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## Cloning, Expression, and Characterization of Fimbrial Operon F9 from Enterohemorrhagic *Escherichia coli* O157:H7

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Recent transposon mutagenesis studies with two enterohemorrhagic *Escherichia coli* (EHEC) strains, a serotype O26:H- strain and a serotype O157:H7 strain, led to identification of a putative fimbrial operon that promotes colonization of young calves (1 to 2 weeks old). The distribution of the gene encoding the major fimbrial subunit present in O-island 61 of EHEC O157:H7 in a characterized set of 78 diarrheagenic *E. coli* strains was determined, and this gene was found in 87.2% of the strains and is therefore not an EHEC-specific region. The cluster was amplified by long-range PCR and cloned into the inducible expression vector pBAD18. Induced expression in *E. coli* K-12 led to production of fimbriae, as demonstrated by transmission electron microscopy and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. The fimbriae were purified, and sera to the purified major subunit were raised and used to demonstrate expression from wild-type *E. coli* O157:H7 strains. Induced expression of the fimbriae, designated F9 fimbriae, was used to characterize binding to bovine epithelial cells, bovine gastrointestinal tissue explants, and extracellular matrix components. The fimbriae promoted increases in the levels of *E. coli* K-12 binding only to bovine epithelial cells. In contrast, induced expression of F9 fimbriae in *E. coli* O157:H7 significantly reduced adherence of the bacteria to bovine gastrointestinal explant tissue. This may have been due to physical hindrance of type III secretion-dependent attachment. The main F9 subunit gene was deleted in *E. coli* O157:H7, and the resulting mutant was compared with the wild-type strain for colonization in weaned cattle. While the shedding levels of the mutant were reduced, the animals were still colonized at the terminal rectum, indicating that the adhesin is not responsible for the rectal tropism observed but may contribute to colonization at other sites, as demonstrated previously with very young animals.

Enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 causes gastrointestinal disease in humans that can be life threatening as a consequence of Shiga toxin activity on kidney and brain vasculature. Ruminants, particularly cattle, are the primary reservoir hosts for this organism (1, 3). Cattle are colonized without any overt symptoms and can shed EHEC O157:H7 in their feces for several weeks at levels up to 10<sup>6</sup> cells per g. Recent work has demonstrated that the predominant colonization site in cattle for *E. coli* O157:H7 is the final few centimeters of the terminal rectum, a site having a high density of lymphoid follicles and lymphoid-associated mucosa (19, 21, 30). A key aim of our research is to identify bacterial factors that drive this tropism for the terminal rectum.

Considerable attention has been focused on type III secretion that leads to intimate attachment, and this has been shown to be important for ruminant colonization in a number of studies (5, 7, 8, 22, 44). For example, in a recent signature-tagged mutagenesis (STM) screening for genes that promote *E. coli* O157:H7 colonization in young calves, the workers obtained multiple transposon inserts in the locus of enterocyte effacement, which contains the majority of the type III secretion apparatus genes, as well as the genes encoding the translocated intimin receptor and intimin, which are required for intimate attachment (11). Type III secretion is therefore central to ruminant colonization. However, bacterial tissue affinity is often a result of fimbrial adhesins that promote initial attachment, and examples of such adhesins in *E. coli* are P, S, F4 (K88), F5 (K99), 987P, and type 1 fimbriae. In the STM study mentioned above (11) the workers obtained an insert in Z2203 that exhibits homology with the usher gene in fimbrial operons, such as the operon encoding type 1 fimbriae. This gene is in a

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cluster that contains genes with homology to genes encoding the classic chaperone/usher class fimbrial adhesins and does not appear to contain pseudogenes, so it should encode functional fimbriae. Deletion of the putative main subunit gene produced a strain that was less competitive for colonization of very young calves (11), although such animals do not exhibit the terminal rectal tropism apparent in older weaned animals. The same putative fimbrial operon was also identified as a relevant operon for colonization in an STM screening of enterohemorrhagic *E. coli* O26:H- in cattle (43). Interestingly, the same study established that failure to down-regulate type 1 fimbria expression in *E. coli* O26 is also a disadvantage for colonization.

Based on an analysis of the previously published sequences (14, 25), *E. coli* O157:H7 contains at least 16 putative fimbrial operons, and expression screening of these operons showed that the operon containing Z2203 in EHEC O157:H7 O-island 61 (OI61) is one of only four operons that show some expression under *in vitro* conditions (19a). Furthermore, O-island 61 is one of five regions in the chromosome of EHEC O157:H7 strain EDL933 encoding putative fimbrial proteins that were described as EHEC-specific fimbria loci (25). A modified version of the cluster is also present in the sequenced uropathogenic *E. coli* isolate CFT073 (45), and a truncated version is present in *E. coli* K-12 strain MG1655 (11). Very little is known about the other putative fimbrial clusters in *E. coli* O157:H7, with the exception of the clusters encoding long polar fimbriae (LPF1 and LPF2), type 1 fimbriae, and curli (4, 10, 18, 28, 38, 39, 42). Type 1 fimbriae cannot be expressed by *E. coli* O157:H7 because of a deletion in the *fim* regulatory region (28). Even if these fimbriae are expressed, the sequenced isolates contain a mutation in FimH that eliminates mannose binding (16). The LPF1 and LPF2 clusters have mutations in the usher and chaperone genes, respectively, although at least the LPF1 cluster still appears to be able to produce fimbriae and may contribute to colonization *in vivo* (38, 39). Curli expression is variable in EHEC O157:H7 isolates.

In addition to type III secretion-related adherence and fimbrial adhesins, *E. coli* O157:H7 has a number of other factors that promote adherence, at least adherence *in vitro* to cell lines. These factors include OmpA, Iha, Cah, ToxB, and a truncated version of Efa1 (Efa1') (23, 35–37, 40, 41). ToxB and Efa1' play no apparent role in colonization of young calves by *E. coli* O157:H7, and it is not clear whether they act as adhesins *per se* or influence *in vitro* adherence via effects on type III secretion (35, 37). The roles of other putative EHEC adhesins in colonization of cattle have not been determined.

The aims of the current study were to learn more about the distribution of O-island 61 and other O-island putative fimbrial gene clusters in *E. coli*, to determine whether the O-island 61 operon (Z2200 to Z2206) from *E. coli* O157:H7 can produce functional fimbriae, to investigate different potential binding substrates, and to establish whether expression of the cluster drives the EHEC O157:H7 tropism for the terminal rectum of cattle.

#### MATERIALS AND METHODS

**Media and bacterial strains.** The three Shiga toxin-negative *E. coli* O157:H7 strains used in this research are *E. coli* EDL933 *stx* (= TUV933-0) (9), ZAP198 (= Walla 3) (24), and NCTC12900. The *E. coli* K-12 derivative AECC185 (2)

was used for the majority of the cloning work and to study induced expression of F9. AECC185 is an *E. coli* K-12  $\Delta$ *recA*  $\Delta$ *fim* strain that does not express fimbriae (2) and that has been used for expression of foreign fimbrial genes (12). The well-characterized reference collection of diarrheagenic *E. coli* (DEC) strains (46) was used as indicated below. Bacteria were cultured either in minimal essential medium with HEPES modification, in M9 minimal medium (with 0.4% glycerol as a carbon source), or in Luria-Bertani (LB) broth. Agar (4%) was added to make appropriate plates. Ampicillin at a concentration of 25  $\mu$ g/ml was used for maintenance of the pBAD18 derivative plasmids, and chloramphenicol at a concentration of 25  $\mu$ g/ml was used for maintenance of pACYC184 derivatives. For arabinose induction 0.2% (wt/vol) arabinose was added to M9 or LB media.

**Detection of putative major fimbria subunit genes encoded on O islands.** The DEC strains (46) were screened for five genes, representing the major fimbrial subunits of five putative EHEC-specific O islands, by PCR and hybridization. The DEC strains were obtained from the STEC Center at Michigan State University (46) and were selected because they included strains representing common clones of enteropathogenic *E. coli* and EHEC in which the evolution of virulence and pathogenic mechanisms has been established previously (27). The genes encoding two of the major fimbrial subunits, *lpfA1* and *lpfA2*, were analyzed previously by Torres et al. (39). Recombinant *Taq* polymerase and a PCR buffer from Gibco-BRL-Invitrogen were employed with 1 U of *Taq* polymerase, 2 mM MgCl<sub>2</sub>, and 1  $\mu$ M oligonucleotide primer in each reaction mixture. All amplifications were performed for 30 cycles of 94°C for 45 s, 45°C for 1 min, and 72°C for 2 min. PCRs were performed with boiled bacterial colonies. To amplify the genes encoding the corresponding major fimbria subunits, the following primer pairs (forward and reverse primers, respectively) were used: for OI141 *lpfA1*, 5'-GGTCGTTTTGCTTAACCGC-3' and 5'-AGGTTGAAATCGACCTGCGC-3'; for OI154 *lpfA2*, 5'-TTATGGCAGGTACCTACAGGC-3' and 5'-TCAACTGTAACTGCGAGTCGG-3'; for OI47 Z1534, 5'-CAAAAAGCAGCTCTGCTGGC-3' and 5'-CGACAGCGGTAATTTACCGGC-3'; for OI61 Z2200, 5'-GATTGTCGTTTCTGTGTTGGCG-3' and 5'-TGAAGGTCGCATCTGCGTTTTG-3'; and for OI27 Z4498, 5'-ACAGCTATTGCGGGCTTACTTTG-3' and 5'-TTAACGACAGCGTCTGCTTCCC-3'. Colony hybridization with digoxigenin (DIG)-labeled probes was performed to validate the PCR results. Fragment probes that were generated by PCR using EHEC O157 strain EDL933 as the template and the primer pairs listed above were used to screen colony blots of all DEC isolates, using 82-mm nylon membranes (Roche Applied Science, Indianapolis, IN). Membranes were denatured in 0.5 M NaOH–1.5 M NaCl, neutralized in 1.5 M NaCl–0.5 M Tris-HCl–1 mM EDTA, dried, and fixed by UV exposure. All probes were purified by gel extraction and DIG labeled by using a commercially available DIG DNA labeling and detection kit (Roche Applied Science) and the manufacturer's instructions. Following 2 h of pre-hybridization at 42°C, the membranes were hybridized with a denatured probe at 42°C with continuous, gentle agitation in a hybridization solution containing 50% formamide, 5 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5% blocking reagent, 0.1% *N*-lauryl sarcosine, and 0.02% sodium dodecyl sulfate (SDS). The membranes were washed three times in 2 $\times$  SSC–0.1% SDS and then three times in 0.1 $\times$  SSC–0.1% SDS at 65°C (high stringency) or 55°C (low stringency). Signals were detected by using a DIG nucleic acid detection kit (Roche) in accordance with the manufacturer's instructions.

