# Bacteriophage Control of Campylobacters in Retail Poultry

By Robert Atterbury B.Sc.

Thesis submitted to the University of Nottingham

for the degree of Doctor of Philosophy, August 2003

Division of Food Sciences
School of Biosciences
University of Nottingham
Sutton Bonington Campus
Loughborough LE12 5RD
United Kingdom

# APPENDIX
# NOT COPIED

# ON INSTRUCTION
# FROM
# THE UNIVERSITY

# TABLE OF CONTENTS

# TABLE OF FIGURES

# TABLE OF TABLES

# TABLE OF ABBREVIATIONS

| | |
|---|---|
| °C | Degrees Celsius |
| ACP | Assured Chicken Production |
| APS | Ammonium persulphate |
| BCA | Bicinchoninic acid |
| bp | Base pair |
| BSA | Bovine serum albumin |
| CBA | Columbia blood agar |
| CCDA | Modified charcoal cefoperazone deoxycholate agar |
| CDSC | Communicable Disease Surveillance Centre |
| CE | Competitive exclusion |
| cfu | Colony-forming unit |
| cm | Centimetre |
| C-terminus | Carboxyl terminus of a protein |
| Da | Dalton |
| DEFRA | Department for the Environment, Food and Raural Affairs |
| DNA | Deoxyribonucleic acid |
| DNase | Deoxyribonuclease |
| dNTP | 2' deoxyribonucleotide 5' triphosphate |
| DTT | Dithiothreitol |
| EDTA | Ethylene diamine tetra-acetic acid |
| eps | Extracellular polysaccharide |
| FPB | FBP growth supplement |
| GBS | Guillain Barré syndrome |
| GSM | Glycerol storage medium |
| ICTV | International Committee for the Taxonomy of Viruses |
| IPTG | Isopropyl-b-D-thiogalactoside |
| kb | kilobase pair |
| LB | Luria-Bertani medium |
| LOS | Lipooligosaccaride |
| LPS | Lipopolysaccharide |
| M | Molar |
| mA | Milliamperes |
| MDR | Multidrug resistant |
| ml | Millilitre |
| MLSTs | Multi-locus sequence types |
| MOI | Multiplicity of infection |
| mol | Mole |
| MRD | Maximum recovery diluent |
| NA | Nutrient agar |
| NB2 | Nutrient broth number 2 |
| NCTC | National Collection of Type Cultures |
| N-terminus | Amino terminus of a protein |
| NZB | NZCYM base medium |
| OD | Optical density |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate buffered saline |
| PCA | Plate count agar |
| PCR | Polymerase chain reaction |
| Pfu | Plaque-forming unit |
| PHLS | Public Health Laboratory Service |
| ppm | Parts per million |
| PVDF | Polyvinylidene difluoride |
| RES | Reticuloendothelial system |
| RNA | Ribonucleic acid |
| RNase | Ribonuclease |
| RO | Reverse osmosis |
| rpm | Revolutions per minute |
| RTP | Room temperature and pressure |
| RVS | Rappaport-Vassiliadis Soya Peptone broth |
| SDS | Sodium dodecyl sulphate |
| SM | SM Buffer |
| STs | Sequence types |
| TAE | Tris acetate EDTA buffer |
| TBE | Tris borate EDTA buffer |
| TBS | Tris Buffered Saline |
| TE | Tris EDTA buffer |
| TEMED | N,N,N',N'-tetramethyl ethylene Diamine |
| TLA | Top layer agar |
| Tris | Tris (hydroxymethyl) aminomethane |
| U | Unit |
| UK | United Kingdom |
| UV | Ultraviolet |
| V | Volt |
| v/v | Volume for volume |
| w/v | Weight for volume |
| X-gal | 5-bromo-4-chloro-3-b-D-Galactopyranoside |
| XLD | Xylose-Lysine desoxycholate agar |

# ACKNOWLEDGEMENTS

Firstly I would like to thank my supervisors Prof. Ian Connerton, Dr. Christine Dodd and Dr. Cath Rees for their support and encouragement throughout my Ph.D. I am particularly grateful to Prof. Connerton for his informal and open attitude and always finding the time to discuss my work and help with publications.

I would like to thank Dr. Pippa Connerton for her invaluable support in the laboratory and stoically reading through my entire thesis (several times) to provide constructive criticism. Thanks go to Eleanor Dillon, Wendy Fielder, Niki Cummings and Dave Fowler who helped with many aspects of my laboratory work. Thanks also go to the undergraduate and postgraduate project students I have supervised for their help in some of the more tedious aspects of my work and (generally) not complaining.

I would like to thank my fellow PhD students and colleagues for their support and encouragement during my time at Nottingham. Particular thanks go to Dr. Pat Landers, a good friend who enriched the lives many people inside and outside of the lab, who tragically died in 2002.

Lastly I would most like to thank my parents and brother for their unwavering support, both moral and financial, and for taking a keen interest in my work (even if they didn't understand some of it!). I am grateful to the University of Nottingham, DEFRA and the. Poultry Consortium for funding this project.

# ABSTRACT

Food-borne disease continues to be a major cause of human morbidity and mortality. During the past few decades, *Campylobacter jejuni* has ascended to become the greatest cause of bacterial enteric disease worldwide. Anecdotal evidence suggests the majority of human campylobacteriosis in industrialised countries is caused by the consumption of undercooked chicken. *Campylobacter* continues to frustrate current control strategies throughout the food chain and in 2001 was responsible for over 56, 000 cases of food poisoning in the U.K. alone. The work presented in this thesis examined the potential of host-specific bacteriophage as a novel measure to control the population of *Campylobacter* in poultry production.

Several surveys in this thesis revealed that campylobacters and their bacteriophage permeate the entire poultry meat supply chain, from chickens in the broiler house to packaged retail products. Characterisation of the bacteriophage recovered from such sources showed that retail poultry isolates exhibited greater similarities in host range than those originating from broiler houses, implying poultry processing selected for a sub-population of phage. Additionally, broiler chickens harbouring bacteriophage in their gastrointestinal tract generally contained fewer campylobacters. All of the phage isolates studied belonged to the Myoviridae virus family as they possessed dsDNA genomes encapsulated in an icosahedral head with a rigid, contractile tail. Fragments of the phage genomes exhibited significant sequence homology with a number of genes involved in DNA replication from phage T4. Studies of the attachment and replication of the phage

isolates *in vitro* suggested that adsorption to the host cell was efficient but the burst size was low (<10 virions per cell). *Campylobacter jejuni* was found to produce membrane vesicles but these did not significantly affect bacteriophage replication *in vitro*.

A series of trials using 'phage therapy' in broiler chickens revealed that *Campylobacter* colonisation can be reduced by $\geq \log_{10}$ 8.0 cfu $g^{-1}$ caecal contents by dosing with specific bacteriophage. However, both the timing and extent of the reduction in *Campylobacter* colonisation showed considerable variation. Additionally, the ability of bacteriophage to infect their host *in vitro* was not a reliable indicator of their efficacy *in vivo*. The direct application of bacteriophage to the surface of chicken skin artificially contaminated with *Campylobacter* led to a significant reduction in the number of recoverable host cells. Host resistance to bacteriophage infection was not detected in either the *in vivo* trials or when recovering *Campylobacter* cells from chicken skin treated with phage.

The work presented in this thesis demonstrates that bacteriophage have considerable potential in the control of *Campylobacter* in poultry production. They already appear to constitute a limiting factor in *Campylobacter* colonisation of the chicken gastrointestinal tract and can be detected with their host on retail products. However, further research is required to fully realise their potential and optimising the timing, level and type of bacteriophage used in dosing will be important for their efficacy *in vivo*.

# CHAPTER 1

# INTRODUCTION

## 1.0 OBJECTIVES

Bacteriophage have successfully been used to control populations of food-borne pathogens such as *Salmonella* and *E. coli* colonising the gastrointestinal tracts of domestic fowl and cattle (**Smith and Huggins, 1983; Berchieri *et al.*, 1991**). However, comparatively little is known about bacteriophage of *Campylobacter*. The work presented in this thesis sought to expand current knowledge about the presence of bacteriophage and their *Campylobacter* host populations in poultry production at various points of the human food chain. This information was then used to evaluate bacteriophage as potential agents for controlling *Campylobacter* in broiler chickens.

## 1.1 CHARACTERISTICS OF THE *CAMPYLOBACTER* GENUS

Campylobacters were first thought to have been observed by Theodor Escherich in 1886 who described non-culturable spiral shaped rods in the stools of children who died from diarrhoeal disease (**Butzler, 2000**). It was almost a century later when Skirrow was able to successfully isolate and culture these organisms (**Skirrow, 1977**). In the near three decades since isolation methods became available, *Campylobacter* has become

recognised as the leading cause of human bacterial enteritis in the developed world **(Tauxe, 2000).**

The *Campylobacter* genus is the largest constituent of the Campylobacteriaeceae family in the order Campylobacteriales. They are the most studied examples of the epsilon subdivision of the proteobacteria phylum. Other important members of the Campylobacteriaceae family are the *Arcobacter* and *Sulfurospirillum* genera. Until recently, *Helicobacter* was also a member of this family but, due to taxonomic dissimilarities, has been reclassified into a new family. There are currently 18 species and subspecies of *Campylobacter* **(Stern *et al.*, 1992; Vandamme, 2000)** of which the most studied and clinically important species are *C. jejuni* subspecies *jejuni*, *C. coli* and *C. lari*. These species have collectively been named the 'thermophilic' campylobacters owing to their reported optimal growth temperature at 42 - 43°C and inability to grow below 25°C **(Phillips, 1995).**

Campylobacters are Gram negative and biochemically characterised as oxidase positive and nitrate reducing. They produce negative results for Methyl Red and Voges – Proskaur tests and are unable to hydrolyse gelatin **(Phillips, 1995).** Since campylobacters are not saccharolytic, classical carbohydrate utilisation tests for Gram negative bacteria have little use in their speciation **(Smibert, 1984).** However, biochemical tests have been developed to assist in preliminary identification **(Stern *et al.*, 1992)** and are core constituents of certain commercial testing kits (e.g. APICampy, bioMérieux, Lyon, France). Probably the most widely used biochemical assay for *Campylobacter* is the hippurate test **(Collins *et al.*, 1995).** At present, only *C. jejuni* has been shown to possess

the *hipO* gene, whose product (hippuricase) hydrolyses hippurate ($C_6H_5.CO.NH.CH_2.CO_2^-$) to benzoate ($C_2H_5O_2^-$) and glycine ($C_2H_5NO_2$). Campylobacters may also be broadly divided according to their ability to produce catalase, which is a characteristic associated with strains which cause human disease (**Stern *et al.*, 1992**). Campylobacters are microaerophiles and capnophilic, growing optimally in a gaseous environment consisting of a reduced atmospheric oxygen tension with a greater concentration of carbon dioxide (**Smibert, 1984**). Typically, these organisms are cultured in an atmosphere consisting of 5% $O_2$, 10% $CO_2$ and 85% $N_2$ which is usually achieved either through evacuation and replacement of the air in a gas jar or the use of commercial chemical-based "gas pack" systems (**Stern *et al.*, 1992**).

In terms of morphology, campylobacters are classified as pleomorphs as their size and shape varies at different stages of their life cycle, ranging from spiral shaped rods to coccoid forms (**Ng *et al.*, 1985; Griffiths, 1993; Thomas *et al.*, 1999**). During exponential growth phase these bacteria usually display a "gull wing" shape with a diameter of 0.2 – 0.5 μm and a length of up to 5 μm (**Singleton and Sainsbury, 1999**). campylobacters possess single or bipolar unsheathed flagella and are highly motile, typically exhibiting a darting motility with occasional "corkscrew" movements (**Smibert, 1984; Singleton and Sainsbury, 1999**).

## 1.2.0 HUMAN CAMPYLOBACTERIOSIS

The Communicable Disease Surveillance Centre (CDSC), at the time of writing part of the Public Health Laboratory Service for England and Wales (PHLS), has monitored the number of human campylobacteriosis cases since 1986. During this time, the annual number of human *Campylobacter* enteritis cases has risen from 24, 809 to 56, 420 in 2001 (http://www.phls.org.uk/topics_az/campylo/menu.htm) costing the UK an estimated £150 m per annum (**Gillespie *et al.*, 2002**). Similar increases have been observed in other Western countries over a comparable time scale (**Tauxe, 2000**). This increase may be explained partly by the development of more sophisticated and sensitive isolation techniques in conjunction with an increase in the awareness of public health professionals (**Phillips, 1995; Tauxe, 2000**). However, there remains a general concern that the increase in reported (or 'visible') *Campylobacter* infections may underestimate the actual occurrence of this disease in the general population. This underestimate is believed to be up to ten times in the UK (**Pebody *et al.*, 1997**) and thirty-eight times in the United States of America (**Tauxe, 2000**). However, this could be an indication that most patients suffering from campylobacteriosis recover adequately by themselves and do not usually require professional medical attention. When such treatment is necessary, macrolide antibiotics (particularly erythromycin) are the drugs of choice although 68% of *C. coli* food isolates are reported to be resistant to this antibiotic (**Kramer *et al.*, 2000**). The mechanism of erythromycin in treating *Campylobacter* infections is equivocal. This drug is known to bind to motilin receptors in the gut, promoting gastrointestinal peristalsis which may play a major part in removing *Campylobacter* from the patient (**Archer and Polk, 2001**). Erythromycin can also directly bind to the 50S subunit of bacterial

ribosomes and inhibit protein synthesis in a range of bacteria (**Archer and Polk, 2001**). Resistance to macrolides is mediated by a plasmid-encoded methylase which modifies ribosomal RNA, preventing the drug binding to its target (**Archer and Polk, 2001**). All of the above factors against the backdrop of a successful campaign to reduce the number of human enteric *Salmonella* infections (**Cogan and Humphrey, 2003**) has made *Campylobacter* a priority in the development of government food safety policy.

### 1.2.1 Symptoms of human *Campylobacter* enteritis

The number of *Campylobacter* cells required to infect humans is thought to be low (**Phillips, 1995**). Reports suggest that as few as 500 - 800 cfu may be sufficient to cause enteric disease in a healthy human adult (**Black *et al.*, 1988**). Following infection the incubation period ranges from 48 – 72 h before the onset of symptoms. Clinical symptoms are usually presented as a fever (40°C) followed by nausea and severe abdominal cramps (**Blaser, 2001**). Diarrhoea often follows rapidly and is characterised by being either watery and profuse or mucoid, sometimes containing blood (**Skirrow and Blaser, 2000**). These symptoms are usually accompanied by acute inflammatory enteritis extending from the small intestine to the colon and rectum. Fatalities resulting from *Campylobacter* infections are rare and consequently current knowledge of the histological changes occurring during such infections has been accrued from proctosigmoidoscopy studies (**Wassenaar and Blaser, 1999**). Such studies clearly demonstrate inflammatory bowel disease is an integral part of many *Campylobacter* infections. This observation is underpinned by data showing that leukocytes and erythrocytes can both be recovered from the stools of patients, even when the diarrhoea is

not bloody (**Wassenaar and Blaser, 1999**). *Campylobacter* invasion of human cells has been demonstrated both *in vitro* and *in vivo* (**Wooldridge and Ketley, 1997; Wassenaar and Blaser, 1999**). Although deep tissue penetration is a possible progression from invasion, the sensitivity of *Campylobacter* to complement-mediated lysis in immunocompetent individuals is thought to limit proliferation in the blood and prevents further systemic dissemination (**Wassenaar and Blaser, 1999**). However, blood samples are not routinely cultured in cases of campylobacteriosis and thus bacteraemia is unlikely to be detected unless it develops into systemic disease, usually in immunocompromised individuals (**Black *et al.*, 1988; Skirrow *et al.*, 1993; Wassenaar and Blaser, 1999**).

It may take up to two weeks for patients to fully recover from the initial symptoms of campylobacteriosis but mild relapses can occur, which are more persistent in immunocompromised patients (**Blaser, 2001**). After the regression of clinical symptoms, patients may continue to excrete campylobacters in their faeces for several weeks. One study revealed the mean post infection shedding period was 37.6 days (**Kapperud *et al.*, 1992**). Although this may present a short term problem for patients who work in the catering, hospitality or healthcare services (**Murphy *et al.*, 1995**), long term asymptomatic carriage as seen with other food-borne diseases such as typhoid fever (**Salyers and Whitt, 1994**) has not been reported for *Campylobacter*.

### 1.2.2 Pathogenesis and Immunity

Campylobacteriosis is usually self-limiting and typical treatment consists of rest and fluid replacement (**Blaser, 2001**). Patients show a marked increase in serological anti-

*Campylobacter* antibodies (**Wassenaar and Blaser, 1999**) which may offer protection from the same or related strains for a period of at least one year (**Cawthraw *et al.*, 2002**). The protective duration of such seroconversion may depend on each individual's response but serum titres of IgG, IgM and IgA usually decline rapidly after clinical symptoms of the disease have subsided. A degree of immunity is developed in people with a continually high level of exposure to this organism (**Cawthraw *et al.*, 2000**) and serious recurring infections in AIDS sufferers suggest cell-mediated immunity is an important factor in long-term protection (**Wassenaar and Blaser, 1999; Blaser, 2001**). The mechanism of *Campylobacter* pathogenesis in humans has yet to be fully understood. Campylobacters exhibit positive chemotaxis to L-fucose and L-serine, both constituents of mucin (**Marchant *et al.*, 2002**). Moreover, this bacterium can subsist on mucin as its sole carbon source (**Schoeni and Doyle, 1992**). Mucous-filled intestinal crypts are the primary colonisation site in chickens (**Beery *et al.*, 1988**) but, due a paucity of analogous animals disease models, this area of *Campylobacter* pathogenesis is poorly understood in man (**Young *et al.*, 2000**). Although campylobacters can produce a range of toxins (**Ketley, 1997; Wassenaar, 1997; Wassenaar and Blaser, 1999**) their role in the disease process is unclear. The extensive range of symptoms experienced by campylobacteriosis sufferers is thought by some to be a consequence of *Campylobacter* producing different toxins in the gut (**Wassenaar, 1997**).

### 1.2.3 Chronic complications of *Campylobacter* infection

In recent years, *Campylobacter* has been increasingly associated with a range of serious long term complications (**Nachamkin, 2002**). The most serious of these is Guillain Barré

syndrome (GBS) which is a potentially life-threatening autoimmune disease. GBS, or acute ascending polyradioculoneuropathy, is a disease affecting the peripheral nerves which, if left untreated, can lead to respiratory paralysis and death (**Nachamkin *et al.*, 2000**). GBS is the most common acute neurological disease in the developed world (**Hughes *et al.*, 1999**). Serological evidence suggests that *Campylobacter* infections occur in up to 40% of GBS patients, even though culture-positive *Campylobacter* can only be isolated from the stools of 15% of these (**Nachamkin, 2002**). Previous studies have suggested that GBS is triggered by complement-mediated damage of neuron myelin sheaths, leading to dymyelination and flaccid paralysis (**Nachamkin, 2002**). Certain *Campylobacter* serotypes (in particular HS:19) are known to possess lipo-oligosaccharide core regions which mimic the sialic acid structures found in the gangliosides of peripheral nerve tissue (**Nachamkin, 2002**). Pathogenic antibodies against GM1 gangliosides are thought to lead to GBS whereas antibodies against other gangliosides may trigger related conditions such as Miller Fisher Syndrome (**Nachamkin, 2002**). However, not all people exposed to HS:19 go on to develop GBS suggesting host response is important in the development of this condition (**Bowes *et al.*, 2002**).

### 1.2.4 Epidemiology

Given the large number of *Campylobacter* enteritis cases reported to the CDSC every year and the relatively low infective dose, it is perhaps surprising that campylobacteriosis is mainly a sporadic disease (**Phillips, 1995; Pebody *et al.*, 1997; Friedman *et al.*, 2000**). Although several well-studied outbreaks have been reported (**Istre *et al.*, 1984; Murphy *et al.*, 1995; Anon, 1998; Evans *et al.*, 1998**) they are still relatively rare, and in

many cases it is difficult to pinpoint the origin of infection. This problem is compounded by the lack of consensus on the most effective epidemiological typing tools (**Wassenaar and Newell, 2000**) coupled with the high degree of genetic heterogeneity in the genus (**Wassenaar *et al.*, 2000; Dorrell *et al.*, 2001**). Data from most first-world countries indicates approximately 95% of human campylobacteriosis is caused by *C. jejuni* and *C. coli* with other species such as *C. lari* responsible for a minor proportion (**Lastovica and Skirrow, 2000; Shane, 2000**). The reason for the predominance of *C. jejuni* in human infection remains undetermined as it appears that many different species of *Campylobacter* have the potential to cause disease (**Lastovica and Skirrow, 2000**). As co-infection with multiple *Campylobacter* species and strains appears to be rare in humans (**Richardson *et al.*, 2001**) ultimately this may be a reflection of exposure, host responses or the ability of specific *Campylobacter* strains to persist in infection reservoirs.

A growing consensus among researchers points to contaminated poultry, in particular chicken, as a significant risk factor in acquiring human *Campylobacter* enteritis (**Jacobs-Reitsma, 2000**). This assertion is founded primarily on anecdotal evidence as no study has yet conclusively proven a 'cause and effect' relationship (**Stern *et al.*, 2003**). Nevertheless, of the outbreaks of *Campylobacter* which have been studied in detail, many strongly implicate undercooked chicken (**Istre *et al.*, 1984; Harris *et al.*, 1986; Evans *et al.*, 1998; Shane, 2000**). Taking this into consideration, a recent risk assessment study predicted that a $\log_{10}$ 2.0 cfu reduction in the *Campylobacter* load on individual retail broiler carcasses could lead to a fall in the number of human campylobacteriosis cases by up to 30 fold (**Rosenquist *et al.*, 2003**).

## 1.3 PREVALENCE OF *CAMPYLOBACTER* IN RETAIL POULTRY PRODUCTS

In the year 2000 the UK chicken market produced 0.7 million tonnes of poultry meat with an estimated value of £1.6 bn, accounting for almost 30% of the £5.4 bn meat market (**Anon, 1999**). The whole and portioned chicken market accounted for the majority of this produce (0.52 m tonnes) with the remainder supplying ingredients for value-added poultry products. The high prevalence of *Campylobacter* in retail poultry products has been widely reported in European countries (**Jacobs-Reitsma, 2000; Kramer *et al.*, 2000; Dominguez *et al.*, 2002; Jorgensen *et al.*, 2002**) and the United States of America (**Friedman *et al.*, 2000; Shane, 2000; Zhao *et al.*, 2001**). Campylobacters are sensitive to freeze-thaw damage (**Chan *et al.*, 2001**) and consequently, frozen poultry products are generally contaminated to a lesser extent than fresh products (**Stern *et al.*, 1984; Jacobs-Reitsma, 2000; Stern *et al.*, 2003**). The rising number of human campylobacteriosis cases together with a growing body of research linking this disease with the consumption of contaminated poultry meat has prompted public-funded research into practical control strategies throughout the food chain (**Anon, 2001b**).

Two surveys of retail poultry meat in the UK concluded that approximately 80% of products were contaminated with *Campylobacter* at levels of up to $10^9$ cfu per product (**Kramer *et al.*, 2000; Jorgensen *et al.*, 2002**). In the year 2000 the FSA commissioned a large survey to examine UK poultry meat for *Campylobacter* and *Salmonella* contamination (**Anon, 2001c**). They found that 50% of fresh chicken produce was contaminated with *Campylobacter* compared with 5.7% for *Salmonella*. Half of all the

*C. jejuni* isolates from this survey were resistant to at least one antibiotic. A concurrent FSA survey assessed consumer attitudes towards food and their knowledge of food safety. They found that, although there was great concern over food safety and hygiene, a considerable number of respondents were not handling and preparing their food safely (**Anon, 2001a**). **Cogan *et al.* (2002)** found that *Campylobacter* contamination in kitchens could be significantly reduced when study participants used a specified cleaning procedure with detergent. However, another study revealed that during undirected food preparation, there was extensive cross contamination of kitchen surfaces with *Campylobacter* and *Salmonella*, which were not significantly decreased by cleaning (**Cogan *et al.*, 1999**). Despite being sensitive to desiccation, *Campylobacter* cells have been found to survive for at least three hours on contaminated domestic surfaces (**Cogan *et al.*, 1999**). Taking the above information into account it is perhaps unsurprising that cross-contamination in food preparation areas has been identified as a major risk factor in acquiring *Campylobacter* infections (**Rosenquist *et al.*, 2003**).

Practices employed in UK poultry abattoirs have, thus far, failed to significantly impact on carcass contamination on the supermarket shelf. The chemical decontaminant hypochlorite has previously been used in the scald water and chill tanks of abattoirs at concentrations of 50 – 250 ppm in an effort to reduce the numbers of pathogenic and spoilage bacteria (**Allen *et al.*, 2000**). Free chlorine in solution is a potent antimicrobial agent, however, under commercial operating conditions its efficacy is poor in reducing carcass contamination (**Allen *et al.*, 2000; Whyte *et al.*, 2001; Yang *et al.*, 2001**). This is purportedly due to inactivation by organic matter in the tanks and the protective effect of chicken skin for bacteria associated with the cuticle (**Berndtson *et al.*, 1996**).

Although still routinely applied in many countries, the use of hypochlorite in EU member states should have ceased in 2001 in accordance with article #9712138.

Unfortunately, far from reducing carcass contamination, current abattoir codes of practice may unwittingly serve to disseminate *Campylobacter* cells. As a consequence of their increased environmental exposure, organic and free-range flocks are more frequently colonised with *Campylobacter* (**Heuer *et al.*, 2001**). However, the code of practice from the body governing organic food production, the Soil Association (www.soilassociation.org), dictates that to maintain their status, organic birds must be slaughtered prior to conventionally reared flocks. Previous studies have demonstrated that flocks testing negative for *Campylobacter* at slaughter can be contaminated by strains found in the caeca of birds slaughtered earlier in the production line (**Miwa *et al.*, 2002**).

A major study of the enclosed Icelandic poultry industry by **Stern *et al.* (2003)** highlighted that a significant fall in human campylobacteriosis was recorded by the Iceland Health Department following a reduction in the number of *Campylobacter* positive broiler chicken carcasses. The Icelandic government's proactive response to the increasing number of human *Campylobacter* infections in their country led to many intervention strategies operating concurrently. Consequently, it was impossible to determine which of these strategies contributed most to reducing human disease.

A study examining the multi-locus sequence types (MLSTs) of campylobacters isolated from human, animal and environmental sources concluded that there was a broad range

of sequence types (STs) colonising chickens whereas most human isolates clustered around ST21 (**Dingle *et al.*, 2001**). Why only certain sequence types should cause disease when humans are probably exposed to many more remains undetermined. It is possible that not all strains of *C. jejuni* are pathogenic to humans or that some strains survive carcass processing better than others. There may also be other important reservoirs of the *Campylobacter* strains that can cause human disease. In any case, if not universally regarded as the most aetiologically important source of human *Campylobacter* infection, poultry products must be considered an important risk factor.

## 1.4 *CAMPYLOBACTER* IN BROILER FLOCKS

Campylobacters are excellent colonisers of the gut of many avian species (**Waldenstrom *et al.*, 2002**). Although some evidence suggests that campylobacters may, in certain circumstances, invade extra-intestinal sites of the chicken (**Young *et al.*, 1999**), the general consensus in the research community is that this bacterium is not an avian pathogen and its primary role is commensal (**Newell and Wagenaar, 2000; Shane, 2000; Waldenstrom *et al.*, 2002**). This commensal relationship is thought to be the result of a long evolutionary history between a parasite and its host (**Waldenstrom *et al.*, 2002**). Campylobacters are not a natural constituent of human gut flora and their pathogenic *modus operandi* in man is suggested to be merely coincidental.

*Campylobacter* is an extremely infectious agent of chickens with maximal colonisation achieved with as few as 35 cfu of some strains (**Cawthraw *et al.*, 1996**). Given this fact it is unsurprising that the increased biosecurity measures implemented so effectively to

combat *Salmonella* (**Cogan and Humphrey, 2003**) did not prevent *Campylobacter* colonisation. Moreover, passage through the chicken gut is known to enhance the colonisation of some *Campylobacter* strains by almost 3,000 fold (**Cawthraw *et al.*, 1996**). This has led to the general resignation of the poultry industry to the prospect that nothing may prevent their flocks becoming infected. This is supported by the fact that even breeder flocks, which are maintained under high levels of biosecurity, can become positive (**Stern *et al.*, 2003**) as can newly-constructed purpose-built facilities (**Gregory *et al.*, 1997**). Despite the difficulty farmers face in dealing with *Campylobacter* colonisation of their flocks, leading figures in food safety have continually called for poultry producers to do more to prevent infection (**Lacey, 1994**).

Although the UK poultry industry is worth an estimated £1.6 bn per annum (**Anon, 1999**), legislation and the continual pressure from retailers to produce cheaper birds have reduced profit margins on the average chicken carcass. In addition, the poultry industry has never received farming subsidies from the EU and bearing these facts in mind, any attempt to control campylobacters at the farm level must be practical but inexpensive (**Anon, 2003**). Enhanced biosecurity may reduce the probability of *Campylobacter* infection in a flock but may be considered too draconian for the poultry industry to bear (**Newell and Wagenaar, 2000**). Moreover, the costs of such measures are likely to be absorbed solely by the poultry industry as retailers would be reluctant to increase prices. In addition to financial pressures, the poultry industry is increasingly constrained by stringent codes of practice and government legislation. Most major retailers insist that the poultry they sell is reared in accordance with the Assured Chicken Production (ACP) scheme (www.asssuredchicken.org). This scheme is supported by the UK government

with the intention of ensuring poultry destined for human consumption in the UK is produced to a consistently high standard with humane treatment of farmed animals.

A large body of research indicates that broiler chicks are not born with gut flora containing viable *Campylobacter* cells (**Gregory *et al.*, 1997**) and that the risk of infection increases with age (**Newell and Wagenaar, 2000**). Campylobacters are able to penetrate intact egg shells (**Jacobs-Reitsma, 2000**) and colonise the reproductive tract of chickens (**Cox *et al.*, 2002b; Hiett *et al.*, 2002**). Although vertical transmission has been reported (**Cox *et al.*, 2002a**), it is generally considered to be a rare event and remains a controversial issue among researchers (**Newell and Wagenaar, 2000**). Arguing against a major role for vertical transmission was a study showing that few offspring of a highly colonised breeder flock had *Campylobacter* positive caeca (**Stern *et al.*, 2003**). There also appears to be a period in the early life of the chick when campylobacters cannot be isolated from the caecum (**Newell and Wagenaar, 2000; Shane, 2000**). However, proponents of vertical transmission highlight research revealing *Campylobacter* DNA can be detected from such caeca using more sensitive non-culture based techniques (**Chuma *et al.*, 1997**).

Previous studies have consistently shown that chicks reared both in commercial broiler houses and under controlled conditions are not colonised by *Campylobacter* for a period of 2 – 3 weeks (**Newell and Wagenaar, 2000; Shane, 2000**). This period has been called the "lag phase" and is currently the subject of much debate in the *Campylobacter* research community. As *Campylobacter* is ubiquitous in the environment and an excellent coloniser of chickens it seems unlikely that the lag phase can be explained by a

lack of exposure (**Newell and Wagenaar, 2000**). There is some evidence suggesting maternal anti-*Campylobacter* antibodies are responsible for this phenomenon (**Sahin *et al.*, 2001**). However, if experimentally inoculated with a high enough dose, day old chicks may still become colonised by *Campylobacter* (**Young *et al.*, 1999**). The genetic diversity (**Dorrell *et al.*, 2001**) and variable surface structures of *Campylobacter* (**Parkhill *et al.*, 2000**) would suggest that antibodies probably could not account for the universality of the lag phase in chickens. Moreover, breeder flocks possessing anti-*Campylobacter* antibodies are still intestinally colonized with this bacterium (**Widders *et al.*, 1996**) suggesting such antibodies are either not cross-protective or only prevent invasion, not colonization. Other potential explanations of the lag phase include an inhospitable gut environment created by competitive microflora (**Humphrey *et al.*, 1989**), changes in feed composition or the possibility that, as for *Salmonella*, some birds have an innate resistance to infection (**Newell and Wagenaar, 2000**).

Although the role of vertical transmission remains equivocal there is little disagreement among researchers about the importance of horizontal infection. Once one bird in a broiler house is infected, it is common for the entire flock to become positive within days (**Shreeve *et al.*, 2000**). *Campylobacter* can quickly colonise the chicken caecum to levels greater than $10^{10}$ cfu $g^{-1}$ (**Ahmed *et al.*, 2002**) and has a multitude of transmission routes at its disposal including aerosols (**Berndtson *et al.*, 1996**), water (**Gregory *et al.*, 1997**) or vector-mediated infection (**Jacobs-Reitsma *et al.*, 1995**). As chickens are coprophagic, the main transmission route between birds is likely to be faecal – oral (**Newell and Wagenaar, 2000**). Conventionally reared flocks in the UK tend to be colonised by a restricted number of *Campylobacter* subtypes (**Ayling *et al.*, 1996**)

although multiple strain infections have been reported more frequently in other countries (**Jacobs-Reitsma *et al.*, 1995**). The probability of infection appears to increase with bird age (**Evans and Sayers, 2000**) and exposure to the environment since organic birds appear to be colonised with the greatest variety of *Campylobacter* strains (**Heuer *et al.*, 2001**).

The source of initial flock infection is still a highly contentious issue and the wide range of environmental reservoirs in which *Campylobacter* can persist frustrates efforts to focus on specific critical control points. Different parts of the world rear poultry in different ways and the relative importance of specific environmental reservoirs in *Campylobacter* contamination will change according to their particular circumstances. Consequently, there is considerable disparity in the positivity of poultry flocks from different countries (**Shane, 2000**) although the sensitivity and frequency of *Campylobacter* surveillance may also have an influence. In addition, some countries experience a seasonality in human and poultry *Campylobacter* infections (**Newell and Wagenaar, 2000**). This is still an equivocal issue in the UK with some research failing to find any such pattern (**Humphrey *et al.*, 1993**).

Many environmental reservoirs have been implicated as sources of *Campylobacter* infection of broiler flocks (**Gregory *et al.*, 1997**). Certain taxa of migrating birds are known to carry *Campylobacter* species (**Waldenstrom *et al.*, 2002**), although their role as a source in broiler infection remains debatable (**Petersen *et al.*, 2001**). Insects, in particular flying insects, have also been implicated as a possible source (**Jacobs-Reitsma *et al.*, 1995; Gregory *et al.*, 1997**). Most farms have effective vermin control

programmes and consequently rodents are not considered a major source (**Evans and Sayers, 2000**). The presence of other domestic animals on the farm is associated with an increased risk of broiler flock infection with *Campylobacter* (**Jacobs-Reitsma *et al.*, 1995; Gregory *et al.*, 1997**). As campylobacters are highly sensitive to desiccation (**Shane, 2000**), feed is not considered a major source of *Campylobacter*. Similarly, spent litter is not thought to sustain campylobacters long enough to cause infection (**Payne *et al.*, 1999; Shane, 2000**). *Campylobacter* has been shown to survive well in aqueous environments at low temperature over an extended period of time (**Buswell *et al.*, 1998**). Despite this, water is not generally highlighted as a major infection source for broiler flocks. Although supplying potable or sanitised water to broiler houses is a suggested critical control point for preventing flock infection in the UK (**Anon, 1999**), research in the U.S.A. suggests chlorination of water seems to have little impact on flock positivity (**Stern *et al.*, 2002**). Under experimental conditions, campylobacters are able to survive in biofilms (**Trachoo *et al.*, 2002**) which have been shown to imbue bacteria with greater resistance to chemical decontaminants (**Dunne, 2002**).

A persistent risk of flock infection on a farm is the relatively frequent access of farm workers in the broiler house (**Newell and Wagenaar, 2000**). Asymptomatic carriage of campylobacters in humans is rare and thus the main infection hazard will be horizontal transfer from environmental reservoirs into the broiler house. **Berndtson *et al.* (1996)** reported that human-mediated transfer was a possible source of *Campylobacter* infection of flocks. Broiler house cleaning procedures appear to adequately remove *Campylobacter* between flocks (**Evans and Sayers, 2000**). Nevertheless, successive

flock rotations can become infected by the same strain (**Petersen and Wedderkopp, 2001**) which suggests a common environmental reservoir.

One practice consistently highlighted as an infection risk for flocks is thinning (**Anon, 1999; Anon, 2003**). This procedure involves the periodic removal of birds from the broiler house at certain ages to satisfy the market demand for birds of different sizes and to comply with UK laws on animal stocking density. Thinning may take place up to three weeks prior to final depopulation and, given the high infectivity of *Campylobacter* in poultry, provides ample opportunity for colonisation of the entire flock before slaughter. Moreover, abattoir transport crates used in this procedure are usually not thoroughly washed and decontaminated and can act as a source of contamination to the birds either directly or via abattoir staff (**Berndtson *et al.*, 1996; Newell *et al.*, 2001**).

## 1.5.0 METHODS OF CONTROLLING *CAMPYLOBACTER* ON THE FARM

### 1.5.1 Biosecurity

Controlling *Campylobacter* contamination of poultry products in the human food chain has principally targeted three areas: the farm, the abattoir and the consumer. Irradiation (or 'cold pasteurisation') of the final food product is often seen by scientists as a definitive solution to food-borne infections (**Corry *et al.*, 1995a**). However, recent research suggests that *Campylobacter* may be able to initiate an inflammatory response irrespective of the viability of the ingested organism (**Mellits *et al.*, 2002**) and public opposition to irradiated food is considerable (**Corry *et al.*, 1995a**). Consequently, it

would be sagacious to reduce the presence of *Campylobacter* at the beginning of the food chain, i.e. on the farm. Increasing the level of biosecurity on farms to exclude pathogenic organisms from food animals is a primary aim of the UK government (**Anon, 2003**). However, stringent biosecurity is difficult and expensive to maintain and only serves to limit, not eliminate infection (**Newell and Wagenaar, 2000**). Although some success has been achieved using this approach with *Campylobacter* (**Gibbens *et al.*, 2001**), improving farm biosecurity on a general basis only appears to delay the onset of colonisation. Targeted biosecurity measures may prove to be more effective but a paucity of data on definitive environmental sources of *Campylobacter* has led to general disagreement about which areas should be selected. Ultimately, only longitudinal studies examining the dynamics of *Campylobacter* in the farm environment using molecular epidemiology will resolve this problem (**Newell and Wagenaar, 2000**).

### 1.5.2 Competitive exclusion

Microbial competitive exclusion (CE) is a process by which microflora in a given ecological niche prevent the establishment of other bacterial populations. This is achieved by either competing more successfully for limited resources or by producing inhibitory metabolites. CE was originally described by **Nurmi and Rantala (1973)** who were investigating the resistance of chicks to *Salmonella* infection. They observed a link between the environmental conditions in which the chicks were reared and their susceptibility to infection (**Rantala and Nurmi, 1973**). Chicks reared in more sanitised environments often do not develop an intestinal microflora capable of competing with salmonellas (**Mead, 2000**). **Nurmi and Rantala (1973)** also found that mature

microflora from *Salmonella*-negative birds could be cultured and re-administered to chicks to protect them from *Salmonella* infection. Since then, several defined commercial CE preparations have been developed (**Mead, 2000**) these include: Briolact (Orion corp.); CF-3 (Milk Specialities Co.) and Mucosal Starter Culture (Continental Grain Co.). Undefined preparations (i.e. the bacterial constituents in the product are unknown) include Avigard (Bayer AG); and Avifree (Alltech Ltd.). CE has been relatively successful in preventing caecal colonisation of *Salmonella* but less so for *Campylobacter*. Commercial CE products such as Avian Pac™ (Loveland Industries Inc) have shown modest protection against *Campylobacter* (**Morishita *et al.*, 1997**). However, such protection is uncommon as many commercial products effective against *Salmonella* are not consistently effective against *Campylobacter* (**Chen and Stern, 2001**). This is reported as a consequence of the unique niche occupied by *Campylobacter* in the caecum, where it swims freely in the mucous-filled crypts (**Beery *et al.*, 1988**) as opposed to *Salmonella* which attaches to receptor sites on the mucosal epithelium (**Mead, 2000**). Inoculating chicks with bacteria capable of utilising mucin as a carbon source, thus directly competing with *Campylobacter*, has achieved some success (**Schoeni and Doyle, 1992**). However, other workers have failed to reproduce this when selecting bacterial species using similar criteria (**Mead *et al.*, 1996**). **Stern (1994)** and **Mead *et al.* (1996)** report the ability of undefined anaerobic chicken intestine mucous preparations to prevent *Campylobacter* colonisation.

Interestingly, recent research in this area has focussed on heterologous CE i.e. using campylobacters to prevent the colonisation of other campylobacters (**Newell and Wagenaar, 2000; Chen and Stern, 2001**). If non-pathogenic strains of *Campylobacter*

can be identified, this technique may prove useful. However, given the genomic plasticity of *Campylobacter* (**Wassenaar *et al.*, 2000**) and that natural transformation has been demonstrated *in vitro* (**Wang and Taylor, 1990**) and *in vivo* (**Boer *et al.*, 2002**), a considerable amount of research is required to identify suitably stable strains before this technique is feasible.

### 1.5.3 Vaccination

The dramatic decrease of human salmonellosis in the UK during the past few years is primarily attributed to the control of *Salmonella* in chickens through vaccination (**Cogan and Humphrey, 2003**). However, the development of a vaccine to combat *Campylobacter* in poultry is more problematic for a number of reasons. Firstly, *Campylobacter* contamination of broiler carcasses is considered the major threat to human health compared with eggs for *Salmonella*. Laying hens are generally kept for a considerably longer period than broiler chickens and therefore have a fully-developed immune system capable of mounting a response to a live oral vaccine. The lifespan of the average broiler chicken is only six weeks which gives little time for their immature immune systems to develop a protective response (**Newell and Wagenaar, 2000**). A possible solution to this is passive immunisation (**Newell and Wagenaar, 2000**). However, mature breeding hens often possess circulating anti-*Campylobacter* antibodies (**Sahin *et al.*, 2001**) which do not prevent them from being caecally colonized by *Campylobacter* (**Newell and Wagenaar, 2000**). This raises doubts about the efficacy of such antibodies in preventing colonisation in chicks through maternal inheritance.

However, antibodies administered orally to chicks appear to be effective in both therapeutic and prophylactic trials (**Tsubokura *et al.*, 1997**).

Live vaccines are generally regarded to induce a more comprehensive immune response than killed or subunit vaccines (**Kuby, 1997**) and have been effective against *Salmonella* in chickens (**Cogan and Humphrey, 2003**). However, since a detectable seroconversion accompanies *Campylobacter* presence in most chickens, there is uncertainty as to how this response can be enhanced to prevent colonisation. Killed vaccines may provide some protection when administered parenterally but this may be too time consuming and costly for broilers (**Newell and Wagenaar, 2000**).

A recent trend in vaccine development is the utilization of recombinant vector vaccines. These vaccines are produced by introducing genes encoding major antigens of virulent pathogens into an attenuated bacterium or virus. A number of attenuated *Salmonella* mutants are able to interact with the lymphoid tissues in Peyer's patches but are not able to cause systemic disease (**Cardenas and Clements, 1992**). One such mutant of *Salmonella* Typhimurium has been used to express *Campylobacter* flagellin and offered some protection from *Campylobacter* colonisation when administered orally to chickens (**Kauc and Nachamkin, 1998**). Nevertheless, a vaccine eliciting an immune response in the chicken capable of consistently preventing *Campylobacter* colonisation has, thus far, proven elusive.

## 1.6.0 BACTERIOPHAGE AND THEIR PRESENCE IN THE FOOD CHAIN

### 1.6.1 The discovery and nature of bacteriophage

Bacteriophage (phage) are viruses which exclusively infect bacterial cells. They were independently discovered by **Twort (1915)** and **D' Herelle (1917)** although initial observations of these viruses date back to Hankin in 1896 **(Kutter, 1997; Sulakvelidze *et al.*, 2001)**. Although D' Herelle was quickly convinced of the viral nature of phage, many scientists believed their bactericidal effect was the result of an enzyme **(Kutter, 1997; Sulakvelidze *et al.*, 2001)**. Indeed during this period, leading microbiologists such as Bordet and Gratia engaged in increasingly acrimonious arguments about the nature of these agents (reviewed by **Stent and Adelberg, 1960)**.

Definitive proof that phage are able to multiply at the expense of their host came when **Ellis and Delbruck (1939)** performed their classic one-step growth curve. They went on to explain how Bordet and Gratia misinterpreted phage-induced bacterial death as an autolysis event. Delbruck elegantly demonstrated that if many phage attack one bacterium simultaneously, they lyse their host without replication (lysis from without). However, if a single phage infects a cell it multiplies inside its host until the progeny lyse the host from the inside and diffuse into the culture medium (lysis from within or "lytic infection"). A more detailed examination of the intracellular replication of *E. coli* phage T4 was undertaken in 1951 by **Doermann (1951)**. This series of experiments determined that phage replication within the host cell could be divided into four phases (Fig. 1A). However, although lytic infection was beginning to be characterised, it became clear that

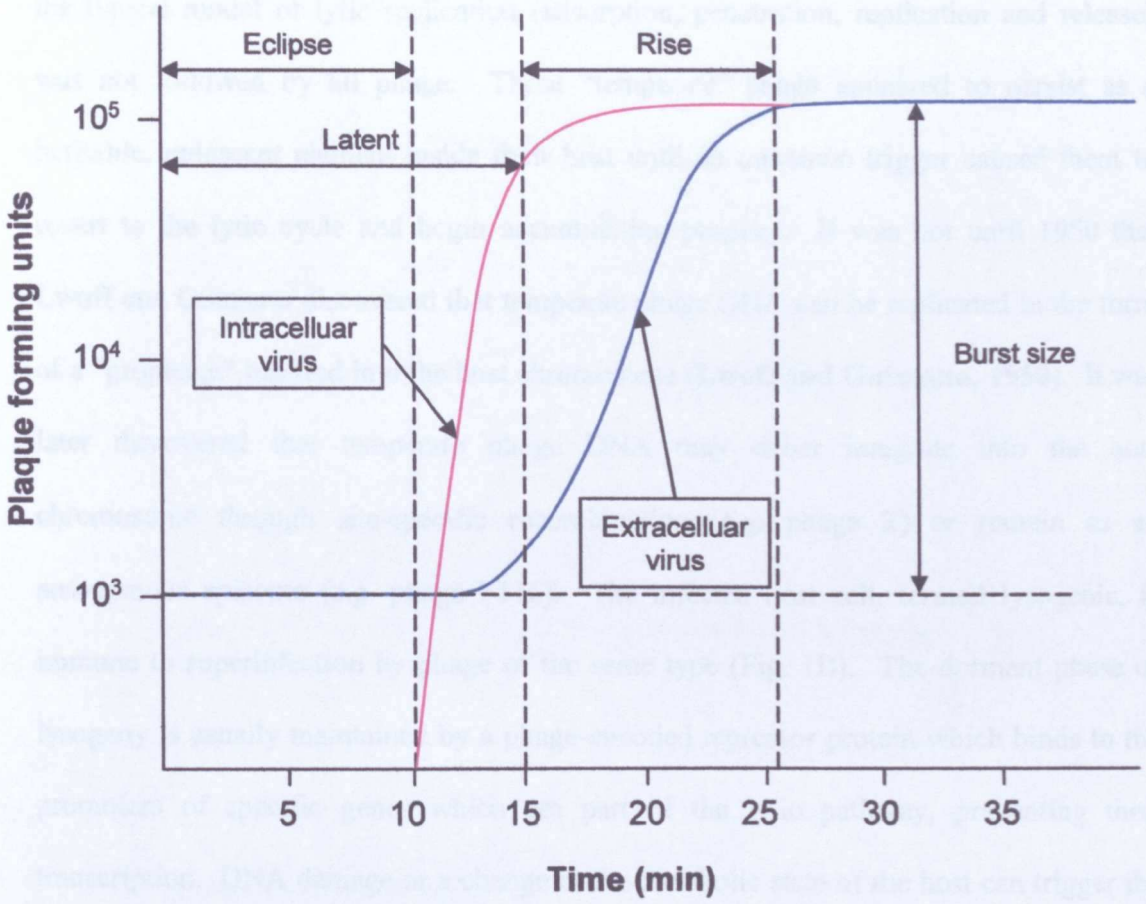


**Figure 1A. Bacteriophage One-step Growth Curve.**
In a one-step growth curve, phage are mixed with their hosts and allowed to adsorb. Any unadsorbed phage are inactivated by specific antisera and the culture is diluted 1,000 fold to ensure progeny phage cannot initiate secondary replication cycles. Following adsorption, the phase where mature progeny cannot be isolated from intracellular fractions is called the eclipse period. Eventually, mature viruses begin to accumulate in the cell but are not detected in the extracellular medium (latent period) until the cells lyse and release their progeny (rise period). The difference between the initial and final number of plaques is known as the burst size and can be estimated on a per-cell basis.

the typical model of lytic replication (adsorption, penetration, replication and release) was not followed by all phage. These "temperate" phage appeared to persist as a heritable, quiescent element inside their host until an unknown trigger caused them to revert to the lytic cycle and begin accumulating progeny. It was not until 1950 that Lwoff and Gutmann discovered that temperate phage DNA can be replicated in the form of a "prophage" inserted into the host chromosome (**Lwoff and Gutmann, 1950**). It was later discovered that temperate phage DNA may either integrate into the host chromosome through site-specific recombination (e.g. phage λ) or remain as an autonomous episome (e.g. phage F116). The infected host cell, termed lysogenic, is immune to superinfection by phage of the same type (Fig. 1B). The dormant phase of lysogeny is usually maintained by a phage-encoded repressor protein which binds to the promoters of specific genes which are part of the lytic pathway, preventing their transcription. DNA damage or a change in the metabolic state of the host can trigger the lytic pathway by cleavage of the repressor protein by enzymes synthesised *de novo* by the host. Ionising radiation triggers the global transcription of genes involved in the protective SOS response in *E. coli*. The protein RecA is one product of this response and cleaves the cI repressor of phage λ allowing the lytic cascade to commence.

### 1.6.2 Bacteriophage prevalence and use in the human food chain

Bacteriophage are regarded as the most abundant forms of life on the planet with an estimated $10^{31}$ present in the biome at any one time (**Rohwer and Edwards, 2002**). Their presence throughout the food chain has been widely documented with some phage

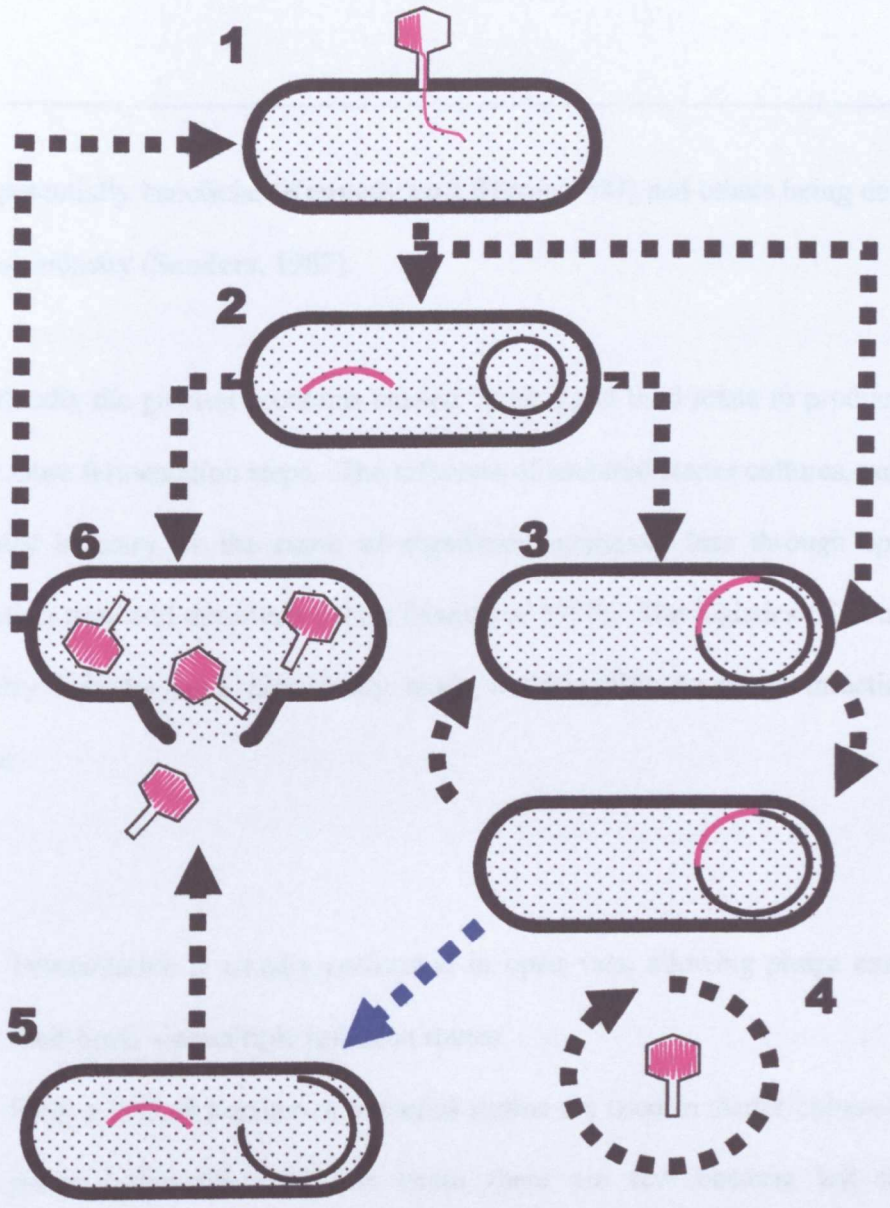


**Figure 1B. Bacteriophage infection via lytic and lysogenic pathways.**

Infection in both pathways is initiated by the adsorption of the virion onto the host cell surface and injection of the viral genome into the cytoplasm (1). Once the phage genome has entered the cell (2), viral replication may proceed via the lytic pathway. This occurs when transcription of phage DNA results in the formation of new virions and eventual lysis of the host cell (6). Alternatively, the phage may integrate into the host genome to form a prophage (3). The prophage element is replicated along with the host chromosome and confers immunity of the lysogenic bacterium to superinfection by phage of the same immunity group (4). Induction results in the excision of the prophage from the host chromosome (5) and initiation of the lytic cycle (6).

being potentially beneficial (**Kennedy and Bitton, 1987**) and others being detrimental to the food industry (**Sanders, 1987**).

Undoubtedly the greatest problems caused by phage in food relate to products requiring one or more fermentation steps. The infection of bacterial starter cultures, particularly in the dairy industry, is the cause of significant economic loss through spoilage, lost production time and decontamination (**Sanders, 1987**). The logistics of fermentation in the dairy industry have historically made it susceptible to phage infection for four reasons:

1. Fermentation is usually performed in open vats, allowing phage easy access to their hosts via multiple infection routes.
2. Only a limited number of bacterial strains are used in starter cultures. Should a phage infect the dominant strain there are few bacteria left to assist in fermentation.
3. Dispersion of phage particles throughout the product is facilitated by the use of multiple mixing steps which encourages secondary infections.
4. The high throughput of products in modern factories results in a very high density of host. As many as $10^{18}$ bacterial cells may be produced on a daily basis and the opportunities for phage multiplication in a host population of this size is clear.

The development of strategies to control phage infection in fermentation has mainly focussed on either elimination of external sources of contamination; developing bacterial strains resistant to infection or the addition of inhibitory agents during processing.

Aside from the burden of disrupted fermentation in the dairy industry, beneficial applications of phage have been devised. Pseudomonads are a primary cause of food spoilage at low temperature (**Jay, 1992**). Psychrophilic phage, capable of active replication on *Pseudomonas* hosts at temperatures as low as 1°C, have been isolated from fresh meat, poultry and fish (**Kennedy and Bitton, 1987**). Moreover, **Greer (1986)** has demonstrated that phage could actively control *Pseudomonas* growth when applied to the surface of contaminated beef products.

Phage usually exhibit a greater resistance to physical and chemical inactivation than vegetative bacterial cells (**Kennedy and Bitton, 1987**). This has allowed their use in various situations to monitor pollution both in the environment and in the food chain. Due to their general association with faecal contamination, phage of coliforms have received particular attention in this area (**Kennedy and Bitton, 1987**). Phage are cheap and sensitive indicators of faecal pollution in foods and have been useful in the detection of contamination in food products exposed to environmental waters, especially shellfish (**Kennedy and Bitton, 1987**). Bacteriophage have also been used to monitor the faecal contamination of groundwater (**Leclerc *et al.*, 2000**).

The use of phage in the food chain is not limited to prevention of food spoilage. Phage have been effectively implemented to limit the growth and presence of food-borne

pathogens on fruit (**Leverentz *et al.*, 2001**), in livestock (**Smith and Huggins, 1983**) and poultry (**Barrow *et al.*, 1998**). To date, most research on the potential of phage to control bacterial populations has focussed on humans and domestic animals, often known as "phage therapy".

## 1.7.0 BACTERIOPHAGE THERAPY

It was D' Herelle who first sought to harness the potential of phage to treat bacterial infections. In 1919 he performed the first successful phage therapy trial on patients suffering from bacterial dysentery (**Sulakvelidze *et al.*, 2001**). This early success was repeated by Bruynoghe and Maisin in 1921 in the treatment of skin infections caused by *Staphylococcus* ssp. (**Kutter, 1997; Sulakvelidze *et al.*, 2001; Summers, 2001**). However, the therapeutic successes with phage were not matched by an understanding of how or why bacterial death occurred. Indeed, an extensive review of phage therapy in the 1930s, conducted by the American Medical Association, concluded that phage were likely to be enzymes rather than viruses (**Eaton and Bayne-Jones, 1934**). Enthusiastic entrepreneurs and medics were keen to exploit phage therapy which resulted in a number of commercial products. However, D' Herelle himself tested twenty such products, some of which he concluded contained no active phage at all (**Kutter, 1997**). The generally poor efficacy of commercial phage preparations led to widespread criticism and disagreement about this new technique. The re-examination of early phage therapy trials by present day virologists suggests many of the failures could be attributed to one or more of the following factors:

1. Paucity of understanding of the heterogeneity and ecology of both the phage and bacterium involved.
2. Failure to select phage of high virulence against the target bacteria present in the patient.
3. Use of single phage in infections involving mixtures of different bacteria.
4. Emergence of resistant bacterial strains.
5. Failure to appropriately characterise or titre phage preparations.
6. Failure to neutralise gastric pH prior to oral administration.
7. Inactivation of phage by specific and non-specific host immune responses.
8. Liberation of endotoxins leading to toxic shock in the patient.
9. The scarcity of reliable bacteriology laboratories to correctly identify the pathogens causing an infection.

