

Isolation and preliminary characterization of bacteriophages of thermophilic *Bacillus* and *Geobacillus* species

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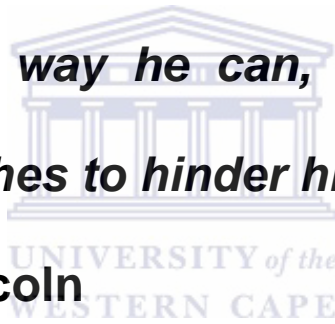
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I declare that this thesis is my own account of my research and that its main content has not previously been submitted for a degree at any tertiary education institution.

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Babele Timothee Emedi

“The way for a young man to rise, is to improve himself in every way he can, never suspecting that anybody wishes to hinder him”.

- Abraham Lincoln



In loving memory of my mother

Nyassa Sipora Balebwa Emedi



UNIVERSITY *of the*
WESTERN CAPE

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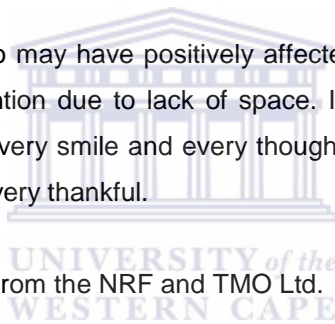
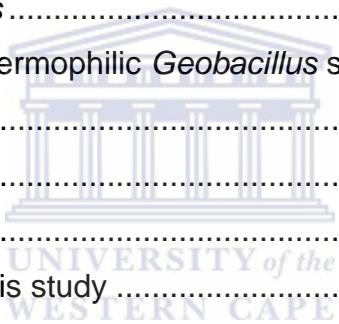


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List of frequently used symbols and abbreviations

Symbol/abbreviation	Expansion/meaning
× g	Centrifugal force
BLAST	Basic local alignment sequencing tool
bp	Base pair
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylenediaminetetra-acetic acid
g	Gram
kV	Kilo volt
L	Liter
LB	Luria Bertani
M	Molar
m	Milli
min	Minute
mM	Millimolar
°C	Degrees Celsius
OD	Optical density
ORF	Open Reading Frame
PEG	Polyethylene glycol
PFU	Plaque-forming unit
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal ribonucleic acid
TAE	Tris-acetate EDTA buffer
TE	Tris EDTA
TEM	Transmission electron microscopy
TSB	Tryptone Soya Broth
v/v	Volume per volume
w/v	Weight per volume
μF	Micro Farad
μl	Microliter

Abstract

Thermophilic bacteriophages provide simple model systems for understanding biochemical and biological adaptation mechanisms at elevated temperatures. The essential objectives of this study were to characterise the physicochemical properties of select *Geobacillus* bacteriophages and to sequence their complete genomes. The later objective is believed to be an essential prerequisite to the engineering of a site-specific integration vector for the stable cloning of exogenous genes into host bacteria. Bacteriophages were assayed at 55°C by the agar overlay technique using dry Karoo soils as source material. A pure strain of bacteriophage called GV1 (for *Geobacillus stearothermophilus* virus 1) was isolated with the strain *Geobacillus stearothermophilus* TAU3A1. Plaques were medium sized (2 to 4 mm diameters), with regular contour, clear, and without resistant cells. Host range specificity study showed that GV1 was lytic on thirteen thermophilic *Bacillus*-like strains tested, including strains of *Geobacillus stearothermophilus*, *G. thermoglucosidasius*, *B. licheniformis*, *Anoxybacillus hidirlerensis*, and *A. kuwalawohkensis*. However, GV1 failed to infect a mesophilic strain of *Bacillus megaterium*. TEM analysis of semi-purified particles revealed that the phage belongs to the family of *Siphoviridae*. Morphological characteristics included a long tail of approximately 100 nm and a hexagonal head of approximately 50 nm diameter. Viability and stability studies showed that the phage was best maintained at -80°C in PMN buffer supplemented with 20% glycerol. It was stable at a pH range of 5.5 to 7.5 and MgCl₂ and CaCl₂ concentration of 0.001 M. Thermostability experiments, conducted over short periods

of time, showed that GV1 was stable over the temperature range 50 to 75°C, with optimum at 55°C. The study of phage-host interactions showed that phage particles inhibited the initial growth of infected cultures in the first six hours post-infection, presumably while mature phages were released. This was followed by a steady recovery of the growth rate. Attempts to obtain pure particles and to extract and sequence phage DNA were unsuccessful due to the low titer nature of the phage.

Keywords: Thermophilic bacteriophages, *Bacillus* species, *Geobacillus* species, *Geobacillus* bacteriophages, physicochemical characterization, phage stability, phage purification, plaque assays, plaque-forming unit (pfu), DNA isolation and sequencing



Chapter I: Literature review

1.1 General Introduction to bacteriophages

1.1.1 The historical outline of phage biology development

Bacterial viruses or bacteriophages, commonly known as phages, were discovered twice at the beginning of the 20th century. Frederick William Twort, a British pathologist in London, first described them in 1915 when he observed a glassy transformation of "*Micrococcus*" by a transmissible agent. This discovery was followed by an unusual and probably independent finding by Felix Hubert d'Herelle, a French Canadian then working at the Pasteur Institute, in 1917 (Campbell, 2003; Flint *et al.*, 2000; Pennazio, 2006; Ackermann, 2003). D'Herelle coined the term bacteriophage (bacteria eater) following his observation of the lysis of *Shigella* by an agent that he later clearly recognized to be viral in nature. He was the first to postulate the intracellular multiplication of viruses and to introduce phage therapy of infectious diseases. The use of phages to fight bacterial pathogens persisted in Eastern European countries and in the former Soviet Union. This kind of phage use has been revived by the threat posed by the increasing resistance to antibiotics (Levin and Bull, 1996; Sulakvelidze *et al.*, 2001). The viral nature of bacteriophages was initially contested and until 1940, following the advent of the electron microscope, wide acceptance of this was not gained (Ackermann, 2003).

Owing to the simplicity of their systems, their short cycle and the wide range and abundance of their host cells (mainly bacteria), which take a relatively short time

period to grow, bacteriophages provided the appropriate tools to study some of the major questions pertaining to life. Bacteriophage research, for example, played an important role in showing that DNA is the hereditary molecule (Weinbauer, 2004). Bacteriophages offered a far more convenient experimental system for analysis than plant and animal viruses (Campbell, 2003). The history of phage discovery and the events that marked the development of phage studies are excellently described in a recent review (Pennazio, 2006).

1.1.2 Definition and properties of bacteriophages

Phages are viruses infecting bacteria and are known to be by far the most abundant of all viral groups. Viruses are very small intracellular (molecular) parasites (20-200nm long) structurally composed of at least a genetic material (consisting of either DNA or RNA, single- or double stranded) encapsulated in a protein coat (capsid). Some viruses also have lipids imbedded in the protein coat (Campbell, 2003; Fuhrman, 1999; Flint *et al.*, 2000). Other definitive properties of viruses are related to their reproduction strategies and can be summarized as follows: the viral genome replicates once inside an appropriate host cell and directs the synthesis, by cellular systems, of other virion components; these newly synthesized components are assembled (the process is called *de novo assembly*) within the host cell; progeny virions assembled during infection are released usually by cell lysis or bursting and form the medium for transmission of the viral genome to the next host cell or organism (Flint *et al.*, 2000).

1.1.3 Phage classification systems

1.1.3.1 Old classification systems

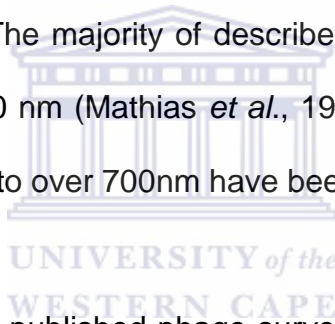
Considerable progress had occurred in the classification of bacteriophages since their discovery nearly a century ago. Early work by D'Herelle classified bacteriophages as a single species with many subsets - the *Bacteriophagum intestinale*. This was followed by the short-lived Holmes's classification system that categorized viruses according to symptoms of diseases. Holmes later proposed that bacteriophages be classified as a distinct suborder, with a single family, and a single genus of the order *Virales*. This gave way to the system proposed by Lwoff, Horne and Tournier based on morphology and nucleic acid type. They suggested the order *Urovirales* for the tailed phages, the family *Inoviridae* for the filamentous phages, and the family *Microviridae* for Φ X-type phages, respectively. Bradley's classification marked a turning point in the history of phage classification. Bradley recognized six basic types: tailed phages, filamentous phages and cubic phages with ssDNA, dsDNA, ssRNA or dsRNA (Ackermann, 2003; Weinbauer, 2004). This simple scheme still forms the basis of phage classification and is used in most microbiology and virology text books (e.g. Prescott *et al.*, 2005). The ICTV (International Committee for Taxonomy of Viruses) has built on the Bradley's basic classification scheme to structure what is known as the modern phage classification systems or the ICTV classification. New phage groups have been introduced over time, with the most recent development being the establishment of the order *Caudovirales* for tailed phages containing 15 genera (Ackermann, 2003).

1.1.3.2 Modern classification

Presently, the ICTV considers viruses to group into three orders, 61 families, and 241 genera (van Regenmortel *et al.*, 2000). Bacteriophages form one order, 13 families and 30 genera (see Table 1.1). Originally viruses were classified based on morphological properties and host type. At present, the ICTV has an “open strategy for virus classification, meaning that every available property may be considered for classification. The current trend utilizes sequence information, which can provide fine mapping of related viruses. The type of genome (i.e. RNA or DNA, single- or double-stranded) is still in use to provide fundamental classification criteria. The species concept has been used to classify viruses. A viral species is defined by a set of properties, some of which may be absent in a particular member (Ackermann, 2003). This is also called the “polythetic species concept”, which defines viral species as a polythetic class of viruses constituting a replicating lineage and occupying specific ecological niches (Weinbauer, 2004). Greek or Latin roots ending in *-virales*, *-viridae*, and *virus* are labels used to construct taxonomic names of orders, families, and genera, respectively (Ackermann, 2003).

1.1.4 Morphology and size

Morphological features of isolated phages have been extensively described (Ackermann and DuBow, 1987; Bradley, 1967). Figure 1.1 shows various known phage morphotypes (Ackermann, 2007). Bacteriophages typically have a head and a tail joined together by a connector. However, the shapes of phages are diverse and include cubic, spindle, lemon-shaped, filamentous or pleomorphic viruses (Ackermann, 2003). A diversity of other structures such as head appendages, collar and tail fibers or spikes are also found in some bacteriophages. It is not uncommon for the capsid diameter and the genome size to vary by a factor of ten between viruses (Weinbauer, 2004). The majority of described phages have a head diameter usually ranging from 30 to 60 nm (Mathias *et al.*, 1995). However, giant viruses with head sizes ranging from 200 to over 700nm have been reported (Gowing, 1993).



According to the most recent published phage survey, at least 5,556 phage particles have been examined by electron microscopy to date (Ackermann, 2007). A majority (96%) of these phages are tailed while only 3.7% are polyhedral, filamentous, or pleomorphic. Species belonging to the order *Caudovirales* have a double-stranded DNA as genetic material and are divided into three families: *Siphoviridae*, *Myoviridae* and *Podoviridae* with long flexible tail (noncontractile tail), contractile tail and very short tail, respectively (Table 1.1.) (Ackermann 2007; 2000). Phages with long, noncontractile tail, belonging to the family *Siphoviridae*, are dominant among the tailed phages (61%). Phages are present in 11 eubacterial and archaeal phyla and

are active on 154 host genera, mostly of the phyla *Actinobacteria*, *Firmicutes*, and *Proteobacteria* (Ackermann, 2007).

1.1.4.1 Novel virus descriptions from hyperthermophiles

Several new phage descriptions have recently been added, however, they still await classification by ICTV (Ackermann, 2007). These are viruses with unusual features not previously observed in nature, which has sanctioned the introduction of novel virus families (see reviews by Zillig *et al.*, 1996, 1998; Prangishvili *et al.*, 2001; Prangishvili and Zillig, 2002). Most of these recently described phages have derived from archaeal species isolated from various extreme- and hyperthermophilic features. The unique and diverse morphologies and genomes of these viruses reinforce that they represent novel virus families (Bettstetter *et al.*, 2001; Prangishvili, 2003). These advances are further discussed under section 1.1.10 (Thermophilic bacteriophages). All the established phage families as well as some of the new ones awaiting ICTV classification are listed in Table 1.1.

Table 1. 1. Overview of phage families (taken from Ackermann, 2007)

Shape	Nucleic acid	Virus group	Particulars	Example
Tailed	DNA, 2, L	<i>Myoviridae</i>	tail contractile	T4
		<i>Siphoviridae</i>	tail long, noncontractile	λ
		<i>Podoviridae</i>	tail short	T7
Polyhedral	DNA, 1, C	<i>Microviridae</i>	conspicuous capsomers	φX174
		<i>Corticoviridae</i>	complex capsid, lipids	PM2
		<i>Tectiviridae</i>	inner lipid vesicle, pseudotail	PRD1
		SHI, group*	inner lipid vesicle	SH1
		STV1 group*	turret-shaped protrusions	STIV
	RNA, 1, L	<i>Leviviridae</i>	poliovirus-like	MS2
		<i>Cystoviridae</i>	envelope, lipids	φ6
Filamentous	DNA, 1, C	<i>Inoviridae</i>	a. long filaments b. short rods	fd MVL1
		<i>Lipothrixviridae</i>	envelope, lipids	TTV1
	2, L	<i>Rudiviridae</i>	TMV-like	SIRV-1
	Pleomorphic	DNA, 2, C, S	<i>Plasmaviridae</i>	envelope, lipids, no capsid
<i>Fuselloviridae</i>			same, lemon-shaped	SSV1
<i>Salterprovirus</i>			same, lemon-shaped	His1
<i>Guttaviridae</i>			droplet-shaped	SNDV
<i>Ampullaviridae</i> *			bottle-shaped	ABV
<i>Bicaudaviridae</i> *			two-tailed, growth cycle	ATV
<i>Globuloviridae</i> *			paramyxovirus-like	PSV

C Circular; L linear; S superhelical; seg segmented; I single-stranded; 2 double-stranded
*Awaiting classification

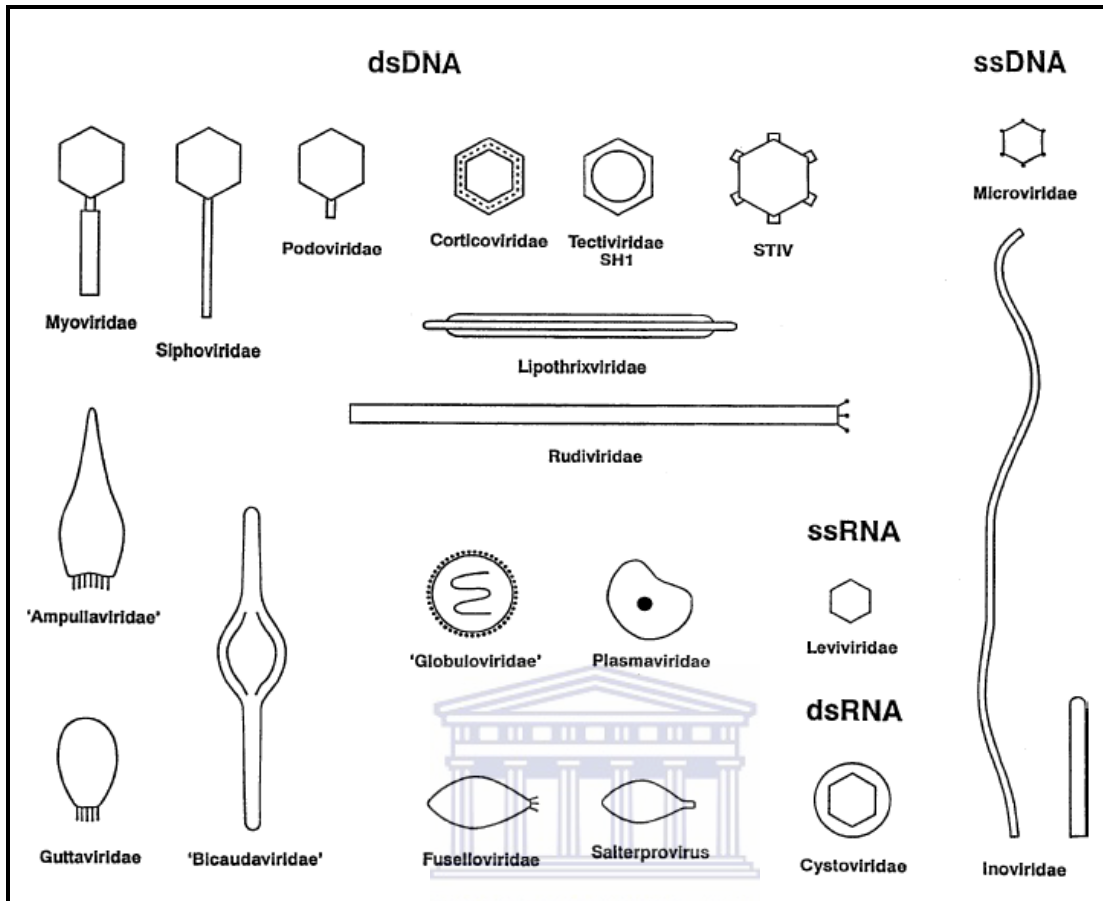


Figure 1. 1 Schematic representation of major phage morphological groups (Ackermann, 2007)

1.1.5 Phage reproduction strategies and life cycles

A series of steps common to viruses have also been identified as part of the bacteriophage life cycle, which include adsorption, penetration of nucleic acids, expression and replication of nucleic acids, virion assembly, release and transmission (Weinbauer, 2004). The bacteriophage life cycle begins when a phage particle enters into contact with its host randomly through passive diffusion and uses the host cell's membrane proteins (often transport proteins) as sites for attachment and entry (Campbell, 2003; Fuhrman, 1999). Following adsorption, the cell wall is rendered

penetrable by specialized phage enzymes expressed in the tail or in the capsid. Thereafter, the phage, or at least its genetic material, enters the cell and initiates a reproduction cycle. The genetic material can either be integrated into the host genome or remain in the cytoplasm (Fuhrman, 1999; Weinbauer, 2004). During the following stage gene expression, genome replication and morphogenesis occur. The final stage of the phage life cycle, release, typically results from the dual action of peptidoglycan hydrolases (endolysins) and holins, but sometimes the release is achieved by budding or extrusion (Weinbauer, 2004).

Four types of life cycles have been shown in viruses: lytic infection, chronic infection, lysogeny and pseudolysogeny (Figure 1.2) (Weinbauer, 2004). Lytic infection may be the sole reproduction strategy for most viruses and thus the most commonly found (Campbell, 2003). This infection cycle initiates when the virus appropriates the reproductive machinery of the host cell upon entry and uses it as a bioreactor or factory for the synthesis of progeny viruses, which are subsequently released as a result of a fatal lysis of the cell. Chronic infection, on the other hand, is a non-lethal process whereby the host cell releases virions by extraction or budding without lysing over several generations. During lysogenic cycle, the virus incorporates its genome into the host chromosome and replicates in the host cell line as a prophage (also known as a provirus) (Fuhrman, 1999; Weinbauer, 2004). During pseudolysogeny, a process not very well known, viral nucleic acid may be retained within a host cell for a few generations before lysis, or cell destruction, can occur. Pseudolysogeny is thought to be related to host starvation, in which the virus adopts an inactive state

and is unable to initiate viral gene expression owing to the low energy state of the cell. Nourishing the cell would result in return of viral activities (Fuhrman, 1999).

