnissii*	-	-	-	-	-	-
MUS 14	*Vibrio* sp.	-	-	-	-	-	-
MUS 15	*Vibrio harveyi*	-	-	-	-	-	-
MV 3	*Vibrio* sp.	-	-	-	-	-	-
MV 7	*Vibrio* sp.	-	-	-	-	-	-
MV 8	*Vibrio* sp.	-	-	-	-	-	-
MV 15	*Vibrio* sp.	-	-	-	-	-	-
MV-5	*Vibrio vulnificus*	+	+	-	-	+	+
ΦMV-5	Vibriophage	+	+	+	-	+	+

Chapter 5

DISCUSSION

Vibriophages are phages infecting *Vibrios* and they are well documented. The earliest records of the isolation of bacteriophages appeared in the 1920s. As typical viruses, phages infect suitable bacterial host cells for proliferation (Josephsen and Neve, 1998). Phages have been isolated from the environment in which host bacterial strains generally survive and can be found in sewage, faeces, soil, and water (Chakrabarti *et al.,* 2000). In most of the earlier studies, the phage was isolated from the same sample as that from which the host bacterium was isolated. In the present study too, a filamentous vibriophage ΦMV-5 was isolated from the Mangalavanam mangrove, Kochi using a host *Vibrio* sp. MV-5, which was also isolated from the same location.

The host for phage ΦMV-5, *Vibrio* sp. MV-5 isolated from the Mangalavanam mangrove closely resembles *V. vulnificus* based on partial 16S rRNA gene analysis. *Vibrio vulnificus* is a naturally occurring estuarine bacteria (DePaola *et al.,* 1994) capable of causing primary septicemia or gastroenteritis after ingestion and secondary septicemia through skin lesions in individuals with underlying chronic diseases. It is abundant in a variety of estuarine habitats, and highest levels have been reported in the intestines of finfish (DePaola *et al.,* 1997). They have also been reported from mangroves throughout the world.

The plaque formed by a particular phage is one of the many important parameters for characterization of the phage. A lot can be determined about a bacteriophage from looking at its plaque size and shape. There are two known forms of phage reproduction, lytic and lysogenic. In a lytic cycle, a bacteriophage

infects a host bacterium and converts it into a "phage factory". The bacteriophages inside eventually destroy or lyse the bacterium and burst out as many as 50 to 200 new phages. Larger plaques mean that closer to 200 phages are produced every time the process occurs or that the phage has had more time on the plate to consume the bacteria. Smaller plaques mean the opposite. A clear plaque is strong evidence of a lytic phage. In the lytic cycle, the host bacterium is eventually destroyed, so there is nothing visible in that area.

The plaques produced by the vibriophage ΦMV-5 were characteristically turbid, which is the typical plaque morphology of temperate phages (Jiang *et al.*, 1998). The turbidity in a turbid plaque arises from the growth of surviving bacteria within the zone of lysis. In a lysogenic cycle, the infecting phage mixes some of its DNA with that of the host bacterium, and becomes immune to any other phage of the same type. These are known as temperate phages, which use both a lytic and lysogenic life cycle. They create turbid plaques because many of the bacteria are not destroyed, just genetically different. If a plaque has both a lytic and lysogenic area, it is an indication of the phage having both lytic and lysogenic life cycles. It also suggests that the phage is temperate.

Phage ΦMV-5 showed characteristic small turbid plaques of the size of a pinhead, but with smooth outline, typical of plaques exhibited by filamentous phages. Phages lytic to *V. vulnificus* have been discovered in the estuarine water samples of Louisiana which included four morphological groups. The plaques were reported to be minute (1 mm or less) with fuzzy outlines and complete lysis was not evident (Pellon *et al.*, 1995). A diverse group of *V. vulnificus* phages, reported to be abundant in Gulf Coast oysters throughout the year, where suggested to be an important agent in the control of microbial populations in estuarine and coastal environments. (DePaola *et al.*, 1997). There are reports of temperate phages for *V. vulnificus*, which showed no evidence of lysis (Marco-Noales *et al.*, 2004).

Previous reports have indicated that lysogenic phages are abundant among marine bacterial isolates (Jiang and Paul, 1994), suggesting the temperate nature of many marine bacteriophages. However, isolation of temperate phages from marine environments was rare. Out of the 300 marine phage isolates only 29 were found to be temperate. However, this phage collection was obtained by liquid nutrient enrichment isolation method which favors isolation of lytic phages (Moebus, 1980). The same approach was used in the present study also. The phage was isolated using the soft agar overlay method used for the isolation of lytic phages.

