Adherence Properties of an *mrkD*-Negative Mutant of *Klebsiella pneumoniae*

DOUGLAS B. HORNICKE,^{1*} JESSIE THOMMANDRU,¹ WILLIAM SMITS,² AND STEVEN CLEGG³

Departments of Internal Medicine,¹ Pediatrics,² and Microbiology,³ University of Iowa College of Medicine,
Iowa City, Iowa 52242-1081

Received 4 November 1994/Returned for modification 28 December 1994/Accepted 13 February 1995

The role of the *mrkD* gene in attachment by a type 3 fimbriate *Klebsiella pneumoniae* strain was further characterized. A clinical isolate, *K. pneumoniae* IA565, was found to contain two copies of the gene encoding the fimbrial subunit, *mrkA*, and one copy of the gene encoding the adhesin subunit, *mrkD*. One copy of *mrkA* was located on the bacterial chromosome, and the other copy was associated with *mrkD* and located on a plasmid. The plasmid-borne *mrk* gene cluster was lost when *K. pneumoniae* IA565 was subcultured serially in broth at 44°C. The resulting *mrkD*-negative strain, designated *K. pneumoniae* IApc35, did not exhibit the following adherence characteristics associated with *K. pneumoniae* possessing MrkD-positive fimbriae: agglutination of tannic acid-treated human erythrocytes and attachment to trypsinized human buccal cells. However, *K. pneumoniae* IApc35 produced type 3 fimbriae that were composed of the characteristic 21.5-kDa major fimbrial subunit, were reactive with specific serum, and were visualized specifically by immunoelectron microscopy. *K. pneumoniae* IApc35 retained a copy of the *mrkA* gene on its chromosome. This *mrkA*-containing gene cluster could be complemented by a recombinant plasmid carrying only the *mrkD* gene, resulting in restoration of the *K. pneumoniae* IA565-like adhesive phenotype and demonstration of type 3 filament-associated MrkD subunits by using colloidal gold labeling and immunoelectron microscopy. These data indicate that *K. pneumoniae* may contain multiple copies of the *mrk* genes which may be present simultaneously on both plasmid and chromosomal DNAs and which may encode fimbriae with different binding specificities.

Klebsiella pneumoniae was first recognized by Friedlander (9) in 1882 as a virulent pneumonic pathogen, and it remains a cause of severe pneumonia, with a mortality rate exceeding 30% despite seemingly appropriate antibiotic therapy (5, 7). Strains of *K. pneumoniae* are implicated in up to 35% of the gram-negative nosocomial pneumonias, and among community-acquired pneumonias they follow *Haemophilus influenzae* as the most frequent cause of gram-negative bacillary pneumonia (3, 10, 30). Colonization precedes pneumonia, and adherence by the bacteria is required to overcome clearance mechanisms of the respiratory tract (5, 16, 18). We have previously demonstrated that recombinant *Escherichia coli* expressing *K. pneumoniae* type 3 fimbriae adheres to respiratory cells (17).

The type 3 fimbriae are detected by specific agglutination of tannic acid-treated erythrocytes, resulting in a mannose-resistant, *Klebsiella*-like hemagglutination (MR/K HA). Six genes of the *mrk* gene cluster have been cloned and sequenced, and they are required for functional expression of *Klebsiella* type 3 fimbriae in recombinant *E. coli* (1). Analogous to the type 1 and P fimbrial systems of *E. coli* (20, 25), a single gene (*mrkA*) encodes the major type 3 fimbrial subunit, whereas the adhesin subunit is encoded by *mrkD*. The other *mrk* genes encode ancillary proteins that facilitate transport and organization of the fimbrial subunits at the outer membrane (1). When expressed by *E. coli* possessing recombinant plasmids, the *mrk* gene products mediate adherence to type V collagen (32), human bronchial cells, and trypsinized human buccal cells (17). The basement membrane region and the basolateral surface of the epithelial cells are specific sites where attachment occurs in both the renal and pulmonary epithelium (17, 32). Similarly, a

phenotypically type 3 fimbriate strain of *K. pneumoniae* demonstrated these binding properties, whereas nonfimbriate bacteria did not (17).

The current study examined adherence to human respiratory cells by *K. pneumoniae* strains differing in the *mrkD* genotype. Most of the previous investigations demonstrating MrkD-mediated adherence to eucaryotic cells were performed with *E. coli* transformed with recombinant plasmids (17, 32). However, the expression of type 3 fimbriae by recombinant strains of *E. coli* may differ quantitatively from that by *K. pneumoniae* IA565, from which the *mrk* genes were cloned (12). Furthermore, although a phenotypically type 3 fimbriate strain of *K. pneumoniae* under specific culture conditions will demonstrate binding properties similar to those of the recombinant *E. coli*, culture conditions which alter type 3 fimbrial expression by *K. pneumoniae* may influence additional surface components and affect attachment. For these reasons an *mrkD*-negative derivative of *K. pneumoniae* IA565 was isolated and used to further define the role of the MrkD adhesin in facilitating attachment to human respiratory cells. We also determined that the clinical isolate *K. pneumoniae* IA565 possesses the type V collagen-binding MrkD protein as the product of a plasmid-borne gene. Loss of the plasmid-encoded MrkD protein results in loss of MR/K HA and adherence to trypsinized human buccal epithelial cells. Complementation of the *mrkD*-negative mutant of *K. pneumoniae* by a recombinant plasmid possessing only *mrkD* results in restoration of the adhesive phenotype.

