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ORIGINAL ARTICLE

Isolation and characterization of lytic bacteriophages against enterohaemorrhagic *Escherichia coli*

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Abstract

Aims: The objective of this study was to isolate, identify and characterize a collection of lytic bacteriophages capable of infecting enterohaemorrhagic *Escherichia coli* (EHEC) serotypes.

Methods and Results: Phages were isolated from dairy and cattle feedlot manure using *E. coli* O157, O26 and O111 strains as hosts. Phages were enriched from faecal slurries by culture in 10× trypticase soy broth at 37°C overnight. Phage plaques were obtained by mixing the filtered culture supernatant with molten tryptone agar containing the phage *E. coli* host strain, pouring the inoculated agar on top of cooled TS agar and incubating the culture overnight. Phages were purified from plaques and screened against additional *E. coli* and EHEC strains by the efficiency of plating method (EOP). Phage CEV2, and five other phages previously isolated, were able to lyse all of the 15 O157 strains tested with EOP values consistently above 0·001. Two phages were found to be highly effective against strains of *E. coli* O157 through EOP tests and against O26 strains through spot tests, but not against the O serogroup 111 strains. A cocktail of eight phage that lyse *E. coli* O157 strains resulted in >5 log CFU ml^{-1} reductions at 37°C. Multiplex-PCR revealed that none of these eight phages carried *stx1*, *stx2*, *hlyA* or *eaeA* genes.

Conclusions: A cocktail of bacteriophages was capable of lysing most strains of two EHEC serotypes.

Significance and Impact of the Study: This collection of phages can be combined and potentially used as an antimicrobial cocktail to inactivate *E. coli* strains from O serogroups 157 and 26 and reduce their incidence in the food chain.

Introduction

In recent years, enterohaemorrhagic *Escherichia coli* (EHEC) have caused multiple foodborne disease outbreaks worldwide related to the consumption of contaminated meat and fresh produce (Bell 2002). One of these serotypes, *E. coli* O157:H7 is a leading cause of produce-related infections, accounting for 20% of the outbreaks in which the etiological agent was identified (Olsen *et al.* 2000). Over the last 10 years, despite the best efforts of the food industry, we have seen an increase in *E. coli* O157:H7 outbreaks associated with the consumption of fresh vegetables, such as the spinach outbreak of 2006, that have stressed the importance of developing new more potent antimicrobial strategies (CDC 2006; Maki 2006). The fresh characteristic of produce limits the number of antimicrobial treatments that can be used.

Bacteriophages are viruses of bacteria which are highly specific in their host-cell recognition often only infecting specific members of a single genus and as such offer an alternative natural method to reduce bacterial pathogens within the food supply chain (Callaway *et al.* 2008). This high level of specificity leads to little disturbance of commensal bacteria through the oral consumption of

phages targeted at pathogens, and while bacteria develop specialized phage-defence mechanisms, phages also continuously adapt to these changed host systems (Hagens and Loessner 2007). Also, to address this issue, it is highly recommended to use cocktails of several phages to obtain sufficient breadth of host range and to reduce the probability of phage resistance (Brussow 2005). Because of their origin, phages are considered 'natural' and therefore acceptable in the production of organic foods.

Advantages of using phages over traditional antimicrobial systems for foods have been reviewed on the preharvest (Barrow and Soothill 1997; Joerger 2003) and postharvest level (Leverentz *et al.* 2001, 2003). Phages are highly specific and their use in agriculture is not likely to select for phage resistance in untargeted bacterial species. Furthermore, bacterial resistance mechanisms against phages and antibiotics differ; thus, the possible emergence of resistance against phages will not affect the susceptibility of bacteria to antibiotics used for humans. In addition, phage preparations can readily be modified in response to changes in bacterial pathogen populations or susceptibility, while antibiotics have a long and expensive development cycle (Sulakvelidze and Barrow 2005).

In 2006, the US Food and Drug Administration announced the approval of bacteriophages as antimicrobial food additives for control of *Listeria monocytogenes* in ready-to-eat meat and poultry products (FDA 2006). The use of specific phages to eliminate or reduce the levels of contaminating bacteria on various foods such as fresh-cut fruits and vegetables is actively being investigated to bring about a reduction in *E. coli* O157:H7. As part of an ongoing study, Sharma *et al.* (2009) tested the effectiveness of a mixture of bacteriophages in reducing *E. coli* O157:H7 gfp 86 on fresh produce. They found that the bacteriophage treatment resulted in significantly lower counts of the pathogen on both the lettuce and cantaloupe compared with the negative control.

