BACTERIOPHAGE-DISPLAYED PEPTIDES FOR THE CONTROL OF PATHOGENS IN SWINE 2C-106

Report prepared for the Co-operative Research Centre for High Integrity Australian Pork

By

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Executive Summary

Escherichia coli are Gram-negative, rod-shaped bacteria that are commonly found in the lower intestine of warm-blooded animals such as swine. Pathogenic *E. coli* strains are also a common cause of post-weaning enteric disorders in young piglets with antibiotics being used for its prophylactic and therapeutic control. Controlling or eliminating pathogenic *E. coli* strains on-farm would lead to a corresponding reduction of post-weaning enteric disorders in young piglets. Novel antimicrobials based on phage displayed peptides are not classified as antibiotics used for human medicine because they have unique modes-of-action against the targeted pathogen.

Receptors/epitopes on the cell surface of *E. coli* interact with the host animal to facilitate its colonisation and persistence in the gastrointestinal tract. The aim of this project was to demonstrate, as an initial proof-of-concept, that we could target the cell surface receptors of *E. coli* 0157 using phage display peptides. We used a subtractive phage display procedure to affinity select for peptides binding to the cell surface of *E. coli* 0157. These phage peptides, selected on the basis of their ability to bind to *E. coli*, were then tested for their potential to act as species-specific antimicrobials using in-vitro assays.

In total, 55 phage displayed peptides were isolated from the eight sub-libraries generated. Sixteen of the phage peptides inhibited the growth of *E. coli* 0157 in-vitro at a theoretical peptide concentration in the nanomolar range (70-200 nM). Three phage peptides completely inhibited the growth of this bacterium.

In this project, the peptides were tested as part of a fusion with the pIII coat protein of the bacteriophage. This made their evaluation difficult because of the toxicity of the displayed peptide on the *E. coli* cells used to amplify them. This was unsurprising given the relatedness of the pathogenic and non-pathogenic *E. coli* strains. Thus it is likely that the peptides would show enhanced antimicrobial activity when synthesized as monomeric or tetrameric peptides, as has previously been demonstrated (Pini et al. 2005).

Using dot-blots we demonstrated that the phage peptides bound to the cell surface of *E. coli* 0157. This could be used as a strategy for the isolation of vaccine candidates.

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1. Introduction

Escherichia coli are Gram-negative, rod-shaped bacteria that are commonly found in the lower intestine of warm blooded animals such as swine. Most *E. coli* strains are harmless but some serotypes are food-borne pathogen of humans. Pathogenic *E. coli* are also one of the most important causes of post-weaning diarrhoea in suckling and post-weaned piglets, resulting in increased morbidity and mortality (Fairbrother et al. 2005).

The post-weaning period in swine is often managed by the prophylactic and therapeutic use of broad-spectrum antibiotics. These antibiotics, designed for human medicine, generally have broad-spectrum activity and fall into a relatively small number of classes with respect to mechanism of action (Butler & Cooper, 2012). Consequently, swine fed those antibiotics end up as a reservoir for antibiotic-resistant bacteria in the gastrointestinal tract, which can then be transmitted to humans. However, the emergence of antibiotic resistant strains of *E. coli* and growing restrictions on the use of broad-spectrum antibiotics in food animals has increased the awareness of pathogen-specific therapies. Such strategies include the development of novel antimicrobials with unique modes of action (Christensen et al. 2001). Ideally, these compounds would need to target multiple strains of pathogenic *E. coli* whilst being highly specific to this microorganism.

Since its invention in 1985 (Smith, 1985), phage display has been successfully applied to many different areas of research, including immunology, cancer research, drug discovery and in infectious disease. It has also been used to identify small peptide ligands inhibiting the function of targeted receptors for a wide range of applications. Phage display has also become a powerful tool for the isolation of peptides binding to the cell surface epitopes on intact live cells because it allows for the isolation of targeting peptides in cases where little is known about the cellular landscape (Fong et al. 1994). Using this technology, therapeutic peptides have been isolated 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 molecules on the cell surface of *E. coli* play a significant role in its colonization and persistence within the swine herd. Consequently, these cell surface epitopes/ 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 phage peptides inhibited the growth of pathogenic *E. coli* by almost 100% in-vitro.

2. Methodology

2.1. Bacterial Methods and Culture Conditions

Escherichia coli 0157 strains were maintained at -75 °C using the 'Protect' Bacterial Preserve Vials (Oxoid, Australia). Cultures were maintained (for < 1 week) on Nutrient agar plates [25 g/L Nutrient broth #2 (Oxoid, Australia), 15 g/L Bacteriological agar (Amresco, Australia)] and stored at 4 °C until needed. *E. coli* 0157 was grown in Nutrient broth (25 g/L Nutrient broth #2, Oxoid, Australia) and

incubated at 37 °C, 200 rpm, until it reached mid-log growth (OD_{595} of ~0.5). In all experiments, bacterial titers were estimated by optical density (OD) measurements and confirmed by quantitative culture by plating on Nutrient agar plates.

