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Molecular Cloning and Characterization of Dr-II, a Nonfimbrial Adhesin-I-Like Adhesin Isolated from Gestational Pyelonephritis-Associated *Escherichia coli* That Binds to Decay-Accelerating Factor

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Bacterial adhesins play an important role in the colonization of the human urogenital tract. *Escherichia coli* Dr family adhesins have been found to be frequently expressed in strains associated with pyelonephritis in pregnant females. The tissue receptor for known Dr adhesins has been localized to the short consensus repeat-3 (SCR-3) domain of decay accelerating factor (DAF), a complement regulatory protein. In this report, we identified and cloned *draE2*, a gene encoding a novel 17-kDa DAF-binding adhesin, Dr-II, from a strain of *E. coli* associated with acute gestational pyelonephritis. Despite the significant sequence diversity between Dr-II and Dr family adhesins, the receptor of Dr-II was found to be the SCR-3 domain of DAF. Sequence analysis of the 186-amino-acid Dr-II open reading frame revealed significant diversity from other members of the Dr adhesin family, including Dr, AFA-I, AFA-III, and F1845, but only an 8-amino-acid difference in sequence from that of the 17-kDa nonfimbrial adhesin NFA-I of unknown receptor specificity. N-terminal peptide sequencing of the purified adhesin confirmed the identity of the open reading frame and indicated cleavage of a 28-amino-acid signal peptide. Antibodies raised against purified Dr-II adhesin exhibited little or no cross-reactivity to Dr adhesin. Characterization of the biological properties demonstrated that like the Dr adhesins, Dr-II was associated with the ability of *E. coli* to bind to tubular basement membranes and Bowman's capsule and to be internalized into HeLa cells.

Urinary tract infections are among the most common bacterial infections in humans. *Escherichia coli*, the dominant etiologic pathogen in urinary tract infections, accounts for more than 80% of all cases. Factors contributing to the virulence of uropathogenic *E. coli* include hemolysins (4), O serotypes (16), and adhesins (4, 11). Adhesins are the most important among these factors in initiating the infection, allowing the bacteria to attach and colonize the uroepithelium and ascend the urinary tract. The Dr family adhesins, which recognize the Dr blood group antigen, have emerged in the last few years to become an important class of adhesins among pyelonephritis-causing *E. coli*, with isolation from at least 30% of pregnant subjects (41) compared to the 6 to 12% frequency reported for pyelonephritis isolates from a nonpregnant population and for control fecal strains (2, 3). The increase may be due partly to the progesterone-mediated upregulation of *E. coli* Dr binding sites (24).

Dr adhesins were originally identified by their agglutination of human erythrocytes expressing the Dr blood group antigen and the inability to hemagglutinate Dr(a−) erythrocytes (38), a rare phenotype of the Cromer blood group system in which the Dr antigen is not expressed (30). The Dr blood group receptor for these adhesins has been mapped to the short consensus repeat-3 (SCR-3) domain of decay accelerating factor (DAF), a cell membrane protein that regulates the complement cascade and protects the cell from lysis by autologous complement by destabilizing the formation of C3 convertases (29). The inability of Dr-bearing *E. coli* to agglutinate Dr(a−) erythrocytes may be due to a point mutation, Ser-165 to Leu, contained in the SCR-3 domain of DAF (30). Receptors for Dr adhesins have been shown to be present in several tissues, including renal tissue (Bowman's capsule and basement membranes) and the uroepithelium of the urinary tract, where they are proposed to facilitate the ascending colonization and chronic interstitial infection of the urinary tract (14, 39).

Dr family adhesins characterized to date include the uropathogenic-associated AFA-I, AFA-III, and Dr as well as the chronic diarrhea-associated F1845 adhesin (7, 26, 36). The genetic organizations of the operons encoding these four adhesins are very similar, since each is composed of a 6- to 8-kb cluster of genes coding for five polypeptides, the furthest downstream of which encodes the 14- to 16-kDa adhesin subunit. Accessory genes necessary for the adhesin biogenesis were found to be highly conserved among the Dr-related operons (45).

Human factors also contribute to the virulence of uropathogenic *E. coli*, since certain population groups such as women, children, and the elderly are more susceptible to urinary tract infections (20, 21, 43). In women, the frequency of acute pyelonephritis has been observed to be elevated during pregnancy (44). This increase has been attributed largely to host factors such as anatomical obstruction of the ureters by the enlarging uterus and the high incidence of vesicoureteral reflux (31), while studies on the bacterial contribution have been lacking. Recently, we examined the frequency of *dra* and *dra*-related operons in *E. coli* isolates associated with gestational pyelonephritis and discovered a diverse population in terms of DAF-binding specificity (41), which may reflect the importance of

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TABLE 1. Bacterial strains used in this study

Strain	Recombinant plasmid[a]	Vector	MRHA[e]	Reference
EC7372[b]	NA[c]	NA	+	This study
TP404	pTP404	pBluescript II SK−	+	This study
TP405	pTP405	pBluescript II SK−	−	This study
TP407	pTP407	pBluescript II SK−	−	This study
TP408	pTP408	pBluescript II SK−	−	This study
TP409	pTP409	pBluescript II SK−	−	This study
TP410	pTP410	pBluescript II SK−	−	This study
TP411	pTP411	pBluescript II SK−	+	This study
TP411A	pTP411A	pGEM-7Zf(+)	+	This study
TP414	pTP414	pGEM-7Zf(+)	−	This study
BN406	pBJN406	pACYC184	−	34
BNT406	pBNT406[d]	pUC19	+	This study
TP420	pTP420	pUC19	+	This study

[a] All recombinant plasmids were maintained in *E. coli* host strain DH5, except pBJN406, which was maintained in HB101.
[b] A clinical gestational pyelonephritis-associated *E. coli* isolate (O25:H?:K?).
[c] NA, not applicable.
[d] Ten-kilobase *Hin*dIII-*Eco*RI fragment from pBJN406 cloned into pUC19.
[e] MRHA, mannose-resistant hemagglutination.

DAF as a ligand for bacterial attachment in the pathogenesis of urinary tract infection during pregnancy. The biological significance of DAF as a bacterial adhesin receptor in pregnant women is further illustrated in this paper since we characterize a novel DAF-binding adhesin from a gestational pyelonephritis-associated strain that displays limited sequence homology to other known DAF-binding adhesins.

MATERIALS AND METHODS

Bacterial strains. *E. coli* clinical isolates were derived from patients at the Clinic of Obstetrics and Gynecology at the University of Texas Medical Branch (41). All strains were stored in 20% glycerol–L broth at −70°C. Bacteria were plated on L agar overnight before being used for all assays. Host strain *E. coli* LE392 (Promega, Madison, Wis.) was used for lambda phage infection. Recombinant cloning vectors pBluescript II SK− (Stratagene, La Jolla, Calif.) and pGEM-7Zf(+) (Promega) were used to construct all the recombinant plasmids (Table 1 and Fig. 1). The recombinant plasmids in this study were maintained in host strain DH5α (Gibco-BRL, Gaithersburg, Md.) grown in Luria broth or on L-agar plates supplemented with ampicillin (100 μg/ml).

Hemagglutination assay. Human erythrocytes in 50% Alsever's solution were obtained from the M. D. Anderson Cancer Center, Science Park, Tex. Dr(a−) erythrocytes were provided by John Moulds (Gamma Biologicals, Houston, Tex.). Bacteria and erythrocytes were prepared for hemagglutination as described previously (35, 41).

