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Role of *Escherichia coli* O157:H7 Virulence Factors in Colonization at the Bovine Terminal Rectal Mucosa

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The human pathogen *Escherichia coli* O157:H7 causes hemorrhagic colitis and life-threatening sequelae and transiently colonizes healthy cattle at the terminal rectal mucosa. This study analyzed virulence factors important for the clinical manifestations of human *E. coli* O157:H7 infection for their contribution to the persistence of *E. coli* in cattle. The colonizing ability of *E. coli* O157:H7 was compared with those of nonpathogenic *E. coli* K-12 and isogenic deletion mutants missing Shiga toxin (Stx), the adhesin intimin, its receptor Tir, hemolysin, or the ~92-kb pO157. Fully ruminant steers received a single rectal application of one *E. coli* strain so that effects of mucosal attachment and survival at the terminal rectum could be measured without the impact of bacterial passage through the entire gastrointestinal tract. Colonization was monitored by sensitive recto-anal junction mucosal swab culture. Nonpathogenic *E. coli* K-12 did not colonize as well as *E. coli* O157:H7 at the bovine terminal rectal mucosa. The *E. coli* O157:H7 best able to persist had intimin, Tir, and the pO157. Strains missing even one of these factors were recovered in lower numbers and were cleared faster than the wild type. In contrast, *E. coli* O157:H7 strains that were missing Stx or hemolysin colonized like the wild type. For these three strains, the number of bacteria increased between days 1 and 4 postapplication and then decreased slowly. In contrast, the numbers of noncolonizing strains (K-12, Δ*tir*, and Δ*eae*) decreased from the day of application. These patterns consistently predicted long-term colonization or clearance of the bacteria from the bovine terminal rectal mucosa.

Enterohemorrhagic *Escherichia coli* (EHEC) strains are a subset of Shiga toxin-producing *E. coli* (STEC) that can cause human disease and are threats to public health worldwide (46, 49). Human illnesses caused by EHEC range from self-limiting watery diarrhea or hemorrhagic colitis to life-threatening sequelae, the hemolytic-uremic syndrome or thrombotic thrombocytopenic purpura. The predominant EHEC serotype associated with the most severe disease in North America, the United Kingdom, and Japan is O157:H7 (23, 42, 44, 46, 59).

Cattle are considered the primary reservoir for *E. coli* O157:H7 and the most common source for food-borne and direct animal contact infections (5, 25, 69). Healthy cattle carry *E. coli* O157:H7 transiently without suffering pathological symptoms (2, 4, 26). Individual animals can passively shed *E. coli* O157:H7 in their feces for a short time (a few days) without establishing a colonized state or can pass fecal *E. coli* O157:H7 for a longer time (a month or more) if the bacteria colonize and persist (22). The conditions that lead to these different host-bacterium interactions are not understood.

It is well accepted that reducing the carriage or prevalence of *E. coli* O157:H7 in cattle would reduce the risk of human exposure to this pathogen (61). Recently, the recto-anal junction (RAJ) mucosa was identified as the primary site of *E. coli* O157:H7 colonization in cattle (47). Based on this finding, we demonstrated that culture of recto-anal mucosal swabs (RAMS) is easier and more sensitive than fecal culture to detect the bacteria (51) and that administration of *E. coli* O157:H7 to the terminal rectal mucosa is a reliable method to establish colonized cattle experimentally (58). Development of effective interventions to reduce bovine carriage of *E. coli* O157:H7 will rely on understanding the bacterial factors involved in *E. coli* O157:H7 colonization and persistence at the RAJ mucosa. Virulence factors that contribute to the clinical manifestations of *E. coli* O157:H7 infection in humans or other mammalian species, such as rabbits or gnotobiotc pigs (40, 52), may be the same factors important for *E. coli* O157:H7 colonization and/or persistence in cattle.

In a previous study, we identified a naturally occurring, RAJ-colonizing STEC strain prevalent in cattle and similar to *E. coli* O157:H7 (57). This *E. coli* ONT:H25 isolate shares at least five putative virulence genes with *E. coli* O157:H7, including stx_2 (encodes Shiga toxin type 2), *eae* (encodes intimin), *tir* (encodes the translocated intimin receptor [Tir]), *exhA* (encodes enterohemolysin), and a pO157-like plasmid (57; unpublished data). We hypothesized that these common virulence factors contribute to the colonization and persistence of the bacteria at the bovine terminal rectal mucosa. Studies in mature, fully ruminant cattle have not investigated the role of these five most prominent virulence factors in EHEC colonization at the RAJ mucosa. Also, it is important to assess whether or not nonpathogenic *E. coli* without any of the aforementioned virulence factors can colonize this site. Does the O157:H7 serotype have an interaction with the terminal rectal tissue that is unique compared with other *E. coli*? Since many nonpathogenic *E. coli* strains can be cultured from RAMS samples, analysis of their persistence would be instructive in understanding the factors required for bacterial colonization.

The purpose of this study was to analyze specific virulence factors and their contribution to the persistence of *E. coli* at the bovine RAJ mucosa. To that end, we (i) compared the RAJ

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TABLE 1. Bacterial strains

Strain	Description	Source or reference
K-12	*E. coli* MG1655 strain	Laboratory stock
K-12 Nal^r	*E. coli* K-12 with nalidixic acid resistance	This work
43894	*E. coli* O157:H7 ATCC 43894, clinical isolate, stx_1^+/stx_2^+	ATCC
43895	*E. coli* O157:H7 ATCC 43895, clinical isolate, stx_1^+/stx_2^+	ATCC
905	*E. coli* O157:H7, clinical isolate, $\Delta stx_1/stx_2^+$	52
43895 Nal^r	*E. coli* O157:H7 ATCC 43895 with nalidixic acid resistance	This work
905 Δstx_2	*E. coli* O157:H7 strain 905 with stx_2 deletion	52
43895 Δeae	*E. coli* O157:H7 ATCC 43895 with intimin deletion	This work
43895 Δtir	*E. coli* O157:H7 ATCC 43895 with translocated intimin receptor deletion	This work
43895 $\Delta ehxA$	*E. coli* O157:H7 ATCC 43895 with hemolysin deletion	This work
43894 $\Delta ehxA$::Kan^r	*E. coli* O157:H7 ATCC 43894 with hemolysin deletion and kanamycin resistance	This work
277	*E. coli* O157:H7 ATCC 43894 with pO157 plasmid cured	J. Shaw[a]
277::pO157 $\Delta ehxA$	*E. coli* O157:H7 strain 277 complementation with pO157 $\Delta ehxA$	This work

