Biological and molecular characterization of South African bacteriophages infective against *Staphylococcus aureus* subsp. *aureus* Rosenbach 1884, causal agent of bovine mastitis

by

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THESIS SUMMARY

Bacteriophage therapy has been exploited for the control of bacterial diseases in fauna, flora and humans. However, the advent of antibiotic therapy lead to a cessation of most phage research. Recently, the problem of antibiotic resistance has rendered many commonly used antibiotics ineffective, thereby renewing interest in phage therapy as an alternative source of control. This is particularly relevant in the case of bovine mastitis, an inflammatory disease of bovine mammary glands, caused by strains such as *Staphylococcus aureus* subsp. *aureus* Rosenbach 1884. Antibiotic resistance (primarily towards penicillin and methicillin) by staphylococcal strains causing mastitis is regularly reported. Phage therapy can provide a stable, effective and affordable system of mastitis control with little to no deleterious effect on the surrounding environment or the affected animal itself. Several studies have delved into the field of biocontrol of bovine mastitis using phages. Results are variable. While some phage-based products have been commercialized for the treatment of *S. aureus*-associated infections in humans, no products have yet been formulated specifically for the strains responsible for bovine mastitis. If the reliability of phage therapy can be resolved, then phages may become a primary form of control for bovine mastitis and other bacterial diseases.

This study investigated the presence of *S. aureus* and its phages in a dairy environment, as well as the lytic ability of phage isolates against antibiotic-resistant strains of mastitic *S. aureus*. The primary goals of the thesis were to review the available literature on bovine mastitis and its associated control, and then to link this information to the use of phages as potential control agents for the disease, to conduct *in vitro* bioassays on the selected phages, to conduct phage sensitivity assays to assess phage activity against different chemical and environmental stresses, to morphologically classify the selected phages using transmission electron microscopy, to characterize the phage proteins using one-dimensional electrophoresis, and lastly, to characterize phage genomes, using both electrophoresis as well as full genome sequencing.

Twenty-eight phages were isolated and screened against four strains of *S. aureus*. Only six phages showed potential for further testing, based on their wide host range, high titres and common growth requirements. Optimal growth conditions for the host *S. aureus* strain was 37°C for 12hr. This allowed for optimal phage replication. At an optimal titre of between 6.2×10^7 to

2.9×10^8 pfu.ml^{-1} (at 10^{-5} dilution of phage stock), these phages were able to reduce live bacterial cell counts by 64-95%. In addition, all six phages showed pathogenicity towards another 18 *S. aureus* strains that were isolated from different milk-producing regions during a farm survey. These six phages were named Sabp-P1, Sabp-P2, Sabp-P3, Sabp-P4, Sabp-P5 and Sabp-P6.

Sensitivity bioassays, towards simulated environmental and formulation stresses were conducted on six identified phages. Phages Sabp-P1, Sabp-P2 and Sabp-P3 showed the most stable replication rates at increasing temperatures (45-70°C), in comparison to phages Sabp-P4, Sabp-P5 and Sabp-P6. The effect of temperature on storage of phages showed that 4ºC was the minimum temperature at which phages could be stored without a significant reduction in their lytic and replication abilities. Furthermore, all phages showed varying levels of sensitivity to chloroform exposure, with Sabp-P5 exhibiting the highest level of reduction in activity (74.23%) in comparison to the other phages. All six phages showed optimal lytic ability at pH 6.0-7.0 and reduced activity at any pH above or below pH 6.0-7.0. Exposure of phages to varying glycerol concentrations (5-100%) produced variable results. All six phages were most stable at a glycerol concentration of 10-15%. Three of the six isolated phages, Sabp-P1, Sabp-P2 and Sabp-P3, performed optimally during the *in vitro* assays and were used for the remainder of the study.

Morphological classification of phages Sabp-P1, Sabp-P2 and Sabp-P3 was carried out using transmission electron microscopy. All three phages appeared structurally similar. Each possessed an icosahedral head separated from a striated, contractile tail region by a constricted neck region. The head capsules ranged in diameter between 90-110nm with the tail length ranging from 150-200nm in the non-contractile state and 100-130nm in the contractile state. Rigid tail fibres were also visible below the striated tail. The major steps in the virus replicative cycle were also documented as electron micrographs. Ultra-thin sections through phage plaques were prepared through a modification of traditional methods to speed up the process, with no negative effects on sample integrity. The major steps that were captured in the phage replicative cycle were (1) attachment to host cells, (2) replication within host cells, and, (3) release from cells. Overall results suggested that all three phages are strains from the order *Caudovirales* and are part of the *Myoviridae* family.

A wealth of information can be derived about an organism based on analysis of its proteomic data. In the current study, one-dimensional electrophoretic methods, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and ultra-thin layer isoelectric focusing (UTLIEF), were used to analyse the proteins of three phages, Sabp-P1, Sabp-P2 and Sabp-P3, in order to determine whether these strains differed from each other. SDS-PAGE analysis produced unique protein profiles for each phage, with band fragments ranging in size from 8.86-171.66kDa. Combined similarity matrices showed an 84.62% similarity between Sabp-P1 and Sabp-P2 and a 73.33% similarity between Sabp-P1 and Sabp-P3. Sabp-P2 showed a 69.23% similarity to Sabp-P3. UTLIEF analysis showed protein isoelectric charges in the range of pI 4.21-8.13, for all three phages. The isoelectric profiles for each phage were distinct from each other. A combined similarity matrix of both SDS-PAGE and UTLIEF data showed an 80.00% similarity between phages Sabp-P1 and Sabp-P2, and a 68.29% similarity between Sabp-P1 and Sabp-P3. Sabp-P2 showed a 70.59% similarity to Sabp-P3. Although the current results are based on putative protein fragments analysis, it can be confirmed that phages Sabp-P1, Sabp-P2 and Sabp-P3 are three distinct phages.

This was further confirmed through genomic characterization of the three staphylococcal phages, Sabp-P1, Sabp-P2 and Sabp-P3, using restriction fragment length analysis and whole genome sequencing. Results showed that the genomes of phages Sabp-P1, Sabp-P2 and Sabp-P3 were all different from each other. Phages Sabp-P1 and Sabp-P3 showed sequence homology to a particular form of *Pseudomonas* phages, called "giant" phages. Phage Sabp-P3 showed sequence homology to a *Clostridium perfringens* phage. Major phage functional proteins (the tail tape measure protein, virion structural proteins, head morphogenesis proteins, and capsid proteins) were identified in all three phages. However, although the level of sequence similarity between the screened phages and those already found on the databases, enabled preliminary classification of the phages into the order *Caudovirales*, family *Myoviridae*, the level of homology was not sufficient enough to assign each phage to a particular type species. These results suggest that phage Sabp-P1 might be a new species of phage within the *Myoviridae* family. One longer-term objective of the study is to carry out complete assembly and annotation of all the contigs for each phage. This will provide definitive conclusions in terms of phage relatedness and classification.

DECLARATION

I, Iona Hershna Basdew, declare that:

(i) The research reported in this thesis, except where otherwise indicated, is my original work.

(ii) This thesis has not been submitted for any degree or examination at any other university.

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THESIS INTRODUCTION

Bovine mastitis is an infectious inflammation of the mammary glands that interferes with the normal flow and quality of milk (Blowey and Edmondson, 2010). This disease is a major cause of economic losses in the dairy industry worldwide. Direct costs can be incurred through discarded milk, and drug and veterinary costs, while indirect costs can be incurred through milk price penalties as a result of increased somatic cell counts (SCC), decreased milk yield due to udder damage, additional labour requirements for treating infected cows, and higher culling and replacement costs (Blowey and Edmondson, 2010; Mubarack *et al.*, 2011). In the United States alone, annual losses are estimated to be 2 billion USD, and worldwide, mastitis is associated with economic losses of up to 35 billion USD annually (Jones and Bailey, 2009; Van den Borne, 2010; Mubarack *et al.*, 2011).

The wide range of organisms that can cause the disease, and the ubiquity of these causal agents, makes complete eradication of the disease from a dairy system virtually impossible. Traditional methods of disease control revolved around the use of antibiotic treatment of infected cows. However, the development of resistance by the major causal organisms, against those antibiotics commonly used to treat the disease, has reinforced the importance of integrated control measures. This scenario is particularly relevant to bovine mastitis induced by *Staphylococcus aureus* subsp. *aureus* Rosenbach 1884. Numerous strains have developed antibiotic resistance. Resistance was noted to have first developed against penicillin, after which methicillin became the treatment of choice (Vanderhaeghen *et al.*, 2010). However, the development of resistance to methicillin shifted treatment to the use of vancomycin, and, as anticipated, some strains of *S. aureus* have now developed resistance towards vancomycin (Vanderhaeghen *et al.*, 2010). As a result, interest has shifted from the more conventional antibiotic therapies towards the field of biological control of the disease.

One such biocontrol option involves the use of lytic bacterial viruses (bacteriophages) that are specific to *S. aureus*. Phage therapy has been applied to different disciplines, ranging from human and veterinary medicine to agricultural settings. There have been increased applications of phages in the food industry to control major food pathogens such as *Listeria monocytogenes* (Murray *et al. 1926*), *Salmonella*, *Campylobacter* and pathogenic *Escherichia coli* (Hagens and Offerhaus, 2008); in human medicine, to treat burns, contaminated wounds,

diarrheal diseases (Soothill, 1994) and for use against methicillin-resistant *Staphylococcus aureus* (Rosenbach) (MRSA) (Capparelli *et al.*, 2007). In the United States, in the past two years alone, several phage applications have been approved for use. The most prominent include Listex (an organic anti-*Listeria* phage*)* (Hagens and Offerhaus, 2008), an anti-*Escherichia coli* wash, and an anti-*Salmonella* wash for the treatment of live animals prior to slaughter (www.omnilytics.com). In addition to phage therapy, phage use can be extended to the field of phage display, vaccine delivery and pathogen detection. There has been significant progress, using animal models, in the field of control of *S. aureus* using phages. However, no single phage-based product has yet been commercialized for use against bovine mastitis, despite the clear need for a more sustainable control option to manage the disease. Phages are specific for their target bacterium and hence create no negative effects on the surrounding mammary tissues or the environment. This, coupled with their ability to multiply up to 1000-fold within a host cell, makes the phage an ideal candidate for the biocontrol of bovine mastitis (Blowey and Edmondson, 2010).

With this background in mind, the long-term objective of the current project was to use phages as a supplementary treatment against bovine mastitis. However, prior to any *in vivo* applications, *in vitro* biological and molecular classification of isolated phages was essential in order to understand the organism before field application. The objectives of the current study were therefore as follows:

1. Review available literature, firstly on bovine mastitis, in order to properly understand the course of infection, treatment protocols and associated drawbacks, and secondly, to link this information to the use of phages as potential biological control agents for the disease.

2. Conduct biological activity assays on phages isolated from the dairy environment, in order to assess their *in vitro* ability to control South African strains of *S. aureus*.

3. Investigation of phage sensitivity, *in vitro*, towards simulated environmental and chemical stresses. This study aimed to contribute to phage formulations for eventual *in vivo* application.

4. Classification of three phages with activity against South African strains of *S. aureus*, according to their morphology and replicative cycle, using transmission electron microscopy.

5. Characterisation of phage proteins using one-dimensional electrophoretic methods, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and ultra-thin layer isoelectric focusing (UTLIEF), in order to determine whether the selected strains differed from each other.

6. Characterization of phage genomes, using both electrophoresis as well as full genome sequencing, in order to verify whether or not each selected strain is indeed different from the others.

This dissertation is comprised of six discreet chapters; one being a review of literature, followed by five research chapters, each covering a specific aspect of phage classification and characterization. Each research chapter has been compiled in the form of a discreet, scientific paper. As a result, there has been some repetition of references between chapters. This is the standard format adopted for dissertations by the University of KwaZulu-Natal. All references have been formatted in accordance with the Journal of General Virology.

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CHAPTER 1

Review of Literature

1.1 Introduction

Bovine mastitis is an infectious inflammation or irritation of the mammary glands that interferes with the normal flow and quality of milk (Blowey and Edmondson, 2010). Although a range of control measures are used, this disease remains a major cause of economic losses in the dairy industry worldwide. While some diseases, such as foot-and-mouth, can be completely eliminated from a system using culling and vaccination protocols, bovine mastitis is an endemic disease that cannot be completely eradicated. The wide range of microorganisms that can cause this disease, and the ubiquity of these organisms, make complete eradication unlikely. Optimum control therefore lies in first understanding the epidemiology of the disease and the causal agents, and then implementing an integrated control strategy.

In recent years, the use of integrated control measures has become paramount, due to the onset of resistance against the antibiotics commonly used to treat the disease. This is particularly relevant to the primary causal bacterium of bovine mastitis, *Staphylococcus aureus* subsp. *aureus* Rosenbach 1884. Numerous strains have developed antibiotic resistance. Resistance was noted to have first developed against penicillin (both β-lactam and non- β-lactam), after which methicillin became the treatment of choice (Vanderhaeghen *et al.* 2010). However, the development of resistance to methicillin shifted treatment to the use of vancomycin, but some strains of *S. aureus* have now developed resistance towards vancomycin. The financial implications of such resistance within a herd of infected cows can be substantial. Losses can exceed 200USD per cow because culling of infected animals serves as the final control measure (Borm *et al.* 2006). As a result, interest has shifted from the more conventional antibiotic therapies towards the field of biological control of the disease.

One such biocontrol option involves the use of lytic bacterial viruses (bacteriophages) that are specific to *S. aureus*. Phage therapy has been applied to different disciplines, ranging from human and veterinary medicine to agricultural settings. There have been increased applications of phages in the food industry to control major food pathogens such as *Listeria monocytogenes* (E. Murray *et al.* 1926), *Salmonella enterica* (ex. Kaufmann and Edwards 1952), *Campylobacter* Sebald and Véron 1963 and pathogenic *Escherichia coli* Castellani and Chalmers

1919 (Hagens and Offerhaus, 2008); in human medicine, to treat burns, contaminated wounds, diarrheal diseases (Soothill, 1994) and for use against methicillin-resistant *S. aureus* (MRSA) (Capparelli *et al.* 2007). In the United States, in the past two years alone, several phage applications have been approved for use. The most prominent include Listex (an organic anti-*Listeria* phage) (Hagens and Offerhaus, 2008), an anti-*E. coli* wash, and an anti-*Salmonella* wash for the treatment of live animals prior to slaughter (www.omnilytics.com). Phage use can be extended to the field of phage display, vaccine delivery and pathogen detection (Chanishvilli *et al.* 2001). In addition, there has been significant progress, using animal models, in the field of control of *S. aureus* using phages (Soothill, 1992; Kelly *et al.* 2011). However, no single phage-based product has yet been commercialized for use against bovine mastitis, despite the clear need for a more sustainable control option to manage the disease.

This review covers the biology and current control of bovine mastitis and assesses the use of phages as an alternative or supplementary therapy for the disease.

1.2 Types of mastitis

Due to the more than 200 recorded organisms that are able to cause mastitis (Ranjan *et al.* 2006) and the varying levels of infection associated with each, there exists a complete lexicon to describe the disease and classify it according to specific organisms. A simple classification method recognizes two distinct forms of the disease, contagious and environmental (Blowey and Edmondson, 2010). It must be understood however, that proper identification of the disease using bacteriological analysis of causal agents must be undertaken in order to definitively classify the type of mastitis that the animal is afflicted with.

1.2.1 Contagious mastitis

Pathogens that are spread from cow to cow primarily during milking are referred to as contagious pathogens (Tyler and Ensminger, 1993). The mammary glands and teat skin serve as reservoirs of infections with colonies establishing at the teat end and slowly growing into the teat canal over 1-3 days (Blowey and Edmondson, 2010). Commonly used control measures include postmilking teat dipping, dry cow therapy, milking hygiene and culling. Among the contagious organisms, *S.*

aureus, *Streptococcus agalactiae* Rosenbach 1884 and *Streptococcus dysgalactiae* Rosenbach 1884 have been identified as the major causes of bovine mastitis in many countries (Rebhun, 1995; Sandgren *et al.* 2008; Nickerson, 2009).

S. aureus organisms are haemolytic, Gram-positive cocci that appear as cream-white to yellow colonies on solid growth media (Madigan *et al.* 2012). The primary reservoir for the bacterium is within the mammary gland itself. Staphylococci are exceptionally difficult to eliminate once infection is established within a system. Trials have shown that a cow releasing *S. aureus* in her milk may contaminate the teats of the next 6 to 8 cows to be milked (Blowey and Edmondson, 2010). It therefore makes sense that if it is present in a herd, certain mandatory precautions should be applied: postmilking teat disinfection is vital; infected cows should be milked last and in a separate group using separate machinery; and teat skin should be maintained in an optimum condition to prevent entry of bacteria through broken/chapped surfaces. Under certain circumstances, *S. aureus* can also cause an acute gangrenous mastitis (Blowey and Edmondson, 2010). This form of mastitis is not caused by a specific acute strain of the bacterium but rather by a change in the immune system of the cow as a result of a chronic *S. aureus* infection (Blowey and Edmondson, 2010). The clinical appearance of gangrenous mastitis is characterized by a blue/black discolouration of teat and udder skin (Rebhun, 1995; Blowey and Edmondson, 2010). The teats and udder also feel cold and sticky to the touch and can develop small blisters (Rebhun, 1995; Blowey and Edmondson, 2010).

Strep. agalactiae is a Gram-positive alpha-haemolytic coccus that appears as very small colonies that exhibit a blue hue on Edwards medium (Madigan *et al.* 2012). Its primary reservoir of infection is in the udder, however, it has been found to colonise the teat canal and even the teat skin, particularly if these surfaces are chapped (Rebhun, 1995; Nickerson, 2009; Petzer *et al.* 2009). These organisms do not cause overt abscesses or fibrosis but will permanently decrease productivity in infected glands (Rebhun, 1995; Nickerson, 2009).

Strep. dysgalactiae is also a Gram-positive haemolytic coccus with very small colonies that cause a green discolouration of Edwards medium (Madigan *et al.* 2012). While this organism does share many of the properties applicable to *S. aureus* and *Strep. agalactiae*, a few distinct differences do exist. *Strep. dysgalactiae* survives well in the environment and may be considered to be halfway between contagious and environmental organisms. It is commonly found on teat

skin, particularly when the surface integrity is compromised by chaps, cuts or bruises (Rebhun, 1995; Nickerson, 2009). *Strep. dysgalactiae* is also present on the tonsils of the cow and hence licking could also transmit infection to the teats (Rebhun, 1995; Nickerson, 2009). Conditions such as teat irritation associated with flies or chapping due to cold weather might encourage an animal to lick its teats and hence transfer infection which gradually colonises the teat canal until clinical mastitis occurs (Rebhun, 1995; Nickerson, 2009).

Contagious mastitis can be divided into three major groups: (a) Clinical mastitis is commonly characterized by gross inflammation of the udder, e.g., swelling, redness, heat, and also by anomalies in the appearance of milk, e.g., clots and changes in the colour and smell of the milk (Rebhun, 1995; Blowey and Edmondson, 2010). Clinical mastitis can be further divided into peracute mastitis which is characterized by gross inflammation, disrupted functions (reduction in milk yields, changes in milk composition) and systemic signs (fever, depression, loss of appetite, shivering, loss of weight) (Figure 1.1); acute mastitis which is similar to the peracute form, but with lesser systemic symptoms (fever and mild depression); sub-acute mastitis where the mammary gland infection is minimal and there are no visible systemic symptoms (Blowey and Edmondson, 2010); (b) Sub-clinical mastitis is commonly characterized by a change in milk composition with no signs of gross inflammation or milk abnormalities (Rebhun, 1995; Blowey and Edmondson, 2010). The changes in milk composition have to be detected using special diagnostic tests such as somatic somatic cell counts (SCC), electrical conductivity tests, culture and pH tests and enzymatic analyses (Viguier *et al.* 2009; Reyher and Dohoo, 2011); (c) Chronic mastitis refers to a form of the disease where inflammation exists for months, and may continue from one lactation to another (Rebhun, 1995; Blowey and Edmondson, 2010). Chronic mastitis, for the most part, exists in a sub-clinical form but may exhibit periodic flare-ups into the acute or sub-acute forms for short periods of time (Rebhun, 1995).

1.2.2 Environmental mastitis

This type of mastitis is caused by organisms that do not normally live on the surface of the skin or in the udder, but which enter the teat canal when the cow comes into contact with a contaminated environment (Blowey and Edmondson, 2010). These pathogens can normally be found in faeces, bedding material, farmyard and feed. They are usually transferred from the reservoir to the teats between milking (Blowey and Edmondson, 2010). Control of environmental

mastitis is usually achieved far more successfully than with the contagious form of the disease by implementing proper environmental hygiene, predipping teats and applying dry period teat sealants. Among the environmental organisms, coliforms (especially *E. coli*) and *Streptococcus uberis* have been identified as the major problematic microbes.

Figure 1.1 Healthy udder (a) alongside a mastitic udder (b); (a) www.indiamart.com; (b) www.valleyveterinarygroup.com.

E. coli is a Gram-negative rod-shaped bacterium which produces grey mucoid colonies in blood agar, with strains that are both haemolytic and non-haemolytic (Madigan *et al.* 2012). *E. coli* is present in high numbers in faeces and hence infection occurs primarily when animal housing conditions are wet, and when hygiene is poor. During lactation, *E. coli* is thought to enter the teat canal by propulsion and hence increased mastitis is seen with dirty teats, suboptimal machinery function or techniques that lead to teat-end impacts (Blowey and Edmondson, 2010). *E. coli* penetration of the teat canal though, does not always result in chronic infection, with a high percentage (80-90%) of infections undergoing self-cure (Blowey and Edmondson, 2010). Typical symptoms include slight damage to the endothelial lining of the teat wall that manifests as flaky clots in milk, and a hard, hot swollen quarter with a watery discharge (Nickerson, 2009; Blowey and Edmondson, 2010). The toxic effects of *E. coli* mastitis are due to the release of an endotoxin (typically lipopolysaccharide) that is derived from the bacterial cell wall. The accumulation of this toxin results in intense haemorrhaging of the endothelial lining of the teat wall (Blowey and Edmondson, 2010). At times, damage to the blood vessels is so severe

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that serum ooze can be seen on the surface of the udder and teat. Such cases can lead to endotoxaemia, extensive gangrene and eventual sloughing of the udder tissue (Nickerson, 2009; Blowey and Edmondson, 2010).

Strep.uberis is a Gram-positive coccus that is non-haemolytic and produces brown colonies on Edwards medium (Madigan et al. 2012). A typical case of Strep. uberis mastitis is often sudden in onset, and produces a hard, swollen udder, very high body temperatures and milk with large white clots (Nickerson, 2009; Blowey and Edmondson, 2010). It is particularly associated with straw yards where as much as 10^6 organisms per gram of straw have been reported (Blowey and Edmondson, 2010). In addition to being found in the environment, Strep. uberis can also be found on a wide range of sites on the animal itself, e.g., mouth, vulva, groin and axilla (Nickerson, 2009; Blowey and Edmondson, 2010). Although present in faeces, levels are not significantly high, and in this respect Strep. uberis differs from E. coli. Following infection, Strep. uberis has been found to remain persistent in the udder for an average of 1.5 months after treatment (Nickerson, 2009). This poor response to treatment and the long refractory period within the udder are possibly due to its resistance to phagocytosis, its ability to survive within animal cells where it is protected from antibiotics and its ability to move into the lymph node from where it maintains a reservoir of infection (Nickerson, 2009).

1.3 The effects of mastitis on milk synthesis and milk proteins

The effects of mastitis on milk yield and composition are of significant economic importance to the dairy farmer. Direct costs can be incurred through discarded milk and drug and veterinary costs, while indirect costs can be incurred through penalties as a result of increased SCC, decreased milk yields due to udder damage, additional labour requirements for treating infected cows, and higher culling and replacement costs (Blowey and Edmondson, 2010; Mubarack et al. 2011). In the United States alone, animal losses are estimated to approach 2 billion USD. Worldwide, it is associated with economic losses of up to 35 billion USD annually (Jones and Bailey, 2009; Van den Borne, 2010; Mubarack et al. 2011). On average, a quarter infected with a major pathogen will yield approximately 30% less milk than an equivalent uninfected quarter of the same cow (Kudi et al. 2009; Blowey and Edmondson, 2010). Infected cows are also culled more quickly either because of repeated clinical infections or reduced milk yields.

In addition, mastitis has a major effect on the taste and quality of milk mainly as a result of its effects on the lactose, protein and fat content of milk; typically affected milk is rancid and slightly salty/bitter to the taste (Table 1.1) (Mubarack *et al.* 2011). As a result of diminished milk quality, overall income is reduced as the farmer receives a lower price (up to 20% less per litre) for such milk (Mubarack *et al.* 2011).

Table 1.1 Effects of mastitis on milk quality (adapted from Hardy, 1995a, b; Batavani *et al.* 2007; Ogola *et al.* 2007; Blowey and Edmondson, 2010; Mubarack *et al.* 2011).

Component	Normal milk (mg.l^{-1})	Sub-clinical mastitic milk (mg.l^{-1})
Calcium	126.29	90.45
Phosphorous	30.59	24.40
Potassium	167.74	15.56
Total proteins (%)		
α-lactalbumin	28.72	22.25
β-lactoglobulin	57.08	34.21
Albumin	6.99	17.21
Pre-albumin	0.18	0.09
Undesirable components		
Plasmin (degrades casein	Low	High
Lipase (breaks down fat)	Low	High
Sodium	Low	High
Immunoglobulin	Low	High

Lactose

Glucose, produced in the cow liver as a result of rumen fermentation, is transferred to the udder where part of it is converted to the simple sugar, galactose (Walstra *et al.* 2006; Blowey and Edmondson, 2010). Then one molecule of glucose combines with one molecule of galactose to form the disaccharide, lactose. Lactose is the main osmotic determinant of milk (Blowey and Edmondson, 2010). Lactose concentration increases and decreases as the concentration of the other milk components varies, in order to maintain milk at the same concentration as blood

(Harding, 1995a; Walstra *et al.* 2006; Blowey and Edmondson, 2010). However, the pH of milk is slightly lower than that of blood, i.e. 6.7 and 7.4, respectively (Blowey and Edmondson, 2010). This difference has been used to attract drugs such as erythromycin, trimethoprim and penethamate into the mammary gland (Walstra *et al.* 2006; Blowey and Edmondson, 2010).

If lactose concentrations within the udder drop, then sodium and chloride levels increase to maintain osmotic potential of the milk (Walstra *et al.* 2006; Blowey and Edmondson, 2010). This is one of the reasons for the bitter and slightly salty taste of mastitic milk. These changes can also be used to assess mastitis status by measuring the electrical conductivity of the milk as sodium and chloride are much better conductors of electricity than lactose is (Harding, 1995a, b; Walstra *et al.* 2006; Blowey and Edmondson, 2010).

Protein

The majority of protein in milk is in the form of casein (Walstra *et al.* 2006; Blowey and Edmondson, 2010). Amino acids are transported to the udder via the bloodstream and transformed into casein by the mammary alveolar cells (Harding 1995a; Blowey and Edmondson, 2010). Other proteins present in milk but in smaller quantities than casein, are albumin and globulins (Harmon, 1995; Ogola *et al.* 2007). These are transferred straight from the blood stream into milk. Mastitic milk on the other hand, contains reduced levels of casein and increased levels of albumin and globulin (Blowey and Edmondson, 2010).

Hence, while the total protein content may remain unchanged, the milk is of poor quality particularly, for manufacturing processes such as cheese and yoghurt production where casein coagulation serves as one of the major starting processes (Harmon, 1995). In addition, mastitic milk contains high levels of the enzyme plasmin, which degrades casein in stored milk. This enzyme is not destroyed during pasteurisation and remains active in milk even at 4°C implying that milk will continue to be degraded even while it is on supermarket shelves (Harmon, 1995).

Milk fat

Milk fat is formed in the udder secretory cells in the alveoli when fatty acids combine with glycerol to form a neutral form of fat called a triglyceride (Walstra *et al.* 2006; Blowey and Edmondson, 2010). Fatty acids are derived from three main sources, i.e., body fat (50% of total

fatty acids), dietary fat and fatty acids synthesized in the udder from acetate (which is absorbed as a product of rumen fermentation) (Ogola *et al.* 2007; Walstra *et al.* 2006; Blowey and Edmondson, 2010). Aside from the enzyme plasmin, mastitic milk also has an increased level of the enzyme lipase (Harding, 1995b). This leads to degradation of milk fat into its fatty acid components and thus imparts a rancid flavour to the milk. Increased levels of fatty acids can also inhibit starter cultures in cheese and yoghurt manufacture and impart an adverse flavour to these products (Harding, 1995b).

1.4 Disease cycle and epidemiology

A sound knowledge of the disease cycle is required as a basis for the implementation of control measures to ensure that the most appropriate options are applied to treat specific phases of the disease. Mastitis occurs in the following sequence: (i) arrival of a reservoir of infection, (ii) transfer of infection from the reservoir to the teat end of the animal, (iii) penetration of the teat canal by the microbe, (iv) colonization of host tissues within the teat and udder (Blowey and Edmondson, 2010).

1.4.1 Arrival of a reservoir of infection

Bovine mastitis has been characterised according to the primary source of organisms that lead to infection. The facilities in which dairy cows are maintained serves as one such reservoir with microbes such as *E. coli*, *Strep. uberis*, certain *Bacillus* spp., fungi and yeasts always being present (Madigan *et al.* 2012). These organisms can inhabit the environment without causing harm to the animals. However, a problem arises when there is a sudden change in environmental conditions which promotes the rapid reproduction of any single species of microbe. This results in an increased challenge of infection on the teat end, especially if the teat is soiled or damaged. Typical environmental reservoirs include dairy workers, housing and bedding quarters, farmyards and milking machines.

Other infections are normally only present in the udder of infected cows (Blowey and Edmondson, 2010). The arrival of a reservoir in this instance indicates either the purchase of a new cow or also an infected cow calving down into a herd. This infection is therefore regarded as

'contagious' as it passes from one cow to the next and includes organisms such as *S. aureus*, *Strep. agalactiae* and *Strep. dysgalactiae* (Blowey and Edmondson, 2010).

1.4.2 Transfer of infection from reservoir to teat end

This generally occurs between milkings for environmental organisms, as the first stage in the establishment of new infection is the transfer of microbes from the environment to the teat end (Blowey and Edmondson, 2010). For contagious mastitis however, transfer occurs during the milking process and vectors (such as udder cloths, milkers' hands, and milking machines that are not properly sanitised) which are needed to carry the bacteria from the infected cow (or quarter) to a non-infected cow (or quarter). Furthermore, after milking, the teat canal remains dilated for 1-2 hours while the canal of a damaged teat may remain partially open (Blowey and Edmondson, 2010). This facilitates movement of organisms from either the environment or surrounding/injured skin into the teat canal.

1.4.3 Penetration of the teat canal

There are two ways in which bacteria commonly penetrate the teat canal. Contagious organisms such as *S. aureus* and *Strep. agalactiae* have strong adhesive factors that enable them to grow into the teat canal by attaching to the teat end (Blowey and Edmondson, 2010; Madigan *et al.* 2012). Here they multiply and colonise the teat-end and then literally grow up through the teat canal and enter the teat sinus. The second method of entry is propulsion through the canal (Blowey and Edmondson, 2010; Madigan *et al.* 2012). Environmental pathogens such as *E. coli* commonly use this method because they lack adhesive properties (Madigan *et al.* 2012). They are, therefore, forced through the canal, usually with a reverse flow of milk. This typically occurs with teat-end impacts which are forces that result in milk particles being propelled from the milk-tubes attached to the cow teats up against the teat-end (Blowey and Edmondson, 2010; Madigan *et al.* 2012). Teat-end impacts usually occur when air enters between the teat and lining of the milking machine, leading to an imbalance of pressure between the teat-end and the milking unit (Blowey and Edmondson, 2010). These two modes of bacterial entry to the teat canal are by no means as distinctive as this. It must be noted that a reverse flow of milk will also assist the

movement of contagious pathogens into the teat canal and that high teat-end challenge immediately after milking could facilitate movement of environmental organisms into the teat canal without the need for reverse flow of milk (Blowey and Edmondson, 2010).

1.4.4 Colonisation of host tissues

Once bacterial infection is established, bacteria multiply rapidly. As they multiply, they enter the glandular tissues and compete with milk-secreting alveolar cells for nourishment (Tyler and Ensminger, 1993). Toxins produced through bacterial metabolism eventually causes death of the mammary alveoli (toxaemia) (Tyler and Ensminger, 1993; Blowey and Edmondson, 2010). In addition, they may mechanically block normal circulation and change the filtering ability of membranes. In chronic infections, the teat duct can become so inflamed that it becomes completely blocked off (Tyler and Ensminger, 1993). If prompt treatment is not implemented at this stage, scar tissue develops and the ducts become permanently blocked. In acute mastitis, blood vessels become greatly dilated, causing stagnation (Tyler and Ensminger, 1993; Blowey and Edmondson, 2010). Milk ducts become compressed so that little or no milk is formed, and the milk that is formed cannot be withdrawn (Tyler and Ensminger, 1993; Blowey and Edmondson, 2010). As a result of these changes, medication cannot be effectively delivered into the gland. Essentially, the longer the mastitis is allowed to persist, the more alveolar cells will be damaged followed by further blockages in the teat ducts and hence significant decreases in milk production potential.

1.5 Teat and udder defences

Although bacteria may penetrate the teat canal and enter the udder, the cow is by no means completely defenceless. There are a variety of ways in which the teats and udder are able to overcome infection.

1.5.1 Teat defences

Teat skin has a thick covering of stratified squamous epithelium, the surface of which consists of dead cells filled with keratin (Tyler and Ensminger, 1993; Walstra *et al.* 2006; Blowey and

Edmondson, 2010). When intact, this provides a hostile environment for bacteria, hence preventing their growth. In addition, there are bacteriostatic fatty acids present on skin that also prevent bacterial growth (Tyler and Ensminger, 1993; Walstra *et al.* 2006; Blowey and Edmondson, 2010). These bacteriostatic properties can be reduced by continual washing of teats after milking, especially with detergents.

The teat canal ranges from 5-13mm long and is lined with keratinised skin epidermis covered in a thin film of lipid keratin (Tyler and Ensminger, 1993; Walstra *et al.* 2006). This lipid has similar bacteriostatic properties to the teat skin. These properties are most effective when contraction of the sphincter muscle leads to canal closure (Tyler and Ensminger, 1993; Walstra *et al.* 2006). At teat closure, the sphincter muscles contract, the folds interdigitate to form a tight seal and the hydrophobic lipid lining ensures that no residual continuous column of milk persists within the canal (Tyler and Ensminger, 1993; Walstra *et al.* 2006; Blowey and Edmondson, 2010). Damage to the canal lining and lipid seal could result in a persistent residual column of milk, which can act as a 'wick' for bacterial entry (Blowey and Edmondson, 2010). The teat canal also contains specialised cells called the Rosette of Furstenberg (Blowey and Edmondson, 2010). This is on the inner side of the teat canal and is formed by a ring of lymphocyte cells that detect invading bacteria and initiate an immune response (Blowey and Edmondson, 2010).

Many bacteria entering the teat between milkings become trapped by the layer of keratin and lipid that lines the teat canal. They are then flushed out at the start of the next milking by the first flow of milk, as this removes the superficial layers of keratin lining the teat canal (Tyler and Ensminger, 1993; Blowey and Edmondson, 2010). This process is known as the 'keratin flush'.

It is very important to ensure that udder preparation and unit attachment are such that milk flows out of the teat when the milking cluster is applied and that there is no reverse flow of milk which could carry infection back into the udder (Tyler and Ensminger, 1993; Blowey and Edmondson, 2010). In addition, during the dry period (i.e., when lactogenesis is stopped), a mixture of wax and keratin accumulates in the teat canal to form a physical plug (Tyler and Ensminger, 1993; Blowey and Edmondson, 2010). This mechanism is important in preventing new infection. However, the plug begins to dissolve as calving approaches (Tyler and Ensminger, 1993), making the udder susceptible to infection once again.

1.5.2 Udder defences

Even if bacteria are able to breach the teat canal and enter the udder, there are several highly efficient systems that exist within the udder that are able to assist in the removal of such bacteria and hence facilitate the reduction of infection. These can be categorised as intrinsic defence mechanisms, which are mechanisms that are continuously present in the udder, and inducible systems, which are only activated in response to bacterial infection (Walstra *et al.* 2006; Blowey and Edmondson, 2010).

a. Intrinsic defence mechanisms

Lactoferrin

Iron is required for bacterial growth (especially that of *E. coli*). In the dry, non-lactating udder, lactoferrin removes iron from udder secretions and in so doing, minimises bacterial multiplication (Walstra *et al.* 2006; Blowey and Edmondson, 2010). Although the risk of new infection by *E. coli* during the dry period is four times greater than in lactation, the presence of lactoferrin ensures that clinical disease onset is minimised (particularly by contagious organisms) (Blowey and Edmondson, 2010). Because it is only present in low concentrations, the bacteriostatic effects of lactoferrin are lost during lactation. This is mainly as a result of high citrate levels in milk which competes with lactoferrin for iron in order to produce iron citrate, which in turn is utilised during bacterial metabolism (Blowey and Edmondson, 2010).

Lactoperoxidase

All milk contains the enzyme lactoperoxidase (LP) (Walstra *et al.* 2006; Blowey and Edmondson, 2010). In the presence of thiocyanate (SCN) and hydrogen peroxide (H_2O_2), LP can inhibit the growth of some bacteria (Gram-positive organisms such as *S. aureus* and *Streptococcus* spp.) and kill others (Gram-negative bacteria such as *E. coli*) (Walstra *et al.* 2006; Blowey and Edmondson, 2010). The level of SCN in milk varies with diet, with levels being particularly high when cows are fed brassicas or legumes (Walstra *et al.* 2006). Hydrogen peroxide can be produced by bacteria themselves. Gram-negative bacteria produce very little H_2O_2 and hence the LP system is not very effective in their control (Walstra *et al.* 2006; Blowey and Edmondson, 2010). However, there is some evidence that Gram-positive bacteria produce

sufficient amounts of H_2O_2 during their metabolism to support the LP system and hence impose partial control (Walstra *et al.* 2006; Blowey and Edmondson, 2010).

Antibodies (immunoglobulins)

It is unlikely that antibodies have a primary effect on mastitis control (Leitner *et al.* 2003; Barrio *et al.* 2003; Blowey and Edmondson, 2010). It is known that colostrum contains very high levels of antibodies, yet freshly calved cows can develop peracute mastitis and frequently get severe mastitis several days after calving (Blowey and Edmondson, 2010). The role of specific antibodies against mastitic bacteria is, to date, not fully understood. It has been postulated that their main role is in the opsonisation of bacterial cells before they are phagocytosed by leukocytes and macrophages (O'Brien *et al.* 2000; Barrio *et al.* 2003). During opsonisation, a portion of the antibody molecule (Fab arm) attaches to the bacteria, leaving the second antibody arm (the Fc fragment) exposed. Leukocytes are activated by the exposed Fc arm and attach to it. Phagocytosis of the bacteria then proceeds more rapidly (Barrio *et al.* 2003).

Cellular response

There are a variety of somatic cell types present in milk, with leukocytes constituting the majority. These leukocytes can be counted and are expressed as the SCC of milk. There are disputes over which cell types are actually present in milk, and their various proportions at different stages of lactation (Table 1.2) (Blowey and Edmondson, 2010). These proportions vary with factors such as level of milk yield, stage of lactation and the presence of infection.

The primary function of macrophages and lymphocytes is to recognise bacteria and then trigger 'alarm' systems that induce a more vigorous host response, eventually leading to huge numbers of polymorphonuclear leukocytes (PMN) (mainly neutrophils) entering the milk. These "alarm systems" comprise the inducible mechanism to be discussed subsequently.

Table 1.2 Ratio of cell types in milk and colostrum as a percentage (adapted from Blowey and Edmondson, 2010).

Type of cell	Stage present	
	Mid-lactation (%)	Colostrum (%)
Polymorphonuclear leukocytes	3	61
Vacuolated macrophages	65	8
Non-vacuolated macrophages	14	25
Lymphocytes	16	3
Duct cells	2	3

b. Inducible defence mechanisms

The inducible mechanisms of defence serve as the final control option when all other pathways have been breached by bacteria. Alarm signals are sent to the body of the cow, which results in a sequence of immunity events, in an attempt to curb infection.

The chemotaxin alarm

The macrophages and PMNs already present in the milk recognise and engulf fragments of dead bacteria and their toxins during phagocytosis (Sordillo *et al.* 1989; Walstra *et al.* 2006). Phagocytosis in turn, leads to the release of various chemotaxins such as interleukin 8 and tumour necrosis factor (TNF) (Walstra *et al.* 2006; Blowey and Edmondson, 2010). These chemotaxins, together with the toxins produced by the bacterial cells themselves that are multiplying within the udder, that act as the inducers of the defence system triggered to control the bacteria (Blowey and Edmondson, 2010).

The inflammatory response

The principal response to chemotaxins is a massive inflow of PMNs from the capillaries in the teat wall and udder into the udder cisterns and ducts (Sordillo *et al.* 1989; Ali-Vehmas *et al.* 1997; Blowey and Edmondson, 2010). The severity of the inflammation is often such that it persists well after the bacteria have been destroyed. The increase in the somatic cell count of milk due to the inflammatory response can be enormous. From a base count of only 10^5 cells.ml^{-1} it may increase to as much as 10^8 to 10^9 cells.ml^{-1} in just a few hours (Blowey and Edmondson,

2012). Bacteria are killed, and concurrently, so many PMNs may have entered the udder that the white cell count of the blood falls to almost zero (Blowey and Edmondson, 2012). This occurs through a variety of processes:

Increased blood flow: blood vessels in the teat wall dilate, increasing the blood flow and supply, and the number of PMNs to the infected quarter/s. Hence a quarter with an acute mastitis infection becomes palpably swollen, hot and painful (Sordillo *et al.* 1989; Ali-Vehmas *et al.* 1997).

