Identification and Characterization of the Locus for Diffuse Adherence, Which Encodes a Novel Afimbrial Adhesin Found in Atypical Enteropathogenic *Escherichia coli*

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The O26 serogroup of enteropathogenic *Escherichia coli* (EPEC) is one of the serogroups most frequently implicated in infant diarrhea and is also common among enterohemorrhagic *E. coli* (EHEC) strains. The most common O26 strains belong to EPEC/EHEC serotype O26:H11 and are generally Shiga toxin (Stx) positive. Stx-negative *E. coli* strains that are negative for the EPEC EAF plasmid and bundle-forming pilus (Bfp) are classified as atypical EPEC. Here, we report a novel adhesin present in an *stx*-negative *bfpA*-negative atypical EPEC O26:H11 strain isolated from an infant with diarrhea. A cloned 15-kb genomic region from this strain, designated the *l*ocus for *d*iffuse *a*dherence (*lda*), confers diffuse adherence on HEp-2 cells when expressed in *E. coli* K-12. Sequence analysis of *lda* revealed a G+C content of 46.8% and 15 open reading frames sharing homology with the *E. coli* K88 *fae* and CS31A *clp* fimbrial operons. The *lda* region is part of a putative 26-kb genomic island inserted into the *proP* gene of the *E. coli* chromosome. Hybridization studies have demonstrated the prevalence of the minor structural subunit gene, *ldaH*, across *E. coli* serogroups O5, O26, O111, and O145. A second plasmid-encoded factor that contributed to the Hep-2 adherence of this strain was also identified but was not characterized. Null mutations that abolish adherence to HEp-2 cells can be restored by plasmid complementation. Antiserum raised against the major structural subunit, LdaG, recognizes a 25-kDa protein from crude heat-extracted protein preparations and inhibits the adherence of the *E. coli* DH5α *lda*+ clone to HEp-2 cells. Electron microscopy revealed a nonfimbrial structure surrounding the bacterial cell.

Diarrheagenic strains of *Escherichia coli* can be subdivided into at least six categories depending on the virulence properties they possess (23, 38). Two of these *E. coli* categories are enteropathogenic *E. coli* (EPEC), a major cause of infant diarrhea, and enterohemorrhagic *E. coli* (EHEC), which produces Shiga toxin (Stx) and is associated with bloody diarrhea and hemolytic uremic syndrome. Multiple serogroups and serotypes of *E. coli* comprise these two categories. One serogroup shared between EPEC and EHEC is O26. The virulence characteristics of serotype O26:H11 strains vary in accordance with their geographical distribution. Many O26:H11 strains isolated in North America, Europe, and Japan produce Stx and are classified as EHEC (28). In Brazil, with the exception of one reported case isolate (17), O26:H11 and O26:H⁻ isolates from infants with diarrhea have consistently been *stx* negative and have not been associated with hemolytic uremic syndrome (42, 48).

A key aspect of enteric infection caused by *E. coli* is the colonization of the gastrointestinal tract, which is mediated by specific adherence factors. EPEC and EHEC strains produce an adherence factor, called intimin, which is chromosomally encoded by the *eae* (EPEC attaching and effacing) gene lo-

cated within the locus for enterocyte effacement (LEE) pathogenicity island (21, 31). The more restrictive designation of EHEC refers to those Stx-producing *E. coli* strains that possess the LEE. In EPEC, a plasmid-encoded type IV bundle-forming pilus (Bfp) has also been shown to be required for intestinal colonization (16). Bfp mediates bacterium-to-bacterium adherence, resulting in the formation of compact microcolonies on HEp-2 epithelial cell monolayers after 3 hours, which is referred to as localized adherence (LA) (11, 46). *E. coli* O26 strains that are LEE+ *stx* negative and EAF⁻ *bfp* negative are classified as atypical EPEC and differ from typical EPEC in several characteristics. Typical EPEC, found primarily in developing countries, affects infants and children under the age of 2 years, whereas atypical EPEC strains have caused outbreaks of diarrheal disease in both children and adults in industrialized countries (22, 54). In the absence of Bfp, atypical EPEC strains can still adhere to HEp-2 cells in either a diffuse-adherence or aggregative-adherence pattern (37). Some clinical isolates of serogroups O26, O55, and O111 show a Bfp-independent localized-adherence pattern similar to the LA, called localized-adherent-like (43). The majority of these strains form microcolonies on HEp-2 epithelial monolayers, but only after 6 hours of infection, whereas LA is apparent after 3 hours. While the presence of Bfp correlates well with LA, not all LA+ EPEC strains are Bfp positive. Studies by other investigators (15, 43, 47) have reported EAF⁻ Bfp⁻ EPEC serotypes that exhibit localized adherence, but so far, no

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TABLE 1. Bacterial strains used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
E. coli strains		
DH5α	Host strain	Invitrogen, Carlsbad, CA
SM10λ*pir*	Host strain	49
MG1655	Sequenced K-12 strain	9
XL-10	Host strain	Stratagene, La Jolla, CA
22	O26:H11 *stx* negative *eae*$^+$ *bfp* negative *exhA* negative	43
ICS1	Δ*ldaG*::*kan* derivative of *E. coli* 22	This study
LDA1	Δ*ldaG* derivative of *E. coli* 22	This study
LDA3	LDA1 cured of pIJ22	This study
ORN103	Δ*fim*	41
Plasmids		
pHM5	Suicide vector; *sacB ori*R6K Ap^r	44
pHC79	Cosmid vector; Ap^r	19
pACYC177	Cloning vector; Km^r Ap^r	10
pACYC184	Cloning vector; Cm^r Tc^r	10
pKD3	Source of Cm^r cassette	12
pKD4	Source of Km^r cassette	12
pCP20	*flp* recombinase	12
pIJ9	pV-B-6 ΔBssHII	This study
pIJ13	Δ*ldaG*::*kan* Ap^r Km^r *oriR6K sacB*	This study
pIJ20	pIJ9 ΔStuI fragment; *lda* minimal subclone; Ap^r	This study
pIJ22-1	*E. coli* 22 plasmid encoding LA; Km^r	This study
pIJ22	Spontaneous deletion product of pIJ22-1; Km^r	This study
pIJ30	pIJ22::*cat*	This study
pV-B-6	pHC79::*lda* adherence$^+$ Ap^r	This study
pV-B-6TN	*ldaH*::*kan* adherence$^-$ Km^r	This study
pBSL180	mini-Tn*10*::*kan* donor plasmid; Km^r	3
pLDAH	pUC18::*ldaH*::*kan* subclone; Km^r	This study

characterization of the factor(s) responsible for this phenotype has been reported. Fischer et al. (13) reported an EAF$^-$ *stx*-negative *eae*$^+$ O26:NM strain isolated from a calf with diarrhea that was LA$^+$ on HEp-2 cells, but the adherence factor was not identified. *E. coli* 22, an O26:H11 isolate originally obtained from an infant with diarrhea in Brazil, is *stx* negative *eae*$^+$ and exhibits the LA phenotype within 3 hours, identical to the Bfp-mediated phenotype. However, *E. coli* 22 does not harbor the EAF plasmid or the *bfpA* gene. The aim of this study was to identify the genetic determinants conferring adherence in this strain and to compare it to those encoding other known *E. coli* adherence factors. We report the cloning, nucleotide sequence, and organization of a chromosomal locus encoding an afimbrial adhesin with homologies to the operons encoding enterotoxigenic *E. coli* (ETEC) K88 and CS31A fimbriae and demonstrate its role in mediating bacterial adherence to epithelial cells. In addition, we present data showing the prevalence of these gene sequences across diverse *E. coli* serogroups. Our data indicate that this new fimbria is a member of the K88 family of adhesins.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and media. The bacterial strains and plasmids used in constructions are listed in Table 1. Wild-type *E. coli* strains tested for the presence of *ldaH* were obtained from the bacterial culture collection at the Center for Vaccine Development. All bacterial strains were maintained in Luria-Bertani medium or tryptic soy broth. Antibiotics were used at the following concentrations unless otherwise indicated: ampicillin (Ap), 100 μg/ml; chloramphenicol (Cm), 20 μg/ml; and kanamycin (Km), 50 μg/ml.

Standard methods of DNA manipulation and transformation were employed (45). Plasmid isolations were performed using alkaline lysis and CsCl$_2$ gradient centrifugation (45). Restriction nucleases and enzymes were obtained from Invitrogen (Carlsbad, CA).

Primer sequences are listed in Table 2. The extension times for primer pairs used for determining the upstream junction of the LDA island were 2 min (K1/K3), 30 s (K2/K3), 3 min (K4/K6), 2 min (K5/K7), and 7 min (K3/K2792).

Cell culture adhesion and adhesion inhibition assays. The HEp-2 cell adhesion assay was performed as described previously (46). Monolayers of ca. 10^5 HEp-2 cells were grown in 24-well tissue culture plates in Dulbecco modified Eagle medium containing 10% fetal bovine serum. Bacterial strains were grown statically in 3 ml of tryptic soy broth or Luria broth for 16 to 18 h at 37°C. The monolayers were infected with 40 μl of bacterial culture and incubated for 3

TABLE 2. Primers used in PCR amplifications and plasmid constructions

Primer	Sequence
K1	5′-ACATCCGCGAATTGATGAATAA
K2	5′-GACGCTGATCAGCTCAAACA
K3	5′-CGACGCCATAATAATCAGGG
K4	5′-ATCCGCAGCCTGGTAATACT
K5	5′-TGGCTACCCGCTTTATTTACA
K6	5′-GACGTAATTTATTGTCACGAAG
K7	5′-ATTGCTGCAAACAAAAATGTCAT
K2792	5′-GCATGCCTGTACTCAGTGCG
K2937	5′-ATATATCTAGACATATGAATATCCTCCTTA GTTCCT
K2938	5′-ATATATCTAGATGTGTAGGCTGGAGCTG CTTC
K3094	5′-AAAGATCTGTGATGAGGTTCAGGTGAAG
K3095	5′-AAATCTAGATGCAGACGCAACTACAGCCA
K3096	5′-AAATCTAGAGCTAACAGTACCTTGGT TATG
K3097	5′-AAAGAGCTCACGATGCATAAGCTGC AGCA

hours at 37°C with 5% CO_2 in the presence of 0.5% D-mannose. The cells were then washed six times with phosphate-buffered saline (PBS; pH 7.2), fixed with methanol, stained with May-Grünwald-Giemsa stain, and examined under a light microscope. For the inhibition assay, 50 μl of an overnight bacterial culture was mixed with a 1:50 dilution of LdaG rabbit antiserum and incubated for 2 h at 37°C prior to the adherence assay (7). The effect of the antiserum upon bacterial adherence to HEp-2 cells was determined by comparing bacteria preincubated with preimmune antiserum to bacteria preincubated with LdaG antisera under the same conditions. All experiments were performed in triplicate.

