A Double, Long Polar Fimbria Mutant of *Escherichia coli* O157:H7 Expresses Curli and Exhibits Reduced *In Vivo* Colonization

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Escherichia coli O157:H7 causes food and waterborne enteric infections that can result in hemorrhagic colitis and life-threatening hemolytic uremic syndrome. Intimate adherence of the bacteria to intestinal epithelial cells is mediated by intimin, but *E. coli* O157:H7 also possess several other putative adhesins, including curli and two operons that encode long polar fimbriae (Lpf). To assess the importance of Lpf for intestinal colonization, we performed competition experiments between *E. coli* O157:H7 and an isogenic Δ*lpfA1* Δ*lpfA2* double mutant in the infant rabbit model. The mutant was outcompeted in the ileum, cecum, and midcolon, suggesting that Lpf contributes to intestinal colonization. In contrast, the Δ*lpfA1* Δ*lpfA2* mutant showed increased adherence to colonic epithelial cells *in vitro*. Transmission electron microscopy revealed curli-like structures on the surface of the Δ*lpfA1* Δ*lpfA2* mutant, and the presence of curli was confirmed by Congo red binding, immunogold-labeling electron microscopy, immunoblotting, and quantitative real-time reverse transcription-PCR (qRT-PCR) measuring *csgA* expression. However, deletion of *csgA*, which encodes the major curli subunit, does not appear to affect intestinal colonization. In addition to suggesting that Lpf can contribute to EHEC intestinal colonization, our observations indicate that the regulatory pathways governing the expression of Lpf and curli are interdependent.

nfection with enterohemorrhagic *Escherichia coli* (EHEC) causes abdominal cramping and nonbloody diarrhea that is usually self-limiting but can progress to hemorrhagic colitis and hemolytic uremic syndrome (HUS). EHEC is transmitted through food or water contaminated by feces, usually from cattle, the major reservoir of EHEC. *E. coli* O157:H7 is the most common EHEC serotype linked to diarrheal disease in the United States, the United Kingdom, Canada, Japan, and Argentina (2, 5, 13, 17, 19). EHEC organisms belong to a group of bacteria that cause attaching and effacing (A/E) lesions on intestinal epithelial cells. Formation of A/E lesions is dependent on the locus of enterocyte effacement (LEE), which encodes a type III secretion system and numerous effector proteins. In particular, tight adherence of EHEC to intestinal cells requires transfer of the bacterial receptor Tir to host cells and expression of the Tir-binding adhesin, intimin (9). However, evidence suggests that an additional factor(s) comprised of both fimbrial and nonfimbrial adhesins may act to promote the initial loose attachment of EHEC to intestinal cells (reviewed in reference 28).

E. coli O157:H7 possesses two tightly regulated operons encoding long polar fimbriae (Lpf), which are induced *in vitro* in tissue culture media (24) or under iron-restricted conditions (25) and which have been found to influence adherence *in vitro* and colonization *in vivo* both for O157 strains and for other pathogenic *E. coli* strains (4, 6, 8, 26). Mutation of the *lpf1* operon in *E. coli* O157:H7 results in a reduced number of cells adhering to cultured HeLa cells (approximately one-fifth fewer than the number of adhering wild-type cells), as well as a reduction in microcolony formation (24). Similarly, O157:H7 *lpf2* mutants show somewhat reduced adherence to Caco-2 cells, and a possible role for *lpf2* in early adherence has been suggested (25). *In vivo* colonization assays using sheep and pigs have also suggested that the absence of Lpf impairs EHEC colonization (8). However, analyses of binding performed with human tissue, using *in vitro* organ culture,

showed that EHEC strains with *lpfA* mutations colonized intestinal regions that were not normally bound by a wild-type strain (6). Given the relatively subtle effects of *lpfA* mutations on adherence *in vitro*, coupled with the divergent findings regarding tissue colonization, the precise role of Lpf in EHEC adherence has remained somewhat ambiguous.

E. coli O157:H7 has the ability to produce, in addition to Lpf, thin aggregative fimbriae termed curli. These appendages may promote adhesion under specific conditions (10), and they have also been implicated in biofilm formation, invasion, and the induction of inflammation (reviewed in reference 1). However, curli are expressed in relatively few *E. coli* O157:H7 strains mainly in response to certain environmental conditions (29). For example, some strains have been reported to produce curli at 37°C under salt- and nutrient- or oxygen-limiting conditions (reviewed in reference 1). In addition, mutants with altered expression of curli have been described (29). The relationship between the expression of curli and other fimbriae has largely not been explored; however, there is evidence for cross talk in the regulation of other fimbrial structures (22, 30).