MATERIALS AND METHODS

Bacterial strains. *K. pneumoniae* IA565 was originally obtained from the University of Iowa Hospitals and Clinics Special Microbiology Laboratory. Recombinant plasmids were maintained in *E. coli* HB101 (12). *K. pneumoniae* IApc35 is an *mrkD*-negative derivative of *K. pneumoniae* IA565, and its isolation is described below.

Manipulation of recombinant plasmids and *Klebsiella*-derived plasmids. The recombinant plasmid pFK10 contains a 6.8-kb fragment derived from *K. pneu-*

* Corresponding author. Mailing address: Department of Internal Medicine, University of Iowa College of Medicine, Room C33-C GH UIHC, 200 Hawkins Dr., Iowa City, IA 52242-1081. Phone: (319) 356-8266. Fax: (319) 353-6406.

moniae IA565 that encodes the phenotypic expression of type 3 fimbriae. The plasmid pFK52 contains a 1.5-kb insert derived from pFK10 and functionally consists of only the adhesin gene, *mrkD* (0.9 kb). Both of these plasmids have been described in detail previously (11, 13). Plasmid DNA was isolated by a commercially available method (Qiagen, Chatsworth, Calif.).

An *mrkD*-negative derivative of *K. pneumoniae* IA565 was obtained by subculture in Luria broth at 44°C. Organisms were plated for isolated colonies on L agar after being cultured at an elevated temperature, and individual colonies were screened for loss of the MR/K HA phenotype. The loss of this phenotype was confirmed by subculture on glycerol-Casamino Acids (GCAA) agar. GCAA agar has previously been shown to favor expression of type 3 fimbriae (12).

The introduction of recombinant plasmids into *K. pneumoniae* IApc35 was achieved with an ECM 600 electroporation pulse generator (BTX Inc., San Diego, Calif.) by adapting the methods of Dower et al. (8). Bacteria were prepared for electroporation by extensive washing in deionized water at 2°C and frozen at -80°C in deionized water containing 10% glycerol at a concentration of 10^{11} cells per ml. Transformation rates of 2×10^7 CFU/ μ g of DNA were obtained at a field strength of 20 kV/cm and time constants of 0.6 to 1.2 ms.

Southern hybridization. Genomic DNA was prepared according to standard procedures (G-Nome; Bio101, La Jolla, Calif.). DNA preparations (approximately 5 to 10 μ g) were restricted with commercially obtained enzymes according to the manufacturers' instructions. After electrophoresis through a 0.5% agarose gel, the DNA was denatured with 0.5 N NaOH-1.5 M NaCl, neutralized with 1.0 M Tris-1.5 M NaCl, and transferred by capillary action to a positively charged nylon membrane (Boehringer Mannheim, Indianapolis, Ind.). DNA was fixed to the membrane by UV cross-linking (Stratalinker 1800; Stratagene, La Jolla, Calif.). Nucleotide probes, approximately 400 bases in length, specific for genes *mrkA* and *mrkD* were prepared as described elsewhere (29) by PCR with primers derived from sequences within each gene. After the probes were labeled with a nonradioactive label (Genius system; Boehringer Mannheim), hybridization was performed under high-stringency conditions. Regions of homology were detected by autoradiography with the Lumiphos reagent according to the manufacturer's instructions (Genius system; Boehringer Mannheim).

Hemagglutination and immunologic assay. Bacterial suspensions (approximately 10^{11} CFU/ml) were prepared from organisms subcultured on GCAA agar. MR/K HA was detected by D-mannose-resistant agglutination of a 3% (vol/vol) suspension of human erythrocytes (type A) pretreated with 0.01% tannic acid (26). The presence of fimbrial antigens on the surface of the bacteria was detected with fimbria-specific polyclonal antiserum as previously described (12).

Analysis of type 3 fimbriae. Surface-associated fimbrial appendages were observed by transmission electron microscopy (Hitachi H7000) of bacteria that were negatively stained with 2% phosphotungstic acid. This procedure has been described in detail elsewhere (12). Colloidal gold beads (5-nm diameter) coupled to anti-rabbit immunoglobulin G (Auroprobe EM, Amersham, United Kingdom) at a 1:50 dilution in 0.01 M phosphate-buffered saline (PBS) supplemented with 0.5% bovine serum albumin and 0.1% sodium azide were used to detect polyclonal type 3 fimbria-specific or MrkD-specific antiserum coating the filaments.