As observed under the Transmission Electron Microscope (TEM), phage ΦMV-5 morphology was found to be typical that of a filamentous phage. Each phage particle consisted of single, long filament that was approximately 1.1 μm in length and 0.03 μm in diameter. The phage also shows a pointed head and a blunt tail, characteristic of all filamentous phages, and may be considered to be an inovirus-like bacteriophage.

Filamentous phages constitute a large family of bacterial viruses that infect many gram-negative bacteria. Their defining characteristic is a circular, ss DNA genome encased in a long somewhat flexible tube composed of thousands of copies of a single major coat protein, with a few minor proteins at the tips. The genome is small-a dozen or fewer closely packed genes and an intergenic region that contains sequences necessary for DNA replication and encapsidation. Unlike most bacterial viruses, filamentous phages are produced and secreted from infected bacteria without cell killing or lysis.

The ends of the phage particle ΦMV-5 were clearly distinguishable in electron micrographs. There is a pointed head and a blunt edge. All filamentous phages that have been characterized use pili, which are long and slender cell surface appendages that resemble the phage themselves as receptors. Many

vibriophages reported are found to have similar filamentous nature. EM of phage 493 of *V. cholerae* O139 revealed the filamentous nature of the particle with an estimated width of 2.6 nm and variable length. In some preparations, one end of the phage appeared to carry slender fibers. The phage was resistant to pH values of 2.8-11.8 and to heat treatment up to 60°C for 10 min, but it was totally inactivated at 75°C and by chloroform treatment (Jouravleva *et al.*, 1998).

Vibrio cholerae is known to be a host to a variety of bacteriophages (vibriophages), which include virulent phages as well as temperate phages, represented by the kappa-type phages produced by most strains of the El Tor biotype (Guidolin and Manning, 1987; Takeya, 1974). Another group includes the filamentous phages, which have a single-stranded DNA (ssDNA) genome (Campos *et al.*, 2003a; Ehara *et al.*, 1997; Ikema and Honma, 1998; Kar *et al.*, 1996; Waldor and Mekalanos, 1996). Several of the *V. cholerae*-specific filamentous phages have been implicated in virulence gene transfer among *V. cholerae* strains (Campos *et al.*, 2003b; Davis and Waldor, 2003; Waldor and Mekalanos, 1996). Filamentous phages of *V. cholerae* have also been found to be distinct from the well-characterized filamentous coliphages, in that some of these phages can form lysogens (Campos *et al.*, 2003a; Kar *et al.*, 1996; Waldor and Mekalanos, 1996). The most remarkable of these phages is CTXΦ (Waldor and Mekalanos, 1996), which exists as a prophage in toxigenic *V. cholerae* and encodes the cholera toxin (CT).

The vibriophage ΦMV-5 isolated was concentrated using polyethylene glycol (PEG). It is a high-molecular-weight, water-soluble polymer, and has been used to concentrate viruses from aqueous suspensions. This method is termed as a phase separation rather than a precipitation (Yamamoto *et al.*, 1970) and was initially used in combination with NaCl and another polymer, dextran sulfate (DS), to concentrate preparations of poliovirus (Norrby and Albertsson, 1960),

bacteriophage, adenovirus etc. Subsequent studies indicated that PEG is equally effective when used in combination with only NaCl to concentrate bacteriophage (Yamamoto *et al.*, 1970).

The concentrations of PEG and NaCl were varied to determine the optimum conditions for the recovery of infectious ΦMV-5 in the pellets and the 20% conc. of PEG 6000 was found to be the optimum for the phase separation of this phage. The purpose of this study was to determine the optimum conditions, in terms of the properties and relative recovery of virus particles, for concentrating the phage. The results demonstrated that although maximum recovery was obtained with 40% PEG 6000 for ΦMV-5, maximum purity was obtained at 20%.

Although PEG precipitation could be used to concentrate the bacteriophage, it was reported that the bacteriophage concentrated using PEG 6000, were more often broken into head and tail components and would obviously not be viable for phage VHML infecting *V. harveyi* (Oakey and Owens, 2000). But, in the present study, viable phage particles with maximum purity could be retrieved through PEG precipitation. This showed the greater stability of the phage ΦMV-5.