2.2. Phage Display Methods

2.2.1. Phage Libraries and General Laboratory Procedures

The Ph.D-12 phage display library, purchased from New England Biolabs (NEB, Australia), was used to affinity select for *E. coli*-binding phages as described in Section 2.3. This library was based on the M13 filamentous bacteriophage vector that was modified for the pentavalent display of 12-mer peptides as N-terminal fusions to minor coat protein pIII. This library contained a complexity of 10^9 peptide variants. The laboratory strain of *E. coli* (ER2378), also provided by NEB (Australia), was used for the propagation of the individual phage clones.

2.2.2. Phage Growth, Purification and Titering in *E. coli*

The general procedures for recombinant phage production and buffers used in this study have been described (Smith & Scott, 1993; Yu & Smith, 1996). In brief: phage was purified twice using 20% polyethylene glycol, 8000-2.5 M NaCl (PEG/NaCl) precipitation and re-suspended in Tris-borate saline buffer (TBS buffer; 50 mM Tris-Cl, pH 7.5, 150 mM NaCl) (Smith & Scott, 1993; Yu & Smith, 1996). The concentration of phage particles (virions/µL) was determined spectrophotometrically as previously described (Barbas et al. 2001) using the following formula:

Virions/mL = (A_{269} x 6 x 10^{16})/ number of nucleotides in the phage genome.

The concentration of infective phages was determined by infecting *E. coli* ER2378, that had grown to mid-log phase and plating dilutions onto Luria Bertani (LB) plates containing X-gal and isopropyl ß-D-1-thiogalactopyranoside (IPTG) and incubating overnight at 37 °C. The number of resulting colonies on a plate was determined as plaque forming units (PFU) and corresponded to the biological titer of the phage.

2.2.3. Affinity Selection of the Phage Displayed Peptides

In the affinity selection procedures, phage displayed peptides were selected on the basis of their ability to bind to *E. coli* 0157. Two different selection strategies were used, based on progressive pre-adsorption of the phage library against the cell surface of non-pathogenic *E. coli* (ER2378 and JM109 for selection schemes 1 and 2, respectively). Further subtractions were carried out against the cell surface of *E. coli* 0157, based on the rationale that progressive pre-adsorption against the cell surface epitopes of *E. coli* progressively captures peptides binding to the less dominant receptors. Further sub-selections involved different wash strategies to remove non-binding and loosely adherent phage from *E. coli* 0157. These were either a high salt [TBS pH 7.5 containing 0.1% Tween20 (TBST)] or low salt [phosphate buffered saline pH 7.4 containing 0.1% gelatin (PBSg)] buffer. This resulted in eight sub-libraries being generated as depicted below.

In brief:
In selection scheme 1A, the Ph.D-12 phage library was pre-adsorbed once against a non-pathogenic strain of *E. coli* (ER2378), the host strain of the phage library (Section 2.1), prior to being used to select for peptides binding to the cell surface of *E. coli* 0157. The non-binding phage was removed using either a TBST (pH 7.5)

or PBSg (pH 7.4) buffer, resulting in the generation of EBT3/ and EBP3/ sub-libraries , respectively (Figure 1A).

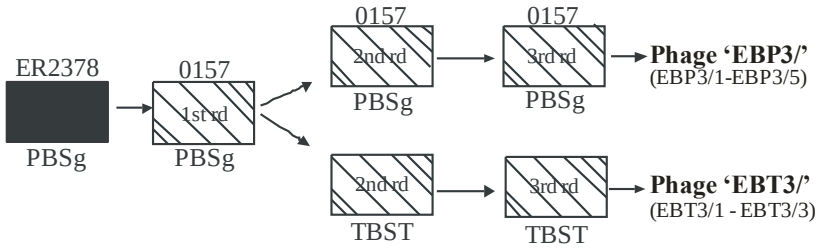

Figure 1A: Selection scheme 1A
The Ph.D phage display library was subtracted once against *E. coli* ER2378 cells (black box), prior to three affinity selections against *E. coli* 0157 (hatched boxes). This resulted in the isolation of EBP3/ and EBT3/ pools of phage peptides.

In selection scheme 1B, the Ph.D-12 phage library was pre-adsorbed twice (once against *E. coli* ER2378 cells and once against *E. coli* 0157) prior to being affinity selected for peptides binding to the cell surface of *E. coli* 0157. This generated ECT3/ and ECP3/ sub-libraries for those washed in TBST (pH 7.5) and PBSg (pH 7.4), respectively (Figure 1B).

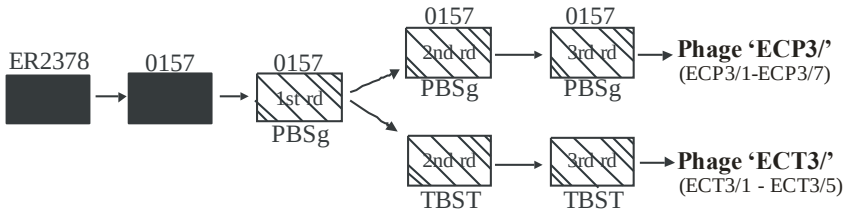

Figure 1B: Selection scheme 1B
The Ph.D phage display library was subtracted against *E. coli* ER2378 cells and *E. coli* 0157 (black boxes), prior to three affinity selections against *E. coli* 0157 (hatched boxes). This resulted in the isolation of ECP3/ and ECT3/ sub-libraries being generated.

