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Characterization of the Type 3 Fimbrial Adhesins of *Klebsiella* Strains

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The *Klebsiella pneumoniae* fimbrial adhesin, MrkD, mediates adherence to the basolateral surfaces of renal and pulmonary epithelia and to the basement membranes of tissues. Although all isolates possessing the MrkD adhesin mediate the agglutination, in vitro, of erythrocytes treated with tannic acid, the *mrkD* gene is not conserved within species. The ability of a plasmid-borne *mrkD* gene product to mediate binding to type V collagen is associated frequently with strains of *K. oxytoca* and rarely with strains of *K. pneumoniae*. In *K. pneumoniae*, the MrkD adhesin is located within a chromosomally borne gene cluster and mediates binding to collagen types IV and V. The plasmid-borne determinant, $mrkD_{1P}$, and the chromosomally borne gene, $mrkD_{1C}$, are not genetically related. Some strains of enterobacteria possess a $mrkD_{1C}$ allele that is associated with hemagglutinating activity but does not bind to either type IV or type V collagen.

Type 3 fimbriae are produced by many members of the *Enterobacteriaceae*, including *Klebsiella*, *Enterobacter*, *Proteus*, *Providencia*, and *Serratia* species (5, 7, 10, 19, 22, 23, 25). This fimbrial type is detected by agglutination, in vitro, of erythrocytes treated with tannic acid, and hemagglutination can occur in the presence or absence of D-mannose (7, 21). This characteristic was originally demonstrated in *Klebsiella*, and the associated adherence phenotype is often referred to as the mannose-resistant *Klebsiella*-like hemagglutination (MR/KHA) reaction (7, 24, 26). MR/KHA activity is mediated by the MrkD adhesin polypeptide of the type 3 fimbrial gene cluster (1, 5, 16), and the adhesin facilitates binding to the basement membranes of human tissues (14, 33).

The expression of type 3 fimbriae in *Klebsiella* requires the presence of at least six *mrk* genes which have been cloned and sequenced (1, 5). The gene (*mrkD*) encoding the fimbrial adhesin is distinct from that which encodes the major fimbrial subunit (*mrkA*) (1, 5). In one strain of *Klebsiella pneumoniae*, the MrkD adhesin has been shown to mediate adherence to human basement membrane and basolateral surfaces of renal and pulmonary epithelia (14, 16). Specifically, this adhesin has been shown to bind to type V collagen and is an extracellular matrix binding protein (32). However, we have previously demonstrated that the *mrkD* gene is not conserved among all fimbriate and hemagglutinating strains of *Klebsiella* (28). Southern hybridization analysis indicated that in one isolate of *K. pneumoniae*, the *mrkD* gene is present on a large native plasmid (16). It has not been determined whether most isolates of *K. pneumoniae* possess a plasmid-borne copy of the *mrk* gene cluster. Also, since all *mrkD* genes are not identical, the ability of different *mrkD* gene products to bind to type V collagen has not been investigated. In the studies described below, the MrkD-mediated receptor-binding specificity of type 3 fimbria-associated adhesins encoded by *mrkD* alleles is reported. Differences in receptor-binding specificities can be associated with variability in the amino acid sequences of the MrkD adhesin.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The sources of the clinical and environmental isolates of *Klebsiella* strains used in this study have previously been described (14, 15, 28). Table 1 lists the recombinant plasmids used, and these plasmids were maintained in *Escherichia coli* HB101 (4), JM109 (36), or DH12S (20). Transformations were performed by electroporation with an ECM600 pulse generator (BTX Inc., San Diego, Calif.), and transformants were selected following growth in L-broth supplemented with the appropriate antibiotics.

Optimal expression of type 3 fimbriae in *Klebsiella* strains was achieved by cultivation on glycerol-Casamino Acids agar as previously described (15, 16). All strains were incubated at 37°C for 18 to 24 h. Large, native plasmids from *Klebsiella* isolates were purified by a commercially available technique (Bigger Prep; 5 prime-3 prime, Inc., Boulder, Colo.). Plasmids were restricted with endonucleases from commercial sources according to the manufacturers' instructions. Restriction fragments were analyzed by agarose gel electrophoresis as described elsewhere (27).

Southern hybridization analysis. Total genomic DNA was prepared by standard procedures (3), and Southern hybridization analysis with both total genomic DNA and plasmid DNA preparations was performed as previously described (28). DNA probes were constructed by PCR amplification with relevant plasmids as the template (Table 1) and primers derived from sequences within appropriate *mrk* genes. The construction and use of the *mrkA* gene probe and one of the *mrkD* probes have been described elsewhere (15, 28). All DNA probes were nonradioactively labeled according to standard procedures (Genius System; Boehringer Mannheim, Indianapolis, Ind.), and hybridization was carried out under high-stringency conditions as previously described (28).

Isolation of $mrkD_{1C}$ genes. The *mrkD* genes from *K. pneumoniae* UIR079 and 43816 were isolated with a gene probe derived from *K. pneumoniae* IApc35 (Table 1) (16). The probe was prepared with the primers 5′-TTCTGCACAGC GGTCCC-3′ and 5′-GATACCCGGCGTTTTCGTTAC-3′ and comprises 581 bp within the region flanked by *mrkC* and *mrkF* on the chromosome of *K. pneumoniae* IApc35 (Fig. 1, probe B). Following isolation of genomic DNA from the two *K. pneumoniae* strains (3), the DNA was partially digested with *Eco*RI, and DNA fragments of approximately 5 to 7 kb in size were isolated (27). Subsequently, these fragments were ligated into the *Eco*RI site of pBluescript KS (Stratagene, La Jolla, Calif.), and transformants in *E. coli* JM109 or DH12S were isolated by conventional techniques (27). Recombinants possessing *Klebsiella*-derived DNA were identified with the *mrkD* gene probe and by colony hybridization techniques described in detail elsewhere (3, 28).

The nucleotide sequences of the *mrkD* genes from *K. pneumoniae* UIR079 and 43816 were determined with the fmol sequencing reagents (Promega Corporation, Madison, Wis.) according to the manufacturer's instructions. The plasmids pTS83 and pTS84 (Table 1 and Fig. 1) were used as templates for these reactions, and the DNA sequences of both strands were determined. The predicted amino acid sequences of the *mrkD* determinants were derived, and all amino acid sequence comparisons were performed with the basic local alignment search tool (BLAST) program (2) or with Eugene software from UNIX systems (Molecular Biology Information Resource, Baylor College of Medicine, Houston, Tex.).