The protein profiles of the ΦMV-5 showed a single band with a molecular weight of ~45 kDa suggesting that there is only one major protein. Invariably, the major protein is the Major Coat Protein (mcp).

SDS-PAGE of *Vibrio cholerae* typing phage e5 had displayed 13 structural polypeptides with molecular sizes ranging from 21.5 kDa to 90 kDa. The major component had a molecular size of 50 kDa (Basu *et al.*, 1993).

In the present study, the focus was also on the interaction of the vibriophage with its host cell. The influence of physicochemical parameters on phage viability and adsorption to sensitive cells were especially investigated. The phage ΦMV-5 was also characterized for its propagation and adsorption parameters.

The first step in the growth of bacteriophage is its attachment to susceptible bacteria. The rate of this attachment can be readily measured by centrifuging the bacteria out of a suspension containing phage, at various times, and determining the amount of phage which remains unattached in the supernatant. Phage cannot multiply except when attached to bacteria, therefore, the rate of attachment may under certain conditions, limit the rate of growth (Ellis and Delbruck, 1939).

The MOI resulting in highest phage titer under standard conditions was considered as the optimal MOI and used in subsequent large scale phage production. From the data obtained, the optimal multiplicity of infection was observed to be 5 PFU/ml.

Previous research revealed the potential importance of latent period, burst size, and the ability to produce lysogens as "strategies" by which a phage might optimize its ability to survive in nature (Abedon, 1989; Stewart and Levin, 1984; Wang *et al.*, 1996).The infection cycle of ΦMV-5 was characterized for their phage multiplication parameters: latent period was found to be 30 minutes and the rise period was 50 minutes. From the calculations, it was found that the burst size was 60 phages per cell. On comparison it was found that the burst size and latent time values varied from that previously reported for vibriophages. An earlier report involving a survey of 52 cultured marine phages, found that there is great variation

in the burst size; the average marine phage burst size was 185, and burst sizes range from 5 to 610 (Borsheim, 1993; Jiang *et al.*, 1998).

In phage e5, a *Vibrio El Tor* typing phage, growth was characterized by a latent period of 15 min, a rise period of 13 min and a burst size of around 100 pfu per cell (Basu *et al.*, 1993). Corresponding values for Ph-1, the only other cholera phage belonging to the same family and species were 50 min and 10^3 pfu per cell (Mukherjee, 1978).

The latent periods of phages nt-1 and nt-6, isolated from a salt marsh, are 50 and 60 min respectively, under optimal conditions. However, the latent periods were shown an increase to 170 and 120 min respectively, when the temperature was 10°C below optimal (Zachary, 1978). A phage infecting *Vibrio fischeri* MJ-1 had a latent period of 25 min (Levisohn *et al.*, 1987), while the latent periods for two bacteriophages isolated from the North Sea were 150 and 180 min (Chen *et al.*, 1966). The latent periods of phage ΦMV-5 are comparable to those previously reported for other vibriophages.

The phage latent period is typically under the control of a phage protein complex known as a holin. Holins restrain the activity of cell-wall-digesting endolysins, and mutations in holin genes can significantly modify the timing of host cell lysis (Young *et al.*, 2000). Increasing rates of phage exponential growth-larger burst sizes, shorter generation times, or, for well-mixed cultures (Yin and McCaskill, 1992), faster phage adsorption-should lead to faster phage-mediated exploitation of host populations. Both burst size and the phage generation time, however, are controlled by the phage latent period, with greater burst sizes associated with longer latent periods but shorter generation times associated with shorter latent periods. This conflict between burst size enlargement and generation time reduction complicates phage latent-period optimization (Abedon *et al.*, 2001).

In order to design adsorption assays, the stability of phages was examined during 30 min at 37°C. ΦMV-5 phage showed maximum adsorption at 30 min.

The influence of physical parameters on the process of adsorption was also studied in addition to propagation factors. The lytic cycle of a phage begins with its adsorption on the cell wall receptors of a sensitive bacterial host, a highly specific event (Quiberoni *et al.*, 2004a). The maximum burst size will be obtained only under conditions of maximum adsorption. Therefore, the effect of temperature, Ca^{2+}, pH and NaCl on the adsorption process was also studied. Regarding the effect of temperature on the adsorption process, the results were highly dependent on the system studied.

