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Type 3 Fimbrial Shaft (MrkA) of *Klebsiella pneumoniae*, but Not the Fimbrial Adhesin (MrkD), Facilitates Biofilm Formation

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Isolates of *Klebsiella pneumoniae* are responsible for opportunistic infections, particularly of the urinary tract and respiratory tract, in humans. These bacteria express type 3 fimbriae that have been implicated in binding to eucaryotic cells and matrix proteins. The type 3 fimbriae mediate binding to target tissue using the MrkD adhesin that is associated with the fimbrial shaft comprised of the MrkA protein. The formation of biofilms in vitro by strains of *K. pneumoniae* was shown to be affected by the production of fimbriae on the bacterial surface. However, a functional MrkD adhesin was not necessary for efficient biofilm formation. Nonfimbriate strains were impaired in their ability to form biofilms. Using isogenic fimbriate and nonfimbriate strains of *K. pneumoniae* expressing green fluorescent protein it was possible to demonstrate that the presence of type 3 fimbriae facilitated the formation of dense biofilms in a continuous-flowthrough chamber. Transformation of nonfimbriate mutants with a plasmid possessing an intact *mrk* gene cluster restored the fimbrial phenotype and the rapid ability to form biofilms.

The ability of bacteria to form biofilms has been suggested to play a role in the pathogenesis of numerous bacterial species (4, 5, 31). The formation of biofilms by bacteria is a complex process, and even the production of a single-species biofilm requires several stages (23). A genetic analysis of factors facilitating the production of biofilms has been performed in some bacterial species, including the pathogens *Pseudomonas aeruginosa* (22) and *Vibrio cholerae* (32). These studies have suggested that both flagella and type IV pili play a role in the initial stages of biofilm formation on inanimate surfaces. Also, the production of an exopolysaccharide capsule by *P. aeruginosa* is influential on the establishment of biofilms, and exopolysaccharide production is increased in biofilm-associated bacteria compared to planktonic bacteria (6). In pulmonary infections of patients with cystic fibrosis, the production of the biofilm matrix is believed to play a role in protection of *P. aeruginosa* against host defense mechanisms and also prevents therapeutic concentrations of antibiotics from reaching the microbial cell.

Klebsiella pneumoniae is an opportunistic pathogen frequently associated with nosocomially acquired infections of the respiratory and urinary tract in compromised individuals (33). *K. pneumoniae* is characterized by its ability to produce several distinct types of adherence factors and also copious amounts of an acidic polysaccharide capsule (29). Growth of *Klebsiella* strains as a biofilm mass would be expected to occur under a variety of environmental conditions, because the organism can be isolated from both clinical and nonclinical sources. However, a genetic analysis of biofilm formation and the bacterial attributes produced by *K. pneumoniae* responsible for this formation has not been performed.

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The type 3 fimbriae are produced by most strains of *Klebsiella* and have been shown to play a role in binding in vitro to several types of cells and extracellular matrix proteins (ECMPs) (27). These fimbriae are produced using the chaperone-usher assembly pathway to form fimbrial appendages that are routinely detected by hemagglutination tests (14, 15). The complete nucleotide sequence of the gene cluster (*mrk*) encoding type 3 fimbrial expression has been reported by our group (2). The MrkA protein is the major structural component of type 3 fimbriae, whereas binding to collagen molecules is determined by the presence of the MrkD adhesin (27). Three allelic variants of the *mrkD* gene of *K. pneumoniae* have been reported and mediate binding to various components of the extracellular matrix. We have previously described the construction of *K. pneumoniae* MrkA and MrkD mutants that are either phenotypically nonfimbriate and nonadhesive (Fim^- Adh^-) or fimbriate but nonadhesive (Fim^+ Adh^-), respectively (27, 30). The role of type 3 fimbriae in biofilm formation by *K. pneumoniae* using these mutants was investigated. In addition, we constructed a bank of Tn*5* transposon mutants of *K. pneumoniae* to isolate mutants that were impaired in biofilm formation in vitro. Also, because phenotypic variation of type 3 fimbrial expression has been reported in *K. pneumoniae* (11), we compared the abilities of numerous fimbriate- and nonfimbriate-phase *K. pneumoniae* isolates, from diverse sources, to form biofilms using an in vitro assay.