**Cloning of F9 fimbrial operon.** To determine if the O-island 61 cluster was able to express fimbriae, the cluster was amplified by long-range PCR (Roche) in two sections, and the sections were then cloned and combined in pBAD18 (13), an arabinose-inducible vector, to create pASL04. The first section was amplified using primers 5'CCGAGCTCCTTAATTGTTAACTCTCTTTATTTCAGC (SacI restriction site underlined) and 5'CTGTCCACCCGGGCCCTTTACTATTG (XmaI restriction site underlined) and cloned using the SacI and XmaI restriction sites. The second section was amplified using primers 5'CAATAGTAAAA GGCCCGGGTGGAAACAG (XmaI restriction site underlined) and 5'GCTAG TCTAGAGATTTTCTTACAAGTGTAGGATAATAG (XbaI restriction site underlined) and cloned using the XmaI and XbaI restriction sites.

**Construction and measurement of an F9 translational fusion to eGFP.** The putative promoter region of Z2200 was amplified by PCR from *E. coli* O157:H7 strain Walla 3 using primers 5'CGAGGATCCGCCATATAAACCATTAGTAGCC (BamHI restriction site underlined) and 5'GCCGGTACCATGTTTGA GTTTCATAATTCTCC (KpnI restriction site underlined) and cloned into pAJR70 (29), which contains the enhanced green fluorescent protein (eGFP) gene, using the BamHI and KpnI restriction sites. This created a translational fusion to the start of the Z2200 open reading frame. This plasmid was transformed into *E. coli* O157:H7 strain Walla 3, and fluorescence levels were measured by analyzing 100- $\mu$ l aliquots of cultures using a fluorescent plate reader

(Fluorostar Optima; BMG) during growth of the bacteria in LB medium at 28, 34, and 37°C.

**Electron microscopy.** To visualize fimbrial structures on the bacterial cell surface, *E. coli* K-12 containing either pBAD18 or pASL04 was induced for 2 h with 0.2% (wt/vol) arabinose in M9 medium with glycerol. A drop of bacterial suspension was placed onto a carbon-Formvar-coated electron microscope grid and left for 2 min. The suspension was removed with filter paper, and 20  $\mu$ l of gluteraldehyde fixative (4%) was placed on the slide and left for 2 min. The fixative was then removed, and the slide was allowed to air dry before negative staining was carried out. The bacteria adsorbed to carbon-Formvar-coated electron microscope grids were negatively stained with either 2% aqueous phosphotungstic acid (pH 6.8) or 2% aqueous ammonium molybdate. The grids were transferred to a Philips CM120 Biotwin microscope for examination and subsequent photography. Kodak SO163 TEM film was used and processed according to the manufacturer's recommendations. The negatives were scanned with an Epson Perfection 4870 scanner.

**Method for preparation of F9 fimbriae.** The method used for purification of fimbriae was adapted from the method of Korhonen et al. (17). Briefly, bacteria (strain AAEC185 containing pASL04) were grown in LB broth (180 rpm, 37°C) under inducing conditions (0.2% [wt/vol] arabinose). The cells were harvested by centrifugation (6,000  $\times$  g, 4°C, 15 min) and suspended in cold Tris buffer (10 mM Tris-HCl, pH 7.5). The fimbriae were detached from the cells by heating the suspension at 60°C with magnetic stirring for 1 h. After centrifugation (4,000  $\times$  g, 4°C, 15 min) crystalline ammonium sulfate was added to the supernatant to 50% saturation, and the suspension was stirred overnight at 4°C. The precipitate was collected by centrifugation (10,000  $\times$  g, 4°C, 1 h), suspended in Tris buffer, and dialyzed (100,000-molecular-weight cutoff dialysis tubing [Medicell International Ltd., London, United Kingdom]) for 48 h against Tris buffer with several changes of the buffer. After dialysis, sodium deoxycholate was added to the suspension to a final concentration of 0.5% (wt/vol), and the suspension was dialyzed against DOC buffer (Tris buffer containing 0.5% sodium deoxycholate) for 48 h. The suspension was centrifuged (10,000  $\times$  g, 4°C, 10 min), and the supernatant was collected. The supernatant was designated DOC-soluble material, and the pellet was designated DOC-insoluble material. According to Korhonen et al. (17), no pilus protein should remain in DOC-insoluble material, whereas most of the outer membrane proteins remained in this fraction. The DOC-soluble material was dialyzed for 48 h against Tris buffer with several changes of buffer to remove the sodium deoxycholate from the fimbrial preparations. A control preparation obtained by starting with just induced pBAD18 in *E. coli* K-12 was processed in an identical manner using one-half the volumes.

**Western blotting.** Wild-type strains and the F9 deletion mutant were cultured overnight in 1 liter of LB medium at the relevant temperature. The fimbriae were removed as described above, except that they were precipitated with trichloroacetic acid (TCA) added to a final volume of 10% (vol/vol). The TCA precipitates were incubated overnight at 4°C, and the precipitated material was collected by centrifugation at 15,000  $\times$  g at 4°C for 30 min. The pellets were suspended in 10 mM Tris-HCl buffer (pH 7.5), and the volume depended on the optical density of the original culture. Purified fimbriae (2.36 mg/ml) were prepared for SDS-polyacrylamide gel electrophoresis (PAGE) as described below. The fimbrial samples were acid treated by adding 2  $\mu$ l of 1 M HCl and 2  $\mu$ l of 10% SDS to 16  $\mu$ l of each fimbrial sample and boiling the preparation for 3 min to break up the fimbriae into subunits. Gel loading buffer was then added to the sample, and the preparation was neutralized with NaOH (1 M) and then boiled for a further 3 min before it was loaded onto a 12% SDS-PAGE gel, which was run under standard conditions. The proteins were transferred to a nitrocellulose membrane for Western blotting using standard procedures. The blot was blocked with milk powder (10%) in phosphate-buffered saline (PBS). The primary antibody used to identify the F9 subunit proteins is described below and was used at a 1:100 dilution in PBS. The blot was then washed three times with PBS containing Tween 20 (0.05%). A horseradish peroxidase-conjugated secondary antibody (Dako) against rabbit immunoglobulins was used as the secondary antibody at a dilution of 1:500 in PBS.

**Cloning, expression, and purification of the putative major F9 fimbrial subunit, Z2200.** The Z2200 open reading frame, including initiation and stop codons, was amplified by PCR using VENT proofreading DNA polymerase and primers MFSLic-for (5' GACGACGACAAGATGAACTCAAACATGT TGGT 3') and MFSLic-rev (5' GAGGAGAAGCCCGTTATAGATATTGA ACTTTG 3'). Following treatment with T4 DNA polymerase, the PCR product was cloned by ligation-independent cloning into the pET-30 Ek/LIC bacterial expression vector (Novagen, United Kingdom) according to the manufacturer's protocol. We selected recombinants which expressed a Z2200 fusion protein with N-terminal His and S tags for detection and purification. The resulting plasmid was transformed into *E. coli* BL21(DE3) cells (Novagen, United Kingdom), and

the strain was cultured in 50 ml LB medium containing 2% (wt/vol) D-(+)-glucose to the mid-logarithmic phase at 37°C prior to induction with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) overnight at 37°C with shaking at 200 rpm. Cells were collected by centrifugation and resuspended in 10 ml Tris-buffered saline prior to disruption by sonication. The supernatant and pellet fractions were separated by low-speed centrifugation (ca. 3,000  $\times$  g), and the insoluble (pellet) fraction was resuspended in denaturing binding buffer (8 M urea, 0.1 M sodium phosphate buffer, 0.01 M Tris-Cl; pH 8.0) and mixed with Ni-nitrilotriacetic acid His $\cdot$  Bind resin (Novagen, United Kingdom). The fusion protein was allowed to bind to the resin at the ambient temperature overnight with gentle rotation, and the resin was recovered by centrifugation at ca. 1,500  $\times$  g for 5 min at the ambient temperature before repeated washing (0.1 M sodium phosphate buffer, 0.01 M Tris-Cl; pH 6.3) and elution of the protein (0.1 M sodium phosphate buffer, 0.01 M Tris-Cl; pH 4.5). The purity of the protein was assessed by 4 to 15% gradient SDS-PAGE.