There are some recognized factors affecting the phage adsorption process, such as the presence of Ca^{2+} ions, the physiological state of bacterial cells, pH and temperature. The role of inorganic cations like Ca^{2+} and Mg^{2+} is noteworthy and it was observed that 10 mM $CaCl_2$ was optimum for the propagation of the ΦMV-5 phage. Beyond this concentration also, the effect was the same. 1 mM $CaCl_2$ showed a reduced effect than 10 mM $CaCl_2$ on adsorption, but enhanced final PFU and faster adsorption as compared to the control. These results obtained for ΦMV-5 phage in the present study, showed that Ca^{2-} was not indispensable either for the adsorption or for the completion of the lytic cycle, although lysis was faster in the presence of the cation. Regarding the effect of calcium, similar results were obtained for some *Streptococcus thermophilus* and *Lact. Delbrueckii* subsp. *Bulgaricus* phages (Binetti *et al.*, 2002; Quiberoni *et al.*, 2004b). The requirement for Ca^{2-} (or Mg^{2+}) not only stabilizes the coiled DNA inside the phage capsid, but also greatly improves the adsorption rate, and controls the penetration efficiency of phage DNA into the bacterial cells (Se´chaud *et al.*, 1988). Calcium ions were required for the penetration of phage genomes into the host cells of *Lactobacillus casei* (Watanabe and Takesue, 1972).

Phage ΦMV-5 was shown to tolerate temperatures up to 70°C and was rapidly inactivated at temperatures above 70°C. Usually vibriophages are associated with high temperature tolerance since they are present in higher numbers during summer season.

Thermal inactivation profiles of phage have been quite extensively studied for vibriophages (Mukherjee, 1978; Yamamoto *et al.*, 1970). The half life of thermal inactivation of e5 at 60°C was found to be 12.5 min (Basu *et al.*, 1993) compared to 2.5 min of that found for Ph-1 (Mukherjee, 1978).

The phage ΦMV-5 was viable over a wide pH range of 5 to 11 at 37°C. The viral suspension was completely inactivated after 30 min at pH 2- 4 indicating high sensitivity to lower pH. Salinity, pH and temperature are factors that play an important role in the inactivation of *Vibrio vulnificus* and *Vibrio parahaemolyticus* bacteriophages. The optimum pH for *vibrio* bacteriophages was around a neutral pH of 6 to 8. Most proteins are stable at around neutral pH. However, at extreme pH values some proteins begin to swell and unfold (Fennema, 1996). Bacteriophages are composed of a core of nucleic acid covered by a protein coat. A phage capable of infecting a bacterium attaches to a receptor site on the cell surface then uses lysozyme that is located in the phage tail to weaken the bacterial cell wall. A phage tail fiber injects the DNA through the weakened wall into the bacterial cell. This process is very important for phage reproduction. However, pH can interfere with lysozyme enzyme or protein coat thus preventing phage to attach to receptor sites of the host cell (Leverentz *et al.*, 2001; Leverentz *et al.*, 2004).

Phages adsorb to specific receptor sites on the bacterial cell wall. In gram-negative bacteria the receptors have been identified as protein and lipopolysaccharide components of the outer membrane layer surrounding the peptidoglycan. A particular phage or group of phages will adsorb to a specific site,

and different phages adsorb to different sites. Thus, on the surface of a given bacterial cell a variety of receptors are present, each type being present in large numbers (Chakrabarti *et al.*, 2000).

The effect of sugars on the viability of phage ΦMV-5 was studied using several sugars. All the sugars were found to exhibit a very high rate of inhibition on the phage ranging between 80 to 100%. 100% phage viability was lost with glucoseamine. This actually points towards the role of sugars in the process of adsorption and infection of ΦMV-5. These sugars may be acting as a part of the receptors which help in the process of their infection into the host cell.

Significant inhibition of phage was observed when it was incubated with sugars. Most of the sugars tested are known to be a part of bacterial cell wall. This fact suggests that these may be an essential component of phage receptor structures or that their conformation is recognized by phages. For *Lactobacillus casei* phages, it was reported that the receptor was the rhamnose of the cell wall polysaccharide (Watanabe *et al.*, 1993).

There was 10% adsorption of phage ΦMV-5 to its host at 0.01M NaCl. This was followed by 65%, 60%, 61% and 60% for NaCl concentrations from 0.25 to 1M NaCl.