Type 3 fimbriae were purified from *K. pneumoniae* IA565 and IApc35 as previously described (12). The fimbrial preparations were examined by electron microscopy, and the mobilities and molecular weights of the major fimbrial subunit (MrkA) from each were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15% acrylamide, 0.3% bisacrylamide) with Coomassie brilliant blue staining.

Surface hydrophobicity assay. Bacteria were harvested from agar plates and suspended in PBS. The hydrophobicities of bacterial suspensions were determined by partition between aqueous and organic phases by the methods of Rosenberg et al. (28).

Bacterial adherence assays. Bacteria were labeled with [35 S]methionine as described previously (17). All *K. pneumoniae* strains used in the adherence assays were subcultured on GCAA agar, and the appropriate fimbrial phenotype was confirmed prior to labeling. Also, the addition of 3% α -methylmannoside to the adherence assay had no impact on the results.

(i) **Adherence to human buccal cells.** Previous investigations indicated that the optimal adherence to human buccal cells by type 3 fimbriate bacteria occurs after the buccal cells are trypsinized (17). Washed, trypsinized buccal cells were mixed with 35 S-labeled bacteria in a bacterium-to-cell ratio of 1,000:1 and were incubated for 90 min at 37°C. The adherence assay and quantification of the results were performed as previously described (17).

(ii) **Adherence to human bronchial epithelial cells.** A human bronchial epithelial cell line (HBE) was kindly provided by Dieter Gruenert (Cardiovascular and Cancer Research Institute, San Francisco, Calif.). This cell line was derived from human segmental bronchial epithelial cells transformed with the large T antigen of an origin-defective simian virus 40 (14). The HBE cells were seeded at 10^5 cells per cm 2 on the 0.6-cm 2 surface of type IV collagen-coated (Sigma, St. Louis, Mo.) Millicell-HA inserts (Millipore, Bedford, Mass.) and were maintained in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μ g of streptomycin per ml. HBE cells express a polarized phenotype and can maintain resistances of greater than 1,000 Ω when cultured on permeable supports. HBE cells were utilized for experiments between passages 3 and 20.

FIG. 1. Analysis of plasmid DNA. Photographic reversals of ethidium bromide-stained DNA electrophoresed in agarose gels are shown. (A) Uncut plasmid DNA from *K. pneumoniae* IA565 (lane 1) and *K. pneumoniae* IApc35 (lane 2). The plasmid in lane 1 and the corresponding plasmid in lane 2 (arrow) have identical mobilities. The *mrkD* probe hybridized to the plasmid DNA (arrowhead) prepared from *K. pneumoniae* IA565 and did not hybridize to plasmid DNA from *K. pneumoniae* IApc35 (see Fig. 2). (B) Plasmid DNA isolated from *K. pneumoniae* IA565 (lanes 1 and 3) or *K. pneumoniae* IApc35 (lanes 2 and 4). DNA was restriction digested with *Eco*RI (lanes 1 and 2) or *Hind*III (lanes 3 and 4). Fewer restriction fragments were present in plasmid DNA from *K. pneumoniae* IApc35.

Adherence of bacteria to HBE cells was determined by using bacteria labeled with [35 S]methionine as described above. HBE cells grown on the permeable inserts were washed twice with serum-free medium prior to being overlaid with bacteria resuspended in Hanks balanced salt solution plus 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer. The bacteria and HBE cells were incubated at a ratio of 1,000:1 at 37°C on a shaker platform. At the end of the incubation period, the HBE cell monolayers were washed with 6 volumes of PBS to remove nonadherent bacteria. The cells with adherent bacteria and the permeable support were dissolved with 0.5 N NaOH, and the counts associated with the cells were determined with a liquid scintillation counter (Beckman Instruments, Fullerton, Calif.). *E. coli* HB101, which does not adhere to HBE cells or to the collagen support, served as a negative control for these experiments. To model denuded epithelium, assays were performed in a similar manner after cells were removed by 25 mM EGTA [ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid]. This method was chosen because it is an effective means of removing cells without causing damage to the collagen matrix on which the HBE cells are grown (24, 33).

Statistical analysis. For the adherence assays, arithmetic means and standard deviations were calculated. Significant differences were determined by using Student's *t* test for independent variables. *P* values reported were for two-tailed tests.

RESULTS

Characteristics of *K. pneumoniae* IApc35. Following five passages of *K. pneumoniae* IA565 by serial subculturing in L broth at 44°C, approximately two-thirds of the colonies isolated on L agar at 37°C and subsequently subcultured on GCAA agar no longer demonstrated MR/K HA. *K. pneumoniae* IApc35 is a nonhemagglutinating derivative of *K. pneumoniae* IA565. *K. pneumoniae* IApc35 did not differ from *K. pneumoniae* IA565 in biotyping characteristics, antibiogram profile, or hydrophobicity characteristics.