**Construction of Z2200 insertion and nonpolar deletion mutants of *E. coli* O157:H7.** Replacement of the Z2200 gene with a kanamycin resistance cassette was achieved by integration of linear DNA following transient expression of bacteriophage  $\lambda$  Red recombinase as described by Datsenko and Wanner (6). Briefly, a kanamycin resistance cassette was amplified from plasmid pKD4 using primers MFS1P1 (5'-AATTGTTAACTCTCTTTATTCAGCTACTTAAATAT AAATTTTGGGAATGTGTAGGCTGGAGCTGCTTC-3') and MFS2P2 (5'-CACACACCCCTGTTTAAATTTTCAGATGCCGTATAGCCACCTTAATA TTGAACATATGAATATCCTCCTTAG-3'). The 5' termini of these primers comprise 50 nucleotides identical to the sequences flanking the predicted start and stop codons of the Z2200 gene, and the 3'-terminal 20 nucleotides prime from pKD4. The amplified product was concentrated on QIAGEN spin columns, digested with DpnI for 1 h at 37°C, and then purified following agarose gel electrophoresis using a QIAGEN gel purification kit. A nalidixic acid-resistant variant of *E. coli* O157:H7 strain Walla 3 (21) was transformed with the temperature-sensitive  $\lambda$  Red-encoding plasmid pKD46 with selection for ampicillin resistance at 30°C. The Z2200::Kan<sup>r</sup> PCR product was used to transform electrocompetent Walla 3 Nal<sup>r</sup>(pKD46) following induction of expression of  $\lambda$  Red recombinase by growth in LB medium in the presence of 10 mM L-arabinose at 30°C as described previously (6). Mutants were selected on LB agar containing nalidixic acid and kanamycin and were confirmed to carry the insertion by PCR using the Z2200 flanking primers MFS-for (5'-CTCTCTTAATTTCAGCTACTT-3') and MFS-rev (5'-TTCAGATGCCGTATAGCCAC-3'). Plasmid pKD46 was cured from the mutant by overnight growth at 37°C, and the mutant was confirmed to be ampicillin sensitive. An *E. coli* O157:H7 strain EDL933 Nal<sup>r</sup> Z2200::Kan<sup>r</sup> mutant recently characterized in calves harbored a mutation identical to the mutation constructed here (11).

A Z2200 nonpolar deletion mutant was selected by transient expression of FLP recombinase in strain Walla 3 Nal<sup>r</sup> Z2200::Kan<sup>r</sup>, which excised the kanamycin resistance cassette via recombination between FLP recognition target sites flanking the Kan<sup>r</sup> gene, leaving an 84-nucleotide "scar." This was accomplished by transformation of Walla 3 Nal<sup>r</sup> Z2200::Kan<sup>r</sup> with the temperature-sensitive plasmid pFT-A (26) and transient induction of FLP using 20  $\mu$ g/ml heat-inactivated chlortetracycline during logarithmic growth at 30°C as described previously (26). Bacteria were plated onto LB medium and replica plated onto media with and without kanamycin to identify recombinants. Successful excision of the cassette was confirmed by PCR with the flanking primers MFS-for and MFS-rev (see above) and sequencing of the PCR product (Lark Technologies, United Kingdom) (data not shown). Plasmid pFT-A was cured by growth at 37°C in the absence of ampicillin.

We also wanted to compare the genetic structure of the mutant with that of the parent strain approximately 10 kb proximal and distal to the site of mutation. Chromosomal DNA was isolated from both wild-type strain Walla 3 Nal<sup>r</sup> and mutant Walla 3 Nal<sup>r</sup>  $\Delta$  Z2200. A 20-kb sequence that included the Z2200 gene was retrieved from Colibase (<http://colibase.bham.ac.uk>), and primers were designed to amplify approximately 10-kb regions on either side of the Z2200 gene. The proximal region of the Z2200 gene was amplified from both wild-type and mutant strains with primers Prox 5 (5'-AGTACTACTGATGGCGCTGGAT-3') and Prox 3 (5'-CTTGATTGGTTGTTGCCCTGAGA-3') to generate approximately 9.9-kb fragments, using Herculase Hotstart DNA polymerase (Stratagene). Similarly, the distal region was amplified from both wild-type and mutant strains with primers Dist 5 (5'-TTAAGGTGGCTATACGGCATCTG-3') and Dist 3 (5'-GTGGTTGACTACTCGTTAGCA-3') to generate an approximately 10-kb fragment. Fragments that were similar sizes were generated for both strains (data not shown). To confirm that these fragments represented similar DNA sequences, both proximal and distal amplified fragments from wild-type and mutant strains were gel purified (QIAGEN) and digested with 10 U of EcoRI for 3 h. The digests were resolved on 0.7% agarose alongside undigested DNA, and

the results revealed expected numbers and sizes of fragments following EcoRI digestion (data not shown).

**Generation of rabbit polyclonal antiserum against purified Z2200 protein.** Approximately 25 µg highly purified Z2200 fusion protein was resolved on an SDS-PAGE gel and eluted from a gel slice by maceration in 1 ml phosphate-buffered saline. After centrifugation at 10,500 × g to remove gel fragments, 600 µl of the protein suspension was mixed with 600 µl TiterMax Gold adjuvant (CytRx Corp., Norcross GA), and an emulsion was prepared. A 10-week-old New Zealand White rabbit was immunized subcutaneously with 250 µl of this emulsion at four different sites (ca. 13 µg protein per immunization), and the immunization was repeated twice at 3-week intervals (for a total of three immunizations). Ten days after the final immunization the blood was collected by cardiac puncture under terminal anesthesia, and serum was collected. Seropositivity and the specificity of the antiserum for Z2200 were confirmed by Western blot analysis using lysates of the Z2200 expression strain and the control BL21(DE3) parent strain (data not shown).

**Immunofluorescence microscopy.** Bacteria were fixed by mixing them with an equal volume of 4% paraformaldehyde. Aliquots of fixed bacteria were air dried onto slides and incubated with the primary anti-O157 serum (1:100; MAST Diagnostics) or anti-F9 serum (1:100) (this study) in PBS–0.1% bovine serum albumin (BSA) for 60 min at room temperature. After three washes with PBS–0.1% BSA, the samples were incubated with either fluorescein isothiocyanate-conjugated (1/500; Dako), tetramethyl rhodamine isocyanate-conjugated (1/500; Dako), or Alexa Fluor 594-conjugated (1/500; Molecular Probes) anti-rabbit immunoglobulin secondary antibody for 30 min, and the slide was washed three times with PBS–0.1% BSA or PBS–0.1% Tween 20. The slide was then examined by fluorescence microscopy using a Leica DMBL fluorescence microscope and appropriate filter sets. The images were captured using Leica or Impropvision software.

**Bacterial binding to purified substrates.** To identify a substrate for F9, a series of binding experiments were carried out using purified bovine substrates. Solutions (1 mg/ml) were prepared for the following substrates: BSA, fibrinogen, fibronectin, type I collagen, type III collagen, calf skin collagen, elastin, bovine skin gelatin, globulins, hyaluronic acid, bovine serum, and chondroitin sulfate A (all obtained from Sigma-Aldrich). Then 150 µl of each of these substrates was left on glass slides overnight at 4°C to coat the slides. The suspensions were then removed and washed once with PBS–Tween 20. Bacterial cultures in M9 medium with glycerol (with selective antibiotics) were diluted 1:50 into fresh M9 medium with glycerol (with selective antibiotics) and allowed to reach an optical density at 600 nm of 0.25 before induction with 0.2% arabinose for 2 h. The optical density at 600 nm of both cultures were adjusted to 0.5 before the assays were carried out, and 150 µl was added to the substrate-coated slides. The binding reaction was allowed to take place at 37°C for 1 h. The slides were then prepared for immunofluorescence microscopy as described above.

**Epithelial cell binding assays.** Epithelial bovine lung (EBL) cells were seeded into eight-chamber microscope slides (Becton and Dickinson) at a density of  $1 \times 10^5$  cells/chamber and were incubated at 37°C in 5% CO<sub>2</sub> for 24 to 48 h prior to binding assays. For some assays the *E. coli* strains were also transformed with the *rpsM::GFP*<sup>+</sup> fusion plasmid pAJR145 (29) to simplify quantification of adherent bacteria. In other assays the bacteria were imaged by anti-O157 lipopolysaccharide staining as described above for the substrate binding assays. Induced and control cultures were prepared as described above for the substrate binding assays. The culture growth medium (minimal essential medium) was removed from the EBL cells, and bacteria were added to the appropriate well of each multichamber slide (300 µl per chamber). The bacteria were centrifuged onto the cells at 2,000 rpm for 5 min. The cells were then incubated at 37°C in the presence of 5% CO<sub>2</sub> for 2 h. The bacterial suspension was then removed, and the wells were gently washed three times with PBS before addition of 4% paraformaldehyde to fix the cells. The slides were then viewed with the microscope using the appropriate filter, and the numbers of bacteria attached to cells were recorded.

Binding assays were also carried out with primary cultures derived from bovine terminal rectal epithelium. The primary cultures were prepared as described by Mahajan et al. (20) and then treated and quantified as described above for the EBL cell assay.