Genomic library construction and cloning of Dr-II adhesin. Standard molecular biology methods were used (5). Chromosomal DNA was isolated from gestational pyelonephritis-associated *E. coli* 7732 (O25:K?:H?) and partially digested with *Sau*3A to yield fragments of 10 to 25 kb. The cut DNA then was ligated to *Bam*HI arms of Lambda Gem-11 and packaged with phage packaging extract (Promega). *E. coli* LE392 was used as the host for infection on agar plates. Approximately 5,000 plaques were screened for clones hybridizing with the 1.1-kb *Pst*I *dra*B-containing fragment. The fragment was isolated from pBN406 plasmid and gel purified prior to labelling. The probe (200 ng) was labelled with fluorescein with the ECL random-prime labelling and detection system (Amersham, Arlington Heights, Ill.). Labelling and detection were performed as described in the manufacturer's instructions. Positive plaques identified by hybridization were further used for reinfection for at least two more rounds to establish that they were from the same clones. Four different phage clones with inserts of 11 to 20 kb were identified. Phage DNA was isolated for Southern blot analysis and further subcloning with a Lambda DNA Extraction kit (Qiagen, Chatsworth, Calif.). Various phage inserts or insert fragments were then subcloned into the plasmid vector pBluescript II SK− (Stratagene) or pGEM-7Zf(+) (Promega). The plasmid constructs were used to transform competent *E. coli* DH5α and screen for hemagglutination of Dr(a+) erythrocytes. The first hemagglutinating clone obtained, TP404, was used subsequently to test for binding specificity on erythrocytes, DAF cDNA-transfected Chinese hamster ovary (CHO) cells, and human kidney cryosections and for the ability to be internalized by HeLa cells. Plasmid DNA from this strain was isolated for DNA sequencing and deletional analysis. The dideoxy-chain termination DNA sequencing method was performed with Sequenase T7 DNA polymerase (U.S. Biochemicals, Cleveland, Ohio). For regions not within sequencing distance of the universal primers, oligonucleotide primers were designed (Bio Synthesis, Lewisville, Tex.) from known adjacent sequences. The sequence data were analyzed by PCgene DNA analysis software (IntelliGenetics, Mountain View, Calif.). The nucleotide sequence of the gene encoding Dr-II structural adhesin, *draE2*, presented in this paper has been submitted to GenBank (accession number U95163).

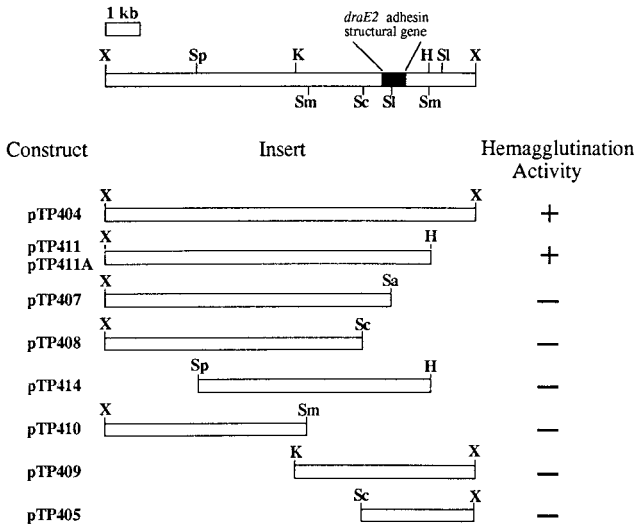

FIG. 1. Restriction map of the operon of the gene encoding DR-II and analysis of deletion mutants. The 11-kb *Xho*I fragment of construct pTP411 was isolated from a phage clone. Restriction site abbreviations: X, *Xho*I; Sp, *Sph*I; K, *Kpn*I; Sm, *Sma*I; Sc, *Sac*I; Sl, *Sal*I; H, *Hin*dIII. Restriction sites were not found for *Eco*RI, *Bam*HI, *Cla*I, and *Xba*I. All constructs were subcloned into pBluescript II SK−, with the exception of pTP411A and pTP414, which were subcloned into pGEM-7Zf(+). *E. coli* DH5α was used as the host strain for all constructs. The recombinant strains were tested for hemagglutination of Dr(a+) erythrocytes.

Purification of Dr-II adhesin protein. Recombinant strain TP404 bearing Dr-II adhesin was grown overnight on L-agar plates containing ampicillin (100 μg/ml). The bacteria were harvested by scraping and then suspended in phosphate-buffered saline (PBS). The suspension was vortexed for 5 min prior to centrifugation for 10 min at 10,000 × *g* in SS-34 centrifuge tubes. The supernatants were filtered through a 0.22-μm-pore-size membrane. Adhesin protein was purified from the filtrate by ammonium sulfate precipitation and size exclusion chromatography (13). The column was connected to the Econo low-pressure liquid chromatography system (Bio-Rad, Hercules, Calif.). The eluted adhesin was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 15% acrylamide).

N-terminal protein microsequencing. Purified adhesin protein (50 μg) was electrophoresed on an SDS–15% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad) prior to staining with 0.1% Coomassie blue R-250 (Sigma, St. Louis, Mo.). Following destaining in 50% methanol, the stained 17-kDa band on the membrane was excised. The primary structure of the N-terminal region of the Dr-II adhesin protein was determined by microsequencing analysis with an Applied Biosystems 475A protein-peptide sequencer in the Protein Chemistry Laboratory at the University of Texas Medical Branch.

***dra*/*dra2* operon fusion.** The 6-kb *Kpn*I-*Hin*dIII *draE*-containing fragment of plasmid pBNT406, which contained the 10-kb *Hin*dIII-*Eco*RI *dra* insert-containing fragment from pBJN406 cloned into the pUC19 plasmid vector, was replaced with the 4-kb *Kpn*I-*Hin*dIII *draE2*-containing fragment. The latter fragment was isolated from the plasmid construct pTP411. Both fragments were purified from agarose gels prior to ligation. The resulting plasmid construct, pTP420, was used to transform *E. coli* DH5α and to test for hemagglutination specificity.

Production of anti-Dr-II antibodies. New Zealand White rabbits (Charles River, Wilmington, Mass.) were immunized subcutaneously with 100 μg of either purified Dr or Dr-II adhesin. The immunogens were prepared by slicing the Coomassie blue-stained bands from SDS–15% polyacrylamide gels containing purified Dr-II or Dr protein. This procedure was used to minimize bacterial contaminants in the preparation. The gel fragments were homogenized in incomplete Freund's adjuvant for immunization. Injections were given every week for the first 3 weeks and every 2 weeks thereafter. One week after the sixth immunization, sera were collected by ear vein bleeding and tested for anti-Dr and anti-Dr-II immunoreactivity by Western blotting (immunoblotting) and an enzyme-linked immunoadsorbent assay (ELISA). All animals were bled via cardiac puncture and sacrificed 3 days later, and approximately 100 ml of serum was

