INFECTION AND IMMUNITY, May 1995, p. 1663–1668 Vol. 63, No. 5
0019-9567/95/$04.00+0

dra-Related X Adhesins of Gestational Pyelonephritis-Associated *Escherichia coli* Recognize SCR-3 and SCR-4 Domains of Recombinant Decay-Accelerating Factor

TUAN PHAM,[1] ANIL KAUL,[2] AUDREY HART,[2] PAWEL GOLUSZKO,[2] JOHN MOULDS,[3] STELLA NOWICKI,[1,2] DOUGLAS M. LUBLIN,[4] AND BOGDAN J. NOWICKI[1,2]*

Department of Microbiology & Immunology[1] and Department of Obstetrics & Gynecology,[2] The University of Texas Medical Branch at Galveston, Galveston, Texas 77555; Gamma Biologicals Inc., Houston, Texas 77092[3]; and Departments of Pathology and Internal Medicine, Washington University School of Medicine, St. Louis, Missouri 63110[4]

Received 6 October 1994/Returned for modification 22 November 1994/Accepted 6 February 1995

Bacterial adhesins are important virulence factors that allow colonization of the human urogenital tract by *Escherichia coli*. Adhesins of the Dr family have been found to be more frequently expressed in strains associated with symptomatic urinary tract infections. Because of the high frequency of symptomatic urinary tract infections during pregnancy, we screened *E. coli* isolates from 64 gestational pyelonephritis patients for the expression of Dr and X adhesins to address their potential virulence roles in this population. Using PCR and primers for the *afaB* gene, we detected *dra*-related operons in 17 isolates (27%). On the basis of the lack of hemagglutination of Dr(a$^-$) erythrocytes containing a point mutation in the decay-accelerating factor (DAF) short consensus repeat-3 (SCR-3) domain, 12 of these strains were categorized as classical Dr adhesins. The hemagglutination of O erythrocytes by Dr$^+$ strains was blocked or reduced by a monoclonal antibody to the DAF SCR-3 domain. The remaining five *dra*-positive strains agglutinated Dr(a$^-$) erythrocytes. Monoclonal antibody to the DAF SCR-3 domain failed to block O-erythrocyte hemagglutination. Adhesins in these strains did not fulfill criteria for Dr hemagglutinins because of the undefined receptor specificities and were categorized as X. *E. coli* strains bearing *dra*-related X adhesins bound to DAF cDNA-transfected Chinese hamster ovary cells. Three of these *dra*-related X-adhesin-bearing *E. coli* strains failed to attach to the SCR-3Δ deletion transfectant, which suggested that binding sites were located in the SCR-3 domain but outside the region blocked by the monoclonal anti-SCR-3 immunoglobulin G. The binding sites of the remaining two *dra*-related X adhesin strains were localized to the SCR-4 domain, as the attachment was shown to be abolished on an SCR-4Δ mutant but unaffected by an SCR-3Δ deletion. The heterogeneity in the binding sites of *E. coli* DAF (Dr) family adhesins from gestational pyelonephritis isolates may reflect the ability of the adhesins to evolve to recognize alternate peptide epitopes for efficient colonization.

Escherichia coli is the most common etiologic agent of urinary tract infection (UTI). It has been estimated that at least 10 to 20% of women and 12% of men experience an acute symptomatic UTI at some time in their lives, and many more develop asymptomatic bacteriuria (10, 18). *E. coli* infections of the female genitourinary tract may lead to severe sequelae such as acute and chronic pyelonephritis, end-stage renal disease, septicemia, and preterm labor (23). Among the *E. coli* virulence factors causing UTI, adhesins enable the bacteria to attach, establish colonization, and perpetuate inflammatory responses (1, 8, 9, 33). It was initially observed that UTI-associated *E. coli* organisms hemagglutinate human erythrocytes and bind to uroepithelial cells (7, 32, 33) via ligands expressed on host tissues (13, 30).