Margination: small carbohydrate projections (selectins) appear on the inner surface of cells lining the capillary walls. These attract PMNs out towards the sides of the capillaries and help force them between the capillary cells and out through the wall (Sordillo *et al.* 1989; Blowey and Edmondson, 2010).

Loosening of endothelial cell junctions: under the influence of specific chemotaxins, the endothelial cells lining the capillaries, and teat and udder cisterns move apart to facilitate more rapid passage of PMNs into the infected milk. Once the PMNs have passed through, the cells come together once again (Sordillo *et al.* 1989; Ali-Vehmas *et al.* 1997; Walstra *et al.* 2006; Blowey and Edmondson, 2010).

Diapedesis: in this process, PMNs squeeze through the walls of the capillaries, across the tissue of the teat wall and udder, through the endothelial lining and into the milk, where phagocytosis takes place (Sordillo *et al.* 1989; Smits *et al.* 1998).

Serum ooze from blood vessels: as a result of the opening of the junctions between endothelial cells in the capillary walls to allow for the passage of PMNs, serum can also flow into the tissues (Sordillo *et al.* 1989). Udder tissues are therefore stretched and dilated by fluid and appear swollen. This phenomenon is particularly apparent with acute *E. coli* infections, where leakage of serum is so pronounced that it flows directly into the milk and produces a yellow, watery secretion. Occasionally serum ooze may even be seen on the skin surface as small reddish-brown droplets (Sordillo *et al.* 1989; Jackson *et al.* 1990).

Phagocytosis: once they have passed into the milk, the PMNs released in response to the chemotaxin alarm, start to engulf whole bacteria (Jackson *et al.* 1990). Inside the PMN, the bacteria are destroyed by a process of oxidative metabolism that is induced by the PMNs. This results in the production of several microbicidal agents within the PMNs such as hydrogen

peroxide, hydroxyl radical and singlet oxygen, which oxidise lipids in the bacterial membranes and causes lysis of the bacteria (Jackson *et al.* 1990).

1.6 Mastitis and its control through the ages

1.6.1 Antibiotic therapy

Under optimal conditions, the natural defence mechanisms (self-cure) discussed above, in conjunction with cultural control measures such as clean bedding, proper milking sanitation and vigilant inspection of lactating cows, may prove effective in curbing disease onset and severity. However, the real problem arises when these intrinsic defences and cultural measures are compromised by infection. Antibiotic therapy has traditionally served as the next option to achieve effective control of the disease. Overall benefits of antibiotic therapy include a more rapid elimination of bacterial pathogens than self-cure, a reduced probability of chronic recurrent infections, a reduced depression in milk yield, and a more rapid return to an acceptable SCC and hence to saleable milk (Barkema *et al.* 2006; Nickerson, 2009).

Antibiotic treatments can be administered both during lactation (lactation cow therapy) and the period during which the cow is dried off (dry cow therapy), via intramammary or parenteral means (Blowey and Edmondson, 2010). However, despite much success with antibiotics, it remains debatable as to whether this therapy is indeed worthwhile. There are several conflicting views on this (Murchan *et al.* 2004; Borm *et al.* 2006; Sandgren *et al.* 2008; Nickerson, 2009; Blowey and Edmondson, 2010; Vanderhaeghen *et al.* 2010), many of which revolve around *S. aureus* and its ability to develop antibiotic resistance. Of the large number of organisms that are able to cause bovine mastitis, *S. aureus* is the etiological agent most commonly associated with the disease (clinical, subclinical and chronic forms) (Pereira *et al.* 2011). However, the cure rate of antibiotic treatments against this pathogen is low and, therefore, the disease cannot be effectively eliminated and/or controlled in infected herds by using antibiotics (Sutra *et al.* 1993; Carter *et al.* 2003; Murchan *et al.* 2004; Shi *et al.* 2010).

1.6.2 Antibiotic resistance and *S. aureus*

Antibiotic resistance can be attributed to several factors. *S. aureus* forms abscesses within the udder that are surrounded by a thick fibrous capsule (Almeida *et al.* 1996; Villar *et al.* 2011). This prevents sufficient concentrations of antibiotic from entering the abscess itself and hence blocks the effective destruction of viable bacteria. Some strains of *S. aureus* can live within cells such as macrophages. Most antibiotics are only able to circulate in the body fluids surrounding cells and cannot penetrate within cells themselves, hence these staphylococci are protected from the majority of antibiotics (Herbert *et al.* 2000; Villar *et al.* 2011). Many strains of *S. aureus* produce beta-lactamase, rendering them resistant to certain formulations of penicillin, in addition to other antibiotics that have been deemed to be more effective (such as certain aminoglycosides, cephalosporins and tetracyclines) (Herbert *et al.* 2000; Blowey and Edmondson, 2010; Cabrera *et al.* 2011). Some strains of *S. aureus* can persist in a state of bacterial dormancy within a mucoid capsule and completely cease all replication (Blowey and Edmondson, 2010; Cabrera *et al.* 2011). In this state, they are not killed by antibiotics and are able to reactivate once favourable conditions prevail.

The spread of virulent methicillin-resistant *S. aureus* (MRSA) (Goñi *et al.* 2004; Murchan *et al.* 2004; Nickerson, 2009; Shi *et al.* 2010; Vanderhaeghen *et al.* 2010), coupled with the development of resistance to two new antibiotics (daptomycin and linezolid) recently approved for clinical use against Gram-positive bacteria (Mangili *et al.* 2005), has shown that *S. aureus* is indeed a formidable pathogen. Antibiotic resistance is however, not exclusive to *S. aureus*. In the past three decades, we have witnessed a rise in the populations of bacteria carrying extended spectrum β-lactamases, which are mutants of enzymes that previously could only inactivate penicillins but now have gained activity against many cephalosporins too (Wright, 2010); plasmid-mediated (and hence horizontally transmitted) resistance to fluoroquinolone antibiotics (Silver, 1993); the rise of multi-drug resistant *Neisseria gonorrhoeae* Zopf 1885 (Tapsall, 2009); the emergence and global dissemination of multi-drug resistant *Acinetobacter baumannii* Bouvet and Grimont 1986, *Pseudomonas aeruginosa* Schröter 1872, *Klebsiella pneumonia* Schroeter 1886 and *Enterobacteriaceae* Rahn 1937(Kumarasamy *et al.* 2010; Wright, 2010); and, the spread of extensively drug-resistant *Mycobacterium tuberculosis* Zopf 1883 (Wright, 2010). This being said however, the development of new antibiotics is still ongoing. Tigecycline, a third-

generation, semi-synthetic tetracycline antibiotic approved in 2005, has been shown to have activity against MRSA when used in conjunction with rifampin (Raad *et al.* 2007). In addition, ceftobiprole, a fifth-generation cephalosporin, has shown in clinical trials, to provide significant control over MRSA (Zhanel *et al.* 2008). The core problem is that the rate of development of new antibiotics is unable to keep up with the pace of microbial evolution and hence resistance easily develops against common antibiotics. As a result, the latest antibiotics being used are themselves the source of the evolutionary pressure that will render them obsolete.

1.6.3 Alternative therapies for mastitis

Due to these treatment limitations, research on the control of bovine mastitis has shifted to alternative therapies, such as the development of vaccines (Sutra, 1993; Herbert *et al.* 2000; Pereira *et al.* 2011) and biological control options such as the use of botanical extracts (Akinyemi *et al.* 2005; Fawole, 2009). Vaccines for *S. aureus* have produced varied levels of success. This has been a result of the type of vaccine used, adjuvants and other factors such as age of the cow and environmental conditions (Hoedemaker *et al.* 2001, Pereira *et al.* 2011). A recent study has shown that a commercial bacterin (vaccine developed from killed bacteria) did not result in effective protection from new infections of *S. aureus* (Middleton *et al.* 2009). Better results have been obtained with a toxoid produced from three bacterial strains (with each strain demonstrating a different haemolysis pattern) added to the bacterin, which resulted in greater than 50% protection in the experimental challenge (Leitner *et al.* 2006; Pereira *et al.* 2011). Besides the conventional bacterins, new technologies are currently being used in the development of *S. aureus* vaccines. DNA and recombinant protein vaccines have been tested for the main factors of *S. aureus* virulence that are related to infections in the mammary gland (Middleton, 2008). A DNA vaccine with a booster dose of recombinant protein has proven to significantly increase humoral and cellular immune response and has achieved promising results in protecting mammary glands from new *S. aureus* infections (Shkreta *et al.* 2004). However, due to the wide variety of vaccines available against *S. aureus* and the variable clinical results associated with each, there remains no general consensus on which immunotherapeutic protocol is the most efficient.

1.7 Bacteriophages and disease control

There remains however, a biological resource for the control of *S. aureus* that has not been thoroughly tapped. This involves the use of bacterial viruses (bacteriophages) for the biological control of *S. aureus*. Phages are ubiquitous in the environment - from the oceans, soil, deep sea vents, hot-springs, the water we consume and the food we eat (Kutter and Sulakvelidze, 2005; Miedzybrodski *et al.* 2005). The discovery of these organisms dates back to the early part of the twentieth century (Jones *et al.* 2007). The discoveries by Twort and d'Herelle (1915) allowed for phage research to develop, such that phage therapy has been applied to different disciplines ranging from human and veterinary medicine to plant pathology (Sulakvelidze and Barrow, 2005). It was initially used successfully to treat a variety of diseases ranging from dysentery, typhoid and paratyphoid fevers, cholera and pyogenic urinary tract infections (Hagens and Offerhaus, 2008). A further facet of phage technology is that appropriate phages can control plant bacterial pathogens, which antibiotics do not do well. Circumstances in which phage therapy of plants or plant products has been attempted include control of *Salmonella* associated with fresh-cut fruit (Leverentz *et al.* 2001), to disinfest *Streptomyces scabies* Rosenbach 1884-infected potato seed tubers (McKenna *et al.* 2001), against bacterial spot of mungbeans caused by *Xanthomonas axonopodis* pv. *vignaeradiatae* (Borah *et al.* 2000), against *Xanthomonas pruni* (Smith) Dowson associated with bacterial soft rot of peaches (Randhawa and Civerolo, 1986), against *X. campestris* pv. *pruni* (Smith) Dye infections on peach trees, cabbage and pepper diseases (Randhawa and Civerolo, 1986), to control *Ralstonia solanacearum* Smith 1896 (causal organism of bacterial wilt) (Fujiwara *et al.* 2011) and to control soft rot and fire blight associated with *Erwinia* spp. Winslow *et al.* 1920 (Gill and Abedon, 2009). These findings have all supported the further development of phage therapeutics (Table 1.3; http://www.phageinternational.com).

1.7.1 Bacteriophage classification

Phages have been found to infect more than 140 bacterial genera including (1) aerobes and anaerobes; (2) exospore and exospore formers; (3) cyanobacteria, spirochetes, mycoplasmas, chlamydias; (4) budding, gliding, ramified, stalked and sheathed bacteria; (5) extreme halophiles and methanogens; and (6) hyperthermophilic Archaea (Ackermann, 2005). Phages have been

found to be extremely heterogeneous in their structural, physicochemical and biological properties, which could suggest that they are polyphyletic in nature (Ackermann, 2005). However, their primary mode of action is similar. Virions differ significantly as well; occurring as tailed, polyhedral, filamentous or pleomorphic structures with genomic make-ups ranging dsDNA, ssDNA, dsRNA and ssRNA (Ackermann, 2005; 2007). By 2007, at least 5568 phages had been examined through electron microscopy (Table 1.4) (Ackermann, 2007). Results derived from genomic and morphological studies have been used to categorise phages into 1 order, 13 families and 31 genera (Ackermann, 2005; 2007).

Table 1.3 Commercial phage-based products and companies (adapted from Monk *et al.* (2010)).

Product	Target organism	Company	Country
AgriPhage™	*Xanthomonas campestris* pv. *vesicatoria* or *Pseudomonas syringae* pv. Tomato	Omnilytics www.omnilytics,com	Israel
BioTector	*Salmonella* spp. in poultry	CheiIJdang Corporation http://eng.cjcheiljedang.com/	China
EcoShield™	*Escherichia coli* in foods and food processing facilities	Intralytix www.intralytix.com	USA
FASTPlaque-Response™	Detection of rifampicin resistance in *Mycobacterium tuberculosis*	Biotech Laboratories www.biotec.com	United Kingdom
FASTPlaqueTB™	Detection of *M. tuberculosis*	Biotech Laboratories www.biotec.com	United Kingdom
ListShield™	*Listeria monocytogenes* in foods and food processing facilities	Intralytix www.intralytix.com	USA
LISTEX™ P100	Targets *L. monocytogenes* strains on food products	EBI Food Safety www.micreosfoodsafety.com	The Netherlands
MRSA/MSSA blood culture test	Detects *Staphylococcus aureus* methicillin resistance/susceptibility	Microphage www.microphage.com	USA
MRSA screening test	MRSA	Microphage www.microphage.com	USA
MicroPhage MRSA/MSSA test	Differentiation of methicillin resistant (MRSA) and methicillin-susceptible (MSSA) *Staph. aureus*	Microphage	USA

Tailed phages of the order *Caudovirales* constitute the most extensive group of bacterial viruses, comprising 96% of phages identified so far (Table 1.4; Figure 1.2) (Ackermann, 2007; Gutiérrez *et al.* 2011). These phages consist of a protein shell and linear dsDNA, and lack any type of envelope (Ackermann, 2005; 2007). Phage heads are icosahedral and tails are true helices that consist of stacked discs. These tails usually possess terminal adsorption structures such as base plates, spikes or tail fibres (Ackermann, 2005). Although these phages constitute a monophyletic group possessing related properties (morphological, physicochemical, physiological) and have been classified into as a single order, *Caudovirales*, their individual properties are rather varied. Typically, they differ in terms of dimension and fine structure, DNA content and composition, nature of constitutive proteins, serology and host range (Table 1.4; Figure 1.2) (Ackermann, 2005). Ackermann (2005) has devised helpful criteria by which to distinguish between these phages:

Myoviridae: contractile tails consisting of a sheath and central tube; approximately 25% of tailed phages;

Siphoviridae: long, non-contractile tails; approximately 61% of tailed phages; and,

Podoviridae: short, non-contractile tails; approximately 14% of tailed phages.

The DNA composition of tailed phages generally resembles that of their specific host bacteria (Ackermann, 2005). Phage genomes are large, complex and usually organized into interchangeable modules, with genes for related functions clustered together (Ackermann, 2005; 2007). Replicating DNA tends to form large, branched concatemers, which are then cut into unit lengths and inserted into preformed capsids (Ackermann, 2005). Virion assembly of individual components (heads, tails, tail fibres) takes places via separate pathways that form the last stage of the maturation process (Ackermann, 2005). Newly assembled phage particles are then liberated into the surrounding environment via lysis of the host bacteria (Ackermann, 2005).

While tailed phages do comprise the majority of bacterial viruses, tailless phages also occur (Table 1.4; Figure 1.2). These include only about 190 known viruses, corresponding to less than 4% of the currently recognised bacterial viruses (Ackermann, 2005). They are classified into 10 small families, occur enveloped or non-enveloped, and are of three types: polyhedral phages that are icosahedral with cubic symmetry and either DNA or RNA constituted, filamentous

phages with helical symmetry that are DNA constituted, and, a few pleomorphic types without obvious symmetry axes that are also DNA constituted (Ackermann, 2005).

Table 1.4 Overview of phage families (Ackermann, 2007).

Shape	Nucleic acid	Virus group	Particulars	Example
Tailed	DNA, 2, L	*Myoviridae*	tail contractile	T4
		Siphoviridae	tail long, noncontractile	λ
		Podoviridae	tail short	T7
Polyhedral	DNA, 1, C	*Microviridae*	conspicuous capsomers	φX174
	2, C, S	*Corticoviridae*	complex capsids, lipids	PM2
	2, L	*Tectiviridae*	inner lipid vesicle,	PRD1
	2, L	SHI, group*	pseudotail	SH1
	2, C	STV1 group*	inner lipid vesicle	STIV
	RNA, 1, L	*Leviviridae*	turret-shaped protrusion	MS2
	2, L, seg	*Cystoviridae*	poliovirus-like	Φ6
			envelope, lipids	
Filamentous	DNA, 1, C	*Inoviridae*	(1)long filaments (2)short	(1)fd
	2, L	*Lipothrixviridae*	rods	(2)MVL1
	2, L	*Rudiviridae*	envelope, lipids	TTV1
			TMV-like	SIRV-1
Pleomorphic	DNA, 2, C, S	*Plasmaviridae*	envelope, lipids, no capsid	L2
	2, C, S	*Fuselloviridae*	same, lemon shaped	SSV1
	2, L, S	*Salterprovirus*	same, lemon-shaped	His1
	2, C, S	*Guttavirudae*	droplet-shaped	SNDV
	2, L	*Ampullaviridae**	bottle-shaped	ABV
	2, C	*Bicaudaviridae**	two-tailed, growth cycle	ATV
	2, L	*Globuloviridae**	paramyxovirus-like	PSV

Key: C=circular; L=linear; S=superhelical; seg=segmented; 1=single-stranded; 2=double-stranded; *awaiting classification
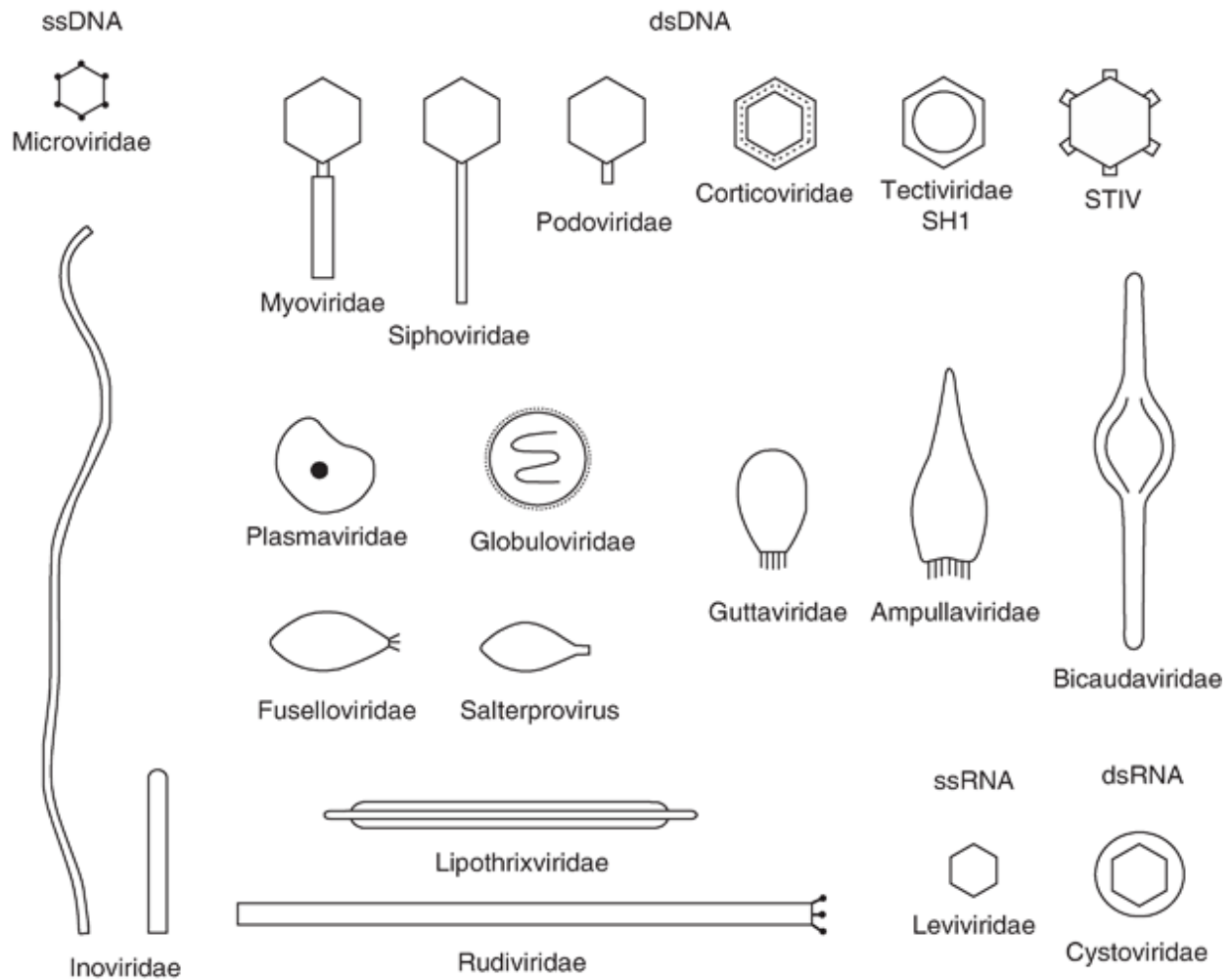
Figure 1.2 Morphotypes of phages (Ackermann, 2007).

1.7.2. Genomic studies on *S. aureus* bacteriophages

Phages have been found to play a fundamental role in the evolution of their host. Whole genome sequencing of bacteria has shown that phage elements contribute significantly to sequence diversity and can potentially influence pathogenicity (Monk *et al.* 2010). To date, there are approximately 967 phage genome sequences available in the NCBI phage database, with genomes ranging from 15-500kbp in size (http://www.ncbi.nlm.nih.gov). Generally, phage genomes are organized in modular structures with each set of modules containing a set of genes which carry out a biological function (O'Flaherty *et al.* 2004). Phage evolution can occur by the

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exchange of modules between phages that have access to the same gene pool (O'Flaherty *et al.* 2004).

To date, the genomes of more than 50 *S. aureus* phages are currently available in public databases (García *et al.* 2009). The number of known, strictly lytic phages is limited to the close-knit *Myoviridae* genus of the SPO1-like viruses which also contains phages K, Twort and G1 (O'Flaherty *et al.* 2004; García *et al.* 2009). Apart from this group, a large number of genomes from unclassified *Siphoviridae* in lysogenic *S. aureus* strains are available (Kwan *et al.* 2005). Some lysogenic phages may play an important role in the pathogenicity of *S. aureus* by carrying virulence factors, mediating lateral gene transfer and possibly facilitating adaptation of the pathogen during infection (Górski *et al.* 2009). Kwan *et al.* (2005) sequenced the complete genomes and proteomes of 27 *S. aureus* phages. Comparative nucleotide and protein sequence analysis was used to group these phages into three classes: <20kbp, ~40kbp and >125kbp. García *et al.* (2009) also presented a detailed genomic and molecular characterisation of two *S. aureus* lytic phages. Other studies conducted by various researchers also on sequence analysis of *S. aureus* phages (O'Flaherty *et al.* 2004; Rashel *et al.* 2008; Vandersteegen *et al.* 2011; Gu *et al.* 2012; Kwiatek *et al.* 2012) have provided a solid background for future comparative studies.

1.7.3 Bacteriophages and their mode of action

Phages can display one of two types of life cycle: lytic or temperate. Lytic phages bring about rapid lysis and death of the host bacterium whereas the temperate forms spend part of their life cycle in a quiescent state (prophage) (Monk *et al.* 2010). In this lysogenic cycle, viral DNA is integrated into host cell DNA. Prophage DNA will then be replicated when the host cell genome replicates and hence daughter cells will inherit the viral DNA (Fischetti, 2005). This temperate form however, has very little value in terms of phage therapy applications. During a typical lytic cycle, the virus attaches to the host cell via specific receptor sites that may be one of a variety of cell surface components including protein, oligosaccharide, teichoic acid, peptidoglycan and lipopolysaccharide (Monk *et al.* 2010). Phage genetic material is then transferred into the host cell and usually occurs via contraction of the virus tail and formation of a hole within the bacterial cell wall (Fischetti, 2005; Monk *et al.* 2010). The viral genome is then transcribed by host cell RNA polymerase, producing early mRNA that has the effect of taking over the

metabolic machinery of the bacterium (Monk *et al.* 2010). In so doing, the metabolic processes within the host cell are redirected towards the manufacture of new viral components which are then assembled into new virions (Monk *et al.* 2010). Following construction and assembly of the new virions, there remains the matter of release from the host cell. Nearly all dsDNA phages have developed enzymes that attack bacterial peptidoglycans (Fischetti, 2005; Monk *et al.* 2010). These muralytic enzymes (or endolysins) are produced within the cytoplasm and require another enzyme to enable them to cross the cytoplasmic membrane to reach their substrate. This enzyme is a holin that disrupts the membrane, allowing the lysin to degrade the peptidoglycan (Fischetti, 2005). In this way, the holin controls the timing of cell lysis and the release of viral progeny.

This intricate cascade of events was not well understood during the early period of phage discovery. This lack of in-depth knowledge, coupled with the advent of antibiotics, lead to the early demise of phage therapy. However, the surge in antibiotic resistance and the wide pool of information now available on phage biology has proven pivotal in re-establishment of interest in phage therapy. It is easy to see why there is an increased trend towards phages as therapeutic agents because they offer several advantages over the use of antibiotics. Phages target only the pathogens of interest and the normal microflora of a system remains unaffected (Monk *et al.* 2010). Their mechanism of action is completely different from all available antibiotics and they are effective against bacterial strains exhibiting resistance towards multiple antibiotics (Monk *et al.* 2010). As a result, even if they are not used as the first line of defence, phages represent a very useful last line of defence (Monk *et al.* 2010). Furthermore, work presented in the early 1980's by Smith and Huggins (1982), demonstrated the superiority of phage treatments against *E. coli* infections in mice in comparison to antibiotics. This provided further support for the renewed interest in phage therapy.

The effects of phage therapy are all localised at the infection site whereas antibiotics do not necessarily concentrate at the initial site of infection (Veiga-Crespo *et al.* 2010). The pharmokinetics of phage therapy is such that the initial dose increases exponentially as the virus multiplies within the susceptible bacterial host and is subsequently released (Monk *et al.* 2010). Often all that is required thereafter is a 'booster' dose of phage to ensure elimination of any residual bacterial cells. In addition, there is evidence that phages are able to penetrate poorly vascularised tissues and can even cross the blood-brain barrier (Alisky *et al.* 1998). Extensive

clinical experience in the former Soviet Union and Eastern Europe has revealed very few cases of side-effects or allergic reactions (Deresinski *et al.* 2008; Monk *et al.* 2010). It is less certain however, what immunological effects might arise upon multiple dosages (Deresinski *et al.* 2008; Monk *et al.* 2010). In spite of these positive factors however, part of the evolutionary capability of the target bacteria is to develop resistance to specific phages. The solution is to recognise this as a normal process and to put into place a phage discovery and screening system to constantly supply more strains of phages to replace the newly ineffective strains. This has been put into practise in spin-off companies from the world renowned George Eliava Institute of Bacteriophage, Microbiology and Virology in Georgian Republic (www.eliava-institute.org). Furthermore, production costs of phages are significantly lower than those associated with antibiotic manufacture (Veiga-Crespo *et al.* 2010).

1.8 Bacteriophage therapy and bovine mastitis

Considering the wealth of information at our disposal, phage therapy appears to be one of the most sustainable measures for control of bovine mastitis. It is essential when looking at the treatment of bovine mastitis with phages that one has a full grasp of the disease cycle and where phages occur within this cycle (Figure 1.3).

A limited number of studies have identified various phages with lytic capabilities towards *S. aureus* (O'Flaherty *et al.* 2005b; Gill *et al.* 2006a; García *et al.* 2007; García *et al.* 2008; Synott *et al.* 2009). In studies by Gill *et al.* (2006a), the effectiveness of phage therapy was evaluated for the control of mastitis during lactation. The study was designed to treat already established intramammary infections in cows housed in a commercial production environment. Infected cows were treated with a 5-day course of intramammary infusions with either the lytic phage (called Phage K) or a saline placebo. The effects of phage infusion into healthy quarters were also examined in order to assess the pharmacokinetics of infused phages and its impact on milk quality. Phage infusion was able to elicit a heightened immune response as exhibited by an increase in the SCC of treated udders and resulted in a cure rate of 16.7%. In addition, phages were detectable in milk for up to 36hrs post-infusion but at significantly lower rates, indicating that degradation or inactivation of the infused phage occurred within the gland. While this study proved that Phage K was able to control *S. aureus* in infected udders, several limiting factors

were also detected. Phage inactivation in the udder could have taken place as a result of the presence of milk proteins and fats.

Infected udder

Contagious mastitic bacteria:
Staphylococcus aureus Streptococcus agalactiae Streptococcus dysgalactiae

Environmental mastitic bacteria:
Escherichia coli
Streptococcus uberis
Bacillus spp.
Fungi, Yeasts

- bacteria occur and survive in milk and feed off milk protein
- attach to duct end, duct walls

*Bacterial reservoirs:
- mammary glands
- teat skin
- dairy worker hands
- milking facilities

*Bacterial reservoirs:
- farmyard
- cowsheds
- faeces
- bedding
- milking facilities

Healthy udder

= bacteriophage occurrence

Phages occur *with* bacteria:
- in teats in milk
- on dairy floors
- on teat surfaces
- on farmyards

Transferred from * via:
- splashing off dairy floors
- worker gloves
- insect pests

Figure 1.3 Disease cycle of bovine mastitis indicating phage occurrence.

It has been found that whey proteins attach to *S. aureus* cell surfaces which inhibit phage binding (O'Flaherty *et al.* 2005a; Gill *et al.* 2006b). In addition, *S. aureus* has been shown to aggregate when it is grown in milk or milk whey, which could also confer some protection for bacteria against phage attack (O'Flaherty *et al.* 2005b). However, another study by O'Flaherty *et*

al. (2005b) showed that an infusion of a cocktail of three phages at 10^8pfu.ml^{-1} into live cow teats resulted in no detectable increase in SCC, indicating that there was no localised immune response to high numbers of phage. This is in stark contrast to the study by Gill *et al.* (2006a) where inflammation of the udder as a result of a high SCC was noted. *In vivo* studies by Wills *et al.* (2005) in rabbits, showed increased recovery of viable phage from treated animals than was initially administered, implying that phage multiplication occurred successfully within animal tissues. Capparelli *et al.* (2007) also tested phage against *S. aureus* in mice. Findings included a 97% recovery of infected animals over a 10-day period. In addition, phage delivery into macrophages by *S. aureus*, was found to kill intracellular staphylococci both *in vivo* and *in vitro*.

1.9 Conclusions and future prospects

Phages are specific for their target bacterium and hence create no negative effects on the surrounding mammary tissues or the environment. This, coupled with their ability to multiply up to 1000-fold within a host cell, makes the phage an ideal candidate for the biocontrol of bovine mastitis (Blowey and Edmondson, 2010). While phages do provide solutions to bacterial diseases, there are still several areas of research that require further investigation. Studies into the detailed effects of whey proteins on bacterial activity and aggregation, proper administration of phage cocktails into animal tissues and the development of phage formulations to facilitate optimal delivery and activity within intramammary tissues, are ongoing. Further studies also need to be conducted on the pharmacokinetics and pharmacodynamics of these organisms in order to properly understand lytic events and to exploit them to their full potential. While some are of the opinion that phage therapy for bovine mastitis has limited potential (Johnson *et al.* 2008), the advances made thus far, and the global occurrence of antibiotic resistance, has re-opened the doorway for phage therapy.

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CHAPTER 2

Biological and pathogenicity assays of bacteriophages specific for *Staphylococcus aureus* strains, associated with bovine mastitis

Abstract

Bovine mastitis is an infectious disease of the mammary glands of dairy cattle. One of the primary causal agents for the disease is the bacterium, *Staphylococcus aureus* subsp. *aureus* Rosenbach1884. Traditional control of this organism was through the use of antibiotics. However, *S. aureus* is developing resistance towards these chemotherapeutic agents faster than they are being developed. Bacteriophages can serve as an alternative control measure for the disease. This study investigated the prevalence of phages and *S. aureus* in the dairy environment, as well as pathogenicity of phage isolates against antibiotic-resistant *S. aureus*. Twenty-eight phages were isolated and screened against four strains of antibiotic-resistant *S. aureus*. Only six phages showed potential for further testing based on their wide host range, high titres and common growth requirements. Optimal growth conditions for the host *S. aureus* strain was 37°C for 12hr. This allowed for optimal phage multiplication. At an optimal titre of between 6.2×10^7 to 2.9×10^8 pfu.ml^{-1} (at 10^{-5} dilution of phage stock), these phages were able to reduce live bacterial cell counts between 64-95%. In addition, these six phages showed further pathogenicity towards 18 *S. aureus* strains that were isolated from a different milk-producing region during a farm survey. The phages isolated in this study show great potential for *in vivo* applications. However, lytic ability must be evaluated when the phages are exposed to elements that will be involved in their mass production and formulation, prior to such application.

2.1 Introduction

Bovine mastitis is an infectious inflammation or irritation of the mammary glands that interferes with the normal flow and quality of milk (Blowey and Edmondson, 2010). Although a range of control measures are used, this disease remains a major cause of economic losses in the dairy industry worldwide. Direct costs can be incurred through discarded milk, and drug and

veterinary costs, while indirect costs can be incurred through penalties as a result of increased somatic cell counts (SCC), decreased milk yield due to udder damage, additional labour requirements for treating infected cows, and higher culling and replacement costs (Blowey and Edmondson, 2010; Mubarack *et al.* 2011). In the United States alone, losses are estimated to approach 2 billion USD and worldwide, mastitis is associated with economic losses of up to 35 billion USD annually (Jones and Bailey, 2009; Van den Borne, 2010; Mubarack *et al.* 2011).

On average, a quarter of an udder infected with a major pathogen will yield approximately 30% less milk than an equivalent uninfected quarter of the same cow (Kudi *et al.* 2009; Blowey and Edmondson, 2010). Infected cows are also culled more quickly, either because of repeated clinical infections, or reduced milk yields. In addition, mastitis has a major effect on the taste and quality of milk, mainly as a result of its effects on the lactose, protein and fat content of milk; typically affected milk is rancid and slightly salty/bitter to the taste (Mubarack *et al.* 2011). As a result of diminished milk quality, overall profitability is reduced as the farmer receives a lower price (up to 20% less per litre) for such milk (Mubarack *et al.* 2011).

In recent years, the use of integrated control measures for the control of bovine mastitis has become paramount due to the onset of resistance against the antibiotics commonly used to treat the disease. This is particularly relevant to the primary causal bacterium of bovine mastitis, *Staphylococcus aureus* subsp. *aureus* Rosenbach 1884. Numerous strains have developed antibiotic resistance (Smith and Huggins, 1982; Borm *et al.* 2006; Vanderhaeghen *et al.* 2010), with the result that interest has shifted from antibiotic therapies towards the field of biological control of the disease.

One such biocontrol option involves the use of lytic bacterial viruses (bacteriophages) that are specific to *S. aureus*. Phage therapy has been applied to different disciplines, ranging from human and veterinary medicine to agricultural settings (Soothill, 1994; Capparelli *et al.* 2007; Hagens and Offerhaus, 2008). However, no single phage-based product has yet been commercialized for use against bovine mastitis. This is despite the clear need for a more sustainable control option to manage the disease. Phages have several associated benefits. They are specific for their target bacterium and hence create no negative effects on the surrounding mammary tissues or the environment. This, coupled with their ability to multiply up to 1000-fold

within a host cell (Blowey and Edmondson, 2010), makes the phage an ideal candidate for the biocontrol of bovine mastitis.

This study looked at the isolation of lytic anti-staphylococcal phages that were isolated from the local dairy environment in the province of KwaZulu-Natal (Republic of South Africa). These phages were further investigated for their *in vitro* ability to control South African strains of *S. aureus*. It is envisaged that these results will contribute towards a final goal of creating a phage-based biological control product to supplement current control methods for bovine mastitis in dairy cows.

2.2 Materials and Methods

2.2.1 Bacterial host strains and their isolation

Four *S. aureus* strains were used for phage isolation and propagation, i.e., *SaB1*, *SaB2*, *SaB3* and *SaB4*. Three strains (*SaB1*, *SaB2* and *SaB3*) were isolated from raw bovine milk collected from dairy farms in the province of KwaZulu-Natal, RSA (Republic of South Africa). One strain (*SaB4*) was from the American Type Culture Collection (ATCC Strain 6538; *S. aureus* subsp. *aureus* Rosenbach). The milk samples from which *S. aureus* was isolated were selected at random from mastitis-affected dairy cows.

S. aureus was initially isolated on blood agar. Cultures required for phage propagation were maintained on tryptone soy agar (TSA) or typtone soy broth (TSB). Identity of each bacterial isolate as a *S. aureus* strain was confirmed by hemolysis on blood agar, catalase reaction, Gram reaction and arrangement of bacterial cells upon Gram staining, reaction on Baird Parker agar (augmented with egg yellow tellurite) (www.merck.com) and coagulase reaction using EDTA-rabbit plasma (www.merck.com). All four strains of *S. aureus* were used for phage titre and host range screening. *SaB1* was used as the representative strain for all subsequent screening.

2.2.2 Antibiotic resistance assay of *S. aureus*

The Kirby-Bauer disc diffusion method (Jorgensen and Turnidge, 2007), was used to screen the four *S. aureus* strains for resistance against a range of antibiotics applied against bovine mastitis. Antibiotics were selected from six major groups: β-lactam (ampicillin, penicillin G, methicillin and amoxycillin), macrolides (erythromycin), aminoglycosides (streptomycin), tetracyclines (tetracycline, oxytetracycline), glycopeptides (vancomycin), and non-β-lactam (trimethoprim). Antibiotic discs were used for all screening, and all assays were run on Mueller-Hinton agar (4mm agar thickness; pH 7.2). Four discs were used per plate and all assays were carried out in triplicate. Plates were incubated for 12hrs at 37ºC for all antibiotics, excluding vancomycin. Vancomycin plates were incubated for 24hr at 37ºC. Zones of inhibition (mm) were recorded after incubation. All assays were done in triplicate and standard deviations were determined where zones of inhibition did develop.

2.2.3 Phage isolation, propagation and purification

Raw bovine milk samples collected from dairy farms in the province of KwaZulu-Natal, RSA (Republic of South Africa) served as the initial material from which to isolate phages. These milk samples were chosen at random and included milk with both high (>400,000cells.ml^{-1}) and low somatic cell counts (SCC). All milk samples were stored on ice during transit, or at 4ºC in the laboratory.

Phages were isolated using the spot-test method on double-layer agar (Sambrook *et al.* 1989; Harley and Prescott, 1993). Overnight *S. aureus* cultures, *SaB1* to *SaB4*, at ~1 x 10^{8} cfu.ml^{-1}, were incorporated into 7% top agar that was supplemented with 1M $CaCl_2$. Raw milk samples were filtered through a 0.45μm syringe filter. Filtered samples were then spotted onto the surface of solidified top agar at 10μl per spot. Plates were allowed to dry for 2hr, followed by incubation for 12hr at 37ºC. Zones of clearing (plaques) were indicative of phage activity. Plaques were removed from top agar and soaked in phage buffer (Appendix 1) for 12hr with gentle agitation (150rpm) at 4ºC. The resulting suspension was centrifuged using an Avanti J-26 XPI (www.beckmancoulter.com) at 10,000g x 10min at 4ºC. The supernatant was filtered using a 0.45μm syringe filter and stored as phage stock at 4ºC. Subsequent phage was grown

from this stock in liquid broth culture or using the double-layer agar method (Sambrook *et al.* 1989).

Isolated phages were purified through a modification of standard methods (Sambrook *et al.* 1989; Harley and Prescott, 1993). Filter-sterilised phage stock isolated from either liquid broth culture or double-layer agar method, were subjected to centrifugation using an Avanti J-26 XPI (Beckman-Coulter)[1] at 75,600g x 3hrs x 10ºC. Resulting phage pellets were re-suspended in fresh phage buffer and a second centrifugation was conducted (Avanti J-26 XPI (Beckman-Coulter) at 75,600g x 3hrs x 10ºC). The phage pellets were then re-suspended in fresh phage buffer at 1/10 of the original volume that was processed. All stocks were stored at 4ºC.

A total 28 phages were isolated however, only six showed consistency with regard to host cell lysis and growth requirements. These six phages were used for subsequent screening and were named Sabp-P1, Sabp-P2, Sabp-P3, Sabp-P4, Sabp-P5 and Sabp-P6. Multiplicity of infection (MOI) was calculated for each phage.

2.2.4 Phage titre

A dilution series (10^0 -10^{-10}) was made up for each of Sabp-P1-Sabp-P6. The standard double-layer plating method as described by Sambrook *et al.* (1989) was followed. All six phages were titred against all four bacterial cultures. Plates were incubated for 12hr at 37ºC. Plaques were counted after 12hr and titres were calculated using the formula: Number of plaque forming units.ml^{-1} (pfu.ml^{-1}) = number of plaques x reciprocal of dilution x dilution factor (Sambrook *et al.* 1989).

2.2.5 Phage host range analysis

Host range analysis was carried out using the spot-test method (Harley and Prescott, 1993). An overnight culture of each *S. aureus* (*SaB1* to *SaB4*) was made up on TSA plates. Colonies were scraped off the TSA and re-suspended in 0.9% saline. Bacterial suspension (+ 1M $CaCl_2$) was incorporated into pre-warmed top agar (0.7%). Top agar was immediately poured into previously prepared bottom layer agar. Plates were left to dry for 1hr. Undiluted phage stock of Sabp-P1 to Sabp-P6, were screened against *SaB1-SaB4* by spotting 10µl droplets of each phage onto the

surface of a prepared double-layer plate inoculated with *S. aureus*. All plating was carried out in triplicate. The plates were left to dry for 2hr followed by incubation for 12hr at 37ºC. The control treatments consisted of sterile phage buffer that was spotted onto individual lawns of *SaB1* to *SaB4*. After overnight incubation, any plates exhibiting zones of clearing were regarded as a positive result.

