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STUDIES TOWARDS THE DEVELOPMENT OF *SALMONELLA*-SPECIFIC BACTERIOPHAGES FOR SANITATION IN THE FOOD INDUSTRY

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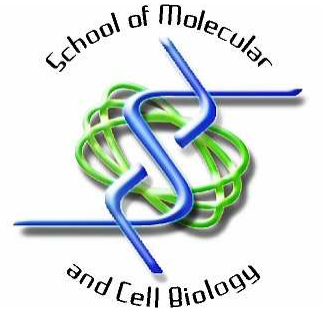

Candidate: Angela Hobbs
Supervisor: Dr. Steven I. Durbach
Co-supervisor: Dr. Denise Lindsay



University of the Witwatersrand

STUDIES TOWARDS THE DEVELOPMENT OF *SALMONELLA*-SPECIFIC BACTERIOPHAGES FOR SANITATION IN THE FOOD INDUSTRY

Angela Hobbs

A research report submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirement for the degree of Master of Science in Biotechnology.

Johannesburg, 2007

DECLARATION

I declare that this research report is my own, unaided work. It is being submitted for the degree of Master of Science in Biotechnology in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university.

_____

Angela Hobbs

_____ Day of March 2007

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ABSTRACT

Bacteriophages have sparked interest as novel ways to control foodborne pathogens. The application of *Salmonella*-specific phages as antimicrobial agents was tested against relevant *Salmonella* isolates of poultry origin. Two different *Salmonella*-specific phages, A and C, were isolated from enriched sewage. They displayed differences in their host-range but exhibited virulent behaviour towards *Salmonella enterica* serovar Typhimurium ATCC 13311. Toxicity studies were conducted with individual and combined applications of phages A and C, at an MOI of 1, on *Salmonella* ATCC 13311. Following 3 hour exposure, both applications were equally effective at reducing *Salmonella* by approximately 1 x 10^5 CFU/ml. Similar toxicity profiles were observed with both applications, however, a delay occurred with phage A. We propose that phages A and C have similar infective specificities and that during combination competition for the receptor is overcome by phage C. Neither application eliminated *Salmonella* to undetectable levels. The presence of phage-resistant mutants is a fundamental issue that will hamper the use of phages as alternate antimicrobial agents.

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ABBREVIATIONS

Abi - Abortive infection

ATCC – American type culture collection

BCF – Battery chicken faeces

bp – Base pairs

CFU – Colony forming units

CFU/ml – Colony forming units per millilitre

CG – Chlorhexidine gluconate-containing sanitizer

CIP - Cleaning-in-place

cm^2 – Centimetre squared

DNA - Deoxyribonucleic acid

ds – Double stranded

EPS - Extracellular polymeric substances

EU – European Union

FCF – Free-range chicken faeces

G + C - Guanine and Cytosine

g – Grams

HACCP - Hazard Analysis Critical Control Point

hr – Hours

I - Iodophor

L - Litre

LA – Luria-Bertani agar

LB – Luria-Bertani broth

LLO - Listeriolysin O

m - Million

min – Minutes

ml – Millilitre

mM – Millimolar

mm - Millimetre

MOI - Multiplicity of infection

MSc – Master of Science

N/A – Not Applicable

NCBI - National Centre for Biotechnology Information

OF - Oxidation-fermentation

Omp - Outer membrane protein

O/N – Overnight

PAH – Peracetic acid/hydrogen peroxide combination sanitizer

PCR – Polymerase chain reaction

PFU – Plaque forming units

PFU/ml – Plaque forming units per millilitre

pp. - Page

ppm – Parts per million

Quats - Quaternary ammonium compounds

rDNA – ribosomal DNA

RNA - Ribonucleic acid

rpm – Revolutions per minute

S.D. – Standard deviation

spp. – Species

ss – Single stranded

SS – Sewage

TBE - Tris-Borate-EDTA

TEM – Transmission electron microscopy

TSB – Tryptone soy broth

USA - United States of America

US – United States

USDA - US Department of Agriculture

V – Receptor variant

δ - Sigma

µl – Microlitre

µm - Micron

CHAPTER ONE

1. INTRODUCTION

1.1 FOODBORNE ILLNESS

Foodborne illnesses arise from the consumption of food or water that has been contaminated with microbiological pathogens or toxic chemicals (Sanders, 1996; Sanders, 1999). Various groups of foodborne pathogens exist (Table 1), which if present in contaminated food products, can lead to significant impacts on human health and industrial economies (Sanders, 1999; Helms *et al*., 2003). The symptoms of foodborne illnesses, resulting from the consumption of pathogen contaminated foods, can range from mild to more severe indications such as diarrhea, fever, nausea, vomiting, abdominal cramps, dehydration, meningitis, endocarditis, kidney failure, and septicaemia (Darwin and Miller, 1999). In the United States of America (USA), roughly 76 million cases of foodborne illnesses with 325,000 hospital admissions and 5,000 deaths are said to occur annually (Mead *et al*., 2006). *Salmonella*, *Toxoplasma* and *Listeria monocytogenes* cause approximately 1,500 deaths every year (Mead *et al*., 2006). In Australia it is estimated that 5.4 million cases of foodborne illnesses occur annually and that the main pathogens involved are *Salmonella*, *E. coli*, *Campylobacter* and Norovirus (Hall *et al*., 2005). The main foodborne pathogens that result in foodborne illnesses in the USA are depicted in Figure 1. One of the major concerns associated with foodborne pathogens in developing countries is the high incidence of diarrheal diseases among children caused by *E. coli* and Rotavirus, which is estimated to be higher than the combined fatalities of AIDS, malaria and TB in children under 5 (Gerlin, 2006). There are no statistics on the incidence of foodborne illnesses in South Africa, however, as this is a third-world country a similar or perhaps worsened situation is expected compared to those experienced in first-world countries such as in the USA.

The economic implications of foodborne illnesses result in financial losses through litigations, medical expenses, expenses on product recall and disposal of contaminated products, and decreases in productivity (Whyte *et al*., 2004; Normanno *et al*., 2005). For example, decreases in productivity, caused by *Clostridium perfringens* infections, are reported to cost the US economy $12.5 billion (Norvak and Juneja, 2002). In the USA, patient-related costs for treatment of foodborne infections are estimated at around $6.7 billion annually (Tauxe, 2001). Foodborne diseases caused by staphylococci cost the US economy an estimated $1.5 billion annually (Normanno *et al*., 2005).

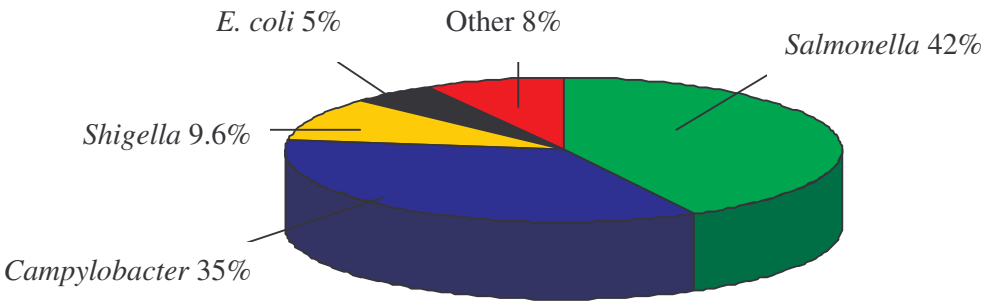

Figure 1: Causes of foodborne illnesses in the USA. (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm4910a1.htm).

The onset of bacterial foodborne disease will occur if a sufficient quantity of pathogenic bacteria is ingested (Jay *et al*., 2005). The bacteria subsequently invade the host via the small and large intestines, or they may cause disease through toxin production within the host (Pierson and Corlett, 1992). Alternatively disease may arise from intoxication through the ingestion of pre-formed toxins, which the pathogenic bacteria produce and excrete while contained in the food (Pierson and Corlett, 1992).

Table 1: Groups of foodborne pathogens

Foodborne pathogens	**Examples**
Bacteria	Gram positive: *Staphylococcus aureus, S. epidermidis* *Bacillus cereus* *B. anthracis* *Clostridium botulinum* *C. argentinensis* *C. perfringens* *Listeria monocytogenes* *Mycobacterium avium subsp.paratuberculosis* Gram negative: *Escherichia coli* O157:H7 *Salmonella typhimurium, S. enteritidis* *Shigella sonnei, S. flexneri* *Yersinia enterocolitica* *Vibrio parahaemolyticus* *Brucella melitensis* *Campylobacter jejuni*
Viruses	Noroviruses Hepatitis A Rotaviruses
Prions	Creutzfeldt-Jacob disease Bovine spongiform encephalopathy (Mad cow disease)
Toxigenic phytoplanktons	Paralytic shellfish poison Domoic acid Ciguatoxin
Fungi- mycotoxin producers	Aflatoxins Fumonisins Alternaria toxins Ochratoxins
Protozoa	*Cyclospora* *Cryptosporidium* *Giardia lamblia* *Entamoeba* *Toxoplasma* *Sarcocystis*
Roundworms	*Trichinella* *Ascaris* *Anisakis* *Pseudoterranova* *Toxocara*
Flatworms	Flukes: *Fasciola* *Fasciolopsis* *Paragonimus* *Clonorchis* Tapeworms: *Diphyllobothrium* *Taenia*

(Jay *et al*., 2005).

In order for an intestinal pathogen to successfully invade its host and cause disease, several obstacles must be overcome. Firstly, the pathogen must survive the low-pH environment of the stomach (Jay, 2000). This may be achieved by several acid tolerance mechanisms, such as the RpoS stress response. The *rpoS* gene, also known as *KatF*, encodes the sigma factor, sigma-38 or δ^{38}, which is a subunit of bacterial RNA polymerase that varies in size and regulates at least 30 proteins (Jay, 2000). The rpoS protein is specifically involved in the stationary growth phase of enteric bacteria such as *E. coli*, *Shigella*, *Salmonella*, and *Y. enterocolitica*, and it confers survival of these bacteria at pH 2.2 for more than 2 hr (Hengge-Aronis *et al*., 1991; Siegele and Kolter, 1992; Gorden and Small, 1993; Small *et al*., 1994). The underlying mechanism is the induction of those genes that control extreme alkaline resistance and acidic pH, along with the control of thermotolerance, starvation survival, hydrogen peroxide resistance, glycogen synthesis and osmotic stress (Jay, 2000). The RpoS stress response is also said to increases the expression of certain virulence determinants as seen with *Salmonella* spp. (Hengge-Aronis *et al*., 1991; Siegele and Kolter, 1992; Gorden and Small, 1993; Small *et al*., 1994). Another means of evading the acidic environment of the stomach is through the protective effect of food (Jay, 2000).

In addition to surviving low pH in the stomach, intestinal pathogens, such as *Listeria monocytogenes*, must be capable of adhering to and colonising the intestinal mucosa. In order to accomplish this, the pathogen must overcome the mucus layer of the intestinal mucosa (Coconnier *et al*., 1998). *L. monocytogenes* is able to remove the mucus layer with the aid of listeriolysin O or LLO (Coconnier *et al*., 1998). Furthermore, once present in the gut, the pathogen must be able to compete with the natural microbiota of the gut, as well as overcome host defence mechanisms, such as the gut-associated lymphoid tissue (Jay, 2000). Once the pathogen has successfully adhered to and colonised the intestinal mucosa, it must be able to cross the epithelial wall and enter phagocytic or somatic cells (e.g., *L. monocytogenes*) or it must synthesise and release its toxins (e.g., *Vibrio cholerae* non-01) (Jay, 2000). As a result, the small intestine malfunctions, causing the intestinal contents to be discharged as watery or bloody stools (Gerlin, 2006). The loss of fluid leads to dehydration that may be accompanied with a dry or sticky mouth, less and darker urine and dizziness. Kidney failure, septicaemia and death occur with sever dehydration (Gerlin, 2006).

The contamination of food by pathogenic micro-organisms can arise from several sources (Jay, 2000). Foodborne pathogens (viruses and enteropathogenic protozoa and bacteria) or their preformed toxic products are usually contracted via the faecal-oral route. Dissemination of pathogens may occur from contaminated faeces by means of water, crawling or flying insects, or by the fingers of food handlers that practice poor personal hygiene (Jay, 2000). In addition to this, transmission may arise from poorly sanitised food processing equipment (surfaces and utensils) or from water or ice used in food processing and preparation (Jay, 2000).

1.2 CLEANING-IN-PLACE SYSTEMS USED IN FOOD PROCESSING

The prevention of microbiological foodborne diseases can be achieved by the food industry through effective cleaning-in-place (CIP) procedures, and training and educating staff in 'Good Manufacturing Practice' (Wirtanen *et al*., 2000). Sanitation programmes and Hazard Analysis Critical Control Point (HACCP) systems are also important components in food processing for the control of microbiological hazards and for food safety improvement. HACCP systems monitor the whole manufacturing process by assessing microbiological hazards and risks, and enforce appropriate control measures (Sanders, 1999).

Various cleaning methods that are used in food processing plants depend on the nature of the contaminant (known as soil) that is to be removed (Troller, 1983). The soil can either be protein, fat, sugar, mineral salts and/or organic matter (Troller, 1983; Wirtanen *et al*., 2000). Fat can be removed using thermal treatment at temperatures above their melting point (Troller, 1983). Even though carbohydrates and sugars are water-soluble at elevated temperatures, thermal treatment should be avoided with sugar as elevated temperatures cause caramelization (Wirtanen *et al*., 2000). Micro-organisms can then make use of food soils for growth. Examples include dried fruit juice or mould growing on equipment surfaces in fruit processing plants, and field dirt, frying oil, starch and bacterial slimes in potato processing plants (Troller, 1983).

CIP operations are usually automatically controlled, lasting as long as 1 hr and requires the recirculation of cleaning solutions for repeated exposure and for conservation of energy and cleaning compounds (Marriott, 1985). An effective CIP system includes the following operations in order of sequence: 1. preliminary rinse to remove gross soil; 2. detergent to remove the residual soil; 3. rinse to remove the cleaning compound; 4. sanitizer to destroy the residual micro-organisms; 5. final rinse for removal of cleaning solutions and sanitizers (Marriott, 1985). Drying is also usually done as a final step as wet surfaces can provide favourable conditions for microbial growth (Wirtanen *et al*., 2000).

(i) Detergents

A detergent is a surface-active agent that deflocculates and suspends soil particles by lowering their surface tension (Troller, 1983). There are several types of detergents available for use in food processing plants (Table 2). Alkaline detergents are divided into 3 groups, 1. Strong alkaline detergents; 2. Heavy-duty alkaline detergents; 3. Mild alkaline detergents (Marriott, 1985). Strong alkaline detergents, such as sodium hydroxide, are very corrosive agents that are used for the removal of heavy soils but are ineffective against mineral deposits (Marriott, 1985). Heavy-duty alkaline detergents, such as sodium pyrophosphate, are mildly to non-corrosive and are frequently used in CIP systems for the removal of fats, but as with strong alkaline detergents, they are ineffective against mineral deposits (Marriott, 1985). Mild alkaline detergents, such as sodium carbonate, are used for cleaning lightly soiled areas and are also ineffective against mineral deposits (Marriott, 1985).