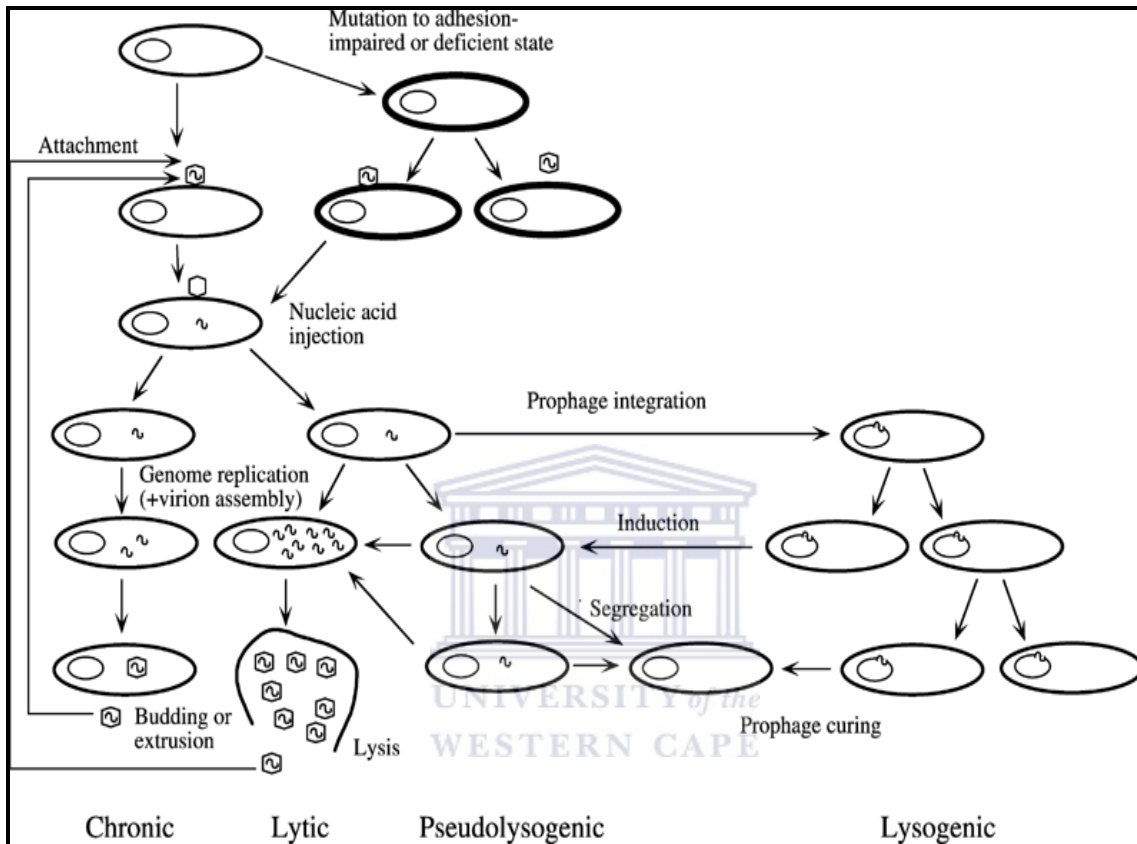
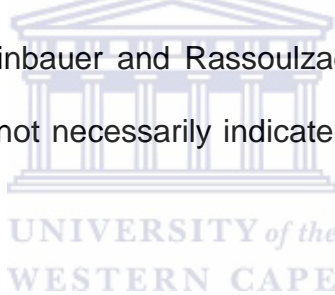


Figure 1. 2 Four types of phage life cycles (taken from Weinbauer (2004))

1.1.6 Phage ecology: abundance, diversity and environmental implications

1.1.6.1 Abundance and diversity

Viruses are among the most dominant and diverse groups of organisms, with a global population on the order of 10^{31} (Hendrix, 2003). Much of the understanding of viral abundance and diversity has emanated from studies on marine ecosystems, where there are, on average, ten million viruses per ml (Bergh *et al.*, 1989; Wommack and Colwell, 2000). The immensity of viral diversity in aquatic environments has been supported by many studies (Wilhelm and Suttle, 1999; Suttle, 1994; Thingstad *et al.*, 1993; Bratbak *et al.*, 1994; Proctor, 1997; Fuhrman, 1999; Wommack and Colwell, 2000; Weinbauer, 2004; Weinbauer and Rassoulzadegan, 2004). A high local viral diversity, nevertheless, may not necessarily indicate a high global diversity (Wilhelm and Suttle, 1999).



1.1.6.2 Global and community diversity

The global diversity of bacteriophages appears to be relatively low, in part because viruses move between environments (Breitbart and Rohwer, 2005; Hambly and Suttle, 2005). There are two possible explanations for low global phage diversity: i) different environments can contain identical microbial hosts and ii) viruses often have a broad host range (although some are known to be strictly host-specific). The second possibility is supported by most studies and is more likely given the high frequency of cross-infecting viruses (also termed polyvalent viruses) (Sano *et al.*, 2004). Most natural environments are therefore not composed of unique endemic

viral populations. Conserved gene studies have helped bring further clarification by showing that identical, or nearly identical, phage-encoded sequences are present in different biomes (e.g. marine versus freshwater versus soil). These sequences must have moved between environments within recent evolutionary time. A good example would be the phage-encoded DNA polymerase sequence HECTOR, which was found in marine water, soil rumen, in association with corals and in solar saltern water (Breitbart and Rohwer, 2005).

The diversity of phage community structures was first demonstrated by electron microscopy even before viruses were first reported to be highly abundant in the sea (Torella and Marita, 1979). Cyanophages that infect *Synechococcus* were the first viruses isolated that infect primary producers in the sea (Suttle *et al.*, 1990; Suttle and Chan, 1993; Waterbury and Valois, 1993). Other commonly isolated viruses include myoviruses, podoviruses and siphoviruses. A gap still persists between the numbers of species isolated and characterized and the estimated viral richness in the marine ecosystem (Hambly and Suttle, 2005).

1.1.6.3 Mechanisms influencing phage diversity

The mechanisms controlling viral diversity have not been well documented and therefore not clearly understood. Certainly, one of the most important factors affecting phage diversity is the availability and quality of host systems (Weinbauer, 2004). The removal of viruses by grazing of heterotrophic nanoflagellates has been well studied (Gonzalez and Suttle, 1993). However, grazing rates are highest at high viral

abundance, and grazing can only effectively influence viral diversity when various viral types are not grazed at the same rate (Weinbauer, 2004). At an evolutionary level, phage diversity is significantly influenced by gene exchange between phages and host during transduction and between co-infecting phages (Moineau *et al.*, 2004). The fact that gene transfer happens more frequently in lysogenic phages than in lytic phages suggests that lysogenic phages should be more diverse than lytic ones (Chen and Lu, 2002). In addition to biological factors described above, a variety of physicochemical factors characteristic of the phage ecological niches may influence viral diversity. These factors include adsorption to particles, UV light, and temperature (Weinbauer, 2004).



1.1.6.3 Methods used to study phage diversity

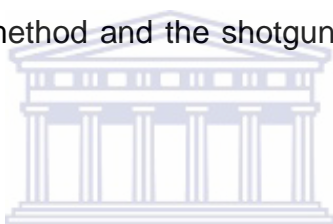
a. Culture-based versus culture-independent studies of viral diversity and abundance

Culture-based methods are the traditional basis for microbial diversity studies (Hambly and Suttle, 2005). Usually performed by “plaquing”, these methods have been used to demonstrate that at least one phage type, and usually multiple types, can infect any microbial host. For example, more than fifty phage types can infect *E. coli* (Breitbart and Rohwer, 2005). Nevertheless, some bacteriophages are specific to certain species or even strains of bacteria.

The current estimates indicate that less than 1% of microorganisms in a given environment are cultivatable (Streit and Schmitz, 2004). Therefore, culture-based

strategies have been ineffective in identifying viral diversity. This problem would be remedied with the implementation of new culturing strategies which would yield an increasing number of novel bacterial species. The rapid development of molecular phylogenetic tools for assessment of microbial diversity will not, however, have an equivalent impact on understandings of viral diversity largely because there is no single genetic element shared by all viruses (Breitbart and Rohwer, 2005).

The solution to these limitations comes with the development of alternative DNA-based strategies (using genome comparison methods), two of which will be reviewed here: the conserved genes method and the shotgun libraries (or viral metagenomic) strategy.



b. Conserved genes method

The discovery and the use of conserved genes (or signature genes) shared by certain viral taxonomic subgroups has helped to study diversity in both cultured isolates and in the environment (Breitbart and Rohwer, 2005; Giovannoni and Rappe, 2000). These conserved genes are mainly sequences encoding for structural proteins such as capsid proteins. This method involves PCR-amplification of a segment of the target gene, separation of fragments by denaturing gel electrophoresis, sequencing of selected fragments and phylogenetic analyses. This approach has been recently used in many studies to evaluate the diversity of cyanophages. All these studies indicated that environmental viral diversity is high and essentially uncharacterized (Short and Suttle, 2005; Dorigo *et al.*, 2004; Zhong *et al.*, 2002). For example, the g20

gene was used to investigate genetic richness in natural cyanophage communities and to determine genetic similarity between viruses using samples obtained from various environments. Four previously uncharacterized groups of g20 clusters were discovered, two of which were entirely found in freshwater. Moreover, sequences with >99% identities were recovered from environments that differed greatly in temperature and salinity. These results have suggested that closely related hosts and the viruses infecting them are distributed widely across environments or that horizontal gene exchange occurs among phage communities from very different environments (Short and Suttle, 2005).

c. The metagenomic or the shotgun libraries approach

Although the conserved genes method is useful in studying the genetic diversity of phages, it is still limited in application, because it only targets genes that are known to be present in cultured phages. Therefore, this method cannot address the question of the overall diversity of phage communities. In contrast, a metagenomic approach allowing quantification of the genetic diversity of the entire viral community has the potential to access a much wider range of viral genomes (Hambly and Suttle, 2005). This approach uses sequence analysis of shotgun libraries of the total viral DNA, and can provide insights into the extent of the genetic diversity in natural viral communities (Breitbart and Rohwer, 2005). Recently, Cann *et al.* (2005) conducted a viral metagenome study in which shotgun libraries from equine feces were sequenced. This study showed that more than 75% of the sequences of these viral

metagenomes did not have any match in the database, supporting the view that most viral diversity remains uncharacterized.

1.1.6.4 Ecological implications of bacteriophages

a. Agents of bacterial mortality

Viruses and phages are significant agents of microbial mortality (Hambly and Suttle, 2005). Aside from protists, which are primarily nanoflagellates, bacteriophages are important pathogens of marine bacteria and are estimated to account for 4-50% of bacterial mortality. In addition, viral abundance often corresponds with microbial abundance with a relatively constant ratio of 5-10 viral particles per bacterium (Breitbart and Rohwer, 2005). Directly or indirectly, bacteriophages also play a role in nutrient and energy cycles, in control of community composition and in lateral gene transfer, which collectively characterize the principal environmental interactions between bacteriophages and their hosts (Hambly and Suttle, 2005).

b. Implications of bacteriophages in food web and biogeochemical cycles

To date, much of the available information on the interaction of viral communities with food web processes and biogeochemical cycles has been gathered from aquatic systems (Weinbauer, 2004). However, the principles identified may also be applicable to soils. Viral lysis converts large portion of bacterial cells into viruses and cell debris (Fuhrman, 1999; Weinbauer, 2004). This debris is composed of dissolved organic molecules (DOM) (monomers, oligomers and polymers) which becomes available for bacterial consumption (Proctor and Fuhrman, 1990; Fuhrman, 1999; Bratbak *et al.*,

1990). Prokaryotes are also grazed upon by small grazers such as flagellates and ciliates which are in turn consumed by larger grazers, allowing the organic matter to be recycled back into the grazing food web. This flow of organic carbon from DOM via prokaryotes and small eukaryotes back to the grazing food chain is called microbial loop (Fuhrman, 1999; Weinbauer, 2004). Figure 1.3 shows a representation of the grazing food chain with emphasis on the semi-closed loop connecting prokaryotes, viruses and DOM (Fuhrman, 1999). The composition, source and degradation of DOM has recently drawn considerable interest in biogeochemistry and microbial ecology, since DOM or DOC (dissolved organic carbon) contribute to regional or global carbon cycling, thus having an impact on atmospheric carbon dioxide (Weinbauer, 2004).



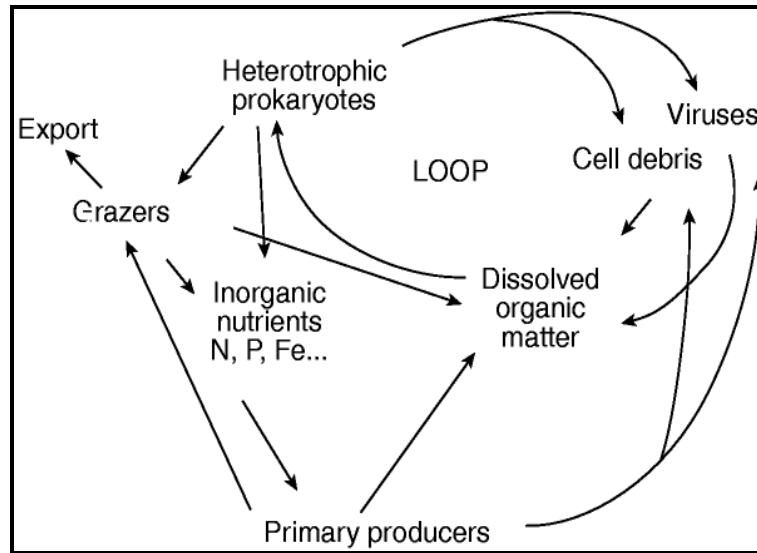
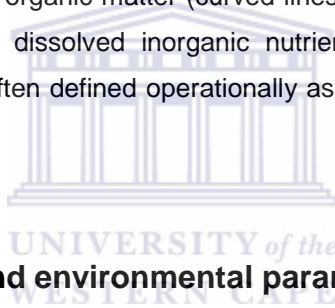


Figure 1. 3 Schematic diagram of an aquatic food web, showing the semi-closed loop connecting prokaryotes, viruses and dissolved organic matter (curved lines). Note that the loop has the net effect of converting organic matter into dissolved inorganic nutrients. The viruses and cell debris are separated for illustration, but are often defined operationally as dissolved organic matter. (Taken from Fuhrmann (1999)).



1.1.6.5 Ecological niches and environmental parameters

The concept of a viral niche is characterized by more than the habitat of a population -- it includes all the important ecological factors, dimensions, conditions, and resources that determine the survival of a population (Hutchington, 1957). Environmental viral niches have not been studied extensively, hence the lack of comprehensive data. However, incomplete information on niche dimensions has been obtained from the study of virus-host isolated systems and from *in situ* analyses (Weinbauer, 2004, Wommack and Cowell, 2000). Environmental conditions and resources that influence the survival of bacteriophages include water, temperature, radiation, the ionic environment, oxygen, pH, organic matter and host availability (Weinbauer, 2004).

a. Water

Water is essential for viral transmission in aqueous environments. Water availability is a particular problem for soil bacteriophages. Host populations, which depend on water availability, indirectly affect the abundance and distribution of phages (Williams *et al.*, 1987; Farrah, 1987; Moebus, 1987).

b. Temperature

Although tests indicate that phages are capable of tolerating broad temperature ranges, in natural systems phages appear only to occur within constrained temperature ranges (Weinbauer, 2004; Spencer, 1955). In soil, temperature varies strongly with season, depth and probably with micro-habitats. The varying temperature tolerance of phages indicates that temperature is an important niche dimension in both soil and marine systems (Borsheim, 1993; Williams *et al.*, 1987 and Farrah, 1987). Temperature sensitivity can therefore be a characteristic of a phage population found in a particular environment. For example, marine bacteriophages have been shown to be more sensitive to heat than phages from other environments (Spencer, 1955).

c. pH and ionic environments

Chemical parameters, such as pH and ionic environments, also constitute important niche parameters. Marine pH is typically stable. These phages are generally only affected by pH values deviating from that of the surrounding sea water (Farrah, 1987). Lymnetic systems cover a broad range of pH values from alkaline (e.g. in salt

lakes) to acidic (e.g. lakes and fens). The ability of these phages to absorb host cells is tied to pH (Farrah, 1987). Drastic pH variations within a single system have been observed in soil pH tests. Marine pH is typically stable, although it can vary considerably in lymnetic systems from alkaline (e.g. in salt lakes) to acidic (e.g. in particular lakes or fens). Nevertheless, some isolates proved to remain stable for months over a wide range of pH values (Borsheim, 1993). Moreover, tolerance of many cyanophages against a broad range of pH values was a good indication that changes in pH do not affect their distribution in fresh water (Suttle, 2000).

Phages from fresh water, marine and soil are generally affected by ionic environment at all stages of the life cycle, such as adsorption, replication, lytic activity and survival (Williams *et al.*, 1987; Farrah, 1987; Moebus, 1987). The effect of divalent cations such as Mg^{+2} or $Mg^{+2} + Ca^{+2}$ is usually positive on phages, whereas the effect of Na^{+} is quite variable. Such observations seem to emphasize the importance of ionic environment in the definition of the ecological niche of phages (Farrah, 1987; Weinbauer, 2004).

d. Light, wind, wave and currents

Physical forces such as light, wind, wave and currents are known to affect bacteriophages. Light can stimulate adsorption to hosts, a phenomenon that is thought to be mediated by charge neutralization at the cell surface or by changes in ionic environment near the cell (Suttle, 2000). Wind, wave and currents affect phages in their environmental distribution, for example, by transferring them from liquid to

gaseous phases. Phage distribution in marine environments can also be mediated by their attachment to mobile organisms or to sinking particles (phages are too small to sink in water) (Ruiz *et al.*, 2000). In the atmosphere, phage's transportation or dispersion can be facilitated by air current and by attachment to flying organisms such as insects or birds (Weinbauer, 2004).

1.1.7 Bacteriophage genomics

1.1.7.1 The role of comparative genomics in understanding phage genomics

Comparative genomic studies, supported by environmental studies, have started to address questions about the genetic structure and the genomic evolution of the global phage population (Juhala *et al.*, 2000; Ravin *et al.*, 2000; Clark *et al.*, 2001; Hendrix, 2003). Work in this area is presently restrained by the small number of phage genomic sequences available. Nevertheless, sequence comparisons, together with environmental studies, have provided a new picture of the size, genetic structure and dynamics of phage populations, as well as the mechanisms through which their genomes have evolved (Hendrix, 2003; Casjens, 2005).

1.1.7.2 Phage genomic structure

The phage genome is composed of modules (the basic units of organization in phage genomes) that include extra genes termed MORONs (for more DNAs) arising from other phages or from hosts (Campbell and Bostein, 1983). Comparative genomic studies have revealed that the trademark of phage genomic structure is genetic

mosaicism originating from ancestral sequences (Juhala, *et al.*, 2000). Modules of mosaicism, first discovered in 'lambdoid' phages infecting enteric bacteria, are technically different units (or gene clusters), with the ability to move around while retaining their functionality, that have been assembled by mixing and matching through both homologous and nonhomologous recombinations (Hendrix, 2003). Figure 1.4 shows a schematic comparison of the genomes of five enteric phages, showing various mosaic modules. It is currently believed that wide circulation of genetic elements among phages and between phages and distantly related hosts take place via lateral gene transfer. This is shown by the presence of identical sequences in phage groups from extremely different environments (Hambly and Suttle, 2005; Breitbart and Rohwer, 2005).



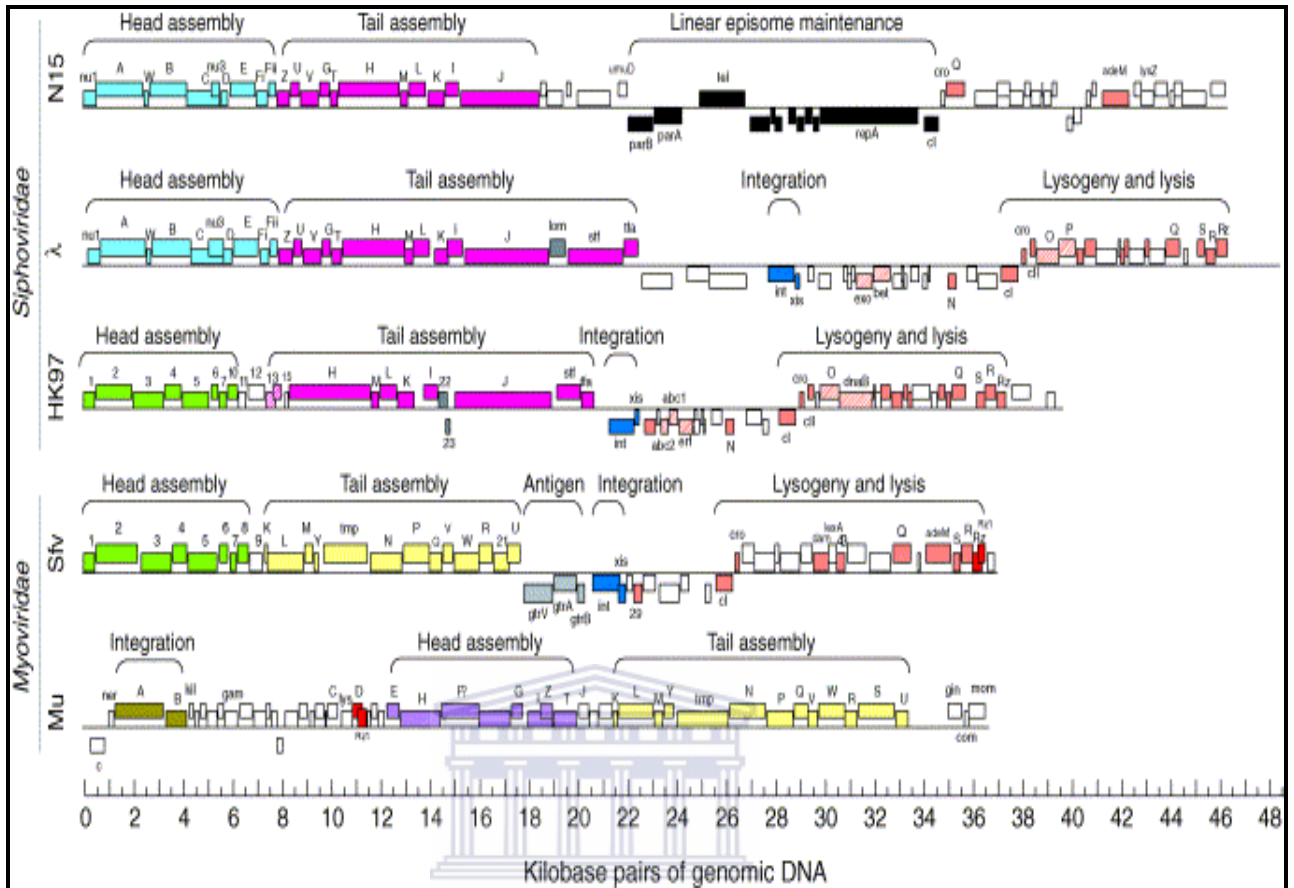


Figure 1. 4 Schematic comparison of five genomes of phages infecting enteric bacterial hosts. Head genes are clustered together as a single mosaic module. Tail genes also form clusters. The color coding indicates which genes belong to the same sequence families. Sequences that are shared between two or more of the phages: shaded genes. Genes that are analogous but not homologous: striped fill.

