Escherichia coli O157:H7 Strain Origin, Lineage, and Shiga Toxin 2 Expression Affect Colonization of Cattle^{∇†}

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Enterohemorrhagic *Escherichia coli* O157:H7 has evolved into an important human pathogen with cattle as the main reservoir. The recent discovery of *E. coli* O157:H7-induced pathologies in challenged cattle has suggested that previously discounted bacterial virulence factors may contribute to the colonization of cattle. The objective of the present study was to examine the impact of lineage type, cytotoxin activity, and cytotoxin expression on the amount of *E. coli* O157:H7 colonization of cattle tissue and cells in vitro. Using selected bovine- and human-origin strains, we determined that lineage type predicted the amount of *E. coli* O157:H7 strain colonization: lineage I > intermediate lineages > lineage II. All *E. coli* O157:H7 strain colonization was dose dependent, with threshold colonization at 10³ to 10⁵ CFU and maximum colonization at 10⁷ CFU. We also determined that an as-yet-unknown factor of strain origin was the most dominant predictor of the amount of strain colonization in vitro. The amount of *E. coli* O157:H7 colonization was also influenced by strain cytotoxin activity and the inclusion of cytotoxins from lineage I or intermediate lineage strains increased colonization of a lineage II strain. There was a higher level of expression of the Shiga toxin 1 gene (*stx*₁) in human-origin strains than in bovine-origin strains. In addition, lineage I strains expressed higher levels of the Shiga toxin 2 gene (*stx*₂). The present study supports a role for strain origin, lineage type, cytotoxin activity, and *stx*₂ expression in modulating the amount of *E. coli* O157:H7 colonization of cattle.

Enterohemorrhagic *Escherichia coli* O157:H7 is a bacterium that causes serious human disease outbreaks through the consumption of contaminated food or water (39). Mature cattle are considered the primary reservoir for *E. coli* O157:H7 and historically were reported to have no symptoms or pathologies (17, 23, 38); this was attributed both to a lack of receptors for a critical *E. coli* O157:H7 virulence factor, Shiga toxin 1 (Stx1 [29]), and to a differential expression of type III protein secretion system effector molecules such as EspA, EspD, and Iha (25, 30) in cattle compared to humans. In 2008, it was established for the first time that *E. coli* O157:H7 causes mild to severe intestinal pathology in persistent shedding cattle (5, 26) and that the secreted cytotoxins enhanced *E. coli* O157:H7 colonization of intestinal tissues of cattle (6). This suggested that cattle were susceptible to *E. coli* O157:H7 infection and that previously discounted virulence factors could influence the amount of colonization in cattle.

Three distinct *E. coli* O157:H7 lineages have been identified based on the lineage specific polymorphism assay (LSPA-6) that suggests both the evolutionary history of the strain and their propensity to be present among animals, the environment, and clinical human isolates (21, 22, 24, 33, 40, 42). Typically, two predominant lineages have been described, lineages I and II (22, 40) and, more recently, intermediate lin-

eages that have characteristics of lineage I and/or II have been reported at higher frequency among cattle (34). Although all *E. coli* O157:H7 lineages have been isolated from feedlot cattle, the predominant recovery of lineage I from clinical human illnesses suggests that this particular lineage type has unique expression patterns that may contribute to its preferential colonization of humans. There is some evidence to suggest that lineage I strains do not express certain virulence factors in bovine hosts, whereas other factors such as cytotoxins are expressed equally irrespective of host (30). One virulence factor associated with all lineages is the bacterium's ability to form intimate attaching-and-effacing lesions or colonization sites in the ilea of susceptible animals (28). The amount of colonization is enhanced by the expression of Shiga toxin 2 (Stx2) through both an increase in the expression of alternative non-TIR (translocated intimin receptor) colonization sites (31) and toxicity to the absorptive epithelial cells (32). In cattle, attaching-and-effacing lesions are also formed (5), and Stx2 increases colonization but is not cytotoxic to epithelial cells from the jejunum and descending colons of cattle (4). Differential expression of *stx*₂ among *E. coli* O157:H7 lineages is also linked to the increased pathogenicity of lineage I strains in humans (25), and this may affect cattle similarly. Together, this information suggests that at least some similar virulence factors affecting *E. coli* O157:H7 colonization in humans also function in cattle.