In selection scheme 2A, the Ph.D-12 phage library was pre-adsorbed against *E. coli* JM109, a general laboratory non-pathogenic strain of *E. coli*, prior to being affinity selected for peptides binding to *E. coli* 0157. This generated sub-libraries JBT3/ and JBP3/ respectively (Figure 1C).

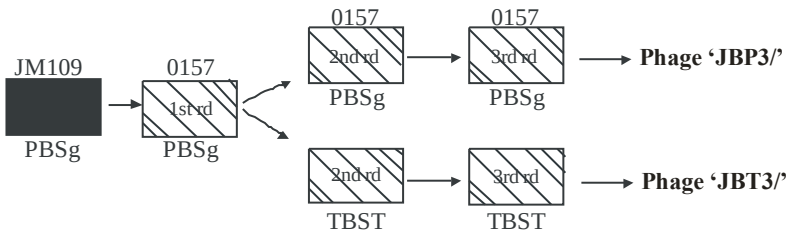

Figure 1C: Selection scheme 2A
The Ph.D phage display library was subtracted once against *E. coli* JM109 cells (black box) before being affinity selected against *E. coli* 0157 (hatched boxes), resulting in the generation of JBP3/ and JBT3/ sub-libraries.

In selection scheme 2B, the phage library was pre-adsorbed twice; once against the *E. coli* JM109 (as for Selection scheme 2A) and once against *E. coli* 0157. The removal of non-binding phage using TBST (pH 7.5) or PBSg (pH 7.4) resulted in the generation of sub-libraries JCT3/ and JCP3/ (Figure 1D).

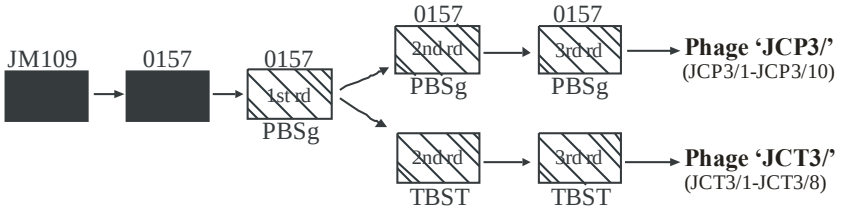

Figure 1D: Selection scheme 2B
The Ph.D phage display library was subtracted once against *E. coli* JM109 cells and once against *E. coli* 0157 (black boxes), prior to three affinity selections against *E. coli* 0157 (hatched boxes), resulting in the generation of the JCP3/ and JCT3/ sub-libraries.

Detailed methodologies for the affinity selections are presented below for Selection scheme 1A. This methodology was generally the same for the other sub-libraries generated in selection schemes 1B, 2A and 2B, except that different bacteria were used for the subtractions.

Selection Scheme 1A

E. coli strains ER2378 and 0157 were separately grown in Nutrient broth (Section 1.0) twice precipitated by centrifugation (5,000 rpm, 2 min) and re-suspended in PBSg (pH 7.4) to a concentration of 10^9 CFU/mL.

In this subtraction step, 2 x 10^{11} PFU of the Ph.D 12-mer phage display library was added to freshly prepared 10^9 CFU of *E. coli* ER2378 in a total volume of 3 mL PBSg (pH 7.4) in a Petri dish (Sarstedt, Australia). This bacterial-phage solution was agitated on an orbital shaker at room temperature so that the phage library could bind to the ER2378 cells in solution. After 1 hr, bacteria containing surface-bound phages were precipitated by centrifugation (10,000 rpm for 2 min). The non-binding phage in solution, representing the subtracted phage library, were removed and then used to affinity select for phage peptides binding to the cell surface epitopes of *E. coli* 0157.

In the affinity selection procedure, the subtracted phage library, depleted of clones binding to ER2378, was added to 10^9 CFU of freshly prepared *E. coli* 0157 in a total volume of 3 mL PBSg (pH 7.4). This bacterial-phage mixture was agitated as above on an orbital shaker for 30 min at room temperature. *E. coli* 0157 containing bound phage were separated from non-binding phage in solution through a series of washing and centrifugation steps (6,000 rpm 2 min) using 1 mL of PBSg each time (for this first round of affinity selection; Figure 1A). The phage remaining bound to *E. coli* 0157 were eluted from cell surface epitopes of the pelleted bacteria by the addition of Elution Buffer (0.1 N HCl, pH 2.2 with glycine; 1 g/L bovine serum albumin) for 10 min at 4 °C and then neutralized by the addition of 1 M Tris-HCl (pH 9.0). The eluted phage was titered as PFU (Section 2.2) in *E. coli* ER2378 cells so that the yield of phage binding to *E. coli* 0157 could be determined (calculated as output phage/input phage x 100; Yu & Smith, 1996). The remaining phage was amplified by *E. coli* ER2378 infection, twice purified by PEG/NaCl precipitation and re-suspended in TBS buffer (pH 7.5) as described in Section 2.2.

