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Biocontrol of *Escherichia coli* O157 with O157-Specific Bacteriophages

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***Escherichia coli* O157 antigen-specific bacteriophages were isolated and tested to determine their ability to lyse laboratory cultures of *Escherichia coli* O157:H7. A total of 53 bovine or ovine fecal samples were enriched for phage, and 5 of these samples were found to contain lytic phages that grow on *E. coli* O157:H7. Three bacteriophages, designated KH1, KH4, and KH5, were evaluated. At 37 or 4°C, a mixture of these three O157-specific phages lysed all of the *E. coli* O157 cultures tested and none of the non-O157 *E. coli* or non-*E. coli* cultures tested. These results required culture aeration and a high multiplicity of infection. Without aeration, complete lysis of the bacterial cells occurred only after 5 days of incubation and only at 4°C. Phage infection and plaque formation were influenced by the nature of the host cell O157 lipopolysaccharide (LPS). Strains that did not express the O157 antigen or expressed a truncated LPS were not susceptible to plaque formation or lysis by phage. In addition, strains that expressed abundant mid-range-molecular-weight LPS did not support plaque formation but were lysed in liquid culture. Virulent O157 antigen-specific phages could play a role in biocontrol of *E. coli* O157:H7 in animals and fresh foods without compromising the viability of other normal flora or food quality.**

Serotype O157:H7 Shiga toxin-producing *Escherichia coli* is commonly associated with hemorrhagic colitis and its secondary systemic sequelae (15, 32). Few therapeutic alternatives and poor prognoses for severe sequelae have led to intensive research targeting elimination of this human pathogen from its sources (15, 32). Cattle and sheep transiently harbor *E. coli* O157:H7, and many disease outbreaks have been linked to contaminated bovine food products (16, 20, 30). In addition, deer, horses, dogs, and birds can also transiently harbor these bacteria (16, 19, 26, 37). Feces from these animals or from humans may contaminate food or water and pose a risk for human infection (15, 32, 33).

Elimination of *E. coli* O157:H7 at the preharvest stage could play a significant role in preventing the introduction of this human pathogen into the food chain (16, 36). Diet and probiotic therapy have been evaluated as preharvest management strategies that may reduce the risk of culture-positive animals (16, 22, 40). Other more direct alternatives include vaccination or the use of colicins (16, 24). Recolonization of previously infected animals with the same or different strains of *E. coli* O157:H7 has been demonstrated and may limit these approaches (13, 22). In addition, serotype specificity is not a characteristic of colicin activity (24). Elimination of *E. coli* O157:H7 during food processing is effective, and pasteurization is a common technique that can assure the safety of dairy products and apple cider (7, 8, 18, 33). In addition, agents that can effectively eliminate *E. coli* O157:H7 from water and vegetables without compromising the quality of these materials are being evaluated (33).

Bactericidal bacteriophages (phages) may provide a natural, nontoxic, feasible approach for controlling several human pathogens (2). Phages are parts of both gastrointestinal and environmental ecosystems (34). In fact, early in this century, phages were used to treat bacterial infections before the advent of chemical antibiotics (2). Early studies suggested that phage therapy may be effective against a broad range of human infections caused by members of the genera *Staphylococcus*, *Salmonella*, *Klebsiella*, *Escherichia*, *Proteus*, and *Pseudomonas* (2, 4). In addition, in experimental animal studies workers analyzed conditions that influence the in vivo activities of various phages (2, 4). The lack of techniques to counter phage sequestration, resistance, and conversion eventually led to replacement of phage therapy with antibiotic treatment (2, 4). However, with our present knowledge of phage and bacterial genetics, it may be possible to circumvent the problems encountered in previous attempts to use phages as natural antimicrobial agents (2, 4).

In this study, we isolated phages and evaluated the ability of these phages to lyse laboratory cultures of *E. coli* O157. O-antigen-specific virulent phages may provide an economical tool for controlling *E. coli* O157:H7 in environmental settings without compromising food quality, health, or the viability of other normal flora. Only one other report has described the isolation and characterization of a phage capable of lysing *E. coli* O157:H7, and this coliphage (AR1) is toxin specific and lyses other Shiga toxin-producing *E. coli* and *Shigella dysenteriae* (27). Therefore, we attempted to isolate O157-specific phages and assess the ability of these phages to cause bacterial death.