(modified from **Kutter, 1997**)

These factors, along with the discovery of antibiotics in the 1940s and the ensuing Second World War led to the sidelining and subsequent abandonment of phage therapy in the West. However, research and practical application of phage therapy continued in various institutes of Eastern Europe and the old U.S.S.R. The most famous of these are the Bacteriophage Institute in Tbilisi, Georgia and the Polish Academy of Sciences in Wroclaw, Poland.

### 1.7.1 Reappraisal of phage therapy

The renaissance of phage therapy in the West was initiated by a series of veterinary experiments by Smith and Huggins in the 1980s. They found that *E. coli* infections of calves, lambs and piglets could be cleared or severely retarded by the oral dosing of cocktails of lytic coliphage (**Smith and Huggins, 1983**). Emerging multidrug resistant (MDR) bacteria causing clinical and veterinary infections has led to research comparing the efficacy of treating such infections with phage instead of antibiotics (**Smith and Huggins, 1982**). The greater understanding of bacteriophage biology today has led to some notable successes in phage therapy. **Berchieri *et al.* (1991)** used phage isolated from human sewage to successfully treat experimental *Salmonella* Typhimurium infections in chickens. In a demonstration of the potential benefits of phage over chemotherapy, **Biswas *et al.* (2002)** were able to prevent the death of mice inoculated with vancomycin-resistant *Enterococcus faecium* by intraperitoneal injection of bacteriophage. Work by **Cerveny *et al.* (2002)** showed that bacteriophage could protect mice which had been intravenously injected with over ten times the lethal dose of *Vibrio vulnificus*. Bacteriophage are imbued with several traits which give them unique advantages over antibiotics. Phage are both self-replicating and self-limiting, they will only persist as long as susceptible hosts are present. They can be targeted more specifically than antibiotics which not only limits widespread phage resistance but prevents the disruption of commensal microflora often seen with broad-spectrum antibiotics. The selection of phage which adsorb to bacterial virulence determinants has shown that mutants which are resistant to phage infection are also less virulent (**Smith and Huggins, 1983**). The long-term use of phage to treat human infections in Eastern

Europe with few ill effects demonstrates that, unlike some antibiotics, phage can be applied without the development of toxicosis or allergy. Phage may be used prophylactically, therapeutically and in the sanitising of surfaces. Producing antibiotics at a high concentration and purity is difficult and expensive, requiring substantial capital investment in manufacturing and development. Conversely, phage are very cheap to produce and purify and require only basic fermentation and purification equipment. Another disadvantage of antibiotics is their diffusion from the site of administration. Antibiotics become less effective with time as their power to penetrate deep into tissues is limited by their initial concentration. However, as bacteriophage multiply in the presence of their host, their penetrative power is only limited, in theory, by the presence of their hosts. Bacteriophage are able to circulate in the bloodstream following both oral and parenteral administration and can effectively pass through the blood-brain barrier (**Barrow and Soothill, 1997**). A key advantage over antibiotics is the ability of phage to mutate to evade bacterial resistance mechanisms making them far more versatile agents.

## 1.7.2 Potential drawbacks of phage therapy

### *1.7.2.1 Safety in mammalian and avian species*

Data from the extensive human phage therapy trials of Eastern Europe and the veterinary trials recently performed in the West suggest that phage are innocuous to mammals and avians (**Barrow and Soothill, 1997; Sulakvelidze *et al.*, 2001**). This is supported by the ubiquity of these organisms in the environment (**Bergh *et al.*, 1989**) and their regular

consumption in foods without notable adverse effects. The main problem from a therapeutic perspective is not the damage the phage can cause the animal but *vice versa.*

When phage are administered to the subject, especially intravenously, the reticuloendothelial system (RES) often sequesters the virions before they are able to achieve maximal effect on the target bacterial population (**Barrow and Soothill, 1997**). One report indicated phage may become resistant to RES sequestration by serial passage and selection for virions which do not evoke a strong immune response (**Merril *et al.*, 1996**). However, the efficacy of this technique was recently challenged by **Barrow and Soothill (1997)** and may be too time-consuming for clinical applications. Phage have been shown to elicit humoral immunity in humans which presents another barrier to phage therapy (**Kucharewicz-Krukowska and Slopek, 1987; Barrow and Soothill, 1997**). The nature of the humoral response to phage has not been fully determined but will undoubtedly play a major part in defining the breadth of scenarios in which phage therapy can be applied. If phage are shown to induce a cell-mediated response by the host, which allows the development of immunological memory, repeated use of phage for subsequent infections may prove ineffective (**Barrow and Soothill, 1997**).

The liberation of endotoxins following the death of bacterial cells is a major concern with antibiotic chemotherapy. This process, known as the Herxheimer reaction, may also occur with bacteriophage-mediated lysis. Whilst antibiotics will diffuse from the site of administration and gradually lose potency, bacteriophage will penetrate the tissues of the subject in increasing numbers and potentially liberate more endotoxin. The replication of phage in susceptible populations of bacteria within a subject would not be under the

control of the therapist and may present a considerable obstacle to the widespread use of phage therapy. Data from the phage therapy institutes in Poland and Georgia may prove invaluable during an assessment of these risks.

### *1.7.2.2 Development of bacterial resistance to phage infection*

Natural selection for resistance in bacterial populations is a problem akin to both antibiotic and phage therapeutics. Although bacterial resistance to phage infection is an inevitable consequence of widespread phage therapy, such resistance is thought to occur at a markedly lower frequency than that for antibiotics (**Carlton, 1999**).

## 1.8.0 MECHANISMS OF PHAGE RESISTANCE

The means by which bacteria have evolved resistance to phage are as diverse as those for antibiotics. Such resistance mechanisms should not be confused with lysogeny where short-term immunity to phage of the same group is granted by the resident prophage. The initial adsorption of the phage to the host cell can be prevented by alteration, loss or concealment of receptor sites. Bacteriophage often utilize highly conserved structures on the host cell surface as their receptors (e.g. phage λ uses maltose binding protein on *E. coli*). This introduces a counter-evolutionary pressure on the host cell since a mutation in a vital gene may be either lethal or render the bacterium incapable of competing successfully in its ecological niche. **Baggesen *et al.* (1997)** demonstrated that the loss of the lipopolysaccharide receptor in strains of *Salmonella* Enteritidis altered the phage type of the bacterium but did not prevent infection by other phage suggesting the use of an

alternate receptor. **Deveau *et al.* (2002)** found that phage resistant strains of lactococci produced greater amounts of extracellular polysaccharide than sensitive strains, which possibly obscured receptor sites. Other structures which have been implicated as phage receptors include lipopolysaccharide (**Nesper *et al.*, 2000**), outer membrane proteins (**Ho and Slauch, 2001**) and pili (**Roncero *et al.*, 1990**).

### 1.8.1 Restriction modification

After the phage successfully adsorbs to the host cell and injects its genetic material into the cytoplasm, it has to contend with possible attack by host restriction enzymes. Bacterial genomes sometimes encode methylases which modify the host DNA by covalently bonding a methyl group to specific cytosine or adenine residues in a particular recognition sequence. Such methylases recognise the same DNA sequences as their respective restriction endonucleases, rendering the modified bacterial DNA impervious to digestion within the bacterium. However any foreign DNA entering the cell which does not possess the same methylation pattern will be subject to digestion. Indeed the term "restriction" was originally derived from the ability of these enzymes to limit the host range of some bacteriophage. Nevertheless, bacteriophage have evolved methods of evading such resistance mechanisms. For example, the genome of coliphage T7 encodes a protein which binds to Type I restriction endonucleases of the host, leading to irreversible inactivation of these enzymes. An alternative approach by phage T4 is to methylate its own DNA to prevent cleavage. Even in the absence of such countermeasures, host restriction modification is not an absolute method of protection. Evolutionary selection of point mutations in recognition sequences on the phage DNA

may render host restriction enzymes ineffective. Negligence by host endonucleases also occurs where there is a high multiplicity of infection and the host is overwhelmed by viral DNA. Only one phage genome need survive digestion and it will be modified with the host's methylation pattern and be able to infect other cells of the same strain *ad libitum*.

No matter which mechanism is used, the development of phage resistance can be minimised by using a cocktail of different phage which are able to infect the bacterium in diverse ways. Antibiotics were used successfully in this way for many decades, although it is generally accepted that multiple drug resistance in bacterial pathogens is becoming more common. However, unlike resistance to antibiotics, phage resistance may not be entirely disadvantageous and can be combated more easily. Altering or entirely losing a phage receptor site may render the host bacterium immune to phage infection but could also reduce pathogenicity or the ability to compete in an ecological niche (**Smith and Huggins, 1983**). Phage typing schemes have been successfully applied to numerous pathogenic bacteria with few, if any, strains being universally resistant to infection. Moreover, previous work has demonstrated that the development of resistance to one phage may result in susceptibility to infection by others (**Baggesen *et al.*, 1997**). As such, the co-evolutionary relationship between a virus and its host can be worked to the advantage of the phage therapist in ways which are not available to the chemotherapist.

## 1.9 TRANSDUCTION

The bacteriophage-mediated movement of DNA from a donor bacterial cell to a recipient is known as transduction and is arguably the greatest obstacle to the widespread use of phage therapeutically. Currently, there are two known mechanisms of transduction, generalised and specialised. Generalised transduction is possible with both lytic and temperate phage and has the potential to transfer any part of the donor chromosome to the recipient cell. The digestion of the host chromosome constitutes a part of the lytic phase in several phage families. After replication of the phage genome is complete, the viral DNA is translocated and packaged into a preformed protein shell, the prohead (**Fujisawa and Morita, 1997**). The mechanisms of DNA packaging vary between phage but proceeds either through recognition of specific sequences (such as the *pac* genes in T7); a pure "head full" mechanism where DNA of a certain size is packed into the prohead (e.g. T-even phage) or, in the case of phage μ, a combination of both (**Fujisawa and Morita, 1997**). Host DNA fragments of a similar size to the phage genome may be accidentally packaged into proheads of T-even phage. With *Salmonella* phage P22, some regions of the host chromosome may resemble *pac* sites on the phage genome and be accidentally packaged into the prohead (**Singleton and Sainsbury, 1999**). The phage resulting from such events, called transductants, are able to infect other host cells but in many cases cannot complete their lytic cycle. Once injected into the donor cell, the DNA may either be digested by restriction endonucleases; undergo recombination and stably integrate into a host chromosome or plasmid (complete transduction) or persist as an extra-chromosomal element, which is usually lost after several divisions of the host cell (abortive transduction).

Specialised transduction is undertaken only by temperate phage whose genomes are incorporated into the host chromosome. The integration of temperate phage genomes into the host chromosome is the result of a single site-specific recombination event. For example, phage λ integrates at the *att* sites in-between the *gal* and *bio* genes of *E. coli* **(Lewin, 1997)**. Aberrant excision of the integrated prophage may result in DNA surrounding the *gal* or *bio* genes being incorporated into a prohead instead of part of the λ genome. Infection of a recipient cell by the transductant may then lead to stable integration of that gene fragment into the host chromosome. Alternatively, the DNA could be degraded as with generalised transduction. Whether or not the transducing phage is fully-functional and capable of both lysis and lysogeny depends on the viral gene(s) 'sacrificed' during the acquisition of bacterial host DNA.

Transduction is generally a rare event, occurring at a frequency of $10^{-7}$ to $10^{-5}$ depending on the phage and host systems **(Singleton and Sainsbury, 1999)**. Successful (i.e. stable) transduction events occur more frequently with closely related bacterial strains and decline with genomic distance. Nevertheless, there are many examples of genes derived from phage which are stably integrated into the chromosomes or plasmids of pathogenic bacteria. Historically, toxin production has been the most widely recognised bacterial characteristic linked to bacteriophage infection **(Bishai and Murphy, 1988)**. Examples include the toxins of *Clostridium botulinum*, *Corynebacterium diptheriae*, *Vibrio cholerae* and *Streptococcus pyogenes* **(Waldor, 1998)**. However, more recent work has demonstrated that phage are able to influence bacterial pathogenicity by encoding products resulting in a wide diversity of virulence determinants. Examples include the

toxin-coregulated pili of *V. cholerae*; fibrinolysin in *Staphylococcus aureus* and serum resistance in *Shigella dysenteriae* (**Waldor, 1998**). Widespread use of phage for therapeutic purposes would undoubtedly lead to an increase in transduction frequency. Specialised transduction can be eliminated by only using lytic phage. However, generalised transduction is much more difficult to control and remains an unresolved issue of concern in the scientific community.

## 1.10 *CAMPYLOBACTER* BACTERIOPHAGE

Very little information is available for phage of campylobacters. Over 170 phage for this genus have been reported and most are classified as members of the Myoviridae and Siphoviridae families (Table 1A). Thus far, there has been no evidence of lysogeny in the *Campylobacter* genus. Indeed the recently-completed genome sequence of *C. jejuni* NCTC 11168 was unusual among bacteria in that it contained no phage-like elements or insertion sequences (**Parkhill *et al.*, 2000**). Whilst phage therapy of *Salmonella* in chickens has achieved some success (**Berchieri *et al.*, 1991**) the primary use of *Campylobacter* phage has been in typing schemes (**Frost *et al.*, 1999**). Phage used in such typing schemes either originated from abattoir effluent or poultry faeces (**Grajewski *et al.*, 1985; Sails *et al.*, 1998**). The *Campylobacter* phage typing scheme adopted by the PHLS in the UK is the result of comprehensive trials with the 170 *Campylobacter* phage reported to date. A total of sixteen phage were selected for this scheme on the basis of producing reproducible lytic profiles whilst providing the greatest discriminatory power between *Campylobacter* strains. These sixteen phage were also characterised with respect to their morphological and genetic characteristics. They were found to all be

| Family or Group | Genera | Type Member | Particle Morphology | Envelope | Genome |
|---|---|---|---|---|---|
| Corticoviridae | Corticovirus | PM2 | icosahedral | No | supercoiled d/s DNA |
| Cystoviridae | Cystovirus | Ø6 | icosahedral | Yes | 3 segments d/s RNA |
| Inoviridae | Inovirus | coliphage fd | rod | No | circular s/s DNA |
| | Plectrovirus | Acholeplasma phage | | | |
| Leviviridae | Levivirus | coliphage MS2 | icosahedral | No | 1 (+)strand RNA |
| | Allolevirus | coliphage Qbeta | | | |
| Lipothrixviridae | Lipothrixvirus | Thermoproteus phage 1 | rod | Yes | linear d/s DNA |
| Microviridae | Microvirus | coliphage ØX174 | icosahedral | No | circular s/s DNA |
| | Spirovirus | Spiroplasma phages | | | |
| | | Mac-1 phage | | | |
| Myoviridae | | coliphage T4 | tailed phage | No | linear d/s DNA |
| Plasmaviridae | Plasmavirus | Acholeplasma phage | pleiomorphic | Yes | Circular d/s DNA |
| Podoviridae | | coliphage T7 | tailed phage | No | linear d/s DNA |
| Siphoviridae | lambda phage group | coliphage lambda | tailed phage | No | linear d/s DNA |
| Sulpholobus shibatae virus | | SSV-1 | lemon-shaped | No | circular d/s DNA |
| Tectiviridae | Tectivirus | phage PRD1 | icosahedral | No | linear d/s dna |

**Table 1A. Current names and attributes of bacteriophage families recognised by the International Committee on the Taxonomy of Viruses (Cann, 1993).**

members of the Myoviridae family and possessed unusually large genomes (up to 320 kb in size). Phage typing schemes such as this provide a cheap and easy method of discriminating between bacterial strains and requires only minimal equipment. In addition to phage typing, the potential to exploit bacteriophage as biocontrol agents has prompted interest from several companies wishing to develop and use phage therapy to control bacterial pathogens, including *Campylobacter*, in humans and animals. These include BioPhage Inc. (www.biophage.com); Intralytix (www.intralytix.com); and Phage Therapeutics International Inc. (www.phagetx.com). However, the results of their endeavours are not yet known.

# CHAPTER 2

## GENERAL MATERIALS AND METHODS

### 2.0 SOLUTIONS

All solutions were prepared in reverse osmosis (RO) water. Chemicals were obtained from Fisher Scientific (Loughborough, Leicestershire) and biochemicals from Sigma Gillingham, Dorset) unless otherwise stated. All media were prepared using RO water and sterilized at 15 psi for 20 min unless otherwise stated.

#### 2.0.1 Magnesium sulphate solution

A 1 M stock solution of $MgSO_4$ was prepared and filter sterilized using a sterile 0.2 µm pore cellulose acetate membrane filter (Minisart™, Sartorius, Gottingen, Germany). This was stored at room temperature and pressure (RTP) until required. For suspension of *Campylobacter* cells, a 10 mM $MgSO_4$ solution was prepared by adding 100 µl of the 1 M stock solution to 10 ml of sterile RO water.

#### 2.0.2 Maximum recovery diluent (MRD)

MRD, a peptone saline diluent, was used for all serial dilutions of bacterial cell suspensions unless otherwise stated. MRD medium (CM733, Oxoid, Basingstoke, UK) was dissolved in 500 ml of RO water and sterilized according to the manufacturer's instructions. Final quantities of ingredients were: peptone (1 g $l^{-1}$); NaCl (8.5 g $l^{-1}$) pH 7.0.

### 2.0.3 Phosphate buffered saline (PBS)

Five PBS tablets (BR14a, Oxoid) were dissolved in 500 ml of RO water and sterilized according to the manufacturer's instructions. PBS was stored at RTP until required. Final quantities of constituents were: NaCl (8 g $l^{-1}$), KCl 0.2 g $l^{-1}$, disodium hydrogen phosphate (1.15 g $l^{-1}$), potassium dihydrogen phosphate (0.2 g $l^{-1}$) pH 7.3.

### 2.0.4 SM Buffer (SM)

SM buffer was used for all bacteriophage dilutions unless otherwise stated. SM was prepared by adding Trizma base (2-amino-2-hydroxymethyl-1,3-propanediol, 3 g), NaCl (2.9 g), $MgSO_4.7H_2O$ (1.0 g) and gelatin (5 ml of a 1% solution) to 500 ml of RO water. The pH of the solution was adjusted to 7.5 with concentrated hydrochloric acid. This was sterilised by autoclaving at 15 psi for 20 min.

### 2.0.5 Tris acetate EDTA buffer (TAE) pH 8.0

A 10X TAE buffer stock solution was prepared by adding Trizma base (48.4 g); glacial acetic acid (10 ml) and EDTA (2.92 g) to 1 l of RO water. This solution was diluted 1:9 in RO water when required.

### 2.0.6 Tris EDTA buffer (TE) pH 7.5

A 10X TE buffer stock solution was prepared by adding Trizma base (12.1 g) and EDTA (3.7 g) to 1 l of RO water. This solution was diluted 1:9 in RO water when required.

### 2.0.7 Tris borate EDTA buffer (TBE) pH 8.0

A 5X TBE buffer stock solution was prepared by adding Trizma base (54.0 g); boric acid (27.5 g) and EDTA (2.93 g) to 1 l of RO water. This solution was diluted 1:4 in RO water when required.

## 2.1 BACTERIAL STRAINS AND THEIR STORAGE

*Campylobacter* reference strains obtained from the National Collection of Type Cultures (NCTC) are listed in Table 2A. Typically, strains were subcultured on Columbia blood agar plates (CBA, **2.3.1.1**) and incubated for 24 h at 42°C in microaerobic conditions (**2.3.3.3**). These subcultures were stored on a short term basis (<1 month) under microaerobic conditions at 4°C. For long term storage a sterile cotton swab was used to harvest blood plate growth after 24 h. This swab was then transferred to a cryovial containing 1 ml of glycerol storage medium (**2.3.1.2**) and frozen at -80°C until required. Resuscitation was performed by thawing the cryovial contents then using the swab to inoculate a fresh plate of CBA which was then incubated under microaerobic conditions at 42°C for 24 - 48 h.

## 2.2 BACTERIOPHAGE STOCKS AND THEIR STORAGE

*Campylobacter* bacteriophage reference strains used by the Central Public Health Laboratories (CPHL, Colindale, London) in their phage typing scheme were obtained from the NCTC and are listed in Table 2B. Bacteriophage stocks were propagated using a modification of the agar overlay technique described by **Sambrook *et al.* (1989)** (**2.3.6**). Bacteriophage stocks were stored in SM buffer (**2.0.4**) at 4°C unless stated otherwise.

| *Campylobacter* species | Reference number | Source |
|---|---|---|
| *C. jejuni* | NCTC 11168 | NCTC |
| *C. jejuni* | NCTC 12658 | NCTC |
| *C. jejuni* | NCTC 12659 | NCTC |
| *C. jejuni* | NCTC 12660 | NCTC |
| *C. jejuni* | NCTC 12661 | NCTC |
| *C. jejuni* | NCTC 12662 | NCTC |
| *C. jejuni* | NCTC 12663 | NCTC |
| *C. jejuni* | NCTC 12664 | NCTC |
| *C. jejuni* | NCTC 12665 | NCTC |
| *C. jejuni* | NCTC 81176 | NCTC |
| *C. jejuni* | PT1 | NCTC |
| *C. jejuni* | PT2 | NCTC |
| *C. jejuni* | PT5 | NCTC |
| *C. jejuni* | PT6 | NCTC |
| *C. jejuni* | PT19 | NCTC |
| *C. jejuni* | PT33 | NCTC |
| *C. jejuni* | PT35 | NCTC |
| *C. jejuni* | PT44 | NCTC |
| *C. coli* | NCTC 12666 | NCTC |
| *C. coli* | NCTC 12667 | NCTC |
| *C. coli* | NCTC 12668 | NCTC |
| *C. lari* | NCTC 11352 | NCTC |

**Table 2A. Names of bacterial strains used.**
Names of bacterial strains used in experiments. NCTC = National Collection of Type Cultures (Colindale, London, UK).

| Bacteriophage | Reference | Propagating strain |
|---|---|---|
| ϕ1 | NCTC 12673 | *C. jejuni* NCTC 12661 |
| ϕ2 | NCTC 12674 | *C. jejuni* NCTC 12661 |
| ϕ3 | NCTC 12682 | *C. coli* NCTC 12667 |
| ϕ4 | NCTC 12676 | *C. jejuni* NCTC 12663 |
| ϕ5 | NCTC 12678 | *C. jejuni* NCTC 12664 |
| ϕ6 | NCTC 12680 | *C. jejuni* NCTC 12665 |
| ϕ7 | NCTC 12671 | *C. jejuni* NCTC 12660 |
| ϕ8 | NCTC 12681 | *C. coli* NCTC 12666 |
| ϕ9 | NCTC 12669 | *C. jejuni* NCTC 12658 |
| ϕ10 | NCTC 12683 | *C. coli* NCTC 12668 |
| ϕ11 | NCTC 12679 | *C. jejuni* NCTC 12664 |
| ϕ12 | NCTC 12677 | *C. jejuni* NCTC 12663 |
| ϕ13 | NCTC 12672 | *C. jejuni* NCTC 12660 |
| ϕ14 | NCTC 12675 | *C. coli* NCTC 12668 |
| ϕ15 | NCTC 12684 | *C. jejuni* NCTC 12659 |
| ϕ16 | NCTC 12670 | *C. jejuni* NCTC 12662 |

**Table 2B. Names of bacteriophage strains used.**
Names of bacteriophage strains used in experiments. The *Campylobacter* host typically used in the propagation of each phage is shown in the far right column. NCTC = National Collection of Type Cultures (Colindale, London, UK).

## 2.3 METHODS FOR THE CULTIVATION OF BACTERIAL STRAINS AND PROPAGATION OF BACTERIOPHAGE

### 2.3.1 Growth media

Unless otherwise stated, all media were prepared in RO water and sterilized at 15 psi for 20 min. Following sterilization, all media were tempered to 50°C and dispensed (15 - 20 ml) in to sterile Petri dishes. The Petri dishes were then dried in a laminar flow cabinet for 10 h before storage in the dark at 4°C for up to one month.

#### *2.3.1.1 Columbia blood agar (CBA)*

Columbia blood agar base (CM 331, Oxoid) was prepared and sterilized according to the manufacturer's instructions. Laked horse blood (SR48, Oxoid) was added to a final concentration of 5% v/v before dispensing into Petri dishes.

#### *2.3.1.2 Glycerol storage medium (GSM)*

Nutrient broth No. 2 (NB2, 2.3.1.6) supplemented with 20% v/v glycerol was dispensed (500 µl) into 2 ml cryovials and sterilised according to the manufacturer's instructions. Following sterilisation the cryovials were stored at RTP until required.

#### *2.3.1.3 FBP growth supplement*

FBP consisted of $FeSO_4.7H_2O$ (10% w/v), sodium pyruvate (10% w/v) and sodium metabisulphite (10% w/v) in RO water. The solution was heated to 50°C and stirred continuously on a heated magnetic stirring platform until the ingredients had dissolved. The solution was then either used immediately or stored at -20°C until required.

### 2.3.1.4 Modified charcoal cefoperazone deoxycholate agar (CCDA)

Blood free *Campylobacter* selective agar base (Lab 112, Lab M, Bury, UK) was prepared and sterilized according to the manufacturer's instructions. Following sterilization, *Campylobacter* selective supplement (PL 450, Prolab diagnostics, Cheshire, UK) was added according to the manufacturer's instructions to give a final concentration of the following constituents: cefoperazone (32 mg $l^{-1}$), amphotericin B (10 mg $l^{-1}$). The medium was then dispensed into sterile Petri dishes. CCDA was used in the primary isolation of *Campylobacter* from all sources.

### 2.3.1.5 Nutrient agar (NA)

Nutrient agar (CM 3, Oxoid) was prepared and sterilized according to the manufacturer's instructions and dispensed into Petri dishes.

### 2.3.1.6 Nutrient broth number 2 (NB2)

Nutrient broth number 2 (CM 67, Oxoid) was prepared and sterilized according to the manufacturer's instructions and used within 24 h of preparation. Where stated, *Campylobacter* growth was optimized by the addition of the FBP supplement (**2.3.1.3**) to a final concentration of 0.05% v/v.

### 2.3.1.7 NZCYM base medium (NZB)

NZCYM broth (0688-17-0, Difco, Oxford, UK) supplemented with 1.2% w/v Agar No. 3 (L13, Oxoid) was prepared and sterilized according to the manufacturer's instructions and dispensed into Petri dishes. NZB was used as the growth support medium for *Campylobacter* strains used in bacteriophage propagation.

### 2.3.1.8 Plate count agar (PCA)

Bacto™ Plate Count Agar (0479-01-1, Difco) was prepared and sterilized according to the manufacturer's instructions and dispensed into Petri dishes.

### 2.3.1.9 Rappaport-Vassiliadis soya peptone broth (RVS)

RVS broth (CM 866, Oxoid) was prepared and sterilized in 10 ml volumes according to the manufacturer's instructions and stored at RTP for up to 48 h before use. RVS was used for the selective enrichment of *Salmonella* ssp. from faeces.

### 2.3.1.10 Top layer agar (TLA)

NZCYM broth (0688-17-0, Difco) supplemented with 0.6% w/v Agar No. 3 (L13, Oxoid) was prepared and sterilized according to the manufacturer's instructions. Volumes (500 ml) of this medium were stored at RTP for up to one month. When required, the TLA was reheated in a microwave and tempered to 50°C in a water bath before dispensing 5 ml volumes into sterile universal tubes. The 5 ml TLA aliquots were stored in a water bath at 50°C for up to one hour before use.

### 2.3.1.11 Xylose-Lysine desoxycholate agar (XLD)

XLD medium (CM 469, Oxoid) was prepared in 500 ml volumes and heated until reaching boiling point where upon it was tempered to 50°C and dispensed into sterile Petri dishes.

## 2.3.2 Preparation of McFarland turbidity standards

The McFarland standards (**McFarland, 1907**) uses mixtures of 1% w/v anhydrous $BaCl_2$ and a 1% v/v $H_2SO_4$ to form a turbid suspension from the white precipitate ($BaSO_4$). A

McFarland scale was prepared by mixing the solutions in the volumes given in Table 2C. The standards were shaken thoroughly before use and stored for no longer than six months.

### 2.3.3 Bulk cultivation of *Campylobacter* ssp.

#### *2.3.3.1 Cultivation on solid media*

Columbia blood agar (CBA, **2.3.1.1**) was used for growth of all pure *Campylobacter* cultures unless otherwise stated. A sterile cotton swab was used to harvest growth from broth or plate cultures and then used to uniformly inoculate the surface of a CBA plate. Plates were inverted and placed in a gas jar and incubated at 42°C for 18 - 24 h in microaerobic conditions (**2.3.3.3**) unless otherwise stated.

#### *2.3.3.2 Cultivation in broth*

Nutrient broth No. 2 (NB2, **2.3.1.6**) was used for growth of *Campylobacter* ssp. in all cases. During growth curves and other situations requiring access to the same culture at multiple time points, FBP supplement addition was necessary to buffer oxygen tension in the broth and prevent erratic growth. During standard overnight (15 - 20 h) incubation of cultures, the addition of FBP was not necessary. A loopful of biomass was taken from a fresh blood plate culture and used to inoculate a flask of sterile NB2. Flask aeration was maintained at five times the broth volume (100 ml of broth in a 500 ml flask unless stated otherwise). The flask was transferred to a gas jar and the internal atmosphere adjusted to microaerobic conditions (**2.3.3.3**). Gas jars were secured in a shaking platform incubator at 42°C and 150 rpm unless otherwise stated.

| McFarland Scale No. | 1% $BaCl_2$ (ml) | 1% $H_2SO_4$ (ml) |
|---|---|---|
| 0.5 | 0.05 | 9.95 |
| 1.0 | 0.1 | 9.9 |
| 2.0 | 0.2 | 9.8 |
| 3.0 | 0.3 | 9.7 |
| 4.0 | 0.4 | 9.6 |
| 5.0 | 0.5 | 9.5 |
| 6.0 | 0.6 | 9.4 |
| 7.0 | 0.7 | 9.3 |
| 8.0 | 0.8 | 9.2 |
| 9.0 | 0.9 | 9.1 |
| 10.0 | 1.0 | 9.0 |

**Table 2C. McFarland turbidity standards**
The McFarland standards are prepared by mixing different ratios of 1% w/v anhydrous $BaCl_2$ and a 1% v/v $H_2SO_4$ to form a turbid suspension from the white precipitate ($BaSO_4$).

#### 2.3.3.3 Establishment of a microaerobic atmosphere

Rubber-sealed 3.5 l evacuable gas jars were used for all growth conditions requiring a modified atmosphere. Modification of the jar's atmosphere was achieved by evacuating the chamber to -20 Hg and replacing with an anaerobic gas mixture (5% $H_2$, 10% $CO_2$, 85% $N_2$) to give a final microaerobic atmosphere consisting of approximately 7% $O_2$, 3.3% $H_2$, 6.6% $CO_2$ and 82.2% $N_2$.

### 2.3.4 Speciation and enumeration of *Campylobacter*

#### 2.3.4.1 Morphology

Colonies on CCDA plates **(2.3.1.4)** conforming to the description of typical *Campylobacter* growth on this medium were subcultured on CBA **(2.3.1.1)**. Typical colonies are defined as grey, moist, flat and spreading **(Bridson, 1998)**. Subcultures were Gram stained and examined by light microscopy for typical spiral morphology **(Smibert, 1984)**. Motility was examined by suspending a small quantity of culture in 30 µl of MRD **(2.0.2)** on a clean glass slide. The cell suspension was overlaid with a cover slip and examined by light microscopy for the typical darting and corkscrew motility characteristics of *Campylobacter* **(Smibert, 1984)**.

#### 2.3.4.2 Biochemical speciation

Biochemical tests on *Campylobacter* isolates focused on (a) confirmation of the isolate being a member of the *Campylobacter* genus (catalase and oxidase tests) and (b) Identification of *C. jejuni* using the hippurate test.

### 2.3.4.2.1 Catalase test

This test detects the presence of catalase, an oxidoreductase which catalyses the formation of $H_2O$ and $O_2$ from $H_2O_2$. The presence of catalase was determined by suspending one colony in 30 µl of MRD (**2.0.2**) on a clean glass slide. Thermophilic *Campylobacter* species are catalase positive (**Stern *et al.*, 1992**). Ten microlitres of 30% $H_2O_2$ solution was added to the slide and overlaid with a cover slip. The formation of bubbles under the cover slip indicated a catalase positive reaction.

### 2.3.4.2.2 Oxidase test

This is a test used to detect the presence of cytochrome C and its associated oxidase (**Stern *et al.*, 1992**). To do this, 0.1 g of oxidase reagent (tetra methyl-p-phenylenediamine dihydrochloride) was dissolved in 10 ml of sterile RO water. The solution was used to wet a sterile swab which, in turn, was used to harvest growth from the surface of an overnight CBA plate (**2.3.1.1**). A positive result was indicated by the development of a deep blue colour within 10 sec. No colour change or a slight blue tinge was considered negative. All members of the *Campylobacter* genus are oxidase positive.

### 2.3.4.2.3 Hippurate test

Hippuricase is an enzyme only found in *C. jejuni* which hydrolyses hippurate ($C_6H_5.CO.NH.CH_2.CO_2^-$) to benzoate ($C_2H_5O_2^-$) and glycine ($C_2H_5NO_2$). A 1% w/v solution of sodium hippurate was prepared in RO water, filter sterilized, dispensed into sterile 1.5 ml Eppendorf tubes in 500 µl volumes and stored at -20°C (**Stern *et al.*, 1992**). Upon thawing, the hippurate vials were heavily inoculated with growth from an overnight CBA plate (**2.3.1.1**) and incubated aerobically at 37°C for 2 h. Hippurate activity was detected by the addition of 100 µl of 7% w/v 1,2,3-triketohydrindene monohydrate

(ninhydrin reagent). A positive result was indicated by the development of a deep blue colour. No colour change or a mild blue/lilac colour was considered negative.

#### *2.3.4.3 Additional confirmation and speciation*

When required, further confirmation and speciation tests were implemented. These consisted of a latex agglutination test (*Campylobacter* Test Kit, DR0150, Oxoid) and the API Campy kit developed by bioMérieux. Both were used according to the manufacturer's instructions.

#### *2.3.4.4 Enumeration of Campylobacter ssp. in pure culture*

*Campylobacter* enumeration was performed essentially as described by **Miles and Misra (1938)** unless otherwise stated. Briefly, a cell suspension was serially diluted in ten fold steps down to a dilution of $10^{-10}$. Five drops (10 µl) of each dilution were spotted onto the surface of CCDA plates (**2.3.1.4**) without the selective supplement. CCDA was used instead of CBA (**2.3.1.1**) as colonies on the latter tended to spread and be difficult to count. Plates were left to dry for 30 min then inverted and incubated in microaerobic conditions (**2.3.3.3**) at 42°C. Plates were examined for growth at 24 h and 48 h if further incubation was required. Dilutions giving rise to 3 - 30 colonies were used for enumeration. The number of colony forming units (cfu) per 10 µl of the undiluted suspension was calculated by dividing the mean number of colonies for a particular dilution by the dilution factor. This was then multiplied by 100 to convert to cfu per ml.

### 2.3.5 Propagation of *Bdellovibrio* ssp.

A single colony of *E. coli* K12 from a nutrient agar plate (**2.3.1.5**) was used to inoculate 100 ml of Tripticase Soy Broth (211768, Difco) and incubated for 24 h at 30°C. An aliquot of this culture (3 ml) was mixed with 1 ml of approximately $10^6$ pfu $ml^{-1}$

*Bdellovibrio bacteriovorous* 109J prepared in HEPES Ca buffer (HEPES 5.94 g $l^{-1}$; $CaCl_2$ 0.294 g $l^{-1}$ pH 7.8). This suspension was then added to 50 ml of HEPES Ca buffer and incubated aerobically for 48 h at 30°C in an orbital shaking incubator (50 rpm). The resulting lysate was filtered through a 0.45 µm pore size filter (Sartorius) and stored at 4°C for up to four weeks.

### 2.3.6 Propagation of bacteriophage

Bacteriophage isolates were subjected to a minimum of three rounds of serial plaque purification (**2.3.6.1**) prior to storage at 4°C. The *Campylobacter* strain used for the propagation of the phage was subcultured on CBA (**2.3.1.1**). The resulting growth of five plates was harvested into 10 ml volumes of 10 mM $MgSO_4$ suspension (**2.0.1**) of which ten 500 µl volumes were dispensed into sterile 1.5 ml Eppendorf tubes. Phage suspension (100 µl) was then added to each Eppendorf tube and incubated aerobically at 37°C for 20 min to allow for phage adsorption. The suspension was then added to 5 ml of molten TLA (**2.3.1.10**) tempered to 50°C and briefly mixed before pouring onto NZB (**2.3.1.7**). Plates were swirled to ensure an even distribution of TLA on the surface of the base agar. After allowing the agar to set (10 min RTP) the plates were incubated at 42°C for 24 h in microaerobic conditions. Following incubation the plates were examined for lysis compared with a control lawn of the host bacterium. Bacteriophage were eluted from the TLA by the addition of 5 ml of SM buffer (**2.0.4**) to the surface of the plates and gently shaking (60 cycles $min^{-1}$) on an orbital platform shaker for 18 - 20 h at 4°C. The eluate was pooled and filtered through a 0.45 µm pore membrane filter (Sartorius) and stored at 4°C until required.

### 2.3.6.1 Serial plaque purification

Single plaques resulting from bacteriophage infection were excised from top layer agar using the cut off tip of a sterile polypropylene 1000 µl-capacity micropipette. The agar plug was transferred to 200 µl of SM buffer (**2.0.4**) and phage were allowed to elute from the plaque over 24 h at 4°C. An aliquot of the eluate (100 µl) was then used for bacteriophage propagation (**2.3.6**) and repeated selection of individual plaques. Serial plaque purification was performed a minimum of three times for each bacteriophage isolate prior to storage at 4°C.

### 2.3.6.2 Purification of Bacteriophage particles using CsCl density gradient centrifugation

Purification of bacteriophage particles was performed using the CsCl density gradient centrifugation technique (**Sambrook *et al.*, 1989**). CsCl was added to a $10^9$ pfu $ml^{-1}$ suspension of phage to a final concentration of 1.5 g $ml^{-1}$. This suspension was then subjected to centrifugation at 70,000 rpm at 4°C for 24 h in a Beckman TL-100 ultracentrifuge using a TLA 100.3 rotor. Formation of a thin blue band following centrifugation indicated the location of concentrated phage particles. This was extracted by piercing the centrifuge tube with a hypodermic needle at a level just below the blue band. The concentrated phage particles were siphoned off into a fresh Eppendorf tube prior to titration and storage at 4°C.

### 2.3.6.3 Concentration of bacteriophage stocks

Bacteriophage stocks were concentrated using the centrifugation technique described by **Sambrook *et al.* (1989)**. Briefly, the filtered phage lysate eluates (**2.3.6**) were subjected to centrifugation at 40,000 *g* for 2 h in a Beckman JA20 rotor using a Beckman J2-21 centrifuge (Beckman, High Wycombe, UK). Following centrifugation, the supernatant

was discarded and the pellet resuspended in 1 ml of SM buffer (**2.0.4**). The centrifuge tube was stored at 4°C for 24 h to allow any phage attached to the surface of the tube to elute into the buffer solution. The phage eluate was then transferred to sterile Eppendorf tubes prior to enumeration (**2.3.6.4**) and storage at 4°C.

### *2.3.6.4 Enumeration of bacteriophage in purified lysates*

Unless otherwise stated, bacteriophage were enumerated using the surface droplet technique (**Salama *et al.*, 1990a**). This technique is a modification of the protocol devised for bacterial enumeration by **Miles and Misra (1938)**. Briefly, the bacteriophage suspension was decimally diluted in ten fold steps in SM buffer (**2.0.4**) down to $10^{-8}$. Lawns of the host *Campylobacter* strain were prepared by adding 500 µl of a $10^9$ cfu $ml^{-1}$ in 10 mM $MgSO_4$ (**2.0.1**) to 5 ml of molten TLA (**2.3.1.10**) tempered to 50°C and poured onto the surface of NZB (**2.3.1.7**). After allowing 5 min for the TLA to set, the plates were inverted in a 37°C incubator and the lids slightly opened to allow the surface of the plates to dry for 20 min. Following drying, the plates were inoculated with 5 drops (10 µl) of each dilution and left to dry for 20 min. The plates were then incubated microaerobically at 42°C for 18 - 20 h before examining for plaque formation. Dilutions giving rise to 3 – 30 plaques were used for enumeration. The number of plaque forming units (pfu) per 10 µl of undiluted suspension was calculated by dividing the mean number of plaques for a particular dilution by the dilution factor. This figure was then multiplied by 100 to convert to pfu $ml^{-1}$.

### *2.3.6.5 Determination of Campylobacter susceptibility to phage infection*

To determine the susceptibility of a *Campylobacter* strain to phage infection, each strain was cultured on CBA (**2.3.1.1**) prior to suspension in 10 mM $MgSO_4$ (**2.0.1**). Lawns of each strain were prepared for bacteriophage inoculation as described in **2.3.6.** The

bacteriophage isolates were plaque purified (**2.3.6.1**) and titres adjusted to $10^7$ pfu $ml^{-1}$ (titres determined on the propagating strain) by dilution in SM buffer (**2.0.4**). Three drops (10 µl) of each phage suspension were dispensed onto the surface of each *Campylobacter* lawn and left to dry for up to 30 min. Following drying the plates were inverted and incubated in microaerobic conditions (**2.3.3.3**) for 24 h before examination for plaques.

## 2.4 ANALYSIS OF BACTERIAL AND VIRAL PROTEINS

### 2.4.1 Protein quantitation

#### *2.4.1.1 Bradford Coomassie Plus*

Standard protein quantitation was performed using the Coomassie Plus® Kit (BioRad), based on the Bradford Coomassie protein quantitation assay. The binding of Coomassie dye in an acidic solution is accompanied by an absorbance shift from 465 nm to 595 nm, changing the colour from red/brown to blue. Protein standards were prepared by diluting a 2 mg $ml^{-1}$ bovine serum albumen (BSA) suspension in the same diluent as the sample (e.g. PBS, **2.0.3**) to give concentrations of 50 µg, 100 µg, 200 µg, 500 µg and 1000 µg $ml^{-1}$. Samples were diluted to give a reading predicted to be within the expected standard range. Volumes of the samples and standards (500 µl) were dispensed into 1 ml cuvettes and each mixed with an equal volume of Coomassie Plus® reagent. Absorbance at 595 nm was measured compared with a 'blank' (Coomassie Plus® reagent plus diluent) using a Cecil CE2021 spectrophotometer (Cecil Instruments Ltd, Cambridge). All standards and samples were tested in duplicate with the mean value used to calculate protein concentrations from a standard curve plotted using Microsoft Excel XP.

### 2.4.1.2 Bicinchoninic acid (BCA)

The bicinchoninic acid (BCA) assay has been shown to produce more accurate estimates of protein concentration in bacterial membrane preparations than other techniques based on the Bradford assay. In these circumstances, protein quantitation was performed using a method based on the BCA assay (BCA Protein Assay, Pierce biotechnology, Rockford, Illinois, USA). Protein reduces $Cu^{2+}$ to $Cu^{+}$ in an alkaline medium (the biuret reaction). A water-soluble complex is formed when two molecules of BCA chelate one cuprous cation, causing a strong absorbance shift to 562 nm which is almost linear with increasing protein concentrations over a broad working range (20-2,000 μg $ml^{-1}$). The BCA 'working reagent' was prepared in a sterile test tube according to the manufacturer's instructions and mixed (2 ml) with sample (0.1 ml) before incubating for 30 min at 37°C. Subsequently, all tubes were cooled to RTP before measurement of absorbance against the blank at 562 nm using a Cecil CE2021 spectrophotometer. Protein standards were prepared in the range of 20 – 2000 μg $ml^{-1}$ from a 2 mg $ml^{-1}$ stock of BSA provided by the manufacturer. All standards and samples were tested in duplicate with the mean value used to calculate protein concentrations from a standard curve plotted using Microsoft Excel XP.

## 2.4.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

All protein gels were prepared using the protocols described by **Sambrook *et al.* (1989)** using a Mini Protean II kit (Biorad) unless otherwise stated. Separating gels were prepared at 12% and stacking gels at 5% unless otherwise stated. Separating gels were prepared in quantities of 5 ml per gel cast by mixing ingredients in the following order: sterile RO water (1.9 ml); 30% w/v acrylamide, 1% w/v bisacrylamide (1.7 ml); 1.5M

Tris, pH 8.8 (1.3 ml) ; 10% w/v SDS (0.05 ml) ; 10% ammonium persulphate (0.05 ml); N, N, N', N'-tetramethylethylenediamine, TEMED (0.002 ml).

Once poured into the cast, the gel was allowed to set at RTP for 10 – 15 min under a layer of water saturated butanol. Following polymerization the butanol was removed and the exposed surface of the gel was washed with sterile RO water. Filter paper was inserted into the gel cast to remove traces of RO water. Volumes (3 ml) of stacking gel were prepared by mixing ingredients in the following order: sterile RO water (2.1 ml); 30% w/v acrylamide, 1% w/v bisacrylamide (0.5 ml); 1.5M Tris, pH 6.8 (0.38 ml) ; 10% w/v SDS (0.03 ml); 10% ammonium persulphate (0.03 ml); N, N, N', N'-tetramethylethylenediamine, TEMED (0.003 ml).

A well-forming comb was inserted into the stacking gel after pouring and left to set at RTP for 10 – 15 min. Following polymerization of the stacking gel, the comb was carefully removed and each of the wells was washed five times with sterile RO water to remove any unpolymerised acrylamide. The electrophoresis chamber was then assembled and flooded with running buffer (25 mM Tris, 250 mM glycine (electrophoresis grade) pH 8.3, 0.1% SDS).

Protein samples were prepared by mixing 5X loading buffer (250 mM Tris.Cl, pH 6.8; 10% w/v SDS; 0.5% w/v bromophenol blue; 50% v/v glycerol; 5% v/v β-mercaptoethanol) with the sample in a 1:4 ratio (buffer:sample). Samples were then boiled for three min before loading 10 - 20 µl per well on the gel. Electrophoresis was performed at 200 V for 30 - 40 min unless otherwise specified.

### 2.4.3 Staining of SDS-PAGE gels

Following electrophoresis (**2.4.2**), the acrylamide gels were removed from the casts and the stacking gel excised using a sterile scalpel blade. The gel was briefly washed in RO

water to remove running buffer and then either stained (Coomassie blue, **2.4.3.1**; silver stain, **2.4.3.2**) or prepared for electroblotting to a solid support (**2.4.4**).

### *2.4.3.1 Coomassie blue staining*

Polyacrylamide gels were stained using Coomassie blue according to the method described by **Sambrook *et al.* (1989)**. Gels were immersed in 50 ml of Coomassie blue stain (Coomassie Brilliant Blue R250, 0.125 g; methanol:$H_2O$ (1:1 v/v), 45 ml; glacial acetic acid, 5 ml) for 1 h with gentle agitation on an orbital platform shaker. The staining solution was then removed and the gel rinsed with RO water before immersion in destaining solution (methanol, 40% v/v; glacial acetic acid, 10% v/v). The destaining solution was replaced every 30 min until protein bands were visible.

### *2.4.3.2 Silver staining*

Silver staining was performed using the Plus One Silver Staining Kit according to the manufacturer's instructions (Pharmacia, Milton Keynes, UK). The gel was initially placed in a fixing solution (ethanol, 40% v/v; glacial acetic acid, 10% v/v) for 30 min. Removal of the fixative was immediately followed by addition of the sensitizing solution (ethanol, 30% v/v; gluteraldehyde, 0.125% w/v; sodium thiosulphate, 0.2% w/v; sodium acetate, 0.5 M) for 30 min. The gel was then washed for 3 x 5 min in RO water prior to initiation of the silver reaction by immersion in the staining solution (silver nitrate, 0.25% w/v; formaldehyde, 0.015% v/v) for 20 min. The gel was then washed for 2 x 1 min in RO water and developed by immersion in a solution containing 0.25 M sodium carbonate and 0.007% formaldehyde. Development of the silver stain was arrested using 0.04 M EDTA solution.

### 2.4.4 Transfer of proteins from SDS-PAGE gels to solid supports

Proteins separated using SDS-PAGE (**2.4.2**) were transferred onto a solid polyvinylidene difluoride (PVDF) membrane using a SemiPhor semi-dry electroblotting chamber according to the manufacturer's instructions (Pharmacia). Briefly, the PVDF membrane was immersed in 100% methanol for 5 min, rinsed in RO water then immersed for 5 min in transfer buffer (39 mM glycine, 48 mM Tris, 0.037% w/v lauryl sulphate (electrophoresis grade), 20% v/v methanol). The gel was then placed on top of the membrane with air bubbles in-between them being removed using a glass rod. Pieces of blotting paper cut to match the size of the gel were pre-soaked for 5 min in transfer buffer before being placed either side of the gel and membrane to form a "Transphor Sandwich". The anode and cathode plates of the SemiPhor chamber were liberally flushed with transfer buffer prior to centrally placing the Transphor sandwich in-between them. Protein transfer was performed at a current of 32 mA for 1 h. Following transfer, the PVDF membrane was stored in-between pieces of filter paper in the dark at 4°C until required.

### 2.4.5 Western blotting

A PVDF membrane containing proteins electrotransferred from an SDS-PAGE gel (**2.4.2**) were immersed in 10 ml of blocking solution (Tris Buffered Saline, TBS containing 2% w/v skimmed milk powder) for 30 min at 37°C. The membrane was then rinsed in 10 ml of washing buffer (TBS containing 0.05% v/v Tween 20 (P-7949, Sigma) for 5 min on a gyratory platform shaker at 60 rpm. After rinsing, the membrane was transferred to a heat-sealable plastic bag. To this bag was added 10 ml of blocking solution containing 5 µl a 1/1000 dilution of primary antibody (polyclonal antisera raised in rabbits against acid-glycine extractable proteins from *C. jejuni*, obtained from Dr. P. Connerton). The plastic bag was sealed and incubated at 37°C for 1 h. Following

incubation the membrane was immersed for 3 x 5 min in washing buffer prior to transferral to a new heat-sealable bag. To this bag was added 10 ml of blocking buffer containing 2 µl of the secondary antibody (goat anti-rabbit IgG alkaline phosphatase conjugate) prior to sealing and incubation at 37°C for 1 h. Following incubation the membrane was immersed for 3 x 5 min in washing buffer and 1 x 5 min in TBS prior to transferral to a square Petri dish (Sterlin UK). The membrane was then immersed in 10 ml of developing solution (50 µl 5-bromo-4-chloro-3-indolyl phosphate, BCIP; 50 µl nitroblue tetrazolium, NBT; in 10 ml TBS) and incubated in the dark at RTP until the desired level of development was achieved. The membrane was then washed for 3 x 5 min in RO water prior to photographic recording using a HP Scanjet 4c digital scanner and associated PC software.

### 2.4.6 Profiling of bacteriophage proteins

Bacteriophage proteins were separated on a 12% SDS-PAGE gel (**2.4.2**) and stained using Coomassie blue (**2.4.3.1**). Selected bands were excised from the gel and sent for mass spectrometry sequencing by Dr. Kevin Bailey at the Queen's Medical Centre, Nottingham. Polypeptides resulting from a tryptic digest of the bands were spray ionized and sequenced using a Micromass Q-TOF-2 working on a Matrix-assisted Laser Desorption Ionisation Time-of-Flight (MALDI-TOF) system. The peptide fingerprints were used to interrogate the ExPasy database (www.expasy.org) for protein identification.

## 2.5 ANALYSIS OF BACTERIAL AND VIRAL NUCLEIC ACID

### 2.5.1 Extraction of bacterial chromosomal DNA

Chromosomal DNA was extracted using the protocol determined by (**Pitcher *et al.*, 1989**) Briefly, 1.5 ml of overnight broth culture (**2.3.3.2**) was transferred to a 1.5 ml

Eppendorf tube and the cells pelleted by centrifugation at 13,000 *g* for 2 min. The supernatant was discarded and the pellet washed once in 1 ml of ice cold lysis buffer (25 mM Tris-HCl pH8.0, 10 mM EDTA, 50 mM sucrose). The cells were then resuspended in 100µl of lysis buffer and mixed with 500 µl of GES solution (5 M guanidium thoiocyanate, 0.1 M EDTA, 0.5% v/v sarkosyl). The solution was incubated for 5 min or until the solution cleared. The lysate was cooled on ice for 2 min after which 0.25 ml of ice cold 7.5 M ammonium acetate was added. The lysate was vortexed and incubated on ice for 10 min. Following incubation 0.5 ml of chloroform:isoamylalcohol (24:1) was added, the solution vortexed and subjected to centrifugation at 13,000 *g* for 10 min. The upper phase (850 µl) was removed and added to 540 µl of ice cold isopropanol, mixed for 1 min and centrifuged at 13,000 *g* for 1 min to pellet the DNA. The pellet was washed three times in 70% ethanol without resuspending and then air dried for 30 min. The DNA was then resuspended in 50 µl of sterile RO water.

### 2.5.2 Extraction of Bacteriophage DNA

SM buffer (**2.0.4**, 200 µl) supplemented with 1 mg $ml^{-1}$ Proteinase K was added to the same volume of a $10^{10}$ pfu $ml^{-1}$ suspension of phage and incubated for 2 h at 55°C in order to digest the viral capsid. All proteins were then denatured by the addition of an equal volume of Tris-saturated phenol (pH 8.0). The mixture was vortexed until an emulsion formed after which it was subjected to centrifugation at 5,000 *g* for 5 min. The aqueous phase was removed and treated with a 1:1 mixture of equilibrated phenol:chloroform prior to repeating the vortex and centrifugation steps. The upper phase was then treated once with an equal volume of chloroform and, following centrifugation, was mixed with 100 µl of 7.5 M ammonium acetate and 1 ml of ice-cold absolute ethanol. The DNA was left to precipitate for 1 h prior to centrifugation at 13,000 *g* for 5 min. The resulting pellet was then washed three times (without

resuspending) in 70% v/v ethanol and air dried at 37°C for 30 min. Finally, the pellet was resuspended in 100 µl of TE buffer (**2.0.6**) and stored at 4°C until required.

### 2.5.3 Preparation of plasmid DNA

A single white colony was transferred to 5 ml of LB broth supplemented with 50 µg $ml^{-1}$ ampicillin and incubated at 37°C overnight shaking at 100 rpm. Following incubation, plasmids were extracted from the cells using a QIAprep® Miniprep kit according to the manufacturer's instructions (QIAGEN ltd, West Sussex).

### 2.5.4 Digestion of DNA using endonucleases

Typically, preparations of genomic (**2.5.1, 2.5.2**) and plasmid (**2.5.2**) DNA were digested in volumes of 20 µl. Digestion of DNA by restriction enzymes was carried out using the conditions recommended by the enzyme supplier (Promega Ltd, Southampton, UK). Digestions were performed at 37°C for 2 h using approximately 0.5 - 2 units of enzyme per 1 µg of DNA using the appropriate restriction buffer unless stated otherwise.

### 2.5.5 Preparation of agarose gels

Agarose gels were prepared at a concentration of 0.8 – 2% w/v by suspending agarose powder (Hi-pure low EEO agarose, Biogene, Cambridge, UK) in TAE buffer (**2.0.5**) and heating in a microwave oven until the suspension had cleared. The suspension was then cooled to ~50°C and ethidium bromide added to a final concentration of 1 µg $ml^{-1}$. The suspension was then poured into a minigel cast (Anachem, Luton, Bedfordshire) and allowed to set following insertion of 1.5 mm well combs. After the gel was set, the well combs were removed and the gel transferred to an electrophoresis chamber flooded with TAE buffer (**2.1.5**).

### 2.5.6 DNA sample preparation for agarose gel electrophoresis

DNA extracts were mixed with loading buffer (glycerol, 50% v/v; EDTA, 0.025 M pH 8.0; bromophenol blue, 0.01% w/v) in a ratio of 4:1 (sample:buffer) and loaded on the gel. Typically, electrophoresis was performed at 70 V for 30 - 45 min. The progress of electrophoresis was monitored using the migration of the bromophenol blue dye in the loading buffer. On completion of electrophoresis the gel was removed from the buffer tank and photographed using a Pharmacia SE 400 digital imaging system.

### 2.5.7 Detection of *Bdellovibrio* ssp. using PCR.

Bdellovibrio were detected using the genus specific primers described by **Jurkevitch and Ramati (2000)**. The base sequence of each primer was as follows:

**FORWARD PRIMER 842** (5' – CGWCACTGAAGGGGTCAA – 3')

**REVERSE PRIMER 343R** (5' – ATTCCCCTGCGACAGAG – 3')

Each PCR reaction was performed in 50 µl volumes consisting of sterile RO water (38 µl); 10X PCR buffer (ABgene, 5µl); $MgCl_2$ [25 mM], (1 µl); dNTP mixture [25 mM], AB-0241, ABgene, (1µl); primer 842, 1 µg $ml^{-1}$, (0.5 µl); primer 343R, 1 µg $ml^{-1}$, (0.5 µl); DNA template (1 µl); Taq DNA polymerase, AB-01921B, Abgene, 1U $ml^{-1}$ (1 µl). Reaction mixtures were placed in a Techne Progene® thermocycler and subjected to one heating cycle at 94°C for 240 sec to denature the DNA template. This was followed by 34 cycles of (denaturation, 94°C, 60 sec; primer annealing, 50°C, 60 sec and elongation, 72°C, 130 sec). The amplification program was completed by one final elongation cycle at 72°C for 300 sec. PCR amplicons were then resolved on a 1% agarose gel (**2.5.5**).