Purification of type 3 fimbriae. Fimbriae were purified from *Klebsiella* isolates as previously reported (11). Briefly, bacteria were grown under conditions to optimize fimbrial production, harvested, resuspended in 5 mM Tris-HCl (pH

TABLE 1. *Klebsiella* strains and recombinant plasmids used in this study

Strain, vector, or plasmid	Relevant genotype or description	Source or reference[a]
K. pneumoniae		
IA565	Chromosomal and plasmid-borne *mrk* gene clusters; fimbriate and hemagglutinating	9
IApc35	IA565 lacking the plasmid-borne gene cluster; fimbriate and nonhemagglutinating	16
UIR079	Fimbriate and hemagglutinating, with no detectable plasmid	UIHC
43816	Fimbriate and hemagglutinating, with no detectable plasmid	ATCC
Cloning vectors		
pBluescript II		Stratagene
pACYC184		New England Biolabs
Recombinant plasmids		
pFK10	The plasmid-borne *mrk* gene cluster of *K. pneumoniae* IA565 cloned into pACYC184	1
pTSΔT3	6.2-kb *Eco*RI DNA fragment possessing *mrk* gene cluster from *K. pneumoniae* IApc35 cloned into pACYC184	This work
pTS83	6.2-kb *Eco*RI DNA fragment possessing *mrk* gene cluster from *K. pneumoniae* UIR079 cloned into pBluescript II	This work
pTS84	6.0-kb *Eco*RI DNA fragment possessing *mrk* gene cluster from *K. pneumoniae* 43816 cloned into pBluescript II	This work

[a] UIHC, University of Iowa Hospital and Clinics; ATCC, American Type Culture Collection.

7.5), and homogenized in a Waring blender at 4°C. After homogenization, the bacteria were removed by centrifugation, and following a second homogenization and centrifugation step, ammonium sulfate (10% [wt/vol]) was added to the supernatant. After 30 min at ambient temperature, the precipitate was collected by centrifugation and discarded. Ammonium sulfate (30% [wt/vol]) was added to the supernatant, and the suspension was allowed to stand at 4°C for 18 h. The precipitate was subsequently collected and dissolved in distilled water. Cesium chloride was added (42% [wt/vol]), and the protein solution was centrifuged in a vertical angle rotor for 6 h at 55,000 × *g*. The fimbriae were collected, concentrated, and resuspended in sterile distilled water.

Detection of type 3 fimbriae and fimbria-associated proteins. MR/KHA activity was determined as previously described with tanned erythrocytes (26). The presence of type 3 fimbriae on the surface of bacteria was detected with fimbria-specific antiserum as described elsewhere (22, 26). Transmission electron microscopy was used to confirm phenotypic expression of type 3 fimbriae by bacteria (24, 26).

The purity and size of fimbrial polypeptides were determined by sodium dodecyl sulfate-polyacrylamide electrophoresis. Western blotting was performed by standard procedures with either anti-type 3 fimbrial serum or serum raised against a synthetic oligopeptide representing the first 10 amino acids of the mature MrkD adhesin (12, 28).

Binding to ECMPs. An enzyme-linked immunosorbent assay was developed to demonstrate specific binding mediated by type 3 fimbriae. The wells of flat-bottom microtiter plates were coated following incubation overnight at 4°C with optimal concentrations of extracellular matrix proteins (ECMPs) diluted in carbonate-bicarbonate buffer, pH 9.6 (34). Stock solutions of commercially available purified types I, IV, V, and X collagens, fibronectin, and laminin were prepared as recommended by the manufacturer, and the optimal coating concentrations were determined as described elsewhere (32). Prior to incubation with bacteria or purified fimbriae, nonspecific binding sites were blocked by incubation for 2 h at 22°C with a 1% (wt/vol) solution of bovine serum albumin. Subsequently, 100 μl of serial twofold dilutions of either bacterial suspensions (10^{10} bacteria/ml) or purified type 3 fimbriae (50 μg), prepared in phosphate-buffered saline-Tween 20 (PBS-T) (pH 7.4; 0.5 ml Tween 20 in 1 liter of PBS), was added to the wells. Following incubation for 2 h at 22°C with gentle shaking, unattached bacteria were removed by washing three times in PBS-T. For each well, the adherence assay was developed with a rabbit monospecific antifimbrial serum (100 μl) diluted in PBS-T. Then, after being washed in PBS-T, 100 μl of goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase was added to the wells and allowed to incubate for 1 h at 37°C. Finally, the plates were washed thoroughly, *p*-nitrophenyl phosphate (5 mg/ml in diethanolamine buffer) (Sigma Chemical Co., St. Louis, Mo.) was added, and the reaction was allowed to proceed for 40 min at 37°C. All tests were performed in triplicate, and color development was determined with an enzyme-linked immunosorbent assay plate reader set to an optical density of 405 nm.

RESULTS

Plasmid-borne and chromosomally borne *mrkD* genes. Of 44 strains of *Klebsiella* examined, all 10 isolates of *K. oxytoca* and 6 of 34 *K. pneumoniae* strains possessed a gene related to the *mrkD* determinant carried on pFK10 (Fig. 1 and Table 2). All of these strains express type 3 fimbriae and demonstrated the characteristic MR/KHA activity. Seven of the *K. oxytoca*