DNA hybridization. Colony blot and Southern blot hybridizations (45) were performed at 65°C. Gene probes, *bfpA* (15), *stx* (39), *ehxA* (pO157 plasmid sequences) (28), *ldaG*, *ldaH*, and kanamycin were generated either by PCR or by restriction digestion, gel purified, and labeled with [32P]dCTP using a Rediprime kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions.

Transposon mutagenesis. Mini-Tn10::kan was introduced into *E. coli* 22 or *E. coli* DH5α(pV-B-6) by electroporation with pBSL180 (3). Isolates were selected on L agar with kanamycin or kanamycin-ampicillin and tested for adherence in the HEp-2 assay.

Cloning of LDA locus. Chromosomal DNA was isolated from *E. coli* 22 using the EZ-DNA kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and then partially digested with Sau3AI and ligated into the BamHI-digested cosmid vector pHC79. The ligation was packaged into bacteriophage lambda particles using the GigaPak III Gold packaging kit (Stratagene), transfected into *E. coli* DH5α, and plated onto L agar with ampicillin. Colonies were tested for localized adherence in the HEp-2 adherence assay. One Ap^r LA$^+$ colony harboring a cosmid, designated pV-B-6, was selected for further analysis.

A minimal-size subclone that still possessed the ability to adhere to HEp-2 cells was obtained by digesting pV-B-6 with BssHII, followed by self-ligation. The resulting clone, pIJ9, was digested with StuI and self-ligated, yielding pIJ20, which is ca. 21.5 kb in size. pIJ20 was transformed into *E. coli* DH5α and tested for HEp-2 adherence.

Construction of bacterial deletion mutants and plasmids. A kanamycin-resistant subclone of plasmid pV-B-6-Tn, designated pLDAH, was generated by ligating an EcoRI-HindIII restriction fragment that contained the *kan* gene (present in the mini Tn10::kan cassette) and flanking *lda* sequences into pUC18.

A 699-bp nonpolar chromosomal deletion in *ldaG* was constructed in *E. coli* 22 by inserting sequences flanking the *ldaG* gene into suicide plasmid pHM5 (44). Primer sets K3094/K3095 and K3096/K3097 were used in conjunction with *Pfx* polymerase to generate fragments containing 950 bp upstream and 920 bp downstream of the *ldaG* gene, respectively. The fragments were ligated into suicide vector pHM5 to create pIJ12. The deletion was marked by the insertion of a kanamycin gene cassette that contained flanking inverted-repeat FLP sites amplified from pKD4 (12). A 1.6-kb kanamycin cassette was amplified from pKD4 using primer set K2938/K2937 and blunt-end ligated into the XbaI-digested/Klenow-treated site of pIJ12, yielding pIJ13. Plasmid pIJ13 was introduced into *E. coli* 22 by conjugal mating with *E. coli* SM10λ*pir* and directly plated onto modified L agar containing 5% sucrose and 25 μg/ml kanamycin. Individual colonies were then patched onto McConkey media to distinguish *E. coli* 22 (*lac$^+$*) from *E. coli* SM10λ*pir* (*lac* negative). One lactose-positive isolate exhibiting Km^r and Ap^s was called ICS1. The kanamycin marker was then eliminated from the chromosome with plasmid pCP20, which harbors the *flp* recombinase gene on a temperature-sensitive replicon (12). *E. coli* ICS1 was electroporated with pCP20 and plated on L agar with ampicillin. After overnight incubation at 30°C, colonies were picked and grown under nonselective pressure on L agar at 42°C. Colonies were separately patched onto media containing kanamycin or ampicillin to check for loss of the kanamycin cassette and pCP20, respectively. One Km^s Ap^s isolate that tested negative for *ldaG* by Southern blotting was designated LDA1.

A pIJ22-1 plasmid-cured derivative of LDA1 was created using plasmid incompatibility. pIJ22 was digested with EcoRI and ligated to a chloramphenicol gene cassette (*cat*), creating pIJ30. Plasmid pIJ30 was electroporated into the *ldaG* mutant LDA1 and plated onto L agar with chloramphenicol. A Cm^r Km^s mutant that was negative for Hep-2 adherence was called LDA3.

Sequencing and analysis. The nucleotide sequence of the cosmid pV-B-6 was determined by first subcloning small overlapping PstI, BamHI, or EcoRI restriction fragments into plasmid vector pAYCY177 or pACYC184. Plasmid DNA was prepared by cesium chloride density gradient centrifugation, and cycle sequencing was performed using the ABI 3100 Gene Analyzer. Nucleotide sequences were analyzed for open reading frames (ORFs) using Redasoft Visual Cloning 2000 DNA software (Whitehead Institute for Biomedical Research). BLAST searches and comparisons, as well as DNA-protein analysis, were conducted using databases at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/), The Institute for Genomic Research (http://www.tigr.org), and the CLUSTAL W (http://clustalw.genome.jp) websites.

Preparation of whole-cell lysates and heat-extracted proteins and identification of LdaG by N-terminal sequencing. *E. coli* strains grown on MacConkey agar at 37°C were harvested from the plates with 1 ml PBS (pH 7.4). The samples were aliquoted into portions of approximately 4.0×10^8 bacteria for whole-cell lysates and 4.0×10^9 bacteria for heat-extracted proteins. The whole-cell lysate samples were harvested by centrifugation at $12,000 \times g$ for 5 min at room temperature, washed in 1 ml PBS (pH 7.4), centrifuged again, resuspended in 200 μl of sodium dodecyl sulfate (SDS) sample buffer, and lysed at 100°C for 10 min. The cell debris was removed by centrifugation, and the supernatant was transferred to a fresh tube and stored at −20°C. The heat-extracted protein samples were harvested by centrifugation at $3,000 \times g$ for 10 min, resuspended in 160 μl of PBS (pH 7.4), and incubated at 60°C for 30 min. The samples were then pelleted by centrifugation at $3,000 \times g$ for 10 min, and the supernatant was transferred to a fresh tube; 40 μl of 5× SDS sample buffer was added, and the samples were boiled at 100°C for 5 min. The samples were separated by SDS-12% PAGE minigels, and proteins were stained with Coomassie blue. The 25-kDa protein that was highly expressed in strain 22 was excised from the gel (whole-cell lysate and heat-extracted proteins) and sent for N-terminal sequencing at the Stanford University sequencing facility (Palo Alto, CA).

Production of LdaG antiserum. A heat extraction of *E. coli* DH5α(pV-B-6) was prepared and separated on a 12% SDS-PAGE gel and stained with Coomassie blue. The 25-kDa LdaG protein was excised from the gel, eluted using a Mini Whole Gel Eluter (Bio-Rad), and extensively dialyzed in water. New Zealand White rabbits were immunized subcutaneously with 100 μg of purified LdaG protein conjugated with complete Freund's adjuvant. The animals were inoculated 3 weeks later with the same dose of protein in complete Freund's adjuvant. Ten days later, blood was drawn and the serum was collected.

Western blot assay. Heat-extracted proteins were separated on a 12% SDS-PAGE gel and transferred to Immobilon-P membranes (53). The membranes were blocked overnight with 5% bovine serum albumin in Tris-buffered saline (pH 7.4) plus 0.1% Tween 20 (TBS-Tween 20). Rabbit anti-LdaG antiserum (1:10,000) was incubated for 1 h and washed with TBS-Tween 20. Goat anti-rabbit immunoglobulin G-horseradish peroxidase (1:50,000) (Sigma-Aldrich, St. Louis, MO) was incubated for 1 h and washed with TBS-Tween 20. The protein was detected using the ECL Chemiluminescent Western Blotting Detection kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions.

Immunogold labeling and electron microscopy. Strains were grown statically overnight at 37°C in MacConkey broth (Difco), pelleted at $5,000 \times g$, resuspended in PBS (pH 7.4), and allowed to adhere to Formvar-carbon-coated copper grids (200 mesh; Electron Microscopy Sciences). The grids were floated on drops of rabbit anti-LdaG antiserum (1:100 dilution) for 30 min. After being washed, the grids were placed onto 10-nm-gold-labeled goat anti-rabbit sera (1:20 dilution; Electron Microscopy Sciences) for 30 min. After being further washed in PBS and distilled water, the grids were negatively stained with 1% phosphotungstic acid, pH 6.8 (Kodak), and visualized in a Joel JEM 1200 EX II transmission electron microscope at 80 kV.

Nucleotide sequence accession number. The GenBank accession number for the *lda* gene sequence is AY858803.

RESULTS

Identification and cloning of a locus for diffuse adherence. In a previous report (43), *Escherichia coli* strains isolated from Brazilian children with watery diarrhea were tested for adherence to HEp-2 cell monolayers. Isolates that exhibited the LA phenotype in 3 h were then analyzed by colony hybridization for the presence/absence of sequences encoding EPEC Bfp (*bfpA*), EHEC Stx (*stx*), and hemolysin (*exhA-hlyA*), the last serving as a marker for the pO157 plasmid. One LA$^+$ *bfpA*-negative *stx*-negative *exhA*-negative isolate, *E. coli* 22, was selected for use in this study (Fig. 1A).