### 2.5.8 *fla* typing of *Campylobacter* isolates

The *fla* typing scheme used was the specified protocol of the CampyNet *fla* gene typing subcommittee (http://campynet.vetinst.sk/Fla.html). *Campylobacter* strains were subcultured on CBA (**2.3.1.1**) and genomic DNA extracted using the GES protocol (**2.5.1**). Amplification of the *fla*A gene using the polymerase chain reaction (PCR) used the following primers:

**FORWARD PRIMER A1:** 5' GGA TTT CGT ATT AAC ACA AAT GGT GC 3'

**REVERSE PRIMER A2:** 5' CTG TAG TAA TCT TAA AAC ATT TTG 3'

PCR was performed in 50 µl volumes unless otherwise stated. Reactants were mixed in a 0.2 ml PCR tube in the following concentrations: sterile RO water (38 µl); 10X PCR buffer, ABgene (5 µl); $MgCl_2$, 50 mM (1 µl); dNTP mixture, 25 mM, (1 µl); primer A1, 1µg $ml^{-1}$, (0.5 µl); primer A2, 1µg $ml^{-1}$, (0.5 µl); DNA (1 µl); ABgene Taq DNA polymerase, 1U $ml^{-1}$ (1 µl). Reaction mixtures were placed in a Techne Progene® thermocycler and subjected to one heating cycle at 94°C for 60 sec to denature the DNA template. This was followed by 30 cycles of (denaturation, 94°C, 30 sec; primer annealing, 45°C, 60 sec and elongation, 72°C, 120 sec). The amplification program was completed by one final elongation cycle at 72°C for 300 sec. Following amplification, the PCR product was digested using restriction endonuclease *Dde*I (Promega) and resolved on a 1% agarose gel (**2.5.5**).

### 2.5.9 Preparation of *Campylobacter* for pulsed field gel electrophoresis (PFGE)

Agarose plugs were prepared by first adding 25 µl of a 20 mg $ml^{-1}$ stock of Proteinase K (prepared in RO water) to 400 µl of an approximately $10^9$ cfu $ml^{-1}$ suspension of

*Campylobacter* strain in PBS and mixing by gentle inversion. A 400 µl volume of molten 1% PFGE-grade agarose (BioRad, Hemel Hempstead, Hertfordshire) prepared in TE buffer (**2.0.6**) was subsequently added to the cell suspension and mixed by gentle pipetting before dispensing into plug moulds (BioRad) and allowed to set at room temperature and pressure (RTP) for 10 – 15 min. The plugs were then transferred to 5 ml of lysis buffer (50 mM Tris, 50 mM EDTA, pH 8.0, 1% w/v sarcosine, 0.1 mg $ml^{-1}$ Proteinase K) and incubated for 15 min at 54°C in an orbital shaking incubator (150 rpm). Following lysis, the plugs were washed a total of four times (once in sterile RO water, three times in TE buffer), all washing steps taking place for 15 min at 54°C in an orbital shaking incubator (150 rpm).

### *2.5.9.1 Macrorestriction profiling of Campylobacter genomes using PFGE*

Macrorestriction profiling was carried out essentially as described by **Ribot *et al.* (2001)**. For restriction digestion, a 2 mm slice was cut from each agarose plug (**2.5.9**) with a sterile scalpel and transferred to an Eppendorf tube containing 1X restriction buffer solution (buffer A for *Sma*I; buffer L for *Kpn*I, both obtained from Roche, Lewes, East Sussex) and incubated at RTP for 5 min. This pre-digestion solution was removed by pipette and replaced with 200 µl of 1 x restriction buffer solution containing 40U of endonuclease (either *Sma*I or *Kpn*I, Roche, Lewes, East Sussex) prior to incubation at 30°C (*Sma*I) or 37°C (*Kpn*I) for 2 h. Following incubation the plug slices were transferred to an Eppendorf tube and washed in 200 µl of 0.5X TBE buffer (**2.0.7**) for 5 min before loading onto a 1% agarose (PFGE grade) gel prepared in 0.5X TBE buffer. The gel was then subjected to electrophoresis for 18 h in 0.5X TBE buffer using a BioRad CHEF-DR® II system with a switch time of 6.75 – 38.35 sec (gradient = 6 V $cm^{-1}$). This allowed optimal resolution of digestion fragments in the 50 – 400 kbp range. Following electrophoresis the gel was stained for 20 min in staining solution (RO water,

200 ml, containing 50 μg $ml^{-1}$ EtBr) and visualised using a Pharmacia SE 400 digital imaging system under U.V. illumination.

## 2.5.10 Preparation of bacteriophage for Pulsed Field Gel Electrophoresis (PFGE)

Bacteriophage were prepared for PFGE using a modification of a bacterial protocol devised by **Liu and Sanderson (1996)**. Briefly, an aliquot (10 μl) of a $10^{10}$ pfu $ml^{-1}$ suspension of phage was diluted in 40 μl of TE buffer **(2.0.6)**. This was mixed with an equal volume of 1.4% molten agarose (PFGE grade) in TE buffer and dispensed into plug moulds (BioRad). The plugs were allowed to set at room temperature then transferred to Petri dishes containing 5 ml of lysis buffer (100 mM EDTA, 10 mM Tris pH 7.2, 1% Sarkosyl w/v, 0.1 mg $ml^{-1}$ Proteinase K). The plates were incubated at 55°C for 18 h with gentle shaking to lyse the phage capsids and digest the protein components. The lysis solution was discarded and Proteinase K was inactivated by addition of 5 ml of 1 mM Phenylmethylsulfonyl fluoride (PMSF) in wash buffer (50 mM EDTA, 20 mM Tris pH 7.2) and incubated for 1 h at room temperature with gentle shaking. The plugs were then washed three times for 20 min each with successive changes of wash buffer at room temperature with gentle shaking. A 2 mm slice of each plug was then inserted into the wells of a 1% w/v agarose gel. The gel was run using a BioRad CHEF DRII system in 0.5 TBE **(2.0.7)** for 18 h at 200 V with a switch time of 30 – 60 sec. Lambda concatomers (BioRad) were used as markers.

### *2.5.10.1 Macrorestriction profiling of bacteriophage genomes using PFGE*

For restriction endonuclease digests a 2 mm slice of each plug was incubated at 37°C overnight with 10 U of restriction enzyme (Table 4E) in 100 μl of digestion buffer prepared according to the manufacturer's instructions (Promega). The plug digests were

then transferred to a 1% w/v agarose gel (**2.5.10**) and run using BioRad CHEF DRII system as described above but with a switch time of 2 - 10 sec for better resolution of the smaller DNA fragments. PFGE gels were stained for 20 – 30 min in a TBE solution containing 1 μg $ml^{-1}$ ethidium bromide before photographing under U.V. illumination using a Pharmacia SE 400 digital imaging system.

### 2.5.11 Cloning and sequencing of bacteriophage DNA

Bacteriophage genomic DNA was extracted as described in **2.5.1.** Following extraction, the DNA was digested using endonuclease *Dra*I (Pharmacia). Reagents were mixed in 20 μl volumes containing the following: sterile RO water, 16 μl; 10X One-Phor-All buffer (Pharmacia), 2 μl; *Dra*I (10U $\mu l^{-1}$), 1 μl; DNA (approximately 1 μg $ml^{-1}$), 1 μl. This mixture was then incubated at 37°C for 90 min.

Following digestion, the endonuclease was inactivated by heating at 70°C for 10 min. The products of the digest were then added to a tube of Ready-To-Go pUC18 (27-5266-01, Pharmacia) and incubated overnight at 16°C according to the manufacturer's instructions. The Ready-To-Go kit uses plasmid pUC18 pre-digested with *Sma*I to allow cloning of any fragment digested with an enzyme giving blunt ends (e.g. *Dra*I). The kit also contains T4 DNA ligase so the cloning reaction is one-step. The ligation product (2 μl) was then added to chemically competent *E. coli* cells (18 μl) obtained from the TOPO TA Cloning® Kit (*E. coli* TOP10 One Shot®, Invitrogen, California) and transformation performed according to the manufacturer's instructions.

The transformed cells were diluted in 250 μl of Luria-Bertani (LB) broth (244610, Difco) and incubated at 37°C for 1 h. Following incubation, volumes (50 μl and 200 μl) of cell suspension were spread onto Luria-Bertani Agar (LBA) plates supplemented with 50 μg $ml^{-1}$ ampicillin, 50 μl of a 2% w/v solution of 5-bromo-4-chloro-3-indolyl-β-D-

galactopyranosine (X-gal), and 50 µl of a 100 mM solution of isopropyl-β-D-thiogalactopyranoside (IPTG). The plates were incubated aerobically at 37°C for 24 h prior to selection of successful transformants using blue/white screening.

## 2.6 METHODS FOR THE RECOVERY OF *CAMPYLOBACTER* AND BACTERIOPHAGE FROM RETAIL POULTRY

### 2.6.1 Protocols for the isolation of *Campylobacter* from chicken skin

#### *2.6.1.1 Protocol A*

For each sampling location on the carcass a cotton-tip swab was moistened in maximum recovery diluent (MRD, **2.0.2**) and used to wipe the surface of the skin. The swab was then used to inoculate a plate of CCDA (**2.3.1.4**) prior to streaking for single colonies. The plates were incubated in microaerobic conditions (**2.3.3.3**) at 42°C and examined for typical colonies (**2.3.4.1**) after 24 and 48 h.

#### *2.6.1.2 Protocol B*

For each sampling location on the carcass a cotton-tip swab was moistened in MRD (**2.0.2**) and used to wipe the surface of the skin. The swab was then used to inoculate a plate of modified Blaser-Wang selective agar (CBA, **2.3.1.1**) with selective supplement SR98 (Oxoid) prior to streaking for single colonies. The Blaser-Wang supplement was added according to the manufacturer's instructions to give final concentrations of the following constituents: vancomycin (0.01 mg $ml^{-1}$); polymixin B (2.5 IU $ml^{-1}$); trimethoprim (0.005 mg $ml^{-1}$); amphotericin B (0.002 mg $ml^{-1}$) and cephalothin (0.015 mg $ml^{-1}$). The plates were incubated in microaerobic conditions (**2.3.3.3**) at 42°C and examined for typical colonies after 24 and 48 h.

### 2.6.1.3 Protocol C

A section of skin from each sampling site was weighed (10 g) and macerated into 0.2 – 0.5 $cm^2$ sections using clean flame-sterilised scissors. These sections were then added to 90 ml of Park and Sanders broth (tryptone, 10 g $l^{-1}$; peptone P, 10 g $l^{-1}$; glucose, 1 g $l^{-1}$; yeast extract, 2 g $l^{-1}$; sodium citrate, 1 g $l^{-1}$; NaCl, 5 g $l^{-1}$; sodium metabisulphite, 0.1 g $l^{-1}$; sodium pyruvate, 0.25 g $l^{-1}$) containing selective supplement A (vancomycin, 0.1 g; trimethoprim, 0.1 g in 50 ml Brucella broth, 0495-17-3, Difco) and incubated in a 250 ml capacity flask at 32°C for 2 h. Following incubation, 7.5 ml of antibiotic supplement B was added (cefoperazone, 0.032 g; cyclohexamide, 0.1 g in 50 ml Brucella broth) and the suspension was incubated at 38°C for a further 2 h. After this period, incubation continued at 42°C for 40 h before streaking for single colonies onto the surface of a CCDA plate (**2.3.1.4**). All incubation steps were performed under microaerobic conditions (**2.3.3.3**) in a shaking platform incubator (100 rpm).

## 2.6.2 Detection of *Campylobacter* on chicken skin using Exeter enrichment

A 25 ml aliquot of carcass rinse fluid (**2.6.3**) was diluted in 225 ml of modified Exeter broth (Nutrient broth 25 g $l^{-1}$, CM1, Oxoid, Basingstoke; sodium metabisulphate 250 mg $l^{-1}$; sodium pyruvate 250 mg $l^{-1}$; ferrous sulphate 250 mg $l^{-1}$; trimethoprim 10 mg $l^{-1}$; rifampicin 5 mg $l^{-1}$, polymyxin B 2500 IU $l^{-1}$; cefaperazone 15 mg $l^{-1}$, amphotericin B 2 mg $l^{-1}$; lysed horse blood (SR48, Oxoid) 10 ml $l^{-1}$) and incubated aerobically at 37°C for 48 h. Subsequently, an aliquot of the broth (10 µl) was streaked onto CCDA agar (**2.3.1.4**) and incubated in a microaerobic atmosphere at 37°C for 48 h (**2.3.3.3**). Presumptive *Campylobacter* growth was confirmed using standard morphological and biochemical tests (**2.3.4.1**).

### 2.6.3 Isolation of *Campylobacter* from broiler carcass rinses

Each whole chicken carcass was transferred to a sterile polythene bag containing 300 ml of buffered peptone water (CM509, Oxoid). The carcass was vigorously shaken inside the bag for approximately 2 min. An aliquot of this rinse fluid (25 ml) was transferred to a sterile 500 ml flask for enrichment using the Exeter method (**2.6.2**). In parallel to enrichment, three volumes of carcass rinse (0.5 ml each) were spread-plated onto modified CCDA agar (**2.3.1.4**) and incubated in microaerobic conditions (**2.3.3.3**) for 24 h at 42°C before examination for growth. Presumptive *Campylobacter* colonies were confirmed using morphological (**2.3.4.1**) and biochemical (**2.3.4.2**) assays.

### 2.6.4 Methods for the isolation of *Campylobacter* bacteriophage from chicken skin

Sections of chicken skin (10 $cm^2$) were cut from standard class retail chicken thigh portions using flame-sterilised scissors. Ten chicken skin sections were used for the assessment of each recovery protocol. The skin sections were inoculated with $10^7$ pfu of *Campylobacter* bacteriophage $\phi 2$ and left to dry for 30 min prior to implementing the recovery method. Unless otherwise stated, enumeration of bacteriophage was performed using the Miles and Misra method described in section **2.3.6.4.**

#### *2.6.4.1 Protocol I*

Cotton-tip swabs were moistened in SM buffer (**2.0.4**) and used to wipe the surface of the inoculated skin five times. The swab was then snapped off into 5 ml of SM buffer and left at 4°C for 24 h. Following this period the swab was removed and the SM buffer filtered through a 0.45 µm pore size membrane filter (Sartorius). Decimal dilutions of the filtrate were made in SM buffer down to $10^{-8}$ and used to inoculate the lawn of a host bacterium in a Miles and Misra titration series.

### 2.6.4.2 Protocol II

Each chicken skin section was transferred to a sterile 50 ml capacity Falcon tube containing 20 ml of SM buffer (**2.0.4**) . The tube was gently inverted approximately 20 times prior to filtration using a 0.45 μm pore size membrane filter. Decimal dilutions of the filtrate were made in SM buffer down to $10^{-8}$ and used to inoculate the lawn of a host bacterium in a Miles and Misra titration series.

### 2.6.4.3 Protocol III

Each chicken skin section was transferred to a sterile filter stomacher bag (BA6041/STR, Seward, Norfolk, UK) containing 10 ml of SM buffer (**2.0.4**). The skin was stomached for 5 min using a LabBlender 400™ (Seward) and then filtered through a 0.45 μm pore size membrane filter. Decimal dilutions of the filtrate were made in SM buffer down to $10^{-8}$ and used to inoculate the lawn of a host bacterium in a Miles and Misra titration series.

### 2.6.4.4 Protocol IV

Each chicken skin section was transferred to a sterile filter stomacher bag (Seward) containing 10 ml of SM buffer (**2.0.4**). The skin was stomached for 5 min using a LabBlender 400™ and the resulting stomachate subjected to centrifugation at 2.5 *g* for 10 min at RTP prior to filtration through a 0.45 μm pore size membrane filter. The filtrate was then diluted in ten fold steps down to $10^{-4}$ in SM buffer. Aliquots of each dilution (100 μl) were transferred to a sterile Eppendorf tube and mixed with 400 μl of PT14 suspension and subsequently incubated aerobically for 30 min at 42°C. After incubation, the suspension was added to 5 ml of NZCYM overlay agar (TLA, **2.3.1.10**), gently shaken and added to pre-warmed (42°C, 30 min) NZCYM base agar (NZB, **2.3.1.7**).

Plates were incubated microaerobically at 42°C for 24 h and subsequently examined for the presence of plaques.

#### *2.6.4.5 Protocol V*

Each chicken skin section was transferred to a sterile filter stomacher bag (Seward) containing 10 ml of SM buffer (**2.0.4**). The skin was stomached for 5 min using a LabBlender 400™. An aliquot of this stomachate (1 ml) was transferred to a sterile Eppendorf tube containing 100 µl of chloroform and inverted approximately ten times. The suspension was then subjected to centrifugation at 13, 000 *g* for 5 min and the titre of phage in the supernatant was then determined using the method of **Miles and Misra (1938)**.

### 2.6.5 Isolation of bacteriophage from whole retail chicken carcasses

An aliquot (1 ml) of carcass rinse fluid (**2.6.3**) was subjected to centrifugation at 13, 000 *g* for 5 min to remove debris and bacterial cells. The supernatant was subsequently filtered through a 0.45 µm pore-size membrane filter (Sartorius) into an Eppendorf tube. Volumes of filtrate (100 µl) were then mixed with 400 µl of a $10^9$ cfu $ml^{-1}$ suspension of *C. jejuni* NCTC 12662 phage type 14, PT14, and incubated aerobically at 42°C for 30 min. Following incubation, the suspension was mixed with 5 ml of molten NZCYM top layer agar (**2.3.1.10**) which had been held at 50°C. The molten agar was then evenly poured on the surface of NZCYM base agar (**2.3.1.7**) and left to set at room temperature and pressure (RTP) for approximately 10 min. The plates were then inverted and incubated in a microaerobic atmosphere (**2.3.3.3**) at 42°C for 24 h before examining for plaques.

### 2.6.6 Survey of *Campylobacter* and bacteriophage prevalence in retail chicken portions

Three different classes (free-range, standard and economy) of chilled (4°C) chicken thighs were purchased from a single supermarket over a two-week period. Two different classes (standard and economy) of frozen (-20°C) chicken thighs were purchased from the same supermarket over a one-week period. One hundred samples of each class of fresh meat and seventy-five portions of each class of frozen meat, representing a range of poultry producers, were screened for the presence of *Campylobacter* (**2.6.1.2**) and phage (**2.6.4.4**).

### 2.6.7 Recovery of bacteriophage from the surface of retail chicken products over time

Sections of chicken skin (10 $cm^2$) were cut from standard class retail chicken thigh portions using clean, flame-sterilised scissors. Each section of skin was inoculated with 100 µl of a $10^8$ pfu $ml^{-1}$ suspension of *Campylobacter* bacteriophage and left to dry for 1 h. Triplicate skin sections were inoculated for each sampling point. Following inoculation, the skin sections were individually sealed in sterile containers and stored at 4°C (fresh chicken) or refrozen at –20°C (frozen chicken). Frozen chicken portions were defrosted for 24 h at 4°C prior to inoculation. Samples were initially taken 1 h after inoculation then at 24 h intervals thereafter for up to ten days. Bacteriophage recovery from both fresh and frozen samples was performed using the method described in section **2.6.4.4**.

### 2.6.8 Preparation of chicken skin for *Campylobacter* inoculation

Commercial Ross broiler chickens reared free of *Campylobacter* were obtained from a registered supplier (P. D. Hook, Oxfordshire). At 30 days of age the broiler chickens

were sacrificed using a UK Home Office Schedule I method then manually plucked and the skin removed. The breast skin from each bird was cut into 2 $cm^2$ sections using flame-sterilised scissors and transferred to sterile Petri dishes.

### 2.6.9 Bacteriophage activity on the surface of chicken skin stored under fresh and frozen conditions

Sections of chicken skin (**2.6.8**) were inoculated with a combination of phage and *Campylobacter* titers in the form of a simple 3 x 3 matrix to obtain multiplicities of infection (MOI) ranging from 0.001 to 100,000 (Table 6A). Each specific MOI combination was inoculated in triplicate for each sampling time point. The skin was inoculated with the *Campylobacter* suspension, and following a 30 min drying period, the phage were inoculated. A further 30 min period was allowed for the phage inoculum to dry before incubation at 4°C (fresh) or -20°C (frozen). The initial (day 1) samples were enumerated immediately following the final 30 min drying period and thereafter on days three and five for fresh samples and day five only for frozen samples. At each sampling point, the skin sections were transferred to sterile filter stomacher bags, homogenised in 20 ml MRD (**2.0.2**) and the *Campylobacter* and bacteriophage populations enumerated using the methods described in sections **2.3.4.4** and **2.3.6.4** respectively.

## 2.7 TRANSMISSION ELECTRON MICROSCOPY (TEM)

The negative staining technique uses an electron-opaque staining solution to coat the sample. The stain does not usually penetrate the sample and so areas not in contact with the stain are easily permeable to transmitted electrons, appearing "bright" on a phosphorescent screen. The contrast between light and dark areas is used to determine the structure of the particle. To perform negative staining TEM, 8 µl of a $10^8$ pfu $ml^{-1}$ suspension of phage was added to the surface of a glow-discharged carbon coated

pioloform grid. The specimen was fixed for 2 min by exposure to gluteraldehyde vapour. Excess sample was removed and the grid washed with a drop of double distilled water. Negative staining was performed by adding one drop of 0.5% w/v uranyl acetate to the grid surface, removing excess stain immediately. The grids were allowed to air dry for 20 min then observed using a JOEL 100CX transmission electron microscope.

### 2.7.1 On-grid Immunogold labelling

An aliquot (10 µl) of vesicle suspension was allowed to adsorb to the surface of a carbon-coated pioloform grid for 5 min. This was followed by washing the grid for 1 min with a drop of phosphate buffered saline (PBS, **2.0.3**) followed by thrice washing with PBS containing 1 mg $ml^{-1}$ bovine serum albumen (PBS-BSA). Following the final washing step, excess wash fluid was removed and immediately replaced by 15 µl of primary antibody prior to incubation at RTP for 25 min. Subsequently, the grid was thrice washed for 1 min with PBS-BSA after which excess wash fluid was removed. A 15 µl drop of appropriate anti-antibody-gold conjugate was then placed on the grid surface and incubated at RTP for 20 min. Incubation was followed by thrice washing in PBS and fixation by exposure to a 15 µl drop of 0.5% v/v gluteraldehyde in PBS for 2 min. The grid was then washed thrice in double distilled water (DDW) for 1 min. Negative staining was then performed by adding a drop of 0.5% w/v uranyl acetate (made in DDW) to the grid and immediately draining excess stain with blotting paper. The grid was then flushed with three drops of DDW and allowed to dry at RTP for 30 min prior to examination using a JOEL CX100 transmission electron microscope.

## 2.8 PREPARATION OF *CAMPYLOBACTER* MEMBRANE VESICLES

### 2.8.1 Protocol A.

Cells from a 1 l overnight culture of *Campylobacter* in Nutrient Broth No. 2 (NB2, **2.3.1.6**) were removed from the growth medium by centrifugation at 10,000 g for 20 min at 4°C. Subsequently, over a period of 2 h, 240 g of ammonium sulphate was added to the supernatant prior to centrifugation at 20,000 g for 120 min. The supernatant was then discarded and the pellet was suspended in 16 ml of Tris Buffer A (50 mM Tris, pH 9.5; 0.5 mM dithiothreitol, DTT). This suspension was then dialyzed overnight against 6 l of the same buffer. Membrane vesicles were then pelleted by centrifugation at 27,000 *g* for 40 min prior to resuspension in 1.5 ml of Tris Buffer B (50 mM Tris, pH 7.2) and storage at -20°C.

### 2.8.2 Protocol B

A 1 l overnight culture of *Campylobacter* in NB2 (**2.3.1.6**) was subjected to centrifugation at 10,000 *g* for 10 min to pellet bacterial cells. Membrane vesicles in the supernatant were concentrated by further centrifugation (2.5 *g* for 30 min) through a 30 kDa molecular weight cut-off filter (PL-30, Millipore, Bedford, USA). The retentate was resuspended in 5 ml of phosphate buffered saline (PBS, **2.0.3**) and stored at -20°C.

## 2.9 OUTER MEMBRANE PREPARATION FROM *CAMPYLOBACTER*

An aliquot (20 ml) of a 100 ml overnight *Campylobacter* culture in NB2 (**2.3.1.6**) was subjected to centrifugation at 3, 000 *g* for 25 min to pellet bacterial cells. These cells were then resuspended in 1.5 ml of ice-cold N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (10 mM HEPES, pH 7.4) prior to centrifugation at 13,000 *g* for 3 min. The resulting pellet was resuspended in 1 ml of HEPES buffer before

rupturing of the cells by sonication. Subsequently, the suspension was subjected to centrifugation at 13,000 *g* for 3 min. The supernatant was decanted and outer membrane fragments pelleted by centrifugation at 10,000 *g* for 40 min at 4 °C. The pellet was resuspended in 200 µl HEPES buffer to which was added 200 µl of 2% w/v sarkosyl (prepared in HEPES buffer). This suspension was then incubated at RTP for 30 min prior to centrifugation at 15,600 *g* for 30 min. The resulting pellet was washed (without resuspending) with 0.5 ml of HEPES buffer and resuspended in 25 µl of HEPES before storage at -70°C.

## 2.10 BACTERIOPHAGE ADSORPTION AND REPLICATION *IN VITRO*

### 2.10.1 Adsorption time course

*Campylobacter* cells grown in 1 l NB2 (**2.3.1.6**) were subjected to centrifugation at 10,000 *g* for 10 min and the pellet resuspended in 10 mM $MgSO_4$ (**2.0.1**) to a density equivalent to McFarland No. 3 (**2.3.2**), approximately $10^9$ cfu $ml^{-1}$. The actual titre of *Campylobacter* colony forming units was determined by plating serial dilutions of each suspension on CCDA without antibiotics (**2.3.1.4**). Phage suspension (1 ml of a $10^8$ pfu $ml^{-1}$ stock) was added to 9 ml of *Campylobacter* $MgSO_4$ suspension to give a multiplicity of infection (MOI) of 0.01 and incubated at 42°C in microaerobic conditions. During incubation, 1 ml aliquots of this suspension were taken at regular time points. Bacterial cells in each sample were pelleted by centrifugation at 13,000 *g* for 3 min prior to enumeration of bacteriophage in the supernatant using the Miles and Misra technique (**2.3.4.4**).

### 2.10.2 Bacteriophage replication in liquid culture

A suspension of *Campylobacter* host cells was prepared by subjecting a 1 l overnight culture (**2.3.3.2**) to centrifugation at 10,000 *g* for 10 min and resuspending the pellet in 9

ml of fresh broth to a density equivalent to McFarland No. 3 (approximately $10^9$ cfu $ml^{-1}$) (2.3.2). To this cell suspension, a sample (1 ml) of a $10^9$ pfu $ml^{-1}$ suspension of bacteriophage was added to give an MOI of 0.1 ($10^8$ pfu $ml^{-1}$). The suspension was incubated for 15 min in microaerobic conditions (2.3.3.3) to allow for phage adsorption before diluting 1:100 by the addition of fresh NB2 broth (2.3.1.6, 990 ml) containing the FBP supplement (2.3.1.3).

Following dilution, the culture was incubated microaerobically at 42°C and shaken at 100 rpm. Samples (1 ml) were taken at regular intervals and phage titres recorded as described previously (2.3.6.4). *Campylobacter* titres were determined by washing the pellet 3 x in MRD then resuspending in 1 ml of MRD and spotting serial dilutions on a CCDA plate according to the methods of Miles and Misra (2.3.4.4). The burst size was calculated using the following formula:

$$\frac{(P - x)}{[I - x]}$$

Where: **P** = maximum phage titre after lysis complete (pfu $ml^{-1}$)

**x** = number of phage from the original inoculum which did not adsorb (pfu $ml^{-1}$)

**I** = titre of the original inoculum (pfu $ml^{-1}$)

### 2.10.3 Bacteriophage adsorption to *Campylobacter* membrane vesicles and whole cells

*Campylobacter* cells were harvested from overnight Columbia blood agar plates (**2.3.3.1**) using a sterile cotton-tip swab and resuspended in 20 ml of 10 mM $MgSO_4$ solution (**2.0.1**). Membrane vesicles were prepared either using the precipitation technique (protocol A, **2.8.1**) or molecular filtration technique (protocol B, **2.8.2**). Aliquots (0.5 ml) of a $10^9$ pfu $ml^{-1}$ suspension of bacteriophage in SM buffer (**2.0.4**) were then mixed with 4.5 ml volumes of either membrane vesicles, whole cells ($10^9$ cfu $ml^{-1}$) or diluent (10 mM $MgSO_4$, **2.0.1**, or Nutrient broth No.2, NB2, **2.3.1.6**). Each suspension was then incubated aerobically at 37°C. Aliquots (100 µl) were taken from each suspension at regular time points and diluted in 900 µl of corresponding diluent ($MgSO_4$ or NB2). The diluted samples were then subjected to centrifugation at 40 , 000 *g* for 60 min at 4°C. The titre of phage in the supernatant of each sample was then determined on lawns of susceptible bacteria using the technique of Miles and Misra (**2.3.6.4**).

### 2.10.4 Replication of bacteriophage in the presence of membrane vesicles

A 1 l overnight culture of *Campylobacter* in NB2 (**2.3.3.2**) was subjected to centrifugation at 10 , 000 *g* for 10 min. The pellet was then thrice washed in 50 ml volumes of NB2 prior to resuspension in 9 ml of NB2. This suspension was then divided in to two 4.5 ml volumes. Membrane vesicles were collected from the culture supernatant using the molecular filtration technique (**2.8.2**) prior to resuspension in 4.5 ml of NB2. Subsequently, aliquots (1 ml) of $10^8$ pfu $ml^{-1}$ phage suspension were each mixed with either the membrane vesicle or whole cell suspensions. Following phage treatment, the suspensions were incubated aerobically at 37°C for 15 min. After incubation, the 'phage plus vesicles' suspensions were briefly mixed with the 4.5 ml of uninfected *Campylobacter* suspension prior to transfer to 200 ml of NB2 containing the

FBP supplement (**2.3.1.3**). The 'phage plus whole cells' suspension was briefly mixed with 4.5 ml of NB2 prior to transfer to 200 ml of NB2 + FBP. All the preparations were then incubated in microaerobic conditions (**2.3.3.3**) at 42°C in a gyratory platform incubator set to 100 rpm. The negative control consisted of 1 ml of phage suspension diluted in 9 ml of NB2 which, following a 15 min 'adsorption' period was incubated in 200 ml of NB2 under identical conditions to all other preparations. At regular time points during incubation, 1 ml aliquots were taken from each culture and subjected to centrifugation at 13 , 000 *g* for 5 min. Bacteriophage in the supernatant were enumerated on lawns of susceptible bacteria using the surface droplet technique (**2.3.6.4**). Pellets from samples containing whole cells were thrice washed in MRD (**2.0.2**) prior to enumeration of *Campylobacter* cells on charcoal agar using the Miles and Misra technique.

## 2.11 BACTERIOPHAGE THERAPY TRIALS

### 2.11.1 Source and treatment of broiler chicks prior to commencing trials

One day old commercial Ross broiler chickens reared to be free of *Campylobacter* were obtained from a registered supplier (P.D. Hook Hatcheries Ltd, Oxfordshire) and transported to a single environmentally controlled room at the experimental avian house at the Sutton Bonington campus of the University of Nottingham. The birds were provided with water and food *ad libitum* and otherwise treated in accordance with UK Home Office regulations: Animal (Scientific Procedures) Act, 1986. At fifteen days of age (DOA) the birds were assigned to experimental groups at random with respect to their live weights. Each of these experimental groups was housed in separate environmentally-controlled rooms and individually housed in wire cages. Unless otherwise stated, voided excreta was collected daily from 15 - 19 DOA to examine for *Campylobacter* and *Salmonella* colonisation prior to commencing each trial. Chicks

positive for either organism were culled using a UK Home Office Schedule I method and removed from the group.

### 2.11.2 Isolation of *Salmonella* ssp. from chick excreta.

The isolation of *Salmonella* ssp. was performed according to the method approved by ISO/BSI (*Salmonella* Monograph, Oxoid). Fresh voided excreta (1 g) was resuspended in 9 ml of buffered peptone water (BPW CM509, Oxoid). A pre-enrichment step was performed by incubating this suspension aerobically for 16 – 20 h at 37°C. An aliquot of faecal suspension (1 ml) was subsequently added to 10 ml of Rappaport – Vassiliadis (RV) broth (**2.3.1.9**) for selective enrichment and incubated for 18 – 24 h at 45°C. Following incubation, a 10 µl loopful of suspension was used to inoculate a plate of XLD selective agar (**2.3.11**) and streaked for single colonies before incubation at 37°C for 18 – 24 h. Colonies on XLD were compared with positive controls for characteristic *Salmonella* growth on this medium (red colonies with black centres (**Bridson, 1998**). Positive controls consisted of excreta specimens inoculated with $10^2$ cfu $g^{-1}$ *Salmonella enteritidis* strain WF01 (a human faecal isolate).

### 2.11.3 Preparation of *Campylobacter* for broiler chick oral inoculation.

*Campylobacter* strains were grown on Columbia blood agar (CBA, **2.3.1.1**) and harvested into phosphate buffered saline (PBS, **2.0.3**) using a sterile cotton tip swab to give a final titre of approximately $10^8$ cfu $ml^{-1}$ by comparison with turbidity standards (**2.3.2**). The actual *Campylobacter* titre was determined on CCDA agar plates using the Miles and Misra technique (**2.3.4.4**). Each chick was dosed once with approximately 1 ml of *Campylobacter* suspension by oral gavage.

### 2.11.4 Preparation of bacteriophage for broiler chick oral inoculation.

Bacteriophage were propagated using the plate lysate method (**2.3.6**) and concentrated by centrifugation (**2.3.6.3**). The phage suspension was diluted in 10 mM phosphate buffer (PB) to give a final titre of approximately $10^9$ pfu $ml^{-1}$ (titre determined on propagating host). Unless otherwise stated, the gastric pH of the birds was neutralised by administration of phage in PB containing 30% w/v $CaCO_3$. Each chick was dosed with approximately 1 ml of phage suspension 48 – 72 h following *Campylobacter* administration.

### 2.11.5 *Campylobacter* colonisation and phage therapy trials

At approximately 15 – 20 days of age the broiler chicks were separated into their respective treatment groups and their excreta monitored daily for the presence of natural *Campylobacter* and *Salmonella*. Any chicks found to be colonised with these organisms prior to oral dosing were sacrificed and removed from the trial. Campylobacters were prepared on CBA (**2.3.1.1**) and suspended in PBS (**2.0.3**) to give a titre of approximately $10^8$ cfu $ml^{-1}$. The chicks were dosed with ~ 1 ml of *Campylobacter* suspension by oral gavage. Negative controls were dosed with PBS only. Unless otherwise stated, three days after *Campylobacter* inoculation, the appropriate chick group was dosed with ~ 1 ml of approximately $10^9$ pfu $ml^{-1}$ of bacteriophage prepared in 10 mM PB containing antacid (30% w/v $CaCO_3$). Unless indicated otherwise, at 24 h intervals, random representatives of each treatment group were sacrificed using a UK Home Office Schedule I procedure and transported to the laboratory for dissection (**2.11.6**).

### 2.11.6 Sacrifice and dissection procedure for broiler chicks.

Broiler chicks were sacrificed at specific time points using a United Kingdom Home Office Schedule I approved method and transported to the laboratory. Immediately

following sacrifice the chicks were transferred to individual sealed polythene bags and then stored at 4°C until dissection. All dissections were performed within 6 h of chicken death. Where multiple organs were assessed for *Campylobacter* presence, the intestinal organs were always the last to be removed to reduce the possibility of contamination and care was taken to remove individual organs as aseptically as possible. Wherever practicable, ligatures were tied around organs prior to their removal. Dissection implements were thoroughly cleaned and flame-sterilised between each organ excision. The dissection area was sprayed with a chemical disinfectant (Virkon™, Suffolk, UK) before and in between dissections. Virkon™ was prepared at 1% w/v and used according to the manufacturer's instructions.

### 2.11.7 Enumeration of *Campylobacter* and microflora in broiler chicken caeca

Following broiler chicken dissection (**2.11.6**), the caecal contents (1 g) were transferred to a sterile plastic universal tube (Sterilin) and diluted 1:9 in MRD and resuspended by vortex mixing for approximately 2 min. The resulting suspension was then decimally diluted in the same diluent down to $10^{-5}$. Each dilution series was performed in duplicate. Volumes (100 µl) of each dilution were spread plated onto CCDA (**2.3.1.4**) containing 2% agar, and incubated under microaerobic conditions (**2.3.3.3**) at 42°C. The plates were examined for typical *Campylobacter* colonies after 24 h and 48 h incubation. This procedure was repeated on plate count agar (PCA, **2.3.1.8**) with incubation in aerobic, anaerobic and microaerobic conditions at 37°C to determine the total aerobic, anaerobic and microaerophilic populations in the caeca respectively. Generation of an anaerobic atmosphere was achieved using a rubber-sealed gas jar containing an AnaeroGen™ sachet (AN0035, Oxoid). Bacterial titres were calculated as described in section **2.3.4.4.**

Presumptive *Campylobacter* colonies were Gram stained to confirm morphology (**2.3.4.1**) prior to biochemical speciation (**2.3.4.2**)

### 2.11.8 Enumeration of *Campylobacter* bacteriophage in broiler chicken caeca

Caecal contents were weighed (1 g) then transferred to a sterile universal tube and resuspended in 9 ml of SMB diluent (**2.0.4**) using gentle inversion and pulse vortex mixing for up to 5 min. This suspension was then incubated at 4°C for 24 h with gentle agitation to allow phage to elute into the buffer. An aliquot of the eluate (1 ml) was then transferred to an Eppendorf tube and subjected to centrifugation at 10, 000 *g* for 3 min to remove bulk debris. The supernatant was then transferred to a fresh Eppendorf tube and subjected to a further centrifugation step at 13, 000 *g* for 5 min prior to filtration using a 0.2 μm-pore membrane filter (Sartorius) to remove any remaining bacterial cells. The resulting filtrate was then decimally diluted in SM buffer down to $10^{-6}$. Bacteriophage were subsequently enumerated using the Miles and Misra technique using a lawn of host bacteria cells (**2.3.6.4**).

### 2.11.9 Detection of *Campylobacter* in non-intestinal organs of broiler chickens

Following dissection of the chicken (**2.11.6**), a small section of each organ was excised and aseptically transferred into a sterile universal tube containing 10 ml of maximum recovery diluent (MRD, **2.0.2**) and homogenised for 2 min using a Taurex food homogeniser. Volumes (100 μl) of homogenate were spread plated in triplicate onto CCDA agar plates (**2.3.1.4**) containing a total of 2% w/v agar (to prevent swarming) and incubated in microaerobic conditions (**2.3.3.3**) at 42°C. The plates were examined for

typical *Campylobacter* colonies after 24 h and 48 h incubation. Presumptive *Campylobacter* colonies were Gram stained to confirm typical morphology (**2.3.4.1**).

### 2.11.10 Measurement of relative protease activity in chicken caeca

The caecal contents (1 g) were transferred to a sterile plastic universal tube (Sterilin) and diluted 1:9 in MRD (**2.0.2**) and resuspended by vortex mixing for approximately 2 min An aliquot of this suspension (1 ml) was then transferred to an Eppendorf tube and subjected to centrifugation at 10, 000 *g* for 3 min to remove bulk debris. The supernatant was then transferred to a fresh Eppendorf tube and subjected to a further centrifugation step at 13, 000 *g* for 5 min prior to filtration through a 0.2 μm-pore membrane filter (Minisart, Sartorius) to remove any remaining bacterial cells. The EnzChek® Protease Assay Kit (E-6638, Molecular Probes, Leiden, Netherlands) was used to determine the total protease activity in these samples according to the manufacturer's instructions using a Victor 1420 multilabel photoluminometer (Perkin-Elmer UK Ltd) with excitation and emission wavelengths of 485 nm 530nm respectively. This kit is based on the protease-catalysed hydrolysis of a casein protein substrate labelled with green fluorescent dye (BODIPY® FL). Once the dye is released, an increase in fluorescence is recorded which is proportional to the total protease activity in a sample. A standard curve of protease activity was generated using concentrations of trypsin (1 – 5 μg $ml^{-1}$) as directed by the manufacturer.

## 2.12 DATA TRANSFORMATION AND STATISTICAL ANALYSIS

All statistical analyses were performed using the data analysis add-in for Microsoft Excel™ XP. Unless otherwise stated, all bacterial and bacteriophage titres were transformed to $\log_{10}$ values prior to the application of significance tests.

Significant differences between two data groups was assessed using the Student's t- test. For three or more data groups, a single factor analysis of variance (ANOVA) test was applied. For all statistical tests, the confidence limit was set at 95% with the assumption that the data possessed a normal distribution and the variance between different data sets was equivalent.

# CHAPTER 3

## SURVIVAL AND ISOLATION OF *CAMPYLOBACTER* BACTERIOPHAGE ON RETAIL POULTRY PRODUCTS

### 3.0 INTRODUCTION

Studies of several *Campylobacter* outbreaks in humans have shown undercooked poultry, specifically chicken, to be the source of infection (**Istre *et al.*, 1984; Murphy *et al.*, 1995; Evans *et al.*, 1998**). Campylobacters readily colonise many species of poultry but the dissemination of the bacteria from these birds is exacerbated during slaughter, as negative flocks are often mixed with positive flocks leading to large-scale cross contamination (**Newell *et al.*, 2001**). As up to 76% of flocks sent to slaughter are *Campylobacter* positive (**Humphrey *et al.*, 1993**), the control of contamination remains a major challenge to the industry. Whilst post-processing treatment and storage conditions may reduce *Campylobacter* contamination on broiler carcasses, in some cases these treatments are considered to have a negligible impact on human exposure to this organism (**Jacobs-Reitsma, 2000**). A recent survey in the UK demonstrated that 83.3% of samples taken from retail chicken were *Campylobacter* positive (**Kramer *et al.*, 2000**).

In addition, *Campylobacter* is able to survive on kitchen surfaces for several hours after contamination (**Cogan *et al.*, 1999**) leading to cross contamination events in the home.

*Campylobacter* bacteriophage have previously been isolated from sewage and abattoir effluent (**Salama *et al.*, 1989**). Some of these phage isolates have been utilised in typing schemes to complement various other typing methods (**Grajewski *et al.*, 1985; Salama *et al.*, 1990b; Frost *et al.*, 1999**). The lytic capability and host specificity of phage also present the opportunity of using them to reduce the numbers of campylobacters emanating from animal sources and subsequently reduce cross contamination during processing. Generally, a reduction in the numbers of campylobacters entering the human food chain is likely to have a beneficial effect on the disease burden (**Rosenquist *et al.*, 2003**). As a first step towards implementing these strategies it was necessary to establish if bacteriophage were already present on retail poultry in order to gauge the possible impact of intervention and to establish if these strategies would be introducing any new biological elements into the human food chain.

To date there have been no reports of the isolation of *Campylobacter* phage from retail chicken products. The aims of this study were to establish and validate recovery methods for *Campylobacter* and bacteriophage from poultry meat samples, and to determine the incidence of phage from fresh and frozen retail poultry in the UK. The ability of a subset of phage isolates to survive on the surface of fresh and frozen poultry products was also investigated.

## 3.1 RESULTS

### 3.1.1 Evaluation of methods for *Campylobacter* isolation from chicken skin

Three protocols (A, B and C, **2.6.1**) were compared for their ability to isolate *Campylobacter* cells from various sites on the surface of fifty standard medium weight broiler chicken carcasses (Fig. 3A). Briefly, areas of chicken skin were wiped with a sterile swab which was subsequently used to inoculate plates of mCCDA (protocol A) or Blaser – Wang Agar (protocol B). For protocol C, 10 g of chicken skin from each sampling location were macerated and enriched for *Campylobacter* using the Park and Sanders method. Each isolation method was used on different areas of skin within the same sampling zones on individual carcasses (Fig. 3A). Care was taken not to sample the same skin area twice. Presumptive *Campylobacter* colonies ($n = 5$) recovered using each method from each carcass were confirmed using morphological (**2.3.4.1**) and biochemical (**2.3.4.2**) assays. The efficacy of each isolation protocol for each sampling site is presented in Table 3A. Direct plating onto Blaser-Wang agar (protocol B) was consistently less successful in *Campylobacter* isolation than either of the other methods. However, this medium also resulted in the fewest number of contaminants. Overall, enrichment (protocol C) was the most successful method, isolating *Campylobacter* from 234 of the 300 sites tested (78%) compared with 210 (70%) for direct plating onto CCDA (protocol A). However, it should be noted that some samples positive by direct plating were not positive by enrichment and *vice versa*. The cavity, neck skin, thigh and back were the sites most likely to harbour campylobacters with the mean recovery from the 50 chickens sampled of 77% (39), 76% (38), 72% (36) and 71% (35) respectively

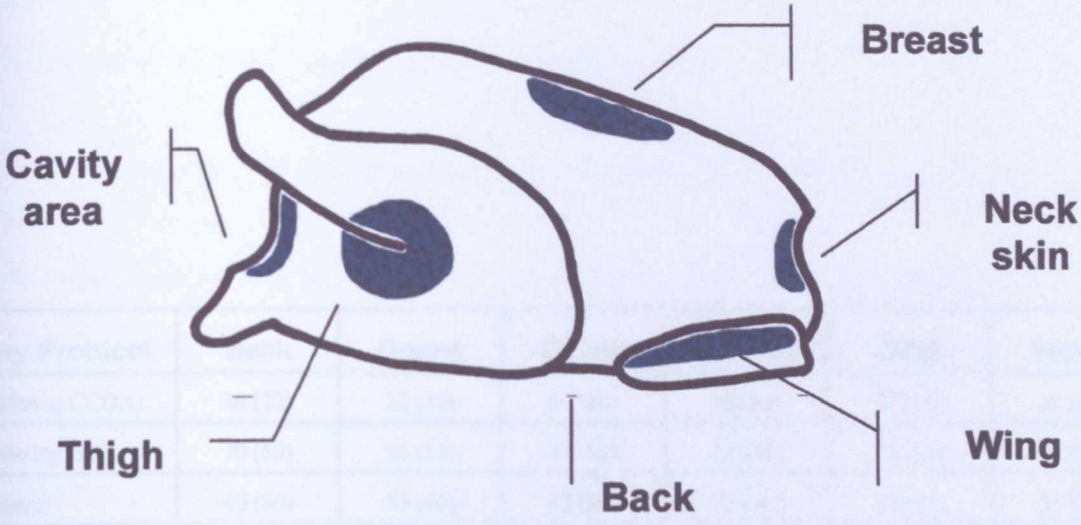


**Figure 3A. Diagram of carcass sampling sites.**

Diagram indicating the sites sampled on a standard retail broiler chicken carcass to determined the efficacy of three *Campylobacter* recovery protocols.

| Recovery Protocol | Back | Breast | Cavity | Neck Skin | Thigh | Wings |
|---|---|---|---|---|---|---|
| A (direct plating CCDA) | 36 (72) | 22 (44) | 40 (80) | 40 (80) | 37 (78) | 36 (72) |
| B (direct plating BW) | 30 (60) | 26 (52) | 33 (66) | 31 (62) | 28 (56) | 28 (56) |
| C (enrichment) | 40 (80) | 33 (66) | 42 (84) | 43 (86) | 41 (82) | 35 (70) |

**Table 3A. Recovery of *Campylobacter* from broiler chicken carcasses.**
Table showing the recovery of *Campylobacter* spp. from six sites on the surface of fifty retail broiler chicken carcasses. Recovery is given as the number, and the percentage in parentheses, of *Campylobacter* positive samples.

(calculated from positive samples using all methods). Of the isolated colonies which were confirmed using morphological and biochemical assays, 98% were hippurate positive (*C. jejuni*) with the remainder likely to be *C. coli* based on previous reports (**Kramer *et al.*, 2000; Jorgensen *et al.*, 2002**).

### 3.1.2 Evaluation of *Campylobacter* bacteriophage isolation methods.

*Campylobacter jejuni* NCTC 12662 Phage Type 14 (PT14) was supplied by the Central Public Health Laboratory (CPHL, Colindale, London) and used as the host in all primary phage isolation and recovery from chicken skin. PT14 is sensitive to all the phage employed in the current UK phage-typing scheme (**Frost *et al.*, 1999**) and was considered the most suitable strain to use for phage recovery. Bacteriophage ϕ2 (NCTC 12674, ATCC 35922-B2) was also supplied by the CPHL and is a constituent of their phage-typing scheme (**Frost *et al.*, 1999**). Phage ϕ2 was used as a positive control to estimate the recovery efficiency from chicken skin. This phage consistently gives clear, distinct plaques on the *C. jejuni* PT14 propagating strain. Five protocols (I, II, III, IV and V, **2.6.4**) were compared for their ability to recover $10^7$ pfu of *Campylobacter* bacteriophage ϕ2 from the skin surface of retail chicken thigh portions. A brief overview of each protocol is presented below.

| PROTOCOL | DESCRIPTION |
|---|---|
| I | Chicken skin surface was swabbed and placed in 5 ml of SM buffer overnight to allow the phage to elute. The resulting eluate was filtered and phage in the filtrate titred using the surface droplet technique on a lawn of susceptible bacteria. |
| II | Skin sections were washed in 20 ml of SM buffer prior to filtration and titration as described for protocol I. |
| III | Skin sections were stomached for 5 min in 10 ml of SM buffer prior to filtration and titration as described for protocol I |
| IV | Skin sections were stomached for 5 min in 10 ml of SM buffer. The stomachate was then subjected to centrifugation (2.5 *g*, 10 min) and filtration. Titration was then performed by mixing dilutions of filtrate with host cells and transferring to molten soft agar which was overlaid on to base agar (modified pour plate technique). |
| V | Skin sections were stomached for 5 min in 10 ml of SM buffer. A 1 ml aliquot of stomachate was treated with 100 µl of chloroform and subjected to centrifugation. The titre of phage in the supernatant was determined using the Miles and Misra technique as with protocol I. |

The comparative efficacy of each method in recovering bacteriophage from the surface of inoculated chicken skin is presented in Table 3B. Recovery was less efficient using swabbing and washing techniques (protocols I and II) with only 2.2% and 0.5% of the inoculated phage titre recovered from the skin surface using each of these methods

| Recovery Protocol | Percentage Recovery | Recovery Range | Standard Deviation |
|---|---|---|---|
| I (swab, filter) | 2.2 | 0.03 - 5.21 | 1.8 |
| II (wash, filter) | 0.5 | 0.02 - 1.10 | 0.4 |
| III (stomach, filter) | 27.7 | 16.5 - 41.4 | 7.6 |
| IV (stomach, centrifuge, filter) | 67.3 | 59.2 - 73.8 | 4.9 |
| V (stomach, chloroform treat) | 48.6 | 39.6 - 59.9 | 7.9 |

**Table 3B. Efficiency of bacteriophage recovery from chicken skin.**
Table showing the recovery of *Campylobacter* bacteriophage $\phi 2$ from the surface of inoculated chicken skin using five protocols. The percentage recovery of the initial inoculum is presented along with the recovery range and standard deviation for each method used. These results are based on ten replicates.

respectively. In contrast, protocols III, IV and V, all of which used stomaching, demonstrated a consistently higher percentage of recovery (27.7%, 67.3% and 48.6% respectively) than seen with protocols I and II. The direct filtration of stomached samples described in protocol III was often problematic due to the high fat content of the stomachate in some samples. This was alleviated by the additional centrifugation step in protocol IV. Chloroform treatment (protocol V) reduced the number of bacterial contaminants and did not appear to have any adverse effects on the bacteriophage, suggesting $\phi 2$ is not sensitive to this agent. Based on ten replications, the recovery of bacteriophage from chicken skin was most successful and least variable using protocol IV. Consequently, this protocol was selected for use in all subsequent phage recovery from chicken skin.

In order to estimate the lower limit of phage detection on chicken skin, the recovery efficiency of protocol IV was examined using *Campylobacter* phage $\phi 2$. Table 3C shows the lower detection limit determined for the recovery of this phage following addition of serial dilutions onto retail chicken skin sections as described above. Based on three replicates, this method will reliably detect at least $10^3$ pfu of $\phi 2$ per 10 $cm^2$ area of chicken skin.

### 3.1.3 Recovery of phage $\phi 2$ from the surface of chicken skin over time.

As a prerequisite to a survey of retail chicken for the presence of *Campylobacter* phage it was necessary to establish that phage could survive *in situ* on chicken skin under retail storage conditions. To ensure that any incumbent *Campylobacter* phage would not influence the recovery results, uninoculated samples were examined for environmental

| Inoculum ($log_{10}$ pfu) | Mean Recovery ($log_{10}$ pfu) | Average Recovery (% ± SD) |
|---|---|---|
| 7.3 | 6.6 | 18.7 ± 4.0 |
| 6.3 | 6.0 | 42.3 ± 7.8 |
| 5.3 | 5.0 | 43.3 ± 4.5 |
| 4.3 | 4.0 | 20.3 ± 4.1 |
| 3.3 | 2.6 | 17.3 ± 2.5 |
| 2.3 | 2.0 | 9.6 ± 4.1 |
| 1.3 | -0.2 | 3 ± 5.2 |

**Table 3C. Validation of the bacteriophage recovery method.**
The left column shows the number of phage inoculated onto the surface of retail chicken thighs (10 $cm^2$). The central column shows the mean number of phage recovered from the surface of the inoculated chicken skin which is also shown as a percentage ± standard deviation in the right column. These results are based on three replicates.

phage. If phage recovery was successful, the sample was discarded so that the results were not influenced by the presence of natural phage. Sections of skin were then excised from fresh and frozen chicken thighs not containing natural phage. These skin samples were then inoculated with $10^7$ pfu of phage $\phi 2$ and, following a 1 h drying period, the skin sections were individually stored in sterile containers and incubated at either 4°C or -20°C. Triplicate skin sections were taken from each storage condition commencing 1 h after inoculation and then at 24 h intervals thereafter for a period of ten days. Bacteriophage on each skin sample were enumerated using recovery protocol IV as described previously **(2.6.4.4)**. Data showing the recovery of the inoculated *Campylobacter* phage from the surface of fresh and frozen chicken thigh skin over a ten day period are presented in Figure 3BI. Phage recovery remained constant at 42 - 44% of the initial inoculated titre over a six day period for skin stored at 4°C. At day seven the titre fell from 44% ± 0.8 to 34% ± 7.0 and continued to fall for the duration of the experiment until reaching 17% ± 1.8 at day ten. In contrast, the recovery of phage from thawed frozen chicken was initially close to 100% (1 h) but following refreezing and subsequent thaw the titre fell to 22% ± 0.1. Phage recovery from chicken portions thawed at 24 h intervals thereafter were in the range of 17 - 34% over a ten day period. In order to determine if the presence of viable *Campylobacter* cells on the surface of chicken skin could affect the recovery of inoculated phage, 5 $cm^2$ areas of skin from each chicken portion were also screened for the presence of *Campylobacter* by swabbing and plating directly onto CCDA selective agar **(2.6.1)**. The recovery of phage from skin without detectable *Campylobacter* did not significantly differ from skin with *Campylobacter*

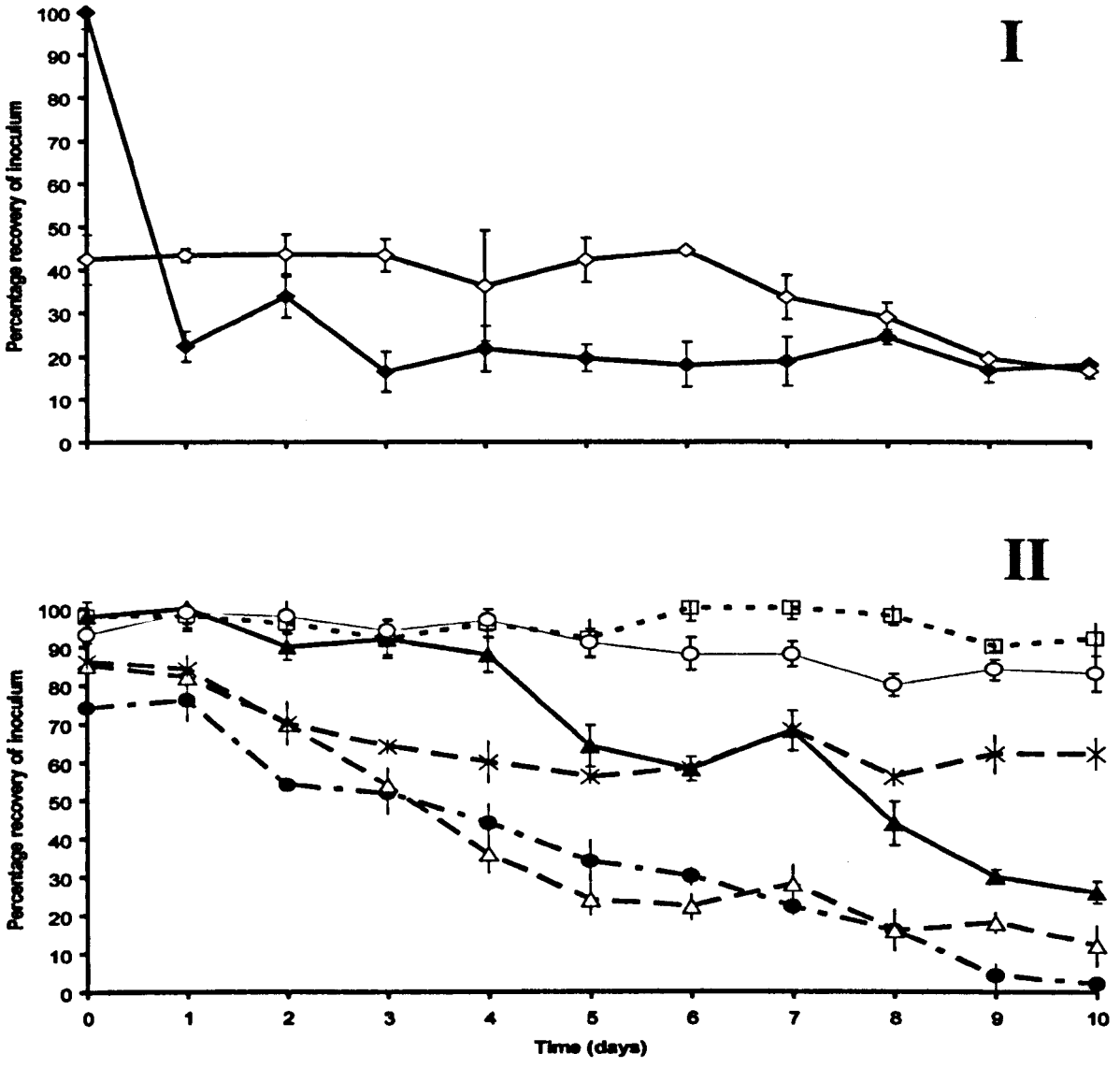


**Figure 3B. Recovery of phage from chicken skin stored at 4°C and -20°C.** (I) Recovery of phage ϕ2 (NCTC 12674) from chicken skin stored for ten days under fresh (4°C; ◇) and frozen (-20°C; ◆) conditions. Triplicate 10 $cm^2$ section of chicken thigh skin were inoculated with $10^8$ pfu of ϕ2 then percentage recovery of this inoculum (± SD) was recorded at 24 h intervals. (II) Recovery of six *Campylobacter* bacteriophage chicken skin isolates exhibiting different lytic spectra. $10^8$ pfu of each phage were applied to fresh chicken thigh skin in triplicate and stored for ten days at 4°C. The percentage recovery of the initial inoculum (± SD) was recorded at 24 h intervals. Key: (▲)w2; (●) w3; (△) w4; (□) w5; (X) w8 and (o) w10. In all cases the initial samples (day one) were collected one hour after inoculation.