In order to gain a better understanding of the factors modulating *E. coli* O157:H7 colonization in cattle, we compared the ability of lineage I, lineage II, and intermediate lineages isolated from human sources to colonize the jejunum tissue and a colonic cell line from cattle. We hypothesized that the bovine colonic cell line could be used as a model system to

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TABLE 1. Host source, lineage type, LSPA-6 genotype, and virulence gene characteristics of the strains used in this study

Strain	Source	Lineage; LSPA-6 genotype ^a	Virulence gene characterization ^b				
			<i>stx</i> ₁	<i>stx</i> ₂	<i>ehxA</i>	<i>eae</i>	<i>tir</i>
Sakai	Human	I; 111111	+	+	+	+	+
24	Bovine	I; 111111	+	+	+	+	+
ECI 1717	Human	II; 222222	+	+	+	+	+
25	Bovine	II; 222222	+	+	+	+	+
84	Human	Intermediate lineage; 211111	+	+	+	+	+
12	Bovine	Intermediate lineage; 211111	+	+	+	+	+
86	Human	Intermediate lineage; 222212	+	+	+	+	–
138	Bovine	Intermediate lineage; 222212	+	+	+	+	–
197	Human	Intermediate lineage; 110111*	+	+	+	+	+
H3-2R	Bovine	Intermediate lineage; 100111*	+	+	+	+	+

^a *, isolates of the selected intermediate lineage type were not available from both bovine and human hosts.

^b The presence (+) or absence (–) of the gene is indicated.

reflect *E. coli* O157:H7 colonization of tissue. To confirm the value of this model, the role of strain origin in colonization of cattle was examined. In order to understand the differences in colonization associated with lineage and strain origins, we assessed cytotoxin expression, secreted cytotoxin activity, and cytotoxin-induced changes in *E. coli* O157:H7 colonization. Given the known lack of *Stx*₁ activity in cattle, we examined the effects of LSPA-6 genotype, strain origin (human versus bovine), and cytotoxin activity on *E. coli* O157:H7 colonization of cattle.

MATERIALS AND METHODS

***E. coli* O157:H7 strains and culture conditions.** The *E. coli* O157:H7 lineage I (Sakai and 24), lineage II (ECI 1717 and 25), and intermediate-lineage (84, 12, 86, 138, 197, and H3-2R) strains used in the present study (Table 1) were obtained and characterized from a previous study (33). These strains were maintained at –80°C in 25% glycerol–75% TSB (Becton Dickinson, Oakville, Ontario, Canada) and were grown overnight at 37°C in LB broth (Fisher Scientific, Ottawa, Ontario, Canada) when required. Each strain was serially diluted to the desired concentration with phosphate-buffered saline (PBS). Bacterial cell counts were determined by plating on sorbitol MacConkey agar (SMAC; Dalynn, Calgary, Alberta, Canada) and examined for non-sorbitol-fermenting colonies that appeared as colorless colonies.

Cytotoxin isolation. *E. coli* O157:H7 secreted cytotoxins were isolated and concentrated by using a combination of centrifugation and ultrafiltration as described previously (6). Briefly, the strains were grown in 15 ml of M9 media for 5 days. After growth, the cultures were centrifuged, and the supernatant containing the secreted cytotoxins was concentrated. The protein content of secreted cytotoxins was assessed by using a QuickStart Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA). The concentrated complex of cytotoxins were then serially diluted in a 1:1 ratio for eight dilutions.

In vitro organ culture (IVOC) *E. coli* O157:H7 adherence assay. Necropsy samples were obtained from eight Hereford × Angus steers using standard methods (5, 6, 38). Jejunum tissues (30 cm) were removed within 2 min of release of the intestinal tract from the carcass and each piece was placed in 200 ml of oxygenated Dulbecco modified Eagle medium (DMEM; high glucose; HyClone, Logan, UT) supplemented with bovine albumin. The tissue was maintained at 4°C and transported back to the laboratory. Upon arrival, the tissue was cut open and washed using PBS at 4°C, and 2.5-cm² pieces were excised. The tissue pieces were placed in PBS at 4°C until all sites were processed (about 20 min/animal). Experiments were started within 2 h of collecting the tissue sample.