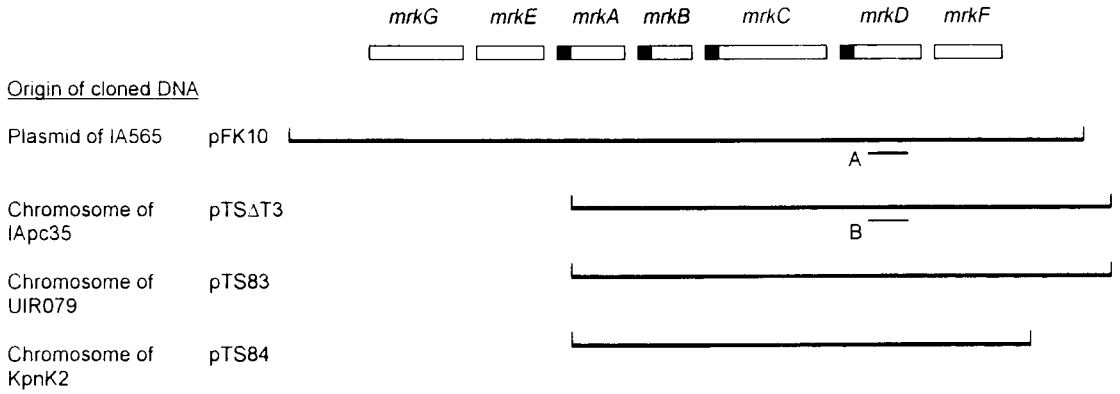

FIG. 1. Genetic organization of the *mrk* gene cluster. The *Klebsiella*-derived DNA fragments of recombinant plasmids are shown by the solid lines. The regions and sources of the $mrkD_{1P}$-specific gene probe (A) and the $mrkD_{1C}$-specific gene probe (B) are as indicated. The function of the *mrk* gene products has previously been reviewed (5).

TABLE 2. Genetic relatedness of *mrkD* genes in bacterial strains

Bacterium	No. of strains	No. of strains exhibiting hybridization to indicated gene probe:		
		$mrkD_{1P}$	$mrkD_{1C}$	both *mrkD*
K. pneumoniae	34	3	28	3
K. oxytoca	10	10	0	0
E. cloacae	8	1	7	0

strains were shown to possess a plasmid-borne *mrkD* gene, designated $mrkD_{1P}$, since plasmid preparations from these strains possessed sequences homologous to the gene probe (Fig. 1, probe A). Three *K. pneumoniae* strains also possessed an $mrkD_{1P}$ determinant on a plasmid isolated from these strains. The remaining 28 isolates of *K. pneumoniae* were MR/KHA positive and produce type 3 fimbriae but do not possess sequences related to the *mrkD* gene carried on pFK10.

K. pneumoniae IApc35 is a derivative of *K. pneumoniae* IA565 that has lost its plasmid-borne copy of the *mrk* gene cluster but retains a copy on the chromosome (16). Strain IApc35 was used to prepare an *mrkD* gene probe comprising sequences from within the chromosomally borne *mrkD* (Fig. 1, probe B). Plasmid pTSΔT3 carries the *mrkD* gene, designated $mrkD_{1C}$, cloned from *K. pneumoniae* IApc35 (Table 1 and Fig. 1). A 581-bp DNA fragment consisting solely of *mrkD*-derived nucleotides was used as a gene probe to detect homologous sequences among the *Klebsiella* strains. With this gene probe, 31 of 34 *K. pneumoniae* isolates could be shown to carry homologous sequences, whereas none of the type 3 fimbria-producing strains of *K. oxytoca* possess related nucleotide sequences (Table 2). Three of the 34 isolates of *K. pneumoniae* did not possess nucleotides related to the probe derived from pTSΔT3, although these three strains do retain an $mrkD_{1P}$ gene homologous to that carried on pFK10 (Table 2). Also, in all three of these strains, the $mrkD_{1P}$ gene was detected in plasmid DNA preparations. Three strains of *K. pneumoniae*, including strain IA565, from which the *mrk* gene cluster was originally cloned (9, 12), possess sequences related to both *mrkD* gene probes.

A total of eight fimbriate isolates of *Enterobacter cloacae* were also examined for the presence of *mrkD* genes because this genus expresses type 3 fimbriae related to those of *Klebsiella* (22). Seven isolates possess a gene comprised of sequences related to those derived from $mrkD_{1C}$, whereas one strain carried a gene similar to the $mrkD_{1P}$ DNA probe. In this latter strain, the *mrkD* gene was carried on DNA isolated either as total genomic DNA or from plasmid preparations.

Characterization of *mrkD* sequences and their gene products. *K. pneumoniae* IApc35 is a nonhemagglutinating derivative of IA565 (16). Therefore, this strain and *K. pneumoniae* UIR079, an MR/KHA-positive isolate, were used as a source of DNA to determine the nucleotide sequences of their $mrkD_{1C}$ genes. The *mrkD* gene of *K. pneumoniae* 43816 was also isolated, since this gene is closely related to that of strain IApc35 as determined by Southern hybridization, and the bacteria are hemagglutinating but do not bind to collagen (see below).

The plasmid pTS83 (Table 1 and Fig. 1) contains a 6.2-kb DNA fragment derived from *K. pneumoniae* UIR079 and possesses an *mrkD* determinant that is 978 bp in length. The predicted size of the MrkD polypeptide is 34.9 kDa, which is slightly larger than the 33.8-kDa gene product encoded by the $mrkD_{1P}$ gene carried on pFK10 (Fig. 1) (5). The *mrkD* gene of *K. pneumoniae* 43816 is carried on a 6.0-kb DNA fragment of plasmid pTS84 and comprises an open reading frame of 987 bp. The predicted size of the MrkD polypeptide encoded by this region is 35.4 kDa, and a comparison of the MrkD proteins of *K. pneumoniae* UIR079 and 43816 with that of the previously described plasmid-borne MrkD adhesin (5, 12) is shown in Fig. 2. At the amino acid level, $MrkD_{1C}$ of *K. pneumoniae* UIR079 is 55% identical to the $MrkD_{1P}$ polypeptide, and the two genes encoding these molecules possess 62.5% identity at the nucleotide level. A comparison of the amino acid and nucleotide sequences of the $MrkD_{1C}$ polypeptides and their genes from *K. pneumoniae* UIR079 and 43816 indicates 74 and 86% identity, respectively.

Bacterial binding to ECMPs. The results of the ECMP-binding assays for strains possessing the *mrkD* genes are summarized in Table 3 and Fig. 3. None of the *Klebsiella* isolates adhere, in vitro, to type I or type X collagen, fibronectin, laminin, or bovine serum albumin. All nine *Klebsiella* strains tested that possess the $mrkD_{1P}$ gene related to that carried on pFK10 bound only to type V collagen (Table 3). Nineteen of 28 *K. pneumoniae* strains that are type 3 fimbriate and exhibit MR/KHA but do not possess the $mrkD_{1P}$ gene bind to type IV and type V collagen. All of these strains possess $mrkD_{1C}$ sequences related to those isolated from *K. pneumoniae* IApc35 and UIR079. *E. coli* transformants possessing pTS83 also adhere to type IV and type V collagens, whereas transformants lacking the *mrk* gene cluster do not. Nine hemagglutinating and fimbriate isolates of *K. pneumoniae*, including strain 43816, possess nucleotide sequences homologous to those of strain IApc35 but did not bind to any of the ECMPs used in the assays. Six of the 10 *K. oxytoca* strains that were found to possess sequences homologous to the pFK10-derived *mrkD* gene probe were also examined for their ability to bind collagens. All six exhibited binding only to type V collagen.