2.2.6 Single step growth curve

The 10^{-5} pfu.ml^{-1} dilution of each phage was used to determine the single step growth curve as this dilution produced the most distinctive number of plaques without "webbing" of the plaques. Only *S. aureus*, *SaB1*, was used for this assay. The protocol was carried out according to Harley and Prescott (1993). Phage starting solution (10^{-5}) was made up in 9.9ml TSB. Then 100μl of overnight *SaB1* (+ 1M CaCl$_2$) was added to the phage dilution and the mixture was incubated at 37ºC with gentle agitation (150rpm). This was recorded as Time Zero. When 19 minutes had elapsed, 100μl phage suspension was aliquoted onto pre-warmed top-layer agar. This was followed by the addition of 100μl overnight SaB1 culture to the same top-layer agar. The top-layer agar was immediately poured onto previously prepared bottom-layer agar. The plate was left to dry for 2hr, followed by incubation for 12hr at 37ºC. This process was repeated for the following intervals: 25min, 30min, 35min, 40min, 45min, 50min, 55min and 60min. The control treatment consisted of 100μl *SaB1* + 100μl phage buffer (+ 1M CaCl$_2$). Plaques were counted on each plate after 12hr incubation.

2.2.7 Lethal dose assay

Only *SaB1* was used for this assay. Phages were all used at the 10^{-5} pfu.ml^{-1} dilution. *SaB1* cultures were grown in six different batches in TSB with 12hr incubation at 37ºC. Bacterial cell counts were determined after overnight incubation using dilution plating. Thereafter, each batch was inoculated with each phage. Final reaction mixtures each consisted of 50ml *SaB1* + 5ml phage (+ 1M CaCl$_2$). Inoculated cultures were incubated for 12hr at 37ºC. After 12hr incubation, live bacterial cell counts were determined using dilution plating.

2.2.8 Farm survey

A preliminary survey looking at the prevalence of *S. aureus* and its associated phages within a commercial dairy setting was carried out. This survey was primarily conducted in order to assess the bacteria: phage dynamics in an *in vivo* system, and to investigate the prevalence of phages, even if the host bacterium is absent. The dairy was located in the Karkloof Valley, KwaZulu-Natal, Republic of South Africa. Sampling was carried out twice. Composite milk samples were drawn from cows with a high somatic cell count ($>400,000$cells.ml^{-1}). Milk samples were screened for the presence of *S. aureus* using hemolysis plating, catalase testing and Gram reaction. Milk was screened for phages using the spot-test method. *S. aureus*, *SaB1*, was used as the host organism. In addition, phages Sabp-P1 to Sabp-P6 were screened for virulence against the fresh *S. aureus* cultures that were isolated from the raw milk. The spot-test method was used to determine susceptibility of each *S. aureus* strain to each phage isolate.

2.2.9 Statistical analyses

Differences between treatments were determined by analysis of variance (ANOVA) using Genstat 14th Edition. Fisher's least significant difference test (LSD), F-value and coefficient of variance (CV%) were determined for each set of experiments. All assays were carried out in triplicate.

2.3 Results

2.3.1 *S. aureus* isolation and screening

Hemolysis patterns on blood agar, catalase reactions using hydrogen peroxide (5%), Gram reaction, coagulase tests and colony formation on Baird-Parker agar (amended with egg yellow tellurite) were sufficient to distinguish *S. aureus* cultures from other bacteria in milk samples (Figure 1, Appendix 1).

2.3.2 Phage isolation

Of the 28 phages that were isolated, only six showed consistency with regard to lysis of all four strains of *S. aureus* (*SaB1* to *SaB4*), and under similar growth conditions, i.e., 10-12hr incubation period at 37ºC. Of the 22 phages that were not selected, 13 showed variable lytic ability towards the bacterial hosts; this was in terms of time to lyse cells and efficiency of lysis. Furthermore, the remaining 9 phages became lysogenic after multiple culturing. The six phages that showed enhanced activity in comparison to the other 22, were used for subsequent screening: phage titre, host range analysis, single step growth curves, lethal dose assay and the farm survey. These six phages were named: Sabp-P1, Sabp-P2, Sabp-P3, Sabp-P4, Sabp-P5 and Sabp-P6

2.3.3 Phage titre

Phages Sabp-P1, Sabp-P2 and Sabp-P3 appeared to be highly virulent and produced higher plaque counts than Sabp-P4, Sabp-P5 or Sabp-P6. Plaque counts were most accurate when phage stock suspensions were diluted to $1x10^{-5}$ phages.ml^{-1}. At this dilution, plaques were distinct and there was no "webbing" or adjoining of neighbouring plaques (Figure 2, Appendix1). This dilution was used for all subsequent screening. Phages Sabp-P1, Sabp-P2 and Sabp-P3 produced titres of $2.91x10^8$, $3.04x10^8$ and $3.12x10^8$ pfu.ml^{-1}, respectively. Phages Sabp-P4, Sabp-P5 and Sabp-P6 produced much lower titres of $1.9x10^7$, $1.76x10^7$ and $2.6x10^7$ pfu.ml^{-1}. Phage MOI was determined for each phage: Sabp-P1=3, Sabp-P2=3, Sabp-P3=3, Sabp-P4=0.2, Sabp-P5=0.1 and Sabp-P6=0.2.

2.3.4 Phage host range analysis and antibiotic resistance

SaB1, *SaB2* and *SaB3* isolated from different regions of the major milk-producing areas in KwaZulu-Natal showed susceptibility to lysis by all 28 phages that were isolated. The commercial *S. aureus* strain (*SaB4*), ATCC 6538, showed susceptibility to only 22 of the isolated phages. As a result, the six most effective phages were selected for all further screening. Selection criteria were primarily based on time to reach optimal titre and ability to lyse all four bacterial hosts, within a 12hr incubation period.

Coupled to the phage host range assay, an antibiotic resistance assay was carried out where the same four bacterial strains were screened against the major chemotherapeutic agents used *in vivo*. These bacterial strains were found to be resistant to the most commonly applied antibiotics used in the dairy industry (Table 2.1). However, all four bacterial strains showed susceptibility towards antibiotics in the tetracycline group, i.e., tetracycline and oxytetracycline.

Table 2.1 Antibiotic resistance screening of *S. aureus* strains, *SaB1*, *SaB2*, *SaB3* and *SaB4*.

Antibiotics			Average zone of inhibition (mm)			
Group	**Antibiotic**	**Abbr.**	*SaB1*	*SaB2*	*SaB3*	*SaB4*
β-lactamase	ampicillin	AMP5	nz	nz	nz	nz
	penicillin G	P10	nz	nz	nz	nz
	methicillin	Met10	nz	nz	nz	nz
	amoxycillin/flucloxacillin	A/F	nz	nz	nz	nz
Macrolides	erythromycin	E5	nz	nz	nz	nz
Aminoglycosides	streptomycin	S25	nz	nz	nz	nz
Tetracyclines	Tetracycline[*]	TE10	11.6	16.7	11.4	6.7
	Oxytetracycline[**]	OB	25	13	6.7	7.6
Glycopeptides	vancomycin	Vc	nz	nz	nz	nz
Non- β-lactamase	trimethoprim	Tr	nz	nz	nz	nz

nz = no zone of inhibition;[*]Standard deviation *SaB1*=0.29, *SaB2*=0.29; *SaB3*=0.29; *SaB4*=0.29; [**]Standard deviation *SaB1*=0.5, *SaB2*=0.61, *SaB3*=0.21, *SaB4*=0.25.

2.3.5 Single step growth curve

The single step growth curve for phages, Sabp-P1 to Sabp-P6, is presented in Figure 2.1 (Table 1, Appendix 1); *SaB1* was used as the host bacterium. ANOVA showed highly significant (p<0.001) differences between the activity of each phage over the duration of the growth cycle. Sabp-P1 to Sabp-P3 showed a significant (p<0.001) increase in phage multiplication and entered the exponential phase of growth after 40-50min of incubation. Sabp-P4, Sabp-P5 and Sabp-P6 do not exhibit a clear exponential phase throughout their growth cycle. Phage counts at the exponential phase (40-50min) for Sabp-P1, Sabp-P2 and Sabp-P3 were not statistically different from each other, but were significantly different from that of Sabp-P4, Sabp-P5 and Sabp-P6. All

six phages entered a phase with limited replication from 50min onwards. In general, Sabp-P4, Sabp-P5 and Sabp-P6 appear to be low titre phages.
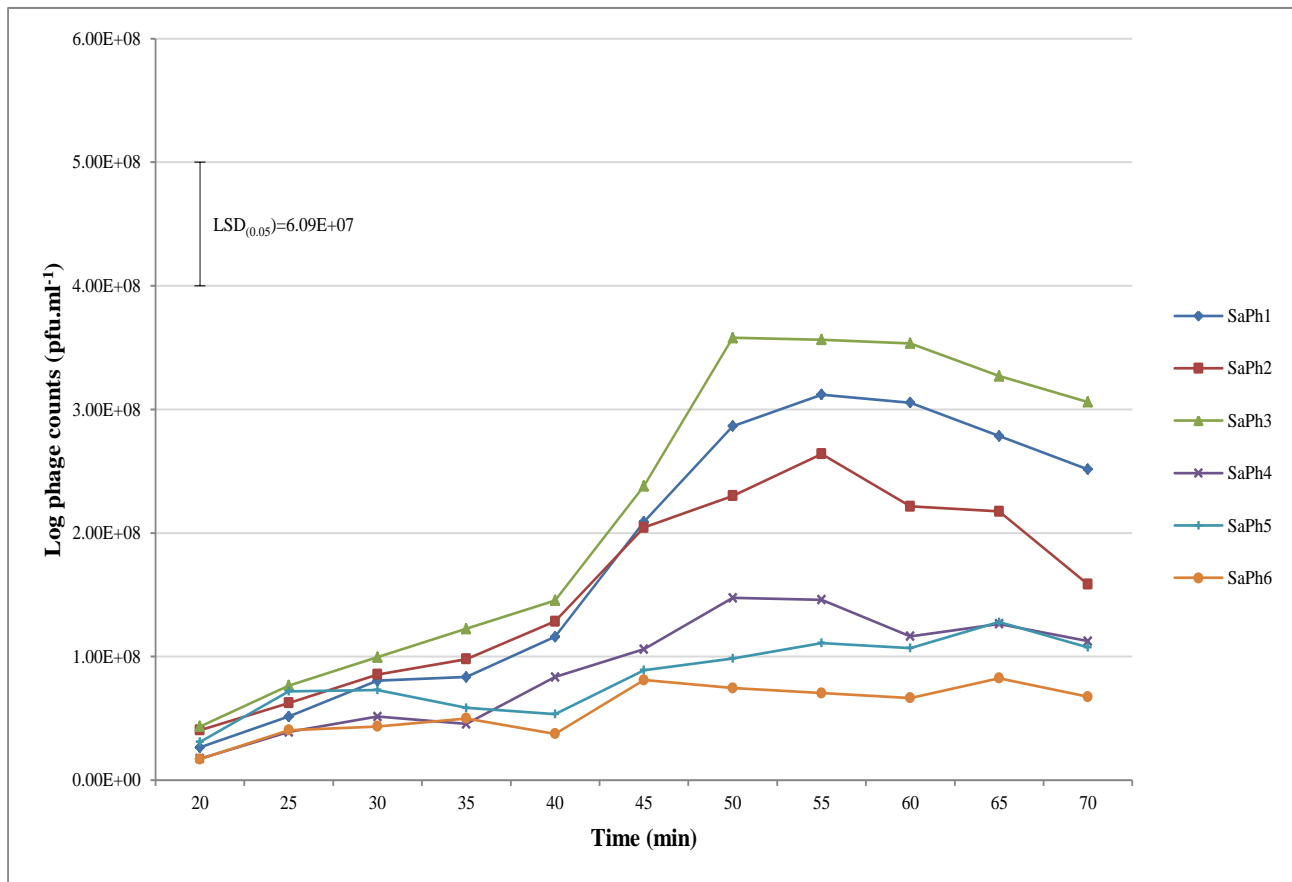
Figure 2.1 Infection curve for *S. aureus* phages. Data points are the means of three independent experiments. The vertical bar represents the LSD $_{(0.05)}$ when comparing any phage count x time combination.

2.3.6 Lethal dose assay

The lethal dose assay was carried out to investigate phage efficacy in reducing live bacterial cell counts after incubation for 12hr. Phages Sabp-P1, Sabp-P2, Sabp-P3, Sabp-P4, Sabp-P5 and Sabp-P6 were tested against *S. aureus* strain, *SaB1*. ANOVA showed highly significant ($p<0.001$) differences in bacterial cell counts when *SaB1* was inoculated with phages in comparison to un-inoculated *SaB1*. Live cell counts decreased significantly upon introduction of phages into the bacterial culture (Figure 2.2; Table 2 in Appendix 1). No significant differences were observed in lytic activity between Sabp-P1, Sabp-P2 and Sabp-P3. Similarly, no significant

difference was noted between lytic activity of Sabp-P4, Sabp-P5 and Sabp-P6. However, significant (p<0.001) differences were apparent in lytic activity between the two groups of phages, i.e., Sabp-P1, Sabp-P2 and Sabp-P3 compared to Sabp-P4, Sabp-P5 and Sabp-P6. Phages Sabp-P1, Sabp-P2 and Sabp-P3 were able to reduce live bacterial cell count up to 95.32% (Table 2.2). Phages Sabp-P4, Sabp-P5 and Sabp-P6 performed poorly, with reductions ranging between 64-68%.
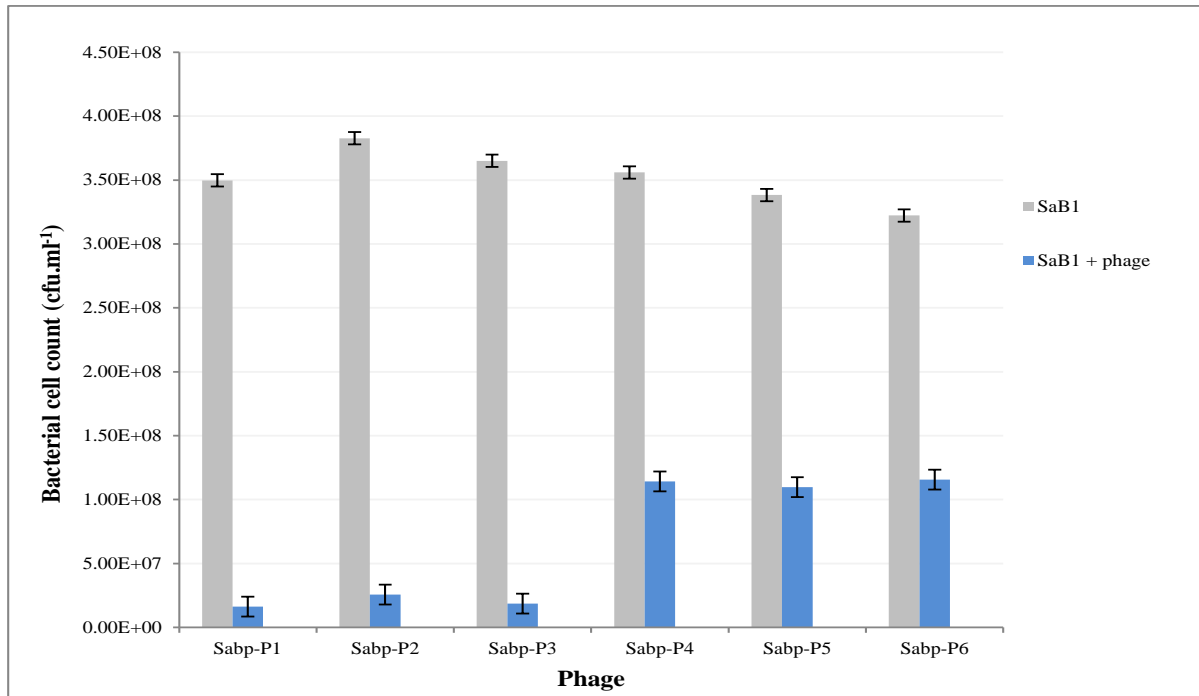
Figure 2.2 Lethal dose assay showing *S. aureus* (*SaB1*) growth with and without addition of phages. Each bar represents the means of three independent experiments. The vertical bar represents the LSD $_{(0.05)}$ when comparing any phage x bacterium combination.

Table 2.2 Reduction in live bacterial cell count after addition of phages to bacterial cultures relative to the control.

Phage	Reduction in growth of *SaB1* (%)
Sabp-P1	95.32
Sabp-P2	93.30
Sabp-P3	94.87
Sabp-P4	67.90
Sabp-P5	67.54
Sabp-P6	64.11
Mean	80.51
F-value	<0.001
LSD	4.094
CV%	2.8

2.3.7 Farm survey

Results of the farm survey differed significantly in terms of bacterial species and number of phages that were isolated between each sampling (Table 2.3). Although the cows SCC values were high (>400,000 cells.ml^{-1}), a great percentage of samples showed zero bacterial growth (Table 2.3) even after 24hr incubation at 37°C. In Sampling 1, 80% of the samples tested positive for presence of phage. In Sampling 2, 71% of the samples tested positive for presence of phage. *S. aureus* was only present in 13.33% and 17.31% of the milk samples in Sampling 1 and Sampling 2, respectively.

Phage activity of Sabp-P1, Sabp-P2, Sabp-P3, Sabp-P4, Sabp-P5 and Sabp-P6 was also tested against the *S. aureus* strains isolated from both samplings. Of the two *S. aureus* strains isolated from Sampling 1, only one showed susceptibility to all six phages; the other showed susceptibility to only Sabp-P1, Sabp-P2, Sabp-P4, and Sabp-P5. From Sampling 2, where 21 *S. aureus* strains were isolated, 18 showed susceptibility to all six phages. Three strains showed resistance to all six phages.

Table 2.3 Prevalence of bacterial species in raw milk, drawn from cows with a high (>400,000 cells.ml^{-1}) somatic cell count.

Bacterial species	Prevalence of bacteria	
	Sampling 1 (%)	Sampling 2 (%)
Staphylococcous aureus	13.33	17.31
Streptococcus agalactiae	6.67	5.77
Coliform bacteria	20.00	1.92
Micrococcus spp.	26.67	13.46
Bacilliform bacteria	0	1.92
Zero bacteria	33.33	59.62
% of samples that contained phages	80	71

2.4 Discussion

Phages have potential as biological control agents of *S. aureus*. The ubiquity of phages in any environment, their ability to replicate exponentially within their hosts and their specificity, make them ideal candidates for more sustainable mastitis control. In particular, the results of the current study demonstrate the potential of South African phage isolates for biological control of bovine mastitis. The six phages that were screened showed pathogenicity towards 18 *S. aureus* strains that were isolated from different milk-producing regions. Additionally, phages were readily isolated from raw milk from different geographical locations. It is this ubiquity that distinguishes phage potential for disease control from other measures.

Farmers today are frequently faced with the problem of antibiotic resistance, which essentially breeds a "super-parasite" that cannot be controlled using conventional antibiotics. This resistance can be attributed to several factors. *S. aureus* has the ability to form abscesses within the udder that are surrounded by thick fibrous tissue (Almeida *et al.* 1996; Villar *et al.* 2011). This fibrous layer inhibits translocation of antibiotics into the abscess, thereby preventing destruction of bacterial cells. In addition, some strains of *S. aureus* can survive within animal cells as macrophages (Herbert *et al.* 2000; Villar *et al.* 2011). Most antibiotics do not have the ability to penetrate within cells themselves, hence these staphylococci remain protected and infectious. Many strains of *S. aureus* produce beta-lactamase which confers resistance to certain formulations of penicillin, in addition to other antibiotics that have been deemed more effective

(such as certain aminoglycosides, cephalosporins and tetracyclines) (Herbert *et al.* 2000; Blowey and Edmondson, 2010; Cabrera *et al.* 2011). Some strains of *S. aureus* can persist in a state of bacterial dormancy with a mucoid capsule and completely cease all replication (Blowey and Edmondson, 2010; Cabrera *et al.* 2011). In this state, they are not killed by antibiotics and are able to reactivate once favourable conditions prevail.

The current study also identified a significant drawback in the application of SCC to identify mastitic cows. While SCC is an accurate estimate of the white blood cell count in raw milk, it does not necessarily imply that that milk has a high bacterial cell count, or that the cow requires antibiotic treatment. When faced with a high SCC, farmers are encouraged to treat those affected animals with antibiotics. Over time, this misuse could potentially lead to selection for resistance in bacteria that occur within the dairy system.

Antibiotic resistance is however, not exclusive to *S. aureus*. In the past three decades, we have witnessed a rise in bacteria carrying extended spectrum β-lactamases, which are mutants of enzymes that previously could only inactivate penicillins but now have gained activity against many cephalosporins too (Wright, 2010); plasmid-mediated (and hence horizontally transmitted) resistance to fluoroquinolone antibiotics (Silver, 1993); the rise of multi-drug resistant *Neisseria gonorrhoeae* (Tapsall, 2009); the emergence and global dissemination of multi-drug resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Enterobacteriaceae* (Kumarasamy *et al.* 2010; Wright, 2010); and the spread of extensively drug-resistant *Mycobacterium tuberculosis* (Wright, 2010). This being said however, the development of new antibiotics is ongoing. Tigecycline, a third-generation, semi-synthetic tetracycline antibiotic approved in 2005, has been shown to have activity against MRSA when used in conjunction with rifampin (Raad *et al.* 2007). In addition, ceftobiprole, a fifth-generation cephalosporin, has shown in clinical trials to provide significant control over MRSA (Zhanel *et al.* 2008). The core problem is that the rate of development of new antibiotics is unable to keep up with the pace of microbial evolution for antibiotic resistance. As a result, the newest antibiotics being used are themselves the source of the evolutionary pressure that will render them obsolete.

Development of phages as a biocontrol option could obviate this problem. Their ubiquity implies a readily available source of control agents should bacterial hosts overcome the control

provided by already developed phages. Their mode of attachment and replication (Kutter and Sulakvelidze, 2005) means that they need not permeate the bacterial cell wall in the way that an antibiotic would. As a result, they are able to readily inject their DNA into bacterial hosts. Furthermore, the exponential rate at which phages are able to multiply within a bacterial cell, while simultaneously killing the target host, is an added benefit. Phage specificity is also a significant advantage. Target bacteria are lysed by their appropriate phages, while other commensal (or non-target) organisms remain unaffected. Studies have shown that production costs for commercial phages are significantly lower than those associated with development of new antibiotics or vaccines (Gill *et al.* 2007; Veiga-Crespo *et al.* 2010).

2.5 Conclusion

Of the six phages screened in this study, three have shown significant potential for further development. It is essential that these phages undergo further screening in order to classify them into appropriate taxonomic groups. Lytic ability must also be evaluated when the phages are exposed to elements that will be involved in their mass production, formulation and final *in vivo* application. With the onset of antibiotic resistance amongst South African dairy cattle, it is also essential that diagnostic methods evolve in order to include accurate bacterial cell counts in raw milk, and not just white blood cells. These results have shown that phages are already abundant in the dairy environment. The next step that remains is to develop this resource and make it readily available as a supplementary treatment for affected dairy cattle.

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CHAPTER 3

Stress sensitivity assays of bacteriophages associated with *Staphylococcus aureus*, causal organism of bovine mastitis

Abstract

Bacteriophages can provide an alternative measure for the control of *Staphylococcus aureus*, the major causal agent for bovine mastitis. This study looked at the sensitivity of six phages towards simulated environmental and formulation stresses. Phages Sabp-P1, Sabp-P2 and Sabp-P3 showed the most stable replication rates at increasing temperatures (45-70ºC), in comparison to phages Sabp-P4, Sabp-P5 and Sabp-P6. The effect of temperature on storage of phages showed that 4ºC was the minimum temperature at which phages could be stored without a significant reduction in their lytic and replication abilities. Furthermore, all phages showed varying levels of sensitivity to chloroform exposure, with Sabp-P5 exhibiting the highest level of reduction in activity (74.23%) in comparison to the other phages. All six phages showed optimal lytic ability at pH 6-7 and reduced activity at any pH above or below pH 6-7. Exposure of phages to varying glycerol concentrations (5-100%) produced variable results. All six phages were most stable at a glycerol concentration between 10-15%. Three of the six isolated phages, Sabp-P1, Sabp-P2 and Sabp-P3, performed optimally during the *in vitro* assays and have considerable potential for *in vivo* applications to treat mastitis-infected dairy cattle.

3.1 Introduction

Staphylococcus aureus-induced bovine mastitis in dairy herds is one of the most widespread and destructive diseases of dairy cows. It has far reaching consequences that affect milk quality and yield, health of the dairy cow, and the economics associated with the processing of milk and milk products. Under optimal conditions, the natural defence mechanisms of the cow itself, in conjunction with cultural control measures, may prove adequate in curbing disease onset and severity. However, the real problem arises when these intrinsic defences and cultural measures are compromised by infection. Antibiotic therapy has traditionally served as the next option to achieve effective control of the disease. Overall benefits of antibiotic therapy include a more rapid elimination of bacterial pathogens than self-cure, a reduced probability of chronic recurrent

infections, a reduced depression in milk yield and a more rapid return to an acceptable somatic cell count and hence to saleable milk (Barkema *et al.* 2006; Nickerson, 2009).

Despite the documented success associated with antibiotic usage, it remains debatable as to whether this therapy is indeed positive in the long term. There are several conflicting views on this (Murchan *et al.* 2004; Borm *et al.* 2006; Sandgren *et al.* 2008; Nickerson, 2009; Blowey and Edmondson, 2010; Vanderhaeghen *et al.* 2010), many of which revolve around *S. aureus* and its ability to develop antibiotic resistance. In the long term, the cure rate of antibiotic treatments against this pathogen is low and, therefore, the disease cannot be effectively eliminated and/or controlled in infected herds by using antibiotics alone (Sutra *et al.* 1993; Carter *et al.* 2003; Murchan *et al.* 2004; Shi *et al.* 2010).

Antibiotic resistance can be attributed to several factors, ranging from structural features that protect the bacterium, to production of chemicals that can neutralise antibiotics (Almeida *et al.* 1996; Herbert *et al.* 2000; Blowey and Edmondson, 2010; Cabrera *et al.* 2011; Villar *et al.* 2011). The spread of virulent methicillin-resistant *S. aureus* (MRSA) (Goñi *et al.* 2004; Murchan *et al.* 2004; Nickerson, 2009; Shi *et al.* 2010; Vanderhaeghen *et al.* 2010), coupled with the development of resistance to two new antibiotics (daptomycin and linezolid) recently approved for clinical use against Gram-positive bacteria (Mangili *et al.* 2005), has shown that *S. aureus* is indeed a formidable pathogen.

Due to these treatment limitations, research focus into the control of bovine mastitis has shifted to alternative therapies, such as the development of vaccines (Sutra, 1993; Herbert *et al.* 2000; Pereira *et al.* 2011) and biological control options, such as the use of botanical extracts (Akinyemi *et al.* 2005; Fawole, 2009) or phage therapy (O'Flaherty *et al.* 2005b; Sulakvelidze and Barrow, 2005; Gill *et al.* 2006a; García *et al.* 2007; Jones *et al.* 2007; García *et al.* 2008; Synott *et al.* 2009). Considering the wealth of information at our disposal, phage therapy appears to be one of the most sustainable measures for control of bovine mastitis. Several studies have identified various phages with lytic capabilities towards *S. aureus* (O'Flaherty *et al.* 2005a; Gill *et al.* 2006a; García *et al.* 2007; García *et al.* 2008; Synott *et al.* 2009). While each study did present noteworthy results, it must be noted that there are limitations which must be overcome. Phage inactivation can be triggered by milk proteins and fats (O'Flaherty *et al.* 2005b; Gill *et al.* 2006b), aggregation of *S. aureus* cells within milk (O'Flaherty *et al.* 2005b), and intrinsic

immune factors within the cow itself (O'Flaherty *et al.* 2005a). While these limitations do exist, the solutions to these problems are merely a product of time. Further studies into the detailed effects of whey proteins on bacterial activity and aggregation, proper delivery of phage cocktails into animal tissues, and the development of phage formulations to facilitate optimal delivery and activity within intramammary tissues, are ongoing.

The primary focus of the current study was to investigate phage sensitivity, *in vitro*, towards simulated environmental and chemical stresses. This study aimed to contribute to phage formulations for *in vivo* application.

3.2 Materials and Methods

3.2.1 Bacterial host strains and their isolation

Strain SaB1 of *S. aureus* was used for phage isolation and propagation. This strain was isolated from raw bovine milk collected from dairy farms in the province of KwaZulu-Natal, RSA (Republic of South Africa). *SaB1* was initially isolated on blood agar. Cultures required for phage propagation were maintained on tryptone soy agar (TSA) or typtone soy broth (TSB).

3.2.2 Phage isolation, propagation and purification

Raw bovine milk samples collected from dairy farms in the province of KwaZulu-Natal, RSA (Republic of South Africa) served as the initial material from which to isolate phages. These milk samples were stored at 4ºC. Phages were isolated using the spot-plate method on double-layer agar (Sambrook *et al.* 1989; Harley and Prescott, 1993). Overnight *SaB1* cultures (~1 x 10^8 cfu.ml^{-1}) were incorporated into 7% top agar that was supplemented with 1M CaCl$_2$. Raw milk samples were filtered through a 0.45μm syringe filter. Filtered samples were then spotted onto the surface of the solidified top agar at 10μl per spot. Plates were allowed to dry for 2hr, followed by incubation for 12hr at 37ºC. Zones of clearing (plaques) were indicative of phage activity. Plaques were removed from top agar and soaked in phage buffer (Appendix 1) for 12hr with gentle agitation (150rpm) at 4ºC. The resulting suspension was centrifuged using an Avanti J-26 XPI (www.beckmancoulter.com) at 10,000g x 10min at 4ºC. The supernatant was filtered using a 0.45μm syringe filter and stored as phage stock at 4ºC. Subsequent phage was grown

from this stock in liquid broth culture or using the double-layer agar method (Sambrook *et al.* 1989).

Isolated phages were purified through a modification of standard methods (Sambrook *et al.* 1989; Harley and Prescott, 1993). Filter-sterilised phage stock isolated from either liquid broth culture or double-layer agar method, were subjected to centrifugation using the Avanti J-26 XPI (Beckman-Coulter) at 75,600g x 3hrs x 10ºC. The resulting phage pellets were re-suspended in fresh phage buffer and a second centrifugation was conducted (Avanti J-26 XPI (Beckman-Coulter) at 75,600g x 3hrs x 10ºC). The phage pellets were then re-suspended in fresh phage buffer at 1/10 of the original volume that was processed. All stocks were stored at 4ºC. Six phages were isolated and named Sabp-P1, Sabp-P2, Sabp-P3, Sabp-P4, Sabp-P5 and Sabp-P6. Multiplicity of infection (MOI) was calculated for each phage. Phage MOI was determined for each phage: Sabp-P1=3, Sabp-P2=3, Sabp-P3=3, Sabp-P4=0.2, Sabp-P5=0.1 and Sabp-P6=0.2.

3.2.3 Temperature sensitivity

a. Heat sensitivity

This method was a modification of that described by Harley and Prescott (1993). Optimal phage dilutions of 10^{-5} pfu.ml^{-1} were prepared for each phage for this assay. Phage dilutions were carried out in phage buffer. Diluted phages, Sabp-P1 to Sabp-P6, were placed into a water-bath at 45°C. 100µl aliquots were removed at each of the following time intervals: 0min (control), 5min, 10min, 20min, 30min, 40min, 50min and 60min. Each aliquot was plated using the double-layer plating technique (Sambrook *et al.* 1989), with an overnight culture of *SaB1* as the host bacterium. The experiment was repeated for temperatures 50°C, 55°C, 60°C, 65°C and 70°C. Phage diluted in phage buffer was used as a control. All plates were incubated at 37°C for 12hr. After 12hr, the number of plaques was counted and phage titre was calculated for each time and its associated temperature. All assays were carried out in duplicate.

b. Cold sensitivity (storage feasibility of phages)

This assay was conducted in order to see the opposite effects of heat treatment on phages and to test their storage capability over time. Optimal phage dilutions of 10^{-5} pfu.ml^{-1} were prepared for each phage. Phage dilutions were carried out in phage buffer. This experiment was ongoing over

a period of 6mo. Overnight *SaB1* was used as the host bacterium. Phages Sabp-P1 to Sabp-P6 were screened for stability at various storage temperatures, i.e., 4°C, -20°C and -80°C, with different durations at each temperature, i.e., 1hr, 8hr, 12hr, 1wk, 2wk, 3mo and 6mo. All phages were stored in sterile phage buffer (supplemented with 5% glycerol) for the duration of the experiment. At the end of each storage duration, at each temperature, a 100μl aliquot of phage was removed and immediately plated using the double-layer plating technique. An overnight culture of *SaB1* was used as the host bacterium, hence final reaction contained 100μl phage + 100μl SaB1 (+ 1M $CaCl_2$). This was followed by double-layer plating as previously described (Sambrook *et al.* 1989). Fresh phage diluted in phage buffer was used as a control. Plates were incubated for 12hr at 37°C. The total number of plaques was counted and phage titre was calculated for each phage, at each time and at each associated temperature. The same phage sample was stored and screened for the entire duration of the experiment (6mo). All assays were carried out in triplicate.

3.2.4 pH sensitivity

This method is a modification of that described by Harley and Prescott (1993). TSB was calibrated according to the following pH range: 2, 4, 6, 8, 10, 12 and 14, and 900μl of each was aliquoted into 2ml Eppendorf vials. Phage stock was added to the calibrated TSB to bring the final phage dilution to 10^{-5} pfu.ml^{-1}; i.e., approximately 2.91 x 10^8 pfu.ml^{-1}, 3.04 x 10^8 pfu.ml^{-1}, 3.12 x 10^8 pfu.ml^{-1}, 1.9 x 10^7 pfu.ml^{-1}, 1.76 x 10^7 pfu.ml^{-1}, 2.6 x 10^7 pfu.ml^{-1}, for Sabp-P1, Sabp-P2, Sabp-P3, Sabp-P4, Sabp-P5 and Sabp-P6 respectively. The vials were gently inverted in order to mix the phage within the TSB medium. All vials were left to stand at room temperature (22°C) for 60min. At 60min, 100μl of phage suspension was removed and mixed with 100μl of an overnight SaB1 culture (+ 1M $CaCl_2$). This was followed by double-layer plating as previously described (Sambrook *et al.* 1989). Phage diluted in phage buffer was used as a control. All plates were incubated at 37°C for 12hr. After 12hr, the number of plaques was counted and phage titre was calculated for each pH. All assays were carried out in triplicate.

3.2.5 Chloroform sensitivity

This method is a modification of that described by Harley and Prescott (1993). Optimal phage dilutions of 10^{-5} pfu/ml were prepared for each phage. Phage dilutions were carried out in phage buffer (Appendix 1). Overnight *SaB1* was used as the host bacterium. 1ml each, of phages Sabp-P1 to Sabp-P6, were treated with 5% (v/v) chloroform. This suspension was gently shaken (100rpm) at room temperature (22°C) for 15min, followed by centrifugation at 10,000g x 10min at 4°C. The aqueous phase was withdrawn and phages were titred using standard double-layer plating. Phage diluted in phage buffer was used as the control. All plates were incubated at 37°C for 12hr. After 12hr, the number of plaques was counted and phage titre was calculated. All assays were carried out in triplicate.

3.2.6 Glycerol sensitivity

This assay was adapted from Santos *et al.* (2009). Optimal phage dilutions of 10^{-5} pfu.ml^{-1} were prepared for each phage. Phage dilutions were carried out in phage buffer. Overnight *SaB1* was used as the host bacterium. Glycerol was made up in sterile distilled water at the following concentrations (v/v): 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%. Phage stock was added to the 900µl of each glycerol solution to bring the final phage dilution to 10^{-5} pfu/ml. This was left at room temperature (22°C) for 1hr. Incubation was followed by standard double-plating and incubation of all plates at 37°C for 12hr. After incubation, the number of plaques was counted and phage titre was calculated. Phage diluted in phage buffer was used as a control. All assays were carried out in triplicate.

3.2.7 Statistical analyses

Differences between treatments were determined by analysis of variance (ANOVA) using Genstat 14th Edition.

3.3 Results

3.3.1 Heat assay

ANOVA of the phage titres showed highly significant ($p<0.001$) differences for phage titers at different temperatures, and a highly significant phage x temperature x exposure time interaction.

It was found that an increase in temperature, coupled with increased exposure time to that temperature, reduced phage reproductive activity (Figure 3.1; Table 1 in Appendix 3).

Phage counts dropped significantly from 0min exposure to high temperature, to 10min exposure to the same temperature (across the complete temperature range of 45-70ºC). After 10min, phage reproductive activity stabilised across all temperature ranges, but remained low (Figure 3.1). Significant differences were noted between phage activity at each temperature and exposure time (<0.001), as well as between each phage itself (<0.001). In general, Sabp-P1, Sabp-P2 and Sabp-P3 were more stable, and titers remained higher than those for Sabp-P4, Sabp-P5 and Sabp-P6. Exposure to 70°C was the most damaging with a 92-96% reduction in phage titers. Overall results show that propagation of these phages is negatively affected by increased exposure to high temperatures.

3.3.2 Cold assay (storage feasibility of phages)

Storage temperature played an important role in influencing phage titre. ANOVA showed highly significant differences (p<0.001) in phage counts when the phages were stored at different temperatures for different durations (Figure 3.2; Table 2 in Appendix 3). Storage of phages at temperatures below 4°C reduced phage activity significantly. Sabp-P1, Sabp-P2 and Sabp-P3 displayed more stability at all three temperature ranges (4°C, -20°C, -80°C), although phage titre did decrease significantly at -80°C. The titres for the other three phages Sabp-P4, Sabp-P5 and Sabp-P6, were generally low throughout these studies. However, these three phages showed stability during storage at all durations and temperatures. While the study did not proceed beyond a screening period of 6mo, it appears that the optimal storage temperature for the isolated phages (in phage buffer) is in the region of 4°C.
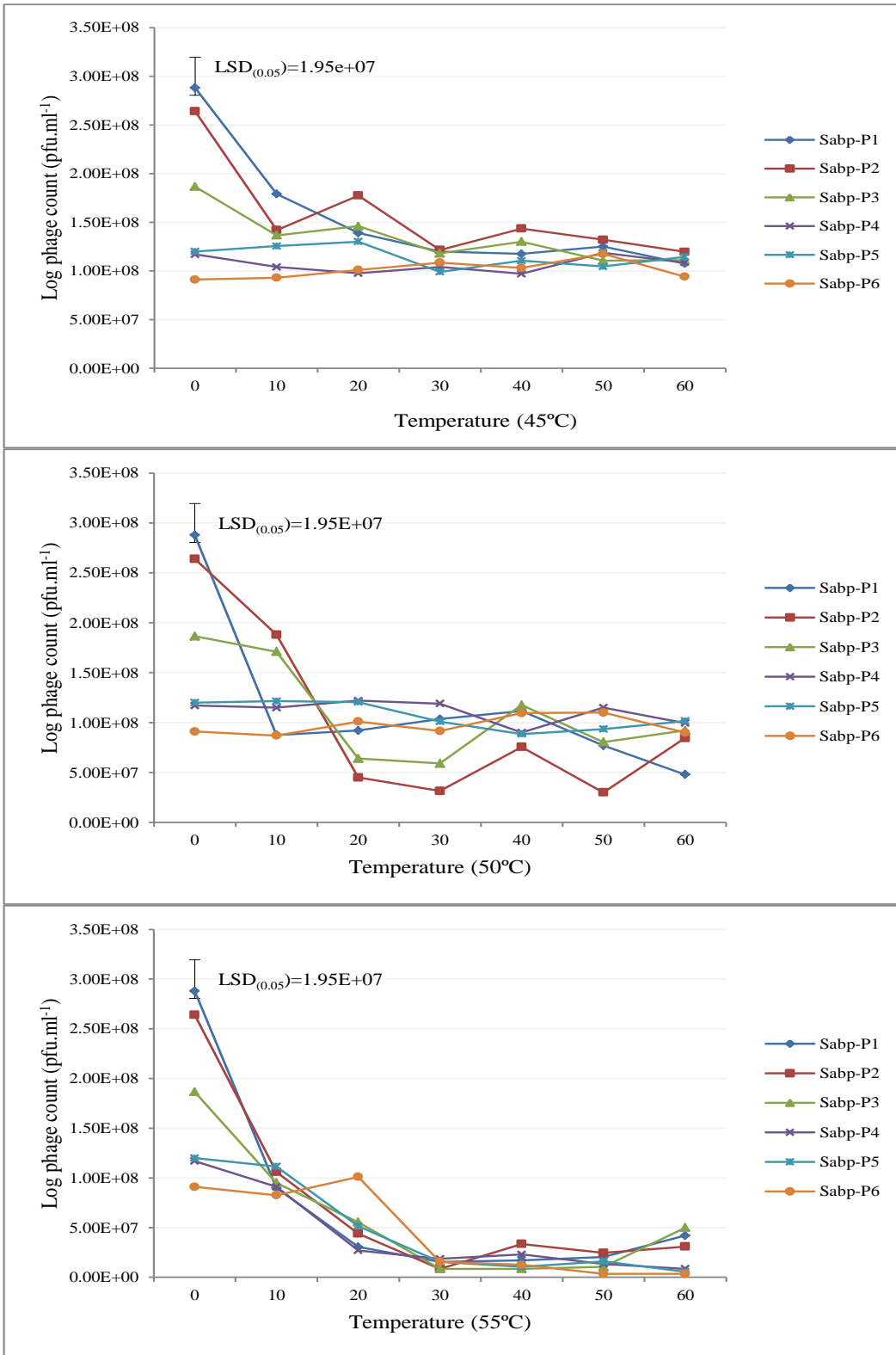
Figure 3.1 Heat assay screening the titres of six phages over a range of different temperatures. *SaB1* was used as the host bacterium for phage propagation; Data points are the means of two independent experiments. The vertical bar represents the LSD $_{(0.05)}$ when comparing any phage x temperature x time combination.