In this study, we have further explored the role of Lpf in *E. coli* O157:H7 adherence to, and colonization of, intestinal tissue. Col-

TABLE 1 Bacterial strains used in this study

Strain	Description	Reference or source
E. coli		
86-24	EHEC O157:H7 strain 86-24 Sm^r Nal^r	23
86-24∆lacZ	86-24 ∆*lacZ* Sm^r	This study
CVD468	86-24 ∆*lpfA1* Sm^r Cm^r	24
AGT201	86-24 ∆*lpfA2* Sm^r Tc^r	25
AGT210	86-24 ∆*lpfA1* ∆*lpfA2* Sm^r Cm^r Tc^r	25
SJL102	86-24 ∆*csgA* Sm^r Cm^r	This study
SJL101	86-24 ∆*lpfA1* ∆*lpfA2* ∆*csgA* Sm^r Cm^r Tc^r Km^r	This study
SM10 (λ *pir*)	(pCVD442 *csgA::kan*) Ap^r Km^r Suc^s	This study
S. Typhimurium	Strain 2157	Torres' collection

onization was assessed using the infant rabbit model of EHEC infection, which reproduces many aspects of intestinal disease seen during human EHEC infection (15). We found that a strain lacking both *lpfA1* and *lpfA2* (and thus lacking Lpf) is outcompeted in the rabbit intestine by its parental strain, suggesting that Lpf might contribute to intestinal adhesion. In contrast, the ∆*lpfA1* ∆*lpfA2* mutant showed increased adherence to cultured intestinal epithelial cells, perhaps due to overexpression of curli in this strain. Thus, analyses of the roles of Lpf and curli, both *in vivo* and *in vitro*, are likely to be complicated by regulatory cross talk between these adhesins. Nonetheless, our data suggest that both of these factors can promote EHEC interaction with the intestinal epithelium.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains are listed in Table 1 and were grown in Luria broth at 37°C and 225 to 250 rpm. All *E. coli* O157:H7 strains used in this study are derivatives of strain 86-24 (23). Antibiotics were used at the following concentrations: ampicillin (Ap), 100 µg/ml; chloramphenicol (Cm), 30 µg/ml; kanamycin (Km), 50 µg/ml; streptomycin (Sm), 100 µg/ml; and tetracycline (Tc), 12.5 µg/ml.

Animal experiments. Litters of 3-day-old New Zealand White infant rabbits were infected as described previously (15, 16). Briefly, the animals were orogastrically inoculated (~5 × 10^8 CFU per 90 g of rabbit) with the wild-type strain 86-24, the ∆*csgA* mutant, the ∆*lpfA1* ∆*lpfA2* mutant, or the ∆*csgA* ∆*lpfA1* ∆*lpfA2* mutant or with 1:1 mixtures of 86-24 and the aforementioned mutants. The bacterial inoculum was prepared by growing the strains overnight in L broth at 37°C in the presence of antibiotics, harvesting the cells by centrifugation, and then resuspending the cell pellet in sterile phosphate-buffered saline (PBS; pH 7.2) to give a final cell density of ~10^9 CFU/ml. The rabbits were necropsied 5 days postinoculation, tissue samples were taken from the small and large intestines, and the cecum, as well as fecal material, were collected for microbiologic analyses. In the single-infection experiments, the number of EHEC CFU recovered in the samples was determined by plating homogenates on media containing the appropriate antibiotics. In the competition experiments, we used two approaches to determine the number of wild-type and mutant cells present in the intestinal tissue. In some experiments, EHEC was recovered by plating on media containing Sm (to detect total numbers of EHEC), followed by replica plating onto media containing the appropriate antibiotic(s) to select for the mutants. In other experiments, a *lacZ* mutant of 86-24 was used as the wild-type strain to allow simultaneous detection of colonies based on their ability to utilize bromo-chloro-indolyl-galactopyranoside (also known as X-gal). This second approach was taken in case the use of multiple antibiotics affected the number of mutant cells that were recovered following *in vivo* passage. Preliminary studies showed that the 86-24 ∆*lacZ* mutant competed equally with the parental strain in

all regions of the intestine (data not shown). Experiments with mutants that exhibited reduced colonization in the rabbit intestine compared to colonization by the wild type were repeated in a second independent litter. In samples where no colonies were detected at the lowest dilution plated, the limit of detection (~10^2 CFU/g) was used as a value. Competition indices (CI) were calculated as the ratio of the mutant output to the wild-type output divided by the ratio of the mutant to the wild-type input. The CI ratios for each intestinal segment were compared to a theoretical value of 1 using the one-sample *t* test. In addition, for each intestinal segment, the CI obtained by the two different detection methods were compared using Student's two-way *t* test (GraphPad Prism version 5.02).

