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06-9:
Phage displayed peptides for controlling and diagnosing Campylobacter infection in poultry

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Phage-displayed peptides for diagnosing and controlling Campylobacter infection in poultry
Project No. 06-9

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Executive summary

In developed countries, *Campylobacter jejuni* is a leading cause of zoonotic bacterial gastroenteritis in humans with chicken meat implicated as a source of infection. *Campylobacter jejuni* colonises the lower gastrointestinal tract of poultry and during processing is spread from the gastrointestinal tract onto the surface of dressed carcasses. Controlling or eliminating *C. jejuni* on-farm is considered to be one of the best strategies for reducing human infection due to this organism. Molecules on the cell surface of *C. jejuni* interact with the host to facilitate its colonisation and persistence in the gastrointestinal tract of poultry.

We used a subtractive phage-display protocol to affinity select for peptides binding to the cell surface of a poultry isolate of *C. jejuni* with the aim of finding peptides that could be used as a therapeutic to control this organism in chickens. In total, 44 phage-displayed peptides were isolated, representing 27 unique clones. Sixteen of these 27 unique phage peptides were found to inhibit the growth of *C. jejuni* by up to 97% *in-vitro*. One phage clone (CP3/2) was bacteridical, reducing the initial inoculum of *C. jejuni* by 84% *in-vitro*. These phage clones were novel in that unlike current antimicrobials, they were highly specific in their activity towards *C. jejuni*; none of them showed any activity against Gram-negative and Gram-positive bacteria tested. Unfortunately, these phage peptides also had a tendency to be strain specific; showing antimicrobial activity in only four of the five *C. jejuni* strains tested indicating that the targeted epitope was not present in all *C. jejuni* strains.

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Introduction

Campylobacter jejuni is a small Gram-negative bacterium that is a common cause of zoonotic bacterial gastroenteritis in humans (Blaser, 1997). In industrialised countries, most cases of *C. jejuni* infection are self-limiting and characterised by diarrhoea, fever and abdominal cramps; however, in a small number of cases, post-infection complications can arise, including the peripheral neuropathies such as Guillain-Barré and Miller-Fisher syndromes (Allos, 2001; Altekruse et al. 1999). The mishandling of raw poultry and consumption of under-cooked poultry meat are often precursors for human infection (Wingstrand et al. 2006).

C. jejuni naturally colonises the gastrointestinal tract of many birds (including chickens), where it establishes a commensal relationship with its host. In chickens, *C. jejuni* is often associated with the mucus layer of the deep crypts of the caecum, where it colonises to high levels (up to 1×10^9 CFU/g of intestine), without invading host tissues. In contrast, *C. jejuni* is pathogenic to humans, resulting in an acute gastroenteritis even at a low infectious dose.

Reducing *C. jejuni* carriage in chickens is considered to be one of the most important approaches for reducing human infection (Rosenquist et al. 2003). However, the routes to infection for chickens remain unclear and thus difficult to control (Havelaar et al. 2007) due to the ubiquitous presence of this organism in the intestines of many animals, pets, birds and insects (Bates et al. 2004; Broman et al. 2004; Gregory et al. 1997). Chickens usually become colonised by *C. jejuni* between 4 to 7 weeks of age (Denis et al. 2001; Evans & Sayers 2000) and the bacteria is rapidly transmitted to the remaining flock within a few days via horizontal transmission.

During poultry processing, *Campylobacter* is spread from the gastrointestinal tract of chickens onto the surface of dressed carcasses (Berrang et al. 2006). Studies have shown that in most developed countries, approximately 20-73% of retail chicken meat is surface contaminated with thermophilic *Campylobacter* spp. (Altekruse et al. 1999; Cason et al. 1997; Pointon et al. 2008).

To date, there has been no single, effective strategy for controlling the colonisation of chickens on-farm by *Campylobacter*. For example, on-farm biosecurity practices that are usually considered to be an important method for reducing *Campylobacter* infection in chickens, have usually achieved up to a 50% reduction of infection in broiler flocks (Gibbens et al. 2001). Other methods such as probiotics and competitive exclusion with beneficial bacteria have reduced *Campylobacter* colonisation by 2-27% (Morishita et al. 1997; Stern et al. 2005).

Therapeutics targeted at eliminating or reducing *C. jejuni* from the gastrointestinal tracts of chickens, prior to slaughter, would markedly improve the chances of producing carcasses free from these organisms. Current antibiotics, designed for human medicine, generally have broad-spectrum activity and fall into a relatively small number of classes with respect to mechanisms of action. Consequently, chickens fed these antibiotics end up as a reservoir for antibiotic-resistant bacteria in the gastrointestinal tract, which can then end up being transmitted to humans via contaminated poultry in the retail store. A strategy for overcoming this problem would be the development of novel antimicrobials with unique modes of action (Christensen et al. 2001). Ideally, these compounds would target multiple strains of *C. jejuni* whilst being highly specific to this organism. One example of this is the use of bacteriocins. Stern et al. (2005) demonstrated that bacteriocins, purified from *Paenibacillus polymyxa* significantly reduced the presence of *C. jejuni* in the gastrointestinal tract of chicks.

Since it was first reported (Smith, 1985), combinatorial technologies such as phage-display have become routinely used to affinity select for peptides binding to small molecules (Saggio & Laufer, 1993), enzymes (Kay & Hamilton, 2001) and purified receptors (White et al. 2005). It has also become a powerful tool for the affinity selection of peptides binding to the cell surface epitopes on intact cells because it allows for the isolation of targeting peptides in cases where little is known about the cellular landscape (Fong et al. 1994). It has therefore been used to generate diagnostic and therapeutic targeting peptides for a variety of pathogens including plant fungal zoospores (Bishop-Hurley et al.

2002; Fang et al. 2006), bacteria (Bishop-Hurley et al. 2005; Carnazza et al. 2008; Sorokulova et al. 2005) and insects (Ghosh et al. 2002).

Protein and other surface molecules on bacteria such as *C. jejuni* play a significant role in the persistence and colonisation of these organisms within the host. Consequently, these cell surface molecules/ receptors represent a significant target for disruption by inhibitory antagonistic molecules without prior knowledge of what these receptors/ epitopes are. In this project, we demonstrate that these "unknown" receptors/ cell surface molecules could be targeted using phage-display libraries. Furthermore, we found that the isolated peptides were highly specific towards *C. jejuni*, inhibiting its growth by almost 100% in a species-specific manner. We also demonstrate that the free peptides in solution were active towards *C. jejuni*.

Objectives

This project spanned two granting periods as detailed below.

A. July 1st 2006-30th June 2007

B. 1st July 2007 – 1st December 2009. Please note that this grant also included a one-year proposal on phage peptides for real-time detection of *C. jejuni*. This part of the project finished on 30th June 2008 and a final report was submitted.

Part A: 1st July 2006 – 30th June 2007

Reducing or eliminating the presence of *Campylobacter jejuni* in the gastrointestinal tract of chickens pre-slaughter would correspondingly reduce zoonotic food-borne illnesses due to this microorganism in humans (Rosenquist et al. 2003). Protein and other surface molecules on *C. jejuni* play a significant role in the colonisation and persistence of this organism in the gastrointestinal tract of chickens. Consequently, these cell surface molecules/receptors represented a significant target for disruption by inhibitory antagonistic molecules.

The aim of this one year proof-of-concept project was to demonstrate, as an initial proof of concept, that we could target these "unknown" cell surface receptors/ molecules using peptides selected from random phage-display libraries. These peptides were selected on the basis of their ability to bind to live *C. jejuni* and then tested for their potential to act as species-specific antimicrobials using *in-vitro* assays involving strains of *C. jejuni* isolated from chickens and other gram-negative and gram-positive bacteria.

In particular, the specific aims of Part A were to:

Specific Aim 1: Isolate phage-displayed peptides binding to live *C. jejuni* bacteria

***Aim 1A*:** Establish methods for growing pure cultures of *C. jejuni* on Campylobacter agar plates and in liquid broth.

***Aim 1B*:** Affinity-select phage-displayed peptides binding to outer surface receptors of *C. jejuni*

***Aim 1C*:** Sequence phage-displayed peptides that have been isolated through the affinity selection procedure in Aim 1B.

Specific Aim 2: To test selected phage-displayed peptides isolated from Specific Aim 1 for antimicrobial/ bactericidal activities against *C. jejuni*

***Aim 2A*:** To test phage-displayed peptides for potential inhibitory growth activities against *C. jejuni*

***Aim 2B*:** To determine/ characterise the antimicrobial/ bactericidal activities of the phage-displayed peptides

***Aim 2C*:** To determine the specificity of antimicrobial/ bactericidal activity of the phage-displayed peptides towards *C. jejuni*

Part B: 1st July 2007 – 1st December 2009

In Part A (1st July 2006 - 30th June 2007), we isolated a set of phage peptides that inhibited the growth of poultry isolates of *C. jejuni in-vitro*. Some of these peptides were greater than 99% effective in inhibiting the growth of *C. jejuni*. The overall aim of this follow-up project was to develop data to allow a full patent application to be filed and to develop proof-of-concept showing that these phage peptides were effective as vaccines or therapeutic agents against *C. jejuni*. In particular, we fused selected peptides with a suitable carrier system so that the activities of the peptides were retained. We also aimed to determine the effects of the peptides on *C. jejuni* colonisation and carrying out animal trials to assess the effects of the peptides on *C. jejuni*.

In particular, the specific aims of part B were to:

Specific Aim 1: Further isolate bactericidal and antimicrobial phage clones and test them for *in-vitro* activity

***Aim 1A*:** Isolation of bactericidal phage clones

***Aim 1B*:** Antimicrobial activity of the phage clones

***Aim 1C*:** Testing the specificity of binding towards *C. jejuni* and other bacterial spp. using Western blots and co-precipitation assays

Specific Aim 2: Mode of action of phage peptides.

Specific Aim 3: Activity of the phage peptides outside of the phage coat

Specific Aim 4: Testing of the phage clones in an animal trial

Methodology

1.0 Bacterial methods

1.1 Bacterial strains used in this work

The bacterial strains used in this work are detailed in Table 1. In brief: *Campylobacter jejuni* strains, isolated from Queensland poultry houses were kindly provided by Dr Pat Blackall (QDPI&F, Australia). A reference strain of *C. jejuni* subsp. *jejuni* (ACM 3393, equivalent to NCTC 11351) was purchased from the Australian Collection of Microorganisms (ACM; University of Queensland, QLD, Australia). *Salmonella enterica* subsp. *enterica* (ATCC 14028), *Enterococcus faecalis* (ATCC 29212) and *Proteus mirabilis* (ATCC 43071) were obtained from the American Type Culture Collection (ATCC; Oxoid, Australia) as Culti-Loops®. *Escherichia coli* 026 was originally isolated from cattle (Queensland, Australia) and provided by Dr Chris McSweeney (CSIRO-Livestock Industries, St. Lucia).

1.2 Growth of bacteria on solid media

All bacterial strains were maintained at -75 °C using the "Protect" Bacterial Preserve Vials (Oxoid, Australia). *C. jejuni* strains were streaked onto Campylobacter agar plates (purchased from Oxoid, Australia; Catalogue No. PP2005) and incubated at 37 °C for 24-48 hr. A microaerophilic atmosphere was provided by the use of CampyGen sachets (Oxoid, Australia; Catalogue No. CN0025) in Oxoid 2.5 L anaerobe jars. Alternatively, GasPakE2 Campy Pouches (BD Biosciences, Australia; Catalogue No. 260-685) were used to provide a microaerophilic environment.

C. jejuni was also grown on blood agar plates [consisting of 40 g/l Blood Agar Base No. 2 (Oxoid, Catalogue No. CM0217B), supplemented with 5% (v/v) defibrinated horse blood (Oxoid, Catalogue No. HB250) and Campylobacter growth supplement (Oxoid, Catalogue No. SR0232E)] and incubated as above. *S. typhimurium*, *E. faecalis*, *P. mirabilis* and *E. coli* 026 were grown at 37 °C for 24 hr using the same media, except that they were grown aerobically and the Campylobacter growth supplement was omitted.

1.3 Growth of bacteria in liquid media

C. jejuni strains incubated overnight on blood agar plates were re-suspended in Nutrient Broth No. 2 (Oxoid, Australia; Catalogue No. CM0067) to an OD_{595} of 0.2 (equated to approximately 5-7 x 10^8 CFU/ml). Aliquots of *C. jejuni* (~10^6 CFU/ml) were transferred to 15 ml polypropylene tubes (120 x 17 mm; Sarstedt, Australia) containing 10 ml Nutrient Broth and 4 µl Campylobacter growth supplement (Oxoid, Australia). These tubes were incubated for 48 hr at 37 °C with shaking (180 rpm) with an internal microaerophilic environment provided by the use of GasPakE2 Campy Pouches (BD Biosciences, Australia). *S. typhimurium*, *E. faecalis*, *P. mirabilis* and *E. coli* 026 were grown at 37 °C for 24 hr using the same media, except that they were grown aerobically and the Campylobacter growth supplement was omitted.

Bacterial strains	**Characteristics**	**Source**
C. jejuni	ACM 3393 (NCTC 11351)	Australian Collection of Microorganisms
	C338 poultry isolate	Pat Blackall, QDPI&F
	C838 poultry isolate	
	C1208 poultry isolate	
	C1209 poultry isolate	
	C1212 poultry isolate	
	C858 poultry isolate	
	C654 poultry isolate	
	C660 poultry isolate	
	C627 poultry isolate	
	C541 poultry isolate	
E. coli	026; cattle isolate	Chris McSweeney, CSIRO-LI
E. coli	K91BlueKan	George Smith, University of Missouri
S. enterica subsp. *enterica*	ATCC 14028; chicken isolate	Oxoid, Australia
E. faecalis	ATCC 29212; urine isolate	Oxoid, Australia
P. mirabilis	ATCC 43071; rectal isolate	Oxoid, Australia

Table 1: Bacterial strains used in this study

1.4 Growth of *C. jejuni* strains in 96-well plates

C. jejuni strains were grown in 96-well plates for the *in-vitro* microtiter broth dilution assay. Each well in a 96 well polystyrene microtiter plate (Sarstedt, Australia) was inoculated with 150 µl of *C. jejuni* (equivalent to 1-5 x 10^5 CFU/ml) and sealed with a gas permeable cover (AeraSeal; Sigma, Australia). After incubation in a microaerophilic environment for 48 hr at 37 ºC, the optical densities of the bacterial cultures were measured in a plate reader at A_{595} nm (Wallac Victor2, Perkin Elmer, Australia).

2.0 Phage-display methods

2.1 Phage libraries and general laboratory procedures

The f88-4/15-mer peptide phage-display library (provided by Dr G. P. Smith, University of Missouri, Columbia, USA) was used for the affinity selection experiments. This library consisted of filamentous bacterophage fd-tet displaying 15-mer amino acid peptides fused to 150-300 copies of major coat protein pVIII. It contained a complexity of 2.0 x 10^9 peptide variants/ml and was used to affinity select for peptides binding to the cell surface of *C. jejuni* (Figure 1). *E. coli* K91BlueKan was also provided by Dr G. P. Smith (University of Missouri, Columbia, USA) and this was used for the propagation of the individual phage clones (Yu & Smith, 1996). Details of the library construction are detailed by Smith & Scott (1993).

2.2 Phage growth, purification and titering in *E. coli*

Phage was purified using the double polyethylene glycol (PEG)-NaCl precipitation (16.7%, 3.3 M), as described by Yu & Smith (1996) and resuspended in Tris-borate Saline buffer (TBS; 50 mM Tris-Cl, pH 7.5; 150 mM NaCl). The concentration of phage particles in the solution was determined spectrophotometrically, assuming that an absorbance of 1.0 at A_{269} corresponded to 6.7 x 10^{12} virions/ml for the 9273 bp f88-4/15-mer phage library.

The number of infective phage in solution was determined by infecting starved *E. coli* K91BlueKan cells with phage and spreading them onto Luria broth (LB) plates containing 40 µg/ml tetracycline and 1 mM isopropyl β-D-1 thiogalactopyranoside (IPTG). The number of resultant colonies on the plate were determined as tetracycline transducing units (TU) and corresponded to the biological titre of the phage. These general procedures are described fully by Smith & Scott (1993) and Yu & Smith (1996).