After the first round of affinity selection, this amplified library was divided into two sub-libraries based on the solution used to wash off non-binding phage (either PBSg or TBST). Amplified phages (10^{11} PFU) generated from the first round of affinity selection against *E. coli* 0157 were separately added to two Petri dishes, each containing 10^9 CFU of *E. coli* 0157 in 3 mL of PBSg. The entire process of affinity selections was repeated as above, except that the phage-bacteria complex from one Petri dish was washed with PBSg (pH 7.4) and the other with TBST (pH 7.5) The phages from these sub-libraries were eluted off the cell surface of *E. coli* 0157 as described above and independently propagated in *E. coli* ER2378 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* ER2378 cells and plated on LB agar plates containing X-gal/IPTG plates as described above. Bacterial colonies, each containing a unique phage clone, were randomly selected for propagation and subsequent analyses. The phages from these two sub-libraries were named EBP3/ and EBT3/ for those washed with PBSg and TBST, respectively.

2.3. In-vitro Antimicrobial Assay

Representative affinity-selected phage displayed peptides were tested for their ability to inhibit the growth of *E. coli* 0157 using the microtiter broth dilution assay. Each phage clone was amplified by infecting *E. coli* ER2378 cells, purified by PEG/NaCl precipitation before being resuspended in TBS (pH 7.5). Phage concentrations were calculated spectrophotometrically as virions/µL (Section 2.2). *E. coli* 0157 was grown in Nutrient broth (Section 2.2) to mid-log phase (A_{595} nm of ~0.5). Bacterial titers were estimated by measuring the OD's on a spectrophotometer at A_{595} nm (Wallac Victor2, Perkin Elmer, Australia) and confirmed by plating dilutions on Nutrient agar plates.

The microtiter broth dilution assay was carried out in 96-well flat bottomed polystyrene microtiter plates (Sarstedt, Australia). In this assay, each well was inoculated with 150 µL of *E. coli* 0157 (equivalent to 1-5 x 10^5 CFU/mL) and 45 µL of individual phage displayed peptides and incubated for 12-16 hr at 37 °C without shaking. The OD's of the bacterial cultures were measured on a spectrophotometer (Wallac Victor2, Perkin Elmer, Australia), which indirectly quantifies the number of bacteria present by optical absorbance. The number of viable bacteria in the 96-well plates was also determined by performing colony counts on the diluted inoculums by plating dilutions on Nutrient agar plates.

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

2.4. Immunoblotting

Candidate phage displayed peptides were individually tested for their ability to bind to the cell surface of *E. coli* 0157. Individual phage clones were amplified as described in Section 2.2. Freshly prepared *E. coli* 0157 (grown according to Section 1.0) was re-suspended to an OD_{595} nm of 0.6 in PBS (pH 7.4). Two microliters of this bacterial suspension was concentrated by precipitation (6,000 rpm for 2 min) and re-suspended in 200 µL of PBS (pH 7.4). Six microliters of this suspension (equating to ~2 x 10^7 cells) was dot-blotted onto nitrocellulose membrane strips (Hybond-N; Amersham, Australia) in 2 µL aliquots. These nitrocellulose membranes were blocked for 2 hr at room temperature in blocking solution (5% skim milk, 10 mM Tris-HCL pH 7.4, 100 mM NaCl, 0.1% Tween20) prior to being incubated with 2 x 10^{12} virions of phage displayed peptides in TBS (pH 7.5) for 1 hr at room temperature. After being washed in PBS (pH 7.4) containing 0.1% Tween20 (PBST) for 2 x 5 min, the membranes were incubated with a 1:2500

dilution of mouse anti-M13 antibody conjugated to horseradish peroxidase (HRP; Amersham) in blocking buffer for 1 hr at room temperature. The membranes were washed as above and then incubated with 1-step TMB-blotting substrate (Thermoscientific, Australia) for detection. The negative control was *E. coli* 0157 cells that were incubated with a non-related phage control.

3. Outcomes

3.1. Phage Selections

A phage library, pentavalently displaying 12-mer peptides on minor coat protein pIII, was used to affinity select for peptides binding to the receptors/ epitopes on the cell surface of *E. coli* 0157. Without prior knowledge of the cell surface receptors/epitopes involved in pathogenesis, the aim was to maximize the number of cell surface receptors targeted by progressively pre-adsorbing the phage library so that the less abundant epitopes were also targeted.

The starting library was pre-adsorbed against laboratory strains of *E. coli* (ER2378 and JM109 for selection schemes 1 and 2, respectively; Section 2.2). This was followed by a further pre-adsorption against the cell surface of *E. coli* 0157 (selection schemes 1B and 2B), prior to being used to affinity select for peptides binding to *E. coli* 0157. Without these pre-adsorption steps, phage clones binding to the more abundant/accessible epitopes shared by the non-pathogenic/pathogenic *E. coli* strains would have been the predominant outcome during the affinity selection steps.

Furthermore, the amplified first rounds of each of the affinity selections were split into sub-libraries so that solutions of different ionic strengths could be explored in the second and third rounds of selections (Section 2.2; Bishop-Hurley et al. 2010). Hydrophobic and ionic interactions of the peptides with their targets tend to be favored under conditions of high and low ionic strengths, respectively.

In total eight sub-libraries were generated in this project. Phage clones were isolated from each of these sub-libraries and tested for potential antimicrobial activity (as described in Section 2.2).