A genomic cosmid library of *E. coli* 22 was generated in *E. coli* K-12, and the resulting clones were screened for localized adherence to HEp-2 cells. One cosmid clone, pV-B-6, exhibited adherence in a diffuse pattern over the entire epithelial cell rather than the LA microcolony formation seen with *E. coli*
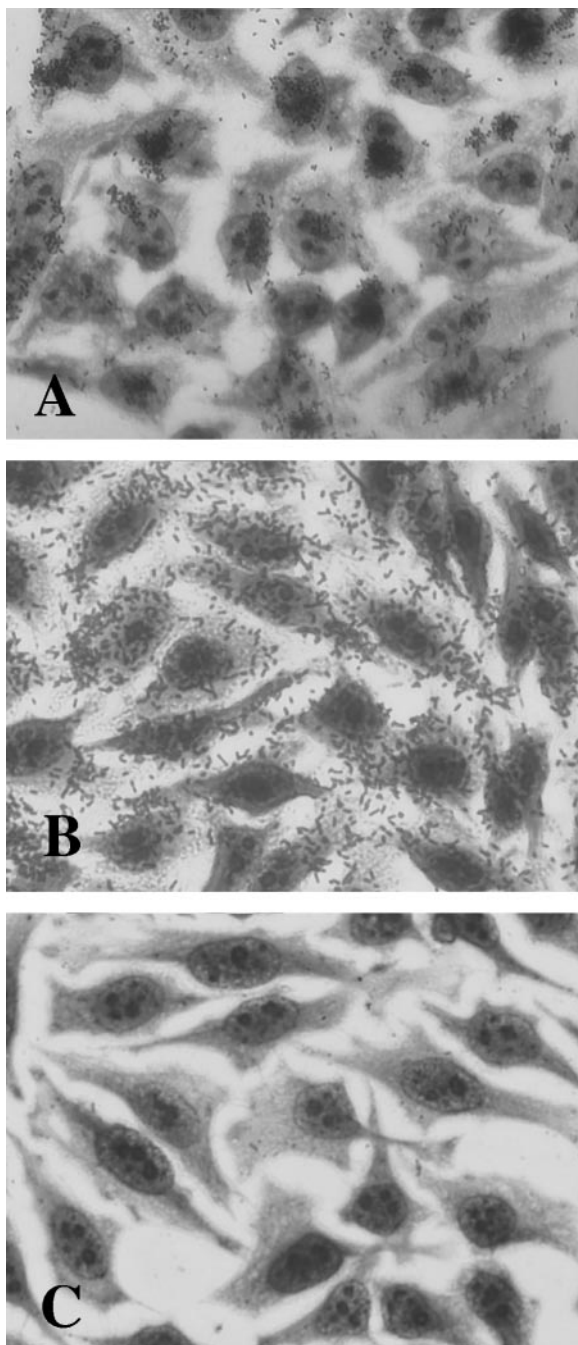
FIG. 1. HEp-2 adherence assay. (A) *E. coli* 22 wild type. (B) *E. coli* DH5α(pV-B-6). (C) *E. coli* DH5α(pV-B-6-Tn).

22 (Fig. 1B). Transposon mutagenesis was utilized to identify the region of cosmid pV-B-6 responsible for the adhesive phenotype. Plasmid pBSL180 carrying mini-Tn*10*::*kan* was electroporated into *E. coli* DH5α(pV-B-6), and isolates were selected on L agar with kanamycin and ampicillin. One isolate resistant to ampicillin and kanamycin that tested negative for adherence (pV-B-6-Tn) was chosen for further analysis (Fig. 1C). DNA sequencing of a subclone of pV-B-6 (pLDAH) revealed an open reading frame, *ldaH*, whose predicted protein product shares 73% amino acid identity with that of the *E.*

coli K88 fimbrial gene *faeH* (6), suggesting that this region might include genes involved in a novel adherence factor (Fig. 2). This region was named the *l*ocus for *d*iffuse *a*dherence (*lda*).

Sequence analysis of *lda* and homology with other fimbrial operons. Subsequent mapping and subcloning of pV-B-6 using restriction enzymes HindIII, PstI, EcoRI, and BamHI generated overlapping clones, which were purified and whose DNA sequences were determined. Sequence analysis revealed a 15-kb region having a G+C content of 46.8% comprised of 15 ORFs whose predicted protein products are similar to those of the K88 *fae* (33) and CS31A (14, 32) operons. Because of its well-characterized nature, we have chosen to use the K88 operon as the family prototype of chaperone-usher fimbria in which to compare the LDA operon (Fig. 3). Putative functions of the predicted proteins have been assigned on the basis of their homology to other proteins (Table 3). To maintain parallel nomenclature, we have named the *lda* genes to correspond to their counterparts in the K88 *fae* operon. These proteins include a structural subunit (LdaG), an outer membrane usher protein (LdaD), a periplasmic chaperone (LdaE), and four accessory proteins (LdaC, LdaF, LdaH, and LdaI). LdaC shows 85% amino acid identity to FaeC and is predicted to be a minor-subunit protein. Although slightly shorter than FaeD (812 amino acids), LdaD (790 amino acids) shares 82% amino acid identity and is predicted to be an outer membrane usher protein. Like FaeD, LdaD contains four conserved cysteine residues, two at the N terminus and two at the C terminus, which are necessary for proper folding of the protein (32). Compared to the periplasmic chaperone protein FaeE, LdaE is slightly larger and shares ca. 75% identity. Like other chaperone proteins within the gram-negative immunoglobulin superfamily, LdaE contains the highly conserved Arg-8, Lys-112, and Met-172 residues (numbered according to the PapD sequence) necessary for pilin production, as demonstrated by site-directed mutagenesis in PapD (20). The predicted minor subunit LdaF shows 72% identity to FaeF. The predicted major structural subunit, LdaG, shows 56% identity to the K99 adhesin, F41 (4), and shows only 23% identity with the K88ac major subunit. On the nucleotide level, *ldaG* shares no striking homology with any bacterial genes in the National Center for Biotechnology Information GenBank database. The predicted minor pilin subunits LdaH and LdaI show 73% and 60% identity, respectively, to FaeH/ClpH and FaeI/ClpL.

There are two ORFs with similarity to transcriptional regulators. The first ORF, LdaA, has 53% and 49% identity to YdeR of ETEC O159:H4 and AfrR of rabbit diarrheagenic *E. coli* type 1, respectively. The second ORF, LdaB, has 73% and 38% amino acid identity to a putative transcriptional regulator in *E. coli* O157:H7 and to EPEC plasmid regulator PerC, respectively. Interestingly, LdaA and LdaB have little identity (<11%) to either of their regulatory counterparts, FaeA and FaeB, and unlike the *fae* operon, in which the *faeA* and *faeB* regulator genes are next to each other, *ldaA* and *ldaB* are separated by the structural genes of the *lda* locus.

There are four ORFs (ORF1, -2, -5, and -6) that share 37 to 39% nucleotide homology with genes encoding transposases and insertion sequence-like sequences, suggesting that this region could have been horizontally acquired from another organism. ORF3 shows 87% identity to the hypothetical *E. coli*

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LdaH    MNNMKVIRYYRIFHAALIMVVLFYMTTSRADIIDGGEIQFHGFVTDEAPKWTWQIGSSEQ
FaeH    ---MKITHHYKSLLSAIISVALFYSAAPHADILDGGEIQFNGFVTDDAPKWTWQISSPDQ
           **:  ::*:  : :*:* *.*** ::..:***:*******:*****:********.*..:*

LdaH    SWAVDTADARVESGQFIFDLHDKGSLPFLEGHLFEVAERGGPGFTPFVSFSSNGQPFTVT
FaeH    TWAVDTADARTENGQLVFDLSDKGPLPFLEGYLYEVAERGGPGFTPFITFSSNGRPFAVK
           :*********.*.**:.*** ***.******:*.:************:.*****.**:*.

LdaH    DGNSTTAQHFRASVPVSNPENGQVTGRLYFTINQGMAVSTGVQEGGVSVPAGMSLVSGQS
FaeH    EGSDTSVQRFRASVPVRDPETGNVSGQLSFTLNQGMAVSTGKQEEGASTPSGMSLVSGQS
           :*..*:.*:******* :**.*:*:*:* **:********* ** *.*.*:*********

LdaH    VTNVQSDSLPQELKNRLSSLLLMNQNFGAGMSAVDNGQVISQGVLSDGRVTNLAAAYASS
FaeH    VTDVQSGSLPQGLKNRLSALLLMNKGFGNGMSAVDNGQVITQGVLADGRVMNLAAAYASA
           **:***.**** ******:*****:.** ***********:****:**** ********:

LdaH    VSDFELHLPAEGTPAQWQAKLNVTVTVQ
FaeH    VSDFELRLPAEGTPARWQAGLNVTVTVQ
           ******:*******:*** ********
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FIG. 2. Comparison of predicted amino acid sequence of LdaH with *E. coli* K88 fimbrial subunit FaeH. Identical residues in both proteins are indicated by asterisks, and conserved residues are indicated by dots (:, strongly similar; ., weakly similar). The alignment was created by CLUSTAL W.

YbdN protein (9), which is a member of the phosphoadenosine phosphosulfate reductase family and may play a role in amino acid transport and metabolism. Lastly, ORF4 is similar to conserved hypothetical proteins of unknown function but shows some limited amino acid identity to YdbM (9), so it may function similarly to ORF3.

The *lda* locus is part of a putative pathogenicity island. The flanking transposase-like sequences and lower G+C content