($P>0.2$). This suggested that the presence of potential *Campylobacter* hosts on the chicken skin surface did not appreciably influence the recovery of bacteriophage.

### 3.1.4 *Campylobacter* and bacteriophage isolation from retail chicken portions.

In order to assess the presence of phage and their hosts on commercial products, a total of 450 retail chicken thigh portions (300 fresh, 150 frozen) were purchased from a UK supermarket (**2.6.6**). These portions were selected on the basis of different product bar codes and processing unit numbers to give a cross section of samples from six UK producers. For the isolation of *Campylobacter* ssp., areas of chicken skin (10 $cm^2$) were wiped with a sterile swab which was used to inoculate a plate of CCDA selective agar (as described in section **2.6.1**). Presumptive *Campylobacter* colonies were Gram stained to confirm morphology prior to biochemical speciation (**2.3.4.2**). Bacteriophage were isolated from samples using isolation protocol IV described previously (**2.6.4.4**). *Campylobacter* was isolated from 100% of free range chicken portions compared with 75% and 62% for standard and economy products respectively. It was noted that *Campylobacter* was isolated at a lower frequency from frozen chicken samples with 9% of standard and 12% of economy products being positive. The majority of campylobacters isolated (96%) were *C. jejuni* with the rest likely to be *C. coli* or *C. lari* based on previous studies (**Jorgensen *et al.*, 2002**). *Campylobacter* phage were recovered from the skin of 34 fresh chicken thighs out of the 300 tested (11%). Twenty seven (79%) of the phage-positive chicken thighs were from free-range chicken products as compared with five (15%) and two (6%) from standard and economy products

respectively. A one way ANOVA (**2.12**) demonstrated that the observed incidence on the free-range product is significantly different ($P<0.001$) to that of the other products. The mean number of phage isolated from the whole chicken skin surface was $\log_{10}$ 5.7 pfu (recovery ranged from $\log_{10}$ 2.0 to 6.6 pfu). Phage recovery from frozen chicken thighs was unsuccessful. Given the 80% reduction in the recovery of phage ϕ2 from inoculated frozen samples (Fig. 3BI) this result was not unexpected.

### 3.1.5 Variation in the rates of recovery of phage isolates from chicken skin.

The relative recovery of representative phage isolates (characterized on the basis of lytic spectrum, genome size and morphology, see Chapter 4), from the surface of inoculated chicken skin were examined using recovery protocol IV (**2.6.4.4**). The phage used in this experiment (w2, w3, w4, w5, w8 and w10) represented lytic spectrum classes I to VI respectively (Chapter 4). The recovery of these phage following inoculation onto chicken skin and incubation at 4°C showed considerable variation when recorded at 24 h intervals over a ten day period (Fig. 3BII). At least 90% of the phage w5 inoculum could be recovered over the full course of the experiment compared with only 2% of the w3 inoculum. However, the recovery of all phage from the surface of frozen chicken did not differ significantly from the pattern recorded for phage ϕ2 (Fig. 3BI). High recovery titres were recorded prior to freezing (ranging from 84% for w3 to 98% for w5), at which time the recovery decreased to 18% - 30% and thereafter remained stable for the rest of the time course.

## 3.2 DISCUSSION

The efficacy of *Campylobacter* isolation methods has been debated since this organism was successfully isolated from human stools in the 1970s (**Skirrow, 1977**). None of the current methods for the isolation of *Campylobacter* from food have been universally approved and implemented, making it difficult to compare the prevalence of this organism in different geographical areas. In the present study, enrichment using the Park and Sanders method recovered *Campylobacter* from the largest number of samples. However, the time, expense and man-hours required to apply this method make it impractical for larger studies. Direct plating onto CCDA recovered *Campylobacter* from marginally fewer sites than enrichment but resulted in less contamination, and proved to be far quicker and cheaper. Direct plating onto Blaser-Wang medium resulted in minimal contamination but the recovery of *Campylobacter* was appreciably less than the other methods. Sub-lethally injured *Campylobacter* cells are reported to be sensitive to polymyxin but not other antibiotics in selective media (**Corry *et al.*, 1995b**). As the Blaser-Wang agar contained polymyxin this may explain the lower sensitivity of this medium in *Campylobacter* isolation.

There was a wide variation in the efficacy of the protocols used to recover bacteriophage from chicken skin. Generally, swabbing or washing the surface of the skin was less effective than more vigorous treatments such as stomaching. This may indicate that the bacteriophage become closely associated with the skin following inoculation. Association with chicken skin is known to protect *Campylobacter* from chemical decontamination procedures in slaughter houses (**Whyte *et al.*, 2001**; **Yang *et al.*, 2001**)

and is likely to provide non-specific protection for phage as well. Chloroform is known to inactivate certain phage which are members of the Tectiviridae family as well as certain Plectiviruses and Ionoviruses. This is thought to occur by the disruption of the hydrophobic bonds which maintain capsid protein integrity (**Ackermann and DuBow, 1987**). Chloroform did not appear to have any appreciable effect on the recovery of *Campylobacter* bacteriophage ϕ2.

The successful isolation of *Campylobacter* phage from 34/300 fresh retail chicken samples demonstrated that the methods developed in this study are practically applicable. Since the detection limit of the recovery method was determined to be $10^3$ pfu/10cm$^2$ of chicken skin, isolation of phage from these products implies that at least this many bacteriophage have survived retail poultry processing and packaging. The phage isolated in this study are likely to originate from the caecal contents of chickens, particularly since *Campylobacter* phage have previously been recovered from abattoir effluent and chicken excreta (**Grajewski *et al.*, 1985**). Contamination of carcasses from chicken intestinal contents both preceding and following abattoir processing has been reported previously.

The *Campylobacter* phage isolated in the present study were from fresh chicken thighs within their prescribed shelf life. However, survival experiments demonstrated that bacteriophage can be recovered up to ten days following inoculation, which is well beyond their stated shelf life. Phage ϕ2 could be efficiently recovered from the surface of frozen retail chicken which had been thawed prior to phage application. However, subsequent freeze and thaw resulted in a fall in the recovery by almost 80%. Birds produced for freezing are scalded at a higher temperature (**Humphrey *et al.*, 1988**) and

that, together with the subsequent freeze-thaw is known to change the skin surface structure (**Adams and Moss, 1995**). The results in the present study suggest that phage may not attach as well to skin surfaces following such treatment, leading to higher initial recovery rates (99.8%). The subsequent fall in recovery is likely the result of reduced phage viability on freezing, to which members of the Myoviridae family are particularly susceptible (**Ackermann and DuBow, 1987**).

The lytic spectra of the individual phage isolates (Chapter 4) revealed similarities in host range. Phage exhibiting the same lytic spectrum were placed in one of eight host range classes. The host ranges of all the retail poultry isolates differed markedly from the lytic spectrum of the $\phi$2 positive control (Chapter 4). The similarity in lytic profiles of phage isolated from different chicken samples may suggest some form of selection has taken place. This may reflect the ability of the bacteriophage to survive on the chicken surface during processing and their ability to survive the storage conditions applied. Previous studies suggest that certain *Campylobacter fla*-types are able to survive abattoir processing better than others (**Newell *et al.*, 2001**), and this may be a factor in determining the survival of any predator phage that are associated with them.

Free-range chickens are subjected to greater environmental exposure than conventionally-reared birds and are therefore more likely to encounter a wider range of campylobacters and their phage. A significantly greater percentage of free-range products were phage positive compared with standard and economy classes which may, in part, be explained by the frequency of *Campylobacter* positive carcasses originating from the free-range sector. Greater exposure to the environment consistent with organic and free

range farming practices is purportedly the cause of a generally higher incidence of *Campylobacter* in these types of flocks (**Hald *et al.*, 2001**). The probability of phage isolation will increase with the presence of a susceptible host, thus chickens with higher rates of carriage of campylobacters are more likely to be a source of phage. However, with widespread cross-contamination in the abattoir, it is likely that the *Campylobacter* and phage found on a single carcass arise from more than one source. In addition to this the recovery of phage isolates representing different lytic spectra classes inoculated onto chicken skin show marked variation. Four of the six chicken skin isolates tested yielded a higher rate of recovery over a 10 day period when applied to chicken skin and retained at 4°C (Fig. 3BII) when compared with the ϕ2 control (Fig. 3BI). Most notably phage w5 could be recovered at >90% of the inoculum throughout the course of the experiment.

Phage were only recovered from chicken samples which also harboured campylobacters, a finding that is not surprising as the phage will originate from an environment containing host cells. Cross contamination may occur but the numbers of phage involved could be below the limit of detection. Survival data for phage inoculated onto chicken skin and incubated in retail storage conditions shows a general decline, suggesting that replication of the phage is not occurring on the surface of the samples. This is not surprising as *Campylobacter* do not grow on food samples stored under these conditions and hence will not have an active metabolism. There are multiple variables dictating the survival of both phage and *Campylobacter* throughout commercial poultry production. These factors may not necessarily allow both the phage and its host to survive at the end of processing. The multiple cross contamination events and survival variables of *Campylobacter* and

phage in the processing plant would make any attempt to correlate the phage with a host from a common chicken source of little value.

This study has demonstrated that *Campylobacter* phage can survive on fresh and frozen retail poultry products. An isolation protocol was developed and successfully implemented to recover *Campylobacter* phage from 34 independent retail chicken samples, a source previously not examined for the presence of *Campylobacter* phage. The finding that phage are present on retail poultry products demonstrates that the use of phage as a biocontrol agent would constitute a minimum intervention that would not ultimately introduce any entity into the food product which is not already present.

# CHAPTER 4

# CHARACTERISATION OF BACTERIOPHAGE ISOLATED FROM RETAIL POULTRY PRODUCTS

## 4.0 INTRODUCTION

The characterization of the bacteriophage isolated in Chapter 3 is of paramount importance should they be implemented in any future biocontrol strategies against *Campylobacter*. Bacteriophage characterization has historically been based on morphology, genetic similarities and host range (**Ackermann *et al.*, 1992**). These primary traits are still fundamental features used in the categorization of phage by the International Committee on the Taxonomy of Viruses (ICTV). The binomial nomenclature system developed from the work of Linnaeus and successfully used for many organisms is not easily applied in viral taxonomy (**Ackermann *et al.*, 1992**). Another major problem is that, unlike bacteria, phage do not have one single conserved gene amongst them (**Ackermann *et al.*, 1992**). As a consequence, the classification of viruses by using, for example, a similar technique to rDNA profiling in bacteria is not possible with bacteriophage. More recently there have been attempts to modernize traditional bacteriophage taxonomy, with one approach basing nomenclature on predicted

protein homology from known phage DNA sequences combined with "signature genes" found in particular families of viruses (**Rohwer and Edwards, 2002**). Nevertheless, the traditional system prevails as the accepted standard and phage are currently ascribed to one of twelve families (Table 1A).

Although the morphology and genetic similarities between phage may provide useful indications as to the lifestyle of the virus, the most critical aspects from a therapeutic perspective are the host range and replication dynamics. These particular qualities were often overlooked when phage therapy was in its infancy in the early 20$^{th}$ century, often leading to failure (**Summers, 2001**). The work of Ellis and Delbrück on the growth of phage in the 1930s, in particular the "one step growth curve" (**Ellis and Delbruck, 1939**), forms the cornerstone of modern phage therapy models (**Payne and Jansen, 2001**).

Proceeding from this principle, the lytic activity of the bacteriophage isolates from Chapter 3 against *Campylobacter* hosts from three sources was investigated. Subsequently, a subset of phage exhibiting different lytic profiles and the broadest host range were selected for further characterisation which consisted of examining:

- Virion morphology using negative staining transmission electron microscopy
- Phage replication dynamics (adsorption efficiency, replication time)
- Major phage proteins using SDS PAGE and mass spectrometry sequencing
- Genome attributes (genome size, nature of the nucleic acid, macro restriction profiles, sequencing of cloned fragments)

The aim of these analyses was to collate information on different aspects of the phage isolates prior to selection of the most suitable candidates for therapeutic interventions in broiler chickens.

## 4.1 RESULTS

### 4.1.1 Host range and lytic profiling

The success of bacteriophage therapy in an industrial scenario is more likely if the viruses applied have a broad host range within the targeted bacterial genus. With the aim of selecting the most suitable candidates for future *in vivo* therapeutic endeavours, 34 phage isolated from retail poultry (see Chapter 3) were screened against a total of 109 strains of *Campylobacter*. The phage isolates were screened for lytic activity against two groups of campylobacters (A and B) by using the surface droplet technique (**2.3.6.5**). Group A ($n$ = 11) consisted of National Collection of Type Cultures (NCTC) strains used as propagating hosts for different phage in the *Campylobacter* phage typing scheme adopted in the UK (**Frost *et al.*, 1999**). Group B ($n$ = 18) consisted of a subset of campylobacters, which were isolated contemporaneously with the bacteriophage (Chapter 3), that possessed different major flagellin gene (*flaA*) profiles. A subset of bacteriophage exhibiting the greatest host range with groups A and B were subsequently sent to the Central Public Health Laboratories (CPHL, Colindale, London) to be profiled using 80 different clinical *Campylobacter* isolates (Group C).

#### *4.1.1.1 Results using Group A*

The results using the eleven host strains of *Campylobacter* from the NCTC separated the phage isolates into eight lytic spectrum classes (Table 4AI). None of these classes corresponded to the profile of the positive control, NCTC phage ϕ2. The only strains to

| | | *Bacteriophage Class* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *CPHL PT (REF)* | I | II | III | IV | V | VI | VII | VIII | ϕ2 |
| **GROUP A** | PT 1 (C605) | - | - | - | - | - | - | - | - | - |
| | PT2 (C682) | + | + | + | + | + | - | - | - | - |
| | PT5 (C856) | - | + | - | - | - | - | - | + | - |
| | PT6 (C594) | - | + | - | - | - | - | - | - | + |
| | PT14 (NCTC 12661) | + | + | + | + | + | + | + | + | + |
| | PT14 (NCTC 12662) | + | + | + | + | + | + | + | + | + |
| | PT19 (C11288) | - | + | + | + | + | (+) | - | - | - |
| | PT33 (C1312) | - | - | - | + | - | - | - | - | - |
| | PT35 (C13553) | - | - | - | - | - | - | - | - | - |
| | PT44 (C10131) | - | - | - | - | - | - | - | - | + |
| | NCTC 11168 | - | - | - | - | - | - | - | + | (+) |
| | *FLA TYPED ISOLATES* | | | | | | | | | |
| **GROUP B** | 1 | - | + | + | + | + | + | - | - | - |
| | 2 | - | + | + | + | + | + | - | - | - |
| | 7 | - | + | + | + | + | + | - | - | - |
| | 12 | + | - | - | - | - | - | - | - | - |
| | 17 | - | - | - | - | - | - | - | - | - |
| | 19 | - | + | + | + | + | + | - | - | - |
| | 29 | - | - | - | - | + | (+) | - | - | + |
| | 31 | - | - | - | - | - | - | - | - | - |
| | 33 | - | - | + | - | - | + | - | - | - |
| | 34 | - | - | - | - | - | - | - | - | - |
| | 35 | - | - | - | - | - | - | - | - | - |
| | 39 | - | - | - | - | - | - | - | - | (+) |
| | 77 | - | + | + | + | + | + | - | - | - |
| | 190 | - | - | (+) | - | - | - | - | - | - |
| | 191 | - | - | - | - | - | - | - | - | - |
| | 192 | - | - | - | - | - | - | - | - | - |
| | 193 | - | - | - | - | - | - | - | - | - |
| | 194 | - | - | - | - | - | - | - | - | - |
| | TOTAL STRAINS INFECTED | 4 | 11 | 11 | 10 | 10 | 10 | 2 | 4 | 7 |

**Table 4A I. Phage lytic spectra using group A and B *Campylobacter* hosts**

Lytic spectra of 8 phage isolates belonging to different lytic spectra classes. Lytic activity against group A and B hosts are shown as + (clear lysis of host); (+) (opalescent lysis) and – (no lysis). The total number of strains lysed by each lytic class [ + and (+)] is given at the bottom of the table.

be infected and confluently lysed by all phage were NCTC 12661 and NCTC 12662, both of which were CPHL phage type (PT) 14. *Campylobacter* strains of phage types 1 and 35 were not lysed by any of the phage isolates.

### *4.1.1.2 Results using Group B*

A subset ($n$ = 60) of *Campylobacter* isolates from retail chicken thigh portions were typed using the restriction fragment length polymorphism patterns of the *flaA* gene (*fla* typing, **2.5.8**). Campylobacters exhibiting different *fla* types ($n$ = 18) were selected as a representative panel of strains isolated from retail chicken. An example of the differences in *fla* type observed among a subset of these isolates is presented in Figure 4A.

In contrast with the lytic activity recorded against the NCTC strains, no one *fla* type was lysed by all of the phage. Of the eighteen *fla* types used, eight were not lysed by any of the bacteriophage (*fla* types 17, 31, 34, 35, 191, 192, 193 and 194). The phage isolates could be ascribed to eight distinct lytic spectra according to their ability to infect the combined panel of hosts from groups A and B (Table 4AI).

### *4.1.1.3 Results using Group C*

Only two patterns of lytic activity were observed when the subset of phage were tested against 80 human *Campylobacter* isolates. The first consisted of isolates w1, w2, w6 and w7, the second consisted of w3, w4, w5, w8-18 (Table 4AII). Seventeen of the

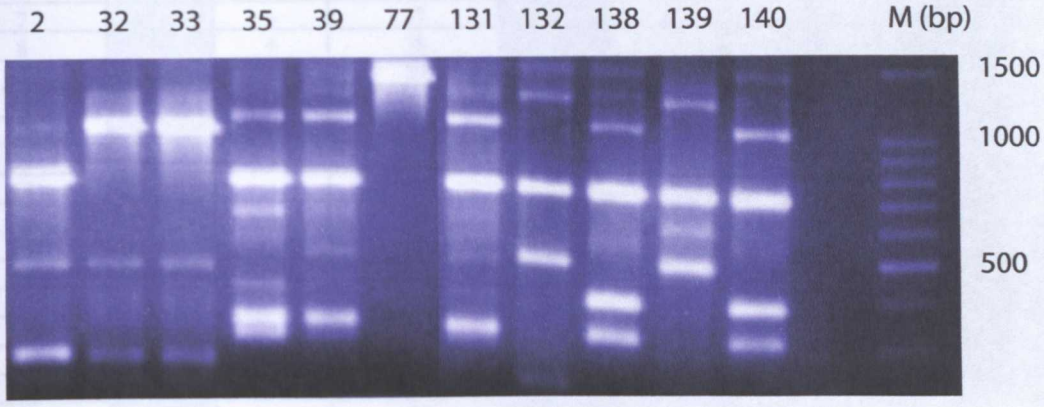


**Figure 4A. *flaA* profiles of campylobacters isolated from retail chicken.** Agarose gel showing the *flaA* amplicons from campylobacters isolated from retail chicken when digested with endonuclease *Dde*I. Eight unique restriction profiles could be discerned from the eleven *Campylobacter* strains shown here. The strain reference is shown at the top of each lane along with a 100 bp marker (M) on the far right of the figure.

| Phage type | Class I | Class II | Phage type | Class I | Class II | Phage type | Class I | Class II |
|---|---|---|---|---|---|---|---|---|
| 1 | - | - | 31 | + | - | 61 | - | - |
| 2 | - | + | 32 | - | + | 62 | - | - |
| 3 | + | + | 33 | + | - | 63 | - | - |
| 4 | + | + | 34 | - | - | 64 | - | - |
| 5 | - | - | 35 | - | - | 65 | + | + |
| 6 | + | - | 36 | + | - | 66 | + | + |
| 7 | - | - | 37 | - | + | 67 | (+) | - |
| 8 | - | + | 38 | + | - | 68 | - | - |
| 9 | + | + | 39 | - | - | 69 | (+) | + |
| 10 | - | - | 40 | (+) | - | 70 | + | + |
| 11 | + | - | 41 | - | - | 71 | + | + |
| 12 | - | + | 42 | - | - | 72 | + | - |
| 13 | + | + | 43 | - | - | 73 | - | - |
| 14 | + | + | 44 | - | - | 74 | - | - |
| 15 | + | - | 45 | - | - | 75 | + | + |
| 16 | + | + | 46 | - | + | 76 | - | + |
| 17 | - | + | 47 | + | + | 77 | + | - |
| 18 | - | + | 48 | + | - | 78 | + | - |
| 19 | - | + | 49 | - | - | 79 | - | + |
| 20 | + | - | 50 | - | - | 80 | + | - |
| 21 | - | - | 51 | - | - | | | |
| 22 | - | - | 52 | - | - | | | |
| 23 | - | - | 53 | - | + | | | |
| 24 | - | - | 54 | + | + | | | |
| 25 | + | + | 55 | + | + | | | |
| 26 | + | - | 56 | + | + | | | |
| 27 | + | + | 57 | (+) | - | | | |
| 28 | + | - | 58 | - | - | | | |
| 29 | - | - | 59 | - | - | | | |
| 30 | + | + | 60 | (+) | - | | | |
| **STRAINS INFECTED** | | | | | | | **38** | **31** |

**Table 4A II. Lytic spectra classes obtained using group C *Campylobacter* hosts**

Lytic spectra of 8 phage isolates belonging to different lytic spectra classes against human *Campylobacter* isolates (group C hosts). Only two lytic patterns were observed for these hosts (I and II).

eighty strains tested (21%) were infected and lysed by all of the isolates whilst twenty nine (36%) were not lysed by any.

### 4.1.2 Morphological analysis by Transmission Electron Microscopy (TEM)

Morphological analysis of bacteriophage is one of the key criteria used in the classification of viruses by the ICTV. Previously characterised *Campylobacter* phage were found to be members of the Myoviridae family with unusually large genomes (**Sails *et al.*, 1998**). Elucidating the morphology of phage isolated in the present study would provide important indications as to their lifestyle and thus potential use in phage therapy. The physical structure of the bacteriophage isolates was determined by negative staining transmission electron microscopy (**2.7**). Representative electron micrographs taken of phage isolates from different lytic spectra are presented in Figure 4B. All the phage had icosahedral heads and rigid contractile tails, suggesting they were all members of the Myoviridae family. As with the phage characterised by **Sails *et al.* (1998)**, differences in the head and tail dimensions of the phage isolated in the present study suggested these isolates could be subdivided. A summary of these measurements with observed standard deviations is presented in Table 4B.

### 4.1.3 Profiling of bacteriophage proteins

With the aim of further confirming the differences between the lytic spectra of the phage isolates, bacteriophage suspensions were subjected to SDS-PAGE to obtain profiles of

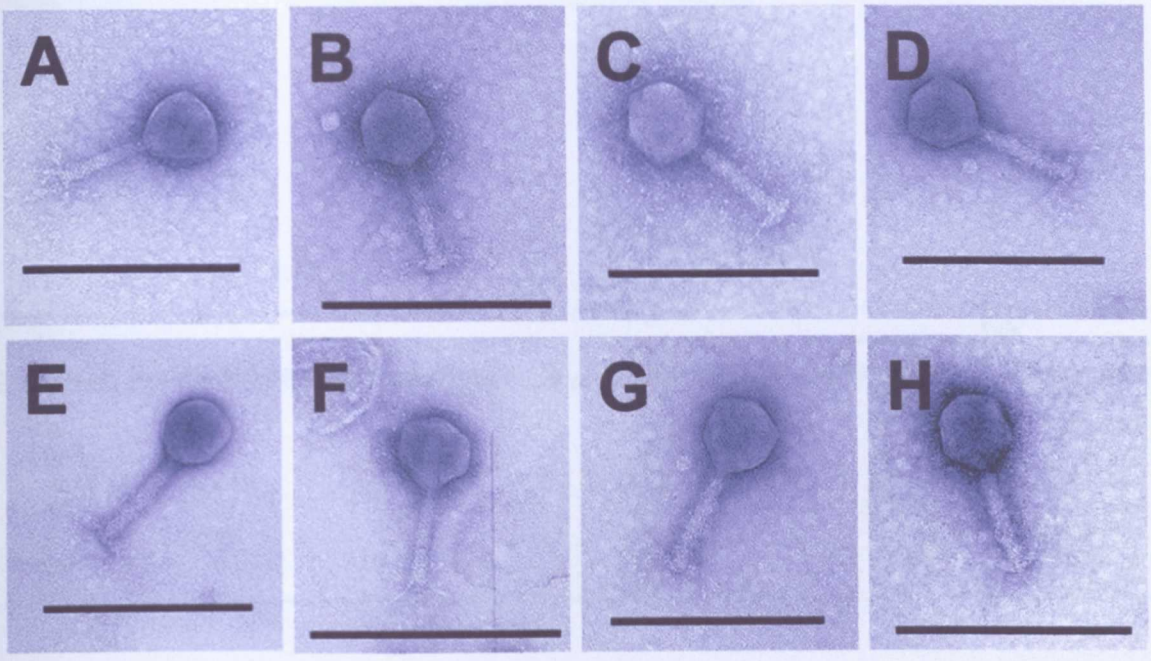


**Figure 4B. Electron micrographs of phage isolated from fresh chicken skin representing eight lytic spectra classes.**
The icosahedral head and rigid contractile tail are typical features of phage from the Myoviridae family. A = w2, B = w3, C = w4, D = w5, E = w8, F = w10, G = w19, H = w20. The bar represents 250 nm. All electron micrographs were taken at 100, 000 x magnification using a JOEL 100 CX transmission electron microscope.

| | | | | Tail | | |
|---|---|---|---|---|---|---|
| Lytic Class | Genome Size (kb) | Restriction Pattern | Head diameter, nm ± SD | Diameter, nm ± SD | Length, nm ± SD | *n* |
| I | 150 | 1 | 85 ± 1.8 | 16 ± 3.6 | 103 ± 1.9 | 6 |
| II | 190 | uncut | 83 ± 1.3 | 20 ± 2.8 | 124 ± 2.9 | 5 |
| III | 190 | uncut | 92 ± 4.3 | 19 ± 2.4 | 116 ± 5.8 | 5 |
| IV | 195 + 135 | 2 | 91 ± 1.9 | 20 ± 2.8 | 112 ± 3.8 | 6 |
| V | 230 | uncut | 89 ± 4.0 | 21 ± 2.4 | 130 ± 3.3 | 6 |
| VI | 230 | 3 | 88 ± 4.5 | 21 ± 2.4 | 125 ± 2.1 | 7 |
| VII | U | U | 90 ± 2.4 | 19 ± 1.5 | 115 ± 3.6 | 6 |
| VIII | U | U | 91 ± 2.6 | 19 ± 2.1 | 116 ± 2.7 | 5 |

**Table 4B. Morphological and genetic characteristics of phage grouped according to their lytic spectra.**

Genome sizes were ascertained by Pulsed Field Gel Electrophoresis (PFGE). Genomes of phage which failed to yield defined bands on a PFGE gel are labeled U (unable to determine). Digestion of the phage genomes with endonuclease *Hha*I yielded 3 RFLP patterns. Genomes refractory to digestion with *Hha*I are designated "uncut". Morphological features were observed by negative staining electron microscopy. Measurements of phage structure dimensions are recorded as the mean (nm) ± standard deviation for *n* numbers of phage examined from each lytic class.

their major proteins (**2.4.2**). This technique has previously been used to assist in the discrimination of *Listeria* phage (**Zink and Loessner, 1992**).

### *4.1.3.1 SDS PAGE*

Attempts were made to purify the *Campylobacter* bacteriophage isolates using a CsCl gradient (**2.3.6.2**) prior to separation by SDS-PAGE (**2.4.2**). Purification was attempted a minimum of four times per isolate but was unsuccessful for all phage derived from retail poultry despite several changes to the protocol. However, one phage isolated from chicken faeces as part of another study (gift from Ms. Catherine Loc-Carrillo) was successfully purified. This CsCl-purified sample was separated along with non-purified isolates by SDS-PAGE.

The protein profiles of six bacteriophage isolates representing a subset of the different lytic spectrum classes is presented in Figure 4C. This subset was selected from lytic spectrum classes which could be characterised according to their genome size and restriction pattern when digested with endonuclease *Hha*I (Table 4B). The protein profiles of these phage all possessed a single protein (about 34 kDa) in common which corresponds to a band from the host cell-only preparation. This is presumed to be the major outer membrane protein of *C. jejuni* although this protein appears to be smaller than published values (**Logan and Trust, 1982**). The other major band visibly differs in size with respect to the individual bacteriophage isolates (black arrows). There were also differences in the profiles of higher molecular weight bands although these were more difficult to see (blue arrows). In order to gain insight into the identity of the major

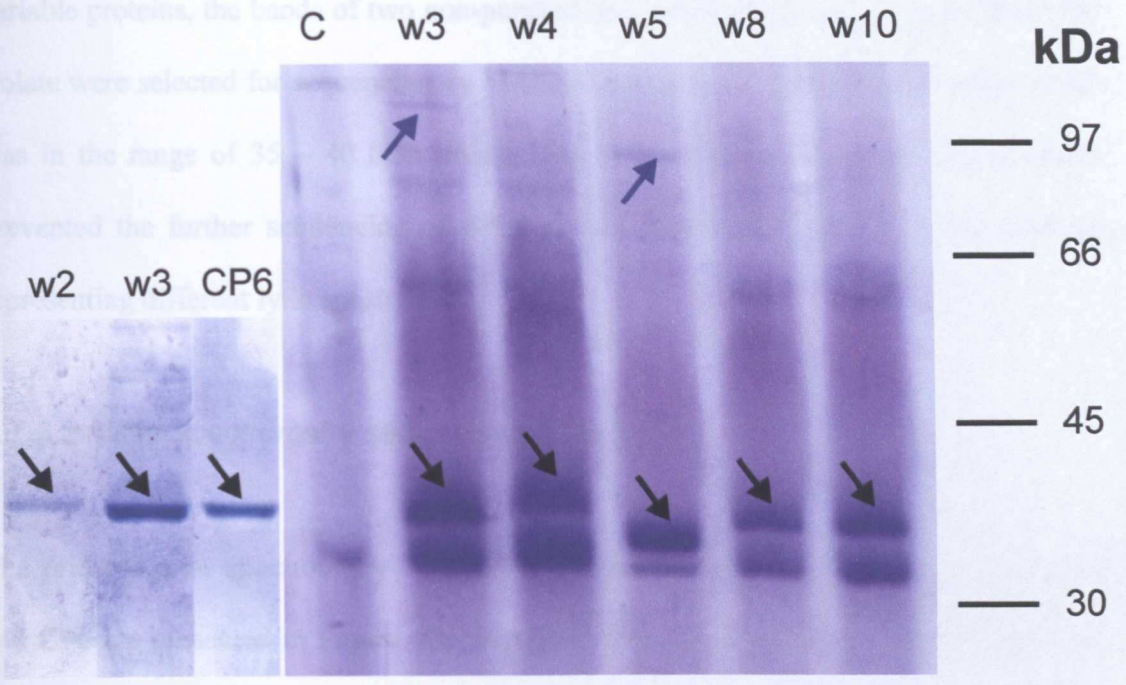


**Figure 4C. Bacteriophage protein profiles**
A 12% SDS-PAGE gel showing the migration profiles of proteins present in concentrated bacteriophage plate lysates of phage w2, w3, CP6, w4, w5, w8 and w10 compared with a host-cell-only preparation (C). Representative molecular weight markers are shown at the far right of the gel. The black arrows and blue arrows highlight proteins of varying sizes which differ between phage isolates. Phage w2, w3 and CP6 were selected for MALDI-TOF sequencing.

variable proteins, the bands of two non-purified (w2, w3) isolates and one purified (CP6) isolate were selected for sequencing by MALDI-TOF (**2.4.6**). Each of these major bands was in the range of 35 – 40 kDa in size (Fig. 4C). Constraints on project resources prevented the further sequencing of other protein bands from w2, w3, CP6 or phage representing different lytic spectra.

### *4.1.3.2 Mass Spectrometry sequencing of proteins*

The primary mass spectrometry 'finger prints' obtained from tryptic digests of w2, w3 and CP6 are presented in Figure 4D. None of the fingerprints matched anything in the ExPasy protein mass spectrum database (**2.4.6**). The black arrows on each fingerprint (Fig. 4D) indicate the tryptic fragments selected for fragmentation (sequence) analysis. Sequences were obtained from five of the nine fragments (indicated by an *). Table 4C summarises the homology searches performed on these sequences using the BLAST search engine for protein short or near exact sequences (NCBI). Four out of the five protein sequences exhibited homology with viral proteins, with 40% specific to phage proteins. Interestingly, a fragment sequenced from w2 is almost exactly the same (92.3% identity) as a fragment from the CsCl-purified bacteriophage CP6 (Table 4C).

Interrogation of the NCBI database using the BLASTp search engine revealed that these sequences exhibited homology with an as-yet uncharacterised phage-related protein from *Clostridium acetobutylium*. In addition, the w2 fragment exhibited homology with gp16.1, a protein of unknown function found in temperate *Staphylococcus* phage ϕETA. The CP6 fragment exhibited homology to a 19.3 kDa protein of unknown function in

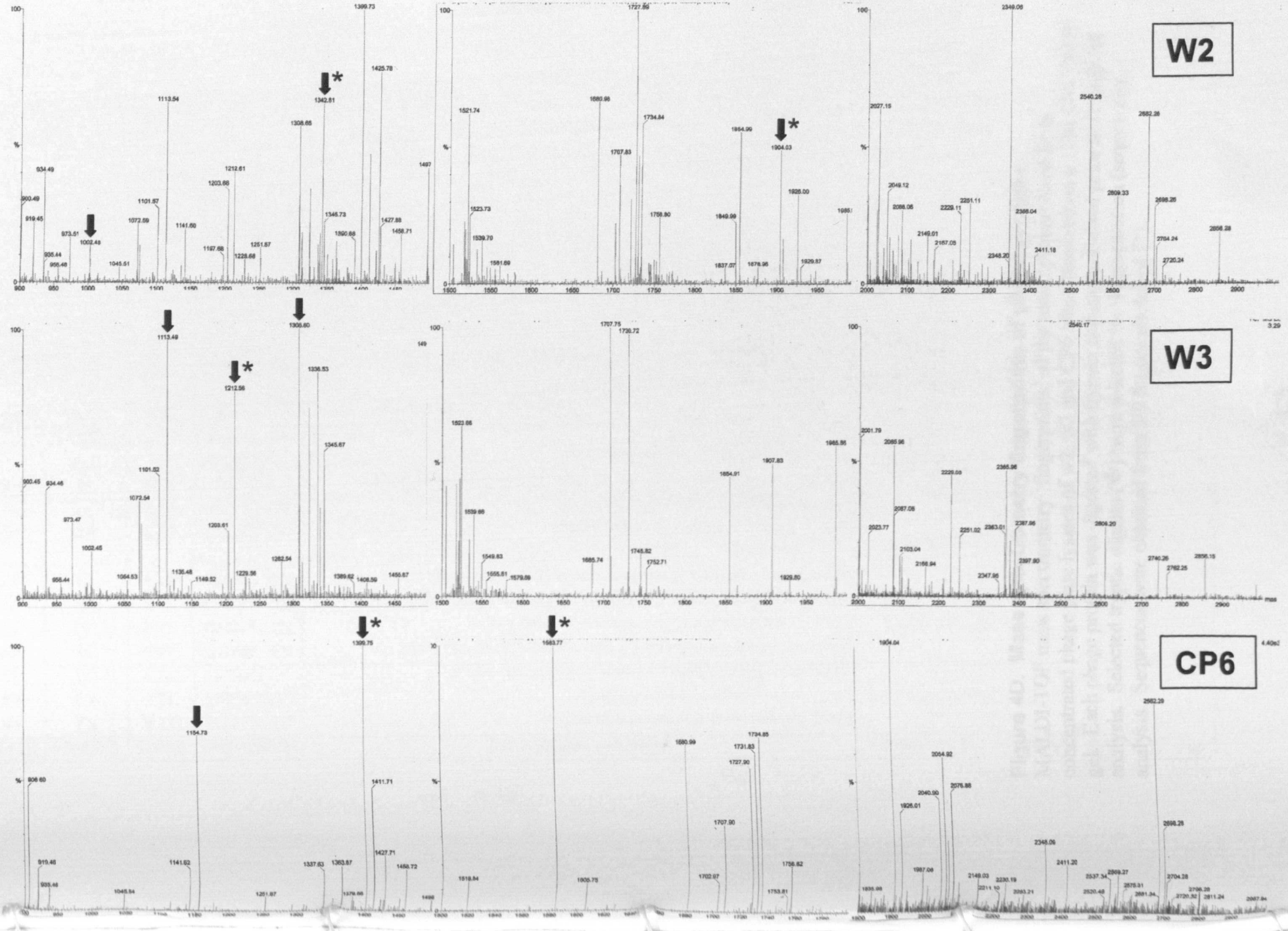
W2
W3
CP6

**Figure 4D. Mass spectrometry fingerprints of phage proteins.**
MALDI-TOF mass spectrometry 'fingerprints' of the major protein band from concentrated phage plate lysates of w2, w3 and CP6 when resolved on a 12% SDS PAGE gel. Each phage protein was digested with trypsin and spray-ionised prior to fingerprint analysis. Selected tryptic digests (↓) were selected for fragmentation (sequencing) analysis. Sequences were obtained from 5/9 fragments tested (*).

| Phage Isolate | Amino Acid sequence | Relevant homologies | Accession | Score | E Value | ID (% |
|---|---|---|---|---|---|---|
| w2 | VATLLDELPPPYPLLPR | **Mast cell protease 6 [Mus musculus]** | **NP_034911** | **29.5** | **5.2** | **90** |
| | | UL77 protein [human herpesvirus 5] | AAC40817 | 28.6 | 9.3 | 64 |
| | | gag protein [Recombinant M-MuLV/RaLV retrovirus] | AAO46142 | 26.9 | 30 | 80 |
| | NYTGPYATAAGEK | **Proteasome alpha subunit C6 [Encephalitozoon cuniculi]** | **NP_586170** | **26.9** | **31** | **100** |
| | | Phage-related protein [Clostridium acetobutylicum] | NP_346709 | 24.8 | 133 | 87 |
| | | ORF48~similar to phage Spp1 gp16.1 [Bacteriophage phi ETA] | NP_510942 | 22.7 | 578 | 75 |
| w3 | YNVLVDQHPK | **hypothetical protein [Pseudomonas syringae pv. tomato str. DC3000]** | **NP_793458** | **27.4** | **23** | **87** |
| | | EsV-1-79 [Ectocarpus siliculosus virus] | NP_077564 | 25.2 | 100 | 77 |
| | | ORF113 [Xestia c-nigrum granulovirus] | NP_059261 | 23.1 | 435 | 66 |
| CP6 | QAGTDDVVNVLLR | **Colicin E7 immunity protein - Escherichia coli plasmid ColE7** | **S27394** | **26.9** | **31** | **66** |
| | | Polyprotein [Oat necrotic mottle virus] | AF454460 | 24 | 178 | 80 |
| | | m047R [Myxoma virus] | NP_051761 | 24 | 239 | 77 |
| | NYTGPYATAAGEALGK | **Transcriptional regulatory protein [Schizosaccharomyces pombe]** | **NP_596012** | **26.1** | **54** | **72** |
| | | ORF205; putative [Bacteriophage LL-H] | AAL77553 | 26.1 | 54 | 76 |
| | | Phage-related protein [Clostridium acetobutylicum] | NP_346709 | 24.8 | 132 | 87 |



**Table 4C. BLAST search results for bacteriophage protein sequences**
Homology of bacteriophage protein sequences obtained from MALDI-TOF fragmentation analysis with proteins currently found in the NCBI database using the BLAST protein sequence search engine for short, near-exact matches. The amino acid sequence is shown in column 2 along with selected database matches in column 3 (the highest match is shown in bold). Columns 5, 6 and 7 show the hommology score (in bits); the E-value and percentage homology respectively.

virulent *Lactococcus* phage LL-H. Interestingly, the second fragment sequence from CP6 showed homology with *E. coli* colicin immunity protein. This 9.9 kDa protein is encoded by the *cei* gene on the ColE7 plasmid and binds to the colicin produced by the bacterium to prevent autolysis (**Chak *et al.*, 1991**). The remaining sequences for w2, w3 and CP6 showed some general homology to eukaryotic virus proteins of unknown function.

### 4.1.4 Analysis of the phage genome

The type of nucleic acid a virus possesses is now an important component of phage taxonomy. Such data is important not only for the classification of viruses but also provides information about their lifestyle. In order to determine the nature of the nucleic acid present in the phage isolates, genomic preparations (**2.5.2**) were first treated with RNase for 30 min at 37°C to digest the genome should it be composed of RNA. Subsequently, the genomic preparations were digested with a selection of endonucleases (**2.5.4**) in order to differentiate between genomes composed of single stranded and double stranded DNA (restriction endonucleases cleave only dsDNA). A 1% agarose gel showing the results of bacteriophage subjected to these treatments is presented in Figure 4E. The nucleic acid was not digested when treated with RNaseA indicating that the viral genome did not consist of RNA. In addition, digestion with *Cfo*I (and other endonucleases tested, Table 4E) revealed the DNA was double stranded. These data supported the former indications that the phage isolates were members of the Myoviridae family (Table 4D).

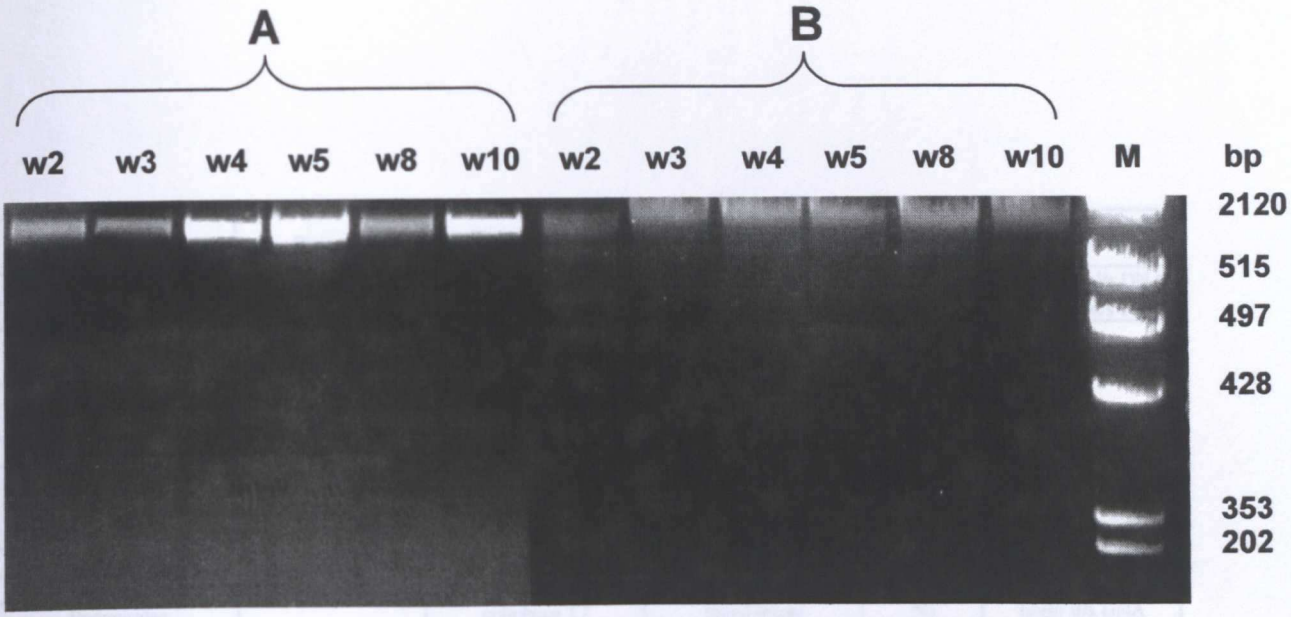


**Figure 4E. Restriction profiles of RNase-treated bacteriophage genomic extracts.**

A 1% agarose gel showing the migration of genomic preparations from bacteriophage w2, w3, w4, w5, w8 and w10 after treatment with RNase (A) and digestion with endonuclease *Cfo*I (B). λ/*Hin*dIII digest markers are shown at the far right of the figure (M) with sizes shown in base pairs (bp).

| Family or Group | Genera | Type Member | Particle Morphology | Envelope | Genome |
|---|---|---|---|---|---|
| Corticoviridae | Corticovirus | PM2 | icosahedral | No | supercoiled d/s DNA |
| Cystoviridae | Cystovirus | Ø6 | icosahedral | Yes | 3 segments d/s RNA |
| Inoviridae | Inovirus | coliphage fd | rod | No | circular s/s DNA |
| | Plectrovirus | Acholeplasma phage | | | |
| Leviviridae | Levivirus | coliphage MS2 | icosahedral | No | 1 (+)strand RNA |
| | Allolevirus | coliphage Qbeta | | | |
| Lipothrixviridae | Lipothrixvirus | Thermoproteus phage 1 | rod | Yes | linear d/s DNA |
| Microviridae | Microvirus | coliphage ØX174 | icosahedral | No | circular s/s DNA |
| | Spirovirus | Spiroplasma phages | | | |
| | | Mac-1 phage | | | |
| Myoviridae | | coliphage T4 | tailed phage | No | linear d/s DNA |
| Plasmaviridae | Plasmavirus | Acholeplasma phage | pleiomorphic | Yes | Circular d/s DNA |
| Podoviridae | | coliphage T7 | tailed phage | No | linear d/s DNA |
| Siphoviridae | lambda phage group | coliphage lambda | tailed phage | No | linear d/s DNA |
| Sulpholobus shibatae virus | | SSV-1 | lemon-shaped | No | circular d/s DNA |
| Tectiviridae | Tectivirus | phage PRD1 | icosahedral | No | linear d/s dna |

**Table 4D. Current names and attributes of bacteriophage families recognised by the International Committee on the Taxonomy of Viruses (ICTV).**

| Endonuclease | Recognition site |
|---|---|
| *Taq* I | T/CGA |
| *Dpn* I | GA/TC |
| *Ssp* I | AAT/AAT |
| *Mse* I | T/TAA |
| *Hae* III | GG/CC |
| *Dra* I | TTT/AAA |
| *Mbo* I | /GATC |
| *Hin* dIII | A/AGCTT |
| *Pst* I | CTGCA/G |
| *Eco* RI | G/AATTC |
| *Eco* RV | GAT/ATC |
| *Cfo* I | GCG/C |
| *Hha* I | GCG/C |

**Table 4E. Endonucleases used on bacteriophage DNA preparations which were unable to produce discriminatory restriction patterns on a pulsed field gel.**

### *4.1.4.1 Whole genome analysis of phage isolates by Pulsed Field Gel Electrophoresis (PFGE)*

PFGE has been used in a previous study to determine the genome size of *Campylobacter* bacteriophage (**Sails *et al.*, 1998**). It allows the separation of DNA fragments greater than 50 kb in size by periodically alternating the orientation of the electrical field imposed on the gel. A method for the preparation of phage genomic DNA for PFGE was modified from standard protocols for the preparation of bacterial genomic DNA (**2.5.10**). A pulsed field gel showing the genome sizes of bacteriophage representing six lytic spectra classes is presented in Figure 4F. The phage genome sizes ranged from 150 kb to 230 kb. Phage w5 was unusual in that it appeared to have two bands present (135 kb and 195kb). A minimum of six successive rounds of plaque purification performed at the University of Nottingham and the Central Public Health Laboratories failed to separate this phage into two clones with single bands.

As the genomes of the phage isolates were previously determined to consist of dsDNA, they could be discriminated on the basis of their susceptibility to restriction endonucleases. Genomic digests were performed using thirteen different endonucleases (Table 4E). Of the restriction enzymes used, only treatment with *Hha*I and its isoschitsomer *Cfo*I yielded discriminatory profiles when the digests were separated by PFGE. Other restriction enzymes used either did not cleave the phage DNA or cleaved too frequently which resulted in a smeared pattern on the gel. The phage could be ascribed to three categories according to the susceptibility of their genomic DNA to digestion with endonuclease *Hha*I (Fig. 4G).

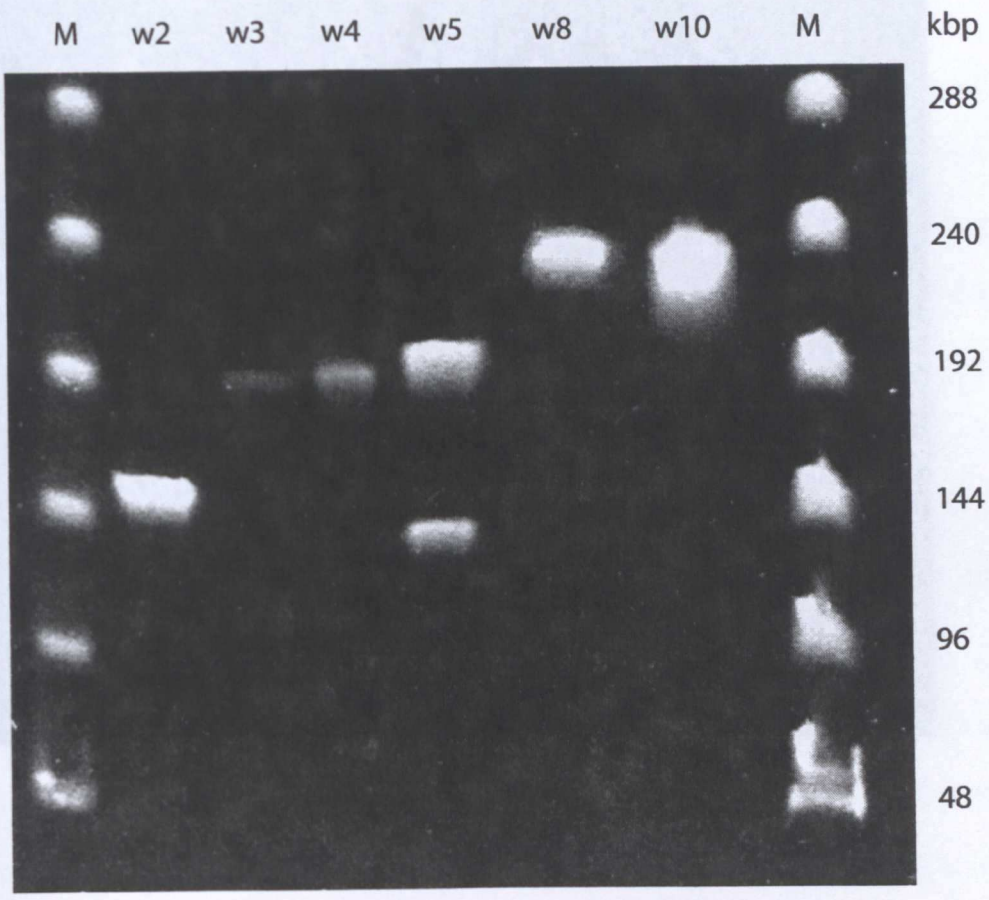


**Figure 4F. Genome size of bacteriophage isolates.**
Pulsed Field Gel of undigested bacteriophage genomic preparations representing different lytic spectra classes (w2, w3, w4, w5, w8, w10). Lambda concatomer markers (M) are shown flanking the phage DNA, sizes given in kilobase pairs (kbp).

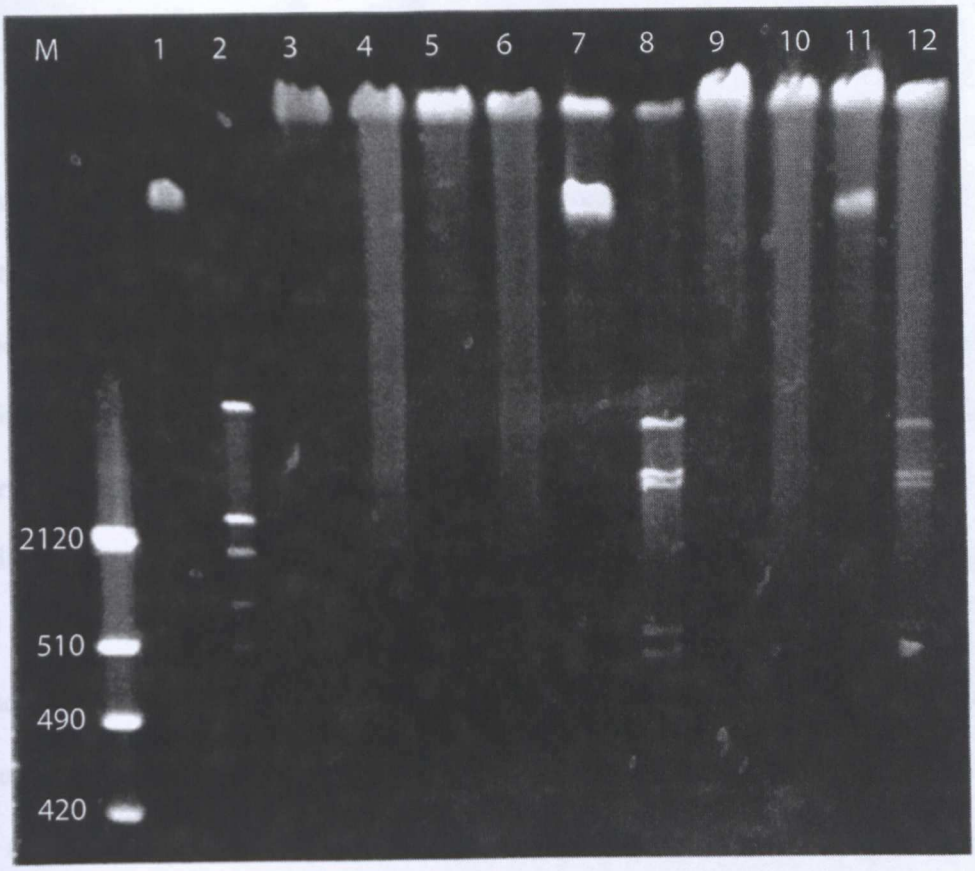


**Figure 4G. Bacteriophage macrorestriction profiles.**
Gel showing restriction fragments generated from digesting phage genomes with endonuclease *Hha*I. Lanes are as follows: M = λ /*Hin*dIII marker; 1 = undigested w2; 2 = digested w2; 3 = undigested w3; 4 = digested w3; 5 = undigested w4; 6 = digested w4; 7 = undigested w5; 8 = digested w5; 9 = undigested w8; 10 = digested w8; 11 = undigested w10; 2 = digested w10.

### 4.1.4.2 Phage DNA sequence analysis

The sequencing of genomic DNA fragments from the phage isolates would allow interrogation of databases for homologous sequences from phage genes of known function. This may assist in identification of the phage and also provide information on the GC content of the genome and possibly the lifecycle of the virus. To this end, bacteriophage DNA was extracted (**2.5.2**) and digested using endonuclease *Dra*I (**2.5.4**). The digestion products were then cloned into plasmid vector pUC18 using a Ready-To-Go™ commercial cloning kit (27-5266-01, Pharmacia) according to the manufacturer's instructions by Dr. P. Connerton. Successful transformant colonies, selected on the basis of blue/white screening, were incubated overnight in LB broth (**2.5.11**) supplemented with ampicillin (50 μg $ml^{-1}$) prior to plasmid extraction (**2.5.3**) using a QIAprep® Miniprep kit (QIAGEN Ltd, West Sussex). Plasmid preparations were then digested with a combination of *Eco*RI and *Bam*HI endonucleases (**2.5.4**) to cleave the plasmid DNA at sites flanking the insertion site. Plasmid digests showing inserted fragments when resolved on a 1% agarose gel (**2.5.6**) were sent for sequencing using a CEQ® 2000 dye-terminator cycle system (Beckman Coulter) at the Plant Sciences Department, University of Nottingham. Sequences obtained from phage DNA inserts into a pUC18 vector (**2.5.11**) were used to interrogate the National Center for Bioinformatics Information (NCBI) database using the BLAST search engine. *Campylobacter* host DNA was present in 6/21 of plasmid inserts, which was unsurprising considering that the DNA arose from non-CsCl purified filtered bacteriophage lysates. Of the unique DNA sequences, 6/15 possessed translated amino acid sequences showing identity to the deduced primary structure of proteins from phage infecting other bacterial genera (Table 4F).

| Bacteriophage | Code | Primer | Relevant homologies | Accession | Score | E Value | ID (%) |
|---|---|---|---|---|---|---|---|
| w2 | A | M13F | **primase-helicase [Enterobacteria phage T4]** | **NP_049654** | **46** | **1.00E-04** | **28** |
| | | | Gp41 [Bacteriophage RB49] | AAL87839 | 45 | 2.00E-04 | 26 |
| | | | ORFA [Bacteriophage Lc-Nu] | AAG43560 | 30 | 6 | 39 |
| | B | M13F | no match | | NA | NA | NA |
| w5 | A | M13F | **hypothetical protein [Plasmodium falciparum 3D7]** | **NP_705188** | **37** | **0.054** | **30** |
| | | | ORF1 [TT Virus] | BAA93586 | 31 | 3 | 27 |
| | B | M13F | no match | | NA | NA | NA |
| | C | M13F | **hypothetical protein [Corynebacterium glutamicum ATCC 13032]** | **NP_600985** | **53.5** | **7.00E-07** | **32** |
| | | | envelope glycoprotein [Human immunodeficiency virus type 1] | AAK82668 | 32 | 1.7 | 27 |
| | | | ORF MSV204 ALI motif gene family protein [Melanoplus sanguinipes entomopoxvirus] | NP_048275 | 30 | 8.3 | 25 |
| | D | M13F | **hypothetical protein [Plasmodium falciparum 3D7]** | **NP_705188** | **37** | **0.054** | **30** |
| | | | envelope protein p74 [Lymantria dispar nucleopolyhedrovirus] | NP_047663 | 31 | 3 | 29 |
| | | | ORF1 [TT virus] | BAA93586 | 31 | 3 | 27 |
| | E | M13R | no match | | NA | NA | NA |
| | F | M13F | **bll0063 [Bradyrhizobium japonicum]** | **NP_766703** | **33.5** | **1.1** | **23** |
| | | | hypothetical protein [Bacteriophage Mx8] | NP_203437 | 32 | 2.3 | 44 |
| | | | p23 [Bacteriophage Mx8] | AAK94358 | 32 | 2.3 | 44 |
| | G | M13R | **unknown [Bacteriophage SPBc2]** | **NP 046579** | **45** | **2.00E-04** | **32** |
| | | | hypothetical protein yomD - Bacillus subtilis phage SPBc2 | T12791 | 45 | 2.00E-04 | 32 |
| | | | host specificity protein [Streptococcus thermophilus bacteriophage MD2] | AAK83242 | 31 | 4.6 | 29 |
| | H | M13F | **unknown [Bacteriophage SPBc2]** | **NP 046579** | **45** | **2.00E-04** | **32** |
| | | | hypothetical protein yomD - Bacillus subtilis phage SPBc2 | T12791 | 45 | 2.00E-04 | 32 |
| | I | M13F | no match | | NA | NA | NA |
| | J | M13R | no match | | NA | NA | NA |
| | K | M13R | **hypothetical protein [Corynebacterium glutamicum ATCC 13032]** | **NP_600985** | **55** | **5.00E-07** | **30** |
| | | | DNA gyrase subunit B [Buchnera aphidicola] | NP_660371 | 35 | 0.51 | 27 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| w8 | A | M13F | **topoisomerase [Enterobacteria phage T4]** | **NP_049621** | **134** | **5.00E-31** | **48** |
| | | | DNA topoisomerase large subunit [Enterobacteria phage T4] | CAA29569 | 134 | 5.00E-31 | 48 |
| | | | DNA topoisomerase (ATP-hydrolyzing) (EC 5.99.1.3) large chain - phage T4 | ISBPT4 | 134 | 5.00E-31 | 48 |
| | B | M13R | **gp21v [Bacteriophage KVP40]** | **BAA77380** | **58** | **3.00E-08** | **45** |
| | | | prohead protease gp21 [cyanophage S-PM2] | AAL09969 | 41 | 3.00E-07 | 35 |
| | | | prohead protease [Enterobacteria phage T4] | NP_049785 | 49 | 2.00E-05 | 38 |



**Table 4F. Homology of translated bacteriophage nucleotide sequences (BLASTx).**
Table showing homology of translated nucleotide sequences from cloned bacteriophage DNA fragments when used to interrogate the NCBI database using the BLASTx search engine. Each translated sequence was assigned a one-letter code (column 2), with the primer used for the individual sequencing reactions noted in column 3. Relevant homologies to translated nucleotide sequences in the NCBI database are given in column 4 with the top match in each search highlighted in **bold**. The score (in bits), E-value and percentage identity with each database match are presented in columns 6, 7 and 8 respectively.