1.1.7.3 Marine phage genome: the largest untapped reservoir of genomic information

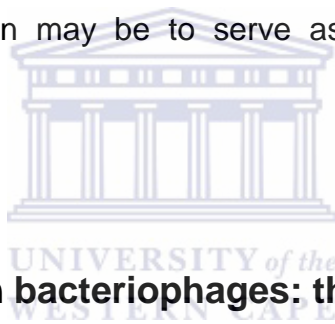
Marine bacteriophages form the largest uncharacterized reservoir of genomic information (Hambly and Suttle, 2005). To date, only 17 marine phage genomic sequences have been published in GenBank. Nevertheless, these sequences have revealed interesting features such as the presence of photosynthetic genes in

cyanophage and common patterns of genomic organization (Mann *et al.*, 2003; Lindell *et al.*, 2004). Furthermore, the study of uncultivated phage community genomes (phage metagenomics) through the shotgun cloning approach has led to novel observations. For example, 60-80% of marine phage ORFs showed no similarity to ORFs already present in GenBank. By contrast, well studied phages such as coliphages and streptococcal phages, showed over 80% homology to previously cultivated phages and a highly conserved gene order (Chibani-Chennoufi *et al.*, 2004).

1.1.7.4 The role phage genome in lateral gene transfer

Phages increase their genome size by acquisition of extra genes termed MORONs (for more DNAs) which are phage- or host-originated (Campbell and Bostein, 1983). MORONs are inserted via various recombination mechanisms that do not involve the functional disruption of the modules (Fischer and Eisenberg, 1999). Their deletion or maintenance by natural selection occurs over evolutionary time. Their maintenance may drastically affect the behavior of both the phage and the host. For example, the acquisition of phage-derived exotoxin genes by certain phage-infected bacteria has been shown to convert the host bacteria into pathogens (Davis and Waldor, 2002). Moreover, reviews indicate that bacteriophages are important vectors for transmission of virulence genes within bacterial populations through lateral gene transfer (Boyd *et al.*, 2001, Lawrence, 1999; Dilmann and Brussow, 2003).

Phages adapt to new environments by acquiring ecologically important genes as MORONs. For example, it has been shown that cyanophages infecting *Synechococcus* and *Prochlorococcus* have acquired genes involved in photosynthesis (e.g. the *psbA* gene that encodes the D1 protein, a rate limiting photosynthesis protein) (Mann *et al.*, 2003; Lindell *et al.*, 2004). Expression of these phage-encoded proteins enables the phage to maintain photosynthesis throughout the infection cycle, presumably providing the phage with energy (Bailey *et al.*, 2004). The presence of photosynthesis genes in phages is a good example of their contribution to lateral gene transfer. These observations support the assumption that a primary role of host populations in phage ecology and evolution may be to serve as vectors for genetic exchange (Hambly and Suttle, 2005).



1.1.8 Gene regulation in bacteriophages: the genetic switch model in bacteriophage lambda

Much of the early understanding of gene regulation in bacteriophages emanated from work on bacteriophage lambda (Dodd *et al.*, 2005). Lambda has particularly helped elucidate the mechanism of molecular switches (a model of gene regulation very common in prokaryotes). Some of the advances in understanding gene control, particularly in λ , have been revisited in recent reviews (Friedman and Court, 2001; Hochschild, 2002; Patshne, 2004; Kobilier *et al.*, 2004). Lambda is considered as the prototype of bacteriophages with the ability to select between alternative, stable modes of development. For example, bacteriophage λ may choose to adopt the lytic

growth cycle when *Escherichia coli* host cells are healthy and numerous, or the lysogenic pathway (a better strategy used when growth conditions are not favorable). Phages can switch from lysogeny to lytic growth in response to external stimuli (for review, see Johnson *et al.*, 1981). Through a process known as prophage induction, triggered by exposure to UV light and other DNA damaging agents, lysogenic phages can be induced to switch to lytic growth (Roberts and Devoret, 1983).

The molecule central to gene regulation in bacteriophage lambda is the cI protein (λcI), which acts as both the repressor and the activator of transcription. The cI protein is required for the maintenance of lysogeny (Ptashne, 1992). The mechanism of lysis/lysogeny decision was initially described by Ptashne and coworkers as depending essentially on the repressor's interaction with the right operator sites ($\text{O}_{\text{R}1}$ through $\text{O}_{\text{R}3}$). However, recent studies have discovered that λcI also interacts with sites on the leftward operator O_{L} ($\text{O}_{\text{L}1}$ through $\text{O}_{\text{L}3}$), which is located approximately 3-kb away from O_{R} (Revet *et al.*, 1999; Dodd *et al.*, 2001; Ptashne and Gann, 2002) (Figure 1.5(A)). The three O_{R} sites are flanked by two promoters, P_{R} and P_{RM} , which direct transcription of early genes and cI gene, respectively. O_{L} , on the other hand, contains a single promoter, P_{L} , which, like P_{R} , directs the direct transcription of early genes. The repressor, therefore, binds to both sets of operator sites, forming a tetramer as required for cooperative binding (Senear *et al.*, 1993). It has also been shown that the cooperatively bound pair of cI dimers at $\text{O}_{\text{R}1}$ and $\text{O}_{\text{R}2}$ interacts with the cooperatively bound pair at OL1 and OL2 . This leads to the formation of a 2.4 kb DNA loop held together by a cI octamer that facilitates cI dimers binding cooperatively

to O_{R3} and O_{L3} (Figure 1.5(B)) (Dodd *et al.*, 2001). Therefore, O_R and O_L adopt conformations that facilitate the repression of the *Cro* gene and other lytic genes, while simultaneously activating transcription of the *cl* gene. In this way λ cl acts as a positive autoregulator of its own gene (Ptashne, 1992). Through this mechanism, the lysogenic *cl* level is kept approximately 10-fold higher than the lytic transition threshold (Aurell, 2002). However, it is thought that λ cl may repress its own production (negative autoregulation) through interaction with a low affinity binding site (O_{R3}) (Maurer *et al.*, 1980).

On the other hand, the switching of the phage into 'lytic state' is facilitated by the autoproteolysis of λ cl, which is initiated by the host DNA-repair protein *recA*. This disruption of the repressor protein results in derepression of the *Cro* gene and the subsequent production of the Cro protein (a secondary repressor). The Cro protein then occupies the operator site O_{R3} , thereby putting off the transcription of *cl* gene, while simultaneously inducing its own synthesis (Campbell, 2003; Dodd *et al.*, 2005). This view may seem to suggest that the race between Cro and λ cl for the O_{R3} site is the only factor influencing the establishment of repression. Nevertheless, a much earlier study revealed that another protein *cII*, plays an equally important role in repression establishment. The production of *cII* is thought to be decreased by the binding of Cro to O_R sites (O_{R1} and O_{R2}) (Reichardt and Kaiser, 1971).

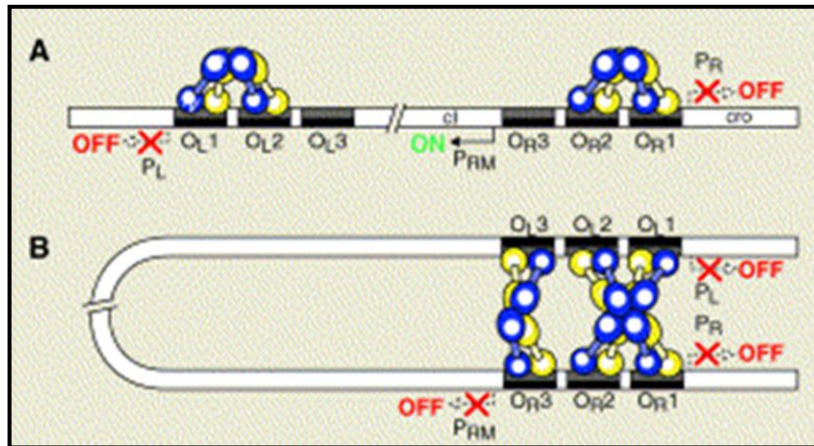


Figure 1. 5 Interactions of λ cl at the right and left operator regions. (A) Pairs of cl dimers bind cooperatively at OR and OL. Cooperative binding of dimers to OR1 and OR2 results in repression of transcription from PR, while the dimer at OR2 also activates transcription from PRM. Cooperative binding of dimers to OL1 and OL2 represses transcription from PL. (B) Higher-order looped complex. Interaction between the cooperatively bound pair of cl dimers at OR interacts and the cooperatively bound pair at OL, forming an octameric complex and a 2.4 kb DNA loop. (Taken from Dodd et al., 2001.)

1.1.9 Biotechnological applications of bacteriophages

Since their discovery, bacteriophages have frequently been used as research tools. However, recent years have witnessed renewed interests and advances in the applied use of bacteriophages (Clark and March, 2006). For example, bacteriophages have been used in phage therapy (as antibacterial agents), in phage display (where proteins, peptides and antibodies are exposed on the phage surface), in the development of phage-delivered vaccines, as vectors in gene therapy and in bacterial typing (Clark and March, 2006; Petty *et al.*, 2006).

1.1.9.1 Phage therapy

Phage therapy is defined as the treatment of bacterial infections with intact phages (as substitutes to antibiotics) (Petty *et al.*, 2006). Early attempts of phage therapy yielded inconsistent and unsuccessful results mainly owing to lack of understanding of phage biology, poor quality control and the absence of properly controlled studies. This, together with the discovery of antibiotics, led to a general reluctance to develop phage therapy in many western countries. However, this trend is shifting (Summers, 1999; Kutter and Sulakvelidze, 2005; Sulakvelidze *et al.*, 2001). Although the goal of totally replacing antibiotics with bacteriophages is far from being achieved, there is hope that, with the removal of problems encountered initially, phage therapy will one day become at least an effective alternative or addition to antibiotics (Clark and March, 2006). Moreover, a renewal of interest in phage therapy has been observed in recent years as a result of the emergence of antibiotic-resistant bacteria and the occurrence of highly difficult pathogens that are resistant to intensive treatments (Petty *et al.*, 2006).

1.1.9.2 Phage display

First described more than twenty years ago, phage display has been one of the major tools in the study of protein-ligand interactions (Mullen *et al.*, 2006). Phage display uses transcriptional fusion with a coat-protein encoding gene to display a heterologous protein or peptide on the surface of a phage. This results in novel phage particles with a wide range of potential uses (Smith, 1985). Phage display has proven a robust and successful technique for generating libraries containing millions, perhaps

billions of different peptides or proteins. Phage display has applications in antibody engineering and epitope determination, in screening for receptor antagonists, in enzyme substrates selection and in engineering of nano-wires (in nanotechnology) (Petty *et al.*, 2006; Kay *et al.*, 1996; Griffiths and Duncan, 1998). Most reported phage display methods are based on the use of M13 and related filamentous phages of *Escherichia coli*. However, other *E. coli* phages such as lambda and T7 have also been used (Benhar, 2001; Willats, 2002).

1.1.9.3 Phages as vectors for vaccine delivery

Bacteriophages have been used as potential vectors for vaccine delivery. Two distinct strategies have been established to achieve this. The first is by directly vaccinating with whole phage particles to deliver vaccines in the form of immunogenic peptides attached to modified phage coat proteins (a variant of phage display). The second is by using a phage vector containing a eukaryotic promoter-driven gene as a vehicle for the delivery of DNA vaccines (Clark and March, 2004a).

The use of intact phages to deliver DNA vaccines is among the most recent developments in immunization technology (Clark and March, 2004b; March *et al.*, 2004; Jepson and March, 2004). For example bacteriophage genetic immunization uses expression cassettes containing a vaccine gene under the control of an appropriate eukaryotic promoter (e.g. cytomegalovirus [CMV] promoter), and cloned into a suitable bacteriophage (e.g. lambda phage) (March, *et al.*, 2006; Clark and March, 2006). The undamaged bacteriophage is then purified and injected into an *E.*

coli host for propagation. Encapsulated in the phage capsid, the vaccine DNA is protected against possible deterioration when the recombinant phage is injected into a host (phage vaccination). This method has been shown to be more efficient than the standard 'naked DNA' vaccination and it has resulted in superior antibody response in mice and rabbits (Clark and March, 2004b; March *et al.*, 2004). Similar to phage display libraries, genome libraries in phages can be screened with convalescent serum resulting in identification of potential vaccines (Jepson and March, 2004). These can be used for direct vaccination.

1.1.9.4 Phage-based bacterial detection

Bacteriophages have been used for many decades for the typing of bacterial strains and for the detection of low numbers of pathogenic bacteria (Clark and March, 2006). Although other established bacterial detection methods exist, phage-based strategies present several advantages over other methods, which include low-cost, ready production in large scale and specificity for a target bacterial species. Other biomolecules may confer specificity for molecular recognition but are associated with a number of disadvantages, such as cost, and antibody sensitivity to harsh environmental conditions. Perhaps the greatest advantage that phage systems have over other methods is their ability to exclusively target living bacteria, therefore minimizing the number of false positives associated with other systems such as PCR (Petty *et al.*, 2006).

Applications of phage-based bacterial detection methods are found in food and water industries, in bioterrorism disinfection, in hospitals and in agriculture. Classical identification methods include phage typing, enrichment on selective growth media and a series of biochemical tests. Such procedures can be lengthy and associated with costly, or even deadly, implications in hospitals and in food industries. The current knowledge of bacterial and phage biology has enabled the development of, quicker, cheaper and more sensitive assays than before (Petty *et al.*, 2006).

1.1.9.5 Phage as transformation vector systems

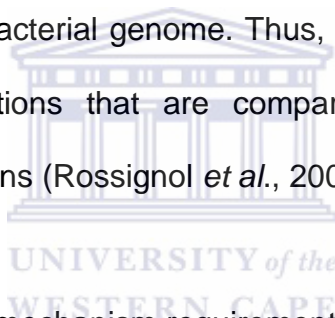
Cloning vectors are generally classified as plasmid and phage vectors. The engineering of viral DNA for use as cloning vectors goes as far back as 1974, with the first viral vector having originated from a lambda bacteriophage (Barnum, 1998). Endowed with amazing integration mechanisms, lysogenic bacteriophages such as lambda have attracted much interest and have been used as vehicles for introducing novel genes into bacteria (Lee *et al.*, 1991a).

The choice of a vector is primarily based on i) the size of the fragment to be inserted, ii) the restriction enzymes to be used, iii) the necessity for expression of the cloned fragment, and iv) the screening method to be used to select the desired clones (Chauthaiwale, 1992). Bacteriophage vectors present many advantages over plasmid-based vectors, and some of these advantages are discussed in following sections. Nevertheless, no single phage vector contains all the desired features.

1.1.9.5.1 Advantages of phage vectors over plasmid-based vectors

1. Integration of a single copy of DNA

Plasmid transformation systems result in high plasmid copy numbers, a trait that may be useful for identification of weak promoters and for studying gene structure and function. However, multi-copy plasmids lead to high-copy-number artifacts, especially during physiological studies (Hoang *et al.*, 2000). Moreover, gene expression studies on high-copy-number plasmids are difficult to control, particularly when there is a single copy regulator gene that's chromosomally encoded (Rossignol *et al.*, 2002). On the other hand, integration vectors mediate the introduction of a single copy of heterologous DNA into the bacterial genome. Thus, they allow expression studies to be conducted under conditions that are comparable to those for single-copy chromosomal genes or operons (Rossignol *et al.*, 2002; Yang *et al.*, 2002).



2. Exclusion of selective mechanism requirement for transformed organisms

Selective markers are a requirement in plasmid systems for both the identification of transformed bacteria and the maintenance of plasmid within the host. Although useful in laboratory context, such markers may have negative effects on the environment or on host cells. For example, they are associated with the propagation of antibiotic resistance. Moreover, maintaining several plasmids using multiple antibiotics may be even more damaging. The need for selective pressure can be overcome through chromosomal insertion of a stable integrative vector (Shimizu-Kadota, 2001).

3. Cloning of greater insert size

The cloning of large insert fragments is one of the major hurdles encountered through the use of plasmids (Lee *et al.*, 1991a; Shimizu-Kadota, 2001; Lauer *et al.*, 2002). Larger inserts are more likely to contribute towards destabilizing the plasmid in the host cell. In contrast, with the use integrative vectors, it is plausible to insert fragments of DNA at least as large as a phage genome. For example, the SP β c2 bacteriophage genome, despite being 134 kb in size, can be stably incorporated in its host genome (Lazaveric *et al.*, 1999; Yang *et al.*, 2002; Shimizu-Kadota, 2001). However, it has been proposed that, in some integrative vectors that do not necessitate the packaging of DNA into the phage head, there is no upper size limit for the introduced DNA (Yang *et al.*, 2002).

1.1.9.5.2 The principle behind bacteriophage integration vectors

The development of phage vectors for targeted insertion of DNA has been facilitated by the availability of genome sequences. Such vector systems, known as site-specific insertion, integration, or recombination vectors, take advantage of the integrase protein and the *attP* site of temperate bacteriophages or prophages identified by genomic studies (Groth and Calos, 2004). The recombination (or integration) reaction is established through the interaction of a recombinase (which may belong either to the λ integrase-like or to the resolvase/invertase family depending on amino acid sequence similarity and mechanisms of catalysis) with a specific site in the DNA (Sadowski, 1986). The enzyme mediates the meeting between the phage-borne *AttP* site and the chromosomal *attB* locus in a synapse and then catalyses the strand

exchange, resulting in DNA cleavage and religation to partner sequences (Sadowski, 1986; Stark *et al.*, 1992; Argos *et al.*, 1986). This reaction results in the formation of the recombinant *attL* and *attR* sites flanking the inserted DNA sequence. Much of the understanding of the mechanism of site-specific recombination has emanated from various studies on the well-documented lambdoid phage family (for example Campbell, 1962; Weisberg and Landy, 1983). These studies have led to the knowledge and the technology necessary for the engineering of site-specific insertion vectors.

1.1.9.5.3 Recent advances in integrative vectors

The engineering of phage-based vectors is primarily dependent on cloning the *attP* and *Int* genes and the removal of origins of replication (Hoang *et al.*, 2000). This has allowed the insertion of a single copy of exogenous DNA into the targeted host bacterium. Nonetheless, much of the structure of these vectors is composed of plasmid sequences. The recent focus of integrative vector research has been on evolving a single copy, stable insertion system that's free of plasmid backbone sequences (Hoang *et al.*, 2000; Shimizu-Kadota, 2001; Rossignol *et al.*, 2002). Three methods have been recently developed for this purpose in *P. aeruginosa*, *Lactobacillus casei* and *E. coli* (Hoang *et al.*, 2000; Shimizu-kadota, 2001; Rossignol *et al.*, 2002).

In *P. aeruginosa* integrative vectors mini-CTX1 and mini-CTX2, which carry yeast FRT sites, the removal of unwanted plasmid sequences, including the antibiotic

resistance gene and the *Int* gene, is catalyzed by the yeast FLP recombinase provided by an additional helper plasmid, pFLP2 (Hoang *et al.*, 1998). Following integration of mini-CTX-based vector, the helper plasmid, which also contains a negative selective marker, the sucrose (*sacB*) gene, is removed by culturing the transformants on sucrose based medium (Hoang *et al.*, 1998, 2000).

