## 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$^{o}$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$^{o}$C to precipitate the DNA. The DNA was pelleted by centrifugation at 13,000 rpm, at 4$^{o}$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$^{o}$C), then air-dried at 37$^{o}$C for up to 10 min. The DNA pellet was resuspended in 50 µl TE buffer and stored at -20$^{o}$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$^{o}$C for 20 min. Plasmid DNA was pelleted by centrifugation at 15000 rpm and 4$^{o}$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$^{o}$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$^{o}$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 *Hind*III, *Pst*I or *BamH*I). Visualization of gels was achieved using AlphaImager 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 µl and adjusted proportionally according to requirements. The reactions contained appropriate 1 x reaction buffer, 5-10 U of enzyme per µg of plasmid or genomic DNA and $ddH_2O$. They were incubated in a water bath at $37^oC$ 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 *Mbo*I. Genomic DNA (1µg in 20 µl volume) was cut with *Mbo*I diluted 100 folds from a stock concentration of 10 u/µl. The reaction was incubated at $37^oC$ 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$^{TM}$ 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, $ddH_2O$, 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$^{o}$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$^{o}$C.

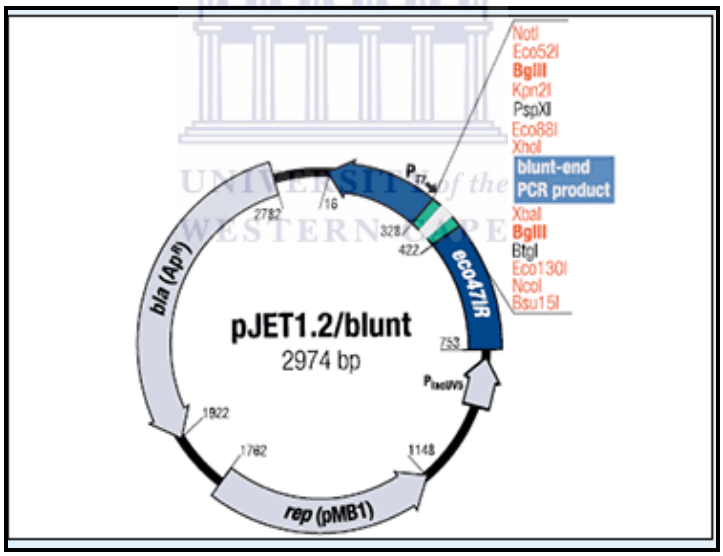


**Figure 2. 1** Map and features of pJET1.2/blunt cloning vector

### 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$^{o}$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$^{o}$C with shaking at 150 rpm until the culture $OD_{600}$ 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$^{o}$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$^{o}$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$^{o}$C freezer. The electro-competent cells were stored at -80$^{o}$C.



### 2.9.6. Transformation of electro-competent cells

Electro-component *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$^{TM}$ (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 37ºC for 1 hour. Thereafter, 100 µl of the transformed cultures were plated out onto LBA plates supplemented with appropriate antibiotics and incubated at 37ºC 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 $CaCl_2$ and $MgCl_2$ 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 (Farelly *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 (Invitek) 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** | *Anoxybacillus* sp. Q-Y1 | 0.0 | 97% | EU740973.1 |
| | *Anoxybacillus kualawohkensis* strain KW 12 | 0.0 | 97% | DQ401072.1 |
| | *Bacillus* sp. CCR3 16S, isolate CCR3 | 0.0 | 97% | AJ810551.1 |
| **Fur6A2** | *Bacillus* sp. CCR3 | 0.0 | 99% | AJ810551.1 |
| | *Anoxybacillus kualawohkensis* strain KW 12 | 0.0 | 99% | DQ401072.1 |
| | *Anoxybacillus kamchatkensis* | 0.0 | 99% | AF510985.1 |
| **Fur12A2** | *Bacillus* sp. CCR3 | 0.0 | 99% | AJ810551.1 |
| | *Anoxybacillus kualawohkensis* strain KW 12 | 0.0 | 99% | DQ401072.1 |
| | *Anoxybacillus kamchatkensis* | 0.0 | 99% | AF510985.1 |
| **TAU3A1** | *Geobacillus thermocatenulatus* strain BCRC | 0.0 | 99% | EU484351.1 |
| | *Geobacillus kaustophilus* isolate KKUA1 | 0.0 | 98% | DQ836047.1 |
| | *Geobacillus stearothermophilus* strain GBPI-10 | 0.0 | 98% | EU381192.1 |
| **G18A9** | *Bacillus* sp. CCR3 1 | 0.0 | 99% | AJ810551.1 |
| | *Anoxybacillus kualawohkensis* strain KW 12 | 0.0 | 99% | DQ401072.1 |
| | *Anoxybacillus kamchatkensis* | 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.