suggested that this region could be part of a mobile element introduced as a block into the *E. coli* chromosome. To determine the chromosomal location of the *lda* region in *E. coli* 22, we examined the sequences flanking the 5′ and 3′ ends of the *lda* locus in cosmid clone pV-B-6 and compared the sequence to the published *E. coli* K-12 MG1655 genome (9). DNA sequence analysis revealed that the upstream LDA junction was within the *proP* gene (Fig. 4). Unfortunately, sequences within
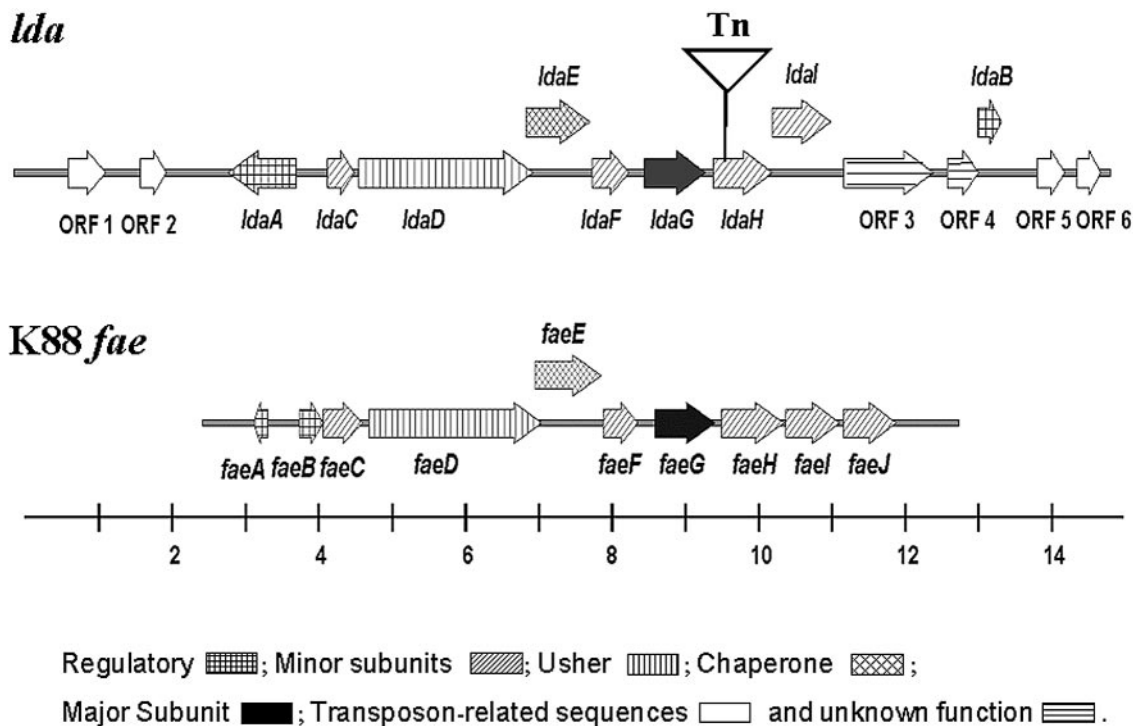

FIG. 3. Maps of *lda* and K88 *fae* loci (drawn to scale). The locations and directions of genes are indicated by arrows, and the point of Tn5 insertion in pV-B-6-Tn is shown by an inverted triangle. A line scale drawn below indicates the size in kb. The shading patterns within the arrows indicate the predicted functions of the proteins as indicated.

TABLE 3. Description of *lda* open reading frames

Protein or ORF	No. of amino acids	Protein mass (kDa)	% Identity	Similar protein(s) and putative function	Protein accession no.
ORF1	513	20	38	Putative transposase; *Yersinia pestis*	NP_669742.1
			37	Hypothetical protein encoded within IS*1240* of *Pseudomonas syringae*	AAB81643.1
ORF2	118	13.5	63	Putative transposase; *Pseudomonas syringae*	AAB81642.1
			54	Putative transposase; *Yersinia pestis*	CAC90690.1
LdaA	309	36.4	53	CS12 transcriptional regulator (YdeR)	AAK09057.1
			49	Transcriptional activator (AfrR) in *E. coli* RDEC-1[a]	AAC28314.1
			39	Caf1 of *Yersinia pestis*	S19097
LdaC	119	12.8	85	*E. coli* K88 fimbrial protein (FaeC)	AAL47165.1
			54	*E. coli* CS31A fimbrial protein (ClpC)	AAB39316.1
LdaD	790	83.5	82	*E. coli* K88 outer membrane usher (FaeD)	CAA27310.1
			60	RalD of rabbit *E. coli* (REPEC)	AAB97611
LdaE	287	31.9	82	*E. coli* CS31A periplasmic chaperone (ClpE)	I41172
			75	*E. coli* K88 periplasmic chaperone (FaeE)	S15227
LdaF	163	17.8	72	*E. coli* CS31A minor subunit (ClpF)	AAA23584.1
			63	*E. coli* K88 minor fimbrial subunit (FaeF)	B45725
LdaG	277	29	56	*E. coli* K99 adhesin F41	I41116
			27	CS31A fimbrial subunit of *E. coli* K88 (ClpG)	A41663
			23	*E. coli* K88 fimbrial subunit K88ac (FaeG)	I41318
LdaH	268	28.9	73	*E. coli* K88 minor fimbrial subunit (FaeH)	S24810
			73	CS31A minor subunit (ClpH)	AAA23780.1
			71	Minor fimbrial subunit; *Salmonella enterica*	AAC09003.1
LdaI	265	28.9	61	*E. coli* minor subunit CS31A (ClpL)	AAA23586.1
			59	*E. coli* K88 minor fimbrial subunit (FaeI)	F45725
			59	Minor fimbrial subunit; *Salmonella enterica*	AAC09004.1
ORF3	407	48.2	87	Conserved hypothetical protein (YdbN); *E. coli*	NP754377
ORF4	143	16.4	85	Hypothetical protein; *Shigella flexneri*	AAK18415.1
			82	Hypothetical protein; *Salmonella enterica*	NP_458668.1
			79	Hypothetical protein; *E. coli* O157:H7	NP_286739.1
LdaB	108	12.7	73	Putative transcriptional regulator; *E. coli* O157:H7	NP_309415.1
			38	PerC transcriptional regulator; *E. coli*	AAG16672.1
ORF5	126	14.3	55	ISEc8-related protein within prophage CP-933X of *E. coli* O157:H7	NP_286015.1
ORF6	107	12.3	55	ISEc8-related protein within prophage CP-933X of *E. coli* O157:H7	NP_286015.1

[a] RDEC-1, rabbit diarrheagenic *E. coli* type 1.

pV-B-6 did not extend far enough downstream, and so we relied on PCR analysis to determine the downstream junction. Based on *E. coli* K-12 and LDA sequence data, we designed primers downstream of the *proP* gene and one primer (K2792) within the region of *lda* in cosmid pV-B-6. Primer sets K1/K3, K2/K3, K4/K6, and K5/K7 all yielded the same size products from *E. coli* MG1655 and *E. coli* 22, indicating that the region downstream of *proP* was the same in both strains. PCR amplification using primer set K3/K2792 yielded a 6.5-kb product with *E. coli* 22. No PCR product was obtained with *E. coli* MG1655, indicating that this region was not present in K-12. These results confirmed that the *lda* island (ca. 26 kb) is inserted within the *proP* gene.

Presence of *ldaH* and *ldaG* in other *E. coli* serotypes. To determine whether the *lda* locus was specific to our O26:H11 strain or if it is present in other *E. coli* strains, we performed colony blots and HEp-2 adherence assays on a collection of *eae*[+] *bfp*-negative *E. coli* isolates representing 22 O serogroups. Nineteen strains in four groups, O5, O26, O111, and O145, were positive for the *ldaH* gene probe, indicating that this adhesin is not limited to O26:H11 (Table 4). All *ldaH* probe-positive isolates were positive for HEp-2 localized-adherence assays, but not all LA[+] strains were *ldaH* probe positive, suggesting the presence of another adherence factor(s) in these isolates. *ldaH* was found in both *stx*-positive and -negative isolates. Because the CS31A and K88 operons are re-
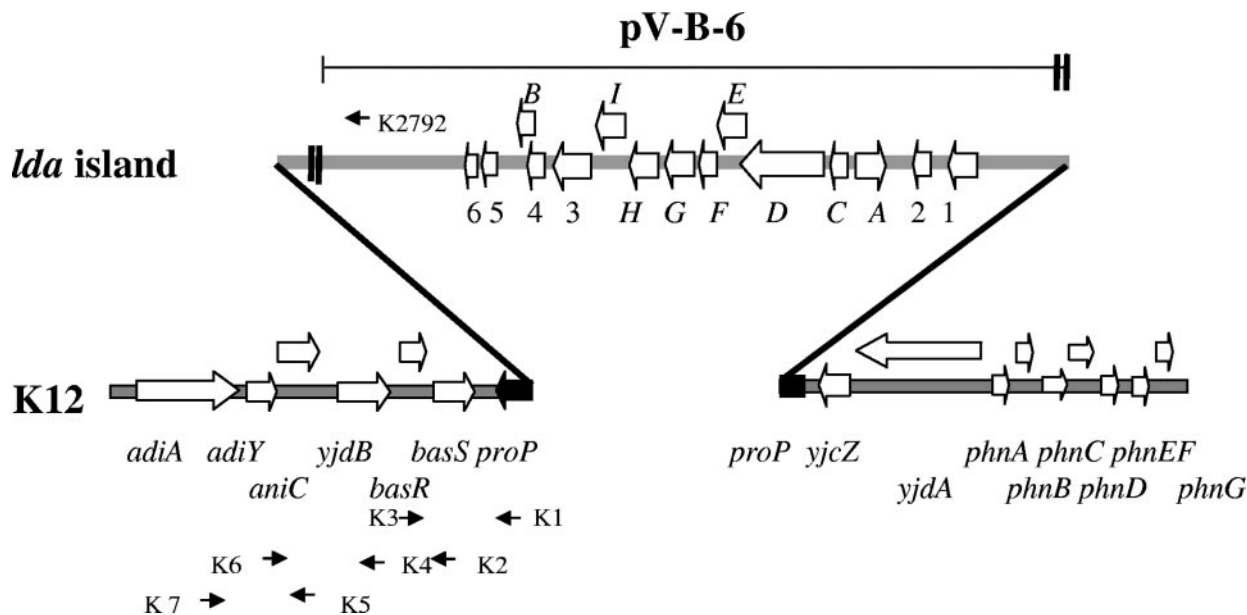
FIG. 4. Chromosomal location of LDA island. Wide arrows indicate the locations and directions of ORFs and genes. The interrupted *proP* gene is represented as a discontinuous filled arrow. The small arrows indicate the locations of PCR primers. The region encompassing the cosmid clone is represented by a thin horizontal line. Double bold vertical lines indicate gaps in the sequence.

ported to share high homology between coding regions of the accessory proteins but to have distinct structural genes (30, 36), we tested for the presence of the major structural subunit gene. Six *ldaH*+ isolates, including those representing O26:H strains and all non-O26 *lda*+ strains, were tested with an *ldaG* gene probe. *E. coli* strains 22 (O26:H11), 86-280 (O26:H11), and 84-8198 (O26:H−) were positive and 88-0643 (O5:H−), 85-0839 (O111:H−), and 87-1713 (O145:H16) were negative with the *ldaG* gene probe, indicating that while there are homologies within a minor subunit (*ldaH*) among serotypes, the major structural subunit (*ldaG*) may be specific for the O26 serogroup. Control strains harboring genes for K88 and F41 fimbriae were all negative (data not shown).