[a] Armstrong Laboratory, Brooks Air Force Base, Texas.

mucosa-colonizing ability of nonpathogenic *E. coli* K-12 with that of wild-type *E. coli* O157:H7, (ii) engineered isogenic gene deletion mutants that were each missing one of four virulence factors (*eae*, *tir*, *ehxA*, and the pO157), and (iii) compared each deletion mutant and a previously constructed Stx-negative strain with the corresponding wild-type *E. coli* O157:H7 strain for the ability to colonize the bovine RAJ mucosa. In all trials, animals received a single rectal application of one *E. coli* strain and colonization was monitored by the highly sensitive RAMS culture technique.

MATERIALS AND METHODS

Bacterial strains and reagents. The bacteria used in this study are shown in Table 1. *E. coli* O157:H7 ATCC 43894 is a human isolate from a patient with hemorrhagic colitis, and ATCC 43895 is an isolate from raw hamburger meat implicated in a hemorrhagic colitis outbreak. Strain 905 is a human isolate from a patient with hemolytic-uremic syndrome (52, 53). The reagents were used as follows: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 35 μg/ml; nalidixic acid, 25 μg/ml; cefixime, 50 ng/ml; potassium tellurite, 2.5 μg/ml; 4-methylunbelliferyl-β-D-glucuronide (MUG), 0.1 mg/ml. Antibiotics and MUG were purchased from Sigma Aldrich (St. Louis, MO). Oligonucleotide primers were purchased from Invitrogen (Carlsbad, CA).

Construction and confirmation of virulence gene deletion mutants. The *eae*, *tir*, and *ehxA* genes of *E. coli* O157:H7 ATCC 43895 or ATCC 43894 were deleted by the λ Red recombinase system described by Datsenko and Wanner (7). The primers used for deletion of *eae*, *tir*, and *ehxA* were described by Ritchie et al. (52). Briefly, the primers contained 40 to 45 nucleotides corresponding to the regions adjacent to the genes targeted for deletion and 20 additional nucleotides that annealed to the template DNA from pKD4. This plasmid carries a kanamycin resistance gene flanked by FRT sites. Electrocompetent cells were prepared by growing *E. coli* O157:H7 carrying pKD46 in Luria-Bertani (LB) broth (Difco, Detroit, Mich.) containing 0.1 mM L-arabinose, and the PCR products were introduced by electroporation (15). The kanamycin resistance gene was eliminated by introducing the plasmid pCP20 encoding FLP recombinase. Plasmids pKD46 and pCP20 were then cured at 42°C. This procedure resulted in a deletion of the target gene and no remaining antibiotic resistance gene. The gene deletions were analyzed by PCR and confirmed by DNA sequencing on a Perkin-Elmer thermocycler using ABI Big Dye sequencing mix according to the manufacturer's instructions and an ABI no. 3730 genetic analyzer.

To compare the hemolysin activities of wild-type 43895 and 43895 Δ*ehxA*, the strains were grown on tryptose agar plates (Difco Laboratories, Detroit, Mich.) supplemented with 10 mM $CaCl_2$ and 5% defibrinated sheep blood cells, as described previously (57). Inoculated plates were observed for hemolysis after overnight incubation in ambient air at 37°C. To test Stx production in wild-type 905 and 905 Δstx_2, cells were grown in LB broth and an enzyme immunoassay for Stx (Premier EHEC; Meridian Diagnostics, Inc., Cincinnati, Ohio) was performed according to the manufacturer's instructions. Results were analyzed spectrophotometrically at two wavelengths, 450 and 630 nm, in a PowerWave XS reader (Bio-Tek, Winooski, Vermont). In both the hemolysin and toxin assays, *E. coli* K-12 (MG1655) and ATCC 43894 were used as negative and positive controls, respectively.

Extraction of pO157 and construction of a pO157-complemented strain. The pO157-cured *E. coli* O157:H7 strain 277 used in this study was a gift from J. Shaw (Brooks Air Force Base, Texas). This strain was cured using standard mini-F incompatibility, a procedure that did not mutate chromosomal DNA. The complemented pO157 strain was constructed with the pO157 isolated from *E. coli* O157:H7 ATCC 43894 Δ*ehxA* retaining the inserted kanamycin resistance gene (constructed as described above). The kanamycin resistance gene that replaced *ehxA* on this pO157 served as the selection marker. The pO157 was extracted from this strain by the method described by Kado and Liu (34) with slight modifications. Briefly, the *E. coli* O157:H7 43894 Δ*ehxA* :: Kan^r was streaked on a 1-cm^2 area of an LB-Kan agar plate and incubated at 37°C overnight. About one-fourth of the colonies on the 1-cm^2 area were scraped off and suspended in 100 μl lysing solution (3% sodium dodecyl sulfate, 50 mM Tris, with pH 12.6 adjusted by 2 N NaOH). The suspension was incubated at 55°C for 60 min and emulsified in an equal volume of phenol-chloroform. After centrifugation at 6,000 × *g* for 5 min at 4°C, the upper aqueous phase was transferred to a new tube. A portion of the sample was electrophoresed through a 0.7% agarose gel for plasmid detection. The remaining sample was extracted with diethyl ether. Traces of ether were removed by heating the solution to 68°C for 5 min and transferring the solution to a new tube. The plasmid DNA was precipitated by the tRNA/ethanol method described by Zhu and Dean (70). Briefly, 30 μl of DNA solution was mixed with 5 μg/μl yeast tRNA (Invitrogen) and 2 volumes of 100% cold ethanol was added to precipitate the plasmid DNA. The sample was cooled on crushed dry ice for 10 min and centrifuged at 14,000 × *g* for 10 min at room temperature. The pellet was washed with 80% ethanol twice, air dried, and resuspended in 2 μl of ultrapure water. The purified plasmid was transformed into the pO157-cured strain 277 by electroporation to make a complemented mutation. The complemented 277::pO157 Δ*ehxA* was confirmed by plasmid extraction and PCR for the existence of the plasmid-encoded *eae*-conserved fragment gene (*ecf*) and lack of *exhA* on the pO157.