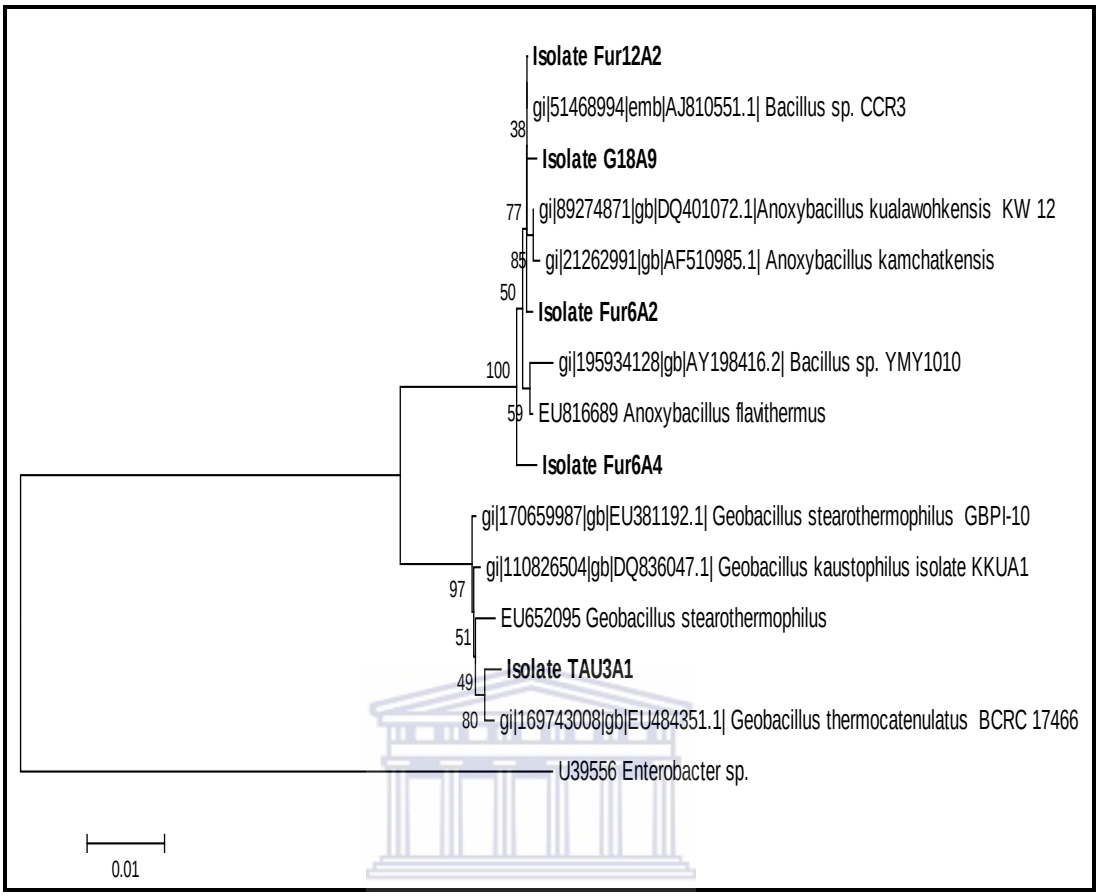




**Figure 3. 1** Phylsogenetic analysis based on the alignment of 16S rRNA sequences of isolates Fur6A4, Fur6A2, Fur12A2, TAU3A1 and G18A9 and ten GenBank sequences. The tree was constructed by the neighbor joining method with 1000 bootstrap replicates using the MEGA 4.0 software. The 16S rRNA gene sequence 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 |
| *G. stearothermophilus* RS 239 | - | - |
| *G. kaustophilus* G18A6 | + | - |
| *G. stearothermophilus* NCA 1503 (05330) | + | - |
| *G. stearothermophilus* NCA 1503 (05329) | + | - |
| *G. stearothermophilus* RS 242 | - | - |
| *G. stearothermophilus* TAU3A1 | + | - |
| *Anoxybacillus kuwalawohkensis* 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).

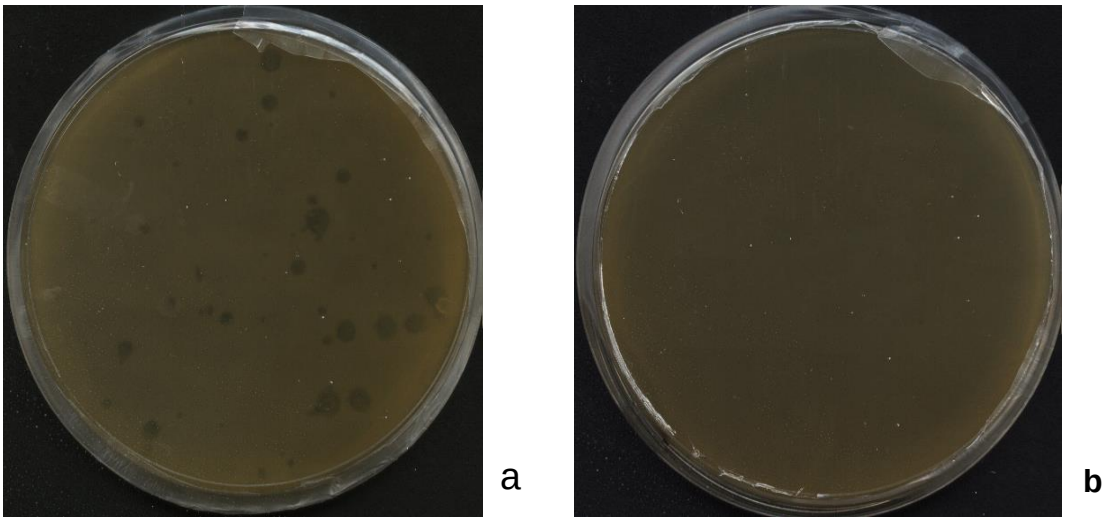


**Figure 3. 2** Plaque assay plates. a) A typical bacteriophage assay plate containing plaques of mixed morphologies dotting the lawn of *G. stearothermophilus* TAU3A1 host cells in the overlay agar after a fourth round of reinfection from