Cell culture and adherence assays. T84 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)-F12 at 37°C and 5% CO$_2$. Medium was supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic (Invitrogen/Gibco, Carlsbad, CA). For adhesion assays, 24-well plates were seeded with 10^5 cells per well and incubated as described above until ~80% confluent. Monolayers were washed twice with 1 ml of PBS, 1 ml of medium with no supplements was added to each well, and then wells were inoculated with 10^7 bacterial cells from overnight cultures (multiplicity of infection [MOI], 100). Inoculated monolayers were incubated for 3 h at 37°C and 5% CO$_2$. After incubation, cells were washed twice with PBS, and then 200 µl of 0.1% Triton X-100 in PBS was added to each well. Plates were incubated at 37°C and 5% CO$_2$ until monolayers detached from the bottom of the plate. Monolayers were homogenized by pipetting. Tenfold dilutions were plated onto LB agar using the drop plate technique described by Chen et al. (3). The percentage of bacteria recovered was calculated as the number of CFU/ml recovered divided by the initial number of CFU/ml multiplied by 100. The experiments were repeated 3 times in triplicate. Mean percentages of each mutant recovered were compared to the mean percentage of the wild type recovered using Student's *t* test. If all group means were compared, a one-way analysis of variance (ANOVA), followed by Holm-Sidak *post hoc* testing, was used. The *in vitro* competition assays were performed as described above except that cells were inoculated with 5 × 10^6 cells each of mutant and wild-type bacteria (the total number of bacteria/well was 10^7 cells). Numbers of CFU/ml for the mutant and wild type were determined by plating the bacterial mixture onto both LB agar and plates with appropriate antibiotics to select for the mutant. CI were calculated as described above and analyzed using one-way ANOVA and Holm-Sidak *post hoc* testing.

Gene disruption by allelic exchange. ∆*csgA* mutants were created with *E. coli* O157:H7 strain 86-24 and the isogenic double *lpfA* mutant AGT210 by allelic exchange using the *E. coli* SM10 (λ *pir*) strain carrying the suicide vector pCVD442 with the *csgA* gene from *E. coli* O157:H7 interrupted by a kanamycin resistance gene (pCVD442 *csgA::kan*). The suicide vector was introduced into the recipient strains by conjugation, and recombinants were selected by plating strains on the appropriate antibiotics and sucrose and then testing for ampicillin sensitivity to confirm the loss of pCVD442, as previously described (24). Interruption of *csgA* was confirmed by PCR, Congo red binding, and immunoblotting for curli. The *lacZ* mutant of 86-24 was also constructed by allelic exchange using *E. coli* SM10λ *pir* containing pES10 (a kind gift from Elizabeth Shaknovich). Integration of this vector results in deletion of amino acids 359 to 637 of the *lacZ* gene product.

Congo red binding assay. Congo red binding assays were performed as previously described (7). Briefly, bacterial cultures were grown overnight in Luria broth at 37°C and 225 rpm. Cultures were diluted 1:1 in sterile distilled water, and then 6-µl drops were spotted onto Congo red indicator (CRI) plates containing LB agar with no salt (LBNS), 40 µg/ml Congo red, and 20 µg/ml Coomassie brilliant blue. Plates were incubated at 30°C or 37°C and photographed at 24 h.

SDS-PAGE and immunoblotting. Cultures were grown on LB agar with no salt as described above for the Congo red binding assay. At 24 h and 48 h, bacteria were resuspended in 100 µl of 1× PBS. Samples were equilibrated to 10^9 cells as determined by measurements of optical density

TABLE 2 qRT-PCR primers used in this study

Primer name	Sequence (5′–3′)	Target gene	Reference or source
5RTRRSB	TGCAAGTCGAACGGTAACAG	*rrsB*	11
3RTRRSB	AGTTATCCCCCTCCATCAGG	*rrsB*	11
rpoS Fw	AGTCAGAATACGCTGAAAGTTCATG	*rpoS*	21
rpoS Rv	AAGGTAAAGCTGAGTCGCGTC	*rpoS*	21
csgAFw	TGGTAACAGCGCCACTCTTG	*csgA*	This study
csgARv	GACGGTGGAATTAGATGCAGTC	*csgA*	This study

at 600 nm (OD_{600}). Cells were spun down and resuspended in 70 μl of formic acid. The formic acid was evaporated off, and the pellets were resuspended in 200 μl 1× sample buffer, 10 μl of which was loaded onto a 15% SDS-PAGE gel. Semidry transfer onto a polyvinylidene difluoride (PVDF) membrane was performed according to standard procedures. The membrane was incubated overnight at 4°C with the primary antibody against purified curli (rabbit anti-curli at 1:7,000; kindly donated by Matt Chapman) and then incubated for 1 h at room temperature with the secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG at 1:10,000). Signal was detected using Amersham ECL Plus Western blotting detection agents (GE Healthcare, Buckinghamshire, United Kingdom) according to the manufacturer's instructions.

Transmission electron microscopy. Samples were grown statically at 37°C overnight in DMEM-high glucose, spun down, and resuspended in 30 μl 1× PBS. Formvar- and carbon-coated nickel grids (200 mesh) were floated on the cell suspension and stained with 2% potassium-phosphotungstic acid (pH 6.8) to visualize surface structures. For immunogold labeling, grids were prepared as described above but, prior to being stained with phosphotungstic acid, were incubated with rabbit anticurli at 1:1,000 in 1% bovine serum albumin (BSA)-PBS for 30 min at room temperature, washed in 1% BSA-PBS, and then incubated with goat anti-rabbit IgG antibody (1:20 in 1% BSA-PBS) labeled with 15-nm gold particles (Electron Microscopy Sciences, Hatfield, PA) for 30 min at room temperature, washed again with deionized water, fixed in 2% aqueous glutaraldehyde for 10 min, and washed in deionized water. Flagella and curli were visualized using a Phillips 201 electron microscope.