2.3 Peptide synthesis

Monomeric 15 amino acid peptide sequences, corresponding to the selected phage peptide sequences were custom synthesised by AusPep Pty Ltd (VIC, Australia).

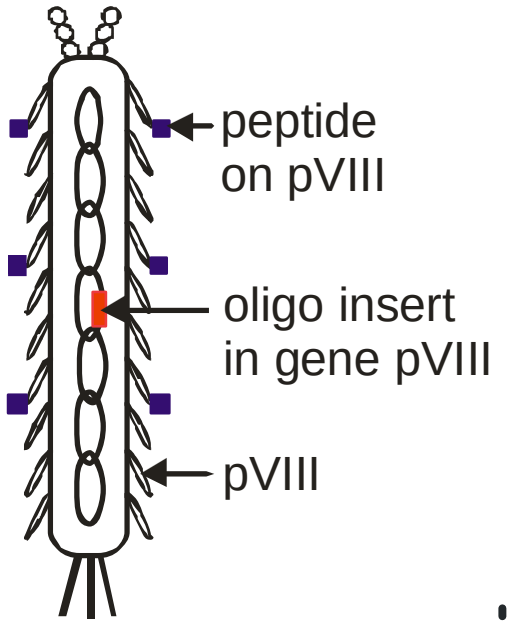


Figure 1: **Peptide display on filamentous bacteriophage.** In this f88 library, foreign peptides fused to the pVIII gene are displayed on major coat protein at 150-300 copies/ virion.

2.4 Affinity selection of phage-displayed peptides binding to *C. jejuni*

In the affinity selection procedures, phage-displayed peptides were selected on the basis of their ability to bind to *C. jejuni*. Three different selection strategies were used based on progressive pre-adsorption of the phage library against the cell surface of *C. jejuni* (Figure 2). This was based on the rationale that progressive pre-adsorption against the cell surface of *C. jejuni* progressively captures peptides binding to less dominant epitopes.

In selection scheme 1, the f88-4/15-mer phage library was directly selected against cell surface of *C. jejuni*, with the resulting phage peptides designated 'A' (Figure 2A). In selection scheme 2, the phage library was subtracted once against *C. jejuni* strain ACM 3393, prior to three rounds of affinity selection against C338. The resultant phage peptides were designated 'B' (Figure 2B). In selection scheme 3, the phage library was subtracted twice against strain ACM 3393, prior to three rounds of affinity selection against C338. These phage peptides were designated 'C' (Figure 2C). In selection scheme 4, the phage library was subtracted twice against ACM 3393 and once against C338, prior to three rounds of affinity selection against C338. These phage peptides were designated 'D' (Figure 2D).

Selection scheme 1

C. jejuni strain ACM 3393 was grown in liquid culture for 48 hr at 37 °C at 180 rpm under microaerophilic conditions. This culture was washed once by centrifugation (5,000 rpm for 10 min) and resuspended in phosphate buffered saline (PBS, pH 7.4) containing 0.1% gelatine (PBSg) to a concentration of 10^9 CFU/ml. In the affinity selection procedure, 10^{10} transducing units (TU; Smith & Scott, 1993) of the f88-4/15-mer phage library was incubated with 10^9 CFU of ACM 3393 in a total volume of 3 ml PBSg (pH 7.4) in a Petri dish (Sarstedt, Australia). This *C. jejuni*-phage mixture was kept in solution by gentle agitation on an orbital shaker at room temperature to allow binding of phage to *Campylobacter* in solution. After 45 min incubation, phage binding to *C. jejuni* was separated from unbound phage through a series of washing and centrifugation steps (5,000 rpm for 2 min) using 1 ml PBSg (pH 7.4) each time. After the final wash, the phage binding to ACM 3393 was eluted non-specifically from the pelleted ACM 3393 by the addition of 200 µl of elution buffer (0.1 N HCl, pH 2.2, 1 g/l bovine serum albumin, pH adjusted to 2.2 with glycine) and incubated for 10 min at room temperature. The phage eluted from the outer surface of *C. jejuni* was immediately neutralised with 1 M Tris.Cl (pH 9.0). This phage was titered as transducing units in *E. coli* K91BlueKan cells so that the yield of phage binding to *C. jejuni* could be determined (calculated as output/input x 100; Yu & Smith, 1996). The remaining phage was amplified by *E. coli* K91BlueKan infection, twice purified by double (PEG)-NaCl precipitation and resuspended in TBS buffer (Smith & Scott, 1993; Yu & Smith, 1996).

After the first round of affinity selection, it became apparent that we had problems with non-specific binding of the phage to *C. jejuni*. Thus, we explored the use of polyoxyethylene sorbitan monolaurate (Tween-20) for removing non-specific binding of the phage in the second and third rounds of affinity selection. Amplified phage (10^{10} TU) from the first round of affinity selection were separately added to two Petri dishes, each containing 10^9 CFU of *C. jejuni* strain ACM 3393 (Figure 2A). The entire process of affinity selection was repeated as above for the first round of affinity selection, except that the phage-bacteria complex from one Petri dish was washed with PBSg (pH 7.4) and the other phage-bacteria mix washed with TBS buffer (pH 7.5) containing 0.5% (v/v) Tween-20 (TBST). The phage from these two sub-libraries were eluted off the cell surface of *C. jejuni* and independently propagated in *E. coli* K91BlueKan cells for the third round of affinity selection. After the third and final round of affinity selection, phages were used to separately infect *E. coli* K91BlueKan cells and plated on LB plates containing 40 µg/ml tetracycline and 1 mM IPTG to select for bacteria colonies containing phage. Bacterial colonies, each containing a unique phage clone, were randomly selected for

propagation and subsequent analysis. The phages from these two sub-libraries were named AP3/ and AT3/ for those washed using PBSg and TBST, respectively (Figure 2A).

Selection scheme 2

This selection scheme was essentially the same as for selection scheme 1, except that the phage library was subtracted once of clones binding to ACM 3393, prior to three rounds of affinity selection for clones binding to ACM 3393 (as detailed in Figure 2B). In the subtraction step, 10^{10} TU of the primary library was added to 10^{9} CFU of *C. jejuni* ACM 3393. This bacterial-phage solution was agitated on an orbital shaker (as for selection scheme 1). The phage clones NOT binding to ACM 3393 were removed via a brief centrifugation step and added to freshly prepared ACM 3393 for the first round of affinity selection. The affinity selection of these phage peptides for binding to the cell surface of *C. jejuni* ACM 3393 were then carried out as detailed for selection scheme 1. Again because of problems with non-specific binding, the eluted phages from the first round of affinity selection against ACM 3393 were divided into two sub-libraries and independently propagated. The resulting phage peptides from these two sub-libraries were named BP3/ and BT3/ (Figure 2B).

Selection scheme 3

In selection scheme 3, the phage library was depleted twice of phage clones binding to ACM 3393 prior to three rounds of affinity selection against C. jejuni strain C338 (Figure 2C). The subtractions and affinity selections were carried out as per above selection schemes. After the third and final round of affinity selection, phages eluted from these two sub-libraries were named CP3/ and CT3/ for those washed using PBSg and TBST, respectively (Figure 2C).

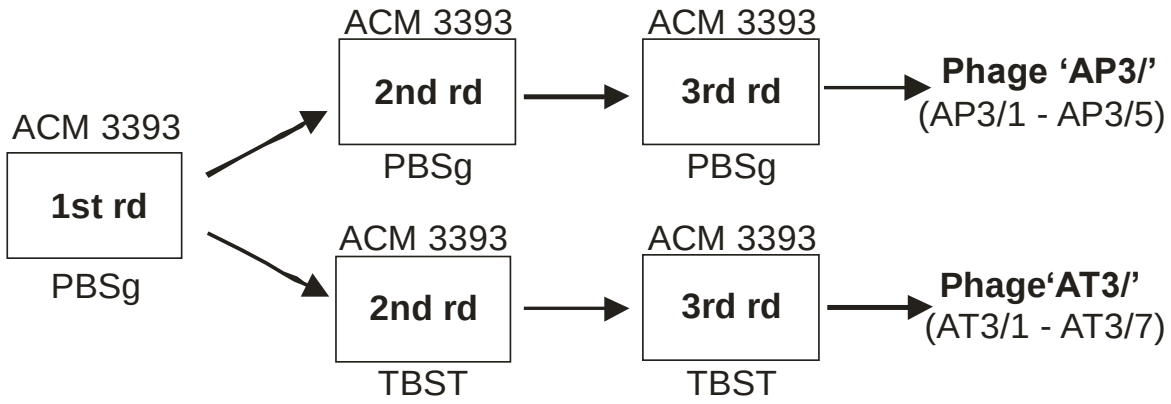
Selection scheme 4

In this selection scheme, the phage-display library was subtracted twice against ACM 3393 and once against C338 (Figure 2D). The subtractions and affinity selections were carried out as per above selection schemes. After the third and final round of affinity selection, phages eluted were named DP3/ and DT3/ respectively (Figure 2D).

Selection scheme 5

This selection scheme was for Part B of the report and is based on selection scheme 1. The outputs from the second round of selection against ACM 3393 was amplified and selected against C338 for two rounds of selection (resulting in a total of four rounds of affinity selection; Figure 1E). These phage clones were washed using PBSg (AP4/). This selection scheme in Figure 1F was identical except that the phage clones were washed with TBST (AT4/).

A:


ACM 3393
1st rd
PBSg
ACM 3393
2nd rd
PBSg
ACM 3393
3rd rd
PBSg
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(AP3/1 - AP3/5)
ACM 3393
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TBST
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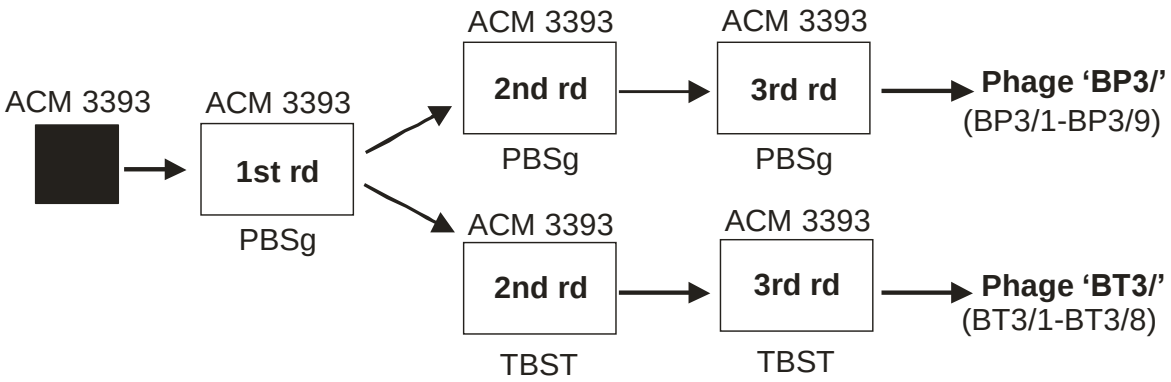
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ACM 3393
1st rd
PBSg
ACM 3393
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PBSg
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PBSg
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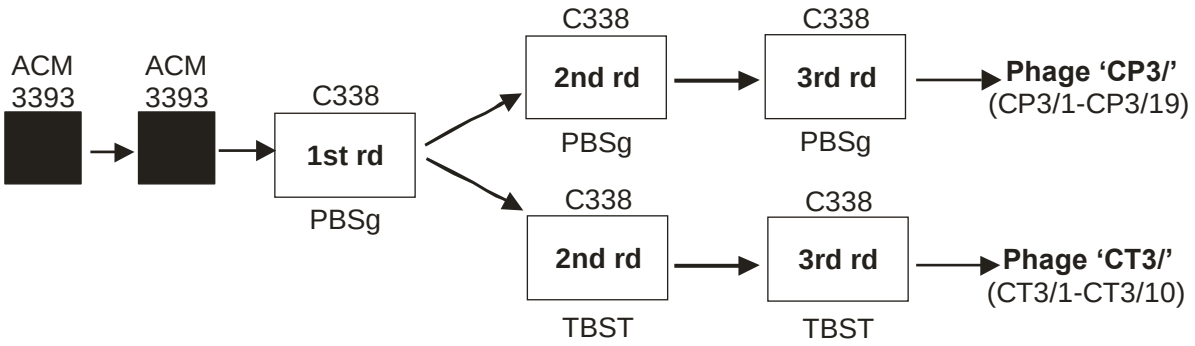
C:


ACM 3393
ACM 3393
C338
1st rd
PBSg
C338
2nd rd
PBSg
C338
3rd rd
PBSg
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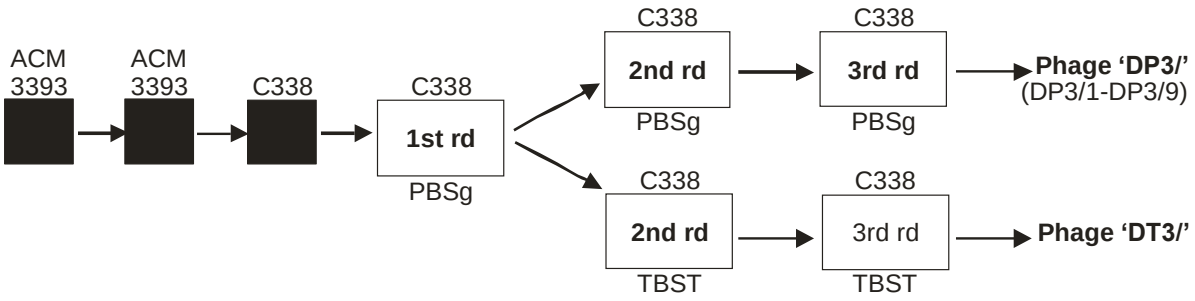
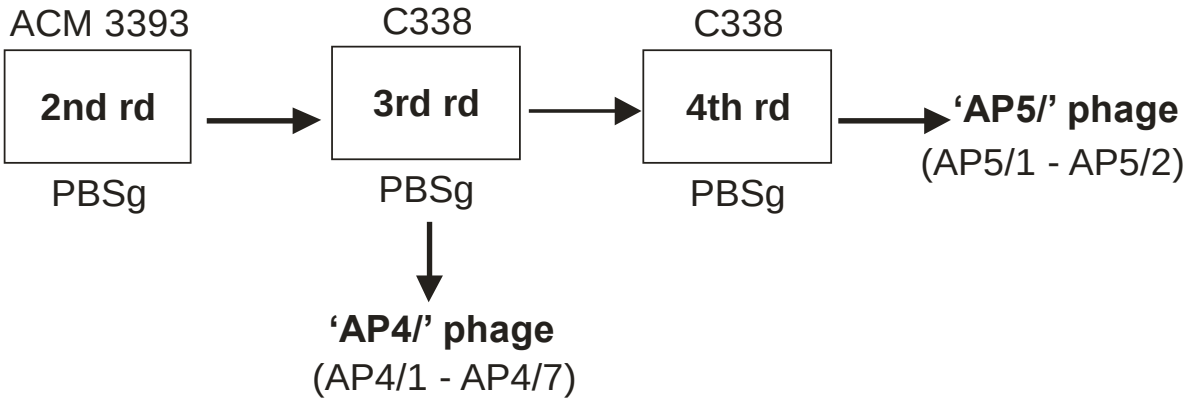
D:


ACM 3393
ACM 3393
C338
C338
1st rd
PBSg
C338
2nd rd
PBSg
C338
3rd rd
PBSg
Phage 'DP3/'
(DP3/1-DP3/9)
C338
2nd rd
TBST
C338
3rd rd
TBST
Phage 'DT3/'

E:



F:

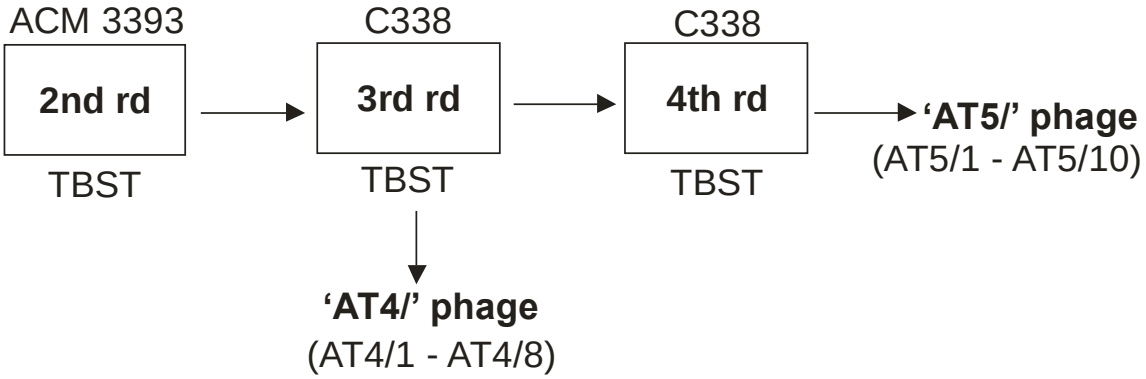


Figure 2: Selection strategies for isolating phage peptides binding to *C. jejuni*

A. Phage peptides from the f88-4/15-mer library were directly selected against *C. jejuni* ACM 3393, resulting in "AP" and "AT" pools of phage peptides

B. The phage-display library was subtracted once against an ATCC strain of *C. jejuni* (black box), prior to three affinity selections against *C. jejuni* ATCC. This resulted in "BP" and "BT" phage peptides.