A study by Niu *et al.* (2009a) evaluated the host range and lytic capability of four phages against Shiga toxin-producing *E. coli* (STEC) O157 from cattle and humans. They found that the phages were effective against the majority of the bovine and human *E. coli* O157 isolates and suggested that lytic capability and host range should be considered when selecting a therapeutic phage for on-farm control of *E. coli* O157. Furthermore, they advocated for the use of phage cocktails as an effective mitigating approach for *E. coli* O157 because of the observation that some *E. coli* O157 isolates exhibited resistance to some but not all phages.

This study is part of a larger research effort focused on the development and application of mixtures of bacteriophages against foodborne pathogens. The specific objective of this study was to develop a collection of bacteriophages capable of infecting different serotypes of EHEC, determine their spectrum of activity and characterize them. This could eventually lead to the creation of a mixture of bacteriophages that would potentially be used in food and food-processing environments.

Materials and methods

Phage isolation, purification and propagation

Phages CEV2 and CBA65 which were shown to lyse *E. coli* O157:H7 NCTC 12900 and ATCC 43895 were isolated from sheep and cattle faecal samples at The Evergreen State College, as per their standard protocol (Raya *et al.* 2006; Oot *et al.* 2007). Dr Lawrence Goodridge (Colorado State University) provided eight pure phages and 31 lysate mixtures from his collection. Additional phages were isolated from dairy and feedlot manure using EHEC O157 (*E. coli* O157:H7 NCTC 12900 and ATCC 43895), O26 (no. 41) and O111 (no. 10) and *E. coli* B strains as hosts as listed in Table 1. Stock cultures were stored in glycerol at −50°C and cultivated in tryptic soy broth (TSB) prior to use in experiments. Six batches of liquid sewage slurry, collected in August and September were used as the initial source for phage enrichment and isolation. Samples (10 ml) were mixed with 1 ml of 10× TSB (Neogen Corp., Lansing, MI, USA), containing 1 mmol l^{-1} $CaCl_2$ to assist in phage attachment (Ashelford *et al.* 2003) and 1 ml of exponentially growing cultures of specific bacterial hosts, and incubated overnight at 37°C. This initial phage enrichment culture was then centrifuged for 5 min at 14 500 ***g*** and filtered through a 0·45-μm filter before being plated with the specific host strain (0·2 ml) in tryptone top agar (TTA) on tryptic soy agar (TSA). Twenty-four hours later, individual phage plaques were plucked and resuspended, before being replated. Phages were considered purified after at least three passages of this process, and stored in phage storage buffer comprised of pH 7·2 phosphate buffer saline (PBS) with added gelatin (0·4% w/v). The phage suspension titres were determined through mixing serial dilutions with liquid cultures of the bacterial host strains in TTA. Phages were propagated by mixing 1 ml of each phage strain (approx. 10^8 PFU ml^{-1}), 10 ml TSB with 1 mmol l^{-1} $CaCl_2$, and 100 μl of the mid-exponential phase (approx. 10^7 CFU ml^{-1}) host bacterial strain and incubating overnight while shaking at 37°C in 50-ml centrifuge tubes. Equal amounts of $CHCl_3$ were added to the tubes, these were vortexed for 5 s, and centrifuged for 5 min at 14 500 ***g***. The supernatant was then filtered through a 0·45-μm pore-size filter and stored in 50-ml centrifuge tubes at 4°C until ready for use.