RNA isolation and cDNA synthesis. Total RNA was obtained from bacteria in contact with monolayers of T84 cells. One ml of DMEM with no supplements was added to each well, followed by infection with 2×10^8 bacterial cells obtained from overnight cultures. Infected monolayers were incubated for 1 and 2 h at 37°C and 5% CO_2. The supernatant was

pooled from 6 wells; the samples were stabilized with RNAprotect reagent (Qiagen, Valencia, CA) and harvested by centrifugation at 3,500 rpm for 20 min. Then, the samples were resuspended in 10 mM Tris-HCl (in 0.1% diethyl pyrocarbonate [DEPC]-H_2O). RNA was purified using the High Pure RNA isolation kit and treated with DNase I (Roche, Mannheim, Germany), quantified, and qualitatively analyzed on 2% agarose gels. Five micrograms of total RNA was used for cDNA synthesis by employing the SuperScript first-strand synthesis system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. A negative control with no reverse transcriptase was also included. The resulting cDNA was utilized for quantitative real-time reverse transcription-PCR (qRT-PCR).

qRT-PCR. qRT-PCR was performed by using the iQ SYBR green Supermix on the CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA). The *rrsB* and *rpoS* genes were used to normalize our data, and a value of 1 was used to standardize *csgA* gene expression to the wild-type strain; the primers used are listed in Table 2. For each reaction, 1 μl of reverse-transcribed cDNA was subjected to PCR amplification in a 12.5-μl final volume containing a 500 nM concentration of each primer and 6.5 μl of 2× SYBR iQ SYBR green Supermix. The following conditions were used for amplification: 1 cycle at 95°C for 30 s and then 40 cycles at 95°C for 5 s and 60°C for 30 s. To ensure the specificity of the PCR products, melting curve analysis was performed by heating products from 65°C to 95°C in increments of 0.5°C every 5 s while monitoring the fluorescence. Expression of *csgA* in each mutant strain at different time points was compared to that of the wild-type strain at 1 h of incubation with T84 cells. These assays were performed in duplicate for each strain. A Student's *t* test was used for statistical analysis.

RESULTS

The Δ*lpfA1* Δ*lpfA2* mutant is outcompeted by wild-type EHEC in an infant rabbit model of infection. Single-strain infections and competition assays between the wild-type O157:H7 strain 86-24 and mutants lacking either one copy or both copies of *lpfA* (which encodes the major fimbrial subunits) were performed with infant rabbits. Rabbits inoculated with the wild-type strain or either of the single *lpfA* mutants (CVD468 and AGT201) or the double *lpfA* mutant (AGT210) developed diarrhea and were colonized (data not shown). However, when rabbits were coinfected with a 1:1 mixture of the double *lpfA* mutant AGT210 and wild-type EHEC, there were approximately 10-fold-fewer mutant CFU recovered from all regions of the intestine and in the stool than with the other strains. Thus, the geometric mean CI were around
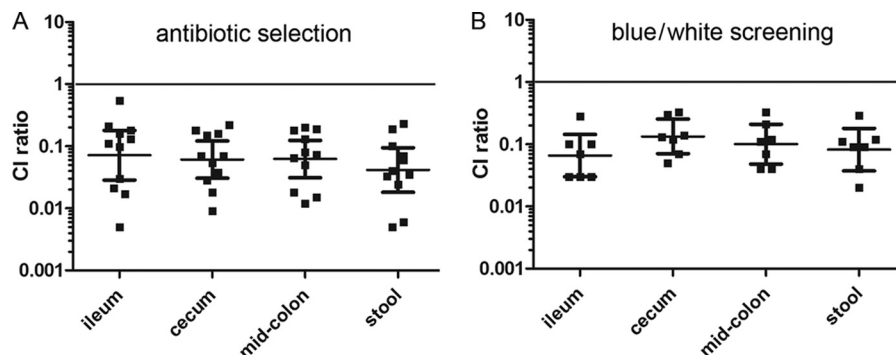

FIG 1 The Δ*lpfA1* Δ*lpfA2* mutant is outcompeted by 86-24 in the infant rabbit intestine. Three-day-old rabbits were orogastrically inoculated with 1:1 mixtures of *E. coli* O157:H7 strain 86-24 and an isogenic Δ*lpfA1* Δ*lpfA2* mutant, AGT210 (A), or 86-24Δ*lacZ* and the Δ*lpfA1* Δ*lpfA2* mutant (B). Animals were euthanized after 5 days. The number of colonies counted on antibiotic-containing medium or medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was used to calculate competition indexes (CI). A CI is the ratio of the number of mutant CFU to the number of wild-type CFU in output samples divided by the number of mutant to wild-type CFU in the inoculum. A CI value of 1 (shown by the black line) indicates that the mutant competes equally with the wild-type strain. Bars represent the geometric means with 95% confidence intervals. The CI of samples from the same intestinal site but with different selection criteria used were compared by Student's *t* test and not found to be significantly different.