MATERIALS AND METHODS

Bacterial strains. *E. coli* O157:H7 strain ATCC 43894 (stx_1^+/stx_2^+) (American Type Culture Collection, Manassas, Va.) was used as a representative *E. coli* O157:H7 strain for phage isolation, propagation, and evaluation. *E. coli* O111:NM and O5:NM were used along with ATCC 43894 to isolate phages that require the O157 antigen for adsorption. Fifty-seven bacterial strains (see Table 1) were tested for phage sensitivity. These strains included a variety of *E. coli* strains that have O157 and non-O157 serotypes and express a variety of flagellar

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H antigens and bacteria that belong to different genera and have O antigens similar to O157. In addition, nalidixic acid-resistant (Nal^r) *E. coli* O157:H7 strain 86-24 Nal^r and O-antigen-deficient derivatives of this strain were tested (see Table 2).

Phage isolation. To isolate *E. coli* O157:H7-specific phages, 53 bovine or ovine fecal samples were collected from four separate cattle and sheep farms in Cottonwood and Moscow, Idaho, and Pullman, Wash. One-gram portions of each sample were inoculated into 5-ml exponential cultures of *E. coli* ATCC 43894 (2×10^8 CFU/ml), and the cultures were grown in Luria-Bertani (LB) medium supplemented with 5 mM $MgSO_4$ (LBM) overnight at 37°C. After overnight growth, 0.5 ml of chloroform was added to each culture, and the cultures were vortexed, centrifuged at 15,000 × *g* for 10 min, and filtered (pore size, 0.45 μm) to remove cellular debris and fecal material. Single plaques were isolated from aliquots of the filtrates that were plated on lawns of *E. coli* ATCC 43894. Based on a preliminary screening for O157 specificity, three phages were selected for further analysis and designated KH1, KH4, and KH5. A derivative of KH1 has been deposited in the American Type Culture Collection under accession no. ATCC 55952.

Preparation of phage stocks. *E. coli* ATCC 43894 in lambda, R, or LB medium, each with and without 5 mM $MgSO_4$, was evaluated for propagation of O157-specific phages (28). Distinct plaques and high-titer stocks were obtained only with LBM. Sterile salt-magnesium (SM) buffer was used to prepare all phage dilutions and suspensions.

Low-titer phage stocks were prepared by using the soft agar overlay technique (28). Each phage isolate was diluted and plated onto LBM along with 10^7 CFU of *E. coli* O157:H7 suspended in a soft agar (0.75% LBM agar) overlay. After overnight incubation at 37°C, single plaques were suspended in SM buffer, incubated at 37°C for 1 h, and serially diluted. Phage suspensions were mixed with 10^7 CFU of *E. coli* O157:H7, adsorbed at 37°C for 15 min, and plated. After overnight incubation at 37°C, SM buffer was added to plates exhibiting semiconfluent lysis, and the top agar was harvested. The soft agar slurry was centrifuged twice at 12,000 × *g* for 20 min to collect the phage-rich supernatant (lysate), which was then treated with 0.05 volume of chloroform. High-titer phage stocks were prepared from the lysates by liquid infection (28). For each phage, 1 ml of low-titer lysate was mixed with 10^7 CFU of *E. coli* O157:H7, adsorbed, added to 200 ml of LBM broth, and incubated overnight at 37°C with aeration. After additional incubation at 25°C without aeration for 24 h, the cultures were each treated with 0.05 volume of chloroform. Lysates were harvested by centrifugation (12,000 × *g*, 20 min) twice and by filtration with 0.2-μm-pore-size filters. Phage stocks were stored in 0.05 volume of chloroform at 4°C. Cultures prepared to determine whether there were bacteria in phage stocks were consistently negative.

Phage infection. To determine the optimal multiplicity of infection (MOI) for phage infection, *E. coli* O157:H7 strain ATCC 43894 was mixed with KH1 at ratios ranging from 10^{-3} to 10^3 PFU/CFU. All assays were done in duplicate. KH1 was selected as the test phage in these experiments because it formed clear, medium-sized plaques on the ATCC 43894 host cells. Bacteria were grown in LBM broth at 37°C without agitation to an absorbance at 600 nm of 1.0 to 1.3 ($\sim 10^8$ CFU/ml). Suspensions were adsorbed and incubated at 37 or 4°C with or without aeration, and the viable cell counts were determined in triplicate by serial dilution and spread plate culturing on LBM agar. Phage-free cultures (containing only bacteria) and cell-free cultures (containing only phage) were used as controls in all experiments to demonstrate the absence of contamination. The deduced optimal MOI (10^3 PFU/CFU) was used in subsequent tests performed with phages KH4 and KH5 and phage mixtures and with ATCC 43894 and all of the other bacteria tested.

E. coli O157:H7 isolates recovered after phage treatment were analyzed for phage resistance by spotting the surface of a bacterial lawn with 10 μl of phage stock. Plaque formation and the absence of plaque formation were taken to indicate susceptibility and resistance to phage, respectively.