Table 1 Bacterial strains used in this study

Bacteria	Strain designation	Strain no.	Source
Escherichia coli O26	36, 37, 38, 39, 40, 41, 42, 43, 44, 45	10	FSML
E. coli O111	1, 2, 3, 4, 5, 6, 7, 8, 9, 10	10	FSML
	I 2005003658	1	MDH
E. coli	ATCC 43895, ATCC 43890, ATCC 35150	3	ATCC
O157:H7	A02, A03, A04, A05, A06, A07, A08, A09, A10, A11, A12, A13, A14, A15, A16, A17, A18, A19, A20, A21, A22, A24, A25, A26, A28, A29, A30, A31, A32, A33, A34, A35, A36, A37, A38, A39, A40, A41, A44, A47, A51, A52, A53, A54, A55, A57, A60, A61, A63, A64, A66, A68, A69, A70, A71, A72, A73, E10, E11, E12, E48, E56, EC261, EC263, EC265, (r)3W, (L)OD, (r)OD, (UC)7W, (M)3W, (c)OD, (M)OD	72	FSML
	M4882, M4489, M4522, 4477, 2027, 2336, 6058, 1144, 1145, 1146, 1147, 1148, 1149, 1150, 1151, 1152, 1153, 1154, 1155, 1156, 1157, 1158, 1159, 1160, 1161, 1162, 2026, 2027, 2028, 2029, 2030, 2031, 2079, 2206, 2255, 2257, 2266, 2309, 2317, 2321, 2324, 3081, MAC, SEA1 31388, 2727 (8727), 4489, 4719, NCTC 12900	49	ARS
	EK1 TWO8609, EK27 TWO8635, E32511 TWO2883, EHEC1, EHEC2, EHEC3, EHEC4, EHEC5, EHEC6, EHEC7, EHEC8	11	MSU
O55	EHEC9, EHEC10, EHEC11, EHEC12	4	MSU
E. coli (nonpathogenic)	KS(gr)2, KS(S)1, KS(S)2, F(S)1, PG(GR)3, PG(gr)2, PG(gr)1, CSH50, SD(L)2, DJ(L)1, KS(S)2, GR(L)1, GR(c)1, F(S)3, KS(L)2, PG(gr)4, K12, W3110, DH5α,	18	FSML
	ATCC 25922, C, B, CR63	3	CSU
Salmonella Typhimurium	I649, I503, E2009005811, I535, I536, UK1, I527, I740, I600, 3010907, I4028, I598, I534, I526, 758	16	FSML
	ATCC700408	1	ATCC
Salmonella Newport	B4442, AM07073, AMO7076, AMO5104, 2006036	5	CDC
Salmonella Enteritidis	2009595, I823, 95657613	3	
Salmonella Montevideo	95573473	1	MDH
Salmonella Saintpaul	E200	1	MDH
Salmonella Agona		1	FSML

FSML, Food Safety Microbiology Laboratory, University of Minnesota; MSU, Microbial Evolution Laboratory, Michigan State University; MDH, Minnesota Department of Health; CSU, Colorado State University; ARS, Agricultural Research Service's Southern Plains Agricultural Research Center; CDC, Centers for Disease Control and Prevention; EHEC, enterohaemorrhagic *E. coli*.
*The secondary strain designations for EK1, EK27 and E32511 are TWO8609, TWO8635 and TWO2883, respectively.

Initial screening/efficiency of plating

All phages were screened against EHEC strains (15 strains O157; 1 strain O26) using the efficiency of plating method (EOP = phage titre on target bacterium/phage titre on bacterium) to determine the effectiveness of each phage against a variety of target bacteria. Ten fold serial dilutions of phage suspensions (100 μl) were mixed with 200 μl of the target or host bacterium and incubated for 5 min at room temperature (24°C) before being mixed with 400 μl of molten TTA and plated as double layers on TSA (Viscardi *et al.* 2007). The *E. coli* strains used for screening included *E. coli* O157:H7 NCTC 12900, ATCC 43895, M4882, M4489, M4522, 4477, 2027, 2309, 2321, 2336, 6058, I 2005003658, EK1, EK27, E32511, *E. coli* O26 no. 41 and the nonpathogenic *E. coli* strains C, B and CR63.

Host range/spot tests

Phages with the highest EOPs were spot tested against 123 *E. coli* O157: H7 strains, 10 O26 strains, 10 O111 strains, 4 O55 strains, 19 *E. coli* nonpathogenic in humans, and 27 *Salmonella* strains (Table 1). Phage activity was examined by a spot test assay that entailed placing 20 μl of each phage (10^5 PFU ml^{-1}) on TSA seeded with each strain of *E. coli*. The plates were checked for clearance zones after incubating for 18–24 h at 37°C. The presence of a lytic zone was considered evidence of phage susceptibility; no lysis was considered evidence of phage resistance.

As a result of an initial screening of phage preparations provided by other researchers and recently isolated phages, as many as 70 lytic phages comprised the collection (Table 2). Isolated phages that resulted in cloudy