The IVOC adherence assay was conducted as previously described (6) using the human-origin *E. coli* O157:H7 strains. This assay has been established as representing in vivo colonization (3, 10, 12, 28). To compare *E. coli* O157:H7 lineage adhesion to cattle intestinal tissue, various concentrations (final exposure doses of 10³, 10⁴, 10⁵, 10⁶, 10⁷, and 10⁸ CFU/ml) of each *E. coli* O157:H7 strain was applied to the mucosal surface. The tissue pieces were incubated for 4 h under standard culture conditions (37°C, 95% humidity, and 5% CO₂). After incubation, each tissue piece was washed six times with 10 ml of PBS to remove any unattached bacteria. The tissue was then turned mucosa-side-down in 3 to 4

ml of PBS supplemented with 1% Triton X-100 (Sigma-Aldrich) and incubated at 4°C overnight. The next day, the released bacteria were quantified by plating serial dilutions on SMAC agar and counting the non-sorbitol-fermenting colorless colonies. The assay was replicated eight times using the jejunum from eight different animals.

In vitro cell culture (IVCC) *E. coli* O157:H7 adherence assay. A colonic cell line was developed from a persistent *E. coli* O157:H7 shedding steer (F1112) in 2006 and has since been maintained in our laboratory. The cell line was cultured in 75-cm² tissue culture flasks (Fisher Scientific) in DMEM supplemented with 10% fetal bovine serum (FBS; HyClone) and gentamicin (50 µg/ml; Sigma-Aldrich) under standard culture conditions. Cells were subcultured by trypsinization of the monolayer with 5 to 10 ml of 0.25% trypsin-EDTA solution (Sigma-Aldrich) for 2 to 5 min and collecting and then centrifuging the cell suspension at 2,087 × *g* for 10 min. The supernatant was decanted, and the cell pellet was resuspended in the culture medium. Cells were added to six-well multiwell Falcon plates (Fisher Scientific) at a density of ~10³ cells. Cells were cultured until confluent (approximately 1 week), and the culture medium was changed every 3 days.

The IVCC adherence assay was performed with all human- and bovine-origin *E. coli* O157:H7 strains. The same methodology was used as described above for the IVOC *E. coli* O157:H7 adherence assay, with a few modifications to accommodate the use of the colonic cell line. To compare *E. coli* O157:H7 strain colonization to the colonic cell line, each well of confluent cells was washed once with DMEM, after which 3 ml of DMEM was added to each well to remove any residual FBS or antibiotic that could affect bacterial adherence. To each well, a 100-µl aliquot of each *E. coli* O157:H7 strain was added to deliver final exposure doses of 10³, 10⁴, 10⁵, 10⁶, 10⁷, and 10⁸ CFU/ml. The cell cultures were incubated for 4 h under standard culture conditions. The medium was replaced for the highest dose after 2 h to maintain pH. At the end of the experiment, cell monolayers were washed four times with 10 ml of PBS to remove any unattached bacteria. To release the bacteria from the cells, 2 ml of PBS supplemented with 1% Triton X-100 was added to each well, followed by incubation overnight at 4°C. Serial dilutions of the released bacteria were performed the next day and quantified by plating onto SMAC plates. Plates were incubated at 37°C overnight, and *E. coli* O157:H7 was quantified by counting the non-sorbitol-fermenting colonies that appeared colorless. The IVCC assay was replicated five times for each strain with a minimum of three different culture dates for the colonic cell line.

The secreted cytotoxins from a human-origin *E. coli* lineage I strain have been shown to enhance the colonization of a bovine-origin *E. coli* lineage I strain that had significantly lower colonization on its own (6). To establish the role, if any, of the secreted cytotoxins produced by *E. coli* lineages in tissue colonization, the colonic cell line was exposed to about 80 ng of secreted cytotoxins from each of the *E. coli* O157:H7 strains in conjunction with 10⁶ CFU/ml of the least adherent *E. coli* O157:H7 strain, ECI 1717. This assay was replicated three times for the secreted cytotoxins produced from all strains except for ECI 1717.

Lawn assay. The lawn assay was used to compare the toxicity of secreted cytotoxins from the *E. coli* O157:H7 strains to epithelial cells. Epithelial cells were extracted from the jejunum, and the lawn assay was performed using the cytotoxins from human and bovine *E. coli* O157:H7 strains as described previously (7). Briefly, a 1% SeaKem Agarose (Mandel Scientific, Guelph, Ontario, Canada) support gel was poured into a petri dish. Next, the lawn agarose (3 ml of 3.7% SeaPlaque agarose [Mandel Scientific]) was mixed with 3 ml of cell

suspension and poured over the support agarose. Each toxin dilution (3 μ l) was applied, and the treated lawn was incubated for 4 h under standard culture conditions. The amount of total cytotoxin applied was 0.1 to 120 ng. The lawn was stained with 0.1% trypan blue (Sigma-Aldrich) and destained using 1.84% KCl. Plates were scored the same day, and the amount of cytotoxin activity was defined as the threshold dose (ng) of secreted proteins in the dilution series to cause a blue spot on the lawn.