Sequence A from phage w2 showed significant homology to a primase-helicase from coliphage T4. Primase-helicase is encoded by genes *gp61* and *gp41* respectively in phage T4 and is required for the synthesis of primers for Okazaki fragments and unwinding of the phage DNA during replication (**Miller *et al.*, 2003**). Supporting this was additional homology with primase gene *gp41* from coliphage RB49 (**Desplats *et al.*, 2002**) and a DNA helicase gene from phage Lc-Nu (**Brandt *et al.*, 2001**). Primase-helicase is part of the core replisome in phage T4 (**Miller *et al.*, 2003**).

Sequences from phage w5 exhibited some general homology with proteins found in eukaryotic viruses (HIV, Entomopoxvirus and TT virus) along with more significant homology to bacteriophage proteins. These included the product of gene *p23* from *Myxococcus* phage Mx8 (**Tojo *et al.*, 1996**), unknown and host specificity proteins from *Streptococcus* phage MD2 (**Duplessis and Moineau, 2001**) and a hypothetical protein (YomD) from *Bacillus subtilis* phage SPBc2 (**Lazarevic *et al.*, 1998**). However, none of these proteins are sufficiently well characterised to deduce any further meaningful conclusions.

Of the sequences used to interrogate the database, those from phage w8 produced the most significant matches. The first of the two sequences produced a Score of 134 bits and E-value of $5 \times 10^{-31}$ for homology with phage T4 topoisomerase. This enzyme is composed of three subunits in phage T4, the products of genes *gp39*, *gp52* and *gp60* (**Miller *et al.*, 2003**). T4 possesses a Type II topoisomerase which is capable of relaxing positive or negative supercoiling in DNA (**Lewin, 1997**). The second sequence had high homology with the prohead proteases of phage KVP40 (**Matsuzaki *et al.*, 1998**),

cyanophage S-PM2 (**Hambly *et al.*, 2001**) and phage T4 (**Miller *et al.*, 2003**). In all these cases, the enzyme is transcribed from gene *gp21* which, in phage T4, is responsible for degrading scaffolding proteins to create space in the cavity of the prohead during virion formation (**Miller *et al.*, 2003**).

### 4.1.5 Replication dynamics

The dynamics of phage replication *in vitro* are useful indicators of their possible efficacy *in vivo*. All the phage in the present study were isolated from retail poultry using a strain of *Campylobacter* (*C. jejuni* NCTC 12662 PT14) susceptible to many different types of phage. This is useful as it allows the direct comparison of the replication of phage from different lytic spectra classes using a common host.

#### *4.1.5.1 Adsorption to host cells*

A time course was performed to determine the dynamics of attachment of phage representing six different lytic spectra to their host *in vitro* (**2.10.1**). As the first step in all phage replication, the ability to adsorb and the efficiency of this process will greatly influence the therapeutic potential of phage. The titre of the phage in the control experiment without host cells showed no appreciable differences throughout the time course indicating that any non-specific attachment of the phage to the polypropylene test tube was negligible.

All phage, regardless of the lytic spectrum, attached to the host cell with similar efficiency with the exception of phage w5, which did not demonstrate any appreciable adsorption over the time course. An example of the adsorption data from one of these isolates (phage w2, lytic spectrum class II) to the host *Campylobacter* (*C. jejuni* NCTC 12662) over a 90 min period is presented in Figure 4H. Approximately 90% of the phage adsorbed to the host within the first 30 sec of exposure. No further changes in adsorption occurred for the remainder of the time course. The adsorption experiments were repeated using *Campylobacter* species/strains not permissive to infection by any of the phage (*C. coli* NCTC 12666, *C. coli* NCTC 12667, *C. coli* NCTC 12668 and *C. lari* NCTC 11352). No fall in titre was recorded, compared with the controls, using any combination of phage with any of these hosts indicating that phage do not attach to the non-permissive strains tested here. An example of the adsorption data from one of these isolates (phage w2, lytic spectrum class II) to *Campylobacter* strains not permissive to infection is presented in Figure 4I.

### *4.1.5.2 Analysis of bacteriophage replication: one step growth*

The replication of bacteriophage isolates from different lytic spectra classes in liquid culture was determined using a one step growth experiment **(2.10.2)**. This was performed by preparing a suspension of *Campylobacter* host cells from a 1 l overnight culture by centrifugation at 10, 000 *g* for 10 min and resuspending the pellet in 9 ml of fresh broth to a density equivalent to McFarland No. 3 (approximately $10^9$ cfu $ml^{-1}$). To this cell suspension was added 1 ml of a $10^9$ pfu $ml^{-1}$ suspension of bacteriophage to give an MOI of 0.1 ($10^8$ pfu $ml^{-1}$). The suspension was incubated for 15 min in microaerobic

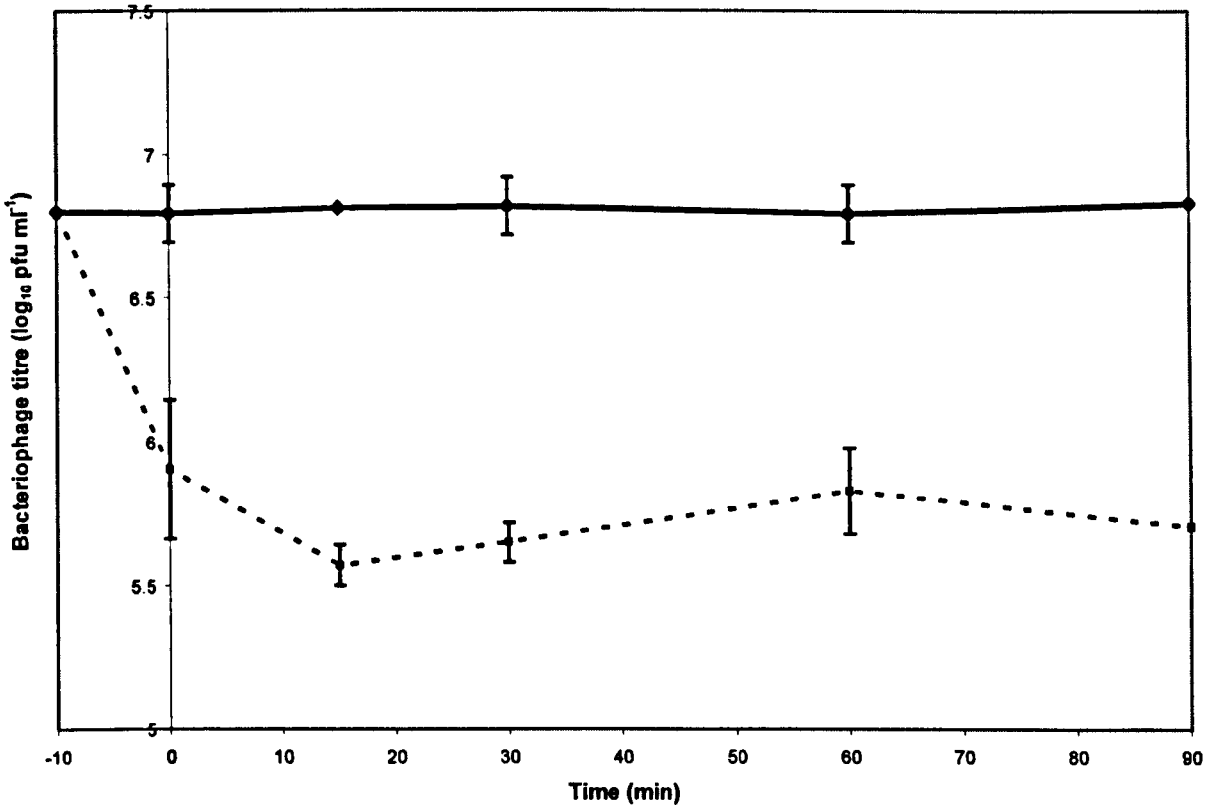


**Figure 4H. Adsorption of phage w2 to host cells**
Graph showing the adsorption of phage w2 in the presence of host cells (*C. jejuni* NCTC 12662 PT14) (---) over a period of 90 min compared with the control (— ) containing phage particles only. These results are based on six replicates.

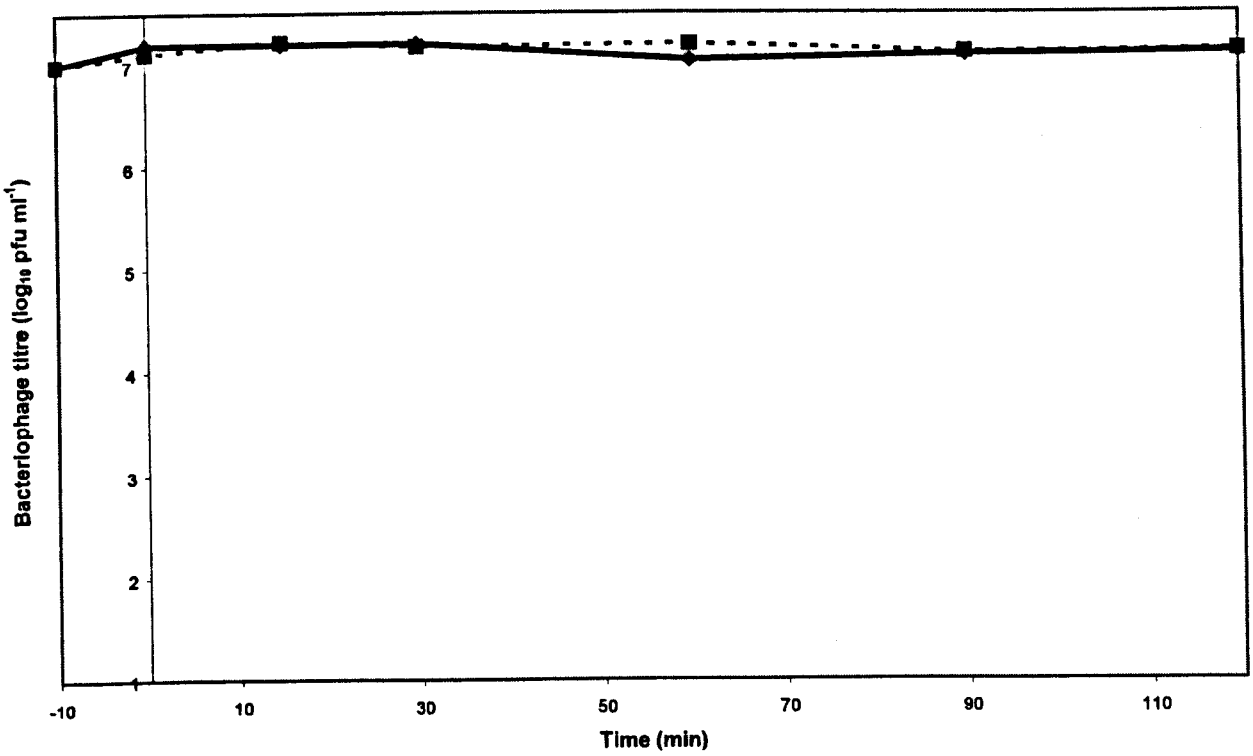


**Figure 4l. Adsorption of phage w2 to non-host cells**
Graph showing the adsorption of phage w2 in the presence of non-host cells (*C. coli* NCTC 12667) (---) over a period of 120 min compared with the control (—) containing phage particles only. These results are based on six replicates.

conditions to allow for phage adsorption before diluting 1:100 by the addition of fresh NB2 broth (990 ml) + FBP (**2.3.1.3**). Following dilution, the culture was incubated microaerobically at 42°C and shaken at 100 rpm. Samples (1 ml) were taken at regular intervals with phage and *Campylobacter* titres being recorded as described previously (**2.3.6.4** and **2.3.4.4**).

The data for the replication of phage w2 when exposed to its host (*C. jejuni* NCTC 12662) in liquid culture over a 2 h period is presented in Figure 4J. As in the adsorption study (**4.1.5.1**) there was an initial fall in phage titre compared with the control. However, the 'one step' growth curve originally defined by Ellis and Delbruck (see Chapter 1) was not observed. Instead of the expected rapid increase in free phage present in the supernatant following the latent period there appeared to be a gradual increase from the 60 min time point onwards. Extending the incubation time to 300 min resulted in the phage titre stabilising at $\log_{10}$ 0.5 - 1.2 higher than the baseline at approximately 120 min. This pattern was similar for all the bacteriophage examined (w2, w3, w4, w8, w10, $\phi$2) with the exception of phage w5 which did not show any increase in virion concentration. Extending the incubation period for over 24 h did not significantly alter the bacteriophage yield. The growth of the *Campylobacter* host cells during this period did not differ appreciably from the control (Fig. 4K). Repeated alterations in the experimental protocol outlined above failed to result in a one step growth curve. These alterations are presented below.

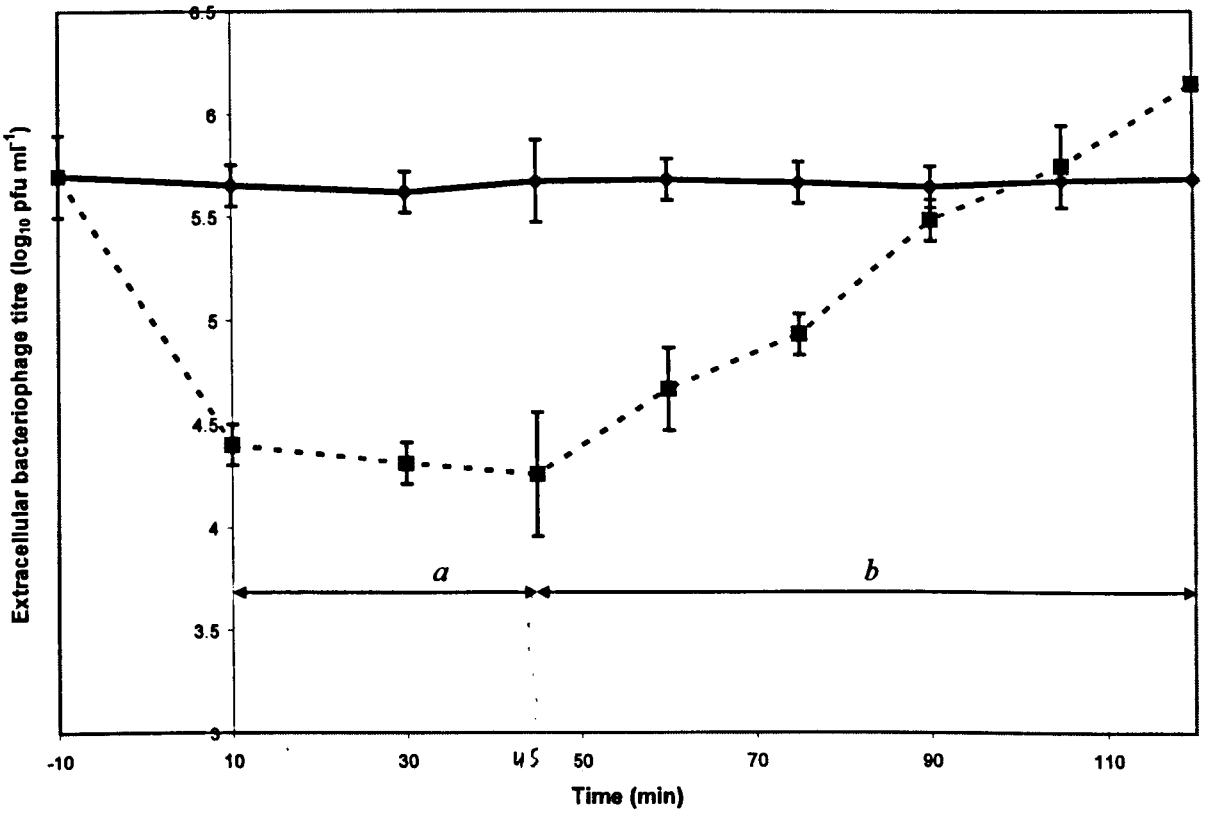


**Figure 4J. *Campylobacter* 'One-step' growth curve**
Graph showing changes in the titre unadsorbed phage w2 when incubated with host *C. jejuni* NCTC 12662 PT14 (---) and without host (—) in liquid culture. The latent period (*a*) was determined to be approximately 45 min and the rise period (*b*) approximately 75 min. These results are based on six replicates.

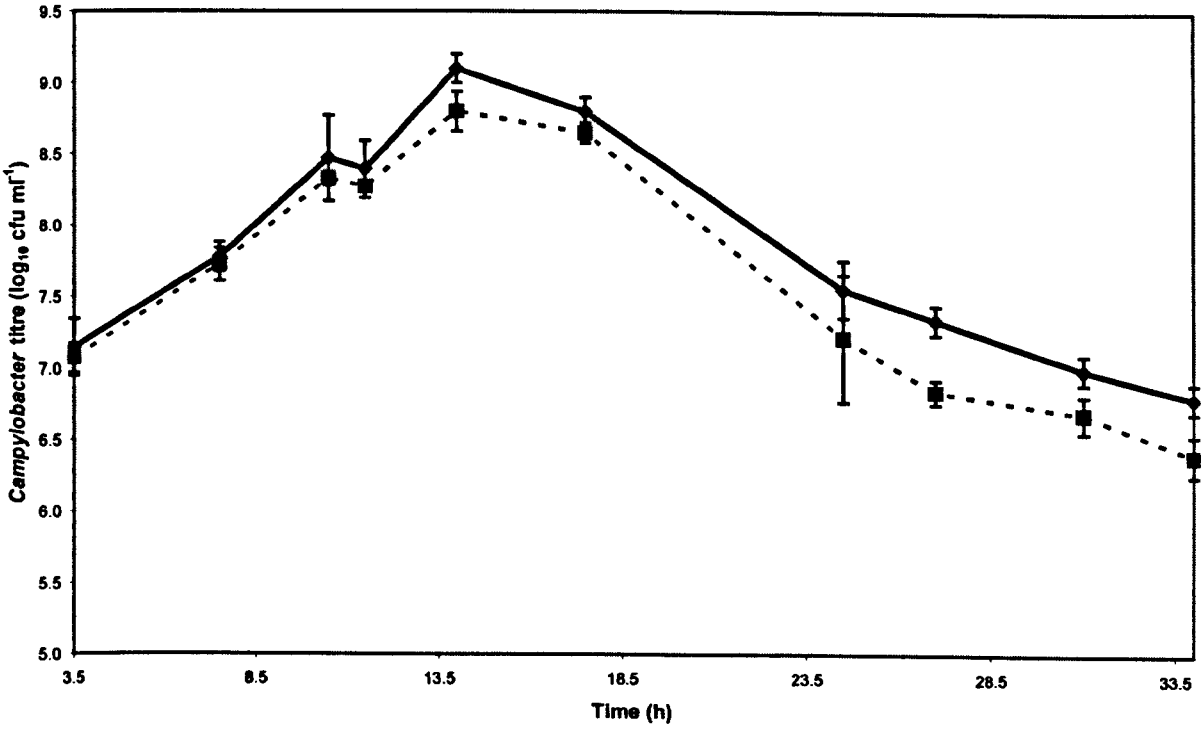


**Figure 4K. Growth *Campylobacter* cultures infected and uninfected with bacteriophage w2**

Graph showing the growth of *Campylobacter jejuni* NCTC 12662 PT14 over a 33 h period when incubated with (---) and without (— ) $10^8$ pfu $ml^{-1}$ bacteriophage w2 (MOI = 0.01). These results are based on six replicates.

#### 4.1.5.2.1 Alterations in growth conditions and media composition

The growth of the *Campylobacter* host would depend partly on the medium used and this may have subsequently affected bacteriophage replication. Four different media were used for bacterial growth (Brain-Heart Infusion, CM225, Oxoid; Müller-Hinton CM425, Oxoid; Nutrient Broth, CM1, Oxoid), none of which resulted in phage replication appreciably different from that presented in Figure 4J. Unpublished data from the CPHL suggested that *Campylobacter* host cells incubated at 37°C rather than 42°C were more susceptible to phage infection (Dr. J. Richardson, pers. comm.). However, incubation at the lower temperature did not appear to significantly affect phage replication.

#### 4.1.5.2.2 Alteration of adsorption conditions and treatment of host cells

In the original replication protocol, dilution of the host/phage suspension immediately following the adsorption period was necessary to limit the probability of unadsorbed free phage initiating new replication cycles. If dilution was not performed there would be a continual release of viral progeny and skewing of burst size calculations. Ellis and Delbruck were able to inactivate unadsorbed phage using antisera. Antisera was not available for the *Campylobacter* phage isolates in the present study and so several trials were performed with experimental viricide products donated by Dr. R. Mole. Three preparations were used, the first was based on a pomegranate extract, the second on a tea extract and the third on ferric compounds. The commercially sensitive nature of these products meant that the active ingredient(s) in each preparation could not be divulged. Each viricide was applied immediately following the adsorption period so that phage

which had successfully initiated an infection would be unaffected. However, only the ferric compounds were effective in reducing the bacteriophage titre in suspension, but only by $\log_{10}$ 0.5 after 1 h and so were not potent enough to be used in the growth curve experiments. The treatment of the host cells prior to adsorption was also altered by taking cells from a 15 h culture (mid logarithmic growth) and thrice washing them in MRD. This was performed to limit the number of membrane vesicles in the host cell preparations which could potentially act as molecular decoys in phage adsorption. These washing steps did not appear to affect the replication of *Campylobacter* phage on their hosts. However, the effect of membrane vesicles on phage replication was investigated further in Chapter 7.

The prolonged rise phase in the lifecycle of the *Campylobacter* bacteriophage isolates made attempts to calculate the burst size impossible since virions will continually initiate new infection cycles after release. The latent period of infection was determined to be approximately 45 min (Fig. 4J) which falls within the range observed for Myoviridae phage infecting other bacterial genera **(Ackermann and DuBow, 1987)**.

## 4.3 DISCUSSION

The lytic spectra of the individual phage isolates revealed similarities in host range, implying that they are related. The activity of the phage against *Campylobacter* isolates recovered from the same samples was disappointing with respect to their potential as therapeutic agents. Interestingly, only two lytic profiles were observed when the phage were used to infect the 80 human *Campylobacter* isolates. This clearly highlights the limitations of relying on a single source of host for phage typing purposes. The greater number of lytic spectra recorded for the bacteriophage isolates used to infect the NCTC and *fla*-typed *Campylobacter* strains was supported by morphological differences and distinct protein profiles and genome sizes between the phage lytic classes. Bacteriophage lytic activity against the NCTC strains was greatest with 73% of strains being infected by at least one of the isolates. This was followed by infection of human strains (64%) and poultry strains (56%). Although poultry isolates seemed least susceptible to infection, the human isolates accounted for a larger proportion of the total number of *Campylobacter* hosts tested. Profiling phage against three sources of *Campylobacter* has clear benefits with respect to selection of the best candidates for future therapeutic endeavours. However, it also demonstrates that caution is required with this approach as differences between phage may not always be reflected as different lytic spectra. If lytic profiling is to be used as the sole method for discriminating between phage isolates is it recommended to use a panel of hosts from diverse sources to minimise bias and improve the sensitivity of the technique.

The electron microscopy data coupled with analysis of the genome revealed all the phage isolates were members of the Myoviridae family. A previous study characterising *Campylobacter* bacteriophage by (**Sails *et al.*, 1998**) found their isolates were also members of the Myoviridae family with similar characteristics to the phage isolated in the present study. Lysogeny has not yet been reported in *Campylobacter* and the sequencing of the *C. jejuni* NCTC 11168 genome failed to demonstrate phage-like sequences or insertion elements which might indicate the presence of a prophage (**Parkhill *et al.*, 2000**). *Campylobacter* phage belonging to the Siphoviridae family (lambda like) have been reported (**Firehammer and Border, 1968**) but no studies thus far have shown conclusively if these phage have a temperate lifecycle.

The physical dimensions of the phage are of particular interest when considering their use as therapeutic agents. Campylobacters are pleomorphic organisms, with the cell morphology changing at various stages of growth (**Griffiths, 1993**). During exponential phase the cell is usually a spiral or 'gull wing' shaped slender rod with a diameter of approximately 0.25 μm and a length ranging from 2 - 3 μm. In contrast, the smallest bacteriophage isolate reported in the present study has a diameter of 0.085 μm (head) and a length of 0.188 μm (head diameter + tail length). Assuming the bacterial cell shape to be cylindrical, the theoretical maximum burst size would be 105, using the entire volume of the host cell and assuming the smallest phage dimensions and the largest host cell dimensions respectively. This compares with a theoretical maximum burst size of 1650 for phage T4 in an average-sized *E. coli* host (phage dimensions: 0.065 μm x 0.190 μm; *E. coli* dimensions: 0.75 μm x 3 μm). However, a previous study revealed a maximum burst size of only 444 for T4 *in vitro* (**Hadas *et al.*, 1997**) with most cells yielding <300

virions. If the T4/*E. coli* model can be extrapolated to estimate *Campylobacter* phage burst sizes, the number of viral progeny per cell may well be less than ten. This has clear implications for phage therapy if active replication is the principal method of controlling the host bacterium population. The concentration of phage particles *in vivo* may become too diffuse to have any effect on the *Campylobacter* population.

With the exception of phage w5, all of the phage isolates representing different lytic spectra classes were able to rapidly and specifically adsorb to the host cell. Significant attachment to non-host cells and polypropylene test tubes was not observed. However, graph plots of phage replication did not indicate a one step curve typical of a lytic phage lifecycle. The asynchronous release of phage is unlikely to be a consequence of asynchronous attachment since the experiments presented earlier suggest adsorption is rapid and specific using the same host at the same cell density. Interestingly, phage replication seems to have, at most, a negligible effect on host replication when compared with the control (Fig. 4K). Filamentous phage such as M13 may be released without cell lysis (**Turner *et al.*, 1998**) but this type of lifestyle has never been reported for members of the Myoviridae family.

The phenomenon of lysis inhibition has been documented in the well-studied Myoviridae phage T4, amongst others (**Miller *et al.*, 2003**). Lysis is delayed if superinfection occurs after five minutes of the primary phage infection (**Miller *et al.*, 2003**). The mechanism of delay is poorly understood but the signal appears to be mediated by the rI protein which regulates holin assembly (**Paddison *et al.*, 1998**). Lysis inhibition itself is thought to be the consequence of a breakdown in cellular energetics during the normal lysis period

(**Miller *et al.*, 2003**). The delay could be a mechanism used by phage to maximise host utility, awaiting the accumulation of additional host bacteria before the release of viral progeny. With regard to *Campylobacter* phage this may be a plausible explanation for the asynchronous release of virions as the burst time would be dependent on the bioenergetics of individual host cells. However, this is unlikely to fully explain the asynchronous release of phage in the 'one-step growth curve' experiments in the present study as the MOI was deliberately maintained at 0.01 to minimise multiple infections of a single cell. Bacteriophage such as T7 are able to regulate their replication rate by encoding a duel-function protein (T7 lysozyme). This protein forms a ternary complex with RNA polymerase and selected promoters on the phage DNA Class II genes to reduce the transcription of genes involved in the replication of viral DNA (**Kumar and Patel, 1997**). Moreover, the inhibitory effect is influenced by the volume and metabolic state of the host cell as well as the concentration of host ribosomes, which have been shown to interfere with the transcription of T7 Class III (late) genes (**You *et al.*, 2002**). Bearing this in mind, the pleomorphic nature of *Campylobacter* observed in liquid culture (**Griffiths, 1993**) could go some way to explain the asynchronous release of phage from infected cells.

Protein profiling has been successfully applied to *Listeria* phage (**Zink and Loessner, 1992**) and this study demonstrates its applicability to *Campylobacter* phage. The protein profiles of the phage lysates revealed that at least one major protein varies between the isolates from different lytic spectra. The results from sequencing of a selection of these variable protein bands suggest they could be of viral origin based on homology of some of the sequences to proteins of bacteriophage ϕETA and LL-H. However, these

homologies were fairly weak and did not include any matches to phage capsid proteins of which the sequenced bands were most likely to be. The fact that two of the phage (w2 and CP6), isolated using the same host, shared one protein tryptic fragment sequence is interesting, particularly since they were isolated from different sources. Moreover, since few genes are shared between phage **(Rohwer and Edwards, 2002)** this may prove useful in sequencing the genomes of multiple phage or even identifying "signature" genes which could be used in bacteriophage taxonomy.

PFGE has been a valuable tool in the subtyping of *Campylobacter* due to its sensitivity and ability to resolve large DNA molecules. Previous work has shown that PFGE may also be applied to *Campylobacter* phage. Many bacteriophage, as a consequence of their lifestyle, are refractory to digestion with endonucleases. This is problematic when searching for enzymes suitable for RFLP profiling to distinguish between phage genomes. The endonuclease *Hha*I, and its isoschitsomer *Cfo*I, were the only enzymes which produced discriminatory RFLP profiles. The paucity of suitable enzymes for restriction analysis is probably because the phage DNA nucleotides are modified in a similar way to phage T4. The genome of this phage encodes DNA methyl transferases which catalyse the conversion of cytosine to hydroxymethyl cytosine which protects its DNA from digestion by host and phage-encoded endonucleases.

The genome sizes of all the phage isolates were larger than the average for a Myoviridae virus (~ 100 kb, source: NCBI). The sequenced strain of *Campylobacter jejuni* (NCTC 11168) is 1.6 Mbp and the replication of a viral genome up to 14% the size of its own

may impose considerable stress on the host which further supports prior indications of a small burst size.

No relevant nucleotide-nucleotide matches were obtained when interrogating the NCBI database with randomly-cloned DNA fragments from bacteriophage lysates using the BLASTn search engine. However when using the BLASTx search engine, which translates the nucleotide sequence into an amino acid sequence, some significant matches could be discerned for the translated products of genes from other bacteriophage. Of particular interest was the homology to T4 prohead protease, topoisomerase and primase-helicase. All of these enzymes play a role in the replication of T4 DNA and are good candidates for a conserved sequences in the Myoviridae family. These data may prove useful in the design of PCR primers for further characterisation of the phage genomes in the future.

# CHAPTER 5

## POPULATIONS OF *CAMPYLOBACTER* AND BACTERIOPHAGE ON RETAIL CHICKEN PRODUCTS AND IN BROILER CHICKEN CAECA

### 5.0 INTRODUCTION

Previous studies have determined the extent to which broiler flocks are colonised with *Campylobacter* (**Newell and Wagenaar, 2000**). However, little or nothing is known of *Campylobacter* bacteriophage throughout the poultry meat supply chain. Over 170 *Campylobacter* bacteriophage have been isolated to date (**Sails *et al.*, 1998**). Most have been recovered from poultry excreta or abattoir effluent (**Grajewski *et al.*, 1985; Salama *et al.*, 1989**). The major application of these isolates so far has been integration into typing schemes (**Grajewski *et al.*, 1985; Salama *et al.*, 1990b; Frost *et al.*, 1999**). The renaissance of phage therapy seen in western countries in recent years (**Kutter, 1997**) has prompted several academic and commercial projects to exploit the potential of phage to control *Campylobacter* in poultry production (www.intralytix.com; http://192.211.16.12/user/T4/phagebiotics.html; www.phage-biotech.com).

An important step in the development of a *Campylobacter* phage intervention strategy in broiler chickens is to examine natural populations of the virus and its host *in vivo*.

Understanding the ability of each organism to survive in the chicken gut and throughout the food chain is vital in the formulation of a targeted and effective phage therapy programme.

The aim of this study was to expand on the bacteriophage isolation and survival data reported in Chapter 3 by examining commercial broiler chicken caeca and whole retail chicken carcasses for the presence of both phage and host. The lytic spectra of phage from both sources was determined using 80 different phage-typed *Campylobacter* reference strains originating from humans. A subset of *C. jejuni* strains isolated from broiler caeca either with or without bacteriophage were discriminated using pulsed field gel electrophoresis. The relationship between the presence of *Campylobacter* and bacteriophage with the total aerobic, anaerobic and microaerobic counts in chicken caeca was also determined.

## 5.1 RESULTS

### 5.1.1 Isolation of *Campylobacter* from retail broiler carcasses

In order to assess the relationship between phage and their hosts on retail poultry products, broiler carcasses were screened for the presence of both virus and its bacterial host. Retail broiler carcasses ($n$ = 688) representing six producers in the UK and one producer in France were rinsed (**2.6.3**) and screened for the presence of *Campylobacter* ssp. by Norpath Ltd (a UKAS-accredited commercial food microbiology laboratory). *Campylobacter* isolations were performed using two BSI-approved standard methods which consisted of enrichment using the Exeter technique (**2.6.2**) and direct plating of wash fluid onto mCCDA selective agar (**2.3.4.1**). *Campylobacter* was isolated from 163/688 (24%) of samples using a combination of both methods. The *Campylobacter* isolates were predominantly *C. jejuni* ($n$ = 150, 92%) with the remainder identified as *C. coli* using biochemical speciation (**2.3.4.3**). The prevalence of *Campylobacter* in the products from each supplier is presented in Table 5A. There was considerable variation in the number of *Campylobacter*-positive carcasses from each of the sources. At the extremes, an average of 70% of carcasses from producer D were contaminated with *Campylobacter* compared with only 3% from producer A.

| Producer | Total Samples | Percent Total | Campylobacter Isolates | Percent Positive |
|---|---|---|---|---|
| A | 60 | 8.7 | 2 | 3.3 |
| B | 60 | 8.7 | 4 | 6.7 |
| C | 60 | 8.7 | 7 | 11.7 |
| D | 129 | 18.8 | 90 | 69.8 |
| E | 120 | 17.4 | 9 | 7.5 |
| F | 60 | 8.7 | 20 | 33.3 |
| G | 180 | 26.0 | 27 | 15.0 |
| H | 20 | 2.9 | 4 | 20.0 |
| **TOTAL** | **689** | **100.0** | **163** | **100.0** |

**Table 5A. Prevalence of *Campylobacter* in retail broiler carcass rinses.** Table showing the number of *Campylobacter* positive broiler carcasses from eight producers based in the UK or France. The first column indicates the producer code followed in the second column by the number of samples from each producer which is also represented as a percentage of the total number of samples in the third column. The fourth column shows the number of *Campylobacter* positive carcasses from each producer which is also given as a percentage of the total number of samples in the fifth column.

### 5.1.2 Isolation of *Campylobacter* bacteriophage from retail broiler carcasses

Aliquots (1 ml) of retail broiler carcass rinses (**2.6.3**) were examined for the presence of bacteriophage (**2.6.5**). Of the 688 samples tested, 41 (6%) harboured bacteriophage capable of infecting host strain *C. jejuni* NCTC 12662 phage-type 14 (PT14). Of the 41 phage-positive carcasses, 35 also harboured *Campylobacter*. However, none of these campylobacters were found to be permissive to infection by bacteriophage isolated from the same carcass when using drops of phage suspension on a soft agar bacterial lawn (**2.3.6.5**). Although the rinses of 41 broiler carcasses contained suspected phage which could form plaques on PT14, 23 such samples (56%) could not be subsequently propagated using this strain. The lytic zones of these plaques were unusually large (>5 mm diameter). To establish whether these plaques may have been the result of infection by a non-viral predator, DNA extractions from the plaque eluates were used in a PCR assay to detect the presence of the Gram negative predatory bacterium, *Bdellovibrio bacteriovorous* (**2.5.7**) using the oligonucleotide primers described by **Jurkevitch and Ramati (2000)**. *B. bacteriovorous* 109J was propagated using plate lysates with *E. coli* K-12 as the host (**2.3.5**). The desired product size from the PCR assay (approximately 830 bp) was obtained from the *B. bacteriovorous* 109J control but not from any of the plaque eluate DNA extractions used ($n$ = 12). In an attempt to visualise the agent responsible for the lytic zones, volumes (10 µl) of the plaque eluates were examined by negative staining electron microscopy (**2.7**). However, neither phage nor *Bdellovibrio*-like organisms were observed. This may well have been a consequence of the small volumes of sample used in electron microscopy.

### 5.1.3 Isolation of *Campylobacter* from chicken caeca

In order to assess the relationship between phage and their hosts in the intestinal tract of chickens, the presence and numbers of each were determined in the caeca of commercial broilers. Broiler chickens ($n$ = 205; mean age = 34 days ± 12 d) from three national poultry producers representing 22 farms were dissected within 48 h of death (storage at 4°C until dissection) and the caeca removed using flame-sterilised implements with the methods described in section **2.11.6.** The caecal contents were then examined for the presence and numbers of *Campylobacter* ssp **(2.11.7).** Of the chickens examined in this study, 129/205 (69%) harboured *Campylobacter* with a mean colonisation level of $\log_{10}$ 6.7 ± 1.1 cfu $g^{-1}$ caecal contents. All of these isolates (129/129, 100%) were identified as *C. jejuni* through biochemical speciation **(2.3.4.2).** Four to eight well-isolated colonies were taken from the positive plates representing the caeca of birds with ($n$ = 9) and without ($n$ = 9) bacteriophage and passaged once on Columbia blood agar plates (CBA, **2.3.1.1).** DNA from these strains ($n$ = 124) was then prepared for pulsed field gel electrophoresis (PFGE) using the protocol described in **2.5.9.** PFGE was performed by Mrs. E. Dillion (research technician) according to the protocol devised by **Ribot *et al.*, 2001).** Sections cut from the agarose DNA blocks (approximately 2 mm) were individually digested with either *Sma*I or *Kpn*I endonucleases and resolved on a 1% agarose gel **(2.5.9.1).** The macrorestriction profiles of 18 *C. jejuni* isolates digested with *Sma*I are presented in Figure 5A. The 124 single colony isolates assessed using PFGE could be ascribed to 16 unique macrorestriction profiles on the basis of digestion with

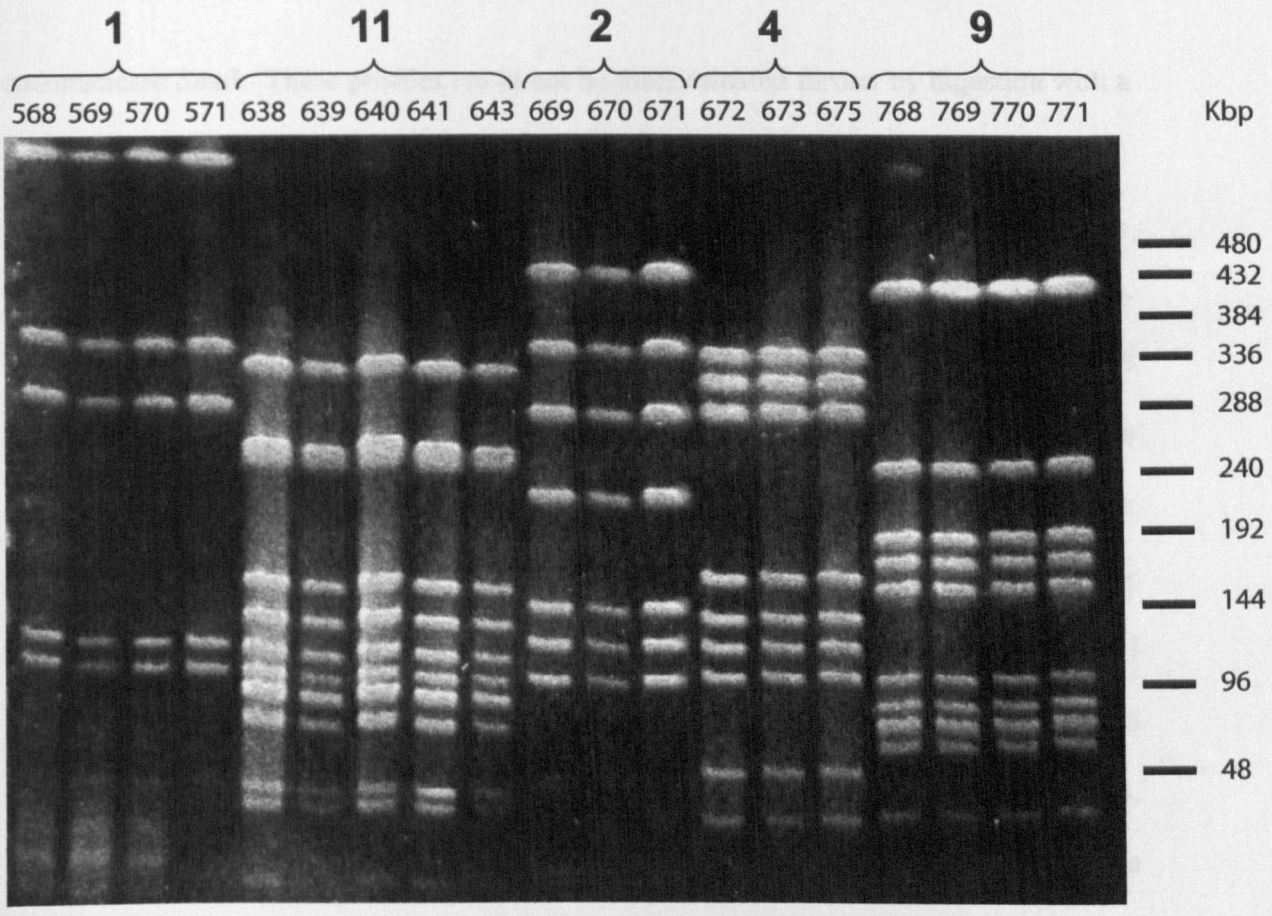


**Figure 5A. Macrorestriction profiles of *Campylobacter* isolates from chicken caeca**

A 1% agarose gel showing the macrorestriction profiles of *Campylobacter* strains digested with endonuclease *Sma*I and subjected to PFGE. The *Campylobacter* strain number is shown at the top of the gel, grouped together according to matching restriction profiles (parentheses). Lambda concatomer markers (Kbp) are shown at far right of the gel.

endonuclease *Sma*I. These profiles could not be discriminated further by digestion with a second endonuclease, *Kpn*I.

Of the 22 farms tested in this study, 13 (59%) harboured *Campylobacter*-positive flocks. With respect to individual farms, campylobacters were either isolated from all the birds on a farm or none (i.e. flock positivity was either 100% or 0%). No farm housed a mixture of positive and negative birds. At least two *C. jejuni* strains were recovered from the houses of 12/13 (92%) positive farms with eight of these (62%) being colonised by three or more strains. PFGE *Sma*I profile 4 (PSP 4) was present in all of the farms of producers A and B. All other macrorestriction profiles were unique to the farms belonging to the individual producers. However, seven strains (PSP 1, PSP 2, PSP 4, PSP 5, PSP 6, PSP 9 and PSP 12) were isolated from chickens from more than one farm within each producer's base. Generally, the macrorestriction profiles of campylobacters isolated from the same bird were identical, with only three birds possessing caeca with two or more different strains of *C. jejuni*.

### 5.1.4 Isolation of *Campylobacter* bacteriophage from commercial broiler chickens

The caecal contents obtained from the broiler chickens in section 5.1.3 were also examined for the presence of *Campylobacter* bacteriophage using PT14 and contemporary *Campylobacter* isolates as hosts (**2.11.8**). Of the 205 chicken caeca examined, 41 (16.5%) harboured phage capable of infecting PT14. Only four birds possessed phage capable of infecting *Campylobacter* isolates originating from the same

caecal contents as their predator. However, all of these phage were capable of infecting PT14 and all subsequent phage purification and propagation was performed using this strain as the host. There was considerable variation in the numbers of phage present in the caeca of individual birds (mean presence = $\log_{10}$ 3.9 ± 1.2 pfu $g^{-1}$ caecal contents) with an observed range from $\log_{10}$ 1.5 to 6.9 pfu $g^{-1}$ caecal contents. Interestingly, chicken caeca harbouring bacteriophage generally contained fewer campylobacters despite the majority of *Campylobacter* isolates not being susceptible to infection by phage isolated from the same sample. Additionally, *Campylobacter* colonisation was below the limit of detection ($\log_{10}$ 2.0 cfu $g^{-1}$ caecal contents) in 29/41 (71%) of birds which also contained bacteriophage. This difference was significant ($P < 0.001$) using a student's T-test (**2.12**). There was no apparent association between *Campylobacter* PFGE macrorestriction profiles and the presence of bacteriophage in the same sample.

### 5.1.5 Host range of phage isolates

In order to further characterise the bacteriophage isolates from retail poultry and chicken caeca for their potential use in therapeutic trials, the phage were first screened for their ability to infect ten NCTC *Campylobacter* strains of different phage types supplied by the Central Public Health Laboratories, Colindale, London (CPHL). Phage exhibiting unique lytic spectra on these strains were selected for further lytic profiling against 80 different clinical *Campylobacter* isolates at the Central Public Health Laboratories, Colindale, London using the protocol defined by **Frost *et al.* (1999)**. Phage isolates ($n$ = 16) exhibiting different lytic spectra from retail poultry ($n$ = 7) and intestinal ($n$ = 9) sources are presented in Table 5B.

The phage isolated from broiler chicken caeca generally infected a greater number of *Campylobacter* strains (mean = 39 ± 11 strains) than those isolated from retail poultry (mean = 18 ± 6 strains). In addition, there was a greater similarity between the lytic profiles of phage isolated from retail chicken compared with broiler caeca. Analysis of the lytic profiles from the retail chicken phage isolates revealed that 18/80 (23%) of the different phage-typed *Campylobacter* strains were infected in total. Of these eighteen strains, thirteen (72%) were commonly lysed by all the retail chicken phage isolates. This compared with only three (4.6%) commonly lysed from the total of 59 strains infected by the broiler phage isolates. Three *Campylobacter* phage types (PT3, PT14 and PT25) were infected by all phage, regardless of the source. Conversely, fourteen phage types were not infected by any of the phage isolates (PT5, PT7, PT20, PT22, PT23, PT24, PT39, PT48, PT57, PT60, PT67, PT75, PT77 and PT78).

### 5.1.6 Populations of aerobic, anaerobic and microaerobic organisms in the chicken caeca.

To investigate the possibility of other bacterial populations affecting the colonisation of *Campylobacter* or the presence of bacteriophage, the total aerobic, anaerobic and microaerobic counts of the caecal contents were determined. Enumeration was performed using the methods endorsed by the Steering Group Report on the Microbiological Safety of Food (**2.11.7**). Briefly, this consisted of spread-plating serial dilutions of caecal suspensions

| PT | PT code | F10 | F11 | F19 | F21 | F7 | F9 | F20 | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1010 | scl | scl | scl | scl | | scl | scl | | | | | | | | | |
| 2 | 3156 | | | | | scl | | | | scl | scl | scl | | | +++ | scl | scl |
| 3 | DEFF | scl | <scl | <scl | <scl | scl | scl | scl | scl | +++ | +++ | scl | <scl | scl | scl | scl | scl |
| 4 | FEB9 | s | s | s | s | scl | s | s | scl | <scl | +++ | scl | s | scl | scl | scl | scl |
| 5 | 1A98 | | | | | | | | | | | | | | | | |
| 6 | DC38 | | | | | | | | scl | | | | | scl | scl | ++ | |
| 7 | 1152 | | | | | | | | | | | | | | | | |
| 8 | 3014 | | | | | <scl | | | | + | + | +++ | | | | <scl | <scl |
| 9 | DE38 | | | | | scl | | | scl | <scl | <scl | <scl | | <scl | <scl | scl | scl |
| 10 | 1C39 | | | | | | | | scl | | | | | scl | scl | ++ | |
| 11 | FF5F | | | | | scl | | | | | | scl | | | | scl | scl |
| 12 | 317E | | | | | scl | | | | <scl | <scl | scl | | | +++ | scl | scl |
| 13 | FEFF | scl | scl | scl | scl | scl | scl | scl | scl | +++ | +++ | <scl | scl | scl | scl | scl | scl |
| 14 | FFFF | scl | scl | scl | scl | scl | scl | scl | scl | <scl | <scl | scl | scl | scl | scl | scl | scl |
| 15 | DC98 | | | | | | | | scl | | | | scl | scl | scl | ++ | |
| 16 | FE99 | | | | | scl | | | scl | +++ | +++ | scl | | <scl | scl | scl | scl |
| 17 | 3056 | | | | | scl | | | | scl | scl | scl | | | +++ | scl | scl |
| 18 | 3116 | | | | | scl | | | | scl | scl | scl | | | +++ | scl | scl |
| 19 | 3FFF | | | | | scl | | | scl | scl | scl | scl | scl | scl | scl | scl | scl |
| 20 | DC18 | | | | | | | | | | | | | | | | |
| 21 | 1030 | | | | | | | | | | | | | | | | |
| 22 | 1A90 | | | | | | | | | | | | | | | | |
| 23 | 1C90 | | | | | | | | | | | | | | | | |
| 24 | 3016 | | | | | | | | | | | | | | | | |
| 25 | FFBD | scl | scl | scl | scl | scl | scl | scl | scl | scl | scl | scl | scl | scl | scl | scl | scl |
| 26 | FC99 | | | | | | | | scl | | | +++ | scl | <scl | scl | ++ | |
| 27 | FCDB | | | | | scl | | | | +++ | +++ | scl | | | ++ | scl | scl |
| 28 | FFFE | scl | scl | scl | scl | | scl | scl | | | | s | scl | | | s | s |
| 29 | 1012 | | | | | | | | scl | | | | | scl | scl | scl | |
| 30 | FF78 | | | | | +++ | | | scl | | | <scl | | 10 | <scl | scl | <scl |
| 31 | DEBD | scl | scl | scl | scl | | scl | scl | scl | + | | +++ | scl | <scl | scl | + | |
| 32 | 3152 | | | | | scl | | | | scl | scl | scl | | | <scl | scl | scl |
| 33 | DEB9 | scl | scl | scl | scl | | scl | scl | scl | ++ | | <scl | scl | scl | scl | ++ | |
| 34 | 1A99 | | | | | | | | scl | ++ | | | | scl | scl | ++ | |
| 35 | DE99 | | | | | | | | scl | | | | | scl | scl | <scl | |
| 36 | DE98 | ++ | +++ | +++ | +++ | | scl | <scl | | | | | | | | | |
| 37 | FDFF | | | | | s | | | s | s | s | <scl | s | s | +++ | <scl | s |
| 38 | DC99 | | | ++ | ++ | | ++ | | scl | | | | scl | ++ | <scl | + | |
| 39 | 3114 | | | | | | | | | | | | | | | | |
| 40 | DEB8 | +++ | +++ | +++ | +++ | | scl | <scl | | | | | | | | | |
| 41 | 1C98 | | | | | | | | scl | | | | | scl | scl | ++ | |
| 42 | 1091 | | | | <scl | | scl | | scl | | | ++ | ++ | ++ | +++ | + | |
| 43 | 1898 | | | | | | | | scl | + | | | | ++ | | | |
| 44 | 1052 | | | | | | | | scl | | | <scl | | scl | scl | ++ | |
| 45 | 1011 | | ++ | | scl | | scl | | scl | ++ | | | scl | <scl | | | |
| 46 | 3FFE | <scl | <scl | <scl | <scl | scl | scl | scl | scl | +++ | +++ | scl | | scl | scl | scl | scl |
| 47 | F5FF | | | | | scl | | | | | scl | scl | scl | | scl | scl | scl |
| 48 | DCB8 | | | | | | | | | | | | | | | | |
| 49 | 1098 | | ++ | | scl | | scl | | | + | | | | | | | |
| 50 | 1AB8 | | | | | | | | | | | | scl | scl | scl | | |

| PT | PT code | F10 | F11 | F19 | F21 | F7 | F9 | F20 | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 51 | 1C18 | | | | | | | | | | | | | | | | |
| 52 | 1C99 | | | | | | | | scl | | | scl | | scl | scl | ++ | |
| 53 | 3FDF | | | | | scl | | | scl | scl | scl | scl | scl | scl | scl | scl | scl |
| 54 | DCDB | | | | | | +++ | | | | | +++ | | | | +++ | +++ |
| 55 | DDFA | | | | | scl | | | scl | scl | scl | scl | <scl | <scl | <scl | scl | scl |
| 56 | FF7E | | | | | scl | | | scl | +++ | +++ | <scl | | ++ | <scl | scl | scl |
| 57 | 1018 | | | | | | | | | | | | | | | | |
| 58 | 1899 | | | | | | | | +++ | ++ | | | | ++ | +++ | | |
| 59 | 1090 | | | | | | | | | + | | | | | | | |
| 60 | 1810 | | | | | | | | | | | | | | | | |
| 61 | 1AB9 | | | | | scl | | | scl | | | +++ | | <scl | <scl | <scl | scl |
| 62 | 1EB9 | | | | | | | | scl | | | +++ | | scl | scl | ++ | |
| 63 | 1E98 | | | | | | | | scl | | | | | scl | <scl | ++ | |
| 64 | 1EB8 | | | | | | | | scl | | | ++ | | <scl | scl | + | |
| 65 | FFDF | +++ | +++ | +++ | +++ | scl | scl | +++ | scl | scl | scl | scl | | +++ | <scl | scl | scl |
| 66 | DEDB | | | | | scl | | | | +++ | +++ | +++ | | | +++ | scl | scl |
| 67 | 1C10 | | | | | | | | | | | | | | | | |
| 68 | 3BDF | | ++ | | scl | | scl | | scl | ++ | | +++ | scl | scl | scl | + | |
| 69 | 1031 | | | | | scl | | | +++ | +++ | +++ | scl | | +++ | +++ | scl | scl |
| 70 | DCDA | | | | | scl | | | | +++ | +++ | +++ | scl | | ++ | scl | scl |
| 71 | DEFB | scl | scl | scl | scl | + | scl | scl | scl | + | | <scl | scl | + | scl | ++ | +++ |
| 72 | FD38 | | | | | | | | scl | | | | | <scl | scl | ++ | |
| 73 | 1C30 | | | | | | | | scl | | | | | +++ | +++ | + | |
| 74 | 1811 | | | | | | scl | | scl | | | scl | scl | scl | scl | ++ | |
| 75 | 1E99 | | | | | | | | | | | | | | | | |
| 76 | 3EFF | | | | | scl | | | | scl | <scl | scl | scl | scl | scl | scl | |
| 77 | DE18 | | | | | | | | | | | | | | | | |
| 78 | DC19 | | | | | | | | | | | | | | | | |
| 79 | F57E | | | | | scl | | | | scl | scl | scl | | | +++ | scl | scl |
| 80 | FD3C | | | | | | | | scl | | | | | <scl | scl | + | |

| KEY | |
|---|---|
| s | shadow lysis |
| + | 10-25 plaques |
| ++ | 25-50 plaques |
| +++ | 50-100 plaques |
| <slcw | <scl (weak) |
| scl | semi-confluent lysis |
| <cl | slightly less than confluent lysis |
| cl | confluent lysis |

**Table 5B. Lytic spectra of (F)ood and (C)aeca phage isolates.**
Table showing the different lytic activity of seven phage isolated from chicken and nine phage isolated from broiler chicken caeca against eighty human *Campylobacter* isolates exhibiting different phage types (PT).

onto Plate Count Agar (**2.3.1.8**) and incubating for 24 – 48 h under the different atmospheric conditions. The total aerobic population remained relatively stable between different birds from the various farms in the survey (mean colonisation = $\log_{10}$ 7.4 ± 0.6 cfu $g^{-1}$ caecal contents). However, the microaerobic and anaerobic counts were more variable with mean colonisation levels recorded as $\log_{10}$ 7.5 ± 2.4 and $\log_{10}$ 8.9 ± 2.1 cfu $g^{-1}$ caecal contents respectively. No association between either the aerobic, anaerobic or microaerobic populations with *Campylobacter* or phage could be discerned.