Many of the urinary tract isolates recognize the Dr blood group antigen and have been designated Dr hemagglutinins (25, 28). This family of adhesins includes the fimbrial Dr adhesin, the afimbrial adhesins AFA-I and AFA-III of uropathogenic bacteria, and the fimbrial diarrhea-associated F1845 adhesin (26). The genetic organizations of these four operons are very similar, and the nucleotide sequence of the Dr hemagglutinin fimbrial subunit is homologous with those of F1845 (34), AFA-I, and AFA-III (15, 17). The receptor for the Dr adhesin family has recently been located on the short consensus repeat-3 (SCR-3) domain of the decay-accelerating factor (DAF) molecule (25), a cell membrane protein that regulates the complement cascade and prevents tissue lysis by autologous complement (20). A single-point substitution in the DAF SCR-3 domain, Ser-165 to Leu, [a rare polymorphism of DAF designated Dr(a$^-$) (21)] causes complete abolition of Dr adhesins binding to Dr(a$^-$) erythrocytes (24). Dr adhesins were postulated to be expressed by 30 to 50% of cystitis-associated *E. coli* (4, 29) and 50% of protracted diarrhea-associated *E. coli* (6). The binding sites for Dr adhesins have been shown to be present in urogenital tissues (12, 13, 30), including the uroepithelium of the upper and lower urinary tracts and renal interstitial tissues, and have been proposed to support ascending infection, possibly leading to chronic interstitial nephritis (30).

Numerous studies have implicated *E. coli* adhesins as virulence factors in UTI in the nonpregnant population (2–4, 7, 9, 11, 31). Limited information has been accumulated, especially with respect to Dr and X adhesins (adhesins with undefined receptor specificity) in UTIs in pregnant women (27, 32). The mechanical compression of the ureters by the enlarging uterus seems to be the major factor contributing to increased frequency of pyelonephritis during pregnancy (22). Although an-

* Corresponding author. Mailing address: Department of Obstetrics & Gynecology, The University of Texas Medical Branch at Galveston, 301 University Blvd., Galveston, TX 77555-1062. Phone: (409) 772-7599. Fax: (409) 747-0475.

atomical changes are likely to contribute to the higher occurrence of UTI in the pregnant females, pathogenic virulence factors may play an important contributory role. Recent data from our laboratory indicated a gestational age-dependent distribution of type 1, P, and Dr fimbriae in pregnant patients with pyelonephritis (27). In this report, we characterize receptor specificity and frequency of *dra*-related adhesins in *E. coli* isolates from pregnant subjects with pyelonephritis. *dra*-positive strains were identified by PCR, using primers in the *afaB* gene of the AFA-I operon. This gene is necessary for the biogenesis of the adhesin and is conserved among the *dra*-related operons (17). Using recombinant DAF (rDAF) and DAFΔ deletion mutants, we identified a group of *dra*-related X adhesins that recognize the SCR-3 and SCR-4 domains of DAF.

MATERIALS AND METHODS

Bacterial strains. *E. coli* pyelonephritis isolates were derived from 64 pregnant patients hospitalized at the University of Texas Medical Branch at Galveston. Strains were selected on the basis of positive urine culture and clinical symptoms. 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. Laboratory *E. coli* strains expressing recombinant adhesins Dr (29), AFA-I, AFA-III (14, 15, 17), and F1845 (34) were plated on L agar containing appropriate antibiotics.

PCR. Overnight, L-broth cultures of 1 ml of *E. coli* were centrifuged and resuspended in 200 μl of water. The samples were boiled at 100°C for 20 min. After a 5-min microcentrifugation of the lysates, the supernatants were removed and stored at −20°C for use as PCR templates. The primers were identical to those previously used to detect Dr and related operons and were designed from the internal sequence of a 1.1-kb *Pst*I fragment of the AFA-I operon (15, 16), which contains the *afaB* gene. The sequences of the primers were as follows: for AFA-I, 5′-GCTGGGCAGCAAACTGATAACTCTC-3′; and for AFA-II, 5′-CATCAAGCTGTTTGTTCGTCCGCCG-3′. PCR was done with the Gene Amp PCR Reagent Kit (Perkin-Elmer Corp., Norwalk, Conn.). The reaction mixtures were composed of 2 μl of DNA template, 200 μM (each) deoxynucleoside triphosphate, 2.5 U of AmpliTaq polymerase, and 1.0 μM (each) primer in a total volume of 100 μl. The reaction mixtures were overlaid with 50 μl of mineral oil. Amplification reactions consisted of 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 55°C, and a 1-min extension at 72°C. An aliquot of 10 μl from each tube was removed for analysis on a 2% agarose gel.

