Application of Bacteriophages To Control Intestinal *Escherichia coli* O157:H7 Levels in Ruminants

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A previously characterized O157-specific lytic bacteriophage KH1 and a newly isolated phage designated SH1 were tested, alone or in combination, for reducing intestinal *Escherichia coli* O157:H7 in animals. Oral treatment with phage KH1 did not reduce the intestinal *E. coli* O157:H7 in sheep. Phage SH1 formed clear and relatively larger plaques on lawns of all 12 *E. coli* O157:H7 isolates tested and had a broader host range than phage KH1, lysing O55:H6 and 18 of 120 non-O157 *E. coli* isolates tested. In vitro, mucin or bovine mucus did not inhibit bacterial lysis by phage SH1 or KH1. A phage treatment protocol was optimized using a mouse model of *E. coli* O157:H7 intestinal carriage. Oral treatment with SH1 or a mixture of SH1 and KH1 at phage/bacterium ratios $\geq 10^2$ terminated the presence of fecal *E. coli* O157:H7 within 2 to 6 days after phage treatment. Untreated control mice remained culture positive for >10 days. To optimize bacterial carriage and phage delivery in cattle, *E. coli* O157:H7 was applied rectally to Holstein steers 7 days before the administration of 10^{10} PFU SH1 and KH1. Phages were applied directly to the rectoanal junction mucosa at phage/bacterium ratios calculated to be $\geq 10^2$. In addition, phages were maintained at 10^6 PFU/ml in the drinking water of the phage treatment group. This phage therapy reduced the average number of *E. coli* O157:H7 CFU among phage-treated steers compared to control steers ($P < 0.05$); however, it did not eliminate the bacteria from the majority of steers.

Since its association with disease in 1982 (33), *Escherichia coli* O157:H7 has become a worldwide threat to public health and is one of today's most troubling food-borne pathogens. Human illness with *E. coli* O157:H7 ranges from self-limited watery diarrhea and hemorrhagic colitis to life-threatening manifestations such as the hemolytic uremic syndrome and thrombotic thrombocytopenic purpura. Cattle and sheep, important domestic ruminants, are the primary reservoirs for this human pathogen and are the most common sources for food-borne, waterborne, and direct-animal-contact infections (9–11, 18, 48, 51). In contrast to humans, the carrier ruminants remain healthy, and *E. coli* serotype O157:H7 is a transient member of the ruminant gastrointestinal flora. Persistence of *E. coli* O157:H7 carriage in naturally and experimentally infected animals varies from days to months (6, 8, 12, 13, 19, 35, 38).

Successful strategies to control or reduce the carriage and prevalence of *E. coli* O157:H7 in live ruminants would reduce the risk of human exposure to this pathogen. There is currently no reliable intervention or animal vaccine available to curb carriage of *E. coli* O157:H7. Probiotics aimed at creating an intestinal microenvironment inhibitory to *E. coli* O157:H7 have been tested, but without consistent success (14, 22, 28, 29, 50). A lytic bacteriophage that would specifically target the *E. coli* O157 serotype is another appealing approach because phage therapy has been successful in animal trials against a broad range of bacterial pathogens such as enteropathogenic

E. coli (3, 39), *Staphylococcus aureus* (23, 41), *Pseudomonas aeruginosa* (41), and vancomycin-resistant *Enterococcus faecium* (7).

Isolating *E. coli* O157-specific phages and testing their use to control *E. coli* O157:H7 in vivo and in vitro have been done by our laboratory and by others (15, 21, 25, 27, 44). However, control of *E. coli* O157:H7 with phage treatment in natural farm settings is limited. To our knowledge, the only published data on the assessment of the ability of phage to control *E. coli* O157:H7 in live animals are by Bach et al. for sheep (2) and Tanji et al. (45) for mice. In the sheep study, lambs orally dosed with *E. coli* O157:H7 and then treated with a single dose of O157-specific phage DC22 did not clear the pathogen, although phage DC22 was effective at controlling *E. coli* O157:H7 in an artificial rumen system (2). In the mouse study, daily administration of a phage cocktail (SP15-21-22) reduced the number of fecal *E. coli* O157:H7 CFU among the treated mice compared to the untreated mice but did not clear the bacteria from the intestinal tract (45).

Two recent breakthroughs in understanding the relationship between ruminants and *E. coli* O157:H7 will likely impact the ability of phage treatment as an intervention strategy. The primary site of *E. coli* O157:H7 colonization in cattle is the rectoanal junction (RAJ) (16, 26). Also, application of *E. coli* O157:H7 directly to the bovine RAJ mucosa, as opposed to an oral dose, is a method that reliably colonizes cattle with these bacteria (38).

Using phage to control *E. coli* O157:H7 has been a goal of our laboratory for years (21). Here we investigated various phage treatments to reduce or eliminate *E. coli* O157:H7 in vivo. Experiments included (i) testing the previously characterized O157-specific lytic phage KH1 (21) for its ability to limit *E. coli* O157 carriage in sheep experimentally infected

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with an oral dose of *E. coli* O157:H7, (ii) the isolation and characterization of a new lytic phage designated SH1, (iii) the use of a mixture of the phages KH1 and SH1 to limit *E. coli* O157:H7 carriage in a mouse model, and (iv) the use of a novel phage treatment that placed a mixture of KH1 and SHI directly onto the bovine RAJ mucosa.

MATERIALS AND METHODS

All personnel followed strict biosafety procedures when handling *E. coli* O157:H7 and/or animals, and all procedures were approved by the Institutional Animal Care and Use and Biosafety committees.

Bacteria. *E. coli* O157:H7 strains included clinical human isolate ATCC 43894 (American Type Culture Collection, Manassas, Va.) and bovine isolates WSU180, WSU400, and WSU588 (32). All four *E. coli* O157:H7 strains have distinct pulsed-field gel electrophoresis profiles and possess the Shiga toxin genes (*stx1* and *stx2*). When mixtures of bacteria were administered to animals, the bacteria were prepared individually in 250-ml flasks and equal volumes were combined just before they were given to the animals. In addition, *E. coli* strains JM103, O55:H6, O111:H12, O111:H21, O111:NM, and O113:H21 and 120 bovine non-O157 *E. coli* strains were used to test phage host range. Bacterial cultures were grown in Luria-Bertani (LB) broth (BBL/Becton Dickinson, Detroit, Mich.) at 37°C for 18 h with aeration (150 rpm).