In some lysogenic systems the spontaneous rate of phage production cannot be modified. In other systems production of phage can be induced at will by the action of some physical and chemical agents. Inducing agents do not unmask a masked phage particle. Lysogeny is perpetuated in the form of prophage. By definition, as long as the prophage remains prophage, it is unable to develop into phage. Therefore in order that a lysogenic bacterium may be able to produce phage, the prophage must lose its nature of prophage. Inducing agents seem to act

in altering the chromosome-prophage equilibrium. This could be achieved by an alteration of the chromosome, an alteration of the prophage, or a modification of the environment of the chromosome-prophage system (Lwoff, 1953). The effect of inducing agents would be to increase the probability of a change of the bacterial chromosome, the change being responsible for the detachment of the prophage.

The effect of a DNA damaging agent, Mitomycin C could induce the phage ΦMV-5 and the titer was found to be ~ 250% higher after induction. Typically, chemical means or UV C radiation (UV-C) is used to damage DNA or inhibit DNA replication; this activates DNA repair mechanisms, including the RecA protein which cleaves a repressor and induces prophages to enter the lytic cycle. One of the most effective and widely used inducing agents is mitomycin C (Ackermann and DuBow, 1987). It has been used by a number of investigators to examine lysogeny in marine viral communities and isolates (Jiang and Paul, 1994; Jiang and Paul, 1995; Tapper and Hicks, 1994). Although some prophages cannot be induced by mitomycin C or UV-C (Calendar, 1970), there is no evidence that such lysogenic associations occur in high relative abundance in the sea or elsewhere. There are several reports which confirms the inducing capacity of Mitomycin C. The increased titer of phages may be because all lysogenic prophages are induced to become lytic. (Jiang and Paul, 1995; Oakey and Owens, 2000; Tapper and Hicks, 1994; Weinbauer and Suttle, 1996) Hence, in the absence of data which indicate otherwise, it is reasonable to assume that mitomycin C and UV-C are also effective inducing agents for marine lysogens.

Broth clearance experiment was used to confirm the nature of the phage. It was observed that no significant lysis occurred in the culture. Active phages could be isolated from the broth upon filtration. This indicates that the phage is filamentous, and therefore may not cause complete lysis of the bacterial host. The filamentous nature of ΦMV-5 was also confirmed by TEM. Considering the

growth parameters obtained from the one-step growth curves, it was noteworthy that latent and burst times obtained were shorter than those determined for the other systems studied. This fact also adds to the lack of clearing of the broth.

The host specificity of phage ΦMV-5 involved using 225 strains of environmental *Vibrios* and 7 clinical strains. Phage ΦMV-5 showed specificity to only 22 environmental strains and none at all towards the clinical isolates. Phage ΦMV-5 could effectively exhibit the lysogenic and lytic cycle characteristic of filamentous phages in the environmental isolates of *Vibrio cholerae, V. furnissii, V. harveyi, V. alginolyticus, V. parahaemolyticus* etc. This suggested that the phage has a broad host range within the *Vibrio* group.

Experimental evidence of phages exerting a strong selective pressure on microbial populations comes from host-range analysis of phage isolates and the observation that very closely related bacterial species and even strains of the same species are infected by different phages (Moebus, 1991; Suttle and Chan, 1993; Waterbury and Valois, 1993). Of the many vibriophages described, phage KVP40 differed in having a broad host range; it has been reported to infect eight *Vibrio* species, including *Vibrio cholerae* and *Vibrio parahaemolyticus*, the non-pathogenic species *Vibrio natriegens*, and *Photobacterium leiognathi* (Matsuzaki *et al.*, 1992). The presence of alternative hosts is a mechanism to tide over unfavourable seasons. Similar results are obtained for a number of marine phages. Seasonal shifts in patterns of host range of phages during winter and the persistence of these viruses in oysters during winter supported by hosts other than *V. parahaemolyticus* was reported by Comeau *et al* (2005). These results imply that there can be strong coupling between phage and host populations that occur in different environments and that a broad host range can explain the occurrence of phage populations even during periods when their hosts are apparently absent.