As shown in Table 3, six of the seven *Enterobacter* strains examined possess sequences homologous to IApc35-derived $mrkD_{1C}$ and bound to type IV and type V collagen. One *E. cloacae* isolate demonstrated binding only to type V collagen, and DNA from this strain hybridized to the pFK10-derived $mrkD_{1P}$ gene probe.

Binding of purified fimbriae to ECMPs. Cell-free fimbriae from four representative isolates of *Klebsiella* were prepared. Fimbriae from *K. pneumoniae* IA565 and its nonhemagglutinating derivative, IApc35, were used in the binding assay. Also, purified type 3 fimbriae from *K. pneumoniae* UIR079 and 43816 were prepared. The fimbriae of *K. pneumoniae* UIR079 binds to type IV and type V collagen, whereas fimbriae from 43816 do not bind to either collagen type, and both strains are strongly hemagglutinating. Also, both strains possess $mrkD_{1C}$ sequences related to those carried on the chromosome of *K. pneumoniae* IApc35. The pattern of collagen binding by the purified fimbriae was identical to that found for whole bacteria (Fig. 4). Fimbriae from strain IA565 bound only to type V collagen, whereas those isolated from *K. pneumoniae* IApc35 did not adhere to either collagen type.

The presence of a 34-kDa polypeptide in the fimbrial preparations, encoded by the *mrkD* adhesin carried on pFK10, was determined with a mrkD-specific antiserum (28). Western blot analyses indicated the presence of this polypeptide in fimbrial preparations from *K. pneumoniae* IA565 but not in those from other strains (Fig. 5). All fimbrial preparations were recognized by serum raised against the MrkA major fimbrial subunit protein of strain IA565.

DISCUSSION

We have previously demonstrated that *K. pneumoniae* IA565 possesses two *mrk* gene clusters that are located on a plasmid

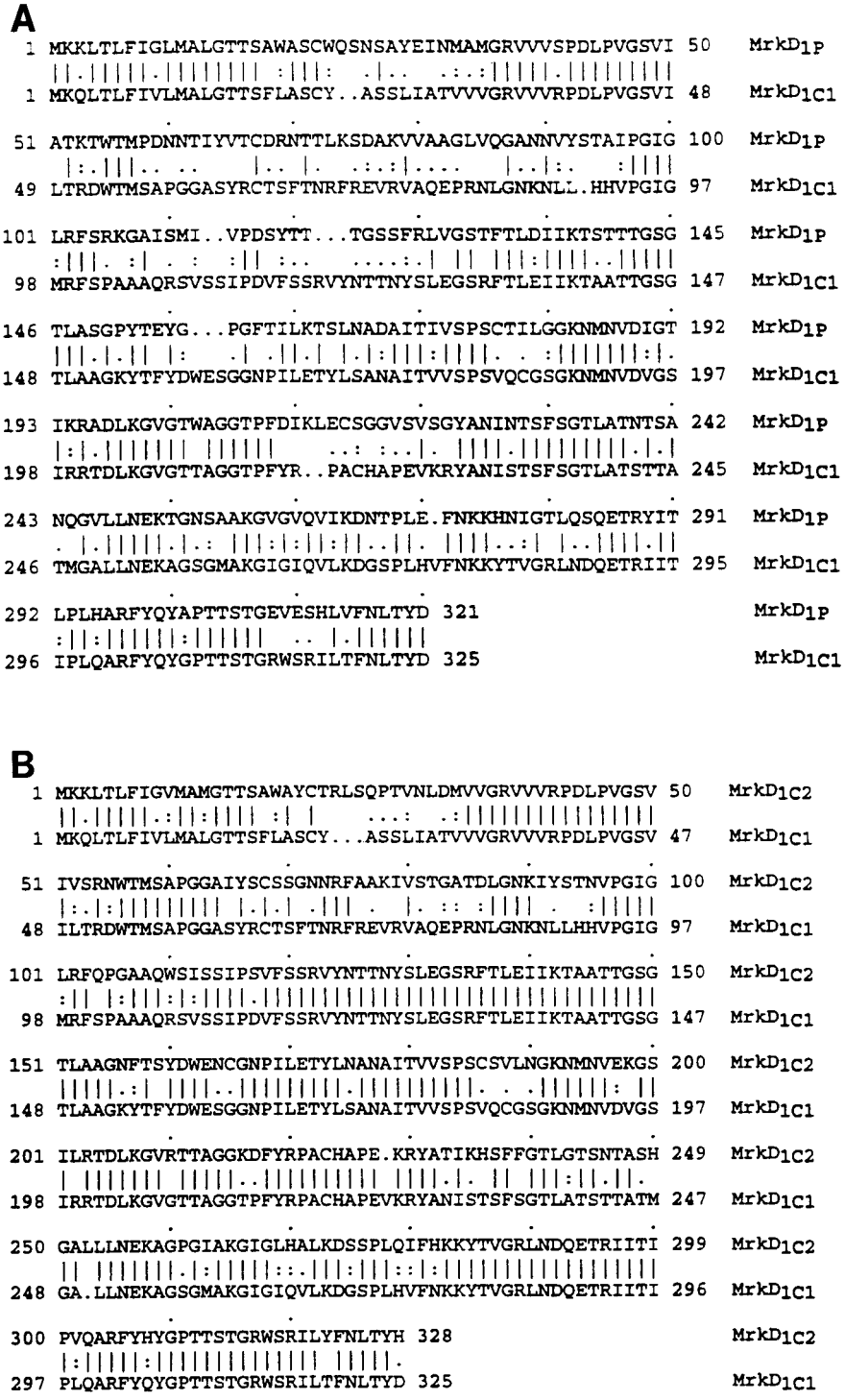

FIG. 2. Comparison of the deduced amino acid sequences of the MrkD polypeptides. $MrkD_{1P}$ is the gene product encoded by a plasmid of *K. pneumoniae* IA565; $MrkD_{1C1}$ is encoded by a chromosomally borne gene of *K. pneumoniae* UIR079; $MrkD_{1C2}$ is the gene product of the *mrkD* allele located on the chromosome of *K. pneumoniae* 43816. Identical amino acids are indicated by solid lines, and isofunctional amino acids are represented by dots.