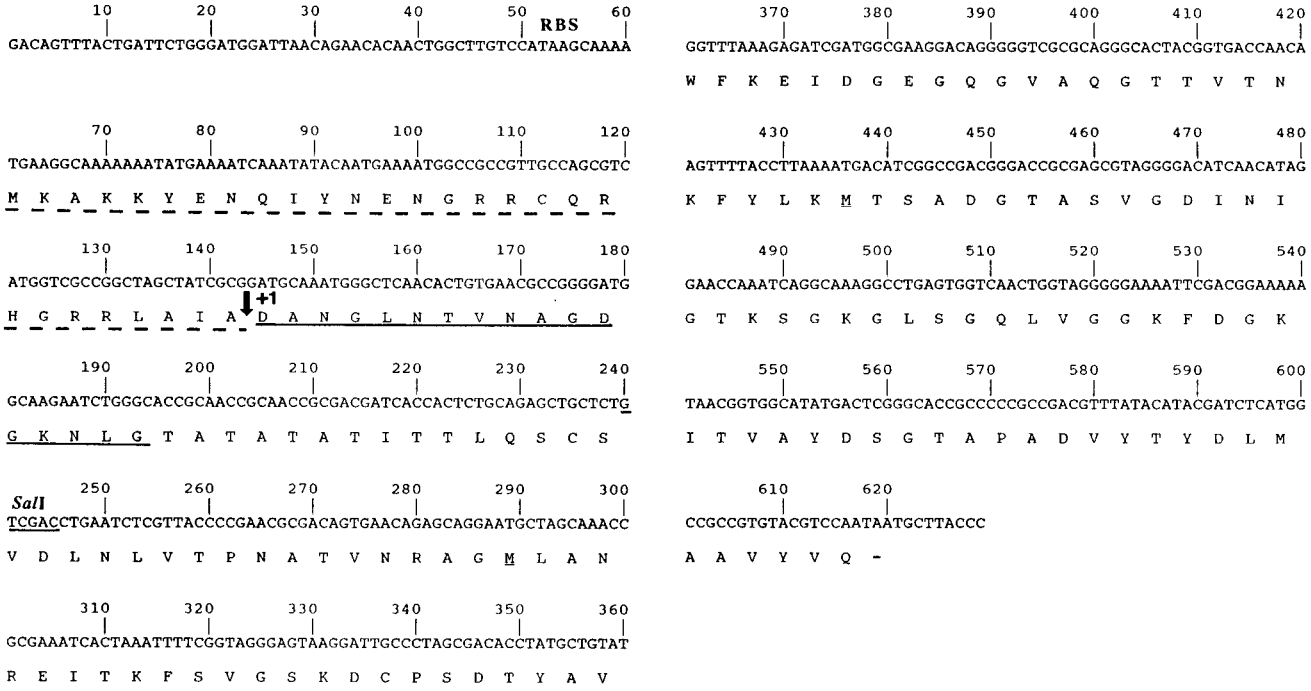

FIG. 2. Open reading frame of the Dr-II adhesin encoded by the gene *draE2*. The 17 amino acid residues determined by N-terminal microsequencing of the purified 17-kDa adhesin are underlined. The deduced 28-amino-acid signal peptide is indicated by a dashed line. An arrow indicates the signal peptide cleavage site, and +1 indicates the first amino acid of the mature protein. A weak ribosome binding site (RBS) just before the start codon is also indicated.

collected from each. Sera were analyzed by Western blot analysis and ELISA against purified Dr-II or Dr adhesin.

Immunoassays. Recombinant *E. coli* strains bearing Dr-II (TP404) or Dr (BNT406) were resuspended in water to an optical density (OD) at 600 nm of 0.5 and then boiled for 5 min. The supernatants were separated by SDS–15% PAGE and transferred electrophoretically to a nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.) for immunoblotting. For the ELISA, 96-well polystyrene ELISA plates (Corning Glass Works, Corning, N.Y.) were coated with purified Dr-II and Dr adhesin proteins at a concentration of 1 μg/ml overnight at 4°C. After blocking with PBS containing 3% bovine serum albumin, primary and secondary antibodies (anti-rabbit IgG-alkaline phosphatase) were added for successive 1-h incubations at room temperature. Washes with PBS containing 0.1% Tween 20 were done (three times) after each incubation. The wells and nitrocellulose strips were developed with alkaline phosphatase substrate kits (Bio-Rad). Plates were read with a microplate reader (model 150; Bio-Rad) at 405 nm.

Immunogold staining. Fresh bacteria grown overnight on an agar plate were suspended in PBS and transferred to nickel support grids (Electron Microscopy Sciences, Fort Washington, Pa.). The grids were incubated in a 1:10 dilution of anti-Dr-II antibody for 30 min in a moist chamber at room temperature. After four PBS washes, they were incubated in a 1:10 dilution of gold-labeled (particles with 5-nm diameter) goat anti-rabbit immunoglobulin G (IgG; Amersham) at room temperature for 30 min. The grids were fixed in 2% glutaraldehyde for 10 min and washed three times in deionized water for 1 min. After negative staining in 2% phosphotungstic acid (pH 6.8) for 1 min and air drying, the grids were examined and photographed on a Philips Electron Optics (Eindhoven, The Netherlands) 201 electron microscope.

CHO cell binding assay. CHO cells stably transfected with cDNA for human DAF or DAF deletion mutants (9) were cultured in Ham's F10 medium with 10% fetal calf serum and grown to 75% confluency in eight-chamber slides (Nunc, Inc., Naperville, Ill.). The binding assay was performed as described previously (35).

Dr-II adhesin protein binding to kidney cryosections. Purified bacterial Dr-II adhesin was used to detect binding to kidney substructures on human kidney cryosections prepared from autopsy specimens (provided by S. Kumar, Department of Pathology, University of Texas Medical Branch) as described previously (39). Briefly, purified Dr-II adhesin (250 μg/ml) was added to the slides, and the binding was detected by use of a rabbit anti-Dr-II antibody (from this study) and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Sigma; both at 1:100). The slides were mounted, examined under a fluorescence microscope (Leica), and photographed with Ektachrome 160 tungsten film (Kodak, Rochester, N.Y.) at a magnification of ×400.

Bacterial internalization assay (invasion assay). HeLa cells (10^5) were seeded in each well of 24-well plates (Corning Glass Works) and grown overnight in minimal essential medium (MEM; Sigma) supplemented with 5% fetal bovine serum (Summit Biotechnology, Fort Collins, Colo.). The well were washed three times the next day and replaced with 500 μl of MEM. Bacteria from each group were added in triplicate (100 μl of a suspension with an OD at 600 nm of 0.5), and the plates were incubated at 37°C in a 5% CO_2 incubator for 3 h. The wells were then washed and incubated with 500 μl of MEM containing 200 μg of gentamicin per ml. After a 1-h incubation, the wells were washed five times with PBS. Lysis buffer (20 mM Tris-HCl [pH 7.5], 2 mM EDTA, 2% Triton X-100) was added to each well, and the wells were allowed to incubate at room temperature for 5 min. A small volume (50 μl) was removed from each well and diluted prior to being plated on Luria broth agar. Colonies were counted 24 h later to determine the number of viable intracellular bacteria. The results were expressed as the number of bacterial CFU per 10^6 HeLa cells.

RESULTS

Cloning of the *E. coli* Dr-II adhesin-encoding gene (*draE2*). Initial Southern blot analysis of both plasmid and genomic DNA of gestational pyelonephritis-associated *E. coli* clinical strain EC7372 showed the hybridization of the *dra*B probe with only genomic DNA (data not shown), indicating that the operon for the Dr-related adhesin is located on chromosomal DNA. Genomic DNA isolated from the strain was partially digested with *Sau*3A and ligated to the arms of lambda phage LamdaGem-11 to construct a genomic DNA library and clone the *dra*-related operon. Screening of the library with the 1.1-kb *dra*B probe resulted in four phage clones with inserts ranging from 10 to 20 kb of DNA. Phage inserts or insert fragments were subcloned into the plasmid pBluescript II SK− and tested for hemagglutination. The first hemagglutinating recombinant clone isolated, TP404, contained a 11-kb *Xho*I fragment from phage clone 2.

Restriction analysis and analysis of deletion mutants. The mappable restriction sites of the 11-kb *Xho*I fragment are shown in Fig. 1. *Eco*RI, *Bam*HI, *Cla*I, and *Xba*I were found to have no restriction sites within the fragment. Deletion mutants were constructed from pTP404, which contained the 11-kb

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Dr-II   MKAKKYENQIYNENGRRCQRHGRRLAIADANGLNTVNAGDGKNLGTATAT -50
        ||||||||||||||||||||||||||||||||||||||||||||||||||
NFA-I   MKAKKYENQIYNENGRRCQRHGRRLAIADANGLNTVNAGDGKNLGTATA- -49

Dr-II   ATITTLQSCSVDLNLVTPNATVNRAGMLANREITKFSVGSKDCPSDTYAV -100
          ||||||||||||||||||||||||||||||||| ||||||||||||||
NFA-I   -IITTLQSCSVDLNLVTPNATVNRAGMLANREITLFSVGSKDCPSDTYAV -98

Dr-II   WFKEIDGEGQGVAQGTTVTNKFYLKMTSADGTASVGDINIGTKSGKGLSG -150
        || ||||||||||||||||||||||||||||||||||||||||||||||||
NFA-I   WFLEIDGEGQGVAQGTTVTNKFYLKMTSADGTASVGDINIGTKSGKGLSG -148

Dr-II   QLVGGKFDGKITVAYDSGTAPADVYTYDLMAAVYVQ -186
        ||||||||||||||| |||||||| ||||||||
NFA-I   QLVGGKFDGKITVAYDSATAPADVYTTDLMAAVYVN -184
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FIG. 3. Amino acid sequence similarity between the open reading frame for Dr-II and the nonfimbrial adhesin NFA-I (1) of uropathogenic *E. coli*. Identical aligned residues are indicated by a vertical line. The receptor for NFA-I has not been identified. The 96% identity suggests that NFA-I may also bind to DAF.