Phage isolation, preparation, and titration. *E. coli* O157:H7-specific lytic phage KH1 was previously isolated from bovine feces (21). Phage SH1 was isolated by a standard enrichment procedure (37) from raw sewage taken from a municipal sewage treatment system (Pullman, WA). Briefly, 15-ml sewage samples were centrifuged at $3,500 \times g$ for 25 min at 10°C. The supernatants were filtered through a 0.45-μm-pore-size filter, 8.6 ml of filtrate was added to 1 ml $10\times$ LB broth, and 100 μl ($\sim 10^8$ CFU) of each *E. coli* O157:H7 strain (ATCC 43894, WSU180, WSU400, and WSU588) was added. The mixture was incubated at 37°C for 18 h with aeration (150 rpm). Debris and bacteria were removed by centrifugation at $10,000 \times g$ for 10 min, and supernatants were filtered through a 0.22-μm-pore-size filter. Phage activity in the supernatant was tested by a spot assay that entailed placing 5 μl of the supernatant on LB agar seeded with *E. coli* O157:H7. The plates were checked for plaques after 18 h at 37°C. Lysis-positive supernatants were serially diluted, and plaques were isolated and purified using a soft agar overlay technique with *E. coli* O157:H7 ATCC 43894 (34). One phage that produced plaques on all four *E. coli* O157:H7 strains (ATCC 43894, WSU180, WSU400, and WSU588) was selected for further study and designated SH1.

Large-scale high-titer stocks of KH1 and SH1 were prepared by the plate lysate method using large petri plates (150 mm by 15 mm), as previously described (34). To remove debris, the lysate and top agar were pooled and centrifuged at $10,000 \times g$ for 10 min at 4°C. Due to the difficulty of filtering the suspension through a 0.45-μm-pore-size filter, the supernatant was heated at 60°C for 25 min to kill *E. coli* O157:H7. Chloroform was avoided at all preparation steps since phages were to be used in animals. The heated phage stock was titrated and stored at 4°C and the titer determined again, just prior to use.

A phage-free mock lysate to be used as a control for phage treatment was prepared as follows. Similar bacterial cultures were grown without phages and centrifuged at $9,000 \times g$ for 10 min at 4°C. The cell pellets were mock lysed by grinding with liquid nitrogen and a prefrozen mortar and pestle. The same volume of supernatant (as used to suspend the phage pellet) was used to resuspend the lysed cells. Debris was removed by centrifugation; the supernatant was heated at 60°C for 25 min to kill viable bacteria, and the success of this step was confirmed by plating on LB agar plates and overnight incubation.

Phage SH1 and KH1 *E. coli* host range. *E. coli* strain JM103, enterohemorrhagic *E. coli* O55:H6, O111:H12, O111:H21, O111:NM, and O113:H21, and 120 bovine non-O157 *E. coli* strains were tested for phage susceptibility. The non-O157 *E. coli* strains were isolated from cattle by plating rectoanal mucosal swab (RAMS) samples (32) from four *E. coli* O157 culture-negative cattle on violet-red bile agar (VRBA; BBL/Becton Dickinson, Detroit, Mich.) supplemented with 4-methylumbelliferyl-β-D-glucuronide (MUG). *E. coli* colonies were distinguished by a purple-red color and bluish fluorescence under long-wave UV light. The sensitivity of each bacterial strain to KH1 and SH1 was determined using the spot assay, as described above. The presence of a lytic zone was considered evidence of phage susceptibility; no lysis was considered evidence of phage resistance.

Determination of phage MOI in *E. coli* O157:H7. The optimal multiplicity of infection (MOI) for *E. coli* O157:H7 strain ATCC 43894 was determined by mixing bacteria and phage SH1 at ratios ranging from 10^{-1} to 10^5 PFU/CFU in tubes containing 10^4 CFU of *E. coli* O157:H7/ml. Phage-free cultures (containing only bacteria) and cell-free cultures (containing only phage) were used as controls. The tubes were incubated at 37°C without aeration for 24 h, and the number of viable cells was determined in triplicate by plating on LB agar following concentration by centrifugation. MOI determinations were done in duplicate.

Phage infection in the presence of mucus. Commercial porcine gastric mucin (PGM) (type III; Sigma, St. Louis, Mo.) was prepared to a final concentration of 10% (wt/vol) in 50 ml phosphate-buffered saline (PBS). Bovine mucus was collected from the RAJ of two 9-month-old steers as follows. The animals were held in a squeeze chute, feces in the terminal rectum were removed by rectal palpation, and the terminal rectum was rinsed with PBS. A soft rubber spatula was use to scrape mucus from the RAJ mucosa tissue. Approximately 3 ml of highly viscous mucus was obtained from each animal, pooled in a 50-ml conical tube, held on ice, and immediately transported to the laboratory. The bovine mucus was dissolved 1:1 (vol/vol) in PBS. Bovine mucus and PGM solutions were sterilized by autoclaving, a procedure known not to change the biological activity of these substances (4). One percent PGM, 1% bovine mucus, or PBS (200 μl) was mixed with 100 μl of an overnight culture of *E. coli* O157:H7 (ATCC 43894). The mixtures were incubated at 37°C for 25 min, added to 2.5 ml LB soft agar (0.75%) containing 10^2 PFU of phage SH1 or KH1, and overlaid onto an LB agar (1.5%) plate (21). Plates were incubated overnight at 37°C and plaques enumerated.

Experimental animals. All animals were acclimated for at least 2 weeks prior to receiving *E. coli* O157:H7.