and the chromosome (16). Also, *K. pneumoniae* IApc35, a derivative of strain IA565, has lost the plasmid-borne *mrk* sequences but retained the chromosomal copy of the *mrk* gene cluster (16). Using an *mrkD* gene probe derived from the plasmid-borne gene cluster, we demonstrated that this determinant was not highly conserved among isolates of *K. pneumoniae*, even though most of these strains express type 3 fimbriae. However, this plasmid-borne *mrkD* gene was found with a high frequency among fimbriate strains of *K. oxytoca* and more rarely among *K. pneumoniae* isolates (28). Therefore, even though most strains of *K. pneumoniae* express the type 3 fimbrial MR/KHA, they must possess an MrkD adhesin that is

TABLE 3. Relationship between collagen binding and *mrkD* genotype

Bacterium	No. of strains examined	MR/KHA	$mrkD_{1P}$[a]	$mrkD_{1C}$[a]	Binding to indicated collagen:	
					Type IV	Type V
K. pneumoniae	19	+	−	+	+	+
	9	+	−	+	−	−
	3	+	+	−	−	+
K. oxytoca	6	+	+	−	−	+
E. cloacae	6	+	−	+	+	+
	1	+	+	−	−	+

[a] Hybridization to the $mrkD_{1P}$- or $mrkD_{1C}$-specific gene probes.

not closely related to that encoded by the plasmid of *K. pneumoniae* IA565. In order to determine whether strains of *K. pneumoniae* possess a highly conserved *mrkD* gene, this determinant was isolated and characterized.

Using an *mrkD* gene probe derived from *K. pneumoniae* IApc35, a strain lacking the plasmid-borne *mrk* gene cluster (16), we demonstrated that most isolates of *K. pneumoniae* possess sequences related to those of the gene probe and that these sequences were not associated with plasmids. The results of Southern hybridization analysis indicate that fimbriate *K. pneumoniae* isolates do retain a highly conserved *mrkD* determinant that is different from that associated with the plasmid-borne adhesin gene. The plasmid-borne adhesin gene is carried more frequently by strains of *K. oxytoca*, often on large native plasmids. Similarly, in the small number of *K. pneumoniae* isolates that do possess an *mrkD* gene related to that carried on the recombinant plasmid pFK10, the gene is commonly carried on a plasmid. Therefore, the plasmid-borne gene found in most isolates of *K. oxytoca* has been designated $mrkD_{1P}$, and that associated with the chromosome of *K. pneumoniae* is termed $mrkD_{1C}$. The presence of the $mrkD_{1P}$ gene in a small number of *K. pneumoniae* strains may be due to horizontal transfer from *K. oxytoca*. We have previously demonstrated that the remaining *mrk* genes of the fimbrial gene cluster are highly conserved regardless of whether they are present on the chromosome or a plasmid in *Klebsiella* (15, 16). Therefore, the two genes encoding the MR/KHA of the type 3 fimbriae have undergone evolutionary divergence. Similarly, in fimbriate isolates of *E. cloacae*, the two distinct *mrkD* genes can be found, and in the strain possessing $mrkD_{1P}$, this determinant is carried on a plasmid.

Although it could be demonstrated by PCR analysis that *K. pneumoniae* IApc35 retains a region of DNA between its chromosomally borne *mrkC* and *mrkF* genes that is approximately 1,200 nucleotides in length, this strain expresses nonadhesive fimbriae (16). If, in fact, the *mrkD* adhesin gene is present in this location, *K. pneumoniae* IApc35 should be hemagglutinating. However, nucleotide sequencing demonstrated that a translation termination codon was present in the middle of this region of DNA, and therefore this strain will synthesize a truncated MrkD polypeptide. Consequently, a hemagglutinating isolate of *K. pneumoniae*, strain UIR079, that possessed

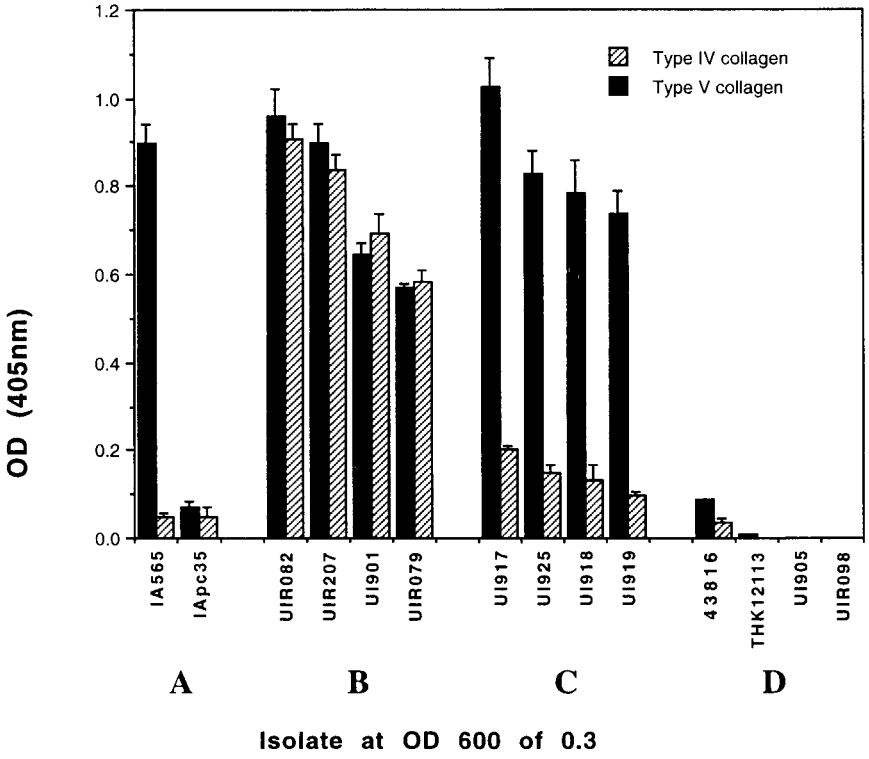

FIG. 3. Binding of *Klebsiella* strains to collagen. Binding to type V collagen is indicated by the solid bars, and type IV collagen binding is represented by the striped bars. All bacterial suspensions were normalized to an optical density at 600 nm (OD_{600}) of 0.3 corresponding to approximately 6×10^8 bacteria/ml. (A) *K. pneumoniae* IA565 and its derivative, IApc35, lacking $mrkD_{1P}$. (B) Representative strains possessing $mrkD_{1C}$ without $mrkD_{1P}$. (C) *Klebsiella* isolates possessing $mrkD_{1P}$ but no $mrkD_{1C}$. (D) Strains of *Klebsiella* with $mrkD_{1C}$ and no $mrkD_{1P}$ but not binding to collagen. For each column, the value is expressed as the mean ± standard error of the mean; all tests were performed at least three times.