C. The phage-display library was subtracted twice against *C. jejuni* strain ATCC, prior to three affinity selections against *C. jejuni* strain C338 (poultry strain). This resulted in "CP" and "CT" phage peptides.

D. The phage-display library was subtracted twice against *C. jejuni* ATCC and once against *C. jejuni* strain C338, prior to three affinity selections against C338. This resulted in "DP" and "DT" phage peptides.

E. The outputs from the second round of selection against ACM 3393 in selection scheme 1 was amplified and used to select against C338, resulting in "AP4/" phage.

F. The outputs from the second round of selection against ACM 3393 in selection scheme 1 was amplified and used to select against C338, resulting in "AT4/" phage

3.0 Sequencing and analysis of the phage clones

Single-stranded DNA was isolated from individual phage clones and sequenced from the 3' end of the pVIII gene using the primer (5'-AGTAGCAGAAGCCTGAAGA-3'; Invitrogen, Australia) at Macquarie University Sequencing Facility (NSW, Australia). The DNA sequences were conceptually translated into amino acids using the 'translate' program available on the proteomics server at the Swiss Institute of Bioinformatics Expert Protein Analysis System (ExPASy) (http://www.expasy.ch). The theoretical isoelectric points of the predicted peptide sequences were calculated using the 'compute MW/pI' tool, also available from the ExPASy server (Bjellqvist et al. 1993). The amino acids were aligned and shaded according to similarity using the ClustalW sequence alignment program (Larkin et al. 2007) and viewed using GeneDoc (http://www.psc.edu/biomed/genedoc; Nicholas et al. 1997). MIMOX (http://web.kuicr.kyoto-u.ac.jp/~hjian/mimox/) was used for the bioinformatical analysis of the peptide sequences. Homologies of the translated peptide sequences with known proteins were obtained using the Basic Local Alignment Search Tool (BLAST) for short peptides using the algorithm 'blastp for short nearly exact matches' (NCBI; http://www.ncbi.nlm.nih.gov/blast).

4.0 *In-vitro* antimicrobial assay

The Clinical and Laboratory Standards Institute (CLSI) recognises only the agar dilution method as the standardized method for *in-vitro* antimicrobial susceptibility testing of *Campylobacter* spp. However, because this method is labour intensive and cost-prohibitive when small numbers of isolates are being tested, two laboratories, including the FDA and Campylobacter Susceptibility Testing Group, have published protocols for the use of broth microdilution susceptibility testing of *C. jejuni* (Luber et al. 2003; McDermott et al. 2005). These published methods were used as the basis for our work.

Representative affinity-selected phage-displayed peptides were tested for their ability to inhibit the growth of selected bacterial stains *in-vitro* using the microtiter broth dilution assay. Each phage clone was amplified by *E. coli* infection, twice purified by (PEG)-NaCl precipitation, as detailed above, and resuspended in TBS buffer (pH 7.5). Phage concentrations were calculated spectrophotometrically as virions/µl using ultraviolet (UV) absorbance. Bacteria were grown on blood agar plates for 18 hr at 37 °C and resuspended to an OD_{595} of 0.2 in Nutrient Broth. The bacteria were further diluted to 1-5 x 10^5 CFU/ml using the same media.

For each experiment, individual wells in a flat-bottomed 96-well polystyrene microtiter plate (Sarstedt, Australia) were inoculated with 150 µl of 1-5 x 10^5 CFU/ml of bacteria and 45 µl of phage-displayed peptides (in TBS, pH 7.5). Controls included bacteria containing 45 µl TBS (pH 7.5) without phage-displayed peptides and bacteria containing dilutions of an irrelevant phage peptide displaying the 15 amino acid sequence HSAIYYKNFGSSLFR (Tek1/6). This phage peptide showed the same low level of binding as the unselected phage-display library f88-4/15 and so was used as the control for all experiments. After 16-18 hr incubation at 37 °C without shaking, the bacterial cell growth was assessed by measuring the optical density of the culture at A_{595} nm on a Wallac Multi-label Counter (Victor2, Perkin Elmer Life-Sciences, Australia).

The minimal inhibitory concentration (MIC) was determined by monitoring the growth inhibition using two-times serial dilutions of the phage-displayed peptides and was considered to be the lowest concentration at which there was no visible growth spectrophotometrically when measured at A_{595} nm. The number of viable bacteria in the 96-well plates was also determined by performing colony counts on the diluted inoculum by plating onto blood agar plates. The number of input bacteria for each experiment was also enumerated by plating dilutions onto blood agar plates.

A Gram-Stain kit (BD Biosciences, Australia) was also used to investigate changes in bacterial morphology after incubation with the phage peptides.

4.1 Measuring salt sensitivity of the phage clones

Selected phage clones were grown and re-suspended in low salt buffer (10 mM sodium phosphate buffer, pH 7.4). These phage clones were then tested for their ability to inhibit the growth of *C. jejuni* strain C338 using the microtiter broth dilution assay. In brief: each well in a 96-well microtiter plate was inoculated with 150 µl of 1-5 x 10^5 CFU/ml of bacteria and 45 µl of phage-displayed peptides in 10 mM sodium phosphate buffer (pH 7.4). Controls included bacteria without phage-peptides and bacteria containing an irrelevant peptide. After an overnight incubation at 37 ºC in microaerophilic conditions without shaking, the optical density of the bacterial cultures was measured at OD_{595} in a plate reader. The number of viable *C. jejuni* remaining in the 96-well microtiter plates was determined by plating onto solid blood agar plates and compared with the number of colonies of a control phage peptide.

4.3 Effect of growth phase on antimicrobial activity

Each well in a 96-well plate was inoculated with 1 x 10^5 CFU/ml of *C. jejuni* strain C338 and selected phage-displayed peptides. Controls included *C. jejuni* with an irrelevant phage peptide. These were incubated at 37 °C under microaerophilic conditions and at various intervals, the amount of bacterial growth was assessed in the 96-well plates by measuring the absorbance at A_{595} nm.

4.3 Experimentally induced resistance to phage peptides

Using the *in-vitro* microtiter broth dilution assay, each well of a 96-well plate was inoculated with 1 x 10^5 CFU/ml of *C. jejuni* at concentrations sufficient to induce 100% inhibition of growth. Controls included *C. jejuni* without phage peptides (45 µl of TBS, pH 7.5) and *C. jejuni* incubated with an irrelevant phage peptide (displaying the sequence HSAIYYKNFGSSLFR; Tek1/6). After an overnight incubation at 37 °C, the bacteria grown in the presence of the phage peptides were enumerated by performing colony counts on blood agar plates. The number of resulting colonies was compared with *C. jejuni* grown in the presence of the control phage peptide.

Three separate colonies of *C. jejuni* were chosen from the output blood agar plates and termed passage 1 because they were grown in the presence of the inhibitory phage peptides. These output *C. jejuni* were re-tested for potential resistance against the selected inhibitory phage peptides and the process repeated with each output *C. jejuni* termed passage 1, 2, 3 etc.

5.0 Binding of phage clones to *C. jejuni*

5.1 Phage-capture ELISA

In this assay, wells of a 96-well ELISA plate were coated overnight at 37 °C with 100 µl suspensions of *C. jejuni* strain C338 (OD_{595} 1.0) in PBS (pH 7.4) and incubated in a humidified chamber. The wells were aspirated and then incubated with 200 µl blocking buffer (1% BSA in TBS, pH 7.5) for 1.5 hr at room temperature. The wells were again aspirated and then incubated with individual phage clones (1×10^{12} virions in 100 µl of binding buffer (TBS containing 1% BSA and 0.1% Tween-20). This was incubated for one hour at room temperature. The wells were washed twice with 200 µl of wash buffer (TBS containing 0.1% Tween-20) each time and then incubated with 100 µl of a 1:5000 dilution of anti-phage antibody conjugated to HRP (Amersham Biosciences, Australia) in blocking buffer. This was incubated for one hour at room temperature and then washed as above before incubating with 100 µl Ultra TMB substrate (Pierce, Australia) for 30 min at room temperature. The reaction was stopped with 50 µl of 2 M sulphuric acid and the optical absorbance recorded at 450 nm (Wallac Victor 2; Perkin-Elmer, Australia). Control phage served as a negative control for evaluation of background from non-specific binding.

6.0 Mode-of-action of phage-displayed peptides

6.1 Autoagglutination assay

Autoagglutination assays were carried out as described by Misawa & Blaser (2000). *C. jejuni* was grown on blood agar plates for 48 hr at 37 ºC under microaerophilic conditions and then resuspended in PBS (pH 7.4) to an OD_{595} nm of 1.0. An aliquot (2.5 ml) was transferred to sterile borosilicate glass tubes (13 mm x 100 mm) along with 1×10^{13} virions of phage-displayed peptides (in 500 µL TBS, pH 7.5). Controls included TBS (pH 7.5) without phage-displayed peptides and TBS containing an irrelevant control phage-displayed peptide. The tubes were covered with parafilm and incubated at room temperature for 24 hr. One mL from the top of each tube was carefully removed and the OD_{595} nm measured on a spectrophotometer. A drop in the OD_{595} from the initial setting of 1.0 reflected the degree of autoagglutination. Each assay was done at least in quadruplicate.

6.2 Motility assay

C. jejuni was grown on blood agar plates for 48 hr at 37 °C in microaerophilic conditions. It was then resuspended in Muller-Hinton broth (Oxoid, Australia) to an OD_{595} of 0.6 - 0.7. To 150 µl of this suspension was added either 45 µl of phage-displayed peptides, TBS control or phage control containing an irrelevant peptide. These were incubated at room temperature for 2 hr prior to stabbing 1 µl of suspension into Muller-Hinton semi-solid agar plates containing 0.4% agar. These plates were set up in duplicate and a control (*C. jejuni* containing MH broth and TBS) was added to each plate. A plate with an irrelevant phage clone was also included as a control. The ability of *C. jejuni* to swarm was assessed after incubation under microaerophilic conditions at either 37 °C or 42 °C for up to 48 hrs.

7.0 Activity of phage peptides outside of the phage coat

7.1 Partial denaturation of phage-displayed peptides with chloroform

Selected phage clones were partially denatured by chloroform, according to Petrenko & Smith 2000. In brief; individual phage clones were purified twice by (PEG)-NaCl precipitation and resuspended in 0.75% TBS buffer (pH 7.5) to a concentration of 3 x 10^{13} virions/ml in a microfuge tube. An equal volume of chloroform:isoamyl alcohol (24:1) was added, vortex mixed for 1 min and then centrifuged for 1 min at 13,000 g. The upper aqueous phase was transferred to a new microfuge tube and the concentration of virions assessed spectrophotometrically as virions/ml. Loss of infectivity of the phage was assessed by infecting *E. coli* K91BlueKan cells and determining the number of TU by spreading onto LB plates containing tetracycline and IPTG.

7.2 Purification of pVIII coat proteins

Selected phage clones were solubilised using the procedure outlined by Jayanna et al. 2009. In brief: 350 µl phage in 1 x TBS was mixed with 700 µl 120 mM cholate in 10 mM Tris-Cl, 0.2 mM EDTA and chloroform (2.5% v/v final concentration). The suspension was incubated for 1 hr at 37 °C and then applied to a sepharose 6B-CL column and eluted with 10 mM cholate in 10 mM Tris-Cl, 0.2 mM EDTA, pH 8.0. to separate major coat proteins from viral DNA. Concentration of protein samples was determined spectrophotometrically.

Chapter 1: Results obtained from Part A

In this chapter we present the results from the first grant spanning 1st July 2006 – 30th June 2007. Results are presented under each of the Specific Aims (as outlined in the objectives).

Specific Aim 1: Isolate phage-displayed peptides that bind to *C. jejuni*

Specific Aim 1A: Establish methods for growing *C. jejuni*

The aim was to establish reliably growth *C. jejuni* strains on solid agar plates and in liquid broth (in 96-well plates and culture tubes). We obtained ten Australian poultry isolates of *C. jejuni* from Dr. Pat Blackall (QDPI&F, Australia), as detailed in Table 1. Dr. Pat Blackall had obtained these strains from Queensland poultry farms and these strains represented the 10 different *fla* groupings of *C. jejuni* (J. Templeton, pers. comm.; DPI&F). We also obtained a reference strain of *C. jejuni* (ACM 3393) from the Australian Collection of Microorganisms (ACM, University of Queensland, Australia), as detailed in Methods (Section 1.1).

Oxoid (Australia) recommended the use of blood agar base for routine growth of *C. jejuni* on solid agar plates. They also recommended the addition of Campylobacter growth supplement, which helps to improve the aerotolerance of *C. jejuni*. We also chose to incubate our cultures at 37 °C for between 24-48 hr (Methods, Section 1.2). Using these conditions, we were able to reliably grow all of the *C. jejuni* strains on solid agar media. This media was used for all of our routine growth of *C. jejuni*.

We used Nutrient Broth containing Campylobacter growth supplement for all of our routine growth of *C. jejuni* in liquid media (Methods, Section 1.3). We found that shaking the cultures at 180 rpm in 15 ml polypropylene tubes (120 x 17 mm; Sarstedt, Australia) tubes markedly improved the growth of *C. jejuni* as measured spectrophotometrically at A_{595}. For example, the absorbance of the cultures at A_{595} nm with shaking at 180 rpm was 0.181 ± 0.038. With no shaking the absorbance at A_{595} nm was 0.037 ± 0.0045; n = 3 tubes).

We were able to readily grow all of the *C. jejuni* strains in 96-well plates (Methods, Section 1.4). We found that there was no difference in growth between the *C. jejuni* strains; however, the amount of growth depended on the initial inoculum added to the wells (Figure 3).