(i) Sheep. Six 7-month-old Suffolk ewes were housed in a containment facility at Washington State University (Pullman). Two groups ($n = 3$) were separated in two different rooms without cross contamination. Ewes were fed pelleted alfalfa twice daily, with drinking water ad libitum. No supplements were included in their diets.

(ii) Mice. Twelve 8-week-old female Swiss Webster mice were obtained from Simonsen Laboratories, Inc. (Gilroy, CA). Mice were randomly allocated to cages with three mice each and housed under standard day length, temperature, and humidity conditions. Water and pelleted food were offered ad libitum.

(iii) Steers. Six-month-old Holstein steers were housed in a quarantined facility at the University of Idaho. Two groups ($n = 5$) were separated in different containment pens without contact between groups and without cross contamination for the phage treatment trial. Steers were fed alfalfa hay twice daily and grain pellets once daily and had drinking water ad libitum.

Bacterial challenge and phage treatment. Six sheep were given a single oral dose of 3.5×10^{10} CFU *E. coli* O157:H7 ATCC 43894/animal on day −1. On days 0, 8, 9, and 10, three ewes were given oral doses of 1.3×10^{11} PFU KH1/animal/day (treatment group) and three ewes were given similar oral doses of medium without phage (control group).

Six mice were given a single oral dose of 10^8 CFU *E. coli* O157:H7 ATCC 43894/animal on day −1. On days 0, 1, and 2 after phage treatment, three mice were given 10^{10} PFU SH1 (phage 1) and three mice were given 100 μl mock lysate (control 1). Another six mice were given a single oral dose of 10^8 CFU of a four-strain mixture of *E. coli* O157:H7 containing strains ATCC 43894, WSU180, WSU400, and WSU588 (the number of CFU of each strain was confirmed prior to mixing by measuring the optical density at 600 nm). On days 0, 1, and 2 after phage treatment, three mice received an oral dose of 10^{10} PFU of both KH1 and SH1 (phage 2) or the mock lysate (control 2). To improve the palatability of the solutions, the bacterial cultures, mock lysate, and phage stocks were mixed with sucrose (final concentration of 20%) prior to oral administration. All oral doses were administered while the mice were held in a supine position with the head up, allowing the animals to suck the fluid from the tip of a micropipette.

Ten Holstein steers were given a single rectal application of a mixture of 10^{10} CFU of each of four strains of *E. coli* O157:H7 (ATCC 43894, WSU180, WSU400, and WSU588, the same strains used in the mouse experiment above) on day −7. Rectal application of bacteria was carried out as described previously (38) with a slight modification as follows: instead of using a large cylindrical sponge, a sterile foam-tipped applicator (catalog no. 10812-022; VWR International, Buffalo Grove, Ill.) was saturated with *E. coli* O157:H7 overnight culture, and the swab was inserted into the terminal rectum and gently rubbed against the rectoanal junction mucosa. On days 0, 1, 2, and 4 after phage treatment, five steers were given phage rectally (treatment group). The anuses of the steers were digitally palpated to open, and feces were removed. The mixture of phages SH1 and KH1 (25 ml of 10^{10} PFU/ml) was delivered into the anus with a syringe and a 10-cm by 3.5-cm-diameter cylindrical sponge (Rubbermaid, St. Francis, WI) with a wooden handle. The sponge was saturated with 5 ml of the phage mixture and was inserted and gently swabbed against the wall of the RAJ mucosa. The

TABLE 1. Duration and number of fecal *E. coli* O157:H7 CFU in sheep with and without phage treatment

| Day after phage treatment[a] | No. of CFU/g of feces in animal[b] treated with: | | | | | |
| | KH1 in treatment group: | | | Mock lysate in control group: | | |
	P1	P2	P3	C1	C2	C3
0	2.55×10^6	1.15×10^6	5.25×10^6	2.50×10^5	1.50×10^6	5.10×10^6
2	6.00×10^4	1.05×10^5	5.00×10^3	1.50×10^3	5.00×10^3	1.00×10^2
4	1.35×10^3	5.00×10^3	3.00×10^1	E+[c]	5.00×10^4	E+
6	E+	0	E+	E+	4.50×10^4	E+
8	E+	0	E+	E+	E+	E+
9	E+	0	E+	E+	E+	E+
10	0	0	E+	E+	0	E+
11	0	0	E+	E+	0	0
12	0	0	E+	E+	0	0
14	0	0	E+	E+	0	0
21	0	0	E+	E+	0	0

[a] On days 0, 8, 9, and 10, ewes were given an oral dose of 1.3×10^{11} PFU phage KH1/animal.

[b] Animals were 7-month-old Suffolk ewes and were given a single oral dose of 3.5×10^{10} CFU *E. coli* O157:H7 (ATCC 43894)/animal on day −1.

[c] E+, sample was positive by enrichment culture technique but not by direct culture technique, indicating that the number of *E. coli* O157:H7 CFU in the sample was ≤10 CFU/g.

tails of the treated steers were held down for 10 min immediately following withdrawal of the swab. This was done to prevent animals from defecating and presumably to allow the phage interactions without immediate passage of fecal material. Phages were also administered via drinking water by adding phage daily to a final concentration of 1.8×10^6 to 5.4×10^6 PFU/ml starting on day 0, and as animals drank the phage concentration was maintained at that level through the end of the experiment. Similarly, five steers were given the mock lysate rectally and in their drinking water (control group).

Sample collection for enumeration of *E. coli* O157 CFU and phage. Sheep fecal samples were collected from each ewe by a previously described method on the days indicated in Table 1 (20). Briefly, fecal samples were collected aseptically, placed in Whirl-pak bags, and kept on ice until culture. Ten grams of feces was diluted into 50 ml Trypticase soy broth (TSB; BBL/Becton Dickinson, Detroit, Mich.) supplemented with cefixime (50 ng/ml; generously provided by D. D. Hancock, Washington State University), potassium tellurite (2.5 mg/liter; Sigma Chemical Co., St. Louis, Mo.), and vancomycin (40 mg/liter; Sigma) (referred to hereafter as TSB-CTV). Mouse fecal samples were collected daily as three freshly voided pellets from each mouse and placed into 15-ml conical tubes containing 3 ml ice-cold TSB, suspended by vortexing, and kept on ice until the culture procedure. Cattle RAMS samples were collected by the previously described method (32). Briefly, RAMS samples from individual steers were placed into 15-ml conical tubes containing 3 ml ice-cold TSB, mixed by vortexing, and kept on ice until the culture procedure. All fecal and RAMS samples were processed within 3 h of collection. On day 0 after phage treatment and twice weekly thereafter, RAMS samples were taken from each individual steer for culture of *E. coli* O157:H7.

