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December 2007

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SHORT COMMUNICATION

Reduced intestinal colonization of adult beef cattle by *Escherichia coli* O157:H7 *tir* deletion and nalidixic-acid-resistant mutants lacking flagellar expression

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Abstract

The importance of the *Escherichia coli* O157:H7 translocated intimin receptor (Tir) protein in intestinal colonization and the effect of infection with Tir⁺ strains on protection against subsequent challenge was studied in adult beef cattle. Cattle were orally inoculated (C1) with a Shiga toxin-2⁺ *E. coli* O157:H7 strain that was Tir⁺ or Tir⁻, and 42 days later were re-challenged (C2) with the nalidixic acid (Nal)^R parent strain to test whether prior infection had any effect on fecal shedding. During the first 14 days post-C1, the Nal^S wildtype (WT) strain was shed at significantly higher levels and for a longer period than the other strains; however, the mean levels of shedding of the Nal^R and Δtir complemented strains were not significantly different from that of the Tir⁻ strains. The Δtir , Tir complemented mutant, and Δtir vector control strains inadvertently did not express flagellin, and did not effectively colonize the intestine. We were unable to determine whether Tir exposure at C1 had any effect on protection. Further, those given an initial inoculation with a non-flagellated variant of *E. coli* O157:H7 were more susceptible to a second challenge with motile *E. coli* O157:H7 than those originally inoculated with motile strains. The cause of the loss of expression of flagellin was not addressed. We suggest that either the flagellum or a factor that regulates both its production and that of some other effector has a significant function in colonization.

Keywords: *Escherichia coli* O157:H7, cattle, colonization, translocated intimin receptor, flagella

1. Introduction

Escherichia coli O157:H7 is an important food-borne pathogen that causes hemorrhagic colitis and hemolytic uremic syndrome in humans (Griffin *et al.*, 1988). Cat-

tle are an important reservoir host of *E. coli* O157:H7, in which the organism colonizes the intestinal tract and is shed in the feces (Cray and Moon, 1995). The mucosa of the terminal rectum is a principal site of colonization (Naylor *et al.*, 2003). Colonization of this site involves

the formation of attaching-effacing (A/E) lesions (Naylor *et al.*, 2005). The bacterial outer membrane protein intimin and proteins secreted via the type III secretion system (TTSS), viz., the translocated-intimin receptor (Tir) and several *E. coli*-secreted proteins (Esp), have significant functions in A/E lesion induction (Hartland *et al.*, 2000).

Cattle are susceptible to re-infection under natural conditions (Khaita *et al.*, 2003). However, experimentally, young calves shed fewer organisms upon re-exposure to the homologous strain (Naylor *et al.*, 2007). The objectives of this study were to determine whether inactivation of *tir* reduces the magnitude and duration of *E. coli* O157:H7 fecal shedding in adult cattle, and to compare the effects of initial infection with Tir⁺ and Tir⁻ strains on shedding following a second challenge.

2. Materials and methods

2.1. Experimental animals

Thirty adult beef cattle (mean age, ~16 months) that tested negative for *E. coli* O157:H7, *Salmonella* spp., coccidia and nematodes in the feces, and negative for bovine viral diarrhoea virus in the serum were included in the study. Cattle were individually housed in bio-safety level (BL)-2 containment rooms that had individual floor drains. The floors and walls of each room were thoroughly washed once daily.

2.2. Inoculum strains and culture methods

Strains used in this study were cultured in LB with appropriate antibiotic selection (Table 1). Cultures were incubated overnight at 37 °C with shaking, unless otherwise stated. For motility tests, LB broth cultures were incubated overnight on blood agar, then stabbed and incubated at 37 °C overnight in tube motility medium or 12 h in tryptone agar plates.

2.3. Western blots and electron microscopy

Each strain was tested for Tir expression by Western blot as previously described (DeVinney *et al.*, 1999) with minor modifications. Whole-bacterial extracts were used to test for H7 flagellin expression (Girón *et al.*, 2002). Nitrocellulose membranes were incubated with a 1:1000 dilution of a commercial rabbit anti-H7 antiserum (Difco), followed by a 1:2000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (Gibco BRL®). LB cultures, processed by routine methods for transmission electron microscopy (TEM) were examined for flagella.