Hemagglutination assay. Human O and Dr(a$^-$) erythrocytes in 50% Alsever's solution were washed three times in phosphate-buffered saline (PBS). A 3% (vol/vol) suspension was made from packed erythrocytes in PBS plus 2% α-methylmannose. *E. coli* isolates, as well as the recombinant BN406 Dr hemagglutinin control strain, were suspended in PBS at an optical density at 600 nm of 0.6 and were mixed with equal volumes of erythrocytes: approximately 15 μl of each type. Each mixture was rotated on an angular rotator for 5 min at room temperature. Agglutination was scored as either positive or negative. If agglutination was observed, the isolates were tested for the presence of Dr specificity with human erythrocytes that had been pretreated with monoclonal antibody 1H4 to the SCR-3 domain of DAF. For the DAF SCR-3 inhibition assay, antibody was mixed with the 3% O-erythrocyte suspension at 1:16 and the mixture was incubated for 10 min at room temperature. The cells were then washed three times with PBS and resuspended at a 3% (vol/vol) concentration.

CHO cell binding assay. CHO cells stably transfected with cDNA encoding human DAF or DAFΔ deletion mutants (5, 20), including SCR-1Δ, SCR-2Δ, SCR-3Δ, SCR-4Δ, DAF S/TΔ, and DAF S/TΔ + HLA (where S/T is serine/threonine), and control CHO cells transfected with the expression vector SFFV-.neo only were cultured in Ham's F12 medium containing 10% fetal bovine serum and were grown to 75% confluency on eight-chamber slides. Suspensions of live *E. coli* (300 μl in PBS with 2% α-methylmannose) were added to the chambers containing CHO cells, incubated for 30 min, washed five times with PBS, fixed with acetone for 10 min, stained with safranin for 15 s, and then air dried. The stained chambers were examined under a phase-contrast microscope (Leica, Inc., Deerfield, Ill.), and bacteria binding to 100 CHO cells per well were counted. The results were expressed as the mean number of bacterial cells per CHO cell. Use of the phase-contrast microscope for the stained samples allowed excellent visualization of bacterial cells that appeared dark while CHO cells remained pink or reddish. Statistical analysis (*t* test) for all bacterial strains tested was performed to evaluate the binding difference between control- and DAF-transfected CHO cells.

RESULTS

PCR primers were designed to detect the 750-bp *afaB* gene of the AFA-I operon and related accessory genes in other Dr family operons. By PCR, we detected the presence of *dra* and related DNA regions (Fig. 1) in 17 pyelonephritis-associated strains (27%). Phenotypically, 12 of these 17 strains were categorized as Dr adhesins on the basis of agglutination of human O erythrocytes and lack of hemagglutination of Dr(a$^-$) erythrocytes (Table 1). This hemagglutination pattern was also observed for control strains expressing Dr, AFA-I, AFA-III, and F1845 adhesins of the Dr family (26). With O erythrocytes pretreated with anti-SCR-3 immunoglobulin G, hemagglutination of 12 *E. coli*-bearing Dr adhesins, as well as that of strains carrying prototypic Dr adhesins, was either inhibited or substantially reduced. For some strains tested (Table 1), hemagglutination was negative following 3 min of slide rotation; however, weak reactivity indicated by weak granulation of erythrocytes was observed after 5 min. This may have resulted from either the existence of unknown hemagglutinins or the monoclonal antibody that only partially blocked the binding epitope on DAF. The remaining five *dra* PCR-positive strains, unlike other strains of the Dr family characterized to date, agglutinated Dr(a$^-$) and O erythrocytes, including erythrocytes pretreated with anti-SCR-3 monoclonal antibody, and were designated X*dra* (genotype of *E. coli* carrying *dra*-related sequences and expressing adhesins of undetermined specificities). None of the PCR-negative strains exhibited Dr-specific hemagglutination.

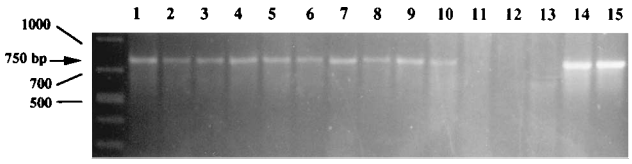

FIG. 1. Detection of *dra* and *dra*-related operons in clinical *E. coli* isolates by PCR. The gel illustrates the amplified products from Dr fimbriated strains (lanes 1 to 5) and X-adhesin strains (lanes 6 to 10). Lanes 11 to 13 were negative controls, containing reaction products from strains with type 1 and P adhesins. Positive control reaction samples were loaded in lanes 14 and 15. Lane 14 was loaded with PCR product from strain HI11128, a Dr$^+$ clinical strain. Lane 15 was loaded with PCR product from the recombinant plasmid pBJN406 containing the *dra* operon.