Figure 3.1 continued
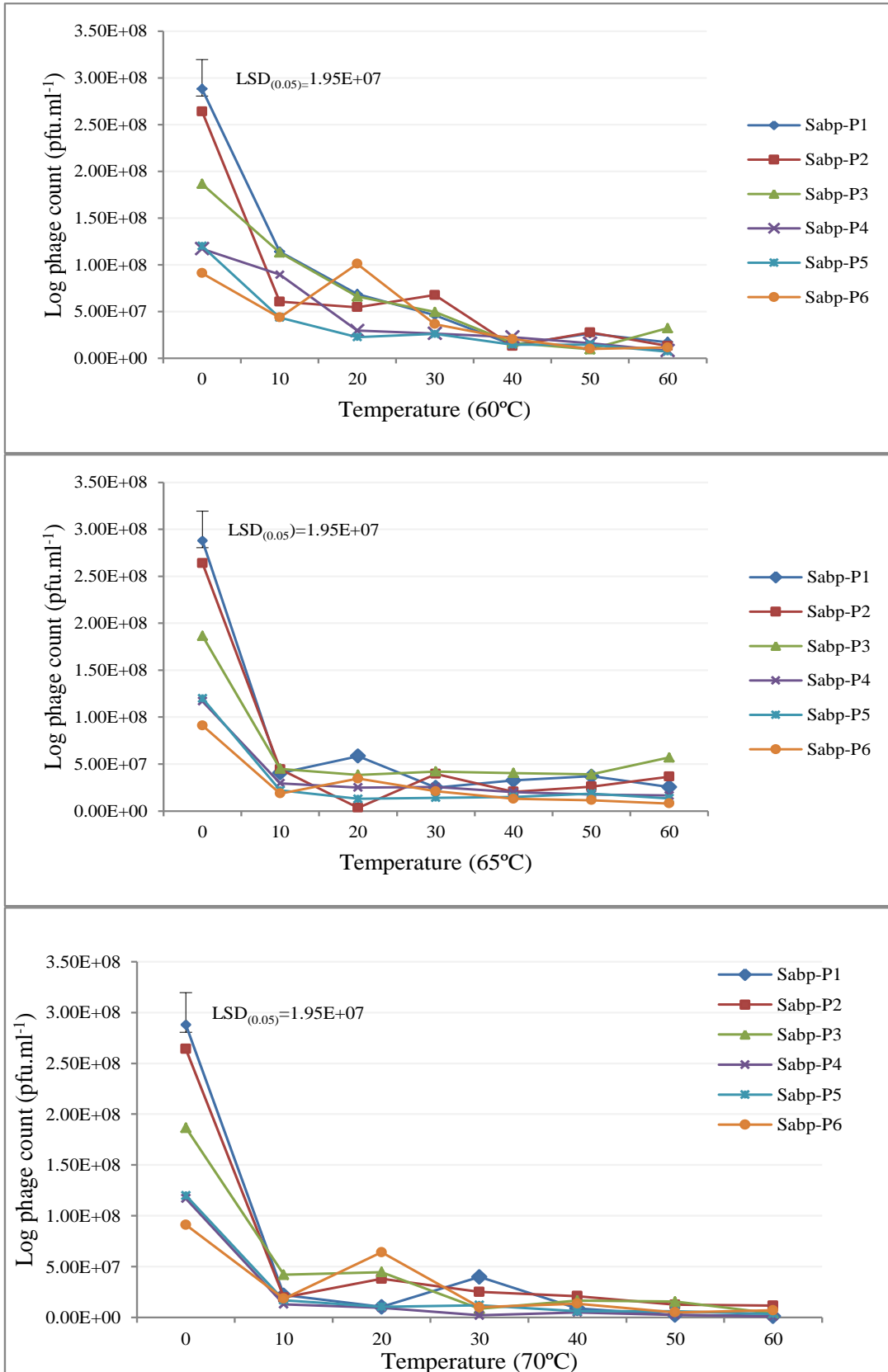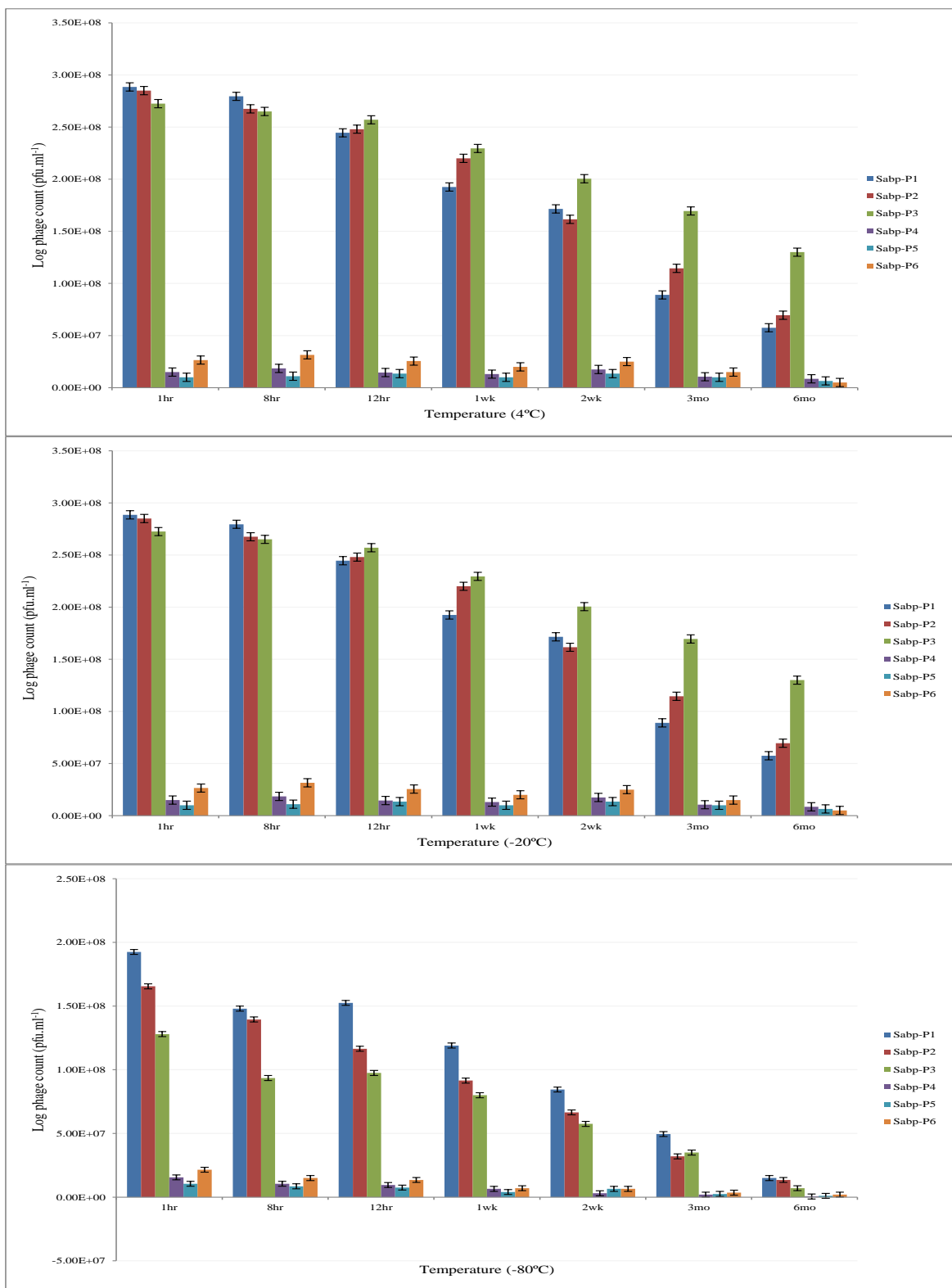
Figure 3.2 Screening the storage ability of six phages over a range of low temperatures. *SaB1* was used as the host bacterium for phage propagation.Each bar represents the means of three independent experiments. The vertical bar represents the LSD $_{(0.05)}$=3.93E+06 when comparing any phage x temperature combination.

3.3.3 Chloroform Assay

Chloroform exposure imposed negative effects on phage titers (Figure 3.3). ANOVA showed that significant (p<0.001) differences occurred between the titers of Sabp-P1, Sabp-P2 and Sabp-P3 in comparison to phages Sabp-P4, Sabp-P5 and Sabp-P6. Sabp-P1, Sabp-P2 and Sabp-P3 appeared more stable and titres did not reduce as significantly upon exposure to chloroform, as they did for Sabp-P4 and Sabp-P5. Sabp-P6 showed the most stability over time upon exposure to chloroform. However, a general reduction in phage titers was noted for each phage (Table 3.1).
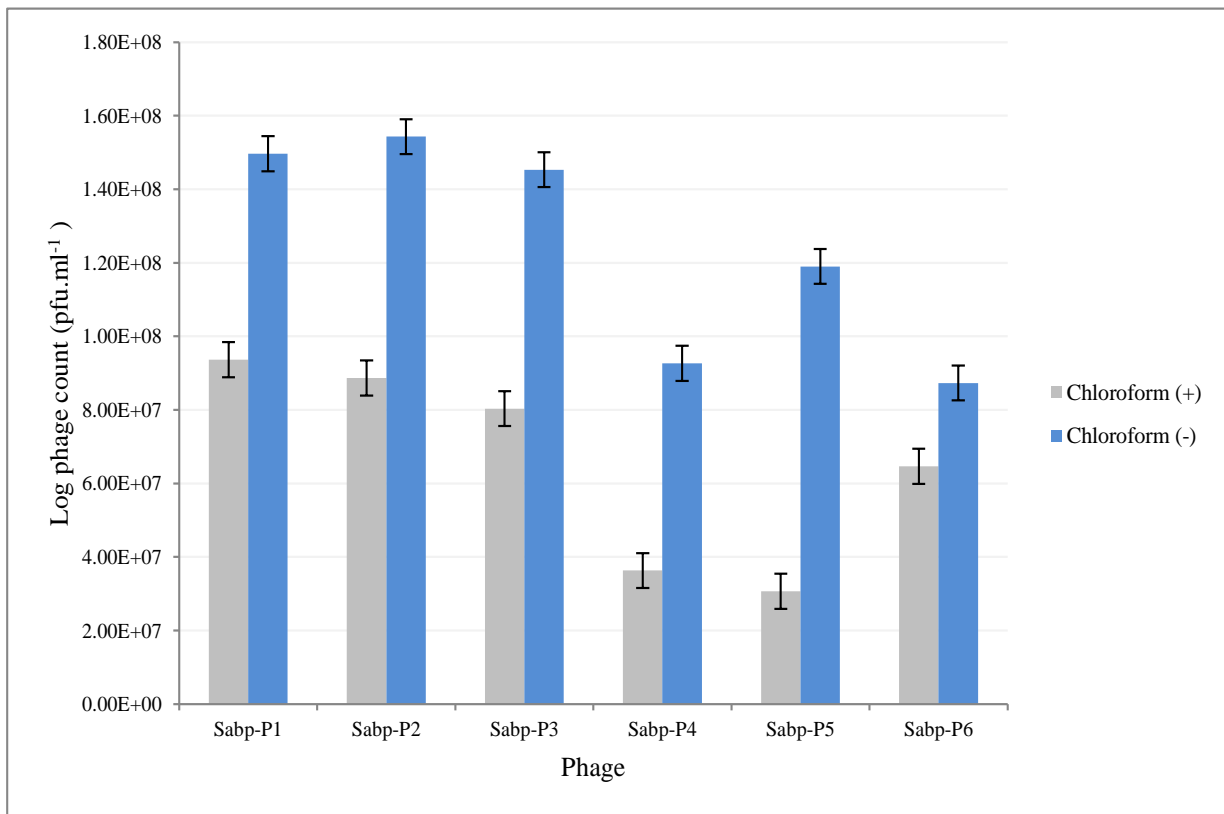
Figure 3.3 Effect of chloroform on phage titers. *SaB1* was used as the host bacterium for phage propagation.

Each bar represents the means of three independent experiments. The vertical bar represents the LSD $_{(0.05)}$=9.5E+07 when comparing any phage x bacterium combination.

Table 3.1 Reduction in phage titers upon exposure to chloroform

Phage	Reduction in phage activity (%)
Sabp-P1	37.42
Sabp-P2	42.55
Sabp-P3	44.73
Sabp-P4	60.80
Sabp-P5	74.23
Sabp-P6	25.95
Mean	47.61
LSD	9.50
CV%	0.4

3.3.4 pH assay

All of the screened phages (Sabp-P1, Sabp-P2, Sabp-P3, Sabp-P4, Sabp-P5, Sabp-P6) showed sensitivity to changes in the pH of the surrounding growth media (Figure 3.4). ANOVA showed significant differences ($p < 0.001$) in activity between phages as pH increased, as well as significant difference in interactions between the phages themselves. Sabp-P1, Sabp-P2 and Sabp-P3 showed the highest increase in phage titer between pH 6-7. Thereafter, a sharp decline in phage titre was noted from pH 7.2-12. Phages, Sabp-P4, Sabp-P5 and Sabp-P6, showed a similar pattern.. However, titres were significantly lower from those of Sabp-P1, Sabp-P2 and Sabp-P3. There was no significant difference in activity between Sabp-P1, Sabp-P2 and Sabp-P3, or, between activity of Sabp-P4, Sabp-P5 and Sabp-P6. However, each group differed significantly from each other.
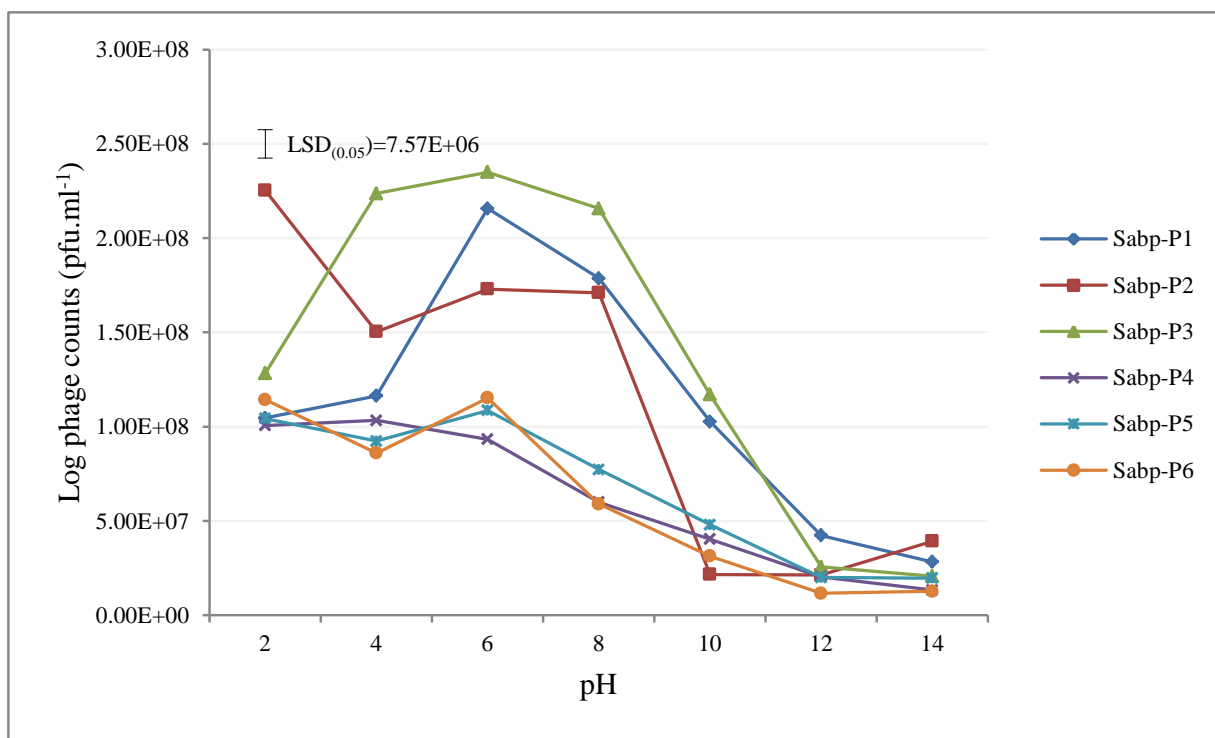
Figure 3.4 Effect of increasing pH on the titers of six phages. *SaB1* was used as the host bacterium for phage propagation. Data points are the means of three independent experiments. The vertical bar represents the LSD $_{(0.05)}$ when comparing any phage x pH combination.

3.3.5 Glycerol Assay

A general trend of reduced phage activity was observed with an increasing concentration of glycerol (Figure 3.5). ANOVA showed significant differences (p<0.001) in activity between phages as glycerol concentrations increased from 5-100%. Phages Sabp-P2 and Sabp-P3 are stable and able to replicate up to a glycerol concentration of 25%. Thereafter, titres dropped significantly and no further increase was noted. Phages Sabp-P1, Sabp-P4, Sabp-P5 and Sabp-P6 showed a dramatic decline in titre as glycerol concentration increased from 10% upwards. This differed significantly from the activity of Sabp-P2 and Sabp-P3 (<0.001). Exposure of the selected phages to high concentrations of glycerol resulted in a dramatic decrease in titre that ranged from 52-94% (Table 3.2).
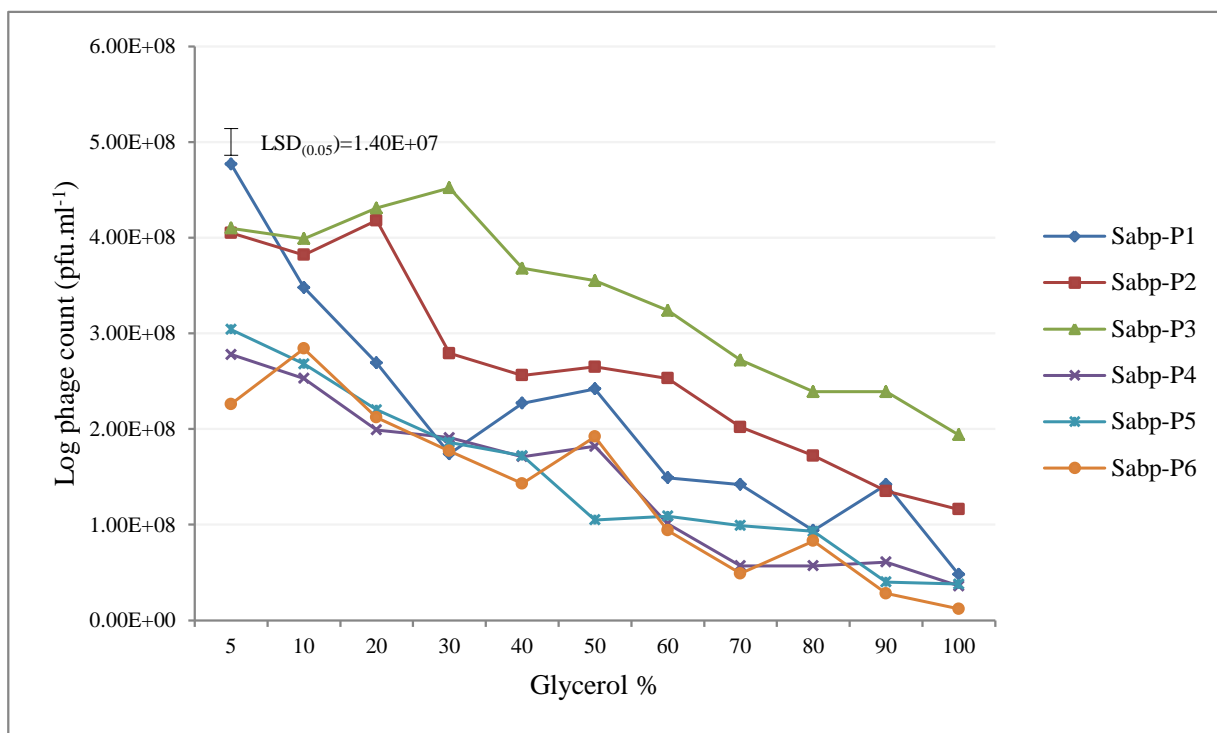
Figure 3.5 Effects of increasing glycerol concentration on phage titers. *SaB1* was used as the host bacterium for phage propagation. Data points are the means of three independent experiments. The vertical bar represents the LSD $_{(0.05)}$ when comparing any phage x glycerol % combination.

Table 3.2 Reduction in phage titers after exposure to increasing concentration ranges of glycerol

Phage	Reduction in phage activity (%)	Range of increase in glycerol concentration that limits phage growth
Sabp-P1	89.94	10-100%
Sabp-P2	71.36	25-100%
Sabp-P3	52.68	25-100%
Sabp-P4	87.05	10-100%
Sabp-P5	87.5	10-100%
Sabp-P6	94.69	10-100%
Mean	80.54	
LSD	7.01	
CV%	1.5	

3.4 Discussion

The onset of antibiotic resistance in *S. aureus* and its implications for the treatment of bovine mastitis have raised awareness of the need for alternative control therapies. Phages provide such an alternative. However, associated with this therapy are several challenges, particularly in terms of developing stable formulations and optimizing storage and *in vivo* application of phage products. The primary objective of the current study was to evaluate phage titers upon exposure to simulated stresses that might be encountered during the formulation of the phage or during *in vivo* application of the formulated phages.

Phage activity was assessed *in vitro*, in a heat assay to determine the temperature at which the selected phages are most stable, for the longest duration. *In vivo* application of phages requires resistance (or tolerance) to high temperatures. Temperature fluctuations in an *in vivo* system could possibly arise from changes in weather patterns, physiological changes in the body of the cow itself, or even changes in the storage conditions of the phage. All six phages that were screened showed reduced lytic ability from as early as 10min exposure to 45ºC. This is significant, especially in terms of formulation of phages into commercial products. Certain formulation procedures may require high temperatures during the manufacturing process. This has to be undertaken with caution in order to minimize any reduction in the replication ability of the phages. Phages are primarily composed of protein (Kutter and Sulakvelidze, 2005). Any prolonged exposure to high temperature may cause denaturation of phage proteins responsible for infection of a bacterial host. Compromised physical structure of an otherwise infective phage, then reduces the control potential that that phage could have imposed on a target host. These results correlate with previous studies where phage lytic ability was shown to be reduced by prolonged exposure to high temperatures (Da Silva and Janes, 2005; Bryant *et al.* 2007; Chandra *et al.* 2011).

The selected phages also showed reduced lytic ability after exposure to temperatures below 4ºC. This reduced pathogenicity could be attributed to the effect that freezing/thawing may have on phage ultrastructure. This is particularly relevant for tailed phages from the *Myoviridae* family. Delicate tail and tail fibres could become dissociated from the virus head due to changes in osmotic pressure (Jończyk *et al.* 2011). This sort of dissociation renders the phage ineffective as a control agent. The results of this study contradict those of Hsieh *et al.* (2011),

where phages were found to have maintained stability even after one year of storage at -85ºC. Hsieh *et al.* (2011) stored all phages in 7% dimethyl sulfoxide, in comparison to phage buffer (+ glycerol) in the current study. The choice of storage medium might therefore influence phage stability over time. For the purposes of an *in vivo* study, long- and short-term storage of phage products at 4ºC, in a more protective medium (such as glycerol) seems the most likely way forward.

When phages are grown in large-scale liquid broth cultures, either for laboratory or *in vivo* assays, chloroform is usually added to the growing medium to kill any live bacterial cells that remained un-lysed (Sambrook *et al.* 1989). The isolated phages from this study showed extreme sensitivity to chloroform treatments. While phages Sabp-P1, Sabp-P2 and Sabp-P3 demonstrated significant tolerance to chloroform treatment, their lytic ability was still compromised. In light of this, an alternative method has to be employed in order to remove host cells from a culture suspension. Micro-filtration serves as one of the least-damaging methods to apply, as phage simply pass directly through the filter while un-lysed bacterial cells as well as large bacterial debris, are retained. The use of micro-filters was implemented throughout the current study.

The pH of the medium in which a phage is propagated is also an area of importance. Depending on where a phage has been isolated from, the pH of its growth medium should ideally mimic that of its natural environment in order to promote optimal phage replication. The pH of milk is between pH 6.6-6.7 (Blowey and Edmondson, 2010). Optimal phage replication occurred at pH 6-7, followed by a sharp decline at higher pH. This was also noted in a study by Da Silva and Janes (2005), where phages specific to *Vibrio* spp. (infective on oysters) were screened at various pH ranges. The Vibrio phages were most stable at that pH range which best mimicked the pH of the oyster system (pH 7-8).

The formulation of phage/s into a product that can be applied in an *in vivo* system requires storage of the phages in a medium that maintains phage stability, is itself stable over time, and does not damage the teat upon application. Dairy farmers implement both pre- and post-dipping disinfectants during the milking process (Blowey and Edmondson, 2010). Teat skin has relatively few sebaceous glands, and continual washing and drying of the teats can remove the limited amount of fatty acids that maintain the skin barrier (Blowey and Edmondson, 2010).

This can result in severe cracking/breaking of the teat skin, leaving the teat exposed to entry of pathogens. In an attempt to maintain healthy teat skin, additives are used in post-dipping formulations. Emollients (such as lanolin) and humectants (such as glycerin) are the most commonly applied additives (Blowey and Edmondson, 2010). The current study investigated the effect of glycerol concentrations on phage activity. Phage lytic ability was optimal in a suspension of up to 15% glycerol, but dropped thereafter. Phage suspension in a 10% glycerol solution could be applied as an *in vivo* treatment for the treatment of bovine mastitis. Such an application could have a two-fold benefit: (1) the protective effect of phages against *S. aureus* on teat surfaces; (2) the moisturizing effect of glycerol on sensitive teat skin. Note that phages have not been shown to have any negative effects on either the actual health of the cow (O'Flaherty *et al.* 2005b; Gill *et al.* 2006a) or the quality of milk produced (Hudson *et al.* 2005; García *et al.* 2008).

3.5 Conclusion

This study showed the optimum storage and growth conditions necessary in order to maintain high tire phage cultures. Slight deviations from the standard growth requirements (37°C for optimal growth; storage at 4°C, culture medium pH of 6-7) for the selected phages resulted in dramatic reductions in phage activity. Phages Sabp-P1, Sabp-P2 and Sabp-P3 showed more vigour and were more robust than Sabp-P4, Sabp-P5 or Sabp-P6. Sabp-P1, Sabp-P2 and Sabp-P3 would be preferred candidates when investigating *in vivo* applications of the phages as a control treatment against staphylococcal bovine mastitis.

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CHAPTER 4

Morphological classification of three novel South African bacteriophages infective against *Staphylococcus aureus*, causal agent of bovine mastitis

Abstract

Morphological classification of unknown biological entities forms an integral step in their proper nomenclature and grouping. Bacteriophages can usually be placed into a group based on their physical structure. The aim of the current study was to morphologically characterise three novel South African phages and to investigate their infection cycle within the bacterial host, using transmission electron microscopy. The phages were isolated from raw milk drawn off dairy cows that had tested positive for *Staphylococcus aureus*-induced bovine mastitis. Results showed that all three phages appeared structurally similar. Each possessed an icosahedral head separated from a striated, contractile tail region by a constricted neck region. The head capsules ranged in diameter between 90-110nm with the tail length ranging from 150-200nm in the non-contractile state and 100-130nm in the contractile state. Rigid tail fibres were also visible below the striated tail. The major steps in the virus replicative cycle were also documented. Ultra-thin sections through phage plaques were prepared through a modification of traditional methods, with no negative effects on sample integrity. The major steps that were reported in the phage replicative cycle were (1) attachment to host cells, (2) replication within host cells, and, (3) release from cells. Overall results suggested that the three phages are strains from the order *Caudovirales* and are part of the *Myoviridae* family.

4.1 Introduction

Phages have been found to infect more than 140 bacterial genera including (1) aerobes and anaerobes; (2) exospore and endospore formers; (3) cyanobacteria, spirochetes, mycoplasmas, chlamydias; (4) budding, gliding, ramified, stalked and sheathed bacteria; (5) extreme halophiles and methanogens; and, (6) hyperthermophilic Archaea (Ackermann, 2005). Phages have been found to be extremely heterogeneous in their structural, physicochemical and biological properties, which could suggest that they are polyphyletic in nature (Ackermann, 2005).

However, their primary mode of action is similar. Virions differ significantly as well, occurring as tailed, polyhedral, filamentous or pleomorphic, with heterogenous genomes ranging from dsDNA, ssDNA, dsRNA and ssRNA (Ackermann, 2005; 2007). By 2007, at least 5568 phages had been examined through electron microscopy (Ackermann, 2007). Results derived from genomic and morphological studies have been used to categorise phages into one order, 13 families and 31 genera (Ackermann, 2005; 2007).

Tailed phages of the order *Caudovirales* constitute the most extensive group of bacterial viruses comprising 96% of phages identified so far (Ackermann, 2007; Gutiérrez *et al.* 2011). These phages consist of a protein shell and linear dsDNA and lack any type of envelope (Ackermann, 2005; 2007). Phage heads are icosahedral and tails are true helices that consist of stacked discs. These tails usually possess terminal adsorption structures such as base plates, spikes or tail fibres (Ackermann, 2005). Although these phages constitute a monophyletic group possessing related properties (morphological, physicochemical, physiological) and have been classified into as a single order, *Caudovirales*, their individual properties are rather varied. Typically, they differ in terms of dimension and fine structure, DNA content and composition, nature of constitutive proteins, serology and host range (Ackermann, 2005). Ackermann (2005) has devised criteria to distinguish between these phages: *Myoviridae*, with contractile tails consisting of a sheath and central tube, approximately 25% of tailed phages; *Siphoviridae*, with long, non-contractile tails, approximately 61% of tailed phages; and, *Podoviridae*, with short, non-contractile tails, approximately 14% of tailed phages.

While tailed phages do comprise the majority of bacterial viruses, tailless phages also occur. These include only about 190 known viruses, corresponding to less than 4% of the currently recognised bacterial viruses (Ackermann, 2005). They have been classified into 10 small families, occur enveloped or non-enveloped, and are of three types: (1) polyhedral phages that are icosahedral with cubic symmetry and either DNA or RNA constituted; (2) filamentous phages with helical symmetry that are DNA constituted; and, (3) a few pleomorphic types without obvious symmetry axes that are also DNA constituted (Ackermann, 2005).

The current study involved the morphological classification of three phages with specific lytic activity against *Staphylococcus aureus*, the causal organism of bovine mastitis. Bovine mastitis is an infectious inflammation or irritation of the mammary glands that interferes with the

normal flow and quality of milk (Blowey and Edmondson, 2010). Although a range of control measures are used, this disease remains a major cause of economic losses in the dairy industry both within South Africa and worldwide. In recent years, the use of integrated control measures has become paramount, due to the onset of resistance against the antibiotics commonly used to treat the disease. Phages provide an alternative control measure because antibiotic resistance is particularly relevant to *S. aureus*-induced bovine mastitis.

Against this background, three phages with activity against South African strains of *S. aureus*, were characterised according to their morphology and replicative cycle, using transmission electron microscopy. Other than genomic analysis, electron microscopy is often the fastest method for phage identification. It can be used to assign an unknown phage to a specific family, based on head and tail morphology, and to differentiate between different phage types.

4.2 Materials and Methods

4.2.1 Bacterial strains

Four *S. aureus* strains were used for phage isolation, i.e., *SaB1*, *SaB2*, *SaB3* and *SaB4*. These strains were isolated from raw bovine milk collected from dairy farms in the province of KwaZulu-Natal, RSA (Republic of South Africa). *S. aureus* was initially isolated on blood agar. Thereafter, cultures were maintained on tryptone soy agar (TSA). *S. aureus* cultures used for phage propagation were grown on TSA for 12hr at 37ºC.

4.2.2 Phage isolation

Raw bovine milk samples collected from dairy farms in the province of KwaZulu-Natal, RSA (Republic of South Africa) served as the initial material from which to isolate phages. These milk samples were stored at 4ºC. Phages were isolated using the spot-plate method on double-layer agar (Sambrook *et al.* 1989). Overnight *S. aureus* cultures (~1 x 10^8 cfu.ml^{-1}) were incorporated into 7% top agar that was supplemented with 1M $CaCl_2$. Raw milk samples were filtered through a 0.45µm syringe filter. Filtered samples were then spotted onto the surface of the solidified top agar at 10µl per spot. Plates were allowed to dry for 2hr, followed by

incubation for 12hr at 37ºC. Zones of clearing (plaques) were indicative of phage activity. Plaques were removed from top agar and soaked in phage buffer for 12hr with gentle agitation (150rpm) at 4ºC. The resulting suspension was centrifuged using the Avanti J-26 XPI (www.beckmancoulter.com) at 10,000g x 10min at 4ºC. The supernatant was filtered using a 0.45μm syringe filter and stored as phage stock at 4ºC.

4.2.3 Phage preparation for microscopy

Two different phage preparations were used during the study. Phage was propagated via standard double-plate technique (Sambrook *et al.* 1989). Resulting plaques that developed on the top agar were cut into quarters along the margin between the clear zone and the bacterial lawn. These quarters were processed further for microscopy. Phage was also propagated using enrichment procedures described previously (Gill *et al.* 2006). Enrichment protocols involved inoculation of an overnight broth culture of *SaB1* with phage eluted directly from plaques. Thereafter, phages were concentrated through centrifugation at 75,600g using Avanti J-26 XPI (Beckman-Coulter) for 3hrs at 10ºC. The resulting virus pellet was resuspended in phage buffer (Appendix 1) and stored at 4ºC.

4.2.4 Microscopy

a. Embedding and sectioning

Agar quarters containing both phage and bacterial cells were prepared for transmission electron microscopy analysis over a 4d period (Table 4.1). The methodology described in Table 4.1 represents a modification of methods of Hajibagheri and Trenton (1999). Resin embedded samples were sectioned using the Reichert-Jung Ultracut ultramicrotome (www.reichert.com). These sections were collected on formvar coated copper mesh grids and stained with 2% uranyl acetate (10min), followed by 2% lead citrate (10min). The grids were viewed using the JEOL JEM-1400 (www.jeol.com) at an operating voltage of 100kV. Images were captured using the Orius Camera (SC600A) (www.gatan.com) operating off Gatan Digital Micrograph (version 2.02.800.0) software (www.gatan.com).

b. Negative staining

Phages incubated with live bacterial cells were viewed using a negative staining technique. Bacterial cultures were grown for 12hr at 37ºC in liquid broth medium. The bacterial suspension was centrifuged at 10,000g x 10min at 4ºC. The supernatant was discarded and the bacterial pellet was re-suspended in 1M $MgSO_4$ amended with 1M $CaCl_2$. Previously isolated phage was then added to the bacterial suspension and incubated at 37ºC. Composite samples were negatively stained at 20min, 8hr and 12hr, post incubation. Formvar coated copper mesh grids (both 200 mesh) were inverted, for 1min each, onto 20µl sample droplets representative of an incubation period. Excess sample was wicked off and grids were stained with either uranyl acetate (2%) or phosphotungstic acid (2%) for 30sec. All stains were freshly prepared and used within 2wk. Grids were left to air-dry, followed by viewing using the JEOL JEM-1400 at an operating voltage of 100kV. Images were captured using the Orius Camera (SC600A) operating off Gatan Digital Micrograph (Version 2.02.800.0) software.

4.3 Results

Transmission electron microscopy proved to be a useful tool for the identification of key features of the isolated phages. Negative staining of the viral wet mounts using phosphotungstic acid provided more clarity than staining with uranyl acetate. However, staining of ultra-thin sections using uranyl acetate plus lead citrate provided optimal resolution of samples. The three phages were named Sabp-P1, Sabp-P2 and Sabp-P3.

Figures 4.1a-f shows that each of the three phages possesses an icosahedral head that is separated from a rigid, striated, contractile tail region by a constricted neck region. The head capsule ranged in diameter from 90-110nm, with the tail ranging in length from 150-200nm in the non-contractile state and 100-130nm in the contractile state.

Table 4.1 Amended protocol for transmission electron microscopy preparation of phage plaques at room temperature (unless otherwise stated).

Day	Procedure	Time
1	Fixation of agar plugs in 3% buffered glutaraldehyde	2hr
	Washing in 0.05M sodium cacodylate buffer	2 x 30min
	Post-fixation in 2% osmium tetroxide	2hr
	Washing in 0.05M sodium cacodylate buffer	1 x 30min (sample left overnight in fresh buffer)
2	Alcohol dehydration series: 30%, 70%, 100%	10min per alcohol solution for 30% and 70%
		3 x 10min for 100%
	Removal of residual alcohol using propylene oxide	1 x 10min
	Embedding in epon/araldite and propylene oxide (+DMP):	
	25% epon: 75% propylene oxide	2hr (in Eppendorf tube)
	50% epon: 50% propylene oxide	2hr (in Eppendorf tube)
	75% epon: 25% propylene oxide	2hr (in Eppendorf tube)
	100% epon	overnight (in Eppendorf tube)
3	Polymerization using 100% epon + DMP	14hr in oven at 70ºC (open tube)
4	Trim, section, stain, view	

Rigid tail fibres were also visible below the striated tail. Head capsules manifested in two different states – a collapsed state and an intact state (Figure 4.1e). The hexagonal shape of the head capsule is clearly distinguishable in the intact state, as are the tail components, whereas the virus particle in the collapsed state appears circular and has a contracted tail. Figure 4.2a shows the distinctive icosahedral virus head, but with a contracted tail. An anomaly that was noted was the presence of viral proteins that ranged in length from 150-450nm (Figure 4.2b). These viral polyrods were found associated with virus stock that had been stored in phage buffer, at 4ºC, for more than 2wk.

Two methods were employed in order to gain information on the key stages of the virus replication cycle after inoculation of *S. aureus*: (1) embedding and sectioning of a phage plaque

12hr post inoculation (Figure 4.3); and (2) wet mount analysis of phage activity at 20min, 8hr and 12hr post inoculation (Figure 4.4). The observations reported below are for phage Sabp-P3 only. Modification of the traditional embedding protocol proved efficient as sample processing was reduced from 7 days to 4 days, with no compromise in sample integrity.

Staining of the ultra-thin sections using uranyl acetate and lead citrate provided optimal resolution (Figures 4.3a-d). Typical signs of phage activity included the presence of bacterial cell wall debris, and cells with indistinct internal structures that appeared to have collapsed. Figures 4.3a-d shows sections through the marginal region of a phage plaque. In Figure 4.3a, residual intercellular annuli can be seen. Cells with swollen protoplasts and partially digested membranes are also visible. Figure 4.3b shows extrusion of a bubble of cytoplasm from a bacterial cell shortly after rupture of the cell wall. Figures 4.3c and 4.3d show lysed cells and cells that are packed with newly formed phages. In addition, a single phage particle can be seen attached to the outer membrane of a bacterial cell that is already at an advanced stage of infection.

Figures 4.4a-f show the various stages of virus replication, using the wet mount and negative staining technique. Figures 4.4a and 4.4b show attachment of virus particles to the surface of bacterial cell walls 20min post inoculation. Figures 4.4c and 4.4d show virus infection at a more advanced stage 8hr post inoculation. Bacterial cells have begun to lose cell wall integrity and the cell cytoplasm has begun to ooze from areas of the cell wall that have already ruptured (Figure 4.4c). Virus particles are also packed within bacterial cells and are ready for release. Figure 4.4d shows the presence of thin fibrils among the virus particles. Figures 4.4e-f show virus replication at 12hr post inoculation.