2.4. Experimental design

Six cattle each were inoculated with a Tir⁺ or Tir⁻ *E. coli* O157:H7 strain (Table 1) and re-challenged 42 days later (C2) with the Tir⁺ Nal^R parent strain. In all cases,

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

Strains	Characteristics				Comments	Reference and/or source
	Tir ^a	Nal ^b	Cm ^c	Tet ^d		
Wild-Type 86-24	+	S	S	S		Griffin <i>et al.</i> (1988)
86-24 Nal ^R	+	R	S	S	Spontaneous Nal ^R mutant	DeVinney <i>et al.</i> (1999)
86-24 Δtir	-	R	S	S	Non-polar <i>tir</i> deletion	DeVinney <i>et al.</i> (1999)
86-24 Δtir (pEH86) ^e	+	R	R	S	<i>tir</i> gene cloned into pACYC184	DeVinney <i>et al.</i> (2001)
86-24 Δtir (pACYC184)	-	R	R	R	86-24 Δtir transformed with pACYC184	This study

^a Presence (+) or absence (-) of *tir* gene.

^b Nalidixic acid (Nal).

^c Chloramphenicol (Cm).

^d Tetracycline (Tet). Sensitivity (S = sensitive and R = resistant).

^e pEH86 is pACYC184 with *tir* cloned into BamHI-SalI sites. *tir* is under the control of the tetracycline resistance gene promoter.

the inoculum dose was 1×10^9 CFU given in the feed, and cattle were observed to consume the entire feed sample. Fecal samples were collected daily from the centers of fresh pats on the floor for 14 days after C1 and C2, and on alternate days from days 14 to 42 after C1.

2.5. Fecal cultures and identification of isolates

Each fecal sample was cultured by direct plating (DP) for quantification of shedding levels (CFU/g), and GN broth enrichment with antibiotics followed by O157 immunomagnetic separation (E-IMS) and plating to detect levels below that detectable by DP. E-IMS was done as described (Smith *et al.*, 2005), with the exception of Nal and chloramphenicol (Cm) selection at the plating step where appropriate. For DP, 1 g of feces was added to 9 mL buffered peptone water and serial 10-fold diluted to 10^{-5} . One hundred microliter of each dilution was spread onto each of two sorbitol MacConkey (SMAC) plates containing cefixime (0.5 μ g/ml) and potassium tellurite (2.5 μ g/ml) to quantify WT, or 40 μ g/ml Nal to quantify all other strains. Plates were incubated 18–24 h at 37 °C. One to three isolates on each E-IMS or DP count plate were confirmed to be *E. coli* O157:H7 by standard testing (Smith *et al.*, 2005). Non-sorbitol-fermenting isolates on SMAC-Nal plates from cattle inoculated with Δtir (pEH86) or Δtir (pACYC184) were tested for the chloramphenicol acetyltransferase (*cat*) gene by colony hybridization and subculture onto SMAC-Cm (40 μ g/ml) agar. Hybridizations used a horseradish peroxidase-labeled EcoRI–XbaI (1425-bp) fragment of pACYC184, and a chemiluminescence system (ECL, Amersham Pharmacia).

2.6. Statistical analysis

The mean magnitude and duration of fecal shedding was estimated for the first 14 days post-C1 and post-C2. The magnitude of shedding was determined as the geometric mean expressed as \log_{10} CFU/g (wet weight) of feces. The duration of shedding based on DP or E-IMS, expressed as the percentage of days in which *E. coli* O157:H7 was detected was determined. Magnitude and duration of fecal shedding, when the latter was assessed either by DP or E-IMS, was assessed by Spearman's correlation analysis (Proc CORR, SAS, 1999). Data were analyzed as described by La Ragione *et al.* (2005).

3. Results

3.1. Phenotypic characterization of the inoculum strains

The *in vitro* growth rates of the different strains were not significantly different ($P > 0.05$). The predicted Tir⁺ and Tir⁻ status of each strain (Table 1) was confirmed by Western blot (data not shown). The Nal^R Δtir and derivative strains were non-flagellated, lacking evidence of flagellin by Western blot (Figure 1) and flagella by TEM (data not shown). Moreover, the Nal^R parent strain produced less flagellin (Figure 1) and was less motile than the wild-type (WT; Nal-sensitive) strain (data not shown).