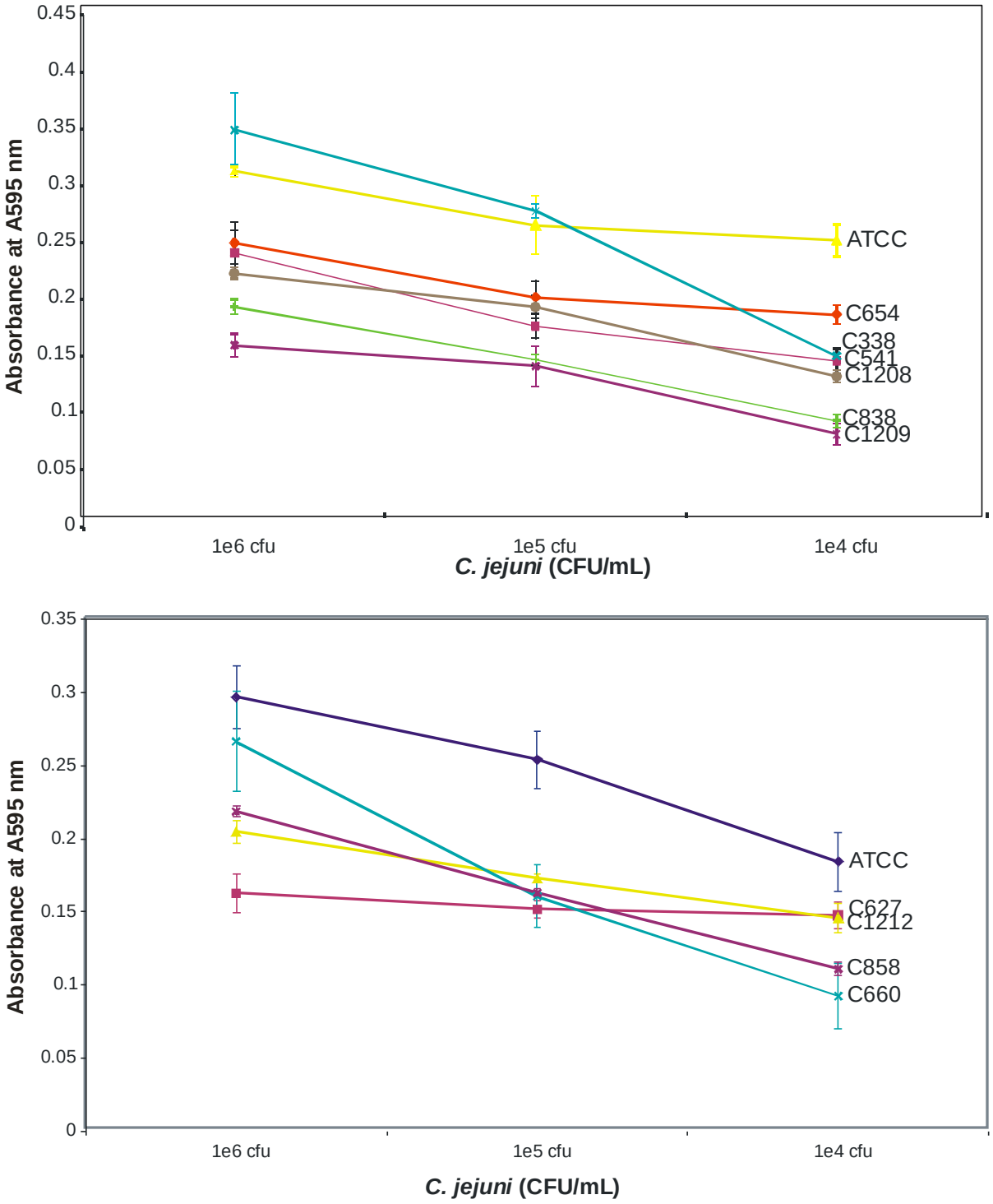


Figure 3: Growth of *C. jejuni* strains in 96-well plates
Each well in a 96 well polystyrene microtiter plate (Iwaki) was inoculated with 150 µl of *C. jejuni* (at 10^6, 10^5 or 10^4 CFU/ml) and sealed with a gas permeable cover (AeraSeal; Sigma). After incubation in a microaerophilic environment for 48 hr at 37 ºC, the optical densities of the bacterial cultures were measured spectrophotometrically in a plate reader at A_{595} nm (Wallac Victor2, Perkin Elmer).

Specific Aim 1B: Affinity select phage peptides binding to live *C. jejuni*

A phage library displaying 15 amino acid peptides on major coat protein pVIII (f88-4/15) was used to affinity select for peptides binding to the cell surface of a poultry strain of *C. jejuni* (strain C338). Without prior information on the epitopes involved in pathogenesis, the aim was to maximise the number of epitopes targeted by progressive subtraction of the library against the cell surface of *C. jejuni* (Figure 2) with the aim that both the abundant and less predominant epitopes to be targeted.

In the original proposal we mentioned that we would be using two different phage libraries; however, because of on-going problems with non-specific binding of the phage library to *C. jejuni*, we chose to split the amplified first round of affinity selection into two sub-libraries, so that different wash strategies (PBSg versus TBST) could be explored in the subsequent second and third rounds of selections (Methods, Section 2.4; Figure 2).

Selection scheme 1

Selection scheme 1 represented a direct selection strategy, as detailed in Section 2.4 of methods where the phage peptides were directly selected against the cell surface epitopes of ACM 3393 (Figure 2A). From the AP3/ phage sub-library, five clones were randomly selected from the eluted phage population and independently propagated. Single-stranded DNA was isolated from these clones and the peptide encoding region on gene 8 sequenced. From these sequenced clones, four clones contained a stop codon (UAG; data not shown) and were discarded. The remaining one clone (AP3/5; RTFGAKPPNIPFPRR) encoded a unique peptide sequence (Table 2).

From the sub-library designated AT3/, seven individual phage clones were randomly selected and sequenced. Four of these clones contained a single stop codon (UAG) and were discarded. The remaining three phage clones (AT3/3, AT3/4 and AT3/5) encoded unique peptide sequences, as detailed in Table 2.

Selection scheme 2

In selection scheme 2, the phage library was subtracted once against ACM 3393, prior to three rounds of affinity selection against ACM 3393 (Figure 2B). After the third round of affinity selection, nine phage clones from the BP3/ sub-library were randomly selected and sequenced. Only two of these phage clones did not contain a stop codon (BP3/5 and BP3/9) and are represented in Table 2.

From the BT3/ sub-library, eight phage clones were sequenced; three of these did not contain a stop codon (BT3/3, BT3/4 and BT3/7) and represent unique sequences (Table 2).

Selection scheme 3

In selection scheme 3, the phage library was subtracted twice against ACM 3393, prior to three rounds of selection against C338 (Figure 2C). From the CP3/ phage sub-library, 19 individual phage clones were randomly selected from the eluted phage population, independently propagated and sequenced. Twelve of these clones encoded the same predicted peptide sequence, as represented by CP3/3 (FLIDSPLASIGPTSM; Table 2). They also contained the same genomic sequence, indicating that they were likely to have arisen from the same clone. Likewise two clones, represented by CP3/4 also

encoded the same predicted peptide sequence (FMIDSPLASIGPTSM; Table 2) and contained the same genomic sequence. The remaining five clones encoded unique peptide sequences (Table 2).

From the sub-library designated CT3/, ten individual phage clones were randomly selected from the eluted phage population and sequenced. Four of the phage clones contained a single stop codon (UAG) (data not shown) and were discarded. Two of the remaining six phage clones encoded the same peptide sequences (represented by CT3/9; CFNDPLDIVPPMLLL), whilst the remaining four clones encoded unique peptide sequences and are detailed in Table 2.

Selection scheme 4

In selection scheme 4, the phage library was subtracted twice against ACM 3393 and once against C338 prior to three rounds of affinity selection against C338 (Figure 2D). From the DP3/ phage pool, nine clones were randomly selected and sequenced. Five of these clones encoded the same predicted peptide sequence as represented by DP3/5 (FLSDPPAPPTSPGVV; Table 2). The remaining four clones contained a single stop codon UAG (data not shown).

Specific Aim 1C: Sequence analysis of the phage clones

Results of sequencing the phage clones are presented in Table 2. When the CP3/ and DP3/ phage clones were aligned and shaded according to their physical-chemical properties, it was evident that there was a strong selection for preferred groups of amino acids (Figure 4A). For example, there was a strong selection for hydrophobic amino acids at positions 3, 4 and 17 of the alignment and for a small amino acid inserted at position 15. Using MIMOX, the consensus sequence for CP3/ and DP3/ phage were FLIDSPLAPI(GS)PX(LS)(ML).

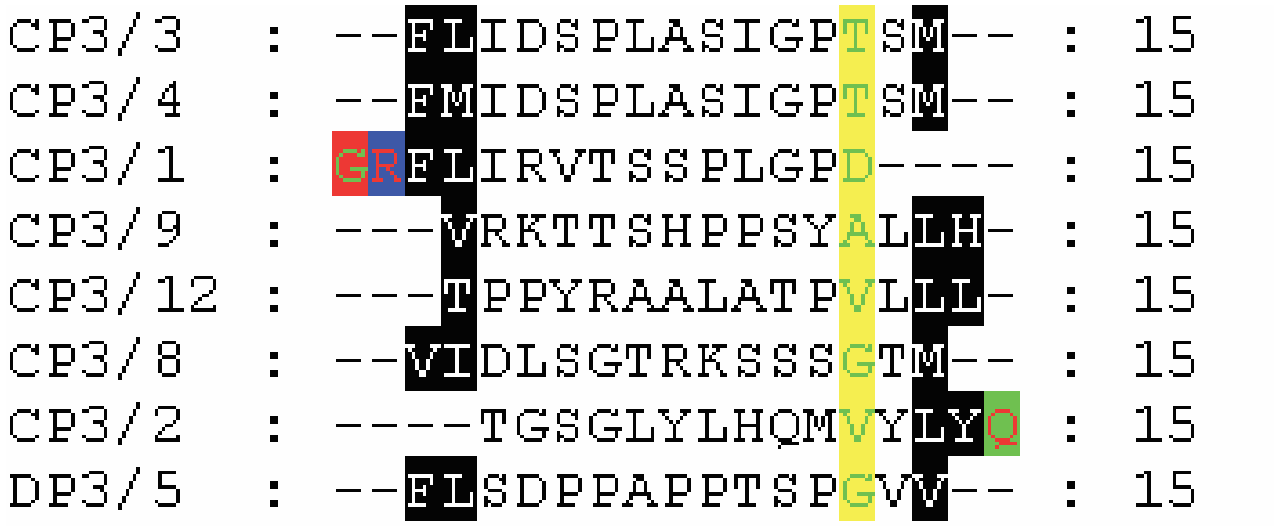
When the CT3/ phage were aligned and shaded according to their physical-chemical properties, they contained a conserved polar amino acid residue at position 3 of the alignment (Figure 4B). Hydrophobic amino acids were also strongly conserved at positions 8, 12, 14, 16 and 17 of the alignment. These conserved amino acid residues were not present in the CP3/ and DP3/ phage, indicating that the strategy for removing non-specific binding phage selected for different groups of amino acid residues.

Clone	Freq.[a]	Amino acid sequence	pI[b]	% growth inhib.	MIC[c](virions/μl)[c]
AT3-3	1	EGVLGAALSAFSFDS	3.67	Not Active	Not Active
AT3-4	1	QAQPCPNQPDGSVYA	3.80	Not Active	Not Active
AT3-5	1	STALPLWSNYSYDSA	3.8	Not Active	Not Active
AP3-5	1	RTFGAKPPNIPFPRR	12.30	Not Active	Not Active
BT3/3	1	KAIAFQSSPGSPVLL	8.75	Not Active	Not Active
BT3/4	1	TCNLSDYTLPRARVL	7.89	60%	Not tested
BT3/7	1	NFLCPSPYPKVQPGL	8.20	Not Active	Not Active
BP3-5	1	VPESIHKASLVCYRF	8.18	Not Active	Not Active
BP3-9	1	SRPIARFYTWSQNTT	10.83	Not Active	Not Active
CT3/2	1	FSPFRISELVYTLHP	6.75	98-99%	1×10^{10} (4.1 μM[d])
CT3/4	1	LPFNLAKPELYIFVQ	6.00	Not Active	Not Active
CT3/6	1	LSAPSPMFLPPVNPH	6.74	99%	2.5×10^{10} (10.3)
CT3/7	1	HRPVKTPANAPTTMM	11	99%	1.5×10^{10} (6.1)
CT3/9	2	CFNDPLDIVPPMLLL	3.56	95-99%	1.9×10^{10} (7.8)
CP3/1	1	GRFLIRVTSSPLGPD	9.60	96%	3.7×10^{9} (1.5)
CP3/2	1	TGSGLYLHQMVYLYQ	6.4		
CP3/3	12	FLIDSPLASIGPTSM	3.80	91-97%	7.6×10^{9} (3.1)
CP3/4	2	FMIDSPLASIGPTSM	3.80	Not Active	Not Active
CP3/8	1	VIDLSGTRKSSSGTM	8.72	93-99%	6.6×10^{9} (2.7)
CP3/9	1	VRKTTSHPPSYALLH	9.99	99%	3.8×10^{9} (1.5)
CP3/12	1	TPPYRAALATPVLLL	8.41	80-90%	1×10^{10} (4.1)
DP3/5	5	FLSDPPAPPTSPGVV	3.80	99%	9.2×10^{9} (3.8)
CP2/5	1	VLAWFSPLTLESSRL		99%	7.2×10^{9} (2.97)
AP4/2	1	KVQIIPKDTLAPLPP	8.59	99%	6.9×10^{9} (2.85)
AT4/3	1	AHAPCSLFFPLSLRP	8.30	97-99%	6.2×10^{9} (2.56)
AT4/5	1	KALYALHVPSMQVFA	8.60	97-99%	1.3×10^{10} (2.56)
AT4/8	1	RPSRWPWQEPLPISI	9.60	99%	1.1×10^{10} (4.54)

Table 2: Deduced amino acid sequences of the phage-displayed peptides and their antimicrobial activities

[a] frequency of the phage clones isolated from the f88-4/15-mer library
[b] pI, calculated isoelectric points of the "displayed" peptide
[c] MIC, minimal inhibitory concentrations of the phage clones, resulting in no detectable growth.
[d] the molar concentration of the phage-displayed peptides, assuming that all 150 copies displayed on pVIII are antimicrobial

A.



B.

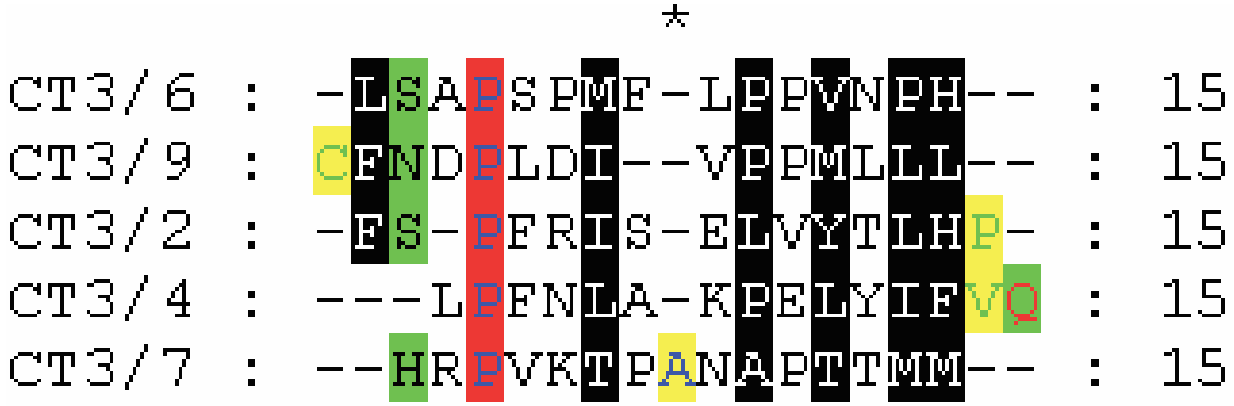


Figure 4: Alignment of the phage-displayed peptides

The deduced amino acid sequences of CP3/1, CP3/2, CP3/3, CP3/4, CP3/8, CP3/9, CP3/12 and DP3/5 (Figure 4A) and CT3/2, CT3/4, CT3/6, CT3/7 and CT3/9 (Figure 4B) were aligned using ClustalW. Dashes indicate gaps used to maximise the alignment. The physical-chemical mode of GeneDoc was used to assign each column of the alignment to one of 12 pre-defined groups of physical-chemical properties. White writing on a black background indicates hydrophobic amino acids; green writing on a yellow background indicates small amino acids; black writing on a green background indicates polar amino acids; blue writing on red background indicates prolines.

Specific Aim 2: Testing phage peptides for antimicrobial activity

Specific Aim 2A: Testing the phage clones for potential inhibitory activities

In Specific Aim 1, a total of 44 phage clones were isolated, representing 27 unique peptide sequences (Table 2). In this Specific Aim, an *in-vitro* microdilution susceptibility test was used to identify potential phage clones inhibiting the growth of *C. jejuni* in 96-well plates. *C. jejuni* strain C338 was grown on blood agar plates (Methods, Section 1.2), resuspended to a concentration of 1 x 10^5 CFU/ml in Nutrient Broth (Methods, Section 1.4) and individually incubated with each of the 27 unique phage peptides from Table 2 (Methods, Section 4). Phage bearing an irrelevant 15 amino acid peptide sequence HSAIYYKNFGSSLFR was incorporated as a control (Tek1/6).

Overall none of the phage clones from selection scheme 1 showed any evidence of inhibiting the growth of *C. jejuni* (Figure 5). A Gram-stain also showed there was no evidence of the phage peptides affecting the morphology of *C. jejuni* (data not shown).