Due to the *dra* relatedness of the five *E. coli* strains bearing X adhesins, we investigated whether new binding sites may exist outside the DAF region affected by the point mutation in the SCR-3 domain and the region blocked by the monoclonal antibody. We recently established a novel system for testing *E. coli* Dr adhesins on an rDAF receptor expressed in CHO cells (24), and this system was used here to map the structure-function relationships of the X-adhesin–DAF ligand interaction. Binding of *dra*-related *E. coli* X strains was examined with human DAF cDNA-transfected CHO cells and control CHO DAF-negative cells transfected with the expression vector SFFV.neo only (Fig. 2A). There was no significant binding to control CHO cells of any of the X strains tested (DAF negative). In contrast, all five X strains, as well as the recombinant Dr hemagglutinin-bearing *E. coli* BN406 and related recombinant AFAI-, AFA-III-, and F1845-bearing strains, were shown to bind to DAF transfectants, indicating that *dra*-related X adhesins bind to DAF.

We next attempted to localize the binding sites of X adhesins on the DAF molecule by using a series of DAF deletion mutants. Most of the extracellular domains of DAF are composed of four SCR domains, an S/T-rich region, and a glucosylphosphoinositol (GPI) domain anchoring DAF to the cell membrane (19) (Fig. 3). A set of deletion mutants that individually lack one of the domains was expressed in CHO cells,

TABLE 1. Hemagglutination patterns of *dra*-positive *E. coli* strains associated with gestational pyelonephritis

Strain	Dr(a$^-$) agglutination	O-erythrocyte agglutination		Binding to DAF-transfected CHO cells	Adhesin specificity
		Without anti-SCR-3	With anti-SCR-3		
8635	−	+	−	+	Dr
7031	−	+	−[a]	+	Dr
0366	−	+	−[a]	+	Dr
6081	−	+	−[a]	+	Dr
6510-1	−	+	−[a]	+	Dr
8369	−	+	−[a]	+	Dr
2722	−	+	−	+	Dr
7001	−	+	−[a]	+	Dr
5439	−	+	−	+	Dr
7222	−	+	−[a]	+	Dr
1498	−	+	−	+	Dr
7847	−	+	−[a]	+	Dr
7077	+	+	+	+	X (DAF SCR-3)
7048	+	+	+	+	X (DAF SCR-3)
7900	+	+	+	+	X (DAF SCR-3)
7372	+	+	+	+	X (DAF SCR-4)
8826[b]	+	+	+	+	X (DAF SCR-4)
Control					
11128	−	+	−	+	Dr
F1845	−	+	−	+	Dr
AFA-I	−	+	−	+	Dr
AFA-III	−	+	−	+	Dr

[a] Strains inhibited by anti-SCR-3 immunoglobulin G following 3 min of incubation showed weak aggregation of erythrocytes, observed after 5 min.
[b] Variation in phenotypic expression occurred. Strain required at least two subcultures for hemagglutination.

with the amounts of surface DAF approximately equal for each mutant (5). DAFΔ deletion mutants SCR-1Δ, SCR-2Δ, SCR-3Δ, SCR-4Δ, DAF S/TΔ, and DAF S/TΔ + HLA were used in binding experiments. Similar to the results seen in our previous binding study with Dr, F1845, AFA-I, and AFA-III strains (24), the Dr strains in this study and the three X strains did not bind to the DAF SCR-3Δ mutant (Fig. 2B). The remaining two X strains, however, showed efficient binding to the SCR-3Δ mutant. It was further shown with the CHO-DAF SCR-4Δ mutant that this domain was essential for the binding of *E. coli* 7372 and 8826 (Fig. 2C), as the binding of these strains (but not that of the remaining X and Dr strains) was abolished. While there were differences in the binding to the SCR-3 and SCR-4 domains of DAF among the *dra*-related X-adhesin-bearing strains, they all exhibited binding to DAF-SCR-2Δ-transfected CHO cells (Fig. 2D). This mutation has been previously shown to inhibit the binding of Dr, F1845, and AFA-III adhesins but not the AFA-I adhesin (24). The DAF SCR-1Δ mutant did not abolish the binding of any of the strains (not shown), as observed in our earlier study with Dr, F1845, AFA-I, and AFA-III strains.