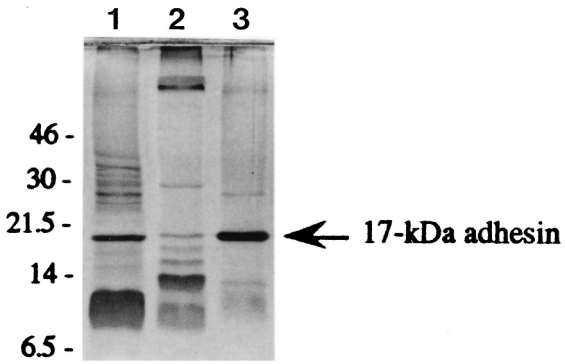

FIG. 5. Purification of Dr-II adhesin subunit from bacterial outer membrane. Samples were analyzed by SDS–15% PAGE and silver stained. Molecular weights of markers (in thousands) are shown on the left. Lanes: 1 and 2, crude heat-shocked preparations of the *E. coli* recombinant strain (TP404) and wild-type strain (EC7372), respectively; 3, gel chromatography-purified preparation. An arrow indicates the 17-kDa adhesin protein. N-terminal protein sequencing of the purified adhesin confirmed the open reading frame deduced from the cloned DNA sequence.

*Xho*I insert within the vector pBluescript II SK−, to localize the cluster of genes necessary for the biogenesis of the adhesin. The smallest constructs with positive hemagglutinin expression contained a 1.2-kb *Hin*dIII-*Xho*I fragment deletion (pTP411 and pTP411A). Recombinant strains with larger deletions up to the *Sal*I and *Sma*I sites (pTP407 and pTP410, respectively) did not hemagglutinate. The construct containing the smallest deletion from the 5′ end, the 2.5-kb *Xho*I-*Sph*I fragment, also failed to express hemagglutination activity (pTP414).

Identification of the 186-amino-acid Dr-II open reading frame. Since deletions of sequence up to the *Sal*I and *Sph*I sites in constructs pTP407 and pTP414, respectively, resulted in the loss of hemagglutination activity, it was possible that either of these truncations may have occurred within the adhesin structural gene, which lies furthest downstream among the genes in the *dra* or *dra*-related operons. Initial DNA sequencing extending into the *Sal*I site of construct pTP407 by use of a reverse universal primer yielded sequences with a high degree of homology to *nfaE*, which encodes the nonfimbrial adhesin NFA-I (1). DNA sequencing in both directions from the *Sal*I site to obtain the complete coding sequence was performed with a pTP404 plasmid template. A 186-amino-acid open reading frame was found for the Dr-II adhesin encoded by *draE2* (Fig. 2). A putative weak ribosome recognition Shine-Dalgarno sequence (UAAG) was identified just upstream from the initiator (AUG) codon. The location of the *Sal*I restriction site is also indicated. By amino acid sequence alignment analysis, the open reading frame was found to have 96% identity to that of the NFA-I adhesin (Fig. 3), for which the receptor specificity has not yet been determined (1). Similar to the Dr adhesin, the NFA-I-like adhesin was found to recognize the Dr blood group antigen and was termed Dr-II.

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Dr      MK----------KLAIMAAASMVFAVSSAHAGFTPSGTT   29
AFA-III MK----------KLAIMAAASMVFAVSSAHAGFTPSGTT   29
F1845   MK----------KLAIMAAASMIFTVGSAQATFQASGTT   29
AFA-I   MK----------KLAIIGATSVMMMTGTAQANFTSSGTN   29
Dr-II   MKAKKYENQIYNENGRRCQRHGRRLAIADANGL--NTVNAGDGKNLGTAT   48
        **                  .*** .*...   . .* ..   ....

Dr      GTTKLTVTEECQVRVGDLT--VAKTRGQLTDAAPIGPVTVQALGCD-ARQ   76
AFA-III GTTKLTVTEECQVRVGDLT--VAKTRGQLTDAAPIGPVTVQALGCN-ARQ   76
F1845   GITTLTVTEECRVQVGNVT--ATLARSKLKDDTAIGVIGVTALGCN-GLQ   76
AFA-I   GKVDLTITEECRVTVESKS--ESFLRSGLVANRHITNLGIQSTGCGTGQR   77
Dr-II   ATATITTLQSCSVDLNLVTPNATVNRAGMLANREITKFSVGSKDCPSDTY   98
        . ...*. ..* * ..  .   .  *. . ..  *.  .. . .* .

Dr      VALKADTDNFEQGK-FFLISDNNRDKLYVNIRPTDNSAWTTDNGVFYKND  125
AFA-III VALKADTDNFEQGK-FFLISDNNRDKLYVNIRPMDNSAWTTDNGVFYKND  125
F1845   AALQADPDNYDATN-LYMTS-RNHDKLNVKLKATDGSSWTYGNGVFYKTE  124
AFA-I   VALKLGAGSYDDTNGAHMTHENGTDKLLVSMGSATGDG-TQDGGVYY---  123
Dr-II   AVWFKEIDGEGQGVA---QGTTVTNKFYLKMTSADGTASVGDINIGTKSG  145
        ..   . .. ...           .*. ... . .... . . ..

Dr      VGSWGGIIGIYVDGQQT------NTPPGNYTLTLTGGY---WAK   160
AFA-III VGSWGGTIGIYVDGQQT------NTPPGNYTLTLTGGY---WAK   160
F1845   GGNWGGHVGISVDGNQT------DKPTGEYTLNLTGGY---WTN   159
AFA-I   ----------INRDGTGT------GRWCSSYEMT-NSTYQPASTP   151
Dr-II   KGLSGQLVGGKFDGKITVAYDSGTAPADVYTYDLMAA---VYVQ   186
                  **  *      .   . *. .  ..     .
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FIG. 4. Alignment of amino acid sequences of Dr, AFA-II, F1845, AFA-I and Dr-II by use of PCgene sequence analysis software. Conserved residues among the sequences are indicated by asterisks and similar residues are indicated by periods below the sequences. A higher degree of homology, especially among the first four adhesins, is observed in the N-terminal portion. Most similar to Dr is AFA-III, which differs by only three amino acids, followed by F1845, AFA-I, and Dr-II. Downward arrows indicate the positions of signal peptide cleavage.

Sequence alignment analysis. The results of protein sequence alignment analysis of Dr-II and the Dr family adhesins Dr, AFA-III, F1845, and AFA-I are shown in Fig. 4; identical amino acid residues among the sequences in the alignment performed by PCgene (IntelliGenetics) multiple sequence alignment analysis are indicated. Only 18 amino acid residues were found to be identical between Dr-II and other members of the Dr adhesin family; most notable among these are the two cysteine residues that are 32 to 34 amino acids apart. Also similar between the sequences of the Dr family adhesins and that of Dr-II are the two tyrosine residues at the C terminus. When the sequence of Dr-II was aligned individually with the sequences of other Dr family adhesins, i.e., Dr, AFA-III, F1845, and AFA-I, 17 to 20% amino acid identity was demonstrated (31 to 38 identical amino acids out of 186). This was much lower than the 98% sequence identity observed between Dr and AFA-III (157 identical amino acids out of 160), the 57% identity between Dr and F1845 (91 out of 160 amino acids), and the 32% identity between Dr and AFA-I (51 out of 160 amino acids).