### 5.1.7 Protease activity in the chicken caeca

Preliminary investigations indicated that the skin and caecal contents of birds examined in a previous study contained a ultra-filtratable agent capable of inhibiting the growth of *Campylobacter* on lawns in a concentration-dependent manner. Serial dilution of the filtrate failed to result in the formation of isolated plaques on *Campylobacter* lawns using the surface droplet technique (**Salama *et al.*, 1990a**) suggesting the agent responsible for the inhibition of growth was not a bacteriophage. Further investigations revealed this agent was inactive after 48 h storage at 4°C, after one freeze-thaw cycle at -20°C or after treatment with Proteinase K. Moreover, the inhibitory activity was eliminated after incubation with 4-amidino-phenyl-methane-sulfonyl fluoride (APMSF, a specific irreversible serine proteinase inhibitor) but not when incubated with inhibitors of other protease classes such as EDTA (metallo proteases) or E64 (cysteine proteases). To determine if *Campylobacter* populations in the chicken caeca could be correlated with protease activity, the EnzChek® kit (**2.11.10**) was used to determine the total relative protease activity in caecal filtrates. This kit is based on the protease-catalysed hydrolysis

of a casein protein substrate labelled with green fluorescent dye (BODIPY® FL). Once the dye is released, an increase in fluorescence is recorded which is proportional to the total protease activity in a sample. The protease activity measured from the caecal contents of the chickens was highly variable with samples producing fluorescence counts ranging from 12,453 to 154,173. As the variety and quantity of each protease in the chicken caeca was unknown, specific units of protease activity could not be determined and thus the fluorescence of each sample can only be used as a guide to relative total protease activity. A reading of 500 fluorescence units was recorded for 5 $\mu g\ ml^{-1}$ of trypsin on a standard curve prepared and measured at the same time as the caecal samples. However, high fluorescence counts (and therefore protease activity) could not be correlated with the presence or absence of detectable *Campylobacter* cells. None of the caecal contents in the present study contained any ultra-filtratable agent capable of inhibiting *Campylobacter* growth using the surface droplet technique.

## 5.2 DISCUSSION

Previous studies have shown that there is a high prevalence of *Campylobacter* in broiler chicken flocks (**Humphrey *et al.*, 1993; Evans and Sayers, 2000; Newell and Wagenaar, 2000**) which are able to survive abattoir processing and contaminate retail products (**Newell *et al.*, 2001**). The present study demonstrates that *Campylobacter* bacteriophage can also survive processing and are recoverable from whole commercial chicken carcasses. *Campylobacter* was detected on 24% of products surveyed in this study which is lower than recent published values in the UK (**Kramer *et al.*, 2000; Jorgensen *et al.*, 2002**). The wide variation in the *Campylobacter* positivity of carcasses from different retailers emphasises the need for representative sampling to obtain accurate data on the prevalence of this bacterium on retail poultry meat.

Bacteriophage are obligate intracellular parasites, and thus are likely to be present on chicken products which also harbour a greater number and diversity of their hosts. It was interesting that samples from the poultry producer supplying the highest number of *Campylobacter*-positive carcasses did not harbour any recoverable phage and the reason for this is unclear. Certain *Campylobacter fla* types are known to survive abattoir processing better than others (**Newell *et al.*, 2001**) and this may also be case for their associated phage. This is supported by data from Chapter 3 which demonstrates the variability in survival of phage isolates on the surface of experimentally inoculated chicken skin. This may assist in explaining the different characteristics (e.g. host range) of the phage isolated from retail poultry compared with those from other sources. It was also important to note that bacteriophage isolated from broiler chicken caeca were able to

infect a greater number of *Campylobacter* strains than those isolated from retail chicken portions. Considering the overall aims of this project, efforts to isolate bacteriophage of potential therapeutic benefit at the farm level are probably best focussed on the chicken gastrointestinal tract. However, the ability of bacteriophage to remain viable on retail chicken products would become important if phage were to be applied as a post-processing treatment. This is explored further in Chapter 6.

Although the presence of *Campylobacter* and their phage in the chicken intestine has been known for some time (**Grajewski *et al.*, 1985; Salama *et al.*, 1989; Sails *et al.*, 1998**) there has never been any attempt to correlate naturally-occurring populations of the two in commercial broiler chickens. The results from the present study suggest that the *Campylobacter* population of the chicken caecum is generally lower when phage are also present. Indeed, 29/41 phage positive chickens harboured *Campylobacter* below detectable limits. The wide range of bacteriophage titres recorded in the caecum may reflect the availability of hosts due to the dynamic nature of *Campylobacter* populations in the chicken gut. Previous studies with *Salmonella typhi* and *E. coli* have shown that phage do not always eliminate their prey (**Alexander, 1981**). Often in these cases, the titres of predator and prey increase and decline with characteristic out of phase population oscillations (**Van Den Ende, 1973**). This phenomenon has also been described in predatory protozoa (**Van Den Ende, 1973**) and *Bdellovibrio* (**Alexander, 1981**). Providing such interactions occur between phage and *Campylobacter* in the chicken gut, the numbers of phage recovered would vary according to which stage of the oscillatory cycle the sample was taken from.

There was considerable variation in the macrorestriction profiles produced by PFGE of *Sma*I digested genomic DNA observed from different birds from the same and different farms. This supports previous studies which emphasise the genetic diversity of this bacterium even in this specialised niche (**Newell and Wagenaar, 2000**).

The high frequency of plaques from which phage could not be propagated was interesting. Gram negative bacterial pathogens such as *Bdellovibrio* ssp. were unlikely to be the cause of this phenomenon as they could not be detected using PCR with genus-specific oligonucleotide primer sets and were not visible by electron microscopy. Another possible explanation is the production of a bacteriocin by a bacterial contaminant. However, this is also unlikely as the centrifugation and filtration steps in the phage isolation protocol should have been sufficient to remove these bacteria. Moreover, no colonies were observed at the centre of the plaques when visualised under a dissecting microscope. Potentially, the phage themselves may be defective in some part of their life cycle and may only produce components capable of lysis but not complete replication. Ultimately, this study was unable to determine what was responsible for this phenomenon, which would make an interesting area of further research.

An interesting observation in preliminary experiments led to an investigation of a possible lytic agent present in the skin and caeca of broiler chickens. Several studies have shown that the chicken gut may contain organisms which are inhibitory to *Campylobacter* growth (**Humphrey *et al.*, 1989**), only a fraction of which have been characterised to any degree (**Mead, 2000**). This inhibition is usually due to production of secondary metabolites or direct competition for colonisation sites in the gut (**Mead,**

2000). In the present study, the total aerobic, anaerobic or microaerobic populations showed no association with *Campylobacter* or phage populations. Previous studies suggested that filtered caecal suspensions sometimes have anti-*Campylobacter* properties (**Humphrey *et al.*, 1989**). However, despite these preliminary findings, dilutions of filtered caecal suspensions in the present study failed to demonstrate any lytic activity when used to inoculate lawns of PT14. This indicates the 'hit or miss' nature of this lytic phenomenon. The total protease activity of the broiler caeca was measured as this was thought to be one possible mechanism of *Campylobacter* inhibition, not necessarily resulting in cell lysis. The relative protease activity in the caeca varied appreciably from bird to bird but no campylobactericidal activity was observed in the caecal suspension filtrates (other than that caused by phage infection).

The most significant association between populations was with the reduced presence, or even absence, of *Campylobacter* in caeca also harbouring bacteriophage. It should be noted that only a small number of *Campylobacter* strains were actually sensitive to contemporaneous bacteriophage isolates. This may be a consequence of: bias towards recovery of certain phage and *Campylobacter* strains; changes in host susceptibility upon subculture or a non-susceptible *Campylobacter* strain displacing the original host. Another explanation would be that a proportion of the *Campylobacter* population became resistant due to exposure to the phage. Resistance to phage infection would confer a competitive advantage on the mutant and allow a greater colonisation of the chicken gut. In turn, this would increase the probability of isolating a *Campylobacter* resistant to infection by phage in the same sample. Previous studies have shown phage resistant mutants of *E. coli* to be less virulent and less efficient colonisers than the wild type

(**Smith and Huggins, 1983**). This is also supported by data in Chapter 8 and may partly explain why fewer campylobacters were isolated from caeca also containing phage.

# CHAPTER 6

# APPLICATION OF HOST-SPECIFIC BACTERIOPHAGE TO THE SURFACE OF CHICKEN SKIN LEADS TO A REDUCTION IN RECOVERY OF *CAMPYLOBACTER*

## 6.0 INTRODUCTION

Campylobacters can be frequently isolated from the feathers and skin of broiler chickens (**Berrang *et al.*, 2000**). Reducing this contamination would be a step towards reducing *Campylobacter* presence on retail poultry meat. One common method of reducing broiler carcass contamination involves the use of hypochlorite in scalding water carcass washes and chilled water tanks. Previous studies have shown this treatment to be ineffective in substantially reducing the numbers of human pathogens, including *Campylobacter*, associated with the chicken skin when used under standard commercial operating conditions (**Whyte *et al.*, 2001**). Increasing the concentration of hypochlorite does marginally increase its efficacy, but reduces the quality of the end product which is unacceptable to consumers.

Host-specific bacteriophage have been used previously in the treatment of *E. coli* infections in piglets (**Smith and Huggins, 1983**) and more recently in *Enterococcus* (**Biswas *et al.*, 2002**) and *Vibrio* (**Cerveny *et al.*, 2002**) infections. Their efficacy in experimental enteric

and systemic infections has already been demonstrated. Additionally, other studies have shown that the spoilage of beef by *Pseudomonas* ssp. can be reduced by the application of phage (**Greer, 1986**). However, the ability of host-specific phage to reduce the presence of a food-borne pathogen which is not actively replicating on the surface of food has yet to be investigated. This study assessed the ability of specific bacteriophage to reduce the numbers of *Campylobacter* on experimentally inoculated chicken skin.

## 6.1 RESULTS

### 6.1.1 Survival of *Campylobacter* NCTC 12662 on the surface of chicken skin

The survival of *Campylobacter* bacteriophage ϕ2 on the surface of chicken skin stored at 4°C and –20°C was determined in Chapter 3. In order to gauge the effect of phage on populations of their hosts when stored under these conditions, the present study examined the recovery of *Campylobacter jejuni* NCTC 12662 from the surface of chicken skin. The caecal contents and skin of broiler chicks reared to be free of campylobacters were confirmed *Campylobacter*-negative by the direct plating of samples onto modified charcoal cefoperazone deoxycholate agar (mCCDA, **2.3.1.4**). Sections of chicken skin (2 $cm^2$) from these birds (**2.6.8**) were inoculated with $log_{10}$ 6.0 cfu *Campylobacter* and individually stored under fresh (4°C) and frozen (-20°C) conditions over a ten day period. Triplicate skin sections were removed from each storage condition at 24 h intervals for the enumeration of *Campylobacter*. Enumeration was performed by stomaching the skin in MRD (20 ml) and spread-plating serial dilutions of the stomachate onto triplicate mCCDA plates. Representatives of typical *Campylobacter* colonies from each sample were sub-cultured onto Columbia blood agar plates (CBA, **2.3.1.1**) before Gram staining to confirm typical *Campylobacter* cell morphology (**2.3.4.1**).

The recovery of *C. jejuni* NCTC 12662 over ten days under each storage condition is presented in Figure 6A. *Campylobacter* recovery at the initial time point was identical ($log_{10}$ 5.4 cfu per section of skin) for samples stored under both conditions. However, recovery from the fresh samples continued to gradually decline to reach a base level of $log_{10}$ 5.1 ± 0.04 cfu at the end of the experiment. In contrast, the freeze-thaw treatment

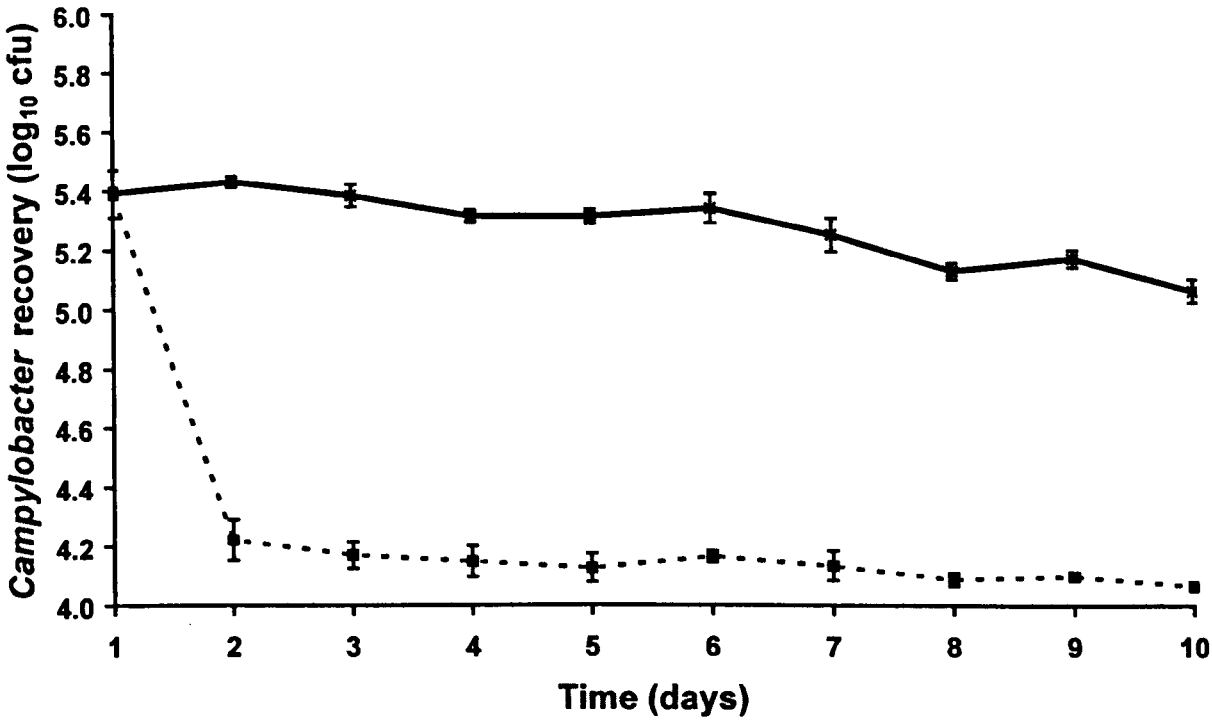


**Figure 6A. Recovery of *Campylobacter* from chicken skin stored at different temperatures.**

Graph showing the recovery of *C. jejuni* NCTC 12662 from the surface of chicken skin inoculated with $10^6$ cfu and stored at 4°C ( ■ ) and -20°C ( □ ) over a ten day period. Recovery is presented as the mean $\log_{10}$ cfu ± SD. These results are based on three replicates.

of the skin resulted in a fall in *Campylobacter* recovery to $\log_{10}$ 4.2 ± 0.08 cfu on day two compared with $\log_{10}$ 5.4 ± 0.02 cfu for the fresh chicken skin at the same time point. In parallel to the pattern of recovery recorded for phage from skin stored at –20°C (Chapter 3), the level of *Campylobacter* recovery remained at approximately the same level for the rest of the experiment with only a slight decline to $\log_{10}$ 4.1 ± 0.01 cfu on day ten.

### 6.1.2 Activity of phage inoculated onto chicken skin artificially contaminated with *Campylobacter*

Once the independent recovery and survival of *Campylobacter* and bacteriophage on chicken skin was established, the ability of phage ϕ2 to reduce the number of recoverable *Campylobacter* cells on the surface of inoculated chicken skin under these conditions was determined. Sections of chicken skin (2 $cm^2$) from birds reared to be free of *Campylobacter* (**2.6.8**) were inoculated with a matrix of phage and host titre combinations (Table 6A) in triplicate prior to incubation at 4°C or –20°C. Campylobacters and bacteriophage were recovered from the chicken skin one, three and five days after inoculation using the methods described in section **2.6.9**. Chicken skin sections each inoculated with either bacteriophage or *Campylobacter* served as positive controls for the experimental recovery of each. Uninoculated chicken skin was used as a negative control. The recovery of *Campylobacter* from the surface of chicken skin following treatment with different titres of bacteriophage is presented in Table 6B. Recovery is shown as mean $\log_{10}$ *Campylobacter* cfu ± standard deviation for all treatments compared with control inoculations. The statistical significance of phage

| *Campylobacter* inoculum (cfu) | | Bacteriophage inoculum (pfu) | | |
|---|---|---|---|---|
| | | $10^7$ | $10^5$ | $10^4$ |
| | $10^6$ | 10 | 0.1 | 0.01 |
| | $10^4$ | 1000 | 10 | 1 |
| | $10^2$ | 100000 | 1000 | 10 |

**Table 6A. Bacteriophage and *Campylobacter* inoculation matrix**
Matrix showing the combinations of bacteriophage and *Campylobacter* titres used to inoculate chicken skin. The grid shows the multiplicity of infection (MOI) for each combination.

| *Campylobacter* inoculum (cfu) | Bacteriophage Inoculum (pfu) | | | | | | | | | Control Recovery | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $10^7$ | | | $10^5$ | | | $10^3$ | | | | | |
| | 1 | 3 | 5 | 1 | 3 | 5 | 1 | 3 | 5 | 1 | 3 | 5 |
| $10^6$ | 4.2 ± 0.2 | 4.2 ± 0.1 | 4.2 ± 0.1 | 4.9 ± 0.1 | 4.9 ± 0.1 | 4.9 ± 0.1 | 5.0 ± 0.1 | 4.9 ± 0.1 | 5.0 ± 0.1 | 5.3 ± 0.1 | 5.4 ± 0.1 | 5.2 ± 0.1 |
| $10^4$ | 1.9 ± 0.1 | 2.1 ± 0.0 | 2.3 ± 0.0 | 3.4 ± 0.2 | 3.5 ± 0.0 | 3.1 ± 0.1 | 3.3 ± 0.1 | 3.4 ± 0.2 | 3.4 ± 0.1 | 3.1 ± 0.4 | 3.4 ± 0.2 | 3.4 ± 0.2 |
| $10^2$ | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 1.8 ± 1.3 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.8 ± 1.3 | 0.0 ± 0.0 | 0.0 ± 0.0 |

**Table 6B. Recovery of *Campylobacter* from the surface of phage-treated chicken skin**

*C. jejuni* NCTC 12662 from the surface of chicken skin treated with bacteriophage ϕ2 and stored at 4°C. Results are shown as the $\log_{10}$ cfu recovery ± SD for different combinations of phage and host inocula recorded at days 1, 3 and 5 compared with controls.

treatment (2.12) on *Campylobacter* numbers was assessed using the one-tailed T-test with a confidence limit of 95%.

With the lowest phage titre applied ($10^3$ pfu) there was no statistically significant reduction in the numbers of *Campylobacter* recovered from fresh or frozen chicken skin compared with the control. *Campylobacter* recovery from controls inoculated with $10^6$ and $10^4$ cfu remained consistent for the course of the experiment with recovery ranging from $\log_{10}$ 5.2 - 5.4 cfu ($10^6$ cfu inoculation) and $\log_{10}$ 3.1 - 3.4 cfu ($10^4$ inoculation). In all cases of the highest titre phage treatment ($10^7$ pfu) there was a significant reduction in *Campylobacter* recovery by $\log_{10}$ 1.1 - 1.2 cfu ($10^6$ inoculation) and $\log_{10}$ 1.1 - 1.3 cfu ($10^4$ inoculation). Analysis of the data using a Student T-test showed that the reduction in numbers of recovered campylobacters was significant ($P<0.0001$). The difference in recovery was even greater in the frozen chicken skin samples where the application of $10^7$ pfu resulted in a reduction of $\log_{10}$ 2.3 and 2.5 cfu compared with the controls for *Campylobacter* inoculations of $10^6$ and $10^4$ cfu respectively. These reductions were also found to be significant ($P<0.0001$) using a one-tailed T-test.

### 6.1.3 Properties of recovered Campylobacters

One explanation for the recovery of *Campylobacter* cells from phage-treated skin could be the *in situ* selection for a sub-population of resistant bacteria. To determine if this was the case, campylobacters recovered from phage-treated and control chicken skin sections were used to produce lawns on NZCYM plates (2.3.1.7). These lawns were then re-inoculated with decimal dilutions of the phage $\phi$2 suspension used in the original skin inoculation using the surface droplet technique (2.3.6.4). All of the campylobacters

recovered from phage-treated and control chicken skin remained sensitive to ϕ2. The titres obtained on lawns of these bacteria did not differ significantly from those obtained on the original host strain ($P = 0.2$). The PFGE patterns produced by *Sma*I digestion of the genomic DNAs of the recovered *Campylobacter* strains (**2.5.9.1**) were indistinguishable from the inoculated strain (Fig. 6B).

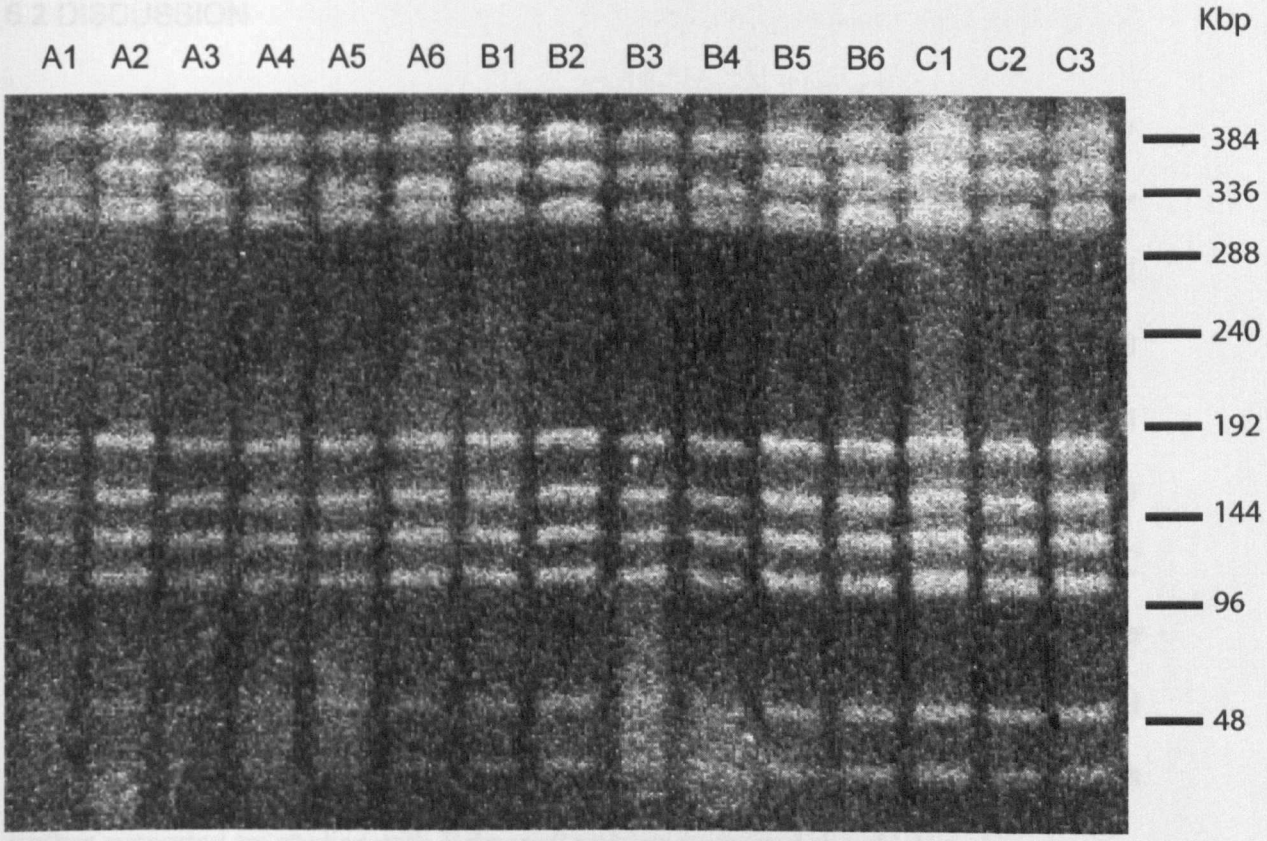


**Figure 6B. Macrorestriction profiles of *Campylobacter* from phage treated chicken skin**

A 1% agarose gel showing the macrorestriction profiles of whole genome preparations from *Campylobacter* colonies recovered from chicken skin treated with bacteriophage (A1 – A6). DNA digestion was performed using endonuclease *Sma*I prior to resolution of genomic fragments by PFGE. The profiles of campylobacters recovered from skin not treated with bacteriophage (B1 – B6) and from campylobacters not inoculated onto chicken skin (C1 – C3) are also shown. Lambda concatomer size markers (Kbp) are presented to the far right of the gel.

## 6.2 DISCUSSION

Recent studies have demonstrated the widespread contamination of retail poultry products with *Campylobacter* (**Jacobs-Reitsma, 2000; Kramer *et al.*, 2000; Berrang *et al.*, 2001; Jorgensen *et al.*, 2002**). Investigations into the survival of clinical *Campylobacter* isolates stored in suspension at different temperatures noted a $\log_{10}$ 3.0 cfu $ml^{-1}$ drop in viable cells upon freezing (**Chan *et al.*, 2001**). Data from the present study suggests this may not be the case for campylobacters on the surface of chicken skin where the reduction was closer to $\log_{10}$ 1.0 or $\log_{10}$ 2.0 for skin stored respectively at 4°C and -20°C. It is therefore likely that chicken skin has a protective effect on the *Campylobacter* cells. The protective effect may extend to different types of chemical and physical carcass decontamination methods (**Humphrey and Lanning, 1987**). This is further supported by studies with *Salmonella* which suggested the chicken skin provides an environment conferring non-specific protection to bacteria from potentially harmful treatments (**Humphrey and Lanning, 1987; Whyte *et al.*, 2001**). Such protection is most likely to stem from the presence of feather follicles and folds on the epidermal surface. Together with naturally-occurring oils and fats, this may offer some protection from ice crystal formation which is known to be a major cause of damage to cells undergoing the freezing process. Antecedent reports suggest that chicken carcasses can be contaminated with up to $10^9$ cfu *Campylobacter* (**Jorgensen *et al.*, 2002**). Since the infective dose of *Campylobacter* for humans is thought to be $\leq$ 500 cells (**Black *et al.*, 1988**), the fact that enhanced survival of *Campylobacter* on chicken skin was occurring under these conditions is of importance to food safety.

Supporting the data collated in Chapter 3, the present study demonstrated the ability of phage to survive on the surface of chicken skin for a period of at least ten days when stored under either fresh or frozen conditions. This is important when considering the potential efficacy and environmental impact of phage biocontrol on animals that carry *Campylobacter*. Phage of *Campylobacter* along with their hosts can potentially survive on the retail product until well after its prescribed shelf life. This may have an important influence on the opinions of the general public should *Campylobacter* phage therapy in poultry be commercialized.

A high titre of *Campylobacter*-specific bacteriophage applied to the surface of chicken skin inoculated with *Campylobacter* significantly reduced the number of recoverable host cells. This reduction was consistent over the course of the experiment with a fall in recovery of $\log_{10}$ 1.0 cfu for inoculated skin stored at 4°C. A larger reduction would be desirable if this practice was to yield commercial benefit in the future as a sole control measure. However, in its current state it could contribute to the overall reduction when applied with other decontamination practices. There was a significantly greater reduction in *Campylobacter* recovery associated with the combination of freezing and phage treatment. Countries such as Iceland routinely freeze *Campylobacter* positive carcasses in an attempt to reduce contamination. Combining this freezing with phage treatment could result in further reductions in *Campylobacter* prevalence on broiler carcasses.

It is generally accepted that campylobacters do not replicate when incubated at 4°C. Our data support this since the number of campylobacters which could be recovered from the surface of the skin stored at this temperature fell over time. There was also no increase in

bacteriophage numbers on any of the chicken skin samples inoculated with *Campylobacter*. Recovery of bacteriophage on all of the samples declined with time but did not differ significantly from the control. In the absence of active replication, the phage are unlikely to reduce the number of *Campylobacter* cells *in situ*. Consequently the most likely explanation for the observed reduction in *Campylobacter* recovery is that upon inoculation a proportion of the phage successfully adsorb to the surface of the bacteria but do not replicate until the bacterium itself increases its metabolic activity. This is supported by the data in Chapter 4 showing there is no reduction in either bacteriophage or *Campylobacter* titre when the phage is mixed with a non-permissive host in suspension, suggesting that non-specific adsorption or lysis by the phage is not taking place. This has implications for using phage as decontaminating agents as only populations of specifically susceptible bacteria would be affected. Effective cocktails of broad host range phage would be required for this technique to be practically applicable.

The campylobacters recovered following phage treatment of the chicken skin were diluted before placing them under conditions that will allow growth. This procedure enabled the enumeration of the surviving bacteria. Under these circumstances any bacterial cells with phage adsorbed would perish upon growth but the viral progeny resulting from such an infection could not easily disseminate to other host bacteria. However, in a situation where the phage-adsorbed bacteria are part of a larger, localized population of host cells, the phage progeny could initiate new infection cycles due to the propinquity of susceptible cells. If the conditions permissive for growth were a human or animal gut, then the initial viral infective dose would be reduced with the prospect of

further control of the host bacterium by the initiation of secondary and tertiary infection cycles.

Bacterial resistance to bacteriophage infection can be readily demonstrated in the laboratory and is postulated to be a major drawback for the use of phage in biocontrol. There was no evidence of resistance arising in the *Campylobacter* recovered following phage treatment in this study. The campylobacters recovered were confirmed as being genotypically identical to the inoculation strain by PFGE of *Sma*I restriction fragments of genomic DNA. However, in the absence of replication these studies do not rule out the generation of new mutational events selected post-treatment.

In conclusion this study has demonstrated the ability of a characterized NCTC *Campylobacter* and its bacteriophage predator to survive independently on the surface of chicken skin. Phage inoculated onto the surface of skin contaminated with *Campylobacter* exhibit a control effect even in the absence of host growth. Further development of this study could lead to the use of bacteriophage in connection with other measures to control chicken contaminated with *Campylobacter*.

# CHAPTER 7

# THE EFFECT OF *CAMPYLOBACTER* MEMBRANE VESICLES ON THE ADSORPTION AND REPLICATION OF BACTERIOPHAGE

## 7.0 INTRODUCTION

Many different genera of Gram negative bacteria have the potential to produce membrane vesicles (MV) at some point during their lifecycle (**Mayrand and Grenier, 1989; Zhou *et al.*, 1998**). During normal bacterial growth the rigid cell wall must be broken down, extended and then re-annealed. This process primarily consists of digesting peptidoglycan in the cell wall which liberates muramyl peptides into the periplasmic space. These peptides are unable to diffuse out of the cell which consequently leads to their accumulation in the periplasm, imposing a turgor pressure on the outer membrane (**Zhou *et al.*, 1998**). If this pressure is not alleviated, the outer membrane begins to herneate (bleb) and finally releases a MV from the cell (Fig. 7A).

MVs retain many of the surface structures of the cells from which they emanate, though their exact composition depends on the site at which the vesicle is most likely to form (**Beveridge, 1999**). Once liberated from the cell, mature MVs, once liberated from the cell, may interact with other Gram negative or Gram positive species in several ways.

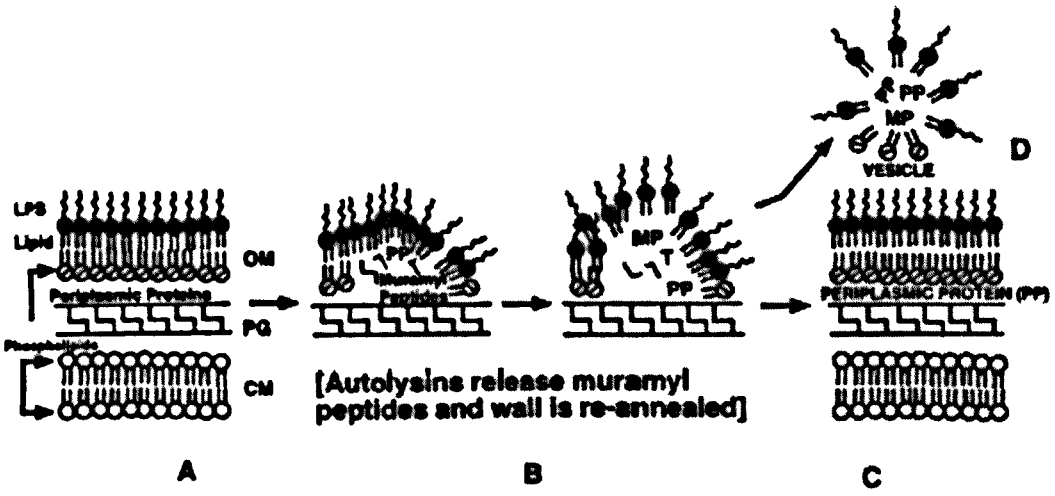


**Figure 7A. Origin of extracellular membrane vesicles in Gram-negative bacteria**

During growth, cell wall is excised and released from the peptidoglycan. The released muramyl peptides create a turgor pressure on the outer membrane. If the pressure is not alleviated by uptake of the muramyl peptides, the blebs will continue to grow and ultimately be shed into the growth medium. A: Idealized cross-section of Gram-negative envelope showing cytoplasmic membrane, periplasmic spaces, peptidoglycan and outer membrane. B: Turnover of peptidoglycan and initial herniation of outer membrane. C: Released vesicle containing components of outer membrane, periplasmic space and peptidoglycan components. CM, cytoplasmic membrane; PG, peptidoglycan; PP, periplasmic proteins; OM, outer membrane; LPS, lipopolysaccharide. Taken from Zhou *et al.* (1999).

The vesicles cannot fuse with Gram positive bacteria as they do not possess an outer membrane. Consequently, the vesicles often rupture and release their contents directly into the Gram positive cell wall. The contents of MVs usually include periplasmic proteins which are entrapped in their lumen during formation. Some of these proteins, e.g. peptidoglycan hydrolases, can cause direct damage to the Gram positive cell wall and have been shown to cause death in specific circumstances (**Li *et al.*, 1998**).

Fusion of MVs with Gram negative cells offers a greater range of possible outcomes. As with Gram positive cells, peptidoglycan hydrolases may attack the cell wall should the target cell not possess the appropriate homologous regulatory systems. Alternatively, MV periplasmic enzymes which inactivate or digest antibiotics (e.g. β-lactamase) may provide the recipient cell with a form of 'passive resistance' to antimicrobial agents without acquisition of resistance genes (**Beveridge, 1999**).

As MVs are predominantly formed from the Gram negative outer membrane they should possess many of the surface receptors necessary for bacteriophage adsorption. Bacteria may produce MVs which could act as molecular decoys, providing receptors in an attempt to protect the cell from antibiotics or phage. Campylobacters are known to produce MVs with components reflecting those present in the outer membrane (**Blaser *et al.*, 1983**). This study sought to establish if bacteriophage could adsorb to MVs produced by several *Campylobacter* species and whether binding impacted on the ability of phage to replicate in the host.

## 7.1 RESULTS

### 7.1.1 Analysis of *Campylobacter* membrane vesicle proteins

As a precursor to studying the effect of membrane vesicles on the adsorption of phage to host cells, *Campylobacter* MVs were prepared and characterised with respect to their biochemical, morphological and immunological properties. MVs were purified from overnight *Campylobacter* broth cultures using two protocols (A and B). Protocol A (**2.8.1**) consisted of sequential filtration, precipitation and centrifugation steps according to the method of (**Zhou *et al.*, 1998**). Protocol B (**2.8.2**) involved centrifugation of a culture filtrate through a 30 kDa molecular size cut off filter as a modification of the method used by (**Mayrand and Grenier, 1989**). The total protein content of each vesicle preparation was determined using the BCA assay (Pierce Biotech, **2.4.1.2**). A comparison of the total protein content of ten vesicle preparations from *C. jejuni* NCTC 12662 using protocols A and B is presented in Table 7A. The total quantity of protein in the vesicle preparations of the present study were generally inferior to those of other workers (**Mayrand and Grenier, 1989**). A possible reason for this is the use of quantitation assays which tend to overestimate the protein content in membranes (**Zhou *et al.*, 1998**). Vesicles prepared using protocol A contained an average of 2 mg $ml^{-1}$ protein which compared favourably with the 0.4 mg $ml^{-1}$ average using protocol B.

In order to determine the profile of proteins present in the MVs, the vesicle preparations were subjected to SDS-PAGE (**2.4.2**) alongside preparations of outer membrane proteins

| Preparation | Method A | Method B |
|---|---|---|
| 1 | 1.30 | 0.50 |
| 2 | 2.10 | 0.50 |
| 3 | 2.30 | 0.31 |
| 4 | 2.00 | 0.30 |
| 5 | 1.80 | 0.27 |
| 6 | 2.10 | 0.29 |
| 7 | 2.50 | 0.34 |
| 8 | 1.60 | 0.61 |
| 9 | 1.90 | 0.54 |
| 10 | 2.30 | 0.32 |
| **Mean** | **2.0 ± 0.4** | **0.4 ± 0.1** |

**Table 7A. Protein content of vesicle preparations.**
Table showing the total protein content of ten membrane vesicle suspensions prepared from *C. jejuni* NCTC 12662 using a molecular filtration technique (method A) and a precipitation technique (method B). Protein concentrations were measured using the BCA assay kit (Pierce Biotechnology) and are presented as mg $ml^{-1}$. Mean protein content ± SD are presented at the bottom of the table.

(2.9) and whole cells from the same strain used to prepare the vesicles. PAGE gels were initially stained with Coomassie blue (2.4.3.1) followed by silver staining (2.4.3.2) to detect differences in glycoprotein content. The protein profiles of vesicles prepared from *C. jejuni* NCTC 12662 using protocol A following Coomassie blue and silver staining are presented in Figure 7B. Protein profiles of vesicles prepared from the same strain using protocol B are presented in Figure 7C.

Both Coomassie and silver staining revealed that several proteins were present in both the vesicle and outer membrane preparations at a higher concentration than others. Based on previous studies, a band present at approximately 42 kDa is likely to be the major outer membrane protein (**Blaser *et al.*, 1983**). Likewise, bands present at 62 kDa are likely to be flagellin. Significantly fewer protein bands were observed for vesicles prepared using method B compared with method A. However, this is probably a reflection of the significant differences in total protein concentrations between vesicles prepared using the different methods. This is supported by the presence of weaker MOMP and flagellin bands in method B vesicle preparations compared with their method A counterparts.

With the aim of confirming the origin of the vesicles, polyclonal rabbit antisera raised against acid-glycine extractable proteins from *C. jejuni* was used in on-grid Immunogold labelling (2.7.1). Examination of these grids by transmission electron microscopy revealed that gold particles were clearly associated with MVs (Fig. 7C). Immunoblots (performed by Dr. P. Connerton) using the same antisera against preparations of *Campylobacter* whole cells, vesicles and outer membranes (2.4.5) confirmed antibody binding to proteins present in all three (Fig. 7C). This indicated the MVs contained

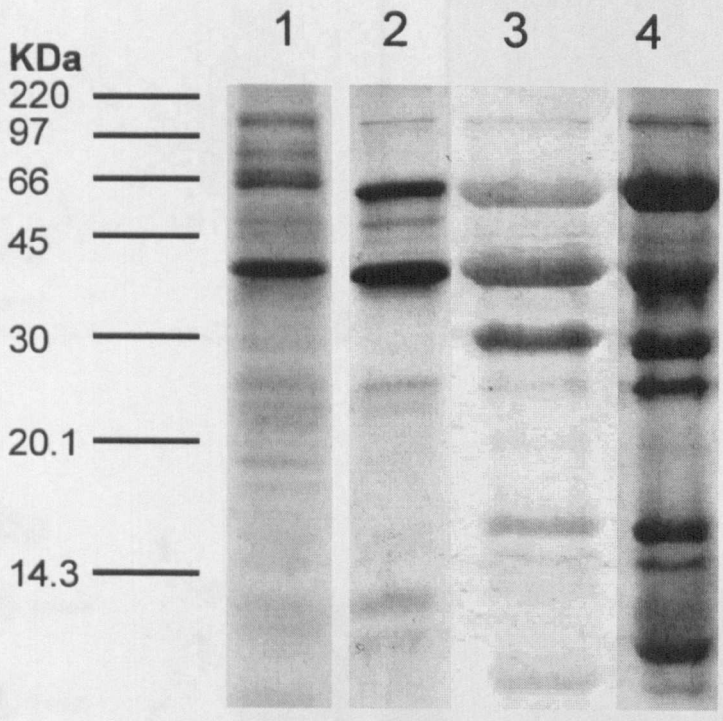


**Figure 7B. Protein profiles of outer membranes, membrane vesicles and whole cells of *Campylobacter*.**

A 12% SDS-PAGE gel showing the protein profiles of whole cells (1); outer membrane (2) and membrane vesicle (3) preparations following Coomassie blue staining. Lane 4 shows the appearance of the membrane vesicle preparation following silver staining. Molecular weight markers are shown on the left hand side of the figure.

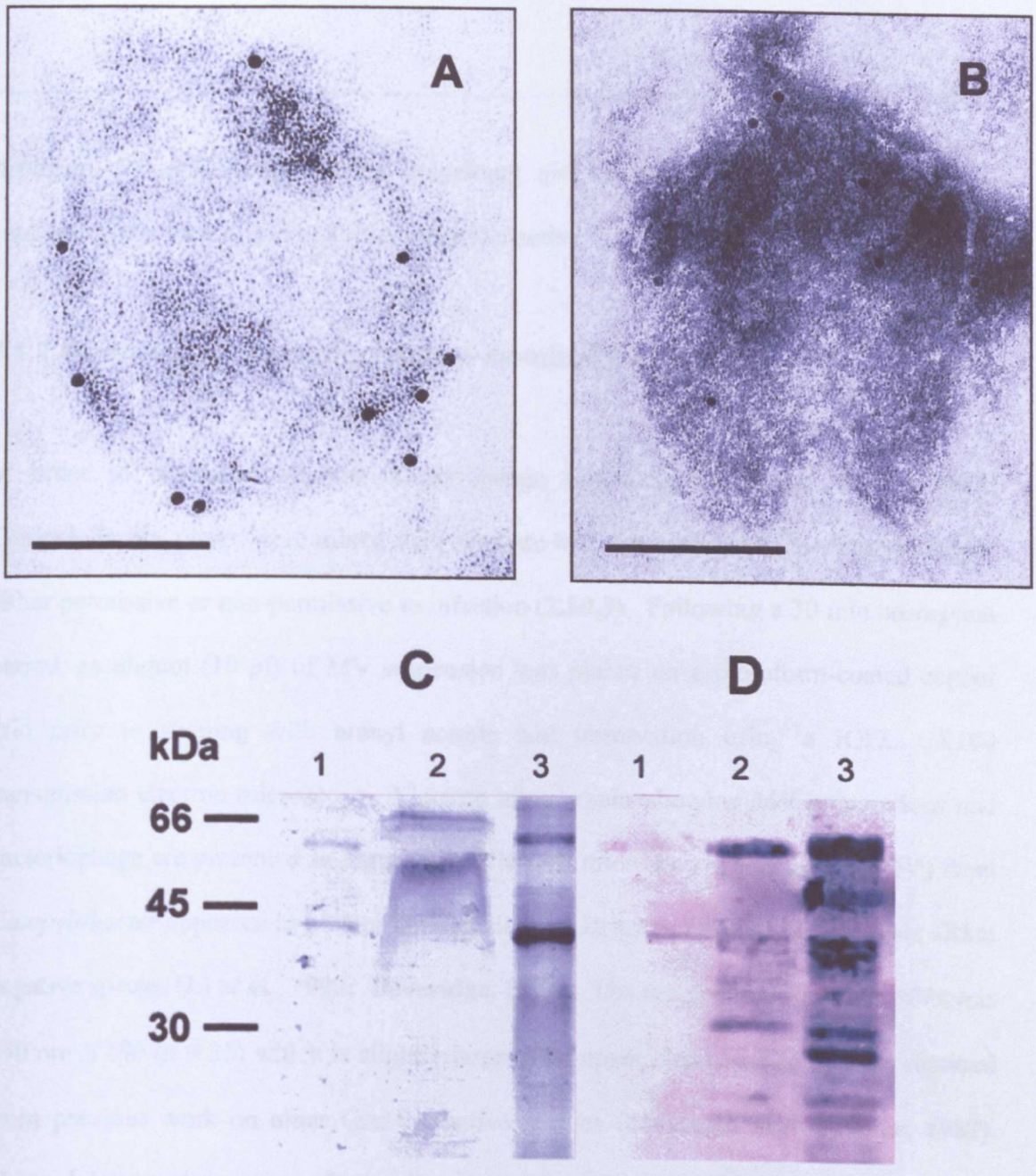


**Figure 7C. Immunogold staining of *Campylobacter* membrane vesicles**
The top half of the figure shows two electron micrographs of membrane vesicles from *Campylobacter jejuni* NCTC 12662 stained with gold particles (A and B). The membrane vesicles were incubated with antisera raised from a rabbit against a *Campylobacter* acid-glycine extractable proteins (anti-GE). Labelling was then performed using gold particles conjugated to goat anti-rabbit sera. Bar = 200 nm. The lower half of the figure outer membrane (1); membrane vesicle (2) and whole cell (3) preparations of *C. jejuni* NCTC 12662 resolved on a 10% SDS-PAGE gel stained with Coomassie blue (C) and immunoblotted using anti-GE antisera (D).

structural epitopes recognised by polyclonal anti-*Campylobacter* sera from rabbits, confirming that they originated from campylobacters.

### 7.1.2 Adsorption of bacteriophage to membrane vesicles

In order to determine whether bacteriophage bound to membrane vesicles from *Campylobacter*, phage were mixed with separate MV preparations from campylobacters either permissive or non-permissive to infection (**2.10.3**). Following a 30 min adsorption period, an aliquot (10 µl) of MV suspension was placed on a pioloform-coated copper grid prior to staining with uranyl acetate and observation using a JOEL CX100 transmission electron microscope. Electron micrographs showing MV preparations and bacteriophage are presented in Figure 7D. Electron microscopy revealed that MVs from *Campylobacter* appeared to be morphologically similar to those observed for other Gram negative species (**Li *et al.*, 1998; Beveridge, 1999**). The mean diameter of the MVs was 590 nm ± 190 ($n$ = 35) which is slightly larger than upper size range of 500 nm reported from previous work on other Gram negative species (**Mayrand and Grenier, 1989**). *Campylobacter* phage were observed associating with membrane vesicles of their host and possibly penetrating the vesicle wall to inject their genetic material into the lumen (Fig. 7D). However, phage were not observed adsorbing to the surface of vesicles purified from *Campylobacter* strains not permissive to infection.

Subsequent to these findings, a series of *in vitro* experiments were performed in order to gauge the possible impact of MVs on phage adsorption. MVs were prepared from three *C. jejuni* strains (NCTC 12660, NCTC 12661, NCTC 12662); one *C. coli* strain (NCTC

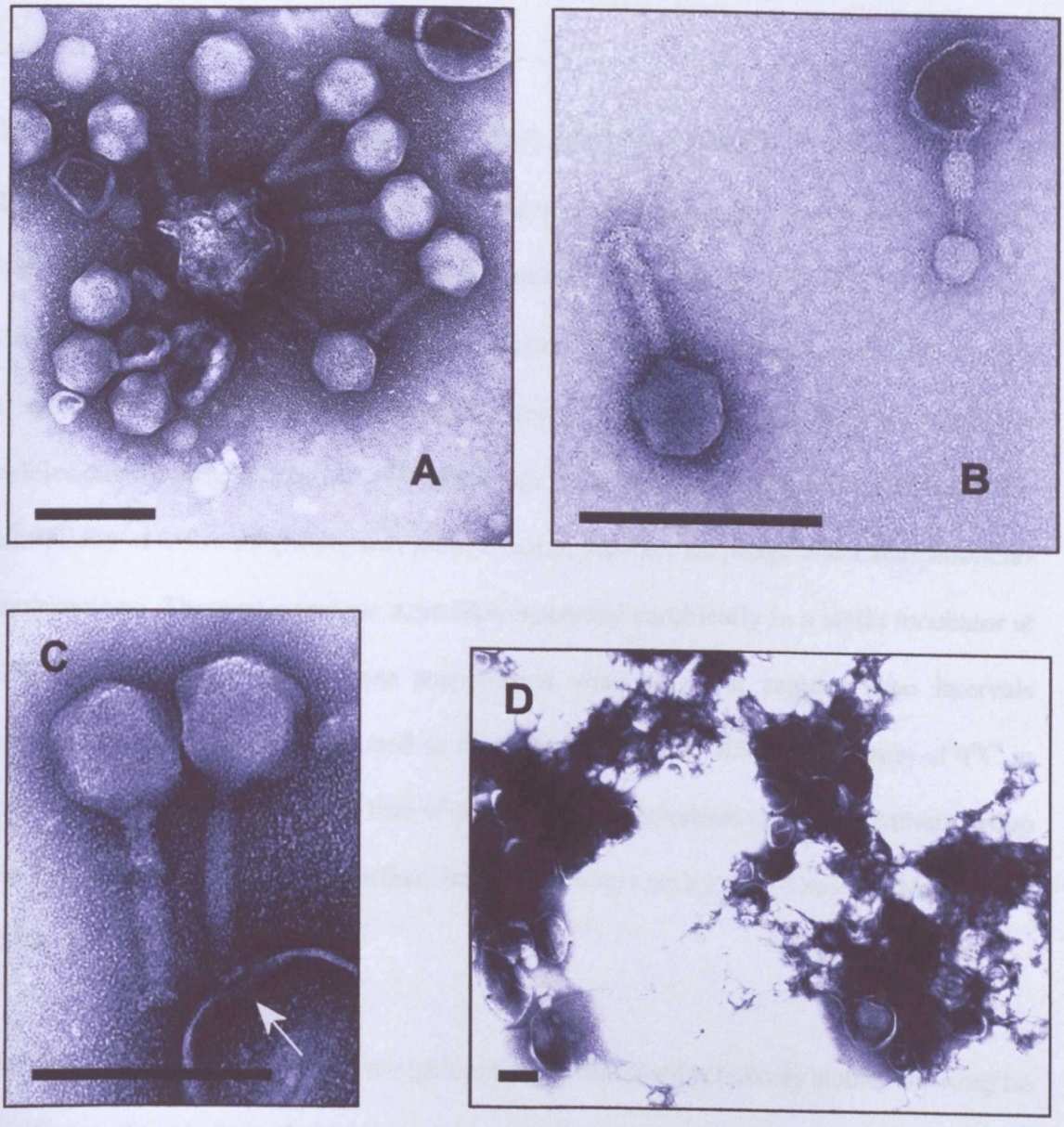


**Figure 7D. Bacteriophage associated with membrane vesicles.**
Electron micrographs showing the attachment of *Campylobacter* bacteriophage w2 to membrane vesicles derived from *C. jejuni* NCTC 12662 PT14 (A); Unadsorbed and ghost phage of w3 (B); Phage w5 adsorbed and penetrating (white arrow) membrane vesicle (C) and a purified vesicle preparation (D). Bar = 250 nm.

12666) and one *C. lari* strain (NCTC 11352) using the methods described previously (**2.8**). All of the bacteriophage used in this experiment (ϕ2, w2, w3, w4, w5, w8, w10, CP8) were known to infect the *C. jejuni* strains but not the *C. coli* or *C. lari* strains. Aliquots (0.5 ml) of different phage (titre approximately $10^8$ pfu $ml^{-1}$) were added to 4.5 ml volumes of either whole cells (approximately $10^9$ cfu $ml^{-1}$); whole cells plus vesicles; vesicles only or diluent only (10 mM $MgSO_4$ or Nutrient broth No. 2, NB2, **2.3.1.6**). The multiplicity of infection (MOI) was maintained at 0.01 for all phage and *Campylobacter* combinations. These suspensions were then incubated aerobically in a static incubator at 42°C. Aliquots (1 ml) of these suspensions were taken at regular time intervals throughout incubation and subjected to centrifugation at 40, 000 *g* for 60 min at 4°C in order to pellet the vesicles. The titre of phage in the supernatant of each treatment group was then determined using the surface droplet technique on a lawn of susceptible bacteria (**2.3.6.4**).

The titre of all phage in the controls (diluent only) remained relatively stable, showing no significant changes over the time courses. Adsorption to the 'host cell-only' and 'host cell with vesicles' preparations was rapid with all phage except w5. Titres of phage in the 'vesicle only' preparation resembled that of the diluent control and did not appear to be influenced by the either of the protocols used to prepare the vesicles. This suggested that either phage attachment to vesicles was a relatively rare event or that attachment was reversible. Additionally, the number of free phage in the supernatant of 'whole cell only' and 'whole cell plus vesicle' preparations was similar, suggesting whole cells were responsible for the majority of phage adsorption taking place. Generally, the pattern of phage adsorption observed for the different treatment groups did not vary appreciably

between the various phage and susceptible host combinations used. All showed a $\log_{10}$ 0.7 – 1.5 pfu $ml^{-1}$ fall in supernatant phage titre within 30 seconds of mixing with host cells. The exception was for phage w5 in which no significant adsorption to host *C. jejuni* NCTC 12662 was observed (Fig. 7E). This phenomenon has also been observed for *Campylobacter* phage ϕ5 (Dr. R. Mole, unpublished data). Changing the diluent medium (either 10 mM $MgSO_4$ or NB2) did not have any appreciable effect on the phage adsorption time. A representative graph showing the attachment dynamics of phage w2 to vesicle and whole cell combinations of host strain *C. jejuni* NCTC 12662 is presented in Figure 7E.

### 7.1.3 Replication of bacteriophage in the presence of membrane vesicles

The effect of membrane vesicles on the *in vitro* replication of bacteriophage was determined using strains *C. jejuni* NCTC 12660, 12661 and 12662 with phage w2, w5 and ϕ2 **(2.10.4)**. Whole cells were harvested from a one litre overnight culture by centrifugation and washing thrice in NB2 prior to resuspension in 9 ml NB2. This cell suspension was then divided into two 4.5 ml volumes. Vesicles were collected from the supernatant of the overnight culture using the centrifugation and filtration technique described previously **(2.8.2)** and made up to a total volume of 4.5 ml with NB2. Aliquots (1 ml) of phage suspension (titre approximately $10^8$ pfu $ml^{-1}$) were then each mixed with either the MV suspension or one of the whole cell preparations. Following a 15 min adsorption period, the 'phage plus whole cell' suspension was briefly mixed with 4.5 ml of NB2 prior to transfer to 200 ml of NB2. The 'phage plus MV' suspension was incubated in an identical fashion but was mixed with 4.5 ml of the 'uninfected'

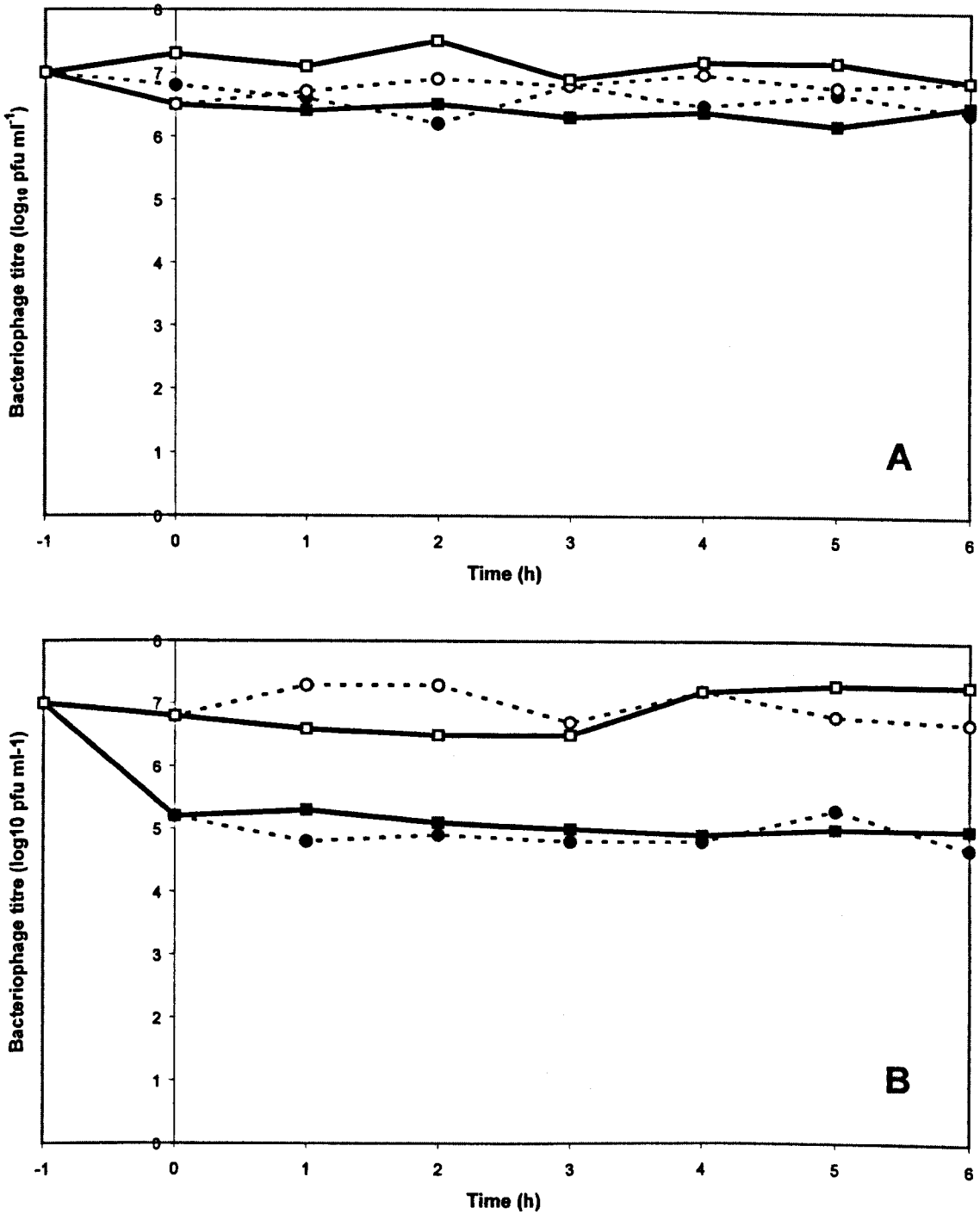


**Figure 7E. Adsorption of bacteriophage to host cells in the presence of membrane vesicles**

Bacteriophage were mixed with suspensions of host cells (■); host cells plus membrane vesicles (●); diluent only (□) and membrane vesicles only (○). These graphs show the titre of bacteriophage in the supernatant of each suspension following centrifugation. Graphs A and B show the respective adsorption of phage w5 and w2 to whole cell and vesicle suspensions derived from propagating strain *C. jejuni* NCTC 12662 over a period of 6 h. These results are based on three replicates.