a single plaque; b) A control plate of an uninfected *G. stearothermophilus* 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$^{o}$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$^{o}$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$^{o}$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 |
|---|---|
| *Anoxybacillus hidirlerensis* FUR6A2 | $1x10^6$ |
| *Anoxybacillus kuwalawohkensis G18A9* | $>10^7$ |
| FUR6A5 (unidentified) | $7x10^4$ |
| *Anoxybacillus hidirlerensis* FUR12A2 | $1x10^5$ |
| *Geobacillus stearothermophilus* RS93 | ----- |
| *G. stearothermophilus* RS239 | $1x10^5$ |
| *G. stearothermophilus* RS240 | $3x10^6$ |
| *G. stearothermophilus* RS241 | $3x10^7$ |
| *G. stearothermophilus* RS242 | $2x10^6$ |
| *G. stearothermophilus* NCA1503 (CAMR05329) | $5x10^7$ |
| *G. stearothermophilus* NCA1503 (CAMR05330) | $3x10^7$ |
| *G. stearothermophilus* TAU3A1 | $1x10^5$ |
| *G. thermoglucosidasius* 11955 | $1x10^5$ |
| DSM406 | $1x10^7$ |
| *Bacillus megaterium* | ----- |
| *Bacillus licheniformis* | $5x10^7$ |