Determination of bacterium and phage concentrations. To remove free phage before bacterial enumeration, all samples in TSB or TSB-CTV were centrifuged at $10,000 \times g$ for 5 min at 4°C to separate the supernatant from the solid particles and cells. Serial dilutions of supernatants were used in a standard soft-agar technique to determine the titer of phage in corresponding samples (34). The pellets were washed and resuspended into an equal volume of fresh TSB or TSB-CTV, correspondingly, for direct or enrichment procedures to determine the number of *E. coli* O157:H7 CFU.

Samples were cultured for *E. coli* O157:H7 by direct and enrichment procedures as previously described (32). Briefly, initial suspensions were made with 10-g fecal samples from sheep, three-pellet feces from mice, or RAMS from cattle. Direct cultures were prepared by plating serial dilutions made in sterile saline (0.15 M NaCl) onto sorbitol-MacConkey agar supplemented with MUG, cefixime, potassium tellurite, and vancomycin (SMAC-CTVM). Plates were incubated at 37°C overnight and observed for colonies not fermenting sorbitol (white colonies) and not hydrolyzing MUG (no florescence at 363 nm). Presumptive *E. coli* O157:H7 colonies were confirmed by latex agglutination (Pro-Lab Diagnostics, Toronto, Canada). Positive cultures from this direct-plating technique result in a quantitative measure of *E. coli* O157:H7 bacteria in CFU/gram feces for sheep, CFU/fecal pellet for mice, or CFU/swab for cattle. Cultures negative by these direct methods were further analyzed by an enrichment procedure. Mouse feces in TSB, RAMS in TSB, or sheep feces in TSB-CTV were

incubated at 37°C with aeration for 18 h, serial dilutions were plated onto SMAC-CTVM, and presumptive *E. coli* O157:H7 colonies were confirmed by latex agglutination.

Analysis of colony morphology. *E. coli* O157:H7 colony morphologies were analyzed visually from direct cultures of murine feces or bovine RAMS using SMAC-CTMV. To determine the consistency of colony morphologies, irregular colonies were picked, subcultured by restreaking onto SMAC-CTMV, and reanalyzed visually.

Statistical analysis. The number of *E. coli* O157:H7 CFU recovered from individual samples was determined as the average of duplicate plate counts. Values from experiments were transformed to $\log_{10}$ CFU/pellet for the mouse experiments and $\log_{10}$ CFU/swab for the cattle experiments. Samples positive only by enrichment procedure were assigned a value of 5 CFU/pellet for the mice and 10 CFU/swab for the cattle. Significant differences between the phage-treated and control groups were determined using the statistical analysis's *t* test procedure (36).

RESULTS

All animals in this study were healthy throughout the trials and had no ill effects of carrying *E. coli* O157:H7 or phage. All animals were culture negative for *E. coli* O157:H7 prior to experimental challenge with the bacteria.

Oral phage treatment by KH1 did not reduce intestinal *E. coli* O157:H7 in sheep. To test whether the previously characterized O157-specific lytic phage KH1 could eliminate *E. coli* O157:H7 in vivo, phage KH1 was applied to sheep given a single oral dose of *E. coli* O157:H7 (Table 1). All sheep had *E. coli* O157:H7 culture-positive feces 1 day after the bacterial dose. A single oral dose of phage KH1 was given to sheep in the phage treatment group on day 0. Although one ewe in the phage treatment group became culture negative for *E. coli* O157:H7 on day 6 after phage treatment and remained so for the duration of the experiment, the average number of fecal *E. coli* O157:H7 CFU for ewes in the phage treatment group was not significantly different from that for the ewes in the control group ($P = 0.74$ for the first 8 days after phage treatment) (Table 1). The numbers of fecal *E. coli* O157:H7 CFU and duration of carriage among both the control and phage-treated animals were typical for orally dosed ruminants. Thus, we can conclude that phage did not clear carriage of *E. coli* O157 in these sheep. Following the single oral dose of 10^{11} PFU of

KH1 on day 0, there was a variable number of phage found in fecal samples, and on days 5 through 8 all phage-treated animals had between 10^5 and 10^6 PFU/g feces. The apparent ratios of *E. coli* O157:H7/KH1 ranged from <1 to >10^3 (data not shown). Three more oral doses of phage KH1 were administered on days 8, 9, and 10 to the animals in the phage treatment group. Although phage concentrations measured in fecal samples after these repeated treatments were as high as 10^6 PFU/g, the numbers of fecal *E. coli* O157:H7 CFU were similar to the untreated control group (Table 1 and data not shown).