Determination of bacterial and phage concentrations. Survival of *E. coli* O157 in phage-infected cultures was determined by plating serial dilutions of cultures onto LB agar. At various times after phage infection, the surviving *E. coli* O157:H7 cells were enumerated and compared to the number of bacteria at the start of the experiment and the number of bacteria in a phage-free *E. coli* O157:H7 control culture. After overnight incubation at 37°C, colonies were confirmed to have the O157 serotype serologically (ProLab Diagnostics, Richmond Hill, Ontario, Canada). Cultures that produced no colonies were analyzed further by transferring 1 ml of the bacterium-phage mixture into 5 ml of an enrichment medium and incubating the preparation with aeration at 37°C; colonies were confirmed to have the O157 serotype serologically, as previously described (21).

Phages were enumerated by using the soft agar overlay technique described above. One milliliter of each culture was treated with 0.05 volume of chloroform and centrifuged. The supernatants were serially diluted with sterile SM buffer, mixed with 10^7 CFU of *E. coli* O157:H7, and overlaid on LBM plates. After overnight incubation at 37°C, the plates were examined for plaques.

Preparation of LPS. Overnight cultures of bacteria were grown in LBM at 37°C without aeration. The viable cell count was determined for each culture as described above. Lipopolysaccharide (LPS) was extracted by using the protocol described by Inzana (17). Bacterial cells were pelleted and washed in phosphate-buffered saline (PBS) containing 0.15 mM $CaCl_2$ and 0.5 M $MgCl_2$ prior to hot phenol extraction of LPS. The final LPS pellet obtained after ethanol precipitation was resuspended in 50 μl of sterile distilled water (17).

Immunoblot analysis of LPS. The LPS of *E. coli* O157 strains that were resistant and susceptible to plaque formation by the three phages were compared by performing an immunoblot analysis. The following strains were analyzed: CDC 3004-89, CDC G5933, FDA 13A80, 7A, 7C, 7D, and 7E, which were resistant to plaque formation, and ATCC 43894, ATCC 43888, ATCC 43889, FDA 13A81, and 86-24 Nal^r, which were susceptible to plaque formation (Tables 1 and 2).

LPS were separated on sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis gels and stained with silver (35) (results not shown) or transferred electrophoretically to Immobilon polyvinylidene difluoride membranes (Millipore Corp., Bedford, Mass.). Following this transfer, the membranes were incubated overnight at 4°C in blocking solution containing 5% (wt/vol) nonfat dry milk, 0.05% (vol/vol) Tween 20 (Sigma), and 0.02% (wt/vol) sodium azide (Sigma). The membranes were then placed in PBS containing 0.05% (vol/vol) Tween 20 and 1:100 (vol/vol) rabbit anti-O157 antibody (Difco Laboratories, Detroit, Mich.) for 1 h at room temperature on a rocking platform. After the membranes were washed three times in PBS containing 0.05% (vol/vol) Tween 20 at room temperature with rocking, they were incubated in PBS containing 0.05% (vol/vol) Tween 20 and goat anti-rabbit immunoglobulin G peroxidase (1:12,000 dilution; Boehringer-Mannheim, Indianapolis, Ind.). Immunoreactive LPS were visualized with the SuperSignal chemiluminescent Western blot substrate (Pierce, Rockford, Ill.) used according to the manufacturer's instructions, and the membranes were exposed to X-ray film, which was then developed.

RESULTS

Isolation of phages that form plaques on an *E. coli* O157 host. Fifty-three independent bovine and ovine fecal samples were enriched for phage, and five samples contained lytic phages that produced plaques on lawns of *E. coli* O157:H7 strain ATCC 43894. Three phages (KH1, KH4, and KH5) produced plaques on this strain but not on Shiga toxin-producing *E. coli* strains O111:NM and O5:NM. KH1 formed medium-size (0.8-mm-diameter) clear plaques on *E. coli* O157:H7 strain ATCC 43894, whereas KH4 and KH5 formed smaller (0.4- to 0.5-mm-diameter) clear plaques. High-titer stocks prepared for each phage contained phage particles at concentrations between 10^9 and 10^{10} PFU/ml.

KH1, KH4, and KH5 coliphages are O157 antigen specific. Fifty-seven *E. coli* isolates of human or animal origin were tested to determine the O-antigen specificity of the phages (Table 1). KH1, KH4, and KH5 lysed all *E. coli* isolates with the O157 serotype and did not lyse any of the non-O157 strains tested (Table 1). Likewise, they did not form plaques on any non-O157 strain tested; however, 7 of the 57 *E. coli* O157 strains tested did not support plaque formation (Table 1).