Broad-host-range (polyvalent) phages are common among marine temperate phages. Indeed, some observations suggest that phages isolated from nutrient-poor marine environments showed a trend towards increased polyvalency, possibly representing an adaptation to low host cell concentrations (Chibani-Chennoufi *et al.*, 2004). The polyvalent phages infecting different genera in the *Enterobacteriaceae* must be regarded with some caution because this family is such a closely related bacterial group. Even data on marine phages indicate that most of them are host species specific, many even show strain specificity. Polyvalence was more prevalent in cyanophages, but fluorescence-labeled cyanophages attached specifically only to their host and not other bacteria of the natural consortium (Hennes *et al.*, 1995). Data from the ocean showed that polyvalency was correlated with phage morphology. Phages isolated from high-light-adapted *Prochlorococcus* hosts yielded exclusively *Podoviridae* that were strain specific (Sullivan *et al.*, 2003). In contrast, low-light-adapted *Prochlorococcus* hosts yielded *Myoviridae* that also infected *Synechococcus* spp., a phylogenetically related cyanobacterium. Similarly, *Synechococcus*-infecting *Myoviridae* also cross-infected *Prochlorococcus* spp., lending some support to the polyvalency concept in the marine environment. Also, in other environments, *Myoviridae* showed a broader host range than *Siphoviridae* and *Podoviridae* (Chibani-Chennoufi *et al.*, 2004).

A detailed understanding of the biology of vibriophages represents an alternative to study the genetics of these species, as phage genomes can be exploited to develop genetic tools for biotechnological applications. Furthermore, knowledge on environmental factors that influence their binding to sensitive cells is very important to develop strategies for their industrial applications and use in phage therapy.

The phage ΦMV-5 DNA stained green with acridine orange, suggesting the double stranded nature of its DNA, as against orange for single strandedness. This may turn out to be a unique report since all the filamentous phages of *Vibrios* are known to be single stranded DNA molecule. ΦMV-5 DNA was not digested by the restriction enzymes *Eco* RI, *Bam* HI, *Bgl* II, *Hind* III, *Not* I, *Pst* I and *Sau* 3AI and therefore may be resistant to all these enzymes. It is observed that most of the vibriophages are resistant to restriction enzymes (Sen and Ghosh, 2005). Similar results of resistance were also observed for phage e5 (Basu *et al.*, 1993). The nucleic acid type and gross morphology are the most important properties for phage description and classification and less emphasis should be placed on molecular weight and restriction endonuclease patterns (Ackermann and DuBow, 1987). Other reports have shown that none of the phage DNAs isolated could be digested by *Bam*HI and most were found to be resistant to restriction digestion by *Xba*I, *Kpn*I, *Sal*I, *Pst*I, *Sac*I, and *Sma*I, but the reason for this resistance was not yet determined (Jiang *et al.*, 1998).

One reason for this resistance may be that phages often modify their genomic DNA to avoid host restriction systems or to target their DNA for activity by specialized phage-encoded enzymes. *V. parahaemolyticus* Φ TB16T and Φ TB16C could not be cloned using standard approaches (e.g. enzymatic digestion and cloning) (Rohwer *et al.*, 2001).

In order to study the relationship of the major coat protein of phage ΦMV-5 with other phages, a set of primers were designed and a small portion of the mcp gene was amplified, sequenced and compared with other reported phages. The BLAST results, showed that phage ΦMV-5 gave a hit to five reported Vibriophages VpV262, VP2, VP5, Φ JL001 and KVP40. It was not possible to compare with *V. vulnificus* phage sequences and other filamentous phage mcps

since reports on gene sequences of these organisms are currently not available on the NCBI database.

Mcps of morphologically similar phages of vibrios and enterobacteria have been fairly conserved during the course of evolution of the phages and their host bacteria (Matsuzaki *et al.,* 1998). Conservation of Mcps was also recognized by (Monod *et al.,* 1997) in classical T-even and certain "pseudo-T-even" coliphages.

A major goal of phage genomic sequencing projects should be to provide the information necessary to classify marine phage into guilds that reflect their biology (Paul *et al.,* 2002). Current phage taxonomy relies on the morphological characteristics of the free phage particle as established by the International Committee on Taxonomy of Viruses (ICTV) (Murphy *et al.,* 1995). The ICTV classification, however, provides very little information about the ecological niches or lifestyles of phage. Additionally, the ICTV system does not have sufficient resolution to address phage biodiversity questions, nor will it be useful for analyzing uncultured marine phage or prophage genomes. In response to these shortcomings, numerous groups are actively constructing phage taxonomical systems based on completed genomic sequences (Lawrence *et al.,* 2002; Rohwer and Edwards, 2002). These systems will help classify marine phages into families that provide information about their lifecycles and ecological roles, as well as identify phage types that deserve more detailed analyses.