In the second method, the plasmid pMSK761 has been used in a mechanism involving *Lactobacillus casei* phage Φ FSW site specific recombination in conjunction with homologous recombination in order to achieve chromosomal integration and removal of unwanted vector sequences (Shimizu-Kadota, 2001). In this mechanism, pMSK761, which contains an ori designed to allow replication in *E.coli* but not in *L. casei*, is inserted into the *L. casei* genome by means of the Φ FSW *Int* gene and *attP* site. Then, the stability of the integration is maintained through endogenous recombination with two copies of the gene being integrated and the resultant elimination of vector sequences not required in the final integrant (Shimizu-Kadota, 2001).

The third integration system is specific for *E. coli* transformation and requires first a helper plasmid, pHK-Int, as a source for the *Int* gene, and second a non-replicating integrative plasmid (pHK11, containing an *attP* and antibiotic resistance gene) for delivery of genetic material into the host genome (Rossignol *et al.*, 2002). In this strategy, the *Int* gene has a thermo-sensitive replicon that is lost due to long term exposure to 42°C and whose expression is inhibited at low temperatures (about

32°C). However, the basal level for *Int* expression is at 37°C (Rossignol *et al.*, 2002). *E. coli* cells are first transformed with helper plasmid, pHK-Int, and high pHK-Int copy numbers are increased by growing transformants at 37°C. Then the pHK11 plasmid is introduced and suppression of the *Int* gene is achieved by growing cells at 30°C. The level of *Int* produced before suppression, the basal level, is enough to facilitate pHK11 integration into the *E. coli* chromosome. The removal of the unwanted helper plasmid is then achieved by incubating transformants at 42°C overnight (Rossignol *et al.*, 2002).

1.1.9.5.4 Summary of transformation systems that are based on phage integrative recombination process

Some examples of the successful use of bacteriophage integration mechanisms in bacterial cells include the stable transformation of various strains of *Corynebacterium glutamicum* using a vector derived from corynephage Φ16 (Moreau *et al.*, 1999; Oram *et al.*, 2006) and the engineering of *Mycobacterium tuberculosis* and *M. smegmatis* using the tyrosine integrase proteins of Φ Rv1 and L5 (Bibb and Hatfull, 2002; Lee *et al.*, 1991a). This system has also been used in other bacterial genera which are summarized in Table 1.2.

Table 1. 2. A list of integrative vectors developed for bacteria.

Bacteria	Species/strain	Phage	Plasmid
<i>Staphylococcus</i>	<i>S. aureus</i> RN4220	L54a	pCL55 (Lee <i>et al.</i> , 1991a)*
	<i>S. aureus</i> CYL316	L54a	pY1112Δ19/pCL83 or pCL84 (Lee <i>et al.</i> , 1991a)1)*
<i>Mycobacterium</i>	<i>M. smegmatis</i>	L5	pMH94 (Lee <i>et al.</i> , 1991b)1) pbluescriptint/pBS37 and pBSS33 (Saviola and Bishai, 2004)2)*
	<i>M. smegmatis</i> , <i>M. vaccae</i> , <i>M. bovis</i> BCG, <i>M. tuberculosis</i> H37Ra	Ms6	pAV1 (Freitas-Vieira <i>et al.</i> , 1998)
<i>Streptomyces</i>	Various <i>Streptomyces</i> species	ΦC31	pKC796 (Kuhstoss <i>et al.</i> , 1991) pSET 152 (Bierman <i>et al.</i> , 1992)
	<i>S. rimosus</i> R6-554	RP3	pKG2 (Gabriel <i>et al.</i> , 1995)
	<i>S. venezuelae</i> ETH14603	VWB	pKT02 (Van Mellaert <i>et al.</i> , 1998)
<i>Pseudomonas</i>	Various <i>Streptomyces</i> species	ΦBT1	pRT801 (Gregory <i>et al.</i> , 2003)
	<i>P. aeruginosa</i>	ΦCTX	pIBH/pTABF, p1000, p400 (Wang <i>et al.</i> , 1995) mini-CTX1, mini-CTX2 with pFLP2 (Hoang <i>et al.</i> , 2000)
<i>Streptococcus</i>	<i>S. pyogenes</i>	T12	pWM139, pWM245, p7INT (McShan <i>et al.</i> , 1998) pIAPU1 (Gindreau <i>et al.</i> , 2000)
<i>Listeria</i>	<i>L. monocytogenes</i>	U153	pPL1 (Lauer <i>et al.</i> , 2002)
<i>Enterococcus</i>	<i>E. faecalis</i> KBL707	PSA	pPL2 (Lauer <i>et al.</i> , 2002)
		ΦFC1	pEMJ1-1 (Yang <i>et al.</i> , 2002)
<i>Rhizobium</i>	<i>R. meliloti</i> 41	16-3	Patt164, patt202 (Hermesz <i>et al.</i> , 1992) pSEM102/pEP226, pEP228, pEP181, pEP184 (Elo <i>et al.</i> , 1998) pSEM167 (Semsey <i>et al.</i> , 1999; 2002) pGSG1 (Ferenczi <i>et al.</i> , 2004)
<i>Lactobacillus</i>	<i>Lactobacillus casei</i> YIT9029	ΦFSW	pMSK761 (Shimizu-Kadota, 2001)1)
<i>Escherichia</i>	<i>E. coli</i>	HK022	pHK-Int/pHK11 (Rossignol <i>et al.</i> , 2002)
<i>Bacteroides</i>	<i>B. thetaiotaomicron</i> AR29	ΦAR29	pBA (Wong <i>et al.</i> , 2003)

Note *: require two plasmids to insert plasmid, 1) plasmid carrying *Int* and 2) plasmid carrying *attP* site (cloned integrase plasmid/plasmid with *attP*).

1.1.10 Thermophilic bacteriophages

The body of research describing viruses that infect thermophilic bacterial strains (i.e. moderate thermophilic, extreme-thermophilic, hyperthermophilic) is limited. (Bettstetter *et al.*, 2003; Rice *et al.*, 2004). However, there is increasing interests in these viruses because they are potential model systems for understanding

biochemical and biological adaptations at elevated temperatures. Moreover, they have an impact on biogeochemical and ecological processes (Fuhrman, 1999; Suttle, 2005). Among the few viruses to be isolated from thermophilic bacteria and archaea are *Geobacillus stearothermophilus*, *Thermus scotoductus*, *Thermus thermophilus* and some genera of archaea such as *Sulfolobus* and *Thermoproteus* (Prangishvili *et al.*, 2001; Rice *et al.*, 2004; Epstein and Campbell, 1974; Sakaki and Oshima, 1974; Geslin *et al.*, 2003; Blondal *et al.*, 2005). Sampling sites for thermophilic viruses include hot springs, mud pools, and solfataric fields (Rice *et al.*, 2004; Rachel *et al.*, 2002; Prangishvili, 2003). Bacteriophage species have been recently isolated from a deep-sea thermophilic bacterium (Liu *et al.*, 2006).



1.1.10.1 Phages of extreme- and hyperthermophilic habitats: a remarkable morphological and genomic diversity

Archaeobacteria, once the dominant form of life on Earth, are now relegated, but still well represented in extreme- and hyperthermal environments. Of the three fundamental domains of life, Archaea are least understood (Stetter, 1999). Until recently, viruses associated with Archaea have not been well characterized (Rice *et al.*, 2004). Of a total of approximately 5,500 viruses identified to date, only 36 have been isolated from Archaea [International Committee on Taxonomy of viruses, www.ncbi.nlm.nih.gov/ICTV] (Ackermann, 2007). Archaeal species are capable of growth at some of the highest possible temperatures and most acidic pH found in natural environments (Rachel *et al.*, 2002; Zillig *et al.*, 1998; Rice *et al.*, 2001). These organisms are commonly referred to as extreme thermophiles (optimum growth in the

range of 55°C and can tolerate up to 75-80°C) and hyperthermophiles (grow optimally above 80°C). Recently, novel viruses were isolated from hyperthermo-acidophilic archaea obtained from Yellowstone National Park (YNP) and from other thermal features from Japan, Iceland and Russia, giving a more comprehensive picture of viral diversity in hot habitats (Bettstetter *et al.*, 2003; Rice *et al.*, 2004).

Studies on extreme-thermophilic and hyperthermophilic bacteriophages were pioneered by Wolfram Zillig (Bettstetter *et al.*, 2003). The work in his laboratory has been very influential in the establishment of novel viral families, most of which having been isolated from extreme- and hyperthermophilic Archaea of the orders Sulfolobales and Thermoproteales (Rice *et al.*, 2003). These studies have resulted in a more remarkable picture of viral diversity in various hot environments and their discoveries have been excellently reported, and include: *Fuselloviridae* comprising spindle-shaped enveloped particles with circular double-stranded DNA genomes (SSV1, SSV2, and SSV3); *Lipothrixviridae*, enveloped lipid-containing filamentous viruses with linear double-stranded genomes (TTV1, TTV2, TTV3, DAFV, and SIFV), and unenveloped, stiff-rod-shaped *Rudiviridae* with linear double-stranded DNA genomes (SIRV1 and SIRV2) (see Zillig *et al.*, 1996, 1998; Prangishvili *et al.*, Prangishvili and Zillig, 2002). A fourth family was proposed albeit still in the process of being approved by the ICTV, the *Guttaviridae*, bearded-droplet-shaped particles with double-stranded DNA genome (SNDV) (Arnold *et al.*, 2000). More recently, the first virus to infect the hyperthermophilic archaeal genus *Acidianus* was reported. The

virus was named AFV1, meaning *Acidianus* filamentous virus 1 (Bettstetter *et al.*, 2003)

1.1.10.2 Phages of *Geobacillus* species

Bacteriophages infecting *Geobacillus* species have not been widely characterized. Since the introduction of electron microscopy in 1959, roughly 5,500 phages have been examined morphologically (Ackermann, 2003, 2007). Of these, only 13 are phages of *Geobacillus* species, and all of them are tailed phages belonging to the family of *Siphoviridae* (Ackermann, 2007). On 7 March 2007, only 403 phage genomes were available on the NCBI website, which is a total of a little over 17.5 Mb, that is, the equivalent of only 3.5 *Escherichia coli* K-12 chromosomes (Lima-Mendez, 2007). No genomic sequence of a bacteriophage of a *Geobacillus* species was then found in public databases. Nonetheless, the EMBL-EBI phage genome page, a repertoire of phage genomes from 429 organisms, contains to date two genomes from phages of *Geobacillus* species - *Geobacillus* phage GBSV1 and *Geobacillus* virus E2 (EMBL-EBI phage genomes, <http://www.ebi.ac.uk/genomes/phage.html>). These observations evoke not only that phage genomics is still in its infancy but also that there is great need to sequence and analyze more genomes from phages of *Geobacillus* species using modern genome analysis technologies. Such investigations would yield insights into viral phylogenetic and evolutionary relationships and have the potential to increase industrial, medical or biotechnological applications of these phages as has been the case for the well-studied Mycobacteriophages (Ford *et al.*, 2002).

Earlier studies of bacteriophages of thermophilic *Bacillus* strains reported their preliminary characterization, which generally included host specificity, stability and morphology studies (Sharp *et al.*, 1986). Some of these phages were probably phages of *Geobacillus* species because the genus *Bacillus* underwent an extensive reclassification in 2001, and the thermophiles belonging to *Bacillus* genetic group 5 were reclassified as members of *Geobacillus* gen. nov., with the well-studied *Geobacillus (Bacillus) stearothermophilus* as the type strain (Ash *et al.*, 2001; Nazina *et al.*, 2001). In 1982, an important study extended the research on these bacteriophages by characterizing 24 bacteriophages of thermophilic *Bacillus* (*Geobacillus*-like) species (Sharp *et al.*, 1986). In that study, bacteriophages were isolated from diverse sources such as compost, soil, silage and rotting straw. The phages varied considerably in morphology and size and were most stable at 50°C for 4-5 hours, but at 70°C the plaque-forming units decreased by between 10²- and 10⁷-fold in 2 hours. Their morphological description fitted best that of *Siphoviridae* (icosahedral heads and long non-contractile tails) (Sharp *et al.*, 1986).

Despite the growing awareness of viral abundance in marine systems and the roles that viruses may play in various biogeochemical and ecological processes, there was no published report of a virion obtained from a deep-sea hydrothermal microorganism before 2006. However, this fact changed when, for the first time, a thermophilic bacteriophage, *Geobacillus* virus E1 (GVE1) from *Geobacillus* sp. E26323, was purified from deep sea hydrothermal fields. It was a typical *Syphoviridae* with double stranded DNA (Liu *et al.*, 2006). A more recent study of a deep-sea thermophilic

bacteriophage *Geobacillus* virus E2 (GVE2) led to the identification of single stranded binding (SSB) protein genes. SSB proteins, which were then discovered for the first from a deep-sea hydrothermal bacteriophage, are known to be important for the survival of most microorganisms and have numerous potential applications in diverse molecular biology and analytical methods (Wei and Zhang, 2008).



1.2 Thermophilic *Geobacillus*

1.2.1 Reclassification of thermophilic *Geobacillus* strains

Bacteria belonging to the genus *Bacillus* form a large grouping of aerobic and facultatively anaerobic, rod-shaped Gram-positive bacteria. They include psychrophiles, thermophiles, acidophiles, alkalophiles and halophilic bacteria maintaining heterotrophic or autotrophic growth strategies. The thermophilic subset of this genus has undergone considerable reclassification as a direct consequence of phylogenetic analysis (Ash *et al.*, 2001). Most thermophilic aerobic spore-forming bacteria (45 to 70°C optima) were previously classified into genera such as *Alicyclobacillus*, *Brevibacillus*, *Aneuribacillus*, *Sulfobacillus*, *Thermoactinomyces* and *Thermobacillus* (Wisotzkey *et al.*, 1992; Dufresne *et al.*, 1996; Hendrix *et al.*, 1997; Touzel *et al.*, 2000). These genera were divided into *Bacillus* genetic groups 1 and 5 based on molecular analyses (Ash *et al.*, 2001; Rainey *et al.*, 1994). Group 5 contained phenotypically and phylogenetically coherent bacilli (98.5-99.2% sequence similarity). Subsequently, in 2001, *Bacillus* genetic group 5 was separated from the genus *Bacillus* and reclassified as members of *Geobacillus* gen. nov., and the well-studied *Geobacillus (Bacillus) stearotherophilus* was assigned as the type strain. At least 12 well-described thermophilic *Geobacillus* species have been reclassified from the genus *Bacillus* (Nazina *et al.*, 2001).

1.2.2 Habitat

The global distribution of thermophilic bacilli such as *Geobacillus* in terrestrial habitats extends across all continents (Sharp *et al.*, 1992). The successful isolation of *Geobacilli* from various geothermal areas as well as from shallow marine hot spring and from deep-sea hydrothermal vents has been reported (Maugeri *et al.*, 2002). Members of *Geobacillus* have been retrieved from different artificial high-temperature biotopes such as hot water pipelines, heat exchangers, waste treatment plants, burning coal refuse piles and bioremediation biopiles (Maugeri *et al.*, 2001; Obojska *et al.*, 2002). Their isolation from temperate and permanently cold environments suggests their ability to adapt to disparate environments (McMullan *et al.*, 2004).



1.2.3 Genomics

To date, the Comprehensive Microbial Resource (CMR) database at The Institute for Genomic Research (www.tigr.org) contains whole genome sequences for 23 members of the *Bacillaceae* family, including: *Bacillus anthracis* (9 strains), *Bacillus cereus* (3 strains), *Bacillus licheniformis* (2 strains), *Bacillus subtilis*, and *Bacillus thuringiensis* subsp. *konkukian* (Graham *et al.*, 2006). This genomic information has created an excellent platform for comparative genomic studies between phylogenetically similar bacterial species. Comparative genomics has been used to extract candidate genes responsible for adaptation to extreme environments (Takami *et al.*, 2004). For example, reverse gyrase, the enzyme which is thought to help the DNA function optimally at high temperatures by increasing topological links between

the two DNA strands, has been identified by comparison of a variety of hyperthermophilic genome sequences (Forterre, 2002). Current genome sequencing projects are contributing towards increasing the amount of genomic information available from the newly described genus *Geobacillus*. For example, genome sequencing projects of *G. stearothermophilus* and *G. kaustophilus* are being carried out at Oklahoma State University and the Japan Marine Science and Technology Centre, respectively (McMullan *et al.*, 2004).

1.2.4 Applications

The potential application of *Geobacillus* species has generated significant scientific attention in the area of biotechnological processes as source of various thermostable enzymes, such as proteases, amylases, lipases and pullanases (Sookheo *et al.*, 2000; Uma Maheswar Rao and Satyanarayana, 2003; Lee *et al.*, 2001). *Geobacillus* species have potential industrial application in the production of exopolysaccharides (Schiano Moreillo *et al.*, 2003). The production of bacteriocins from two strains of *G. thermoleovorans* has been described. These bacteriocins exhibit lytic activity on various bacteria including medically important ones such as *Salmonella typhimurium* (Novoty and Perry, 1992). The ability of *Geobacillus* species to hydrolyse various hydrocarbons has afforded them various potential industrial biotechnology applications (Bustard *et al.*, 2002).

1.3 Aims and objectives of this study

The main aim of this project was to sequence and map the entire genome of a *Geobacillus*-specific bacteriophage isolated from South African terrestrial sites – Tankwa Karoo and Klein Karoo. The approach is to use established methods (or modifications thereof) to isolate and propagate bacteriophages of *Geobacillus* species and to sequence the genome of at least one phage isolate using a shotgun strategy. The project also seeks to ascribe functions to bacteriophage genomic modules using a set of bioinformatic tools. Investigating the genome organization of phages of *Geobacillus* species will elucidate the predicted mosaic nature of bacteriophage genomes and provide insights into their phylogenetic relationships. Furthermore, this project has the potential to enlighten future research towards integrative vector engineering for stable transformation of *Geobacillus* species. To realize these goals the following objectives have been set:

- To isolate *Geobacillus* species-specific bacteriophages using plaque assay methods;
- To investigate their morphology using TEM technology and, subsequently, assign them to viral families;
- To isolate bacteriophage DNA using standard methods;
- To sequence a bacteriophage genome using a shotgun cloning approach;
- To assemble and edit sequencing data using the Phred-Phrap or similar software packages;
- To identify open reading frames (ORFs) using GenMark and FramePlot programs;

- To predict gene functions through homology studies using BLASTP and Pfam databases.



Chapter II: Materials and methods

2.1. General and specialized chemicals

Unless specified, all chemicals used in this study were analytical reagent grade and did not require further purification. Both general and specialized chemicals were purchased from suppliers such as Sigma Chemical Co or Merck Laboratory Supplies. Other materials including enzymes and antibiotics were obtained from Kimix, Oxoid, Fluka/Sigma, Bio Basic Inc./Sigma, Roch/Sigma, Kimix Laboratory Supplies and BDH.

2.2. Culture media and buffers

2.2.1. General growth medium

TSB (Tryptone Soya Broth)

Constituent	g L⁻¹
Pancreatic digest of casein	17.0
Papaic digest of soybean meal	3.0
Sodium chloride	5.0
Potassium dihydrogen phosphate	2.5
Glucose	2.5
ddH ₂ O up to	1000 ml

Geobacillus and other thermophilic strains used in this study were routinely cultured in TSB (Tryptone Soya Broth) or TSBA (Tryptone Soya Broth Agar). The broths were prepared by mixing all constituents, adding, and adjusting the pH to 7.4 and autoclaving 121°C for 30 min. TSBA media were prepared in a similar manner but contained 1.5 % agar. The agar overlay used in plaque assays contained 0.6% agar and 1 mM CaCl₂.

2.2.2. Other media

SOB (Super Optimal Broth)

Constituent

Tryptone

L⁻¹

20 g

Yeast extract

5 g

NaCl

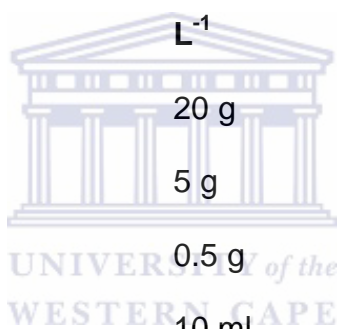
0.5 g

KCl (250 mM)

10 ml

ddH₂O up to

1000ml



The pH was adjusted to 7.0 with 10M NaOH (100µl) before autoclaving. After autoclaving, the broth was cooled to ~50°C and the following filter sterilized solutions added aseptically: 5 ml of MgCl₂ (2M) and 20ml Glucose (1M). This medium was used to prepare the starter culture for the preparation of electro-competent *E. coli* cells.