-----: 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).

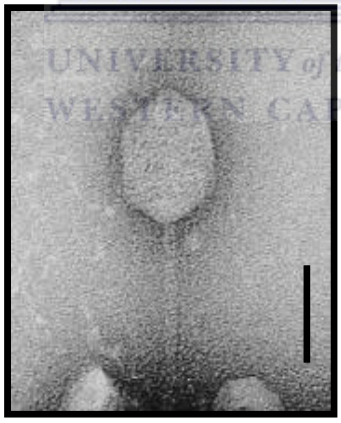



**Figure 3. 3** Transmission electron micrograph of a negatively stained GV1 particle.

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.

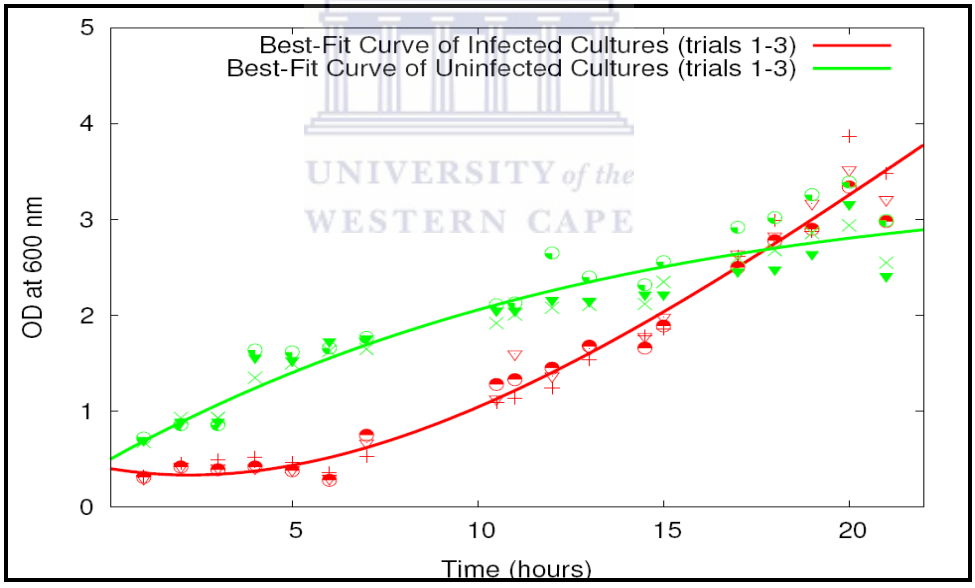




**Figure 3. 4** Growth of uninfected (green line) and infected (red line) cells of *G. stearothermophilus* RS241 with GV1 particles. Experiments were carried out in triplicate and data corresponding to the mean and the standard deviation were plotted as OD at 600 nm versus time (hours).