Isolation, host range, and plaque-forming ability of phage SH1. Despite the success of phage KH1 at lysing *E. coli* O157:H7 in vitro (21), repeated oral application of high titers of phage KH1 was not effective at eliminating intestinal *E. coli* O157:H7 from sheep (Table 1). For this reason, new phages were isolated and characterized. Phage SH1, isolated from a sewage sample, was highly lytic and formed large clear plaques in all 12 *E. coli* O157:H7 strains tested (four human isolates and eight bovine isolates; data not shown). In anticipation of its use in animals, bacterium-free phage preparations were made by heat treatment rather than chloroform extraction. The numbers of viable phage after heating were decreased from ~6×10^{11} PFU/ml to ~5.5×10^{10} PFU/ml. We compared the lytic activities of the phages KH1 and SH1 on the endogenous *E. coli* RAJ flora isolated from a group of four 6-month-old steers. Thirty random *E. coli* isolates from fecal cultures were selected per animal, and a total of 120 non-O157:H7 *E. coli* isolates were tested for sensitivity to the two phages in a plate lysis test. KH1 did not form plaques on any of the non-O157 *E. coli* isolates (data not shown). SH1 formed plaques on 18 of 120 non-O157:H7 *E. coli* isolates (data not shown). The result indicated that the host range of SH1 was broader than KH1, which is highly specific for *E. coli* O157:H7 (21). The theoretical host range of phage SH1 for 120 representative *E. coli* isolates recovered from RAMS samples was eight (6.7%). Neither SH1 nor KH1 formed plaques on *E. coli* JM103 or enterohemorrhagic *E. coli* O111:H12, O111:H21, O111:NM, or O113:H21. SH1 produced plaques on O55:H6 (data not shown). All four strains of *E. coli* O157:H7 (ATCC 43894, WSU180, WSU400, and WSU588) used to challenge animals in this study were sensitive to phages SH1 and KHI. The plaques produced by SH1 (2 mm) were larger than those produced by KH1 (<1 mm) on the all tested *E. coli* O157:H7 lawns (data not shown).

Phage SH1 at an MOI of $\geq 10^2$ PFU/CFU eliminated *E. coli* O157:H7 ATCC 43894 in LB broth. To further characterize SH1 and to prepare for its use in animal trials, the optimal MOI for the elimination of *E. coli* O157:H7 in broth was determined. There was no bacterial growth after phage infections with MOIs of $\geq 10^2$ PFU/CFU. However, at an MOI of 10 PFU/CFU, an average of 9.0×10^3 CFU *E. coli* O157:H7/ml were recovered (data not shown). The bacteria that survived phage infection were all sensitive to phage SH1 in subsequent assays (data not shown). From these results, animal experiments with phage/*E. coli* O157:H7 ratios $\geq 10^2$ were planned.

Mucin did not inhibit the lysis activities of phage SH1 and KH1. The mucus layer in the gastrointestinal tract may provide a protective barrier shielding *E. coli* cells from phage lysis (31, 46). SH1 and KH1 lysis of *E. coli* O157:H7 was tested in
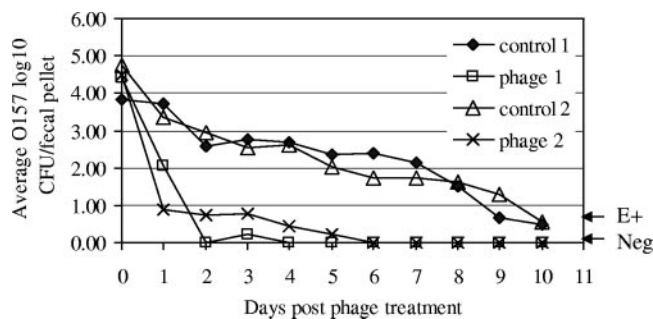

FIG. 1. The effect of oral phage treatment on *E. coli* O157:H7 in mice. Two groups of three mice (control 1 and phage 1) received a single oral dose of 10^8 CFU *E. coli* O157:H7 ATCC 43894 on day −1. On days 0, 1, and 2, mice in phage treatment group 1 were given an oral dose of 10^{10} PFU of SH1 phage. Another two groups of three mice (control 2 and phage 2) received a single oral dose of 10^8 CFU *E. coli* O157:H7 containing equal numbers of four strains (ATCC 43894, WSU180, WSU400, and WSU 588) on day −1. On days 0, 1, and 2 the treated mice were given oral doses of 10^{10} PFU of both SH1 and KH1. Untreated control mice received 100 μl of a mock bacterial lysate without phage. Data points below E+ indicate samples positive for *E. coli* O157:H7 only by an enrichment procedure and with total numbers <10 CFU/fecal pellet. Standard errors ranged from 0.5 log to 1.2 logs. Neg indicates RAMS cultures that were negative by both direct and enrichment culture procedures.

medium containing porcine gastric mucin or bovine intestinal mucus. No differences were observed in broth lysis or plaque formation on the plates in terms of the number of cells killed or the number or size of the plaques when mucin or bovine mucus was present (data not shown).

Oral treatment with phage SH1 alone or mixtures of SH1 and KH1 eliminated intestinal *E. coli* O157:H7 in mice. Phage SH1 and KH1 were tested in a murine model of *E. coli* O157:H7 intestinal carriage using a single strain (ATCC 43894) or a mixture of four strains (ATCC43894, WSU180, WSU400, and WSU588). After a single oral dose of 10^8 CFU *E. coli* O157: H7, the mice in both the control groups and phage treatment groups remained active and ostensibly normal over the period of experimentation. In the untreated control groups (control 1 and 2) fecal *E. coli* O157:H7 was cultured from most animals on all sample days (Fig. 1). The numbers of *E. coli* O157 CFU/fecal pellet were highest after the oral dose and declined slowly over the course of the experiment. The number of fecal *E. coli* O157 CFU was reduced by 1.7 log in phage treatment group 1 (phage 1) and 2.4 log in phage treatment group 2 (phage 2) within 24 h after phage treatment, compared to the number of fecal *E. coli* O157:H7 CFU in the respective control groups (Fig. 1). After the second and third phage treatments on days 1 and 2, the numbers of fecal *E. coli* O157:H7 CFU were reduced further and were either undetectable or detected only by enrichment culture. At this time, all untreated control mice continued to have >2 log *E. coli* O157 CFU/fecal pellet (Fig. 1). By day 6, all phage-treated mice were culture negative for *E. coli* O157:H7 (Fig. 1). Phage titers in the feces of treated mice were 10^6 PFU/fecal pellet on days 1, 2, and 3 after phage treatment and declined to 10^3 PFU/fecal pellet on day 5 (data not shown). Thus, the calculated ratio of phage/bacteria was $\geq 10^2$ PFU/CFU at the beginning of the trial and throughout the experiment. The average log10 fecal

E. coli O157 CFU among the phage treatment groups was significantly less at day 1 posttreatment and on all following days compared to the control groups ($P < 0.01$).

