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Abstract

Isogenic strains of *Escherichia coli* O157:H7, missing either stx_2 or the entire Stx2-encoding phage, were compared with the parent strain for their abilities to colonize sheep. The absence of the phage or of the Shiga toxin did not significantly impact the magnitude or duration of shedding of *E. coli* O157:H7.

Disciplines

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Comments

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Shiga Toxin and Shiga Toxin-Encoding Phage Do Not Facilitate *Escherichia coli* O157:H7 Colonization in Sheep[▽]

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Isogenic strains of *Escherichia coli* O157:H7, missing either stx_2 or the entire Stx2-encoding phage, were compared with the parent strain for their abilities to colonize sheep. The absence of the phage or of the Shiga toxin did not significantly impact the magnitude or duration of shedding of *E. coli* O157:H7.

The major reservoirs for Shiga toxin-producing *Escherichia coli* (STEC) are cattle and other ruminants. These bacteria are carried in the gastrointestinal tract and do not cause disease in mature ruminants even when administered at very high doses (2). It is not known why the majority of ruminants are colonized by STEC in contrast to other animal species. One of the major virulence factors of STEC is Shiga toxin (Stx), an A_1B_5 toxin which is encoded by lysogenic bacteriophages. The preferred receptor for the toxin is globotriaosylceramide (Gb_3). It has been shown that cattle lack Gb_3 receptors in their vascular endothelium, and it is thought that this may protect them from the systemic effects of Stx (9, 14). It has also been proposed that Stx may reduce the severities of viral infections such as bovine leukemia virus (4, 5) or modulate intestinal inflammation (12) in cattle.

The objective of this study was to determine whether the Stx2-encoding bacteriophage or the Shiga toxin itself contributed to the magnitude or duration of colonization by *E. coli* O157:H7 in ruminants.

(A preliminary report of this work was presented at the symposium Beyond Antimicrobials, the Future of Gut Microbiology, Aberdeen, Scotland, 2002.)

The inoculum stains used in this study are listed in Table 1. Spontaneous nalidixic acid-, novobiocin-, and/or streptomycin-resistant mutants were selected from parent strain 86-24 and its isogenic derivatives. Strain 86-24 Δϕ933 was made as previously described by site-directed mutagenesis to delete stx_2 (15). Some mutants not only lost the stx_2 operon but suffered deletions in the region flanking the deleted stx_2 operon, suggesting that these mutants may have lost the entire ϕ933 phage. Loss of the phage was confirmed by a PCR assay that utilized several sets of primers (designed based on the published nucleotide sequence for ϕ933 [13]) to amplify approximately 1-kb portions from different regions of the ϕ933 genome. The loss of ϕ933 in strain 86-24 Δϕ933 was also verified by challenging exponentially growing broth cultures of this strain and a ϕ933-positive *E. coli* O157:H7 strain with subinhibitory amounts of norfloxacin (11). Inoculum strains were grown overnight in Trypticase soy broth and frozen in glycerol at −80°C until use.

All experiments complied with the Iowa State University Animal Care and Use guidelines. Young adult sheep (4 to 12 months old, 80 to 120 lb) were purchased from a local source, housed two per pen under biosafety level 2 conditions, and acclimated to a diet of commercial sheep feed concentrate (1 lb/animal/day) and alfalfa/grass hay ad libitum for 2 weeks prior to inoculation as described previously (1). Sheep were randomly divided into three groups and inoculated with 10^{10} CFU of either wild-type *E. coli* O157:H7 (strain 86-24, six sheep), an isogenic stx_2 deletion mutant (strain TUV-86-2, eight sheep), or an isogenic phage-cured mutant (strain 86-24 Δϕ933, eight sheep). Fecal samples were collected on days 2, 3, 4 (initial period), 13, 14, 15 (2 weeks), 28, 29, 30 (1 month), 58, 59, and 60 (2 months) postinoculation (p.i.) and cultured for the inoculum strains. Briefly, 5 g of fresh fecal pellets was diluted 1:5 in phosphate-buffered saline, processed in a stomacher, and then serially diluted 10-fold in phosphate-buffered saline. Dilutions (100 μl) were spread onto selective medium as described previously (1). A 10-g sample was also incubated overnight in enrichment broth (100 ml Trypticase soy broth with 0.15% bile salts), concentrated using immunomagnetic beads (Dynal Biotek, Oslo, Norway), and cultured in selective medium. Data from individual sheep, collected over each 3-day time period, were averaged together using geometric means. Differences in the magnitudes of shedding between groups of sheep were evaluated using repeated measures of analysis of variance. A power analysis was used to determine the likelihood of obtaining statistically significant results. This analysis determined that using eight sheep per group would give an 80% probability of detecting significant treatment differences (in this case, strains) at P values of ≤ 0.05.