2YT (2X tryptone and yeast extract)

Constituent	gL⁻¹
Tryptone	16
Yeast extract	10
NaCl	5
ddH ₂ O up to	1000ml

The pH was adjusted to 7.0 with 10M NaOH and the volume to 1L with H₂O. The medium was sterilized by autoclaving and stored at room temperature. 2YT medium was used to culture *E. coli* cells for the preparation of electro-competent cells.

LB (*Luria-Bertani*)

Constituent	L⁻¹
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
ddH ₂ O up to	1000ml

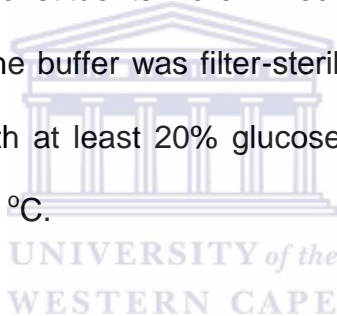
The pH was adjusted to pH 7.0 with 10 M NaOH and the medium was sterilized by autoclaving. LBA medium was prepared in a similar manner but contained 1.5 % (w/v) agar used to grow *E. coli* cells in polystyrene petri dishes. When necessary, the appropriate antibiotic was added after cooling the medium.

2.2.3. Buffers

PMN buffer

Constituent	Molarity
KH ₂ PO ₄	0.01
NaCl	0.05
MgCl ₂	0.001

This buffer was used for eluting bacteriophages from agar and as a phage preservation medium. The constituents were mixed in sterile water and the pH was adjusted to 7.4. Thereafter the buffer was filter-sterilized using a 0.22 µm filter. The buffer was supplemented with at least 20% glucose when used to store phages at temperatures of -20 °C or -70 °C.



Phage disruption buffer

Constituent	Concentration
Proteinase K	5 g.ml ⁻¹
SDS	2%
EDTA	0.01M
Tris pH 8.0	0.1M

The phage disruption buffer was used in isolation of virus DNA and contained Proteinase K and SDS for digesting virus capsid proteins, which was required prior to extraction or purification with phenol and chloroform.

Ammonium acetate buffer

The ammonium acetate buffer was a solution containing 0.1 M ammonium acetate (pH 7.0) served as a washing buffer in the phage purification method (Fortier and Moineau, 2007).

1x Tris-EDTA (TE)

The 1x Tris-EDTA buffer was made by adding 10ml of 1M stock of Tris-Cl (pH 7.5) to 2ml 0.5M stock of EDTA (pH 8.0), mixing and adjusting the volume to 1L with H₂O. The solution was sterilized by autoclaving and was stored at room temperature. TE buffer was used for storage of DNA.



TENS buffer

TENS buffer was used to disrupt *E. coli* cells during plasmid mini-prep.

Constituent	Volume (µl)
20 %SDS	250
10M NaOH	100
1M Tris (pH 7.5)	100
0.5M EDTA (pH 8.0)	20
ddH ₂ O	9530

2.3. Bacteriophage isolation, maintenance and propagations

methods

2.3.1. Soil samples and bacterial strains

Soil samples were collected into 50 ml polypropylene tubes from desert areas of Tanqua Karoo (TK) and Klein Karoo (KK) and stored at -80°C. Samples were used as source material for screening for *Geobacillus*-specific bacteriophages by standard plaque assays using 8 *Bacillus*-like strains. For host range studies, a total of 14 thermophilic *Bacillus* strains were used, including *Geobacillus stearothermophilus* strains (DSM 406, RS 93, RS 239, RS 240, RS 241, NCA 05329, NCA 05330 and TAU3A1), *Geobacillus kaustophilus* strain G18A6, *Anoxybacillus hidilerensis* strains Fur6A4 and Fur6A2 and unidentified isolates Fur6A5 and G18A9. For comparative reasons a mesophilic isolate identified as belonging to *Bacillus megaterium* species was included in host-range studies. All strains were routinely grown aerobically as small scale cultures in TSB (tryptone soya broth) and incubated at 55°C in an orbital shaking incubator or on TSA (tryptone soya agar). For long term storage all bacterial isolates were stored in TSB containing 40% glycerol at -80°C.

2.3.2. Primary isolation of bacteriophage and maintenance

Infective bacteriophage particles were isolated from soil samples by the agar overlay technique (Adams, 1959). Soil samples (0.5 g) were mixed with 5 ml TSB media in glass test tubes with loose metal caps and incubated overnight (about 18 hours) at 55°C and 150 rpm. Cultures were then transferred into 15 ml polypropylene tubes and

cells and debris were removed by centrifugation at 5000g for 20 minutes at 4°C (in an Eppendorf Centrifuge 5810R). The supernatant was filtered through a 0.45 µm disposable filter to remove cells and debris. Ten-fold serial dilutions of supernatants were prepared from undiluted stocks and 300µl of dilutions were mixed with 0.7 ml of *Geobacillus* strains grown to mid-exponential growth phase. Mixtures were incubated at 55°C with intermittent shaking and then added to 3ml soft TSB agar (0.6% w/v agar) at 55°C. After mixing, the soft agar and *Geobacillus* mixtures were poured over the surface of previously cast TSB agar plates (1.5% w/v agar). Following solidification and drying, plates corresponding to each dilution, including a plate containing uninfected cells, were incubated at 55°C up to 24 hours. Plates were subsequently surveyed for the presence of lysis areas (plaques) in the bacterial lawn, which are indicative of viral infection. The concentration of infective viral particles in soil suspensions (and later in phage suspensions) was expressed as plaque-forming units (PFU) per milliliter. Assay reactions included uninfected plates that allowed checking for bacteriocin effect. Bacteriophages were stored for short periods as suspensions in PMN buffer or on plates at 4° C. For long term storage, bacteriophages were maintained as 20% glycerol stocks at -80° C.

2.3.3. Single plaque purification and titration

Plaque purification was conducted by successive rounds of reinfection from single plaques until homogeneity was observed. This was carried out by carefully gauging out a distinct plaque from the overlay agar using a p1000 pipette with a widened tip and transferring the plaque into 100 µl of PMN buffer. The plaque and buffer mixture

was incubated overnight at 4°C on a rocking platform to allow bacteriophages to diffuse out of the agar and into the buffer. The agar was then sedimented by centrifugation at 13,000 rpm on a bench top centrifuge (Eppendorf Centrifuge 5810R) and the supernatant gently transferred into a sterile 1ml tube. This constituted a primary phage stock and was used in subsequent plaque assays and for the production of high titer lysates. Subsequently, bacteriophage titres were determined by making serial dilutions of phage suspensions in PMN buffer (10^0 to 10^{10} dilutions) and plaque assays using the agar overlay method as described above.

2.4. Phage-host interaction studies

2.4.1. Host specificity determination

Host range or specificity study was carried out using both the quick spot and the agar overlay techniques. The experiments were restricted to 15 *Bacillus* strains, of which 14 were thermophilic, and one mesophilic, a *Bacillus megaterium* strain. The quick spot test was performed by spotting 10 µl of GV1 suspension onto the surface of the agar overlay of respective indicator plates. Following overnight incubation at 55°C, the plates were screen by eye for lysis regions. The standard overlay technique allowed determining phage titers obtained with different host and was used as an indication of phage-competency or sensitivity of host bacteria. The phage was considered to possess lytic activity against the test strains if a well-defined margin on the circumference and clear or turbid center (due to resistant) was observed on the spotted area or plaque.

2.4.2. Study of phage of interactions by spectrophotometry

Two sets *G. stearothermophilus* RS 241 cultures were grown at 55°C and each set contained triplicate cultures (25 ml volume in 100 ml volume flasks). When cells reached an optical density (OD) of 0.35 nm one set was infected with about 0.1 volume of a phage solution with a titer of about 10^7 pfu per ml. Changes in the OD at 600 nm in both sets of cultures were monitored intermittently over a period of 21 hours. Data corresponding to the mean and the standard deviation were plotted as OD at 600 nm versus time (hours) and allowed observing deviations in the growth cycle of infected cultures in respect to uninfected ones.

2.5. Bacteriophage stability studies

2.5.1. Thermostability of GV1

Single freshly picked plaques were resuspended each in 0.1 ml PMN buffer, and from these stocks' serial dilutions were made up to 10^{-8} dilutions. Different phage dilution sets were incubated at 50, 55, 60, 65, 70 and 75°C for 1 h in water baths. Following heat treatment, the titers of phages were determined as described previously. Stability was calculated as the ratio of viable phage titer before and after heat treatment.

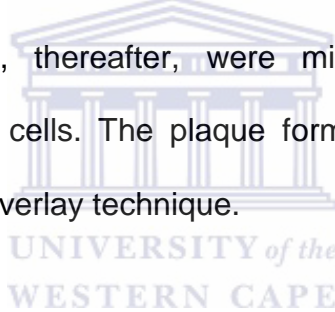
2.5.2. Effect MgCl₂ and CaCl₂ on phage stability and production

RS 241 cells were grown in TSB (pH 7.0) and 900 µl aliquots of actively growing cultures were mixed with 100 µl of bacteriophage dilutions and incubated at

55°C without shaking. The number of PFU produced per milliliter was assayed by the agar overlay technique as previously described. The overlaid agar contained concentrations of MgCl₂ between 0.001 M and 0.1 M.

2.5.3. Effect of pH of the medium on phage stability and production

The TSB medium was adjusted to different pH values (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5) in separate conical flasks (25 ml of medium per 100 ml flask). One-hundred µl aliquot of phage suspension was mixed with 900 µl of each of the seven pH adjusted TSB broths in Eppendorf tubes. The solutions were left at room temperature for up to four hours and, thereafter, were mixed with actively growing *G. stearothermophilus* TAU3A1 cells. The plaque forming unit (pfu) per milliliter was determined by the soft agar overlay technique.



2.6. Methods for mass production of bacteriophages

2.6.1. Cell-free lysate production in liquid cultures and on plates

Larger volumes of cell-free lysates containing bacteriophages were obtained on plates and in liquid cultures. Plate lysis was achieved by the agar overlay method as described above. Bacteriophage stocks were diluted so as to produce confluent lysis, and plates showing complete lysis, following overnight incubation at 55°C, were flooded with PMN buffer. Next, plates were incubated overnight at 4°C on a gently shaking platform, and the liquid was collected together with the top agar. In the case of liquid culture lysis, thermophilic cultures were grown to early exponential phase,

infected with 10% volume of bacteriophage inoculums and grown overnight. Cell lysates obtained in liquid cultures or on plates were freed of cellular debris by centrifuging for 10 minutes at 5000 g and 4°C in the refrigerated Eppendorf Centrifuge 5810R. Remaining cellular debris was removed from resultant supernatants by filter-sterilizing using 0.22 µm syringe mounted disposable filters. The filtrate was stored at 4°C for subsequent work.

2.7. Phage purification and visualization methods

2.7.1. Lysate purification by ammonium acetate centrifugation

Lysates were purified as previously described (Fortier and Moineau, 2007). Cell lysates obtained from liquid cultures or plates (usually 5 ml volume lysate) were centrifuged for 10 minutes at 5000 g and 4°C in the refrigerated Eppendorf Centrifuge 5810R centrifuge. The resultant supernatants were filter-sterilized using 0.4 µm syringe mounted disposable filters and 2% chloroform, which served as a preservative. A 1.5 ml aliquot of the filtrate was transferred into a 1.5 ml high speed micro-centrifuge tube (Eppendorf Centrifuge 5417R) and this was centrifuged for 1 h, at 25000 x g, at 4°C. Approximately 1.4 ml of the supernatant was discarded from the tube by gently pouring out and replaced with ammonium acetate (0.1 M, pH 7.5). This was incubated at 4°C on a shaking platform (120 rpm) for up to 16 h to allow resuspension of the bacteriophage pellet. Centrifugation and resuspension steps were repeated at least twice as described above. After the final washing step,

bacteriophages were resuspended in 20-40 μ l ammonium acetate buffers and were prepared for subsequent transmission electron microscopy.

2.7.2. Transmission electron microscopy (TEM)

Bacteriophage stocks purified as described above were placed onto carbon-coated copper grids and negatively stained with 1% (wt/vol) uranyl acetate. Particles were visualized by using Cleo1 transmission electron microscope at University of Cape Town's electron microscopy unit.

2.7.3. Phage purification by PEG centrifugation

DNase 1 and RNase were added to a filtered lysate each to a final concentration of 1 μ g/ml. The reaction mixture was incubated at room temperature before addition of sodium chloride to a final concentration of 1 M. After incubation on ice for approximately an hour, cell debris was removed by centrifugation (11,000g, 10 min and 4°C). This was followed by the addition of PEG 8000 to a final concentration of 10% w/v, with gently shaking the bottle (100-120 rpm, room temperature). Bacteriophages were precipitated by incubation at 4°C overnight. A PEG pellet, containing phage particles, was obtained by centrifugation at 11,000g for 10 minutes and at 4°C. The pellet was resuspended by adding 5 ml PMN buffer and gently rotating the tube at about 100 rpm, 4°C, overnight. The PEG suspension was stored at 4°C.

2.8. DNA isolation, quantification and visualization methods

2.8.1. Extraction of viral DNA

DNA was extracted from a PEG pellet using a version of the phenol/chloroform method described by Sambrook *et al.* (1989). A 200 µl volume of resuspended virus preparation in TE buffer was added to 200 µl of phage disruption buffer (5 mg/Proteinase K, 2% SDS, 1mM EDTA, 0.1M Tris, pH 8.0). The reaction tube was incubated at 56°C for at least an hour allowing Proteinase K to disrupt the virus capsid. Thereafter, an equal volume of phenol/chloroform was added. The reaction was vigorously mixed by vortexing for 10 seconds and centrifuged for 2 min at 13,000 rpm in a bench top centrifuge (Eppendorf Centrifuge 5810R). The upper, aqueous layer was carefully removed and transferred to a sterile tube. Two times volumes of ethanol plus a tenth volume of 3M sodium acetate were added, and the solution was incubated overnight at 20°C to precipitate the DNA. The DNA was pelleted by centrifugation at 13,000 rpm, at 4°C and for 30 min. DNA pellet was washed by adding 500 µl of chilled 70% ethanol and centrifuging (13,000 rpm, 30 sec, 4°C), then air-dried at 37°C for up to 10 min. The DNA pellet was resuspended in 50 µl TE buffer and stored at -20°C.

2.8.2. Plasmid DNA extraction using TENS buffer

Plasmid DNA was extracted from *E. coli* cells using an alkaline lysis method. A 300 µl volume of TENS buffer (TENS buffer preparation is described in section 2.2.3) was added to a cell pellet obtained from a 4 ml overnight culture. After re-suspending the cells by vortexing, 150 µl of sodium acetate (3M) was added and the mixture was

vortexed and then centrifuged at 13000 rpm for 5 min (Eppendorf 5415 D). The supernatant was transferred into new tubes and re-centrifuged at the same speed and duration. The supernatant was transferred into new tubes, and plasmid DNA was precipitated by adding ice-cold 0.9 ml of 100% absolute ethanol and incubating at -20°C for 20 min. Plasmid DNA was pelleted by centrifugation at 15000 rpm and 4°C (Eppendorf 5417 R). The pellet was air-dried and re-suspended in TE buffer supplemented with 200 µg.ml⁻¹ RNAase A. Isolated plasmids were kept at -20°C for subsequent analyses.

2.8.3. Invisorb® Spin Plasmid Mini Two system

An overnight *E. coli* culture (4 ml) was harvested by centrifugation into a 2 ml microcentrifuge tube. The cells were re-suspended in 250 µl of “Solution A” and lysed with 250 µl volumes of “Solution B” (lysis solution) for 5 minutes at room temperature. Then “Solution C” was added, mixed by inversion 4 times and centrifuged for 5 minutes at 15000 x g. The supernatant was collected and transferred to a mini-column filter, incubated for 1 minute and spun for 1 minute at 10000 x g. The filtrate was discarded and the column containing the sample was washed with 750 µl of “Wash Solution”. Residual ethanol was removed by centrifugation at 16000 x g for 2 minutes. The mini-column was placed into new tubes, and 50 µl of “Elution Solution” was added at the center of the filter. Subsequently, the mini-column was incubated for up to 5 minutes at room temperature and centrifuged at 10000 x g for 1 minute to collect the purified plasmid. Isolated plasmids were stored at -20°C.

2.8.4. Nucleic acid quantification

The Nanodrop ND-1000 system

Extracted and air-dried DNA was resuspended in double distilled water and allowed to stand overnight. DNA quantification was performed using the Nanodrop ND-1000. The instrument was blanked using 1 µl of the same double distilled water used for DNA resuspension. An aliquot of 1 µl of resuspended DNA was then loaded onto the scanning platform and the DNA concentration recorded.

Qubit™ DNA assay

A more accurate quantification of DNA was achieved using the Qubit™ DNA assay kit (Invitrogen), a technology that uses a fluorometric dye specific to the DNA molecule. The Quant-iT™ working solution was prepared by diluting 200 folds the Quant-iT™ reagent with the Quant-iT™ buffer. The working solution was then mixed separately with two provided standard solutions and with the DNA samples (usually 1-20 µl DNA in 200 µl total volume per reaction) by vortexing shortly. Following two minutes incubation at room temperature, absorbance readings were recorded in the Qubit™ fluorometer. The concentration of DNA in the original sample was determined by multiplying the reading by the dilution factor.

2.8.5 Agarose gel electrophoresis

DNA preparations were analyzed in 0.8-1% (w/v) gels prepared in 0.5 x TAE buffer containing 0.5 µg/ml ethidium bromide solution (Sambrook and Russel, 2001). DNA samples were loaded together with a 6 x loading dye, and electrophoresis was

performed in tanks flooded with 0.5 x TAE at voltage range of 20-100 V using GNA 100 power pack (Amersham Bioscience, Sweden). The current was stopped when the dye had migrated two third the length of the gel. The sizes of nucleic acid fragments were estimated by comparison with DNA markers of precisely known fragment sizes (λ DNA digested with *HindIII*, *PstI* or *BamHI*). Visualization of gels was achieved using Alphamager 2000 (USA) digital imaging system, with UV illumination at a peak wavelength of 302 nm.

2.9. Construction of genomic library

2.9.1. Restriction endonuclease digestion

DNA was digested with restriction endonucleases in sterile Eppendorf tubes according to the manufacturer's instructions. Reactions were set to final volumes of 10-20 μ l and adjusted proportionally according to requirements. The reactions contained appropriate 1 x reaction buffer, 5-10 U of enzyme per μ g of plasmid or genomic DNA and ddH₂O. They were incubated in a water bath at 37°C for at least 2 hours. The digestion products were analyzed by gel electrophoresis in 0.6-1% agarose gels as described earlier.

2.9.2. Partial digestion of genomic DNA

Partial endonuclease digestion of genomic DNA was carried out using the four base cutter *Mbol*. Genomic DNA (1 μ g in 20 μ l volume) was cut with *Mbol* diluted 100 folds from a stock concentration of 10 u/ μ l. The reaction was incubated at 37°C for 30

minutes. At 1, 5, 10, 20, and 30 min time points, a 2 µl aliquot was removed and placed on ice to deactivate the enzyme. Thereafter, digestion fragments were separated on 0.8% gels for 10 hours at 30V. The appropriate time period for recovery of 5-10kb fragments was selected for large scale digestion and fragments were recovered for gel band purification.

2.9.3. DNA end-repair reaction

Sheared and damaged DNA was end-repaired using end-repair reagents from the CopyControl™ Fosmid Library Production Kit (Epicenter) according to the instruction manual. Reaction components were mixed in an Eppendorf tube to achieve concentrations of 5 µg DNA, 1 x End-Repair buffer, 0.25 mM dNTP mix, 0.1 mM ATP and 8 µl of End-Repair enzyme mix (T4 DNA polymerase and T4 polynucleotide kinase). The reactions were incubated at room temperature for 2 hours and then run on a 0.8% agarose overnight at 30V. DNA smear found within 4-12 kb range was cut from gel and purified using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare).

2.9.4. DNA blunt cloning

Blunt cloning of gel-purified DNA was achieved using the CloneJet™ PCR Cloning Kit (Fermentas). Figure 2.1 shows the map and features of pJET1.2/blunt cloning vector, the positive selection system used, containing a lethal gene (*eco47IR*) and a gene conferring antibiotic resistance (*bla* (Ap^R)), allowing positive selection and

maintenance of recombinant *E. coli* cells. Prior to cloning, the DNA was blunted using a DNA blunting enzyme that removes 3'-overhangs and fills in 5'-overhangs. Nucleotides for the reaction were supplied in the reaction buffer. The DNA blunting reaction components (DNA, 2x reaction buffer, ddH₂O, DNA blunting enzyme) were mixed as prescribed by the manual. The reaction set included a positive control containing a provided DNA fragment and a negative control without DNA. The mixtures were incubated at 70°C for 5 min, and then chilled on ice. This was followed by the ligation reaction, which was set by adding to the blunting reaction mixtures 1ul of pJET1.2/blunt cloning vector (50ng/μl) and T4 DNA ligase (5u/μl). Ligation reactions were mixed by vortexing shortly and were incubated overnight at 16°C.