Real-time PCR expression. To determine which cytotoxins may be expressed by the strains according to origin and lineage type, expression of *stx*₁, *stx*₂, and *ehxA* were quantified from all strains by using real-time PCR. Total RNA was extracted by using an RNeasy minikit (Qiagen, Inc., Mississauga, Ontario, Canada), and expression analysis was performed by using a one-step Brilliant II QRT-PCR master mix kit (Stratagene, La Jolla, CA). Relative quantification was performed on a Mx3000p (Stratagene) with previously described primers, probes, and cycling conditions for *stx*₁ (35), *stx*₂, and *ehxA* (30). Each plate setup included a standard curve generated by reverse transcribing known concentrations of Sakai RNA. Relative gene expression was analyzed by using the built-in comparative threshold cycle (C_T) methods ($\Delta\Delta C_T$) of MxPro real-time software (version 4.01) normalized using the housekeeping gene *gnd* and calibrated to strain Sakai. All real-time PCRs were conducted in triplicate.

Statistical analysis. The IVOC adherence assay and the IVCC adherence assay data were log transformed to normalize the data prior to analysis. All data were analyzed using analysis of variance, followed by a post hoc Tukey's test for comparison of the means. For the IVOC, the model consisted of two factors: the lineage type and the dose. For the IVCC, the model consisted of two factors: the strain origin and the lineage type. Individual strain colonization was analyzed with dose as a factor. For all data analysis, $P < 0.05$ was considered significant, and $P > 0.05$ was not significant.

RESULTS

IVOC *E. coli* O157:H7 adherence assay. There was a significant difference in *E. coli* O157:H7 human-origin strain colonization in the jejunum ($P = 0.001$, Fig. 1A) and a significant dose-dependent response to *E. coli* lineage colonization ($P = 0.001$, Fig. 1B). The strain hierarchy for colonization of intestinal tissue was as follows: Sakai, 84, ECI 1717 > 86, 197. The strains Sakai, ECI 1717, and 84 colonized equally ($P = 0.653$), while strains 86 and 197 exhibited similar levels of colonization ($P = 0.502$). Strains Sakai and 84 had significantly higher colonization compared to strains 86 and 197 ($P < 0.05$). Strain ECI 1717 showed significantly higher colonization than strain 86 ($P = 0.01$), but ECI 1717 was not significantly different from strain 197 ($P > 0.524$). This apparent contrast between strain ECI 1717 (lineage II) and strains Sakai (lineage I) and 84 (intermediate lineage) was related to a consistent but not significantly lower colonization in all assays. At the highest doses, strains Sakai and 84 had approximately 10 times higher colonization than the rest of the strains (Fig. 1A). At 10^6 CFU/ml, Sakai had almost 10 times more colonization than the rest of the examined strains. At 10^3 to 10^5 CFU, strain 84 had the highest colonization, approximately 10 times higher than the next highest strain, ECI 1717. Throughout the dose range, the lineage I strain, Sakai, was the most adherent strain, while the lineage II strain, ECI 1717, provided variable adherence depending on the replicate. This variability was also observed for the intermediate lineage strains, but the extent of variability was dependent on the exposure dose. This variability among tissues has been previously reported (6) and appears to be related to the expression of mucosal factors that assist colonization. The removal of this variable allows for an examination of the ability of strains to colonize colonic cells, thus supporting a role for a cell culture model in understanding *E. coli* O157:H7 infections in cattle. In addition, the intermediate lineages (strains 84, 86, and 197) had an ~ 10 -fold lower ad-

FIG. 1. Evaluation of *E. coli* O157:H7 lineage and strain colonization of the jejunum from cattle ($n = 8$) using an IVOC adherence assay. Lineage I (Sakai), lineage II (ECI 1717), and intermediate lineages (84, 86, and 197) were assessed for colonization. (A) Comparison of individual strains; (B) comparison of individual lineage types.

herence relative to lineage I (Sakai) and lineage II (ECI 1717) strains. Within environmental exposure dose ranges (10^3 to 10^5 CFU/ml) for *E. coli* O157:H7, there was a significant but low colonization of the jejunum by all lineages (Fig. 1B). The lineage II strain had the equivalent of a lineage I or lineage II colonization, depending upon the replicate or animal tissue used, and this may be due to the uncharacterized mucosal factor that has been previously described as affecting bovine colonization (5).