Both the phage ΦMV-5 DNA and its host DNA were screened for the presence of virulence genes using six primers including *tcpA, toxR, ace, zot, ctxA* and *sxt* employing PCR. Toxin co-regulated pili are the sites of attachment for the temperate phage, CTXΦ in *V. cholerae*. Its expression is co-regulated by the expression of cholera toxin genes ctxA and ctxB (Mukhopadhyay *et al.,* 2001). Whole pili structure are coded by many genes and *tcpA* codes for the A subunit of

a cluster of proteins making toxin-co-regulated pili. This gene is directly responsible for virulence in *Vibrio cholerae*. The presence of this gene was surprising in the phage ΦMV-5 since the host was not *V. cholerae*. The indication of this gene in the host *Vibrio* sp. MV-5 may be because of the lysogenisation of the phage DNA.

There was also positive amplification for *tcpA* and *zot* in the phage ΦMV-5 genome and in the host genome. These toxin genes are all key virulence factors of *Vibrio cholerae*. Other significant virulence factors like the *ace* and *ctx A* did not yield any amplification in the host indicating their absence. *ace* was amplified in the phage ΦMV-5.

toxR code for a transmembrane transcription controlling protein. Expression of more than seventeen virulence genes is under the co-ordinate control of the ToxR protein. ToxR is a transmembrane protein and it binds to and activates the promoter of the operon encoding cholera toxin. ToxR controls transcription of toxT, whose product in turn is directly responsible for activation of several virulence genes under ToxR control (Victor *et al.*, 1991). Many environmental signals act via the ToxR system. The presence of ToxR was also revealed by the PCR amplication in both phage ΦMV-5 and host system.

Sxt, an integrative conjugative element of around 100kb size, was originally reported from *V. cholerae* (Waldor and Mekalanos, 1996). The SXT element has an integrase gene and many other genes of unknown function (John *et al.*, 2002). SXT integrase, *int* from *V. cholerae* is about 1200bp long. This was the gene that was targeted in order to check for the presence of SXT element in the host and the phage. Both the phage ΦMV-5 as well as its host were shown to reveal the presence of *sxt*.

Within the last few years, there has been a major increase in the genetic analysis of *V. cholerae* and its phages. Much of this effort has been associated with the acceptance of the need for a live oral vaccine against cholera (Guidolin and Manning, 1987). The importance of other vibriophages also is gaining more attention.

The phage ΦMV-5 was found to have broad host range infecting 7 different species of *Vibrios* including *Vibrio cholerae, V. parahaemolyticus, V. alginolyticus, V. harveyi, V. vulnificus,* and *V. furnissi* which do not harbor *tcpA, toxR, ace, ctxA, zot* and *sxt* except *V. cholerae* which harbored *toxR* and *sxt.* The specific host, *V. vulnificus* MV-5, of phage ΦMV-5 showed the presence of *tcpA, toxR, zot* and *sxt.* The presence of these genes in the host may be because of the lysogenic association with the phage. Since the phage can lysogenize a number of species within the *Vibrio* group, it may act as a potential source of horizontal gene transfer among these environmental bacterial species and may lead to their evolution into potential pathogens.

Chapter 6

SUMMARY AND CONCLUSION

A vibriophage ΦMV-5 was isolated from the water sample of the Mangalavanam mangrove of Kerala using the host *Vibrio* sp.MV-5, which was first isolated from the same location, and later found to resemble *Vibrio vulnificus* on the basis of partial 16s RNA sequence.

The phage ΦMV-5 was purified, concentrated and stored. Some of the structural, physicochemical and genomic characteristics of the phage was studied. Transmission electron microscopic studies suggest that the vibriophage ΦMV-5 is filamentous in nature. They appear to be single long filaments that are approximately 1.1 μm in length and 0.03 μm in diameter and are considered to be inovirus-like bacteriophages. The phage also showed a pointed head and a blunt tail characteristic of all filamentous phages.

The protein profile was characterized by reductive and non-reductive SDS-PAGE. The optimal multiplicity of infection was observed to be 5 PFU/ml.

The parameters effecting phage multiplication were calculated from the one-step growth curve and the latent period for the phage ΦMV-5 was found to be about 30 minutes and the rise period was 50 minutes. The burst size was 60 phages per cell. The phage showed 100% adsorption within 30 minutes of incubation with the host cells.