## 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 | $6.0 \times 10^3$ |
| 5.5 | $1.3 \times 10^6$ |
| 6.0 | $4.3 \times 10^6$ |
| 6.5 | $3.1 \times 10^7$ |
| 7.0 | $2.0 \times 10^6$ |
| 7.5 | $6.5 \times 10^6$ |
| 8.0 | $2.4 \times 10^4$ |
| 8.5 | $2.2 \times 10^2$ |



## 3.10. Effects of $CaCl_2$ and $MgCl_2$ on phage stability

The effects of concentrations of $CaCl_2$ and $MgCl_2$ on phage stability was investigated by adding actively growing *G. stearothermophilus* TAU3A1 cells to bacteriophage dilutions and transferring the mixture into TSBA soft agar supplemented with $CaCl_2$ or $MgCl_2$ concentrations ranging from 0 to 0.1 M. Phage titers were determined as previously described. Results showed that the incorporation of $CaCl_2$ 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 $MgCl_2$ addition to the assay medium (see Table 3.5).

**Table 3. 5** Effects of $CaC_2$ and $MgCl_2$ 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 | $2.1 \times 10^6$ | $3.2 \times 10^6$ |
| 0.001 | $3.3 \times 10^7$ | $6.1 \times 10^6$ |
| 0.01 | $1.3 \times 10^6$ | $3.9 \times 10^6$ |
| 0.1 | $3.0 \times 10^6$ | $2.5 \times 10^6$ |

## 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 $10^7$ pfu per ml were incubated at 50, 55, 60, 65, 70 and 75$^o$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$^o$C. Results showed that decreasing the incubation temperature resulted in an increase in titers, especially at temperatures below 60$^o$C (Table 3.6). It was evident that 55$^o$C was the optimum temperature for phage production and that there was a significant decrease in titers at temperatures above 60$^o$C.

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

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

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 | $1.8 \times 10^7$ | $1.4 \times 10^7$ |
| 20 | $1.5 \times 10^4$ | $2.3 \times 10^6$ |
| 30 | $4.0 \times 10^3$ | $3.7 \times 10^5$ |
| 60 | $3.3 \times 10^2$ | $1.1 \times 10^3$ |

## 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 ($5x10^7$), 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 µg/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 ng/µl to a 0.162 ng/µl. 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.



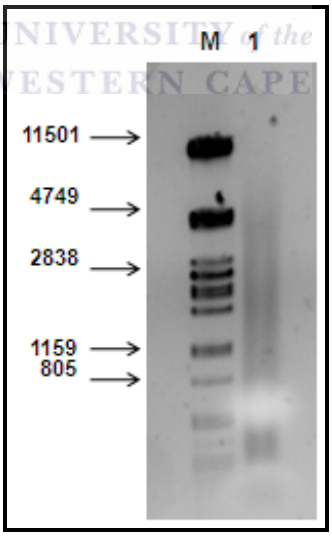


**Figure 3. 5** 0.8% agarose gel picture of sheared DNA obtained from purified *B. licheniformis* lysate. Lane M, Lambda phage DNA digested with *Pst*I; lanes 1, DNA sample. Arrows indicate specific markers' size.

## 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 *Mbo*I confirmed that the extracted DNA was already sheared, as no difference was found between the *Mbo*I 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.

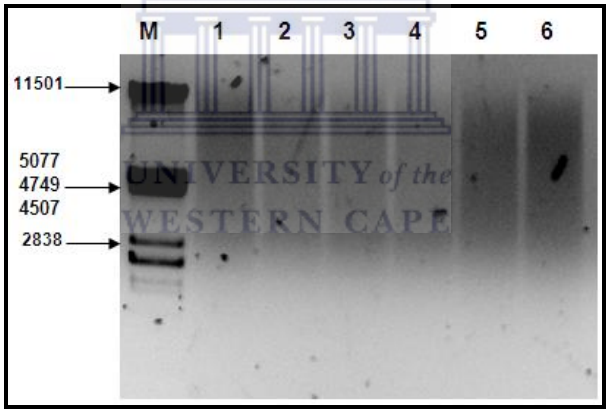


**Figure 3. 6** Partial digestion of DNA with *MboI.* Lane M, Lambda phage DNA digested with *Pst*I; lane 1, undigested DNA. Lanes 2-6, DNA digested with *MboI* for 1, 5, 10, 20 and 30 minutes respectively. Arrows indicate specific markers' size.