Table 2: Types of detergents

Type	**Function**
Alkaline	Prevents mineral build-up
Chlorinated caustic and alkaline	Enhances cleaning and decolourisation
Neutral	Used where surface deterioration may occur
Acid	Removes mineral build-up
Solvented	Removes grease and fats

(Troller, 1983)

(ii) Chemical sanitizers

Chemical disinfectants are sanitizers that are used to remove micro-organisms present on food-contact surfaces and in so doing, prevent cross contamination to raw materials and food products with spoilage and pathogenic organisms (Langsrud *et al*., 2003). There are several types of sanitizers available for use in food processing plants (Table 3). Sanitizers such as chlorine and hypochlorites are commonly used in the food industry (Marriott, 1985). The effectiveness of chlorine compounds as sanitizers are said to increase with decrease in temperature and decrease in pH. Iodophors are the most popular of the iodine-based sanitizers and have effective bactericidal activity when used in acidic conditions (Marriott, 1985). Bromine compounds are often used in combination with chlorine compounds as they are unaffected by alkaline pH ranges and are effective against *Bacillus cereus* spores (Marriott, 1985). Quaternary ammonium compounds or "quats" are commonly used sanitizers as they are more stable than chlorine and iodine sanitizers when exposed to organic matter (Marriott, 1985). Quats are used for sanitation of porous surfaces (Marriott, 1985).

Table 3: The various types of chemical sanitizers used for food sanitation

Type	**Examples**	**Reference**
Chlorine Compounds	Chlorine Hypochlorites Chlorine dioxide	Marriott, 1985
Iodine Compounds	Iodophors Alcohol-iodine solutions Aqueous iodine solutions	
Bromine Compounds	Bromine	
Quaternary Ammonium Compounds	Alkyldimethylbenzylammonium chloride Alkyldimethylethylbenzylammonuim chloride	

Non-chemical sanitation of rooms and equipment may be accomplished through thermal and other forms of radiation (Marriott, 1985). The effectiveness of thermal treatment is dependent on the temperature and the exposure time (Marriott, 1985). This is a relatively inefficient means of sanitation as it requires a large amount of energy (Marriott, 1985). Adequate penetration of heat to areas is dictated by the design of the plant and its' equipment, as well as by the method of dispending (Marriott, 1985). Thermal treatment may be applied as steam or hot water, neither of which is very effective (Marriott, 1985).

In the USA, the purchase of chemical antimicrobials that are used to ensure food safety has been estimated at around $161.7 million in 2005, and is expected to increase to $215.8 million by the year 2012 (ElAmin, 2007a). Worldwide, the expenditure on toxic chemicals used by food and non-food industries in the killing of pathogens is estimated at around €5.6 billion (ElAmin, 2007a).

1.3 BACTERIAL BIOFILMS IN FOOD PROCESSING

Micro-organisms can grow by one of two growth states: planktonic or sessile (Poulsen, 1999). The planktonic growth state is used to describe bacteria that grow as individual, free-floating organisms, while sessile growth describes bacteria that grow as a community and are irreversibly attached to surfaces and are known as biofilms (Poulsen, 1999). In natural and manmade environments the prevailing lifestyle of micro-organisms is that of bacterial biofilms (Lindsay and von Holy, 2006).

Biofilms consist of surface-adhered microbial cells; bacteria, fungi, and /or protozoa, that grow alone (mono-species) or in combination (multi-species or multi-kingdom) and are embedded in a glycocalyx (Poulsen, 1999). The glycocaylx or extracellular matrix is primarily composed of extracellular polymeric substances (EPS), which the microbial cells secrete (Jay *et al*., 2005). The EPS plays an important role in the initial attachment and anchorage of bacteria to surfaces, and is able to retain water, which protects the biofilm cells against desiccation (Kumar and Anand, 1998). In addition to this, in some biofilms EPS protects biofilm cells from the adverse effects of antimicrobial agents, as they bind macromolecules and prevent their diffusion through the biofilm (Kumar and Anand, 1998; Poulsen, 1999). EPS has also been shown to act as a reservoir for nutrients (Kumar and Anand, 1998). Within this microenvironment are microcolonies and water channels that form between and around the microcolonies. The water channels provide a means of transporting nutrients to the microcolonies and toxic by-products away from them (Jay *et al*., 2005).

In natural and industrial settings, the presence of biofilms can have positive impacts. For example, in the human gut biofilms comprise mixed consortia of commensal bacteria that adhere to gut epithelial cells and form a protective barrier against foodborne pathogens ((Sadine, 1979; Lee *et al*., 2000). In sewage treatment plants

mixed-species biofilms are useful in bioremediation processes of human wastes (Characklis, 1973), and they are also used in the commercial production of lactic and acetic acid (Qureshi *et al*., 2005). However, biofilms may also cause adverse effects. In medical settings biofilms occur on medical devices (Dankert *et al*., 1986) such as on endoscopes (Pajkos *et al*., 2004) and catheters (Camargo *et al*., 2005), and as a result may lead to human infections. The dental plaque on teeth consists of diverse bacterial biofilms that may lead to dental decay (Gibbons and van Houte, 1980). On the inner surface of industrial pipe systems biofilms cause fouling as they harbour oxidising bacteria and sulphur-reducing bacteria, which corrode metal surfaces by cathodic depolarisation and acid production (LeChevallier *et al*., 1987; Parsek and Fuqua, 2003).

Biofilms harbouring food spoilage or pathogenic bacteria have significance in the food industry as they accumulate on foods, food utensils and other food-contact surfaces and are difficult to remove, and may consequently result in food spoilage and foodborne illnesses (Jay *et al*., 2005). Biofilms associated with food processing environments can lead to product contamination during food processing, which subsequently affects product shelf life and following the consumption of contaminated foods, may lead to foodborne illnesses (Holah *et al*., 1989).

Foodborne pathogens can persist as biofilms on food contact surfaces, such as aluminium, stainless steel, glass, nylon materials, Buna-N and Tephlon seals (Kumar and Anand, 1998). For example, *Bacillus cereus* spores found in milk have been shown to attach to stainless steel surfaces of milk processing equipment, such as the inside surfaces of pipes, and can eventually lead to blockages that reduce flow through the pipes (Poulsen, 1999). Biofilms that cause blockages can also lead to energy losses in condenser tubes, water and wastewater circuits, and heat exchange systems in milk processing plants (Wirtanen *et al*., 2000). For example, *Streptococcus thermophilus* biofilms in heat exchange systems can lead to reduction in heat transmission (Poulsen, 1999). Biofilms present on food processing equipment may cross contaminate food products and can also lead to corrosion of pipes as acid is produced within the biofilm (Poulsen, 1999).

(i) Biofilm formation on surfaces

The ability of micro-organisms to adhere to and grow on food-contact surfaces as biofilms is of major importance to the food industry worldwide (Hughes *et al*., 1998). In food processing environments bacteria and organic or inorganic matter (e.g. proteins from meat) adhere to surfaces and are referred to as a conditioning film (Hood and Zottola, 1997). The nutrients occurring in this film stimulate biofilm formation (Jeong and Frank, 1994). Biofilm formation can also be induced by bacteria that have become trapped on surfaces in cracks and deep channels, which also protect the cells from mechanical shearing (Wirtanen *et al*., 1996).

Attachment of the bacterium to the conditioning film is influenced by the properties of the surrounding fluid phase (e.g. gravity, fluid dynamics and diffusion) and is dependent on whether the bacterium is motile (Kumar and Anand, 1998). Attachment to surfaces occurs by reversible attachment and is followed by irreversible attachment (von Loosdrecht *et al*., 1990). Cells that are reversibly attached to surfaces can easily be removed by rinsing as they exhibit Brownian movement. With regards to irreversible attachment, the bacterial appendages, pili, frimbriae, flagella, and EPS frimbrils form an irreversible association between the bacterium and the surface (von Loosdrecht *et al*., 1990). These cells are difficult to remove and require mechanical shearing such as scrubbing and scrapping (Marshall *et al*., 1971). The nutrients present in the conditioning film and surrounding fluid allow irreversibly attached cells to grow and divide into microcolonies (Hood and Zottola, 1997). The cells produce EPS to aid in anchorage of the cells to the substratum and for protection against environmental stresses (Meyer, 2003). Cells occurring in the surrounding fluid phase continually attach to the primary colonisers and eventually result in multilayers of bacterial cells that are embedded in EPS (Kumar and Anand, 1998).

(ii) Virulence factors in biofilm formation

Biofilm formation by several pathogenic bacteria is said to be dependent on their virulence factors (Lindsay and von Holy, 2006). Virulence factors are features of micro-organisms that enable them to infect their host and include virulence factors such as flagella, lipopolysaccharides, pili, exotoxin-A, quorum-sensing auto-inducer

and alginate, all of which are involved in biofilm formation (reviewed by Lindsay and von Holy, 2006).

(iii) Removal of bacterial biofilms

Surface adhered micro-organisms are more resistant to sanitising compounds than their free-living planktonic counterparts and as a consequence, any viable cells that persist after sanitation may be transferred to foods when they desorb from the surface. This can result in foodborne diseases or food spoilage as the biofilm may harbour food spoilage or pathogenic bacteria.

- Choice of surfaces

Biofilm formation occurs on almost all surfaces that have rich nutrient environments. The microtopography of surfaces does influence the formation of biofilms. For example, biofilms commonly occur in gaskets, dead corners, cracks, crevices, and joints in pipe materials (Poulsen, 1999). The choice of surface materials, their finish and design are thus important for reducing biofilm formation. Surface material selection depends on the R-factor (roughness) of the material (Poulsen, 1999). For example, a surface with a small R-value signifies the presence of a few cracks. Such material surfaces are ideal for use. Examples of surfaces with small R-values are glass and polished steel. Untreated and treated steel on the other hand, that has undergone mechanical grinding and electrolytic polishing have high R-values (Poulsen, 1999), and are unsuitable as they are vulnerable to microbial colonisation. The pH and temperature of surfaces also influence microbial adhesion (Kumar and Anand, 1998; Norwood and Gilmour, 2001). This is seen with *Pseudomonas fragi* which optimally adsorbs within the pH range 7-8 (Kumar and Anand, 1998).

- Cleaning methods

Effective cleaning methods include mechanical rinsing and treatment with chlorine or monochloramine (Poulsen, 1999; Kreske *et al*., 2006). The age of the biofilm however, plays a role in its susceptibility to cleaning and antimicrobial agents, as resistance is directly proportional to the age of the biofilm. This emphasises the need for immediate treatment (Poulsen, 1999).

(iv) Biofilm susceptibility to chemical sanitizers

Poultry-associated *Salmonella* are able to form biofilms on food contact surfaces such as plastic, cement and stainless steel (Georgnaras *et al*., 1995; Joseph *et al*., 2001). Several studies have evaluated the densities of biofilms formed on these food contact surfaces, as well as the effectiveness of the sanitizer's hypochlorite and iodophor, in reducing both planktonic and sessile growth forms (Lindsay and von Holy, 1999; Joseph *et al*., 2001). One study, conducted by Joseph *et al* (2001), formed Biofilms on 4cm^2 surfaces, which were suspended in tryptone soy broth (TSB) that contained the *Salmonella* cultures. These were incubated at 28 ± 2ºC for 48 hr before being washed and placed into fresh TSB. This was repeated 5 times, so that the total duration for biofilm formation amounted to 10 days. Surfaces were swabbed and cells were enumerated via spread plates. *Salmonella* Weltevreden and *Salmonella* FCM 40 were both found to form biofilms at high cell densities in the order of 10^7 CFU/cm^2 (colony forming units/ cm^2) on plastic, followed by 10^6 CFU/cm^2 on cement, and 10^5 CFU/cm^2 on stainless steel (Joseph *et al*., 2001).

Joseph *et al* (2001) also evaluated the strength of the sanitizers, Cl_2 and I_2 on the *Salmonella* biofilms discussed above. Surfaces containing biofilms were dipped in varying concentrations of sanitizers, and were subsequently washed in a neutralising solution before they were swabbed and enumerated at varying times. Planktonic cells of both cultures were highly susceptible to both sanitizers as they were killed within 10 min exposure to 10 ppm Cl_2, and 5 min exposure to 10 ppm I_2 (Joseph *et al*., 2001). Biofilm cells however are more resistant to the effects of sanitizers as longer durations of exposure and higher concentrations are required for biofilm cell inactivation (Chavant *et al*., 2004; Folsom and Frank, 2006). This did however vary across the surfaces that were used in the Joseph *et al* study. For instance *Salmonella* FCM 40 biofilms on plastic was resistant to 100 ppm (parts per million) Cl_2, but *Salmonella* FCM 40 biofilms on cement and stainless steel was killed and inactivated to undetectable levels, with 20 min exposure to 100 ppm Cl_2. *S.* Weltevreden biofilms on plastic and cement were killed after 20 min exposure to 100 ppm Cl_2, while *S.* Weltevreden biofilms on stainless steel were killed after 15 min exposure to 100 ppm Cl_2. *S.* Weltevreden biofilms on plastic were partially resistant to iodine as cells were not completely inactivated by the highest concentration, which was 50 ppm I_2, however, *S.* Weltevreden biofilms on cement and stainless steel were killed after 25

min and 20 min exposure to 50 ppm I_2, respectively. *Salmonella* FCM 40 was completely inactivated after 25 min exposure to 50 ppm I_2. The lower concentrations of sanitizers were marginally effective in reducing cells (Joseph *et al*., 2001). Ideally a sanitizer should reduce cell counts by 5 or more log units, i.e. by 99.999% (Lindsay and von Holy, 1999). This has been achieved against planktonic *P. fluorescens* during 5 min exposure to PAH (peracetic acid/hydrogen peroxide combination sanitizer), I (Iodophor) and CG (chlorhexidine gluconate-containing sanitizer) (Lindsay and von Holy, 1999). Our interest lies in the complete inactivation to undetectable levels.

(v) Biofilm cells: Factors that confer antimicrobial resistance

One of the main problems faced by food industries is the removal of bacterial biofilms. This has proved to be increasingly difficult as biofilms have acquired resistance to certain sanitizers. Biofilms may protect cells from the effects of certain sanitizers as well as from acid exposure, ultraviolet light, dehydration, phagocytosis and metal toxicity (Hall-Stoodley *et al*., 2004; Jefferson, 2004; Hall-Stooley and Stooley, 2005). *Listeria monocytogenes* biofilms present on food-contact surfaces have been found to be impenetrable by quaternary ammonium compounds and acid anionic sanitizers (Frank and Koffi, 1990). *Listeria monocytogenes* has also been found to be resistant to the lethal effect of heat following treatment by H_2O_2, ethanol, starvation and acid (Toarmina and Beuchat, 2001).

Multiple mechanisms which are believed to act in combination are thought to confer antimicrobial resistance. These include chemical and enzymatic inactivation of sanitizers, the induction of cellular stress responses, diffusional resistance of the EPS matrix and changes in cellular physiologies. These and other mechanisms have been reviewed by Lindsay and von Holy (2006).

1.4 POTENTIAL OF *SALMONELLA* AS A FOODBORNE PATHOGEN

One of the major foodborne pathogens that has raised food safety concerns within the food industry is *Salmonella*. *Salmonellae* are motile, non-spore forming, Gram-negative, rod-shaped bacteria that belong to the family Enterobacteriaceae. The molecular % of the G + C (Guanine and Cytosine) content in their DNA is 50 – 53 (Jay, 2000). There are approximately 2,400 *Salmonella* serovars, which are classified under two species, *S. enterica* and *S. bongori* (Jay *et al*., 2005).