To examine the possibility that the length of the O157 LPS influences plaque formation, we tested the phage susceptibility of an O157 antigen-deficient mutant of *E. coli* O157:H7 strain 86-24 Nal^r, designated F12, and derivatives of this mutant (Table 2). The F12 mutant maintained a transposon insertion in $rfbE_{ECO157:H7}$ that codes for an enzyme required for synthesis of perosamine, a subunit of the O157 LPS (9). Although strain F12(pF12) makes the O157 antigen, as demonstrated serologically, it expresses a shorter O157 LPS chain than the parent strain expresses (9). KH1, KH4, and KH5 did not lyse F12 or its derivatives, F12(pSK^+) (F12 transformed with the Bluescript SK^+ plasmid) and F12(pF12) (F12 transformed with pSK^+ containing the cloned $rfbE_{ECO157:H7}$ gene) (Table 2). The three phages lysed only *E. coli* O157:H7 parent strain 86-24 Nal^r, which produced the full-length O157 antigen.

LPS from *E. coli* O157 strains that were resistant and susceptible to plaque formation were different. The immunoreactive LPS found in plaque-sensitive *E. coli* strains 86-24 Nal^r, ATCC 43888, ATCC 43889, ATCC 43894, and FDA 13A81 were high-molecular-weight (> ~40-kDa) and low-molecular-weight (~24- to 26-kDa) O157-antigenic LPS, whereas plaque-resistant strains CDC G5933, CDC 3004-89, FDA 13A80, 7A, 7C, 7D, and 7E produced high-molecular-weight, low-molec-

TABLE 1. Evaluation of phage specificity with various bacteria

Bacterial isolate(s)	Serotype	Reference(s)	Plaque formation			Lytic efficiency of phage mixture at[a]:		
			KH1	KH4	KH5	37°C		4°C[b]
						Aerated	Nonaerated	
E. coli isolates								
ATCC 43894	O157:H7	12, 39	+	+	+	+	−	+
ATCC 43890	O157:H7	12	+	+	+	+	−	+
ATCC 43889	O157:H7	12, 23	+	+	+	+	−	+
ATCC 43888	O157:H7	12	+	+	+	+	−	+
A-9	O157:H7	20	+	+	+	+	−	+
G-13	O157:H7	20	+	+	+	+	−	+
I-1	O157:H7	20	+	+	+	+	−	+
FDA 13A81	O157:H16	9	+	+	+	+	−	+
FDA 13A82	O157:H16	9	+	+	+	+	−	+
CDC 3005-89	O157:H38	9	+	+	+	+	−	+
FDA 13A83	O157:H45	9	+	+	+	+	−	+
CDC 3004-89	O157:H3	9	−	−	−	+	−	+
CDC G5933	O157:H12	9	−	−	−	+	−	+
FDA 13A80	O157:H16	9	−	−	−	+	−	+
7A, 7C, 7D, 7E	O157:H43	9	−	−	−	+	−	+
5A, 5B, 5C, 5D, 5E	O55:H7	9	−	−	−	−	−	−
Enteropathogenic isolates[c]		10	−	−	−	−	−	−
3812-3	O5:NM[d]	20	−	−	−	−	−	−
Other species								
Yersinia enterocolitica	O9	12, 38	−	−	−	−	−	−
Citrobacter freundii	NA[e]	12	−	−	−	−	−	−
Vibrio cholerae 569B	O1	12, 38	−	−	−	−	−	−
Vibrio cholerae O395	O1	12, 38	−	−	−	−	−	−

[a] The MOI was 10^3 PFU/CFU.
[b] Data for aerated and nonaerated cultures.
[c] A total of 33 different enteropathogenic *E. coli* isolates of assorted serotypes were tested. The serotypes included ON:HN, O8:H41, O12:NM, O15:HN, O18:HN, O23:H15, O25:H2, O26:H2, O26:HN, O26:NM, O46:HN, O55:H7, O66:HN, O75:HN, O85:NM, O86:H34, O96:HN, O103:H6, O111:HN, O115:NM, O118:H8, O125:H6, O127:H40, O128:H2, O131:HN, O145:HN, O153:HN, and O156:H8.
[d] NM, nonmotile.
[e] NA, not applicable.

ular-weight, and abundant mid-range-molecular-weight (~26- to 40-kDa) O157 antigens. The molecular weight distributions of the O157 antigens of representative strains are shown in Fig. 1.

High MOI is required for efficient killing by O157-specific phages. Infection of *E. coli* O157:H7 with KH1, KH4, or KH5 at various MOIs with and without aeration was monitored for 5 days. At MOIs ranging from 10^{-3} to 10^2 PFU/CFU, a minimal decline in bacterial titer was observed (data not shown). A significant reduction in the number of CFU per milliliter was observed at an MOI of 10^3 PFU/CFU (Fig. 2). Infections with KH1 resulted in a 10^5-fold decrease in the number of viable *E. coli* O157:H7 CFU/ml after 8 h (Fig. 2). The *E. coli* O157:H7 concentration (3 CFU/ml) was $>10^8$-fold less than the concentration in the phage-free bacterial control (3.58×10^8 CFU/ml) after 8 h (Fig. 2A). In all subsequent experiments, an MOI of 10^3 PFU/CFU was used.