Location of the *mrkD* gene. Conventional agarose electrophoresis of plasmid DNA preparations from *K. pneumoniae* IA565 and IApc35 indicated that each strain contained more than one plasmid (Fig. 1A). *K. pneumoniae* IA565 contained a larger plasmid which was not present in *K. pneumoniae* IApc35 (Fig. 1A, lane 1). *K. pneumoniae* IApc35 contained a smaller plasmid that was absent in the plasmid preparations from *K. pneumoniae* IA565 (Fig. 1A, lane 2). Both strains possessed one plasmid with identical mobilities. The restriction fragment profile of plasmid DNA from *K. pneumoniae* IApc35 (Fig. 1B,

FIG. 2. Southern hybridization analysis. Plasmid DNA from *K. pneumoniae* IA565 (lanes 1) or *K. pneumoniae* IApc35 (lanes 2) and total (chromosomal and plasmid) DNA from *K. pneumoniae* IA565 (lanes 3) or *K. pneumoniae* IApc35 (lanes 4) were restricted by *Hind*III. (A) Photographic reversal of an ethidium bromide-stained agarose gel containing *Hind*III-digested plasmid DNA. The specific plasmid DNA fragment of *K. pneumoniae* IA565 (lower band of the doublet at 8.5 kb), which hybridizes with *mrk* genes, is absent from the plasmid DNA of *K. pneumoniae* IApc35. (B and C) Results of hybridization with *mrkA* and *mrkD* probes, respectively.

lanes 2 and 4) demonstrated fewer fragments than that of plasmid DNA from *K. pneumoniae* IA565.

An *mrkA*-specific gene probe demonstrated that DNA from *K. pneumoniae* IA565 contained two copies of *mrkA*, while DNA from *K. pneumoniae* IApc35 contained only one copy. The *mrkA* gene probe hybridized to 8.5- and 5.0-kb *Hind*III fragments of total genomic (plasmid and chromosomal) DNA prepared from *K. pneumoniae* IA565, whereas it hybridized only to the 8.5-kb fragment of plasmid DNA preparations from the same strain (Fig. 2B). In contrast, the *mrkA* probe hybridized to the 5.0-kb fragment in genomic DNA but did not hybridize with any nucleotide sequences in plasmid DNA prepared from *K. pneumoniae* IApc35 (Fig. 2B).

Only plasmid DNA from *K. pneumoniae* IA565 demonstrated homology to the *mrkD* gene-specific probe, in contrast to plasmid DNA from *K. pneumoniae* IApc35 (Fig. 1A and 2C). However, the *mrkD* gene was detected in both total genomic and plasmid DNA preparations from *K. pneumoniae* IA565. Since genomic DNA comprises both chromosomal and plasmid DNAs, these results indicate that the loss of plasmid DNA sequences, demonstrated for the MR/K HA-negative strain, results in the absence of hybridization to homologous sequences by the *mrkD* probe.

Hemagglutination and immunologic assay. Type 3 fimbrial expression is greater for *K. pneumoniae* IA565 when it is subcultured on GCAA agar than when it is subcultured on L agar (12). *K. pneumoniae* IApc35 also has this characteristic when subcultured on GCAA agar, expressing high titers of type 3 fimbrial antigens, but it demonstrated no MR/K HA (Table 1). Hemagglutination and reactivity with fimbrial antiserum were demonstrated, however, when *K. pneumoniae* IApc35 was transformed with the recombinant plasmid, pFK10, containing the cloned *mrk* gene cluster. In addition, *K. pneumoniae* IApc35 expressed the fimbrial hemagglutinin when it was transformed with a plasmid that possesses only the *mrkD* gene. The increase in fimbrial expression by *K. pneumoniae* IApc35 transformants subcultured on L agar compared with that by

TABLE 1. Hemagglutination and anti-type 3 fimbrial serum titers^a

<i>K. pneumoniae</i> strain	Recombinant plasmid	MR/K HA titer ^b with:		Anti-type 3 fimbria titer with:	
		L agar	GCAA agar	L agar	GCAA agar
IA565	None	4	32	8	2,048
IApc35	None	0	0	8	4,096
	pFK10	128	256	4,096	4,096
	pFK52	16	32	64	4,096

^a Each value is the average from three tests. The growth medium was L agar or GCAA agar (see Materials and Methods).

^b Expressed as the reciprocal of the highest dilution causing visible MR/K HA. Undiluted specimens contained 10¹¹ CFU/ml.

the parental strain is most likely due to the increased copy number of the recombinant plasmids.