Most members of Enterobacteriaceae have evolved to form a part of the normal gut flora, however some members are pathogenic, as they have acquired pathogenic genetic determinants or virulence factors, which they may have obtained from phages, transposons or plasmids (Darwin and Miller, 1999; Fluit, 2005). As previously mentioned virulence factors are the features of the micro-organism that enable it to infect its host and to avoid the immune response. An example is adhesins, which are macromolecules that occur on the cellular envelope that allow adherence of the pathogen to host cells. There are usually two classes of pathogenic determinants, the first of which is usually common to both pathogenic and non-pathogenic organisms, and are involved in physiological processes that are required for survival of the pathogen inside as well as outside of the host (Groisman and Ochman, 1996). The second class is unique to pathogenic organisms and account for a particular virulence phenotype (Groisman and Ochman, 1996). Several pathogenic determinants or virulence cassettes, also known as pathogenicity islands, have been mapped to the chromosome of pathogenic organisms (Hacker *et al*., 1990; Lee, 1996).

Two pathogenicity islands (Figure 2) have been identified in *Salmonella*, namely SPI-1, which occurs in both *S. enterica* and *S. bongori* (Mills *et al*., 1995; Galán, 1996), and SPI-2, which is unique to *S. enterica* (Ochman *et al*., 1996; Shea *et al*., 1996; reviewed by Groisman and Ochman, 1996).

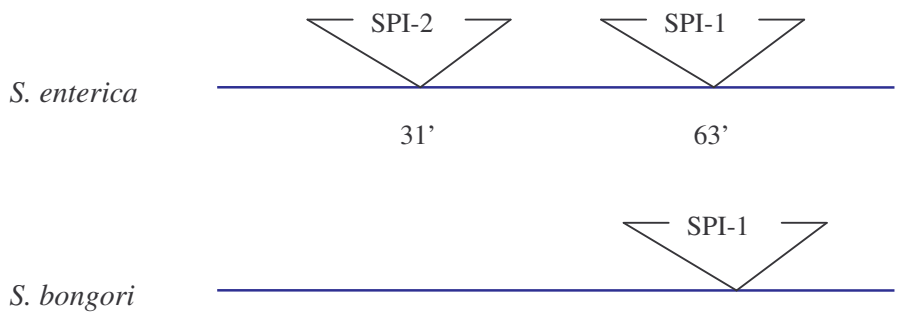

Figure 2: Pathogenicity islands of *Salmonella* (modified from Groisman and Ochman, 1996). Blue lines represent chromosomes and black triangles represent pathogenicity islands.

The two pathogenicity islands SPI-1, located at 63', and SPI-2, located at 31', are approximately 40 kb in length (Groisman *et al*., 1993). SPI-1 is said to confer the ability of *Salmonella* to invade epithelial cells (Mills *et al*., 1995; Galán, 1996; Darwin and Miller, 1999). SPI-2, however, is not required for epithelial cell invasion but rather governs the survival of *Salmonella* within macrophages (Ochman *et al*., 1996; Shea *et al*., 1996). The presence of these islands, which has been crucial in the development of *Salmonella* as an intracellular pathogen, is thought to be attributed to horizontal gene transfer as the G + C contents of both islands differs from that of typical *Salmonella* (Groisman *et al*., 1993). The two pathogenicity islands are thought to be acquired via horizontal gene transfer by phages or plasmids (Bäumler *et al*., 1998).

Infection caused by virulent strains of *S. enterica* commences in non-phagocytic cells (Jay, 2000). Initiation of the infection occurs by the bacterium attaching, via fimbrial adhesions that are encoded by a gene of SPI-1, to the intestinal mucosa (van der Velden *et al*., 1998). The intestinal mucosa is then penetrated, usually at the lymphoid follicles of Peyer's patches and following entry into the ileum of the small intestine, *Salmonella* invades the M cells of Payer's patches (Jones and Falkow, 1994). Once they have reached the vesicles of the M cells of Peyer's patches, they penetrate the lysosome. Production and secretion of the protein, SpiC into the cytoplasm, prevents fusion of vesicles with lysosomes (Jay, 2000).

The primary source of *Salmonella* is the intestinal tract of humans and animals such as farm animals, birds, reptiles and sometimes insects (Jay *et al*., 2005). Transmission of the organism, which is excreted in faeces, may occur via insects that contaminate food or water, or by the unsanitary handling of foods by food handlers (Jay *et al*., 2005). The ingestion of *Salmonella* contaminated foods result in salmonellosis or *Salmonella* poisoning, which consists of a variety of disease syndromes: bacteremia, focal infections, enteric fever and enterocolitis- the most common of all illnesses (Darwin and Miller, 1999). Symptoms that develop include nausea, vomiting, diarrhea, headaches, chills, and abdominal pain. These symptoms are normally associated with drowsiness, faintness, muscle fatigue, fever, restlessness and prostration (Jay *et al*., 2005). Symptoms normally last for 2-3 days in healthy individuals, however, children younger than 1 year of age, persons over 50 years of age, and those who are immune compromised, are most susceptible to salmonellosis, and experience the more severe symptoms (Jay *et al*., 2005, Darwin and Miller, 1999). Fatality rates such as 5.8% and 15 % have occurred in children younger than 1 year and in adults older than 50 years of age, respectively (Jay *et al*., 2005). Overall *Salmonella* is thought to cause 800,000 to 4 million cases of salmonellosis that result in hundreds of fatalities each year in the USA (ASM News, 2003). The number of cells required for the onset of salmonellosis is said to be in the order of 10^7-10^9/g (Jay *et al*., 2005).

Salmonellae occur on fruits and vegetables, poultry carcasses, in poultry processing plants, and in poultry products (van Nierop *et al*., 2005). Recently a *Salmonella* outbreak in England and Wales, which affected 37 people, many of which were children, was said to be caused by Cadbury chocolates that were contaminated with *Salmonella* serotype Montevideo (Times online, 2006). As a consequence, the Food Standards Agency (FSA) ordered Cadbury's to remove 250 tons of chocolates from shop shelves and warehouses (Daily Mail, 2006). It is quite clear that such actions (recall and disposal of chocolates) would have significant economic consequences. The total expense incurred following recalls and modifications in the Cadbury manufacturing plants is estimated at £30m (€44.5m) (ElAmin, 2007b). Prosecution under environmental health laws is expected to cost Cadbury Schweppes more than the recalling expenditure (ElAmin, 2007b). Another outbreak which affected more than 350 people in Britain, occurred in 2004, and was linked to *Salmonella* serotype Newport contaminated lettuce from fast food and catering establishments (Daily Mail, 2004). These and other cases of salmonellosis have enforced a more stringent attitude

towards food products. Regulation tests conducted during March 2005 to July 2006 by the FSA found that one out of every 30 boxes of eggs from those imported into the UK, tested positive for *Salmonella* contamination (ElAmin, 2006c). The highest prevalence of contaminated eggs was found to originate from Spain, with *S. enteritidis* being the most commonly isolated strain. The European Commission prompted efforts to minimize contamination by *Salmonella* and other foodborne pathogens. These included compulsory vaccination of flocks in countries such as Spain and France, which have the highest prevalence of contaminated birds. Secondly trade bans of eggs with high levels of *Salmonella* were also proposed. Such actions have been effective in reducing the number of cases of salmonellosis in the 25 EU states, which was reported at 192,703 cases during 2004 (ElAmin, 2006c).

Poultry products are regarded as the primary vehicles of *Salmonella* transmission (Georgnaras and von Holy., 2000). The birds that are brought to processing factories are a major source of *Salmonella* and *Campylobacter* as they occur on the feathers, skin and in the alimentary tract of the birds. The birds may have acquired *Salmonellae* from contaminated feed, from environmental sources such as wild animals, rodents, birds etc. or through transmission from parent to progeny in hatcheries (Bremner and Johnston, 1996). Due to the liberal use of antibiotics as supplements in animal feed in the poultry industry, an increase in resistance among *Salmonella* has developed (Gouws and Brőzel, 2000). Resistance has also been detected against newer antibiotics (Fluit, 2005), and certain antimicrobial agents have also proved ineffective against *Salmonella* in the food industry. For example, *Salmonella* has shown to exhibit resistance to acid, hypochlorite, iodophor sanitizer treatments as well to antibiotic applications (Georgnaras and von Holy, 2001; Sharma *et al*., 2004).

1.5 BACTERIOPHAGES AND THEIR POTENTIAL AS ANTIMICROBIAL AGENTS

The development of bacterial resistance of biofilms to many of the antimicrobial agents used in food processing is of global importance, especially when dealing with pathogenic bacteria (Chesney *et al*., 1996; Mokgatla *et al*., 1998; Paterson and Bonomo, 2005). Disease outbreaks caused by consuming bacterially- contaminated foods are becoming increasingly difficult to treat (Gouws and Brőzel, 2000). Novel antimicrobial agents are urgently needed in the food industry to limit the growth of

foodborne pathogens. An attractive alternative to the use of chemical sanitizers in reducing food contamination by spoilage and pathogenic bacteria is the use of lytic bacteriophages, the natural viral agents of bacteria.

Bacteriophages are diverse in their structure and morphologies, which relate to how they infect their bacterial hosts (Hayes, 1974). They are composed of a nucleic acid molecule, either double-stranded (ds) or single-stranded (ss) DNA or RNA, which is enclosed and protected by a capsid or protein coat (Hayes, 1974). The capsid consists of subunits called capsomeres, which in turn are composed of smaller protein subunits called protomeres (Hayes, 1974). Certain components of the phage capsid interact with specific receptor sites found on the surface of their bacterial hosts (Hayes, 1974).

Bacteriophages may follow one of two modes of replication (Figure 3), namely the lytic or lysogenic infective cycles (Hayes, 1974). The lytic mode of replication is followed when the host is available at high numbers. However, in the event that the host numbers become limited, then the lysogenic mode of replication is followed. During both forms of infection, the phage adsorbs to the surface of the bacterium, followed by penetration of the bacterial cell and entry of the phage nucleic acid. Unlike temperate bacteriophages, virulent bacteriophages do not lysogenize their DNA into the genome of the host (Hayes, 1974). Phages that pursue the lysogenic state exist as prophages and may confer a selective advantage to the host (Miao and Miller, 1999). For example, Lysogenic conversion could lead to more pathogenic strains of *S. typhimurium* (Mirold *et al*., 1999) since lysogenic phages may transfer DNA between bacteria (Wagner and Waldor, 2002). Temperate phages are thus not suitable as alternative antimicrobial agents.

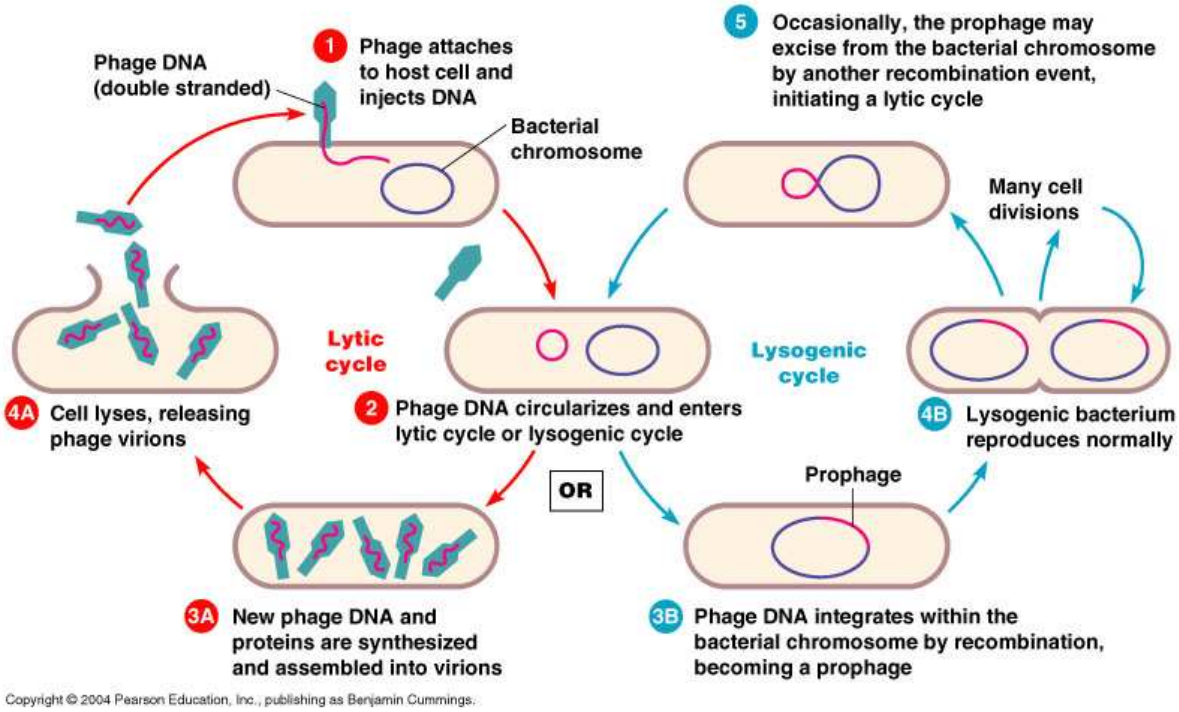

Figure 3. The lytic and lysogenic infective cycles of bacteriophages (http://diverge.hunter.cuny.edu/~weigang/Images/13-12_lysogeniccycle_1.jpg)

(i) Advantages of bacteriophages as potential antimicrobial agents

There are several properties of bacteriophages that make them attractive alternatives to other antimicrobial applications. Bacteriophages are specific for prokaryotes and are relatively safe to humans and the environment, as they generally do not cross the species and genus barrier and will not target commensals in the gastrointestinal tract or the necessary bacteria, such as starter cultures, in foods (Sulakvelidze and Barrow, 2005). Therefore due to their specificity, bacteriophages will target only those bacteria that they are specific for, which may have important implications for not depleting microflora when targeted against a specific pathogen. Phages are the most abundant life forms in the biosphere as approximately 10^{31} tailed bacteriophages are said to exist in aquatic ecosystems (Suttle, 2005), and they are readily isolated from the environment (Hendrix, 2003). Phages represent the most diverse biological entities on earth (Breitbart and Rohwer, 2005; Hatfull *et al*., 2006). The diversity of phages is facilitated by their mode of replication as they are susceptible to the uptake and deposition of host/ phage DNA during the lysogenic infective cycle. The parasitic nature of phages and their diversity makes them attractive candidates for antimicrobial agents and sanitizers.

Since bacteriophages multiply in the presence of their target bacterium, and the resulting progeny are released upon cell lysis, the initial dose of the bacteriophage can be potentially low (Leverentz *et al*., 2001). An increase in phage concentrations will occur over time during the lytic infection cycle, which will cease once all the phage-susceptible cells have been lysed. The number of phages that are introduced into the cell culture, and the period of time during which complete cell lysis will occur, is dependent on the initial concentration of the host. The ratio of phage to host or the number of phage particles that are available for infection of a cell, which is known as the multiplicity of infection (MOI), must be considered. A high MOI should result in more rapid lysis of the host when compared to a low MOI. It has been suggested that the host cells must reach a threshold value before a phage can replicate (Ellis *et al*., 1973; Wiggins and Alexander, 1985). For example, Greer (1988) reported that phage inactivation of a host cell may occur when the host concentration is as low as 46 cells/ cm^2.