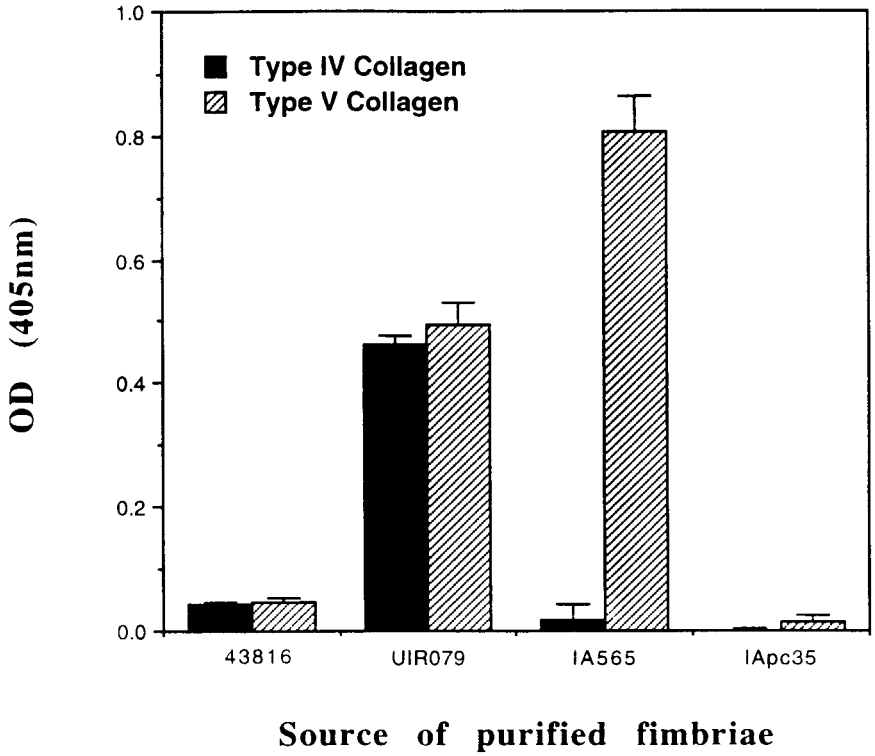

FIG. 4. Binding of purified type 3 fimbriae to collagen. Binding of type V and type IV collagen is shown by the striped and solid bars, respectively. All assays were performed with 12.5 μg of fimbrial protein. Standard errors of the means were calculated after each assay was performed at least three times.

DNA sequences related to that of the $mrkD_{1C}$ gene probe was used to clone and subsequently to determine the nucleotide sequence of a functional $MrkD_{1C}$ adhesin. The $mrkD_{1C}$ and $mrkD_{1P}$ genes are not closely related, and this lack of similarity explains the inability of each gene probe to detect sequences from the heterologous gene. A comparison of the $MrkD_{1C}$ and $MrkD_{1P}$ polypeptides indicates significant differences in the amino acid sequences of these two molecules (Fig. 2), particularly in the N-terminal regions. A greater degree of amino acid sequence conservation is found within the C termini of the two polypeptides. The C-terminal domain of various fimbrial adhesins has been reported to possess four conserved sequence motifs that are thought to be necessary for folding and assembly (13). The MrkD adhesins share these motifs in hydrophobic clusters, which display a typical configuration strongly associated with amphipathic β-strands. As discussed below, *K. pneumoniae* 43816 demonstrates a distinct binding specificity associated with its type 3 fimbriae, and therefore the *mrkD* determinant from this isolate was also cloned and characterized. This specific gene is closely related to that of *K. pneumoniae* UIR079 and IApc35, and a comparison of the amino sequences of the gene products indicates a close similarity (Fig. 2). In fact, the $mrkD_{1C}$ genes are allelic variants, and their gene products differ at only two small regions within the N terminus of the polypeptides. This close relatedness explains why the *K. pneumoniae* 43816 *mrkD* gene is recognized by the gene probe derived from strain IApc35. The lack of reactivity of the *K. pneumoniae* UIR079 and 43816 MrkD proteins with serum raised against the N terminus of MrkD from strain IA565 is consistent with the observed differences in the amino acid sequences at this region. Serum raised against the major fimbrial subunit, MrkA, reacts with the MrkA polypeptides of all fimbriate strains examined (Fig. 5), confirming the serologic relatedness of the type 3 fimbriae reported by Old and Adegbola (22).

We have previously demonstrated that type V collagen-binding specificity is a function of the $MrkD_{1P}$ polypeptide and that all strains of *Klebsiella* possessing this adhesin exhibit identical binding properties (16, 32). We have now demonstrated that the possession of the $MrkD_{1C}$ fimbria-associated protein is frequently correlated with the ability of bacteria to bind to both type IV and type V collagen. Both fimbriate bacteria and cell-free, purified fimbriae exhibited identical binding specificities with those appendages possessing the $MrkD_{1c}$ polypeptide adhering to collagen type IV and type V. Interestingly, the fimbriae isolated from *K. pneumoniae* 43816 could not be shown to bind to any of the extracellular matrix proteins used in our assays. However, the bacteria and purified fimbriae are strongly hemagglutinating, suggesting that these appendages do function in vitro as adhesins. In fact, using the $mrkD_{1P}$ gene, we have previously shown that the MrkD polypeptide is responsible for MR/KHA activity and binding to type V collagen (12). Therefore, hemagglutination by *K. pneumoniae* 43816 is most likely a function of $MrkD_{1C}$. Since the only major differences in the $MrkD_{1C}$ polypeptides of *K. pneumoniae* UIR079 and 43816 are found at two sites within the N termini of these molecules, it is possible that the observed receptor-binding specificity is a function of these regions. Currently, we are investigating this hypothesis, using site-specific mutagenesis of the two *mrkD* genes.