3.2. Phage Isolation and Antimicrobial Results

3.2.1. Overall Summary of Activity

The in-vitro microdilution susceptibility test was used to identify potential phage peptides that displayed antimicrobial activity towards *E. coli* 0157 in functional based assays. Individual phage peptides, isolated from each of the sub-libraries generated in Section 2.2, were incubated with *E. coli* 0157 in Nutrient broth in 96-well format (Section 2.3). After an overnight incubation at 37 °C, 16 individual phage clones were found to inhibit the growth of *E. coli* 0157, as determined by direct measurement of the OD's of the bacterial cultures (Section 2.3; Tables 1-4). The phage clones isolated from the JCP2/ and JCT2/sub-libraries showed the greatest antimicrobial potential, although the toxicity of the displayed peptide on the growth of the phage in the *E. coli* host was a problem in their evaluation.

Because the phage peptides were often tested at below their MIC, they also induced a marked change in the morphology of the target *E. coli* 0157 cells (Fonseca et al. 2004), making the OD measurements of growth inhibition inaccurate. The number of viable bacteria in 96-well plates was more accurately determined for JCP2/1, JCP2/9 and JCT2/5 by performing colony counts on Nutrient agar plates (Section 2.3). Results showed that these phage clones

completely inhibited the growth of *E. coli* 0157, further indicating that measuring the OD's of the bacterial cultures underestimated the inhibition of growth induced by the phage peptides.

A more detailed examination of the phage peptides isolated from each of the selection schemes and their antimicrobial activity is presented below:

3.2.2. Selection Scheme 1A

EBP3/ sub-library

Five phage clones were randomly selected from the EBP3/ sub-library (EBP3/1 – EBP3/5) and independently propagated for antimicrobial testing. None of these phage clones showed evidence of antimicrobial activity and nor were there any changes in the morphology of the bacteria, accompanying antimicrobial testing (Table 1). From this, we concluded that there were unlikely to be any phage clones of therapeutic value from this sub-library generated.

EBT3/ sub-library

Three clones were initially selected from the EBT3/ sub-library (EBT3/1 – EBT3/3) for antimicrobial testing (Table 1). One phage clone, EBT3/2, showed evidence of antimicrobial activity at a theoretical peptide concentration of 0.156 μM. A Gram-stain of the bacteria indicated that there were slight morphological changes to *E. coli* 0157 typical of an antimicrobial acting at below its minimal inhibitory concentration (MIC). The overall results indicated that EBT3/2 showed potential as an antimicrobial if it was taken out of the phage coat for antimicrobial testing.

Table 1: Summary of phage displayed peptides isolated from Selection scheme 1A and their antimicrobial activities

Phage clones	Virion conc./μL	μM conc.[a]	% growth inhibition[b]
EBP3/1	1.71×10^{10}	0.188	Not active
EBP3/2	9.92×10^{9}	0.109	Not active
EBP3/3	1.18×10^{10}	0.130	Not active
EBP3/4	1.08×10^{10}	0.119	Not active
EBP3/5	1.45×10^{10}	0.160	Not active
EBT3/1	1.27×10^{10}	0.140	28%
EBT3/2	1.41×10^{10}	0.156	36%
EBT3/3	1.14×10^{10}	0.126	25%

[a] the μM concentration of the phage peptides, assuming that all five copies displayed on the pIII are binding to the *E. coli* cells

[b] based on a decrease in the optical absorbance of the bacteria at A_{595} nm

3.2.3. Selection Scheme 1B

ECP3/ sub-library

After three rounds of affinity selection against *E. coli* 0157 (Figure 1B), seven phage clones were isolated from the ECP3/ sub-library (ECP3/1 – ECP3/7) and propagated for antimicrobial testing (Table 2). All seven phage peptides were difficult to amplify in the *E. coli* ER2378 host bacteria, and could only be tested in the low nanomolar concentration.

Despite the difficulty in testing these phage clones for antimicrobial activity, four of the seven phage clones (ECP3/3, ECP3/5, ECP3/6 and ECP3/7) showed evidence of antimicrobial activity in the low nanomolar range (Table 2). These peptides would need to be further evaluated as either monomeric or tetrameric peptides.

ECT3/ sub-library

After three rounds of affinity selection against *E. coli* 0157, five phage clones were randomly selected from the ECT3/ sub-library (ECT3/1-ECT3/5) for antimicrobial testing (Table 2). These phage clones were less promising in their antimicrobial potential than the ones isolated from the ECP3/ sub-library.

3.2.4. Selection Scheme 2A

The individual phage clones from the JBP3/ and JBT3/ sub-libraries (Figure 1C) were not evaluated because it was assumed that they would not show antimicrobial activity towards *E. coli* 0157, based on the results from selection scheme 1A.

Table 2: Summary of phage displayed peptides isolated from Selection scheme 1B and their antimicrobial activities

Phage clones	Virion conc./µL	µM conc.[a]	% growth inhibition[b]
ECP3/1	7.62×10^9	0.0838	Not active
ECP3/2	1.11×10^{10}	0.122	27%
ECP3/3	1.08×10^{10}	0.119	37%
ECP3/4	8.54×10^9	0.0939	Not active
ECP3/5	6.23×10^9	0.068	31%
ECP3/6	6.46×10^9	0.0711	35%
ECP3/7	1.07×10^{10}	0.118	42%
ECT3/1	2.31×10^{10}	0.254	38%
ECT3/2	6.23×10^9	0.0685	Not active
ECT3/3	9.55×10^9	0.105	31%
ECT3/4	9.69×10^9	0.107	21%
ECT3/5	1.28×10^{10}	0.141	23%