**Tissue explant binding assays.** The in vitro organ culture conditions were essentially those described previously (22). Prior to the experiment the conditions were optimized for use with bovine tissue explants. Briefly, prewarmed IVOC culture medium (45% [vol/vol] Dulbecco modified Eagle medium [Sigma Aldrich], 45% [vol/vol] NCTC-135 medium [Sigma Aldrich], 10% [vol/vol] newborn calf serum [Sigma Aldrich]) was added until the fluid surface was in contact with the tissue edge. The standard 50 µl of bacterial culture in the mid-logarithmic growth phase was added to each explant, and the samples were incubated at

37°C in an atmosphere containing 95% (vol/vol) O<sub>2</sub> and 5% (vol/vol) CO<sub>2</sub> for 8 h. The culture medium was changed every 2 to 3 h. At the end of the culture period the samples were washed once in fresh culture medium and fixed in 4% (wt/vol) paraformaldehyde in PBS. *E. coli* O157 was visualized by immunostaining as described above for substrate binding.

**Animals and experimental challenge.** Experimental calf challenge with the F9 mutant strain of *E. coli* O157:H7 strain Walla 3 was performed at the Moredun Research Institute (MRI) in containment level 3 large animal housing facilities under Home Office license number 60/3179. Ethical approval was obtained from the MRI Animal Experiments Committee. Calves were reared conventionally on the farm of origin until at least 2 weeks after weaning and transported to MRI, where they were acclimatized for 1 week prior to challenge. Fecal samples obtained from each calf prior to challenge were confirmed to be negative for *E. coli* O157:H7 by immunomagnetic separation, performed according to the manufacturer's instructions (Dynal). At the time of challenge the age of each of the calves was  $21 \pm 2$  weeks. The challenge bacterial strain was grown in LB broth (18 h at 37°C, with aeration) and diluted in sterile PBS to obtain an inoculum containing 10<sup>9</sup> CFU in 20 ml (total volume) for each animal. The inoculum was administered to the calves via a stomach tube and was washed down with 500 ml sterile PBS (21). For enumeration of bacteria in feces, samples were separated into surface and core components as described previously (21). Ten-gram portions of feces were suspended in 90 ml sterile PBS and serially diluted in 10-fold steps in PBS. The serial dilutions were cultured as 100-µl aliquots spread in triplicate onto sorbitol MacConkey agar plates containing 15 µg/ml nalidixic acid (Oxoid). All inoculated media were incubated overnight at 37°C. Non-sorbitol-fermenting colonies were counted, and a colony from each sample was tested for O157 lipopolysaccharide using a latex agglutination test kit (Oxoid). The most probable number of recovered bacteria was determined as described previously (21). Enrichment cultures of samples that were negative as determined by direct culturing were established from 1 ml of an undiluted fecal suspension added to 9 ml of LB medium containing 15 µg ml<sup>-1</sup> nalidixic acid. Following incubation at 37°C for 24 h, 100-µl portions of the enrichment cultures were spread onto sorbitol MacConkey agar plates containing 15 µg/ml nalidixic acid and incubated overnight at 37°C. Strains were compared to *E. coli* O157:H7 parent strain Walla 3 by using data obtained from 19 different calves challenged and sampled in containment level 2 animal housing as described above.

To compare the numbers of surface CFU/g for calves orally inoculated with the F9 deletion mutant and the numbers of surface CFU/g for calves orally inoculated with the wild-type strain, a repeated-measures model was fitted in Genstat, 8th ed. The correlation between observations made with the same calf was modeled using a power metric. A  $\log(x + 1)$  transformation was used in the analysis. For comparisons of the numbers of CFU/g in core and surface fecal samples (which provided an indication of terminal rectal colonization), differences were calculated on a  $\log(x + 1)$  scale.

## RESULTS

**Screening for the EHEC O-island putative fimbrial clusters in a collection of DEC strains.** We determined the presence of the gene encoding the putative major fimbrial subunit found in F9, as well as the presence of the genes encoding four other major fimbrial subunits located in EHEC O157:H7 O islands (O islands are EHEC-specific regions, as described by Perna et al. [25]), in a collection of phylogenetically well-characterized DEC strains (46). The five O islands included OI141, containing genes for long polar fimbria 1 (38); OI154, containing genes for long polar fimbria 2 (39); OI47, containing genes for a putative fimbria exhibiting 30 to 44% identity to CupA1-5 of *Pseudomonas aeruginosa* and *Yersinia pestis*; OI61, containing genes for F9, whose protein products exhibit homology with the well-characterized *E. coli* type 1 fimbriae (see below); and OI127, containing genes for a putative fimbria with 85 to 99% identity to a putative fimbrial locus of *E. coli* K-12. Of the 78 DEC strains selected, 68 (87.2%) Z2200 (F9, OI61)-positive *E. coli* strains were identified by colony dot blot hybridization and were confirmed by PCR (Table 1). Similarly, Z4498 (OI127) was detected in 69 (88.5%) of the strains tested, and Z1534 (OI47) was detected in 65.4% of the strains tested. In contrast,

TABLE 1. Distribution of genes encoding major fimbria subunits in five O157-specific islands in a collection of DEC strains<sup>a</sup>

DEC no. <sup>b</sup>	Serotype	OI141 <i>lpfA1</i> <sup>c</sup>	OI154 <i>lpfA2</i> <sup>c</sup>	OI47 Z1534	OI61 Z2200	OI127 Z4498	DEC no. <sup>b</sup>	Serotype	OI141 <i>lpfA1</i> <sup>c</sup>	OI154 <i>lpfA2</i> <sup>c</sup>	OI47 Z1534	OI61 Z2200	OI127 Z4498
1A	O55:H6	+	-	-	+	-	8C	O111:NM	-	-	+	+	+
1B	O55:H6	+	-	-	+	-	8D	O111:H11	-	-	+	+	+
1C	O55:H6	+	-	-	+	-	8E	O111:H8	-	-	+	+	+
F563-55	O55:H6	+	-	-	-	-	9A	O26:H11	-	-	+	+	+
1E	O55:H6	+	-	-	+	+	9B	O26:N	-	-	+	+	+
2A	O55:H6	+	-	-	+	+	C240-52	O26:N	-	-	+	+	+
2B	O55:NM	+	-	-	+	+	9D	O26:H11	-	-	+	+	+
2C	O55:H6	+	-	+	+	-	45	O26:H11	-	-	+	+	+
2D	O55:H6	+	-	-	+	-	H30	O26:H11	-	-	+	+	+
2E	O55:H6	+	-	-	+	-	10B	O26:H11	-	-	+	+	+
3A	O157:H7	+	+	+	+	+	10C	O26:H11	-	-	+	+	+
3B	O157:H7	+	+	+	+	+	C12-52	O26:H11	-	-	+	+	+
3C	O157:H7	+	-	+	+	+	10E	O26:H11	-	-	+	+	+
3D	O157:H7	+	+	+	+	+	RDEC-1	O15:NM	-	-	+	-	+
3E	O157:H7	+	+	+	+	+	11A	O128a:H2	-	-	+	+	+
493/89	O157:H-	+	+	+	+	+	11B	O128a:H2	-	-	+	+	+
4A	O157:H7	+	+	+	+	+	11C	O45:H2	-	-	+	+	+
4B	O157:H7	+	+	+	+	+	11D	O128:H2	-	-	+	+	+
4C	O157:H7	+	+	+	+	+	11E	O128:H2	-	-	+	+	+
4D	O157:H7	+	+	+	+	+	12A	O111:H2	-	-	+	+	+
4E	O157:H7	+	+	+	+	+	12B	O111:H2	-	-	+	+	+
EDL933	O157:H7	+	+	+	+	+	12C	O111:NM	-	-	+	+	+
5A	O55:H7	+	+	+	+	+	12D	O111:H2	-	-	-	-	+
5B	O55:H7	+	+	+	+	-	12E	O111:N	-	-	-	-	+
5C	O55:H7	+	+	+	+	+	13A	O128:H7	-	-	-	+	+
5D	O55:H7	+	+	+	+	+	13B	O128:H7	-	-	-	+	+
5E	O55:H7	+	+	+	-	+	13C	O128:H7	-	-	-	+	+
6A	O111:H21	-	-	+	-	+	13D	O128:H7	-	-	-	+	+
C142-54	O111:H12	-	-	+	-	+	13E	O128:H47	-	-	-	+	+
6C	O111:H12	-	-	-	-	-	14A	O128:H21	-	-	-	+	+
F436-51	O111:H12	-	-	+	-	+	14B	O128:H21	-	-	-	+	+
6E	O111:NM	-	-	+	-	+	14C	O128a:H21	-	-	-	+	+
7A	O157:H43	-	-	+	+	+	14D	O128:NM	-	-	-	+	+
7B	O149:NM	-	-	+	+	+	14E	O128:H21	-	-	-	+	+
7C	O157:H43	-	-	+	+	+	15A	O111:H21	-	-	-	+	+
7D	O157:H43	-	-	+	+	+	15B	O111:H21	-	-	-	+	+
7E	O157:NM	-	-	+	+	+	15C	O111:H21	-	-	-	+	+
8A	O111a:NM	-	-	+	+	+	15D	O111:H21	-	-	-	+	+
8B	O111:H8	-	-	+	+	+	15E	O111:H21	-	-	-	+	+

<sup>a</sup> The presence of the major fimbrial subunit gene of each O island was confirmed by PCR and colony blot hybridization as described in Materials and Methods.  
<sup>b</sup> DEC classification data were derived from the study of Whittam et al. (46).  
<sup>c</sup> The distribution of the *lpfA1* and *lpfA2* genes is based on data from the study of Torres et al. (39), and the data indicate the distribution of these genes in individual strains.