A small number of the *Klebsiella* isolates examined in this study, as exemplified by *K. pneumoniae* 43816, did not mediate binding to any of the target proteins used in our assays. It is not possible to conclude that these strains represent one group exhibiting an identical binding pattern. However, all of these strains clearly possess an *mrkD* gene that is closely related to

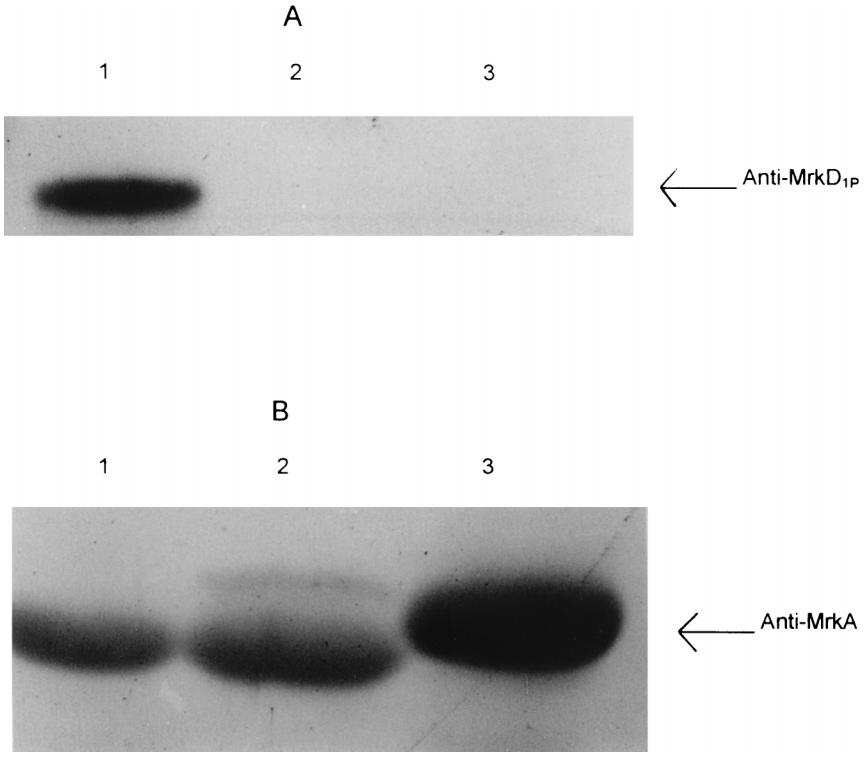

FIG. 5. Immunoblots of purified fimbriae with anti-MrkD (A) and anti-MrkA (B) sera. Fimbriae were purified from *K. pneumoniae* IA565 (lane 1), *K. pneumoniae* IApc35 (lane 2), and *K. pneumoniae* UIR079 (lane 3). The anti-MrkD serum was raised against a synthetic polypeptide representing the first 10 amino acids of $MrkD_{1P}$, and the anti-MrkA serum was raised against fimbriae purified from *E. coli* (pFK10).

mrkD$_{1C}$, since these strains hybridize to this gene probe. Differences in binding specificity have been associated with allelic variation in the adhesin gene of the type 1 fimbrial gene cluster of *E. coli* (29–31). Also, the PapG adhesin of P fimbriae demonstrates variability in binding activity dependent upon the *papG* gene present in the gene cluster (17, 18). The *mrkD*$_{1C}$ genes of the type 3 fimbrial gene cluster are also comprised of at least two allelic variants. However, the plasmid-borne *mrkD*$_{1p}$ gene does not demonstrate a relatedness to the *mrkD*$_{1C}$ alleles, and the nucleotide sequences of these two genes have diverged such that the gene probes do not cross-hybridize.

In summary, we have demonstrated that most strains of *K. pneumoniae* produce type 3 fimbriae possessing an MrkD polypeptide different from that associated with the plasmid-encoded, type-V-collagen-binding molecule (16). This latter MrkD adhesin is found primarily in fimbriate strains of *K. oxytoca* and less frequently among *K. pneumoniae* isolates. Most strains of *K. pneumoniae* produce an MrkD molecule that is not encoded by a plasmid. In these strains, fimbriae carrying one type of MrkD adhesin can be shown to mediate binding to type IV and type V collagen. However, in some *Klebsiella* strains, another MrkD variant present in fimbriae exhibits the characteristic MR/KHA activity but cannot be shown to bind in vitro to a range of ECMPs. Investigations into the molecular biology of these naturally occurring MrkD polypeptides could provide information on the nature of the receptors recognized by type 3 fimbriae. Since type 3 fimbriate enterobacteria are frequent opportunistic pathogens of immunocompromised individuals (6, 8, 15, 35), the role of MrkD molecules in binding to damaged epithelial surfaces is currently being investigated.