The phage clones isolated from selection scheme 2 (BP3/ and BT3/) markedly affected the morphology of *C. jejuni* in a dose-dependent manner. As shown in Figure 6, there was an increase in the absorbance of *C. jejuni* at A_{595} nm relative to the controls. A Gram-stain showed that this increase in absorbance was likely to be attributed to the change in morphology of *C. jejuni*. As shown with BT3/4 (Figure 6), there was a high incidence of thread-like forms of *C. jejuni*, perhaps indicating that the bacteria were under stress. Only one of the phage clones (BT3/4) was antimicrobial, inhibiting the growth of *C. jejuni* by 60%.

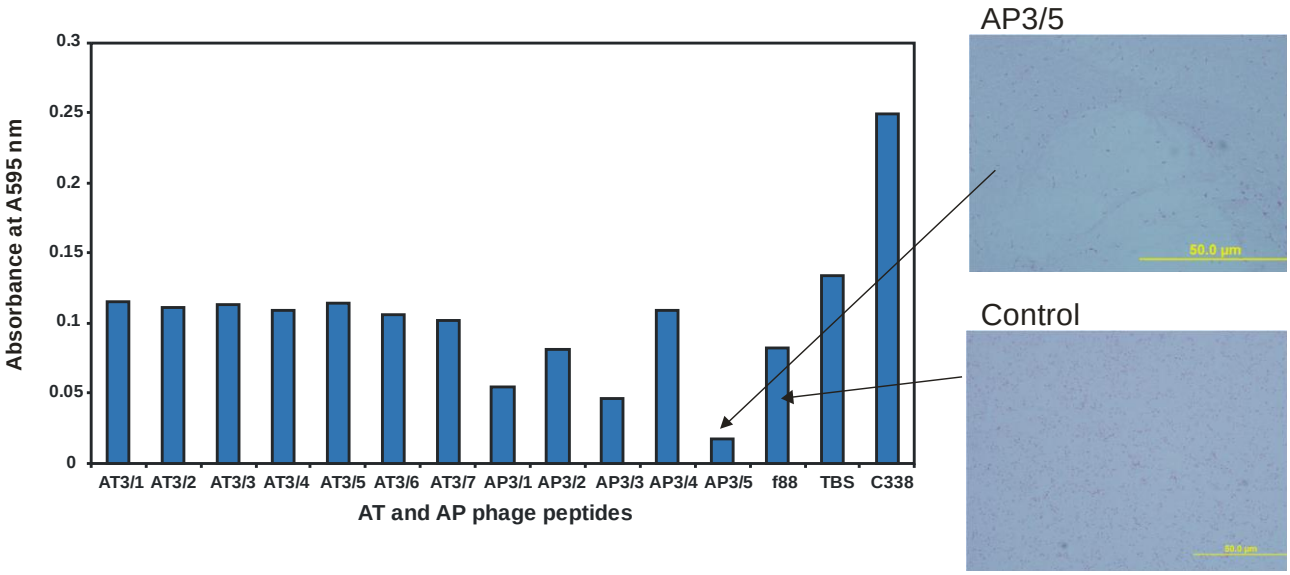


Figure 5: ***In-vitro*** **microdilution susceptibility test to evaluate potential inhibitory activities of selected phage-displayed peptides in 96-well plates**

An *in-vitro* microdilution susceptibility test was used to evaluate potential inhibitory activities of selected phage-displayed peptides. After an overnight growth in 96-well plates, the growth of *C. jejuni* strain C338 was evaluated by measuring the absorbance at A_{595} nm.

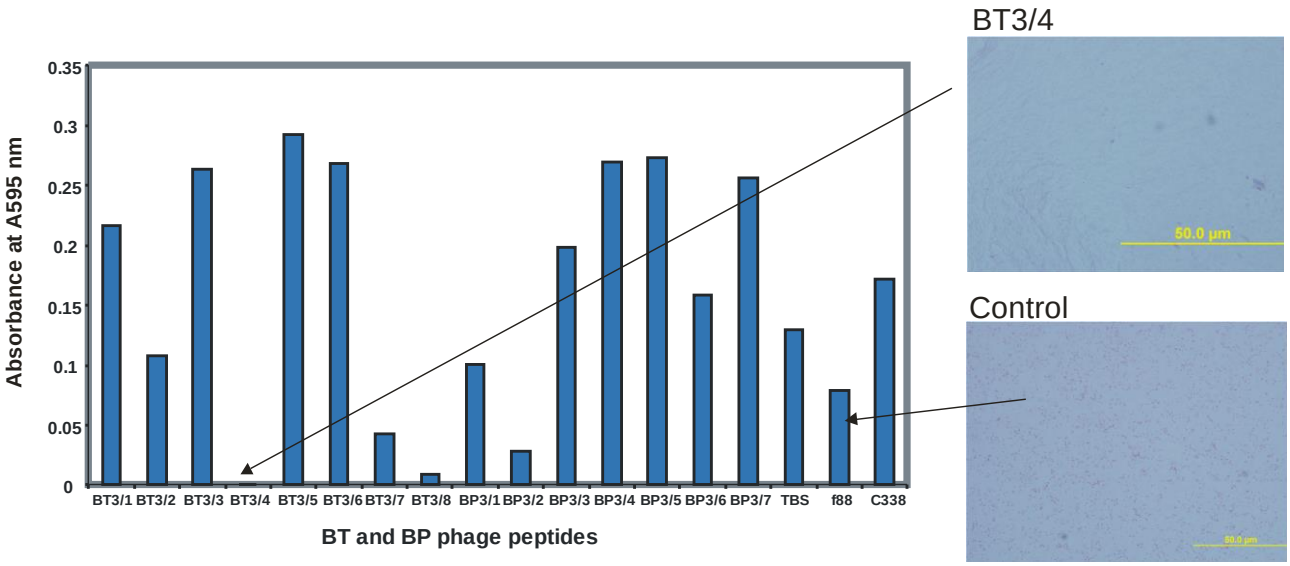


Figure 6: ***In-vitro*** **microdilution susceptibility test to evaluate potential inhibitory activities of selected phage-displayed peptides in 96-well plates**

Ten of the 12 unique phage-displayed peptides (CP3/ and CT3/) that were isolated from selection scheme 3 were found to inhibit the growth of *C. jejuni* C338 in a dose-dependent manner as determined by a decrease in the optical density of the bacterial cultures at A_{595} nm. The remaining two phage clones (CT3/4 and CP3/4) showed no activity against *C. jejuni*, despite CP3/4 differing from CP3/3 by only one amino acid sequence (Table 2).

The single phage clone isolated from selection scheme 4 (DP3/5) inhibited the growth of C338 in a dose-dependent manner (Table 2).

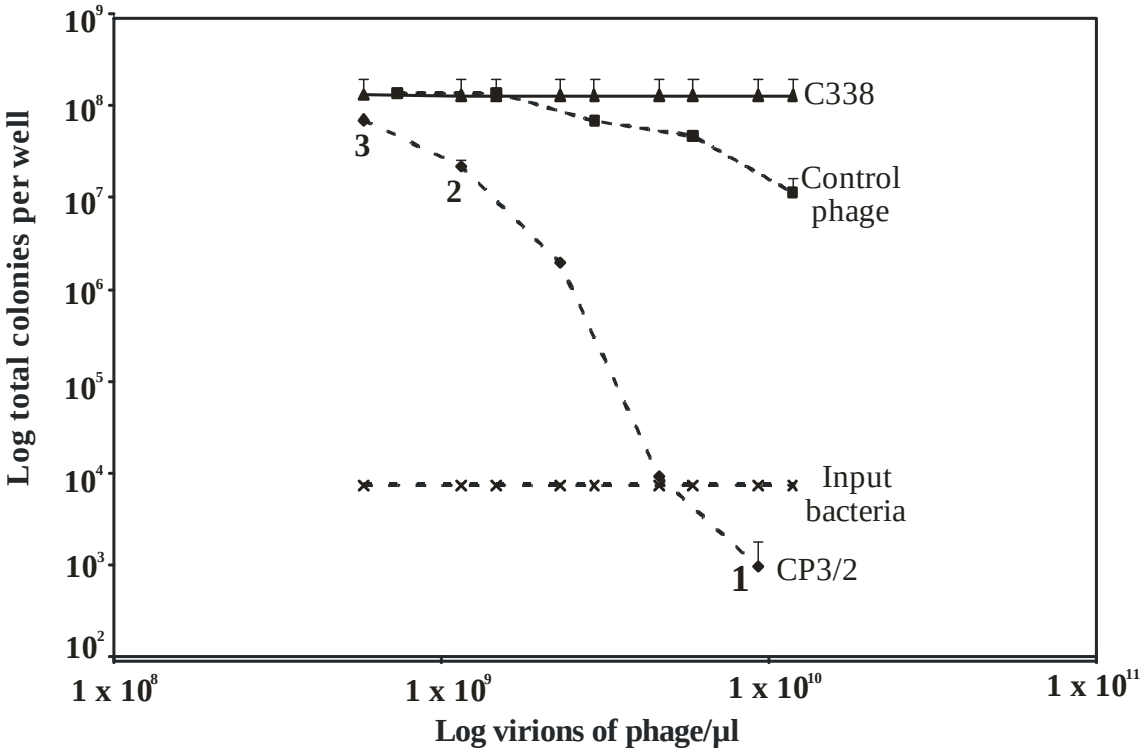
Specific Aim 2B: Determining the antimicrobial/ bactericidal activities of the phage peptides

The microtiter broth dilution assay was used to further characterise the phage clones showing potential activity in Specific Aim 2B. Two-fold dilutions of the 10 phage clones were incubated with *C. jejuni* strain C338 in 96-well plates. After an overnight incubation at 37 °C, the number of CFU remaining in the microtiter plates was determined by plating onto blood agar plates.

One phage clone, CP3/2, was bactericidal in that it reduced the viable counts of strain C338 by up to 87%. It also showed a dose-response effect in that at lower concentrations it inhibited the growth of strain C338 (Figure 7). It also markedly affected the morphology of *C. jejuni*, as depicted in Figure 7B. The remaining nine phage clones were bacteriostatic, reducing the growth of *C. jejuni* by up to 99.9% *in-vitro* and were not bactericidal (Table 2).

The phage clones also varied in their potency against C338 with the CP3/ and DP3/ phage peptides showing higher activity than the CT3/ phage. For example, the CP3/ and DP3/ phage peptides showed MIC values ranging from 3.7×10^9 - 1×10^{10} virions/μl, corresponding to a theoretical peptide concentration between 1.5 and 4.1 μM (Table 2). The CT3/ phage peptides were less active, showing MIC values ranging from 1.0 – 2.5×10^{10} virions/μl, corresponding to a theoretical peptide concentration between 4.1 and 10.3 μM (Table 2). This minimal inhibitory concentration was calculated assuming that all 150-300 copies of the peptide on the phage coat were available for binding *C. jejuni* and was tested using phage from two independent phage preparations.

A.



B.

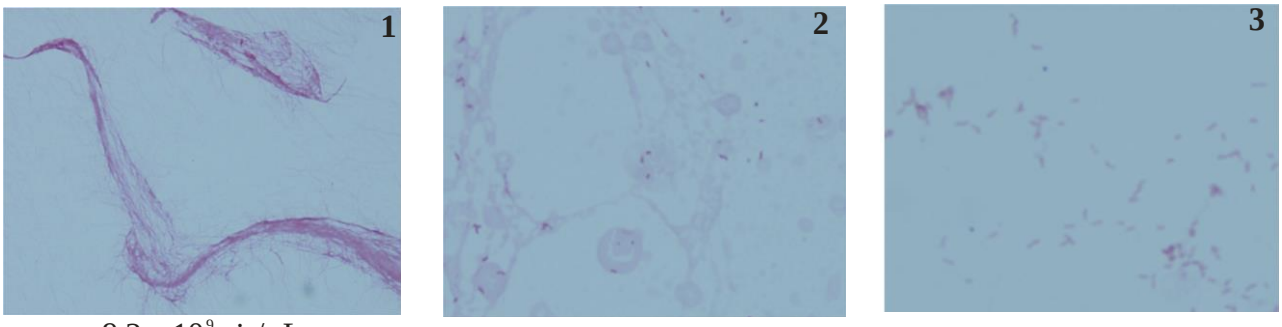


Figure 7: Effect of phage-displayed peptides on the in-vitro survival of *C. jejuni*

Each well in a microtiter plate was inoculated with 150 µl of 1-5 x 10^5 CFU/ml of *C. jejuni* strain C338 and two-fold dilutions of CP3/2 (Methods, Section 4). Controls included C338 without phage-displayed peptides and C338 with dilutions of control phage peptide. The number of viable bacteria was determined by performing colony counts on the diluted inoculum and plating on blood agar plates. Error bars indicate standard deviations for three experiments.

B. Gram-stain showing the effects of CP3/2 on *C. jejuni* strain C338. The numbers 1-3 corresponded to numbers in Figure 7 where a sample was taken for a Gram Stain.

Specific Aim 2C: Specificity of the phage peptides towards *C. jejuni*

An ideal antimicrobial peptide needs to be highly specific towards *C. jejuni* with no activity towards other bacterial spp. The microdilution susceptibility test (Methods, Section 4) was used to evaluate the *in-vitro* antimicrobial activity of all of the antimicrobial phage-peptides from Table 2 against poultry strains of *C. jejuni* as well as other bacterial spp.

All of the phage peptides were highly specific towards *C. jejuni*. None of them showed any antimicrobial activity against Gram-positive (*E. faecalis*), and Gram-negative (*S. typhimurium*, *P. mirabilis* and *E. coli* 026) bacteria at phage concentrations that completely inhibited the growth of *C. jejuni*. There were also no morphological changes in these bacteria, as evidenced by a Gram-stain.

Selected phage peptides were inhibitory towards some of the poultry isolates of *C. jejuni* that were tested; however, they were not inhibitory against all of the poultry isolates. This indicates that they recognised an epitope on *C. jejuni* that was not isolate specific (Table 3).

	C627	**C838**	**C858**	**C1208**	**C1212**
CT3/2	No effect	No effect	Active	Active	98% inhib
CT3/6	N/A	No effect	N/A	N/A	Active
CT3/7	N/A	No effect	N/A	N/A	Active
CT3/9	N/A	No effect	N/A	N/A	Active
CP3/1	No effect	No effect	Active	Active	Active
CP3/2	Active	No effect	Active	No effect	No effect
CP3/3	No effect	No effect	Active	85% inhib	Active
CP3/8	N/A	No effect	N/A	N/A	Active
CP3/9	N/A	No effect	N/A	N/A	Active
CP3/12	N/A	Active	N/A	N/A	Active
DP3/5	No effect	No effect	Active	91% inhib	Active

Table 3: Effect of phage peptides on poultry isolates of *C. jejuni*

Chapter 2: Results obtained from Part B

In this chapter, results are presented from the second grant period, spanning 1st July 2007 – 1st December 2009. Results are presented under each of the Specific Aims that were outlined in the objectives.

Aim 1: Isolation and testing of bactericidal antimicrobial phage clones

In this aim, we wanted to continue isolating phage peptides and testing them for potential antimicrobial activity, as outlined below.

Specific Aim 1A: Isolation of bactericidal phage clones

Selection scheme 5

In this selection scheme, the phage library was directly selected against ACM 3393 for two rounds of affinity selection and then against C338 for the final two rounds of selection (Methods, Section 2.4; Figure 2E and 2F). Seven clones were randomly selected from the eluted phage population from the AP4/ sub-library and independently propagated for sequencing (Figure 2E). Five of the seven peptides contained a stop codon (UAG) and were discarded. Out of the remaining two clones, AP4/2 contained a unique peptide sequence (KVQIIPKDTLAPLPP; Table 2) whereas AP4/5 (CFNDPLDIVPPMLLL) contained the same sequence as CT3/9 (Table 2).

Eight phage clones were randomly selected from the eluted phage population from the AT4/ sub-libararyand sequenced (Figure 2F). Only three of these sequences did not contain a UAG stop codon (AT4/3, AT4/5 and AT4/8) and represented unique sequences (Table 2). The *in-vitro* microdilution susceptibility test was used to identify potential clones inhibiting the growth of *C. jejuni* in 96-well plates. *C. jejuni* strain C338 was grown on blood agar plates (Methods, Section 1.2), resuspended to a concentration of 1 x 10^5 CFU/ml in Nutrient Broth (Methods, Section 1.4) and individually incubated with each of the 27 unique phage peptides from Table 2 (Methods, Section 4). Phage bearing an irrelevant 15 amino acid peptide sequence HSAIYYKNFGSSLFR was incorporated as a control (Tek1/6). All of these phage clones were antimicrobial (see Table 2) with growth inhibitory activities similar to the other CP3/, CT3/ and DP3/ phage clones.