O157-specific phages differ in the ability to kill their hosts. The efficiencies of the phages in reducing the titer of viable *E. coli* O157:H7 varied (Fig. 2). There was a steady increase in the *E. coli* O157:H7 concentrations over time in the phage-free bacterial control (Fig. 2). The bacterial titers in cultures in-

TABLE 2. Phage infection of *E. coli* O157:H7 strain 86-24 Nal^r and its O-antigen-deficient derivatives

E. coli O157:H7 strain[a]	Description	Latex agglutination test (O157 antigen)	Plaque formation			Lytic efficiency of phage mixture at[b]:		
			KH1	KH4	KH5	37°C		4°C[c]
						Aerated	Nonaerated	
86-24 Nal^r	Nalidixic acid-resistant mutant of *E. coli* O157:H7 strain 86-24	+	+	+	+	+	−	+
F12	O157 antigen-deficient mutant derived from strain 86-24 Nal^r	−	−	−	−	−	−	−
F12(pSK$^+$)	F12 transformed with plasmid Bluescript SK$^+$	−	−	−	−	−	−	−
F12(pF12)	F12 transformed with *rfbE*$_{ECO157:H7}$ cloned into pSK$^+$	+	−	−	−	−	−	−

[a] See reference 9.
[b] The MOI was 10^3 PFU/CFU.
[c] Data for aerated and nonaerated cultures.

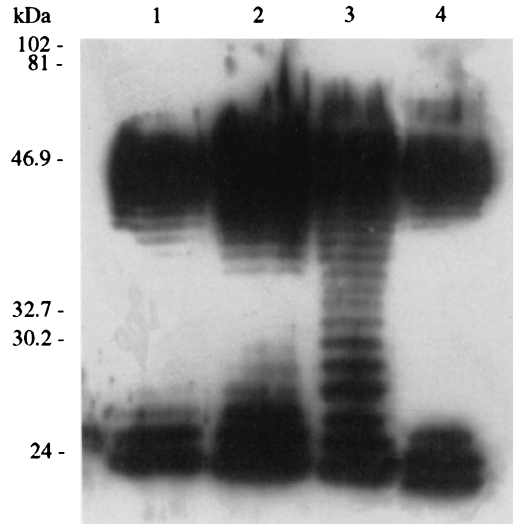

FIG. 1. Immunoblot analysis of LPS produced by strains resistant and susceptible to plaque formation by phage. High- and low-molecular-weight immunoreactive LPS, but not intermediate-molecular-weight immunoreactive LPS, were observed in the strains susceptible to plaque formation. Lanes 1 and 4, *E. coli* O157:H7 strain ATCC 43894; lane 2, *E. coli* O157:H7 strain 86-24 Nal^r. Abundant immunoreactive LPS at all molecular weights were observed in strains resistant to plaque formation. Lane 3 contained representative strain *E. coli* O157:H16 FDA 13A80. The positions of molecular size standards are indicated on the left.

fected with KH1 decreased significantly after 8 h of incubation (see above), while KH4 and KH5 infections resulted in minimal declines 8 h after phage infection (Fig. 2). Thus, at 37°C, although KH1 was more efficient in reducing the number of viable bacteria than KH4 or KH5 was, no single phage was able to eliminate all bacteria.

Bacterial growth was observed in cultures 8, 24, and 48 h after phage infection, but the surviving *E. coli* O157:H7 cells were sensitive to all three phages, which indicated that they had not acquired resistance to phage (data not shown). However, at 5 days after phage infection, the surviving *E. coli* O157:H7 cells were resistant to all three phages (data not shown). The bacterial isolates that survived infection continued to express the O157 antigen, because they were agglutinated with anti-O157 sera.

The phage concentrations in cultures infected at an MOI of 10^3 PFU/CFU consistently increased 10-fold after 8 h of incubation (KH4) or 24 h of incubation (KH1 or KH5), suggesting that the phages replicated (data not shown). The phage concentrations in the cell-free controls decreased about 10-fold after 8 h of incubation at 37°C.

Similar experiments performed at 4°C with KH1, KH4, or KH5 resulted in 0.5- to 0.2-$\log_{10}$ decreases in the titers of viable bacteria compared to the phage-free bacterial control. The phage titers in all of the experiments remained constant throughout the time course of infection (data not shown). At 4°C, no bacterial growth was observed in any culture, and the *E. coli* O157:H7 concentration remained 3×10^4 CFU/ml in the phage-free bacterial control (data not shown).