MATERIALS AND METHODS

Bacterial strains, plasmids and media. The strains and recombinant plasmids used in this study are shown in Table 1. *K. pneumoniae* IA565 and its derivatives have previously been described by our group (27), and *K. pneumoniae* 43816 is a K2-positive capsular serotype obtained from the American Type Culture Collection (Manassas, Va.) and has been demonstrated to produce a severe inflammatory response in the murine model of airway infection (17). The cloning, characterization, and nucleotide sequence of the complete *mrk* gene cluster carried on plasmid pFK12 has been described elsewhere (2). All strains were grown on either L-agar or GCAA agar (9), for optimal type 3 fimbrial expression,

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype and/or relevant feature	Reference or source
K. pneumoniae strains		
IA565	Wild type; complete *mrk* gene cluster possessing collagen-binding $MrkD_{1P}$ adhesin	27
IApc35	Fim^+ Adh^- derivative of IA565 lacking the plasmid-borne $mrkD_{1P}$ gene	27
IAΔT3	*mrkB*::Tn*5*; Fim^- Adh^- derivative of IApc35 possessing a mutation in the fimbrial chaperone	30
43816	Type 3 fimbriate strain with $mrkD_{1C2}$ allele	ATCC; 27
MBM100	Nonfimbriate Tn*5* mutant of 43816	This study
Plasmids		
pFK12	Complete *mrk* gene cluster possessing the $mrkD_{1P}$ allele	2
pUT	Plasmid possessing the Mini-Tn*5Km2* element	8
pBBRMCS-1	GFP expressing, Cm resistant	24

supplemented with appropriate antibiotics when necessary. Cultures were incubated at 37°C for 18 to 24 h. All manipulations of recombinant plasmids were performed by standard techniques (25). The conditions for transformation and characterization of plasmids in strains of *K. pneumoniae* have been described elsewhere (13).

Detection of type 3 fimbriae. The presence of the characteristic MR/K hemagglutinating activity associated with production of the type 3 fimbrial adhesin (MrkD) was detected as previously described using tanned erythrocytes (21). The production of fimbrial appendages and the presence of the major fimbrial subunit (MrkA) on the surface of bacteria were detected using monospecific antiserum as reported by our group (26). Transmission electron microscopy was used to confirm the phenotypic expression of type 3 fimbriae by bacteria (10).

Detection of biofilm formation. The ability of bacteria to form biofilms in vitro was detected using two assays. For the rapid screening of large numbers of strains, the microtiter plate assay developed by O'Toole and Kolter (23) was used to quantify biofilm formation. Unless otherwise stated, bacteria were grown in GCAA broth to optimize type 3 fimbrial expression. Crystal violet was used to detect biofilm-forming bacteria, and quantitation of the biofilm was performed by solubilization of the bound crystal violet in 95% ethanol and measurement of the absorbance at 600 nm as previously described (23). For specific mutants, all assays were performed in duplicate and the results from three independent experiments are reported.

To investigate further the ability of isogenic strains to form biofilms and investigate the nature of these biofilms, a second assay using a flowthrough continuous culture system was used (24). The flowthrough chamber and culture system were identical to those described by Greenberg and colleagues (24). The flow rate was adjusted to 130 μl-min and a 1-to-50 dilution of GCAA broth was used as the culture medium. All bacteria used in this assay were transformed with a plasmid, pBBRMCS-1, expressing green fluorescent protein (GFP) (supplied by E. P. Greenberg, University of Iowa). The imaging of biofilm formation was performed using a Bio-Rad (Hercules, Calif.) MRC600 confocal microscope and appropriate software. Biofilm formation was monitored over a period of 5 days, and all assays were performed at least twice.

Construction of transposon library in *K. pneumoniae* 43816. A mini-Tn*5* element carried on a pUT plasmid in *Escherichia coli* SM10(λ*pir*) was used as a donor to introduce the transposon to the ampicillin-resistant strain *K. pneumoniae* 43816 (8, 18). Following conjugation, kanamycin-resistant mutants of *K. pneumoniae* 43816 were selected on minimal medium supplemented with ampicillin and kanamycin by standard techniques (8). A total of 2,000 mutants were tested for their ability to form biofilms, using the microtiter plate assay described above.