Animals. Five- to eight-month-old, fully ruminant Holstein steers were used in all experiments. Steers were fed alfalfa hay twice and pellet grain feeds once daily and had free access to drinking water. Animals were housed in quarantined facilities in which feed, water, bedding, manure, and waste were not in contact with other animals. All personnel followed strict biosafety procedures, and all procedures were approved by the Institutional Animal Care and Use and Biosafety Committees.

Rectal application of bacteria in cattle. Prior to application of bacteria, the steers were confirmed to be culture negative for *E. coli* O157:H7 by the culture method described below. Bacterial strains were grown separately in LB at 37°C for 18 h with aeration. Bacteria were applied to the bovine terminal rectal mucosa as previously described (58). Briefly, feces were manually cleared from the terminal rectum. A 10-cm by 3.5-cm diameter cylindrical sponge (Rubbermaid, St. Francis, WI) with a wooden handle was saturated with 10 ml overnight culture containing 10^9 CFU bacteria/ml, inserted into the anus, and gently rubbed against the terminal rectal mucosa. After swabbing, defecation was prevented for 10 min by holding the tail firmly against the anus. This presumably ensured that the applied bacteria were retained in the rectal lumen for sufficient time to attach to the mucosa.

Four separate trials were done. In the first trial, nalidixic acid-resistant (Nal^r)

E. coli K-12 and *E. coli* O157:H7 43895 Nal^r were applied rectally to two different groups of four steers. In the second trial, wild-type *E. coli* O157:H7 43895 and three mutants, 43895 Δ*eae*, 43895 Δ*tir*, and 43895 Δ*ehxA*, were applied rectally to each of four different groups of six steers. In the third trial, *E. coli* O157:H7 905 and its isogenic Stx$^-$ mutant 905 Δstx_2 were applied rectally to each of two different groups of four steers. In the fourth trial, *E. coli* O157:H7 43894, 277 (pO157-cured mutant), and 277::pO157 Δ*ehxA* (pO157-complemented strain) were each applied rectally to three different groups of four steers.

Bacterial culture and enumeration. Following bacterial applications, RAMS samples were taken from each steer as previously described (51, 57) on days 1, 4, 7, 11, 18, 25, and 32. Briefly, the RAMS samples were homogenized with 3 ml ice-cold sterile Trypticase soy broth (Difco Laboratories, Detroit, Mich.) and kept ice-cold until processing. Direct and enrichment procedures were used for bacterial culture. For direct culture, serial dilutions of the RAMS homogenates were plated on sorbitol MacConkey agar supplemented with cefixime, potassium tellurite, vancomycin, and MUG (SMAC-CTVM) agar; for culture of the nalidixic acid-resistant strains *E. coli* O157 43895 Nal^r and K-12 Nal^r, LB containing 25 μg/ml nalidixic acid (LB-Nal25) was used. Plates were incubated at 37°C overnight and observed for colonies not fermenting sorbitol (colorless colonies) and not hydrolyzing MUG (no florescence at 363 nm) or colonies resistant to nalidixic acid on LB-Nal25 plates. *E. coli* O157:H7 colonies were confirmed by a latex agglutination test (Pro-Lab Diagnostics, Toronto, Canada), and K-12 colonies recovered from cattle were confirmed by a pulsed-field gel electrophoresis (PFGE) fingerprinting method as described previously (57). *E. coli* O157:H7 isolates from animals that received a deletion mutant were confirmed to be carrying the appropriate gene deletion by PCR. Wild-type and deletion mutant isolates from cattle were periodically confirmed by PFGE to show that no gross chromosomal rearrangements had occurred. The CFU/swab were calculated from plate colony counts, converted to $\log_{10}$, and averaged for each group. Samples negative by direct culture were enriched by incubation at 37°C overnight with aeration, and serial dilutions were plated on SMAC-CTVM (or Nal25-LB where appropriate). For statistical analysis, RAMS samples positive only by enrichment culture were assigned a value of 10 CFU/swab and negative samples were assigned a value of 1 CFU/swab.

Statistical analysis. In the first and third trials, differences in the numbers of the bacteria in RAMS samples between *E. coli* O157:H7 and K-12 or between *E. coli* O157:H7 905 and its isogenic Shiga toxin-negative strain 905 Δstx_2 were evaluated with the Student *t* test. In the second and fourth trials, differences in the number of bacteria in RAMS samples between *E. coli* O157:H7 strains inoculated individually into steers were compared by repeated measures of analysis of variance of the logs of the group geometric mean of CFU/swab from all steers in each group. Graphpad Prism software 4.0 (San Diego, Calif.) was used for this analysis, and a Mantel-Haenszel chi square was used for comparison of the proportion of the steers that cultured positive for *E. coli* O157:H7 or the mutants.