N-terminal amino acid sequencing. The Edman degradation method for N-terminal amino acid sequencing was performed to confirm the open reading frame shown in Fig. 2. Dr-II adhesin was purified from recombinant strain TP411 before electrophoresis and transfer onto a polyvinylidene difluoride membrane for sequencing. Purified Dr-II adhesin was observed to migrate on an SDS-15% PAGE gel as a single dominant band close to 20 kDa (Fig. 5). The purified protein was also observed to have the property of hemagglutination at 50 μg/ml. The 17 amino acid residues identified from the N terminus are indicated in Fig. 2. Identification of the residues on the N terminus of the mature adhesin also allowed for the 28-amino-acid signal peptide cleavage point to be located (Fig. 2). Although migration on an SDS-PAGE gel indicates its molecular size to be close to 20 kDa, the deduced molecular

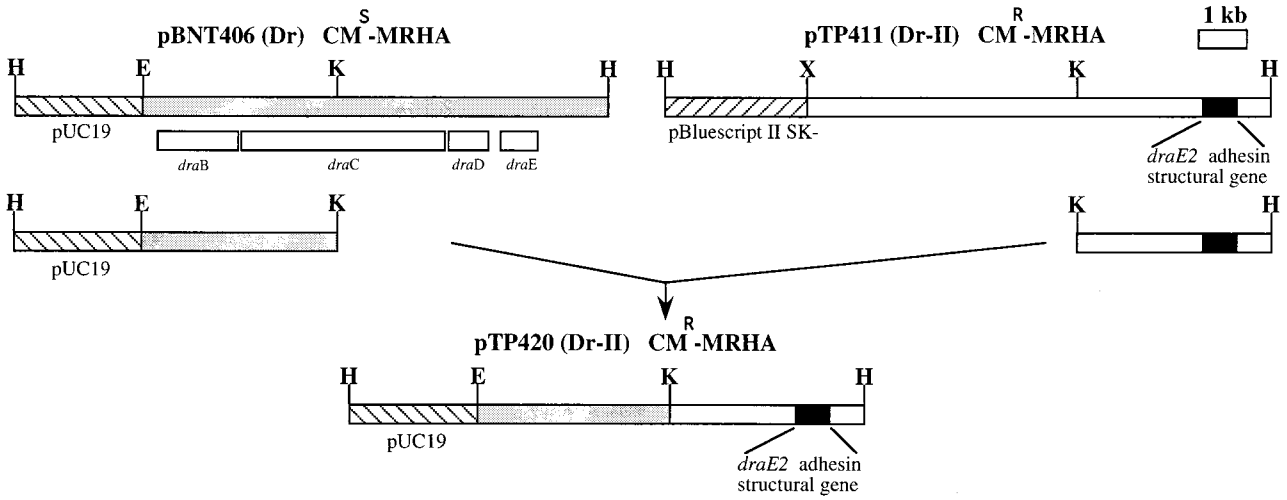

FIG. 6. Fusion of the *dra* and *dra2* operons. The unique *Kpn*I site in the middle of both *dra* and *dra2* operons provided a convenient position for ligating the fragments. Restriction site abbreviations: H, *Hin*dIII; E, *Eco*RI; K, *Kpn*I; X, *Xho*I. The resulting construct, pTP420, yielded fully functional Dr-II expression, shown by chloramphenicol-resistant, mannose-resistant hemagglutination (MRHA).

size of the 158-amino-acid mature Dr-II adhesin is approximately 17 kDa.

***dra*/*dra2* hybrid operon.** Although diversity exists among the adhesin structural genes of Dr family adhesins, there is evidence to suggest that 5- to 7-kb gene clusters upstream which constitute the accessory genes necessary for the assembly and export of the adhesin may be very similar (27, 28, 45). To demonstrate that the accessory gene products of the *dra* operon can support the biogenesis of Dr-II adhesin, we fused the 5′ half of the *dra* operon to the 3′ *draE2*-containing half of the *dra2* operon and tested for hemagglutination activity (Fig. 6). A unique *Kpn*I restriction site in the middle of both the *dra* and *dra2* operons provided a convenient site for joining the operon halves. The recombinant strain transformed with the fusion construct pTP420 not only was found to express functional adhesin but also exhibited mannose-resistant and chloramphenicol-resistant hemagglutination similar to that of the Dr-II-bearing recombinant strain TP404.

Open reading frames from internal DNA sequences. DNA sequencing of the inserts at the *Sph*I site of plasmid template pTP414 and *Kpn*I site of pTP409 resulted in sequences with open reading frames very similar to the internal amino acid sequences of AfaB and AfaC, respectively (12). The 78-amino-acid open reading frame from the short sequence adjacent to the *Sph*I site was found to be 99% (77 of 78 residues) identical to residues 12 to 89 of AfaB by alignment analysis. The 67-amino-acid open reading frame from the short sequence adjacent to the *Kpn*I site was found to be 84% (56 of 67 residues) identical to residues 375 to 441 of AfaC by similar alignment analysis.

Immune cross-reactivity. The purified adhesin allowed us to immunize rabbits and raise specific anti-Dr-II antibody. Antibodies were raised against both Dr and Dr-II adhesins by subcutaneous immunization of New Zealand White rabbits. Immune cross-reactivity between the two adhesins, as well as specific activity to each, was assayed. While all immune sera showed at least a 1:10,000 titer to either Dr or Dr-II purified protein by ELISA (data not shown), only one animal immunized with Dr-II exhibited slight cross-reactivity with Dr as demonstrated by Western blot analysis (Fig. 7).

Immunogold electron microscopy. By use of anti-Dr-II serum, the structure of Dr-II was observed by immunogold staining under transmission electron microscopy at ×35,000 to ×75,000 magnification (Fig. 8). Gold particles were visualized to be closely associated with short globular surface structures on Dr-II-bearing *E. coli* clinical isolate EC7372 and recombinant strain TP404 (Fig. 8A and B, respectively), not typical of the long fiber structure described for the fimbriated adhesins such as type 1 or P (25). The high level of expression of Dr-II can be observed in recombinant strain TP404. An abundance of detached adhesin aggregates, which are present in the recombinant bacterial suspension (gel not shown), could also be

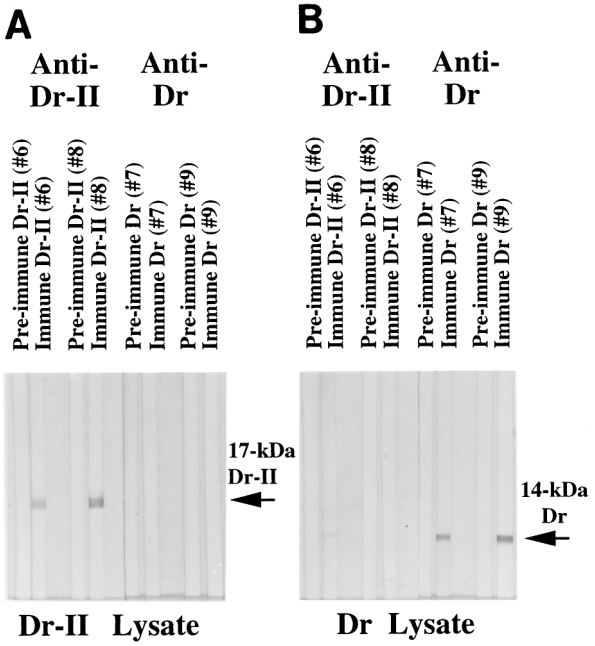

FIG. 7. Western blot analysis of anti-Dr and anti-Dr-II sera against Dr and Dr-II lysates. Nitrocellulose strips contained lysate preparation from Dr-II recombinant strain TP404 (A) and lysate preparation from Dr recombinant strain TP406 (B). The sera were preimmune or immune (1:500) as indicated above the strips. Immune sera had at least 1:10,000 titers against their immunogen measured by ELISA. Almost no cross-reactivity was observed between Dr and Dr-II antisera. The numbers #6 to #9 are rabbit identification numbers.