Binding to ECMPs. Bacterial binding to specific ECMPs and commercially available basement membranes was detected by the assay previously described in detail elsewhere (27). Briefly, suspensions of target molecules were used to coat microtiter plates, and the ability of bacteria to bind to these targets was determined using an enzyme-linked immunosorbent assay. Bacterial binding was detected using *Klebsiella*-specific antiserum and subsequent development with alkaline-conjugated immune serum. All tests were performed in triplicate, and color development was determined using an enzyme-linked immunosorbent assay plate reader set to an optical density of 405 nm (OD_{405}).

RESULTS

Detection of *K. pneumoniae* mini-Tn*5* mutants impaired in biofilm formation. The mini-Tn*5* transposon was used to mutagenize *K. pneumoniae* 43816, and kanamycin-resistant mutants were tested for their ability to form biofilms on the surfaces of polyvinyl chloride microtiter plates according to the procedure of O'Toole and Kolter (23). Bacteria were incubated in GCAA broth for 10 h at 37°C and subsequently removed from the plates, and their ability to form biofilms was detected using crystal violet and comparison to the parental strain. For the initial screening only, strains that exhibited a reduction of 50% or more in the OD_{600} reading compared to *K. pneumoniae* 43816 were considered to be biofilm mutants. A total of 2,000 transposon mutants were screened using this assay, and four (0.2%) exhibited an impaired ability to form a biofilm. These four mutants demonstrated identical growth rates in liquid medium to strain 43816. Three of the four mutants exhibited a characteristic MR/K hemagglutinating reaction, and the expression of type 3 fimbriae could be detected using immune serum. These three strains were not examined further. One of the mutants, designated *K. pneumoniae* MBM100, did not mediate hemagglutination even when relatively large numbers ($>10^{10}$) of bacteria were used and produced no type 3 fimbriae.

The site of the Tn*5* insertion in the chromosome of *K. pneumoniae* MBM100 was determined by cloning this region, as a *Sau*3AI DNA fragment, into the *Bam*HI site of pACYC184 and selecting for kanamycin-resistant transformants in *E. coli* HB101. Plasmid DNA prepared from these transformants, along with a primer specific for one of the termini of the mini-Tn*5*, was used as a template to deduce the nucleotide sequence of the DNA adjacent to the site of insertion. An identical sequence was located on the available genome database for *K. pneumoniae* MGH78578 (http://genome.wustl.edu/gsc/Projects/bacterial/klebsiella/klebsiella.shtml), and comparison to annotated databases indicated that the mini-Tn*5* had inserted into a region most closely related to an *E. coli* hypothetical *yadH* gene of unknown function.

The impaired ability of *K. pneumoniae* MBM100 to form a biofilm in the microtiter plate assay is shown in Fig. 1. The parental strain, *K. pneumoniae* 43816, exhibits good biofilm formation over the incubation period of 8 h. Transformation of strain MBM100 with a plasmid carrying the *mrk* gene cluster restored the ability of this mutant to express type 3 fimbriae. In addition, these transformants were shown to form a biofilm on the surfaces of the microtiter plates (Fig. 1).

Biofilm formation by fimbriate and nonfimbriate strains of *K. pneumoniae*. The isogenic strains *K. pneumoniae* IA565,