TABLE 3 Competitive index values for mutants obtained following colonization of the large intestines of infant rabbits

| Genotype of mutant	*In vivo* colonization	
	Geometric mean	95% confidence interval
Δ*lpfA1*	0.70	0.38–1.32
Δ*lpfA2*	3.29	1.81–5.98
Δ*lpfA1* Δ*lpfA2*	0.07	0.05–0.12
Δ*csgA*	0.92	0.72–1.18
Δ*lpfA1* Δ*lpfA2* Δ*csgA*	0.02	0.007–0.08

0.1, regardless of whether antibiotics (Fig. 1A) or blue/white screening (Fig. 1B) was used to determine the relative numbers of bacteria. The CI ratios in all intestinal sections and in the stool were significantly less than 1 ($P < 0.001$), but there was no significant difference in the CI ratios obtained from the same tissue section using the two detection methods. Deletion of only one of the LpfA subunits was not sufficient to mediate a defect in colonization of the colon (Table 3). The ~10-fold reduction in intestinal colonization of the strain lacking both Lpf genes in the competition experiment suggests that these fimbriae contribute to the capacity of EHEC to colonize the intestine. The absence of a reduction in colonization or disease for the double *lpf* mutant in the single-infection experiments may reflect the limited sensitivity of this assay and/or the presence of additional factors that can substitute for Lpf under these conditions.

The Δ*lpfA1* Δ*lpfA2* mutant shows increased adherence to cultured colonic epithelial cells. Inactivation of both sets of Lpf genes might impair colonization by disrupting adhesion of *E. coli* O157:H7 to the intestinal epithelium. To explore this possibility, we assessed the ability of various strains to adhere to T84 cells. Inoculation of T84 monolayers with single strains resulted in recovery of the single *lpfA1* and *lpfA2* mutants and the Δ*lpfA1* Δ*lpfA2* mutant (34.4% ± 10%, 27.7% ± 4%, and 32.8% ± 7%, respectively) at levels similar to the recovery of wild-type EHEC (29.5% ± 8%, $P = 0.78$). However, when competition adhesion assays were performed using the double *lpfA* mutant AGT210 and wild-type EHEC, the mean CI (1.69 ± 0.12) was significantly

greater than 1($P = 0.03$). This unexpected finding suggested that the strain lacking all Lpf might produce an additional factor that provided a subtle advantage in the adherence assay and/or might be detrimental *in vivo*.

Production of curli is increased in the Δ*lpfA1* Δ*lpfA2* mutant. To further explore potential differences in adhesin production by the wild-type strain and the Δ*lpfA1* Δ*lpfA2* mutant, we used electron microscopy to visualize the surfaces of the two strains. Both negative staining and immunostaining were used to detect the presence of flagella, fimbriae, or other adhesins on bacteria grown in DMEM at 37°C, conditions known to induce Lpf expression. Unexpectedly, micrographs revealed structures like curli on the surface of the Δ*lpfA1* Δ*lpfA2* mutant strain AGT210 (Fig. 2B). These structures were confirmed to be curli by immunogold labeling using sera generated against purified curli (Fig. 2C). Flagella but no other fimbriae were visible on the surface of wild-type EHEC (Fig. 2A).