Construction and complementation of an *ldaG* mutant and identification of a second adherence factor. To demonstrate the requirement for *lda* for adherence in *E. coli* 22, we made a null mutation in the putative structural gene *ldaG* (Table 3) to yield strain LDA1 (see Materials and Methods). The construct was confirmed by Southern blotting and PCR analysis (data not shown). Surprisingly, *E. coli* LDA1 exhibited the same adherence pattern as wild-type *E. coli* 22, albeit to a lesser extent (compare Fig. 5A to Fig. 1A).

Because the cloned LDA region conferred adherence on *E. coli* DH5α and an insertion mutation within the cosmid abolished this phenotype, we hypothesized that the failure to abolish adherence following mutation of *ldaG* in *E. coli* 22 indicated the existence of a second adherence factor in the strain. Plasmid analysis revealed that this strain contains at least five plasmids (Fig. 6), and the possibility of a plasmid-encoded adhesin was investigated. Plasmids were extracted from a collection of transposon-mutagenized *E. coli* 22 isolates (see Materials and Methods), purified by CsCl2 gradient centrifugation, transformed into *E. coli* XL-10, and selected on L agar with kanamycin. The resulting transformants were tested for

adherence on HEp-2 cells, and one Kmr transformant, designated *E. coli*(pIJ22), exhibited a localized adherence phenotype (Fig. 5B). The size of pIJ22 did not correspond to that of any plasmid present in the wild-type strain (taking into account the addition of the mini-Tn10::kan), and we therefore presumed that the parent plasmid had suffered a deletion. To identify which of the five wild-type plasmids encoded adherence in *E. coli* 22 Δ*ldaG* (LDA1), an EcoRI restriction fragment of pIJ22 was used to probe a Southern blot of the unrestricted plasmids isolated from wild-type *E. coli* 22. A large plasmid (pIJ22-1) was identified as containing homologous sequences (Fig. 6) and was targeted for curing by plasmid incompatibility using a chloramphenicol derivative of pIJ22 (see Materials and Methods). The resulting plasmid-cured strain, called LDA3, was negative for adhesion on HEp-2 cells (Fig. 5C) and was confirmed by agarose gel electrophoresis to have lost pIJ22-1 (Fig. 6, lane 2).

To confirm the expression pattern of the LDA phenotype and restore the diffuse-adherence phenotype of LDA3, a minimal subclone of the original cosmid clone pV-B-6 that still possessed the ability to adhere to HEp-2 cells, termed pIJ20 (21.5 kb), was introduced into *E. coli* LDA3 to complement the Δ*ldaG* mutation. As seen in Fig. 5D, LDA3(pIJ20) adheres to the HEp-2 cells in a diffuse pattern identical to that of *E. coli* DH5α(pV-B-6) (compare Fig. 1B and 5D). These results confirm that *E. coli* 22 contains two genetic loci that encode HEp-2 adherence factors. One adhesin, encoded by the chromosomal *lda* locus, expresses diffuse adherence, and the other adhesin (or an essential regulator of adhesion expression), expressing localized adherence, is encoded by a plasmid.

Expression and detection of LdaG. Crude fimbrial preparations of *E. coli* 22 and LDA3 were obtained through whole-cell lysates and heat extracts. A broad band with an apparent molecular mass of 25 kDa was present in the heat extracts of *E.*

TABLE 4. Prevalence of *ldaH* sequences in *E. coli* *eae*[+] *bfp*-negative strains[a]

Serotype	*stx* probe[d]	*ldaH* probe[d]	Localized adherence[b,d]	No. of strains
O26:H⁻	−	−	−	2
O26:H⁻	−	+	+	7
O26:H8	−	−	−	2
O26:H11	−	−	−	6[c]
O26:H11	−	+	+	7
O26:H11	+	−	−	14
O26:H11	+	−	+	5
O26:H11	+	−	+/−	8
O26:H11	+	+	+	2
O26:H25	−	−	−	1
O26:H32	−	−	−	2
O26:H33	−	−	−	2
O1:H6	−	−	−	1
O1:H7	−	−	−	1
O5:H⁻	+	+	+	1
O16:H16	+	−	−	1
O22:H16	+	−	−	1[c]
O38:H21	+	−	−	1
O45:H2	+	−	−	1
O70:H11	+	−	−	1
O82:H8	+	−	−	1
O84:H2	+	−	−	1
O91:H14	+	−	+	1
O103:H2	+	−	+	1
O111:H⁻	+	−	+	1
O111:H⁻	+	+	+	1[c]
O111:H8	+	−	−	2[c]
O111:H34	+	−	−	1
O113:H21	−	−	−	1
O118:H30	+	−	+	1
O121:H⁻	+	−	−	1
O21:H19	−	−	−	1
O126:H	+	−	−	1
O126:H8	+	−	−	1
O128:H12	−	−	+	1
O145:H⁻	+	−	+	1
O145:H⁻	+	−	+/−	1
O145:H16	+	+	+	1[c]
O153:H25	+	−	−	1
O165:H⁻	+	−	−	1
O157:H7	+	−	−	3

[a] All strains are of human fecal origin except for the bovine fecal isolates indicated below. All strains are *bfpA* negative.
[b] Localized adherence assays were recorded at 3 hours with a +/− indicating an equivocal phenotype.
[c] One strain was isolated from bovine feces.
[d] +, positive; −, negative.

coli 22 and to a lesser degree in the whole-cell lysates and was absent in the LDA3 extracts (Fig. 7A). The abundance of this protein is consistent with its being a major fimbrial structural subunit. The 25-kDa band was excised from the gel, and its N-terminal amino acid sequence was determined. The sequence, SDWTDNQPAGDI, corresponds to the mature form of LdaG after a 22-amino-acid putative signal sequence, MKKTLLALAVVASAVVSGSALA, has been removed.

Antibody inhibition of LDA adhesion. To provide further evidence that the LDA adhesin was responsible for the diffuse adherence seen on HEp-2 cells, we raised polyclonal rabbit antiserum against the major structural subunit LdaG. Western blot analysis (Fig. 7B) showed that the antiserum recognized the 25-kDa band present in wild-type *E. coli* 22 and *E. coli* ORN103(pIJ20), but not the LDA3 mutant. To determine

whether the antiserum could inhibit adhesion of the bacteria, overnight bacterial cultures were first incubated with the LdaG antiserum for 2 h at 37°C and then added to the HEp-2 cell monolayer. Addition of the LdaG antiserum at a 1:50 dilution completely inhibited diffuse adherence of *E. coli* DH5α(pV-B-6), containing the cloned *lda* region, to the HEp-2 cells, whereas the preimmune serum from the same rabbit did not inhibit adhesion (Fig. 8A and B). Treatment of wild-type *E. coli* 22 with the antiserum did not inhibit LA adhesion even at a 1:5 dilution, providing further evidence that there are two factors that mediate Hep-2 adherence by this strain (Fig. 8C).

Detection of LDA by electron microscopy. *E. coli* 22 and the LDA isogenic mutant, LDA1, were examined by electron microscopy using immunogold labeling and negative-staining techniques. As seen in Fig. 9A and B, immunogold labeling with LdaG antiserum identified an amorphous surface matrix with no obvious fimbrial structure. At higher magnification (×120,000), very fine wiry fibrils forming mesh-like structures can be visualized, but the details are beyond the resolution of the electron microscope (Fig. 9C). This surface matrix is specific for the presence of LdaG, as 10-nm gold particles can be seen decorating the entire surface of *E. coli* 22 while no gold particles decorate the *ldaG* mutant LDA1 (Fig. 9D). The gold label also recognizes LdaG expressed in the *E. coli* XL-10(pIJ20) subclone (Fig. 9E), which exhibits the same decorative pattern as the wild-type strain (Fig. 9A and B). Controls for nonspecific gold particle binding were performed in which the LdaG primary antibody was omitted prior to incubation with the goat anti-rabbit-labeled 10-nm gold particles. The results were identical to those seen with the null mutant, LDA1 (data not shown). The appearance of LDA is very similar to that seen with other afimbrial adhesins, such as Ral from rabbit EPEC (1), CS6 from ETEC (56), and Afa, originally identified in a uropathogenic *E. coli* serotype O2 strain (26) and later in EPEC strain 132/12 (O55:H⁻) (24). Thus, despite the amino acid homologies to the K88 and CS3A1 fimbriae, LDA appears to be afimbrial in structure.