Analysis of type 3 fimbriae produced by *K. pneumoniae* IApc35. Morphologically identical fimbriae were observed on the surfaces of both *K. pneumoniae* IA565 and IApc35 (Fig. 3). Despite the loss of *mrkD* facilitating MR/K HA activity, *K. pneumoniae* IApc35 still produced fimbriae similar to the typical type 3 fimbriae observed on the surface of the parental strain. These appendages are 2 to 3 nm in diameter and exhibit properties that are consistent with type 3 fimbriae (12, 26). Immunogold particle staining confirmed that the fimbrial appendages observed on the surface of *K. pneumoniae* IApc35 were type 3 fimbriae (Fig. 3E).

Transformation of *K. pneumoniae* IApc35 by pFK52 resulted in fimbriate bacteria that appeared similar to *K. pneumoniae* IA565, whereas the pFK10 transformants were more densely fimbriate than *K. pneumoniae* IA565 (Fig. 3C and D). The *mrkD* gene product, encoded by pFK52, is incorporated into the type 3 fimbrial filament as shown by immunogold particle staining (Fig. 3F).

Fimbriae purified from both *K. pneumoniae* IA565 and *K. pneumoniae* IApc35 were examined by SDS-PAGE and were found to consist predominantly of a major fimbrial subunit, MrkA. Each had the same electrophoretic mobility and a molecular mass of 21.5 kDa (Fig. 4). These results were consistent with prior SDS-PAGE analysis of *K. pneumoniae* type 3 fimbriae (12).

Adherence to respiratory cells. Adherence to trypsinized human buccal cells by *E. coli* HB101 and *K. pneumoniae* IA565, IApc35, and IApc35 containing recombinant plasmids is shown in Table 2. *E. coli* HB101 adhered poorly to trypsinized buccal cells. Type 3 fimbriate *K. pneumoniae* IA565 demonstrated increased adherence to the buccal cells compared with the nonhemagglutinating derivative. Bacterial binding to buccal cells was restored following transformation of *K. pneumoniae* IApc35 with pFK10. Also, adherence to buccal cells was demonstrated by *K. pneumoniae* IApc35 transformed with pFK52.

Bacterial adherence with the cultured HBE monolayers differed from that observed for the isolated human buccal cells (Table 2). *E. coli* HB101 adhered poorly to an intact HBE monolayer and to the substrate exposed after the cell monolayer was removed by 25 mM EGTA. *K. pneumoniae* IA565 also adhered poorly to the intact monolayer, but it bound to the collagen matrix support for the HBE cells after the monolayer was removed. *K. pneumoniae* IApc35 also demonstrated adherence to the exposed collagen matrix and little binding to the intact HBE monolayer (Table 2). In addition, both the pFK10 and pFK52 transformants of *K. pneumoniae* IApc35 adhered to the intact monolayer in low numbers compared with adherence to the exposed matrix support.

FIG. 3. Transmission electron micrographs. (A) *K. pneumoniae* IA565; (B) *K. pneumoniae* IApc35; (C) *K. pneumoniae* IApc35 transformed with pFK52; (D) *K. pneumoniae* IApc35 transformed with pFK10; (E) *K. pneumoniae* IApc35, 1:1,000 dilution of polyclonal anti-type 3 fimbrial antiserum visualized with gold particle (5 nm)-conjugated anti-rabbit immunoglobulin G; and (F) *K. pneumoniae* IApc35 transformed with pFK52, 1:50 dilution of polyclonal anti-MrkD antiserum visualized with gold particle (5nm)-conjugated anti-rabbit immunoglobulin G. Bars, 100 nm.

FIG. 4. SDS-15% polyacrylamide gel stained with Coomassie blue. The type 3 fimbrial subunit (arrow), MrkA, is the major fimbrial protein visualized in the purified type 3 fimbrial preparations from *K. pneumoniae* IApc35 (lane 1) and *K. pneumoniae* IA565 (lane 2). The electrophoretic mobilities of protein standards are shown on the right.

DISCUSSION

We have previously reported that recombinant strains of *E. coli* expressing the *K. pneumoniae* type 3 fimbrial adhesin, MrkD, adhere to trypsinized buccal epithelial cells and the basement membranes of human bronchial epithelium (17). Type V collagen, a component of the epithelial basement membrane, is a target for MrkD, the type 3 fimbrial adhesin expressed by *K. pneumoniae* IA565 (32). In the current study we have isolated an *mrkD*-negative derivative of *K. pneumoniae* IA565 and have used it to further define the role of the MrkD adhesin in facilitating attachment.

A nonhemagglutinating but fimbriate mutant, *K. pneumoniae* IApc35, was derived by repeated subculture of *K. pneumoniae* IA565 in broth at 44°C. *K. pneumoniae* IApc35 produces type 3-like fimbrial appendages, but unlike those of the parental strain, these fimbriae do not mediate MR/K HA of human erythrocytes. Analysis of plasmid DNA from *K. pneumoniae* IApc35, in contrast to that from *K. pneumoniae* IA565, demonstrated the absence of sequences homologous to *mrk* genes including the adhesin gene, *mrkD*. A copy of the *mrkA* determinant, however, was retained on the chromosome of *K. pneumoniae* IApc35. Thus, chromosomal and plasmid DNAs from *K. pneumoniae* IApc35 lack an intact *K. pneumoniae* IA565-like adhesin gene. We suggest that a deletion event occurred to account for these observations. We do not know the precise mechanism by which *mrk* sequences are deleted from the plasmid following growth at 44°C. The elevated tem-

perature of incubation may contribute to the instability of *mrkD*-containing plasmid DNA sequences, since nonhemagglutinating mutants occurred at a high frequency under such conditions.