Bacteriophages also represent a self-cleaning antimicrobial alternative as the bacteriophage population gradually declines once the host bacterium has been eliminated (Leverentz *et al*., 2001). Due to the fact that phages are primarily composed of nucleic acids and proteins, their breakdown products comprise amino acids and nucleic acids and hence, their introduction and collapse within an environment can be seen as an organic process (Sulakvelidze and Barrow, 2005). Even though some bacteriophages are highly specific, others have a broad host-range (Jensen *et al*., 1998; Carlton *et al*., 2005). For example, consider the bacteriophage, K1-5, which was isolated by Scholl *et al* (2001) from sewage effluent obtained from a sewage treatment plant, on *E. coli* K5. The host-range of phage K1-5 demonstrated that phage K1-5 was able to successfully infect and replicate in both *E. coli* K1 and *E. coli* K5 strains. Another example of a single broad host-range phage is phage P100, which has been shown to infect and kill more than 95% of approximately 250 different foodborne *Listeria* isolates of the serovar groups 1,2, 4 (*L. monocytogenes*), and 5 (*L. ivanovii*) (Carlton *et al*., 2005). Felix 01 is another broad host-range phage, as it has shown to lyse 96-99.5% of *Salmonella* serovars (Lindberg, 1967). Broad host-range phages are favourable when targeting several pathogenic bacterial strains.

Phages depend on Brownian movement to come in contact with the host cell, and infection thereof is initiated from the specific interactions between receptors that occur on the surface of the host cell and antireceptors on the phage. Eventually the host cell will mutate, causing modifications in the phage receptors sites, which consequently will lead to phage resistance (reviewed by Hudson *et al*., 2006). The application of a single phage that has a broad host-range for a target bacterial species will eventually lead to the bacterium developing resistance to this phage. The probability of resistance developing to a single phage receptor is greater than resistance developing to an assortment of phage receptors that are used in phage combination treatments. Likewise resistance development would be minimized with the combined use of phages and another antimicrobial agent, such as a sanitizer of antibiotic. Another important feature of using bacteriophages as an antimicrobial agent is that they are able to infect and lyse cells of established biofilms (Hughes *et al*., 1998; Hanlon *et al*., 2001; Doolittle *et al*., 1995).

(ii) Disadvantages of bacteriophages as antimicrobial agents

Due to the presence of non-host bacteria contained in foods that are not sterile, the interaction between the phage and its host may be disrupted (Wilkinson, 2001). A major concern surrounding phages as antimicrobial agents in the food industry is that these infective particles may aid in the transfer of virulence genes from pathogenic bacteria to harmless bacteria (Wagner and Waldor, 2002). This is however a natural process and already exists in food and other environments (reviewed by Hudson *et al*., 2006; Kennedy *et al*., 1986). It may be possible that a phage may follow the lyic cycle within a particular bacterium but may switch to the temperate cycle in another. Since the temperate cycle involves integration of phage DNA into the host genome, virulent genes may be transferred between bacteria.

Infection by virulent phages is generally of short duration, for example, infection of *E. coli* by phage T2 lasts approximately 30 min with rapid virion release upon cell lysis (Hayes, 1974), and they do not incorporate their DNA into the host genome. Prophages can sometimes remain in the lysogenic state for prolonged periods until spontaneous or induced induction has occurred. Following induction of the lytic cycle, the cells are destroyed and the new virion particles are released, however the dangers

of using temperate phages as an antimicrobial alternative, lies in their ability to possibly alter the hosts' phenotype.

(iii) Biofilm susceptibility to bacteriophages

Biofilms were previously thought to be resistant to bacteriophage attack (Corbin *et al*., 2001), however early studies noted the disruption of biofilm morphology by certain bacteriophages (Doolittle *et al*., 1995 Hughes *et al*., 1998) as they were able to penetrate biofilm EPS. This ability is conferred by bacteriophage polysaccharide depolymerase enzymes, which act as surfactants by degrading the polymer chains and thereby reducing EPS viscosity (Hughes *et al*., 1998; Hanlon *et al*., 2001). For example, the bacteriophage SF153b (isolated from sewage) was able to penetrate, infect and lyse *Enterobacter agglomerans* 53b in biofilms (Hughes *et al*., 1998). The bacteriophage was found to possess a polysaccharide depolymerase enzyme that was specific for the EPS of *Enterobacter agglomerans* 53b biofilm cells that were derived from a food processing factory (Hughes *et al*., 1998). Following EPS degradation, the lytic bacteriophage could then reach and infect the bacterial cells, causing them to lyse (Hughes *et al*., 1998). However, when the same bacteriophage preparation was applied to a *Serratia marcescens* Serr biofilm, it was ineffective (Hughes *et al*., 1998). This demonstrated the specificity of bacteriophage glycanases (Hughes *et al*., 1998). Further studies need to assess the use of phages against mixed natural biolfims.

(iv) *In vitro* demonstration of bacteriophages as antimicrobial agents

Recent studies examined the potential use of bacteriophages in preventing/ reducing biofilm formation in medical and food industries. In 2006, Curtin and Donlan demonstrated the use of the lytic *Staphylococcus epidermidis* bacteriophage 456 in reducing *S. epidermidis* 414 biofilm formation on catheters. They pretreated the lumen of a hydrogel-coated catheter with the bacteriophage (10-log-PFU/ml) for 1 hr at 37ºC prior to biofilm formation. The hydrogel provided a means of immobilising the bacteriophage onto the catheter surface (Curtin and Donlan, 2006). Divalent cations were included in bacteriophage pretreatment of catheters as they are often required for adsorption of the bacteriophage to the host cell (Hayes, 1974). Enumeration of viable bacterial counts, following 24 hr phage treatment of the catheter with and without supplementation of $MgCl_2$ and $CaCl_2$, resulted in log

reductions of 4.47 and 2.34 staphylococcal CFU/cm^2, respectively. This study illustrated the potential for lytic bacteriophages in the medical industry for reducing catheter-related infections, such as urinary tract infections, which is of major concern to health care institutions worldwide (Curtin and Donlan, 2006).

With regards to pathogens in the food industry, limited success has been observed with the treatment of biofilms with phage. Sharma *et al* (2004) compared the efficacy of an alkaline sanitizer and a lytic bacteriophage specific for *E. coli* 0157:H7. They formed *E. coli* 0157:H7 biofilms on stainless steel coupons. Successful treatment of *E. coli* 0157:H7 biofilms was observed with 100% alkaline sanitizer (100µg/ml free chlorine pH 11.9) as populations decreased by 5 – 6 $\log_{10}$ CFU/coupon. When coupons with attached cells were treated with phage KH1, reductions of 1.2 $\log_{10}$ CFU/coupon was reported. However, when the phage was applied to established biofilms of *E. coli* 0157:H7, no effect was noted. Reductions associated with phage treatment were not as impressive as those observed with alkaline treatment (Sharma *et al*., 2004).

Disease outbreaks caused by bacterially-contaminated foods, such as fruits and vegetables occur less frequently than those caused by meat, poultry or eggs (Centers for disease control, 1990; Beuchat, 1996). This is said to be due to the peel or rind that provides a protective barrier against the establishment of bacteria on fruits and vegetables (Asplund and Nurmi, 1991; Nguyen-the and Carlin, 1994; Janisiewicz *et al*., 1999). However, fresh-cut fruits and vegetables are susceptible to bacterial contamination, as this protective barrier is removed (Beuchat, 1996). One study noted the persistence of *Salmonella enteritidis* on fresh-cut melon and apples at a range of storage temperatures (Leverentz *et al*., 2001). The study looked at the impact of four lytic bacteriophages on *Salmonella* contaminated fresh cut fruits. Fresh cut apple and melon slices were experimentally contaminated with *Salmonella enteritidis*, followed by treatment with the bacteriophage preparation. The bacteriophages were effective in reducing *Salmonella* populations that were attached on fresh-cut melon, as 3.5 log CFU/g reductions in cell counts were observed. This was twice the reduction than hydrogen peroxide treatments on fresh cut fruits (Liao and Sapers, 2000). This suggested that the bactericidal action of phages may be more effective than certain chemical sanitizers. However, this was not observed on fresh-cut apples as the bacteriophages were thought to be inactivated by the acidic pH of the apples.

Environmental conditions appeared to influence the ability of bacteriophage infection of the surface adhered cells (Leverentz *et al*., 2001). This study indicates the potential of *Salmonella*-specific bacteriophages for sanitation of produce with neutral to alkaline pH, however, it did not investigate the effectiveness of bacteriophages against established biofilms of *Salmonella enteritidis*.

As previously mentioned, bacteriophage treatment may be applied alone or in combination with a sanitizer. For example several lytic bacteriophages were applied in combination with the bacteriocin, nisin to *Listeria monocytogenes* on fresh-cut fruits (Leverentz *et al*., 2003). Comparison of treatments suggested that the combination of phages and nisin was more effective at reducing *L. monocytogenes* populations than sole treatments with phage or nisin (Leverentz *et al*., 2003).

The successful analysis of certain bacteriophages as potential antimicrobial agents has recently resulted in the commercial production of bacteriophage products such as LISTEX™ P100 (http://www.ebifoodsafety.com/en/products-listeria.aspx; ElAmin, 2006a) and LMP-102 (Daniells, 2006). LISTEX™ P100, a product of EBI Food Safety, is a natural (non-genetically modified) product that contains the bacteriophage P100 that is effective against a broad spectrum of *Listeria* strains, and is active at a range of temperatures (7-45˚C), pH range (4-9.5) and high salt conditions (ElAmin, 2006b). The technology behind LISTEX™ P100, which controls *Listeria monocytogenes* in cheese and meat products as well as *Listeria* biofilms on processing equipment, has won EBI Food Safety the Frost & Sullivan award for innovation in food safety (ElAmin, 2006b). LM-102, the FDA approved product produced by the Baltimore-based company Intralytix, targets various *Listeria* strains however, unlike LISTEX™ P100, it comprises six distinct bacteriophages, which reduces resistance development by *Listeria* (Daniells, 2006). Agriphage, another bacteriophage product, recently approved by the US Department of Agriculture (USDA), has been commercially produced for the treatment of *E. coli* 0157:H7 (ElAmin, 2007a). This product is manufactured by OmniLytics and it has a broad application in relation to LISTEX™ P100 and LMP-102, which are used as food additives. Agriphage is used on the hides of live animals prior to slaughtering, as well as for treatment of transportation vehicles, containers and the living quarters or holding area of the animal. This reduces the chance that the meat will become contaminated during processing (ElAmin, 2007a).

In vitro analysis of bacteriophages as potential antimicrobial agents has focused mainly on *Listeria* and *E. coli* populations. Studies have been met with limited success as certain environmental conditions influence their efficacy (Leverentz *et al*., 2001). Few studies have focused on the impacts of different bacteriophages on *Salmonella* populations (Leverentz *et al*., 2001; Higgins *et al*., 2005). It has been shown that *Salmonella*-specific phages can be readily isolated (Carey-Smith *et al*., Poster presentation). We would like to expand on previous studies by assessing the impact of different bacteriophages on relevant *Salmonella* isolates of poultry origin. This study will lay the foundation for future research in determining whether diverse lytic bacteriophages will serve as an alternate means of sanitation against *Salmonella* and other foodborne pathogens in food processing.

RESEARCH QUESTION

Can we isolate different bacteriophages against poultry-associated isolates of *Salmonella*?

OBJECTIVES

- To characterise the poultry-associated *Salmonella* isolates
- To isolate different lytic bacteriophages from poultry farm-associated samples and sewage using different *Salmonella* isolates as sensitive indicator strains.
- To determine the host-range of the different plaque formers.
- To carry out preliminary toxicity studies of the different bacteriophage isolates on planktonic cultures of *E. coli* K12 and *Salmonella* ATCC 13311.

CHAPTER TWO

2. MATERIALS AND METHODS

2.1 BACTERIAL ISOLATES AND BACTERIOPHAGES

The bacterial isolates and bacteriophages used throughout this study are listed in Table 4. Bacteria were cultured in Luria-Bertani (LB) broth and incubated at 37ºC on a Certomat® shaker at 100rpm. Bacterial stocks, containing 15% glycerol were maintained at -20ºC in LB medium.

Table 4: Bacterial isolates and bacteriophages used throughout this study

Isolate	**Source**	**Reference**
Bacteria		
Escherichia coli K12	Laboratory collection	ATCC 29181
Salmonella enterica serovar Typhimurium	Laboratory collection	ATCC 13311
Salmonella isolate 8	Poultry	
Salmonella isolate 16	Poultry	
Salmonella isolate 29	Poultry	Refer to
Salmonella isolate 40	Poultry	legend
Salmonella isolate 47	Poultry	
Salmonella isolate 50	Poultry	
Bacteriophages		
Sewage-associated phages	Cydna Laboratories	N/A
Chicken faeces-associated phages	Battery poultry farm and free-range poultry farm	N/A

All poultry isolates used in this study were previously serotyped (Geornaras and von Holy, 2000; Geornaras, 2000; Geornaras and von Holy, 2001) and were further characterized according to their 16S rDNA similarities.

2.2 SAMPLE COLLECTION AND PREPARATION

Chicken faeces was collected from a free-range and battery poultry farm, located near the Cradle of Humankind in Sterkfontein, Johannesburg. Using gloves, chicken faeces was sampled from the poultry farms and placed into a sterile glass bottle. Sewage, effluent from the Goudkoppies wastewater treatments plant in Johannesburg, was obtained from CYDNA Laboratories, Johannesburg Water (Pty) Ltd.

(i) Sample preparation

The environmental samples were used directly for phage isolation and were also enriched by overnight incubation at 37ºC. By enriching the samples we are initiating a change in the composition, and hence a change in the microbial ecology, of the sample. This may possibly increase the probability of isolating phages that were present in low concentrations prior to the type of enrichment employed. The solid chicken faecal matter was suspended (1:10) in LB medium i.e. an amount of 5g of chicken faeces was suspended in 50ml of LB medium. Sewage was not suspended in 50ml LB medium since it was already in a liquid form, while the faecal matter was not and hence required suspension. Two forms of enrichments were analyzed, both of which involved overnight incubation, however one incorporated divalent ion supplementation of the LB medium. Samples were enriched by supplementation of the LB medium with Mg^{2+} and Ca^{2+} (at a final concentration of 10mM) and were incubated overnight at 37ºC on a labcon Sporn P15 shaker (serial number L25577) at 150rpm. Following incubation, the samples were centrifuged at 6000rpm for 10 min in a Beckman (model J2-21) centrifuge, followed by filtration of the supernatants through sterile Minisart® 0,45µm pore-sized filters (Sartorius AG, www.sartorius.com). The resulting filtrate was used for bacteriophage isolation. The second means of enrichment followed the same procedure discussed above with the exception that no divalent ions were added.

2.3 GROWTH ANALYSIS OF BACTERIAL ISOLATES

The droplet plate technique (Sharpe and Kilsby, 1971; Lindsay and von Holy, 1999) was used to enumerate viable bacteria during 24 hr growth analysis. Primary cultures were incubated overnight for 15 hr and 40 min. The droplet plate technique involved the following. Serial dilutions (1:10) of primary cultures were prepared in 0.85% saline. The dilutions were briefly vortexed and an amount of 50µl was spotted onto LA plates that were segmented to accommodate for the dilutions. LA plates were incubated at 37ºC before CFUs were enumerated. Overnight cultures that were incubated for 15hr, 40min produced approximately 10^9 CFU/ml for all *Salmonella* isolates and approximately 10^8 CFU/ml for *E. coli* K12. These primary cultures were diluted to yield cultures of approximately 10^2 CFU/ml, which were incubated for 24 hr. During this 24 hr period, samples were taken at time 0, 2, 4, 6, 12 and 24 hr. At

these time points cultures were serially diluted in 0.85% saline and were briefly vortexed before 50µl of the dilutions were spotted onto an LA plate. The LA plate was divided into 5 sectors, each representing a specific dilution. LA plates were incubated overnight at 37ºC before CFUs were enumerated.