DISCUSSION

It has long been known that EHEC serogroup O26 is an important pathogen in both human and animal infections (27, 35). The EPEC O26 serogroup has been implicated in human diarrheal infections in several countries worldwide (2, 42, 43), but its incidence in animal infections has only recently been reported (29, 55). Although EPEC differs from EHEC in its inability to produce Stx, both share the same mechanism for intimate attachment and effacement (38). Multiple factors, including Bfp, flagella, and the LEE-encoded proteins, have been implicated in adherence in typical EPEC strains (reviewed in reference 40), but to date, no distinct adhesins have been characterized in atypical EPEC. The work of Szalo et al. (51) identified several *bfpA* *lpfA*-negative O26 bovine isolates that have a gene sequence whose predicted protein sequence shows 89% identity to that of ClpE, the chaperone protein of CS31A pili. These same isolates were negative for the gene encoding the major structural subunit (*clpG*), suggesting the presence of a novel adhesin, but it has yet to be elucidated. Here, we report the identification and characterization of a novel gene cluster, locus for diffuse adherence (*lda*), present in
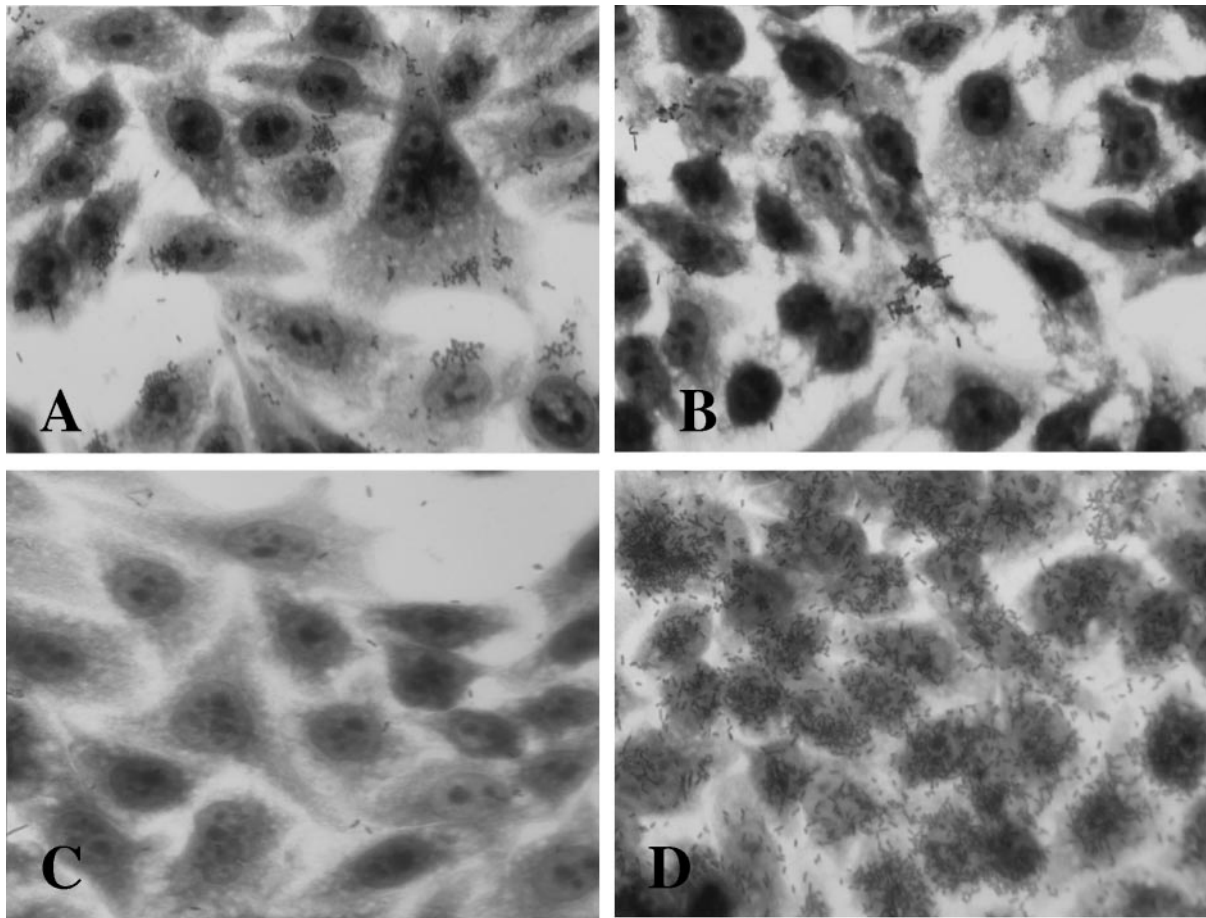
FIG. 5. HEp-2 adherence assay. (A) *E. coli* LDA1. (B) *E. coli* XL-10(pIJ22). (C) *E. coli* LDA3 mutant. (D) *E. coli* LDA3(pIJ20) complement.
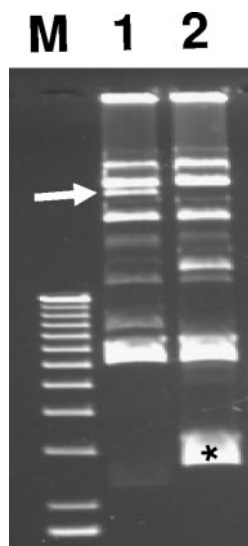

FIG. 6. Plasmid profiles. M, 1-kb DNA ladder markers (Invitrogen); lane 1, *E. coli* 22; lane 2, *E. coli* LDA3. The arrow indicates the location of pIJ22-1. The asterisk in lane 2 identifies pIJ30, used to cure pIJ22-1.

an LA$^+$ *stx*-negative *eae*$^+$ EAF$^-$ *bfpA*-negative atypical O26: H11 *E. coli* strain, that is responsible for mediating diffuse adherence to HEp-2 cells.

Atypical EPEC strain 22 was originally investigated because of its ability to form microcolonies on HEp-2 cells and its lack of hybridization to known fimbrial-gene probes. Our analysis revealed that there are two factors contributing to the adherence phenotype of this strain. The first adhesin is encoded by a chromosomal locus, *lda*, which gives a diffuse pattern of adherence on HEp-2 cells when cloned into a nonadhesive K-12 strain. A chromosomal mutation in the major structural gene, *ldaG*, abated but did not abolish HEp-2 adherence by wild-type *E. coli* 22. The second adhesin is a plasmid-encoded factor, which by itself in *E. coli* XL-10(pIJ22) forms isolated microcolonies on HEp-2 cells, albeit fewer and smaller than those of wild-type *E. coli* 22 (compare Fig. 5B to 1A). Elimination of this plasmid, along with the *ldaG* chromosomal mutation in *E. coli* 22 (LDA3), results in complete lack of adherence to HEp-2 cells. Complementation of the double mutant LDA3 (Δ*ldaG* pIJ22$^-$) with pIJ20 (*lda*$^+$) restored the diffuse-adherence phenotype but not the localized adherence originally observed in the wild-type strain (compare Fig. 5D to 1A). Thus, it is not known if the diffuse-adherence property of LDA is masked by the expression of a second adhesin encoded on
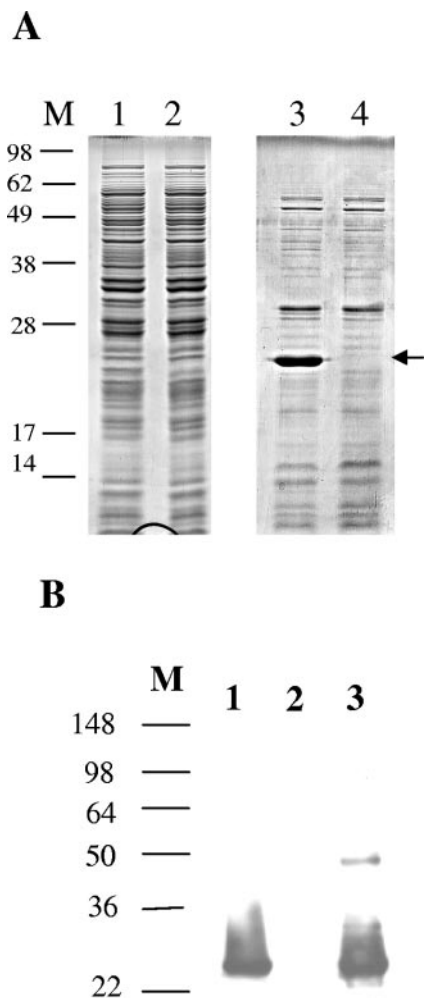
A

B

FIG. 7. Detection of LdaG. (A) SDS-PAGE of whole-cell lysates (lanes 1 and 2) and heat-extracted proteins (lanes 3 and 4) from *E. coli* 22 (lanes 1 and 3) and *E. coli* LDA1 (lanes 2 and 4). The arrow indicates the location of LdaG (lanes 1 and 3). (B) Western blot analysis of LdaG. *E. coli* 22 (lane 1), *E. coli* LDA3 (lane 2), and *E. coli* DH5α(pIJ20) (lane 3) are shown.

FIG. 8. Antibody inhibition of HEp-2 adherence. (A) *E. coli* DH5α(pV-B-6) incubated with preimmune serum (1:50 dilution). (B) *E. coli* DH5α(pV-B-6) incubated with α-LdaG antiserum (1:50 dilution). (C) *E. coli* 22 incubated with α-LdaG antiserum (1:5 dilution).

pIJ22 in wild-type *E. coli* 22 or if these two factors are acting concomitantly to give the full localized-adherence pattern. For example, pIJ22 could be involved in expressing another factor(s) that regulates the LA phenotype. Indeed, one previously described regulator that influences the LA phenotype in EPEC is TrcA; null mutations in *trcA* produced smaller than normal adherent microcolonies compared to wild-type EPEC (52). The nature of this second LA adherence phenotype associated with pIJ22 is unknown and is being investigated.