The production of curli in the wild type and the Δ*lpfA1* Δ*lpfA2* mutant was investigated using immunoblotting and via growth of EHEC on Congo red indicator (CRI) plates. For both assays, the growth medium was LB with no salt because it has been shown that low salt induces maximal expression of curli (1). Expression was monitored at 30°C as well as at 37°C, as low temperature has also been observed to induce expression (1). As can be seen in the electron micrographs of cells grown at 37°C in liquid culture (Fig. 2), these assays indicated that expression of curli was elevated in the Δ*lpfA1* Δ*lpfA2* mutant relative to expression in the wild-type strain at 37°C. No production of CsgA (the 17-kDa major constituent of curli) was observed on Western blots of wild-type cells grown at 37°C, while abundant CsgA was evident in samples from the Δ*lpfA1* Δ*lpfA2* mutant (Fig. 3B). Furthermore, the Δ*lpfA1* Δ*lpfA2* mutant was pink/red when cultures spotted on indicator plates were grown at 37°C, consistent with binding of Congo red by amyloid-cell-like curli. In contrast, spots of the wild-type strain were a light tan, as were those of a strain lacking *csgA* and a Δ*csgA* Δ*lpfA1* Δ*lpfA2* triple mutant, which, as expected, lacked detectable CsgA on immunoblots. As seen with other strains, 86-24 had a slightly darker (but not red) color when grown on CRI plates at
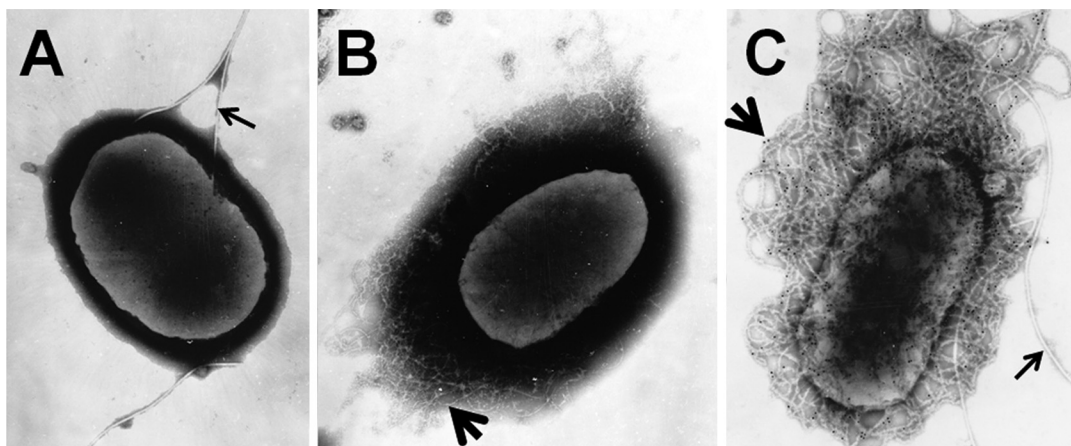
FIG 2 Curli are expressed by the Δ*lpfA1* Δ*lpfA2* mutant. EHEC 86-24 (A) and the Δ*lpfA1* Δ*lpfA2* mutant AGT210 (B) were grown statically overnight at 37°C in DMEM. Samples were spun down, resuspended in PBS, and then stained with phosphotungstic acid (A and B) or incubated with anti-CsgA sera and then with a secondary antibody labeled with 15-nm gold particles (C). Thin arrows indicate flagella (A and C), and thick arrows indicate curli-like fibrils (B) or CsgA-labeled fibrils (C).
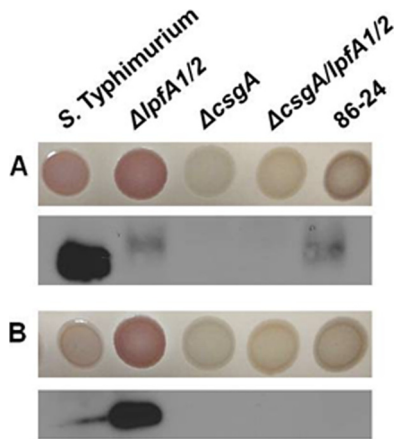
FIG 3 Congo red binding and detection of EHEC curli by immunoblotting. Overnight cultures of wild-type *Salmonella enterica* serovar Typhimurium and EHEC 86-24 and the isogenic mutants AGT210 (Δ*lpfA1* Δ*lpfA2*), SJL102 (Δ*csgA*), and SJL101 (Δ*lpfA1* Δ*lpfA2* Δ*csgA*) were spotted (6 μl) onto LBNS agar containing 40 μg/ml Congo red and 20 μg/ml Coomassie brilliant blue and incubated for 24 h at 30°C (A) or 37°C (B). The positive control for Congo red binding at 30°C was *S.* Typhimurium.

30°C, which is consistent with the slight production of CsgA detected on immunoblots for this condition (Fig. 3A). Interestingly, the Δ*lpfA1* Δ*lpfA2* mutant showed markedly reduced levels of CsgA at 30°C, and on immunoblots, CsgA abundance did not differ from that of the wild-type strain under this condition, suggesting that deletion of *lpfA1* and *lpfA2* has less effect on *csgA* regulation at 30°C than at 37°C. However, for reasons that remain unclear, the Δ*lpfA1* Δ*lpfA2* mutant still appears redder than the wild-type strain on CRI plates at 30°C.

Quantitative real-time PCR was used to support the immunoblot results and to quantify the expression of *csgA* in the wild-type, Δ*lpfA1* Δ*lpfA2* double mutant, and Δ*csgA* Δ*lpfA1* Δ*lpfA2* triple mutant strains while in contact with T84 cell monolayers for 1 or 2 h. Basal expression of *csgA* in the wild-type strain after 1 h of incubation was set at a value of 1, and all the other values of expression at 1 and 2 h were related to this threshold. The expression of *csgA* was 1.63-fold higher in the Δ*lpfA1*Δ *lpfA2* double mutant than in the wild-type strain (Fig. 4) (*P* = 0.01), and threshold expression was observed in the Δ*csgA* Δ*lpfA1* Δ*lpfA2* triple mutant. After 2 h of incubation with T84 cells, the *csgA* expression level in the Δ*lpfA1* Δ*lpfA2* mutant remained approximately the same (1.73-fold), while expression by the wild type increased 1-fold compared to the 1-h baseline value (Fig. 4). Expression of *csgA* in the Δ*csgA* Δ*lpfA1* Δ*lpfA2* mutant remained at baseline levels.