2.4 CHARACTERIZATION OF BACTERIAL ISOLATES

The bacterial isolates were characterized through identification tests such as the Gram stain, oxidase and oxidation-fermentation (OF) tests. The Gram stain was performed as described by Prescott *et al* (2002). For the oxidase test (Kovacs, 1956), a piece of filter paper was impregnated with 1% *N, N, N', N'*-tetramethyl-*p*-phenylenediamine dihydrochlorinde. Using a sterile toothpick, a fresh colony was scraped from a LA plate and smeared onto the filter paper. A colour change to purple or blue was noted within 30 seconds. The OF test (Hugh and Liefson, 1953) involved two test tubes per bacterial isolate, each of which contained semi-solid OF basal medium (Fermentas Life Sciences, www.fermentas.com) and 1% glucose. Both tubes were stab-inoculated with a bacterial isolate, which was immediately followed by pouring a small volume of mineral oil over the OF basal medium in one of the test tubes to produced anaerobic conditions. The test tube overlaid with mineral oil was labelled "closed" while the tube without oil was labelled "open". OF test tubes were incubated for up to 14 days at 37ºC or until a colour change (positive test) was noted.

2.5 BACTERIAL DNA ISOLATION

- DNA extraction

DNA was extracted from each isolate using a modified boiling method described by Scarpellini *et al* (2004). One colony of each isolate from stock LA plates was boiled for 20 min in 40:l sterile distilled and filtered water plus 20:l chloroform, followed by centrifugation at 15000rpm for 5 min. The supernatant was used as the DNA template during PCR reactions.

- PCR and 16S rDNA sequencing

The primer set that was used for the amplification of 16S rDNA was U1392R (5'-ACG GGCGGT GTG TRC-3'; Lane, 1991; Ferris *et al*., 1996; McGarvey *et al*., 2004) and Bac27F (5'-AGA GTT TGA TCM TGG CTC AG-3'; Inagaki *et al*., 2003;

McGarvey *et al*., 2004). Primers were used in combination with 2X PCR Master Mix (Fermentas Life Sciences, www.fermentas.com), according to the manufacturers instructions, and yielded a product of approximately 1300bp. PCR amplifications were performed using the following conditions: initial denaturation of template DNA at 94°C for 3 min; 35 cycles consisting of denaturation (94°C, 30s), annealing (60°C, 45s), extension (1min 30s, 72°C), and a final extension at 72°C for 7 min. The purified PCR product was sequenced and the resulting sequences were analysed by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) against 16S rDNA sequences from GenBank (GenBank database of the National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov/GenBank/). A homology tree of the isolates was constructed using the neighbour joining method and bootstrapping in DNAMAN version 4 (Lynnon Biosoft).

2.6 BACTERIOPHAGE ISOLATION

Based on 16S rDNA similarities among the bacterial isolates, we selected several of these as indicator strains for bacteriophage isolation. Six bacterial isolates were selected: *E. coli* K12, *Salmonella* ATCC 13311, *Salmonella* isolate 29, *Salmonella* isolate 40, *Salmonella* isolate 47, and *Salmonella* isolate 50. Faecal samples were tested directly for phage isolation as well as following enrichment. The standard plaque overlay technique, also known as the double-agar-layer method (Adams, 1959), was used to enumerate bacteriophages present in the samples. This involved the addition of 1000µl of cell cultures grown to mid-log (6 hr incubation) to 3ml of soft agar (0, 8% agar), followed by the addition of 100µl of sample dilution. The soft agar was mixed briefly before the mixture was poured over a layer of 1% agar in a 9cm Petri dish. The LA plates were left to set for approximately 10 min, and were subsequently incubated overnight at 37ºC.

2.7 BACTERIOPHAGE PURIFICATION AND HOST-RANGE ANALYSIS

Selected plaques, designated the letters A to D, were picked using a sterile pipette tip (1000µl) of which the end was cut to yield a larger lumen. This was used to cut out an agar plug containing the plaque, which was subsequently added to and suspended in

900µl phage buffer (pH 7.8). A serial dilution (1:10) of the suspended plaque was prepared up to a 10^{-6} dilution. The standard plaque overlay technique was used to purify plaques by three plaque passages on their original indicator strains. Following purification of each phage their host-range was determined. This was done by the standard plaque overlay technique as described in bacteriophage isolation.

2.8 TEMPERATE BACTERIOPHAGE CONFIRMATION

Plaques were tested for temperateness by spotting a droplet (10µl) of phage lysate onto a fresh bacterial lawn on LA plates, which were subsequently incubated overnight at 37ºC. Turbid growth of bacteria within the phage spot would indicate phage temperateness. A clear spot with a single of few colonies would suggest that the bacteriophage was most likely following the virulent infection cycle and that the colonies contained within the spot would be indicative of a phage resistant bacterium.

2.9 BACTERIOPHAGE AMPLIFICATION

Purified bacteriophages were amplified using the plate lysate stocks protocol described by Sambrook *et al* (1989). This involved the standard plaque overlay technique which was used to make 30 confluent lysis plates per phage. Phage buffer (pH 7.8) was added to the plates, such that the top agar was just covered with buffer. These were left to stand overnight at 4ºC before the phage buffer was harvested and subsequently centrifuged at 5000rpm at 4ºC in a Beckman centrifuge. The supernatant was recovered, filtered and titred before it was maintained at 4ºC until further use. Titres of phage T4, A and C stocks were approximately 1 x10^{11}, 2.5 x 10^{10} and 4.7 x 10^{9} PFU/ml, respectively.

2.10 BACTERIOPHAGE TOXICITY ANALYSIS

Following amplification of the bacteriophages, serial dilutions (1:10) of phage lysate stocks was made in LB medium. Phage T4 was used for toxicity analysis on *E. coli* K12, while phages A and C were used for toxicity analysis on *Salmonella* ATCC 13311. The toxic effect of phages A and C was observed with their use in combination as well as individually.

Equal volumes of the phage and culture were added together and incubated, e.g. an amount of 4.5ml of phage dilution was added to 4.5 ml of mid-log cultures. Toxicity studies mainly focused on a 1:1 ratio of phage: host (i.e. MOI=1). The phage-host suspension was incubated at 37ºC on a labcon Sporn P15 shaker (serial number L25577) at 150rpm for 2 hr with sampling occurring every 15 min. The droplet plate technique was used to enumerate CFU/ml following exposure to phages at various time points. Toxicity studies with phage T4 included supplementation of LB medium with Mg^{2+} and Ca^{2+} ions, as these are required for phage T4 adsorption to *E. coli* K12. Toxicity studies with phages A and C were conducted for 2 hr with sampling occurring every 30 min and no divalent ion supplementation was done. Phage-free cultures were used as controls throughout the toxicity studies.

CHAPTER THREE

3. RESULTS

This study utilized sewage and chicken faecal samples for bacteriophage isolation. Chicken faeces was collected from free-range and battery chicken farms. The chickens from the free-range poultry farm were housed in large enclosures where the earth was covered with sawdust to cohere the excretory products (Figure 4 A to D, pp. 41). Fresh excretory products were sterilely collected for this study. The chickens were in good health and no antibiotics supplemented their feed. The battery farm employed a two-story building, with the upper level housing the chickens in a battery system (Figure 5 A and B, pp. 42) and the lower ground level containing all the excretory products (Figure 5 C, pp. 42), which was used during this study. The battery chickens appeared unhealthy as they lacked feathers in certain bodily areas (Figure 5 D, pp. 42). The battery chicken feed was also devoid of antibiotics.

3.1 CHARACTERIZATION OF THE POULTRY-ASSOCIATED *SALMONELLA* ISOLATES

3.1.1 Growth analysis of bacterial isolates

Prior to bacteriophage isolation from the sewage and chicken faecal samples, the bacterial isolates were characterized. Studies have shown that increased yields of bacteriophages have been isolated from sewage when mid-log cultures are used as opposed to lag or stationary cultures (Havelaar and Hogeboom, 1983). This is due to the expression of certain receptor sites during the logarithmic growth phase, e.g. the receptor sites targeted by male-specific F-RNA coliphages occur on the sex fimbriae of *E. coli* which are only expressed during the logarithmic phase (Havelaar and Hogeboom, 1983). Since we wanted to maximize bacteriophage yields on the various isolates, we needed to identify their mid-log growth phases. This was done at standard laboratory conditions using the droplet plate technique. Bacteria were enumerated every 2 hr for the first 8 hr, followed by the 12th and 24th hr of the growth study. CFU/ml were enumerated following overnight incubation of the droplet plates. The resulting growth patterns of the bacterial isolates are depicted in Figure 6 (pp. 43). The bacterial isolates exhibit similar sigmoidal growth patterns over 24 hr, with the mid-log phase occurring after approximately 6 hr.

3.1.2 Identification tests

In addition to analyzing the growth profiles of the bacterial isolates, we characterized them according to the Gram stain, oxidase and oxidation-fermentation (OF) tests. All of the bacterial isolates were identified as Gram-negative, straight rods that tested positive for oxidation and fermentation of glucose and negative for cytochrome C oxidase. These are characteristics of Enterobacteriaceae.

3.1.3 16S rDNA analysis

Since the poultry-associated isolates were previously serotyped (Georgnaras and von Holy, 2000; Georgnaras, 2000; Georgnaras and von Holy, 2001), we further characterized them by sequencing their 16S rDNA loci, which are highly conserved among bacteria. DNA was extracted from a colony of each isolate, followed by amplification of the 16S rDNA segments. The purified PCR products were resolved by agarose gel electrophoresis (Figure 10, pp. 74). A 1300bp PCR product was conserved among the isolates. The PCR products were subsequently sequenced and compared to sequences within Genbank of the NCBI database by BLAST analysis. From the output sequences and our original query sequences a homology tree (Figure 7, pp. 43) was constructed.

For the bacteriophage experiments we selected *E. coli* K12 and *Salmonella* ATCC 13311 as the standard laboratory strains. In addition to these we selected the outliers of the homology tree, *Salmonella* isolates 29 and 50, as they were the least identical (87%) of the isolates and possibly the most distantly related. *Salmonella* isolates 40 and 47 were also selected as they were >90% identical to the selected *Salmonella* ATCC 13311 (Figure 7, pp. 43).

3.2 ISOLATION OF BACTERIOPHAGES FROM POULTRY FARM-ASSOCIATED SAMPLES AND SEWAGE USING DIFFERENT *SALMONELLA* ISOLATES AS INDICATOR STRAINS

Chicken faeces, which were obtained from a battery and free-range poultry farm, and sewage, effluent from a sewage treatment plant, were used for bacteriophage isolation. In order to assess which sample was the best source of *Salmonella*-specific phages we analyzed them directly and tested whether enrichment of the samples increased the yield of phages. Standard plaque overlays were carried out on the various indicator strains and PFU/ml were enumerated following overnight incubation of plates at 37ºC. The PFU/ml isolated from the samples are depicted in Table 5 (pp. 44).

- Direct isolation:

No plaques were observed on the poultry-associated isolates or on *Salmonella* ATCC 13311. Plaques were isolated on *E. coli* K12. Direct isolation did not yield *Salmonella*-specific phages and since our focus was on the isolation of these phages, we investigated whether enriching the samples would increase their yield on the *Salmonella* isolates.

- Divalent cation supplementation and O/N incubation:

The samples were supplemented with Mg^{2+} and Ca^{2+} ions as certain phages such as phage T4 are dependent on divalent cations for infection (Hayes, 1974; Havelaar and Hogeboom, 1983). These samples were enriched by overnight incubation at 37ºC. Temperature is another environmental factor that affects bacteriophage isolation. Incubation at 37ºC selects for phages that adsorb and replicate optimally at body temperature, e.g. phages that infect bacteria of the intestinal flora in humans and animals (Grabow, 2001).

Similarly to direct phage isolation, no plaques were observed on any of the poultry-associated isolates. Plaques were however isolated on *E. coli* K12 and *Salmonella* ATCC 13311, with the exception that the battery chicken faeces did not contain phages capable of producing plaques on the latter. The phages that were isolated on *Salmonella* ATCC 13311 required Mg^{2+} and Ca^{2+} ions for infection of this isolate. Since we were interested in phages that were able to infect the poultry-associated

isolates, we repeated sample enrichment by overnight incubation but without divalent ion supplementation.

- O/N incubation

As seen with the direct and enriched (divalent ion supplementation) samples, plaques were isolated on *E. coli* K12. The sewage sample that was enriched by overnight incubation produced plaques on *Salmonella* ATCC 13311, and two of the poultry-associated isolates, namely *Salmonella* isolate 29 and *Salmonella* isolate 40. The sewage sample that was enriched by O/N incubation was the best source of *Salmonella*-specific phages. These *Salmonella*-specific phages did not require divalent cations for infection.

With regards to the diversity of *Salmonella*-specific phages isolated from enriched (O/N incubation) sewage, 100 % appeared as clear plaques on *Salmonella* ATCC 13311 and on *Salmonella* isolate 40, while 100% appeared turbid on *Salmonella* isolate 29. Comparison of *Salmonella*-specific phages with regards to plaque morphology identified clear pinprick-sized plaques on *Salmonella* isolate 40 (Figure 11, pp. 75) and small turbid plaques, larger than the pinprick-sized plaques, on *Salmonella* isolate 29 (Figure 11, pp. 75). Plaques isolated on *Salmonella* ATCC 13311 appeared 1mm (74%) or <1mm (26%) in diameter. The turbid plaques isolated on *Salmonella* isolate 29 suggest a temperate infective cycle, and the clear plaques isolated on *Salmonella* ATCC 13311 and *Salmonella* isolate 40 suggest a virulent infective cycle. Further bacteriophage studies focused on these *Salmonella*-specific phages.

3.3 HOST-RANGE ANALYSIS OF THE DIFFERENT PLAQUE FORMERS

Following plaque morphology examination (results not shown), we selected four distinct *Salmonella*-specific phages, namely phages A to D. These were picked and plaque-passaged in triplicate before their host-range was determined by the standard plaque overlay technique. The host-range of the four phages is presented in Table 6 (pp. 45). Phages A and B were isolated on *Salmonella* ATCC 13311, C was isolated on *Salmonella* isolate 29 and D was isolated on *Salmonella* isolate 40.

Phages A and B were able to infect and lyse *E. coli* K12 and *Salmonella* ATCC 13311. Phage C infected and lysed *E. coli* K12, *Salmonella* ATCC 13311 and *Salmonella* isolate 29, while the host-range of phage D included *E. coli* K12 and *Salmonella* isolate 40. Interestingly, phage C displayed temperate activity on *Salmonella* isolate 29 as turbid plaques were observed, however on *Salmonella* ATCC 13311 it displayed virulent activity as clear plaques were observed. Since we wanted to determine the individual and combined toxic impacts of two different *Salmonella*-specific phages, we selected phage A and phage C, as their host-ranges differed from one another and they appeared to follow a virulent cycle on *Salmonella* ATCC 13311. Variations in the host-ranges of phages A and C could be attributed to several explanations, one such example may be the presence or absence of restriction modification systems.

3.4 TOXICITY STUDIES OF THE DIFFERENT BACTERIOPHAGE ISOLATES ON PLANKTONIC CULTURES OF *E. COLI* K12 AND *SALMONELLA* ATCC 13311

Before we conducted toxicity studies with the *Salmonella* sewage-associated phages, a toxicity protocol was established using the well-characterized virulent phage T4 and *E. coli* K12 model. The toxicity study with phage T4 involved incubating equal volumes of phages and mid-log cultures at an MOI of 1 and 0.1. The phage-host mix was incubated and we sampled periodically from this. Serial dilutions were made to minimize contact between the phage and host and these were plated using the droplet plate technique. Following O/N incubation of plates we enumerated CFU/ml.