The studies conducted on the effect exerted by various factors like $CaCl_2$, temperature, pH, sugars and NaCl, on phage viability revealed that 10 mM $CaCl_2$

was optimum for the propagation of the phage. The phage showed temperature tolerance upto 70°C. There was a decrease in the PFU for 50, 60 and 70°C as time of temperature treatment was extended. This also indicated that the phage can tolerate temperatures up to 70°C. Phage titers decreased below the detection limit at 80°C, 90°C and 100°C even within 15 seconds.

The phage was viable over a pH range from 5 to 11 at 37°C. But, the viral suspension was completely inactivated at pH 2- 4 after 30 min, indicating high sensitivity to lower pH. All the sugars tested drastically affected phage viability - inhibition ranging from 80% to 100 % was observed, with glucoseamine completely inhibiting the phage particles.

It was also noted that the phage ΦMV-5 was tolerant up to 3M NaCl, with maximum PFU at 0.25 M NaCl. Beyond this level, the phage titer value decreased.

The effect of pH, temperature and NaCl on the process of adsorption of the phage ΦMV-5 with the host was also studied. The maximum adsorption percentage was seen at pH 7, temperature range from 30°C to 40°C, and NaCl concentration from.0.25 to 1M.

The cumulative effect of all the parameters optimized during the course of this study resulted in the dramatic increase of the burst size from 60 to 121. Mitomycin C led to increase in the burst size indicating that all the prophages can be induced to lysis with this DNA damaging agent of the host.

The result of the broth clearing experiment also pointed towards the filamentous nature of the phage ΦMV-5.The phage ΦMV-5 was found to have a broad host range.

The DNA stained green following acridine orange staining, indicating its double stranded nature. The phage DNA was resistant to the restriction enzymes *Eco* RI, *Bam* HI, *Bgl* II, *Hind* III, *Not* I, *Pst* I and *Sau* 3AI. The major coat protein gene was studied using a set of primer designed. The partial sequence obtained showed similarity to five vibriophages already reported.

The phage ΦMV-5 showed the presence of five of the six virulence genes screened for, which included *tcpA*, *toxR*, *ace*, *zot*, *ctxA* and *sxt*. These virulence genes are characteristic of vibrios, especially that of *V. cholerae* and it was found that except for the *ctxA*, phage possessed all the other virulence factors. The presence of these virulence factors was also checked in the host and it was observed that except *ace* and *ctxA* all genes were present in the *Vibrio* sp. MV-5, found to resemble *V. vulnificus*. Hence it is assumed that this may be due to the presence of phage genome in lysogenic association with the host DNA.

Conclusion

The temperate, filamentous phage ΦMV-5 isolated from Mangalavanam mangrove of Kochi, using the environmental strain of *Vibrio* sp. MV-5 shares many similar properties with other marine phage isolates, while also remaining unique. The study has revealed that the interaction of temperate phages and the microbial population in the marine environment may contribute significantly to microbial genetic diversity and composition by conversion and transduction and which requires greater study.

Prophages contribute a substantial share of the mobile DNA of their bacterial hosts and seem to influence the short-term evolution of pathogenic bacteria. Automated methods for systematic investigation of prophages and other mobile DNA elements in the available bacterial genome sequences will be necessary to understand their role in bacterial genome evolution. In the past, phages were mainly investigated as the simplest model systems in molecular biology. Now it is increasingly realized that phage research will be instrumental in the understanding of bacterial abundance in the environment. One can predict that phage research will impact diverse areas such as geochemistry and medicine. Success will largely depend on integrative multidisciplinary approaches in this field. Clearly, further studies are required to understand how vibriophages interact with *Vibrios* to promote this organism's acquisition of the critical genes which alter its virulence or adaptation to its environmental niche.

It is evident from this study and comparison with those reports cited above that vibriophage ΦMV-5 is a previously unreported bacteriophage. It is recommended that the minimum requirement for reporting a new phage should be novel morphological markers and a description of host range, both of which have been achieved in this study. The detailed description of physicochemical properties are also an advantage for further comparisons (Ackermann *et al.*, 1978).

Chapter 7

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APPENDIX-I

GenBank submissions

EF506946- Vibrio sp.16SrDNA partial sequence.

EF506947- Vibriophage mcp gene partial sequence.