At this stage, virions are packaged in an ordered fashion within bacterial cells that are on the verge of rupture. Bacterial cells have lost cell wall integrity and appear disorganized. Bacterial cell wall debris and annuli are also present as a by-product of those cells that have already ruptured. Virions leaving the ruptured bacterial cells seem to carry with them strands of bacterial cytoplasm (Figure 4.4f). An interesting observation during the release of the virions from lysed bacterial cells was that the virions themselves appeared to aggregate towards each other and create a network of viruses, even if they had been released from neighboring cells that were located a distance away (Figure 4.4f).
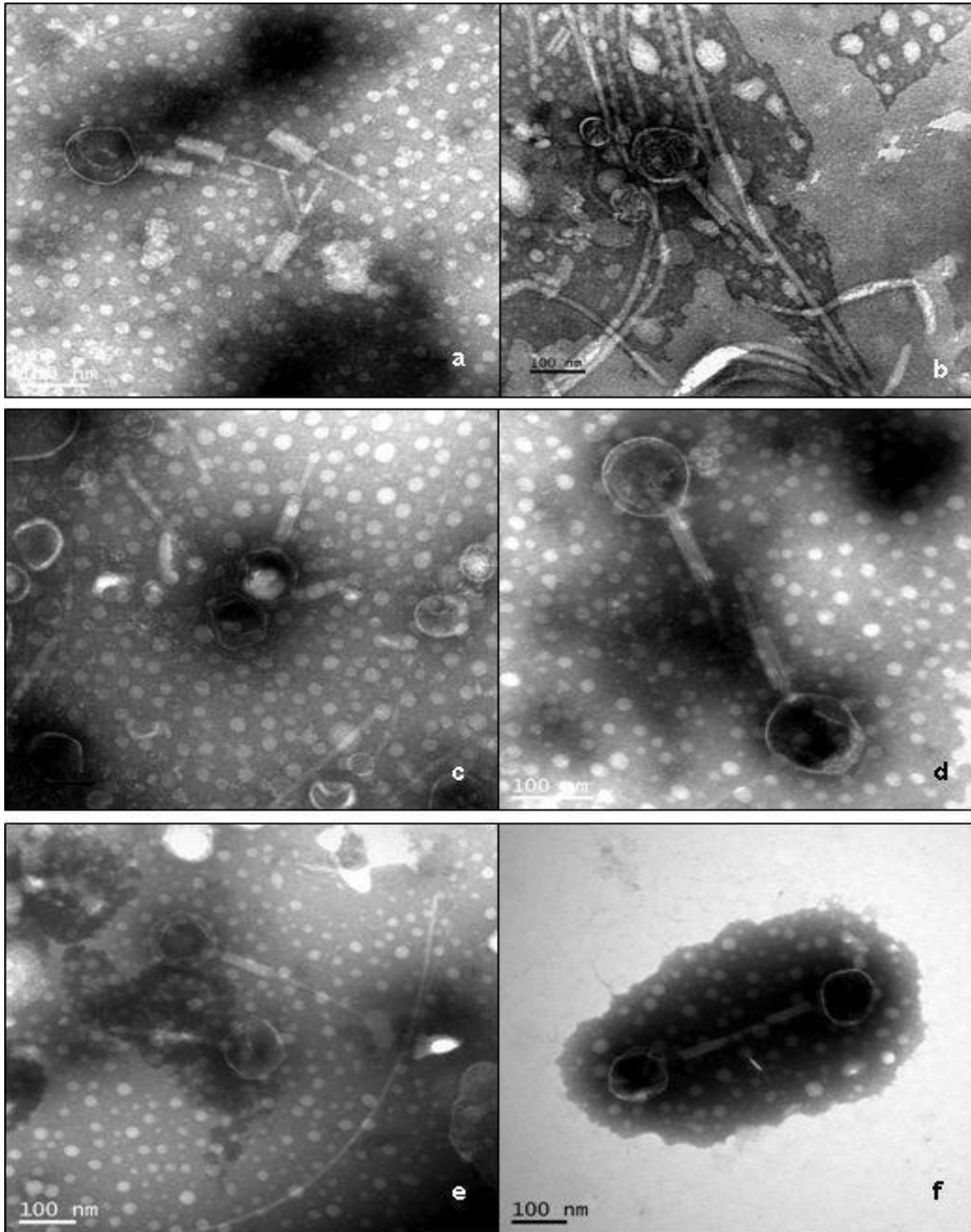
Figure 4.1 Electron micrographs of three different phages derived from *Staphylococcus aureus*. a-b. Sabp-P1; c-d. Sabp-P2; e-f. Sabp-P3. Note the distinctive head and tail regions as well the tail fibres of each phage. All samples were stained using phosphotungstic acid. Magnification 120,000x.
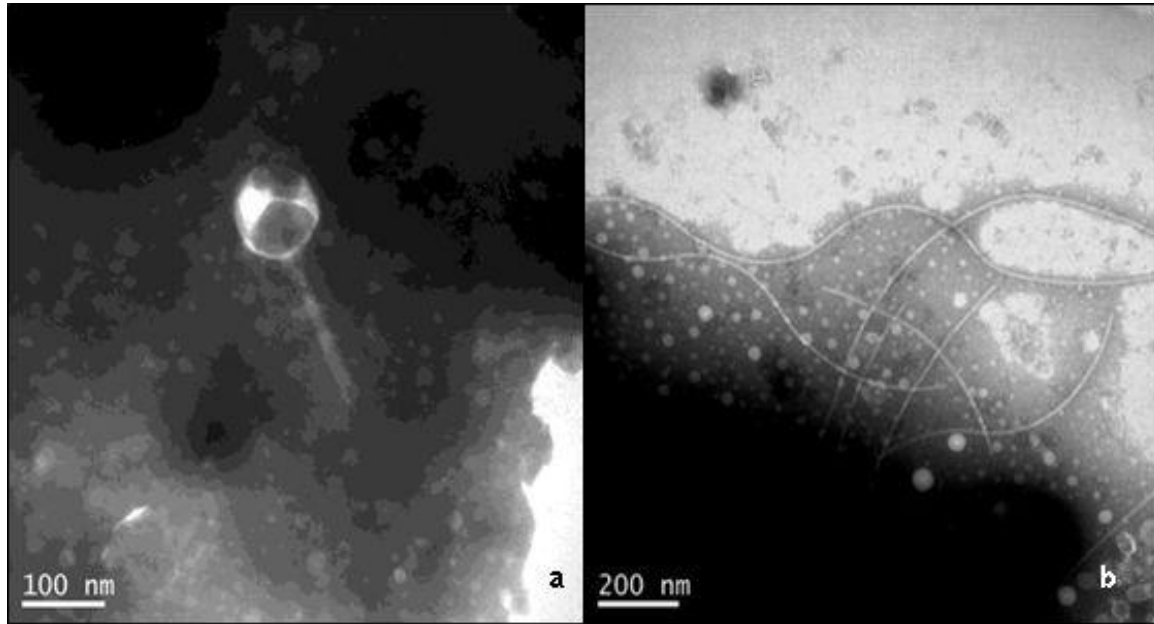
Figure 4.2 Electron micrographs of distinguishing phage structures.

a. Typical hexagonal shape of phage head characteristic to Sabp-P1, Sabp-P2 and Sabp-P3; b. Virus polyrods. All samples were stained using phosphotungstic acid. Magnification 110,000x.
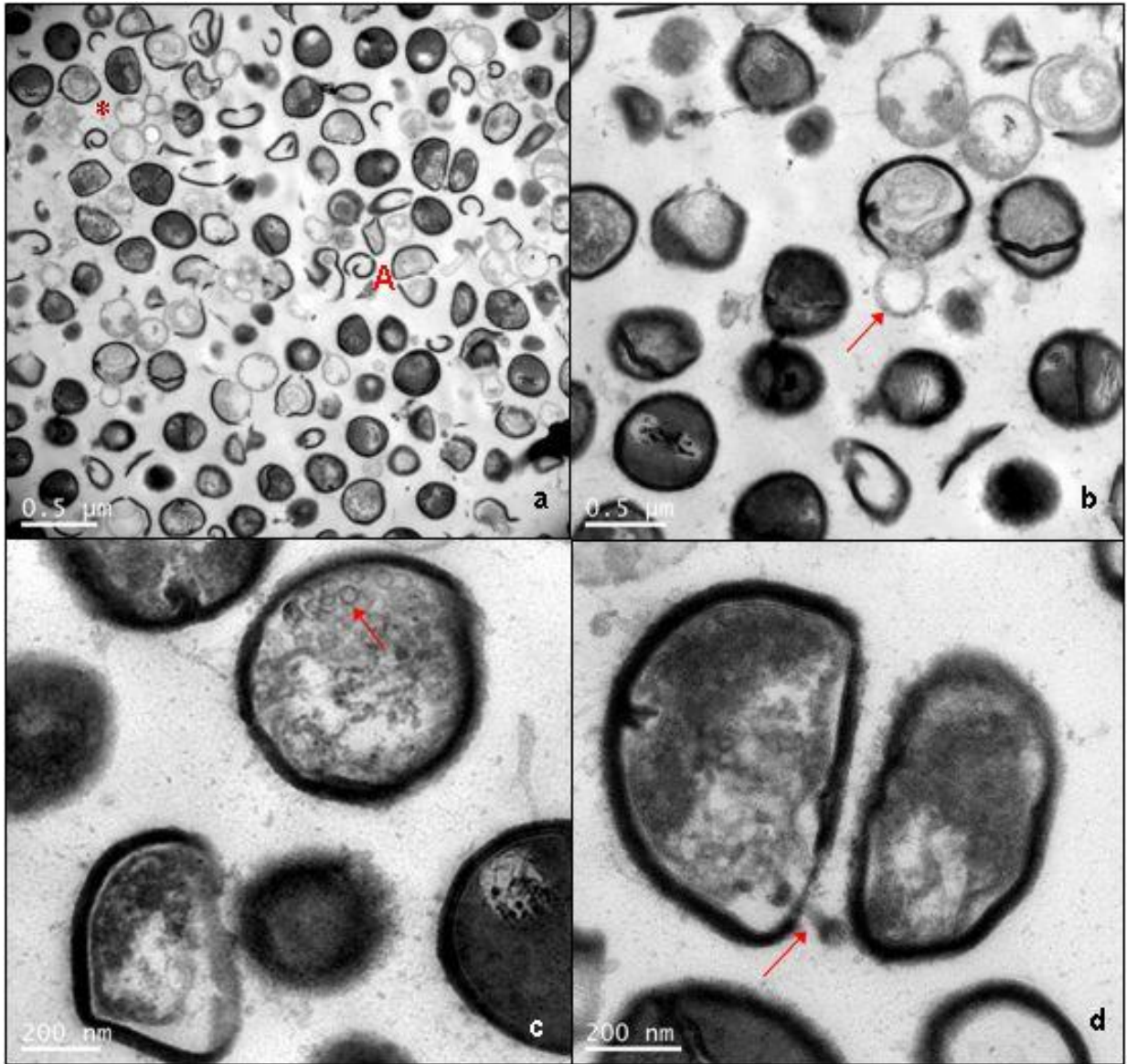
Figure 4.3 Ultra-thin sections through a plaque of phage Sabp-P3 at 12hr post inoculation.

a. Bacterial debris and residual intercellular annulus (A) as well as swollen protoplasts (*); b. Bacterial cell at an advanced stage of phage infection indicated showing extrusion of bubbles of cytoplasm (indicated by arrow); c. Newly formed phage particles packed within bacterial cells (indicated by arrow); d. Phage attached to the outer wall of an infected bacterial cell. All samples were stained using uranyl acetate and lead citrate. Magnification 85,000x.
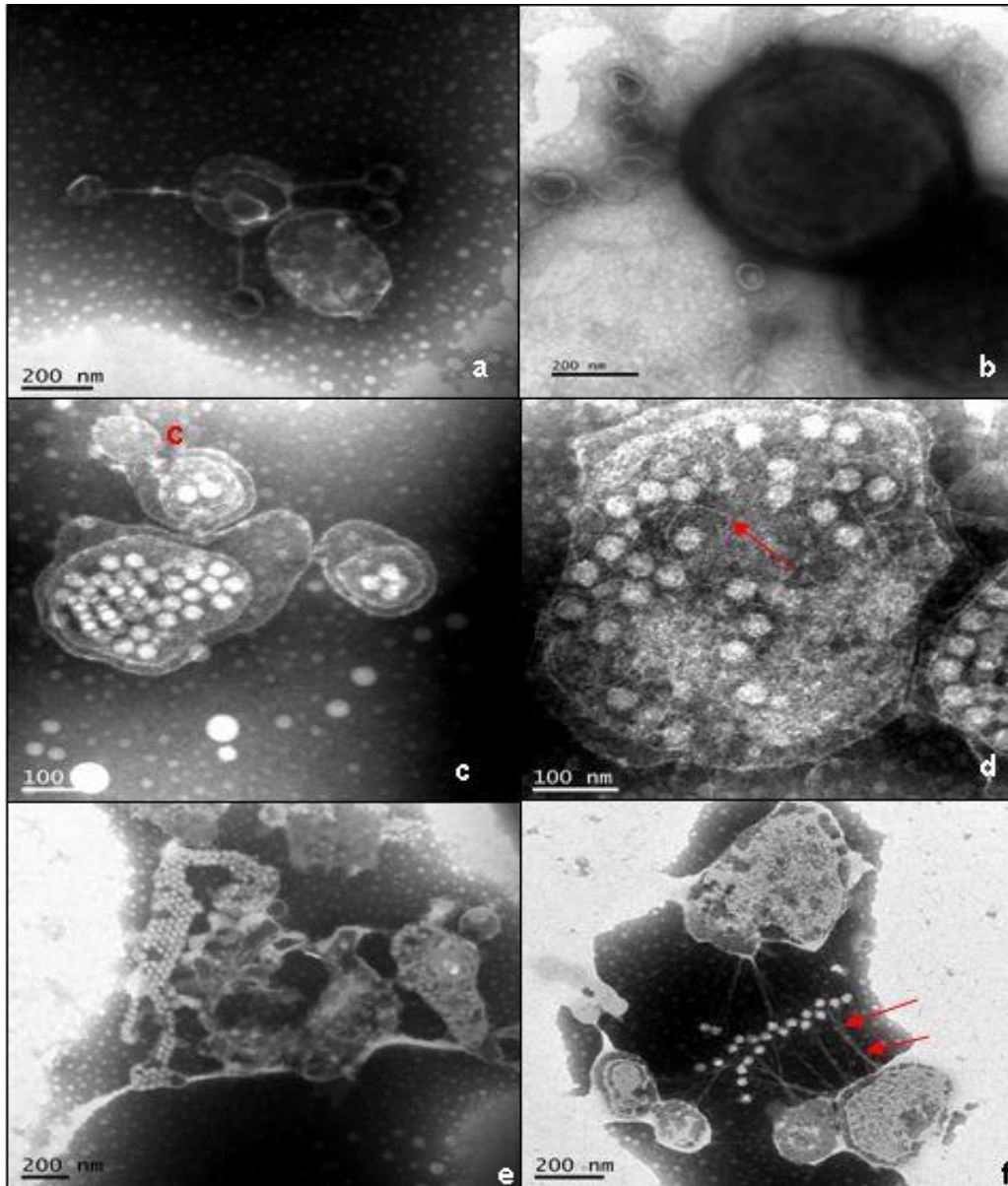
Figure 4.4 Representation of the phage life cycle at 20min, 8hr and 12hr post inoculation onto *Staphylococcus aureus.*

a-b. Phage attachment to bacterial cell wall surface, 20min post inoculation. Note the circular intact form of the cells; c-d. *S. aureus* cells with virus particles packaged within them, 8hr post inoculation, where cell walls have begun to lose integrity and cell cytoplasm (c) can be seen oozing from walls; d. Bacterial cell with virus particles packaged within as well as the presence of viral protein chains/viral polyrods (indicated by arrow); e. Bacterial infection at an advanced stage 12hr post inoculation, where all cell wall integrity has been lost and cell debris from already ruptured cells can be seen; f. Release of virions (v) from ruptured bacterial cells where the virions carry with them a cytoplasm trail as they leave the ruptured cells (indicated by arrow). All samples were stained using phosphotungstic acid. Magnification 120,000x.

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4.4 Discussion

Morphological studies conducted on the unknown phages using transmission electron microscopy revealed their size, shape and basic replication cycle. The length of phage particles including both the head and tail components was between 150-250nm. The presence of a distinct constricted neck region between the head capsule and the tail provided further evidence regarding the placement of the phages into a specific family. The morphological characteristics of the phages reported in this study correlated with those from previous studies. Average phage dimensions were reported in a range between 150-300nm, head capsules were icosahedral, tail structures appeared to end in a base plate, and tail fibers were present in all three phages (Coetzee, 1987; Ackermann, 2005; Hseih, *et al.* 2011). Furthermore, the presence of anomalous viral polyrods were noted. These structures were previously reported by Tikhonenko (1970), who hypothesized that these structures were actually an aggregation of viral tail proteins. This aggregation was reported to have occurred upon phage exposure to high pH (10-11) or prolonged exposure to fluctuating temperatures. In terms of the current study, the latter exposure could have accounted for the development of tail protein aggregation and hence the viral polyrods. These structures were common among all three phages that were examined. Based on these results, the isolated phages can be assigned to the order *Caudovirales*, family *Myoviridae*.

Tailed phages of the order *Caudovirales* constitute the largest and most widespread group of phages, with more than 5000 tailed phages having been identified through electron microscopy (Ackermann, 2005). This order of viruses may be the oldest, dating back to 3.5 billion years (Ackermann, 2005). *Caudovirales* seem to constitute a monophyletic evolutionary group that possesses clearly related morphological, physicochemical and physiological properties (Ackermann, 2005). However, in depth studies have shown that they are actually diverse in their properties, e.g., particle dimensions and fine structure, DNA content and composition, nature of constitutive proteins, serology, host range and physiology (Ackermann, 2005; Abedon, 2008). Ackermann (2005) has proposed a useful criterion based on phage tail structures that can be easily determined via electron microscopy and that is suggestive of virus assembly pathways. Based on this criterion, tailed phages are divided into three families: *Myoviridae*, with long contractile tails consisting of a sheath and a central tube; *Siphoviridae* with long non-contractile tails; and *Podoviridae* with short, non-contractile tails. However, these three families show

extensive overlap in terms of their physicochemical properties and cannot be differentiated solely based on morphology (Ackermann, 2005).

In addition to classification of the newly isolated phages, an image-based representation of the major steps of the virus replication cycle was also documented. Phages can be seen attached to bacterial cells, followed by replication and production of virus protein (fibrils) within cells, and subsequent release from these cells. Tailed phages recognize their host cells through the interaction between attachment sites on their tails and host surface molecules (Kutter *et al.* 2005). Some phages use a single, central fibre as their receptor recognition element (or adhesin), while others use a cluster of 3, 6 or 12 fibres that are associated with the tail structure (Kutter *et al.* 2005). Adsorption to host cells may involve multiple steps, which are quite different for both Gram-positive and Gram-negative bacteria. Little is known about the specific adhesins of phages that are able to infect Gram-positive bacteria such as *S. aureus*, or about the bacterial receptors to which these phages bind. However, studies have been undertaken in an attempt to characterize phage genes involved in this process (Duplessis and Moineau, 2001). Appropriate placement of the phage tail on the cell surface triggers irreversible events that lead to delivery of phage DNA into the host cell. In the case of Myoviruses, although they possess contractile tails, it appears that their tail tubes do not pierce the inner host membrane and that a potential gradient is actually required for DNA entry into the host cell (Molineux, 2001; Letellier *et al.* 2004; Kutter *et al.* 2005). Once phage DNA has entered the host cells, a complex sequence of events follows where phage DNA is replicated, repaired and recombined by using a combination of host and phage mechanisms (Kutter *et al.* 2005). Transcription then follows (Kutter *et al.* 2005), at which stage most of the regulation of phage development takes place. Phage morphogenesis and DNA packaging takes place subsequently.

The *Myoviridae* build some of the most complex virus particles known (Ackermann, 2005; Kutter *et al.* 2005). The well studied phage, T4, devotes more than 40% of its genetic information to the synthesis and assembly of its icosahedral heads, tails with contractile sheaths and tail fibres (Kutter *et al.* 2005). Twenty-five T4 proteins are involved in head morphogenesis, 22 in tail morphogenesis and 7 for tail fibres; in addition, 5 of the 54 proteins are actually assembly catalysts rather than components of final phage structure (Kutter *et al.* 2005). Tail morphogenesis is an intricate step involved in the phage life cycle. For the *Myoviridae*, the many

components of the contractile tail are assembled in a highly ordered fashion. Using T4 as a type example, the baseplate is formed through the associations of six "wedges", each of which consist of seven types of protein, around a "hub" to form a dome-shaped base plate (Kutter *et al.* 2005). Trimeric short tail fibres are located beneath the baseplate with the head of each fibre associating with the tail of the next fibre. These tail fibers serve to irreversibly bind a phage structure to a bacterial cell during infection (Kutter *et al.* 2005).

The last stage in the replication cycle is the release of progeny phage particles from the host cells. Initial assumptions on phage release were that the host cells become packed tightly with virus progeny and then burst as a result of intracellular pressure. However, more recent theories are based on the production of virus lysozymes that facilitate cell rupture and virus release (Kutter *et al.* 2005; Donovan *et al.* 2006). The two major proteins involved are endolysin (to attack the host peptidoglycan layer) and holin (to let the endolysin move from the cell cytoplasm into the periplasmic space in a carefully timed fashion). Kutter *et al.* (2005) discussed how the holin-endolysin combinations facilitated the evolution of several key properties that affect the ability of the phage to compete by regulating the onset of lysis and the speed at which infectious progeny are released.

In addition to morphological classification of phages, molecular analysis of genomic material has been used by others to confirm the identity of specific phages. Fifteen genera have been defined among the tailed phages, based on molecular criteria (Ackermann, 2005). These molecular criteria include the presence/absence of *cos* or *pac* sites, terminal redundancy and circular permutation, DNA or RNA polymerases, and nucleotide sequences (Ackermann, 2005; Abedon, 2008). To date, approximately 260 phage species have been recognized, mostly on the basis of morphology, nucleotide sequencing, protein analysis, serology and DNA-DNA hybridisation (Ackermann, 2005). The results of the current study allow for the preliminary classification of the three phages into a specific order (*Caudovirales)* and family (Myoviridae). Further research is needed to generate the nucleotide sequencing and protein analysis that is required in order to accurately classify the phage to the strain level, as per internationally accepted rules for virus classification and nomenclature (ICTV, 2002).

4.5 Conclusions

This electron microscopy study identified the morphology and parts of the replicative cycle of three novel South African phages infective against *S. aureus* strains responsible for bovine mastitis. In addition, this is the first study involving phages specific against bovine mastitis-causing *S. aureus* that has been undertaken in South Africa. This can serve as a preliminary step in a long-term biocontrol plan to apply these phages as biocontrol agents against *S. aureus*-induced bovine mastitis.

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CHAPTER 5

Protein profile analysis of selected bacteriophages, infective against *Staphylococcus aureus* subsp. *aureus* Rosenbach 1884, causal agent of bovine mastitis

Abstract

A wealth of information can be derived about an organism based on analysis of its proteomic data, and different diagnostic techniques can be employed to derive proteomic data from an organism. The aim of this study was to use one-dimensional electrophoretic methods specifically, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and ultra-thin layer isoelectric focusing (UTLIEF), to analyse the proteins of three strains of staphylococcal bacteriophages, Sabp-P1, Sabp-P2 and Sabp-P3, in order to determine whether these strains differed from each other. SDS-PAGE analysis produced unique protein profiles for each phage, with band fragments ranging in size from 8.86-171.66kDa. Combined similarity matrices showed an 84.62% similarity between Sabp-P1 and Sabp-P2 and a 73.33% similarity between Sabp-P1 and Sabp-P3. Sabp-P2 showed a 69.23% similarity to Sabp-P3. UTLIEF analysis showed protein isoelectric charges in the range of pI 4.21-8.13, for all three phages. The isoelectric profiles for each phage were distinct from each other. A combined similarity matrix of both SDS-PAGE and UTLIEF data showed an 80.00% similarity between phages Sabp-P1 and Sabp-P2, and a 68.29% similarity between Sabp-P1 and Sabp-P3. Sabp-P2 showed a 70.59% similarity to Sabp-P3. Although the current results are based on putative protein fragments analysis, it can be confirmed that phages Sabp-P1, Sabp-P2 and Sabp-P3 are three distinct phage. Complete sequencing of viral genomes is necessary in order to specifically characterize how each phage differs, based on protein chain composition.

5.1 Introduction

Bovine mastitis refers to an infectious inflammation of the mammary glands that interferes with the normal flow and quality of milk (Blowey and Edmondson, 2010). Traditionally, this disease has been managed through the application of chemotherapeutic agents. However, the primary causal organism of the disease, *Staphylococcus aureus* subsp. *aureus* Rosenbach 1884, has with

time, developed resistance towards those antibiotics most commonly applied against it. As a result, interest has shifted to other avenues for control of the disease.

Polyvalent staphylococcal phages of the *Myoviridae* family have received renewed interest with respect to their practical application for the treatment of bovine mastitis. However, due to their ubiquity and associated abundance in an environment, it is paramount to be able to accurately distinguish between each phage, in order to fully exploit its control potential. Analysis of viral proteins serves as one such method of characterisation.

Viral protein analysis can provide further insight into those mechanisms involved in the infection cycle, from attachment of the phage to the host cell wall, to final expulsion of newly-formed phage particles from a lysed host cell. Phages are highly host specific (Kutter and Sulakvelidze, 2005; Tan *et al.* 2008). This specificity is dependant upon the evolution of recognition systems of the viruses, based on the "lock-and-key" theory, where receptors on the host bacterium are recognized by proteins of the phage (Kutter and Sulakvelidae, 2005; Tan *et al.* 2008). To initiate successful infection, an incoming phage must first cross the barrier presented by the bacterial cell wall (Rashel *et al.* 2008). Several studies have identified the proteins associated with initiation of infection associated with Gram-negative bacteria (Kao and McClain, 1980; Caldentey and Bamford, 1992; Sandmeier, 1994).

However, studies into the lytic proteins responsible for initiation of infection of Gram-positive bacteria are rather limited. Kenny *et al.* (2004) showed that a phage specific to *Lactococcus lactis* Lister 1873, Tuc2009, encodes for a tail-associated protein (tal_{2009}) that might be involved in localized cell wall degradation. Eyer *et al.* (2008) reported characterization of a phage (Phage 812) proteome as well the identification of phage virion proteins. It was found that identified proteins of phage 812 showed 99-100% similarity with corresponding proteins of the phage G1, and, approximately 60% similarity with proteins of the phage Twort, all of which are staphylococcal phages. Lood and Collin (2011) also carried out protein analysis on phages of *Propionibacterium acnes* Gilchrist 1900. This study identified major head and tail proteins, as well as three other low molecular weight minor proteins. Monod *et al.* (1997) conducted a study on phages that exhibit T-even morphology but are only distantly related to the T-even phages; these phages were termed "pseudo-T-even". Protein analyses revealed that pseudo-T-even

phages could be a distant source of viral sequences that might be exchanged with the T-even phages, and that each group does in fact differ from the other.

Considering the wealth of information that can be derived from analysis of phage proteins, the primary objective of the current study was to carry out protein characterisation of three novel South African phages isolated from the dairy environment. These phages have shown specific activity towards pathogenic strains of *S. aureus*, associated with bovine mastitis. It is envisaged that these phages will be used in an integrated control program for the control of bovine mastitis in dairy cattle.

5.2 Materials and Methods

5.2.1 Bacterial host strains and their isolation

Staphylococcus aureus strain, SaB1, was used for phage isolation and propagation. This strain was isolated from raw bovine milk collected from dairy farms in the province of KwaZulu-Natal, RSA (Republic of South Africa). SaB1 was initially isolated on blood agar. Cultures required for phage propagation were maintained on tryptone soy agar (TSA) or typtone soy broth (TSB).

5.2.2 Phage isolation, propagation and purification

Raw bovine milk samples collected from dairy farms in the province of KwaZulu-Natal, RSA (Republic of South Africa) served as the initial material from which to isolate phages. These milk samples were stored at 4ºC. Phages were isolated using the spot-plate method on double-layer agar (Sambrook *et al.* 1989; Harley and Prescott, 1993). Overnight *SaB1*cultures (~1 x 10^8 cfu.ml^{-1}) were incorporated into 7% top agar that was supplemented with 1M CaCl$_2$. Raw milk samples were filtered through a 0.45μm syringe filter. Filtered samples were then spotted onto the surface of the solidified top agar at 10μl per spot. Plates were allowed to dry for 2hr, followed by incubation for 12hr at 37ºC. Zones of clearing (plaques) were indicative of phage activity. Plaques were removed from top agar and soaked in phage buffer (Appendix 1) for 12hr with moderate agitation (150rpm) at 4ºC. The resulting suspension was centrifuged using the Avanti J-26 XP1 (www.beckmancoulter.com) at 10,000g x 10min at 4ºC. The supernatant was filtered using a 0.45μm syringe filter and stored as phage stock at 4ºC. Subsequent phage was

grown from this stock in liquid broth culture or using the double-layer agar method (Sambrook *et al.* 1989).

Isolated phages were purified through a modification of standard methods (Sambrook *et al.* 1989; Harley and Prescott, 1993). Filter-sterilised phage stock isolated from either liquid broth culture or double-layer agar method, were subjected to centrifugation using the Avanti J-26 XPI (Beckman-Coulter) at 75,600g x 3hrs x 10ºC. Resulting phage pellets were re-suspended in fresh phage buffer and a second centrifugation was conducted (Avanti J-26 XPI (Beckman-Coulter) at 75,600g x 3hrs x 10ºC). The phage pellets were then re-suspended in fresh phage buffer at 1/10 of the original volume that was processed. All stocks were stored at 4ºC. Six phages were isolated and named Sabp-P1, Sabp-P2, Sabp-P3, Sabp-P4, Sabp-P5 and Sabp-P6. However, only phages Sabp-P1, Sabp-P2 and Sabp-P3, were used in this study. The results of previous screening eliminated the need to characterise all six phages, as only Sabp-P1, Sabp-P2 and Sabp-P3, are intended for use in *in vivo* trials.

5.2.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

a. Sample preparation

Purified phage stock previously prepared through centrifugation was used as the starting material. The protein concentration of each sample was determined using the Nanodrop ND-100 spectrophotometer (www.nanodrop.com). An equal volume of phage sample was mixed with an equal volume of SDS sample reducing buffer (4% (m/v) SDS, 10% (v/v) β-mercaptoethanol, 20% (v/v) glycerol, 125mM Tris-HCl). This suspension was incubated at 55°C for 30min, in order to slowly denature phage proteins. After 30min, all samples were transferred to ice. A marker dye, bromophenol blue (5µl), which migrates with the buffer front, was added to each sample prior to loading onto the gel.

b. Electrophoresis

The proteins were electrophoresed on a standard SDS-PAGE gel (Laemmli, 1970). A 4% stacking gel and a 12.5% separating gel were used to separate the proteins. A total volume of 20µl of each phage was loaded onto the gel; 10µl of protein marker as added to gel (PageRuler™ Prestained Protein Ladder, Thermo Scientific) (www.thermoscientific.com). The gel unit was

connected to a power-pack and the run at 18mA, 100V until the bromophenol blue marker was 0.5cm from the bottom of the gel (~2hr). The gel was removed and stained for 4h using the Coomassie Blue R-250 staining solution (0.125% (m/v) Coomassie R-250 dissolved in 50% (v/v) methanol, 10% (v/v) acetic acid). The gel was then placed overnight in de-stain I (50% (v/v) methanol, 10% (v/v) acetic acid), and thereafter into de-stain II (7% (v/v) methanol, 5% (v/v) methanol). Gels were all shaken at room temperature at 100rpm using a MRC Orbital Shaker (www.mrclab.com). Gels were stored in de-stain II (7% (v/v) methanol, 5% (v/v) methanol), in polyethylene air-tight bags, at 4°C. Gels were photographed using the VersaDoc Gel Imaging System 4000 (www.exetersoftware.com).

5.2.4 Ultra-thin layer isoelectric focussing

a. Sample preparation

Purified phage stock previously prepared through centrifugation was used as the starting material. The protein concentration of each sample was determined using the Nanodrop ND-100 spectrophotometer. An equal volume of phage sample was mixed with an equal volume of isoelectric focussing lysis buffer (Appendix 3) (Berkelman and Stenstedt, 1998). Phage and buffer suspension were incubated at 50°C for 30min. After 30min, all samples were transferred to ice until loaded onto gel. No dye marker was added to any sample.

b. Electrophoresis

All phages samples were applied to ultra-thin iso-electric focusing gels with a wide pI range using large application strips. The gels were supplied by Incotec Proteios South Africa (www.incotech.com). Electrophoresis was performed on a flatbed focuser (Multiphor II electrophoresis system, Pharmacia Biotech) (www.gelifesciences.com) at a pre-cooled temperature of 10°C. The anodal buffer consisted of 25.5mM L^{-1} aspartic acid and 24.5mM L^{-1} glutamic acid in distilled water. The cathodal buffer used was 25.2mM L^{-1} arginine, 24.6 mM L^{-1} lysine and 12% ethylenediamine in distilled water. The gels were run using a single cathode and a single anode, in single direction electrophoresis. The gel was pre-focussed at 200V, 58mA, 12W and 100 volt hours (VH), using a VH integrated electrophoresis power supply, Electrophoresis Power Supply EPS-3501XL (www.gehealthcare.com). 20µl of each phage

sample was loaded into sample wells; 3μl of IEF marker (IEF Markers 3-10, SERVA Liquid Mix) (www.invitrogen.com) was loaded onto each gel. Electrophoresis was carried out using the following run settings: Step 1 = 160V, 46mA, 9.6W, 80VH; Step 2 = 400V, 46mA, 9.6W, 160VH; Step 3 = 1000V, 46mA, 9.6W, 500VH; total run time = ~1hr 20min. After completion of protein focussing, the gels were fixed at room temperature using 20% trichloracetic acid (15min standing, 15min shaking at 50rpm). The gels were rinsed twice in sterile distilled water, and then stained at room temperature for 2hr, using a colloidal Coomassie blue stain (Neuhoff *et al.* 1988; Kang *et al.* 2002) (Appendix 3). After 2hr, the gel was de-stained in sterile distilled water for 8hr, shaking (50rpm) at room temperature using a MRC Orbital Shaker. Each gel was dried in a gel-dryer (Bio-Rad GelAir Dryer; www.bio-rad.com) and then photographed using the VersaDoc Gel Imaging System 4000.

5.2.5 Gel scoring and interpretation

Similarity matrices and dendrograms were generated for each protein profile obtained from SDS-PAGE and UTLIEF. For the similarity matrices construction, bands were scored as present (1) or absent (0). The fragment data was converted to binary data by generating a binary matrix (0, 1) using the banding patterns of each individual. The NTSYS v2.1 software programme (Numerical Taxonomy and Multivariate Analysis for personal computers, Exeter Software, Setauket, NY, U.S.A.) was used to evaluate the similarities between the protein banding patterns for each phage. Pair-wise comparisons were made between phage protein profiles based on DICE (Dice 1945) similarity coefficient; a statistic used for comparison of similarities between two samples. The resultant distance matrix data was used to construct a dendrogram using the agglomerative hierarchical un-weighted pair-group method with an arithmetic average (UPGMA) sub-programme of NYSTS (Rohlf, 1998). Additionally, banding data was used to create standard curves and logarithmic and linear equations were used to extrapolate unknown molecular weights and isoelectric charge, respectively.

5.3 Results

5.3.1 SDS-PAGE

Results derived from SDS-PAGE (Figure 5.1) were used to determine approximate molecular weights (kDa) for phage proteins. In addition, results were subjected to phylogenetic analysis to determine whether phages Sabp-P1, Sabp-P2 and Sabp-P3 differed from each other in terms of their protein content.

Band fragment analysis was carried out using Quantity One software (Bio-Rad)[5]. Results revealed that each phage is comprised of several different sized proteins (Figure 5.1). Phages, Sabp-P1 and Sabp-P3, produced profiles comprising of 17 proteins while Sabp-P2 produced a profile comprising of 15 proteins. While all three phages appear to have several similar sized proteins in common, distinctive differences were also apparent between each phage profile (Figure 5.1 and Table 5.1).
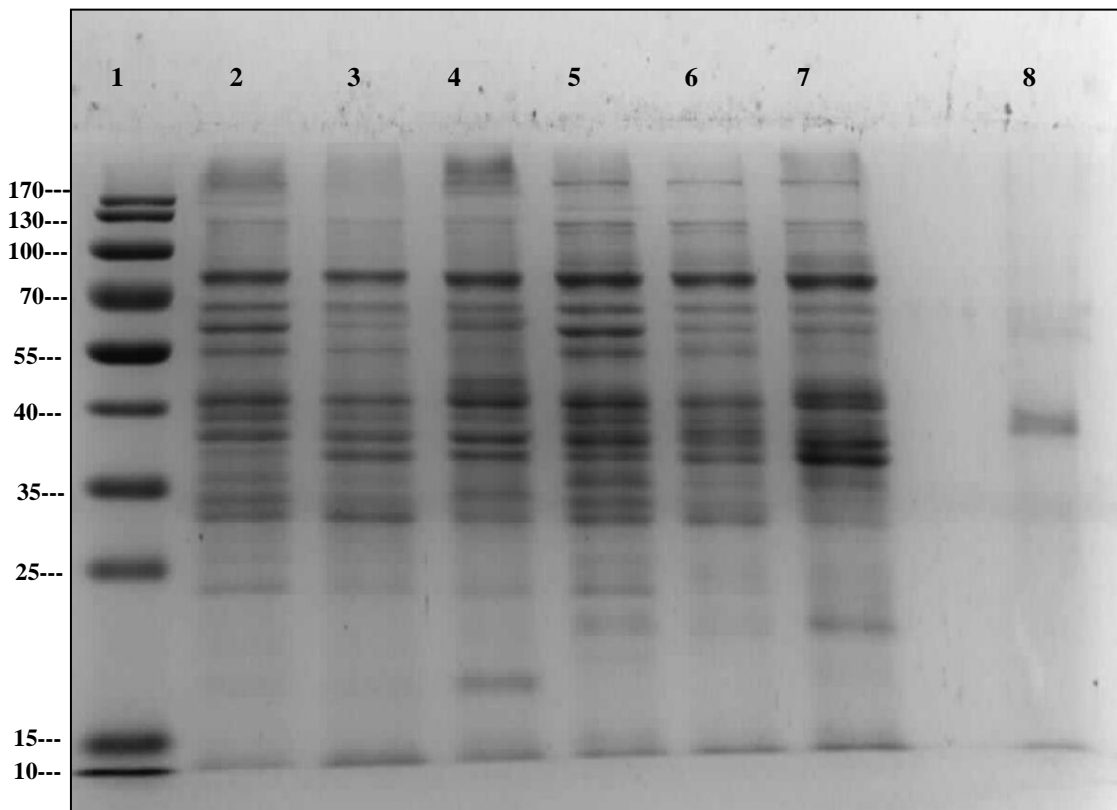
Figure 5.1 SDS-polyacrylamide gel electrophoresis of proteins of phages Sabp-P1, Sabp-P2 and Sabp-P3. Lane identity: 1 = Protein marker; 2 & 5 = Sabp-P1; 3 & 6 = Sabp-P2; 4 & 7 = Sabp-P3; 8 = Positive control (Tobacco mosaic virus).

Table 5.1 Approximate molecular weights for proteins of phages Sabp-P1, Sabp-P2 and Sabp-P3 that were separated during SDS-PAGE.

Protein marker		Approximate MW (kDa) of phage proteins		
Band no.	MW (kDa)	Sabp-P1	Sabp-P2	Sabp-P3
1	170	171.66	171.66	171.66
2	130	143.81	124.94	124.94
3	100	124.94	115.04	115.04
4	70	95.76	92.55	102.74
5	55	81.23	78.71	89.51
6	40	71.73	71.73	78.71
7	35	65.49	65.49	71.73
8	25	53.06	53.06	54.68
9	15	49.94	49.94	51.48
10	10	45.55	46.97	44.15
		42.79	44.15	40.15
		37.63	40.15	36.41
		34.04	36.41	29.57
		30.65	29.57	25.41
		24.41	20.58	19.67
		19.67		15.27
		15.27		8.86

The data of relative mobility and molecular weight of the protein marker were used to plot a standard curve of mobility versus molecular weight (Figure 5.2). The logarithmic equation derived from the standard curve was used to determine approximate molecular weights of proteins of Sabp-P1, Sabp-P2 and Sabp-P3 (Table 5.1). Proteins ranged in size from 15.27-171.66kDa for Sabp-P1, 20.25-171.66kDa for Sabp-P2 and 8.86-171.66kDa for Sabp-P3.
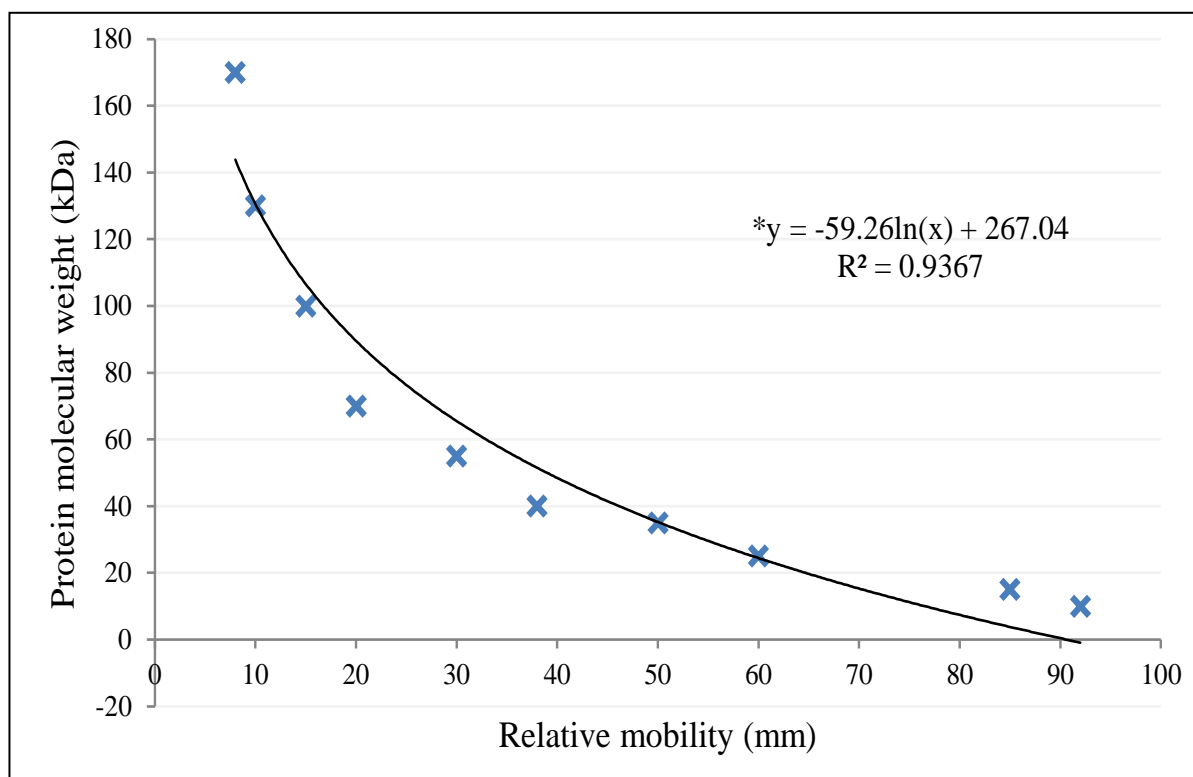
Figure 5.2 Standard curve for proteins separated during SDS-PAGE.
(✖) actual molecular weights for protein marker; (—) fitted logarithmic trendline; (*) logarithmic equation and regression coefficient for extrapolation of unknown protein molecular weights.

Band fragment data (Figure 5.1) was used to construct a dendogram using the UPGMA sub-program on NTSYS (Figure 5.3). Phages Sabp-P1 and Sabp-P2 were clearly distinguishable from Sabp-P3, based on the combined data presented in the dendogram (r^2=96.63%). Combined similarity matrices (Appendix 3) showed an 84.62% similarity between Sabp-P1 and Sabp-P2 and a 73.33% similarity between Sabp-P1 and Sabp-P3. Sabp-P2 showed a 69.23% similarity to Sabp-P3.

Figure 5.3 Phylogenetic tree comparing SDS-PAGE protein profiles for phages Sabp-P1, Sabp-P2 and Sabp-P3.

