Long Polar Fimbriae Contribute to Colonization by *Escherichia coli* O157:H7 In Vivo

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The contribution of long polar fimbriae to intestinal colonization by *Escherichia coli* O157:H7 was evaluated in sheep, conventional pigs, and gnotobiotic piglets. *E. coli* O157:H7 strains with *lpfA1* and *lpfA2* mutated were recovered in significantly lower numbers and caused fewer attachment and effacement lesions than the parent strain.

Escherichia coli O157:H7 is a cause of food-borne illness, mostly due to consumption of undercooked contaminated meat or contaminated vegetables (3, 14, 15, 19; J. Besser-Wiek, D. Boxrud, J. Bender, M. Sullivan, L. Carroll, and F. Leano, Abstr. 96th Gen. Meet. Am. Soc. Microbiol. 1996, abstr. C-364, 1996). *E. coli* O157:H7 can persist in experimentally infected sheep and pigs for two months and in cattle for longer (2, 4, 6). Intimin is imperative for the development of attachment and effacement (A/E) lesions (9, 11, 12, 20) and important for the colonization of cattle and sheep by *E. coli* O157:H7 (3, 5, 8). While the initial bacterial interaction with enterocytes is poorly understood, fimbriae may contribute early in the colonization process.

Long polar (LP) fimbriae are important for the pathogenesis and virulence of *Salmonella enterica* serovar Typhimurium causing murine typhoid (1, 21). *E. coli* O157:H7 contains two *lpf* operons homologous to *lpf* of serovar Typhimurium (16). The sequences of *E. coli* O157:H7 *lpfABCC'DE* operon 1 are comparable and produce proteins with functions similar to those of their serovar Typhimurium counterparts (19). LP fimbriae associated with operon 1 are suggested to enhance attachment to HeLa cells and formation of microcolonies by transformed *E. coli* K-12 (19). LP fimbrial operon 2 in *E. coli* O157:H7 has been recently characterized (10, 16; A. G. Torres, K. J. Kanack, C. B. Tutt, V. Popov, and J. B. Kaper, submitted for publication).

We hypothesized that LP fimbriae contribute to the colonization of sheep and pigs by *E. coli* O157:H7. Our approach compared the ability to colonize of two *lpf* mutant strains to that of the parent strain, *E. coli* O157:H7 86-24. Sheep were used to evaluate the ability of the *lpfA1* and *lpfA1 lpfA2* mutants to persist in a ruminant. Conventional pigs were dually infected to determine if the *lpfA1 lpfA2* mutant can be maintained as long as the parent strain. Neonatal gnotobiotic pigs were used to evaluate the early phase of infection with all three strains.

The inoculum strains were derivatives of *E. coli* O157:H7 strain 86-24 (18). The parent strain was a streptomycin- and nalidixic acid-resistant derivative. A chloramphenicol resistance cassette was inserted into *lpfA1* of the parent strain to create CVD468 (19). AGT210, the double mutant strain, was derived from CVD468 by insertion of a tetracycline cassette into *lpfA2* (Torres et al., submitted). Nonpathogenic *E. coli* strain 123 was used as a negative control (13). All bacteria were grown as overnight cultures in tryptic soy broth with appropriate antibiotics used at the following concentrations: 50 μg of streptomycin/ml, 10 μg of nalidixic acid/ml, 30 μg of chloramphenicol/ml, and 20 μg of tetracycline/ml. The inocula were prepared as previously described (2, 4, 17).

Prolonged colonization. Twenty-two 4- to 12-month-old sheep and eight 10-week-old conventional pigs were obtained, acclimated for 2 weeks, and screened to verify the lack of sorbitol-negative, O157-positive *E. coli* and to characterize the background intestinal flora. The sheep were divided into three groups and inoculated with 10^{10} CFU of either the parent strain (8 animals), CVD468 (6 animals), or AGT210 (8 animals). The pigs were dually inoculated with the parent and AGT210 strains at a dosage of 10^{10} CFU per strain per pig. The inoculum was delivered with feed; the animals were housed in pairs. Fecal samples were collected on three consecutive days from each animal at designated time points postinoculation (PI). There were an initial collection and collections at 2, 4, 6, and 8 weeks PI. The animals were necropsied on day 60 PI. The samples collected from the sheep were from the rumen, ileum, Peyer's patches, spiral colon, and distal colon. The samples collected from the pigs included tonsil, cecum, spiral colon, distal colon, and rectal content. Fecal and tissue samples collected from the animals were processed for bacteriologic evaluation as previously described (2, 4–6; Besser-Wiek et al., Abstr. 96th Gen. Meet. Am. Soc. Microbiol. 1996). A replica plating technique on additional selective media was