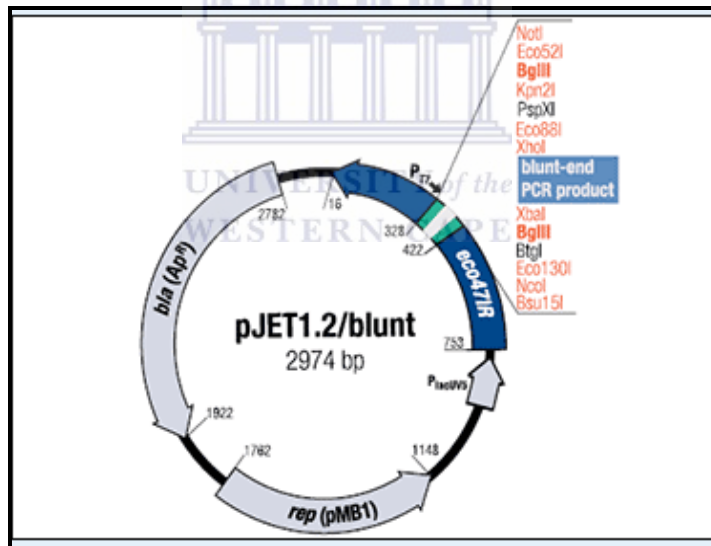


Figure 2. 1 Map and features of pJET1.2/blunt cloning vector

2.9.5. Preparation of *Escherichia coli* electro-competent cells

Electro-competent *E. coli* cells were prepared as follows. A single fresh colony from an LB agar plate was used to inoculate a starter culture of 10 ml SOB medium and

grown overnight at 37°C with shaking at 150 rpm. The overnight starter culture was used to inoculate 500 ml of 2YT medium in a 2 L flask, followed by growth at 37°C with shaking at 150 rpm until the culture OD₆₀₀ reached 0.3-0.6. The cells were chilled on ice for 10 min and then harvested by centrifugation at 4000 rpm for 25 min at 4°C in a JA14 rotor (Beckman, USA). The cells were immediately placed on ice, supernatant removed and pellet re-suspended in 200 ml of ice-cold sterile water and re-centrifuged. A second wash with ice-cold sterile water was applied and the cells were re-centrifuged using the same speed and the duration. The pelleted cells were resuspended in 20 ml of sterile ice-chilled glycerol (15% v/v) and sorbitol (2% w/v) solution and were re-centrifuged in a JA20 rotor at 4000 rpm for 10 min at 4°C (Beckman, USA). Following the removal of the supernatant, the pellet was re-suspended in 1 ml of ice-cold glycerol (15% v/v)-sorbitol (2% w/v) solution. Aliquots of 50 µl were transferred into pre-chilled microcentrifuge tubes and snap-frozen using an ethanol bath freshly removed from -80°C freezer. The electro-competent cells were stored at -80°C.

2.9.6. Transformation of electro-competent cells

Electro-competent *E. coli* strains were transformed as follows. Cells, contained in 50µl in microcentrifuge tubes, were incubated for 5 min on ice prior to electro-poration. They were then mixed with 1 µl (1-10 ng) of the ligation reaction and the mixtures were transferred into 2 mm pathlength electro-poration cuvettes. Cells were pulsed at 25 µF capacitance, 1.8 kV voltages, 200 Ω by-pass resistance using a Gene pulse™ (Bio-Rad Laboratories, Hercules, CA). Subsequently, the 950 µl of

room temperature SOC medium was added, and the cells were transferred into 1.5 ml sterile Eppendorf tubes. The transformed cells were recovered by incubation with shaking (150-200 rpm) at 37°C for 1 hour. Thereafter, 100 µl of the transformed cultures were plated out onto LBA plates supplemented with appropriate antibiotics and incubated at 37°C overnight.

2.10. Sequencing and sequence analysis

Sequencing of cloned insert DNA was performed at Stellenbosch University (South Africa) with an automated sequencer model 373A and a dideoxy chain termination procedure with fluorescein-labeled primers (Perkin Elmer Applied Biosystems). Sequences were edited with BioEdit and DNAMAN and subjected to standard BLAST analyses using the National Center for Bioinformatics Information (NCBI) server (<http://www.ncbi.nlm.nih.gov/blast/>). BLAST results were supported with in-silico gene predictions using GeneMark of Mark Brodovosky (http://exon.biology.gatech.edu/gmhmm2_prok.cgi). GeneMark and GeneMark.hmm are favorable bioinformatics tools for whole genome analysis and for the local analysis of a particular gene and its surrounding regions. GeneMark.hmm makes use of Hidden Markov models for a higher sensitivity of gene detection.

Chapter III: Results

3.1. Introduction

The isolation and characterization of viruses could provide significant insights into their evolutionary and phylogenetic relationships and expand understandings of their ecological roles (Rice *et al.*, 2003). More than 5,000 phage particles have been identified so far, and of those, few are specific for thermophilic bacteria and archaea (Ackermann, 2007). Although bacteriophages are extensively used in biochemistry and molecular biology (Bettstetter *et al.*, 2003; Furmann, 1999; Prangishvili *et al.*, 2001; Prangishvili and Zillig, 2002; Suttle, 2005), a paucity of published studies on bacteriophages that infect thermophilic microorganisms exists. Furthermore, as the most abundant biological entities on earth, bacteriophages play an important role by influencing bacterial abundance, diversity, and pathogenesis (Hendrix, 2003; Weinbauer, 2004; Weinbauer and Rassoulzadegan, 2004).

The effects of environmental factors, such as pH, ionic content and moisture on bacteriophage survival have been investigated in various studies (Ashelford *et al.*, 2000; Burroughs, 2000; Hurst, *et al.*, 1980; Inhong *et al.*, 2005). However, to our knowledge, no such study has been reported on thermophilic bacteriophages infecting *Geobacillus* strains. Phages have been shown to survive for longer periods at lower temperatures than at thermophilic temperatures (Yeager and O'Brien, 1979). This observation suggests that proteins and nucleic acids of thermophilic bacteriophages are endowed with thermostable properties.

This chapter reports results obtained from the isolation of bacteriophages, following the double agar plate technique of Adams (1959) and attempts to purify and sequence phage DNA from bacterial lysates. Here four important outcomes are presented:

1. The morphological characteristics of isolated bacteriophages from crude phage lysates using TEM (Transmission Electron Microscopy),
2. Phage-host specificity, which was characterized through plaque assays using various *Geobacillus* strains and other closely related species/strains as potentially permissible bacteria,
3. Phage stability, which was assessed by assaying for bacteriophage titres at various temperatures, pH and CaCl₂ and MgCl₂ concentrations.
4. Isolation and sequencing of DNA extracted from cell free lysates.

3.2. 16S rRNA gene and phylogenetic analyses of *Bacillus*-like isolates

Six *Bacillus*-like isolates obtained from IMBM culture collection were analyzed by 16S rRNA gene PCR using universal primers E9F (Farely *et al.*, 1995) and U1510R (Raysenbach and Pace, 1995). Following amplification of the 16S rRNA genes from high molecular weight genomic DNA extracted from isolates Fur6A4, Fur6A2, Fur12A2, TAU3A1 and G18A9, DNA fragments corresponding to 1.5 kb PCR products were visualized in a 0.8% agarose gel. Subsequently, the amplified DNA

fragments were gel purified and cloned into pTZ57TR/T cloning vector. Following blue/white screening, recombinant transformants appearing as white colonies were subjected to colony PCR using M13 vector specific primers to screen for the presence of the correct size insert. Plasmid DNA was extracted from clones showing the correct size insert using the Plasmid mini kit (Invitex) and sent for sequencing.

The partial 16S rRNA gene sequences of the six isolates were edited using DNAMAN and BIOEDIT programs and compared to the entire GenBank nucleotide database using BLASTN (<http://www.ncbi.nlm.nih.gov/Blast/>). A summary of the three highest scoring hits for each isolate is shown in Tables 3.1.

The 16S rRNA sequences of the six isolates were aligned against each other as well as other sequences obtained from GenBank using ClustalW. A phylogenetic tree was constructed by the neighbor joining method with 1000 bootstrap replicates using the MEGA 4.0 software (Figure 3.1.). The 16S rRNA gene sequence of *U39556 Enterobacter* sp. was used to root the tree.

Table 3. 1. Partial 16S rRNA gene sequences producing significant alignments

Isolate	Highest blast match	E value	Max identity	Accession number
Fur6A4	<i>Anoxybacillus</i> sp. Q-Y1	0.0	97%	EU740973.1
	<i>Anoxybacillus kualawohkensis</i> strain KW 12	0.0	97%	DQ401072.1
	<i>Bacillus</i> sp. CCR3 16S, isolate CCR3	0.0	97%	AJ810551.1
Fur6A2	<i>Bacillus</i> sp. CCR3	0.0	99%	AJ810551.1
	<i>Anoxybacillus kualawohkensis</i> strain KW 12	0.0	99%	DQ401072.1
	<i>Anoxybacillus kamchatkensis</i>	0.0	99%	AF510985.1
Fur12A2	<i>Bacillus</i> sp. CCR3	0.0	99%	AJ810551.1
	<i>Anoxybacillus kualawohkensis</i> strain KW 12	0.0	99%	DQ401072.1
	<i>Anoxybacillus kamchatkensis</i>	0.0	99%	AF510985.1
TAU3A1	<i>Geobacillus thermocatenulatus</i> strain BCRC	0.0	99%	EU484351.1
	<i>Geobacillus kaustophilus</i> isolate KKUA1	0.0	98%	DQ836047.1
	<i>Geobacillus stearothermophilus</i> strain GBPI-10	0.0	98%	EU381192.1
G18A9	<i>Bacillus</i> sp. CCR3 1	0.0	99%	AJ810551.1
	<i>Anoxybacillus kualawohkensis</i> strain KW 12	0.0	99%	DQ401072.1
	<i>Anoxybacillus kamchatkensis</i>	0.0	99%	AF510985.1

The phylogenetic tree shows that isolates Fur12A2, Fur6A2, Fur6A4 and G18A9 fall within the same cluster as the strains *Bacillus* sp. CCR3, *Bacillus* sp. YMY1010 and some strains of *Anoxybacillus* species. In this cluster, isolates Fur12A2 and 18A9 seem to be more closely related to the strain *Bacillus* sp. CCR3, while isolate Fur6A2 appear to be more associated with *Anoxybacillus* strains. Isolate Fur6A4 is positioned closer to the species *Anoxybacillus flavithermus*. In another cluster, isolate TAU3A1 is associated with *Geobacillus* species and seems closely related to *Geobacillus stearothermophilus* strains.

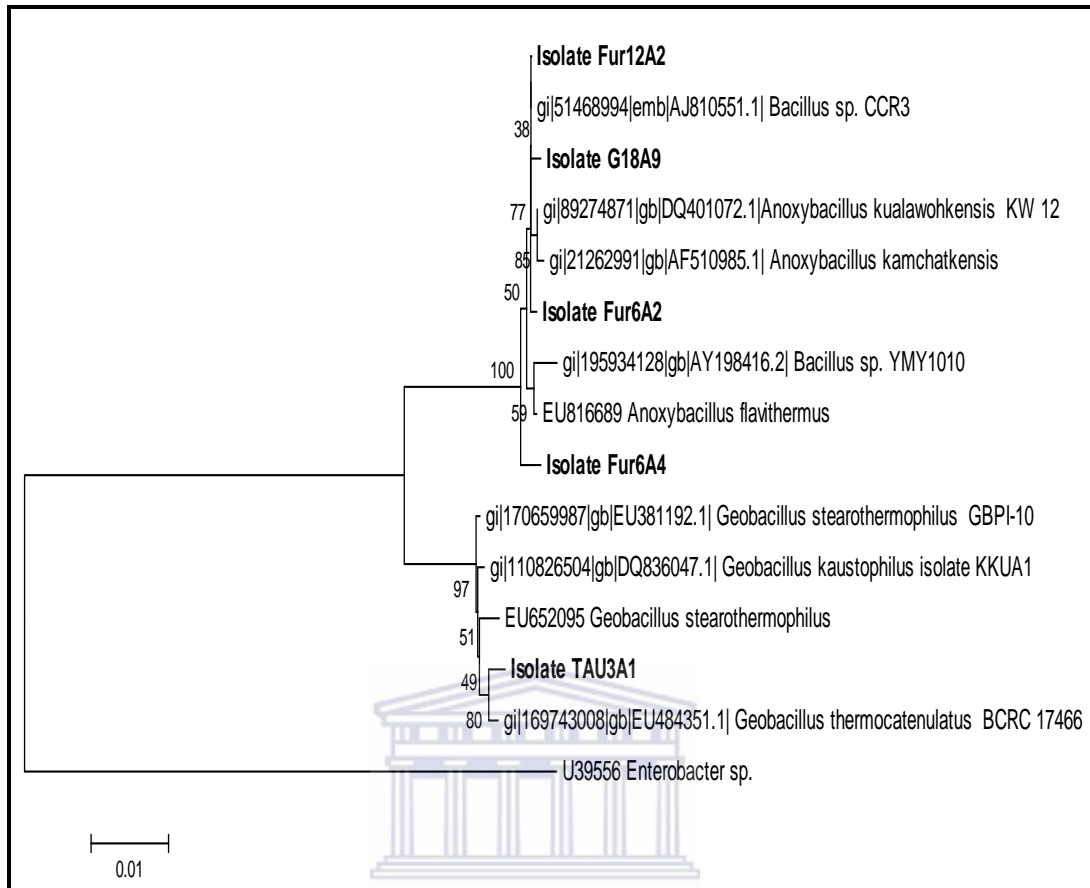


Figure 3. 1 Phylogenetic analysis based on the alignment of 16S rRNA sequences of isolates Fur6A4, Fur6A2, Fur12A2, TAU3A1 and G18A9 and ten GenBank sequences. The tree was constructed by the neighbor joining method with 1000 bootstrap replicates using the MEGA 4.0 software. The 16S rRNA gene sequence of U39556 *Enterobacter* sp. was used to root the tree.

3.3. Primary isolation

Soil samples were collected from arid parts of the Tanqua Karoo (TK) and Klein Karoo (KK) regions. The samples were screened for *Geobacillus*-specific bacteriophages by standard plaque assays using 7 *Bacillus*-like strains according to the protocol described in section 2.3.2. TK soil caused plaque formation with four of the seven indicator strains used, while KK soil caused lysis of one indicator strain (Table 3.2). Positive plates showed distinct plaques with varied morphologies, and

were visible on plates corresponding to 10^{-3} to 10^{-7} dilutions. Plaque purification, carried out through a single plaque transfer process, led to a pure bacteriophage isolate obtained using isolate TAU3A1 (identified to be a strain of *Geobacillus stearothermophilus*) as host. This phage isolate was named GV1 (for *Geobacillus stearothermophilus* virus 1).

Table 3. 2 Thermophilic bacteriophages isolated from environmental samples

Bacterial host strains	Soil samples used	
	TK soil	KK soil
<i>G. stearothermophilus</i> RS 239	-	-
<i>G. kaustophilus</i> G18A6	+	-
<i>G. stearothermophilus</i> NCA 1503 (05330)	+	-
<i>G. stearothermophilus</i> NCA 1503 (05329)	+	-
<i>G. stearothermophilus</i> RS 242	-	-
<i>G. stearothermophilus</i> TAU3A1	+	-
<i>Anoxybacillus kuwalawohkensis</i> G18A9	-	+

+: plaques; -: no plaques. TK was collected in October 2005 in the Tankwa Karoo national park; KK was collected near Oudtshoorn in the Klein Karoo region, in May 2006.

3.4. Plaque morphology

The isolation and propagation of bacteriophages on TSBA (tryptone soya broth agar) plates through agar overlay technique permitted the examination of plaque morphology. Great variations in plaque morphology were observed following primary isolation with different host strains (Figure 3.2.). Plaque descriptions were based on the appearance of the surface, edge and the diameter of the plaque. Initially, plaques were very small (pin head-sized to 2 mm diameter) and with faint edges. With successive reinfection, plaque size increased and plaques of 4-7 mm diameter were observed. The morphology of plaques was standardized by selecting for plaques that were regular in shape, clear and without resistant cells. However, morphological diversity was still observable even after several rounds of plaque transfers, and homogeneity was not observed until the eighth round of single plaque transfers (Figure 3.2.). Moreover, plaque appearance seemed to vary with different host strains. The sizes of the plaques seemed to be influenced by the level of dryness of the plates. Dry plates were observed to yield small plaques whereas wet plates contained larger and watery plaques (with irregular borders). It was also noted that less defined and faint plaques were observed when a two-week or older overlay agar was used. On the other hand, a freshly prepared agar yielded better quality plaques (i.e., with clear surfaces and defined edges).

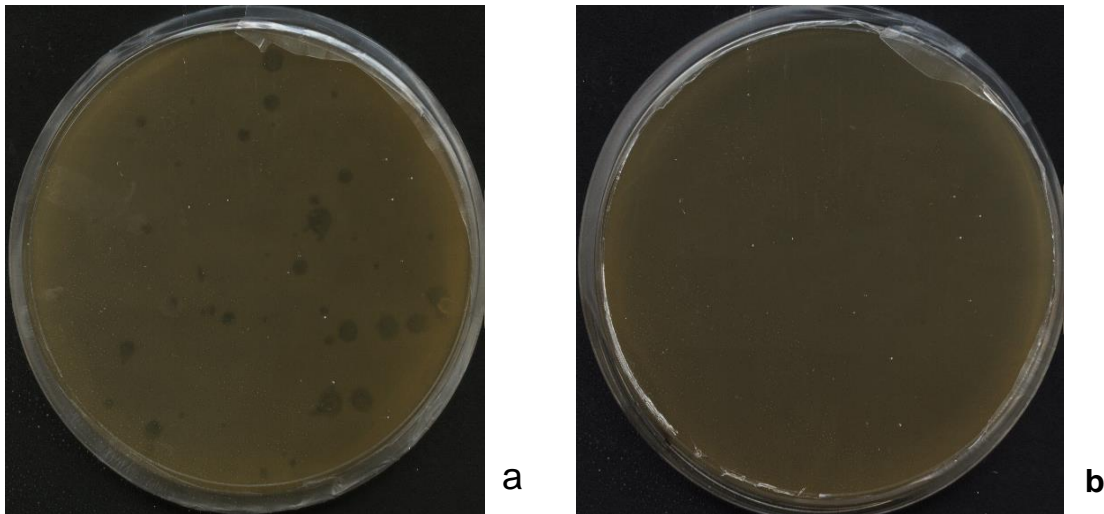


Figure 3. 2 Plaque assay plates. a) A typical bacteriophage assay plate containing plaques of mixed morphologies dotting the lawn of *G. stearotherophilus* TAU3A1 host cells in the overlay agar after a fourth round of reinfection from a single plaque; b) A control plate of an uninfected *G. stearotherophilus* TAU3A1 culture.

3.5. Bacteriophage viability and maintenance

GV1 particles were effectively maintained through sub-culturing as soon as plaques were observed. They were stored on plates as plaques or as suspensions in PMN buffer at -4°C for up to three weeks. A significant decrease in viability (or infectivity) was observed (from 10^7 to 10^1 pfu per ml) when bacteriophage isolates were kept as PMN or TSB suspensions for several months at refrigeration temperatures. Titers were shown to drop by at least one order of magnitude following two days incubation at room temperature. A complete inability to infect was noted after incubation for over a week at this temperature. Storage in 20% glucose at -80°C maintained phage viability for longer periods. However, a decrease by 1 to 3 orders of magnitude was observed when glycerol suspensions were left at -80°C over a year.

3.6. Host range specificity

Bacteriophage host-range studies were conducted using both the quick spot technique and the soft agar overlay method. The quick spot procedure was a rapid method for the identification of phage-competent bacterial strains. It was carried out by spotting 10 µl of a bacteriophage dilution on the surface of a pre-cast soft agar containing potential host bacteria (an indicator plate). The identification of putative hosts through this procedure was indicated by the presence of a clearing region (or lysis region) in the bacterial lawn. Results were later ascertained by the agar overlay method. GV1 particles were able to lyse all 13 thermophilic strains tested (Table 3.3). Higher titers ($>10^7$) were observed with strains *Anoxybacillus hidirlerensis* FUR6A2, *Anoxybacillus kuwalawohkensis* G18A9, *G. stearothermophilus* RS 241, *G. stearothermophilus* NCA1503 (CAMR05329) and *G. stearothermophilus* NCA1503 (CAMR05330). When the phage was tested against a mesophilic strain of *Bacillus megaterium*, it showed no lytic activity. Good activity was noted with a thermophilic isolate identified by 16S rRNA gene analysis as *Bacillus licheniformis* (Table 3.3).