[a] the µM concentration of the phage peptides, assuming that all five copies displayed on the pIII are binding to the *E. coli* cells.
[b] based on a decrease in the optical absorbance at A_{595} nm

3.2.5. Selection Scheme 2B

JCP3/ sub-library

After three rounds of affinity selection against *E. coli* 0157, ten phage clones were randomly selected from the JCP3/ sub-library (JCP3/1- JCP3/10) and amplified for antimicrobial testing. All ten phage clones inhibited the growth of *E. coli* 0157 but at a theoretical peptide concentration in the 100-200 nM range (Table 3). One phage clone (JCP3/9) showed a 58% inhibition of *E. coli* 0157 based on OD measurements of bacterial growth. However, because JCP3/9 also markedly affected the morphology of the bacteria (Figure 2), it is likely that the OD

measurements would have underestimated the inhibition of growth induced by this phage peptide (as shown below for the JCP2/ and JCT2/ sub-libraries).

Some of the phage peptides were tested more than once using different phage cultures; however, for brevity, only one of each phage clone is represented in Table 3.

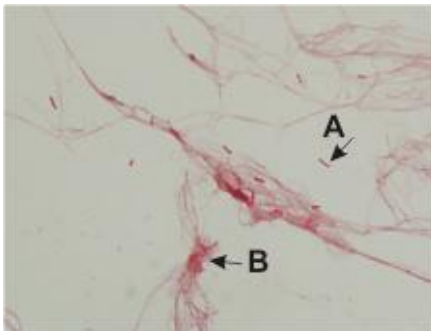

Figure 2: Gram-stain of *E. coli* 0157

Arrows showing: A. normal *E. coli* 0157 morphology and B. atypical morphology, after the addition of JCP3/9.

JCT3/ sub-library

After three rounds of affinity selection against *E. coli* 0157, eight phage clones were evaluated from the JCT3/ sub-library (JCT3/1 – JCT3/8) for antimicrobial testing. None of these phage peptides inhibited the growth of *E. coli* 0157, although JCT3/7 was hard to evaluate due to the difficulty of growing this phage in the *E. coli* ER2378 host bacteria (Table 3).

Table 3: Summary of phage displayed peptides isolated from Selection scheme 2B and their antimicrobial activities

Phage clones	Virion conc./µL	µM conc.[a]	% growth inhibition[b]
JCP3/1	3×10^{10}	0.330	37%
JCP3/2	1.38×10^{10}	0.152	28%
JCP3/3	2.31×10^{10}	0.254	35%
JCP3/4	1.15×10^{10}	0.127	24%
JCP3/5	9.69×10^{9}	0.107	34%
JCP3/6	9.23×10^{9}	0.102	22%
JCP3/7	3×10^{10}	0.330	40%
JCP3/8	3×10^{10}	0.330	40%
JCP3/9	1.98×10^{10}	0.218	58%
JCP3/10	1.85×10^{10}	0.203	40%
JCT3/1	2.24×10^{10}	0.246	18%
JCT3/2	1.25×10^{10}	0.137	Not active
JCT3/3	ND	ND	ND
JCT3/4	ND	ND	ND

Phage clones	Virion conc./µL	µM conc.[a]	% growth inhibition[b]
JCT3/5	1.13×10^{10}	0.124	Not active
JCT3/6	1.11×10^{10}	0.122	Not active
JCT3/7	6.92×10^{9}	0.0762	21%
JCT3/8	1.38×10^{10}	0.152	28%

[a] the µM concentration of the phage peptides, assuming that all five copies displayed on the pIII are binding to the *E. coli* cells
ND is no data
[b] based on changes in the optical absorbance of the bacteria

Phage Peptides from the Second Round of Selection from 2A

Because the individual phage peptides from the JCP3/ and JCT3/ sub-libraries showed antimicrobial activity towards *E. coli* 0157, we decided to also individually clone the phage peptides from the second round of selection. These phage peptides were called JCP2/ and JCT2/ and individually tested for antimicrobial activity (Figure 1D).

JCP2/ sub-library

After two rounds of affinity selection, nine phage clones were isolated from the JCP2/ sub-library (JCP2/1-JCP2/9) and evaluated for antimicrobial potential (Table 4). Five of the nine phage clones (JCP2/1, JCP2/4, JCP2/5, JCP2/7 and JCP2/9) demonstrated considerable antimicrobial activity towards *E. coli* 0157 as a phage displayed peptide. In addition, one phage clone (JCP2/2) could only be amplified to low levels.

JCT2/ sub-library

After two rounds of affinity selection, eight phage clones were randomly isolated from the JCT2/ sub-library (JCT2/1 – JCT2/8) and tested for antimicrobial activity. Three of the eight phage clones (JCT2/1, JCT2/5 and JCT2/7) demonstrated considerable antimicrobial activity towards *E. coli* 0157 (Table 4). Two of the phage clones (JCT2/2 and JCT2/3) were also very difficult to evaluate as a phage peptide due to the toxicity of the displayed peptide on the *E. coli* host bacteria used to amplify it. Nevertheless, even at a theoretical peptide concentration of 11 nM, JCT2/2 showed antimicrobial activity towards *E. coli* 0157 (Table 4).