No binding of X strains to CHO cell transfectants expressing a DAF ΔS/T deletion mutant was observed. CHO transfectants expressing the chimeric molecule DAF ΔS/T+HLA, in which the missing S/T domain was replaced by the HLA-B44 molecule to reposition the SCR domains of DAF (5), however, completely restored the binding (not shown). This observation is consistent with our previous study in which we used the DAF ΔS/T + HLA construct (24) to examine the binding of the Dr adhesin; the results suggested that the DAF-SCR binding domains were projected from the cell membrane by either S/T GPI or chimeric HLA-B44 domains. Taken together with the hemagglutination inhibition data, these results suggest that the *dra* PCR-positive X strains recognize novel sites on DAF. Two of these strains required the SCR-4 domain for binding, indicating further heterogeneity in the *dra* adhesin family.

DISCUSSION

While several studies have implicated the importance of *E. coli* adhesins in pyelonephritis in the nonpregnant population (2–4, 7, 11, 35, 36), little information is available on the importance of bacterial adhesins in establishing UTIs in the pregnant population (27, 32). In the present study, we characterized the occurrence and receptor specificity of Dr and X adhesins of *E. coli* isolates from pregnant subjects with pyelonephritis.

The adhesin-encoding genes differ among the members of the Dr family, which accounts for differences in ligand binding. Accessory genes necessary for the fimbrial biogenesis were found to be highly conserved among the Dr-related operons. One of these *dra*-related genes, *afaB* of the AFA-I operon, was targeted in this report for PCR amplification. By PCR screening, we detected the 750-bp DNA fragment in 17 of 64 (27%) *E. coli* strains isolated from pregnant subjects with acute pyelonephritis. The percentage of *dra E. coli* in gestational pyelonephritis (27%) was higher than the 6 to 12% reported for acute pyelonephritis isolates from a nonpregnant population (2, 3, 26). These results suggest a potential virulence role for Dr-adhesin-bearing *E. coli* in pregnancy-associated pyelonephritis. It is therefore conceivable that, in addition to anatomical obstructions, Dr adhesins may play an important contributory role in enhancing the colonization capacity of the renal tissue for ascending infection during pregnancy.

While verifying the phenotypes of the *dra* PCR-positive strains, we observed that five of these strains did not fulfill criteria for Dr adhesins due to hemagglutination of Dr(a$^-$) erythrocytes. Additionally, the monoclonal antibody to DAF