A fourth group of eight sheep was dually inoculated with 10^{10} CFU of both the wild-type strain and the phage-cured mutant. Fecal samples were collected as described above and plated onto selective agar (1). Differences in the magnitudes of shedding between strains were evaluated using a paired t test. The competitive index (CI) was calculated using the formula $CI = (X - Y)/(X + Y)$, where X is the value for the phage-cured mutant and Y is that for the wild-type strain (10). All sheep were necropsied at 2 months p.i., and 5 g of tissue (rumen, ileum, Peyer's patch, cecum, spiral colon, and distal

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TABLE 1. *E. coli* O157:H7 strains used in this study

Strain	Virulence gene(s)[a]	Antibiotic resistance phenotype	Source	Reference
86-24	stx_2, *eae*	Nal Nov	Human	7
86-24 Δϕ933	*eae*	Str Nal	Construct	15
TUV-86-2	*eae*, stx_2[c]	Nal Nov	Construct	8

[a] A multiplex PCR was used to detect stx_1, stx_2, *eae*, *f41*, *k99*, and *sta* (6).
[b] Nal, nalidixic acid; Nov, novobiocin; Str, streptomycin.
[c] Nonfunctional.

colon) was cultured in enrichment broth for 24 h, followed by immunomagnetic concentration and plating onto selective medium. We did not collect samples from the recto-anal junction since some of the animals in our study were necropsied prior to the description of this location as the primary colonization site of *E. coli* O157:H7.

Portions of the ϕ933 phage were successfully amplified from the DNA of 86-24 containing ϕ933 but not from the DNA of the 86-24 strain that had suffered a deletion of stx_2 and the flanking region (data not shown). While cultures of strain 86-24 Δϕ933 exhibited normal exponential growth for several hours in the presence of norfloxacin, the isogenic parent strain experienced an abrupt decline in culture turbidity in the presence of norfloxacin (data not shown). The phase of abrupt decline in the culture turbidity of the parent strain correlates with phage induction and the release of phage particles due to bacterial cell lysis (17). The magnitudes of colonization by strain 86-24 and its isogenic mutants (TUV-86-2 and 86-24 Δϕ933) were not significantly different between any groups of sheep over the first 2 weeks of the experiment (Fig. 1). At 30 days p.i., *E. coli* O157:H7 was recovered from five of six sheep given the wild-type strain, from seven of eight sheep given the toxin mutant, and from two of eight sheep given the phage-cured mutant (<50 CFU/g). At necropsy (60 days p.i.), strain 86-24 was recovered from the feces of three of six sheep (<50 CFU/g) and from the ileum, spiral colon, and distal colon of one of those sheep. The stx_2 deletion mutant was recovered from the ileum (10^2 CFU/cm), cecum, spiral colon, and distal colon (10^4 CFU/cm) of one of eight sheep. The phage-cured mutant was not recovered from any necropsy samples.

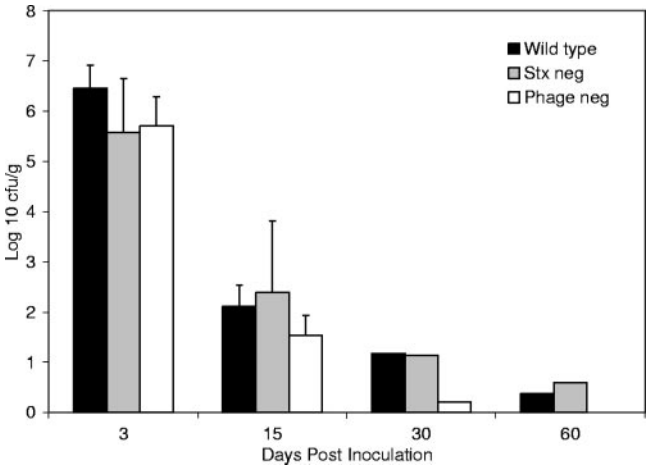

FIG. 1. Mean values for fecal shedding of *E. coli* O157:H7 by sheep inoculated with isogenic strains.