Phage mixtures can eliminate *E. coli* O157. The ability of serial additions of the same phage or different phages to eliminate *E. coli* O157:H7 was assessed. *E. coli* O157:H7 infected with KH1 at an MOI of 10^3 PFU/CFU was superinfected with 10^8 PFU of KH1 per ml (KH1 + KH1), 10^8 PFU of KH4 per ml (KH1 + KH4), or 10^8 PFU of KH5 per ml (KH1 + KH5) after 24 h. The cultures were incubated at 37°C without aeration and were assayed 48 h after the second phage addition. The *E. coli* O157:H7 titers in these superinfected cultures declined by 10^2-fold (KH1 + KH1) to 10^4-fold (KH1 + KH4 or KH1 + KH5) (data not shown). Thus, superinfection with either the same phage or a different phage resulted in a decrease in bacterial survival.

Finally, we studied simultaneous infection of *E. coli* O157:H7 with all three phages at an MOI of 10^3 PFU/CFU. The infected cultures were incubated at 37 or 4°C with or without aeration. In aerated cultures at 37 or 4°C, complete bacterial lysis was observed within 8 h after infection (Fig. 3). No growth occurred on LB agar, and even enrichment of the suspension did not yield viable *E. coli* O157:H7 cells (data not shown). The bacterial concentrations 8 h after phage infection were $>10^9$- and $>10^4$-fold less than the concentrations in the aerated phage-free bacterial controls at 37 and 4°C, respectively (Fig. 3). In nonaerated cultures at 37°C, the bacterial concentrations decreased 10- to 1,000-fold compared to the concentrations in the nonaerated phage-free bacterial controls

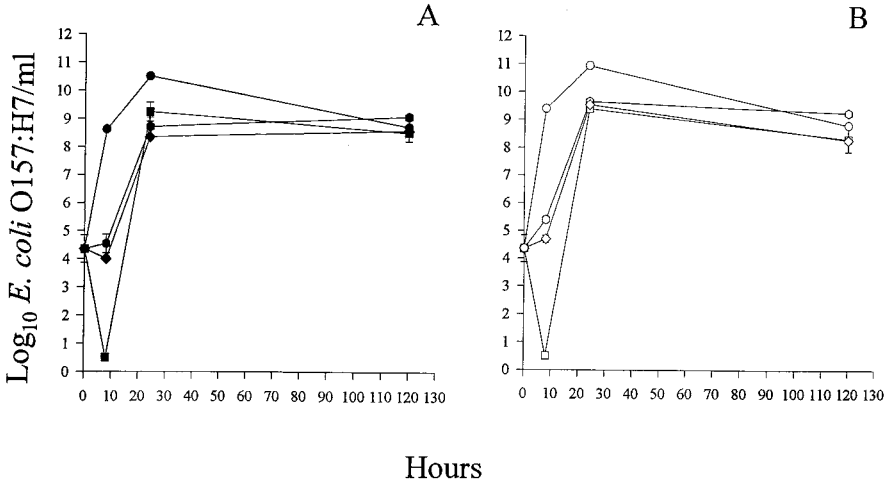

FIG. 2. Comparison of the lytic efficiencies of phages. Cultures were incubated at 37°C without aeration (A) or with aeration (B). *E. coli* O157:H7 (3×10^4 CFU) was treated with KH1 (■ and □), KH4 (● and ○), or KH5 (◆ and ◇). The MOI was 10^3 PFU/CFU. The control contained only *E. coli* O157:H7 (● and ○). The standard errors of the *E. coli* O157:H7 concentrations ranged from 0.03 to 0.5 $\log_{10}$ CFU/ml ($n = 4$).