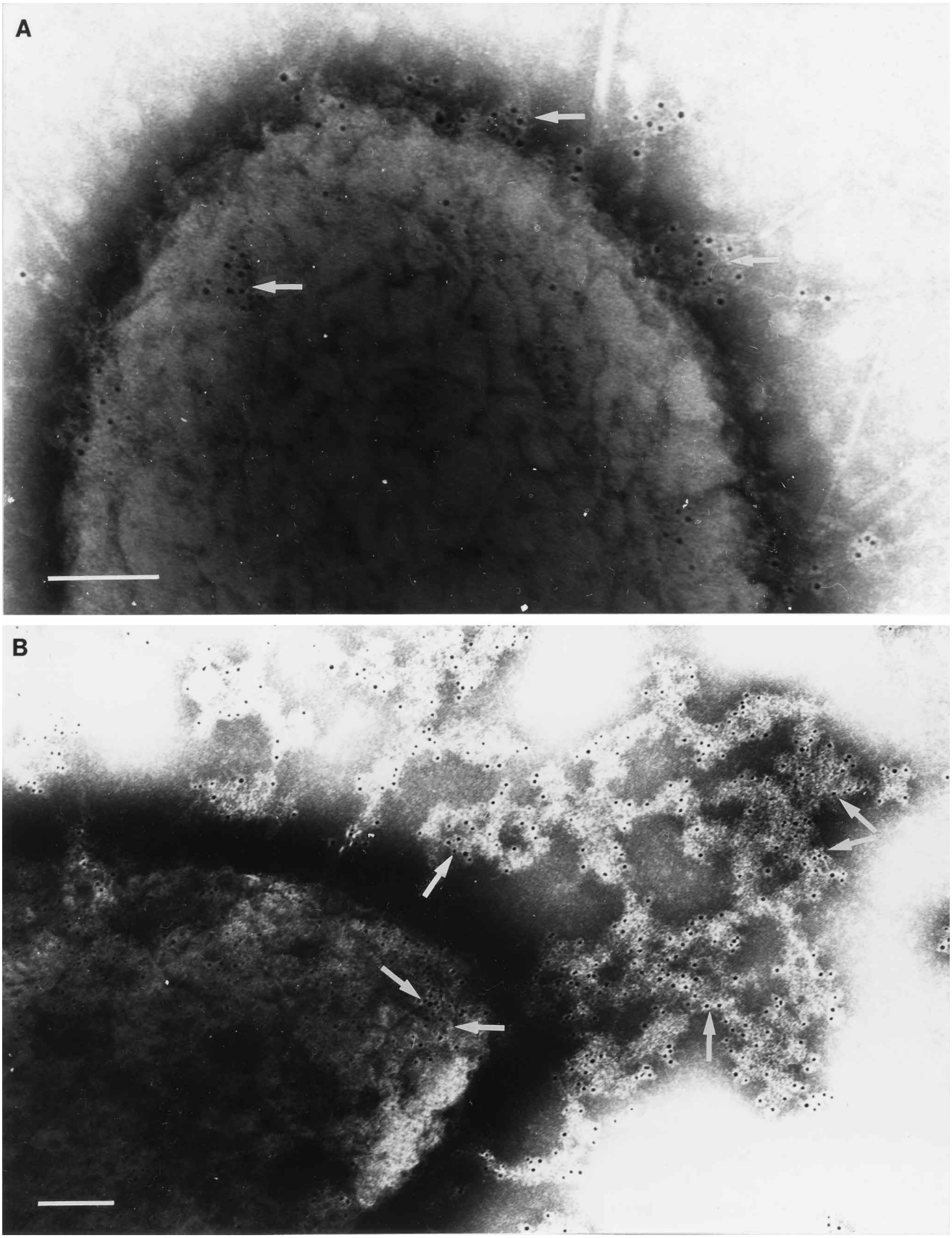

FIG. 8. Immunogold staining of *E. coli* clinical strain EC7372 (A) and recombinant strain TP404 (B) expressing Dr-II adhesin and the control DH5α(pBluescript II SK−) strain (C) observed under a transmission electron microscope at magnification of ×30,000 to ×75,000. Bars, 100 nm. Gold particles are observed to be associated with short globular surface structures, not typical of the long fiber structure associated with fimbriated adhesins, as well as detached aggregates (arrows). The high level of expression of the recombinant adhesin can be observed in the recombinant strain (B). Detached adhesin aggregates are visible surrounding the strain, consistent with the observation that the recombinant adhesin can be easily isolated by vortexing.

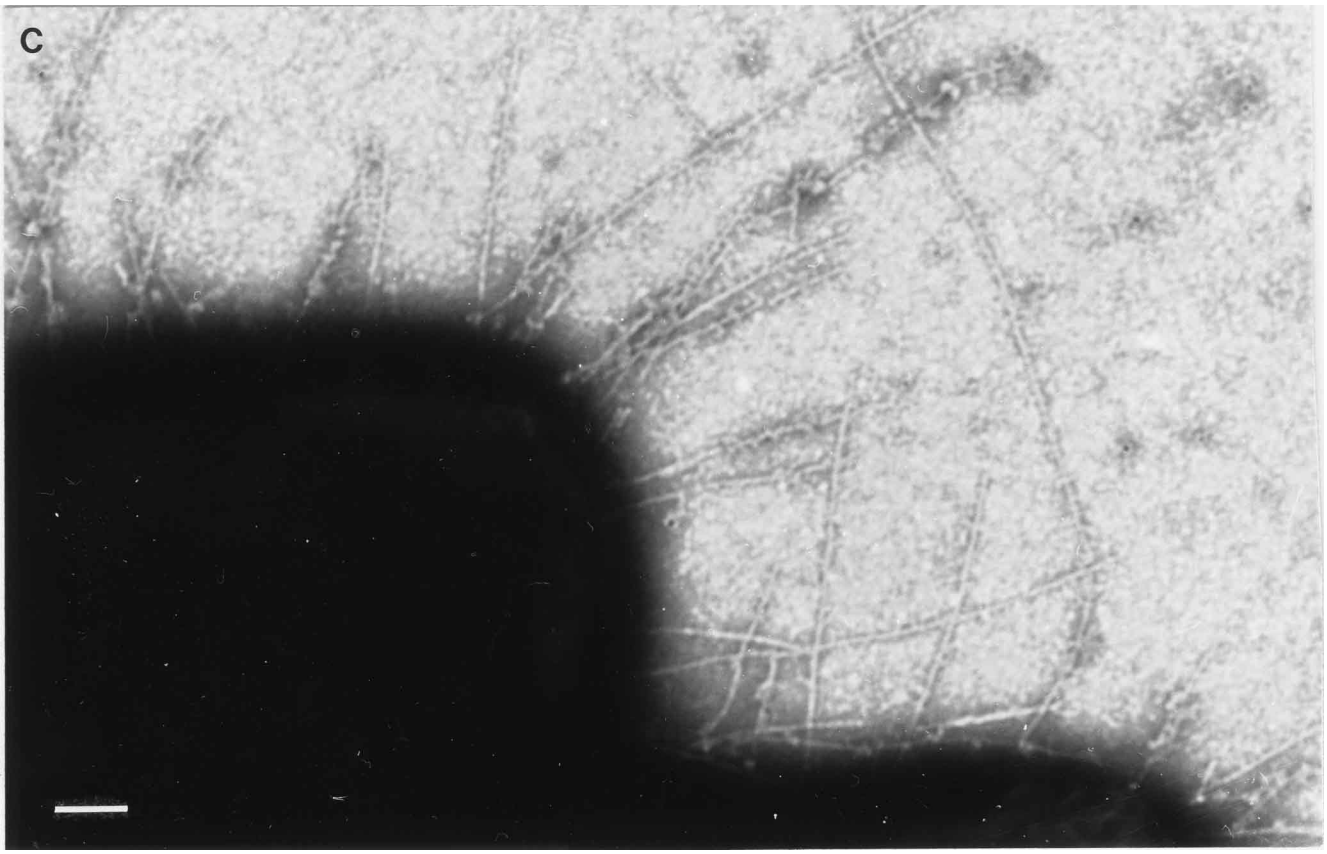

FIG. 8—*Continued.*

observed surrounding the strain. Virtually no gold staining was observed with the control strain DH5α(pBluescript II SK−) (Fig. 8C).