Rectal application of phage reduced the number of *E. coli* O157:H7 CFU but did not clear the pathogen from steers. With the success of phage treatment in mice to clear intestinal *E. coli* O157:H7 (Fig. 1), the combination of SHI and KH1 seemed promising as a therapy for cattle. However, the failure of oral phage treatment in sheep (Table 1) and unsuccessful attempts by others to use oral phage therapy to clear intestinal *E. coli* O157:H7 from calves (D. D. Hancock [Washington State University, Pullman], personal communication) suggested that another approach to phage treatment might be needed to clear intestinal *E. coli* O157:H7 from ruminants. We postulated that the inability of phage therapy to clear intestinal *E. coli* O157 in ruminants may have been due to differences in the numbers of *E. coli* O157:H7 CFU surviving in the intestinal tract after oral dose, bacterium/phage ratios $<10^2$, the large volume of the bovine intestine, and/or differences between the environments of the monogastric and ruminant intestinal tracts. To address the first issues, we designed an experiment to insure that all control animals would carry the bacteria and that the ratio of the phage to bacteria could be maximized. Rectal application of *E. coli* O157:H7 was used to ensure the long-term carriage of bacteria. Ten steers (five controls and five phage treated) were individually dosed with 10^6 CFU of a four-strain mixture of *E. coli* O157 applied to the RAJ mucosa on day −7. Although the dose (10^6 CFU) was well below previous rectal application protocols that use ~10^{10} CFU, long-term carriage of bacteria resulted in all the untreated control animals. All steers were culture positive for the bacteria with 4- to 5-log values of *E. coli* O157:H7 detected in RAMS samples on day 0 (Fig. 2A and B). All untreated control animals carried *E. coli* O157 for the duration of the experiment (Fig. 2B). Phages were applied at the RAJ mucosa of animals in the phage treatment group on days 0, 1, 2, and 4. The average number of *E. coli* O157:H7 CFU in RAMS samples of phage-treated steers declined sharply to 2.4 logs on day 1 after phage treatment, whereas the average number of *E. coli* O157 CFU/swab in the control group was 3.9 logs at this time. The number of *E. coli* O157 CFU cultured from the RAJ among the phage-treated group declined further to an average of 1.4 logs compared to 2.7 logs among the control group 7 days after phage treatment (Fig. 2A and B). Numbers of *E. coli* O157 CFU/swab among the phage-treated steers were significantly less ($P < 0.05$) than among the control group on day 1 and through day 10 posttreatment. Two steers were culture negative for *E. coli* O157 on days 5 and 7, and one remained negative for the pathogen throughout the study. However, the phage treatment did not clear *E. coli* O157 from four of five treated calves.

The titer of phages in the RAMS samples of treated steers was 4 to 6 logs/swab during the first 7 days after phage treatment and remained 2 logs/swab until the end of the study (Fig. 2C). The calculated ratios of phage/*E. coli* O157:H7 in the RAMS samples ranged from 1 to10^4 depending on the sample day and the individual steer.

Some *E. coli* O157 isolates recovered from phage-treated steers displayed unusual colony morphology, and all were sensitive to KH1 and/or SH1. By pulsed-field gel electrophoresis
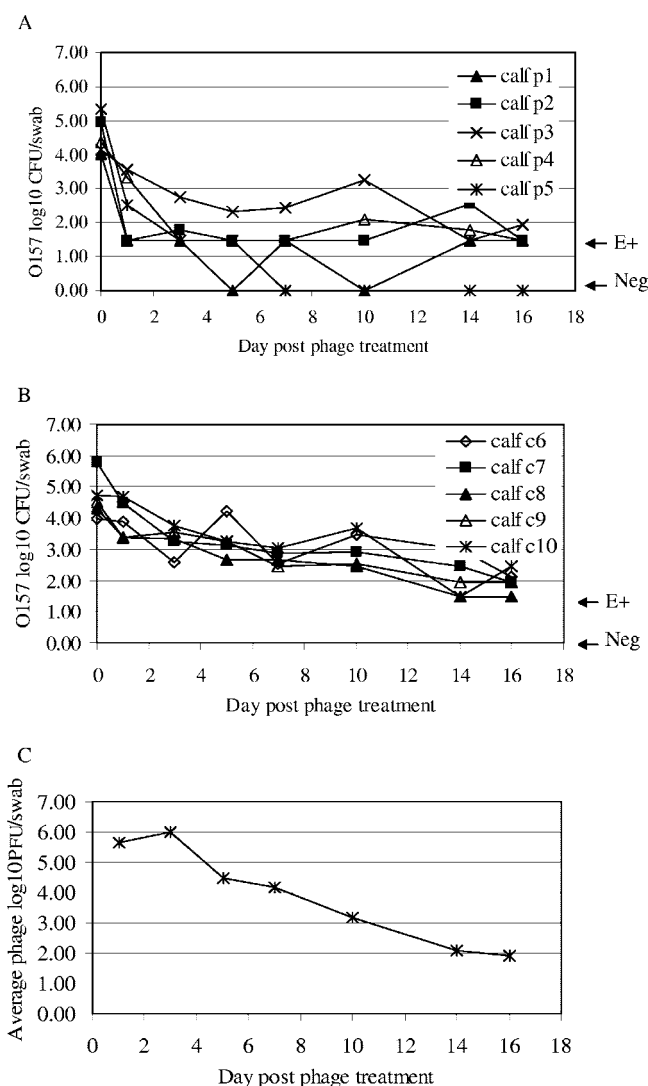

FIG. 2. Effect of phage treatment on the number of *E. coli* O157:H7 CFU in steers. Five phage-treated steers (A) and five untreated control steers (B) received a single rectal application of *E. coli* O157:H7 (approximately 10^6 CFU) containing equal amounts of four strains (ATCC 43894, WSU180, WSU400, and WSU588) on day −7. On days 0, 1, 2, and 4, each steer in the phage treatment group was given rectal applications of 8.1×10^{10} PFU of phages containing equal amounts of SH1 and KH1 in a 30-ml solution. In addition, starting on day 0, drinking water contained SH1 and KH1 (10^6 PFU/ml). The control steers were given 30 ml mock lysate rectally on days 0, 1, 2, and 4, and the drinking water contained the mock lysate without phage. E+ indicates RAMS cultures positive for *E. coli* O157:H7 only by an enrichment procedure and thus contained <30 *E. coli* O157 CFU/swab. Neg indicates RAMS cultures that were negative by both direct and enrichment culture procedures. (C) Average numbers of total SH1 and KH1 phages in RAMS samples from phage-treated steers.