5.3.2 UTLIEF

Band fragment analysis was carried out using Quantity One software (Bio-Rad)[10]. Analysis revealed that proteins of each phage displayed varying isoelectric points along the pH range of the gel. While all three phages appeared to have proteins with similar isoelectric points in common, distinctive differences were also apparent (Figure 5.4 and Table 5.2).
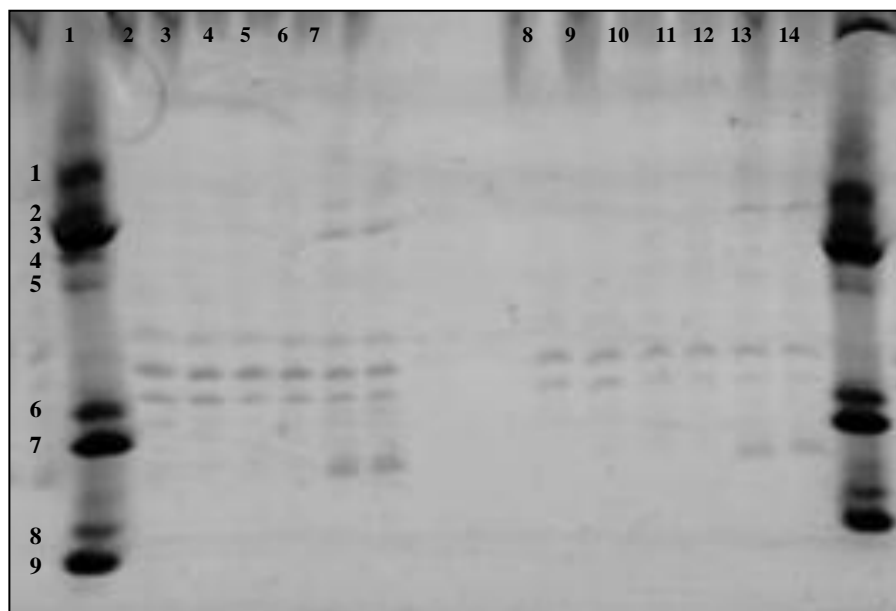
Figure 5.4 UTLIEF of phage proteins. Lane identity: 1 &14 = Markers; 2, 3, 8, 9 = Sabp-P3; 4, 5, 10, 11 = Sabp-P2; 6, 7, 12, 13 = Sabp-P1.

Table 5.2 Approximate isoelectric points (pI) for proteins of phages Sabp-P1, Sabp-P2 and Sabp-P3 that were separated during UTLIEF.

Isoelectric marker		Approximate isoelectric point (pI) of phage proteins		
Band no.	Isoelectric point (kDa)	Sabp-P1	Sabp-P2	Sabp-P3
1	1	6.60	6.26	4.21
2	2	6.94	6.77	4.55
3	3	7.79	7.11	6.26
4	4a[*]			6.60
5	4c[**]			7.11
6	5			8.13
7	6a[*]			
8	6c			
9	7a[*]			
10	7m[***]			
11	7c[**]			
12	8			
13	9			

[*]Migration towards anode; [**]Migration towards cathode; [***]Migration midway between anode and cathode

The data of relative mobility and isoelectric point of the protein marker were used to plot a standard curve of mobility versus isoelectric point (Figure 5.5). The linear equation derived from the standard curve was used to determine approximate isoelectric point of proteins of Sabp-P1, Sabp-P2 and Sabp-P3 (Table 5.2). Approximate isoelectric point for all three phages ranged from a pI of 4.21 to a pI of 8.13.

The graph shows Protein isoelectric point (pI) on the y-axis (0 to 10) versus Relative mobility (mm) on the x-axis (0 to 60), with data points and a fitted trendline. The equation shown is:

$$*y = 0.1703x + 0.297$$
$$R^2 = 0.9689$$
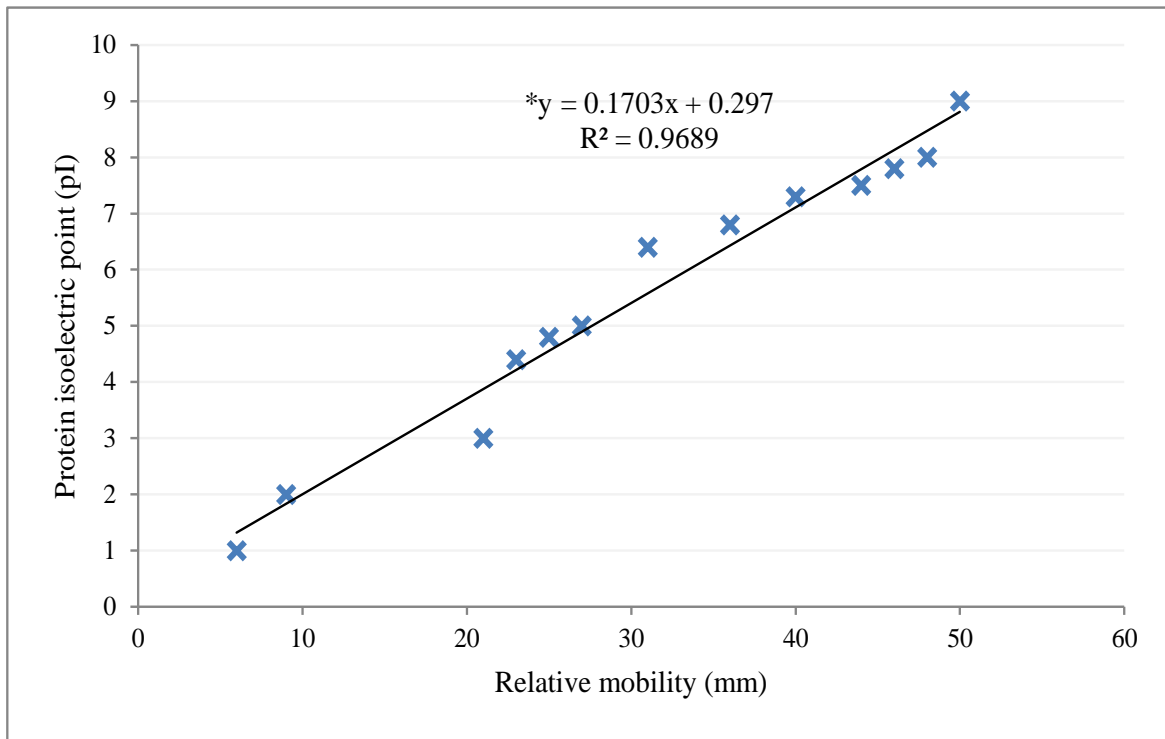
Figure 5.5 Standard curve for migration of proteins according to isoelectric points during ultra-thin isoelectric focusing.

(✗) actual molecular weights for protein marker; (—) fitted linear trendline; (*) linear equation and regression coefficient for extrapolation of unknown protein molecular weights.

Band fragment data from Figure 5.4 was used to construct a dendogram using the UPGMA sub-program on NTSYS (Figure 5.6). Contrary to the results from SDS-PAGE, where phages Sabp-P1 and Sabp-P2 were clearly distinguishable from Sabp-P3, based on relative size of protein fragments, combined analysis of UTLIEF data presented in the dendogram (r^2=80.80%) showed that, based on the isoelectric point of proteins, phages Sabp-P2 and Sabp-P3 showed similarity to each other and were different from Sabp-P1 (Figure 5.6). Combined similarity matrices

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(Appendix 3) showed a 66.67% similarity between phages Sabp-P1 and Sabp-P2 and a 54.55% similarity between Sabp-P1 and Sabp-P3. Sabp-P2 showed a 75.00% similarity to Sabp-P3.
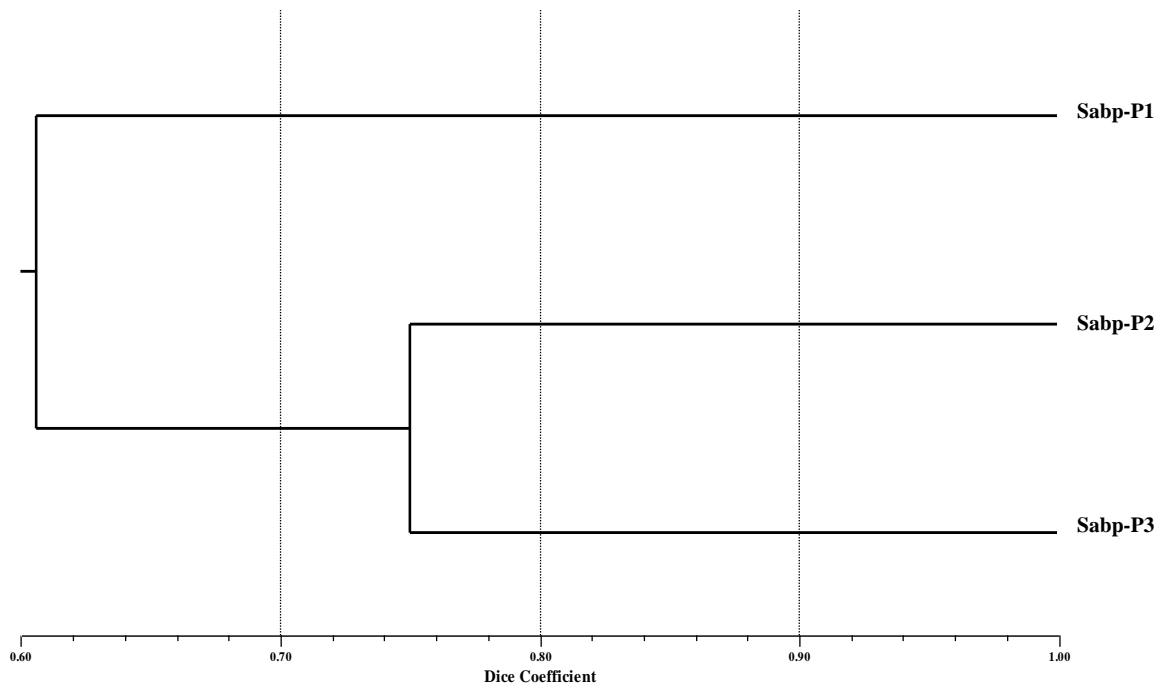
Figure 5.6 Phylogenetic tree comparing relative isoelectric focussing profiles of different phages proteins.

5.3.3 Comparison of SDS-PAGE and UTLIEF

A comparison of all data obtained during both SDS-PAGE and UTLIEF were compared to each other to determine any relatedness between phages Sabp-P1, Sabp-P2 and Sabp-P3. Combined analysis presented in the dendogram (r^2= 98.27%) revealed that Sabp-P1 and Sabp-P2 were more related to each other, than either to Sabp-P3 (Figure 5.7). Combined similarity matrices (Appendix 3) showed an 80.00% similarity between phages Sabp-P1 and Sabp-P2, and a 68.29% similarity between Sabp-P1 and Sabp-P3. Sabp-P2 showed a 70.59% similarity to Sabp-P3.
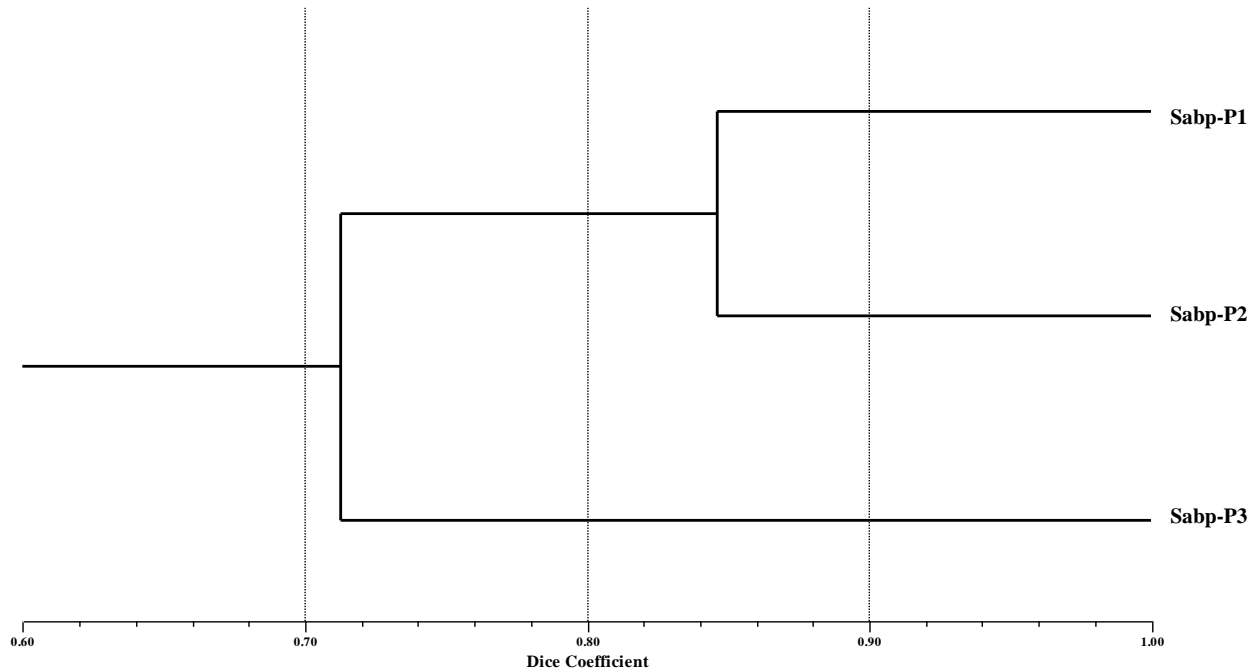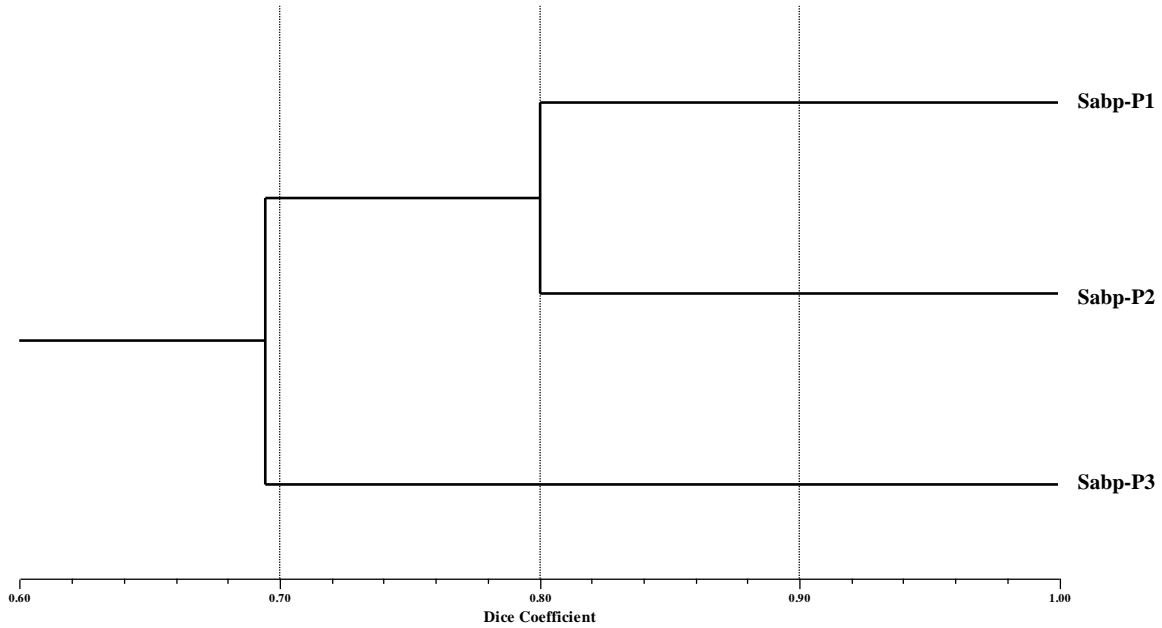
Figure 5.7 Phylogenetic tree comparing combined data from SDS-PAGE and UTLIEF to determine differences between phage strains, Sabp-P1, Sabp-P2 and Sabp-P3.

5.4 Discussion

Characterisation of phages through analysis of their proteomes is a dynamic field of research. This form of characterisation serves as a supplementary technology to genomic analysis and can aid in the characterisation of newly discovered phages. Typical methods of analysis include one-dimensional (SDS-PAGE, IEF) and two-dimensional electrophoresis, spectrophotometric methods (matrix assisted laser desorption/ionization, mass spectrometry), immunoblotting (Western blot) or chromatographic methods (Berkelman and Stenstedt, 1998). Each of these methods characterizes phage proteins, based on different factors ranging from molecular weight, to isoelectric charge, to mass-to-charge ratios (Berkelman and Stenstedt, 1998). The current study investigated one-dimensional electrophoretic analysis of proteins from three different strains of staphylococcal phages, in order to determine whether the phages differed from each other.

Although a majority of proteins encoded by phages with larger genomes have no matches in current sequence databases, and hence have undiscovered functions, there is a core set of

genes that encode for common proteins mainly involved in morphogenesis and nucleotide processing (Eyer *et al.* 2007; Carvalho *et al.* 2012). The most abundant proteins that appear in any electrophoretic profile for phages are the major capsid proteins and the major tail sheath proteins (Lee and Stewart, 1965; Eyer *et al.* 2007; Lood and Collin, 2011). Analysis of phage proteins using SDS-PAGE showed that phages Sabp-P1, Sabp-P2 and Sabp-P3 comprised of proteins that ranged in size from 8.86kDa to 171.66kDa. From the identified protein sizes, nine sizes were common amongst all three phage, with the largest being 171.66kDa. It has been postulated that high molecular weight proteins, in the range from 160kDa up to 500kDa, represent a major head protein, and is probably a complex of 6-8 monomers (Lood and Collins, 2011). Additionally, it appears that this protein complex is not easily disrupted into smaller components, even during lysis by boiling in SDS reducing buffer (Lood and Collin, 2011). Similar results have been reported by Popa *et al.* (1991) with *Enterobacteria* phage HK97, which has complexes of covalently linked major head proteins, instead of a single major head protein that exists as a monomer.

Lood and Collin (2011) also identified several other proteins that showed correlation to phages Sabp-P1, Sabp-P2 and Sabp-P3 (in terms of relative molecular weight). Protein Gp14 (tape measure protein) was estimated to have a molecular weight of approximately 65kDa. This was similar to Sabp-P1 and Sabp-P2 which showed presence of similar sized proteins. Sabp-P1 seemed to lack this protein completely. Additionally, protein Gp3 (~48kDa), or portal protein, showed molecular weight similarity to Sabp-P1 (~49kDa) and Sabp-P2 (~49kDa). Müller *et al.* (1994) identified phage tail tube proteins with molecular weights (~20kDa) that were similar to all three phages. Protein fragments in the range of 70-109kDa have been associated with major tail proteins of phages (Miller *et al.* 2003; Lood and Collin, 2011). Sabp-P1, Sabp-P2 and Sabp-P3 showed both major and minor band fragments in this region. These proteins could be associated with the synthesis of the phage tail and associated structures (tail sheath, tail fibres). Although SDS-PAGE was able to resolve the proteins of each phage and produce a distinctive protein profile for each, proteomic sequence analysis is required in order to fully characterize each derived protein. For the purposes of the current study, SDS-PAGE analysis adequately revealed that, based on protein profiling, each phage represented a unique strain.

The differences between strains were further corroborated through UTLIEF analysis of phage proteins. Sample treatment conditions differed between SDS-PAGE and UTLIEF. Generally, during two-dimensional analysis, where IEF is followed by SDS-PAGE, a single band derived from IEF can yield more than one band when resolved during SDS-PAGE (Brown *et al.* 1980). In the current study, the number of protein fragments generated, differed between the methods, and subsequently, so did the results of UTLIEF phylogenetic analysis. Phage Sabp-P1 differed significantly in terms of relative protein charge, from both Sabp-P2 and Sabp-P3. However, overall similarity matrices showed that although there was a degree of homology between each phage, they were still clearly different from each other. The application of isoelectric focusing for protein resolution is a well documented method (Berkelman and Stenstedt, 1998). However, the use of ultra-thin isoelectric focusing is a new method that has not been previously applied to separate phage proteins. The current study presents novelty in this respect, as it adds a further technique to the spectrum of phage analysis methodology. Although much optimization was required before positive results were obtained, the method was able to produce a unique isoelectric profile for each phage, with good resolution of bands.

5.5 Conclusion

Two different methods of protein profile analysis were successfully applied to differentiate between three strains of staphylococcal phages. Both techniques confirmed that phages Sabp-P1, Sabp-P2 and Sabp-P3 were three different strains of phage. While current results are based on putative protein fragments analysis, complete protein sequencing would be necessary in order to specifically characterize how each phage differs based on protein chain composition.

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CHAPTER 6

Preliminary genomic characterization of bacteriophages with specific lytic activity against *Staphylococcus aureus*, associated with bovine mastitis

Abstract

Staphylococcus aureus subsp. *aureus* Rosenbach 1884 is the primary causal agent of bovine mastitis. Phages were selected for use in a biological control program aimed at reducing staphylococcal-associated infections in dairy cattle. The aim of the current study was to genomically characterize three staphylococcal phages, Sabp-P1, Sabp-P2 and Sabp-P3, using restriction fragment length analysis and whole genome sequencing. Results showed that the genomes of phages Sabp-P1, Sabp-P2 and Sabp-P3 were all different from each other. Phages Sabp-P1 and Sabp-P3 showed sequence homology to a form of *Pseudomonas* phages labelled "giant" phages. Phage Sabp-P3 showed sequence homology to a *Clostridium perfringens* phage. The major functional proteins of phages (tail tape measure protein, virion structural proteins, head morphogenesis proteins, and capsid proteins) were identified in all three phages. However, although the level of sequence similarity between the screened phages and those already found on the databases enabled preliminary classification of the phages into the order *Caudovirales*, family *Myoviridae*, the level of homology was not sufficient to assign each phage to a particular type species. Results suggest that phage Sabp-P1 especially, might be a new species of phage within the *Myoviridae* family. The long-term objective of the sequencing study is to carry out complete assembly and annotation of all the contigs for each phage. This will provide definitive conclusions in terms of phage relatedness and classification.

6.1 Introduction

Genomic characterization serves as one of the most accurate means of distinction between organisms. There are approximately 500 phage genome sequences available in the NCBI phage database with genomes ranging from 15-500kbp in size (Gutiérrez *et al.* 2011). Tailed phages of the order *Caudovirales* constitute the most extensive group of bacterial viruses, comprising up to 96% of phages identified so far (Ackermann, 2007; Gutiérrez *et al.*2011). These phages consist of a protein shell, linear dsDNA, and lack any type of envelope (Ackermann, 2005; 2007). Phage heads are icosahedral and tails are true helices that consist of stacked discs. These tails usually

possess terminal adsorption structures such as base plates, spikes or tail fibres (Ackermann, 2005). Although these phages constitute a monophyletic group possessing related properties (morphological, physicochemical, physiological), and have been classified into a single order, *Caudovirales*, their individual properties are rather varied. Typically, they differ in terms of dimension and fine structure, DNA content and composition, nature of constitutive proteins, serology and host range (Ackermann, 2005). Ackermann (2005) has devised a criterion to distinguish between these phages: (1) *Myoviridae*, with contractile tails consisting of a sheath and central tube, approximately 25% of tailed phages; (2) *Siphoviridae*, with long, non-contractile tails, approximately 61% of tailed phages; and, (3) *Podoviridae*, with short, non-contractile tails, approximately 14% of tailed phages.

Phage genomes are large and complex, and they are usually organized into interchangeable modules, with genes for related functions clustered together (Ackermann, 2005; 2007). Replicating DNA tends to form large, branched concatemers, which are then cut into unit lengths and inserted into preformed capsids (Ackermann, 2005). Virion assembly of individual components (heads, tails, tail fibres) takes places via separate pathways which forms the last stage of the maturation process (Ackermann, 2005). Newly assembled phage particles are then liberated into the surrounding environment via lysis of the host bacterium (Ackermann, 2005).

While tailed phages do comprise the majority of bacterial viruses, tailless phages also occur (Ackermann, 2005). These include only about 190 known viruses corresponding to less than 4% of the currently recognised bacterial viruses (Ackermann, 2005). They are classified into 10 small families, occur enveloped or non-enveloped, and are of three types: polyhedral phages that are icosahedral with cubic symmetry and either DNA or RNA constituted, filamentous phages with helical symmetry that are DNA constituted, and a few pleomorphic types without obvious symmetric axes that are also DNA constituted (Ackermann, 2005).

Several phages specific to mastitis-causing *Staphylococcus aureus* subsp. *aureus* Rosenbach 1884, have been sequenced, either completely or partially (Bon *et al.*1997; O'Flaherty et al, 2004; Kwan *et al.*2005; Garcia *et al.*2009; Lavigne *et al.*2009; Hsieh *et al.*2011; Vandersteegen *et al.*2011). These studies have contributed significantly to understanding the diversity of the phage genome, as well mechanisms such as lateral gene transfer and illegitimate recombination that drive bacterial genetic diversity (Kwan *et al.*2005). The number of known,

strictly lytic phages is limited to the close-knit *Myoviridae* genus of the SPO1-like viruses which also contains phages K, Twort and G1 (O'Flaherty *et al.* 2004; García *et al.* 2009). Apart from this group, a large number of genomes from unclassified *Siphoviridae* in lysogenic *S. aureus* strains are available (Kwan *et al.* 2005). Some lysogenic phages may play an important role in the pathogenicity of *S. aureus* by carrying virulence factors, mediating lateral gene transfer and possibly facilitating adaptation of the pathogen during infection (Górski *et al.* 2009). Kwan *et al.* (2005) sequenced the complete genomes and proteomes of 27 *S. aureus* phages. Comparative nucleotide and protein sequence analysis was used to group these phages into three classes: <20kbp, ~40kbp and >125kbp. García *et al.* (2009) also presented a detailed genomic and molecular characterisation of two *S. aureus* lytic phages. Other studies conducted by various researchers also on sequence analysis of *S. aureus* phages (O'Flaherty *et al.* 2004; Rashel *et al.* 2008; Vandersteegen *et al.* 2011; Gu *et al.* 2012; Kwiatek *et al.* 2012) have provided a solid background for future comparative studies.

While studies into phage genomics, particularly of *S. aureus* phages, have taken place in other parts of the world, within a South African perspective, this remains an area of significant novelty. There are currently no other known staphylococcal phage studies underway, either in terms of genomics or practical application of phages against bovine mastitis in South Africa.

This study served to characterize staphylococcal phages that were isolated from dairy cows diagnosed with bovine mastitis. Through morphological analyses of phage structure and infection cycle, these phages were preliminarily classified into the order *Caudovirales*, family *Myoviridae*. Preliminary genomic characterization was undertaken in order to derive further information on the relatedness of each phage towards each other and to those phages that have already been sequenced worldwide.

6.2 Materials and Methods

6.2.1 Bacterial host strains and their isolation

The *S. aureus* strain, *SaB1*, was used for phage isolation and propagation. This strain was isolated from raw bovine milk collected from dairy farms in the province of KwaZulu-Natal,

RSA (Republic of South Africa). *SaB1* was initially isolated on blood agar. Cultures required for phage propagation were maintained on tryptone soy agar (TSA) or typtone soy broth (TSB).

6.2.2 Phage isolation, propagation and purification

Raw bovine milk samples collected from dairy farms in the province of KwaZulu-Natal, RSA (Republic of South Africa) served as the initial material from which to isolate phages. These milk samples were chosen at random and included milk with both high ($>$400,000cells ml^{-1}) and low somatic cell counts (SCC). All milk samples were stored on ice during transit, or at 4ºC in the laboratory.

Phages were isolated using the spot-test method on double-layer agar (Sambrook *et al.*1989). Overnight *S. aureus* culture, *SaB1*, at ~1 x 10^8 cfu.ml^{-1}, was incorporated into 7% top agar that was supplemented with 1M CaCl$_2$. Raw milk samples were filtered through a 0.45μm syringe filter. Filtered samples were then spotted onto the surface of solidified top agar at 10μl per spot. Plates were allowed to dry for 2hr, followed by incubation for 12hr at 37ºC. Zones of clearing (plaques) were indicative of phage activity. Plaques were removed from top agar and soaked in phage buffer (Appendix 1) for 12hr with gentle agitation (150rpm) at 4ºC. The resulting suspension was centrifuged using the Avanti J-26 XPI (www.beckmancoulter.com) at 10,000g x 10min at 4ºC. The supernatant was filtered using a 0.45μm syringe filter and stored as phage stock at 4ºC. Subsequent phage was grown from this stock in liquid broth culture or using the double-layer agar method (Sambrook *et al.*1989).

Isolated phages were purified through a modification of standard methods (Sambrook *et al.*1989; Harley and Prescott, 1993). Filter-sterilised phage stock isolated from either liquid broth culture or double-layer agar method, were subjected to centrifugation using the Avanti J-26 XPI (Beckman-Coulter, www.beckmancoulter.com) at 75,600g x 3hr at 10ºC. Resulting phage pellets were re-suspended in fresh phage buffer and a second centrifugation was conducted (Avanti J-26 XPI at 75,600g x 3hrs x 10ºC). The phage pellets were then re-suspended in fresh phage buffer at 1/10 of the original volume that was processed. All stocks were stored at 4ºC.

A total of 28 phages were isolated. However, only three showed consistency with regard to host cell lysis and growth requirements. These three phages were used for subsequent screening and were named Sabp-P1, Sabp-P2 and Sabp-P3.

6.2.3 DNA extraction

Phage DNA was extracted using a modification of methods by Sambrook *et al.* (1989). Purified phage pellets were treated with DNase I (5µg.ml^{-1}) (www.fermentas.com) and RNase (10µg.ml^{-1})[2]. The phage suspension was centrifuged at 75,600g x 3hr at 10ºC using the Avanti J-26 XPI (Beckman-Coulter) centrifuge. Phage pellets were re-suspended in 400µl SDS buffer (20%) and incubated at 80ºC x 30min. Thereafter, 400µl cetyltrimethyl ammonium bromide (CTAB) was added and tube was gently shaken. 400µl chloroform: isoamyl alcohol (24:1) with 5% phenol, was added to the tube. The tube was centrifuged at 14,000g x 10min at 4ºC. 600µl of the clear aqueous phase was drawn off, taking care to avoid the impurity-laden organic phase. An equal volume (600µl) of isopropanol was added to the tube, followed by incubation for 1hr at 4ºC. After 1hr, the tube was centrifuged at 14,000g x 10mins at 4ºC. The DNA pellet was washed twice in cold (4ºC) 70% ethanol, air-dried in a laminar flow unit for 30min, followed by final re-suspension in 500µl 1x Tris-EDTA (Appendix 4). All isolated DNA was stored at -20ºC. A Nanodrop-100[1] was used to quantify the concentration and quality of phage DNA. Phage DNA was also analysed using standard agarose gel electrophoresis to check for presence and quality (Appendix 4).

6.2.4 Restriction endonuclease digestion and agarose gel electrophoresis

Phage DNA was treated with the following restriction endonucleases: EcoR1 (Fast Digest) (www.fermentas.com), EcoRV (Fast Digest) (www.fermentas.com), HindIII (www.neb.com), BamH1 (www.neb.com), and PvuII (Fast Digest) (www.fermentas.com). Samples preparations were according to manufacturer instructions. The only amendment that was made for each enzyme was incubation time: all digests, including those using fast-digest enzymes, were left to proceed for 1hr at 37ºC. The O'Gene Ruler 1kb Plus DNA Ladder (www.fermentas.com) was used as the marker.

Restriction products were analysed on a horizontal 1% (w/v) agarose gel with 1xTAE buffer (Appendix 4) (Sambrook and Russell, 2010). All digests were run at 100V for 60min (or until loading dye was 1cm from bottom of gel), using the Labnet Electrophoresis Sub System 70 (Model E-0310) (www.labnetinternational.com) powered off a Bio-Rad Power Pack 300 (www.bio-rad.com). Gels were post-stained in 1x TAE buffer (augmented with 0.01% ethidium

bromide) for 30min. Gels were viewed and photographed using the VersaDoc Gel Imaging System 4000 (www.bio-rad.com).

6.2.5 Gel scoring and intepretation

Quantitative analysis of gel banding patterns was carried out using NtSys (www.exetersoftware.com). Regression coefficients were calculated for data using the Mantel (1967) method. Matrix comparison tests were carried out to quantify differences between banding patterns of each phage. Standard curves were created and logarithmic equations were used to extrapolate unknown molecular weights for each band.

6.2.6 Genome sequencing

a. Sequencing

Approximately 2ug of phage DNA of each sample was sequenced using 454 pyrosequencing technology for a Roche genome sequencer GS FLX system (www.454.com). The Roche 454 technology allowed for sample multiplexing combined with average to long read lengths of each library. The complete sequencing workflow of the GS FLX system comprised of four major steps, leading from purified DNA to analysed results: (1) generation of a single-stranded amplicon DNA library using Ampure (www.454.com) (a high sensitivity QC chip), a qPCR quantitation (www.454.com), and equimolar pooling; (2) emulsion-based clonal amplification of the library where libraries were attached to DNA Capture Beads (www.454.com), with each bead carrying a unique, single-stranded library fragment. Beads were emulsified with amplification reagents in a water-in-oil mixture to trap individual beads in amplification microreactors. The entire emulsion was amplified in parallel to create millions of clonal copies of each library fragment on each bead. Amplified fragments remained bound to their specific beads even after breaking of the emulsion; (3) Data generation via sequencing-by-synthesis, where beads were loaded onto the PicoTiterPlate (www.454.com) (PTP) device which allows only one bead per well. This was followed by a FLX Titanium sequence run on the PTP. Individual nucleotides were flowed in sequence across the wells. Each incorporation of a nucleotide complementary to the template strands on the beads resulted in a chemiluminescent light signal recorded by a

camera; (4) data processing with software that used the signal intensity of each incorporation event at each well position to determine the sequence of all reads in parallel.

b. Analysis of phage genome

Phage genome sequences were analysed using Artemis Release 14.0.0 (www.sanger.ac.uk) program. Genome mapping was then carried out using pDraw 32 (www.acalone.com).

6.3 Results

6.3.1 Restriction fragment length analysis

Analysis of agarose gels post-restriction, revealed that the DNA from all three phages appeared resistant to digestion by the following enzymes: EcoRv (Fast Digest), BamHI, HindIII, and PvuII (Fast Digest). The phage DNA was successfully digested using only EcoRI. Results derived from restriction digestion of phage DNA (Fig. 6.1) were used to determine approximate molecular weights (kbp) for the genome of each phage. In addition, results were subjected to phylogenetic analysis to determine whether phages Sabp-P1, Sabp-P2 and Sabp-P3 differed from each other in terms of the relative sizes of their genomes.

Band fragment analysis was carried out using Quantity One software (Bio-Rad)[5]. Results revealed that each phage possessed a different sized genome (Fig. 6.1, Table 6.1). The data of relative mobility and molecular weight of the DNA marker were used to plot a standard curve of mobility versus molecular weight (Fig. 6.2). The logarithmic equation derived from the standard curve was used to determine approximate molecular weights for individual DNA fragments of Sabp-P1, Sabp-P2 and Sabp-P3 (Table 6.1). Combined sums of each of the range of fragments for each phage showed that Sabp-P1 contained a genome measuring approximately 107kbp, Sabp-P2 contained a genome measuring approximately 70kbp, and Sabp-P3 contained a genome measuring approximately 126kbp.
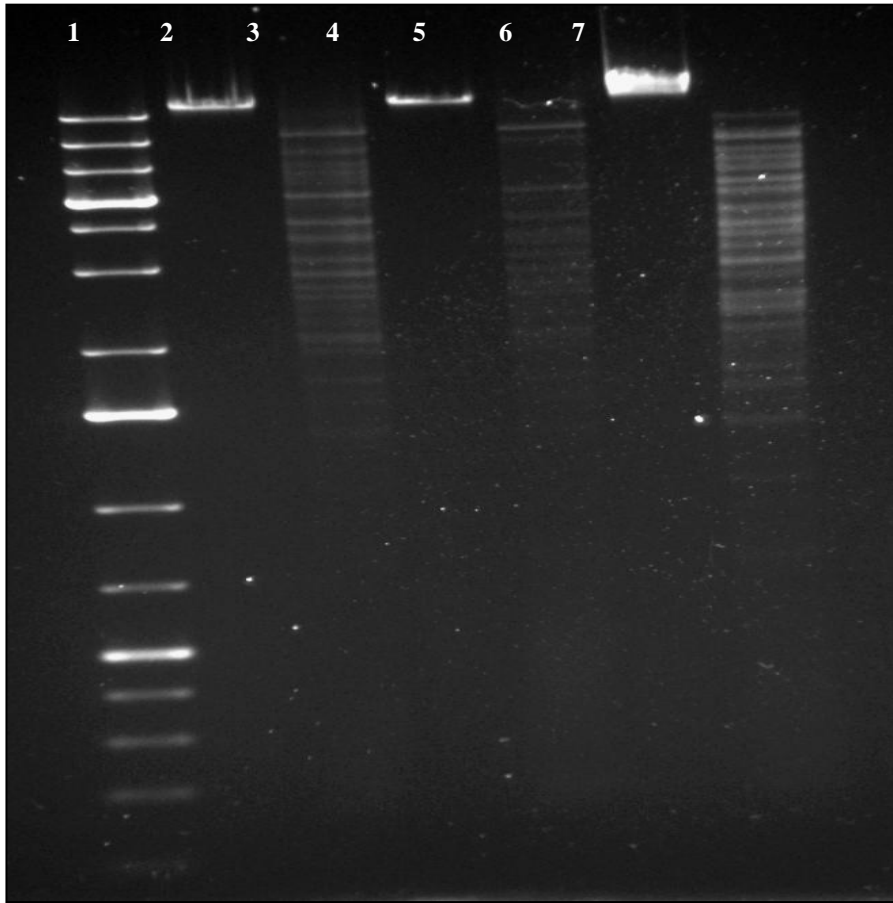
Figure 6.1 Restriction fragments of phage DNA that was digested using the restriction enzyme, EcoRI. Lane identity: 1 = 1kb Plus DNA Ladder; 2 = Sabp-P1 undigested; 3 = Sabp-P1 restriction digest; 4 = Sabp-P2 undigested; 5 = Sabp-P2 restriction digest; 6 = Sabp-P3 undigested; 7 = Sabp-P3 restriction digest.

Table 6.1 Approximate molecular weights (kbp) of phage DNA digested with restriction enzymes

DNA ladder (1kb Plus)		Approximate MW (kbp) of phage genomes		
Band no.	MW (bp)	Sabp-P1	Sabp-P2	Sabp-P3
1	20,000	11,581	12,046	12,555
2	10,000	10,388	10,757	10,757
3	7,000	9,719	7,876	10,043
4	5,000	9,125	7,053	9,414
5	4,000	7,877	6,339	8,851
6	3,000	6,865	5,860	8,341
7	2,000	6,175	5,563	7,876
8	1,500	5,710	5,282	7,247
9	1,000	5,282	4,886	6,684
10	700	5,015	4,400	6,339
11	500	4,673		5,860
12	400	4,400		5,563
13	300	4,172		5,147
14	200	3,954		4,637
15	75	3,745		4,400
		3,161		3,954
		2,805		2,980
		2,470		2,552

Band fragment data (Fig. 6.1) was used to construct a dendogram using the UPGMA sub-program on NTSYS (Fig. 6.3). Phages Sabp-P1 and Sabp-P3 were clearly distinguishable from Sabp-P2 based on combined data presented in the dendogram (r^2=96.82%). Combined similarity matrices (Appendix 4) showed a 70.97% similarity between Sabp-P1 and Sabp-P2 and an 86.50% similarity between Sabp-P1 and Sabp-P3. Sabp-P2 showed a 75.00% similarity to phage Sabp-P3.
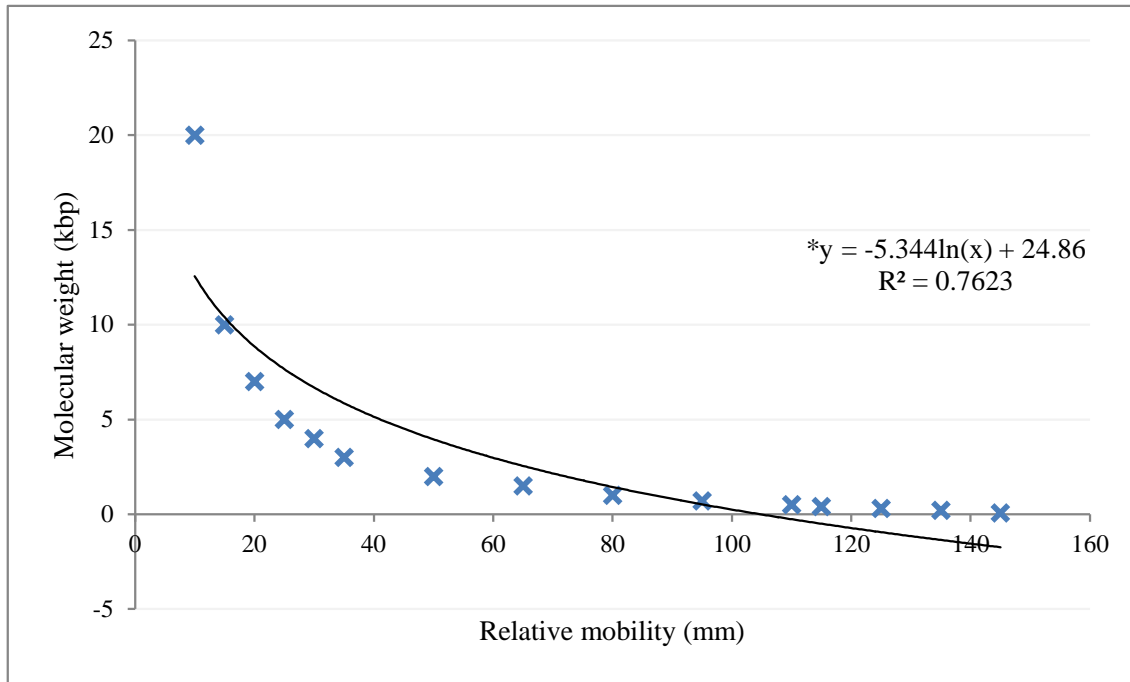
Figure 6.2 Standard curve for molecular weight of DNA for analysis of phage DNA fragments. (✗) actual molecular weights for protein marker; (—) fitted logarithmic trendline; (*) logarithmic equation and regression coefficient for extrapolation of unknown DNA fragment molecular weights.
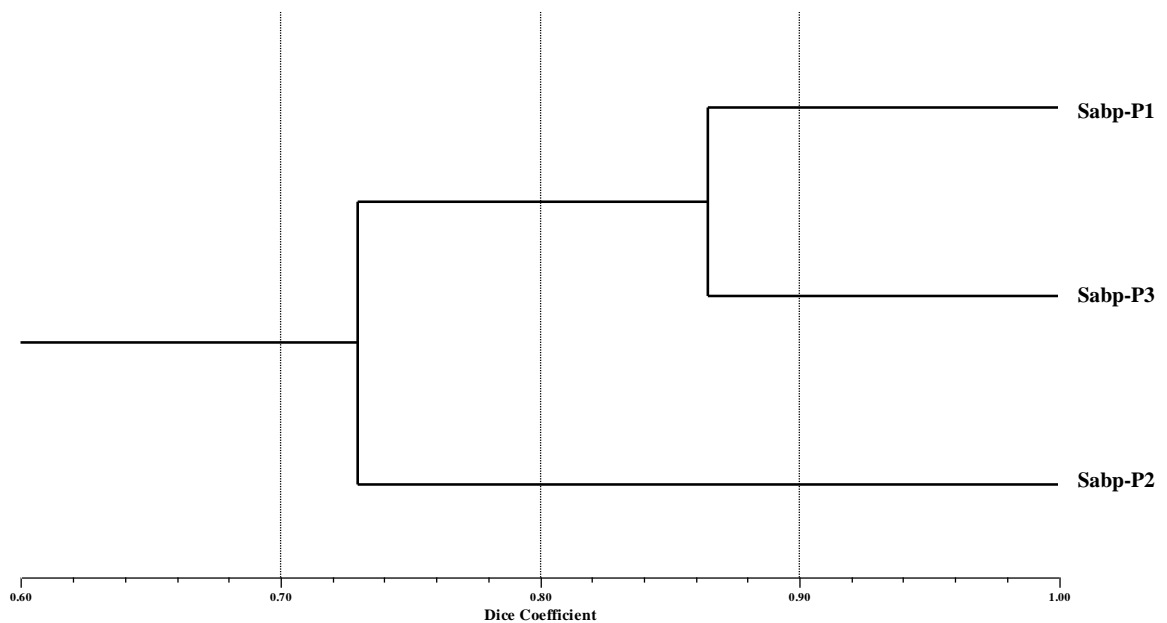
Figure 6.3 Phylogenetic tree comparing relative DNA banding patterns for three different strains of phage, Sabp-P1, Sabp-P2 and Sabp-P3.