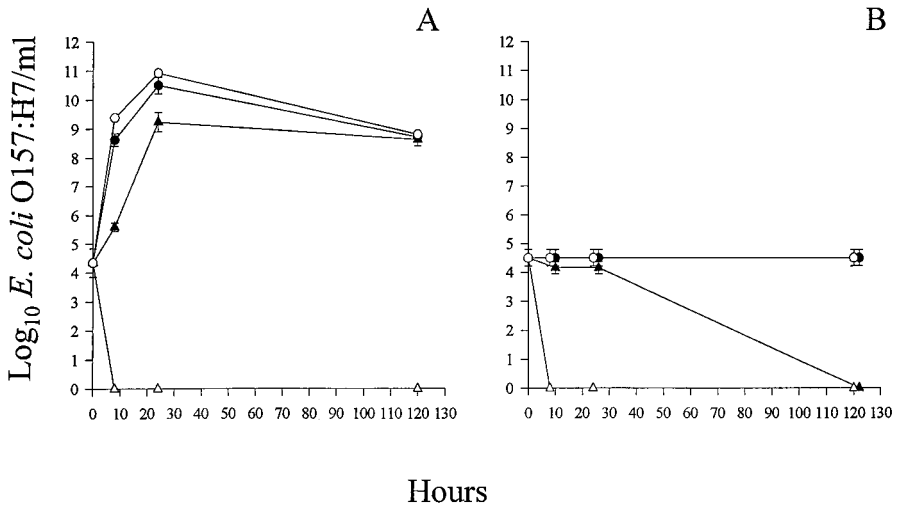

FIG. 3. *E. coli* O157:H7 lysis by a mixture of phages. Cultures were incubated at 37°C (A) or 4°C (B). *E. coli* O157:H7 (3×10^4 CFU) was infected with a mixed phage suspension (10^7 PFU) containing equal concentrations of KH1, KH4, and KH5. Cultures were incubated with (△) or without (▲) aeration. The controls contained only *E. coli* O157:H7 and were either aerated (○) or not aerated (●). The standard errors of the *E. coli* O157:H7 concentrations ranged from 0 to 0.5 $\log_{10}$ CFU/ml ($n = 4$).

by 8 to 24 h after phage infection. However, at 5 days after phage infection, the *E. coli* O157:H7 survivors had grown to a high density (Fig. 3A). In the nonaerated cultures at 4°C, only slight decreases in the bacterial concentrations were observed until 5 days after phage infection, when complete bacterial death occurred (Fig. 3B). In experiments in which all of the *E. coli* O157:H7 cells were killed, the phage titers increased 10-fold or remained the same (data not shown). The phage concentrations in the nonaerated cultures at 37°C decreased 10-fold after 24 h of incubation and then remained at that level (data not shown).

Under similar incubation conditions, the KH1-KH4-KH5 phage mixture killed all of the *E. coli* O157 strains tested (Tables 1 and 2). In contrast, none of the non-O157 *E. coli*, O-antigen-deficient *E. coli* mutant, or non-*E. coli* cultures were killed by the phage mixture (Tables 1 and 2).

DISCUSSION

We found that a mixture of three O157-specific phages is able to eliminate *E. coli* O157 from cultures. The factors that are critical for rapid cell lysis include aeration, incubation at 37°C, a high MOI, and simultaneous infection with the three phages. Phage treatment at 4°C without aeration resulted in bacterial death only after 5 days. The three O157-specific phages are not identical isolates with the same genotype because they behave differently when they kill *E. coli* O157.

Our decision to evaluate phages as anti-*E. coli* O157 agents was based on attempts by other workers to use phages as antibacterial agents. After the discovery of phages in 1915 to 1917, the use of bacterial viruses in human clinical settings became common in Europe (2, 4). Phage therapy was used to combat infections of the skin, bone, gastrointestinal tract, chest, abdomen, head, neck, and other organ systems (2). In fact, phages were also inadvertently responsible for controlling the spread of cholera in several parts of the Indian subcontinent a century ago (4). However, phage therapy was plagued by several controversies, primarily due to the inability to manipulate phage genetics (2, 4). More recently, studies conducted by Smith et al. have shown that phages may have immense potential for controlling *E. coli* infections in cattle (29). These researchers cured or prevented enteropathogenic *E. coli* (10^9 CFU) diarrhea in calves with strain-specific phages administered in a single oral dose (10^5 PFU) or sprayed on the litter (10^2 PFU) (29). However, efficacy was observed only when a phage was administered before or together with the infective bacteria. If the phage was administered after the onset of diarrhea, the disease intensity was decreased but the disease was not cured (29). Similarly, Berchieri et al. controlled an experimental *Salmonella typhimurium* (10^9 CFU) infection in poultry with *Salmonella* phage (10^9 PFU) (6).

Encouraged by these studies and the need for alternative methods of *E. coli* O157:H7 control, we isolated *E. coli* O157-specific phages. Adsorption of phage particles to bacterial cells, the initial step of phage infection, is dependent on the presence of specific receptors on the cell wall (34). Many cell wall receptors can be shared by different bacterial strains and serotypes (34). To obtain O157 antigen-specific phages, we screened for phages that bind to the O157 antigen and against phages that bind to common *E. coli* receptors, such as pili, fimbriae, flagella, LPS cores, and other outer membrane proteins (10, 34). In this way, we hoped to find phages that are lethal to *E. coli* O157 and not to other normal flora. In the one previous report of an *E. coli* O157:H7-specific coliphage, Ronner and Cliver described a phage that was toxin specific and thus lysed other Shiga toxin-producing bacteria, including *S. dysenteriae* (27).