RESULTS

Confirmation of gene deletion and pO157-cured strains. PCR analysis confirmed each engineered gene deletion (data not shown). In addition, sequence analysis confirmed the position of base deletions in the mutant Δ*eae*, Δ*tir*, and Δ*ehxA* genes, the corresponding open reading frames (ORFs) of strain 43895, and the "scar" regions of ~80 bases within each target gene, as previously described (7) (data not shown). The complemented mutant 277::pO157 Δ*ehxA* was confirmed by plasmid extraction and PCRs that were positive for the *ecf* gene and negative for the *exhA* gene (data not shown). Hemolysin assays showed colonies of 43895 Δ*ehxA* with no hemolytic zones, while wild-type colonies had small, clear zones of hemolysis (data not shown). Stx production was not detected from strain 905 Δstx_2 but was detected in the wild-type strain 905 (data not shown). All *E. coli* O157:H7 mutants had growth rates comparable with that of the corresponding parental wild-type strain in the laboratory in either LB or SMAC-CTVM selective medium (data not shown). Also, the two nalidixic acid-resistant *E. coli* strains K-12 and O157:H7 had comparable growth in LB-Nal25 (data not shown).

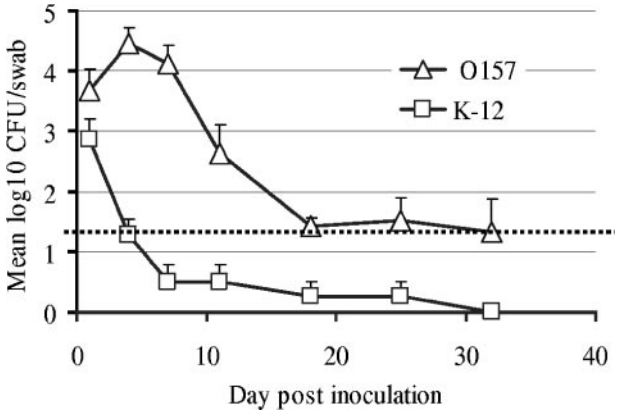

FIG. 1. Comparison of colonization of cattle by nonpathogenic *E. coli* K-12 or *E. coli* O157:H7. Two groups of four Holstein steers received a single rectal application of 1×10^{10} CFU of nalidixic acid-resistant nonpathogenic *E. coli* K-12 or *E. coli* O157:H7 ATCC 43895 Nal^r. The geometric means of log-transformed viable counts (CFU/swab) are shown for each strain cultured from RAMS samples on the indicated days. Values below the dotted line were positive by enrichment culture only, and values on the *x* axis were culture negative. The error bars indicate standard errors of the means.

Animal carriage of *E. coli*. All animals in this study were healthy and did not have ill effects from challenge with the wild type, mutant *E. coli* O157:H7, or *E. coli* K-12. All animals given a single rectal dose of wild-type *E. coli* O157:H7 carried the bacteria in a manner typical of experimental and natural infections with this microorganism (58).

Nonpathogenic *E. coli* K-12 did not colonize as well as *E. coli* O157:H7 at the bovine terminal rectal mucosa. To test the ability of nonpathogenic *E. coli* to colonize and persist at the terminal rectal mucosa, *E. coli* K-12 Nal^r or *E. coli* O157:H7 ATCC 43895 Nal^r was applied rectally to two groups of four steers. Colonization at the RAJ mucosa was monitored by culture of nalidixic acid-resistant bacteria in RAMS samples over the course of the experiment. Isolates were confirmed to be *E. coli* K-12 by a PFGE fingerprinting method as described previously (57) (data not shown).

The number ($P < 0.01$) of *E. coli* K-12 isolates was significantly lower than that of cultured from RAMS samples of *E. coli* O157:H7 isolates cultured over the first 11 days after bacterial application (Fig. 1). Also, distinct patterns in the numbers of *E. coli* K-12 and *E. coli* O157:H7 isolates cultured were seen. Among steers that received *E. coli* O157:H7, the mean number of these bacteria cultured increased between days 1 and 4 from 3.7 log units to 4.5 log units and then gradually decreased on all subsequent sampling days (Fig. 1). This initial increase in the number of *E. coli* O157:H7 between days 1 and 4 indicated that O157 not only successfully attached to the terminal rectal mucosa but also multiplied at that site. In contrast, the pattern of the mean number of *E. coli* K-12 isolates cultured on days 1 and 4 sharply decreased from 2.8 log units to 1.4 log units and continued to decrease on all subsequent sampling days. Three of four steers given *E. coli* O157:H7 continued to be culture positive on day 32, whereas only one of four steers given *E. coli* K-12 continued to be culture positive on day 25 and all four animals in this group eliminated the *E. coli* K-12 by day 32 (Fig. 1). Among the other