The phage peptides had considerably changed the morphology of the *E. coli* 0157 (e.g. Figure 2 for JCP3/9), which would have made the OD measurements of growth inhibition inaccurate. The number of viable bacteria in 96-well plates was more accurately determined for JCP2/1, JCP2/9 and JCT2/5 by performing colony counts on Nutrient agar plates (Section 2.3). All three phage peptides tested in this way were found to completely inhibit the growth of E. coli 0157

For a few select phage clones, the number of viable bacteria in the 96-well plates was more accurately determined by performing colony counts on the diluted inoculum by plating onto Nutrient agar plates. The number of input bacteria was also enumerated in the same way. All three phage peptides tested in this way (JCP2/1, JCP2/9 and JCT2/5) were found to completely inhibit the growth of *E. coli* 0157 at nanomolar concentrations of peptide (Table 4).

Table 4: Summary of phage displayed peptides isolated from the JCP2/ and JCT2/ sub-libraries and their antimicrobial activities

Phage clones	Virion conc./µL	µM conc.[a]	% growth inhibition[b]
JCP2/1	1.39 x 10^{10}	0.153	99%[c]
JCP2/2	5.08 x 10^{9}	0.0558	15%
JCP2/3	1.03 x 10^{10}	0.113	14%
JCP2/4	1.11 x 10^{10}	0.122	46%
JCP2/5	1.95 x 10^{10}	0.215	57%
JCP2/6	1.52 x 10^{10}	0.167	12%
JCP2/7	2.21 x 10^{10}	0.243	59%
JCP2/8	ND	ND	ND
JCP2/9	1.32 x 10^{10}	0.145	**99%**[c]
JCT2/1	2.01 x 10^{10}	00.221	57%
JCT2/2	1.06 x 10^{9}	0.0117	Not active
JCT2/3	2.52 x 10^{9}	0.0277	22%
JCT2/4	ND	ND	ND
JCT2/5	1.78 x 10^{10}	0.195	99%[c]
JCT2/6	2.21 x 10^{10}	0.243	25%
JCT2/7	1.46 x 10^{10}	0.161	39%
JCT2/8	ND	ND	ND

[a] the µM concentration of the phage peptides, assuming that all five copies displayed on the pIII are binding to the *E. coli* cells.
[b] based on changes in the optical absorbance of the bacteria
[c] based on changes in the number of colony forming units

3.3 Binding of the Phage Displayed Peptides to E. coli 0157

A dot-blot assay was used to evaluate the binding of the phage displayed peptides to whole *E. coli* 0157 cells. In this assay, individual phage displayed peptides were added to the *E. coli* cells dot blotted onto nitrocellulose. Following incubation to allow specific binding between the bacteria and the phage, non-binding phage particles were washed away and bound phage detected colorimetrically using an anti-M13 antibody conjugated to horse-radish peroxidase (HRP). A positive reaction was depicted by an insoluble blue precipitate.

Overall, seven phage clones were tested for potential binding to *E. coli* 0157. An irrelevant phage peptide was included as a control. Our results indicated that five of the phage clones (EBP3/4, JCP3/1, JCP3/10, JCP2/3 and JCT2/5 bound to *E. coli* 0157. We could not detect any binding with the JCP2/6 and JCP3/7 phage displayed peptides. It is possible that these phage peptides bind but to a less accessible/dominant epitope. Some of the phage peptides that bound to *E. coli*

0157 were not antimicrobial (i.e. EBP3/4) and may target a different set of cellular receptors than those that were antimicrobial (e.g. JCT2/5).

4. Application of Research

Maintaining herd health in pig production systems is vital for animal welfare, with most antibiotics administered in the water or feed for prophylactic or therapeutic control of disease. This project has produced proof-of-concept in isolating phage peptides that have the potential to be used as pathogen-specific antimicrobials for the therapeutic control of E. coli 0157. Novel antimicrobials, based on phage displayed peptides, are not classified as antibiotics used for human medicine because they have unique modes-of-action against the targeted pathogen.

The commercialisable output for this project would be a final therapeutic compound controlling E. coli in the gastrointestinal tract of swine on-farm. This therapeutic would control the pathogenic strains of E. coli in the gastrointestinal tract without altering the gut microbiota. There is also the potential to develop a vaccine towards the E. coli strains by isolating the unique epitopes/receptors which the phage peptides are targeting on the cell surface.

5. Conclusion

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 being produced. This project has been successful in that 16 phage peptides were isolated that inhibited the growth of E. coli 0157. Some of these phage peptides completely inhibited the growth of this microorganism at a theoretical peptide concentration in the low nanomolar concentration.

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 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 wider 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 selection strategies (selection schemes 1 and 2), which were designed around progressive subtraction of the phage library against the cell surface epitopes of E. coli 0157. We hypothesized that each subtractive step would progressively remove the phage peptides binding to the predominant epitopes, whilst still preserving those binding to the lower density epitopes. This resulted in eight sub-libraries being generated (Section 3.2).

The success of this subtractive strategy was reflected in the high proportion of antimicrobial phage clones isolated.

6. Recommendations

This project has produced proof-of-concept for the feasibility of these peptides. We recommend the following work to be undertaken in order to validate the

peptides and develop them into commercial products to be used by the swine industry. Specifically, the following areas should be addressed.