Contribution of curli to adhesion and intestinal colonization. Given the abundance of curli produced by the Δ*lpfA1* Δ*lpfA2* mutant, we determined whether these might promote its interaction with epithelial cells and thereby account for the enhanced adherence of this strain to T84 cells. As described above, adhesion was assessed using both single-strain and competition assays. In assays of single strains, there was no difference between the levels of adherence of wild-type, Δ*csgA*, Δ*lpfA1* Δ*lpfA2*, and Δ*csgA* Δ*lpfA1* Δ*lpfA2* strains to T84 cells (Fig. 5) (*P* = 0.17). However, a significant difference in adherence for the single Δ*csgA* mutant from that of the Δ*csgA* Δ*lpfA1* Δ*lpfA2* triple mutant (two-way Student's *t* test, *P* = 0.04) was detected. Thus, the lack of Lpf
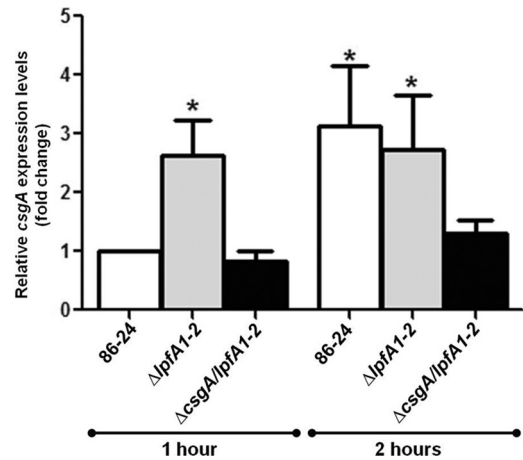
FIG 4 Quantitative real-time RT-PCR analysis of *csgA* expression. The strains were incubated for 1 h and 2 h with T84 cell cultures at 37°C and 5% CO_2. The fold variation of gene expression was obtained by the comparative cycle threshold ($\Delta\Delta C_T$) method. Wild-type EHEC 86-24 *csgA* expression at 1 h is represented as a value of 1, and all other values of expression at 1 h and 2 h are related to this value. The expression of *csgA* was 1.63-fold higher (*, *P* = 0.01) in the Δ*lpfA1* Δ*lpfA2* mutant than in the wild type at 1 h in contact with T84 cells. After 2 h, the expression of *csgA* increased 1-fold in the wild type and remained at ~1.7-fold (*, *P* < 0.05) in the Δ*lpfA1* Δ*lpfA2* mutant. Expression levels of *csgA* in the Δ*csgA* Δ*lpfA1* Δ*lpfA2* triple mutant remained virtually unchanged. Student's *t* test was used for statistical analysis.

impairs, rather than aids, adherence in the absence of curli. Together, these observations suggest that the enhanced adherence of the Δ*lpfA1* Δ*lpfA2* mutant that was observed in initial assays may have been a consequence of the overexpression of curli. However, when expressed at wild-type levels, curli do not appear to play a major role in adherence, based on the results of both the single-strain assays presented above and the competition assays, in which the wild type and Δ*csgA* mutant were recovered in equal numbers (CI, 1.32 ± 0.3; *P* = 0.40). Somewhat unexpectedly, the Δ*csgA* Δ*lpfA1* Δ*lpfA2* triple mutant did not have reduced adhesion rela-
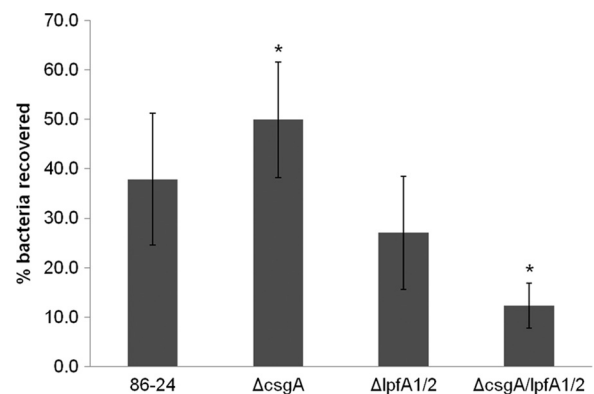
FIG 5 Adherence of *E. coli* O157:H7 and isogenic *lpfA* and/or Δ*csgA* mutants to T84 cells. Monolayers of T84 cells were inoculated with 10^7 bacterial cells from an overnight culture of the wild-type EHEC strain 86-24, AGT210 (Δ*lpfA1* Δ*lpfA2*), SJL102 (Δ*csgA*), and SJL101 (Δ*lpfA1* Δ*lpfA2* Δ*csgA*) and incubated at 37°C and 5% CO_2 for 3 h. T84 cell monolayers were lysed, and 10-fold serial dilutions were plated on LB agar to determine numbers of CFU/ml. Percentages of bacteria recovered were calculated as (final number of CFU per ml/initial number of CFU per ml) × 100. The single Δ*csgA* mutant and the triple mutant were compared by Student's *t* test (*, *P* = 0.04).

tive to that of the Δ*lpfA1* Δ*lpfA2* mutant (CI, 0.96 ± 0.89; $P =$ 0.89), arguing against a role for curli as the sole adhesin promoting attachment in this strain background. However, unlike the Δ*lpfA1* Δ*lpfA2* mutant, the Δ*csgA* Δ*lpfA1* Δ*lpfA2* triple mutant did not display enhanced adherence relative to the wild-type strain (CI, 2.73 ± 1.58; $P = 0.24$), data that support a role for curli in adhesion. Collectively, these data suggest that both Lpf and curli may promote the adherence of EHEC to epithelial cells; however, neither is essential, and interdependence in their expression (and potentially that of other adhesins as well) can complicate analyses of their roles.