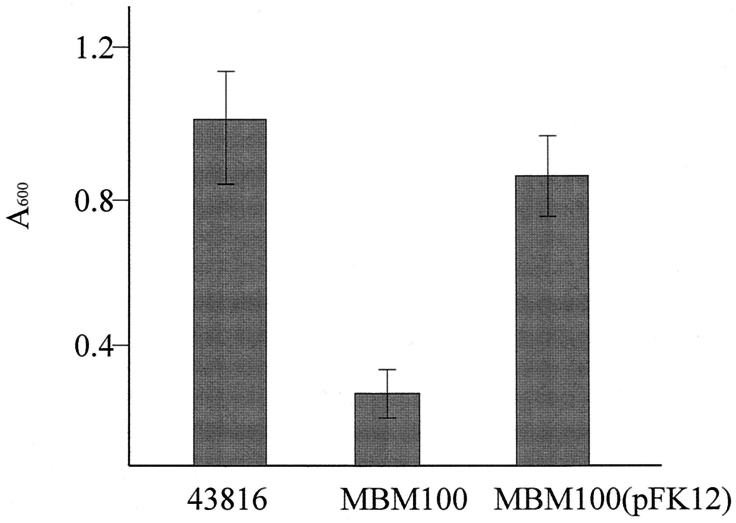

FIG. 1. Production of a biofilm on the surfaces of microtiter plates by *K. pneumoniae* 43816, the nonfimbriate mutant MBM100, and MBM100 transformed with pFK12. Bacteria were incubated in plates containing GCAA broth for 8 h to optimize type 3 fimbrial expression. Crystal violet was used to quantitate biofilm formation as described in Materials and Methods. The bars indicate means ± standard errors of the means (error bars) from three experiments.

IApc35, and IAΔT3 are phenotypically Fim^+ Adh^+, Fim^+ Adh^-, and Fim^- Adh^-, respectively (27). The ability of these strains to form biofilms on the surfaces of microtiter plates following growth for 10 h in GCAA broth is shown in Fig. 2. Only the nonfimbriate strain, IAΔT3, exhibited a reduced ability to form biofilms, and staining with crystal violet indicated an 8- to 10-fold decrease in the OD_{600} compared to the parental strain IA565. There was no difference in the growth rates of all strains tested following incubation in GCAA broth. Biofilm formation could be restored in *K. pneumoniae* IAΔT3 following transformation with a plasmid carrying the *mrk* gene cluster (Fig. 2; Table 2). These transformants were fully fimbriate and exhibited the characteristic MR/K hemagglutinating reaction associated with type-3 fimbrial expression. Also, the expression of a functional MrkD adhesin correlated with bacterial binding to collagen (Table 2). The ability of the *K. pneumoniae* isolates to form biofilms over time is shown in Fig. 3. Optimal biofilm formation occurred after a 10-h incubation period. However, at all time points *K. pneumoniae* IAΔT3 demonstrated impaired biofilm formation compared to the other strains.

Biofilm formation in a once flowthrough continuous culture system. To further compare the biofilms formed by fimbriate and nonfimbriate strains of *K. pneumoniae* a plasmid possess-

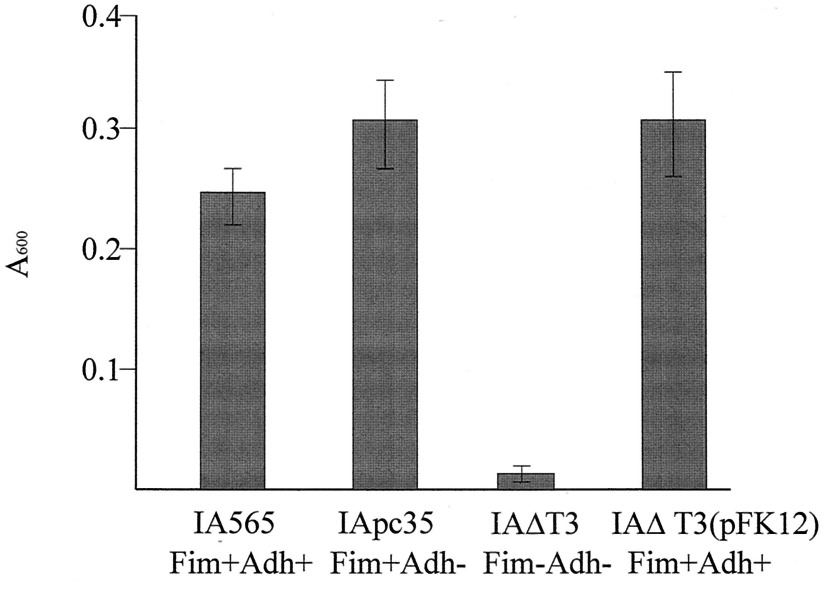

FIG. 2. Biofilm formation in microtiter plates by *K. pneumoniae* IA565 and its derivatives. Biofilm formation was determined after 10 h of incubation for all strains and the results (means ± standard errors of the means [error bars]) of three independent experiments are shown for each strain.