The evidence that supports the conclusion that KH1, KH4, and KH5 are specific for serotype O157 includes the fact that these phages lysed all of the *E. coli* O157 strains tested and did not lyse non-O157 *E. coli*, non-*E. coli*, or O157-deficient mutant *E. coli* strains. In addition, phage infection was dependent on the nature of the O157 LPS. The complement of the *E. coli* O157-deficient mutant, *E. coli* F12(pF12), which produces a truncated O157 LPS (9), was resistant to infection.

Interestingly, although the mixture of phages KH1, KH4, and KH5 lysed (in liquid culture) all of the natural *E. coli* O157 strains tested, we found a few O157 strains that were resistant to plaque formation by individual phages. Like phage infection, plaque formation appears to be influenced by the nature of the O157 LPS. The *E. coli* O157 plaque-resistant strains

produced significantly more mid-range-molecular-weight LPS than the strains susceptible to plaque formation produced (Fig. 1). The excess mid-range-molecular-weight LPS made by the plaque-resistant *E. coli* O157 strains may accumulate around cells in soft agar and influence phage attachment but diffuse from cells in liquid culture. The phenomenon of an LPS requirement for plaque formation is not unique. For example, the *Salmonella* strains susceptible to phage P22 contain around 20 repeats of the common O-antigen trisaccharide unit; strains with shorter O antigens do not support P22 plaque formation (5). Therefore, an appropriate length of the O side chains and an optimal LPS concentration may be necessary to make the receptor available for phage interactions and/or to allow irreversible phage binding (11). It is tempting to speculate that abundant or size-specific immunoreactive O157 LPS competitively inhibits the adherence of the phages under plaquing conditions, but elucidation of the mechanism was beyond the scope of this study. Alternatively, the O157 plaque-resistant strains may not possess auxiliary mechanisms for phage adherence and uptake that are critical for plaque formation. The plaque-resistant O157 strains were non-H7 isolates and belong to lineages quite distant from the lineage to which *E. coli* O157:H7 belongs. It is, however, unlikely that expression of H7 antigen plays a role in plaque resistance since several other O157 non-H7 strains were susceptible to plaque formation by the phages studied.

The presence of magnesium is important for propagation of all three O157-specific phages. Mg^{2+} or Ca^{2+} ions are required for adsorption of a subset of phages (11). Like other coliphages, the O157-specific phages are lytic, and a high MOI favors killing of host cells. Presumably, a high MOI is necessary to ensure that every bacterium is infected by at least one phage. However, even at the optimal MOI (10^3 PFU/CFU), no single phage could clear an *E. coli* O157:H7 culture. Complete bacterial elimination was observed only when cultures were infected with all three phages, indicating that the combined effects of the phages were more than additive. In addition, culture aeration played a critical role in phage induction of rapid (within 8 h postinfection) and complete bacterial death. Aeration may increase the opportunity for phage-bacterium interactions. In nonaerated cultures, complete elimination of bacteria occurred only 5 days after phage infection and only at 4°C. We hypothesize that a low temperature and an absence of bacterial growth may favor better phage adsorption and infection. In contrast, a higher temperature (37°C), cell growth, and the potential for phenotypic variability in expression of the O antigen may favor survival of phage-resistant cells (14).

All of the bacteria that survived phage infection were tested for phage resistance (2, 4), and among the cells that had been exposed to phages for 5 days, we found *E. coli* O157:H7 cells that were resistant to plaque formation with these phages. The factors that contribute to phage resistance include alteration or loss of receptors (2, 4, 11). The finding that all phage-resistant bacteria agglutinate with the O157 antiserum indicates that these organisms have not lost the O side chains completely. These strains, like *E. coli* F12(pF12), may have a shorter O157 antigen. Consistent with this idea, preliminary results indicated that in the phage-resistant *E. coli* O157:H7 strains there may be significant alterations in the length or regulation of LPS production (data not shown). Thus, efficient use of phage to control *E. coli* O157:H7 infections may require isolation of mutant O157-specific phage that can adsorb to hosts that make shorter O side chains.

The phages that we isolated in this study may be used for biocontrol of *E. coli* O157. Based on our finding that killing occurs at 4°C, trials are under way to use these phages to eliminate *E. coli* O157:H7 from fresh vegetables. Several disease outbreaks in the United States have been linked to lettuce contaminated with *E. coli* O157:H7 (1). In addition, contaminated radish sprouts were the likely source of an *E. coli* O157:H7 outbreak in Japan in which more than 10,000 people were infected and 13 people died (25, 31). Because our phages kill efficiently at 4°C, they may be used to eliminate *E. coli* O157:H7 from these types of foods under refrigerated conditions. We have shown that O157-specific phages are prevalent in nature, and therefore it should be possible to isolate additional O157-specific phages or mutants of these phages that can kill under anaerobic conditions and may be used to eliminate *E. coli* O157 from the gastrointestinal tracts of carrier ruminants.

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