3.2. Magnitude and duration of *E. coli* O157:H7 shedding in feces

A highly significant correlation was detected between the magnitude and duration of fecal shedding when the latter was assessed either by DP ($r = 0.99$, $P < 0.0001$, $r^2 = 98\%$, $n = 30$) or E-IMS ($r = 0.92$, $P < 0.0001$, $r^2 = 85\%$, $n = 30$). During the first 14 days post-C1, the WT strain was shed at significantly higher levels and for a longer period ($P < 0.05$) than the other strains, as determined both by DP and E-IMS (Figure 2). The mean levels of shedding of the Nal^R parent and Δtir (pEH86) groups were not significantly different from that of the Tir⁻ groups ($P > 0.05$). However, 1–2 animals in each of these Tir⁺ groups shed at levels comparable to WT, in contrast to no animals shedding at these levels in the Tir⁻ groups (Figure 2). Between days 14 and 42 post-C1, the levels of shedding among the five treatment groups were not significantly different ($P = 0.5$; data not shown). Isolates quantified as Δtir (pEH86) or Δtir (pACYC184) were confirmed to have retained their plasmids. Based on the E-IMS procedure, the percent of positive days of detection of fecal shedding, as a measure of duration

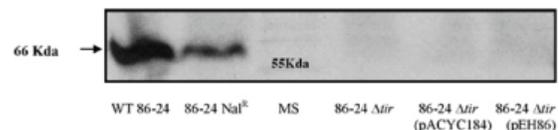


Figure 1. Expression of flagellin by *E. coli* O157:H7 strains. Mass standards (MS) are shown. Molecular weight of H7 flagellin is 66 kDa.

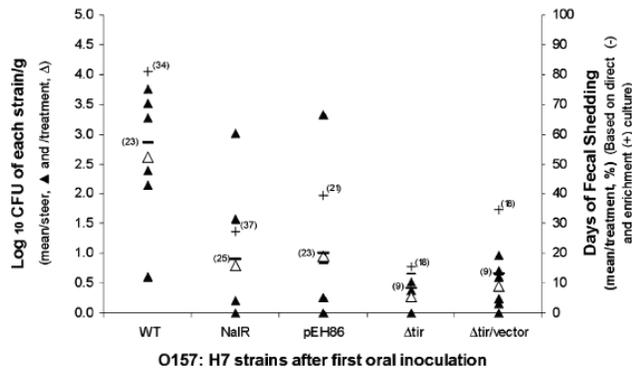


Figure 2. Magnitude (mean per steer, ▲; mean per treatment, Δ; log₁₀ CFU/g) and duration of fecal shedding (mean per treatment; percentage of days positive), as detected by direct plating (DP) and enrichment (E-IMS) culture. Cattle ($n = 6$; ▲ may be overlapped in 0 value) were orally inoculated with Tir⁺ or Tir⁻ *E. coli* O157:H7 strains. Cattle were individually housed and inoculated in isolation rooms and fecal shedding was monitored daily for 14 days. Standard deviations for the duration of fecal shedding based on DP and E-IMS culture are shown in parentheses.

after 14 days post-C1, was 33.0% for WT; 12.0% for Nal^R; 2.4% for Δtir; 11% for Δtir (pEH86); and 3.6% for Δtir (pACYC184). Although these numerical means followed a pattern consistent with the hypothesis that Tir enhances colonization, the results were not significant ($P = 0.2$).

To assess whether previous exposure to Tir protein via enteric infection induces any protection against reinfection, cattle challenged with Tir⁺ or Tir⁻ strains at C1 were re-challenged 42 days later (C2) with the Nal^R parent strain (Figure 3). Cattle inoculated at C1 with WT had numerically lower mean shedding levels following C2, although their mean post-C2 shedding levels were nearly identical to the mean post-C2 shedding levels of cattle inoculated at C1 with the Nal^R strain. Those inoculated at C1 with Δtir (pEH86), Δtir, or Δtir (pACYC184) shed more following C2, although the results were not significant ($P = 0.14$). Cattle inoculated at C1 with the Nal^R parent shed at approximately the same level following C2. However, the results also showed that although the magnitude and duration of shedding of WT post-C1 was significantly greater than that of the Nal^R strain, the latter was able to infect cattle, and was shed at relatively high levels post-C2 in cattle that were exposed at C1 to non-flagellated Tir⁺ [Δtir (pEH86)] or Tir⁻ (Δtir) *E. coli* O157:H7 (Figure 3). Since initial infection with the tir-complemented mutant (pEH86) did not elicit any greater protection against C2 than did initial infection with the