*lpfA1* (OI141) and *lpfA2* (OI154) were found in 34.6% and 20.5% of the strains tested, respectively, and these values are similar to the values described previously (39). Interestingly, serotype-specific distribution patterns were observed (Table 1). Our data indicate that the O islands described above are not EHEC-specific regions and that their distribution in different DEC strains is associated with the genetic rearrangements associated with evolution and the appearance of different serotypes.

**F9 fimbrial cluster.** Figure 1 shows an alignment of the F9 cluster in *E. coli* O157:H7 with the F9 cluster in *E. coli* K-12 strain MG1655. It is clear from this alignment that the main fimbrial subunit gene, the chaperone, and one-half of the usher gene are deleted in *E. coli* K-12. Based on this sequence information, an *E. coli* K-12  $\Delta$ *fim* strain is therefore a logical strain in which to express the cloned cluster from *E. coli* O157:H7. The F9 cluster is most closely related to the F1 (type 1) fimbria cluster of *E. coli* and other *Enterobacteriaceae*. The putative F9 proteins exhibit the following levels of amino acid

identity and similarity with the type 1 fimbrial proteins of *E. coli* K-12 strain MG1655, as determined by the alignment analysis: for the main subunit, 64% identity and 76% similarity; for the chaperone, 56% identity and 70% similarity; for the usher, 56% identity and 69% similarity; and for the adhesin, 47%

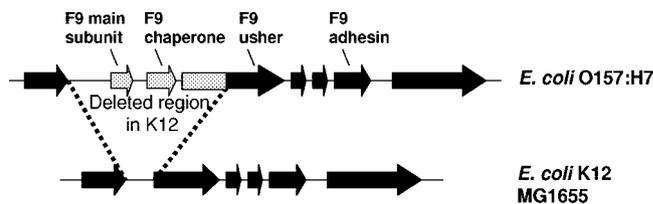


FIG. 1. Comparison of the F9 operon structures in *E. coli* O157:H7 strain EDL933 and *E. coli* K-12 strain MG1655. *E. coli* K-12 has a natural deletion of the first part of the operon and does not express the fimbriae. The sequenced uropathogenic isolate *E. coli* CFT073 contains a homologous region that is 98% identical (at the nucleotide level) to the *E. coli* O157:H7 region (45).

identity and 62% similarity. The most relevant variations are the variations in the main and adhesin subunits, as they determine binding specificity. For comparison, type 1 fimbriae and F1C fimbriae, which have similar structures but different target receptors, differ by 63% and 74%, respectively, in the main subunit and 31% and 46%, respectively, in the adhesin. Our work performed with the reagents described below demonstrated that F9 fimbriae do not cross-react with a type 1 fimbrial antibody (and visa versa) and that F9 fimbriae do not agglutinate yeast in a mannose-resistant or mannose-sensitive manner (data not shown). These data indicate that F9 fimbriae are immunologically and functionally distinct from type 1 fimbriae.

The structural genes for F9 fimbriae are very highly conserved in *E. coli* O157:H7 and the sequenced uropathogenic *E. coli* isolate CFT073 (44); the levels of identity range from 96.6% to 97.8% (at the nucleotide level). In contrast, the upstream sequences that are most likely to contain regulatory regions and regulators bear little resemblance in the two pathotypes. In the O157 F9 cluster, there are three open reading frames upstream of the main structural subunit gene. In the CFT073 F9 cluster there are two divergent open reading frames in the corresponding region, the first of which, *oprR*, encodes a putative LysR-like regulator. The level of identity for this complete region (3,000 bp) is only 45.6%, indicating that while the different pathotypes may share the capacity to express the F9 fimbriae, the regulation mechanisms are likely to be different.

**Cloning of F9 and induced expression in *E. coli* K-12.** The complete cluster, including genes Z2200 to Z2206 (EC2107 to EC2113), was amplified in two parts by long-range PCR using the primers listed in Materials and Methods. The products were cloned into pBAD18 so that expression could be induced by addition of arabinose. The cloned operon was sequenced and was shown to be identical to that found in the genome sequence of *E. coli* O157:H7 strain EDL933 (25). Addition of 0.2% arabinose resulted in production of visible fimbriae on the surface of *E. coli* K-12 strain AAEC185  $\Delta fim$  (Fig. 2A and C). The fimbriae extended from the cell for 1 to 2  $\mu\text{m}$  and were able to form longer bundles that were clearly distinct from flagella (Fig. 2C). These fimbriae were absent in arabinose-induced control (pBAD18) samples (Fig. 2B). To simplify their description, we designated these fimbriae *E. coli* "F9" fimbriae. In order to purify the fimbriae, a modified method developed by Korhonen et al. (17) was used, which relies on heat treatment at 60°C. The fimbriae were then either precipitated by TCA or purified further by ammonium sulfate precipitation and dialysis (see Materials and Methods). The purified fimbriae were then denatured by heating in acid and separated by SDS-PAGE (Fig. 3A). An identical procedure was used for induction of the same strain containing just the pBAD18 plasmid. Expression from the induced cluster produced a clear, abundant protein band at 17 kDa, which corresponded to the expected size of the F9 main subunit. A very weak band that was a similar size was present in the control sample, although clearly at a much reduced level. As shown in Fig. 1, the first part of the F9 cluster was missing in the *E. coli* K-12 strain used. This strain (AAEC185) also lacked type 1 fimbriae. The band may indicate that there was some cross-reaction with yet another fimbrial subunit present in *E. coli* K-12. To confirm

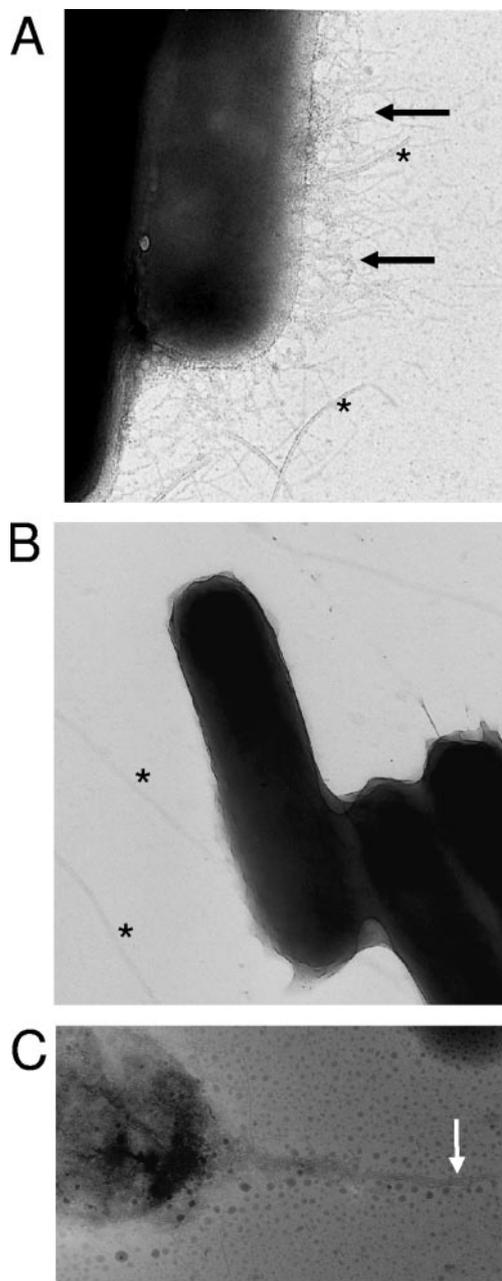


FIG. 2. Transmission electron micrographs of *E. coli* F9 fimbriae. (A) Transmission electron micrograph of *E. coli* K-12 expressing fimbriae (F9<sup>+</sup>, pASL04). The fimbriae are indicated by arrows, and flagellum fragments are indicated by asterisks. Scale, 2  $\mu\text{m}$ . (B) Transmission electron micrograph of *E. coli* K-12 (F9<sup>-</sup>, pBAD18). No fimbriae are visible, although flagella are present (asterisks). Scale, 1.5  $\mu\text{m}$ . (C) Transmission electron micrograph of *E. coli* K-12 expressing fimbriae (F9<sup>+</sup>, pASL04) forming a bundle at the pole of the bacterium. The fimbrial bundle is indicated by an arrow. Scale, 6  $\mu\text{m}$ . All samples were induced with 0.2% arabinose and were prepared as described in Materials and Methods.

that the main 17-kDa protein probably originated from the cloned F9 cluster, serum was raised against the purified main subunit (see Materials and Methods), and antibody from this serum recognized the main fimbrial subunit protein band, as determined by Western blotting (Fig. 3B) and immunofluores-