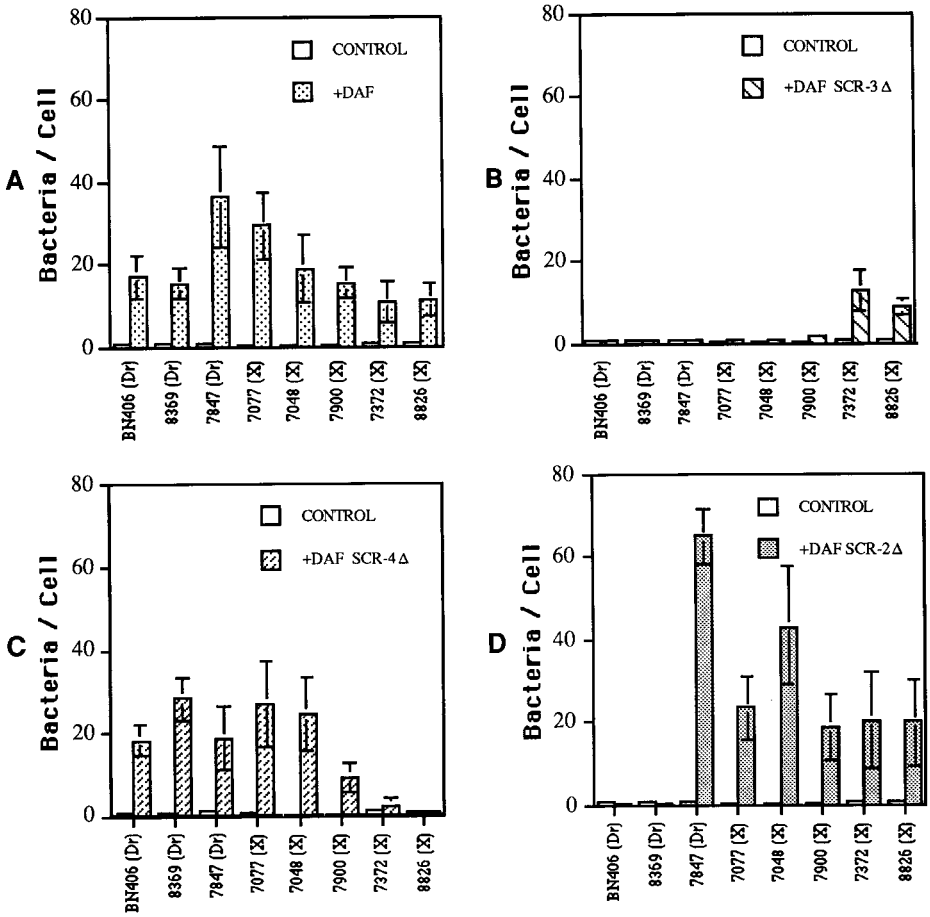

FIG. 2. Binding of *E. coli dra*-positive X strains to CHO cells transfected with different DAF cDNA constructs compared with the Dr strains: (A) full-length DAF cDNA; (B) DAF cDNA with SCR-3 deletion; (C) DAF cDNA with SCR-4 deletion; (D) DAF cDNA with SCR-2 deletion. The adhesin type of each strain is shown in parentheses. BN406 is a recombinant laboratory strain carrying the *dra* operon in the plasmid cloning vector pACYC184. Differences between binding to DAF-transfected and control-transfected CHO cells were statistically significant ($P < 0.001$ by t test).

SCR-3 failed to block hemagglutination by any of these X*dra* strains. Hemagglutination data for the X adhesins suggested novel binding sites different from those recognized by Dr family adhesins. The possible interference by other non-*dra* adhesins hemagglutinating Dr(a^-) erythrocytes was eliminated by the use of rDAF and control DAF-negative CHO cells. Our results, therefore, refer specifically to *dra*-related factors and DAF. Experiments with rDAF and DAF deletion mutants indicated that binding sites for two of five X*dra* strains lie outside the SCR-3 domain. Subsequently, an attachment assay with DAFΔ SCR-4-transfected CHO cells revealed the requirement of this domain for the binding of *E. coli* 7372 and 8026. The remaining three X strains showed a requirement for the SCR-3 domain for binding, most likely outside the Dr(a^+) functional epitope, on the basis of agglutination of Dr(a^-) and anti-SCR-3 immunoglobulin G-treated O erythrocytes. More detailed characterization, however, will be attempted following molecular cloning of these factors, which is currently in progress. Differences in binding to the CHO DAFΔ SCR-2 transfectant were also observed among the X and Dr strains (Fig. 2D). All X strains and one Dr strain showed binding to this mutant similar to AFA-I, while the loss of binding of Dr strain 8369 was similar to the loss of binding seen with Dr, AFA-III, and F1845 (24). The loss of binding suggests that these strains may require SCR-2 for attachment as well. Given

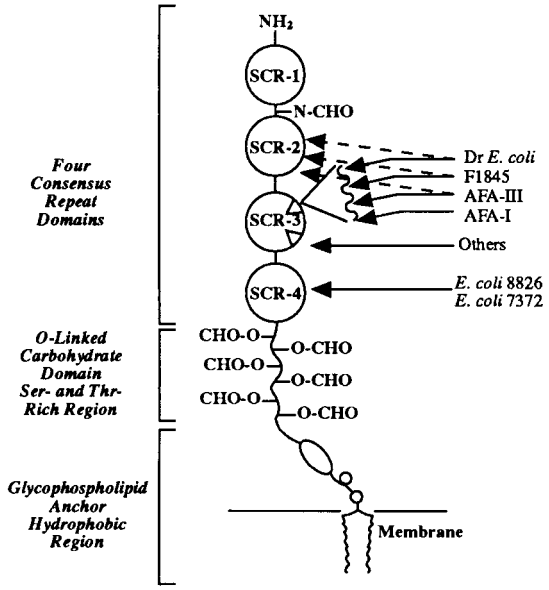

FIG. 3. Simplified structure of DAF containing the hypothetical ligands for the family of adhesins encoded by *dra* and *dra*-related operons (adapted and modified from reference 19). Some Dr family adhesins may also require the SCR-2 domain for binding, as indicated by broken arrows.