Dr-II binding specificity. A series of hemagglutination tests and CHO binding assays were performed to define the receptor specificity of Dr-II. The inhibition of hemagglutination by anti-SCR-3 monoclonal antibody and the inability to agglutinate Dr(a−) erythrocytes (Table 2) clearly indicated binding specificity for the SCR-3 domain of DAF. These results were consistent with the patterns observed for Dr, F1845, AFA-I, and AFA-III adhesins (35). This specificity was further verified by a binding assay using CHO cells transfected with different DAF cDNA constructs, in which the binding to cells carrying the SCR-3 deletion was significantly reduced compared to that to cells expressing the complete construct ($P < 0.001$, Student's *t* test); this was similar to the previously reported results for the Dr family adhesins (35).

Dr-II binding to human kidney cells. Biological functions and properties of Dr-II adhesin were investigated by examining the attachment to renal substructures and invasion of the HeLa cell line. To localize the binding sites of Dr-II to kidney substructures, human kidney cryosections were incubated with the purified Dr-II adhesin (200 μg/ml). The binding then was visualized by immunostaining with anti-Dr-II and FITC-conjugated secondary antibody. Binding was detected at the renal tubular basement membrane and Bowman's capsule in the glomerulus (Fig. 9). Interestingly, no binding was observed on the luminal side of the renal epithelium.

Internalization of Dr-II-bearing *E. coli* by the HeLa cell line. To further evaluate the biological functions of Dr-II-mediated

TABLE 2. Hemagglutination and binding properties of DR-II and Dr adhesins

Adhesin (strain)	Hemagglutination[a] (titer)				Binding to CHO cells[b]	
	Of Dr(a+) erythrocytes	Of Dr(a−)[c] erythrocytes	With anti-DAF SCR-3 MAb[d]	With chloramphenicol	DAF cDNA transfected	DAF cDNA ΔSCR-3 transfected
Dr-II (TP404)	Yes (1:16)	No	No	Yes (1:16)	47 ± 12[e]	7 ± 3[e]
Dr (BN406)	Yes (1:8)	No	No	No	34 ± 9[f]	6 ± 3[f]

[a] Hemagglutination titers of a bacterial suspension with an OD at 600 nm of 0.5.
[b] Binding values expressed as the number of bacteria per CHO cells.
[c] Dr(a−) erythrocytes contain a single point mutation in the SCR-3 domain of DAF (Ser-165 to Leu) and are not able to agglutinate Dr-bearing *E. coli.*
[d] MAb, monoclonal antibody.
[e] $P < 0.001$.
[f] $P < 0.005$.

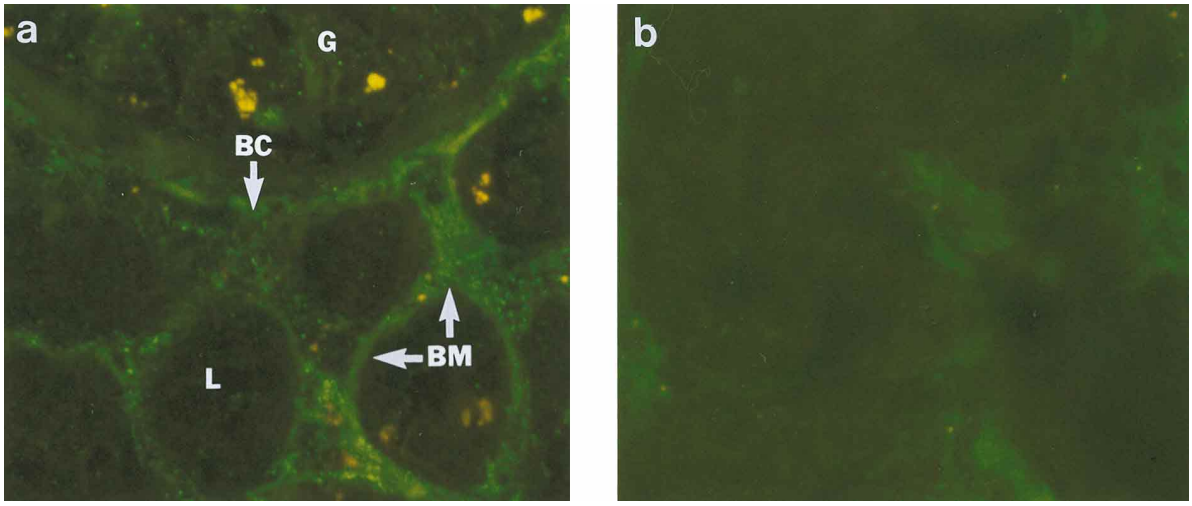

FIG. 9. (a) Binding of purified Dr-II adhesin to renal tubular basement membranes (BM), glomerulus (G), and Bowman's capsule (BC) observed by immunofluorescence microscopy (magnification, ×400). (b) In the control, PBS was used in place of purified Dr-II (200 μg/ml) for the initial incubation with human kidney cryosections. Kidney cryosections were initially incubated with purified Dr-II protein followed by anti-Dr-II and FITC-labelled anti-rabbit IgG antibodies.

virulence in pyelonephritogenic *E. coli*, we utilized the gentamicin protection assay with HeLa cells (15). *E. coli* strains bearing Dr-II adhesin, both recombinant and wild type, were recovered from detergent lysates of HeLa cells. After 1 h of exposure to gentamicin preceded by a 3-h incubation of bacteria and the HeLa cell line, approximately 2,500 to almost 5,000 CFU of Dr-II-bearing *E. coli*, both wild type and recombinant, were recovered per 10^6 HeLa cells. A recombinant strain transformed with truncated *draE2* (TP407) showed almost a complete loss ($P < 0.001$) of internalization (Fig. 10). The data shown in Fig. 10 are from one experiment performed in triplicate. The experiment was repeated twice and resulted in the same magnitude of differences. The invasion of HeLa cells, therefore, appears to depend on the expression of the functional *draE2* adhesin gene.

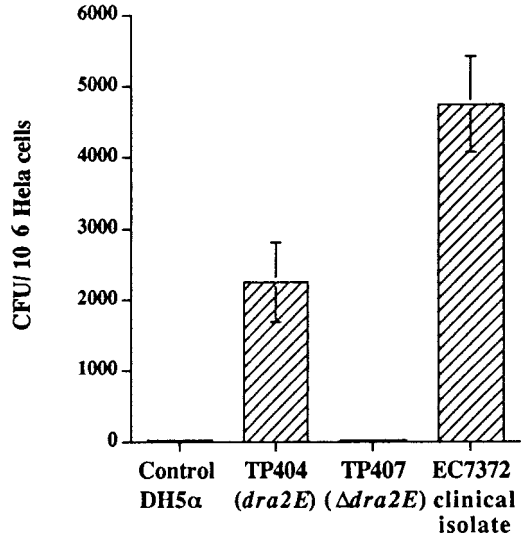

FIG. 10. Internalization of Dr-II-bearing *E. coli* by HeLa cells. *E. coli* DH5α transformed with various Dr-II plasmid constructs and pyelonephritis-associated *E. coli* EC7372 were tested in a gentamicin protection assay. DH5α was transformed with control vector (pBluescript II SK−), pTP404 (carrying the full *dra2* operon), or pTP407 (*draE2* truncation). Error bars represent standard deviations. Internalization is associated with Dr-II expression since only strains TP404 and EC7372 could invade, while control and TP407, which contained the truncated *draE2*, displayed no invasion.