IVCC *E. coli* O157:H7 adherence assay. A similar hierarchy for *E. coli* O157:H7 lineage adherence was obtained with the colonic cell line, as observed for the jejunum. To ensure that this hierarchy was not related to strain origin, five additional *E. coli* O157:H7 strains were selected, four of which represented previously used human LSPA-6 genotypes but were of bovine origin. The strain hierarchy for colonization of the colonic cell line was as follows: 24, H3-2R > Sakai, 25, 12, 138 > 86, 197 > 84 > ECI 1717. However, this type of strain hierarchy is difficult to interpret due to differences in the low-dose colonization between the human- and bovine-origin strains.

We observed a significant dose-dependent response to *E. coli* O157:H7 colonization of the colonic cell line ($P = 0.001$). The human-origin lineage I strain Sakai had a threshold adherence at 10^3 CFU/ml (Fig. 2A). The human-origin strains ECI 1717, 86, and 197 had threshold adherences at 10^4 CFU/ml (Fig. 2A). The human-origin intermediate lineage strain 84 had a threshold adherence at 10^5 CFU/ml (Fig. 2A).

FIG. 2. Evaluation of *E. coli* O157:H7 strain colonization of the colonic cell line from cattle ($n = 5$) using an IVCC colonization assay. Lineage I (Sakai and 24), lineage II (ECI 1717 and 25), and intermediate lineages (84, 86, 197, 12, 138, and H3-2R) were assessed for colonization. (A) Comparison of individual human-origin strains; (B) comparison of individual bovine-origin strains.

This suggests that among human-origin strains of *E. coli* O157:H7, lineage I strains have the highest probability of colonizing at low environmental exposure doses (10^3 to 10^5 CFU/ml). All bovine-origin strains had a threshold adherence at 10^3 CFU/ml, below which there was inconsistent to no adherence (Fig. 2B). All strains had a maximum adherence at 10^7 CFU/ml. There was a significant difference in *E. coli* O157:H7 lineage adherence to the colonic cell line ($P = 0.001$, Fig. 3A). The lineage I strains had greater adherence than the remaining lineages regardless of their origin, human or bovine (Fig. 3A). The lineage II strains had the lowest adherence compared to the other lineages, while intermediate lineages had an adherence that was between the lineage I and II strains (Fig. 3A). Finally, there was a significant difference in *E. coli* O157:H7 strain adherence to the colonic cell line based on strain origin ($P = 0.001$, Fig. 3B). The bovine-origin strains had higher adherence at environmental exposure doses (10^3 to 10^6 CFU/well). In contrast, at higher exposure doses (10^7 to 10^8 CFU/ml), there were no differences between human- and bovine-origin strains (Fig. 3B). This suggests that bovine-origin *E. coli* O157:H7 strains have a higher probability of colonizing cattle

FIG. 3. Effect of *E. coli* O157:H7 lineage and strain origin on colonization of a colonic cell line from cattle ($n = 5$). (A) Comparison of individual lineage types. Lineage I (Sakai and 24), lineage II (ECI 1717 and 25), and intermediate lineage (84, 86, 197, 12, 138, and H3-2R) strains. (B) Comparison of different strain origins. Human-origin strains (Sakai, ECI 1717, 84, 86, and 197) and bovine-origin strains (24, 25, 12, 138, and H3-2R).

than human-origin strains. There was a significant strain-dose interaction ($P = 0.001$) that supports the differences in the slope of the dose-response curves for the bovine-origin strains compared to the human-origin strains.

Sakai had significantly lower adherence than strains 24 and H3-2R at the 10^3 to 10^6 CFU/ml doses ($P < 0.01$) but a similar adherence at the 10^7 to 10^8 CFU/ml doses (Fig. 2). Sakai also had significantly higher adherence than ECI 1717 across all doses ($P = 0.001$). In contrast, Sakai was not significantly different from strains 84, 86, 197, 25, 12, and 138 ($P > 0.142$). ECI 1717 showed significantly less adherence than strains 86, 197, 24, 25, 12, 138, and H3-2R ($P < 0.02$). In contrast, ECI 1717 was not significantly different from strain 84 ($P = 0.14$). Strain 84 demonstrated significantly less adherence than strains 24, 12, 138, and H3-2R ($P < 0.05$). Strain 84 was not significantly different from strains 86, 197, and 25 ($P > 0.35$). Strain 86 had significantly less adherence than strain 24 at the four lowest doses ($P = 0.001$). Strain 86 was not significantly different from strains 197, 25, 12, 138, and H3-2R ($P < 0.06$). Strain 197 showed significantly less adherence than strains 24, H3-2R ($P < 0.01$). Strain 197 was not significantly different from strains 25, 12, and 138 ($P > 0.06$). Strain 24 had significantly higher adherence than strain 25 ($P = 0.001$). Strain 24 was not significantly different from strains 12, 138, and H3-2R