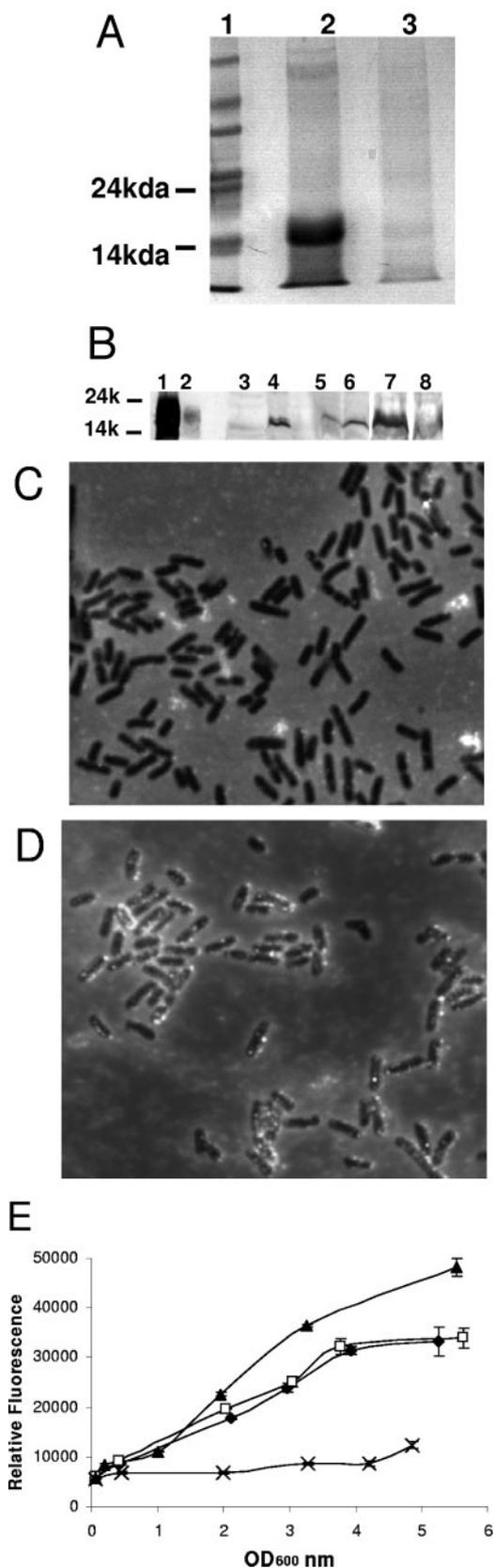


FIG. 3. Expression of F9 fimbriae by *E. coli*. (A) Colloidal blue-stained proteins. Lane 1 contained protein markers; lane 2 contained proteins (20 µg) prepared from *E. coli* K-12 strain AAEC185 expressing

ence microscopy (Fig. 3C and D). Without acid treatment of the samples, the levels of the 17-kDa protein detectable on the gel were reduced, indicating that the acid treatment was required to denature the organelle (data not shown). The cloned cluster therefore encodes a fimbria-like organelle that is predominately made up of 17-kDa protein subunits.

**Expression of F9 by *E. coli* O157:H7.** Ongoing work has shown that a chromosomally integrated β-galactosidase translational fusion to the putative Z2200 promoter region (19a) results in higher levels of expression in rich media, in stationary-phase cultures, and at 28°C than at 37°C. Therefore, fimbrial preparations were made from *E. coli* O157:H7 strain EDL933 (= TUV-933-0) and *E. coli* O157:H7 strain Walla 3 (= ZAP198) cultured at 28°C and 37°C in LB medium until the stationary phase. The fimbriae were prepared as described in Materials and Methods, denatured in acid, and separated by SDS-PAGE, and the fimbrial subunit was detected by Western blotting using the polyclonal rabbit serum raised against the main subunit (Fig. 3B). Preparations from the pBAD18 F9 clone (pASL04) and pBAD18 alone in strain AAEC185 were also included as positive and negative controls, respectively (Fig. 3B). A control preparation produced a weak cross-reacting band (Fig. 3B, lane 2). In order to confirm that the 17-kDa band was the F9 main subunit, a Z2200 (main subunit gene) deletion mutant was constructed using the lambda Red mutagenesis strategy (6). Western blotting of a fimbrial preparation from the *E. coli* O157:H7 deletion strain demonstrated that the 17-kDa subunit band was not present, although there was a cross-reaction with a slightly smaller protein (about 16 kDa) (Fig. 3B, lane). The temperature regulation was confirmed by a plasmid-based eGFP translational fusion (pACK-loc8) to the Z2200 putative promoter region (Fig. 3E), which showed that there was a higher level of expression from the fusion in an *E. coli* O157:H7 background (ZAP198) when the

F9 fimbriae (pASL04); and lane 3 contained proteins prepared from the *E. coli* K-12 strain AAEC185 control (pBAD18). (B) Immunoblot analysis of F9 fimbriae expressed by *E. coli* O157:H7. Serum was raised to the purified main fimbrial subunit as described in Materials and Methods and was used to detect F9 fimbriae by Western blotting. Lane 1 shows detection of purified fimbriae (0.2 µg) from the induced *E. coli* K-12 (F9<sup>+</sup>, pASL04) strain. Lane 2 contained a similar preparation from *E. coli* K-12 containing pBAD18. Lanes 3 and 4 contained a fimbrial preparation from *E. coli* EDL933 *stx* (= TUV933-0) (9) cultured at 37°C (lane 3) and at 28°C (lane 4). Lanes 5 and 6 contained a fimbrial preparation from *E. coli* O157:H7 strain ZAP198 (= Walla 3) cultured at 37°C (lane 5) and at 28°C (lane 6). Lane 7 contained a fimbrial preparation from *E. coli* O157:H7 strain Walla 3 cultured overnight in LB medium at 28°C. Lane 8 contained an identical preparation from the Z2200 (main subunit) mutant of *E. coli* O157:H7 strain Walla 3. Fimbrial preparations were made as described in Materials and Methods. (C and D) The polyclonal antibody raised to the F9 main fimbrial subunit was used to detect F9 fimbriae on the surface of *E. coli* O157:H7 containing induced pBAD18 (F9<sup>-</sup>) (C) and induced pASL04 (F9<sup>+</sup>) (D). (E) Expression analysis of the F9 subunit gene. A translational fusion of Z2200 to the eGFP gene was constructed as described in Materials and Methods. The plasmid construct (pACloc8) was transformed into *E. coli* O157:H7 strain Walla 3, and fluorescence was measured during growth in Luria-Bertani broth at 28°C (▲), 34°C (□), and 37°C (◆). The background fluorescence generated by the *E. coli* O157:H7 strain alone is also shown. OD 600 nm, optical density at 600 nm.

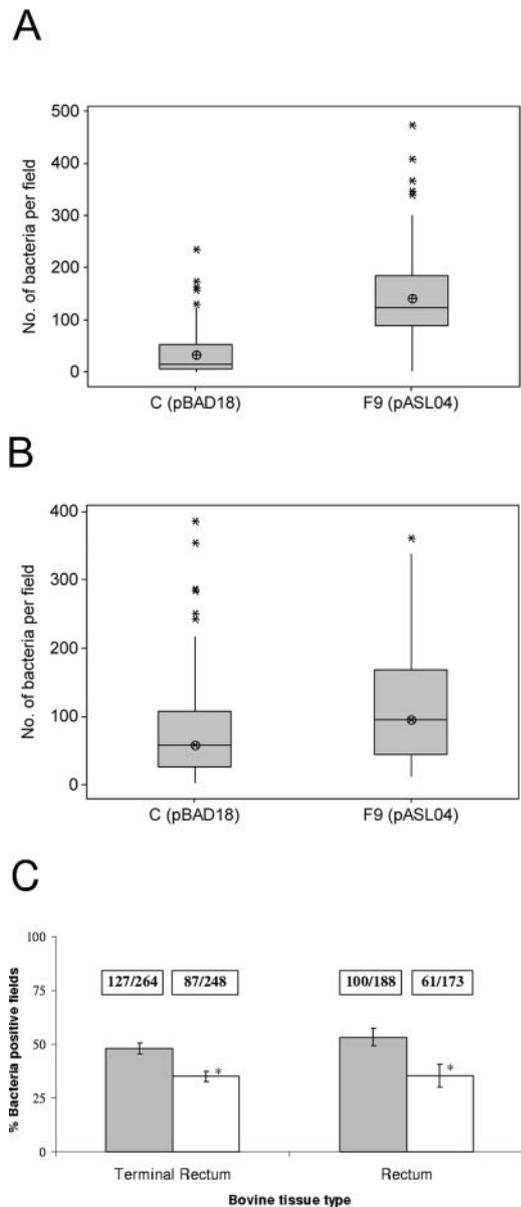


FIG. 4. F9 fimbrial expression and *E. coli* adherence. (A) Expression of F9 by *E. coli* K-12 and adherence to cultured EBL cells. *E. coli* K-12 containing pASL04 (F9<sup>+</sup>) and *E. coli* K-12 containing pBAD18 (F9<sup>-</sup>) were induced with arabinose, and adherence assays with EBL cells were carried out as described in Materials and Methods. A total of 150 fields ( $\times 40$  objective) were quantified for each of the two strains from four different monolayers. A Mann-Whitney U (nonparametric) test showed that the median values of the two data sets were significantly different ( $P < 0.001$ ). (B) Expression of F9 by *E. coli* K-12 and adherence to cultured bovine primary cells from the terminal rectum. Adherence assays were carried out as described in Materials and Methods. A total of 107 fields ( $\times 40$  objective) were counted for *E. coli* K-12 containing pBAD18 (F9<sup>-</sup>), and 136 fields were counted for *E. coli* K-12 containing pASL04 (F9<sup>+</sup>). In panels A and B the boxes indicate the interquartile ranges (25% to 75%) of the data points, and each asterisk indicates an outlying data point. A Mann-Whitney U (nonparametric) test showed that the median values of the two data sets were significantly different ( $P < 0.001$ ). (C) Expression of F9 by *E. coli* O157:H7 and adherence to bovine gastrointestinal explant tissue. Assays for adherence to the explant tissue were carried out as described in Materials and Methods. The open bars show the data for *E. coli* O157:H7 expressing F9 (pASL04), and the shaded bars show the

organisms were cultured at 28°C than when they were cultured at 34°C or 37°C. This construct provided no evidence of heterogeneous or phase-variable expression of F9. *E. coli* O157:H7, therefore, expresses F9 fimbriae, although expression was optimized for lower temperatures in the medium tested. The Western blot data do indicate that there were low levels of expression from the wild-type strains even at 28°C, and this may account for our failure to detect F9 expression on the surface of *E. coli* O157:H7 (ZAP198) by immunofluorescence using the antibody raised against the main subunit. As there was no evidence for phase variation of F9, it may be that only a limited number of fimbriae per cell are present on the bacterial surface. The presence of multiple fimbriae on the bacterial surface was confirmed for *E. coli* O157 containing the induced F9 clone (pASL04) (Fig. 3C and D).