*Campylobacter* cell suspension prior to immediate transfer to 200 ml of NB2. All suspensions were then incubated under microaerobic conditions at 42°C in a gyratory platform incubator set to 100 rpm. Nutrient broth (9ml) containing 1 ml of phage suspension was processed in an identical manner to the other suspensions and served as a negative control for the experiments. Volumes (1 ml) were taken periodically from each 210 ml culture and subjected to centrifugation at 13, 000 *g* for five minutes. Bacteriophage in the supernatant were enumerated on lawns of susceptible bacteria using the surface droplet technique **(2.3.6.4)**. Where applicable, bacterial cells in the pellets following centrifugation were thrice washed in MRD **(2.0.2)** prior to enumeration on charcoal agar **(2.3.1.4)**.

As can be seen in Figure 7F, membrane vesicles had no significant effect on the *in vitro* replication of phage w2 on *C. jejuni* NCTC 12662. A similar pattern was observed for phage ϕ2 but phage w5 failed to demonstrate any appreciable increase in progeny over the time course (Fig. 7F). There are two possible roles of MVs during phage replication in batch culture. Firstly, MVs containing adsorbed phage may be able to initiate infection following rapid re-fusion to susceptible host cells. Alternatively, adsorption to MVs may be reversible in which case specific phage attachment to host cells would be rapid following mixing with a susceptible host population (see Chapter 4).

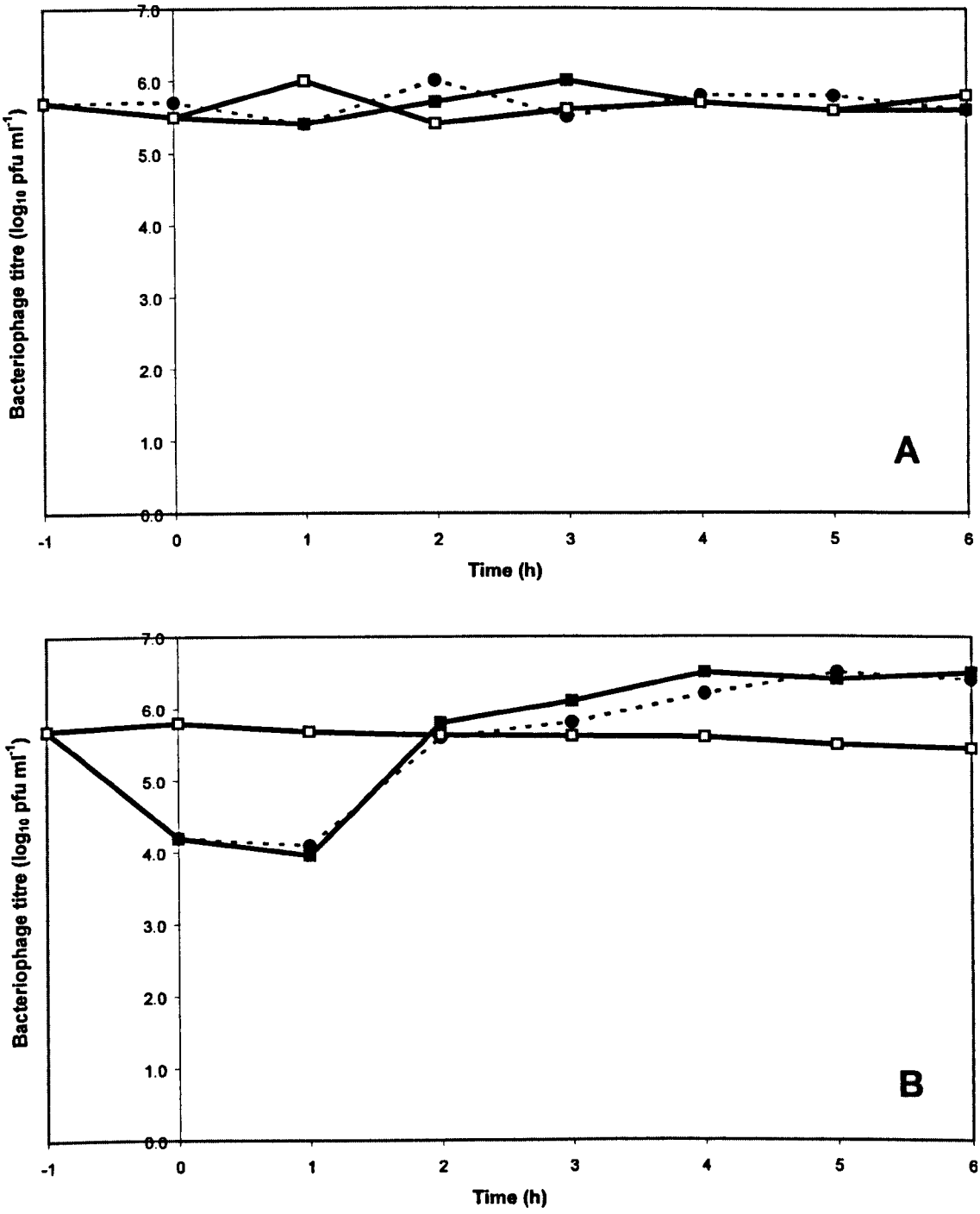


**Figure 7F. Replication of bacteriophage in the presence of membrane vesicles**

Bacteriophage were mixed with suspensions of host cells (■); host cells plus membrane vesicles (●) or diluent only (□). These graphs show the titre of bacteriophage in the supernatant of each suspension following centrifugation. Graphs A and B show the adsorption of phage w5 and w2 respectively to whole cell and vesicle suspensions derived from propagating strain *C. jejuni* NCTC 12662 over a period of 6 h. These results are based on three replicates.

## 7.2 DISCUSSION

The production of MVs by *Campylobacter* was perceived as a potential threat to the efficacy of phage intervention to control populations of this bacterium *in vivo*. This is a phenomenon not previously investigated by researchers with any other phage and host models. Several *Campylobacter* species were shown to produce MVs as part of their normal growth cycle in batch liquid culture. The protein profiles of these vesicles revealed that most of the *Campylobacter* OM proteins were present in MVs. Additional bands in the MV preparations were generally of a low molecular weight (< 42 kDa) and are likely to be periplasmic in origin as they were not observed in pure outer membrane preparations. The membrane polarity and integrity of membrane vesicles is usually maintained and as such many of the epitopes and receptor sites present on the bacterial cell surface are mirrored in the vesicles which they devolve (**Beveridge, 1999**). Immunogold staining along with western blotting confirmed the purified MVs originated from *Campylobacter* cells. Electron microscopy also revealed that phage are able to attach to MVs produced by *Campylobacter* strains susceptible to infection. However, no such attachment was observed for phage mixed with non-host *Campylobacter* strains suggesting such interactions are specific.

A series of *in vitro* studies demonstrated that, although phage adsorption to host cells was rapid, the permanent attachment of virions to purified MV preparations was not appreciable. Together with the electron microscopy data, this suggested that either attachment to membrane vesicles did not occur frequently or that attachment was a reversible process. Myovirus adsorption to the host cell is usually a two-step process,

with the primary association between phage and host being a reversible event (**Ackermann and DuBow, 1987**). Such reversible attachment to *Campylobacter* MVs may arise from a paucity of receptors on the vesicle surface or their relative inaccessibility. Phage such as K20 require the expression of multiple surface receptors (both LPS and OmpF) before full adsorption can take place (**Silverman and Benson, 1987**). Additionally, MVs do not always exactly mirror the outer membrane composition of the cells from which they originate. For example in *Pseudomonas aeruginosa*, where MVs evolve from specific sites with a higher concentration of type B LPS. This leads to vesicles with a highly skewed outer membrane composition containing no type A LPS which is found relatively frequently on the parent cell (**Beveridge, 1999**). Such a system of vesicle blebbing in *C. jejuni* may preclude irreversible adsorption of phage to MVs should some of the necessary receptors be absent. However, without knowing the receptor(s) for phage adsorption, this remains purely speculative.

As MVs did not appear to irreversibly bind phage, it was unsurprising that they had little impact on phage replication in *Campylobacter* batch cultures. *In vivo*, phage will likely be exposed to a number of biotic and abiotic barriers to their host of which the experimenter has little control. The data presented in this study suggests that host-derived membrane vesicles would not present a significant obstacle in the therapeutic use of phage to reduce *Campylobacter* populations.

# CHAPTER 8

## THE ABILITY OF BACTERIOPHAGE TO CONTROL *CAMPYLOBACTER* POPULATIONS IN THE BROILER CHICKEN INTESTINE

### 8.0 INTRODUCTION

The potential of bacteriophage to treat bacterial infections was quickly realised following their independent discovery by **Twort (1915)** and **D' Herelle (1917)**. The first known "phage therapy" study was undertaken by **Bruynoghe and Maisin (1921)** working on staphylococcal skin infections. Although the results of this trial were promising, little more was accomplished in this field during the subsequent years (**Weber-Dabrowska *et al.*, 2000**).

Interest in phage therapy was reignited in the 1930s, although during this time reports of its efficacy were increasingly disparate. Even the very nature of bacteriophage became a contentious issue with some believing an enzyme to be responsible for the bactericidal properties observed (**Eaton and Bayne-Jones, 1934**). This may not be surprising as components of the virion can demonstrate lysis of their hosts without the need for replication (**Nakagawa *et al.*, 1985; Loeffler *et al.*, 2001**). The failure of some of the early therapeutic phage interventions was due to a lack of understanding of viral biology. Present-day virologists have extensively reviewed the problems encountered in early

phage therapy trials (**Kutter, 1997; Sulakvelidze *et al.*, 2001; Summers, 2001**), which have been summarised below.

1. Paucity of understanding the heterogeneity and ecology of both the phage and the bacteria involved.
2. Failure to select virulent phage prior to treatment.
3. Using single phage preparations to treat a mixed bacterial population.
4. Emergence of resistant strains either by selection or lysogeny.
5. Failure to characterise and/or titre phage prior to use.
6. Not neutralising gastric pH prior to phage oral administration.
7. Inactivation of phage by specific and non-specific host immunity.
8. Liberation of endotoxins by lysis (Herxheimer reaction).
9. Lack of reference laboratories for bacterial infections so that suitable virulent phage could be identified.

(Modified from **Kutter, 1997**)

The greater comprehension of bacteriophage today has enabled scientists to exploit the potential benefits of phage therapy (see Chapter 1). The reassessment of bacteriophage as therapeutic agents in the West was spearheaded by an investigation into the treatment of systemic *E. coli* infections in mice (**Smith and Huggins, 1982**) and then calves and piglets in the 1980s (**Smith and Huggins, 1983**). This work was recently repeated and expanded by other workers with similar success (**Bull *et al.*, 2002**). In recent years there

has been a growing archive of literature demonstrating the efficacy of phage therapy *in vivo*. These include work by **Berchieri *et al.* (1991); Soothill (1992); Barrow *et al.* (1998)**; and more recently by **Park *et al.* (2000); Biswas *et al.* (2002)** and **Cerveny *et al.* (2002)**.

The current advances of phage therapy *in vivo* have prompted several theoretical papers on the mathematical dynamics of phage infection **(Wiggins and Alexander, 1985; Payne and Jansen, 2001)**. The models derived from these studies are useful tools in honing phage therapy towards specific objectives for controlling selected bacterial populations. They also highlight that care must be taken to fully characterise the replication dynamics of both phage and host as even minor changes in the timing of phage inoculation and/or the titre administered can have a dramatic effect on the outcome of therapy **(Payne and Jansen, 2001)**. The relative density of the host must also be considered in the design of phage intervention strategies. Both theoretical **(Payne and Jansen, 2000; Payne and Jansen, 2001)** and practical **(Wiggins and Alexander, 1985)** studies report that a critical bacterial density is required to support phage replication *in vivo*.

*Campylobacter* readily colonises the chicken gut **(Beery *et al.*, 1988)** and is highly prevalent in UK flocks **(Humphrey *et al.*, 1993)**. Fresh poultry produce is widely regarded as the major reservoir for human campylobacteriosis **(Jacobs-Reitsma, 2000)**. Bacteriophage-mediated biocontrol of food-borne pathogens has already enjoyed some success with *Salmonella enterica* in poultry **(Barrow *et al.*, 1998)** and fresh fruit **(Leverentz *et al.*, 2001)**, albeit in different circumstances to the present study.

Campylobacters are not generally pathogenic in chickens and so, in the context of this study, "phage therapy" is a misnomer as there is no evidence that the presence or absence of *Campylobacter* in the chicken gut adversely affects the birds' health. The paradigm of this study denotes "phage therapy" as a process utilised to indirectly alleviate a major human public health burden rather than treating a veterinary disease.

The ability of a strain of *Campylobacter* to reliably colonise the chicken gut to within a narrow density range was a prime concern prior to commencing therapeutic phage trials since large fluctuations in host cell density in the targeted organs could result in highly variable efficacy (**Payne and Jansen, 2001**). To this end, the first aim of this study was to determine the ability of several commercial broiler chicken *Campylobacter* isolates to re-colonise broiler chickens under experimental conditions. Once this was complete and suitable hosts were selected, the ability of bacteriophage isolated from chicken excreta (CP8) and retail poultry (w2) to control populations of their hosts in the broiler chicken gastrointestinal tract (Fig. 8A) was investigated. The study concluded by characterising phage-resistant campylobacters re-isolated from phage-treated birds with respect to their colonisation potential and pulsed field gel electrophoresis (PFGE) macro-restriction profiles.

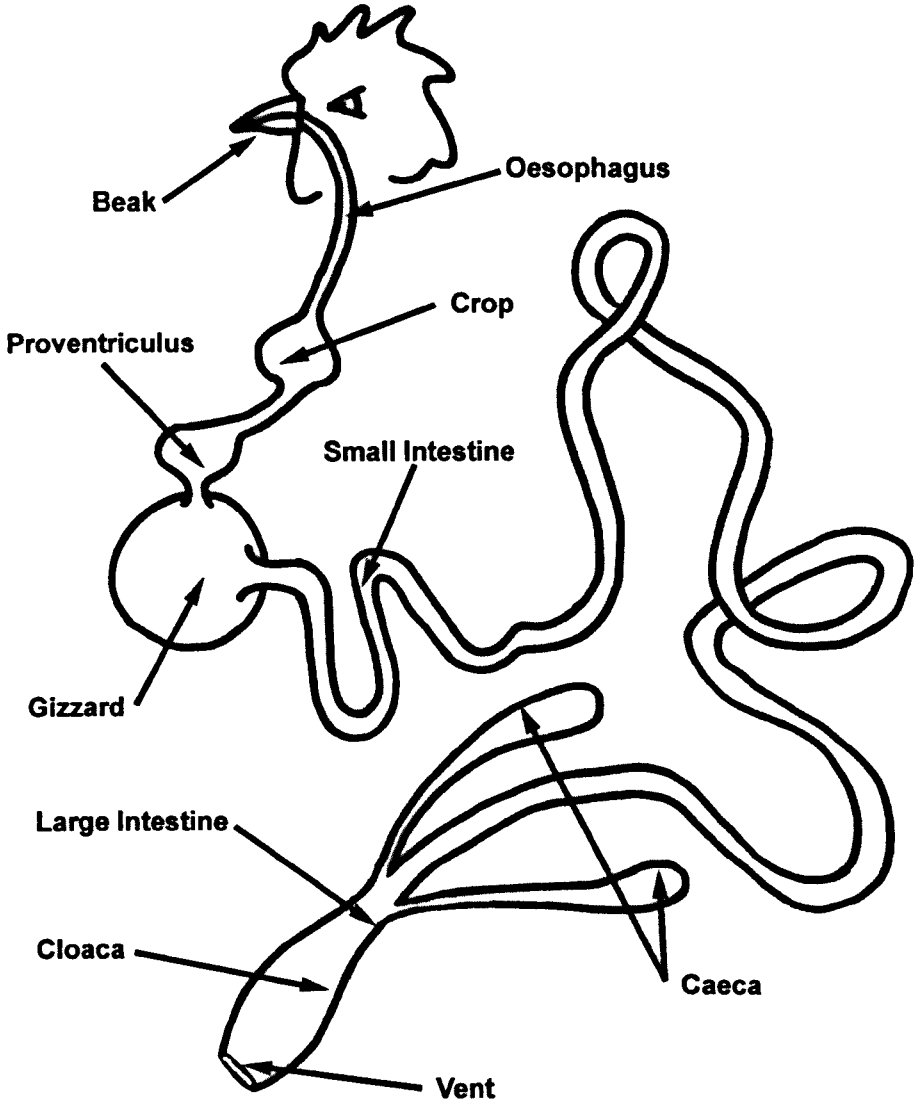


**Figure 8A. Gastrointestinal tract of the domestic chicken.**
Diagram showing the major organs of the chicken gastrointestinal tract. The crop is the major site of food storage and also moistens the food prior to digestion. The proventriuculus produces peptic enzymes which aid the breakdown of ingesta prior to grinding in the gizzard. The small intestine is the main site of enzymatic digestion and nutrient adsorption in the chicken and shares many features with the mammalian equivalent. The caeca function in the adsoption of water, the digestion of fibre and the synthesis of water soluable vitamins. The primary role of the large intestine is water adsorption prior to storage of waste in the cloaca. In females, the cloaca is a junction between the gastrointestinal, urinary and reproductive systems.

## 8.1 RESULTS

### 8.1.1 Isolation of *Campylobacter* and *Salmonella* from broiler excreta prior to and during trials

The chickens used in the phage therapy and *Campylobacter* colonisation trails were reared to be free of *Campylobacter* ssp. and *Salmonella* ssp. **(2.11.1)** but were not specific pathogen free (SPF). Conventionally-reared birds were chosen for the trials to more accurately reflect the probable outcome of phage therapy should it be commercially implemented. Prior to commencing all the trials, samples of excreta were taken periodically from each chick and screened for the presence of *Salmonella* ssp **(2.11.2)** and *Campylobacter* ssp. using enrichment and direct plating techniques specific for each genus. Naturally-occurring *Campylobacter* and *Salmonella* were not recovered from any of the excreta samples taken from the chicks on any of the trials. *Campylobacter* colonies recovered from each experimental and control group ($n$ = 10 per group where applicable) were subjected to *fla* typing **(2.5.8)** to confirm identity with the inoculated strain of *Campylobacter* used in each trial. All recovered *Campylobacter* colonies showed identity to their respective inoculated strains and no 'rogue' *fla* types were detected in any of the trials. No campylobacters or bacteriophage were recovered from 'true negative' chicken controls at any point during any of the trials. These data suggested that the chickens used in the trials were not colonised by environmental *Campylobacter* or bacteriophage strains at detectable levels.

### 8.1.2 Trial 1: Colonisation potential of *Campylobacter* excreta isolates.

Previous reports have recorded considerable variation in the ability of different *Campylobacter* strains to colonise the chicken caecum (**Cawthraw *et al.*, 1996; Ringoir and Korolik, 2003**). The impact of bacteriophage on bacterial populations can be determined more accurately if the colonisation potential of their host is high and shows minimal deviation between birds inoculated simultaneously. As a first step to selecting a host with these traits the colonisation potential of three strains of *Campylobacter* (GIIC8, HPC5 and OCIIC10, previously isolated from chicken excreta by Ms. C. Loc-Carrillo) over a five day period was determined (**2.11.5**). Data showing the colonisation level of these strains in chicken caeca is presented in Figure 8B. The broiler chicken caecum was colonised to average titres ($log_{10}$ cfu $g^{-1}$ caecal contents) of 7.28 ± 0.52 (GIIC8), 6.9 ± 1.31 (HPC5) and 8.08 ± 0.39 (OCIIC10) respectively. Strain OCIIC10 was the most stable and successful coloniser with an average titre of $log_{10}$ 8.08 ± 0.39 cfu $g^{-1}$ caecal contents over the five day period of the trial. Strains GIIC8 and HPC5 colonised the caeca to a comparable level (approximately $log_{10}$ 7.0 cfu $g^{-1}$) but HPC5 showed greater variation (± $log_{10}$ 1.31) than GIIC8 ($log_{10}$ ± 0.52). Figure 8B also shows the comparative colonisation of a well-characterised human faecal isolate (*C. jejuni* 81116) recorded in a concurrent study by Ms. C. Loc-Carrillo. Strain 81116 colonised the chicken caecum to an average level of $log_{10}$ 7.4 ± 0.65 cfu $g^{-1}$ over the course of the trial which was significantly lower ($P<0.001$) than observed by other workers ($log_{10}$ 9.6 cfu $g^{-1}$) using the same strain passaged through the chicken gut (**Cawthraw *et al.*, 1996**). This suggested that maximal colonisation of the chicken caecum would probably be achieved by minimally-passaged *Campylobacter* isolates from the chicken intestine.

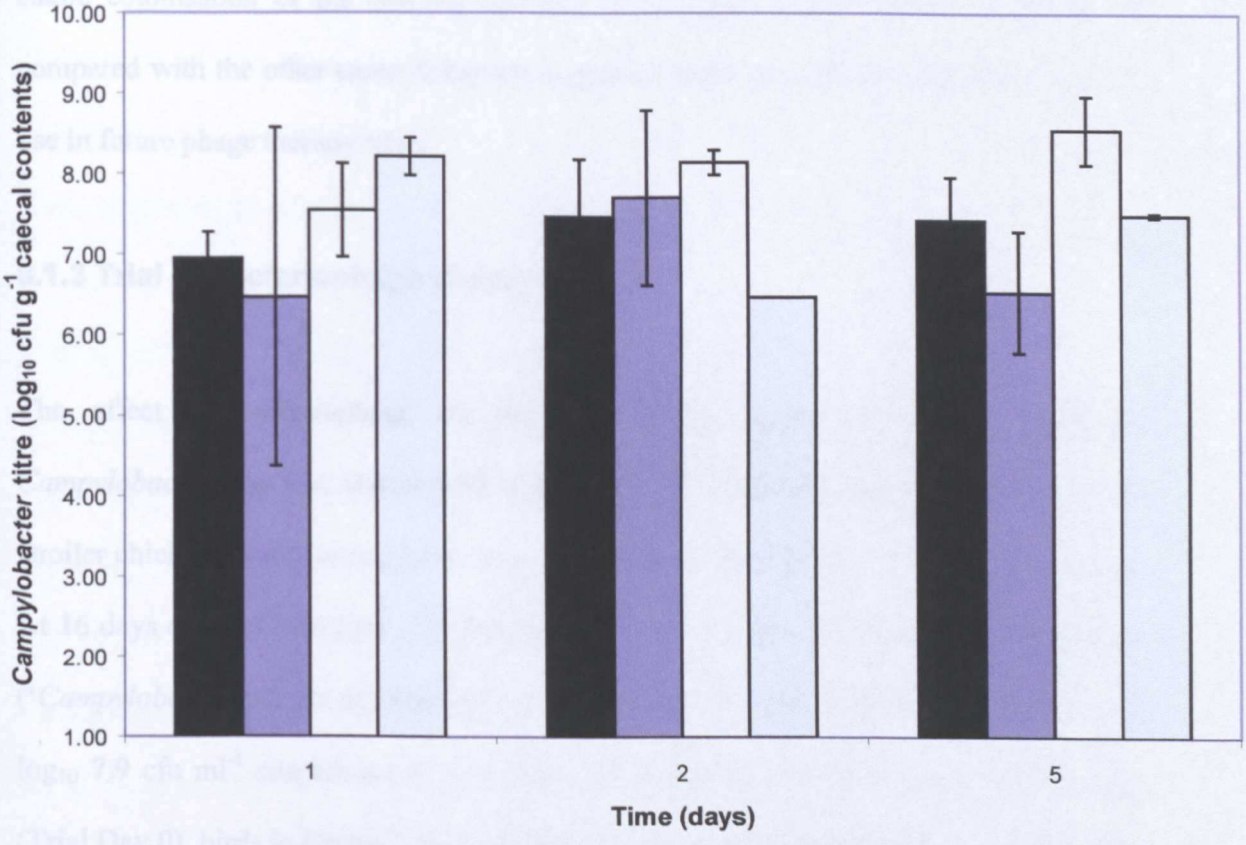


**Figure 8B. Colonisation potential of *Campylobacter jejuni* isolates**
The level to which four strains of *Campylobacter jejuni* colonise the broiler chicken caecum under experimental conditions. Data are presented for chicken isolates GIIC8 ( ■ ); HPC5 ( ▨ ) and OCIIC10 ( □ ) along with human isolate 81116 ( ▨ ). The colonisation level was recorded as $\log_{10}$ cfu $g^{-1}$ caecal contents ± standard deviation. These results are based on three replicates.

*Campylobacter* strains recovered from each experimental and control group showed identical *fla* types to the strains used to inoculate the chickens. The relatively high and stable colonisation of the chicken caecum by *C. jejuni* strains GIIC8 and OCIIC10 compared with the other campylobacters suggested these were the most suitable hosts to use in future phage therapy trials.

### 8.1.3 Trial 2: Bacteriophage therapy trial A.

The effect of bacteriophage on the colonisation of broiler chicken caeca by *Campylobacter* was first investigated using phage CP8 with host strain *C. jejuni* GIIC8. Broiler chicks ($n = 40$) were placed into four treatment groups (4 x 10) at 15 days of age. At 16 days of age (Trial Day -2), birds in Group 2 ('*Campylobacter* only') and Group 4 ('*Campylobacter* plus bacteriophage') were each dosed with approximately 1 ml of a $\log_{10}$ 7.9 cfu $ml^{-1}$ suspension of *C. jejuni* GIIC8 prepared in PBS. At 19 days of age (Trial Day 0), birds in Group 3 ('bacteriophage only') and Group 4 ('*Campylobacter* plus bacteriophage') were each dosed with approximately 1 ml of a $\log_{10}$ 8.7 pfu $ml^{-1}$ suspension of phage CP8 prepared in 10 mM phosphate buffer (PB) supplemented with 30% w/v $CaCO_3$ as an antacid. Birds in Group 1 ('true negative controls') were dosed with PB containing 30% w/v $CaCO_3$. On each of the five days following phage dosing, two birds from each group were sacrificed and the titres of phage (**2.11.8**) and *Campylobacter* (**2.11.7**) in the caeca were determined. The presence of phage and *Campylobacter* in a selection of vital organs was determined by direct plating of tissue samples macerated in maximum recovery diluent (MRD, **2.02**) onto mCCDA selective

agar (**2.3.1.4**). These organs included the heart, liver, kidneys, pancreas, crop, gizzard, proventriculus, small intestine, caeca and large intestine.

The effect of bacteriophage CP8 on the colonisation of broiler chicken caeca by *Campylobacter jejuni* strain GIIC8 is presented in Figure 8C. The '*Campylobacter* only' controls (Group 2) exhibited an average caecal colonisation level of $\log_{10}$ 7.72 ± 0.32 cfu $g^{-1}$ over the period of the trial. This was marginally above the colonisation level observed in trial 1 ($\log_{10}$ 7.28 ± 0.52 cfu $g^{-1}$). In contrast, a fall of $\log_{10}$ 4.85 ± 1.77 cfu $g^{-1}$ was recorded for the Group 4 ('*Campylobacter* plus bacteriophage') on the first day following phage treatment (Trial Day 1) compared with the control. The second day after phage (Trial Day 2) treatment saw the titre of the *Campylobacter* host in the experimental group fall below the limit of detection (<$\log_{10}$ 2.0 cfu $g^{-1}$). Thereafter, the average *Campylobacter* colonisation levels in this group for days three, four, and five were $\log_{10}$ cfu $g^{-1}$ 2.96 ± 0.05, 3.74 ± 0.25 and 3.83 ± 0.11 respectively. The difference in average *Campylobacter* colonisation between Group 2 and Group 4 over the course of the trial was significant ($P < 0.00005$). All of the campylobacters recovered from the chicks were confirmed to be of the same *fla* type as the inoculated strain. None of them showed any appreciable resistance to infection by CP8 in *in vitro* assays (**2.3.6.5**). The titre of phage in the caeca decreased from $\log_{10}$ 6.14 pfu $g^{-1}$ on Trial Day 1 to below detectable limits by Trial Day 3. All *Campylobacter* control birds harboured *Campylobacter* in the small intestine, large intestine and caecum. The crops of 2/10 control birds were *Campylobacter* positive and one bird had a positive gizzard. No *Campylobacter* cells were recovered from any sample site outside of the chicken's gastrointestinal tract. The results from this trial indicated that bacteriophage therapy could potentially reduce the

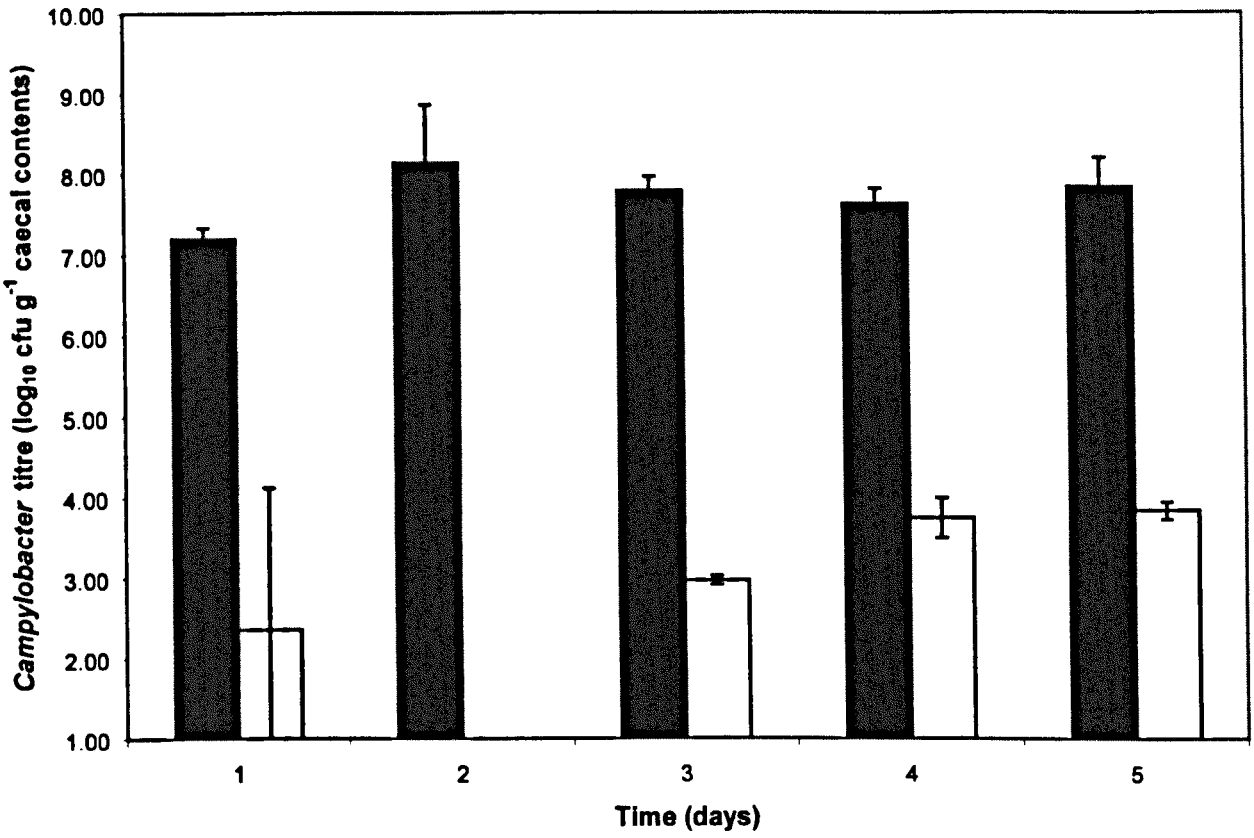


**Figure 8C. Bacteriophage therapy trial A (CP8 and GIIC8)**
The colonisation of *Campylobacter jejuni* strain GIIC8 in the chicken caecum when exposed ( ☐ ) and not exposed ( ■ ) to bacteriophage CP8 at day 0. The colonisation level was recorded as $\log_{10}$ cfu $g^{-1}$ caecal contents ± standard deviation over a 5-day period. These results are based on two replicates.

caecal colonisation of a susceptible *Campylobacter* strain by $\geq \log_{10}$ 8.0 cfu $g^{-1}$. The colonisation of *C. jejuni* GIIC8 in the control group was relatively stable and did not show any indications of invading organs external to the gastrointestinal system.

### 8.1.4 Trial 3: Bacteriophage therapy trial B

This trial sought to determine the reproducibility of the significant reductions in caecal *Campylobacter* colonisation recorded when using bacteriophage CP8 in trial 2. An additional aim of this trial was to establish if antacid administration (30 % $CaCO_3$ suspension) to the chickens had any significant impact on the efficacy of phage therapy. At 15 days of age, broiler chicks ($n = 40$) were placed into one of five groups. At 16 days of age (Trial Day -2), birds in Group 2 ($n$ = 15, '*Campylobacter* plus bacteriophage, no antacid'), Group 4 ($n$ = 10, '*Campylobacter* only' controls) and Group 5 ($n$ = 10, '*Campylobacter* plus bacteriophage, with antacid') were each dosed with approximately 1 ml of a $\log_{10}$ 8.6 cfu $ml^{-1}$ suspension of *C. jejuni* GIIC8. At 19 days of age (Trial Day 0), the birds in Group 2 ( '*Campylobacter* plus bacteriophage, no antacid'), Group 3 ($n$ = 2, 'bacteriophage only with antacid') and Group 5 ('*Campylobacter* plus bacteriophage, with antacid') were each dosed with approximately 1 ml of a $\log_{10}$ 8.7 pfu $ml^{-1}$ suspension of CP8 prepared in PB with or without antacid. Birds in Group 1 ($n$ = 2, 'true negative controls') were each dosed with approximately 1 ml of PB with antacid. During each of the five days following phage dosing, birds were sacrificed from Group 2 ($n$ = 3), Group 4 ($n$ = 2) and Group 5 ($n$ = 2). Birds from Groups 1 and 3 were sacrificed on the final day of the trial (Trial Day 5). The titres of phage (**2.11.8**) and *Campylobacter* (**2.11.7**) in the caeca of the birds was determined as previously described.

The effects of administering phage CP8 with and without $CaCO_3$ supplementation on the colonisation of the broiler chicken caeca by *C. jejuni* GIIC8 are presented in Figure 8D. The average *Campylobacter* colonisation of the caeca in the control group over the course of the trial was $\log_{10}$ 6.7 ± 0.4 cfu $g^{-1}$. This was lower than the average colonisation level recorded in the preceding trial ($\log_{10}$ 7.72 ± 0.32 cfu $g^{-1}$). In contrast with trial two, no significant difference between the experimental groups and the control were observed until day five of the trial ($P < 0.0005$). There was no significant difference ($P = 0.22$) in the efficacy of phage treatment with or without the concurrent dosage of phage with 30% $CaCO_3$ throughout the course of the trial. Again, the titre of phage CP8 in the caeca fell during the course of the experiment from $\log_{10}$ 4.7 ± 0.9 pfu $g^{-1}$ on Trial Day 1 to below the limit of detection by Trial Day 3. All *Campylobacter* control birds harboured *Campylobacter* in the small intestine, large intestine and caecum. All of the campylobacters recovered from the chicks were confirmed to be of the same *fla* type as the inoculated strain. None of them showed any appreciable resistance to infection by CP8 in *in vitro* assays. The results of this trial suggested phage therapy was effective in reducing *Campylobacter* colonisation in a repeat of trial 2. However, the reduction in host population took appreciably longer in trial 3 than trial 2. In addition, although the fall in *Campylobacter* colonisation was significant in trial 3, it was much less pronounced than in the preceding trial. The lower host cell density recorded in the '*Campylobacter* only' control group in trial 3 could have influenced the ability of phage to find a suitable host and thus delayed the onset of the *Campylobacter* population decline.

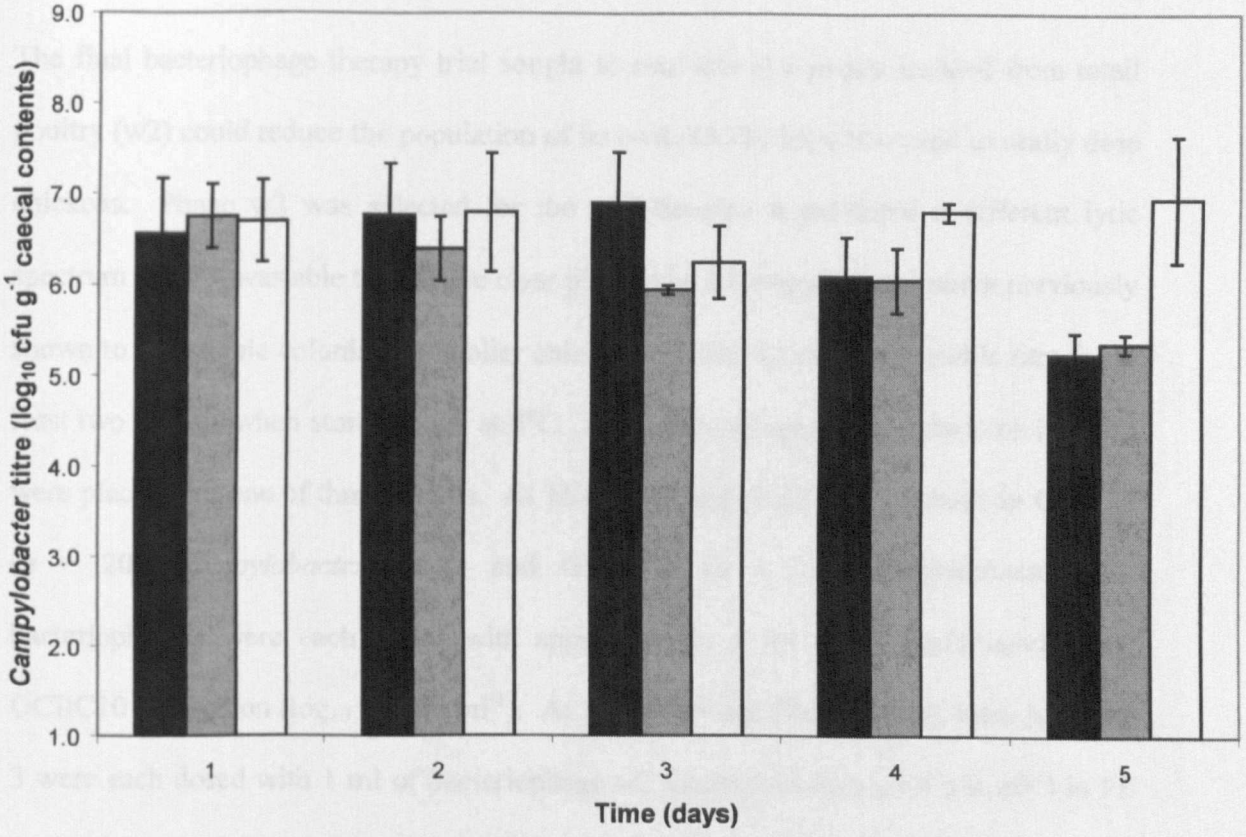


**Figure 8D. Bacteriophage therapy trial B (CP8 and GIIC8)**
The colonisation of *Campylobacter jejuni* strain GIIC8 in the chicken caecum when exposed to bacteriophage CP8 with ( ■ ) and without ( ▒ ) antacid on day 0 compared with the *Campylobacter*-only control ( □ ). The colonisation level was recorded as $\log_{10}$ cfu $g^{-1}$ caecal contents ± standard deviation over a 5-day period. These results are based on three replicates.

### 8.1.5 Trial 4: Bacteriophage therapy trial C.

The final bacteriophage therapy trial sought to establish if a phage isolated from retail poultry (w2) could reduce the population of its host (OCIIC10) when used to orally dose chickens. Phage w2 was selected for the trial because it exhibited a different lytic spectrum to CP8, was able to produce clear plaques on a *Campylobacter* strain previously shown to be a stable coloniser of broiler chickens (OCIIC10) and had a stable titre for at least two months when stored in PB at 4°C. At 15 days of age, broiler chickens ($n$ = 42) were placed into one of three groups. At 16 days of age (Trial day -2), birds in Group 2 ($n$ = 20, '*Campylobacter* only') and Group 3 ($n$ = 20, '*Campylobacter* plus bacteriophage') were each dosed with approximately 1 ml of *Campylobacter jejuni* OCIIC10 suspension ($\log_{10}$ 8.0 cfu $ml^{-1}$). At 19 days of age (Trial Day 0), birds in Group 3 were each dosed with 1 ml of bacteriophage w2 suspension ($\log_{10}$ 9.4 pfu $ml^{-1}$) in PB containing 30% w/v antacid. Birds in Group 1 ($n$ = 2, 'true negative controls') were each dosed with 1 ml of PB. Following *Campylobacter* dosing on Trial Day -2, three birds from Group 2 were sacrificed daily until Trial Day 0 whereafter two birds were sacrificed per day until the end of the trial. Following phage dosing on Trial Day 0, four birds were sacrificed daily from Group 3 until the end of the trial. The birds in Group 1 'true negative controls' were all sacrificed at the end of the trial (Trial Day 5). The titres of *Campylobacter* and bacteriophage in the caeca of the sacrificed broiler chickens were determined using the methods described in sections **2.11.7** and **2.11.8** respectively.

The impact of phage w2 on the colonisation of the broiler chick caeca by *C. jejuni* OCIIC10 is presented in Figure 8E. The *Campylobacter* control group colonised the

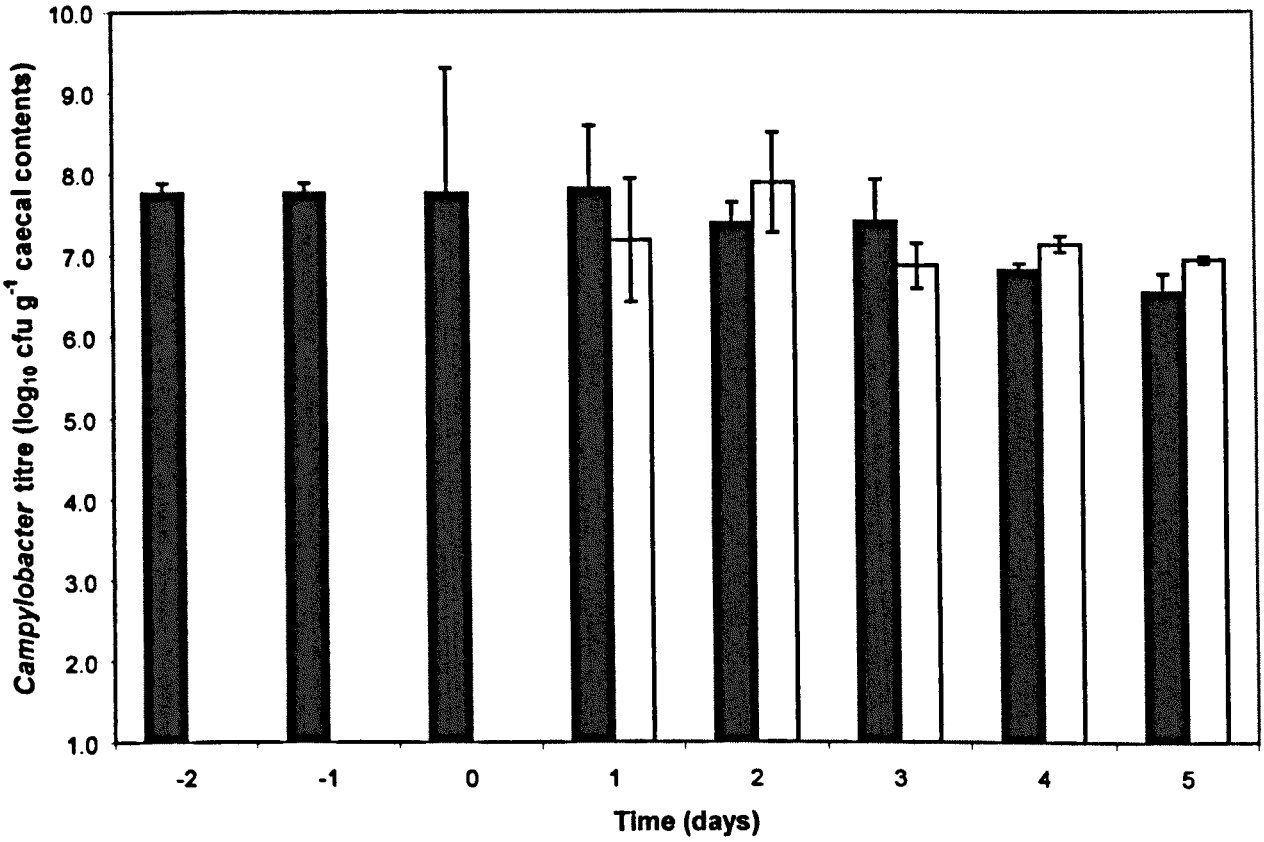


**Figure 8E. Bacteriophage therapy trial C (w2 and OCIIC10).**
The colonisation of *C. jejuni* strain OCIIC10 in the chicken caecum when exposed (□) and not exposed (■) to a single dose of $10^9$ pfu of bacteriophage w2 at day 0. The colonisation level was recorded as $\log_{10}$ cfu $g^{-1}$ caecal contents ± SD over an eight day period. These results are based on four replicates.

caeca to an average level of $\log_{10}$ 7.4 ± 0.5 cfu $g^{-1}$. This was significantly lower ($P <$ 0.02) than the level recorded for trial one ($\log_{10}$ 8.08 ± 0.39 cfu $g^{-1}$). In contrast to the results presented from trials two and three, there was no significant difference in the colonisation of OCIIC10 in the control and experimental groups at any point of the trial for any of the three sites of the intestine sampled ($P = 0.22$, for caeca). As with previous trials, the phage titre in the caecum fell from $\log_{10}$ 5.29 pfu $g^{-1}$ on Trial Day 1 to below detectable levels (<$\log_{10}$ 3.0 pfu $g^{-1}$) by Trial Day 3. No change in the susceptibility of OCIIC10 to w2 *in vitro* for any of the campylobacters recovered could be demonstrated. Three to six random *Campylobacter* colonies recovered from each chick were confirmed to be of the same *fla* type as the inoculated strain. The results of this trial suggest that the ability of bacteriophage to infect *Campylobacter in vitro* is not necessarily an indication of efficacy *in vivo*. There was no evidence for the replication of w2 *in vivo* which may be a prerequisite for the success of phage therapy. The colonisation of *C. jejuni* OCIIC10 in the birds' caeca was significantly lower than that recorded in trial 1. However, based on host caeca colonisation in the two preceding successful phage therapy trials, the level of *Campylobacter* colonisation recorded in trial four should not have been a limiting factor in phage biocontrol.

The development of resistance to bacteriophage infection was always foreseen as a potential problem with this intervention strategy. However, such resistance was not detected in any of the campylobacters recovered from trials two, three or four in the present study. A concurrent study using a phage isolate from chicken excreta (CP34) against *C. jejuni* HPC5 (conducted by Ms C. Loc-Carrillo and Dr. P. Connerton) resulted in the recovery of campylobacters resistant to infection as determined by screening

against agar lawns of bacterial hosts (**2.3.6.5**). Previous phage therapy trials in cattle by other workers has shown that phage-resistant *E. coli* mutants exhibit a reduced colonisation potential when used to re-inoculate calves (**Smith and Huggins, 1983**). In order to determine if this was true in CP34 phage-resistance, single colonies of resistant campylobacters recovered from phage-treated birds were propagated on blood agar (**2.3.3.1**). These were then suspended in PBS and used to orally dose broiler chicks in order to assess any changes in colonisation potential arising in the resistant strains. Four chicks were dosed with 1 ml of a $\log_{10}$ 9.1 cfu $ml^{-1}$ suspension of resistant isolate R14 and five birds were dosed with 1 ml of a $\log_{10}$ 9.0 cfu $ml^{-1}$ suspension of resistant isolate R20. All birds were sacrificed seven days after *Campylobacter* dosing. Following dissection, the titres of *Campylobacter* in the upper intestine, lower intestine and caeca were determined (**2.11.7**). In addition, caecal contents were also tested for the presence of bacteriophage (**2.11.8**). *fla*-typing of *Campylobacter* strains was unlikely to distinguish between phage sensitive and resistant mutants. As such, the macro-restriction profiles of campylobacters recovered from phage-treated birds were compared using PFGE (section **8.2.7**) to determine if resistance was associated with any changes in the genome structure of these strains.

Of the 92 colonies of *Campylobacter* strain HPC5 originally recovered from the phage therapy trial with CP34, 10 (11%) were resistant to phage infection *in vitro*. All colonies recovered from control birds remained sensitive to CP34 infection. PFGE using endonuclease *Sma*I (performed by Ms. E. Dillion) revealed that resistant strains exhibited a different macrorestriction profile to that of the sensitive strains (Fig. 8F). Specifically, extra *Sma*I restriction fragments approximately 390 kb and 230 kb were present in R14

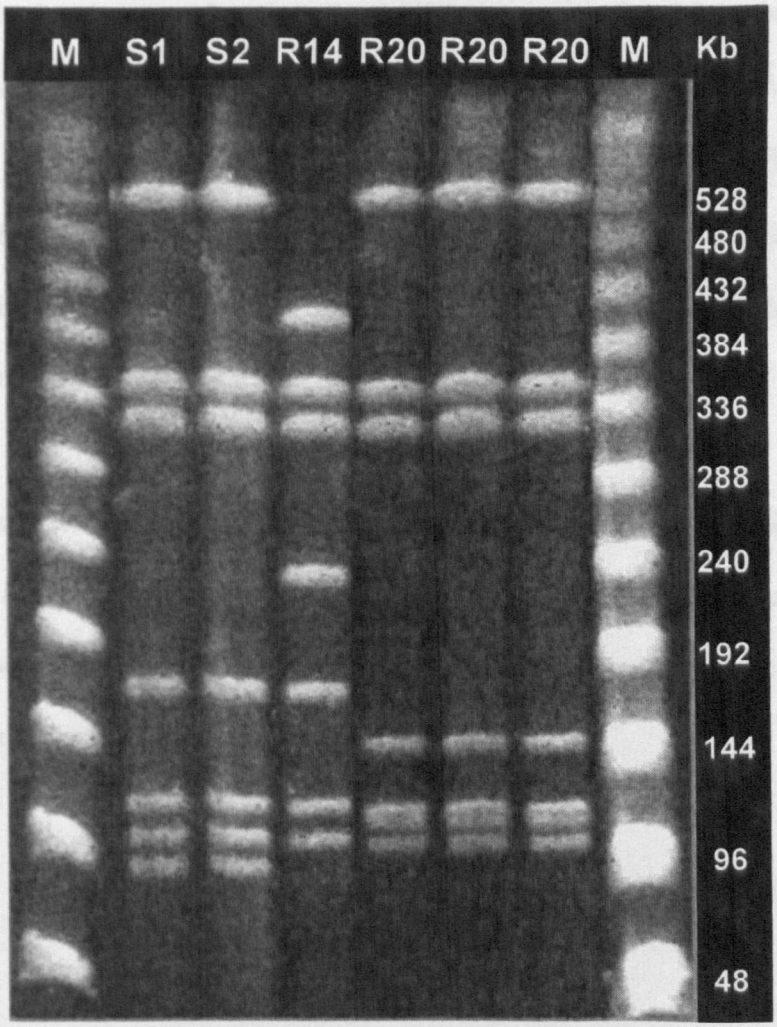


**Figure 8F. PFGE Macro-restriction profiles of phage-resistant and sensitive campylobacters**

A 1% PFGE gel showing whole genome *Sma*I restriction profiles of *Campylobacter* strains recovered from chickens treated with phage CP34. These campylobacters were either sensitive (S1, S2) or resistant (R14, R20) to reinfection by phage CP34. The size of the Lambda concatomer markers (M) are shown in Kb on the far right of the figure.

but not in the controls. A restriction fragment approximately 147 kb was present in R20 whilst a larger DNA band present in the control (170 kb) was absent. Interestingly, both R14 and R20 lacked an 85 kb restriction fragment present in the phage-sensitive control isolates (Fig. 8F). When reintroduced into chickens, average caecal colonisation of the resistant *Campylobacter* strains R14 and R20 was $\log_{10}$ 6.2 ± 1.0 cfu $g^{-1}$ and $\log_{10}$ 5.9 ± 0.7 cfu $g^{-1}$ respectively. This was, on average, $\log_{10}$ 0.7 cfu $g^{-1}$ lower than that recorded for the controls ($\log_{10}$ 6.76 ± 0.3 cfu $g^{-1}$). This difference was significant for R20 ($P <$ 0.02) but not for R14 ($P = 0.13$). However, of the 90 colonies recovered from the chickens recolonised with R14 and R20 in this experiment, only three (3.3%) retained their resistance to infection by phage CP34. Intriguingly, the changes in macrorestriction profiles of the phage-resistant strains recorded in Figure 8F were maintained, implying this profile change in itself does not confer phage resistance.

## 8.2 DISCUSSION

Trial one demonstrated that some *Campylobacter* strains (e.g. OCIIC10) colonise the broiler chick gut to a higher titre and with less variability than other strains (e.g. HPC5). When considering a model for phage therapy, host colonisation to a consistent and high level is crucial if the results of phage intervention are not to be confused with natural variation in host numbers. *C. jejuni* strains GIIC8 and OCIIC10 were stable colonisers of the gut and were deemed suitable hosts for use in phage biocontrol trials. It was interesting to note that the *C. jejuni* 81116 strain passaged *in vitro* colonised the chicken caeca to a significantly lower level than previous reports using the *in vivo* passaged strain 81116P (**Cawthraw *et al.*, 1996**). *C. jejuni* strains cultured *in vitro* tend to have a dose-response colonisation pattern in broiler chickens compared with the "all-or-nothing" colonisation of *in vivo* passaged strains (**Cawthraw *et al.*, 1996**). Considering GIIC8, OCIIC10 and HPC5 were all fresh chicken excreta isolates with minimal *in vitro* passage, they might be expected to colonise the chicken gut to a higher level than 81116 which has been sub-cultured at least 300 times (**Manning *et al.*, 2001**). However, there was no significant difference between the colonisation level of any of the campylobacters isolated from chicken excreta and 81116 ($P = 0.41$, GIIC8; $P = 0.24$, HPC5; $P = 0.15$, OCIIC10). Trial three revealed that the addition of $CaCO_3$ to oral phage preparations has little or no effect on the outcome of phage therapy. Failure to neutralise gastric pH is purported to be a major reason for early phage therapy failures (**Kutter, 1997**). The data presented here suggest that this is not a significant contributory factor to the success of *Campylobacter* phage treatment in broiler chickens.

The discrepancy in the results for trials two and three may reflect the fact that the efficacy of phage intervention relies on optimising the titre and timing of the phage dose (**Payne and Jansen, 2001**). The *Campylobacter* titre in trial three experimental birds fell on day four and continued to fall on day five in relation to the control. The greatest decrease in *Campylobacter* titre in trial three was $\log_{10}$ 1.8 cfu $g^{-1}$ on day five compared with $>\log_{10}$ 8.0 cfu $g^{-1}$ on day one recorded for trial two using the same bacteriophage (CP8). Successful bacteriophage intervention relies not only on the titre of the phage but on the availability and metabolic state of the host (**Payne and Jansen, 2001**). The generally lower caecal colonisation of the *Campylobacter* host in trial three may have contributed to the reduced efficacy of phage CP8. Unlike other predators of bacteria such as *Bdellovibrio*, phage rely solely on diffusion to find their prey (**Wilkinson, 2001**). Mathematical models of phage infection allude to abundance of the host as a critical factor in the success of phage therapy (**Wiggins and Alexander, 1985; Payne and Jansen, 2001**). Since trial three concluded on day five, where the greatest reduction in host population was recorded, it is unknown whether the same reductions observed for trial two would have been repeated if the time course was extended.

Resistance to bacteriophage infection has always been foreseen as a drawback of phage therapy (**Kutter, 1997**). The data presented in this study suggest that resistance to *Campylobacter* phage does occur but may be detected in greater frequency with some bacteriophage/host combinations than others. Classic experiments by Smith and Huggins in 1983 showed that phage resistant *E. coli* mutants were less virulent than their phage-sensitive counterparts (**Smith and Huggins, 1983**). Resistance to infection by one bacteriophage often leads to susceptibility to another, hence the use of mixtures or

"cocktails" of phage in therapeutic trials (**Smith and Huggins, 1983; Bull *et al.*, 2002**). In this study, phage resistant strains of *C. jejuni* HPC5 were poorer colonisers of the broiler chicken gut with the average titre in the caeca of broiler chicks being $\log_{10}$ 0.7 cfu $g^{-1}$ less than the phage-sensitive control. This apparent reduction in colonisation potential was accompanied by a clear shift in macrorestriction patterns observed using PFGE (Fig. 8E) although this was not always associated with resistance to phage infection. The loss of a higher molecular weight band with the gain of a lower band in phage resistant strain R20 suggested a deletion has taken place. Resistance was not maintained in the majority (97%) of strains when used to recolonise chicken indicating a possible selection mechanism against resistance. This would support the findings of Smith and Huggins that phage-resistant strains were less fit (**Smith and Huggins, 1983**).

The declining phage titre in chicken caeca in all of the trials is confusing as active replication on their hosts should be accompanied by an increase in virion concentration. Concurrent trials with phage CP8 and *C. jejuni* HPC5 recorded a stable 'out of phase' cycling of phage and host titres throughout the time course (Dr. P. Connerton pers comm.). However, the decrease in bacterial density in these trials (approximately $\log_{10}$ 1.5 – 2.0 cfu $g^{-1}$ caecal contents) was not as great as that recorded for trial two in the present study. Data presented in Chapter 3 suggest the burst size of *Campylobacter* phage isolated from chicken skin and belonging to the Myoviridae family may be ten or fewer per infected cell. Consequently, the phage may infect and lyse the host when at high titre and then be unable to maintain their population to a level where they can readily find new hosts. In this scenario, the phage would be gradually removed from the

system once host bacteria density had fallen below a critical value required for replication (**Wiggins and Alexander, 1985; Payne and Jansen, 2001**).

The failure of phage w2 to have a significant impact on *C. jejuni* OCIIC10 colonisation in trial 4 was unexpected. Unlike the delayed reduction in host titres recorded for trial 3, in trial 4 there was no such reduction seen at any of the sample sites at any point in the trial. This implied that extending the time scale of the trial would not yield the desired reductions in host colonisation. CP8 is clearly different from the phage isolated from food both in terms of genome size (130 kb) and lytic spectrum (Dr. P. Connerton pers comm.). The therapeutic trials also demonstrate that these phage differ in terms of their ability to control their host populations *in vivo*. Previous work has shown that attachment deficiencies prevent some *Campylobacter* bacteriophage from completing their infection cycle in liquid culture even when they are able to lyse lawns of their hosts on agar plates (Dr. R. Mole pers comm.). The chicken gut contains a plethora of micro organisms of which only a fraction have been fully characterised (**Mead *et al.*, 1996**). Although non-specific attachment of phage w2 to polypropylene test tubes or non-host campylobacters was not observed (Chapter 3) attachment to non-host cells in the chicken's gastrointestinal tract cannot be ruled out. The non-specific adsorption to members of other bacterial genera could be another factor which distinguishes CP8 from w2.