The results reported here indicate that *K. pneumoniae* IA565 possess two *mrkA* genes. One gene is carried on the plasmid in the wild-type strain, comprising part of the gene cluster containing DNA sequences homologous to the *mrkD* gene. This gene cluster is represented on the recombinant plasmid pFK10 (1). The second copy of *mrkA* is located on the chromosome. Other investigations have demonstrated up to four copies of closely related P fimbrial genes on the *E. coli* chromosome (15, 27). It has been hypothesized that multiple copies of the *pap* and *pap*-related gene clusters may occur within the same strain as the result of selection pressure and horizontal transfer of DNA from other *E. coli* organisms (2, 22). That two copies of the *mrk* genes are present in the *K. pneumoniae* IA565 genome suggests that *mrk* genes may be part of a mobile element for genetic exchange in some instances, although we have no specific data supporting this possibility. The data indicating that the chromosomal *mrk* gene cluster is functional and appears to be duplicated (except for the *mrkD* gene) on a plasmid also suggest that genetic transfer of the *mrk* gene cluster may occur. Genes carried on plasmids, however, frequently have very little homology to the chromosomal genes (4). Since the *mrk* gene cluster on the plasmid includes a gene for a distinct adhesin, the duplicated *mrkA* and ancillary genes may have been acquired to gain an additional adherence phenotype. Alternatively, since the plasmid is large, it may contain other genes (currently unidentified) advantageous to the bacterium, and the *mrk* gene cluster may have been acquired coincidentally along with these genes. Recent data suggest that the *mrkD* gene is not common among clinical isolates of *K. pneumoniae* (29). We speculate that the plasmid-borne *mrk* gene cluster containing the *mrkD* gene may have originated among other genera or *Klebsiella* species.

MR/K HA activity can be restored in *K. pneumoniae* IApc35 by complementation with recombinant plasmids possessing *mrkD*, and the plasmid-derived *mrkD* gene product is incorporated into the fimbriae encoded by the chromosomal gene cluster. This suggests that *K. pneumoniae* isolates can incorporate heterologous adhesins into the fimbrial appendage. Recent data examining the *E. coli* type 1 and F1C fimbriae, whose major structural proteins demonstrate approximately 60% homology, indicate that structural components can be exchanged between two fimbrial systems to form hybrid structures that differ in receptor specificity (19). Alternatively, the presence of

TABLE 2. Adherence of *K. pneumoniae* to trypsinized buccal cells and to cultured bronchial epithelial monolayers compared with that to synthetic basal lamina

Bacterial strain	Recombinant plasmid	MR/K HA	No. of bacteria/cell (mean $\pm$ SD) ^a	No. of bacteria/filter (mean $\pm$ SD) (10 ⁶) ^b		
				Confluent HBE monolayer ^c	HBE cells removed ^d	P
<i>E. coli</i> HB101	None	—	2.6 $\pm$ 1.8 ^e	0.98 $\pm$ 0.11	0.92 $\pm$ 0.06	NS ^f
<i>K. pneumoniae</i> IA565	None	+	35.9 $\pm$ 9.7	1.3 $\pm$ 0.4	5.6 $\pm$ 1.7	0.005
<i>K. pneumoniae</i> IApc35	None	—	5.8 $\pm$ 3.6 ^e	1.4 $\pm$ 0.1	3.8 $\pm$ 0.8	<0.001
	pFK10	+	59.0 $\pm$ 15.9 ^g	2.2 $\pm$ 0.4	6.3 $\pm$ 3.4	0.006
	pFK52	+	61.2 $\pm$ 15.2 ^g	1.9 $\pm$ 0.4	9.9 $\pm$ 4.4	<0.001

^a Each adherence assay was performed at least five times with different samples of buccal cells.

^b Each value is the average from eight tests.

^c Only wells in which the transepithelial resistance was >1,000 Ω were used.

^d Cells were removed by 25 mM EGTA.

^e Significantly different from the value for *K. pneumoniae* IA565 ($P = 0.0001$).

^f NS, not significant.

^g Significantly different from the value for *K. pneumoniae* IApc35 ($P = 0.007$).

large amounts of the MrkD adhesin, resulting from the gene being carried on a recombinant plasmid, may influence the adhesive phenotype of these transformants. Thus, it remains possible that *K. pneumoniae* IA565 possesses mechanisms to ensure that only homologous adhesins are integrated into the fimbriae when both gene clusters are present in low copy number.