Table 2 List of phages used in this study, their source and host bacteria

Source	Phage designation	Number of phages	*Escherichia coli* host
Provided by Dr Goodridge	AR1, 38, 39, 41, 42, RB33, RB34, 56, 1, 2, 3, 4, 5, 6, 7, 8, 8a, 9, 10, 10a, 11, 11a, 12, 13, 15, 16, 17, 20, 21, 22, 23, 24, 25, 27, 50, 52, 53, 54, 57	39	B, C or CR63
Provided by Dr Brabban	CEV2, CBA65.	2	O157:H7 NCTC12900
Dairy cattle	ECA1, ECA2, ECC4	3	O157:H7 ATCC 43895
	ECC5, ECC6, ECC7, ECC8a, ECC8b, ECC9, ECC10, ECC11, ECC12, ECC13	10	O26 no. 41
Feedlot cattle	ECB3, ECB4, ECB5, ECB6, ECB7, ECB8, ECB9	7	O157:H7 NCTC 12900
	ECC1, ECC2, ECC3a, ECC3b, ECC14, ECC15	6	O26 no. 41
	ECD1, ECD2, ECD3	3	B, C, or CR63

plaques on the bacterial host were not subjected to further study as incomplete clearing is indicative of temperate phages. EOP values of 0·5–1 were ranked as 'high' efficiency; 0·2–0·5 as 'medium' efficiency; 0·001–0·2 as 'low' efficiency; 0·0 was considered as not effective against the target strain. Phages that were unable to lyse (EOP of 0·0) any of the 15 *E. coli* O157:H7 strains used were not subject to further study (data not shown).

Bacterial challenge tests

The phages with the highest EOP's and a mixture of eight of them designated as 'BEC8' were tested in liquid culture (approx. 10^6 CFU ml^{-1}) of four EHEC O157:H7 strains (EK1, EK27, I 2005003658-462, ATCC 43895). Tubes of TSB were inoculated with each O157 strain and incubated at 37°C overnight; a 100-μl aliquot was transferred in fresh TSB (9 ml) with 10 mmol l^{-1} $CaCl_2$ for 3 h to reach mid-exponential phase. Each tube was inoculated with different concentrations of a phage mixture or TSB to reach MOI of 1, 10 and 100. The tubes were placed at room temperature and 37°C for 5 h, followed by serial dilution in PBS and spread plating on TSA. Standard deviations were determined.

Molecular analysis

Purified phage suspensions (>10^6 PFU ml^{-1}) were heated at 95°C for 5 min, cooled to −20°C for 20 min, heated back up to 95°C for 5 min and stored at 4°C until ready for use. Each phage was examined by multiplex-PCR for the presence of the Shiga toxin 1 (*stx1*), Shiga toxin 2 (*stx2*), intimin (*eae*), and/or the enterohaemolysin (*hly*) gene(s) (Paton and Paton 1998). Samples were subjected to 35 PCR cycles, each consisting of 95°C (1 min); 65°C (2 min) for the first 10 cycles, decrementing to 60°C by cycle 15; 72°C (1·5 min), incrementing to 2·5 min from cycles 25–35. *Escherichia coli* ATCC 43895 and ATCC 43890 were used as positive controls, while *E. coli* ATCC 25922 and sterile water were used as negative and nontemplate controls. With this protocol, approx. 180-, 255-, 384- and 534-bp amplicons were produced for *stx1*, *stx2*, *eaeA*, and *hlyA*, respectively. PCR mixtures were electrophoresed on 2% agarose gels stained with ethidium bromide.

Phage suspensions containing at least 10^9 PFU ml^{-1} were mixed with molten agarose and pulsed-field gel electrophoresis (PFGE) plugs were prepared. Once set the plugs were incubated overnight at 55°C with 2 ml of lysis buffer containing proteinase K (20 mg ml^{-1}), before being washed with TE buffer as previously described (Raya *et al.* 2006).

Statistical analysis

Results reported originated from averaging at least two independent experiments in which duplicate samples with duplicate counts were tested at each time interval. Statistical comparison of counts as log CFU ml^{-1} of inactivation between the individual phages and their mixtures were carried out by ANOVA (PROC MIXED), and the least-squares method was used to determine significant differences ($P < 0·05$) (SAS Statistical Analysis Software, ver. 8.0; SAS Institute, Cary, NC, USA).

Results

Phage activity was detected in as many as six batches of faecal slurry samples, yet only isolates that were active against *E. coli* O157 strain ATCC 4395 and *E. coli* O26 strain no. 41 used in EOP testing were kept in the collection. Specifically, phages using *E. coli* O157:H7 ATCC 43895 and NCTC 12900 and O26 no. 41 as a host were isolated from feedlot cattle faecal slurry samples. No isolates were recovered when using *E. coli* O111 strain no. 1 as a host.