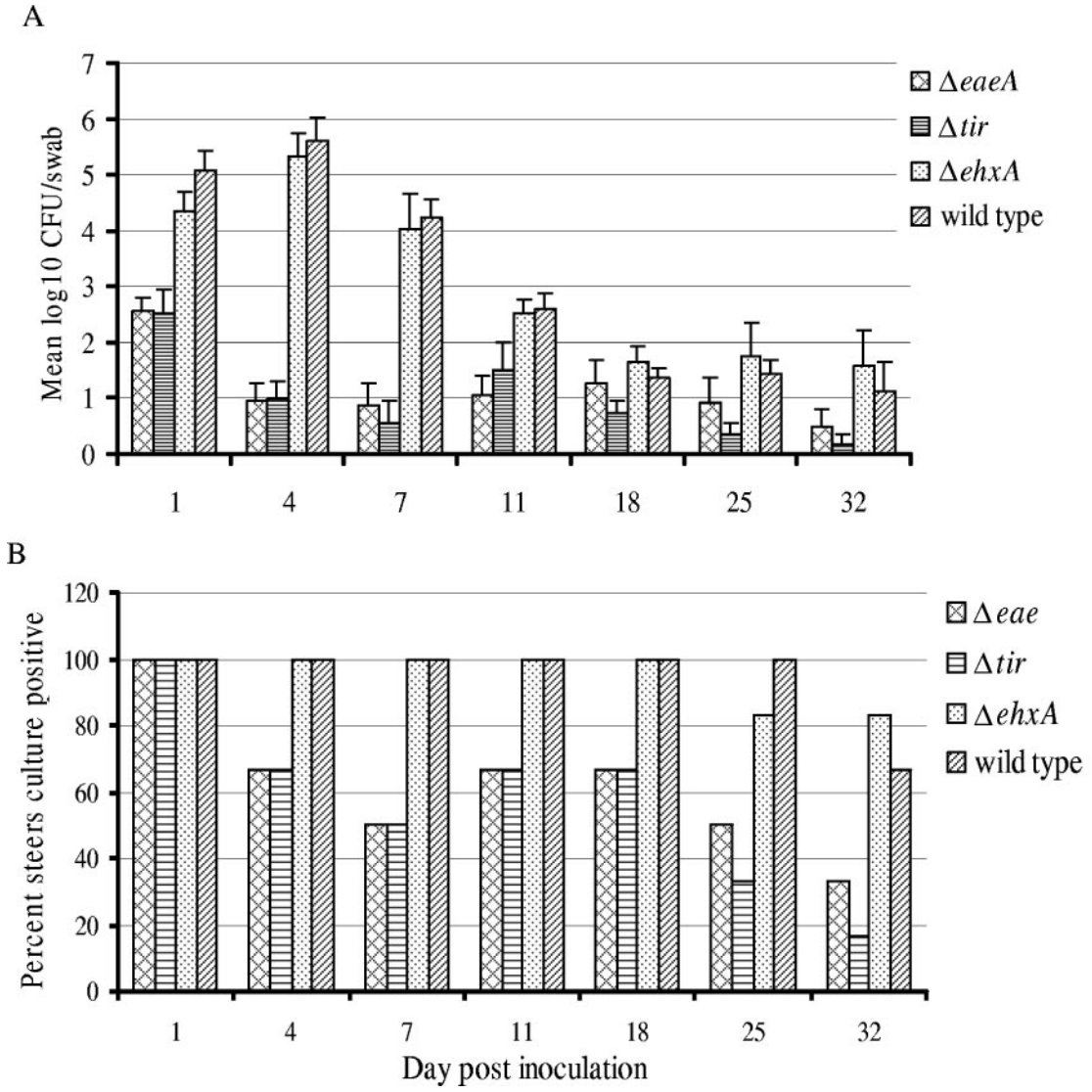

FIG. 2. Colonization of cattle by *E. coli* O157:H7 or deletion mutants missing intimin, the translocated intimin receptor, or hemolysin. (A) Four groups of six Holstein steers received a single rectal application of 1×10^{10} *E. coli* O157:H7 ATCC 43895 wild type or one of the isogenic deletion mutants: 43895 Δ*eae*, 43895 Δ*tir*, or 43895 Δ*ehxA*. Group geometric means of log-transformed viable counts (CFU/swab) are shown for each strain cultured from RAMS samples on the indicated days. (B) Percentages of steers that cultured positive for 43895 wild type, 43895 Δ*eae*, 43895 Δ*tir*, or 43895 Δ*ehxA* on the days indicated.

three animals that received *E. coli* K-12, two cleared the bacteria by day 7 and one cleared the bacteria by day 18. *E. coli* O157:H7 established a persistent colonization at RAJ mucosa, consistent with previous studies (57, 58, 68), and showed a distinct colonization pattern compared with the nonpathogenic *E. coli* K-12.

Intimin and Tir promoted initial adherence and colonization, but hemolysin did not influence colonization at the bovine terminal rectal mucosa. To address the roles of intimin, Tir, and hemolysin in colonization of fully ruminant cattle, four groups of six steers were rectally dosed with *E. coli* O157:H7 43895 (wild-type), 43895 Δ*eae*, 43895 Δ*tir*, or 43895 Δ*ehxA*. On each sampling day, isolates from steers that received a mutant strain of *E. coli* O157:H7 were verified by PCR analysis to be carrying the expected deletion (data not shown). The logs of the geometric means of the CFU of *E. coli* O157:H7/swab in each group and the percentage of culture-positive steers in each group are presented in Fig. 2.

Similar to the pattern seen for the numbers of *E. coli* O157:H7/swab in Fig. 1, the mean numbers of both the wild-type parent strain 43895 and the 43895 Δ*ehxA* strain increased between days 1 and 4 and then gradually decreased on the subsequent sampling days. In contrast, the mean numbers of both the 43895 Δ*eae* and 43895 Δ*tir* strains decreased between days 1 and 4 and continued to decline on the subsequent sampling days (Fig. 2A). This pattern was similar to that of the nonpathogenic *E. coli* K-12 (Fig. 1) and indicated that the ability of *E. coli* O157:H7 to colonize the bovine terminal rectal mucosa was notably impaired with deletion of *eae* or *tir*. A repeated-measures analysis of variance of these data, accounting for the

bacterial strain, day postapplication, and CFU/swab, showed that the differences between 43895 Δ*eae* and the wild type were significant on days 1, 4, 7 ($P < 0.01$), and 11 ($P < 0.05$), while the differences between 43895 Δ*tir* and the wild type were significant on days 1, 4, and 7 ($P < 0.01$). There were no differences ($P > 0.05$) between the CFU of *E. coli* O157 in RAMS samples from animals receiving 43895 Δ*ehxA* or the wild type on any sampling day. The Mantel-Haenszel chi-square comparison of these data indicated that the proportion of steers that were cultured positive for *E. coli* O157:H7 was significantly lower ($P < 0.05$) among animals that received the Δ*eae* or Δ*tir* mutants than among the steers that received the wild type. Similar analysis showed that there was no significant difference ($P > 0.05$) between the proportions of steers cultured positive for the Δ*ehxA* mutant and those culture positive for the wild-type strain.