Given that curli appear to enhance the adhesion of the Δ*lpfA1* Δ*lpfA2* mutant *in vitro*, we assessed whether the absence of curli influences EHEC intestinal colonization in infant rabbits. In a wild-type background, deletion of *csgA* had no effect upon colonization of the colon (Table 3) (geometric mean [95% confidence interval], 0.92 [0.72 to 1.18]), suggesting that basal levels of curli do not make a major contribution to colonization. Furthermore, consistent with our findings above, the CI of the Δ*csgA* Δ*lpfA1* Δ*lpfA2* triple mutant was similar to that of the Δ*lpfA1* Δ*lpfA2* mutant (Table 3) (triple versus double mutant CI geometric mean [95% confidence interval], 0.02 [0.007 to 0.08] versus 0.07 [0.05 to 0.12]), suggesting that even in the Δ*lpfA1* Δ*lpfA2* background, where curli may be overexpressed, this appendage does not contribute to colonization. The single *lpfA* mutants were not outcompeted by the wild type *in vivo* (Table 3), and none of the mutants were outcompeted by the wild type in *in vitro* growth experiments (data not shown), suggesting that the *in vivo* intestinal competition deficits of the double and triple mutants are not due to general growth defects.

DISCUSSION

We have investigated the importance of Lpf and curli in the colonization of and adherence to intestinal epithelial cells by *E. coli* O157:H7, using *in vivo* and *in vitro* assays. We found that a strain lacking both *lpfA1* and *lpfA2* had an impaired ability, relative to the wild-type strain, to colonize the infant rabbit intestinal tract. This result is consistent with previous colonization analyses performed in pigs (8). Unexpectedly, we found that the Δ*lpfA1* Δ*lpfA2* mutant did not exhibit reduced adhesion to cultured intestinal epithelial cells; instead, it showed enhanced adhesion in competition assays against the wild-type strain. Notably, the Δ*lpfA1* Δ*lpfA2* mutant produced elevated amounts of curli, both in liquid culture and on solid media, raising the possibility that curli can compensate for the absence of Lpf under certain conditions. A connection between expression of curli and Lpf in *E. coli* O157:H7 has not previously been described.

Although defects in colonization and adherence were reproducibly observed for the Δ*lpfA1* Δ*lpfA2* mutant in competition assays against a wild-type strain, such deficiencies were not evident in single-strain infections. It is likely that the competition assays enable better detection of relatively subtle colonization defects, as the presence of an internal control (i.e., the wild-type strain) allows compensation for variability between animals, which in a single-infection study may mask the effects of the mutation of interest. Given that the effects of *lpfA1* and *lpfA2* deletion upon colonization are relatively small (~10-fold versus the 10,000-fold effects of a *tir* or an *eae* gene deletion [15]), it is not particularly surprising that the deficiencies of this strain could not be detected in the single-strain analyses. However, it should be noted that competition analyses are not always preferable; for example, *trans*-complementation by a wild-type strain can mask a true deficit in the mutant (14).

Our observation that deletion of *lpfA1* and *lpfA2* results in augmented expression of curli, at least under a subset of growth conditions, may be emblematic of a paradigm for much of fimbrial expression (22, 30). It is clear that *E. coli* O157:H7 strains often encode numerous putative fimbrial adhesins; however, many of these do not appear to be widely expressed in wild-type strains (12). In fact, electron micrograph analyses suggest that some EHEC strains do not synthesize any fimbriae when grown in LB, and therefore, alternate growth conditions are needed to induce expression (20). Overall, the current work strongly supports our previous reports indicating that the production of fimbriae in *E. coli* O157:H7 is tightly regulated (18, 26, 27). However, given that a variety of fimbriae and afimbrial surface structures can promote adhesion, it is possible that the absence of an adhesin often induces EHEC strains to synthesize an alternate structure. Such a compensatory mechanism has also been observed in uropathogenic *E. coli* (UPEC); strains that do not express type 1 and P fimbriae instead use F1C fimbriae to adhere to mammalian kidney cells (22). The possibility of cross talk playing a role in the regulation of fimbrial structures complicates genetic analyses of their roles, as one structure may compensate for the absence of another.

It remains to be determined why the double deletion of *lpfA1* and *lpfA2* alters the production of curli, at least in *E. coli* O157:H7 strain 86-24. However, the absence of Lpf is not sufficient to induce the production of curli, as CsgA production in the Δ*lpfA1* Δ*lpfA2* mutant grown at 30°C was not different from that of the wild type. Thus, it appears that other stimuli also contribute to the production of curli, i.e., after exposure to T84 cells. It is plausible to propose that curli are induced under cell culture conditions or that *csgA* is transcribed as detected (Fig. 4) but that curli are not produced due to posttranscriptional regulation. It would also be interesting to explore whether induction of curli via means other than *lpfA* deletion influences Lpf production, i.e., whether cross talk in the production of these fimbriae is bidirectional. Finally, additional studies to determine the factors and mechanisms that control their expression, the components within epithelial cells that are recognized by these adhesins, and the ways in which adhesion and adhesins change during the course of an intestinal infection are all warranted.

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