Table 3. 3 Host range and plaque morphological characteristics of *Geobacillus stearothermophilus* virus 1 (GV1)

Host strains	PFU per ml
<i>Anoxybacillus hidirlerensis</i> FUR6A2	1x10 ⁶
<i>Anoxybacillus kuwalawohkensis</i> G18A9	>10 ⁷
FUR6A5 (unidentified)	7x10 ⁴
<i>Anoxybacillus hidirlerensis</i> FUR12A2	1x10 ⁵
<i>Geobacillus stearothermophilus</i> RS93	-----
<i>G. stearothermophilus</i> RS239	1x10 ⁵
<i>G. stearothermophilus</i> RS240	3x10 ⁶
<i>G. stearothermophilus</i> RS241	3x10 ⁷
<i>G. stearothermophilus</i> RS242	2x10 ⁶
<i>G. stearothermophilus</i> NCA1503 (CAMR05329)	5x10 ⁷
<i>G. stearothermophilus</i> NCA1503 (CAMR05330)	3x10 ⁷
<i>G. stearothermophilus</i> TAU3A1	1x10 ⁵
<i>G. thermoglucosidasius</i> 11955	1x10 ⁵
DSM406	1x10 ⁷
<i>Bacillus megaterium</i>	-----
<i>Bacillus licheniformis</i>	5x10 ⁷

-----: no lytic activity observed.

3.7. Bacteriophage structure

The morphology of GV1 particles was assessed by transmission electron microscopy (TEM) from a crude lysate purified by ammonium acetate centrifugation as outlined in sections 2.7.1 and 2.7.2. Phage preparations were not of high purity judging by the presence of debris – amorphous structures, broken pieces of flagella and cell membranes, as well as very small whole cells. As a result, phage visualization under TEM was difficult. TEM micrograph (Figure 3.3) showed that GV1 particles had a long tail (approximately 100 nm in length and 10 nm in width) and a hexagonal head (approximately 50 nm in diameter). The particle was therefore a typical *Siphoviridae* phage (Ackermann, 2005).

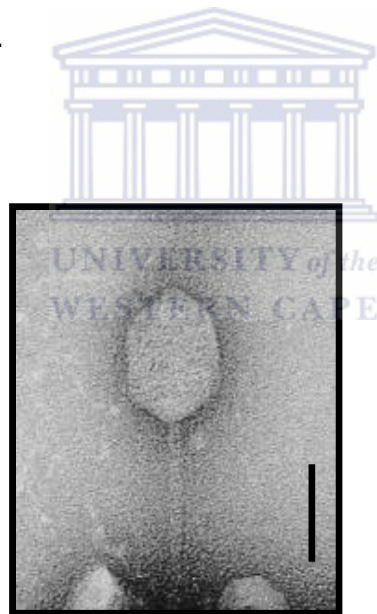


Figure 3. 3 Transmission electron micrograph of a negatively stained GV1 particle.

Bar= approximately 50 nm.

3.8. Virus-host relationships

The study of virus-host interactions was conducted by spectrophotometrically comparing the growth cycles of infected cultures with uninfected ones, as described in Chapter 2. The growth of uninfected cultures (Figure 3.4., green curve) showed a rapid rise over the first 4 hours and steadily, gradually declined thereafter. A strong contrast to this emerged when compared to phage-infected cultures (Figure 3.4., red curve), which showed a stagnant state or no growth over the first 6 hours, but then increased rapidly and exceeded the uninfected cultures by hour 18. A stationary phase was not observed in both cultures after 21 hours of incubation.

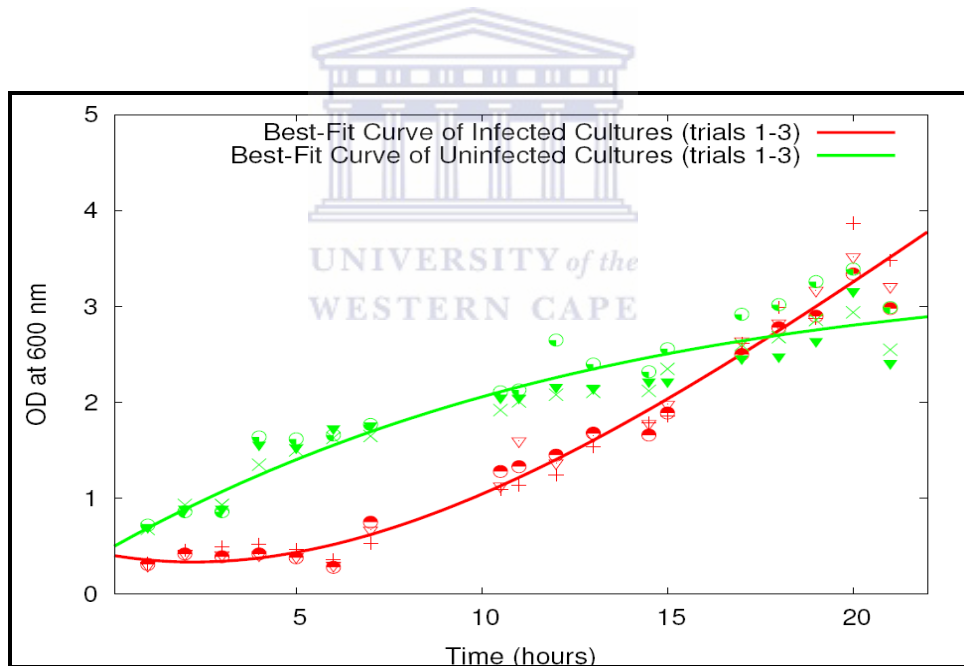


Figure 3. 4 Growth of uninfected (green line) and infected (red line) cells of *G. stearotherophilus* RS241 with GV1 particles. Experiments were carried out in triplicate and data corresponding to the mean and the standard deviation were plotted as OD at 600 nm versus time (hours).

3.9. Effect of pH of the medium on phage stability

To determine the effect that the pH of the medium had on phage stability and production, a fresh bacteriophage suspension was diluted in TSB broth of pre-adjusted pH. The diluted suspensions were incubated at room temperature for up to four hours and phage titers were assayed by the agar overlay method. Particles showed great stability within the pH range of 5.5 to 7.5, with a slight peak at pH 6.5 (Table 3.4). Large decreases in titers were observed at pH values below 5.5 and above 8.0.



Table 3. 4 Effect of pH on phage stability

pH of the medium	PFU per ml
5.0	6.0×10^3
5.5	1.3×10^6
6.0	4.3×10^6
6.5	3.1×10^7
7.0	2.0×10^6
7.5	6.5×10^6
8.0	2.4×10^4
8.5	2.2×10^2

3.10. Effects of CaCl₂ and MgCl₂ on phage stability

The effects of concentrations of CaCl₂ and MgCl₂ on phage stability was investigated by adding actively growing *G. stearotherophilus* TAU3A1 cells to bacteriophage dilutions and transferring the mixture into TSBA soft agar supplemented with CaCl₂ or MgCl₂ concentrations ranging from 0 to 0.1 M. Phage titers were determined as previously described. Results showed that the incorporation of CaCl₂ into the assay medium stimulated bacteriophage production at various concentrations. Optimum increase in pfu was observed at 0.001 M, while the addition of 0.01 and 0.1 M of the compound had less remarkable effect on phage titer. On the other hand, no notable increase in the titer was observed as a result of MgCl₂ addition to the assay medium (see Table 3.5).

Table 3. 5 Effects of CaCl₂ and MgCl₂ on phage GV1's stability

CaCl ₂ and MgCl ₂ added (M)	Phage particles produced per ml in TSBA with	
	CaCl ₂	MgCl ₂
0	2.1 x 10 ⁶	3.2 x 10 ⁶
0.001	3.3 x 10 ⁷	6.1 x 10 ⁶
0.01	1.3 x 10 ⁶	3.9 x 10 ⁶
0.1	3.0 x 10 ⁶	2.5 x 10 ⁶

3.11. Effect of temperature on phage stability

To evaluate the effect of temperature on the stability and the activity of GV1, bacteriophage suspensions containing about 10⁷ pfu per ml were incubated at 50, 55, 60, 65, 70 and 75°C for about 1 h. Thereafter, serial 10-fold dilutions of the suspensions were made and bacteriophage titers were determined by plaque assay at 55°C. Results showed that decreasing the incubation temperature resulted in an increase in titers, especially at temperatures below 60°C (Table 3.6). It was evident that 55°C was the optimum temperature for phage production and that there was a significant decrease in titers at temperatures above 60°C.

Table 3. 6 The effect of temperature on phage GV1's stability

Incubation temperature (°C)	Phage particles produced per ml
50	1.1×10^7
55	3.5×10^7
60	2.2×10^6
65	3.5×10^5
70	1.3×10^3
75	1.2×10^2

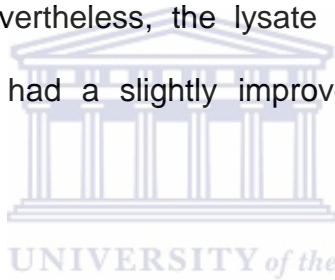
The thermal inactivation profile of GV1 was followed by holding phage suspensions (of about 10^7 pfu per ml) in both TSB and PMN buffer at 75°C and removing aliquots at various time intervals (as specified in Table 3.7) for titer determination. After 1 h incubation at 75°C, the phage proved to be fairly thermostable in TSB, with a decrease of about 4 orders of magnitude. A much sharper decline (of about 5 orders of magnitude) in titers was observed when particles were suspended in PMN buffer.

Table 3. 7 Thermal inactivation of phage GV1 at 75°C

Incubation time (min)	Titer (pfu/ml) observed in	
	PMN	TSB
0	1.8×10^7	1.4×10^7
20	1.5×10^4	2.3×10^6
30	4.0×10^3	3.7×10^5
60	3.3×10^2	1.1×10^3

3.12. Extraction and analysis of DNA obtained from bacteriophage lysate

Three lysates of about 1 L each were separately accumulated by infecting 50-300 ml cultures of isolates TAU3A1, RS241 and *Bacillus licheniformis* with GV1 particles. The lysates were used in numerous attempts to extract pure phage particles. Purified phage suspensions that were free of extraneous cellular extracts, including DNA and RNA, could not be obtained. Moreover, bacteriophage titers obtained with the three cultures proved to be relatively low (10^7 pfu per ml), and therefore unsuitable for phage DNA purification. Nevertheless, the lysate obtained from infected *Bacillus licheniformis* culture, which had a slightly improved titer (5×10^7), was used for subsequent DNA extraction.



B. licheniformis lysate was purified by filtration to remove unlysed cells (which would otherwise multiply during the incubation time) and by treating it with DNAase/RNAase (1 $\mu\text{g/ml}$) to eliminate chromosomal DNA and RNA emanating from lysed cells. This was maximized by a second DNAase/RNAase incubation period, which ultimately resulted in the reduction of DNA from 0.898 $\text{ng}/\mu\text{l}$ to a 0.162 $\text{ng}/\mu\text{l}$. Phage particles were subsequently precipitated out of the lysate by centrifugation with polyethylene glycol (PEG). Purified bacteriophage suspensions showed titers of about 10^6 pfu/ml in volumes of 5 ml. This indicated a decrease in bacteriophage titer and was a cause of concern as high titers are essential for DNA extraction.

A complete elimination of free high molecular weight DNA from lysate was achieved when a lysate was treated repeatedly with a concentration of DNAase/RNAase of up

to 1 mg/ml. However, an extraction experiment conducted using this lysate did not yield any DNA (phage or bacterial). This was a convincing indication that the number of viruses in the lysate was not sufficient to yield detectable DNA.

Despite the evidence showing that the lysate did not have the appropriate concentration of phage particles for DNA extraction, attempts were still made to extract DNA using a lysate that contained a reduced concentration of bacterial DNA. DNA was extracted using a method that involved the removal of phage protein capsid with proteinase K, followed by the standard phenol/chloroform extraction. A gel electrophoretogram of the extracted DNA is shown in Figure 3.5. The DNA appeared to be sheared, but was used to construct a genomic DNA library.

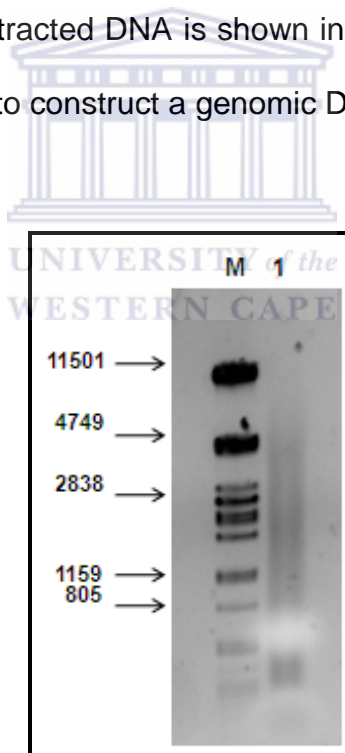


Figure 3. 5 0.8% agarose gel picture of sheared DNA obtained from purified *B. licheniformis* lysate. Lane M, Lambda phage DNA digested with *Pst*I; lanes 1, DNA sample. Arrows indicate specific markers' size.

3.13. Genomic DNA library construction

The construction of DNA library was carried out using the high copy, positive selection cloning vector pJET1.2/blunt. Partial digestion of DNA with *Mbol* confirmed that the extracted DNA was already sheared, as no difference was found between the *Mbol* digested DNA and the undigested sample (Figure 3.6.). The digested DNA was successfully end-repaired and visualized by gel electrophoresis, and the gel region corresponding to 5-10 kb fragments was excised and column-purified (Figure 3.7.). The DNA was then blunt cloned into pJET1.2/blunt and the ligation product was transformed into electro-competent *E. coli* cells.

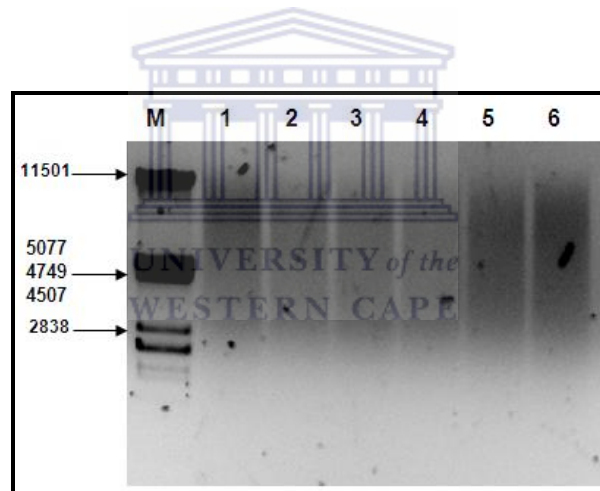


Figure 3. 6 Partial digestion of DNA with *Mbol*. Lane M, Lambda phage DNA digested with *PstI*; lane 1, undigested DNA. Lanes 2-6, DNA digested with *Mbol* for 1, 5, 10, 20 and 30 minutes respectively. Arrows indicate specific markers' size.

High transformation efficiency was achieved and colonies were harvested, sub-cultured and selected for the presence of DNA inserts by plasmid extraction (using a quick alkaline method), endonuclease restriction (using *BglII*) and agarose gel electrophoresis. Figure 3.8 shows a representative gel of the selection experiment.

Positive clones contained much smaller inserts sizes than anticipated (all were about 1000 bp). Plasmids were purified from five positive clones and sequenced (Figure 3.9.).

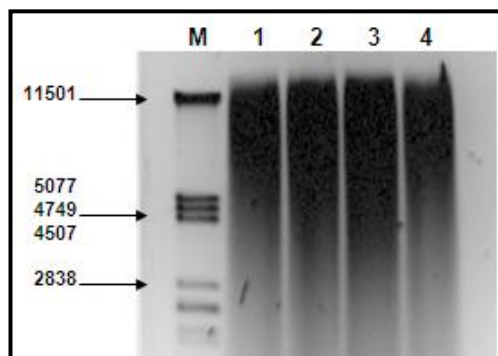


Figure 3. 7 agarose gel showing end repaired DNA. Lane M, Lambda phage DNA digested with *Pst*I; lane 1-6, end repaired DNA. Arrows indicate specific markers' size.

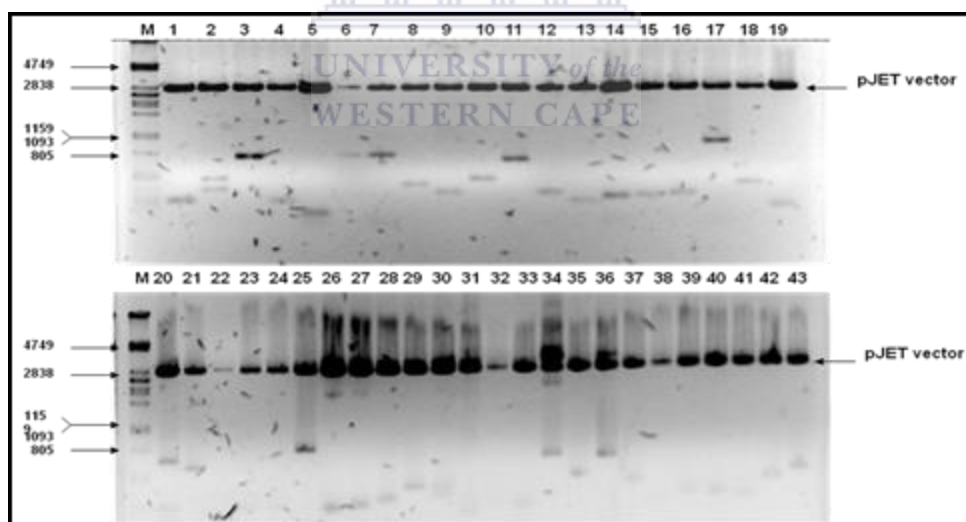


Figure 3. 8 A representative agarose gel (0.8% agarose) showing the selection of positive clones. M, lambda *Pst*I molecular weight maker. Lane 1-43, plasmid mini-prep products cut with *Bgl*II. The pJET vector DNA fragments are shown at position of about 2838 bp while most of the insert DNA fragments appear to be smaller than 1000 bp.

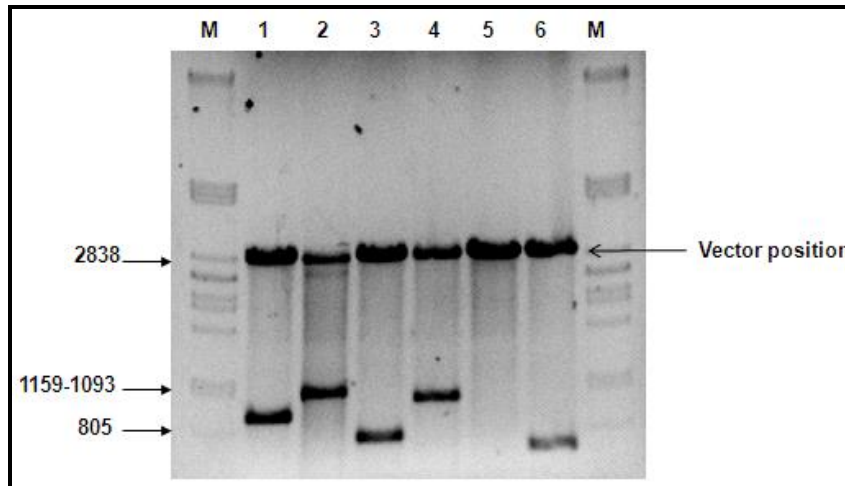


Figure 3. 9 A representative agarose gel electrophoretogram showing five positive clones that were sequenced (lanes 1, 2, 3, 4, and 6). Lane M, Lambda phage DNA digested with *Pst*I; Lanes 1-6, *Bgl*II digested plasmid DNA.

3.14. Sequence analysis

Clones were sequenced using pJET vector primers. Sequences were analyzed using BLAST and GeneMark.hmm for Prokaryotes (Version 2.4). Some of the important characteristics of the sequences, such as gene lengths, putative functions, e values, and percentage identities, are shown in Table 3.8. The positions of ORFs on the sequences are shown in Figure 3.10 (based on GeneMark graphic outputs). Analyses confirmed that insert DNA sequences originated from the same host, which was identified by BLAST search as similar to *Bacillus licheniformis* ATCC 14580. No phage sequences were identified. Clone 1 contained two possible ORFs, the first gene, running on the reverse sequence, showed high sequence identity to isochorismate synthase while the second (found on the direct sequence) had high identity to a ribose ABC transporter (ribose-binding protein). Clone 2 contained two ORFs, one coding for citrate synthase I (on the negative strand) and the other for

adenosylmethionine-8-amino-7-oxononanoate aminotransferase (on the positive strand). Three genes were found in clone 3, one for a conserved membrane protein, another for acyl-CoA dehydrogenase and another for an ABC transporter. Sequence 4 contained two ORFs encoding for a transcriptional regulator and for D-alanine racemase, respectively. A transferase gene (tRNA nucleotidyltransferase and glycosyl transferase gene) was identified in clone 5.