Stx2 did not promote initial adherence and colonization at the bovine terminal rectal mucosa. Controversy surrounding the role of Stx in *E. coli* O157:H7 persistence in ruminants led us to test *E. coli* O157:H7 strain 905 and its isogenic toxin deletion mutant for colonization of cattle. Two different groups of four steers were inoculated rectally with *E. coli* O157:H7 905 ($\Delta stx_1/stx_2^+$) or its isogenic Stx$^-$ strain 905 Δstx_2. On each sampling day, isolates were confirmed by PCR to be carrying the expected *stx* deletion (data not shown). The logs of the geometric means of the CFU of *E. coli* O157:H7/swab in each group are presented in Fig. 3. There was no significant difference ($P > 0.05$) between the CFU of *E. coli* O157:H7/RAMS sample from animals given the Δstx_2 or wild-type strain on any sampling day. The pattern of increasing numbers of *E. coli* O157:H7 between days 1 and 4 was observed for both strains and was similar to the recognized pattern for strains that attached and multiplied at the RAJ mucosa (Fig. 1 and 2). The results indicated that Stx did not influence *E. coli* O157:H7 colonization of cattle.

The pO157 plasmid increased the level of adherence and was required for long-term colonization at the bovine terminal rectal mucosa. To analyze the role of pO157 at the bovine terminal rectal mucosa, three different groups of four steers were given a single rectal application of *E. coli* O157:H7 43894, the plasmid-cured 43894 designated strain 277, or the complemented strain *E. coli* O157:H7 277::pO157. The complemented strain had the *ehxA* hemolysin gene deleted in the process of its construction (see Materials and Methods), but data showed that lack of hemolysin did not affect *E. coli* O157:H7 colonization at the RAJ mucosa (Fig. 2). On each sampling day, isolates were confirmed by PCR to be those originally administered to the animal (data not shown). The logs of the geometric means of the CFU of *E. coli* O157:H7/swab for each group and the percentage of steers cultured positive for *E. coli* O157:H7 for each group are presented in Fig. 4. The pattern of increasing numbers of *E. coli* O157:H7 between days 1 and 4 was observed for both the wild-type and complemented strains and was similar to the recognized pattern for strains that attached and multiplied at the RAJ mucosa (Fig. 1, 2, and 3). This was in contrast to the decreasing CFU of *E. coli* pO157-cured strain/swab and the lower percentage of culture-positive steers than of steers given the wild-type strain. The number of *E. coli* O157:H7 RAMS samples from the steers given the pO157-cured strain 277 was significantly less than those from steers given either the wild-type or complemented strain (277::pO157) on days 4, 7 ($P < 0.01$), and 11 ($P < 0.05$). There was no difference ($P > 0.05$) between the numbers of complemented and wild-type strains/swab on any sampling day. The analysis by Mantel-Haenszel chi square of these data indicated that the proportion of steers positive for the cured strain was significantly lower ($P < 0.05$) than that for the wild-type or complemented strain. This observation indicated that the pO157 increased the level of adherence and was required for long-term colonization at the bovine terminal rectal mucosa.

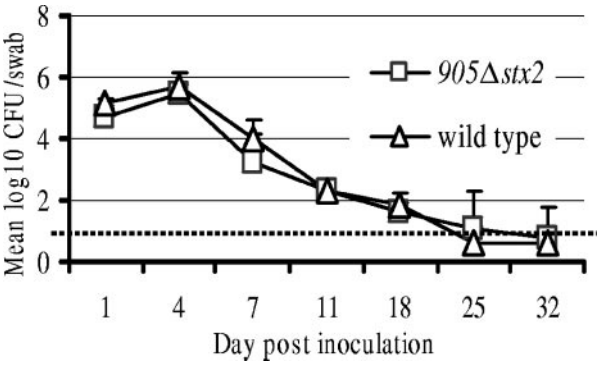

FIG. 3. Colonization of cattle by *E. coli* O157:H7 or a Shiga toxin-negative mutant. Two groups of four Holstein steers received a single rectal application of 1×10^{10} *E. coli* O157:H7 strain 905 or an isogenic Shiga toxin-negative mutant, 905 Δstx_2. The geometric means of log-transformed viable counts (CFU/swab) are shown for each strain cultured from RAMS samples on the indicated days. Values below the dotted line were positive by enrichment culture only, and values on the *x* axis were culture negative. The error bars indicate standard errors of the means.

DISCUSSION

This is the first report that compares the colonization behavior of *E. coli* O157:H7 at the bovine terminal rectal mucosa with a nonpathogenic commensal *E. coli* strain and isogenic deletion mutants missing Stx, intimin, Tir, hemolysin, or pO157. The most important finding was that *E. coli* O157:H7 had a clear advantage compared to the nonpathogenic *E. coli* and that the virulence factors intimin, Tir, and pO157 were each required for efficient *E. coli* O157:H7 colonization of cattle, while Stx and hemolysin were not. Bacterial colonization and persistence at the bovine RAJ mucosa is a dynamic process that likely depends on the ability of bacteria to attach to the epithelial cells and/or use the potentially limited nutrients at this location. A consistent pattern in bacterial numbers was seen for wild-type *E. coli* O157:H7 and strains with hemolysin or Stx deletions. The number of cultured bacteria increased between days 1 and 4 postapplication and then decreased slowly. This pattern was not seen for the nonpathogenic *E. coli* K-12 or mutants missing intimin, Tir, or the pO157, in which bacterial numbers declined rapidly postapplication. These patterns consistently predicted long-term colonization or clearance of the bacteria from the bovine terminal rectal mucosa.

In this study, we used a previously published, novel rectal administration of *Escherichia coli* O157:H7 (57, 58) for assessing colonization factors at the bovine terminal rectal mucosa.