The organization and predicted protein sequence similarities of the *lda* locus closely resemble those of the K88 *fae* operon (6), although it also shows considerable identity to the ETEC CS31A *clp* fimbrial operon (14) (Table 4) and to a lesser extent the rabbit EPEC *ral* (1) fimbrial operon (data not shown). It is interesting that the amino acid sequence of the putative major structural gene, LdaG, is related more closely to the chromosomally encoded F41 adhesin structural subunit (56%) than it is to any of the variants of the plasmid-encoded
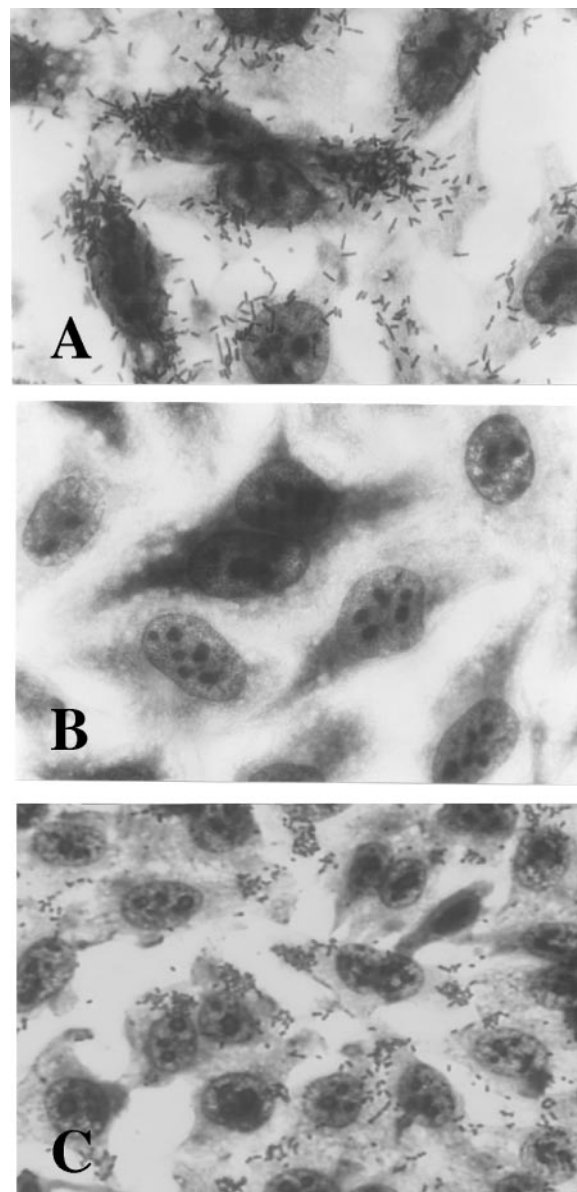
K88 FaeG structural subunits (23%). The specificity of fimbrial adhesins for their host cells is well documented (32, 50). ETEC strains harboring the F41 fimbrial antigen are pathogenic for cattle, swine, and lambs, whereas ETEC K88 strains are pathogenic for swine (34). The importance of LDA-mediated adhesion in the pathogenicity of these strains in human disease is not clear, but the isolation of this O26 isolate from the stool of a patient suffering from diarrhea from whom no other pathogen could be isolated would be consistent with a role in disease. In addition, although our sample size was small (only five strains, each of a different serotype), it is also of interest to note that the two *ldaG*⁺ strains were *stx* negative and the three
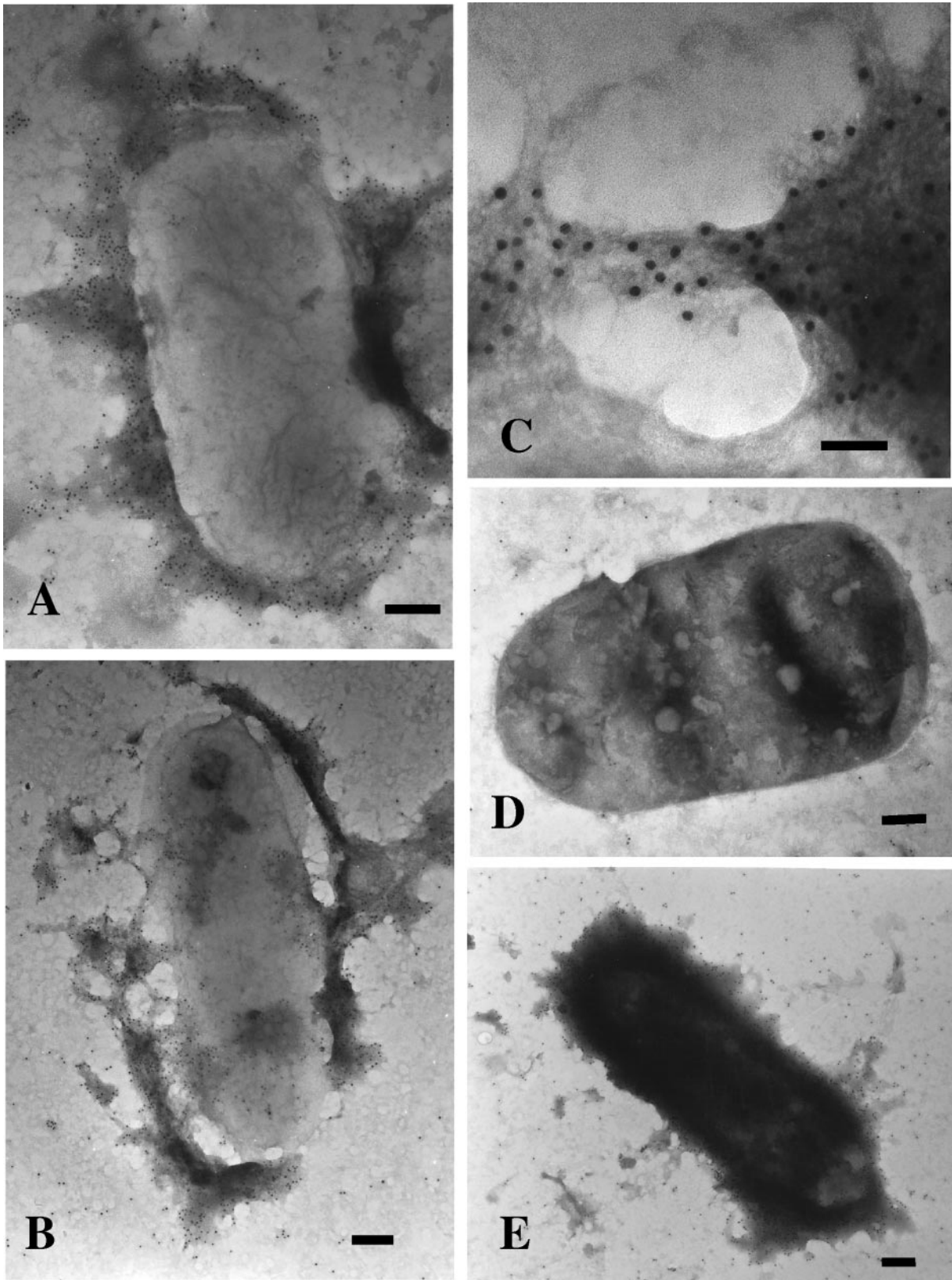
FIG. 9. Immunogold labeling and negative staining of LDA. (A and B) *E. coli* 22. The bars represent 200 nm. (C) *E. coli* 22. The bar represents 50 nm. (D) *E. coli* LDA1. The bar represents 200 nm. (E) *E. coli* XL10(pIJ20). The bar represents 200 nm.

ldaG strains were *stx*[+], further suggesting that Lda might be specific in the pathogenesis of atypical EPEC.

Three of the minor subunits, LdaC, LdaH, and LdaI, show considerable identity (85%, 73%, and 59%) to the FaeC, FaeH, and FaeI proteins of K88 fimbriae, respectively. The closely related FaeH and ClpH proteins show 88% identity to each other. Previous investigations have reported extensive DNA homologies between K88-related ancillary proteins (4, 5, 30), and in fact, some can be used interchangeably for fimbrial expression (25). Therefore, the nucleotide homologies seen within the minor subunit, *ldaH*, among strains of different serotypes were not so surprising (Table 3). LDA appears to belong to a family of fimbrial adhesins that may share a conserved mechanism of pilin production while possessing a unique structural subunit specific for host-fimbria recognition by O26 strains. Indeed, LdaE has striking identity (75% and 82%) to FaeE and ClpE periplasmic chaperones, respectively, suggesting that it too is a member of the chaperone-assisted pilus assembly mechanism, similar to the prototypic Pap operon (8, 50). It is not known how much homology is shared between *E. coli* O26:H11 and the *ldaH*[+] *ldaG*-negative isolates outside the *ldaH* gene, but the possibility that they may be closely related is very intriguing, and we are exploring this hypothesis.

This is the first reported sequence of a novel adhesin found in an atypical EPEC strain. Like many known fimbrial loci, LDA is predicted to contain regulatory genes (*ldaA* and *ldaB*), a periplasmic chaperone (*ldaE*), an usher (*ldaD*), a major structural gene (*ldaG*), and a few ancillary genes (*ldaC*, *ldaF*, *ldaH*, and *ldaI*) thought to aid in the formation of the fimbriae. Unlike its plasmid-borne K88, CS31A, and Ral fimbrial counterparts, Lda is located within a genomic island that is absent from the K-12 strain MG1655 and has not been reported in any of the *E. coli* genomes so far described. The insertion of this region into the *proP* gene, along with the transposon sequences flanking the LDA locus, suggests that this region has been horizontally acquired as a pathogenicity island (18). Additionally, *lda* apparently does not encode a well-defined fimbrial structure but rather one with a flexible morphological appearance similar to nonfimbrial antigens. Like the Ral (1), CS6 (56), and Afa (24) fibrillae, LDA is wiry, unstructured, and rather difficult to visualize under the electron microscope. A comparative exchange of the structural proteins of these distinct fimbriae would provide a useful understanding of the pili and adhesins associated with these *E. coli* strains. Additional work is needed to characterize the regulation and role in virulence of the LDA adhesin.