the structural similarities between SCR-2 and SCR-3, it would not be surprising to see cross-binding between the two domains. Alternatively, it is also possible that the SCR-2 deletion may affect the structural conformation of the binding sites within SCR-3. The lack of binding to the SCR-3 domain in the SCR-2Δ mutant and to the Ser-165 to Leu mutant in SCR-3-positive rDAF (24) supports this possibility. Figure 3 illustrates the hypothetical binding sites of the X*dra E. coli* strains in this study and four Dr adhesins characterized to date. The binding sites of AFA-I, AFA-III, Dr, and F1845 within the DAF SCR-3 domain have previously been proposed on the basis of hemagglutination of enzyme-treated O erythrocytes (26). Since the new *dra* adhesins were defined by the binding to epitopes localized to the SCR-3 and SCR-4 domains of DAF, we propose that both Dr and the X adhesins described here be included in the more diversified DAF family.

Dr adhesin represents a prototype of a unique fimbria with respect to its receptor specificity. Its uniqueness is due to a protein-protein binding which clearly differs from that of other frequent fimbrial types P, 1, and S that recognize carbohydrate ligands. The observed phenomenon indicates the possibility that adhesins of the DAF family evolved to recognize alternate peptide epitopes on DAF, which would result in further diversity and allow options for efficient colonization of a pregnant host. The present study not only has allowed us to identify DAF regions involved in Dr adhesins binding but also has provided evidence of novel adhesins recognizing peptide sequences exclusively. DAF has three forms of carbohydrate modifications (Fig. 3). They include N-linked oligosaccharide at the border of SCR-1 and SCR-2, multiple oligosaccharides at the S/T domain, and a GPI anchor. Deletion of SCR-1 and SCR-2 and replacement of S/T and GPI domains by nonglycosylated HLA-B44 demonstrated that DAF oligosaccharides were not required for Dr-DAF adhesin binding.

Considering the importance of Dr-DAF adhesins in UTI and protracted diarrhea, a rapid detection assay may be a valuable diagnostic tool in determining the potential virulence of *E. coli*. In this study, we demonstrated a fast alternate approach for detecting a family of Dr-DAF adhesins by using, for Dr, a monoclonal antibody blocking the DAF SCR-3 domain and, for novel DAF adhesins, DAF-CHO transfectants and deletion mutants. While a typical hemagglutination assay for P, S, and type 1 fimbrial typing can be done within a few minutes, the limited availability of Dr(a^-) erythrocytes and lack of specific blocking agents have been the major drawbacks in the detection of Dr adhesins. Until now, the specificity of Dr adhesin binding could be demonstrated by the lack of binding to Dr(a^-) erythrocytes, a very rare blood group type known to occur in only a few individuals worldwide (21). Initially, some tyrosine-like compounds such as chloramphenicol were found to inhibit Dr hemagglutination. This inhibition was seen, however, on O75X strains but not other Dr strains (26), which may represent the diversity in the specificity of Dr adhesin ligands. Our proposed simple inhibition method, which uses a monoclonal antibody to the DAF SCR-3 domain, demonstrated some usefulness in detecting Dr adhesins. Strains designated Dr by this method have been verified by the lack of agglutination of Dr(a^-) erythrocytes and by PCR. More importantly, use of a recombinant human DAF ligand expressed in laboratory cells provided a novel option for identification of DAF-specific adhesins. This determination may be useful in evaluating the potential virulence of gestational pyelonephritis-associated and other *E. coli* isolates and may support development of preventive methods for patients at high risk of developing DAF-specific *E. coli* infections.

ACKNOWLEDGMENTS

We thank M. Martens and C. Peyton for assistance in collecting *E. coli* isolates from the obstetrical patients who participated in the study.

This work was supported by National Institutes of Health grants DK42029 to B.J.N. and U01 AI34580 to D.M.L. and by McLaughlin Foundation Fellowship funds to T.P. and A.K.

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