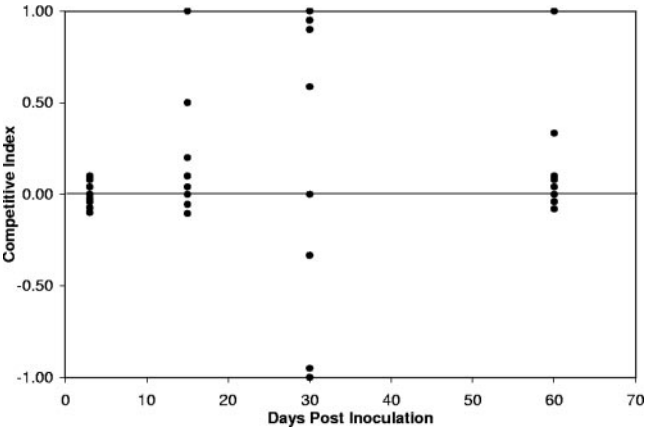

FIG. 2. In vivo competition of isogenic *E. coli* O157:H7 strains in sheep ($n = 8$). Each circle represents a single sheep that was inoculated with both *E. coli* O157:H7 strain 86-24 and an isogenic phage-cured strain (86-24 Δϕ933). CI was calculated by the equation $CI = (X - Y)/(X + Y)$, where X is the value for the phage-cured strain and Y is that for the parent strain. CIs near +1 indicate that the phage-cured mutant was the predominant strain recovered, and CIs near −1 indicate that the parent strain was the predominant strain recovered.

To corroborate whether the apparent difference in persistence at 30 days p.i. between the phage-cured mutant and the other two groups was biologically significant, another group of sheep was dually inoculated with both the wild-type parent strain and the phage-cured mutant. When both of these strains were inoculated into the same animal, the absence of the phage did not significantly inhibit or enhance either the magnitude or the duration of colonization by *E. coli* O157:H7 (Fig. 2). The magnitude of shedding for both strains ranged from 60 to 3.5×10^6 CFU/g during the initial period p.i., from undetectable to 1.8×10^3 CFU/g at 2 weeks p.i., from undetectable to 3×10^4 CFU/g at 1 month p.i., and from undetectable to <50 CFU/g at 2 months p.i.. At the completion of the experiment (2 months p.i.) both *E. coli* O157:H7 strains were recovered from the feces of two of eight sheep, and the phage-cured strain was recovered from the feces of one additional animal. At necropsy, strain 86-24 was recovered from the ileum of one animal and the phage-cured strain was not recovered from any tissues.

We were not able to demonstrate any significant differences in the magnitudes or durations of colonization between wild-type *E. coli* O157:H7, an isogenic stx_2 deletion mutant, and an isogenic phage-cured mutant. However, our experiment was not designed to identify the effects of Stx and the Stx-encoding phage in *trans*. Since the majority of ruminants are colonized by STEC at some point in their lives, it is likely that at least some of our sheep carried STEC and that these strains could have influenced the colonization of our inoculum strains. It has been previously shown that a nontoxigenic *E. coli* O157:H7 strain colonized and persisted in weaned sheep (18), and this occurrence was observed more recently in cattle (16). A pilot study of weaned calves suggested that the presence of the Stx-encoding phage may enhance colonization of *E. coli* O157:H7 in ruminants (3). However, the number of animals in

the pilot study was small, and furthermore, isogenic strains of *E. coli* O157:H7 were not compared in either of the previous studies. Our work using a competitive-colonization approach indicated that there was considerable variability between individual animals (Fig. 2). For example, on day 30 p.i., the phage-cured mutant was recovered in greater numbers from four of eight sheep and the parent strain was recovered in greater numbers from three of eight sheep. By day 60, equal numbers of wild-type and phage-cured-mutant bacteria were recovered from the majority of sheep (six of eight). This suggests that the populations of individual *E. coli* strains are constantly in flux in vivo and that overall, the Stx2-encoding phage did not influence the maintenance of STEC within the gastrointestinal tract of mature sheep.

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