Specific Aim 1B: Antimicrobial activity of the phage clones

In this Specific Aim, we wanted to establish the best conditions for antimicrobial activity of the phage clones. Activity of "natural" antimicrobial peptides is often influenced by environmental factors such as temperature, pH, ionic strength as well as the microbial growth phase of the bacterial cultures. These conditions are often thought to be specific to the target bacterium. Using the *in-vitro* microtiter broth dilution assay, we also tested the potential of *C. jejuni* to become resistant to the selected phage peptides.

1. Testing the effect of ionic strength on antimicrobial activity

Many natural antimicrobial peptides are cationic and electrostatically interact with the surface of negatively charged bacteria. This can be affected by the ionic strength of the solution used to resuspend the peptides, with increasing salt reporting to have an adverse affect on the antimicrobial activity. Some of the phage peptides from Table 2 have attributes that are similar to natural antimicrobial peptides including cationicity and small size.

In this Specific Aim, we wanted to test the effect of a low salt buffer on antimicrobial activity of selected phage peptides by resuspending them in a low salt buffer (10 mM sodium phosphate buffer, pH 7.4) instead of the usual TBS buffer (50 mM Tris.Cl, pH 7.5, 150 mM NaCl), which contains high salt.

Eleven phage clones (from Table 2) were resuspended in 10 mM sodium phosphate buffer, pH 7.4 and tested for their ability to inhibit the growth of *C. jejuni* strain C338 using the microtiter broth dilution assay (Methods, Section 4.1). Our results showed that the use of a low salt buffer resulted in no change in the ability of phage clones CT3/7, CT3/9, CP3/1, CP3/2, CP3/12, DP3/5, CP2/5 and AT4/3 to inhibit the growth of *C. jejuni in-vitro*. These phage clones were able to completely inhibit the growth of *C. jejuni* (Table 4). In contrast, when CT3/2 was resuspended in low salt buffer (10 mM sodium phosphate buffer, pH 7.4), there was an increase in antimicrobial activity. CT3/2 became bactericidal, reducing the initial inoculum of *C. jejuni* by up to 87% (Table 4). Surprisingly, two phage clones (CT3/6 and CP3/3) showed a decrease in activity when resuspended in low salt (10 mM sodium phosphate buffer, pH 7.4). The results from these assays are reported in Table 4.

In conclusion, the majority of the phage clones did not appear to be sensitive to high salt concentrations. This lack of sensitivity to salt may indicate that these phage clones will work in a range of conditions including in-vivo conditions encountered in the chicken gastrointestinal tract.

Clone	Amino acid sequence	Salt Sensitivity[a]	Change in activity[b]
CT3/2	FSPFRISELVYTLHP	87% bac/T	Increased activity
CT3/6	LSAPSPMFLPPVNPH	Not active	Decrease activity
CT3/7	HRPVKTPANAPTTMM	99.96%	Same activity
CT3/9	CFNDPLDIVPPMLLL	Active OD	Same activity
CP3/1	GRFLIRVTSSPLGPD	Active OD	Same activity
CP3/2	TGSGLYLHQMVYLYQ	99.9%	Same activity
CP3/3	FLIDSPLASIGPTSM	Not active	Decrease activity
CP3/8	VIDLSGTRKSSSGTM	ND	
CP3/9	VRKTTSHPPSYALLH	ND	
CP3/12	TPPYRAALATPVLLL	Active OD	Same activity
DP3/5	FLSDPPAPPTSPGVV	99.9%	Same activity
CP2/5	VLAWFSPLTLESSRL	99.9%	Same activity
AP4/2	KVQIIPKDTLAPLPP	ND	
AT4/3	AHAPCSLFFPLSLRP	Active OD	Same activity
AT4/5	KALYALHVPSMQVFA	ND	
AT4/8	RPSRWPWQEPLPISI	ND	

Table 4: Effect of a low salt concentration on antimicrobial activity of selected phage-peptides

[a] Activity of phage peptides in 10 mM sodium phosphate buffer
[b] Change in antimicrobial activity when compared with TBS buffer (pH 7.5) and from Table 2.

2. Effect of microbial growth phase on antimicrobial activity

In general, antimicrobial drug discovery targets the logarithmic phase of bacterial growth; a stage where the cells are actively dividing. Consequently, these antimicrobial drugs have little effect on the non-multiplying stationary phase of growth or on biofilms. The phage peptides isolated in Specific Aim 1 (Part A and B) was isolated against *C. jejuni* that was in the actively growing/dividing stage. In this aim, we wanted to test whether the growth phase of the bacteria affected the antimicrobial activity of the phage peptides, especially since it's unlikely that *C. jejuni* would be present in an actively growing free-form stage in chickens.

Each well of a 96-well plate was inoculated with 1 x 10^5 CFU/ml of *C. jejuni* and CP2/5 phage peptide. Controls included *C. jejuni* with an irrelevant phage peptide. These were incubated at 37 °C under microaerophilic conditions and at various intervals the amount of bacterial growth was assessed in the 96 well plates by measuring the absorbance at A_{595} nm (Methods, Section 4.2). Results showed that *C. jejuni* strain C338 followed a sigmoidal growth pattern, as depicted in Figure 8. The results also indicated that in this assay, exponential growth of *C. jejuni* occurred between 0–36 hrs, stationary phase (36-72 hr) and a decline in growth from 72–144 hr (Figure 8). In this assay, CP2/5 continued to have an inhibitory affect on *C. jejuni* growth, even after 144 hr of incubation at 37 °C. This result has been repeated, indicating that under these *in-vitro* conditions, CP2/5 is likely to be antimicrobial to both exponentially growing bacteria as well as bacteria in the stationary phase of growth.

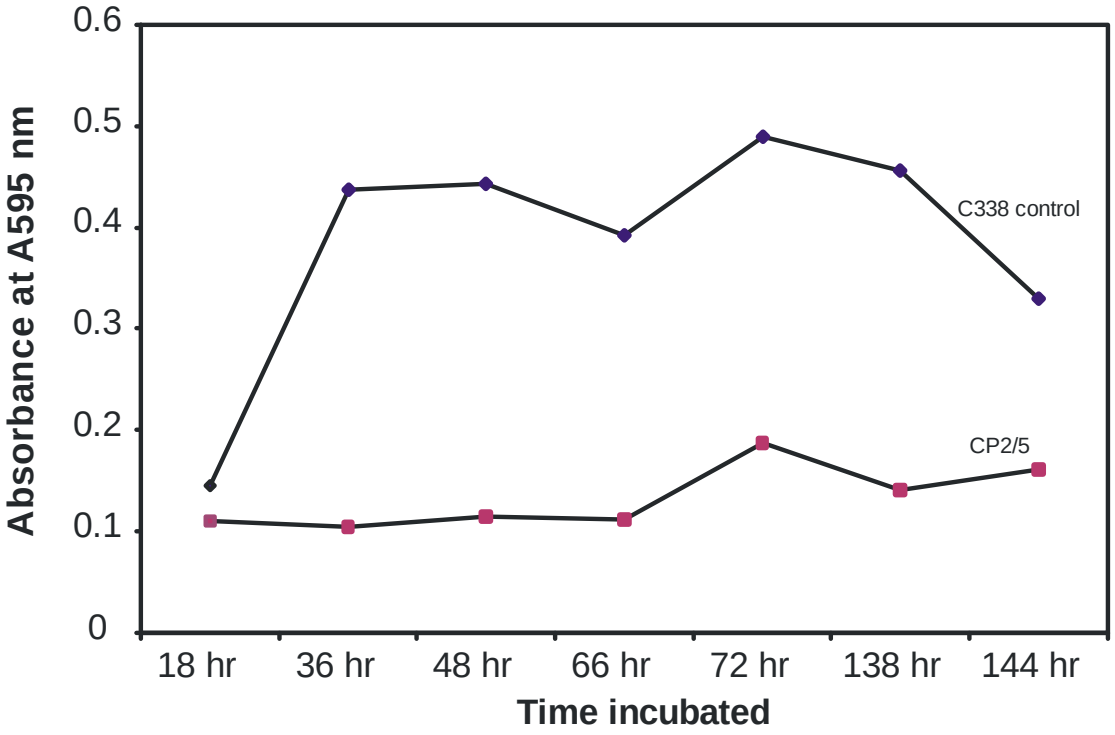


Figure 8: **Effect of CP2/5 on growth of *C. jejuni* up to 144 hr of incubation**

3. Effect of repeated exposure to the phage peptides and development of resistance

It was generally thought that bacteria are unable to develop resistance to natural antimicrobial peptides; however, this theory was initially challenged by Bell & Gouyon in 2003 and then documented by Perron et al. (2006), who demonstrated that resistance to natural antimicrobial peptides can be experimentally induced in *E. coli* and *Pseudomonas aeruginosa*. Research has also shown that if resistance develops to antimicrobial peptides, it tends to be temporary in nature and is quickly lost when the bacteria are no longer exposed to the peptides (Samuelsen et al. 2005).

In this Specific Aim, we wanted to explore the potential of *C. jejuni* to develop resistance to repeated exposure of selected antimicrobial phage peptides. In separate experiments, high concentrations of phage peptides were added to *C. jejuni* strain C338 in 96-well plates (Methods, Section 4.3), with the aim of enhancing the selection of rare resistant mutants that may develop in the bacterial population. We also used high concentrations of the phage peptides because they were more likely to quickly induce experimental resistance.

In separate experiments, *C. jejuni* strain C338 was passaged in the presence of CP2/5, CP3/2 and AT4/3 (Methods, Section 4.3). Our results indicated that under these conditions, none of the phage peptides induced experimental resistance in the *C. jejuni* population, even after several passages (Figure 9). We also initiated experiments with CP3/9, DP3/5 and CT3/7 and none of these phage peptides induced experimental resistance. Together these results suggest that *C. jejuni* is unlikely to become quickly resistant to the phage peptides; however, we cannot conclude that the *C. jejuni* will never form resistance to the phage peptides.

It would be a dangerous assumption to assume that *C. jejuni* are never going to develop resistance to the antimicrobial phage peptides. We could potentially add the safeguard of developing two different phage peptides, targeting different membrane proteins on *C. jejuni* and alternate their use in poultry. In addition, unlike conventional antibiotics, our phage peptides target “novel” receptors on the outer surface of *C. jejuni* (based on the fact that they are species specific) and so they are less likely to cross-react with antibiotics used in human medicine.

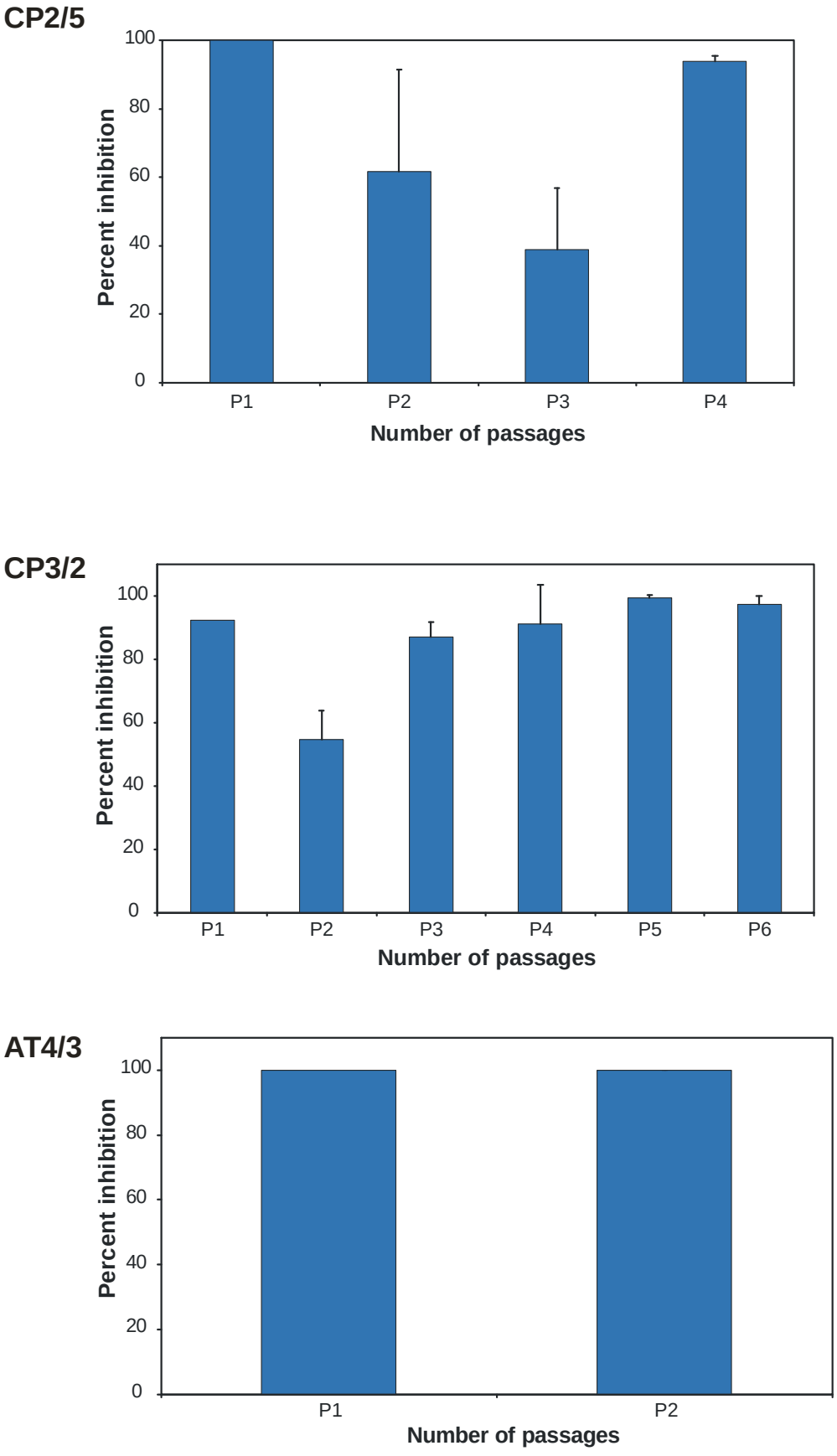


Figure 9: **Induced experimental resistance of *C. jejuni* CP2/5, CP3/2 and AT4/3**

Specific Aim 1C: Testing the specificity of binding towards *C. jejuni* and other bacterial spp.

The isolated phage peptides need to be highly specific in their activity towards *C. jejuni* because broad-spectrum activity would upset the natural balance of the gastrointestinal microflora in chickens. In Part A, we had checked for antimicrobial activity of the phage peptides towards diverse bacterial spp using the *in-vitro* microdilution assay; however, it is possible that the phage peptides may bind to these bacteria and not be active towards them.

A phage-capture ELISA was used to the binding of phage peptides to *C. jejuni* strain C338. In this assay, individual phage clones were added to the wells of a 96-well microtiter plate that was already pre-coated with *C. jejuni* (Methods, Section 5.1). Following incubation to allow specific binding between the bacteria and phage, non-binding phage particles were washed away and bound phage detected colorimetrically using an anti-M13 antibody conjugated to horse-radish peroxidise (HRP).

Overall, 18 phage clones (from Table 2) were tested for potential binding to *C. jejuni* (Figure 10). An rrelevant phage peptide and TBS (pH 7.5) without phage peptides were included as controls. Our results indicated that six clones (CP3/2, CP3/3, CP3/4, CP3/9, CP3/12 and DP3/5) bound *C. jejuni* to a much greater degree compared with an irrelevant phage peptide (Figure 10)

As shown in Figure 11, none of these phage peptides showed any binding to *E. faecalis*, *B. cereus* or *S. typhimurium* using the phage-capture ELISA. These results indicate that the phage peptides are not antimicrobial towards these bacteria because they are not binding to them.