FIG. 4. Effect of *E. coli* O157:H7 secreted cytotoxins on ECI 1717 colonization of a colonic cell line ($n = 3$). The colonic cell line (10^5 cells) was exposed to ~ 80 ng of secreted cytotoxins from each of the *E. coli* O157:H7 strains in conjunction with 10^6 CFU of the least-adherent *E. coli* O157:H7 strain, ECI 1717. ECI 1717 without the cytotoxin addition is denoted as the "control." Detailed strain characteristics are outlined in Table 1.

($P > 0.139$). Strains 25, 12, 138, and H3-2R were not significantly different from each other ($P > 0.47$).

Secreted cytotoxins from all strains differentially affected the colonization of ECI 1717 to a colonic cell line ($P = 0.001$, Fig. 4). The addition of secreted cytotoxins to the same strain from which the toxins were derived was previously found to have little effect on adherence (data not shown); therefore, the strain with the lowest adherence was selected for this assay since it would be expected to have the largest increase in adherence to the cell line. As expected, the secreted cytotoxins from the human-origin lineage I strain Sakai ($P = 0.05$) increased colonization compared to the secreted cytotoxins from all other strains. In addition, secreted cytotoxins from the lineage I strains of human origin (strain Sakai, $P = 0.01$) and bovine origin (strain 24, $P = 0.05$) produced the largest changes in strain ECI 1717 adherence relative to ECI 1717

alone. The secreted cytotoxins from the bovine-origin lineage I strain 24 produced significantly higher colonization than strains 197 ($P = 0.02$), 25 ($P = 0.01$), and H3-2R ($P = 0.03$). The secreted cytotoxins from the human-origin intermediate lineage strains (84, 86, and 197), the bovine origin lineage II strain (25), and the bovine-origin intermediate lineage strains (12, 138, and H3-2R) did not significantly change the adherence relative to ECI 1717 alone.

Lawn assay for secreted cytotoxins. The lawn assay provides a threshold cytotoxin dose that causes epithelial cell death as determined by the presence of a blue spot. The hierarchy for the threshold doses of the secreted cytotoxin activity was as follows: human strains < bovine strains ($P = 0.05$) and lineage I < intermediate lineages < lineage II ($P = 0.05$). The strain hierarchy for the threshold dose (in ng) for the cytotoxin activity was as follows: Sakai < 84, 86, 12 < H3-2R, ECI 1717 < 138 < 24 < 197, 25 ($P < 0.05$, Fig. 5).

Real-time PCR expression. In vitro expression of genes for *stx*₁, *stx*₂, and *ehxA* was quantified, and the data were described relative to the expression of the human-origin lineage I strain, Sakai. The strain hierarchy for average virulence gene expression was as follows: Sakai > 24, 12, 138 > 86, 197 > H3-2R > 84 > 25 (Fig. 6). There was a significant lineage type-strain origin interaction for the expression of *stx*₁ ($P = 0.033$, Fig. 7) whereby human-origin strains showed significantly higher expression of the gene than bovine-origin strains ($P = 0.001$). There were no differences in expression of *stx*₁ between lineage types ($P > 0.700$). We did not find a significant interaction of lineage type and strain origin for the expression of *stx*₂ ($P = 0.059$). For *stx*₂ expression, there was no difference between human- and bovine-origin strains ($P = 0.330$), but the lineage I strains had significantly greater expression than the lineage II and intermediate lineage strains ($P < 0.007$). There was no difference in *stx*₂ expression between lineage II and intermediate lineage strains ($P = 0.992$). We did not find a lineage type-strain origin interaction for expression of *ehxA* ($P = 0.269$). There were no differences in expression of *ehxA* based on strain origin ($P = 0.697$) or lineage type ($P = 0.147$).

FIG. 5. Threshold dose for secreted cytotoxin activity produced by *E. coli* O157:H7 strains using the absorptive epithelial cells from the jejunum of cattle ($n = 7$). (A) Comparison of strains; (B) comparison of strain origin (human-origin strains Sakai, ECI 1717, 84, 86, and 197 and bovine-origin strains 24, 25, 12, 138, and H3-2R) and lineage types (lineage I strains Sakai and 24; lineage II strains ECI 1717 and 25; and intermediate lineage strains 84, 12, 86, 138, 197, and H3-2R).