Among the 70 lytic phage tested through EOP, which compared the phage titre on the test strain to the phage titre on the strain used to isolate the phage, the eight

Table 3 Bacteriophage host range ranking as number of strains falling within each efficiency of plating (EOP) range of the total number of *Escherichia coli* strains used (n = 16) determined using the EOP method

	Phages							
EOP range	38	39	41	AR1	42	CEV2	ECB7	ECA1
0·5–1	6	8	11	13	11	6	10	9
0·2–0·5	5	1	4	3	3	8	1	7
0·001–0·2	5	7	1	0	2	2	4	0
0·0	0	0	0	0	0	0	1	0

most effective phages were phages 38, 39, 41, AR1, 42, CEV2, ECB7 and ECA1 (Table 3). Seven of eight phages were found to be 'highly' effective against more than 50% of the 16 EHEC strains tested. CEV2 had 'high' and 'medium' effectiveness against 40 and 50% of the strains, respectively. 'Medium' effectiveness among the phages ranged from lysis of 6·2–50% of strains, while 'low' effectiveness ranged from lysis of 0–43·8% of strains. ECB7 was the only phage strain among the eight that was not active against *E. coli* O26, but was highly effective against the *E. coli* O157:H7 strains.

Phages 38, 39, 41, AR1, 42, CEV2, ECB7 and ECA1 lysed from 94 to 98% of the *E. coli* O157:H7 (n = 123) strains screened using spot testing, but when tested against O26 strains (n = 10) phages 38, 39 and AR1 inhibited only 70% of the strains. None of the phages were active against the O111 strains (n = 10). The individual phages were poorly lytic against *E. coli* strains nonpathogenic in humans (n = 19) with their effectiveness ranging from 5 to 32% of the strains tested. Spot testing against 27 strains of different serovars of *Salmonella* was used to determine the cross-species infectivity of each phage. Phage lysis of *Salmonella* ranged from 0 to 47% of the 27 strains tested. There were no O157 strains that were resistant to all eight phages.

To investigate the ability of each phage and a mixture of all of them to lyse *E. coli* O157:H7 *in vitro*, challenge tests were performed that included the addition of phage (10^8 PFU ml^{-1}) at MOI of 100 to mid-exponential-phase cultures. The *in vitro E. coli* O157:H7 challenge tests were performed at room temperature and 37°C. Significant decreases in the viability of bacterial strains were observed at both temperatures (Figs 1 and 2); however, the lytic ability was significantly reduced at room temperature. Titres (approx. 10^6 CFU ml^{-1}) of EHEC O157 strains ATCC 43895, EK1, EK27 and 472 were all reduced by each individual phage as well as the phage cocktail 'BEC8'. In general, BEC8 performed nearly as well as or better than the most effective single phage against all four EHEC O157. At 37°C, the eight phage mixture reduced *E. coli* ATCC 43895 and 472 by 5·53 and 5·28 log CFU ml^{-1}, respectively, performing as well as or better than the individual phages. However, BEC8 reduced the population of strain EK1 by 5·06 log CFU ml^{-1} at 37°C, significantly lower than phage 39 that reduced the strain by 5·75 log CFU ml^{-1}, respectively. Similarly, BEC8 reduced strain EK27 by 1 log CFU ml^{-1} less, than phage CEV2 at 37°C. At room temperature, BEC8 was able to perform as well as or better than the individual phages, reducing strains EK1, EK27 and 472 by 3·3, 3·1 and 2·6 log CFU ml^{-1}. However, ECB7 reduced counts of ATTC 43895 by a small but significantly greater amount than BEC8 at room temperature.