The 2 hr toxicity profile of phage T4 on *E. coli* K12 at an MOI of 1 and 0.1 is illustrated in Figure 8 i. Phage T4 resulted in 3.2 x 10^3 CFU/ml and 3.9 x 10^3 CFU/ml reductions in *E. coli* K12 at an MOI of 1 and 0.1, respectively. The reduction by phage T4 at an MOI of 1 occurred 30 min before the reductions observed at an MOI of 0.1. This is expected as there are more phages available for infection at a higher MOI. There is no significant difference between the reductions observed with each MOI however, the time-frame for the reductions differed by 30 min. No further reductions occurred after 60 min, as there was a slight increase from 1.6 x 10^3 CFU/ml to 3.2 x 10^3 CFU/ml.

The individual toxic effects of phages A and C on *Salmonella* ATCC 13311 were next determined. These studies were conducted over 6 hr and focused on an MOI of 1. Results are depicted in Figure 8 ii. At 180 min phages A and C resulted in 5 x 10^5 CFU/ml and 1.6 x 10^5 CFU/ml reductions in *Salmonella* ATCC 13311, respectively. Reductions were first observed with Phage C, as can be seen at time point 150 min, where phage C reduced *Salmonella* ATCC 13311 by 3 x 10^1 CFU/ml as opposed to the reduction of 1.3 CFU/ml that was observed with phage A. Following 180 min *Salmonella* ATCC 13311 increased from approximately 1.3 x 10^2 CFU/ml to 4 x 10^3 CFU/ml. The toxicity profiles of phages A and C are similar to phage T4 which further suggests that these phages are non-lysogenic. The re-ascend following the 10^2 CFU/ml reductions is possibly due to the population of *Salmonella* ATCC 13311 that may have acquired resistance to these phages.

We next determined whether the combined use of phages A and C would result in a further decline than those observed with their individual applications. The toxic impact of phages A and C, in combination, on *Salmonella* ATCC 13311 is illustrated in Figure 8 iii. A similar toxicity profile exists between the individual and combined applications of phages A and C. The individual studies demonstrate that phage C caused a 7.9 x 10^4 CFU/ml reduction in *Salmonella* ATCC 13311, approximately 30 min before the 4 x 10^5 CFU/ml reduction observed with phage A. With the combined use of phages A and C a 1.6 x 10^5 CFU/ml reduction in *Salmonella* ATCC 13311 was observed. During the first 180 min, the profile of the combination study appears to follow the same profile as phage C. Following reductions in *Salmonella* ATCC 13311 cells increased to approximately 10^5 CFU/ml.



Figure 4: The free-range poultry farm.



Figure 5: The battery poultry farm.

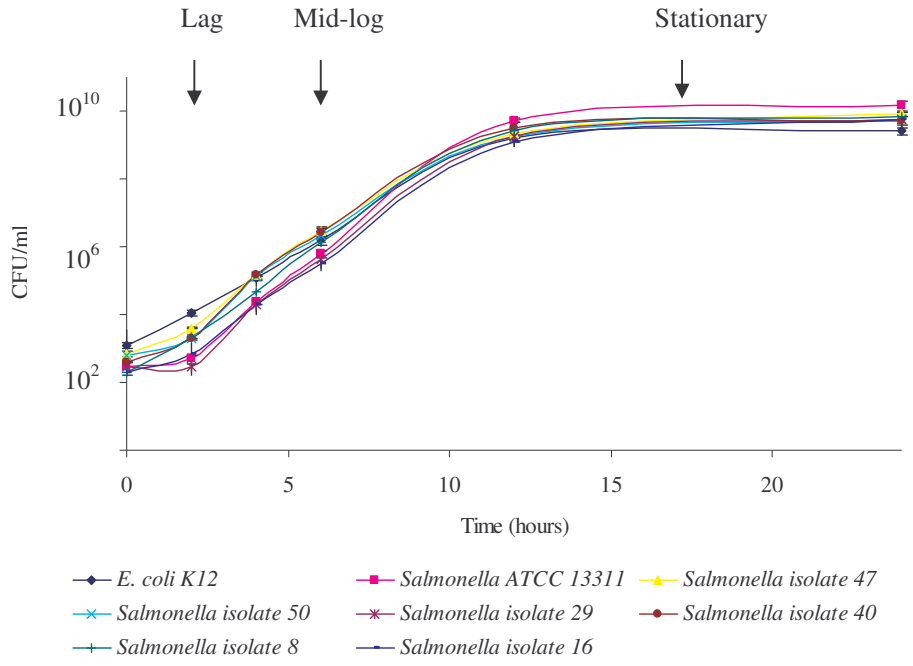

Figure 6: Growth analysis of the bacterial isolates. Data is expressed as mean ± S.D. and were obtained from duplicated growth experiments.

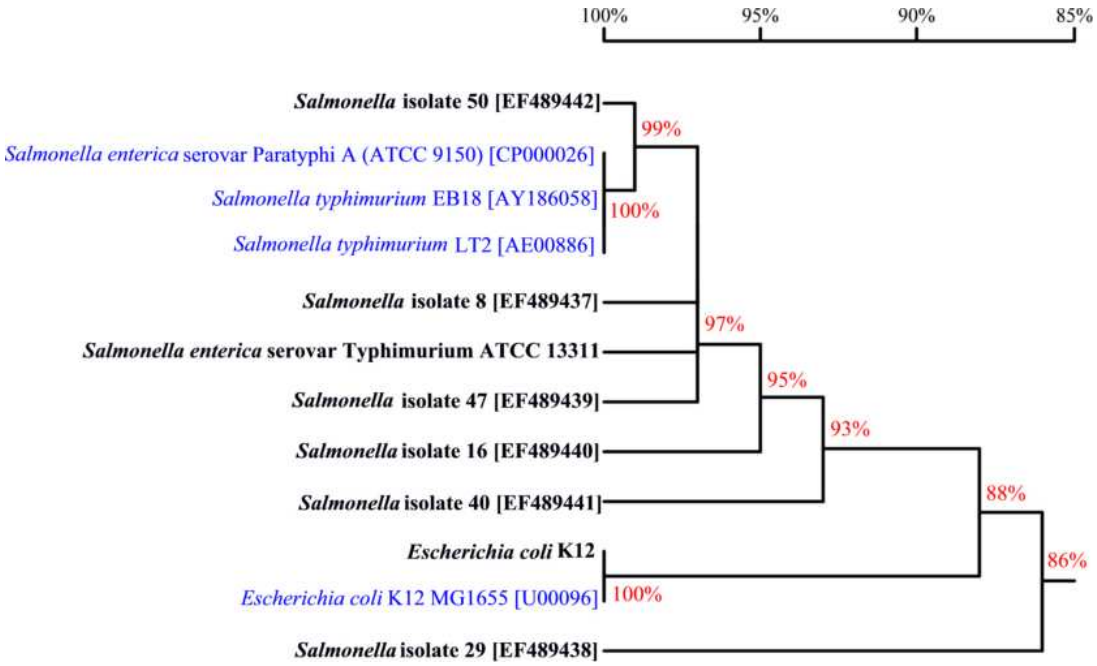

Figure 7: Homology tree of bacterial isolates based on 16S rDNA sequences.
Black bold type refers to bacterial isolates used in this study; blue type refers to their corresponding bacterial counterparts from Genbank of the NCBI database. Accession numbers are in square brackets. The homology tree was constructed with the suite of programmes in DNAMAN version 4 (Lynnon Biosoft).

Table 5: PFU/ml counts isolated from samples that were used directly or following enrichment, on *E. coli* K12, *Salmonella* ATCC (13311) and various poultry-associated *Salmonella* isolates.

Enrichment method	**Sample**	**Bacterial Isolate**					
		E. coli K12	*Salm.* ATCC (13311)	*Salm.* 29	*Salm.* 40	*Salm.* 47	*Salm.* 50
Mg^{2+}, Ca^{2+} & overnight incubation	FF	5.1×10^6	1.2×10^3	/	/	/	/
	BF	2.7×10^5	/	/	/	/	/
	SS	1.8×10^7	2.4×10^3	/	/	/	/
Overnight incubation	FF	1.8×10^7	/	/	/	/	/
	BF	9×10^4	/	/	/	/	/
	SS	8.6×10^6	2.9×10^5	5.6×10^2	4.4×10^3	/	/
Un-enriched	FF	2.8×10^3	/	/	/	/	/
	BF	5×10^4	/	/	/	/	/
	SS	2.7×10^2	/	/	/	/	/

FF denotes free-range chicken faeces; BF denotes battery chicken faeces; SS denotes sewage; / no plaques.

Table 6: Host-range of phages A to D.

Phage	Plaque description	Bacterial isolate			
		E. coli K12	*Salmonella* ATCC 13311	*Salmonella* isolate 29	*Salmonella* isolate 40
A	1 mm clear	○	●	—	—
B	<1mm clear	○	●	—	—
C	1mm	○	○ (clear)	● (turbid)	—
D	<1mm clear	○	—	—	●

● indicates the original strain on which the phage was isolated on; ○ indicates the host-range of the phage. Phages A to D were isolated from sewage that had undergone overnight incubation.

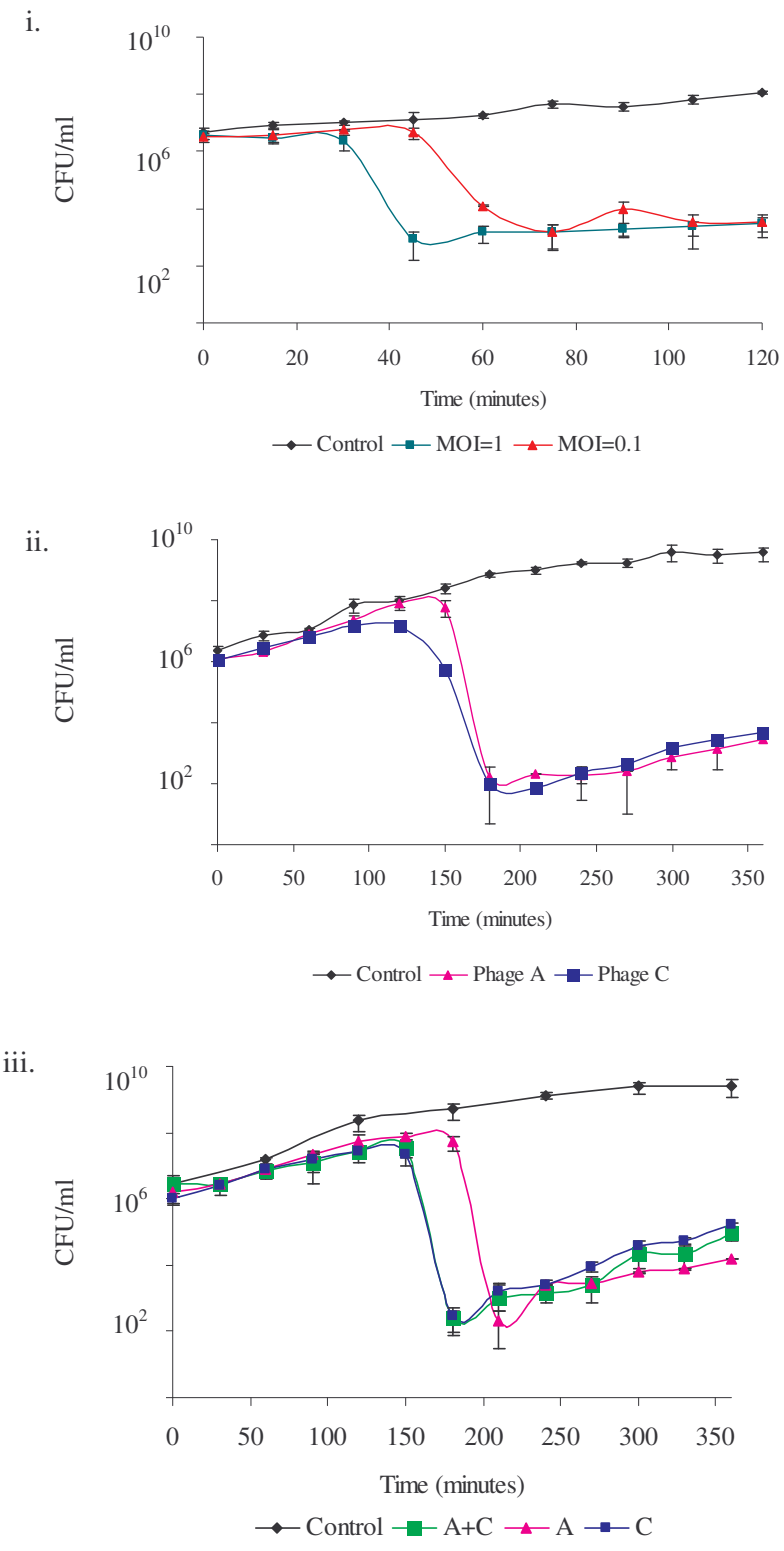

Figure 8: Phage toxicity studies. i. Phage T4 and *E. coli* K12; ii. Phages A and C; iii. Combined effects of phages A and C on *Salmonella* ATCC 13311.

CHAPTER FOUR

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4. DISCUSSION AND CONCLUSION

Two different *Salmonella*-specific bacteriophages (A and C) were isolated from human sewage effluent. Their infective cycles were not dependent on divalent cations and they were able to infect and lyse more than one *Salmonella enterica* isolate. These phages exhibited virulent behavior towards *Salmonella* ATCC 13311 and caused significant reductions of approximately 1 x 10^5 CFU/ml within the first 180 min of application. The individual and combination treatments of these phages displayed similar toxicity profiles on *Salmonella* ATCC 13311. The combined application did not produce more effective reductions in the *Salmonella* population and phage-resistant mutants prevailed following both applications.

4.1 BACTERIOPHAGE ISOLATION

Coliphages were continually isolated from all faecal samples, irrespective of whether they were used directly or following enrichment. Coliphages appeared to outnumber *Salmonella*-specific phages. The number of plaque counts isolated on *E. coli* K12 was on average two times higher than those isolated on the susceptible *Salmonella* isolates. This is consistent with Havelaar *et al* (1986) as they found on average a two to four times higher plaque yield on *E. coli* CN compared to those isolated on *Salmonella enterica* serovar typhimurium phage type 3. Both studies isolated phages from animal and human faecal material. Our study found that human sewage was the best source for *E. coli* and *Salmonella*-specific phages when compared to those isolated from chicken faeces. This is in contrast to the Havelaar *et al* (1986) study as they demonstrated significantly higher plaque counts from broiler chicken faeces compared to human sewage on both indicator strains. Variations in phage yield may be attributed to variations in culturing conditions, the type of indicator strains used and the age of the cultures (Havelaar and Hogeboom, 1983).

The environmental poultry isolates, which were previously serotyped (Georgnaras and von Holy, 2000; Georgnaras, 2000; Georgnaras and von Holy, 2001), seemed less sensitive to phage infection from chicken faeces. The culturing conditions examined may not have been optimal for phage infection. Another possibility is

that the *Salmonella* isolates, which originate from natural *Salmonella* populations (Georgnaras and von Holy, 2000; Georgnaras, 2000; Georgnaras and von Holy, 2001), evolved resistance to poultry-associated phages. This is based on the coevolutionary arms race that is said to exist in natural communities between bacteria defences and phage counter defences (Lenski and Levin, 1985; Weitz *et al*., 2005). This is further supported by the observation that phages were isolated on the laboratory strains, *Salmonella* ATCC 13311 and *E. coli* K12, and seldom on the environmental strains. This is consistent with Lenski and Levin (1985) as they demonstrated that bacteria, recently isolated from natural populations, are usually resistant to phages when compared to laboratory strains that are susceptible to phage infection. This is because laboratory stains have been cultured and stored under highly controlled environments for extended periods in the absence of phage (Lenski and Levin, 1985). The environmental stains are thus more robust than the laboratory stains and hence they evolved resistance against phage attack.