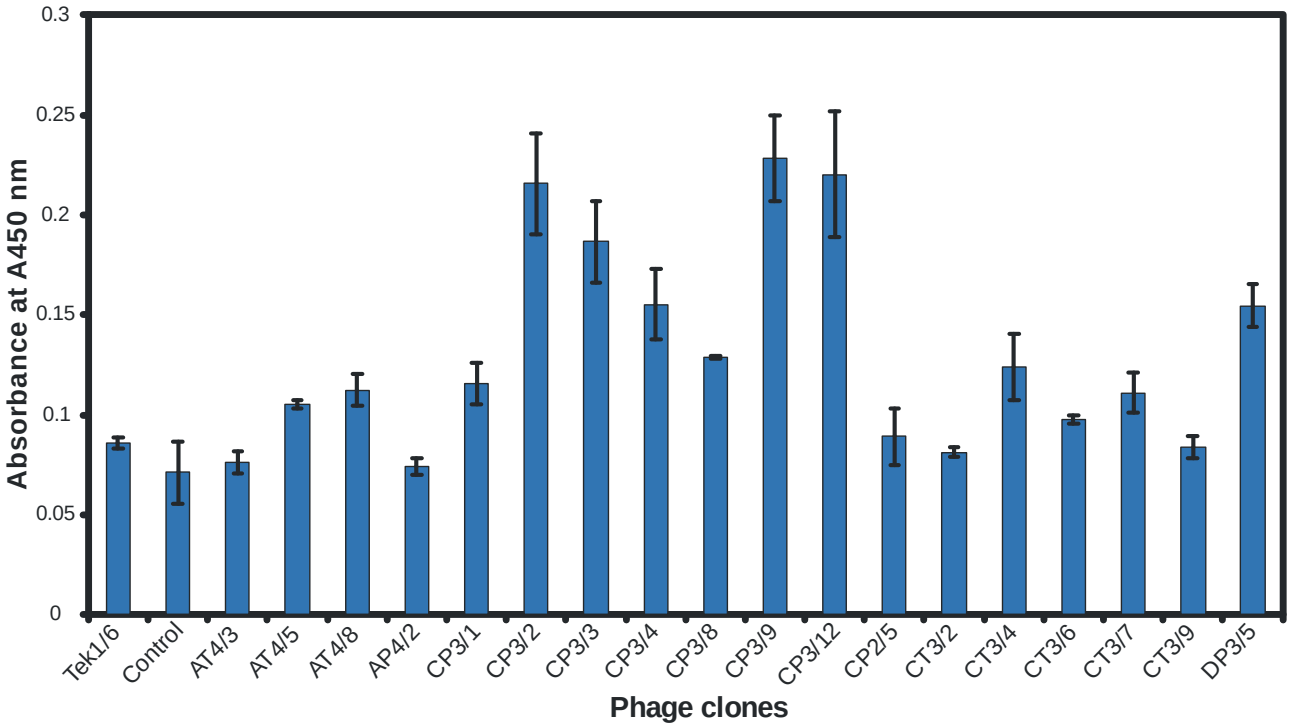


Figure 10: **Binding of selected phage peptides to *C. jejuni* immobilised on a 96-well microtiter plate using phage-capture ELISA.**

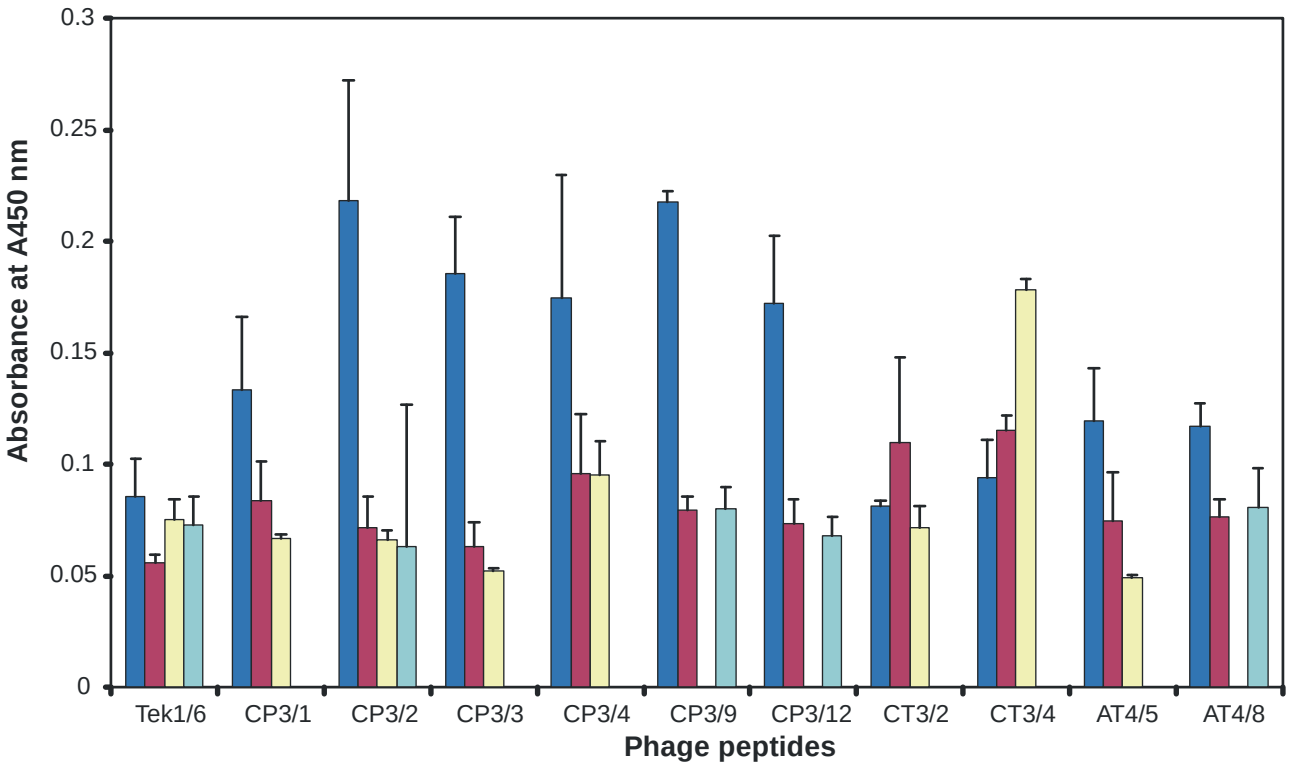


Figure 11: Binding of selected phage clones to bacterial spp. using a phage-capture ELISA

C. jejuni C338, E. faecalis B. cereus; S. typhimurium

Specific Aim 2: Mode of action of phage peptides

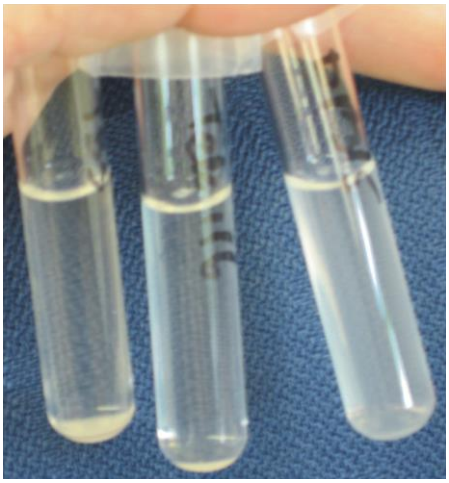
C. jejuni infection in chickens is different to that from humans and not well understood. For example, in chickens, *C. jejuni* establishes a commensal relationship with its host. It is often associated with the mucus layer of the deep crypts of the caecum, where it colonises to high levels (up to 1 x 10^9 CFU/g of infected intestine) without invading host tissues. In contrast, *C. jejuni* is pathogenic to humans, resulting in an acute gastroenteritis at a low infectious dose. Indicators of virulence are also not fully established for *C. jejuni*. For example, studies by Misawa and Blaser (2000), Golden and Acheson (2002), and Guerry et al. (2006) suggest a relationship between virulence and motility, flagella expression, adhesion, invasion and/or autoagglutination. These studies also proposed that autoagglutination was highly dependent on flagella expression (expressed on the cell surface) and flagella structure; properties which also appear to be essential for motility, and also play a part in adhesion to and invasion of chicken submucosal and human intestinal cells.

Although the phage peptides in Table 2 were able to inhibit the growth of *C. jejuni in-vitro*, they may also have other effects such as preventing colonisation in chickens. Thus, in this Specific Aim, we wanted to explore the possibility that the phage peptides may potentially influence the virulence properties of *C. jejuni* and affect its ability to colonise chickens. Because of the central importance of autoagglutination and motility to colonisation of chickens by *C. jejuni* we chose to look at those attributes in this Specific Aim.

2.1 Autoagglutination

The antimicrobial phage peptides in Table 2 were screened for their ability to autoagglutinate *C. jejuni* C338 *in-vitro* (Methods, Section 6.1). Autoagglutination assays were carried as described by Misawar & Blaser (2000) and as documented in Methods, Section 6.1. In this simple assay, *C. jejuni* strains that strongly autoagglutinated sunk to the bottom of a glass tube and mutants that were unable to autoagglutinate remained distributed throughout the glass tube (as shown in Figure 12).

Three phage peptides (CP3/3, CP3/4 and DP3/5) almost completely inhibited the ability of *C. jejuni* to autoagglutinate *in-vitro* as shown in Figure 12 and 13. None of the other phage peptides from Table 2 affected this ability.



Control Tek 1/6 DP3/5

Figure 12: Inhibition of *C. jejuni* autoagglutination by DP3/5
In this assay, DP3/5 inhibited the ability of *C. jejuni* to autoagglutinate; TBS and Tek1/6 were used as controls.

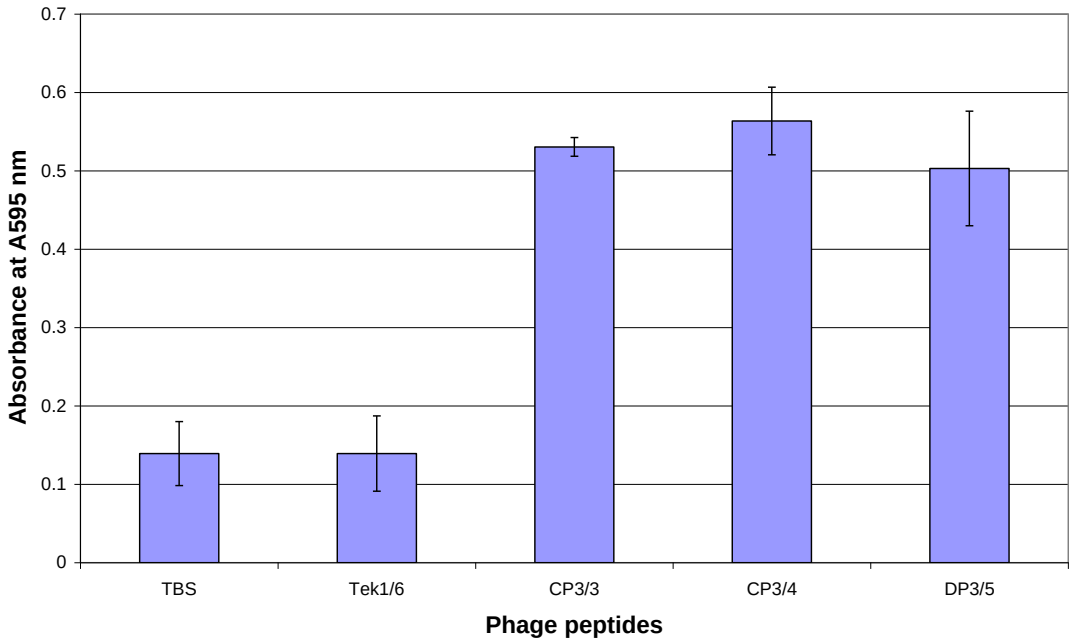


Figure 13: Inhibition of *C. jejuni* autoagglutination by phage peptides CP3/3, CP3/4 and DP3/5.
TBS and phage peptides containing an irrelevant peptide were used as controls**.**

Recent research indicates that the ability of *C. jejuni* to autoagglutinate is related to its ability to colonise chickens; however, this relationship is complex and yet to be fully elucidated. For example, in 2009, mutants defective in flagella protein synthesis (and their glycosylation), quorum sensing (via LuxS gene and production of autoinducer 2), chemotaxis and motility failed to autoagglutinate *in-vitro* as well as showing a reduced ability to colonise chickens.

2.2 Motility

DP3/5 showed similarity to *C. jejuni* flagellar proteins (Accession No's YP_001000562 and ZP_01810255; Figure 14). *C. jejuni* flagellar proteins are related to the ability to autoaggutinate.

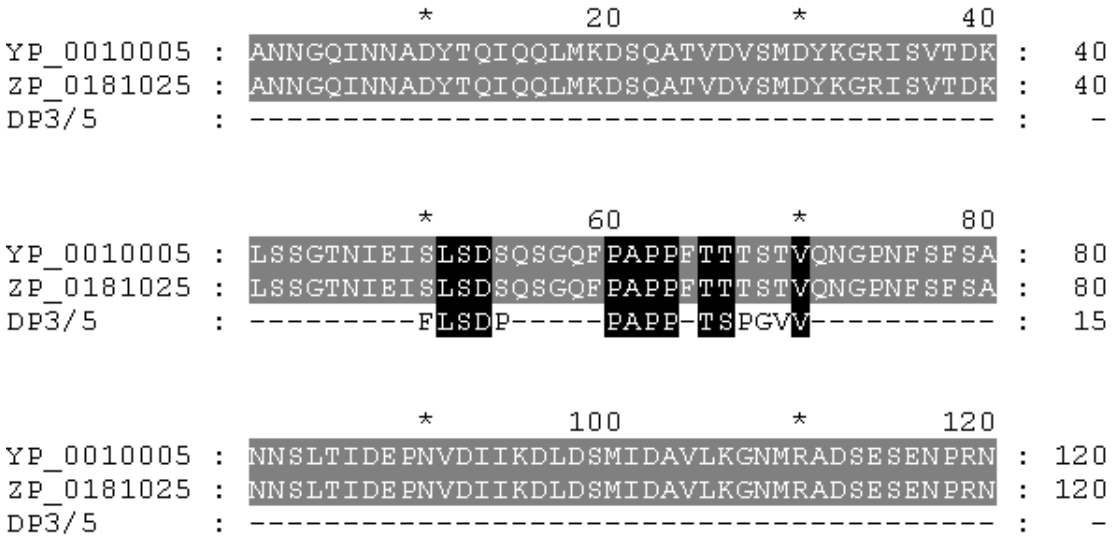


Figure 14: Amino acid alignment of DP3/5 with related proteins

The amino acid sequence deduced from DP3/5, *C. jejuni* flagella proteins (Accession Numbers YP_001000562 and ZP_01810255) were aligned using ClustalW. The shading levels, used to indicate the degree of conservation, were black for 100% amino acid conservation and grey for 75% conservation.

The antimicrobial peptides in Table 2 were evaluated for their ability to inhibit motility of *C. jejuni*. A modified motility assay was designed where the ability of *C. jejuni* to swarm on semi-soft agar plates was assessed after incubation with phage peptides (Methods, Section 6.2). Phage clones that significantly reduced the diameter zone of growth in comparison to the control zone were judged to have some effect on motility of *C. jejuni*.

On 0.4% semi-solid agar plates, visually the halo produced by the phage peptides appeared to be very similar to that produced by the controls. However, when we measured the diameter of the halos, there were slight differences between the phage peptides in their ability to reduce motility. Overall, we found that three phage peptides had a slight inhibitory effect on *C. jejuni* motility, as shown below:
Overall, three phage peptides reduced *C. jejuni* to a small extent:

- BT3/3 exhibited a 10-14% decrease in motility
- BT3/4 exhibited a 18-19% decrease in motility
- DP3/5 exhibited a 25-30% decrease in motility.

These results were repeatable between assays and between replicates; however, it was debatable whether these results were biologically significant. It is also possible, that we hadn't found the best assay conditions for motility.

Specific Aim 3: Activity of peptides outside of the phage coat

The antimicrobial activity of the phage peptides may be retained in the monomeric peptide sequence alone or alternatively, the peptide may only be active when it's part of the structure imposed by being a fusion with major coat protein pVIII (Figure 1). The biological activities of the peptides as described in Table 2 have only been tested as a fusion peptide on the phage coat. Thus, we wanted to move towards developing a carrier system that would be suitable for testing the peptides *in-vivo*.

3.1 Monomeric peptides

We synthesised monomeric peptides and compared their antimicrobial activities with the cognate native phage peptides, where the peptides were present as a fusion with the phage coat. Nine phage peptides (CT3/2, CP3/1, AT4/8, CT3/7, CT3/9, CP3/2, CP3/9, DP3/5 and CP2/5 were custom synthesised by AusPep Pty Ltd (Vic, Australia) as 15 amino acid monomeric peptides. All peptides were synthesised as >80% purity and supplied as 5 mg aliquots (Table 4).