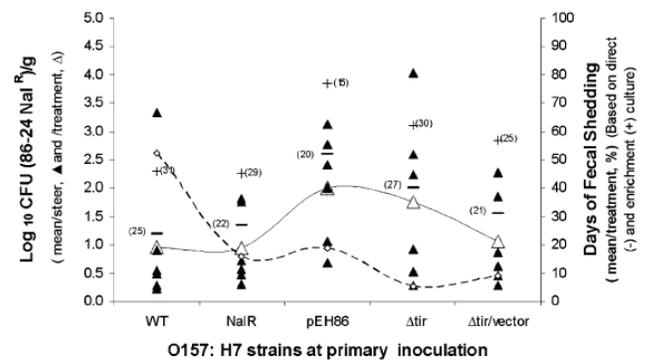


Figure 3. Magnitude (mean per steer, ▲; mean per treatment, Δ; log₁₀ CFU/g) and duration of fecal shedding (mean per treatment; percentage of days), based on direct plating (DP) and enrichment (E-IMS) culture following oral challenge with the Tir⁺ 86-24 Nal^R strain (C2), 42 days after inoculation with one of five isogenic Tir⁺ or Tir⁻ *E. coli* O157:H7 strains, as shown on X axis ($n = 6$). Data points for mean magnitude of fecal shedding after C2 are connected by a solid line (—). Fecal shedding was evaluated daily for 14 days. Mean magnitude of fecal shedding (◇; log₁₀ CFU/g) after primary inoculation (C1), with data points for different treatment groups connected by a dashed line (- - -) is shown. Standard deviations for the duration of fecal shedding based on DP and E-IMS are shown in parentheses.

Δtir and Δtir vector control strains (Figure 3), no definitive role of enteric Tir exposure on protection was detected. It is possible that production of flagella by the inoculum strain played an important role in C1 infection.

4. Discussion

We hypothesized that inactivation of tir in *E. coli* O157:H7 would reduce its ability to colonize the intestine of adult yearling cattle and consequently be shed in the feces at a reduced magnitude and duration. Additionally, we hypothesized that initial infection with a Tir⁺ strain would reduce shedding following a second challenge in experimentally-inoculated cattle. The numerical means were consistent with the hypothesis that Tir expression enhances colonization, but the results were not statistically significant. Further, we were unable to determine whether Tir exposure at C1 had any effect on protection since the lack of flagellar expression by the Δtir strains was a confounder in the study. Regardless, the results support the hypothesis that factors other than Tir-intimin binding have an important function in colonization ([Dobbin *et al.*, 2006] and [Naylor *et al.*, 2005]).

The mechanisms regulating flagellar expression are complex and flagellum biosynthesis is usually co-regu-

lated together with other virulence factors in Gram-negative bacteria (Soutourina and Bertin, 2003). Interestingly, a regulatory connection between the expression of flagella and other virulence factors (Soutourina and Bertin, 2003) including LEE (Sircili *et al.*, 2004) has been identified in enteropathogenic *E. coli*. In the present study, with few exceptions, the non-flagellated strains did not colonize well. This could either point to a role for the flagellum itself in colonization, or a role for a factor that regulates both production of the flagella and some other property that actually impacts colonization.

Dobbin *et al.* (2006) reported that the class I flagellar regulatory gene, *flhC*, and not the flagellin gene, *fliC* impacts *E. coli* O157:H7 colonization of calves. They noted that *flhDC* is a global regulator of 29 operons not related to motility (Dobbin *et al.*, 2006). These authors concluded that a functional flagellum is not necessary for colonization, and hypothesized that the reduced ability of their *E. coli* O157 Δ *flhDC* to colonize the terminal rectum of calves was due to effects on some factor other than flagella (Dobbin *et al.*, 2006). In several bacterial species, flagellum-mediated motility enhances gastrointestinal colonization ([Richardson, 1991] and [Allen-Vercoe and Woodward, 1999]). The flagellum and not intimin has a significant function in persistent *E. coli* O157:H7 infection of chicks (Best *et al.*, 2005).

The WT strain was shed at higher levels and for longer periods than the Nal^R and *tir*-complemented mutant strains. However, in those animals given a C1 inoculum of non-flagellated *E. coli* O157:H7, the Nal^R parent was shed at high levels ($P = 0.14$), comparable to that of the WT strain, following C2. This suggests that the host immunity to flagella might have contributed to the susceptibility of cattle to *E. coli* O157:H7 colonization after C2.

5. Conclusion

Variants of *E. coli* O157:H7 lacking flagellin expression, regardless of whether they expressed Tir, did not effectively colonize the intestines of inoculated cattle. Further, those given an initial inoculation with a non-flagellated variant of *E. coli* O157:H7 were more susceptible to a second challenge with motile *E. coli* O157:H7 than those originally inoculated with motile strains. The cause of the loss of expression of flagellin was not addressed. These findings could either point to a function of the flagellum itself in colonization, or one for a

factor that regulates both production of the flagella and some other effector that actually impacts colonization.

Acknowledgements

The project was supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, grant # 2001-02966, and USDA-CSREES Multi-State Research Project NC-1007.

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