(32), we identified all four *E. coli* O157:H7 strains in similar numbers among the isolates cultured from the phage-treated steers on days 7, 14, and 16. This indicated that all strains were able to survive the phage treatment (data not shown). However, these isolates were each sensitive to KH1 and/or SH1 (data not shown). Very often, every *E. coli* O157:H7 colony recovered from murine fecal or bovine RAMS samples from
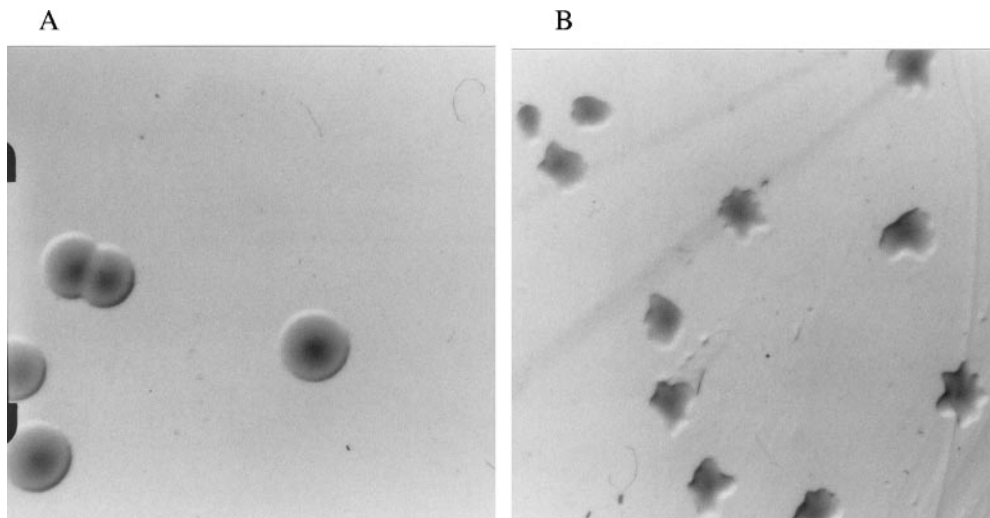
FIG. 3. Digital photographs of representative *E. coli* O157 colonies from (A) control steers or mice and (B) phage-treated steers or mice. Colonies are on SMAC-CTVM plates incubated for 18 h at 37°C.

phage-treated animals differed in morphology from those recovered from control animals (occurring in about one of three cultured samples; data not shown). Colonies of *E. coli* O157:H7 recovered from some mice and steers in the phage treatment groups were relatively small and had irregular star-shaped edges on SMAC-CTVM plates (Fig. 3A). This was in contrast to *E. coli* O157:H7 recovered from control animals, not treated with phage, which had relatively larger colonies with rounded smooth edges (Fig. 3B). Restreaking these irregularly shaped colonies onto LB agar or SMAC-CTVM agar yielded colonies with normal morphology and cells that were susceptible to both KH1 and SH1 phage infections.

DISCUSSION

The most important finding of this study was that a combination of the previously described phage KH1 and a newly isolated and characterized phage SH1 reduced the number of *E. coli* O157:H7 CFU in cattle. This was achieved by applying the phage directly and repeatedly to the RAJ and by maintaining a continuous supply of phage in the drinking water. Although shedding was significantly reduced, the phage treatment did not clear *E. coli* O157 from the majority of steers. This was in contrast to pilot work done in a mouse model of intestinal *E. coli* O157 carriage, in which this phage combination or SH1 phage alone rapidly (4 to 6 days) cleared the bacteria from all test animals.

Ruminants, and especially cattle and sheep, are the major carriers of *E. coli* O157:H7. The recently described and unusual colonization site is the RAJ mucosa (26), and experimental carriage of *E. coli* O157:H7 can be achieved by application of the bacteria directly to this site (38). Since foods of bovine origins have been implicated in numerous outbreaks (1, 5, 17), control measures to reduce or eliminate *E. coli* O157:H7 from cattle would likely reduce food-borne infection but would also have a substantial impact on the presence of the pathogen in the farm/feedlot environments and could reduce waterborne and animal contact infections. Preharvest treatment of cattle

with lytic phage is considered one option for the control of *E. coli* O157:H7 (42). A number of in vitro studies have been carried out in several laboratories to evaluate the abilities of a single phage or phage cocktails to eliminate *E. coli* O157:H7 (2, 21, 44). In beef or in *E. coli* O157:H7 culture-positive sheep or mice, phage treatments reduced the number of bacteria compared to untreated controls but did not eliminate the bacteria (2, 27, 45).

Phage KH1 treatment in sheep did not affect intestinal *E. coli* O157:H7, and the numbers detected by fecal culture among the phage-treated group were not statistically different from the untreated control group. The use of orally dosed sheep is consistent with the mode by which natural infections occur and addresses the survival of *E. coli* O157:H7 throughout the gastrointestinal tract, not just cells attached at the RAJ mucosa, when phage treatment is given. Our results were consistent with work by Bach et al. (2), in which the number of *E. coli* O157:H7 CFU shed by a phage DC22-treated sheep did not differ significantly from an untreated control group. Nonetheless, it was surprising that repeated large oral doses of phage KH1 that resulted in calculated ratio of phage/bacteria estimated as high as $>10^3$ did not affect *E. coli* O157:H7 carriage. This may have been due to specific conditions of the intestinal tract and/or the nature of the KH1 phage.