In conclusion, the data presented here clearly demonstrate that phage therapy has the potential to significantly impact on *Campylobacter* colonisation of the chicken caecum. Nevertheless, more work is required to optimise the timing and level of the phage dosage to maximise the therapeutic effect. The emergence of strains resistant to bacteriophage is

concerning but these strains also seem less able to colonise the chicken caecum. Control of *Campylobacter* contamination of broiler chickens at the farm level, if applied uniformly, is likely to have the greatest impact on contamination on the final product (**Newell and Wagenaar, 2000**) and potentially the disease burden in humans (**Jacobs-Reitsma, 2000**). Recent reports on *Campylobacter* quantitative risk assessment in the food chain suggest a $\log_{10}$ 2.0 cfu reduction of this bacterium on the surface of retail chicken would reduce the human incidence of campylobacteriosis by greater than 30-fold (**Rosenquist *et al.*, 2003**). Slaughtering birds at the point where the bacteriophage have reduced their host's titre to below detectable limits would both limit the emergence of resistant strains and maximise the efficacy of this intervention measure to assist in reaching this goal.

# CHAPTER 9

## DISCUSSION

The work presented in this thesis has provided the underpinning science enabling the use of bacteriophage to control *Campylobacter* in retail poultry production. Successful application of this technique in a wider commercial setting would hopefully lead to a significant reduction in *Campylobacter* contamination of retail chicken products and subsequently lead to a decrease in the incidence of human campylobacteriosis. Recent studies in the West have shown that "phage therapy" can be effectively applied in many situations, from the decontamination of surfaces to the treatment of enteric and systemic bacterial disease in humans and domestic animals. The success of phage therapy to treat *Salmonella* infections in broiler chickens suggests this technique may be transferable to *Campylobacter* in the same host and was the basis for the work presented in this thesis.

Before the broader issues of the *in vivo* efficacy of phage therapy could be addressed, the presence and characteristics of *Campylobacter* bacteriophage already in the poultry food chain needed to be determined. This was crucial if appropriate bacteriophage were to be selected for therapeutic endeavours in the future. To this end, the first aim of this project was to isolate and characterise *Campylobacter* bacteriophage from different parts of the poultry production chain. The successful isolation of phage from retail chicken (Chapter 3) was an important milestone in the broader aims of this project for two reasons. Firstly,

it demonstrated that phage were able to survive abattoir processing and be a potential source of therapeutic phage hitherto not investigated. Secondly, it also showed that phage therapy in broilers would not introduce any new biological entity into the food chain which was not already present. The latter point is particularly important with respect to the acceptability of food treatments to the general public being a prime concern of government. Certainly, there is broad scope for a public-relations disaster if national media coverage of phage therapy in food production was unfavourable.

Ultimately, the phage isolated from the chicken products are likely to originate, like their hosts, from the gastrointestinal tract of the broiler chicken. The variability in survival of different bacteriophage isolates on chicken skin suggests that poultry processing may select for a subset of phage able to survive under those conditions. This data may prove useful should one wish to isolate phage suitable for direct application to decontaminate surfaces. Indeed, the work presented in Chapter 6 indicates that the number of recoverable *Campylobacter* cells from chicken skin can be significantly reduced by the direct application of bacteriophage, even under conditions not permissive for replication of their host. As a consequence of this, phage biocontrol may be applied as a sole or combination treatment in both the farm and the abattoir to achieve maximum reductions in the presence of their host. However, it is unknown as to whether phage used effectively on the farm would be transferable to biocontrol in the abattoir. The use of similar phage at different stages of production would be unwise in any case, as any phage-resistant mutants would be more likely to accumulate in the food chain.

The majority of the 170 *Campylobacter* phage isolated from other studies belong to the Myoviridae family of viruses. Data from Chapter 4 confirmed that all of the phage isolated from retail chicken also belong to this family. The majority of Myoviridae phage follow a lytic lifecycle which bodes well for their potential as therapeutic agents. Lysogeny has not yet been reported in *Campylobacter* and the lack of any phage-like insertion elements in the complete DNA sequence of *C. jejuni* NCTC 11168 suggests this lifecycle is uncommon in *Campylobacter* phage. Specialised transduction mediated by temperate phage must be avoided by the exclusive use of lytic phage for therapy. The prevention of generalised transduction would not be a practical possibility in most cases. However, it could be minimised by careful screening to select lytic phage with a low frequency of transduction.

The similarities in lytic spectra between phage isolates from different chicken samples originating from different producers further supports the hypothesis that poultry processing imposes a selective pressure on the phage. Such pressures are in addition to those imposed in their original environment, which is likely to be the chicken gastrointestinal tract. However, the work in Chapter 4 also highlighted the importance of strain selection in lytic spectrum sensitivity. The large number of strains used by the PHLS for *Campylobacter* phage typing only discriminated two different lytic spectra amongst the phage isolated from retail chicken products. However, far fewer *Campylobacter* strains sourced from retail chicken and the NCTC were able to subdivide these phage into eight lytic classes. Some of these differences could also be correlated with genotypic differences using PFGE and differences in the size of the major proteins. This difference in sensitivity may be a consequence of the relatively narrow source of the

PHLS *Campylobacter* strains, all of which were isolated from human faeces. Techniques such as multilocus sequence typing have shown human *Campylobacter* isolates to cluster around relatively few sequence types whereas isolates from animals show a much broader distribution. This raises important issues regarding the methods and strains which should be used to determine the potential efficacy of phage against certain subgroups of *Campylobacter*. The possibility exists that phage need only be selected against campylobacters of a particular pathogenic subtype. However, in other food-borne pathogens such as *Salmonella*, nearly all subtypes are capable of human disease. With *Salmonella*, infection is determined by the subtype predominating in the food chain at any particular moment.

The work presented in Chapter 5 showed that *Campylobacter* phage were more frequently isolated from chicken caeca that retail poultry products. Perhaps the most interesting conclusion from this work was that chickens harbouring phage were generally colonised by fewer campylobacters. This strongly suggests that phage constitute a restricting force on the level to which campylobacters are able to colonise the chicken caecum under natural conditions. Given the knowledge of predator-prey interactions in nature, this may not be surprising. Should the host and parasite co-exist, the likelihood of isolating a highly virulent phage with a broad host range is minimal as such a phage would quickly eliminate all potential hosts and cause their own eradication. Nevertheless, virulent phage were isolated from chicken caeca which were able to infect the majority of human *Campylobacter* isolates with different phage types employed in the PHLS phage-typing scheme. The highly variable titres of phage recovered and general

insensitivity of their contemporaneous *Campylobacter* isolates to infection suggests a dynamic system in which the type and number of phage isolated can change frequently.

The broiler chickens examined in Chapter 5 were each colonised by a limited number of *Campylobacter* subtypes. This agreed with previous data suggesting that conventionally reared flocks are usually colonised by one or two *Campylobacter* strains. The reason for this is unclear but may be a reflection of the degree of exposure to the environment. This could be advantageous in phage therapy as the colonisation of a flock by a uniform genotype of *Campylobacter* would allow rapid dissemination of an infecting phage throughout the flock. However, this would also present a strong selective pressure for resistant phenotypes of the host which are likely to spread quickly. Consequently, the phenotypic characteristics of campylobacters in phage-infected flocks may be very dynamic and this may not be detected using the more commonly-used genetic subtyping techniques such as *fla* typing unless the flagella themselves are phage receptor sites.

The *in vitro* replication of the *Campylobacter* phage isolates in Chapter 4 suggested these phage have a low burst size and are released asynchronously from their host cells. These properties were not advantageous when selecting appropriate phage for therapeutic trials in chickens. Phage w2 for example, failed to reduce *Campylobacter* colonisation *in vivo* despite showing some promise during *in vitro* assays. During *in vitro* replication on a lawn of susceptible bacteria, the phage are presented with a high concentration of relatively immobile genetically homogenous hosts. This scenario is far removed from the chicken gut where the phage may encounter potentially inactivating extremes of pH, proteolytic enzymes and a plethora of other bacteria which may act as decoys. The data

presented in Chapters 4, 5 and 8 are important for future phage therapy endeavours as phage activity *in vitro* is not necessarily mirrored *in vivo*. In a wider setting, the clinical application of phage therapy needs to take this into account, especially since treatment is often prescribed after screening an infecting bacterial strain against phage *in vitro*. The potential also exists that highly lytic phage *in vivo* are not always detected by current *in vitro* isolation methods.

Another important issue arising from the phage therapy work in Chapter 8 was the variable efficacy of phage CP8 *in vivo*. This phage significantly reduced *Campylobacter* colonisation in two independent trials but by appreciably different degrees and at different times. The reasons for this are unknown but may reflect the physiological state of the birds, the nature of the intestinal microflora, the exact time and quantity of dosing or the titre of *Campylobacter* in the caecum. Further research is required to optimise phage therapy in chickens prior to its commercial application. The emergence of phage-resistant strains, as reported in Chapter 8, was always a predicted outcome of phage therapy. Interestingly, phage-resistant mutants in this study appeared to be less able to colonise the chicken caecum. This difference was statistically significant for only one of the two resistant strains tested. However, previous studies have suggested that phage resistance in *E. coli*, in certain circumstances, can be linked with either a poorer colonisation potential or attenuated virulence.

As a Gram negative bacterium, *Campylobacter* has the potential to produce membrane vesicles (MV) from blebbing of the outer membrane. In other species, such blebs have been shown to perform a variety of functions from causing the death of competing

bacteria to acting as molecular decoys for antibiotics. Such MVs also have the potential to hinder phage infection by acting as decoys for phage attachment. They also have the potential to assist phage as, once infected, they may fuse with the outer membranes of other *Campylobacter* strains which do not possess the "correct" phage receptor site and introduce the viral genome to the cell. The attachment of phage to membrane vesicles produced by *Campylobacter* clearly occurs to some degree as observed by electron microscopy. However, this process either does not affect, or does not occur at an appreciable frequency to affect, replication on the host cell. Therefore it is unlikely that production of membrane vesicles would be deleterious to phage therapy in chickens.

In conclusion, the work presented in this thesis has demonstrated that *Campylobacter* phage are present throughout the production of retail broiler chicken meat. These phage could be ascribed to genotypically and phenotypically distinct classes. Similarities between phage isolates from retail chicken compared with from broiler chicken caeca suggest some selective pressure is taking place during and after abattoir processing. The application of a sufficient quantity of phage directly onto contaminated chicken skin can reduce the numbers of *Campylobacter* recovered under experimental conditions. The presence of bacteriophage has been correlated with a lower titre of *Campylobacter* in the caeca of chickens taken from conventionally-reared flocks. This suggested that phage predation is a controlling factor which limits the colonisation of their hosts in natural systems. Such biocontrol was demonstrated when phage used to dose broiler chicks experimentally infected with *Campylobacter* significantly reduced the presence of their host in various sites of the gastrointestinal tract. Bacteriophage therapy is currently re-emerging in the West. The work presented in this thesis contributes to a growing body of

research highlighting the potential of phage to control bacterial infections in man and animals. If used wisely, with sufficient safeguards, phage therapy could make an invaluable contribution to both veterinary and clinical medicine and be an effective weapon in the fight against bacterial disease.

# CHAPTER 10

## REFERENCES


**Ackermann, H. and DuBow, M. S. (1987)** Viruses of Prokaryotes, CRC Press, Boca Raton.

**Ackermann, H. W., DuBow, M. S., Jarvis, A. W., Jones, L. A., Krylov, V. N., Maniloff, J., Rocourt, J., Safferman, R. S., Schneider, J., Seldin, L. and *et al.* (1992)** The species concept and its application to tailed phages. *Arch Virol,* **124,** 69-82.

**Adams, M. R. and Moss, M. O. (1995)** *Food Microbiology.* Royal Society of Chemistry, Cambridge.

**Ahmed, I. H., Manning, G., Wassenaar, T. M., Cawthraw, S. and Newell, D. G. (2002)** Identification of genetic differences between two *Campylobacter jejuni* strains with different colonization potentials. *Microbiology,* **148,** 1203-12.

**Alexander, M. (1981)** Why microbial predators and parasites do not eliminate their prey and hosts. *Annu Rev Microbiol,* **35,** 113-33.

**Allen, V. M., Corry, J. E., Burton, C. H., Whyte, R. T. and Mead, G. C. (2000)** Hygiene aspects of modern poultry chilling. *Int J Food Microbiol,* **58,** 39-48.

**Anon (1998)** Outbreak of *Campylobacter* enteritis associated with cross-contamination of food - Oklahoma, 1996. *Morbidity and Mortality Weekly Report,* **47,** 129-131.

**Anon (1999)** Working together for the food chain: Views from the food chain group, Ministry of Agriculture, Fisheries and Food, HMSO, London.

**Anon (2001a)** Consumer attitudes to food standards. Food Standards Agency, HMSO, London.

**Anon (2001b)** UK publicly-funded research relating to *Campylobacter*, Food Standards Agency, HMSO, London.

**Anon (2001c)** UK-wide survey of *Salmonella* and *Campylobacter* contamination of fresh and frozen chicken on retail sale, Food Standards Agency, HMSO, London.

**Anon (2003)** Strategy for the control of *Campylobacter* in chickens, Food Standards Agency, HMSO, London.

**Archer, G. L. and Polk, R. E. (2001)** Treatment and prophylaxis of bacterial infections *In* Harrison's Principles of Internal Medicine [CD-ROM] (Eds, Braunwald, E., Fauci, A. S., Kasper, D. L., Hauser, S. L., Longo, D. L. and Jameson, J. L.) McGraw-Hill, New York.

**Ayling, R. D., Woodward, M. J., Evans, S. and Newell, D. G. (1996)** Restriction fragment length polymorphism of polymerase chain reaction products applied to the differentiation of poultry campylobacters for epidemiological investigations. *Res Vet Sci,* **60,** 168-72.

**Baggesen, D. L., Wegener, H. C. and Madsen, M. (1997)** Correlation of conversion of *Salmonella enterica* serovar enteritidis phage type 1, 4, or 6 to phage type 7 with loss of lipopolysaccharide. *J Clin Microbiol,* **35,** 330-3.

**Barrow, P., Lovell, M. and Berchieri, A., Jr. (1998)** Use of lytic bacteriophage for control of experimental *Escherichia coli* septicemia and meningitis in chickens and calves. *Clin Diagn Lab Immunol,* **5,** 294-8.

**Barrow, P. A. and Soothill, J. S. (1997)** Bacteriophage therapy and prophylaxis: rediscovery and renewed assessment of potential. *Trends Microbiol,* **5,** 268-71.

**Beery, J. T., Hugdahl, M. B. and Doyle, M. P. (1988)** Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni. Appl Environ Microbiol,* **54,** 2365-70.

**Berchieri, A., Jr., Lovell, M. A. and Barrow, P. A. (1991)** The activity in the chicken alimentary tract of bacteriophages lytic for *Salmonella* Typhimurium. *Res Microbiol,* **142,** 541-9.

**Bergh, O., Borsheim, K. Y., Bratbak, G. and Heldal, M. (1989)** High abundance of viruses found in aquatic environments. *Nature,* **340,** 467-8.

**Berndtson, E., Danielsson-Tham, M. L. and Engvall, A. (1996)** *Campylobacter* incidence on a chicken farm and the spread of *Campylobacter* during the slaughter process. *Int J Food Microbiol,* **32,** 35-47.

**Berrang, M. E., Buhr, R. J. and Cason, J. A. (2000)** *Campylobacter* recovery from external and internal organs of commercial broiler carcass prior to scalding. *Poult Sci,* **79,** 286-90.

**Berrang, M. E., Ladely, S. R. and Buhr, R. J. (2001)** Presence and level of *Campylobacter,* coliforms, *Escherichia coli,* and total aerobic bacteria recovered from broiler parts with and without skin. *J Food Prot,* **64,** 184-8.

**Beveridge, T. J. (1999)** Structures of Gram-negative cell walls and their derived membrane vesicles. *J Bacteriol,* **181,** 4725-33.

**Bishai, W. R. and Murphy, J. R. (1988)** Bacteriophage gene products that cause human disease *In* The Bacteriophages, Vol. 1 (Ed, Calendar, R.) Plenum Press, New York, pp. 683-724.

**Biswas, B., Adhya, S., Washart, P., Paul, B., Trostel, A. N., Powell, B., Carlton, R. and Merril, C. R. (2002)** Bacteriophage therapy rescues mice bacteremic from a clinical isolate of vancomycin-resistant *Enterococcus faecium. Infect Immun,* **70,** 204-10.

**Black, R. E., Levine, M. M., Clements, M. L., Hughes, T. P. and Blaser, M. J. (1988)** Experimental *Campylobacter jejuni* infection in humans. *J Infect Dis,* **157,** 472-9.

**Blaser, M. J. (2001)** Infections due to *Campylobacter* and related species *In* Harrison's Principles of Internal Medicine [CD-ROM] (Eds, Braunwald, E., Fauci, A. S., Kasper, D. L., Hauser, S. L., Longo, D. L. and Jameson, J. L.) McGraw-Hill, New York.

**Blaser, M. J., Hopkins, J. A., Berka, R. M., Vasil, M. L. and Wang, W. L. (1983)** Identification and characterization of *Campylobacter jejuni* outer membrane proteins. *Infect Immun,* **42,** 276-84.

**Boer, P., Wagenaar, J. A., Achterberg, R. P., Putten, J. P., Schouls, L. M. and Duim, B. (2002)** Generation of *Campylobacter jejuni* genetic diversity *in vivo*. *Mol Microbiol,* **44,** 351-9.

**Bowes, T., Wagner, E. R., Boffey, J., Nicholl, D., Cochrane, L., Benboubetra, M., Conner, J., Furukawa, K. and Willison, H. J. (2002)** Tolerance to self gangliosides is the major factor restricting the antibody response to lipopolysaccharide core oligosaccharides in *Campylobacter jejuni* strains associated with Guillain-Barre syndrome. *Infect Immun,* **70,** 5008-18.

**Brandt, K., Tilsala-Timisjarvi, A. and Alatossava, T. (2001)** Phage-related DNA polymorphism in dairy and probiotic Lactobacillus. *Micron,* **32,** 59-65.

**Bridson, E. Y. (1998)** Oxoid Manual. Oxoid Ltd, Basingstoke.

**Bruynoghe, R. and Maisin, J. (1921)** Essais de therapeutique au moyem du bacteriophage. *C. R. Soc. Biol.,* **85,** 1120-1121.

**Bull, J. J., Levin, B. R., DeRouin, T., Walker, N. and Bloch, C. A. (2002)** Dynamics of success and failure in phage and antibiotic therapy in experimental infections. *BMC Microbiology,* **2,** 35.

**Buswell, C. M., Herlihy, Y. M., Lawrence, L. M., McGuiggan, J. T., Marsh, P. D., Keevil, C. W. and Leach, S. A. (1998)** Extended survival and persistence of *Campylobacter* ssp. in water and aquatic biofilms and their detection by immunofluorescent-antibody and rRNA staining. *Appl Environ Microbiol,* **64,** 733-41.

**Butzler, J. (2000)** Campylobacteriosis in humans (a historical overview) *In* The increasing incidence of human camylobacteriosis. Report and proceedings of a WHO Consultation of ExpertsWorld Health Organisation, Copenhagen, pp. 38-41.

**Cann, A. J. (1993)** Principles of Molecular Virology. Academic Press. New York.

**Cardenas, L. and Clements, J. D. (1992)** Oral immunization using live attenuated *Salmonella* ssp. as carriers of foreign antigens. *Clin Microbiol Rev,* **5,** 328-42.

**Carlton, R. M. (1999)** Phage therapy: past history and future prospects. *Arch Immunol Ther Exp,* **47,** 267-74.

**Cawthraw, S. A., Feldman, R. A., Sayers, A. R. and Newell, D. G. (2002)** Long-term antibody responses following human infection with *Campylobacter jejuni. Clin Exp Immunol,* **130,** 101-6.

**Cawthraw, S. A., Lind, L., Kaijser, B. and Newell, D. G. (2000)** Antibodies, directed towards *Campylobacter jejuni* antigens, in sera from poultry abattoir workers. *Clin Exp Immunol,* **122,** 55-60.

**Cawthraw, S. A., Wassenaar, T. M., Ayling, R. and Newell, D. G. (1996)** Increased colonization potential of *Campylobacter jejuni* strain 81116 after passage through chickens and its implication on the rate of transmission within flocks. *Epidemiol Infect,* **117,** 213-5.

**Cerveny, K. E., DePaola, A., Duckworth, D. H. and Gulig, P. A. (2002)** Phage therapy of local and systemic disease caused by *Vibrio vulnificus* in iron-dextran-treated mice. *Infect Immun,* **70,** 6251-62.

**Chak, K. F., Kuo, W. S., Lu, F. M. and James, R. (1991)** Cloning and characterization of the ColE7 plasmid. *J Gen Microbiol,* **137 (1),** 91-100.

**Chan, K. F., Le Tran, H., Kanenaka, R. Y. and Kathariou, S. (2001)** Survival of clinical and poultry-derived isolates of *Campylobacter jejuni* at a low temperature (4 degrees C). *Appl Environ Microbiol,* **67,** 4186-91.

**Chen, H. C. and Stern, N. J. (2001)** Competitive exclusion of heterologous *Campylobacter* ssp. in chicks. *Appl Environ Microbiol,* **67,** 848-51.

**Chuma, T., Yano, K., Omori, H., Okamoto, K. and Yugi, H. (1997)** Direct detection of *Campylobacter jejuni* in chicken cecal contents by PCR. *J Vet Med Sci,* **59,** 85-87.

**Cogan, T. A., Bloomfield, S. F. and Humphrey, T. J. (1999)** The effectiveness of hygiene procedures for prevention of cross-contamination from chicken carcases in the domestic kitchen. *Lett Appl Microbiol,* **29,** 354-8.

**Cogan, T. A. and Humphrey, T. J. (2003)** The rise and fall of *Salmonella* Enteritidis in the UK. *J Appl Microbiol,* **94 (1),** 114-9.

**Cogan, T. A., Slader, J., Bloomfield, S. F. and Humphrey, T. J. (2002)** Achieving hygiene in the domestic kitchen: the effectiveness of commonly used cleaning procedures. *J Appl Microbiol,* **92,** 885-92.

**Collins, C. H., Lyne, P. M. and Grange, J. M. (1995)** *Campylobacter* and *Helicobacter In:* Microbiological methods, Butterworth-Heinemann Ltd, Oxford, pp. 340-342.

**Corry, J. E., James, C., James, S. J. and Hinton, M. (1995a)** *Salmonella, Campylobacter* and *Escherichia coli* 0157:H7 decontamination techniques for the future. *Int J Food Microbiol,* **28,** 187-196.

**Corry, J. E., Post, D. E., Colin, P. and Laisney, M. J. (1995b)** Culture media for the isolation of campylobacters. *Int J Food Microbiol,* **26,** 43-76.

**Cox, N. A., Stern, N. J., Hiett, K. L. and Berrang, M. E. (2002a)** Identification of a new source of *Campylobacter* contamination in poultry: transmission from breeder hens to broiler chickens. *Avian Dis,* **46,** 535-41.

**Cox, N. A., Stern, N. J., Wilson, J. L., Musgrove, M. T., Buhr, R. J. and Hiett, K. L. (2002b)** Isolation of *Campylobacter* ssp. from semen samples of commercial broiler breeder roosters. *Avian Dis,* **46,** 717-20.

**D' Herelle, F. (1917)** Sur un microbe invisible antagoniste des bacilles dysenteriques. *Compt. rend. Acad. Sci.,* **165,** 373.

**Desplats, C., Dez, C., Tetart, F., Eleaume, H. and Krisch, H. M. (2002)** Snapshot of the genome of the pseudo-T-even bacteriophage RB49. *J Bacteriol,* **184,** 2789-804.

**Deveau, H., Van Calsteren, M. R. and Moineau, S. (2002)** Effect of exopolysaccharides on phage-host interactions in *Lactococcus lactis. Appl Environ Microbiol,* **68,** 4364-9.

**Dingle, K. E., Colles, F. M., Wareing, D. R., Ure, R., Fox, A. J., Bolton, F. E., Bootsma, H. J., Willems, R. J., Urwin, R. and Maiden, M. C. (2001)** Multilocus sequence typing system for *Campylobacter jejuni. J Clin Microbiol,* **39,** 14-23.

**Doermann, A. H. (1951)** The intracellular growth of bacteriophages. *J Gen Physiol,* **35,** 645-656.

**Dominguez, C., Gomez, I. and Zumalacarregui, J. (2002)** Prevalence of *Salmonella* and *Campylobacter* in retail chicken meat in Spain. *Int J Food Microbiol,* **72,** 165-8.

**Dorrell, N., Mangan, J. A., Laing, K. G., Hinds, J., Linton, D., Al-Ghusein, H., Barrell, B. G., Parkhill, J., Stoker, N. G., Karlyshev, A. V., Butcher, P. D. and Wren, B. W. (2001)** Whole genome comparison of *Campylobacter jejuni* human isolates using a low-cost microarray reveals extensive genetic diversity. *Genome Res,* **11,** 1706-15.

**Dunne, W. M., Jr. (2002)** Bacterial adhesion: seen any good biofilms lately? *Clin Microbiol Rev,* **15,** 155-66.

**Duplessis, M. and Moineau, S. (2001)** Identification of a genetic determinant responsible for host specificity in *Streptococcus thermophilus* bacteriophages. *Mol Microbiol,* **41,** 325-36.

**Eaton, M. D. and Bayne-Jones, S. (1934)** Bacteriophage therapy. *Journ. American Med. Assoc.,* **103,** 1769-1776.

**Ellis, E. L. and Delbruck, M. (1939)** The growth of bacteriophage. *Journal of General Physiology,* **22,** 365-384.

**Evans, M. R., Lane, W., Frost, J. A. and Nylen, G. (1998)** A *Campylobacter* outbreak associated with stir-fried food. *Epidemiol Infect,* **121,** 275-9.

**Evans, S. J. and Sayers, A. R. (2000)** A longitudinal study of *Campylobacter* infection of broiler flocks in Great Britain. *Prev Vet Med,* **46,** 209-23.

**Firehammer, B. D. and Border, M. (1968)** Isolation of temperate bacteriophages from *Vibrio fetus. Am J Vet Res,* **29,** 2229-2235.

**Friedman, C. R., Neimann, J., Wegener, H. C. and Tauxe, R. V. (2000)** Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations *In Campylobacter* 2nd Ed (Blaser, M. J. and Nachamkin, I. Eds) American Society for Microbiology, Washington, D.C., pp. 121-138.

**Frost, J. A., Kramer, J. M. and Gillanders, S. A. (1999)** Phage typing of *Campylobacter jejuni* and *Campylobacter coli* and its use as an adjunct to serotyping. *Epidemiol Infect,* **123,** 47-55.

**Fujisawa, H. and Morita, M. (1997)** Phage DNA packaging. *Genes Cells,* **2,** 537-45.

**Gibbens, J. C., Pascoe, S. J., Evans, S. J., Davies, R. H. and Sayers, A. R. (2001)** A trial of biosecurity as a means to control *Campylobacter* infection of broiler chickens. *Prev Vet Med,* **48,** 85-99.

**Gillespie, I. A., O'Brien, S. J., Frost, J. A., Adak, G. K., Horby, P., Swan, A. V., Painter, M. J. and Neal, K. R. (2002)** A case-case comparison of *Campylobacter coli* and *Campylobacter jejuni* infection: a tool for generating hypotheses. *Emerg Infect Dis,* **8,** 937-42.

**Grajewski, B. A., Kusek, J. W. and Gelfand, H. M. (1985)** Development of a bacteriophage typing system for *Campylobacter jejuni* and *Campylobacter coli. J Clin Microbiol,* **22,** 13-8.

**Greer, G. (1986)** Homologous bacteriophage control of *Pseudomonas* growth and beef spoilage. *J Food Prot,* **49,** 104-109.

**Gregory, E., Barnhart, H., Dreesen, D. W., Stern, N. J. and Corn, J. L. (1997)** Epidemiological study of *Campylobacter* ssp. in broilers: source, time of colonization, and prevalence. *Avian Dis,* **41,** 890-8.

**Griffiths, P. L. (1993)** Morphological changes of *Campylobacter jejuni* growing in liquid culture. *Lett Appl Microbiol,* **17,** 152-5.

**Hadas, H., Einav, M., Fishov, I. and Zaritsky, A. (1997)** Bacteriophage T4 development depends on the physiology of its host *Escherichia coli. Microbiology,* **143 (1),** 179-85.

**Hald, B., Rattenborg, E. and Madsen, M. (2001)** Role of batch depletion of broiler houses on the occurrence of *Campylobacter* ssp. in chicken flocks. *Lett Appl Microbiol,* **32,** 253-6.

**Hambly, E., Tetart, F., Desplats, C., Wilson, W. H., Krisch, H. M. and Mann, N. H. (2001)** A conserved genetic module that encodes the major virion components in both the coliphage T4 and the marine cyanophage S-PM2. *Proc Natl Acad Sci U S A,* **98,** 11411-6.

**Harris, N. V., Weiss, N. S. and Nolan, C. M. (1986)** The role of poultry and meats in the etiology of *Campylobacter jejuni/coli* enteritis. *Am J Public Health,* **76,** 407-11.

**Heuer, O. E., Pedersen, K., Andersen, J. S. and Madsen, M. (2001)** Prevalence and antimicrobial susceptibility of thermophilic *Campylobacter* in organic and conventional broiler flocks. *Lett Appl Microbiol,* **33,** 269-74.

**Hiett, K. L., Cox, N. A., Buhr, R. J. and Stern, N. J. (2002)** Genotype analyses of *Campylobacter* isolated from distinct segments of the reproductive tracts of broiler breeder hens. *Curr Microbiol,* **45,** 400-4.

**Ho, T. D. and Slauch, J. M. (2001)** OmpC is the receptor for Gifsy-1 and Gifsy-2 bacteriophages of *Salmonella. J Bacteriol,* **183,** 1495-8.

**Hughes, R. A., Hadden, R. D., Gregson, N. A. and Smith, K. J. (1999)** Pathogenesis of Guillain-Barre syndrome. *J Neuroimmunol,* **100,** 74-97.

**Humphrey, T. J., Henley, A. and Lanning, D. G. (1993)** The colonization of broiler chickens with *Campylobacter jejuni*: some epidemiological investigations. *Epidemiol Infect,* **110,** 601-7.

**Humphrey, T. J. and Lanning, D. G. (1987)** *Salmonella* and *Campylobacter* contamination of broiler chicken carcasses and scald tank water: the influence of water pH. *J Appl Bacteriol,* **63,** 21-5.

**Humphrey, T. J., Lanning, D. G. and Mead, G. C. (1989)** Inhibition of *Campylobacter jejuni in vitro* by broiler chicken caecal contents. *Vet Rec,* **125,** 272-3.

**Humphrey, T. J., Mead, G. and Rowe, B. (1988)** Poultry meat as a source of human salmonellosis in England and Wales. *Epidemiol Infect,* **100,** 175-184.

**Istre, G. R., Blaser, M. J., Shillam, P. and Hopkins, R. S. (1984)** *Campylobacter* enteritis associated with undercooked barbecued chicken. *Am J Public Health,* **74,** 1265-7.

**Jacobs-Reitsma, W. (2000)** *Campylobacter* in the food supply *In Campylobacter* 2nd Ed (Blaser, M. J. and Nachamkin, I. Eds) American Society for Microbiology, Washington, pp. 467-509.

**Jacobs-Reitsma, W. F., van de Giessen, A. W., Bolder, N. M. and Mulder, R. W. (1995)** Epidemiology of *Campylobacter* ssp. at two Dutch broiler farms. *Epidemiol Infect,* **114,** 413-21.

**Jay, J. M. (1992)** Modern Food Microbiology. Chapman and Hall, New York.

**Jorgensen, F., Bailey, R., Williams, S., Henderson, P., Wareing, D. R., Bolton, F. J., Frost, J. A., Ward, L. and Humphrey, T. J. (2002)** Prevalence and numbers of *Salmonella* and *Campylobacter* ssp. on raw, whole chickens in relation to sampling methods. *Int J Food Microbiol,* **76,** 151-64.

**Jurkevitch, E. and Ramati, B. (2000)** Design and uses of *Bdellovibrio* 16S rRNA-targeted oligonucleotides. *FEMS Microbiol Lett,* **184,** 265-271.

**Kapperud, G., Lassen, J., Ostroff, S. M. and Aasen, S. (1992)** Clinical features of sporadic *Campylobacter* infections in Norway. *Scandinavian Journal of Infectious Diseases,* **24,** 741-749.

**Kauc, L. and Nachamkin, I. (1998)** A recombinant *Salmonella* Typhimurium strain expressing *Campylobacter jejuni* flagellin confers partial colonization protection in chicks *In Campylobacter, Helicobacter* and Related Organisms (Lastovica, A. J., Lastovica, E.E. and Newell, D. G. Eds) Institute of Child Health, Cape Town.

**Kennedy, J. E. and Bitton, G. (1987)** Bacteriophages in foods *In* Phage Ecology (Bitton, G., Gerba, G. P. and Goyal, S. M. Eds) John Wiley & Sons, New York, pp. 289-316.

**Ketley, J. M. (1997)** Pathogenesis of enteric infection by *Campylobacter. Microbiology,* **143 (1),** 5-21.

**Kramer, J. M., Frost, J. A., Bolton, F. J. and Wareing, D. R. (2000)** *Campylobacter* contamination of raw meat and poultry at retail sale: identification of multiple types and comparison with isolates from human infection. *J Food Prot,* **63,** 1654-9.

**Kuby, J. (1997)** Immunology. W H Freeman and Co., New York.

**Kucharewicz-Krukowska, A. and Slopek, S. (1987)** Immunogenic effect of bacteriophage in patients subjected to phage therapy. *Arch Immunol Ther Exp (Warsz),* **35,** 553-61.

**Kumar, A. and Patel, S. S. (1997)** Inhibition of T7 RNA polymerase: transcription initiation and transition from initiation to elongation are inhibited by T7 lysozyme via a ternary complex with RNA polymerase and promoter DNA. *Biochemistry,* **36,** 13954-62.

**Kutter, E. (1997),** Phage Therapy: Bacteriophages as antibiotics. Evergreen State College, Olympia.

**Lacey, R. W. (1994)** Hard to Swallow. A brief history of food. Cambridge University Press, Cambridge.

**Lastovica, A. J. and Skirrow, M. B. (2000)** Clinical significance of *Campylobacter* and related species other than *Campylobacter jejuni* and *Campylobacter coli In Campylobacter* 2nd Ed (Blaser, M. J. and Nachamkin, I. Eds) American Society for Microbiology, Washington, D.C., pp. 89-120.

**Lazarevic, V., Soldo, B., Dusterhoft, A., Hilbert, H., Mauel, C. and Karamata, D. (1998)** Introns and intein coding sequence in the ribonucleotide reductase genes of *Bacillus subtilis* temperate bacteriophage SPbeta. *Proc Natl Acad Sci U S A,* **95,** 1692-7.

**Leclerc, H., Edberg, S., Pierzo, V. and Delattre, J. M. (2000)** Bacteriophages as indicators of enteric viruses and public health risk in groundwaters. *J Appl Microbiol,* **88,** 5-21.

**Leverentz, B., Conway, W. S., Alavidze, Z., Janisiewicz, W. J., Fuchs, Y., Camp, M. J., Chighladze, E. and Sulakvelidze, A. (2001)** Examination of bacteriophage as a biocontrol method for *Salmonella* on fresh-cut fruit: a model study. *J Food Prot,* **64,** 1116-1121.

**Lewin, B. (1997)** Genes VI. Oxford University Press, Oxford.

**Li, Z., Clarke, A. J. and Beveridge, T. J. (1998)** Gram-negative bacteria produce membrane vesicles which are capable of killing other bacteria. *J Bacteriol,* **180,** 5478-83.

**Liu, S. L. and Sanderson, K. E. (1996)** Highly plastic chromosomal organization in *Salmonella* Typhi. *Proc Natl Acad Sci U S A,* **93,** 10303-8.

**Loeffler, J. M., Nelson, D. and Fischetti, V. A. (2001)** Rapid killing of *Streptococcus pneumoniae* with a bacteriophage cell wall hydrolase. *Science,* **294,** 2170-2.

**Logan, S. M. and Trust, T. J. (1982)** Outer membrane characteristics of *Campylobacter jejuni. Infect Immun,* **38,** 898-906.

**Lwoff, A. and Gutmann, A. (1950)** Investigations on a lysogenic *Bacillus megaterium. Annales de l'Institut Pasteur,* **78,** 711-739.

**Manning, G., Duim, B., Wassenaar, T., Wagenaar, J. A., Ridley, A. and Newell, D. G. (2001)** Evidence for a genetically stable strain of *Campylobacter jejuni. Appl Environ Microbiol,* **67,** 1185-9.

**Marchant, J., Wren, B. and Ketley, J. (2002)** Exploiting genome sequence: predictions for mechanisms of *Campylobacter* chemotaxis. *Trends Microbiol,* **10,** 155-9.

**Matsuzaki, S., Inoue, T. and Tanaka, S. (1998)** A vibriophage, KVP40, with major capsid protein homologous to gp23* of coliphage T4. *Virology,* **242,** 314-8.

**Mayrand, D. and Grenier, D. (1989)** Biological activities of outer membrane vesicles. *Can J Microbiol,* **35,** 607-13.

**McFarland, J. (1907)** Nephelometer: an instrument for estimating the number of bacteria in suspension used for calculating the opsonic index for vaccines. *Journ. American Med. Assoc.,* **14,** 1176-1178.

**Mead, G. C. (2000)** Prospects for 'competitive exclusion' treatment to control salmonellas and other foodborne pathogens in poultry. *Vet J,* **159,** 111-23.

**Mead, G. C., Scott, M. J., Humphrey, T. J. and McAlpine, K. (1996)** Observations on the control of *Campylobacter jejuni* infection of poultry by 'competetive exclusion'. *Avian Pathology,* **25,** 69-79.

**Mellits, K. H., Mullen, J., Wand, M., Armbruster, G., Patel, A., Connerton, P. L., Skelly, M. and Connerton, I. F. (2002)** Activation of the transcription factor NF-kappaB by *Campylobacter jejuni. Microbiology,* **148,** 2753-63.

**Merril, C. R., Biswas, B., Carlton, R., Jensen, N. C. and Creed, G. J. (1996)** Long-circulating bacteriophage as antibacterial agents. *Proc Natl Acad Sci U S A,* **93,** 3188-3192.

**Miles, A. A. and Misra, S. S. (1938)** The estimation of the bacteriological power of the blood. *J. Hyg.,* **38,** 732.

**Miller, E. S., Kutter, E., Mosig, G., Arisaka, F., Kunisawa, T. and Ruger, W. (2003)** Bacteriophage T4 genome. *Microbiology and Molecular Biology Reviews,* **67,** 86-156.

**Miwa, N., Takegahara, Y., Katsuya, T., Hideo, K. and Takeuchi, T. (2002)** *Campylobacter jejuni* contamination on broiler carcasses of *C. jejuni*-negative flocks during processing in a Japanese slaughterhouse. *Int J Food Microbiol,* **2617,** 1-5.

**Morishita, T. Y., Aye, P. P., Harr, B. S., Cobb, C. W. and Clifford, J. R. (1997)** Evaluation of an avian-specific probiotic to reduce the colonization and shedding of *Campylobacter jejuni* in broilers. *Avian Dis,* **41,** 850-5.

**Murphy, O., Gray, J., Gordon, S. and Bint, A. J. (1995)** An outbreak of *Campylobacter* food poisoning in a health care setting. *J Hosp Infect,* **30,** 225-8.

**Nachamkin, I. (2002)** Chronic effects of *Campylobacter* infection. *Microbes Infect,* **4,** 399-403.

**Nachamkin, I., Allos, B. M. and Ho, T. W. (2000)** *Campylobacter jejuni* infection and the association with Guillain-Barre Syndrome *In Campylobacter* 2nd Ed (Blaser, M. J. and Nachamkin, I. Eds) American Society for Microbiology, Washington, D.C., pp. 155-175.

**Nakagawa, H., Arisaka, F. and Ishii, S. (1985)** Isolation and characterization of the bacteriophage T4 tail-associated lysozyme. *J Virol,* **54,** 460-6.

**Nesper, J., Kapfhammer, D., Klose, K. E., Merkert, H. and Reidl, J. (2000)** Characterization of *Vibrio cholerae* O1 antigen as the bacteriophage K139 receptor and identification of IS1004 insertions aborting O1 antigen biosynthesis. *J Bacteriol,* **182,** 5097-104.

**Newell, D. G., Shreeve, J. E., Toszeghy, M., Domingue, G., Bull, S., Humphrey, T. and Mead, G. (2001)** Changes in the carriage of *Campylobacter* strains by poultry carcasses during processing in abattoirs. *Appl Environ Microbiol,* **67,** 2636-40.

**Newell, D. G. and Wagenaar, J. A. (2000)** Poultry infections and their control at the farm level *In Campylobacter* 2nd Ed (Blaser, M. J. and Nachamkin, I. Eds) ASM Press, Washington, D.C., pp. 497-509.

**Ng, L. K., Sherburne, R., Taylor, D. E. and Stiles, M. E. (1985)** Morphological forms and viability of *Campylobacter* species studied by electron microscopy. *J Bacteriol,* **164,** 338-43.

**Nurmi, E. and Rantala, M. (1973)** New aspects of *Salmonella* infection in broiler production. *Nature,* **241,** 210-1.

**Paddison, P., Abedon, S. T., Dressman, H. K., Gailbreath, K., Tracy, J., Mosser, E., Neitzel, J., Guttman, B. and Kutter, E. (1998)** The roles of the bacteriophage T4 r genes in lysis inhibition and fine-structure genetics: a new perspective. *Genetics,* **148,** 1539-50.

**Park, S. C., Shimamura, I., Fukunaga, M., Mori, K. I. and Nakai, T. (2000)** Isolation of bacteriophages specific to a fish pathogen, *Pseudomonas plecoglossicida,* as a candidate for disease control. *Appl Environ Microbiol,* **66,** 1416-22.

**Parkhill, J., Wren, B. W., Mungall, K., Ketley, J. M., Churcher, C., Basham, D., Chillingworth, T., Davies, R. M., Feltwell, T., Holroyd, S., Jagels, K., Karlyshev, A. V., Moule, S., Pallen, M. J., Penn, C. W., Quail, M. A., Rajandream, M. A., Rutherford, K. M., van Vliet, A. H., Whitehead, S. and Barrell, B. G. (2000)** The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature,* **403,** 665-8.

**Payne, R. E., Lee, M. D., Dreesen, D. W. and Barnhart, H. M. (1999)** Molecular epidemiology of *Campylobacter jejuni* in broiler flocks using randomly amplified polymorphic DNA-PCR and 23S rRNA-PCR and role of litter in its transmission. *Appl Environ Microbiol,* **65,** 260-3.

**Payne, R. J. and Jansen, V. A. (2001)** Understanding bacteriophage therapy as a density-dependent kinetic process. *J Theor Biol,* **208,** 37-48.

**Payne, R. J. H. and Jansen, V. A. (2000)** Phage therapy: the peculiar kinetics of self-replicating pharmaceuticals. *Clin. Pharm. Ther.,* **68,** 225-230.

**Pebody, R. G., Ryan, M. J. and Wall, P. G. (1997)** Outbreaks of *Campylobacter* infection: rare events for a common pathogen. *CDR Review,* **7,** 33-37.

**Petersen, L., Nielsen, E. M., Engberg, J., On, S. L. and Dietz, H. H. (2001)** Comparison of genotypes and serotypes of *Campylobacter jejuni* isolated from Danish wild mammals and birds and from broiler flocks and humans. *Appl Environ Microbiol,* **67,** 3115-21.

**Petersen, L. and Wedderkopp, A. (2001)** Evidence that certain clones of *Campylobacter jejuni* persist during successive broiler flock rotations. *Appl Environ Microbiol,* **67,** 2739-45.

**Phillips, C. A. (1995)** Incidence, epidemiology and prevention of foodborne *Campylobacter* species. *Trends in Food Science and Technology,* **6,** 83-7.

**Pitcher, D. G., Saunders, N. A. and Owen, R. J. (1989)** Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol,* **8,** 151-156.

**Rantala, M. and Nurmi, E. (1973)** Prevention of the growth of *Salmonella infantis* in chicks by the flora of the alimentary tract of chickens. *Br Poult Sci,* **14,** 627-30.

**Ribot, E. M., Fitzgerald, C., Kubota, K., Swaminathan, B. and Barrett, T. J. (2001)** Rapid pulsed-field gel electrophoresis protocol for subtyping of *Campylobacter jejuni. J Clin Microbiol,* **39,** 1889-1894.

**Richardson, J. F., Frost, J. A., Kramer, J. M., Thwaites, R. T., Bolton, F. J., Wareing, D. R. and Gordon, J. A. (2001)** Coinfection with *Campylobacter* species: an epidemiological problem? *J Appl Microbiol,* **91,** 206-11.

**Ringoir, D. D. and Korolik, V. (2003)** Colonisation phenotype and colonisation potential differences in *Campylobacter jejuni* strains in chickens before and after passage *in vivo*. *Vet Microbiol,* **92,** 225-35.

**Rohwer, F. and Edwards, R. (2002)** The Phage Proteomic Tree: a genome-based taxonomy for phage. *J Bacteriol,* **184,** 4529-35.

**Roncero, C., Darzins, A. and Casadaban, M. J. (1990)** *Pseudomonas aeruginosa* transposable bacteriophages D3112 and B3 require pili and surface growth for adsorption. *J Bacteriol,* **172,** 1899-904.

**Rosenquist, H., Nielsen, N. L., Sommer, H. M., Norrung, B. and Christensen, B. B. (2003)** Quantitative risk assessment of human campylobacteriosis associated with thermophilic *Campylobacter* species in chickens. *Int J Food Microbiol,* **83,** 87-103.

**Sahin, O., Zhang, Q., Meitzler, J. C., Harr, B. S., Morishita, T. Y. and Mohan, R. (2001)** Prevalence, antigenic specificity, and bactericidal activity of poultry anti-*Campylobacter* maternal antibodies. *Appl Environ Microbiol,* **67,** 3951-7.

**Sails, A. D., Wareing, D. R. A., Bolton, F. J., Fox, A. J. and Curry, A. (1998)** Characterisation of 16 *Campylobacter jejuni* and *C. coli* typing bacteriophages. *Journal of Medical Microbiology,* **47,** 123-128.

**Salama, S., Bolton, F. J. and Hutchinson, D. N. (1989)** Improved method for the isolation of *Campylobacter jejuni* and *Campylobacter coli* bacteriophages. *Letters in Applied Microbiology,* **8,** 5-7.

**Salama, S., Bolton, F. J. and Hutchinson, D. N. (1990a)** Evaluation of methods for the enumeration of *Campylobacter jejuni* and *Campylobacter coli* bacteriophages. *Letters in Applied Microbiology,* **10,** 193-195.

**Salama, S. M., Bolton, F. J. and Hutchinson, D. N. (1990b)** Application of a new phagetyping scheme to campylobacters isolated during outbreaks. *Epidemiol Infect,* **104,** 405-11.

**Salyers, A. A. and Whitt, D. D. (1994)** Bacterial Pathogenesis: A Molecular Approach. American Society for Microbiology, Washington, D.C.

**Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989)** Molecular cloning: a laboratory manual. Cold Spring Harbor Press, New York.

**Sanders, M. E. (1987)** Bacteriophages of industrial importance *In* Phage Ecology (Bitton, G., Gerba, G. P., and Goyal, S. M. Eds) John Wiley & Sons, New York, pp. 211-244.

**Schoeni, J. L. and Doyle, M. P. (1992)** Reduction of *Campylobacter jejuni* colonization of chicks by cecum-colonizing bacteria producing anti-*C. jejuni* metabolites. *Appl Environ Microbiol,* **58,** 664-70.

**Shane, S. M. (2000)** *Campylobacter* infection of commercial poultry. *Rev Sci Tech,* **19,** 376-95.

**Shreeve, J. E., Toszeghy, M., Pattison, M. and Newell, D. G. (2000)** Sequential spread of *Campylobacter* infection in a multipen broiler house. *Avian Dis,* **44,** 983-8.

**Silverman, J. A. and Benson, S. A. (1987)** Bacteriophage K20 requires both the OmpF porin and lipopolysaccharide for receptor function. *J Bacteriol,* **169,** 4830-3.

**Singleton, P. and Sainsbury, D. (1999)** Dictionary of microbiology and molecular biology. John Wiley and Sons, Trowbridge.

**Skirrow, M. B. (1977)** *Campylobacter* enteritis: a "new" disease. *Br Med J,* **2,** 9-11.

**Skirrow, M. B. and Blaser, M. J. (2000)** Clinical aspects of *Campylobacter* infection *In Campylobacter* 2nd Ed (Blaser, M. J. and Nachamkin, I. Eds) American Society for Microbiology, Washington D.C., pp. 69-87.

**Skirrow, M. B., Jones, D. M., Sutcliffe, E. and Benjamin, J. (1993)** *Campylobacter* bacteraemia in England and Wales, 1981 - 91. *Epidemiol Infect,* **110,** 567-573.

**Smibert, R. M. (1984)** *Campylobacter In* Bergey's Manual of Systematic Bacteriology, Vol. 1 (Holt, J. G. and Krieg, N. R. Eds) The Williams & Wilkins Co., Baltimore.

**Smith, H. W. and Huggins, M. B. (1982)** Successful treatment of experimental *Escherichia coli* infections in mice using phage: its general superiority over antibiotics. *J Gen Microbiol,* **128,** 307-318.

**Smith, H. W. and Huggins, M. B. (1983)** Effectiveness of phages in treating experimental *Escherichia coli* diarrhoea in calves, piglets and lambs. *J Gen Microbiol,* **129 (8),** 2659-75.

**Soothill, J. S. (1992)** Treatment of experimental infections of mice with bacteriophages. *J Med Microbiol,* **37,** 258-61.

**Stent, G. S. and Adelberg, E. A. (1960)** Papers on Bacterial Viruses, Little, Brown and Co., Boston.

**Stern, N. J. (1994)** Mucosal competitive exclusion to diminish colonization of chickens by *Campylobacter jejuni. Poult Sci,* **73,** 402-7.

**Stern, N. J., Green, S. S., Thaker, N., Krout, D. J. and Chiu, J. (1984)** Recovery of *Campylobacter jejuni* from fresh and frozen meat and poultry collected at slaughter. *Journal of Food Protection,* **47,** 372-374.

**Stern, N. J., Hiett, K. L., Alfredsson, G. A., Kristinsson, K. G., Reiersen, J., Hardardottir, H., Briem, H., Gunnarsson, E., Georgsson, F., Lowman, R., Berndtson, E., Lammerding, A. M., Paoli, G. M. and Musgrove, M. T. (2003)** *Campylobacter* ssp. in Icelandic poultry operations and human disease. *Epidemiol Infect,* **130,** 23-32.

**Stern, N. J., Patton, C. M., Doyle, M. P., Park, C. E. and McCardell, B. A. (1992)** *Campylobacter In* Compendium for the microbiological examination of foods (Doores, S., Gabis, D. A., Ito, K. A., Jackson, G. J., Splittstoesser, D. F., Troller, J. A. and Vanderzant, C. Eds) American Public Health Association, Washington D.C., pp. 475-495.

**Stern, N. J., Robach, M. C., Cox, N. A. and Musgrove, M. T. (2002)** Effect of drinking water chlorination on *Campylobacter* ssp. colonization of broilers. *Avian Dis,* **46,** 401-4.

**Sulakvelidze, A., Alavidze, Z. and Morris, J. G., Jr. (2001)** Bacteriophage therapy. *Antimicrob Agents Chemother,* **45,** 649-59.

**Summers, W. C. (2001)** Bacteriophage therapy. *Annu. Rev. Microbiol.,* **55,** 437-51.

**Tauxe, R. V. (2000)** Incidence, trends and sources of campylobacteriosis in developed countries *In* The increasing incidence of human camylobacteriosis. Report and proceedings of a WHO Consultation of Experts. World Health Organisation, Copenhagen, pp. 42-43.

**Thomas, C., Hill, D. J. and Mabey, M. (1999)** Morphological changes of synchronized *Campylobacter jejuni* populations during growth in single phase liquid culture. *Lett Appl Microbiol,* **28,** 194-8.

**Tojo, N., Sanmiya, K., Sugawara, H., Inouye, S. and Komano, T. (1996)** Integration of bacteriophage Mx8 into the *Myxococcus xanthus* chromosome causes a structural alteration at the C-terminal region of the IntP protein. *J Bacteriol,* **178,** 4004-11.

**Trachoo, N., Frank, J. F. and Stern, N. J. (2002)** Survival of *Campylobacter jejuni* in biofilms isolated from chicken houses. *J Food Prot,* **65,** 1110-6.

**Tsubokura, K., Berndtson, E., Bogstedt, A., Kaijser, B., Kim, M., Ozeki, M. and Hammarstrom, L. (1997)** Oral administration of antibodies as prophylaxis and therapy in *Campylobacter jejuni*-infected chickens. *Clin Exp Immunol,* **108,** 451-5.

**Turner, P. C., McLennan, A. G., Bates, A. D. and White, M. R. H. (1998)** Instant notes in molecular biology, Bios Scientific Publishers, Oxford.

**Twort, F. W. (1915)** An investigation on the nature of the ultra microscopic viruses. *Lancet,* **2,** 1241.

**Van Den Ende, P. (1973)** Predator-prey interactions in continuous culture. *Science,* **181,** 562-564.

**Vandamme, P. (2000)** Taxonomy of the family campylobacteriaceae *In Campylobacter* 2nd Ed (Blaser, M. J. and Nachamkin, I. Eds) American Society for Microbiology, Washington, pp. 3-26.

**Waldenstrom, J., Broman, T., Carlsson, I., Hasselquist, D., Achterberg, R. P., Wagenaar, J. A. and Olsen, B. (2002)** Prevalence of *Campylobacter jejuni, Campylobacter lari,* and *Campylobacter coli* in different ecological guilds and taxa of migrating birds. *Appl Environ Microbiol,* **68,** 5911-7.

**Waldor, M. K. (1998)** Bacteriophage biology and bacterial virulence. *Trends Microbiol,* 6, 295-7.

**Wang, Y. and Taylor, D. E. (1990)** Natural transformation in *Campylobacter* species. *J Bacteriol,* 172, 949-55.

**Wassenaar, T., On, S. L. and Meinersmann, R. J. (2000)** Genotyping and the consequences of genetic instability *In Campylobacter* 2nd Ed (Blaser, M. J. and Nachamkin, I. Eds) American Society for Microbiology, Washington, D.C., pp. 369-380.

**Wassenaar, T. M. (1997)** Toxin production by *Campylobacter* ssp. *Clin Microbiol Rev,* 10, 466-76.

**Wassenaar, T. M. and Blaser, M. J. (1999)** Pathophysiology of *Campylobacter jejuni* infections of humans. *Microbes Infect,* 1, 1023-33.

**Wassenaar, T. M. and Newell, D. G. (2000)** Genotyping of *Campylobacter* ssp. *Appl Environ Microbiol,* 66, 1-9.

**Weber-Dabrowska, B., Mulczyk, M. and Gorski, A. (2000)** Bacteriophage therapy of bacterial infections: An update of our institute's experience. *Archivum Immunologiae et Therapiae Experimentalis,* 48, 547-551.

**Whyte, P., Collins, J. D., McGill, K., Monahan, C. and O'Mahony, H. (2001)** Quantitative investigation of the effects of chemical decontamination procedures on the microbiological status of broiler carcasses during processing. *J Food Prot,* 64, 179-83.

**Widders, P. R., Perry, R., Muir, W. I., Husband, A. J. and Long, K. A. (1996)** Immunisation of chickens to reduce intestinal colonisation with *Campylobacter jejuni. Br Poult Sci,* 37, 765-78.

**Wiggins, B. A. and Alexander, M. (1985)** Minimum bacterial density for bacteriophage replication: implications for significance of bacteriophages in natural ecosystems. *Appl Environ Microbiol,* 49, 19-23.

**Wilkinson, M. H. (2001)** Predation in the presence of decoys: an inhibitory factor on pathogen control by bacteriophages or bdellovibrios in dense and diverse ecosystems. *J Theor Biol,* **208,** 27-36.

**Wooldridge, K. G. and Ketley, J. M. (1997)** *Campylobacter* host-cell interactions. *Trends Microbiol,* **5,** 96-102.

**Yang, H., Li, Y. and Johnson, M. G. (2001)** Survival and death of *Salmonella* Typhimurium and *Campylobacter jejuni* in processing water and on chicken skin during poultry scalding and chilling. *J Food Prot,* **64,** 770-6.

**You, L., Suthers, P. F. and Yin, J. (2002)** Effects of *Escherichia coli* physiology on growth of phage T7 *in vivo* and in silico. *J Bacteriol,* **184,** 1888-94.

**Young, C. R., Ziprin, R. L., Hume, M. E. and Stanker, L. H. (1999)** Dose response and organ invasion of day-of-hatch Leghorn chicks by different isolates of *Campylobacter jejuni. Avian Dis,* **43,** 763-7.

**Young, V. B., Schauer, D. B. and Fox, J. G. (2000)** Animal models of *Campylobacter* infection *In Campylobacter* 2nd Ed (Blaser, M. J. and Nachamkin, I. Eds) ASM Press, Washington, D.C., pp. 287-301.

**Zhao, C., Ge, B., De Villena, J., Sudler, R., Yeh, E., Zhao, S., White, D. G., Wagner, D. and Meng, J. (2001)** Prevalence of *Campylobacter* ssp., *Escherichia coli,* and *Salmonella* serovars in retail chicken, turkey, pork, and beef from the Greater Washington, D.C., area. *Appl Environ Microbiol,* **67,** 5431-6.

**Zhou, L., Srisatjaluk, R., Justus, D. E. and Doyle, R. J. (1998)** On the origin of membrane vesicles in Gram-negative bacteria. *FEMS Microbiol Lett,* **163,** 223-8.

**Zink, R. and Loessner, M. J. (1992)** Classification of virulent and temperate bacteriophages of *Listeria* ssp. on the basis of morphology and protein analysis. *Appl Environ Microbiol,* **58,** 296-302.