REFERENCES

1. **Allen, B. L., G.-F. Gerlach, and S. Clegg.** 1991. Nucleotide sequence and functions of *mrk* determinants necessary for expression of type 3 fimbriae in *Klebsiella pneumoniae*. J. Bacteriol. **173:**916–920.
2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. John Wiley and Sons, New York, N.Y.
4. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459–472.
5. **Clegg, S., K. T. Korhonen, B. D. Hornick, and A.-M. Tarkkanen.** 1994. Type 3 fimbriae of the Enterobacteriaceae, p. 97–104. *In* P. Klem (ed.), Fimbriae: adhesion, genetics, biogenesis, and vaccines. CRC Press, Boca Raton, Fla.
6. **Craven, D. E., T. W. Barber, K. A. Steger, and M. A. Montecalvo.** 1990. Nosocomial pneumonia in the 1990s: update of epidemiology and risk factors. Semin. Respir. Infect. **5:**157–172.
7. **Duguid, J. P.** 1959. Fimbriae and adhesive properties in Klebsiella strains. J. Gen. Microbiol. **21:**271–286.
8. **Duma, R. J.** 1985. Gram-negative bacillary infections. Pathogenic and pathophysiologic correlates. Am. J. Med. **78:**154–164.
9. **Gerlach, G.-F., B. L. Allen, and S. Clegg.** 1988. Molecular characterization of the type 3 (MR/K) fimbriae of *Klebsiella pneumoniae*. J. Bacteriol. **170:**3547–3553.
10. **Gerlach, G.-F., B. L. Allen, and S. Clegg.** 1989. Type 3 fimbriae among enterobacteria and the ability of spermidine to inhibit MR/K hemagglutination. Infect. Immun. **57:**219–224.
11. **Gerlach, G. F., and S. Clegg.** 1988. Characterization of two genes encoding antigenically distinct type-1 fimbriae of Klebsiella pneumoniae. Gene **64:** 231–240.
12. **Gerlach, G. F., S. Clegg, and B. L. Allen.** 1989. Identification and characterization of the genes encoding the type 3 and type 1 fimbrial adhesins of *Klebsiella pneumoniae*. J. Bacteriol. **171:**1262–1270.
13. **Girardeau, J. P., and Y. Bertin.** 1995. Pilins of fimbrial adhesins of different member species of Enterobacteriaceae are structurally similar to the C-terminal half of adhesin proteins. FEBS Lett. **357:**103–108.
14. **Hornick, D. B., B. L. Allen, M. A. Horn, and S. Clegg.** 1992. Adherence to

respiratory epithelia by recombinant *Escherichia coli* expressing *Klebsiella pneumoniae* type 3 fimbrial gene products. Infect. Immun. **60:**1577–1588.
15. **Hornick, D. B., B. L. Allen, M. A. Horn, and S. Clegg.** 1991. Fimbrial types among respiratory isolates belonging to the family *Enterobacteriaceae*. J. Clin. Microbiol. **29:**1795–1800.
16. **Hornick, D. B., J. Thommandru, W. Smits, and S. Clegg.** 1995. Adherence properties of an *mrkD*-negative mutant of *Klebsiella pneumoniae*. Infect. Immun. **63:**2026–2032.
17. **Johnson, J. R., and J. J. Brown.** 1996. A novel multiply primed polymerase chain reaction assay for identification of variant papG genes encoding the Gal(alpha 1–4)Gal-binding PapG adhesins of Escherichia coli. J. Infect. Dis. **173:**920–926.
18. **Johnson, J. R., J. L. Swanson, T. J. Barela, and J. J. Brown.** 1997. Receptor specificities of variant Gal(alpha 1–4)Gal-binding PapG adhesins of uropathogenic Escherichia coli as assessed by hemagglutination phenotypes. J. Infect. Dis. **175:**373–381.
19. **Korhonen, T. K., E. Tarkka, H. Ranta, and K. Haahtela.** 1983. Type 3 fimbriae of *Klebsiella* sp.: molecular characterization and role in bacterial adhesion to plant roots. J. Bacteriol. **155:**860–865.
20. **Lin, J. J., M. Smith, J. Jessee, and F. Bloom.** 1992. DH11S: an *Escherichia coli* strain for preparation of single-stranded DNA from phagemid vectors. BioTechniques **12:**718–721.
21. **Ofek, I., and E. H. Beachey.** 1980. Bacterial adherence. Adv. Intern. Med. **25:**503–532.
22. **Old, D. C., and R. A. Adegbola.** 1985. Antigenic relationships among type-3 fimbriae of Enterobacteriaceae revealed by immunoelectronmicroscopy. J. Med. Microbiol. **20:**113–121.
23. **Old, D. C., and R. A. Adegbola.** 1982. Haemagglutinins and fimbriae of Morganella, Proteus and Providencia. J. Med. Microbiol. **15:**551–564.
24. **Old, D. C., and R. A. Adegbola.** 1983. A new mannose-resistant haemagglutinin in Klebsiella. J. Appl. Bacteriol. **55:**165–172.
25. **Old, D. C., and S. S. Scott.** 1981. Hemagglutinins and fimbriae of *Providencia* spp. J. Bacteriol. **146:**404–408.
26. **Old, D. C., A. Tavendale, and B. W. Senior.** 1985. A comparative study of the type-3 fimbriae of Klebsiella species. J. Med. Microbiol. **20:**203–214.
27. **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.
28. **Schurtz, T. A., D. B. Hornick, T. K. Korhonen, and S. Clegg.** 1994. The type 3 fimbrial adhesin gene (*mrkD*) of *Klebsiella* species is not conserved among all fimbriate strains. Infect. Immun. **62:**4186–4191.
29. **Sokurenko, E. V., V. Chesnokova, R. J. Doyle, and D. L. Hasty.** 1997. Diversity of the Escherichia coli type 1 fimbrial lectin. Differential binding to mannosides and uroepithelial cells. J. Biol. Chem. **272:**17880–17886.
30. **Sokurenko, E. V., H. S. Courtney, J. Maslow, A. Siitonen, and D. L. Hasty.** 1995. Quantitative differences in adhesiveness of type 1 fimbriated *Escherichia coli* due to structural differences in *fimH* genes. J. Bacteriol. **177:**3680–3686.
31. **Sokurenko, E. V., H. S. Courtney, D. E. Ohman, P. Klemm, and D. L. Hasty.** 1994. FimH family of type 1 fimbrial adhesins: functional heterogeneity due to minor sequence variations among *fimH* genes. J. Bacteriol. **176:**748–755.
32. **Tarkkanen, A. M., B. L. Allen, B. Westerlund, H. Holthofer, P. Kuusela, L. Risteli, S. Clegg, and T. K. Korhonen.** 1990. Type V collagen as the target for type-3 fimbriae, enterobacterial adherence organelles. Mol. Microbiol. **4:**1353–1361.
33. **Tarkkanen, A.-M., R. Virkola, S. Clegg, and T. K. Korhonen.** 1997. Binding of the type 3 fimbriae of *Klebsiella pneumoniae* to human endothelial and urinary bladder cells. Infect. Immun. **65:**1546–1549.
34. **Voller, A., D. Bidwell, and A. Bartlett.** 1980. Enzyme-linked immunosorbent assay, p. 359–371. *In* N. R. Rose and H. Friedman (ed.), Manual of clinical immunology, 2nd ed. ASM Press, Washington, D.C.
35. **Williams, P., and J. M. Tomas.** 1990. The pathogenicity of Klebsiella pneumoniae. Rev. Med. Microbiol. **1:**196–204.
36. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.

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