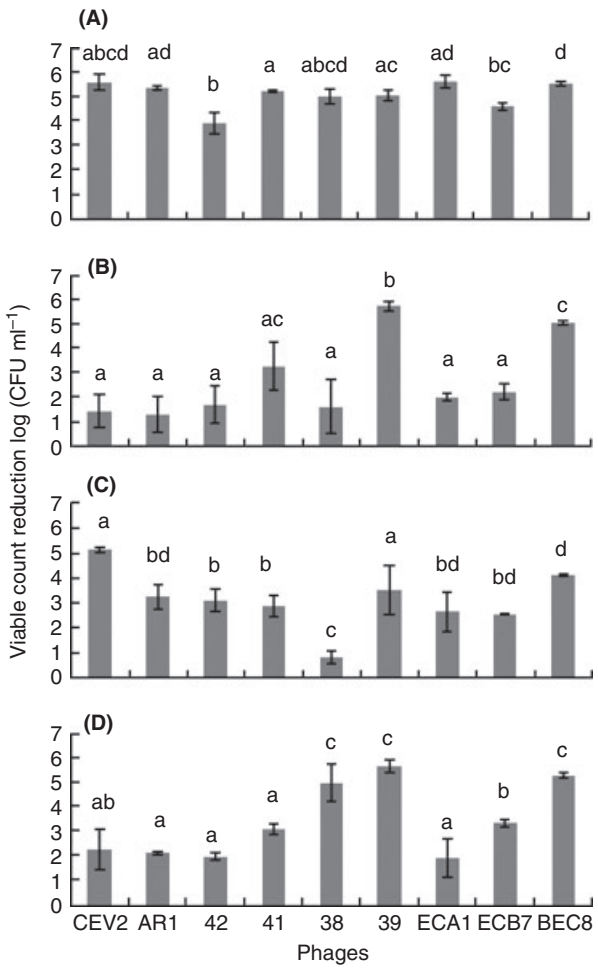

Figure 1 High titre inactivation of *Escherichia coli* O157:H7 strains (A) ATCC 43895, (B) EK1, (C) EK27, and (D) I2005003658 (472) by phages CEV2, AR1, 42, 41, 38, 39, ECA1, ECB7, and the phage mixture BEC8 after 5 h of incubation at 37°C. Bars with different lower-case letter (a through d) indicate statistically significant values. Values show the mean and standard deviation of duplicate measurements from at least two independent experiments.

All phages were subjected to PCR for detection of EHEC virulence factors *stx1*, *stx2*, *eaeA* and *hlyA*. None of

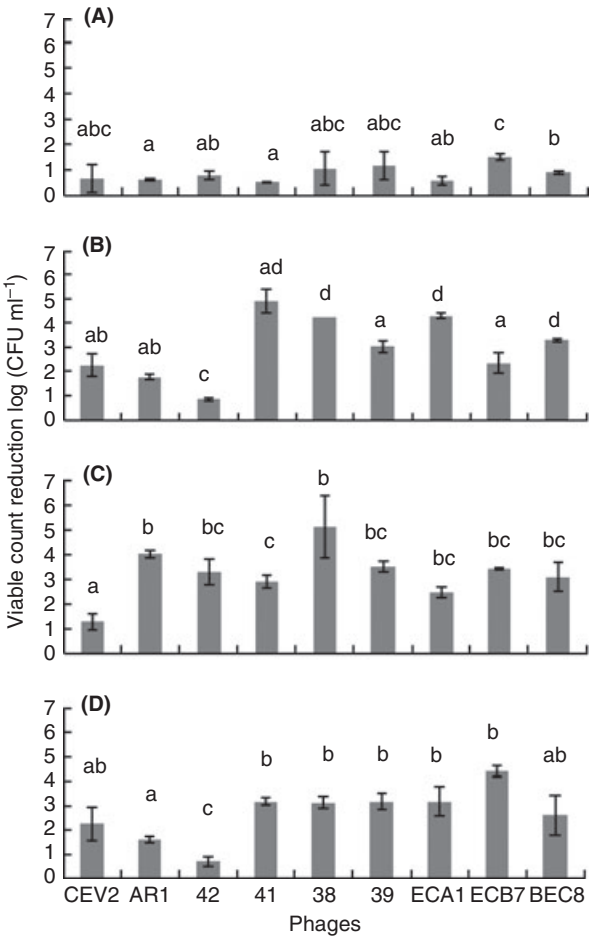

Figure 2 High titre inactivation of *Escherichia coli* O157:H7 strains (A) ATCC 43895, (B) EK1, (C) EK27, and (D) I2005003658 (472) by phages CEV2, AR1, 42, 41, 38, 39, ECA1, ECB7, and the phage mixture BEC8 after 5 h of incubation at room temperature. Bars with different lower-case letter (a through d) indicate statistically significant values. Values show the mean and standard deviation of duplicate measurements from at least two independent experiments.

these virulence factors were detected in any of the BEC8 phages. PFGE analysis revealed the genomic size of the phages was approx. 120 kb for CEV2, 38, ECA1 and ECB7, while the rest of the phages were all approx. 180 kb.

Discussion

Isolation of phages has been reported to be easily accomplished from environmental and faecal samples with enrichment media containing exponentially growing host bacteria (Ronner and Cliver 1990; Morita *et al.* 2002). Because the natural niche of EHEC are ruminants, in one of the first steps of the study phages from faecal slurry samples of dairy and feedlot cattle were isolated (Table 2). The purpose of this study was not to determine the prevalence of EHEC-specific phages from faecal samples. Instead, the study focused on isolating EHEC-specific phages to ultimately create an effective phage cocktail to use to control these pathogens in foods.