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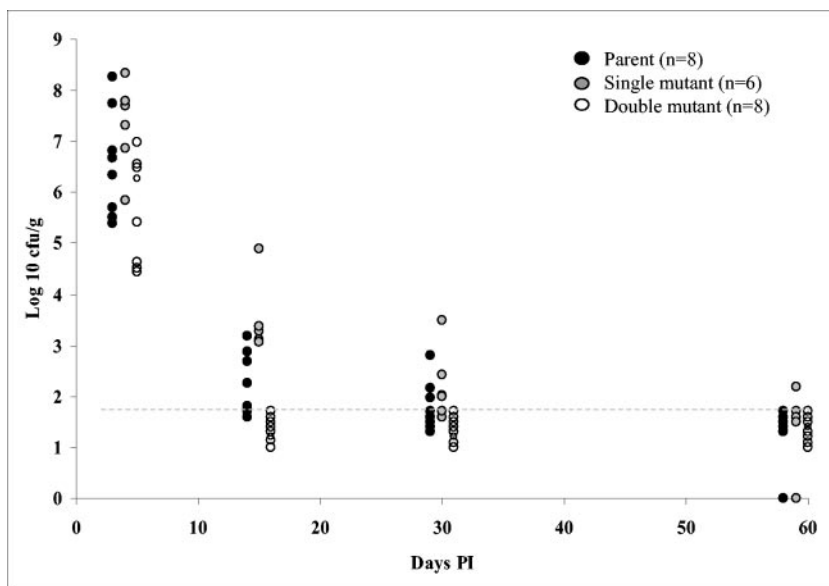
FIG. 1. Recovery of *E. coli* O157:H7 from the feces of sheep inoculated with three isogenic strains. Each symbol represents the geometric mean for one animal over each sampling period. The symbols below the dotted line represent samples that were positive only by enrichment culture. Samples in which no growth was detected by direct plating or enrichment culture are plotted at zero.

used to separate the strains from pigs that were administered the dual inoculum for quantification. Wilcoxon/Kruskal-Wallis nonparameteric tests were done for the bacterial counts. Logistic regression was done to evaluate the decline in shedding. JMP software (version 5.0.1a; SAS Institute, Inc.) was utilized for analysis with *P* values of <0.05.

All three inoculum strains were recovered from the feces of sheep throughout the study (Fig. 1). At 2 weeks PI, the parent strain and CVD468 were recovered from the feces at significantly (*P* < 0.01) higher levels than AGT210. At necropsy, the inoculum strains were recovered primarily from the lower gastrointestinal tract samples and only at enrichment levels (<50 CFU/cm²). The proportion of sheep shedding the inoculum strains was not different throughout the study.

Both inoculum strains were recovered from the feces of all eight conventional pigs during the initial period (Fig. 2). Significantly more (*P* < 0.007) parent-strain than AGT210 bacteria were recovered during the initial and 2-week periods. The proportion of pigs shedding each strain declined significantly (*P* < 0.004) over the course of the study; however, both strains were recovered intermittently from some pigs throughout the study. The parent strain was recovered at necropsy from the cecum, spiral colon, and distal colon of three pigs at levels of <100 CFU/g. One tonsil sample contained 7×10^4 CFU of the parent strain/g. In contrast, even though two pigs were shedding AGT210 in their feces at termination, AGT210 was not recovered from the tissues of any pigs at necropsy and was eliminated from significantly (*P* < 0.003) more pigs than the parent strain.

This study confirmed that *E. coli* O157:H7 can colonize sheep and pigs and further indicated that long-term colonization was facilitated by LP fimbriae. The effect of the double mutation of the LP fimbrial genes was more pronounced in pigs than in sheep. Significantly (*P* < 0.03) more sheep than pigs remained colonized by AGT210 during the study. In both

species, AGT210 tended to be recovered at lower levels than the parent strain; this was demonstrated best at the early time points (≤2 weeks PI). This has also been demonstrated in rabbits infected with a rabbit enteropathogenic E. coli (REPEC) strain with a mutation in *lpf* (15). Furthermore, recovery of AGT210 from the pigs was intermittent and reduced compared to recovery of the parent strain, indicating that LP fimbriae were facilitating its colonization. Intimin-mediated adhesion is one possible mechanism contributing to colonization by AGT210.

Early colonization. Germfree pigs were derived from five litters and orally inoculated at 24 h after birth with 10^5 CFU of bacteria. In aggregate, eleven pigs were inoculated with the parent strain, eight were inoculated with strain CVD468, thirteen were inoculated with strain AGT210, and nine were inoculated with strain 123. Based on a pilot study of 7 days' duration which indicated a possible effect of LP fimbriae early in the period of colonization (<24 h), the pigs were necropsied at 24 h PI. The samples collected were from the ileum, cecum, and spiral colon.