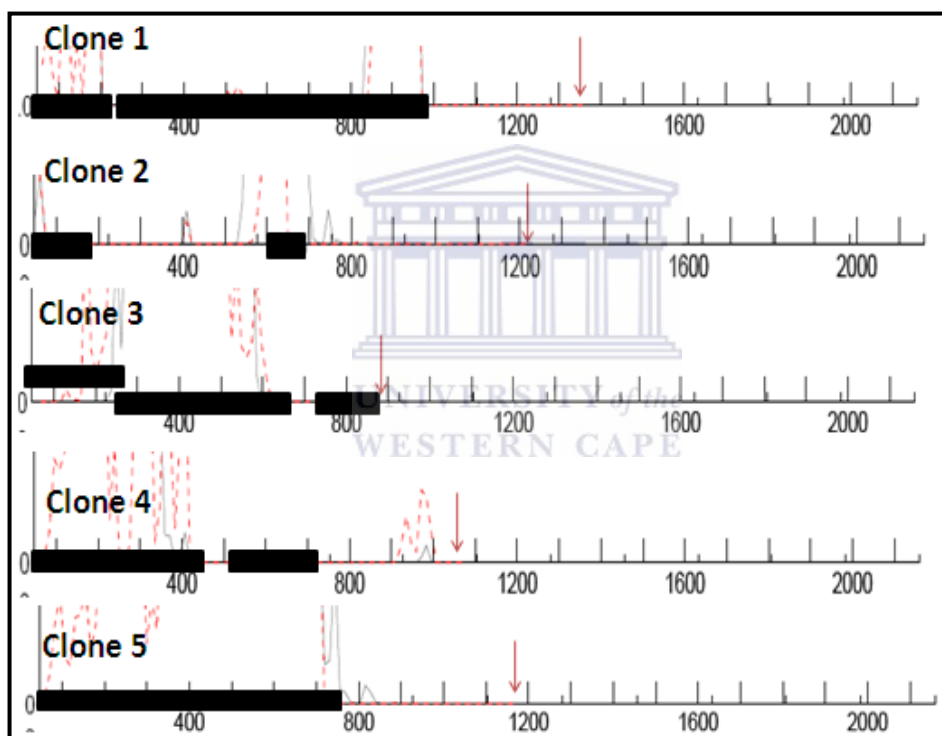


Figure 3. 10 A graphic representation showing the positions of ORFs on the sequences.

Black box (■), ORF; Red arrow (↓), the end of analyzed sequences.

Table 3. 8 Various characteristics of analyzed sequences

Query sequence	Strand	Predicted Genes (from-to)	Amino acids (aa)	Proposed or predicted function	Similarity and % identity	BLAST E value	Reference NCBI accession no.
Clone 1 : Sequence length: 1404 bp; G+C content: 48.01%							
	-	<1-216	54	Isochorismate synthase	179/179 (100%)	7e ⁻⁸⁸	CP000002.3
	+	243-983	247	Ribose ABC transporter (ribose-binding protein)	285/285 (100%)	3e ⁻¹⁴⁶	CP000002.3
Clone 2 : Sequence length: 1261 bp; G+C content: 44.01%							
	-	<3-173	57	Citrate synthase I	138/139 (99%)	2e ⁻⁶³	CP000002.3
	+	616-690	25	Adenosylmethionine-8-amino-7-oxononanoate aminotransferase	65/65 (100%)	4e ⁻²⁵	CP000002.3
Clone 3: Sequence length: 909 bp; G+C content: 48.73 %							
	-	25-261	79	Conserved membrane protein	227/227 (100%)	2e ⁻¹¹⁴	CP000002.3
	+	262- 657	132	Acyl-CoA dehydrogenase	311/311 (100%),	6e ⁻¹⁶¹	CP000002.3
	+	729- 908	60	ABC transporter	179/179 (100%)	6e ⁻⁸⁸	CP000002.3
Clone 4: Sequence length: 1115 bp; G+C Content: 46.55 %							
	+	<3-449	149	Transcriptional regulator	428/430 (99%)	0.0	CP000002.3
	+	525-734	70	D-alanine racemase	183/184 (99%),	5e ⁻⁸⁹	CP000002.3
Clone 5: Sequence length: 1211 bp; G+C Content: 44.43 %							
	+	<1-756	252	tRNA nucleotidyltransferase; Glycosyl transferase, family 4	367/367 (100%)	0.0	CP000002.3

Chapter IV: Discussion

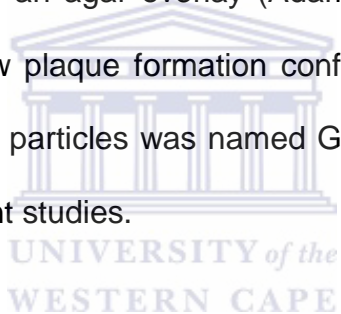
4.1. 16S rRNA characterization

The main objective of this project was to characterize the genome of bacteriophage of *Geobacillus* species. Initial work therefore aimed at identifying *Geobacillus* species among *Bacillus*-like isolates that were present in the lab, as it was necessary that the host bacteria be a *Geobacillus*. Although colony morphologies of these isolates and the fact that they sustained growth at 55°C in a *Geobacillus* growth medium (TSB) prompted the suggestion that they were *Bacillus*-like, it was important that a molecular characterization be performed. For this reason a 16S rRNA gene characterization was performed using universal bacterial primers. Five isolates obtained from the IMBM culture collection were characterized. As shown in Table 3.1, BLAST searches of 16S rRNA gene sequences showed that isolates were highly similar with *Anoxybacillus* sp. Q-Y1 (isolate Fur6A4), *Bacillus* sp. CCR3 (isolates Fur6A2, Fur12A2, and G18A9) and *Geobacillus thermocatenuatus* strain BCRC (isolate TAU3A1). This was followed by a phylogenetic analysis performed based on 16S rRNA sequences, which showed that isolates Fur12A2, Fur6A2, Fur6A4 and G18A9 grouped with *Bacillus* sp. CCR3, *Bacillus* sp. YMY1010 and other strains of *Anoxybacillus* species while isolate Fur6A4 was in the same cluster as *Anoxybacillus flavithermus*. Isolate TAU3A1 grouped with *Geobacillus* species such as *Geobacillus stearothermophilus* and *Geobacillus kaustophilus*. This was selected as the indicator microorganism in the primary isolation of bacteriophages using soil samples from the

Karoo as source material. To increase the probability of isolating “wild bacteriophage” from soil, all six isolates were used in primary isolation experiments.

4.2. Isolation and morphological characterization

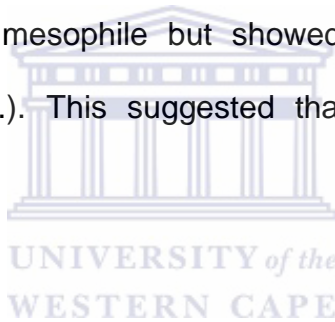
Bacillus- and *Geobacillus*-infecting viruses were readily isolated from arid soils collected from the Karoo region using a relatively simple process known as the agar overlay technique (as described in section 2.3.2). The method allowed the selection of particles with lytic (or virulent) activity and capable of forming plaques in a lawn of bacterial cells immobilized in an agar overlay (Adams, 1959, Romig and Brodesky, 1961). Re-infection and new plaque formation confirmed the virulent nature of the isolated particles. One of the particles was named GV1, for *Geobacillus* virus 1, and was the subject of subsequent studies.



TEM analysis showed that GV1 belongs to the family of *Siphoviridae*, one of the three major phage families belonging to the order *Caudovirales* – tailed bacteriophages (Brussow and Hendrix, 2002). No particles belonging to either *Myoviridae* (with long contractile tail) or *Podoviridae* (with short non-contractile tail) families were identified. Siphoviruses have been extensively characterized and are known to be the most abundant phage morphotype in the environment (Ackermann, 2007). They are double-stranded DNA viruses characterized by a long non-contractile tail and an isometric or prolate capsid (Ackermann, 1996; Brussow and Hendrix, 2002). The bacteriophage tail is an important feature of the particle as it helps it recognize and adsorb to the cell membrane, and insert its DNA into the cytoplasm (Katsura, 1983).

4.3. Host range

Host range studies are used to determine the specificity of phage interactions. First, GV1 was tested against 13 thermophilic strains of *Bacillus* and *Geobacillus* (Table 3.3). The virus proved to be virulent on all thermophilic bacteria tested. This result may suggest that most of the test bacterial strains were closely related. Alternatively, it may indicate that the virus had a broad host range. This trait, if authentic, may make it a good candidate for the engineering of a cloning vector for the transformation of a number of *Geobacillus* species/strains. Further test with a mesophilic strain of *Bacillus megaterium* and a thermophilic strain of *Bacillus licheniformis* showed that GV1 was not lytic on the mesophile but showed improved activity against the thermophile (see Table 3.3.). This suggested that the virus has an affinity for thermophilic strains.



4.4. Phage viability and stability

The viability and stability of bacteriophages under various conditions was investigated by plaque assay. Phages maintained their infectivity for several months following storage as suspensions in PMN buffer supplemented with 20% glycerol at -80°C , but lost it almost completely after three weeks at refrigeration temperatures and after a few days at room temperature. The instability of viral particles could be inherent to their nature, as they are basically heteropolymeric complexes composed of structural proteins and genetic material (Epstein and Campbell, 1975). A phage loses its infectivity once the tail breaks off from its head. Therefore, handling of phage suspensions has to be carried out with extreme care.

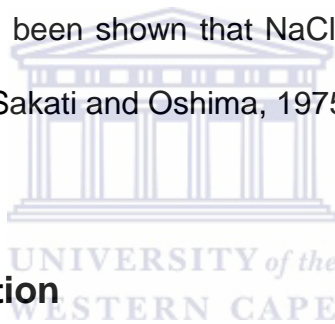
The host bacterium in this experiment, *Geobacillus stearothermophilus*, has been extensively characterized (Ash *et al.*, 2001; Nazina *et al.*, 2001). While the focus of this work was on viruses, growth conditions such as pH, temperature and media composition were deliberately chosen to optimize the growth of the host bacteria. This strategy was motivated by the fact that bacteriophages are obligate intracellular parasites, meaning that they depend completely on their hosts' replicating mechanism for reproduction (Campbell, 2003; Fuhrman, 1999; Flint *et al.*, 2000). Maximal phage production is evidently dependent on media composition and other factors that are important for the growth of the host (Epstein and Campbell, 1975).

GV1 showed greatest stability at pHs between 5.5 and 7.5, with a maximum pick at pH 6.5. Previous studies on the effect of soil pH on bacteriophages have shown that pH is an important factor for phage survival, as it influences adsorption to the host (Farrah and Bitton, 1990). While low pH seems to aid adsorption in a variety of phages, actinophages were not found in soils with pH values below 6.0 (Goyal and Gerba, 1979; Sykes *et al.*, 1981). Changes in the environmental pH affect the phage's adsorptive capability by changing its iso-electric point (i.e. the net charge of the phage) (Dowd *et al.*, 1998).

The effect of CaCl_2 and MgCl_2 on phage production was tested by adding specific concentrations of the salts to the soft agars. Results showed that magnesium ions had an insignificant effect on phage production, while CaCl_2 resulted in slightly increased phage yield. The highest yield was observed at a concentration of 0.001M

for both salts (a titer of 3.3×10^7 pfu per ml for CaCl_2 and 6.1×10^6 pfu per ml for MgCl_2). Previous studies have shown that magnesium and calcium and other divalent cations increase the structural and thermal stability of bacteriophages and prevent the effects of chelating agents such as ethylenediaminetetraacetic acid (EDTA) (Bassel *et al.*, 1971; Saunders and Campbell, 1966). However, it was not evident whether these compounds could improve bacteriophage phage titer. A study of a *Bacillus stearothermophilus* phage concluded that magnesium ions primarily affect phage stability and not production (Epstein and Campbell 1974). In a *Bacillus subtilis* phage study, the addition of calcium, barium or strontium ions to infection media was shown to stabilize and improve bacteriophage titres. Additionally, the study predicted that at least one of the lytic cycles could be calcium dependent and that calcium could be a requirement for phage DNA penetration (Steensma and Blok, 1979). Moreover, a *Staphylococcus* phage study demonstrated that calcium ions were essential for phage DNA injection into the host and that calcium requirement could not be necessarily fulfilled with magnesium or strontium (Rountree, 1951). This later observation was further supported by another study on a thermophilic phage infecting *B. stearothermophilus* strains. The study observed that insufficient calcium concentrations in the broth resulted in small burst sizes (Shafia and Thompson, 1964). In this current study, TSB media contained NaCl, which has also been shown to help the phage adsorb to its host and to initiate lytic cycles in some filamentous phages (Marvin and Hohn, 1969).

Thermostability experiments showed that phage GV1 was stable over a temperature range of 50 to 75°C, with an optimum at 55°C. The thermostable (or thermophilic) nature of the virus was clearly demonstrated by its ability to withstand exposure to 75°C for up to an hour. It is assumed that longer exposure at this temperature could lead to complete inactivation. The thermal inactivation profile of the virus observed at 75°C in TSB medium is similar to the one of $\Phi\mu$ -4 virus at the same temperature in Tris buffer (Shafia and Thompson, 1963). It has also been noted that TSB medium seems to be more stabilizing than PMN buffer, suggesting that the broth contains certain stabilizing factors. This could be attributed to its content of NaCl, KH_2PO_4 and glucose. Nevertheless, it has been shown that NaCl concentrations higher than 2 M could cause viral disruption (Sakati and Oshima, 1975).



4.5. Phage-host interaction

The phage-host interaction study showed that phage particles inhibited the initial growth of infected cultures and that mature phages were released from lysed cells 6 hours post-infection. The growth of infected cultures was thus blocked nearly totally during the first 6 hours following infection, representing the lag phase, which is an important phase in the phage cycle. The fact that complete lysis was not observed in infected cultures may be related to the low titer nature of the phage. After the 6 hour mark, cells slowly began to overcome viral inhibition and growth rapidly increased, and the culture density equaled that of the uninfected cultures by hour 18.

It should be noted that liquid cultures in standard shaking incubators or in chemostats, while providing an acceptable empirical or experimental setting for studying phage-bacteria interactions for most stream-dwelling bacteria such as enteric bacteria (e.g. *Escherichia coli*), do not provide an accurate model to study the population dynamics of soil bacteria and their phages (Pantastico-Caldas *et al.*, 1992). This is because soil phage and bacteria naturally interact in environments that are definitely heterogeneous and almost totally stationary for extensive periods of time. A direct study of phage-bacteria interactions in natural environments is yet to be published. However, laboratory investigations of population dynamics of phages and bacteria under semi-natural culture conditions, such as soil microcosms, have been shown to be more adequate for experimental analyses (Babich and Stotzky, 1980; Stotzky *et al.*, 1981; Williams *et al.*, 1987; Pantastico-Caldas *et al.*, 1992). It is believed that such models may help formulate hypotheses about interactions in nature and lead to more reliable analytical procedures (Pantastico-Caldas *et al.*, 1992).

4.6. DNA analysis and sequencing

Although in principle the process of isolating phage DNA is straightforward, the successful purification of a significant amount of phage DNA is subject to various pitfalls (Scheif and Wensink, 1981; Clokie and Kropinski, 2009). The main difficulty is growing infected cultures with elevated extracellular bacteriophage titers (Sambrook *et al.*, 1989; Brown, 2006). Despite several efforts, lysates with high enough titers could not be obtained in this study. For the well-studied lambda phage, a titer of 10^{10} pfu per ml and about 500-1000 ml of lysate are required to obtain a substantial

amount of DNA (Scheif and Wensink, 1981; Brown, 2006). This implies that it was practically impossible to extract measurable amount of DNA without using high volumes of lysate. Despite having low titer lysates and consequently failing to obtain pure phage particles, DNA was extracted and accumulated from small volume lysates. Smaller volumes of lysates were used because of their manageability.

The extracted DNA was cloned into the positive selection vector pJET1.2/blunt, transformed into *E. coli*, and positive clones were screened and sequenced (see results in section 3.13). For reasons that could not be explained, the cloning vector, which was selected for its ability to take up large DNA fragments (about 5 kb), accepted only smaller fragments (about 1 kb in size). The five clones that were sequenced were shown to contain only bacterial DNA fragments. BLASTN and GeneMark programs allowed identification of ORFs contained in each of these fragments and revealing their respective functions (Table 3.8 and Figure 3.9).

Chapter V: Conclusions, comments and future perspectives

5.1. Main findings

The main objectives of this project, which were to isolate a thermophilic bacteriophage, elucidate some of its physicochemical properties and purify and analyse its DNA, were partially met. Using soil samples from Karoo dry-lands, a thermophilic bacteriophage was isolated using a strain of *Geobacillus stearothermophilus* as host. The phage proved to be able to attack various thermophilic strains belonging to *Bacillus* and *Geobacillus* species. Morphological studies, conducted by TEM experiments, showed that the virus had morphological characteristics of the *Siphoviridae* family, with the hallmark being a long tail and a head with icosahedral symmetry. Viability and stability studies showed that the phage was best maintained at -80°C in PMN buffer supplemented with 20% glycerol. It was stable at a pH range of 5.5 to 7.5 and MgCl_2 and CaCl_2 concentration of 0.001 M. Thermostability experiments, conducted over short periods of time, showed phage GV1 was stable over 50 to 75°C temperature range, with optimum at 55°C . The study of phage-host interactions showed that phage particles inhibited the initial growth of infected cultures in the first six hours post-infection, where, assumedly, mature phages were released. This was followed by a steady recovery of the growth rate. The most disappointing aspect of the virus was definitely the fact that it showed low

titers in *Geobacillus* cultures. As a result, it seemed impossible to obtain pure phage particles and extract measureable quantities of bacteriophage DNA.

5.2. Comments on methodology

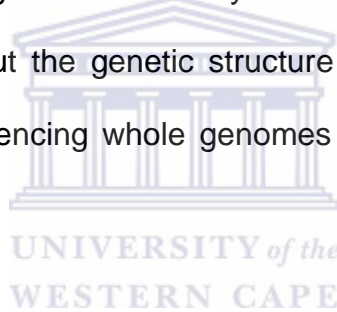
The fact that bacteriophage DNA could not be extracted from infected cultures was anticipated from the moment it was found that the phage displayed relatively low titers. This setback has emphasized the need to ameliorate the methodological approach. A safer and consistent approach would be to accumulate huge volumes of lysates and then concentrate viral particles by PEG precipitation, bringing the phage suspension to a manageable volume (Sambrook *et al.*, 1989; Brown, 2006). Zinc chloride precipitation or other precipitation methods can also be used (Santos, 1991). However, PEG precipitates, as well as precipitates obtained by alternative methods, often contain cellular debris, possibly including cellular DNA (Brown, 2006). This therefore requires an intermediate purification step prior to DNA extraction. CsCl ultra-centrifugation is perhaps the most effective method to separate viral particles from undesired cellular contaminants. It takes advantage of a gradient of CsCl densities and ultracentrifugation to band bacteriophage at a specific density (corresponding to its density) in the gradient. A pure phage preparation is then obtained following removal of CsCl by dialysis (Sambrook *et al.*, 1989; Brown, 2006).

Extracting DNA from purified phage solutions has been proven to be uncomplicated, as a single deproteination step is sufficient to release DNA from phage. This,

however, is usually coupled with the standard phenol/chloroform method to ensure removal of protein contaminants (Sambrook *et al.*, 1989; Brown, 2006).

5.3. Future perspectives

The objective of resolving and analysing the whole genome of GV1 particles is worthy of continued research. By providing preliminary physicochemical characterization of GV1 particles, this work has laid down the groundwork for such an objective. Whole genome sequencing of bacteriophage has the potential to contribute to the understanding of the phage's evolutionary and gene regulation mechanisms. Furthermore, questions about the genetic structure of the global phage population could be answered by sequencing whole genomes of groups of phages previously uncharacterized.



New growth media could be developed to improve the phage titer and efficient purification methods could be used to produce substantial amounts of purified viruses. Modern DNA sequencing technologies will guarantee a rapid and reliable sequencing of viral DNA. The sequence could be analyzed using sophisticated sequence analysis tools that already exist.

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