**Adherence of *E. coli* expressing F9 to bovine epithelial cells and explant tissue.** In order to determine whether expression of F9 fimbriae increases the capacity of *E. coli* to bind to bovine epithelial cells, *E. coli* K-12 strain AAEC185 containing the inducible F9 operon (pASL04) was compared with the same strain containing the pBAD18 vector alone for adherence to EBL cells and to primary cells derived from the bovine terminal rectum. EBL cells and a bovine mammary gland line are the only two immortal bovine epithelial cell lines available for such studies. Following 2 h of arabinose induction of the F9 adhesin, a significant 3.5-fold increase ( $P < 0.001$ ) in bacterial binding to EBL cells was observed after 2 h of incubation of the bacteria with the cells (Fig. 4A). A significant ( $P < 0.001$ ) increase in binding was also observed for the expressed adhesin on the primary cells (Fig. 4B). A mutant with a mutation in the F9 main subunit was also analyzed to determine its capacity to bind to EBL cells and rectal primary cells compared to the capacity of the wild-type strain. No difference in adherence potential between these strains was observed in either assay (data not shown).

Another series of experiments was carried out, in which *E. coli* O157:H7 strain Walla 3 was transformed with the pASL04 (F9<sup>+</sup>) clone and control plasmid pBAD18 and adherence to terminal rectal explant tissue was quantified following 2 h of incubation of the bacteria with the tissue (Fig. 4C). In this case it was clear that induction of the fimbriae had a significant (in both cases  $P < 0.05$ ) negative impact on adherence and F9 expression actually limited binding of the *E. coli* O157:H7 strain.

**Adherence of *E. coli* expressing F9 fimbriae to extracellular matrix components.** Bacterial adhesins that function in a mammalian host have a range of common targets to which they bind. In some cases these targets are specific sugar residues, but in other cases they are proteins such as fibrinogen, fi-

data for *E. coli* O157:H7 containing pBAD18. The values above the bars show the number of positive fields/total number of fields counted for each strain and tissue type. The expression of F9 by *E. coli* O157:H7 was shown to significantly reduce bacterial binding to both tissue types. The binding data for the rectal tissue were analyzed using the Mann-Whitney U test and were found to be significantly different ( $P = 0.012$ ). The data for adherence to the terminal rectal tissue were normally distributed, were analyzed using a two-sample *t* test, and were found to be significantly different ( $P = 0.005$ ).

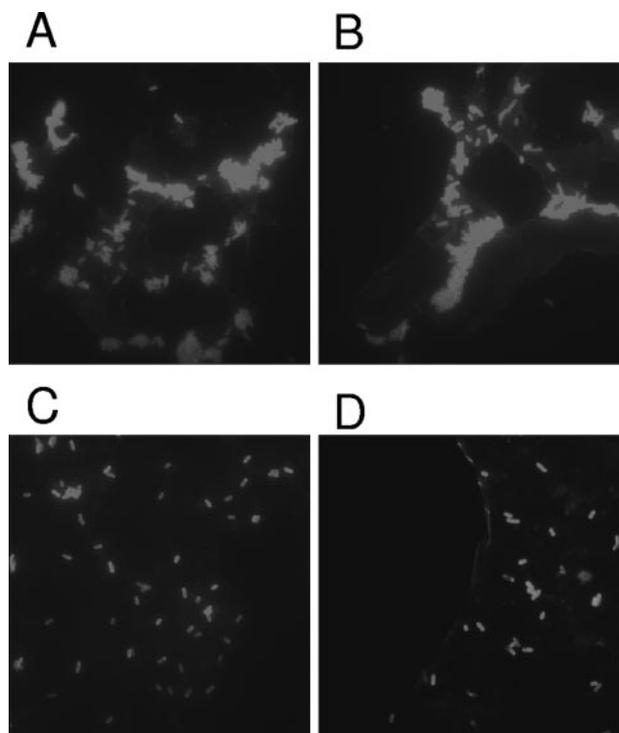


FIG. 5. F9 expression from *E. coli* O157:H7 and binding to bovine fibronectin. (A and B) *E. coli* O157:H7 strain Walla 3 expressing F9 (pASL04) was incubated on slides coated with fibronectin and was visualized by immunofluorescence as described in Materials and Methods. (C and D) *E. coli* O157:H7 containing pBAD18 was incubated and visualized as described above for panels A and B.

bronectin, or collagen. *E. coli* O157:H7 strain Walla 3 (= ZAP198) was transformed with both pASL04 (F9<sup>+</sup>) and the pBAD18 control. The strains were assayed for adherence to a variety of bovine substrates, including fibrinogen, fibronectin, collagen, elastin, and hyaluronic acid, which were coated onto glass slides. Most of the substrates were negative for binding. The only exception was fibronectin. This substrate produced patches of high-density binding (Fig. 5A and B) compared to the control (Fig. 5C and D), indicating that F9 does have an affinity for bovine fibronectin.

**Deletion of F9 and impact on colonization of the bovine terminal rectum.** Previous work has shown that deletion of the F9 main subunit in *E. coli* O157:H7 strain EDL933 reduces the ability to colonize 1- to 2-week old calves. The tropism of *E. coli* O157:H7 for the terminal rectum of cattle does not occur to the same degree in these very young animals, but it is apparent in postweaned calves (6 to 8 weeks) and in adult cattle presented for slaughter (19, 21). It is possible that there is more generalized colonization of the gastrointestinal tract in young calves that obscures any tropism for the terminal rectum and that this is mediated by F9. Alternatively, these animals may differ because the immune inductive sites of the gastrointestinal tract are immature. Thus, we wanted to determine if F9 played a role in the specificity for the terminal rectal site. The main F9 subunit gene, Z2200, was deleted by lambda Red mutagenesis in *E. coli* O157:H7 strain Walla 3 (Nal<sup>r</sup>), and the strain was orally inoculated into seven animals (age, 21 weeks)

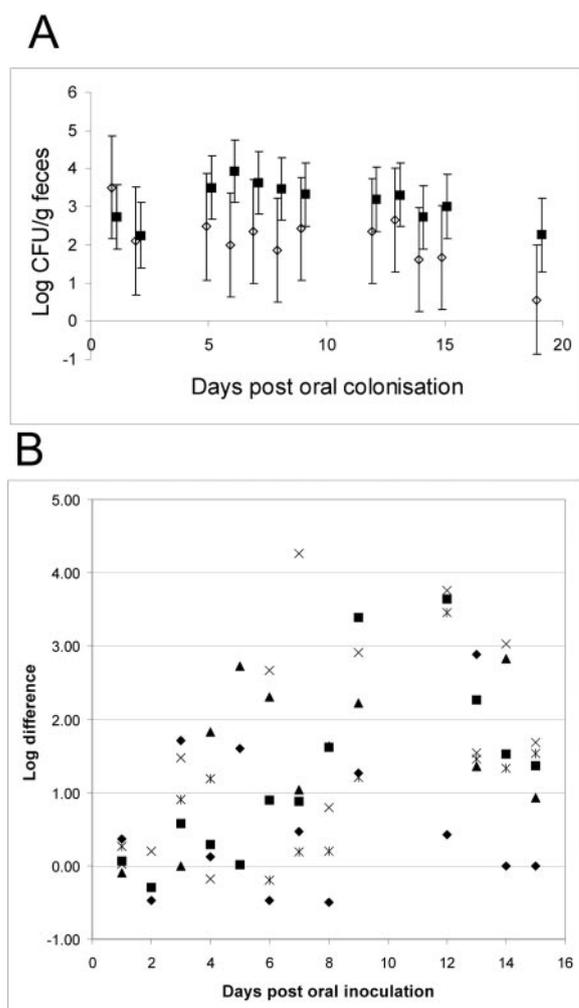


FIG. 6. Role of F9 fimbriae in colonization of the terminal rectum of weaned cattle. (A) Seven calves were orally challenged with *E. coli* O157:H7 (Nal<sup>r</sup>) having a defined deletion of the Z2200 gene (the F9 main fimbrial subunit gene). The mean shedding levels per gram of feces for the mutant ( $\diamond$ ) were compared with the mean values resulting from oral challenges with the parent strain ( $\blacksquare$ ) (data based on 19 orally colonized animals [see Materials and Methods]). Estimated 95% confidence intervals (means  $\pm$  2 standard errors) are indicated. (B) Fecal surface-to-core ratios for *E. coli* O157:H7 CFU as an indicator of terminal rectal colonization. Each symbol indicates data for an individual animal. A  $\log_{10}$  surface-to-core ratio of 0 indicates equivalent distribution, and this result was obtained for the majority of the early samples (days 1 to 3). As colonization was established over days 4 to 10, the ratio increased as localization developed.

as described in Materials and Methods. The shedding profile of the mutant for these seven animals was compared with the shedding profile of the wild-type strain for 19 orally inoculated animals (Fig. 6A). There was no strong evidence that there was a difference in the mean levels of shedding between the groups or that there was a difference in the shedding patterns over time between the groups. However, the possibility that the F9 deletion reduced the levels of shedding should not be ruled out as the mean levels were consistently lower in this group, particularly toward the end of the experiment. For both sets of data there was a large amount of variability in the CFU/g

values both between calves and for the same calf over time. In both groups there were some calves that did not appear to be colonized. A Fisher exact test of the proportion of calves that were not colonized provided no evidence of a difference between the groups (2/7 calves versus 3/19 calves).