6.3.2 Combined analysis of DNA and protein profiling

A comparison of all data obtained during both protein profiling (Chapter 5) and restriction fragment length analysis were compared to each other to determine relatedness between phages Sabp-P1, Sabp-P2 and Sabp-P3. Combined analysis presented in the dendogram (r^2= 71.46%) revealed that Sabp-P1 and Sabp-P3 were more related to each other, than either to Sabp-P2 (Fig. 6.4). Combined similarity matrices (Appendix 4) showed a 75.76% similarity between phages Sabp-P1 and Sabp-P2, and a 76.92% similarity between Sabp-P1 and Sabp-P3. Sabp-P2 showed a 72.73% similarity to Sabp-P3.
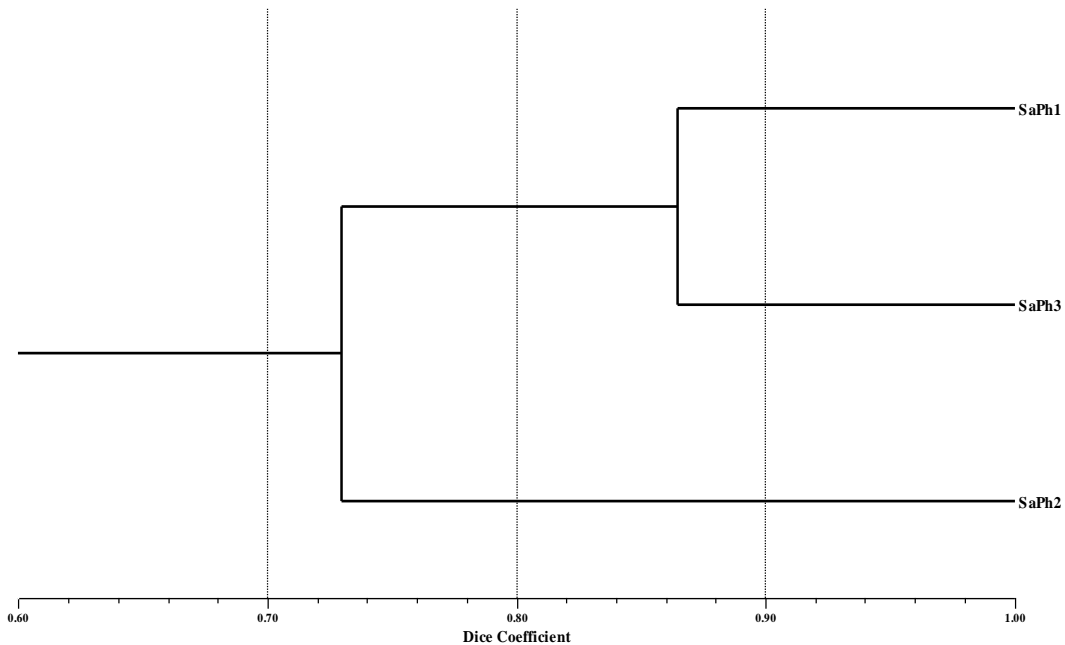
Figure 6.4 Phylogenetic tree comparing combined data from DNA and protein profiling (SDS-PAGE and UTLIEF) to determine differences between phage strains, Sabp-P1, Sabp-P2 and Sabp-P3.

6.3.4 Genome sequencing

Whole-genome shotgun sequencing of the genetic material of phage Sabp-P1 generated 3.8Mb of data that was assembled into 172 contigs with a total size of 367854bp (Table 6.2). Mean contig length was 2138bp, and average G+C content was 47%. With Sabp-P2, a total of 10.47Mb of data was generated that was assembled into 116 contigs with a total size of 425240bp (Table 6.2). Mean contig length was 3665bp, and average G+C content was 38%. The genome of phage

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Sabp-P3 measured 333889bp and comprised of 49 contigs with a mean contig size of 6814bp (Table 6.2). The average G+C content for Sabp-P3 was 39%.

Preliminary comparison of open reading frames (ORFs) of all three phages showed that Sabp-P1 had zero sequence similarity to either Sabp-P2 or Sabp-P3. Phages Sabp-P2 and Sabp-P3 showed genetic relatedness as demonstrated by commonly shared ORFs, but each also possessed divergent ORFs.

Table 6.2 Sequence results from whole genome sequencing of phages Sabp-P1, Sabp-P2 and Sabp-P3.

Data description	Phage		
	Sabp-P1	Sabp-P2	Sabp-P3
Total number of reads	10597	29188	12412
Total genome size (bp)	367854	425240	333889
Number of contigs	172	116	49
Average contig size (bp)	2138	3665	6814
N50 contig size[*]	4990	241493	25278
Largest contig size (bp)	48928	241493	102301
Q40 Plus bases[**]	354908, 96.48%	418478,98.41%	330325, 98.93%
G+C content (%)	47	38	39
Amount of data generated (Mb)	3.8	10.47	4.49

*N50 contig size = >50% of total bases have contigs of 4990 for Sabp-P1, 241493 for Sabp-P2, and 25728 for Sabp-P3 bases or more; **Q40 Plus bases = quality metric where for every 93.48% (Sabp-P1), 98.41% (Sabp-P2) and 98.93% (Sabp-P3) of reads, the error rate was 1 in 10000.

The largest contig for phage Sabp-P1 was analysed against known sequences in genomic databases[1]. Artemis Release 14.0.0[8] was used to predict coding sequences (CDS) on the Sabp-P1 contig. The CDSs were compared to other known sequences using BLASTp[8] analysis. Analysis showed the presence of several key diagnostic proteins that are associated with phage biology and replication. Genes encoding for major proteins associated with head and tail assembly as well several other putative proteins with unknown functions were identified (Table 6.3). Genes

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[1]www.ncbi.nlm.nih.gov

encoding for replicative proteins, such as endonucleases and DNA replicase, were also found. It was also found that Sabp-P1 showed sequence similarity to two pseudomonad phages, isolated from *Pseudomonas fluorescens* Migula, 1895 and *P. aeruginosa* Migula, 1900 (Cornelissen *et al.*2012). Sequence identity, however, was not higher than 51% for any given gene. A noteworthy finding was that Sabp-P1 showed no sequence homology to any other staphylococcal phage that was listed on the genomic database. Sequence homology to key diagnostic proteins, especially the tail tape measure protein, can confirm that this phage is of the order *Caudovirales*, and part of the *Myoviridae* family.

Table 6.3 List of identified proteins and their predicted functions for phage Sabp-P1.

Protein	No. of bases	No. of amino acids	Identity (%)	Predicted protein function	BLASTp best match	Accession reference
1	1221	406	51	Putative virion-associated RNA polymerase beta' subunit	*Pseudomonas* phage OBP	YP_004958184.1
2	1512	503	50	Hypothetical protein PPEV_gp173	*Pseudomonas* phage EL	YP_418206.1
3	453	151	50	Hypothetical protein PAJU2_gp19	*Pseudomonas* phage PAJU2	YP_002284353.1
4	1194	397	49	Putative virion-associated RNA polymerase beta' subunit	*Pseudomonas* phage OBP	YP_004958185.1
5	816	272	49	Hypothetical protein SDSG_00020	*Silicibacter* phage DSS3-P1	AET42286.1
6	687	228	48	Putative virion structural protein	*Pseudomonas* phage OBP	YP_004958175.1
7	1935	645	48	Hypothetical protein BPPAW11_00710	*Pseudomonas* phage YMC/01/01/P52_PAE-BP	AFQ22096.1
8	957	319	48	Putative head morphogenesis protein	*Pseudomonas* phage phi297	YP_005098071.1
9	1077	359	47	Putative coat protein	*Pseudomonas* phage phi297	YP_005098074.1
10	465	155	47	Deoxyuridine 5'-triphosphate nucleotidohydrolase	*Erwinia* phage vB_EamM-Y2	YP_007004707.1
11	2661	887	47	Hypothetical protein PAJU2_gp22	*Pseudomonas* phage PAJU2	YP_002284356.1
12	489	163	46	Hypothetical protein PAJU2_gp20	*Pseudomonas* phage PAJU2	YP_002284354.1
13	1398	466	45	Replicative DNA helicase	*Pseudomonas* phage phi297	YP_005098095.1
14	912	303	45	Hypothetical protein PPEV_gp005	*Pseudomonas* phage EL	YP_418038.1
15	2214	737	45	Hypothetical protein PPEV_gp182	*Pseudomonas* phage EL	YP_418215.1
16	459	152	44	Hypothetical protein PPEV_gp180	*Pseudomonas* phage EL	YP_418213.1
17	519	172	43	Hypothetical protein PPEV_gp194	*Pseudomonas* phage EL	YP_418227.1
18	1842	613	43	Putative virion-associated SNF2-domain containing helicase	*Pseudomonas* phage OBP	YP_004958170.1

Table 6.3 continued

Protein	No. of bases	No. of amino acids	Identity (%)	Predicted protein function	BLASTp best match	Accession reference
19	906	301	43	Virion structural protein	*Pseudomonas* phage 201phi2-1	YP_001956953.1
20	1515	504	42	Hypothetical protein PPEV_gp171	*Pseudomonas* phage EL	YP_418204.1
21	1326	441	42	Hypothetical protein PPEV_gp169	*Pseudomonas* phage EL	YP_418202.1
22	729	243	42	hypothetical protein PMI34_01895	*Pseudomonas* sp. GM74	ZP_10626697.1
23	804	268	42	IstB domain protein ATP-binding protein	*Pseudomonas syringae* pv. *maculicola* str. ES4326	EGH59120.1
24	1542	514	42	Putative T5 A1-like protein	*Caulobacter* phage phiCbK	YP_006988022.1
25	3408	1136	41	Tail tape measure protein	*Pseudomonas* phage phi297	YP_005098093.1
26	492	163	41	Hypothetical protein PPEV_gp178	*Pseudomonas* phage EL	YP_418211.1
27	744	248	41	Hypothetical protein phi297_00054	*Pseudomonas* phage phi297	YP_005098081.1
28	1425	475	41	Hypothetical protein SPN3UB_0003	*Salmonella* phage SPN3UB	YP_007010973.1
29	1029	342	40	Putative endonuclease	*Pseudomonas* phage PhiPA3	AEH03633.1
30	879	292	40	Hypothetical protein PPEV_gp188	*Pseudomonas* phage EL	YP_418221.1
31	738	245	40	Hypothetical protein PPEV_gp191	*Pseudomonas* phage EL	YP_418224.1
32	795	265	39	C-5 cytosine-specific DNA methylase	*Mycobacterium* phage SG4	AER49474.1
33	639	212	38	Putative virion structural protein	*Pseudomonas* phage OBP	YP_004958165.1
34	606	201	38	Dihydrofolate reductase	*Listeria marthii* FSL S4-120	ZP_07871197.1
35	903	300	38	Hypothetical protein PPEV_gp192	*Pseudomonas* phage EL	YP_418225.1
36	3204	1067	38	Hypothetical protein PPEV_gp186	*Pseudomonas* phage EL	YP_418219.1
37	585	195	38	Hypothetical protein	*Rhodococcus* phage REQ2	YP_005087102.1
38	648	216	37	Hypothetical protein AMBK_14	*Salmonella* phage vB_SosS_Oslo	YP_006560821.1

Table 6.3 continued

Protein	No. of bases	No. of amino acids	Identity (%)	Predicted protein function	BLASTp best match	Accession reference
39	2667	888	37	Hypothetical protein PPEV_gp008	*Pseudomonas* phage EL	YP_418041.1
40	2163	720	37	Hypothetical protein PPEV_gp006	*Pseudomonas* phage EL	YP_418039.1
41	7785	2594	37	Putative phage-related lysozyme domain	*Pseudomonas* phage EL	YP_418216.1
42	738	245	37	Thymidine kinase	*Aeromonas* phage Aes508	YP_007010778.1
43	1203	400	36	Putative thymidylate synthase	*Pseudomonas* phage OBP	YP_004958141.1
44	558	186	36	Hypothetical protein ORF003	*Pseudomonas* phage PA11	YP_001294596.1
45	459	153	36	Possible small terminase subunit	*Enterobacteria* phage ES18	YP_224139.1
46	486	162	36	Possible endopeptidase	*Burkholderia* phage DC1	YP_006590002.1
47	843	281	35	Phage-related exonuclease	*Arsenophonus nasoniae*	CBA74387.1
48	882	293	33	Hypothetical protein PPEV_gp007	*Pseudomonas* phage EL	YP_418040.1
49	831	276	32	Putative metal-dependent phosphohydrolase	*Pseudomonas* phage OBP	YP_004958214.1
50	795	265	31	Phage regulatory protein	*Anaerostipes* sp. 3_2_56FAA	ZP_07932474.1
51	1341	447	31	Hypothetical protein	*Cronobacter* phage ES2	AEM24695.1
52	1581	526	30	Putative Gro-EL-like chaperonine protein	*Pseudomonas* phage EL	YP_418179.1
53	543	181	30	Hypothetical protein	*Pseudomonas* phage KPP10	BAK78949.1
54	645	214	29	DNA-binding protein	*Niastella koreensis* GR20-10	YP_005012592.1
55	819	272	29	Hypothetical protein PPEV_gp172	*Pseudomonas* phage EL	YP_418205.1
56	4089	1362	28	Hypothetical protein	*Pseudomonas* phage OBP	YP_004958159.1
57	717	238	27	Hypothetical protein PPEV_gp177	*Pseudomonas* phage EL	YP_418210.1
58	1167	388	27	Putative virion structural protein	*Pseudomonas* phage OBP	YP_004958181.1
59	1566	521	20	Hypothetical protein PPEV_gp190	*Pseudomonas* phage EL	YP_418223.1

The data generated from analysis of the contig for Sabp-P1 using Artemis Release 14.0.0, was used to generate a genome map using pDRAW32 (Figure 6.5). A linear map was created because the whole genome for Sabp-P1 has not yet been annotated. This diagrammatic representation of the ORFs within the contig shows those proteins with highest sequence identity to already sequenced proteins within the BLASTp database. In addition, during the initial stages of the study, the DNA of phage Sabp-P1 was digested using the restriction enzyme, EcoR1, in order to break it into constituent fragments. EcoR1 digests were also used for the construction of the genome map, firstly, to maintain consistency within results, and secondly because initial studies showed that Sabp-P1 remained undigested by restriction enzymes other than EcoR1. A computerized representation of EcoR1 digestion showed 20 restriction sites on the Sabp-P1 contig. This differed from results of the initial studies on restriction fragment length analysis on whole Sabp-P1 DNA, where 18 restriction fragments were identified.

With regard to genomic analysis of Sabp-P2 and Sabp-P3, the contigs of these phages were not analysed in the same detail as Sabp-P1. However, preliminary analysis did provide significant information on the genetic make-up of Sabp-P2 and Sabp-P3. It was found that Sabp-P2 was closely related to a phage of *Clostridium perfringens* Hauduroy *et al.* 1937 (Oakley *et al.* 2011), while Sabp-P3 was closely related to a phage of *Pseudomonas fluorescens* (Cornelissen *et al.*2012). However, as with Sabp-P1, Sabp-P2 and Sabp-P3 showed no sequence homology to any other staphylococcal phages within the genomic databases. Genes encoding for the presence of several different virion associated polymerases and helicases, and DNA polymerase were also noted for both Sabp-P2 and Sabp-P3. Phages Sabp-P2 and Sabp-P3 also showed presence of the tail tape measure protein, which served to confirm their placement in the order *Caudovirales*, and the family *Myoviridae*.

6.4 Discussion

Genomic characterization of three phages specific to *S. aureus* was carried out. However, the results produced, deviated from what was originally expected. Firstly, preliminary analysis of the DNA of phages Sabp-P1, Sabp-P2 and Sabp-P3 using restriction fragment length analysis revealed approximate genome sizes of 107kbp, 70kbp and 126kbp, respectively. This differed significantly from the results obtained after whole genome sequencing using 454 pyrosequencing technology. Phages Sabp-P1, Sabp-P2 and Sabp-P3 were found to contain unusually large genomes of 367kbp, 425kbp and 333kbp, respectively.
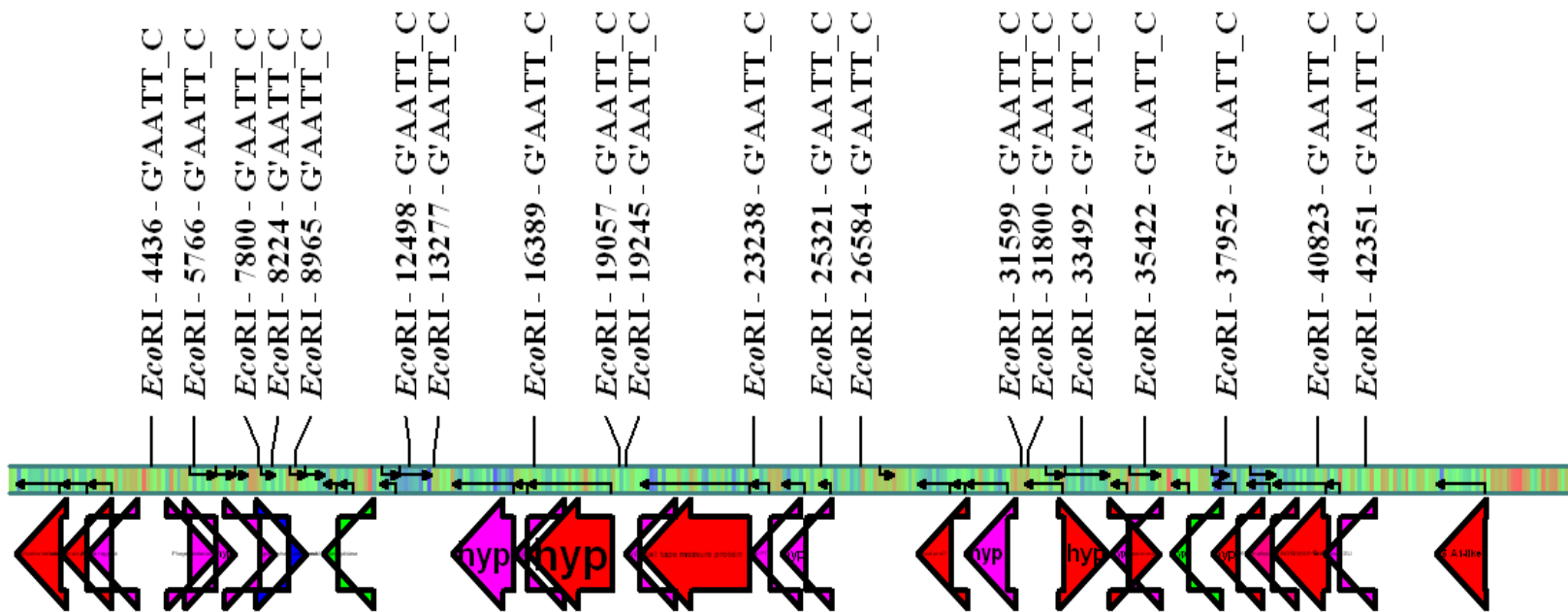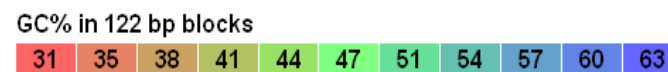
Figure 6.5 Genome map for largest contig of phage Sabp-P1, showing restriction sites using EcoR1 restriction enzyme. Arrows are shown in presumed direction of transcription. The arrows below the GC% block represent the following:

BLASTp sequence identity of >50%

BLASTp sequence identity 40-50%

BLASTp sequence identity 30-40%

BLASTp sequence identity of <30%

Furthermore, during restriction fragment length analysis, it was observed that all three phages were resistant to restriction by the most commonly applied restriction enzymes (EcoRv, BamHI, HindIII, PvuII), and were only digested by EcoRI. It is possible that these three phages lacked the appropriate restriction sites for the respective enzymes. This phenomenon has been previously reported (O'Flaherty *et al.*2004). O'Flaherty *et al.* (2004) found that the genome of staphylococcal Phage K showed little sequence homology to any DNA methylases and that it completely lacked GATC sites which were normally restricted by Sau3AI, BamHI, PvuI and DpnI. A paucity of restriction sites has also been observed in phages that infect lactococci (Moineau *et al.*1993).

Next-generation sequencing technology, particularly pyrosequencing, involves highly precise and sensitive methodologies which allow little chance for error (Henn *et al.*2010). In the current study, results showed high quality metrics for the genomes of all three phages, where for every 93.48% (Sabp-P1), 98.41% (Sabp-P2) and 98.93% (Sabp-P3) of reads, the error rate was 1 in 10000. Although the genome sizes generated from restriction fragment length analysis did not correlate with the results from whole genome sequencing, restriction fragment length analysis can still serve as a good method for the visualization of the relative quality of prepared DNA. Impurities present in a phage DNA preparation can manifest as blotches at the bottom of the gel, indicative of contaminating ribosomal material, as bright smears between individual fragments implying bacterial DNA contamination, or as a thick band band just below the loading well, which is also indicative of bacterial contamination (Sambrook *et al.*1989; Alphey, 1997). The current study showed no manifestation of DNA contamination.

The second set of divergent results pertains to the lack of homologies between the sequence data of the test phages and those already on the genomic databases. It was expected that the phages Sabp-P1, Sabp-P2 and Sabp-P3 would show significant homology to other staphylococcal genomes already sequenced. However, all three phages showed zero homology to any other staphylococcal phage. Instead, phages Sabp-P1 and Sabp-P3 have shown homology to different forms of "giant" phages, particularly those affecting the *Pseudomonas* spp. Phage Sabp-P1 showed 51% sequence identity to the *Pseudomonas* phage OBP (Shaburova *et al.*2006) and phage Sabp-P3 showed 36% sequence identity to *Pseudomonas* phage EL (Krylov *et al.*2004; Adriaenssens *et al.*2012). These "giant" forms of phage have been previously described

(Shaburova *et al.*2006; Adriaenssens *et al.*2012). In a report by Adriaenssen *et al.* (2012), a giant phage pathogenic towards *Ps. putida* was identified and called Pseudomonas Phage Lu11. This phage is related to both *Pseudomonas* phage OBP and *Pseudomonas* phage EL that was previously described by Shaburova *et al.* (2006), is part of the *Myoviridae* family. It has an icosahedral head (~124nm), and a contractile tail (~200nm in length). These particle dimensions correlate with the dimensions of all three test phages as demonstrated in earlier studies (Chapter 4). However, with similarity values of only 51% and 36% for Sabp-P1 and Sabp-P3, respectively, it is likely that these phages are only peripherally related to *Pseudomonas* phages OBP and EL. Phage Sabp-P2 showed only 32% sequence homology to a *Clostridium perfringens* phage that was described by Oakley *et al.* (2011). These results could suggest that phages Sabp-P1, Sabp-P2 and Sabp-P3 are separate species within the *Myoviridae* family. However, only preliminary analysis of the largest contigs of each phage was carried out. Complete assembly of all contigs for all three phages is necessary in order to unequivocally group them.

Although complete annotation and assembly was not within the scope of the current study, preliminary analysis of the functional assignments of proteins making up the largest contig for phage Sabp-P1 was carried out. Analysis revealed the presence of several core proteins involved in phage replication and assembly. Of particular importance were the putative head morphogenesis protein, and the capsid protein. The product of ORF 29 of the Sabp-P1 contig showed 48% similarity to the major head protein of the *Pseudomonas* phage phi297 (Burkal'tseva *et al.*2011). The putative coat protein from ORF 21 of the Sabp-P1 contig, showed 47% similarity, also to the major coat protein of the *Pseudomonas* phage phi297 (Burkal'tseva *et al.*2011). Of the 109 ORFs comprising the Sabp-P1 contig, only 59 showed sequence homology to other known sequences. The percentage similarity between genes encoded by the 59 ORFs and those on the genomic databases are not significantly high enough to deduce that Sabp-P1 may be a specific relative of any given group within the *Myoviridae* family. This further supports the theory that Sabp-P1 could be a new member of the *Myoviridae* family with only distant relatives having been identified already.

Although this study produced unexpected results in terms of genetic relatedness of the test phages to already sequenced phages, the results are still of considerable significance. It was concluded that phages Sabp-P1, Sabp-P2 and Sabp-P3 are of the order *Caudovirales*, and the

family *Myoviridae*. The type species for each of these phages currently appears to be different from anything within the genomic databases. This is particularly relevant from a South African perspective, because this constitutes a first study into characterization of phages pathogenic on mastitis-causing *S. aureus*.

Phages Sabp-P1, Sabp-P2 and Sabp-P3 encoded all of the major functional components expected for a phage, but they still remained significantly divergent from their closest matches. It is acknowledged that much analysis remains in terms of completion of assemblies of all the contigs for all the phages before definitive conclusions can be derived. However, within the scope of the current study, the results were able to meet the objectives such that distinct differences were noted between each of the test phages, and these proved sufficient to distinguish between them.

6.5 Conclusions

Phages Sabp-P1, Sabp-P2 and Sabp-P3 appear to be of the order *Caudovirales*, and the family *Myoviridae*. A particular point of interest is that phages Sabp-P1 and Sabp-P3 appear to be related to a group of already identified "giant" phages. Furthermore, none of the three phages showed sequence homology to any staphylococcal phages already on the database. Only full genome assembly of all phage contigs can definitely address these anomalies.

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Chapter 7

Technical Note: An improved embedding technique for transmission electron microscopy of bacteriophages propagated on solid growth media

Abstract

An improved technique was developed for the embedding of bacteriophages for transmission electron microscopy (TEM). Studies were conducted on phages and their host bacterium, *Staphylococcus aureus*. Phage particles and bacteria were propagated using the standard "double-layer" method. Plaque sections from the top agar were prepared for TEM analysis using both the traditional and an amended embedding method. The amended protocol improved both sample integrity and picture quality. In addition, preparation time which reduced from six days to three days.

7.1 Technical Note

High quality sample preparation for transmission electron microscopy (TEM) is paramount in order to acquire the most amount of information from a given sample. Current methods for studies on phage morphology and infection using TEM include a fixation and embedding protocol over six days (Hajibagheri and Trenton, 1999). The following report presents an amended protocol where sample processing is carried out in half the time, and concurrently, improved sample integrity (Table 7.1).

Standard phage amplification on solid double-layer media was carried out using *Staphylococcus aureus* as the host bacterium (Sambrook *et al.* 1989). Phage plaques that developed on top agar (0.7% w/v) were divided into quarters along the margin between the clear zone and the bacterial lawn. These quarters were processed in two ways, as described in Table 7.1. Resin embedded samples were sectioned using the Reichert-Jung Ultracut ultramicrotome. Sections were collected on formvar-coated copper mesh grids (200 mesh) and stained with 2% uranyl acetate (10min) followed by 2% lead citrate (10min). Grids were viewed using a JEOL JEM-1400[1] at an operating voltage of 100kV. Images were captured using an Orius Camera (SC600A) operating off Gatan Digital Micrograph (version 2.02.800.0) software.

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[1] www.jeol.com

Table 7.1 Comparison of methods for preparation of phage plaques for transmission electron microscopy analysis.

Procedure	Previous protocol*		Amended protocol	
	Time	Day	Time	Day
Fixation of agar plugs in 3% buffered glutaraldehyde	8hr	1	2hr	
Washing in 0.05M sodium cacodylate buffer	2 x 30min (o/n soak in buffer)	2	2 x 30min	1
Post-fixation in 2% osmium tetroxide	4hr		2hr	
Washing in 0.05M sodium cacodylate buffer. Sample left o/n in fresh buffer.	2 x 30min		1 x 30min	
Alcohol dehydration series: 30%, 70%, 100%	10min/alcohol solution for 30%, 70% 3 x 10min for 100%		10min/alcohol solution for 30%, 70% 3 x 10min for 100%	
Removal of residual alcohol using propylene oxide	1 x 10min	3	1 x 10min	
Embedding in epon/araldite and propylene oxide (+DMP):				2
25% epon: 75% propylene oxide	2hr		2hr	
50% epon: 50% propylene oxide	2hr		2hr	
75% epon: 25% propylene oxide	8hr		2hr	
100% epon	overnight	4	overnight	
Polymerization using 100% epon + DMP	48hr	5-6	14hr in oven at 70ºC	3
Trim, section, stain, view				

*as described by (Tikhonenko, 1970; Hajibagheri and Trenton, 1999)

Sample clarity was enhanced in those agar sections prepared using the amended protocol (Figure 7.1a-d) in comparison to those prepared using the traditional method (Figure 7.2a-d).
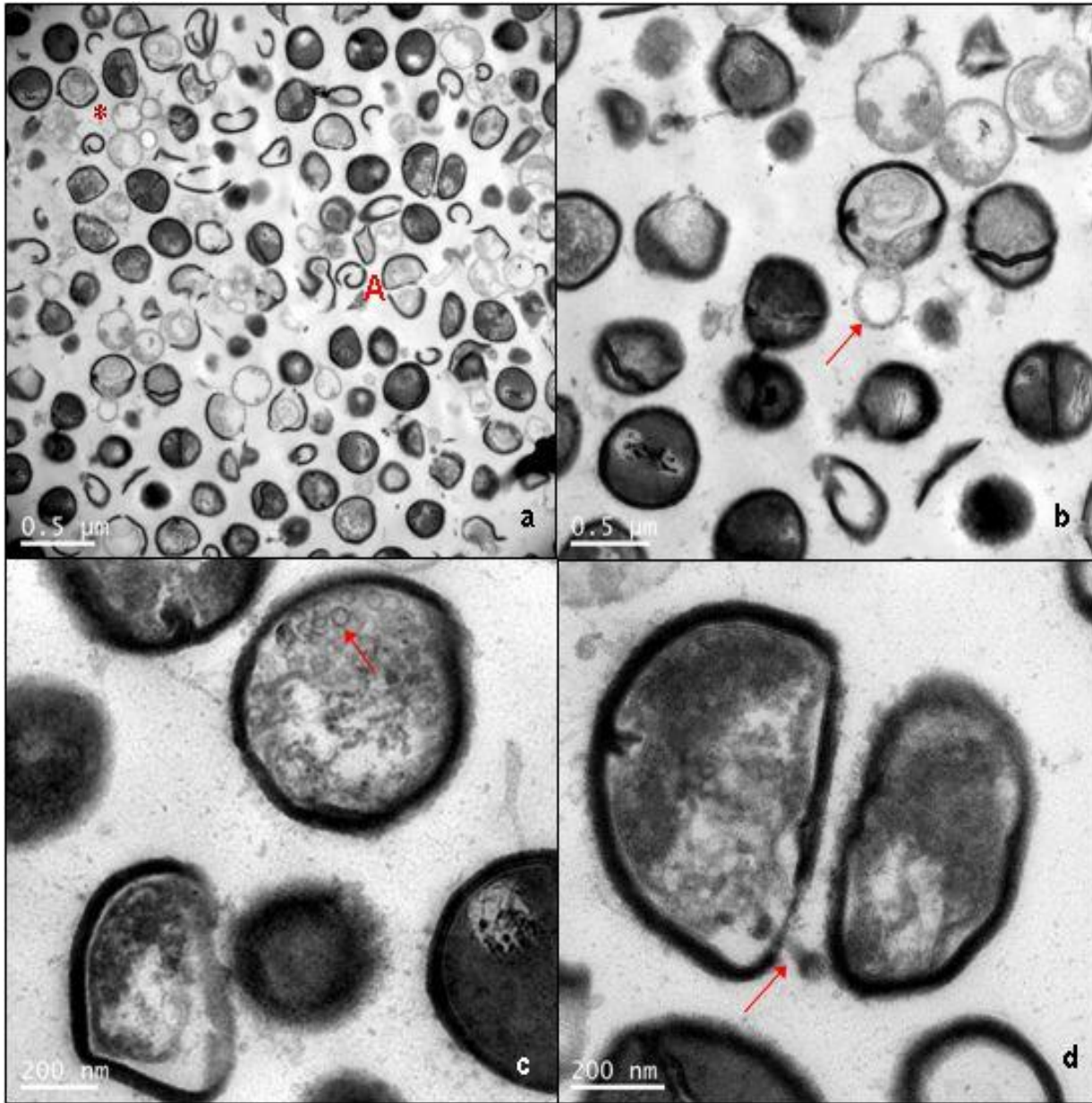
Figure 7.1 Ultra-thin sections through the plaque of a staphylococcal phage, 12hr post inoculation, using an amended embedding protocol for transmission electron microscopy.

a. Bacterial debris and residual intercellular annulus (A) as well as swollen protoplasts (*); b. Bacterial cell at an advanced stage of phage infection indicated showing extrusion of bubbles of cytoplasm (indicated by arrow); c. Newly formed phage particles packed within bacterial cells (indicated by arrow); d. Phage attached to the outer wall of an infected bacterial cell. All samples were stained using uranyl acetate and lead citrate.
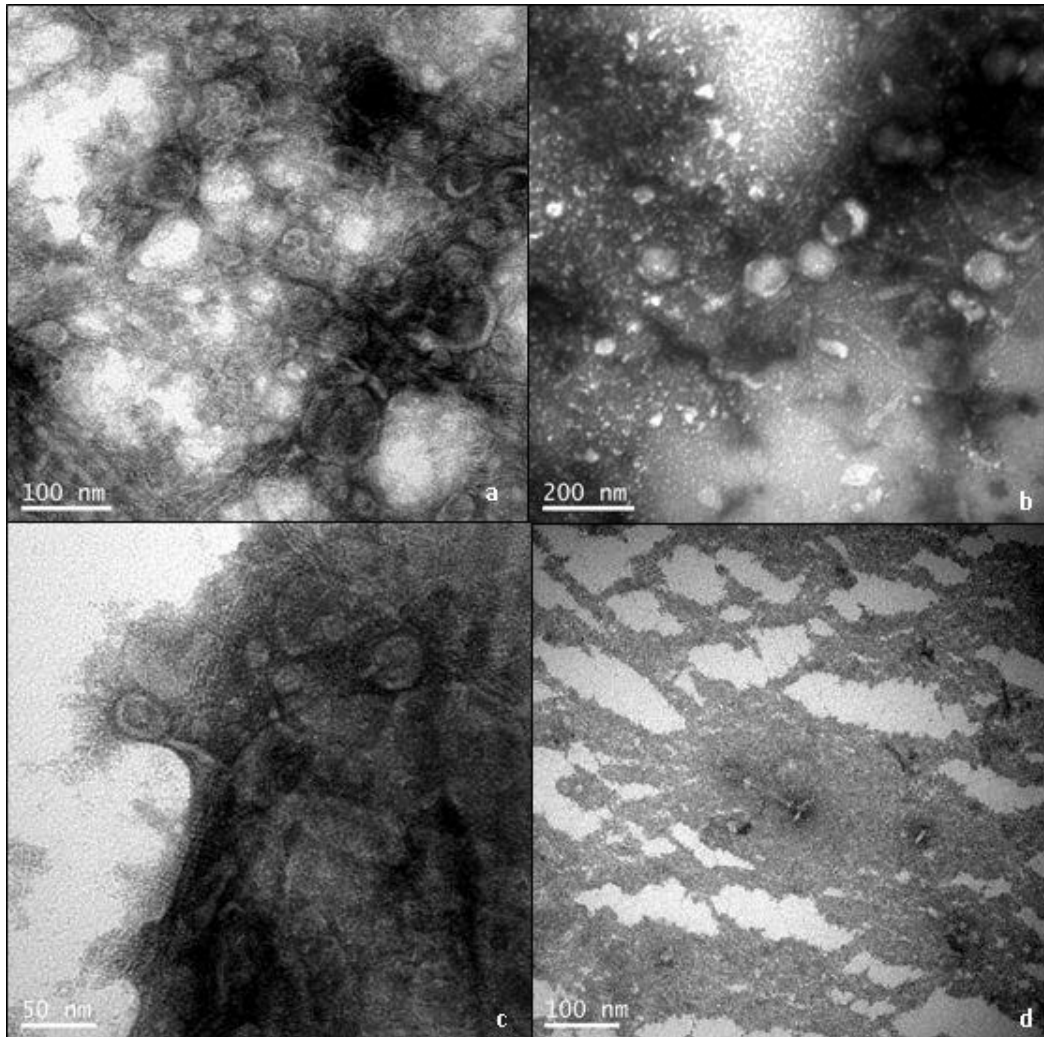
Figure 7.2 Ultra-thin sections through the plaque of a staphylococcal phage, 12hr post inoculation, using traditional methods for sample embedding and fixation for transmission electron microscopy analysis.

a-c. Obscured background and uneven staining in ultra-thin sections. From these images, it is not possible to accurately viral components from bacterial cells; d. Ultra-thin section showing shattering and cracking. All samples were stained using uranyl acetate and lead citrate.

This could be attributed to the porous nature of top agar (0.7% w/v) which may facilitate easier translocation of reactive agents within it. As a result, biological material within the porous medium may be over-exposed to these reactive agents, with the result that visibility becomes obscured. It is also postulated that porosity combined with prolonged periods of exposure results

in samples/sections that are more prone to cracking and shattering, due to over-imbibition of reagents in the embedding procedure (Figure 7.2d).

It is evident from these results that reduced exposure time of agar plaques to reactive agents during the TEM embedding protocol not only maintains superior cell and virus integrity and hence final picture quality, but also adds a valuable time-saving factor. While the amended protocol is specific for phages propagated on soft media, it could also be used on other studies where soft or porous material forms the basic material of the study. Typical examples could include studies in plant and animal tissue culture, or hyperparasitism studies associated with fungi and bacteria.

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CHAPTER 8

THESIS OVERVIEW

8.1 Introduction

Bovine mastitis and its associated ramifications have long plagued dairy cattle. Control measures based on antibiotics provide only temporary relief before they are rendered obsolete by resistance (Du Preez, 2000; Petrovski *et al.*, 2006; Petzer *et al.*, 2009; Schmidt, 2010). The wide range of organisms that are able to cause the disease, coupled to the ubiquity of these organisms, makes disease management a complex process. Antibiotics, integrated with cultural control measures, formed the traditional basis for control of the disease. Global losses associated with bovine mastitis can reach up to 35 billion USD annually (Jones and Bailey, 2009; Van Den Borne, 2010; Mubarack *et al.*, 2011).

Amongst the wide array of microbes that are able to cause bovine mastitis, *Staphylococcus aureus* subsp. *aureus* Rosenbach 1884 remains the major causal agent. Many strains of *S. aureus* has developed antibiotic resistance against those antibiotics most commonly applied to treat bovine mastitis: penicillin, methicillin, and recently vancomycin (Vanderhaeghen *et al.*, 2010). From a South African perspective, penicillin or penicillin+dihydrostreptomycin, make up 97% of the intramammary preparations used within the dairy industry, with the remaining 3% of preparations containing tetracyclines or cephalosporins (Schmidt, 2010). Subsequently, interest has shifted to alternative therapies for the control of bovine mastitis. The application of lytic phages could provide disease control in a far more sustainable manner than either antibiotics or cultural control measures alone. Phage therapy has been applied in several different disciplines ranging from human and veterinary medicine to agricultural settings (Soothill, 1994; Hagens and Offerhaus, 2008; Omnilytics, 2012). In our view, there is a clear niche for the application of phages against *S. aureus*-induced bovine mastitis.

With this in mind, the long-term objective of the current study was to develop phages as a marketable product for use against bovine mastitis, with *S. aureus* being the first target organism. The research presented in this thesis represents *in vitro* screening and characterisation of isolated phages, prior to their development and formulation into commercial bactericides. Such a study is

essential in order to understand the intricacy of phage dynamics relative to its host organism, before application of a phage-based product to infected animals.

8.2 Major findings and their implications

The isolation, screening and characterization of potential phages against *S. aureus*, formed the backbone of this study. Twenty-eight phages were isolated from the dairy environment and raw milk, and screened against four strains of antibiotic-resistant *S. aureus.* However, only six phages showed potential for further screening, based on their wide host range, high titres and common growth requirements. At an optimal titre of between 6.2×10^7 to 2.9×10^8 pfu.ml^{-1} (at 10^{-5} dilution of phage stock), these phages were able to reduce live bacterial cell counts by 64-95%. Additionally, these six phages showed pathogenicity towards a further 18 *S. aureus* strains that were isolated from a different milk-producing region during a farm survey. Phage ubiquity and abundance was also demonstrated during the farm survey, where up to 80% of raw milk samples tested positive for the presence of phages that were specific to the *S. aureus* test strain used throughout the study.