FIG. 6. Relative expression of *stx1*, *stx2*, and *ehxA* from different *E. coli* O157:H7 strains ($n = 10$) and lineages ($n = 6$). (A) Comparison of strains; (B) comparison of individual lineage types (lineage I strains Sakai and 24; lineage II strains ECI 1717 and 25; and intermediate lineage strains 84, 12, 86, 138, 197, and H3-2R).

DISCUSSION

Mature cattle challenged with *E. coli* O157:H7 respond with heterogeneous shedding periods, the duration and magnitude of which are affected by exposure dose (9), animal age (11), and an uncharacterized mucosal factor (5). However, it is still relatively unclear why one animal becomes more heavily colonized than another when exposed to the same dose and strain composition. One possibility is that alternative binding sites exist in some animals that allow for greater colonization. *Stx2*

enhances *E. coli* O157:H7 colonization in cattle (4) and a similar impact has been documented in a less disease-susceptible animal model (31). In the latter system, the *Stx2* enhances the expression of non-TIR-based colonization sites, nucleolin, and inclusion of an *Stx2*-expressing strain with a nonexpressing strain results in an equivalent and higher colonization by both strains, resulting in a longer duration of shedding. Generally, cattle shedding studies have used lineage I strains associated with clinical human disease cases, and typically cattle challenges result in about a 10-day shedding period (1). In multi-strain studies using lineage I (human origin), intermediate lineage (human origin), and lineage I (bovine origin) strains, the shedding period expands to about 45 days (2), suggesting that the bacteria may be using a similar mechanism of alternate binding sites in cattle to facilitate better colonization. Earlier studies using the same multiple strains suggested that cytotoxin activity reflects *E. coli* O157:H7 strain colonization (6, 7); however, a limited number of lineage and origin types were used. In the present study, we examined this question in more detail and determined that bovine-origin strains had less active cytotoxins, as defined by the threshold cytotoxin dose to cause cell death in the lawn assay, compared to human-origin strains, confirming the previous results. However, bovine-origin *E. coli* O157:H7 strains showed much higher colonization than human-origin strains at environmental doses, which is defined as the range of *E. coli* O157:H7 density that cattle would be exposed to under natural circumstances (typically 10^3 to 10^5 CFU/g) (27), suggesting that unknown factors other than secreted cytotoxins are important for cattle colonization. In addition, we examined the role of lineage type in *E. coli* O157:H7 colonization in cattle and found that lineage I strains colonize cattle better than other lineages. The hierarchy for lineage cytotoxin activity was as follows: lineage I > intermediate lineages > lineage II strains. This finding is in agreement with the previously mentioned studies (6, 7) but expands the compari-

FIG. 7. *E. coli* O157:H7 strain origin effect on the relative expression of *stx1*, *stx2*, and *ehxA* ($n = 5$). Human-origin strains (Sakai, ECI 1717, 84, 86, and 197) and bovine-origin strains (24, 25, 12, 138, and H3-2R) are represented.

son to include lineage II *E. coli* O157:H7 strains. It also suggests that cytotoxins are important to *E. coli* O157:H7 colonization of cattle and should be investigated further for their role in colonization.