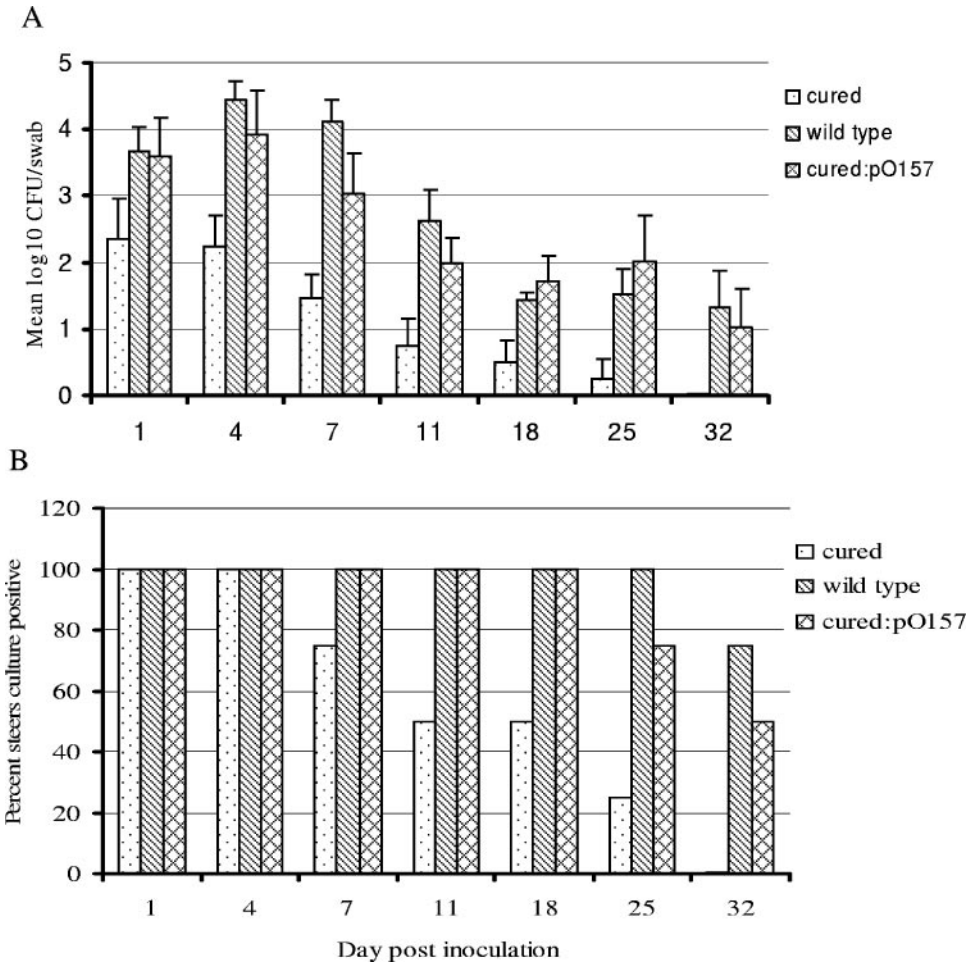

FIG. 4. Colonization of cattle by *E. coli* O157:H7 or a pO157-cured strain. Three groups of four Holstein steers received a single rectal application of 1×10^{10} CFU of *E. coli* O157:H7 ATCC 43894, strain 277 (an isogenic plasmid-cured mutant), or the complemented strain 277::pO157 Δ*ehxA*. (A) Group geometric means of log-transformed viable counts (CFU/swab) of *E. coli* O157:H7 277 (cured), 43894 (wild type), or the complemented strain 277::pO157 Δ*ehxA* are shown for each strain cultured from RAMS samples on the indicated days. (B) Percentages of steers that cultured positive for 43894 wild type, 277 (cured), or the complemented strain 277::pO157 Δ*ehxA* on the days indicated.

The procedure was highly reliable at reproducing the state of the RAJ colonization but also had the advantage of directly measuring the ability of genetically defined *E. coli* O157:H7 mutants to colonize at the RAJ mucosa. The traditional oral administration of *E. coli* O157:H7 to assess colonization factors not only measures survival and attachment to the terminal rectal mucosa but also measures the ability of the bacteria to survive in and pass through the gastrointestinal tract (GIT).

Genomic sequencing of *E. coli* O157:H7 EDL933 (ATCC 43895) reveals that this pathogenic serotype contains 1,387 unique genes not present in the chromosome of nonpathogenic *E. coli* K-12 MG1655 (50). The unique genes are proposed to be related to virulence or benefit the bacteria in some way. An important benefit may be to enhance survival and persistence of *E. coli* O157:H7 in ruminant hosts. Our findings suggested that nonpathogenic *E. coli* K-12, which carries no virulence factors, was more easily passed with the constant movement of digesta/feces and was more easily cleared from the terminal rectal mucosa than was *E. coli* O157:H7. However, it was surprising that *E. coli* K-12 persisted in one animal for >25 days, which suggested that multiple bacterial factors, metabolic capabilities, other flora, and/or host factors likely contribute to the persistence of *E. coli* at this mucosal site. Both *E. coli* K-12 and O157:H7 are able to utilize the same glycolytic substances (45). Nonetheless, this first comparative analysis with a nonpathogenic *E. coli* strain suggested that *E. coli* O157:H7 had a unique attaching ability and/or faster replicating ability at the bovine terminal rectal mucosa than did *E. coli* K-12. Analysis of the RAJ-colonizing ability of bovine commensal *E. coli* strains will be important for a more complete understanding of the host-bacterium dynamics at this site.