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 *Bgl*II) 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.).

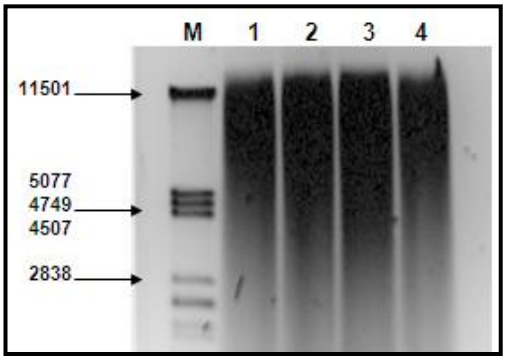


**Figure 3. 7** agarose gel showing end repaired DNA. Lane M, Lambda phage DNA digested with PstI; lane 1-6, end repaired DNA. Arrows indicate specific markers' size.

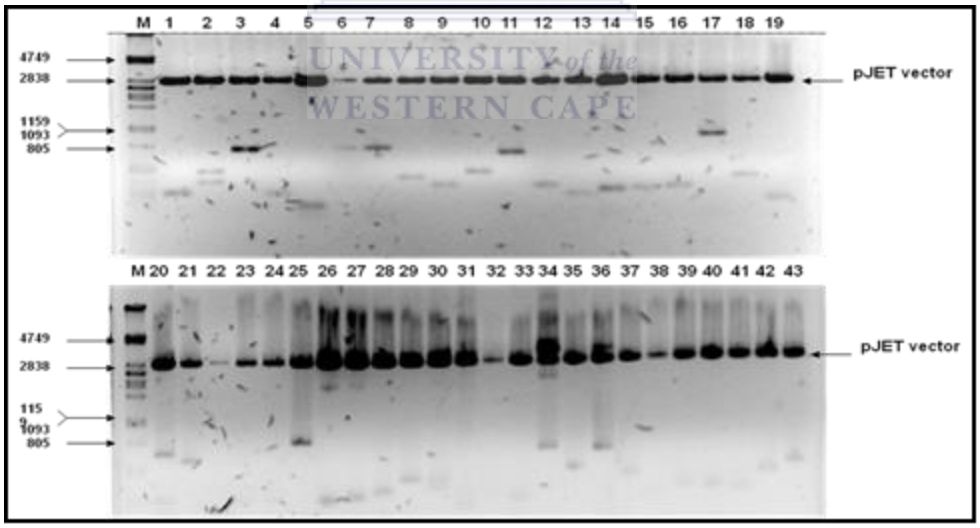


**Figure 3. 8** A representative agarose gel (0.8% agarose) showing the selection of positive clones. M, lambda *PstI* molecular weight maker. Lane 1-43, plasmid mini-prep products cut with *BglII*. The pJET vector DNA fragments are shown at position of about 2838 bp while most of the insert DNA fragments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