In an effort to enhance the phage treatment, a new phage, SH1, was isolated, characterized, and tested with KH1 in a mouse model of intestinal carriage of *E. coli* O157:H7. Mice, similar to cattle, do not become ill with *E. coli* O157:H7, and an oral dose of the bacteria results in transient intestinal carriage (12, 43). It was encouraging that treatment with phage SH1 alone or in combination with KH1 rapidly (within 2 to 6 days) cleared *E. coli* O157:H7 from mice.

Because the oral phage treatment in sheep reported here and previous oral phage trials in sheep and in cattle did not clear the intestinal bacteria and because the site of *E. coli* O157:H7 colonization (the RAJ mucosa) is accessible in live animals, we developed a phage treatment protocol that administered the KH1 and SH1 phages directly to this site. This

treatment entailed repeated applications of high-titer phages and was successful in reducing the number of *E. coli* O157 CFU carried by animals but did not clear *E. coli* O157:H7 from most animals (four of five). This striking difference between the outcomes of phage therapy for the mice and steers could have been due to many factors including the numbers of *E. coli* O157:H7 CFU surviving in the intestinal tract after oral dose, the accessibility of *E. coli* O157:H7 attached to the RAJ mucosa, differences in actual phage/bacterium ratios, and/or differences between the environments of the monogastric and ruminant intestinal tracts.

The nature of *E. coli* O157:H7 carriage in the mouse intestine is different than in cattle and likely contributed to the effectiveness of the phage therapy. Although mice can carry *E. coli* O157:H7 for several weeks following a single oral dose, no specific site of colonization for this serotype has been identified in mice, and it may be that the bacteria persist primarily in the intestinal lumen digesta. Oral doses of *E. coli* O157:H7 to cattle result in brief carriage (<10 days, similar to the mice) or long-term carriage (>1 month), which most often means the animals are colonized at the RAJ mucosa (6, 8, 16, 26, 32). Application of *E. coli* O157:H7 directly to the RAJ mucosa in this study assured that all animals became colonized (38). Also, the timing of phage treatment could be an important factor. The phage treatment in the mice began 24 h after bacterial challenge, whereas the phage treatment in the steers began 7 days after *E. coli* O157:H7 application. This duration was chosen for the cattle trial so that *E. coli* O157:H7 had time to colonize at the RAJ mucosa. However, phage may more easily lyse *E. coli* O157:H7 cells that are free in the digesta and/or effective bacterial contact may be hampered when bacteria are attached to the intestinal mucosa.

The bovine RAJ mucosa is heavily coated with mucin, and this substance may provide a protective barrier for *E. coli* cells (31, 46). Although tests showed that mucin did not block the interaction between the phage and the bacteria, the complex in vivo conditions were not replicated in the laboratory. Studies to localize *E. coli* cells in the murine intestine using fluorescent oligonucleotide probes targeting rRNA in situ show that *E. coli* cells form microcolonies that are embedded in the mucosal material overlying the epithelial cells (31). It is possible that *E. coli* O157 was similarly embedded in the mucous gel layer at the RAJ and that this matrix reduced the efficiency of the phage-bacterium contact. If phage could not penetrate the mucus gel and accumulate to sufficiently high concentrations to result in appropriate phage/bacteria ratios, *E. coli* O157:H7 microcolonies would not be destroyed.

The emergence of *E. coli* O157:H7 insensitive or resistant to phage would also impair phage therapy. Although we did not identify the bacterial receptor for phage SH1, various bacterial outer membrane components have been identified for other phages and include lipopolysaccharide (LPS) and porin protein OmpF for phage K20 in *E. coli* K-12 (47); LPS and OmpC for phage AR1 (49), phage PP01 (25), and phage SP21-22 in *E. coli* O157:H7 (24, 44); and LPS for phage KH1 in *E. coli* O157:H7 (21). The loss of an outer membrane protein and/or alternation of LPS can result in bacteria that are resistant to a particular phage. In this study, we did not find evidence of phage resistance to either KH1 or SH1 among the *E. coli* O157:H7 isolates from animals receiving phage therapy. How-

ever, in some samples we did see unusual colony morphology with highly irregular edges that may have been due to cells resistant to phage, the presence of free phage in the sample, or phage attached to cells. Upon subculture to fresh medium, the colonies appeared normal. Phage, if present, may have been diluted, or resistant cells may have reverted by this restreaking procedure. Although the phenomenon of phage resistance and then reversion to phage sensitivity has been reported by others (27), in these studies, all isolates were sensitive to both KH1 and SH1 phages at every analysis.

Finally, although it is clear that reducing the number of *E. coli* O157:H7 CFU in cattle by phage treatment is possible, efforts to consistently clear *E. coli* O157:H7 from cattle with phage therapy may be unrealistic. The situations in which phage therapy is successful involve infections (3, 7, 23, 39, 41). Good examples of effective phage therapy for *E. coli* infections include treatment of calves and piglets for enteropathogenic *E. coli* diarrhea and treatment of chickens for *E. coli* septicemia and meningitis (3, 39). Similar to the effects of antibiotics, phage therapy reduces the number of bacteria so that the immune response can gain the upper hand and eliminate the infectious agent. However, natural or experimental carriage of *E. coli* O157:H7 is not an infection, and this serotype, as a member of the transient normal flora, does not illicit a strong immune response in cattle or sheep during its natural association with these animals. Thus, even when phage treatment is successful at reducing the number of bacteria, without a strong immune response the bacteria may not be eliminated.

All these factors make it difficult to predict the consequences of a particular phage treatment without empirical analysis. Payne and Jansen (30) use a mathematical model to illustrate that phage therapy is a kinetic process with density-dependent qualities for phage-bacterium interactions. Inoculum size, inoculum timing, phage burst size, appearance of phage-resistant or insensitive mutants, and/or host factors such as antiphage immune response (40) all play roles in this dynamic process. Our work showed that phage therapy would be effective at reducing the number of intestinal *E. coli* O157:H7 CFU in ruminants but highlighted the difficulties of developing an effective phage intervention to eliminate *E. coli* O157:H7.

ACKNOWLEDGMENTS

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