Many studies have implicated intimin and Tir as having roles in *E. coli* O157:H7 intestinal colonization, but no previous study has used isogenic deletion mutants placed at the terminal rectal mucosa in fully ruminant cattle. *E. coli* O157:H7 and many STEC strains contain the locus of enterocyte effacement (LEE) pathogenicity island that encodes a type III secretion system and effector proteins that are homologous to genes in the enteropathogenic *E. coli* (28, 39). Nucleotide sequence analysis identifies five major operons, named LEE1 to LEE5

(16). Intimin and Tir, encoded on the LEE5 operon, are critical for intimate adherence and the formation of characteristic attaching and effacing lesions on cells in tissue culture by EHEC and enteropathogenic *E. coli* (12, 13, 32). Studies to determine the roles of intimin and Tir in animals have used newborn piglets, newborn colostrum-deprived calves, and sheep (6, 8, 10, 11, 14, 33, 40, 60, 63, 65, 67). Recent studies show that *E. coli* O157:H7 forms attaching and effacing lesions at the bovine RAJ and that the LEE4 operon is essential for colonization of *E. coli* O157:H7 in calves (48). Others have shown that intimin can bind to both the bacterium-encoded Tir (11, 27, 36) and to host-encoded receptors referred to as host intimin receptors (10, 19, 27, 41). Our work with 43895 Δ*eae* and 43895 Δ*tir* suggested that intimin and Tir promoted the level of *E. coli* O157:H7 adherence at the terminal rectal mucosa and affected the percentage of colonized steers both short term (2 weeks) and long term (1 month). The Tir deletion mutant was as defective as an intimin deletion mutant in colonizing cattle in this study. Although we did not measure binding to host intimin receptors, this suggested that cattle do not provide an effective intimin receptor at the terminal rectal mucosa and that intimin binding at this site is dependent on the bacterial Tir. A similar result was recently reported in 14-day-old calves using an oral dose model of carriage (65). However, the *eae* gene is not present in all bovine STEC isolates, although they persist in cattle (31, 55, 66), indicating that other factors influence persistence of the bacteria in the bovine GIT.

The role of the pO157 in *E. coli* O157:H7 colonization and persistence in cattle has not been studied previously and is not well understood. The complete nucleotide sequence of the pO157 reveals that it contains 100 ORFs (5), of which fewer than 20 ORFs are characterized. These genes encode enterohemolysin (*ehxA*) (56), lymphocyte inhibitory factor (*lifA and efa*) (30), a putative adhesin (*toxB*) (62), and a zinc metalloprotease (*stcE*) (24). Also, work in our laboratory shows that a myristoyl transferase gene in the plasmid *ecf* operon is associated with lipid A modification. Its product is similar to that of the chromosomal gene *lpxM*. Deletion of both genes leads to reduced survival in the bovine GIT and reduced persistence in water troughs (68). Deletion of *toxB* results in reduced adherence to cultured epithelial cells and influenced the expression and secretion of proteins encoded by LEE but did not affect the colonization of *E. coli* O157:H7 in calves (60, 62). StcE mucinase activity may contribute to intimate adherence of *E. coli* O157:H7 to epithelial cells (24). In vivo and in vitro studies report conflicting results for the role of pO157 in adherence to epithelial cells (20, 35, 64). Fratamico et al. reported that both the plasmid-cured strain and the parent strain produce pili and adhere equally well to HEp-2 and intestinal 407 cells (20). Karch et al. reported that pO157 is associated with the expression of fimbriae that enhance bacterial adherence to epithelial cells in tissue culture (35). Tzipori et al. demonstrated that both plasmid-cured derivatives and the parent strains produce the typical mucosal lesions of bacterial attachment in piglets (64). Differences in cell lines and animal models likely contribute to these conflicting results. In this study, we demonstrated that pO157 was required for efficient colonization at the bovine terminal rectal mucosa.

Shiga toxins are a major EHEC virulence factor with potent cytotoxic activity that is responsible for many patient symptoms and can lead to death. Stx can cause damage to various cell types, including vascular endothelial, renal tubular, glomerular epithelial, and intestinal epithelial cells (29, 37). Conflicting results about the role of Stx in colonization have been reported. Stx-positive strains of *E. coli* O157:H7 colonize and persist longer than a Stx-negative strain in experimentally inoculated weaned calves (9), but the strains used in this study were not isogenic so that differences in colonization may have been due to traits other than Stx production. Robinson et al. showed that the presence of stx_2-converting phage increased adherence of EHEC to epithelial cells, suggesting a role of Stx2 in colonization (54). In contrast, Woodward et al. demonstrated that an Stx-negative *E. coli* O157:H7 persists in weaned sheep, suggesting Shiga toxins are not important in colonization (67). Also, purified Stx1 can inhibit the activation and proliferation of certain bovine lymphocytes (18, 43), which may facilitate colonization of *E. coli* O157:H7 at the bovine RAJ mucosa by modulation of mucosal immune responses. Our findings with 905 and its isogenic Stx^- mutant 905 Δstx_2 showed that that Stx2 did not influence adherence and colonization at the bovine terminal rectal mucosa. Similarly, although nearly all *E. coli* O157:H7 isolated contain the *ehxA* gene and bovine STEC epidemiological data suggest that *ehxA* may be associated with colonization in cattle (1, 3), our findings did not support this conclusion.

Theoretically, bacterial persistence requires the rate of bacterial growth to be at least equal to the rate at which bacteria are flushed from the system by intestinal digesta/fecal passage and/or replication and sloughing of intestinal epithelial cells (21). In a previous study, we suggested that persistence of *E. coli* O157:H7 in the GIT is dependent on the balance between bacterial replication and the rate of epithelial cell proliferation (38). There is no information about the rate of epithelial cell proliferation and sloughing at the terminal rectal mucosa of cattle. In the mouse intestine, complete turnover of cells in intestinal crypts is complete in 2 to 5 days (17). The pattern of an increase in bacterial numbers between days 1 and 4 postapplication suggests that bovine epithelial cell replacement at this bovine location occurs in a similar time frame (days).

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