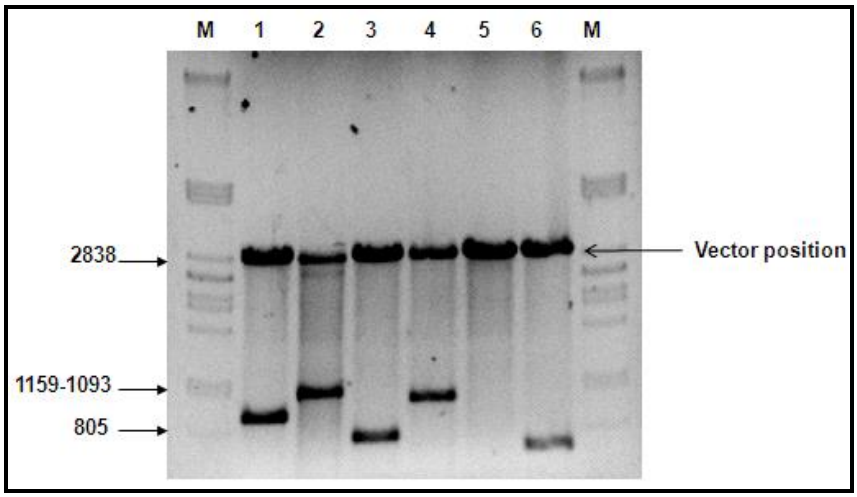


**Figure 3. 9** A representative agarose gel electrophoretogram showing five positive clones that were sequenced (lanes 1, 2, 3, 4, and 6). Lane M, Lambda phage DNA digested with *Pst*I; Lanes 1-6, *BglII* digested plasmid DNA.



## 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.

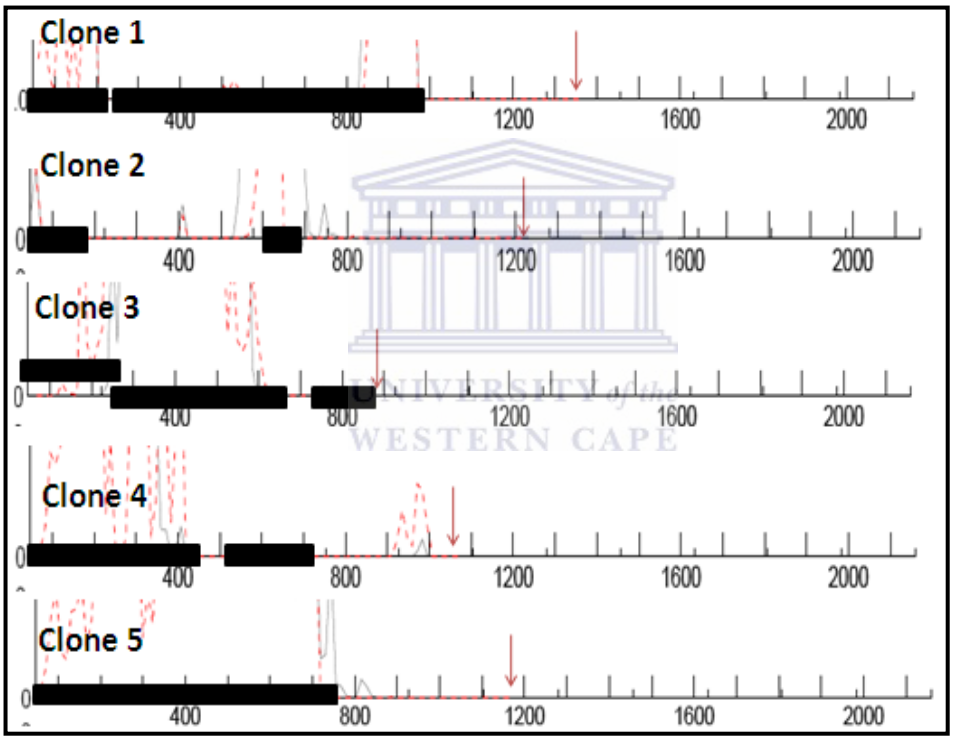


**Figure 3. 10** A graphic representation showing the positions of ORFs on the sequences. Black box (▬), ORF; Red arrow (↓), the end of analyzed sequences.

**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$^{o}$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 thermocatenulatus* strain BCRC (isolate TAU3A1).This was followed by a phylogenetic analysis performed based on 16S rRNA sequences, which showed that isloates 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$^{o}$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 x $10^7$ pfu per ml for $CaCl_2$ and 6.1 x $10^6$ 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$^{o}$C, with an optimum at 55$^{o}$C. The thermostable (or thermophilic) nature of the virus was clearly demonstrated by its ability to withstand exposure to 75$^{o}$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$^{o}$C in TSB medium is similar to the one of Φμ-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$^{o}$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$^{o}$C temperature range, with optimum at 55$^{o}$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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