REFERENCES

1. **Adams, L. M., C. P. Simmons, L. Rezmann, R. A. Strugnell, and R. M. Robins-Browne.** 1997. Identification and characterization of a K88- and CS31A-like operon of a rabbit enteropathogenic *Escherichia coli* strain which encodes fimbriae involved in the colonization of rabbit intestine. Infect. Immun. **65:**5222–5230.
2. **Afset, J. E., K. Bergh, and L. Bevanger.** 2003. High prevalence of atypical enteropathogenic *Escherichia coli* (EPEC) in Norwegian children with diarrhoea. J. Med. Microbiol. **52:**1015–1019.
3. **Alexeyev, M. F., I. N. Shokolenko, and T. P. Croughan.** 1995. New mini-*Tn5* derivatives for insertion mutagenesis and genetic engineering in Gram-negative bacteria. Can. J. Microbiol. **41:**1053–1055.
4. **Anderson, D. G., and S. L. Moseley.** 1988. *Escherichia coli* F41 adhesin: genetic organization, nucleotide sequence, and homology with the K88 determinant. J. Bacteriol. **170:**4890–4896.
5. **Bakker, D., C. E. Vader, B. Roosendaal, F. R. Mooi, B. Oudega, and F. K. de Graaf.** 1991. Structure and function of periplasmic chaperone-like proteins involved in the biosynthesis of K88 and K99 fimbriae in enterotoxigenic *Escherichia coli*. Mol. Microbiol. **5:**875–886.
6. **Bakker, D., P. T. Willemsen, R. H. Willems, T. T. Huisman, F. R. Mooi, B. Oudega, F. Stegehuis, and F. K. de Graaf.** 1992. Identification of minor fimbrial subunits involved in biosynthesis of K88 fimbriae. J. Bacteriol. **174:**6350–6358.
7. **Barros, H. C., M. L. Silva, M. Z. Laporta, R. M. Silva, and L. R. Trabulsi.** 1992. Inhibition of enteropathogenic *Escherichia coli* adherence to HeLa cells by immune rabbit sera. Braz. J. Med. Biol. Res. **25:**809–812.
8. **Bertin, Y., J. P. Girardeau, M. Der Vartanian, and C. Martin.** 1993. The ClpE protein involved in biogenesis of the CS31A capsule-like antigen is a member of a periplasmic chaperone family in gram-negative bacteria. FEMS Microbiol. Lett. **108:**59–67.
9. **Blattner, F. R., G. Plunkett III, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao.** 1997. The complete genome sequence of *Escherichia coli* K-12. Science **277:**1453–1474.
10. **Chang, A. C., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
11. **Cravioto, A., R. J. Gross, S. M. Scotland, and B. Rowe.** 1979. An adhesive factor found in strains of *Escherichia coli* belonging to the traditional infantile enteropathogenic serotypes. Curr. Microbiol. **3:**95–99.
12. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA **97:**6640–6645.
13. **Fischer, J., C. Maddox, R. Moxley, D. Kinden, and M. Miller.** 1994. Pathogenicity of a bovine attaching effacing *Escherichia coli* isolate lacking Shiga-like toxins. Am. J. Vet. Res. **55:**991–999.
14. **Girardeau, J. P., M. Der Vartanian, J. L. Ollier, and M. Contrepois.** 1988. CS31A, a new K88-related fimbrial antigen on bovine enterotoxigenic and septicemic *Escherichia coli* strains. Infect. Immun. **56:**2180–2188.
15. **Girón, J. A., M. S. Donnenberg, W. C. Martin, K. G. Jarvis, and J. B. Kaper.** 1993. Distribution of the bundle-forming pilus structural gene (*bfpA*) among enteropathogenic *Escherichia coli*. J. Infect. Dis. **168:**1037–1041.
16. **Girón, J. A., A. S. Ho, and G. K. Schoolnik.** 1991. An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. Science **254:**710–713.
17. **Guth, B. E., R. Lopes de Souza, T. M. Vaz, and K. Irino.** 2002. First Shiga toxin-producing *Escherichia coli* isolate from a patient with hemolytic uremic syndrome, Brazil. Emerg. Infect. Dis. **8:**535–536.
18. **Hacker, J., and J. B. Kaper.** 1999. The concept of pathogenicity islands, p. 1–11. *In* J. B. Kaper and J. Hacker (ed.), Pathogenicity islands and other mobile virulence elements. American Society for Microbiology, Washington, D.C.
19. **Hohn, B., and J. Collins.** 1980. A small cosmid for efficient cloning of large DNA fragments. Gene **11:**291–298.
20. **Holmgren, A., M. J. Kuehn, C. I. Branden, and S. J. Hultgren.** 1992. Conserved immunoglobulin-like features in a family of periplasmic pilus chaperones in bacteria. EMBO J. **11:**1617–1622.
21. **Jerse, A. E., J. Yu, B. D. Tall, and J. B. Kaper.** 1990. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc. Natl. Acad. Sci. USA **87:**7839–7843.
22. **Kaper, J. B.** 1996. Defining EPEC. Rev. Microbiol. São Paulo **27:**130–133.
23. **Kaper, J. B., J. P. Nataro, and H. L. Mobley.** 2004. Pathogenic *Escherichia coli*. Nat. Rev. Microbiol. **2:**123–140.
24. **Keller, R., J. G. Ordonez, R. R. de Oliveira, L. R. Trabulsi, T. J. Baldwin, and S. Knutton.** 2002. Afa, a diffuse adherence fibrillar adhesin associated with enteropathogenic *Escherichia coli*. Infect. Immun. **70:**2681–2689.
25. **Korth, M. J., J. M. Apostol, Jr., and S. L. Moseley.** 1992. Functional expression of heterologous fimbrial subunits mediated by the F41, K88, and CS31A determinants of *Escherichia coli*. Infect. Immun. **60:**2500–2505.
26. **Labigne-Roussel, A. F., D. Lark, G. Schoolnik, and S. Falkow.** 1984. Cloning

and expression of an afimbrial adhesin (AFA-I) responsible for P blood group-independent, mannose-resistant hemagglutination from a pyelonephritic *Escherichia coli* strain. Infect. Immun. **46:**251–259.

27. Levine, M. M. 1987. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. J. Infect. Dis. **155:**377–389.

28. Levine, M. M., J. G. Xu, J. B. Kaper, H. Lior, V. Prado, B. Tall, J. Nataro, H. Karch, and K. Wachsmuth. 1987. A DNA probe to identify enterohemorrhagic *Escherichia coli* of O157:H7 and other serotypes that cause hemorrhagic colitis and hemolytic uremic syndrome. J. Infect. Dis. **156:**175–182.

29. Mansfield, K. G., K. C. Lin, J. Newman, D. Schauer, J. MacKey, A. A. Lackner, and A. Carville. 2001. Identification of enteropathogenic *Escherichia coli* in simian immunodeficiency virus-infected infant and adult rhesus macaques. J. Clin. Microbiol. **39:**971–976.

30. Martin, C., C. Boeuf, and F. Bousquet. 1991. *Escherichia coli* CS31A fimbriae: molecular cloning, expression and homology with the K88 determinant. Microb. Pathog. **10:**429–442.

31. McDaniel, T. K., and J. B. Kaper. 1997. A cloned pathogenicity island from enteropathogenic *Escherichia coli* confers the attaching and effacing phenotype on *E. coli* K-12. Mol. Microbiol. **23:**399–407.

32. Mol, O., and D. Oudega. 1996. Molecular and structural aspects of fimbriae biosynthesis and assembly in *Escherichia coli*. FEMS Microbiol. Rev. **19:**25–52.

33. Mooi, F. R., N. Harms, D. Bakker, and F. K. de Graaf. 1981. Organization and expression of genes involved in the production of the K88ab antigen. Infect. Immun. **32:**1155–1163.

34. Moon, H. W. 1990. Colonization factor antigens of enterotoxigenic *Escherichia coli* in animals. Curr. Top. Microbiol. Immunol. **151:**147–165.

35. Morris, J. A., and W. J. Sojka. 1985. *Escherichia coli* as a pathogen in animals, p. 44–77. *In* M. Sussman (ed.), The virulence of *Escherichia coli*: reviews and methods. Academic Press, London, United Kingdom.

36. Moseley, S. L., G. Dougan, R. A. Schneider, and H. W. Moon. 1986. Cloning of chromosomal DNA encoding the F41 adhesin of enterotoxigenic *Escherichia coli* and genetic homology between adhesins F41 and K88. J. Bacteriol. **167:**799–804.

37. Nataro, J. P. 2001. Diarrhoeagenic *Escherichia coli*, p. 1463–1504. *In* M. Sussman (ed.), Molecular medical microbiology, vol. 2. Academic Press, London, United Kingdom.

38. Nataro, J. P., and J. B. Kaper. 1998. Diarreagenic *Escherichia coli*. Clin. Microbiol. Rev. **11:**142–201.

39. Newland, J. W., and R. J. Neill. 1988. DNA probes for Shiga-like toxins I and II and for toxin-converting bacteriophages. J. Clin. Microbiol. **26:**1292–1297.

40. Nougayrede, J. P., P. J. Fernandes, and M. S. Donnenberg. 2003. Adhesion of enteropathogenic *Escherichia coli* to host cells. Cell. Microbiol. **5:**359–372.

41. Orndorff, P. E., P. A. Spears, D. Schauer, and S. Falkow. 1985. Two modes of control of *pilA*, the gene encoding type 1 pilin in *Escherichia coli*. J. Bacteriol. **164:**321–330.

42. Peixoto, J. C. C., S. Y. Bando, J. A. G. Ordoñez, B. A. Botelho, L. R. Trabulsi, and C. A. Moreira-Filho. 2001. Genetic differences between *Escherichia coli* O26 strains isolated in Brazil and in other countries. FEMS Microbiol. Lett. **196:**239–244.

43. Pelayo, J. S., I. C. Scaletsky, M. Z. Pedroso, V. Sperandio, J. A. Giron, G. Frankel, and L. R. Trabulsi. 1999. Virulence properties of atypical EPEC strains. J. Med. Microbiol. **48:**41–49.

44. Runyen-Janecky, L. J., M. Hong, and S. M. Payne. 1999. The virulence plasmid-encoded *impCAB* operon enhances survival and induced mutagenesis in *Shigella flexneri* after exposure to UV radiation. Infect. Immun. **67:**1415–1423.

45. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

46. Scaletsky, I. C., M. L. Silva, and L. R. Trabulsi. 1984. Distinctive patterns of adherence of enteropathogenic *Escherichia coli* to HeLa cells. Infect. Immun. **45:**534–536.

47. Scotland, S. M., G. A. Willshaw, H. R. Smith, and B. Rowe. 1990. Properties of strains of *Escherichia coli* O26:H11 in relation to their enteropathogenic or enterohemorrhagic classification. J. Infect. Dis. **162:**1069–1074.

48. Silva, M. L. M., I. C. A. Scaletsky, and L. H. Viotto. 1983. Non-production of cytotoxin among enteropathogenic strains of *Escherichia coli* isolated in São Paulo, Brazil. Rev. Microbiol. **14:**161–162.

49. Simon, R., U. Priefer, and A. Pühler. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. Bio/Technology **1:**784–791.

50. Soto, G. E., and S. J. Hultgren. 1999. Bacterial adhesins: common themes and variations in architecture and assembly. J. Bacteriol. **181:**1059–1071.

51. Szalo, I. M., F. Goffaux, V. Pirson, D. Pierard, H. Ball, and J. Mainil. 2002. Presence in bovine enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) *Escherichia coli* of genes encoding for putative adhesins of human EHEC strains. Res. Microbiol. **153:**653–658.

52. Tobe, T., I. Tatsuno, E. Katayama, C. Y. Wu, G. K. Schoolnik, and C. Sasakawa. 1999. A novel chromosomal locus of enteropathogenic *Escherichia coli* (EPEC), which encodes a *bfpT*-regulated chaperone-like protein, TrcA, involved in microcolony formation by EPEC. Mol. Microbiol. **33:**741–752.

53. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.

54. Trabulsi, L. R., R. Keller, and T. A. Tardelli Gomes. 2002. Typical and atypical enteropathogenic *Escherichia coli*. Emerg. Infect. Dis. **8:**508–513.

55. Wani, S. A., M. A. Bhat, I. Samanta, Y. Nishikawa, and A. S. Buchh. 2003. Isolation and characterization of Shiga toxin-producing *Escherichia coli* (STEC) and enteropathogenic *Escherichia coli* (EPEC) from calves and lambs with diarrhoea in India. Lett. Appl. Microbiol. **37:**121–126.

56. Wolf, M. K., G. P. Andrews, B. D. Tall, M. M. McConnell, M. M. Levine, and E. C. Boedeker. 1989. Characterization of CS4 and CS6 antigenic components of PCF8775, a putative colonization factor complex from enterotoxigenic *Escherichia coli* E8775. Infect. Immun. **57:**164–173.