Since we attempted to isolate phages from the natural environment of the relevant *Salmonella* isolates, the presence of phages within this community would have generated selective pressures, and hence the isolates would have developed mutations, possibly in their receptor sites. By failing to produce the phage receptor sites or by changing the configuration of these, the isolates would be resistant to phage attack (Lenski and Levin, 1985). Other resistance mechanisms include restriction modification systems, abortive infection (Abi) systems, both of which act post-infection, and inhibition and injection blocking which act pre-infection (reviewed by Petty *et al*., 2006). Resistance would prevent phage replication and hence their concentration in the poultry samples would be too low for detection (Lenski and Levin, 1985).

Human sewage effluent was the best source of *Salmonella*-specific phages. The *Salmonella*-specific phages were isolated from sewage that had been enriched by overnight incubation in the absence of divalent cations. There are various types of enrichments and factors such as ion concentration, temperature, and organic

compound concentration, the association of host bacteria and phages to solids, and the densities of host bacteria and phages influence the survival and incidence of phages (Grabow, 2001). For example, the presence of organic matter may cause alterations in the metabolic activities of host bacteria, which consequently influences the replication of phages (Goyal *et al*., 1980; Grabow, 2001). Furthermore, cations such as Ca^{2+} and Mg^{2+} are required by certain phages for adsorption to host bacteria (Hayes, 1974; Havelaar and Hogeboom, 1983).

The incorporation of certain factors into the culturing environment of phages and bacteria may generate favourable or unfavourable conditions for phage infection, and generally selects for those phages that optimally replicate under the specific conditions. The *Salmonella*-specific phages isolated on the environmental *Salmonella* isolates did not require divalent cations for adsorption but required 37ºC for infection of the susceptible *Salmonella*. Secondly, these phages were isolated from an unrelated source to that of the poultry isolates, and hence the lack of phage resistance.

4.2 HOST-RANGE OF PHAGES A TO D

When individual plaques of selected phages were passaged for purification, they yielded plaque morphologies that were not consistent, as they varied in size. A possible explanation for this is variable adsorption rates of the phage to the host. This would cause infection cycles to start at different times after being poured on the LA plate during the standard plaque overlay technique. Variations in plaque morphologies do not confidently distinguish between different phages. Previous work has relied on this for phage differentiation (Higgins *et al*., 2005) but as indicated in this study it should be avoided. Molecular characterization of phages or variations in their host-range are more reliable for differentiating between phages.

Phages A to D were able to infect more than one isolate. *E. coli* K12 was susceptible to infection by all phages, which may reflect its intermediate position on the homology tree. Extended host-range of phages may be explained by the presence of a single antireceptor on the surface of the phage capsid, which targets

a specific receptor that may be conserved among the various host envelopes. For example, phage A may specifically recognise a particular receptor that is conserved between *E. coli* K12 and *Salmonella* ATCC 13311. Alternatively, the phage may produce two different antireceptors, each of which targets a specific receptor on the host envelope. For example *E. coli* K12 and *Salmonella* ATCC 13311 have different cellular receptors and phage A produces two different antireceptors each of which is specific for the particular host receptor. Consider the virulent phage ΦK1-5, isolated from raw sewage (Scholl *et al*., 2001). The genes encoding the tail fibres of this phage comprise two open reading frames for hydrolytic tail fibre proteins that permit infection of *E. coli* strains that have either the K1 or the K5 polysaccharide capsule (Scholl *et al*., 2001). When ΦK1-5 expresses the K5 lyase protein it is able to specifically infect K5 strains of *E. coli*. Once the second endosialidase-like protein is expressed, infection of K1 strains of *E. coli* occurs (Scholl *et al*., 2001). These tail fibre proteins are said to be independently inactivated and hence they play an integral role in coevolving with the specific *E. coli* strain.

Most likely, phages A and C are host-range mutants that have different specificities when exposed to different hosts. Mutational variations in phage antireceptors are advantageous to phages as they expand their host-range. This has been demonstrated by Tétart *et al* (1996), as they isolated host-range mutants of phage T4 that were able to infect *Yersinia pseudotuberculosis* following duplications of a small domain of the T4 tail fibre adhesion. Another example is phage Ox2 which targets the outer membrane protein OmpA on the surface of *E. coli* K12 (Morona and Henning, 1984). The outer membrane proteins OmpA, OmpC and OmpF are generally very different with regards to their structures and functions (Morona and Henning, 1984). Morona and Henning (1984) isolated phage Ox2 that was able to infect OmpA mutants of *E. coli*. It appeared as though Ox2 was recognizing another outer membrane protein. They found that a region within the amino acid sequence of OmpA was homologous to OmpC of T4 susceptible *E. coli*. Phage Ox2 was able to utilize both OmpA and OmpC proteins. Similarly, Moreno and Wandersman (1980) demonstrated that coliphage TuIa,

which used the OmpF protein as its receptor, was able to mutate and target the OmpC or LamB proteins.

Since phages A and C appeared different with regards to their host-range and that they displayed lytic activity towards *Salmonella* ATCC 13311, they were selected for toxicity studies.

4.3 BACTERIOPHAGE TOXICITY ANALYSIS

Before phage A and C toxicity studies were conducted, the toxicity study was optimized using the well-characterized model system of virulent phage T4 and *E. coli* K12. A significant reduction was observed within the first 60 min and thereafter no further decline occurred. This suggests that the remaining population developed resistance to phage T4. A similar result was observed by Mizoguchi *et al* (2003). This group conducted chemostat studies with *E. coli* O157:H7 and the virulent phage PP01. Similarly, phage-resistant mutants occurred following a dramatic reduction in cell concentration. During phage challenge of *E. coli* O157:H7, Mizoguchi *et al* (2003) isolated several phage-resistant mutants. Biochemical analysis of these mutants identified variations in lipopolysaccharide composition and alterations in OmpC production (Mizoguchi *et al*., 2003). These are required, among other envelope structures, for PP01 adsorption and infection (Kudva *et al*., 1999; Ho and Slauch, 2001; Morita *et al*., 2002). This further emphasizes a mutational evolution between a phage and its host, and that survivors will dominate the population (Lenski and Levin, 1985; Mizoguchi *et al*., 2003).

Similar toxicity profiles were observed with phages A and C. A significant reduction occurred during 180 min and thereafter no further decline occurred. Since phage A and C toxicity profiles were consistent to that of phage T4, their infective cycles were confirmed to be virulent. The persistence of cells following reductions, as demonstrated with the phage T4 study, suggest the presence of phage-resistant mutants which dominate the population. Phage C resulted in similar reductions to phage A but these occurred earlier to the latter. The delay in

cell lysis by phage A suggests that phage C may have a more rapid adsorption rate to the cell, or perhaps the burst size of phage A is less than that of phage C. A previous phage study conducted by O'Flynn *et al* (2006) investigated bacterial challenge by the virulent *Salmonella*-specific phages Felix01, st104a and st104b. They demonstrated a 2 hr delay in cell lysis by Felix 01 when compared to individual and combination phage treatments.

The combination study with phages A and C also resulted in reductions during 180 min. The combined toxicity profile followed the profile of the individual study of phage C. The combined and individual studies were equally effective at reducing the *Salmonella* ATCC 13311 population, which suggests similarities in phage A and C infective mechanisms. We have proposed a model for why the combination study trails the phage C toxicity profile (Figure 9).

The difference in host-range of phages A and C is seen with *Salmonella* isolate 29, which is resistant to phage A. The individual applications of phages A and C resulted in the same profile with the exception that the time-frame for reductions caused by phage C were shorter than those for phage A. With the combined use of phages A and C a profile that was almost identical to that of individual phage C applications was observed. This suggests that during combination studies phage C out competed phage A for the host receptor site. Since the host-range of phages A and C differ from one another we propose that *E. coli* K12, *Salmonella* ATCC 13311 and *Salmonella* isolate 29 possess variations of the same receptor on their envelopes, and that phage A is unable to recognise the receptor variant on *Salmonella* isolate 29. Secondly, since phage A was isolated on a laboratory *Salmonella* strain, while phage C was isolated on an environmental strain of *Salmonella*, and because phage C resulted in earlier reductions than phage A, phage C appears to be more robust than phage A.

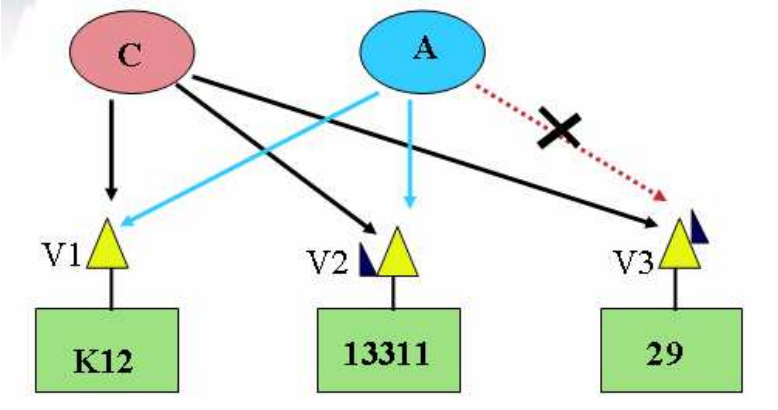

Figure 9: Proposed model of receptor usage by phages A and C.
V1 to V3 represents receptor variants.

In all phage applications phage resistant mutants prevailed. This was also demonstrated by O'Flynn *et al* (2006) since the toxicity studies conducted by this group demonstrated that no further reduction in *Salmonella* counts (initial concentration of 1 x 10^5 CFU/ml) occurred beyond the 1 x 10^2 CFU/ml point. In addition to conducting toxicity tests, they also determined the frequency of phage resistant mutants which were on average 5 x 10^{-4} CFU/ml with the application of phages st104a and st104b and 2 x 10^{-6} with the control phage Felix01. The frequency of phage resistant mutants can thus not be determined directly from toxicity assays and hence frequency tests should be determined separately.

This study isolated two different *Salmonella*-specific bacteriophages, phages A and C, from human sewage effluent. Both phages displayed virulent behaviour towards *Salmonella* ATCC 13311, and caused a reduction of 1 x 10^5 CFU/ml in planktonic cell counts during 180 min exposure, when examined individually or in combination. Phages A and C were thus 99.999% effective at reducing planktonic *Salmonella* ATCC cell counts. This is the percentage efficacy that should be achieved with an ideal sanitizer, as demonstrated with Iodophor and peracetic acid/hydrogen peroxide combination sanitizers, which resulted in 99.999% reductions in planktonic cell counts of *P. fluorescens* following exposure for 1 min (Lindsay and von Holy, 1999). The duration of phage exposure that is required for a 5 log unit reduction is far more than that of sanitizers, however, the

reductions observed in this study makes their use favourable as alternative antimicrobial agents. In addition to this, the specificity of phages favours their antimicrobial application against pathogens. This study also highlighted a number of problems that may compromise the application of phages as novel antimicrobial agents. The formation of phage resistant mutants makes their application ineffective at eliminating the bacterial pathogen. Analysis of combination treatments that comprise lytic phage and another antimicrobial agent such as antibiotics or sanitizers may lead to more significant reductions in bacterial counts. The observation that a particular phage may follow the lytic and lysogenic infective modes in two different hosts further discourages their use in the field as new pathogens may arise from transduction. Rigorous assessments of these issues need to be addressed in future work before their practice can be considered.

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APPENDICES

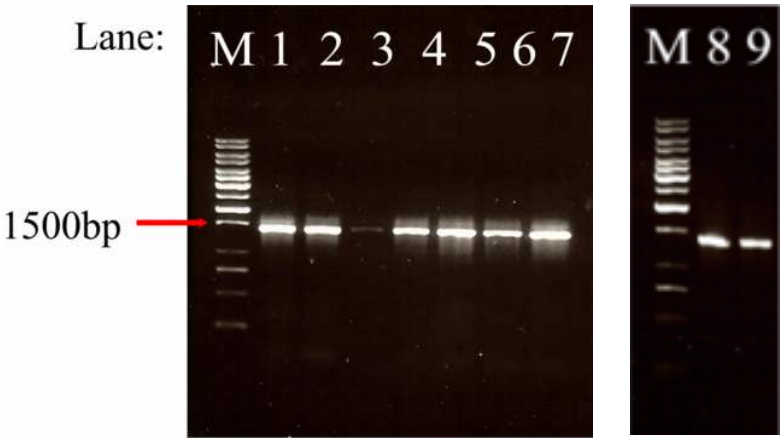

Figure 10: Ethidium-bromide-stained 0.8% agarose gels displaying amplification products obtained from Bac27F and U1392R primers. Lanes: M, 1Kb DNA ladder; 1, *E. coli* K12; 2, *Salmonella* ATCC 13311; 3, *Salmonella* 8; 4, *Salmonella* 16; 5, *Salmonella* 29; 6, *Salmonella* 47; 7, *Salmonella* 50; 8, *Salmonella* 8 (repeat); 9, *Salmonella* 40.

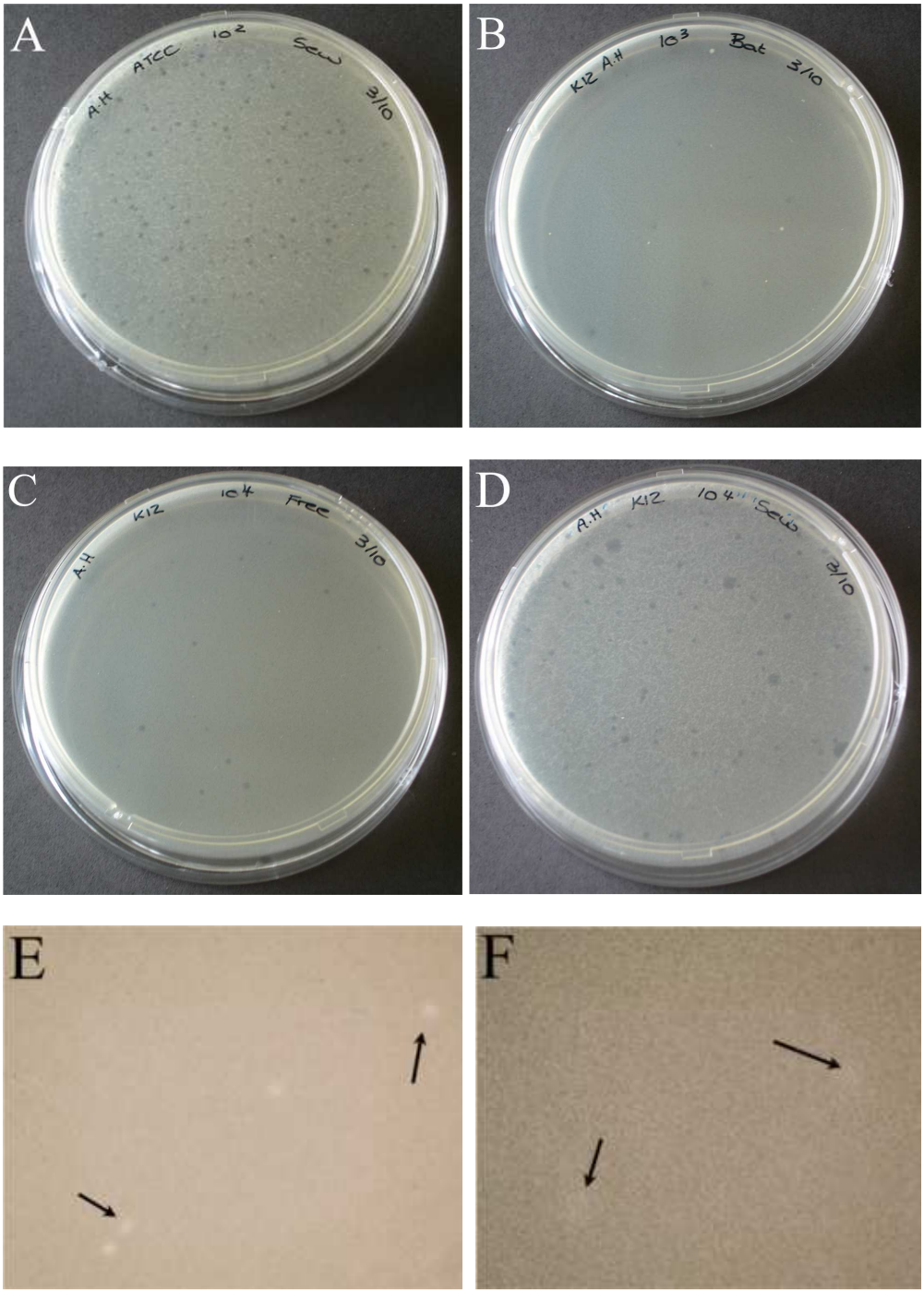

Figure 11: Plaques isolated from samples that were enriched by overnight incubation on (A) *Salmonella* ATCC 13311 (SS sample), (B) *E. coli* K12 (BF sample); (C) *E. coli* K12 (FF sample); (D) *E. coli* K12 (SS sample). (E) and (F) indicate plaques, as seen under a dissection microscope, that were isolated from sewage on *Salmonella* isolate 40 and *Salmonella* isolate 29, respectively.