To determine if F9 is responsible for the terminal rectal tropism, the levels of *E. coli* O157:H7 on the surface of the feces were determined and compared to the levels in the core of the feces (when possible). Terminal rectal colonization resulted in coating of the feces as the animal defecated, and this resulted in surface-to-core ratios that were at least 10-fold higher. As is shown in Fig. 6B, the ratio for the mutant started off around 1:1 as bacteria from the original oral dose were passaged through the gastrointestinal tract. Once colonization was established on day 3, the ratio increased, indicating that there was rectal localization. These data demonstrate that F9 is not essential for localization to the terminal rectal site but may still contribute to the initial colonization, potentially at anterior sites.

## DISCUSSION

The majority of *E. coli* strains associated with human and animal disease express fimbrial adhesins that allow them to colonize epithelium. While sequencing of two enterohemorrhagic *E. coli* O157:H7 strains has revealed the presence of at least 16 putative fimbrial clusters, there have been only a very limited number of reports of fimbriae expressed by this serotype. A signature-tagged mutagenesis screening for *E. coli* O157:H7 genes that promote colonization of young calves was carried out, and an attenuating mutation was identified in a putative fimbrial cluster (Z2200 to Z2206) on O-island 61 (11, 25). A closely related cluster was also identified as relevant for calf colonization in a similar screening of *E. coli* serotype O26:H- (43). Moreover, an initial analysis of the levels of expression from 16 putative fimbrial clusters in *E. coli* O157:H7 identified the O-island 61 cluster as one of four clusters that are expressed in vitro and as a cluster that does contain a complete operon, including a minor FimH-like adhesin gene (submitted for publication). The aims of this study were to determine whether this operon expresses a fimbrial adhesin, to characterize the operon and fimbriae in more detail, and to determine whether the fimbriae are responsible for the tropism that *E. coli* O157:H7 exhibits for the terminal rectum of cattle (21). The fimbriae were designated *E. coli* "F9" fimbriae.

We established the distribution of putative fimbrial genes located in O islands, including the genes for F9 fimbriae present on OI61 of *E. coli* O157:H7. In the case of the OI141 *lpfA1* and OI154 *lpfA2* genes, our data support previous findings indicating that strains belonging to the EHEC O157:H7 serotype or closely related strains (particularly O55:H7 strains) may have acquired putative virulence factors as mobile elements via horizontal transfer and recombination and that these factors could represent EHEC-specific regions (39). In contrast, three of the O islands analyzed were not restricted to EHEC strains. In the case of OI61 Z2200 (F9), this gene was absent only from the *E. coli* strains belonging to the DEC6 group comprising serotypes O111:H21, O111:H12, and O111:NM (46). Similarly, OI47 Z1534 and OI127 Z4498 had a wider distribution,

and a strict association with EHEC strains was not observed. Our results indicate that selective pressure favoring the maintenance of specific combinations of these fimbrial factors presumably enhances the ability of DEC strains to adhere and to colonize specific host niches (e.g., O157:H7 or O26:H- during colonization of calves).

Forced expression of the O-island 61 operon (Z2200 to Z2206) from a plasmid resulted in surface presentation of short fimbriae that were 1 to 2  $\mu\text{m}$  long, although these fimbriae were capable of forming longer bundles (Fig. 2). The F9 fimbriae were removed by heating the bacteria at 60°C, and the main subunit was separated by using SDS-PAGE following denaturation of the fimbriae in acid. Polyclonal antiserum was raised against the purified main subunit, and this antiserum was used to confirm expression of F9 from the fimbrial clone and from two *E. coli* O157:H7 isolates, EDL933 and Walla 3 (9, 24). The fimbriae were detected at both 37°C and 28°C but were not detected by Western blotting in the F9 main subunit deletion strain. Higher levels of fimbriae were produced at the lower temperature in both strains, a finding that correlates with the fluorescence levels produced by a plasmid-based translational fusion of the F9 main subunit promoter region to eGFP in an *E. coli* O157:H7 background (Fig. 3E). Therefore, the operon does encode a fimbrial adhesin and is expressed by *E. coli* O157:H7 but at low levels under the in vitro conditions examined. The higher levels of expression at 28°C and the lack of detectable phase variation are arguments against an important role in vivo.

The expressed fimbriae in *E. coli* K-12 did result in a significant increase in bacterial adherence to cultured bovine epithelial cells. As *E. coli* O157:H7 colonizes the terminal rectum of cattle, cultures of primary cells from this site were prepared, and the adherence of *E. coli* K-12 with F9 and the adherence of *E. coli* K-12 without F9 to these primary cell cultures were compared. A small increase in the number of bacteria bound was observed in the presence of induced F9, but the increase was not significant. However, the increased levels of attachment in both assays did indicate that the fimbriae could confer a colonization advantage.

An interesting result was obtained when F9 fimbriae were expressed from the plasmid clone in an *E. coli* O157:H7 background rather than in *E. coli* K-12. In this background, the expression reduced adherence to explant tissue from the bovine gastrointestinal tract. A number of studies have demonstrated the importance of type III secretion in colonization of ruminant gastrointestinal epithelium, and one possible explanation for this result is that forced expression of a fimbrial adhesin can interfere with type III secretion-based adherence. This highlights the fact that the expressed fimbrial repertoire of EHEC O157:H7 may be quite restricted and means that any fimbrial expression needs to be coordinated with the type III secretion system and be mutually exclusive (15).

Fimbrial adhesins generally bind to either specific sugar residues or extracellular matrix components. *E. coli* K-12 expressing F9 was screened for attachment to a variety of substrates. Some affinity for fibronectin was observed, and it remains to be determined if this is the main substrate for F9 or a subsidiary substrate, as has been demonstrated for other fimbriae (32). The fact that the adhesin cluster has a wide distribution in *E. coli* serotypes implies that a generic adhesive function is not

necessarily linked to the bovine host. This conclusion is supported by the finding that F9 fimbriae are expressed at higher levels at lower temperatures, although fimbrial expression and surface production do occur at 37°C. In recent work on *E. coli* genes up-regulated in urinary tract infections it was found that the F9 gene cluster is up-regulated in human urine (31). While we have focused on colonization of the bovine host and bovine substrates, it is possible that the F9 adhesin could be important in human infections for both *E. coli* O157:H7 and other serotypes. Further research should examine the expression and importance of this adhesin in non-O157 EHEC and possible cross talk with type III secretion.

While STM and deletion analysis do indicate that F9 has a role in colonization of very young calves (1 to 2 weeks old), the terminal rectal tropism observed in older calves and cattle (6 weeks to 2 years) is not as apparent in the young calves. In order to determine if F9 has a role in directing this tropism, the main F9 fimbrial subunit gene was deleted in *E. coli* O157:H7 strain Walla 3 by lambda Red mutagenesis, and the mutant was given orally to seven animals. Five of these animals were colonized by day 5 following the oral challenge. While the mean levels of colonization were slightly lower than the levels of colonization for the wild type, it is clear from the high surface-to-core distribution ratio for the mutant in the feces that colonization of the terminal rectum still occurred. Our previous studies and ongoing studies have shown that when *E. coli* O157:H7 colonizes the terminal rectum, it coats the fecal stool during defecation. On most occasions the surface and core of the feces can be separated, and the bacterial loads can be determined and plotted as a ratio. Following an oral dose, at the early times (days 1 to 3) the surface-to-core ratios are usually around 1. As the terminal rectum site is colonized, the ratio increases to 10 to 100. This increase in the ratio was observed with the F9 mutant. This adhesin is therefore not solely responsible for the terminal rectal tropism but may contribute to colonization at this site and higher sites, especially in young animals in which the immune inductive sites are still developing along with the gastrointestinal flora. Our ongoing work suggests that a lack of fimbrial expression may be an important part of the colonization strategy of *E. coli* O157:H7 in cattle. A lack of generic adhesin expression (particularly expression of type 1 fimbriae) may eliminate colonization of the majority of the gastrointestinal mucosa and therefore focus the bacteria to sites such as follicle-associated epithelium (FAE), which have a reduced mucin layer and therefore potentially fewer competing organisms. The bacteria can colonize these sites using type III secretion-based intimate adherence, perhaps in combination with other adhesins. The terminal rectal tropism may be a result of the factors described above in combination with appropriate regulation of the type III secretion system. It has been demonstrated that the type III secretion system in *E. coli* O157:H7 is regulated by quorum sensing (33, 34), and locus of enterocyte effacement expression is therefore up-regulated in the large intestine, where the bacterial load is highest. Potentially, the terminal rectal FAE is the only FAE site that the bacteria encounter when the system is up-regulated. Other environmental signals, such as the oxygen level, may also be relevant. In future research we will examine the regulatory interplay between surface organelle operons during *E. coli* colonization of epithelium.

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