Shiga toxins are key virulence factors of human disease with both lethal and sublethal effects on target cells (15, 16, 31, 32, 37). Only a few studies are available that have examined the role of lineage or origin on Shiga toxin activities or the promotion of *E. coli* O157:H7 colonization of cattle. Inclusion of Stx2 enhances the colonization of a human-origin lineage I strain by a factor of 100 (4) and, in another study (6), inclusion of the cytotoxin mixture from a human-origin lineage I strain increased colonization of a bovine-origin strain 10-fold compared to a 4-fold change for a human-origin lineage I strain. This suggested that the bovine-origin strains could utilize the cytotoxins to enhance their own colonization. In the present study, we examined the *in vitro* expression of *stx* genes and *ehxA* to determine whether the amount or type of cytotoxin expressed could explain differences in *E. coli* O157:H7 strain origin and lineage colonization of cattle. We found that human-origin strains expressed higher levels of Stx1 than bovine-origin strains, indicating that this toxin is more important to the infection process in humans than in cattle. In cattle, Stx1 is readily degraded in the crypts of the colon (19), and there are no apparent receptors present in the vascular tissue (18), confirming that this toxin is unlikely to be critical for *E. coli* O157:H7 colonization of cattle. Previously, other researchers were unable to find differences in *stx*₂ expression in feces from clinical human cases and cattle that were experimentally inoculated with a human-origin strain of *E. coli* O157:H7 (30). Similarly, in the present study, bovine- and human-origin strains expressed similar levels of *stx*₂, suggesting that this toxin is critical for colonization of cattle and humans. Finally, *ehxA* expression did not differ between human- and bovine-origin strains, making it unclear whether this toxin contributes to the *E. coli* O157:H7 colonization process of cattle. All lineages express *stx*₁ equally, while *stx*₂ is expressed more in lineage I strains than lineage II or intermediate lineages, and this toxin has been shown to increase *E. coli* O157:H7 colonization (4) confirming a link between Stx2 activity and higher strain colonization of cattle. In contrast, *ehxA* was expressed at the highest level in intermediate lineages, with lineage I and II expressing similar amounts. This supports a role for *stx*₂ expression in the higher lineage I colonization of cattle tissue. In our study, *in vitro* expression data may have limitations for application to *in vivo* systems, and we recognize that expression can be affected by numerous factors, such as growth in the presence of epithelial cells (20). Nevertheless, *in vitro* differences observed between strains of various origins or lineage types should not be overlooked since it is plausible that they would also be witnessed *in vivo*. Others, too, have previously used *in vitro* expression analysis to model expression that may occur within the cattle intestinal tract (8, 13, 14). Future *in vivo* expression studies are crucial for determining how expression affects *E. coli* colonization of cattle. Another limitation of the present study was the small strain set used; despite this, the highly significant interactions obtained provide novel insight into *E. coli* O157:H7 pathogenicity.

Tissue- and cell line-based *E. coli* O157:H7 colonization assays did have differences in lineage and strain colonization.

There was significant variability in the colonization of lineage II and intermediate lineage strains in any given tissue where the colonization could be as high as a lineage I strain or as low as a lineage II strain. This variability is likely due to as-yet-undefined mucosal factors (5) which necessitated increased replication to detect differences. This limitation is particularly present at environmental dose ranges (6) and further supports the efficacy of a bovine cell culture model that provides consistent results and yet similar trends to the tissue model. In addition, the lower environmental dose ranges used in the present study gave greater variability than higher doses in *E. coli* O157:H7 colonization of the cattle tissue. This was associated with a difficulty in detecting the pathogen in the presence of the endogenous non-O157 *E. coli*. Again, the IVCC colonization assay did not have this problem since there are no endogenous bacteria allowing for better separation of strain differences. This allowed us, for the first time, to detect the significance of strain origin to *E. coli* O157:H7 colonization of cattle. We conclude that the IVCC assay is a rapid, easy, and sensitive method for evaluating the contribution of *E. coli* O157:H7 virulence factors to colonization of host-specific cells.

The *E. coli* O157:H7 lineage I and II strains are thought to cluster as separate phylogenetic branches and lineage I is the ancestral form, whereas lineage II is derived through changes to or loss of virulence genes (21, 22, 36, 40). *E. coli* O157:H7 lineage I strains cause increased human illness (21, 42) and colonize bovine tissues at a higher level than lineage II strains. The relative abundance of *E. coli* O157:H7 lineage I strains across different geographic areas has been attributed to the rapid dissemination of the newly evolved pathogen into a previously unoccupied ecological niche within human and bovine populations (40). However, different geographic subpopulations of lineage II strains have been identified and, even though the changes from lineage I to lineage II strains are thought to predate the spread of the lineage II strains (22), it is likely that the genetic changes separating these different groups of lineage II strains are due to losses or rearrangements of virulence factors. Indeed, it has been suggested that such genomic changes could have a high frequency due to the relative abundance and homology of numerous prophagelike genomic inserts encoding virulence and regulatory genes within the *E. coli* O157:H7 genome (41). The likelihood of these different lineage II strains becoming or remaining successful within bovine populations despite decreased individual colonization capabilities is increased dramatically due to the previously unknown niche that is supplied by highly colonizing *E. coli* O157:H7 lineage I strains. This mechanism of virulence factor mutation followed by "piggy-back" colonization may have contributed to the different lineage II subgroups that are currently common within feedlot environments and may explain how multiple branches of a phenotype that colonized less has evolved. The present study is the first of its kind to determine that strain origin, lineage type, and *stx*₂ expression greatly influence the amount of *E. coli* O157:H7 colonization in the jejunum and a colonic cell line from cattle.

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