A major finding of the farm survey was that the standard assay of somatic cell count (SCC) of infected milk was not an accurate representation of active infection and the state of mastitis disease. It provided a delayed measurement of an immune response to prior events. However, milk samples with a high SCC ($>400,000$ cells.ml^{-1}) were not necessarily contaminated with bacteria, and many milk samples with a high SCC were complete free of mastitic bacteria. This finding contradicts the traditional expectation of what a high SCC represents, and may invalidate the course of treatment events that usually follow the discovery of a high SCC, such as the injection with antibiotics of all cows with a high SCC. This suggests that a different technique of disease quantitation is essential in order to accurately align treatment protocols with actual mastitis events, in time.

Analysis of phage activity towards simulated environmental and formulation stresses showed that only three of the six screened phages performed well enough to be considered for *in vivo* applications, possibly as a cocktail treatment against mixed *S. aureus* strains. The risk of development of resistance by target bacteria towards their respective phages is ever-present. However, the abundance of phages that are active against *S. aureus* makes the problem of

resistance easier to overcome, both in terms of the time it takes to find new phages, and to incorporate these phages into established formulations. Antibiotic research, on the other hand, requires huge amounts of funding, and years of development research, prior to release of a novel antibiotic product to the consumer market. Coupled with the low economic return on antibiotic usage due to its short treatment duration, most pharmaceutical companies prefer to invest in the sustainability that is associated with production of chronic illness medication (Braine, 2011). This drives the demand for alternative therapies such as the use of phages or phage by-products to treat bacterial illnesses.

Phages demonstrate a unique system of infection and replication within their target bacteria. This intricate network of events is so specific that all other microbes in that environment (pathogenic or commensal) remain unaffected. This, coupled with their ability to multiply between 50-1000 fold within a host bacterium, makes phages an ideal candidate for bacterial disease therapy. This study identified the morphology and the major steps in the replicative cycle of three novel South African phages infective against *S. aureus* strains causing bovine mastitis. These three phages were found to be of the order *Caudovirales*, family *Myoviridae*. The *Myoviridae* comprise approximately 25% of all tailed phages (Ackermann, 2005).

Protein profile analysis of the three phages revealed that proteins ranged in size from 8.86-171.66kDa. Combined similarity matrices based on putative fragment analysis was able to confirm that all three phages were indeed different from each other.

This was further confirmed through analysis of phage genomes. Preliminary sequence data analysis confirmed that the phages Sabp-P1, Sabp-P2 and Sabp-P3 are of the order *Caudovirales*, and the family *Myoviridae*. The type species for each phage currently appears unique from DNA sequences currently found in genomic databases. This is particularly relevant from a South African perspective because this research constitutes a first study into the characterization of phages specifically pathogenic towards mastitic *S. aureus*. Phages Sabp-P1, Sabp-P2 and Sabp-P3 encoded all of the major functional components expected for a phage, but they still remained significantly divergent from their closest matches. A particular point of interest is that phages Sabp-P1 and Sabp-P3 appear related to "giant" phages, and Sabp-P1 appears related to a *Clostridium perfringens* giant phage. None of the three phages showed

sequence homology to any staphylococcal phages already on the database. It is acknowledged that much analysis remains in terms of completion of assemblies of all the contigs for all the phages before definitive conclusions can be derived and these anomalies can be addressed. However, within the scope of the current study, the results were able to meet the objectives such that distinctive differences were noted between each of the test phages, and these proved sufficient to distinguish between them.

8.3 Conclusions and way forward

This study has demonstrated the need for a paradigm shift in the diagnosis and treatment of bovine mastitis, both within South Africa and worldwide. Dairy production in South Africa is faced with the serious problem of antibiotic resistance, with very few treatment alternatives outside of this sphere. The first step in the revolution must be focused on a more precise method for disease diagnosis. Diagnosis of bovine mastitis based on the SCC of raw milk alone, was shown to be an inaccurate method. Alternative methods, such as near-infrared spectrophotometric analysis (NIRA), must be explored. NIRA possesses the ability to detect even trace amounts of target compounds. The effect of mastitis on milk composition is a measurable factor. Another approach could be based on the changes in the lactose content of milk, affecting sodium and chloride levels. Measurement of the electrical conductance of milk may reflect the mastitis status of the cow. Excessive production of certain enzymes (lipase and plasmin) as a result of infection could also be measured. These measurements would supplement the SCC, in order to accurately diagnose the infection status of an animal.

Further studies into the proteomic and genomic make-up of the three test phages should also be undertaken in order to identify specific phage proteins (and/or enzymes) that could be exploited for their control potential.

Finally, *in vivo* application of the isolated phages is the pivotal next step envisaged for the study. These phages have shown stability under variable conditions and when grown in a glycerol-based medium. The application of a phage cocktail infused in a glycerol base, shows promise as a post-milking treatment. These studies all form part of the long-term objective for the project, which is to establish phage therapy as a standard veterinary practice.

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APPENDICES

Appendix 1

Recipe: Phage buffer

Per litre

NaCl	5.8g
MgSO$_4$.7H$_2$O	2g
1M Tris-HCl (pH 7.5)	50ml
2% (w/v) gelatin solution	5ml
Distilled water	Up to 1l

Aliquot buffer into 100ml vials. Sterilize buffer by autoclaving for 20min at 15psi
(1.05kg.cm^{-2}) on liquid cycle. Store buffer at room temperature.
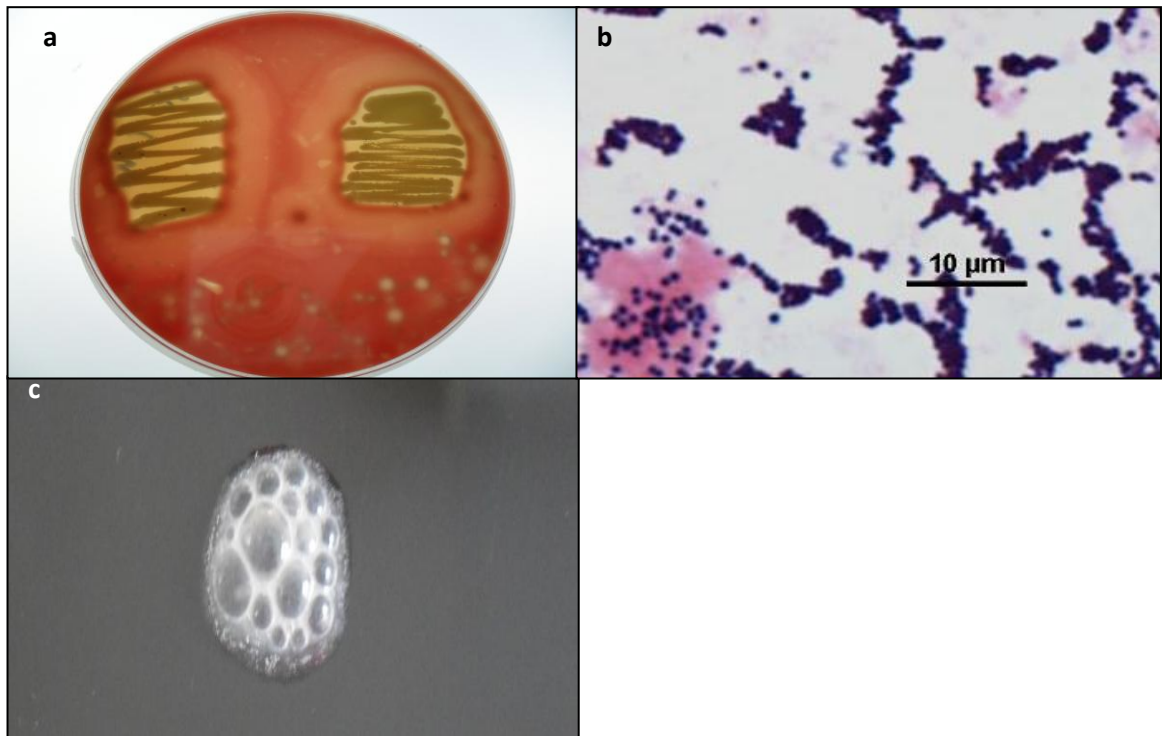
Figure 1. *Staphylococcus aureus* identification tests; a. Typical hemolysis reaction of *S. aureus* on blood agar; b. Gram reaction and coccal arrangement of *S. aureus*; c. Catalase reaction of *S. aureus* with 5% hydrogen peroxide on a glass slide.

Table 1. Single step growth curve data for Sabp-P1, Sabp-P2, Sabp-P3, Sabp-P4, Sabp-P5 and Sabp-P6.

Time (min)	Log phage count (pfu.ml^{-1})					
	Sabp-P1	**Sabp-P2**	**Sabp-P3**	**Sabp-P4**	**Sabp-P5**	**Sabp-P6**
20	5.30×10^7	8.1×10^7	8.7×10^7	3.50×10^7	6.2×10^7	3.4×10^7
25	1.03×10^8	1.25×10^8	1.53×10^8	7.80×10^7	1.44×10^8	8.10×10^7
30	1.61×10^8	1.71×10^8	1.99×10^8	1.03×10^8	1.46×10^8	8.70×10^7
35	1.67×10^8	1.96×10^8	2.45×10^8	9.10×10^7	1.17×10^8	1.00×10^8
40	2.30×10^8	2.57×10^8	2.91×10^8	1.67×10^8	1.07×10^8	7.50×10^7
45	4.18×10^8	4.09×10^8	4.76×10^8	2.12×10^8	8.51×10^8	1.62×10^8
50	5.73×10^8	4.60×10^8	7.16×10^8	2.95×10^8	1.97×10^8	1.49×10^8
55	6.24×10^8	5.28×10^8	7.13×10^8	2.92×10^8	2.22×10^8	1.41×10^8
60	6.11×10^8	4.43×10^8	7.07×10^8	2.33×10^8	2.14×10^8	1.33×10^8
65	5.57×10^8	4.35×10^8	6.54×10^8	2.53×10^8	2.56×10^8	1.65×10^8
70	5.03×10^8	3.17×10^8	6.12×10^8	2.25×10^8	2.15×10^8	1.35×10^8
Mean	3.64×10^8	3.11×10^8	4.41×10^8	1.80×10^8	2.30×10^8	1.15×10^8
F-value	<0.001					
LSD$_{(0.05)}$	1.22E+08					
CV%	5.6					

Table 2. Lethal dose for SaB1 upon treatment with phages (SaB1 only) and without phages (SaB1 + phage).

Phage	*SaB1* only	*SaB1*+ phage
Sabp-P1	3.50×10^8	1.63×10^7
Sabp-P2	3.83×10^8	2.57×10^7
Sabp-P3	3.65×10^8	1.87×10^7
Sabp-P4	3.56×10^8	1.14×10^8
Sabp-P5	3.38×10^8	1.10×10^8
Sabp-P6	3.22×10^8	1.16×10^8
Mean	3.52×10^8	6.67×10^7
F-value	<0.001	
LSD$_{(0.05)}$	9.67×10^6	1.55×10^7
CV%	1.5	12.7
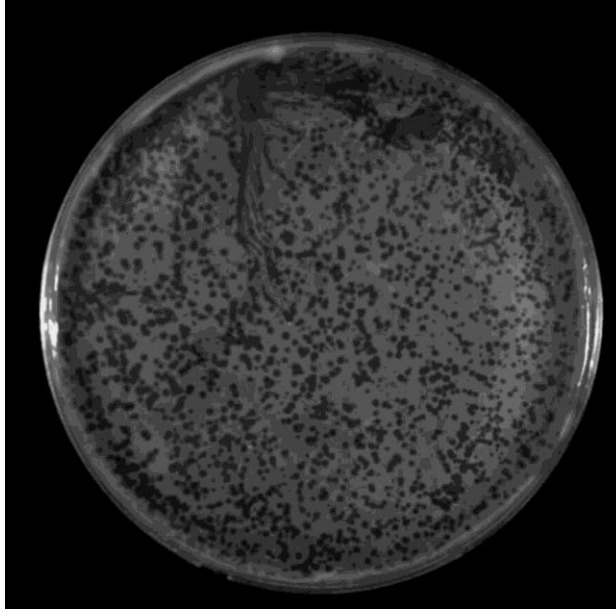
Figure 2. Typical "webbing" or adjoining of neighbouring plaques at high concentrations. This is Sabp-P1 at 1×10^{-4} dilution.

Appendix 2

Table 1. Heat assay data of average phage counts at varied exposure times to different temperatures.

Phage	Time (min)	45	50	55	60	65	70
		Temperature (°C)					
		Log phage count (plaque forming unit.ml^{-1})					
Sabp-P1	0	2.88E+08	2.88E+08	2.88E+08	2.88E+08	2.88E+08	2.88E+08
	10	1.79E+08	8.75E+07	9.00E+07	1.14E+08	4.05E+07	2.20E+07
	20	1.39E+08	9.20E+07	3.05E+07	6.85E+07	5.85E+07	1.05E+07
	30	1.20E+08	1.04E+08	1.55E+07	4.60E+07	2.50E+07	4.00E+07
	40	1.18E+08	1.12E+08	1.70E+07	1.45E+07	3.25E+07	9.00E+06
	50	1.25E+08	7.70E+07	2.05E+07	2.65E+07	3.70E+07	2.50E+06
	60	1.08E+08	4.80E+07	4.20E+07	1.70E+07	2.55E+07	1.50E+06
Sabp-P2	0	2.64E+08	2.64E+08	2.64E+08	2.64E+08	2.64E+08	2.64E+08
	10	1.42E+08	1.88E+08	1.06E+08	6.05E+07	4.45E+07	1.95E+07
	20	1.78E+08	4.50E+07	4.40E+07	5.45E+07	3.50E+06	3.80E+07
	30	1.22E+08	3.15E+07	8.50E+06	6.75E+07	3.95E+07	2.50E+07
	40	1.44E+08	7.55E+07	3.35E+07	1.30E+07	2.05E+07	2.10E+07
	50	1.32E+08	3.00E+07	2.45E+07	2.75E+07	2.60E+07	1.25E+07
	60	1.20E+08	8.45E+07	3.10E+07	1.30E+07	3.65E+07	1.15E+07
Sabp-P3	0	1.87E+08	1.87E+08	1.87E+08	1.87E+08	1.87E+08	1.87E+08
	10	1.37E+08	1.71E+08	9.50E+07	1.13E+08	4.50E+07	4.20E+07
	20	1.46E+08	6.40E+07	5.55E+07	6.60E+07	3.85E+07	4.45E+07
	30	1.18E+08	5.90E+07	8.50E+06	4.95E+07	4.20E+07	8.50E+06
	40	1.30E+08	1.18E+08	8.50E+06	1.65E+07	4.05E+07	1.65E+07
	50	1.11E+08	8.05E+07	1.05E+07	9.50E+06	3.90E+07	1.55E+07
	60	1.11E+08	9.20E+07	5.00E+07	3.20E+07	5.70E+07	4.00E+06
Sabp-P4	0	1.17E+08	1.17E+08	1.17E+08	1.17E+08	1.17E+08	1.17E+08
	10	1.04E+08	1.15E+08	9.10E+07	8.95E+07	2.95E+07	1.30E+07
	20	9.75E+07	1.22E+08	2.70E+07	2.95E+07	2.50E+07	9.50E+06
	30	1.04E+08	1.19E+08	1.85E+07	2.65E+07	2.55E+07	2.00E+06
	40	9.70E+07	9.00E+07	2.30E+07	2.25E+07	2.00E+07	5.00E+06
	50	1.19E+08	1.15E+08	1.35E+07	1.60E+07	1.75E+07	2.50E+06
	60	1.09E+08	9.95E+07	8.50E+06	8.00E+06	1.65E+07	1.00E+06
Sabp-P5	0	1.20E+08	1.20E+08	1.20E+08	1.20E+08	1.20E+08	1.20E+08
	10	1.26E+08	1.22E+08	1.12E+08	4.35E+07	2.20E+07	1.70E+07
	20	1.30E+08	1.21E+08	5.15E+07	2.25E+07	1.30E+07	1.05E+07
	30	9.90E+07	1.01E+08	1.55E+07	2.60E+07	1.40E+07	1.20E+07
	40	1.11E+08	8.85E+07	1.05E+07	1.45E+07	1.50E+07	6.00E+06
	50	1.05E+08	9.35E+07	1.60E+07	1.45E+07	1.85E+07	6.00E+06
	60	1.15E+08	1.02E+08	5.50E+06	7.00E+06	1.35E+07	3.50E+06
Sabp-P6	0	9.10E+07	9.10E+07	9.10E+07	9.10E+07	9.10E+07	9.10E+07

Table 1 continued

	10	9.30E+07	8.70E+07	8.25E+07	4.35E+07	1.85E+07	1.85E+07
	20	1.01E+08	1.01E+08	1.01E+08	1.01E+08	3.44E+07	6.41E+07
	30	1.09E+08	9.15E+07	1.55E+07	3.65E+07	2.10E+07	1.00E+07
	40	1.03E+08	1.10E+08	1.25E+07	2.05E+07	1.30E+07	1.35E+07
	50	1.18E+08	1.10E+08	3.50E+06	1.00E+07	1.15E+07	5.00E+06
	60	9.40E+07	9.00E+07	3.50E+06	1.15E+07	8.00E+06	7.00E+06
LSD$_{(0.05)}$	3.93E+06						
CV%	5.9						
F-value	<0.001						

Table 2. Cold assay data of average phage counts at varied exposure times to different temperatures.

		Time						
		1hr	8hr	12hr	1wk	2wk	3mo	6mo
Phage	**Temp (°C)**	Log phage count (plaque forming unit.ml^{-1})						
Sabp-P1	4	3.24E+08	3.30E+08	3.21E+08	3.31E+08	3.37E+08	2.93E+08	2.58E+08
	-20	2.89E+08	2.80E+08	2.45E+08	1.93E+08	1.72E+08	8.90E+07	5.75E+07
	-80	1.93E+08	1.48E+08	1.53E+08	1.19E+08	8.45E+07	4.95E+07	1.50E+07
Sabp-P2	4	3.30E+08	3.49E+08	3.36E+08	3.19E+08	3.50E+08	2.93E+08	3.03E+08
	-20	2.85E+08	2.68E+08	2.48E+08	2.20E+08	1.62E+08	1.15E+08	6.95E+07
	-80	1.66E+08	1.40E+08	1.17E+08	9.15E+07	6.65E+07	3.20E+07	1.35E+07
Sabp-P3	4	3.26E+08	3.26E+08	3.23E+08	3.31E+08	3.39E+08	3.11E+08	2.96E+08
	-20	2.73E+08	2.65E+08	2.57E+08	2.30E+08	2.01E+08	1.70E+08	1.30E+08
	-80	1.28E+08	9.35E+07	9.75E+07	8.00E+07	5.75E+07	3.50E+07	7.00E+06
Sabp-P4	4	2.90E+07	3.25E+07	3.45E+07	2.25E+07	2.30E+07	2.20E+07	1.40E+07
	-20	1.50E+07	1.85E+07	1.45E+07	1.30E+07	1.75E+07	1.05E+07	8.50E+06
	-80	1.55E+07	1.05E+07	9.50E+06	6.50E+06	3.00E+06	2.00E+06	5.00E+05
Sabp-P5	4	1.65E+07	2.20E+07	1.75E+07	1.45E+07	1.55E+07	8.50E+06	5.50E+06
	-20	1.00E+07	1.10E+07	1.35E+07	1.00E+07	1.35E+07	1.00E+07	6.50E+06
	-80	1.05E+07	8.50E+06	7.50E+06	4.00E+06	6.50E+06	2.50E+06	1.00E+06
Sabp-P6	4	3.90E+07	2.55E+07	3.35E+07	2.95E+07	3.70E+07	2.25E+07	1.65E+07
	-20	2.65E+07	3.15E+07	2.55E+07	2.00E+07	2.50E+07	1.50E+07	5.00E+06
	-80	2.15E+07	1.50E+07	1.35E+07	7.00E+06	6.50E+06	3.50E+06	2.00E+06
LSD$_{(0.05)}$	3.93E+06							
CV%	0.1							
F-value	<0.001							

Table 3. Effects of chloroform on phage replication.

Phage	Chloroform (+)	Chloroform (-)
Sabp-P1	9.37E+07	1.50E+08
Sabp-P2	8.87E+07	1.54E+08
Sabp-P3	8.03E+07	1.45E+08
Sabp-P4	3.63E+07	9.27E+07
Sabp-P5	3.07E+07	1.19E+08
Sabp-P6	6.47E+07	8.73E+07
Mean	6.57E+07	1.25E+08
LSD$_{(0.05)}$	9.47E+06	
CV%	4.00E-01	
F-value	<0.001	

Table 4. Effects of pH on phage replication.

	pH						
Phage	2	4	6	8	10	12	14
Sabp-P1	1.05E+08	1.16E+08	2.16E+08	1.79E+08	1.03E+08	4.23E+07	2.83E+07
Sabp-P2	2.25E+08	1.50E+08	1.73E+08	1.71E+08	2.17E+07	2.13E+07	3.93E+07
Sabp-P3	1.28E+08	2.24E+08	2.35E+08	2.16E+08	1.17E+08	2.57E+07	2.07E+07
Sabp-P4	1.01E+08	1.03E+08	9.33E+07	6.00E+07	4.03E+07	2.03E+07	1.33E+07
Sabp-P5	1.04E+08	9.23E+07	1.09E+08	7.73E+07	4.80E+07	2.00E+07	1.97E+07
Sabp-P6	1.14E+08	8.60E+07	1.15E+08	5.90E+07	3.13E+07	1.17E+07	1.27E+07
LSD$_{(0.05)}$	1.51e+07						
CV%	1.3						
F-value	<0.001						

Table 5. Effects of increasing glycerol concentration on phage replication.

Glycerol %	Phage counts (pfu.ml^{-1})					
	Sabp-P1	Sabp-P2	Sabp-P3	Sabp-P4	Sabp-P5	Sabp-P6
5	4.77E+08	4.05E+08	4.10E+08	2.78E+08	3.04E+08	2.26E+08
10	3.48E+08	3.82E+08	3.99E+08	2.53E+08	2.68E+08	2.84E+08
20	2.69E+08	4.18E+08	4.31E+08	1.99E+08	2.20E+08	2.12E+08
30	1.74E+08	2.79E+08	4.52E+08	1.91E+08	1.86E+08	1.77E+08
40	2.27E+08	2.56E+08	3.68E+08	1.71E+08	1.72E+08	1.43E+08
50	2.42E+08	2.65E+08	3.55E+08	1.82E+08	1.05E+08	1.92E+08
60	1.49E+08	2.53E+08	3.24E+08	1.01E+08	1.09E+08	9.40E+07
70	1.42E+08	2.02E+08	2.72E+08	5.70E+07	9.90E+07	4.90E+07
80	9.40E+07	1.72E+08	2.39E+08	5.70E+07	9.30E+07	8.30E+07
90	1.42E+08	1.35E+08	2.39E+08	6.10E+07	4.00E+07	2.80E+07
100	4.80E+07	1.16E+08	1.94E+08	3.60E+07	3.80E+07	1.20E+07
LSD$_{(0.05)}$	9.50E+07					
CV%	0.4					
F-value	<0.001					

Appendix 3

A. Recipes

1. Colloidal Coomassie blue stain

Ammonium sulphate (10%)	100g
Coomassie G-250	20ml of 5% solution
Ortho-phosphoric acid (3%)	30ml
Ethanol (100%)	200ml
Sterile distilled water	Up to 1l

Store in dark, at room temperature, in tightly sealed container.

2. IEF Lysis buffer

Urea (FW 60.06)	19.2g
Triton-x-100	4ml
Tris-base (FW 121.1)	0.194g
Double distilled water	Up to 40ml

Aliquot buffer and store at -20°C.

B. Similarity Matrices

1. SDS-PAGE

```
========== Section: 1-SimQual ============
SimQual: NTSYSpc 2.11W, (C) 2000-2004, Applied Biostatistics Inc.
Date & time: 11/6/2012 11:06:13 AM
-----------------------------------------
Input parameters:
Read input from file: D:\iona\protein SDS\sds.nts
Compute by: cols
Save results in output file: D:\iona\protein SDS\sdssim.nts
Coefficient: DICE
Positive:   1.0000
Negative:   0.0000

Matrix type = 1, size = 19 by 3, missing value code = 99  (rectangular)

Dis/Similarity matrix (3 by 3) saved in file: D:\iona\protein
SDS\sdssim.nts

Ending date & time: 11/6/2012 11:06:13 AM

========== Section: 2-SAHN ============
SAHN: NTSYSpc 2.11W, (C) 2000-2004, Applied Biostatistics Inc.
Date & time: 11/6/2012 11:06:37 AM
-----------------------------------------
Input parameters:
Read input from file: D:\iona\protein SDS\sdssim.nts
Save result tree in output file: D:\iona\protein SDS\sdstree.NTS
Clustering method: UPGMA
In case of ties: FIND
Max. no. tied trees:         25

Comments:
  SIMQUAL: input=D:\iona\protein SDS\sds.nts, coeff=DICE
  by Cols, += 1.00000, -= 0.00000
Matrix type = 3, size = 3 by 3, missing value code = "none"  (similarity)
Results will be stored in file: D:\iona\protein SDS\sdstree.NTS
Searching for all tied trees

Solution tree number 1
Sorting tree nodes ...
 none needed.
A single tree was found

Ending date & time: 11/6/2012 11:06:37 AM

========== Section: 3-Tree ============
Tree: NTSYSpc 2.11W, (C) 2000-2004, Applied Biostatistics Inc.
Date & time: 11/6/2012 11:07:22 AM
-----------------------------------------
```

Input parameters:
Read input from file: D:\iona\protein SDS\sdstree.NTS

Comments:
  SIMQUAL: input=D:\iona\protein SDS\sds.nts, coeff=DICE
  by Cols, += 1.00000, -= 0.00000
  SAHN: input=D:\iona\protein SDS\sdssim.nts, method=UPGMA, tie=FIND
  Solution tree number 1
Matrix type = 6, size = 3 by 2, missing value code = "none" (tree
(similarity))

Ending date & time: 11/6/2012 11:07:22 AM

========== Section: 4-Output ============
Output: NTSYSpc 2.11W, (C) 2000-2004, Applied Biostatistics Inc.
Date & time: 11/6/2012 11:10:35 AM

Input parameters:
Read input from file: D:\iona\protein SDS\sdssim.nts
Format: width=9 decimals=4
Page width: 80
Field width: 9
Decimal places: 4
Page width: 80
Comments:
  SIMQUAL: input=D:\iona\protein SDS\sds.nts, coeff=DICE
  by Cols, += 1.00000, -= 0.00000
Matrix type = 3, size = 3 by 3, missing value code = "none" (similarity)

          Sabp-P1   Sabp-P2   Sabp-P3

Sabp-P1 | 1.0000
Sabp-P2 | 0.8462   1.0000
Sabp-P3 | 0.7333   0.6923   1.0000

Ending date & time: 11/6/2012 11:10:35 AM

2. UTLIEF

========== Section: 1-SimQual ============
SimQual: NTSYSpc 2.11W, (C) 2000-2004, Applied Biostatistics Inc.
Date & time: 11/6/2012 10:54:29 AM

Input parameters:
Read input from file: D:\iona\protein IEF\ief.nts
Compute by: cols
Save results in output file: D:\iona\protein IEF\iefsim.nts
Coefficient: DICE
Positive: 1.0000
Negative: 0.0000

Matrix type = 1, size = 8 by 3, missing value code = 99 (rectangular)

```
Dis/Similarity matrix (3 by 3) saved in file: D:\iona\protein
IEF\iefsim.nts

Ending date & time: 11/6/2012 10:54:30 AM
========== Section: 2-SAHN ============
SAHN: NTSYSpc 2.11W, (C) 2000-2004, Applied Biostatistics Inc.
Date & time: 11/6/2012 10:55:12 AM
----------------------------------------
Input parameters:
Read input from file: D:\iona\protein IEF\iefsim.nts
Save result tree in output file: D:\iona\protein IEF\ieftree.NTS
Clustering method: UPGMA
In case of ties: FIND
Max. no. tied trees:        25

Comments:
  SIMQUAL: input=D:\iona\protein IEF\ief.nts, coeff=DICE
  by Cols, +=   1.00000, -=   0.00000
Matrix type = 3, size = 3 by 3, missing value code = "none"  (similarity)
Results will be stored in file: D:\iona\protein IEF\ieftree.NTS
Searching for all tied trees

Solution tree number 1
Sorting tree nodes ...
 none needed.
A single tree was found

Ending date & time: 11/6/2012 10:55:12 AM
========== Section: 3-Tree ============
Tree: NTSYSpc 2.11W, (C) 2000-2004, Applied Biostatistics Inc.
Date & time: 11/6/2012 10:55:55 AM
----------------------------------------
Input parameters:
Read input from file: D:\iona\protein IEF\ieftree.NTS

Comments:
  SIMQUAL: input=D:\iona\protein IEF\ief.nts, coeff=DICE
  by Cols, +=   1.00000, -=   0.00000
  SAHN: input=D:\iona\protein IEF\iefsim.nts, method=UPGMA, tie=FIND
  Solution tree number 1
Matrix type = 6, size = 3 by 2, missing value code = "none"  (tree
(similarity))

Ending date & time: 11/6/2012 10:55:55 AM
========== Section: 4-Output ============
Output: NTSYSpc 2.11W, (C) 2000-2004, Applied Biostatistics Inc.
Date & time: 11/6/2012 11:03:20 AM
----------------------------------------
Input parameters:
Read input from file: D:\iona\protein IEF\iefsim.nts
Format: width=9 decimals=4
Page width: 80
```

```
Field width: 9
Decimal places: 4
Page width: 80
Comments:
  SIMQUAL: input=D:\iona\protein IEF\ief.nts, coeff=DICE
  by Cols, +=   1.00000, -=   0.00000
Matrix type = 3, size = 3 by 3, missing value code = "none"  (similarity)


          Sabp-P1     Sabp-P2     Sabp-P3
       ----------------------------
Sabp-P1 |   1.0000
Sabp-P2 |   0.6667    1.0000
Sabp-P3 |   0.5455    0.7500    1.0000


Ending date & time: 11/6/2012 11:03:20 AM
```

3. Combined SDS-PAGE and UTLIEF matrices

```
=========== Section: 1-SimQual ============
SimQual: NTSYSpc 2.11W, (C) 2000-2004, Applied Biostatistics Inc.
Date & time: 11/6/2012 11:33:53 AM
-----------------------------------------
Input parameters:
Read input from file: D:\iona\combined protein\combined protein.nts
Compute by: cols
Save results in output file: D:\iona\combined protein\combined protein
sim.NTS
Coefficient: DICE
Positive:   1.0000
Negative:   0.0000

Matrix type = 1, size = 27 by 3, missing value code = 99   (rectangular)

Dis/Similarity matrix (3 by 3) saved in file: D:\iona\combined
protein\combined protein sim.NTS

Ending date & time: 11/6/2012 11:33:53 AM
=========== Section: 2-SAHN ============
SAHN: NTSYSpc 2.11W, (C) 2000-2004, Applied Biostatistics Inc.
Date & time: 11/6/2012 11:34:14 AM
-----------------------------------------
Input parameters:
Read input from file: D:\iona\combined protein\combined protein sim.NTS
Save result tree in output file: D:\iona\combined protein\combined protein
tree.NTS
Clustering method: UPGMA
In case of ties: FIND
Max. no. tied trees:        25
Comments:
  SIMQUAL: input=D:\iona\combined protein\combined protein.nts, coeff=DICE
  by Cols, +=   1.00000, -=   0.00000
Matrix type = 3, size = 3 by 3, missing value code = "none"  (similarity)
```

Results will be stored in file: D:\iona\combined protein\combined protein
tree.NTS
Searching for all tied trees

Solution tree number 1
Sorting tree nodes ...
 none needed.
A single tree was found

Ending date & time: 11/6/2012 11:34:14 AM
=========== Section: 3-Tree ============
Tree: NTSYSpc 2.11W, (C) 2000-2004, Applied Biostatistics Inc.
Date & time: 11/6/2012 11:34:27 AM

Input parameters:
Read input from file: D:\iona\combined protein\combined protein tree.NTS

Comments:
  SIMQUAL: input=D:\iona\combined protein\combined protein.nts, coeff=DICE
  by Cols, += 1.00000, -= 0.00000
  SAHN: input=D:\iona\combined protein\combined protein sim.NTS,
method=UPGMA, tie=FIND
  Solution tree number 1
Matrix type = 6, size = 3 by 2, missing value code = "none" (tree
(similarity))

Ending date & time: 11/6/2012 11:34:27 AM
=========== Section: 4-Output ============
Output: NTSYSpc 2.11W, (C) 2000-2004, Applied Biostatistics Inc.
Date & time: 11/6/2012 11:36:38 AM

Input parameters:
Read input from file: D:\iona\combined protein\combined protein sim.NTS
Format: width=9 decimals=4
Page width: 80
Field width: 9
Decimal places: 4
Page width: 80
Comments:
  SIMQUAL: input=D:\iona\combined protein\combined protein.nts, coeff=DICE
  by Cols, += 1.00000, -= 0.00000
Matrix type = 3, size = 3 by 3, missing value code = "none" (similarity)

          Sabp-P1   Sabp-P2   Sabp-P3

Sabp-P1 | 1.0000
Sabp-P2 | 0.8000   1.0000
Sabp-P3 | 0.6829   0.7059   1.0000

Ending date & time: 11/6/2012 11:36:38 AM

Appendix 4

1. Tris-EDTA buffer

1M Tris-HCl (pH 7.4)

0.5M EDTA (pH 8.0)

Add 0.5ml 1M Tris-HCl and 0.1ml 0.5M EDTA to a sterile glass bottle and bring volume up to 50ml with sterile distilled water. Store buffer at room temperature.

2. TAE buffer (10x stock solution)

Tris base	48.4g
Glacial acetic acid	11.4ml
EDTA, disodium salt	3.7g
Deionized water	Up to 1l

Dilute buffer to 1x using sterile distilled water. Store 10x stock buffer at room temperature.
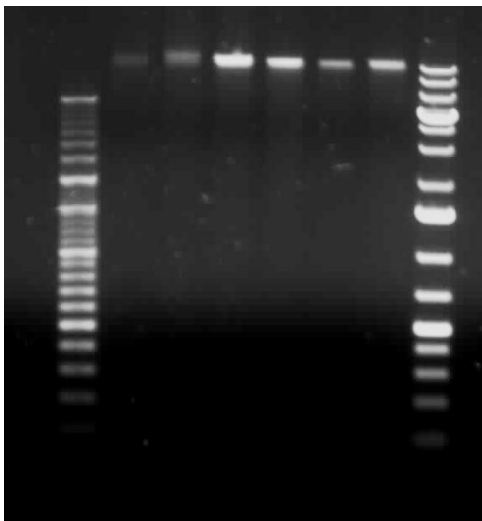
3. Agarose gel electrophoresis

Figure 1. Agarose gel electrophoresis of phage DNA. Bands from left to right: 1. O'Gene Ruler 100bp + 500bp DNA ladder (Fermentas); 2. Sabp-P1 (5µl); 3. Sabp-P2 (5µl); 4. Sabp-P3 (5µl); 5. Sabp-P1 (10µl); 6. Sabp-P2 (10µl); 7. Sabp-P3 (10µl); 8. O'Gene Ruler 1kb Plus DNA Ladder (Fermentas).

4. Similarity matrices

4.1 DNA

```
=========== Section: 1-SimQual ============
SimQual: NTSYSpc 2.11W, (C) 2000-2004, Applied Biostatistics Inc.
Date & time: 9/18/2012 11:45:23 AM
------------------------------------------
Input parameters:
Read input from file: C:\Documents and
Settings\User\Desktop\iona\ecor1.nts
Compute by: cols
Save results in output file: C:\Documents and
Settings\User\Desktop\iona\ecor1sim.nts
Coefficient: DICE
Positive:   1.0000
Negative:   0.0000


Matrix type = 1, size = 21 by 3, missing value code = "none"
(rectangular)


Dis/Similarity matrix (3 by 3) saved in file: C:\Documents and
Settings\User\Desktop\iona\ecor1sim.nts


Ending date & time: 9/18/2012 11:45:23 AM




=========== Section: 2-SAHN ============
SAHN: NTSYSpc 2.11W, (C) 2000-2004, Applied Biostatistics Inc.
Date & time: 9/18/2012 11:46:20 AM
------------------------------------------
Input parameters:
Read input from file: C:\Documents and
Settings\User\Desktop\iona\ecor1sim.nts
Save result tree in output file: C:\Documents and
Settings\User\Desktop\iona\ecor1tree.nts
Clustering method: UPGMA
In case of ties: FIND
Max. no. tied trees:        25


Comments:
  SIMQUAL: input=C:\Documents and Settings\User\Desktop\iona\ecor1.nts,
coeff=DICE
  by Cols, +=   1.00000, -=   0.00000
Matrix type = 3, size = 3 by 3, missing value code = "none"  (similarity)
Results will be stored in file: C:\Documents and
Settings\User\Desktop\iona\ecor1tree.nts
Searching for all tied trees


Solution tree number 1
Sorting tree nodes ...
 none needed.
A single tree was found
```

Ending date & time: 9/18/2012 11:46:20 AM

=========== Section: 3-Tree ============
Tree: NTSYSpc 2.11W, (C) 2000-2004, Applied Biostatistics Inc.
Date & time: 9/18/2012 11:46:42 AM

Input parameters:
Read input from file: C:\Documents and
Settings\User\Desktop\iona\ecor1tree.nts

Comments:
  SIMQUAL: input=C:\Documents and Settings\User\Desktop\iona\ecor1.nts,
coeff=DICE
  by Cols, += 1.00000, -= 0.00000
  SAHN: input=C:\Documents and Settings\User\Desktop\iona\ecor1sim.nts,
method=UPGMA, tie=FIND
  Solution tree number 1
Matrix type = 6, size = 3 by 2, missing value code = "none" (tree
(similarity))

Ending date & time: 9/18/2012 11:46:42 AM

=========== Section: 4-Output ============
Output: NTSYSpc 2.11W, (C) 2000-2004, Applied Biostatistics Inc.
Date & time: 9/18/2012 11:49:15 AM

Input parameters:
Read input from file: C:\Documents and
Settings\User\Desktop\iona\ecor1sim.nts
Format: width=9 decimals=4
Page width: 80
Field width: 9
Decimal places: 4
Page width: 80
Comments:
  SIMQUAL: input=C:\Documents and Settings\User\Desktop\iona\ecor1.nts,
coeff=DICE
  by Cols, += 1.00000, -= 0.00000
Matrix type = 3, size = 3 by 3, missing value code = "none" (similarity)

          118   119   120

Sabp-P1 | 1.0000
Sabp-P2 | 0.7097   1.0000
Sabp-P3 | 0.8649   0.7500   1.0000

Ending date & time: 9/18/2012 11:49:15 AM

4.2 Combined matrix: DNA vs Protein

```
=========== Section: 1-SimQual ============
SimQual: NTSYSpc 2.11W, (C) 2000-2004, Applied Biostatistics Inc.
Date & time: 11/6/2012 11:26:51 AM
------------------------------------------
Input parameters:
Read input from file: D:\iona\combined\combined data.nts
Compute by: cols
Save results in output file: D:\iona\combined\combined data sim.NTS
Coefficient: DICE
Positive:   1.0000
Negative:   0.0000

Matrix type = 1, size = 48 by 3, missing value code = 99  (rectangular)

Dis/Similarity matrix (3 by 3) saved in file: D:\iona\combined\combined
data sim.NTS

Ending date & time: 11/6/2012 11:26:51 AM


=========== Section: 2-SAHN ============
SAHN: NTSYSpc 2.11W, (C) 2000-2004, Applied Biostatistics Inc.
Date & time: 11/6/2012 11:27:22 AM
------------------------------------------
Input parameters:
Read input from file: D:\iona\combined\combined data sim.NTS
Save result tree in output file: D:\iona\combined\combined data tree.NTS
Clustering method: UPGMA
In case of ties: FIND
Max. no. tied trees:        25

Comments:
  SIMQUAL: input=D:\iona\combined\combined data.nts, coeff=DICE
  by Cols, +=   1.00000, -=   0.00000
Matrix type = 3, size = 3 by 3, missing value code = "none"  (similarity)
Results will be stored in file: D:\iona\combined\combined data tree.NTS
Searching for all tied trees

Solution tree number 1
Sorting tree nodes ...
 none needed.
A single tree was found

Ending date & time: 11/6/2012 11:27:22 AM


=========== Section: 3-Tree ============
Tree: NTSYSpc 2.11W, (C) 2000-2004, Applied Biostatistics Inc.
Date & time: 11/6/2012 11:27:44 AM
------------------------------------------
Input parameters:
```

Read input from file: D:\iona\combined\combined data tree.NTS

Comments:
  SIMQUAL: input=D:\iona\combined\combined data.nts, coeff=DICE
  by Cols, += 1.00000, -= 0.00000
  SAHN: input=D:\iona\combined\combined data sim.NTS, method=UPGMA,
tie=FIND
  Solution tree number 1
Matrix type = 6, size = 3 by 2, missing value code = "none" (tree
(similarity))

Ending date & time: 11/6/2012 11:27:44 AM

========== Section: 4-Output ===========
Output: NTSYSpc 2.11W, (C) 2000-2004, Applied Biostatistics Inc.
Date & time: 11/6/2012 11:30:37 AM

Input parameters:
Read input from file: D:\iona\combined\combined data sim.NTS
Format: width=9 decimals=4
Page width: 80
Field width: 9
Decimal places: 4
Page width: 80
Comments:
  SIMQUAL: input=D:\iona\combined\combined data.nts, coeff=DICE
  by Cols, += 1.00000, -= 0.00000
Matrix type = 3, size = 3 by 3, missing value code = "none" (similarity)

           Sabp-P1   Sabp-P2   Sabp-P3

Sabp-P1 | 1.0000
Sabp-P2 | 0.7576   1.0000
Sabp-P3 | 0.7692   0.7273   1.0000

Ending date & time: 11/6/2012 11:30:37 AM