Colonization was defined by the presence of A/E lesions in formalin-fixed tissues collected at necropsy. Tissues were sectioned and stained with hematoxylin and eosin (H&E) and an O157-specific horseradish peroxidase immunohistochemical stain (7). Cross-sections of intestinal segments were evaluated and scored using the criteria in Table 1. Contingency analysis and pair-wise testing of the scored data were done.

The parent and mutant strains formed A/E lesions in cecum and spiral colon. Pigs infected with the parent strain (median lesion score = 1; range, 0 to 2) or CVD468 (median lesion score = 1; range, 0 to 1) had significantly (*P* < 0.02) higher lesion scores for the spiral colon than those infected with AGT210 (median = 0.5; range, 0 to 2). There were no statistical differences in the lesion scores for the cecum (median = 1) or the ileum (median = 0) for any of the pathogenic strains.
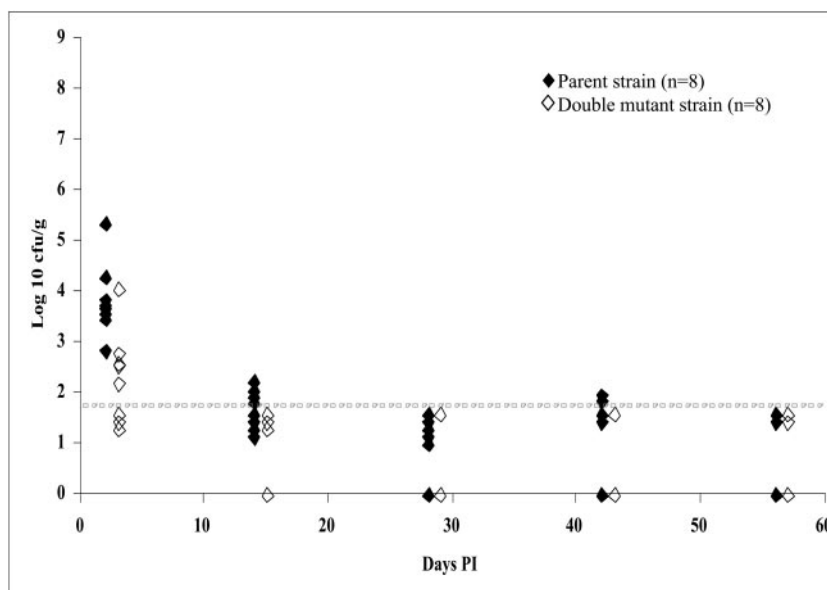
FIG. 2. Recovery of *E. coli* O157:H7 from the feces of conventional pigs dually inoculated with two isogenic strains. Each symbol represents the geometric mean for one animal over each sampling period. The symbols below the dotted line represent samples that were positive only by enrichment culture. Samples in which no growth was detected by direct plating or enrichment culture are plotted at zero.

There were no lesions in pigs inoculated with *E. coli* strain 123. The results were limited by the potential sampling error of histopathology in that only a small portion of intestine can be examined microscopically. Therefore, it is conceivable, but seemingly unlikely, that lesions other than those examined were dependent on *lpf*.

Sections of spiral colon from each group of inoculated pigs were prepared and evaluated by transmission electron microscopy. Upon evaluation, classical A/E lesions (14) were demonstrated in multiple sections of spiral colon from pigs infected with the parent strain, CVD468, and AGT210 (in three of three, one of one, and three of six pigs examined, respectively).

These findings support the hypothesis that LP fimbriae contribute to colonization by *E. coli* O157:H7. Mutations in both LP fimbrial operons diminished the ability of *E. coli* O157:H7 to establish and persist in sheep and pigs for 2 months and reduced, but did not eliminate, A/E lesions. As established for many bacterial species, redundant mechanisms enable bacteria to thrive and survive within many different environments; therefore, mutations in one system, i.e., the *lpf* operon, may not reduce the survival of the bacterial population.

TABLE 1. Criteria for A/E lesion scoring of formalin-fixed intestinal sections

Description of lesion severity	Lesion score
No enterocytes had A/E lesions	0
A/E lesions were detected only with immunohistochemical staining	0.5
<10% of the enterocytes in the section had A/E lesions detected with H&E staining	1
10–50% of the enterocytes in the section had A/E lesions detected with H&E staining	2
>50% of the enterocytes in the section had A/E lesions detected with H&E staining	3

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