DISCUSSION

While the described Dr family adhesins have similar genetic organizations and significant homology among the genes within their operons (28, 45), the Dr-II adhesin described here showed only 20% (37 of 186) amino acid identity to Dr adhesin by PCgene alignment analysis. Despite this limited homology, Dr-II possesses receptor specificity for the SCR-3 domain of DAF (CD55), renal basement membrane binding, and invasiveness similar to that of the Dr family hemagglutinins (15, 39).

Considerable amino acid sequence homology exists among Dr family adhesins characterized to date, including Dr, AFA-III, AFA-I of uropathogenic *E. coli*, and F1845 of diarrheagenic *E. coli*, and the genetic organizations of the operons are very similar (12, 28, 45). Dr family adhesins recognize the Dr blood group antigen (38), which has been mapped to the SCR-3 domain of DAF (35). Dr and AFA-III differ by only three amino acid residues; AFA-I and F1845 display less, but still extensive, homology with Dr (28). The cloning and characterization of the Dr-II adhesin illustrated a much greater structural diversity among the members of the DAF-binding family of adhesins than was previously known. While having only 20% amino acid identity to Dr, Dr-II was found to bind to the SCR-3 domain of DAF as shown by hemagglutination of Dr(a+) erythrocytes and binding to DAF cDNA-transfected CHO cell lines. The only distinction in binding specificity that could be made between Dr and Dr-II was in their sensitivity to chloramphenicol. Unlike Dr-II-bearing *E. coli*, the hemagglutination of Dr-bearing *E. coli* can be inhibited in the presence of chloramphenicol (25 μg/ml), the mechanism of which is not yet understood (36). Site-directed mutagenesis study of Dr adhesin suggested that the charged aspartic acid at residue position 52 in Dr, which immediately follows the second cysteine residue, is involved in the sensitivity to chloramphenicol (8). This hypothesis is consistent with noncharged amino acids found in the same positions in AFA-I, AFA-III, F1845, and now Dr-II (G, N, N, and P, respectively).

A striking similarity exists in the positions of the two cysteine residues in the N-terminal half of Dr family adhesins and Dr-II, separated by 32 to 34 amino acids. Other *E. coli* adhesins containing this two-cysteine motif include the nonfimbrial adhesin I (NFA-I) (1) and the aggregative adherence fimbria I (AAF/I) (42), but it is not clear whether these adhesins bind to DAF. It is however, likely that NFA-I, with its 96% amino acid sequence identity to Dr-II, could have the same receptor specificity in DAF. While only 37 of 186 amino acids (20%) of Dr-II overlap with Dr amino acids in alignment analysis, the fact that they both could recognize the same receptor domain in the SCR-3 region of DAF is remarkable and may reflect the biological significance of DAF-binding *E. coli*. Another common feature among these adhesins is the carboxyl-terminus motif containing the tyrosine and glycine residues, which has been proposed to be the binding site for periplasmic chaperone protein in the transport and assembly of the adhesin subunits of not only Dr but other adhesin families as well (18). The ability of the fusion construct to produce fully functional Dr-II adhesin illustrated that Dr-II accessory genes may be very similar to those in the Dr adhesin family. Given the functional compatibility between *dra* and *dra2* operons, it is not surprising that open reading frames identified from internal DNA sequences of *dra2* should be very similar to accessory gene products of the Dr family, such as AfaB and AfaC, that we have shown here.

Compared to the homology of the accessory proteins, that among Dr family adhesins is not observed to the same extent, especially with regards to Dr-II. The lack of antibody cross-reactivity between Dr-II and Dr is a further example of the diversity among the Dr family members and may be explained by the low degree of homology in their primary sequences, suggesting that antiadhesin antibodies may have a limited role in the protection against other Dr-related adhesins. Attempts to target adhesin subunits for vaccine development may need to be carefully directed to the search for conserved epitopes.

The binding of bacteria to a human complement regulatory molecule was first reported by Nowicki et al., who found that hemagglutinins of pyelonephritis-associated *E. coli* recognized DAF (38). Within the past few years, it has been shown that the microbe-complement regulatory protein family interaction is a widespread phenomenon not unique among Dr-bearing *E. coli*, as other bacteria (40) and viruses (6, 22) have been reported recently to bind to proteins of the complement regulatory protein family, such as DAF and membrane cofactor protein (MCP). It has been proposed that by interacting with cells bearing these molecules, the microorganism could evade host complement attack in the same way that host cells are protected (32). Alternatively, a mechanism by which binding to complement regulatory proteins such as MCP can alter host cytokine production and suppress cell-mediated immunity has also been proposed (23). The emerging picture indicates that DAF and MCP are significant molecules in microbe-host interaction, which may support our finding that despite limited homology between Dr-II and other Dr family adhesins, the capacity to recognize DAF as a receptor is still retained.

Bacterial urogenital infections, including pyelonephritis, in pregnant women represent significant health risks to both the mother and the fetus; these risks include low birth weight, developmental retardation, and preterm labor (10, 34). Pyelonephritis-associated *E. coli* strains from pregnant subjects represent a nonrandom population and are different from strains from nonpregnant subjects and fecal flora in terms of distribution of serotypes, DNA fingerprints, and plasmid profiles (17, 37). These strains may have pregnancy-specific virulence factors, such as Dr-II, that enable them to colonize and establish infection in the host and are different from strains from nonpregnant subjects or from commensal fecal flora. It is not clear whether Dr-II is associated primarily with pregnancy, but its sequence diversity from other DAF-binding adhesins may indicate the existence of a wide range of DAF-binding adhesins to take advantage of the environment changes during pregnancy. One such change is in the expression of DAF in the urinary tract, which may be upregulated by progesterone (24). Thus, while increased DAF expression during pregnancy may play a critical role in protecting the semiallogenic fetus from the cytotoxic action of maternal complements (19), it may also promote increased colonization of Dr family-bearing *E. coli* with diverse DAF-binding adhesins (41). Therefore, potential preventive and therapeutic approaches may need to take into account the heterogeneous distribution of virulence factors in this population of *E. coli.*

Studies using human kidney and urinary tract cryostat sections incubated with purified recombinant Dr-II adhesin protein illustrate the presence of binding sites in different substructures similar to that observed for Dr (39), mainly in the renal tubular basement membranes, glomeruli, and Bowman's capsule. The presence of DAF as receptors within renal tissue indicates that interstitial tropism may play a role in the pathogenesis of gestational pyelonephritis. The lack of receptors on the luminal side of the tubular epithelium raises the question of how bacteria gain entry into the kidney parenchyma. One explanation is that hemolysin may play a role in the cytotoxic destruction of renal tubular epithelial cells and allow the bacteria to penetrate the interstitium, but only about half of the pyelonephritogenic *E. coli* strains have been reported to produce hemolysin (33). The alternative hypothesis is that some uropathogenic *E. coli* strains may be internalized due to the invasive properties (46).

Recent studies of Dr-bearing *E. coli* strains in our lab suggest that the bacteria are internalized into the HeLa cell by a microtubule-dependent process since the internalization was inhibited by microtubule-depolymerizing agent nocodazole (15). Similarly, gestational pyelonephritis isolate *E. coli* 7372 and recombinant strain TP404 expressing Dr-II adhesin displayed invasive properties. Studies on the role of bacterial invasion in pyelonephritis have been limited until recently (15) due to the lack of a suitable animal model. The emerging picture of pathogenesis of gestational pyelonephritis indicates several virulence mechanisms, and ongoing development of a mouse model in our laboratory will allow the pathogenesis process related to Dr-II adhesin to be examined in a more unique context of the pregnant animal.

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