Phage clone	Sequence	pI
CT3/2	N-FSPFRISELVYTLHP-OH	6.75
CT3/7	N-HRPVKTPANAPTTMM-OH	11
CT3/9	N-CFNDPLDIVPPMLLL-OH	3.56
CP3/1	N-GRFLIRVTSSPLGPD-OH	9.60
CP3/2	N-TGSGLYLHQMVYLYQ-OH	6.4
CP3/9	N-VRKTTSHPPSYALLH-OH	9.99
DP3/5	N-FLSDPPAPPTSPGVV-OH	3.80
CP2/5	N-VLAWFSPLTLESSRL-OH	
AT4/8	N-RPSRWPWQEPLPISI-OH	9.60

Table 4: Peptides synthesised by AusPep, Australia

These monomeric peptides were tested for potential antimicrobial activity against *C. jejuni* strain C338 using the microtiter broth dilution assay (Methods, Section 4). At a concentration of 200 μM, CT3/2 inhibited the growth of C338 by 84% *in-vitro*. It also had a marked effect on the morphology of C338. At a concentration of 400 μM, CP3/1 inhibited the growth of C338 by 40% *in-vitro*. At this concentration, peptide AT4/8 also induced morphological changes in *C. jejuni* in a dose-dependent manner even though it had no antimicrobial activity. The remaining monomeric peptides had no detectable activity.

In solution, monomeric peptides are flexible and can adopt different conformations, as opposed to multiple antigenic peptides (MAPs) or phage-displayed peptides, potentially lowering their affinity with the target receptor. Multiple antigenic peptides contain a core of lysine residues with four peptides attached and are frequently used for drug delivery in humans because of increased stability and better antimicrobial activity. We decided not to synthesise MAPs containing the peptides because although we were likely to get improved antimicrobial activity, this route of delivery to chickens would have been cost prohibitive. Likewise, although there are potential delivery mechanisms suitable for the oral delivery of our monomeric peptides within CSIRO, this route of delivery would have been cost-prohibitive for the poultry industry. Instead we have been focusing on the challenge of developing cheap on-farm delivery of antimicrobial phage peptides.

3.2 Denatured phage peptides

Selected phage peptides, once identified need to be harnessed into a suitable drug delivery platform. For the poultry industry, this drug delivery platform would need to be cheap, costing units of a cent per dose. The major coat protein pVIII of phage can be incorporated into miscelles and liposomes, due to its intrinsic function as a membrane protein (Jayanna et al. 2009).

As a start to developing such a platform, we first assessed whether the antimicrobial activity of the peptides could be retained when the phage coat itself was used as part of the delivery vehicle. For this, we chose to use a chloroform extraction protocol (Petrenko & Smith, 2000), which transforms a phage particle from an infectious filament to a non-infective hollow spherical particle (Griffith et al. 1981). Phage peptides CP3/1, CP3/2, CT3/2, CT3/7, and CT3/9 were chloroform extracted (Methods; Section 7.1). Recoveries of the phages were determined spectrophotometrically and loss of infectivity was determined by titrating the phage clones in *E. coli* K91BlueKan cells.

Interestingly, the partially chloroform denatured phage clones were as equally effective as the native phage peptides in inhibiting the growth of *C. jejuni*, indicating that the phage itself could potentially be used as a delivery vehicle for the peptides. The advantage of such an approach is that selection, testing and delivery of the antimicrobial peptides could all be done be done in the context of the phage coat. There would also be no potential loss of antimicrobial activity through chemical synthesis of the peptides or through the use of fusion peptides. Such a delivery vehicle would meet the challenge of providing a low cost vaccine/ therapeutic to the poultry industry.

3.3 Phage as a nanocarrier

As the next stage, we assessed whether the peptides could retain antimicrobial activity when the pVIII protein was stripped from the phage coat. The pVIII proteins containing phage peptides CP3/2 and CT3/2 were stripped from the phage coat according to Methods, Section 7.2. These phage peptides were effective in inhibiting the growth of *C. jejuni* using the microtiter broth dilution assay. Although promising, this assay was fairly crude since we had not evaluated the concentration of the peptides nor evaluated that they had properly separated from the phage coat using Western blotting.

Specific Aim 4: Testing of the phage clones in an animal trial

In this specific aim, the proposed animal work to assess the protective effects of the phage peptides on *C. jejuni* was not undertaken due to time constrains resulting from my laboratory relocating from the JM Rendel laboratory in Rockhampton to the QBP building in Brisbane. This required the relocation of all laboratory equipment and samples and staff. There were also associated problems with samples thawing out during transit which required additional experimentation and further delayed the project.

Patents

Bishop-Hurley, S. L.; McSweeney, C. S. Phage-display peptides are antimicrobial against *Campylobacter* spp. Corporate Owner: The Australian Poultry CRC Pty Ltd. Australian Patent Application 20079903374; International Patent Application PCT/AU2008/00878

Discussion of results

Bacterial pathogens such as *C. jejuni* contain an array of cell surface receptors/ epitopes, enabling them to colonise and persist in the gastrointestinal tract of chickens. Although sequencing and comparative genomic analysis of *Campylobacter* species (Fouts et al. 2005; Parkhill et al. 2000) have provided a starting point for the identification of these cell surface molecules, the proteins involved in the virulence/ colonisation of *C. jejuni* in poultry remain poorly understood. Phage-display can be used to isolate specific ligands capable of disrupting bacterial physiology without prior knowledge of the receptors/ epitopes involved (Bishop-Hurley et al. 2005). In this study, a high avidity phage library was used to isolate peptides binding to the cell surface of a strain of *C. jejuni* isolated from poultry houses. Phage peptides isolated on their ability to bind *C. jejuni* were then individually screened in functional based assays for interference of *C. jejuni* growth and development.

In whole cell screening assays, the affinity selection process is performed in a complex environment where antigens are present at varying densities and accessibilities on the cell surface (Hoogenboom et al. 1999). Although no selective pressure was applied during the affinity selection process, it is nevertheless largely driven by the density and/or accessibility of cell surface antigens (Hoogenboom et al. 1999). Thus, peptides isolated from an affinity selection procedure may all bind to the same cell surface receptor (Oyama et al. 2006). Without prior knowledge of the epitopes/ receptors to target, we designed an affinity selection scheme that would enable us to sample a wide range of receptors rather than the high density/ accessible receptors that would have been the result of a direct selection strategy (Hoogenboom et al. 1999). We used two affinity selection procedures, which were designed around progressive subtraction of the phage library against the cell surface epitopes of *C. jejuni*. We hypothesised that each subtractive step would progressively remove the phage peptides binding to the predominant epitopes, whilst still preserving those binding to lower density epitopes. This strategy was successful in isolating phage peptides that were bactericidal towards *Haemophilus influenzae* (Bishop-Hurley et al. 2005).

The success of this subtractive strategy was reflected in the high proportion of antimicrobial phage clones isolated (16 unique peptides, Table 2). The presence of detergent (Tween-20) in the wash buffer, used for removing non-specific hydrophobic interactions during the affinity selections, appeared to have selected for groups of amino acid residues with different physical-chemical properties to those selected using PBSg (Figure 4). In particular, the aligned CT3/ phage peptides contained a conserved proline and hydrophobic amino acids (Figure 4B). There was also a high incidence of proline residues in the CP3/ and DP3/ phage peptides. Prolines have been shown to be important for protein-protein interactions with some protein interaction domains preferring proline-rich ligands (Kay et al. 2000). Prolines are also known to dominate in some natural antimicrobial peptides such as pyrrhocoricin (Cudic et al. 2002).

In contrast to the CT3/ phage clones, some of the CP3/ and DP3/ phage peptides were selected multiple times. For example, 12 clones were isolated that were represented by the sequence CP3/3 (FLIDSPLASIGPTSM); five clones represented by DP3/5 (FLSDPPAPPTSPGVV) and two clones represented by CP3/4 (FMIDSPLASIGPTSM; Table 2). It was interesting that these phage clones were the only anionic phage clones (i.e. net charge of -1) isolated. They were also the only clones isolated that bound to *C. jejuni* to very high levels (data not shown). The remaining phage clones were either a neutral charge or were cationic (Table 2).

The antimicrobial activity of the phage peptides was dose-dependent with MICs in the low micromolar range (Table 2). However, this was a conservative estimate since the calculations assumed that all 150-300 copies of the peptides displayed on the phage coat were simultaneously accessible for binding to *C. jejuni*. Two (CT3/2, CP3/1) of the nine synthesised monomeric peptides retained antimicrobial activity towards *C. jejuni*; however, they were approximately 50-fold less effective on a molar basis at inhibiting *C. jejuni* growth compared with the corresponding phage sequences. This was not unexpected, since in solution, peptides can adopt many different conformations, which lower their

affinity for their target. Multiple antigen peptides (MAPs) have been found to be more effective than the linear homologue for retaining activity (Pini et al. 2005). This may be related to the multimeric nature of the peptides on the MAPs, which may allow multivalent binding.

Interestingly, the partially chloroform denatured phage clones were as equally effective as the native phage peptides in inhibiting the growth of *C. jejuni*, indicating that the phage itself could potentially be used as a delivery vehicle for the peptides. The advantage of such an approach is that selection, testing and delivery of the antimicrobial peptides could all be done be done in the context of the phage coat. There would also be no potential loss of antimicrobial activity through chemical synthesis of the peptides or through the use of fusion peptides. Such a delivery vehicle would meet the challenge of providing a low cost vaccine/ therapeutic to the poultry industry.

In conclusion, we have described the utility of using live *C. jejuni* for the isolation of species-specific antimicrobial phage-displayed peptides. The phage peptides also had activity in their cognate peptide form indicating that they have the potential to be directly used in drug delivery.

Recommendations for future work

Over the last 30 years, the majority of antimicrobial agents used in human medicine have been analogues of pre-existing compounds with few structurally new compounds produced. This project has been very successful in that 16 unique phage peptides were isolated that inhibited the growth of *C. jejuni* by almost 100% *in-vitro*. These phage peptides were novel in that unlike current antimicrobials, they were specific in their activity towards *C. jejuni*; none of them showed any antimicrobial activity against Gram-positive and Gram-negative bacteria tested. The phage peptides did not bind to these bacteria, indicating that the epitopes on the cell surface of *C. jejuni* targeted by the phage peptides are likely to be unique or structurally different.

This project has produced proof-of-concept for the feasibility of these peptides and the CRC has filed an international patent to protect these peptide sequences. We recommend that the following work be undertaken in order to validate the peptides and develop them into commercial products to be used by the poultry industry. Specifically, the following areas should be addressed.

1. Determination of the peptide binging sites on the cell surface. The phage peptides induced a profound change in morphology of *C. jejuni* in a dose-response manner. Together with their specificity of activity towards *C. jejuni*, it is likely that the peptides are binding to cellular receptors/epitopes, not previously considered as vaccine/ antimicrobial targets. The cellular receptor/epitope(s) that the phage peptides are binding have not been characterised so far. A sequence alignment indicated that the peptides could be divided into two groups, based on their physical-chemical properties. These two groups may each recognise a unique epitope/ receptor on the cell surface of *C. jejuni*. Equally the epitope(s) on the cell surface of *C. jejuni* may not necessarily be a protein molecule but might involve carbohydrate or lipid moieties. Very little is known about the carbohydrate molecules decorating the cell surface of *C. jejuni*; however, recent research suggests that they may actively be involved in virulence. Competition ELISAs using synthetic peptides would allow the phage peptides to be divided into groups, with each group potentially recognising a unique epitope.

Further work should also focus on identifying the receptor/epitope(s) the phage peptides are binding as well as the possible mechanism(s) of action of the phage peptides. This information would support the patent application filed in the first Poultry CRC, as well as being invaluable for the design of peptides with the best possible activity.

2. Assess delivery methods for the peptides. Selected phage peptides, once identified, need to be harnessed into a suitable drug delivery vehicle. The challenge will be the design of a low-cost delivery platform for the peptides that would be easy and cheap to administer, especially since most vaccines are administered via the parenteral route. We have demonstrated that the peptides retained antimicrobial activity as monomeric peptides, which was important since it established that the peptides could retain activity outside of the constraints imposed by the phage pVIII coat protein. Partially denatured phage clones were as equally effective as the native phage peptides in inhibiting the growth of *C. jejuni*, indicating that the phage coat proteins themselves could potentially be incorporated as part of the delivery vehicle. Further work on incorporating the pVIII phage coat protein into a nanoparticle could potentially provide a low-cost route for oral or *in-ova* delivery as well as leading to further patent applications.

Like all antimicrobials isolated, in this work, the peptides were isolated and tested on *C. jejuni* that was in a free-swimming planktonic stage, that is unlikely to exist in poultry. Since 2009, *C. jejuni* was shown to exist as part of a community known as a biofilm. Biofilms are very different from the free-living stage in that they have marked differences in metabolism and cell physiology. Further work should continue on the isolation of peptides, but to develop methods of isolating peptides against *C. jejuni* that are part of this biofilm community.

3. Assessment of efficacy under commercial conditions – animal trials. Experiments described in this report initially developed 16 antimicrobial phage peptides and identified 8 of these as potential candidates for further assessment. An *in-vitro* fermentation assay, developed for *C. jejuni* would allow for the testing of these peptides prior to full scale animal trials. As such, these assays are a predictor of success in animal trials because it evaluates the antimicrobial activity of the peptides in a mixed anaerobic culture of chicken caecal contents containing microorganisms that are largely unculturable. *C. jejuni* in this environment is likely to be in a form that is found in the chicken caeca. In this system, the timing of the peptide addition can be evaluated. This is especially important since the mode-of-action of some peptides may not necessarily be limited to antimicrobial action alone. For example, we found that CP3/3, CP3/4 and DP3/5 appeared to target virulence factors. In addition, the *in-vitro* fermentation assay would allow the evaluation of different peptide constructs as well as the potential activity the phage peptides may have on gut microorganisms.

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Plain English Compendium Summary

As part of the Final Report requirements, authors need to provide a one page, plain English Compendium summary along with each Final Report in electronic and hard copy format. These compendium summaries are published in Poultry CRC's annual Report. A template for the summary can be found below, following the completed example.

Note that this one-page summary will be read by people without expertise in the field of study. It should therefore be as easy to read and understand as possible

Plain English Compendium Summary

Project Title:	
Project No.:	06-9
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Objectives	
Background	*Campylobacter jejuni* is a leading cause of zoonotic food-borne illness in humans with chicken meat implicated as one of the principal sources of infection. *C. jejuni* naturally colonises the lower gastrointestinal tract of poultry and during processing is spread from the gastrointestinal tract onto the surface of dressed carcasses. Reducing *C. jejuni* carriage in chickens is considered to be one of the most important approaches for reducing human infection.
Research	*C. jejuni* contains cell surface receptors that play a significant role in its ability to colonise and persist in chickens. The aim of this project was to isolate peptides that target these 'unknown' cell surface receptors using a method called phage display. In this method, peptides are selected from a library containing billions of different peptide combinations and are selected on the basis of their ability to bind to the outer surface receptors of live *C. jejuni*. Some of these peptides may bind to *C. jejuni* receptors that are involved in its virulence and ability to colonise and persist in the gastrointestinal tract of chickens and thus disrupt these functions. Isolated peptides were screened for their ability to inhibit *C. jejuni* growth using *in-vitro* growth inhibitory assays.
Outcomes	We isolated 16 unique peptide sequences that inhibited the growth of *C. jejuni* by almost 100%. Some of these peptides were also bactericidal in that they were able to reduce the initial inoculum of *C. jejuni* by 84%. These peptides were also highly specific towards *C. jejuni*, showing no antimicrobial activity towards other bacteria species tested. Some of the peptides were able to factors that are associated with virulence in *C. jejuni*, indicating that they may be useful as vaccine candidates. These phage peptides maintained antimicrobial activity when synthesised outside of the phage coat, indicating that they can potentially be administered as part of a low-cost delivery vehicle. An Australian and International Patent Application are being filed on the sequences isolated from this work.
Implications	The phage peptides have potential to be used for the control of *C. jejuni*

	in chickens. A low-cost delivery vehicle will enable the peptides to be administered to chickens either orally or *in-ova*.
Publications	