Solutions

pH Solutions

- Concentrated HCl (12M)

1M HCl (100ml)
8, 3 ml concentrated HCl and 91, 7ml sterile distilled H_2O

- Concentrated NaOH (19M)

10M NaOH (100ml)
52,6ml NaOH concentrate and 47, 4ml sterile H_2O

Bacteriophage isolation and purification

Bacteriophage Buffer pH 7.8

1mM Tris 0,12g
1mM Mg_2SO_4 0,12g
6.8mM NaCl 0,4g
1mM $CaCl_2$

For 1000ml solution:
0,12g Tris
0,12g Mg_2SO4
0,4g NaCl

The above reagents were dissolved and pH to 7.8 in 500ml distilled water before the volume was increased to 999ml. This was followed by sterilisation at 121ºC for 20 min. Once the sterilised solution had cooled an amount of 1000µl of 1M $CaCl_2$ stock was added to give a final concentration of 1mM.

Transmission Electron Microscopy stain

1% Uranyl Acetate in 45 % ethanol

For 10ml Uranyl Acetate stain:
0,1g Uranyl Acetate
4,5ml Absolute Ethanol
5.5ml distilled water

The stain was prepared in falcon tube which was covered in tin foil so as to prevent light from coming in contact with the solution, as uranyl acetate is photo sensitive. The solution was stored at 4ºC.

Bacterial cultivation

Luria-Bertani Agar (100 ml)

1 g Tryptone
0, 5 g NaCl
0, 5 g Yeast Extract
1 g Bacteriological agar
100ml distilled water
Autoclave at 121º for 20 min

Luria-Bertani Broth (100 ml)

1 g Tryptone broth
0, 5 g NaCl
0, 5 g Yeast Extract
100ml distilled water
Autoclave at 121º for 20 min

Molecular Analysis

TBE (5x) pH 8.0 (500 ml)

27 g Tris
13, 7 g Boric Acid
10 ml EDTA (0, 5 M)

The above reagents were dissolved in 250ml distilled water. Once dissolved, which occurred after the pH of the solution was achieved, the volume was increased to a total volume of 500ml before autoclaving at 121ºC for 20 min.

To make 0,5x TBE (500ml):
50ml of 5x TBE in 450ml distilled water

EDTA (0, 5 M) pH 8.0 (100 ml)

14, 6 g EDTA to 100 ml sterile distilled H_2O

Agarose gel

0, 8% Agarose E.g. 0,4g Agarose in 50ml TBE (0,5x)

Microwave and swirl the solution intermittently until the agarose has dissolved. Allow to cool until it is comfortable to touch. Add 1µl Ethidium Bromide (EtBr) and mix by swirling the bottle. Pour this into a gel tray, position the comb and let set.

Bacterial Characterisation

Gram stains

- Crystal violet

5g Crystal violet per 1000ml distilled water

- Iodine

10g of Iodine
20g of Potassium Iodide (KI)
1000ml distilled water

Dissolve the KI and Iodine in 250ml water. Once dissolved the volume is increased to 1L with distilled water.

- Safranin (1%)

1g Safranin in 99ml distilled water

- Decolouriser

70% Acetone
30% Alcohol (100% or 95%)

For a 300ml solution:
210 ml Acetone with 90ml Alcohol

Oxidase test reagent

1% (w/v) tetra methyl p-phenylene diamine dihydrochloride

For a 10ml solution:
0,1g in 10ml distilled water

OF test medium (1000ml)

9,4g OF medium per 1000ml distilled water
1% glucose

Table 7: MSc Biotechnology Budget for the period August 2006- March 2007

MSc Biotechnology Budget: August 2006- February 2007				
Description	**Supplier**	**Cost/unit**	**Quantity**	**Cost**
Laboratory Consumables				
Autoclave tape 12mm	Laboratory & Scientific Equipment Company (Pty) Ltd	R 25.40	1	R 25.40
Bleach (1,5 litres)	Pick 'n Pay	R 13.95	1	R 13.95
Cuvettes standard semi micro plastic (3ml)	Merck Chemicals (Pty) Ltd	R 68.00	1	R 68.00
Dialysis tubing (10,5 m)	Separation Scientific SA (Pty) Ltd	R 1,115.00	1	R 1,115.00
Eppendorfs 1.5 ml (pack of 500)	Laboratory & Scientific Equipment Company (Pty) Ltd	R 131.10	5	R 655.50
Syringe Filters (0,45µm)	Whitehead Sciientific (Pty) Ltd	R 373.92	2	R 373.92
Syringe Filters (0,02µm)	Whitehead Sciientific (Pty) Ltd	R 373.92	1	R 373.92
Filter tips (20µl)	Whitehead Sciientific (Pty) Ltd	R 350.00	1	R 350.00
Filter paper, 3HW, 90mm, 1 box of 100	Lasec SA (Pty) Ltd	R 23.37	1	R 23.37
Foil heavyweight	Pick 'n Pay	R 12.69	1	R 12.69
Glycerol	Merck Chemicals (Pty) Ltd	R 147.20	1	R 147.20
Hand wash Soap I.C.U	Pick 'n Pay	R 13.99	4	R 55.96
Jumbo Roller Towels, single	Cleaning store	R 197.40	1	R 197.40
Latex Gloves (powder-free) small	Merck Chemicals (Pty) Ltd	R 33.06	2	R 66.12
Lighter	Pick 'n Pay	R 5.65	1	R 5.65
Microscope glass slides (50 per box)	Merck Chemicals (Pty) Ltd	R 7.98	1	R 7.98
Parafilm sealing film, length 38m width 100mm	Merck Chemicals (Pty) Ltd	R 213.00	1	R 213.00
Petri Dishes Sterile, box of 500 (90mm)	Plastpro Scientific (Pty) Ltd	R 336.00	5	R 1,680.00
Permanent Marker	Waltons	R 18.64	1	R 18.64
Pipettes (10 ml)	Lasec SA (Pty) Ltd	R 364.80	1	R 364.80
Pipette tips blue (1000 µl)	Plastpro Scientific (Pty) Ltd	R 60.20	6	R 361.20
Pipette tips yellow (200 µl)	Plastpro Scientific (Pty) Ltd	R 49.80	6	R 298.80
Syringes Sterile, 20ml	Kendon Medical Supplies (Pty) Ltd	R 101.46	1	R 101.46
		R 4,036.53	**46**	**R 6,529.96**

Budget continued.

Chemicals and media					
Acetic Acid Glacial, 2.5L	Merck Chemicals (Pty) Ltd	R	147.00	1	R 147.00
Agar bacteriological, 500g	Merck Chemicals (Pty) Ltd	R	189.00	1	R 189.00
Agarose, 100g	Merck Chemicals (Pty) Ltd	R	236.00	1	R 236.00
Anhydrous Magnesium sulphate (MgSO4), 500g	Merck Chemicals (Pty) Ltd	R	21.00	1	R 21.00
Boric acid, 500g	Merck Chemicals (Pty) Ltd	R	315.00	1	R 315.00
Calcium Chloride dihydrate (CaCl2), 500g	Merck Chemicals (Pty) Ltd	R	50.00	1	R 50.00
Caesium Chloride (CsCl), 100g	Merck Chemicals (Pty) Ltd	R	1,402.00	1	R 1,402.00
Chloroform, 2.5L	Merck Chemicals (Pty) Ltd	R	91.00	1	R 91.00
D(+) Glucose anhydrous, 500g	Merck Chemicals (Pty) Ltd	R	28.00	1	R 28.00
Ethanol Absolute 99.9%, 2.5L	Merck Chemicals (Pty) Ltd	R	237.00	4	R 948.00
EDTA Na_2Dihydrate, 100g	Merck Chemicals (Pty) Ltd	R	394.00	1	R 394.00
Ethidium Bromide (EtBr)	Merck Chemicals (Pty) Ltd	R	1,281.00	1	R 1,281.00
Glycerol, 2.5L	Merck Chemicals (Pty) Ltd	R	73.00	1	R 73.00
Hydrochlorous acid (HCl)	Merck Chemicals (Pty) Ltd	R	111.72	1	R 111.72
Hydroxyquinoline-8, 500g	Merck Chemicals (Pty) Ltd	R	390.00	1	R 390.00
Magnesium chloride (MgCl2), 500g	Merck Chemicals (Pty) Ltd	R	38.00	1	R 38.00
Mineral oil, 500ml	Bio-Rad Laboratories Ltd	R	750.00	1	R 750.00
Magnesium sulphate (MgSO4), 500g	Merck Chemicals (Pty) Ltd	R	21.00	1	R 21.00
N, N, N′, N′- tetramethyl-*p*-phenylenediamine dihydrochloride, 10g	Merck Chemicals (Pty) Ltd	R	365.00	1	R 365.00
OF Test medium	Inquaba Biotechnical industries (Pty) Ltd	R	651.00	1	R 651.00
PEG 6000, 500g	Merck Chemicals (Pty) Ltd	R	45.00	1	R 45.00
Phenol crystals, 500g	Merck Chemicals (Pty) Ltd	R	94.00	1	R 94.00
Sodium Chloride (NaCl), 500g	Merck Chemicals (Pty) Ltd	R	13.00	1	R 13.00
Sodium hydroxide (NaOH)	Merck Chemicals (Pty) Ltd	R	221.16	1	R 221.16
Tris (hydroxymethyl) aminomethane, 500g	Merck Chemicals (Pty) Ltd	R	227.00	1	R 227.00
Tryptone powder pancreatic casein, 250g	Merck Chemicals (Pty) Ltd	R	136.00	1	R 136.00
Yeast extract powder, 500g	Merck Chemicals (Pty) Ltd	R	117.00	1	R 117.00
		R	**7,643.88**	**30**	**R 8,354.88**

Budget continued.

Sample analysis				
Full service economic sequencing reactions	Inquaba Biotechnical industries (Pty) Ltd	**R 125.40**	**8**	**R 1,003.20**
Molecular agents				
O'GeneRuler™ 1kb DNA Ladder, ready-to-use	Inquaba Biotechnical Industries (Pty) Ltd	R 1,015.74	1	R 1,015.74
O'GeneRuler™ 1kb DNA Ladder Plus, ready-to-use	Inquaba Biotechnical Industries (Pty) Ltd	R 1,350.90	1	R 1,350.90
PCR kit & Primers	Inqaba Biotechnical Industries (Pty) Ltd	R 961.31	1	R 961.31
		R 3,327.95	**3**	**R 3,327.95**
Total		**R 15,133.76**	**87**	**R 19,215.99**

Please note:

Uranyl acetate is a radioactive substance that is regulated by the government as a license is required for its sale.

Table 8: A timeline of the research conducted during the MSc Biotechnology programme 2006 - 2007

RESEARCH CONDUCTED: AUGUST 2006 – MARCH 2007	
RESEARCH PERIOD	**RESEARCH**
31-Jul-06	Sample collection: battery chicken faeces (BCF)
1 Aug – 4 Aug	Prepared freezer stocks of bacterial isolates
07-Aug	Sample collection: Free-range chicken faeces (FCF)
8 Aug – 18 Aug	Analysed the growth patterns of the bacterial isolates
21 Aug – 24 Aug	Characterised the bacterial isolates: • OF test • Gram-stain • Oxidase test
28 Aug – 4 Sept	Molecular characterisation of bacterial isolates • PCR analysis of 16S rDNA
5 Sept – 15 Sept	Phages were isolated from sewage: compared the use of fresh mid-log cultures to glycerol stocks of mid-log cells
19 Sept - 20 Sept	Analysis of sequenced PCR products, construction of homology tree
22-Sep	Sample collection: BCF, FCF and Sewage
25 Sept – 28 Sept	Phages were isolated from enriched ($MgCl_2$, $CaCl_2$ & overnight (O/N) incubation at 37ºC) samples
2 Oct – 4 Oct	Phages were isolated from enriched (overnight incubation at 37ºC) samples
9 Oct – 11 Oct	Phages were isolated from un-enriched samples
16 Oct – 20 Oct	Determined host-range of phage T4 (Lab supply) and phages A to D- isolated from sewage that was incubated O/N at 37ºC

Timeline continued.

23 Oct – 26 Oct	Repeated host-range
30 Oct – 8 Nov	Performed 1st, 2nd and 3rd plaque passages of phages T4, A and C (we will concentrate on these three phages for the remainder of the study)
13 Nov – 17 Nov	Performed small-scale phage amplification for TEM and host-range confirmation
20 Nov – 24 Nov	Repeated small-scale phage amplification, host-range and TEM
27 Nov – 30 Nov	Titred phage stock
4 Dec – 7 Dec	Performed large-scale phage amplification, titred phage stock. This is to be used for the isolation of phage DNA for restriction analysis.
13 Dec – 15 Dec	Attempted the 2 hr T4 toxicity study on *E. coli* K12 (abandoned experiment due to a 4 hr power failure)
18 Dec – 20 Dec	Repeated the 2 hr T4 toxicity study
21 Dec – 22 Dec	Attempted 6 hr T4 toxicity study (abandoned experiment due to contamination of control media)
27 Dec – 29 Dec	Repeated 6 hr T4 toxicity study
2 Jan 2007 – 5 Jan	Performed a 24 hr T4 toxicity study
9 Jan – 13 Jan	2 hr T4 toxicity study with shaking at 150 rpm. CFU/ml and PFU/ml analysis
14 Jan – 22 Jan	Completed first draft of research report. Photographed chicken farms
23 Jan – 2 Feb	Completed T4 toxicity study with parameters that were determined during pilot study.
6 Feb – 9 March	Completed the individual and combined toxic effects of phages A and C
10 March – 30 March	Completed research report