1. Evaluate the specificity of the peptides by examining their antimicrobial activity against a panel of pathogenic E. coli isolates and other bacterial spp.
2. Assess the delivery methods for the peptides. As part of this we need to synthesise the peptides as either monomeric or tetrameric peptides and demonstrate that they retain antimicrobial activity outside of the constraints imposed by the phage pIII coat protein. It is likely that this would also enhance the antimicrobial activity of the peptides by allowing them to be tested at a sufficiently high concentration. In this project, the peptides were tested as a phage fusion, which made their evaluation difficult as antimicrobials because of the problems in amplifying the phage to a sufficiently high enough concentration due to the toxicity of the displayed peptide on the E. coli host cells. Despite this, the phage peptides showed antimicrobial activity towards E. coli 0157 at low peptide concentrations.
3. As a preliminary to animal trials, test potential antimicrobial activity of the peptides using the in-vitro fermentation assay using a mixed culture of gastrointestinal bacterial to ensure that the peptides retain antimicrobial activity prior to a costly animal trial.

7. References

Barbas, C.F.,III, D.R. Barton, J.K. Scott, and G.J. Silverman. 2001. Phage display: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor: NY.

Bishop-Hurley, S.L., S.A. Mounter, J. Laskey, R.O Morris, J. Elder, P. Roop, C. Rouse, F.J. Schmidt, and J.T. English. 2002. Phage-displayed peptides as developmental agonists for *Phytophthora capsici* zoospores. Appl. Environ. Microbiol. **68:**3315-3320.

Bishop-Hurley, S.L., F.J. Schmidt, A.L. Erwin, and A.L. Smith. 2005. Peptides selected for binding to a virulent strain of *Haemophilus influenzae* by phage-display are bactericidal. Antimicrob. Agents Chemother. **49:**2972-2978.

Bishop-Hurley, S.L., P.J. Rea, and C.S. McSweeney. 2010. Phage-displayed peptides as inhibitors of *Campylobacter jejuni*. Protein Engineering, Design and Selection 23:751-757.

Butler M.S, and M.A. Cooper. 2012. Screening strategies to identify new antibiotics. Curr. Drug Targets **13**(3):373–387.

Carnazza, S., C. Foti, G. Gioffre, F. Felici, and S. Guglielmino. 2008. Specific and selective probes for *Pseudomonas aeruginosa* from phage-displayed random peptide libraries. Biosens. Bioelectron. **23:**1137-1144.

Christensen, D.J., E.B. Gottlin, R.E. Benson, and P.T. Hamilton. 2001. Phage display for target-based antibacterial drug discovery. Drug Discov. Today **6:** 721-727.

Fang, Z.D., J.G. Laskey, S. Huang, K.D. Bilyeu, R.O. Morris, F.J. Schmidt, and J.T.

English. 2006. Combinatorially selected defense peptides protect plant roots from pathogen infection. Proc. Natl. Acad. Sci. USA **103:**18444-18449.

Fong, S., L.V. Doyle, J.J. Devlin, and M.V. Doyle. 1994. Scanning whole cells with phage-display libraries–identification of peptide ligands that modulate cell function. Drug Dev. Res. **33:**64-70.

Fonseca, A.P.; Extremina, C.; Fonseca, A.F.; Sousa, J.C. 2004. Effect of subinhibitory concentration of piperacillin/tazobactam on *Pseudomonas aerugionsa*. J. Med. Microbiol.53:903-910.

Ghosh, A. K., L.A. Moreira, and M. Jacobs-Lorena. 2002. Plasmodium-mosquito interactions, phage-display libraries and transgenic mosquitoes impaired for malaria transmission. Insect Biochem. Mol. Biol. **32:**1325-1331

Hoogenboom, H.R., J.T. Lutgerink, M.M. Pelsers, M. J. Rousch, J. Coote, N. Van Neer, A. De Bruine, F. A. Van Nieuwenhoven, J.F. Glatz, and J.W Arends. 1999. Selection-dominant and non-accessible epitopes on cell-surface receptors revealed by cell-panning with a large phage antibody library. Eur. J. Biochem. **260:**774-784.

Oyama, T., I.T. Rombel, K.N. Samli, X. Zhou, and K.C. Brown. 2006. Isolation of multiple cell-binding ligands from different phage-displayed peptide libraries. Biosens. Bioelectron. **21:**1867-1875.

Pini, A., A. Giuliani, C. Falciani, Y. Runci, C. Ricci, B. Lelli, M. Malossi, P. Neri, G. M. Rossolini, and L. Bracci. 2005. Antimicrobial activity of novel dendrimeric peptides obtained by phage-display selection and rational modification. Antimicrob Agents Chemother. **49:**2665-2672.

Smith, G.P. 1985. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science **228:**1315-1317.

Smith, G.P., and J.K. Scott. 1993. Libraries of peptides and proteins displayed on filamentous phage. Methods Enzymol. **217:**228-257.

Sorokulova, I.B., E.V. Olsen, I.H. Chen, B. Fiebor, J.M. Barbaree, V.J. Vodyanoy, B.A. Chin, and V.A. Petrenko. 2005. Landscape phage probes for *Salmonella typhimurium*. J. Microbiol. Methods **63:**55-72.

Yu, J., and G.P. Smith. 1996. Affinity maturation of phage-displayed peptide ligands. Methods Enzymol. **267:**3-27.