The isolation of a large number of phages specific for pathogenic and nonpathogenic *E. coli* provided a starting point from which an antimicrobial cocktail could be developed. This study sought to investigate the lytic capability and host range of phages specific to *E. coli* O157 and non-O157 strains isolated from various sources. The ease of isolating phages from faecal slurries from cattle is in accordance with a recent study by Niu *et al.* (2009b), in which they found that the prevalence of phages was highest in slurry compared with pooled faecal pats, water with sediment from troughs, and rectal faecal samples. The same study reported that the prevalence of phages fluctuated in a fashion similar to that described for *E. coli* O157:H7. This pattern of occurrence may have contributed to the success of isolating phages in our study, as the faecal slurry samples were obtained during late summer when the prevalence of *E. coli* O157 is highest in cattle (Barkocy-Gallagher *et al.* 2003).

In studies addressing the distribution of lytic *E. coli* O157 phages in samples taken from different pens in a cattle feedlot, no phages were obtained without enrichment, but using enrichment cultures resulted in isolating phages in 65 and 97% of samples specific against *E. coli* O157 and *E. coli* strain B, respectively (Oot *et al.* 2007). In a previous study, different phages were isolated from the same stool samples using different indicator bacterial host cells (Chibani-Chennoufi *et al.* 2004b). Similarly, in our study, enrichment faecal slurries with 10× TSB resulted in many phages being isolated from a small number (six) of slurry samples from dairy and feedlot cattle.

The EOP method was used against a collection of *E. coli* O157:H7 strains to determine which phage were most effective in killing. Ideally, every viral particle attaching to a host cell can enter and result in a plaque on the appropriate bacterial strains under optimum conditions. However, there are a number of factors that affect plating efficiency, such as the specific host strain, thus highlighting the importance of investigating the relative EOP on a variety of susceptible hosts (Kutter 2009). The EOP method was used in our study as a tool to distinguish between efficient and inefficient phages against a collection of *E. coli* O157:H7 strains. This led to the screening of a large number of phage and ultimately to select the phage isolates with EOP values >0·5 and specific for *E. coli* O157:H7 and O26.

The spot testing conducted in this study was used to determine the host range of the eight most effective

Table 4 Determination of host range by using spot testing of bacteriophages against target bacteria

	% Positive							
Target bacterium	38	39	41	AR1	42	CEV2	ECB7	ECA1
O157:H7 (*n* = 130)	96·2	93·1	97·7	93·1	99·2	96·0	93·1	93·1
O26 (*n* = 10)	70·0	70·0	90·0	70·0	80·0	80·0	90·0	100·0
O111 (*n* = 10)	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Commensal *Escherichia coli* (*n* = 19)	26·1	31·6	15·8	15·8	31·6	21·1	21·1	5·3
E. coli O55 (*n* = 4)	25·0	0·0	0·0	75·0	25·0	50·0	50·0	25·0
Salmonella (*n* = 27)	22·2	11·1	7·4	7·4	18·5	0·0	48·2	7·4

phages in the collection (Table 4). All phages were highly effective against strains of EHEC O157 and O26 isolated from sources that historically have posed a threat to the food supply (Pearson 2007). Previous studies have also shown that phage with a broad host range are able to lyse EHEC (Viscardi *et al.* 2007; Niu *et al.* 2009b) and other pathogenic bacteria (Bielke *et al.* 2007; Synnott *et al.* 2009). When the lytic properties of the phages against nonpathogenic *E. coli* were investigated, the phages' lytic ability ranged from 5·3 to 31·6% of the strain collection. Viscardi *et al.* (2008) tested two phages against the ECOR collection of nonpathogenic *E. coli* and found that they lysed 13·9 and 20·8% of the strains. Even though the collection of nonpathogenic *E. coli* strains used in this study was different, a low percentage of these were also lysed by our phage isolates. The spot test allowed us to determine the range of each phage and ultimately create a mixture of phages that would be effective against all O157 strains.

Of particular interest is the cross-genus activity of the phages with the *Salmonella* strains used in the spot tests. Phage ECB7 lysed 48·2% of the *Salmonella* strains, while the rest of the phage lysed from none to 22·2% of the strains of this pathogen. Villegas *et al.* (2009) found that the EHEC O157:H7-specific bacteriophage wV8 had genome characteristics very similar to the *Salmonella*-specific phage Felix O1, yet could not lyse any strains from 12 *Salmonella* serovars through spot testing. In a previous report of an *E. coli* O157:H7-specific coliphage, Ronner and Cliver (1990) described a phage that lysed Shiga toxin-producing bacteria including *Shigella dysenteriae*.