The synthetic basement membrane material underlying the HBE monolayer was exposed without altering its integrity, resulting in adherence to the basement membrane material by the fimbriate strains, including the nonhemagglutinating *K. pneumoniae* IApc35. Attachment by this strain may result from the expression of an adhesin subunit distinct from that of the plasmid-encoded MrkD. Thus, *K. pneumoniae* IA565 may be capable of producing type 3 fimbriae that possess distinct receptor-binding specificities, analogous to the case for *E. coli*. For example, *E. coli* J96 contains both the *pap* and *pap*-related fimbrial operons which encode P fimbriae of the F13 serotype, but they have different adhesin specificities (21, 31). MrkD, the plasmid-borne adhesin of *K. pneumoniae* IA565, binds type V collagen (32), and currently we are determining whether the *mrk* gene cluster on the chromosome possesses a distinct adhesin determinant.

None of the *K. pneumoniae* strains attached to the confluent HBE monolayers in significant numbers; however, these strains attached to the material underlying the monolayers. Type V collagen has been identified as a constituent of epithelium basement membrane (23). Although *K. pneumoniae* IApc35 does not bind to type V collagen (29), another basement membrane matrix compound(s) may be a more suitable target. Our results indicate that the type 3 fimbriate *K. pneumoniae* strains bind to damaged buccal epithelial cells and disrupted bronchial epithelial monolayers, which is consistent with observations that type 3 fimbriate members of the family *Enterobacteriaceae* are most frequently associated with infection of compromised epithelial surfaces. Respiratory tissue injury does occur for multiple reasons in hospitalized or chronically ill patients (6, 18). Such an environment would probably provide a suitable niche for the binding of *K. pneumoniae*.

In this report we have described an *mrkD*-negative mutant of *K. pneumoniae* IA565. The MrkD adhesin and an MrkA fimbrial subunit in *K. pneumoniae* IA565 are encoded by a plasmid-borne gene cluster. This strain also possesses an additional *mrkA* gene that most likely comprises part of a separate *mrk* gene cluster. This assumption is supported by the data showing that the loss of plasmid-borne *mrk* genes does not result in the loss of fimbrial expression but does result in a different adherence phenotype. Currently, we are investigating the diversity and binding specificities of type 3 fimbrial adhesins in *Klebsiella* species in order to determine whether discrete classes of adhesin molecules can be implicated in epithelial colonization of the human host.

ACKNOWLEDGMENTS

We thank Geneva Ford for her editorial help and expert secretarial assistance and the University of Iowa Electron Microscopy Core for their helpful assistance.

This work was supported in part by the American Lung Association, the American Lung Association of Iowa, and the Cystic Fibrosis Foundation RDP at the University of Iowa. D.B.H. is also supported by a clinical investigator award from the NHLBI (K08 HL02559).