The EOP and host range experimental results emphasize the importance of using these techniques when selecting phages for therapeutic or antimicrobial purposes (Viscardi *et al.* 2008). Comparison of the bacterial cocktail against the performance of each individual phage in the mixture was also important. This assay determined whether there was a competition between the phage types rather than a cooperative or synergistic effect. Cocktails of phages may be necessary to obtain sufficient breadth of host range and to significantly reduce the probability of developing resistance (Brussow *et al.* 2004). Host species specificity of phages may be an asset for phage applications, but can also be a liability when a broad coverage of several different subtypes of the pathogen is desired (Denou *et al.* 2009). O'Flynn *et al.* used a three-phage cocktail against 10^3 CFU g^{-1} *E. coli* O157:H7 on beef (O'Flynn *et al.* 2004). In seven of nine samples, no viable cells could be retrieved after storage at 37°C, while in the remaining two samples, counts were below 10 CFU g^{-1}. The *in vitro* challenge test in our study demonstrated that the phage mixture could be used to inactivate strains of pathogenic *E. coli* and has the potential to be used in food applications.

Many *E. coli* O157:H7 strains were used in this study, but some experiments only used single strains while others used more strains. The nature of the experiments required a different number of strains to be used and different types of information were obtained from them. Spot testing and EOP tests used a multitude of single strains to determine the range and efficiency of the phages, respectively, and provided an insight into how to sort through the many different phages that were isolated. The strains that were tested in this study were not intended to be 100% representative of the *E. coli* O157:H7 population, and we do not claim to have tested all clades of the pathogen. The selection of the *E. coli* O157:H7 strains was made based on availability as well as relevancy of their source, i.e. dairy cattle-, fresh produce-, and outbreak-related strains. Similarly, the *Salmonella* strains used are not wholly representative of the entire population, but instead were selected based on availability.

A study by O'Flynn reported that one of the phages isolated that was a member of the *Myoviridae* family had recently acquired the *eaeA* virulence factor from the bacterial host (O'Flynn *et al.* 2004). The lack of four of the important EHEC virulence factors in the phages suggests that they may be safe for use in foods. They do not carry and therefore will not be likely to transfer these genes to other bacteria. However, the possible presence of other virulence factors and phage-encoded genes that may

contribute to virulence was not evaluated. It is important to note that sequencing and bioinformatic analysis of the phage genomes in the future could help in providing a comprehensive assessment of their safety.

Many of the virulence factors of human pathogens can be attributed to the presence of prophages integrated into their genomes (Wagner and Waldor 2002; Brussow *et al.* 2004). Prophages encode Shiga-like toxins, the most important virulence factor of *E. coli* O157:H7. However, T4 phages are considered obligate lytic phages and do not seem to exhibit the same sort of reshuffling of groups of functionally related genes as has been described for lambdoid coliphages (Chibani-Chennoufi *et al.* 2004a). Recent genome analysis and 40 years of genetic research with T4 have shown its chromosome contains no bacterial virulence genes (Miller *et al.* 2003). Early in its infection cycle, T4 rapidly degrades the host genome to the nucleotide level, thus preventing integration of phage DNA into its chromosome (Miller *et al.* 2003). Our study confirmed that there were no toxin genes in these T4-like phage (Brussow 2005). T4 phage appear to differ from lambda-like phages, which have been demonstrated to carry certain virulence genes in T4-like phage (Boyd and Brüssow 2002; Brussow *et al.* 2004).

Conclusion

These recently identified and characterized bacteriophages were highly effective against multiple strains of two EHEC serotypes. None of the phages contained selected EHEC virulence factors as shown through PCR. The combinations of all the phages in a single mixture lead to significant reductions of high numbers of EHEC at 37°C and room temperature, thus suggesting that a phage mixture is feasible and effective against this pathogen. This collection of phages can be grouped and potentially used as an antimicrobial cocktail to inactivate strains of O157 and O26 serotypes and reduce their incidence in the food chain. It has long been recognized that nontransducing, lytic phages are less likely to transfer virulence attributes and may pose a lower risk (Hanlon 2007), and at the same time can be used as a valuable tool for ensuring food safety and public health. However, sequencing and bioinformatic analysis of the phage genomes will be a logical next step in their characterization to assess their safety before they would be put to use in controlling these pathogens in foods (Viscardi *et al.* 2007).

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