REFERENCES

- Allen, B. L., G.-F. Gerlach, and S. Clegg. 1991. The nucleotide sequence and functions of *mrk* determinants necessary for expression of type 3 fimbriae in *Klebsiella pneumoniae*. *J. Bacteriol.* 173:916-920.
- Arthur, M., C. Campanelli, R. D. Arbeit, C. Kim, S. Steinbach, C. E. Johnson, R. H. Rubin, and R. Goldstein. 1989. Structure and copy number of gene clusters related to the *pap* P-adhesin operon of uropathogenic *Escherichia coli*. *Infect. Immun.* 57:314-321.
- Centers for Disease Control. 1986. Nosocomial infections surveillance, 1984. *Morbidity Mortal. Weekly Rep.* 35:17SS-29SS.
- Couturier, M., F. Bex, P. L. Beraquist, and W. K. Maas. 1988. Identification and classification of bacterial plasmids. *Microbiol. Rev.* 52:375-395.
- Craven, D. E., L. M. Kunches, D. A. Lichtenberg, N. R. Kollisch, A. Barry, T. C. Heeren, and W. R. McCabe. 1988. Nosocomial infection and fatality in medical and surgical intensive care unit patients. *Arch. Intern. Med.* 148:1161-1168.
- Dal Nogare, A. R., G. B. Toews, and A. K. Pierce. 1987. Increased salivary protease precedes Gram-negative bacillary colonization in postoperative patients. *Am. Rev. Respir. Dis.* 135:676-681.
- de la Torre, M. G., J. Romero-Vivas, J. Martinez-Veltran, A. Guerrero, et al. 1985. *Klebsiella* bacteremia: an analysis of one hundred cases. *Rev. Infect. Dis.* 7:143-150.
- Dower, W. J., J. F. Miller, and C. W. Ragsdale. 1988. High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res.* 16:6127-6145.
- Friedlander, C. 1882. Über die schizomyceten bei der acuten fibrosen pneumoniae. *Arch. Path. Anat. Physiol. Klin. Med.* 87:319-324.
- Garb, J. L., R. B. Brown, J. R. Garb, and R. W. Tuthill. 1978. Differences in the etiology of pneumonias in the nursing home and community patients. *JAMA* 240:2169-2172.
- 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.
- Gerlach, G.-F., and S. Clegg. 1988. Cloning and characterization of the gene cluster encoding type 3 fimbriae of *Klebsiella pneumoniae*. *FEMS Microbiol. Lett.* 49:377-383.
- 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.
- Gruenert, D. C., C. B. Busbaum, M. J. Welsh, M. Li, W. E. Finkbeiner, and J. A. Nadel. 1988. Characterization of human tracheal epithelial cells transformed by an origin-defective simian virus 40. *Proc. Natl. Acad. Sci. USA* 85:5951-5955.
- High, N. J., B. A. Hales, K. Jann, and G. J. Boulnois. 1988. A block of urovirulence genes encoding multiple fimbriae and hemolysin in *Escherichia coli* O4:K12:H⁻. *Infect. Immun.* 56:513-517.
- Higuchi, J. H., and W. G. Johanson. 1992. Colonization and bronchopulmonary infection. *Clin. Chest Med.* 3:133-142.
- 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.
- Johanson, W. G., A. K. Pierce, J. P. Sanford, and G. R. Thomas. 1972. Nosocomial respiratory infections with gram-negative bacilli: the significance of colonization of the respiratory tract. *Ann. Intern. Med.* 77:701-706.
- Klemm, P., B. Christiansen, B. Kreft, R. Marre, and H. Bergmans. 1994. Reciprocal exchange of minor components of type 1 and F1C fimbriae results in hybrid organelles with changed receptor specificities. *J. Bacteriol.* 176:2227-2234.
- Lund, B., F. Lindberg, B.-I. Marklund, and S. Normark. 1987. The *papG* protein is the α -D-galactopyranosyl-(1 $\rightarrow$ 4)- β -D-galactopyranose-binding adhesin of uropathogenic *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 84:5898-5902.
- Lund, B., B.-I. Marklund, N. Stromberg, F. Lindberg, K.-A. Karlsson, and S. Normark. 1988. Uropathogenic *Escherichia coli* can express serologically identical pili of different receptor binding specificities. *Mol. Microbiol.* 2:255-263.
- Marklund, B.-I., J. M. Tennent, E. Garcia, A. Hamers, M. Baga, F. Lindberg, W. Gastra, and S. Normark. 1992. Horizontal gene transfer of the *Escherichia coli pap* and *prs* pili operons as a mechanism for the development of tissue-specific adhesive properties. *Mol. Microbiol.* 6:2225-2242.
- Martinez-Hernandez, A., and P. Amenta. 1983. The basement membrane in pathology. *Lab. Invest.* 48:656-677.
- Martinez-Palomo, A., I. Meza, G. Beaty, and M. Cerejido. 1980. Experimental modulation of occluding junctions in a cultured transporting epithelium. *J. Cell Biol.* 87:746-754.
- Minion, F. C., S. N. Abraham, E. H. Beachey, and J. D. Goguen. 1986. The genetic determinant of adhesive fraction in type 1 fimbriae of *Escherichia coli* is distinct from the gene encoding fimbrial subunit. *J. Bacteriol.* 165:1033-1036.
- 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.
- Plos, K., T. Carter, S. Hull, R. Hull, and C. Svanborg Eden. 1990. Frequency and organization of *pap* homologous DNA in relation to clinical origin of uropathogenic *Escherichia coli*. *J. Infect. Dis.* 161:518-524.
- Rosenberg, M., D. Gutnick, and E. Rosenberg. 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity.

- FEMS Microbiol. Lett. **9**:29–33.
29. **Shurtz, T. A., D. B. Hornick, T. K. Korhonen, and S. Clegg.** 1994. Type 3 fimbrial adhesin gene (*mrkD*) of *Klebsiella* species is not conserved among all fimbriate strains. *Infect. Immun.* **62**:4186–4191.
30. **Stamm, W. E., S. M. Martin, and J. B. Bennett.** 1977. Epidemiology of nosocomial infections due to Gram-negative bacilli: aspects relevant to development and use of vaccines. *J. Infect. Dis.* **136**(Suppl.):S151–S160.
31. **Stromberg, N., B.-I. Marklund, B. Lund, D. Ilver, A. Hamers, W. Gaastra, K.-A. Karlsson, and S. Normark.** 1990. Host specificity of uropathogenic *Escherichia coli* depends on differences in binding specificity to Gal α 1-4Gal containing isoreceptors. *EMBO J.* **9**:2001–2010.
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. **Volberg, T., B. Geiger, J. Kartenbeck, and W. W. Franke.** 1986. Changes in membrane-microfilament interaction in intercellular adherens junctions upon removal of extracellular Ca²⁺ ions. *J. Cell. Biol.* **102**:1832–1842.