TABLE 2. Phenotypes of *K. pneumoniae* strains[a]

Strain	MR/K HA	Type 3 fimbriae	Binding to ECMP	Biofilm formation[b]
IA565	+++	+++	+	+
IApc35	−	+++	−	+
IAΔT3	−	−	−	−
43816	+++	+++	+	+
MBM100	−	−	−	−
MBM100(pFK12)	+++	+++	+	+

[a] The degree to which each phenotype was exhibited by the indicated strain is represented as follows: +, positive for phenotype; +++, strongly positive for phenotype; −, negative for phenotype.

[b] Biofilm formation determined using the microtiter plate assay after incubation for 8 to 10 h at 37°C.

ing the gene encoding enhanced GFP was introduced by transformation into representative strains. For all strains the plasmid was stable during growth in vitro with no observable loss in fluorescence following repeated subculture. Figures 4 and 5 show the results of epifluorescence and scanning confocal microscopy using *K. pneumoniae* and its derivatives. The results represent biofilm formation after 48 h of incubation since observations at this time point in this assay revealed the greatest difference between strains. After 24 h of incubation relatively little fluorescence was observed for all strains, and at 3 to 5 days of incubation a dense mat of cells was detected for all strains.

Following incubation at 37°C for 48 h, the fimbriate strains *K. pneumoniae* IA565 and IApc35 adhered to the surface of the flowthrough chamber forming dense areas of growth. The nonfimbriate *K. pneumoniae* IAΔT3 exhibited limited growth in the chamber with significantly less fluorescence detectable (Fig. 4, top panels). Composite layers of the *x-y* plane demonstrated that the fimbriate strains formed dense biofilms. Therefore, as shown in Fig. 4 (top panels), imaging of relatively small depths (8 μm) indicated dense bacterial growth, whereas imaging of strain IAΔT3 through a 40 μm depth demonstrated significantly less growth. At a depth of 40 μm, images for both IA565 and IApc35 resulted in fields comprised of complete fluorescence (data not shown). Scanning confocal laser microscopy was used to produce a side view of the biofilms by generation of a *z* series of images (Fig. 4, bottom panels). Both *K. pneumoniae* IA565 and IApc35 produced a biofilm characterized by the presence of pillar- and wall-like regions of growth arising from the surface of the chamber. Random measurements of the biofilm indicated that the maximum depths of the biofilms produced by these two strains were approximately 65 to 70 μm and 70 to 85 μm, respectively. In contrast, no pillar-like structures were observed following growth of *K. pneumoniae* IAΔT3 in the flowthrough chambers. Occasionally, strands of bacteria approximately 10 μm in length were observed (Fig. 4, bottom panels) after 48 h of incubation, but no dense bacterial growth was found to occur. Even following prolonged incubation in the chambers (>5 days) the bacteria grew as a flat mat of cells (maximum detectable depth of 9 μm).

Similar results were observed for *K. pneumoniae* 43816 and its nonfimbriate derivative (Fig. 5). The fully fimbriate strain was able to rapidly form a biofilm in the chamber, whereas the nonfimbriate mutant could not. Biofilm production was associated with the formation of bacterial pillar-like structures associated with the growth on the flowthrough chamber surface. Using composite imaging of the *x-y* plane, the greatest depth of the biofilm produced by *K. pneumoniae* 43816 was observed to be 20 μm. The nonfimbriate mutant demonstrated no ability to form a biofilm in the chamber. Observations of nonfimbriate, fluorescent bacteria indicated that no dense mat of organisms formed over the incubation period (Fig. 5B). Individual bacteria could be observed in the chamber but did not grow on the chamber surface to the density observed for the fimbriate, fluorescent strain (Fig. 5A).

Phenotypic variation of type 3 fimbriae in *K. pneumoniae* is associated with biofilm formation. Phenotypic variation of type 3 fimbrial expression was observed to be associated with the ability to form biofilms. We examined four distinct phase-

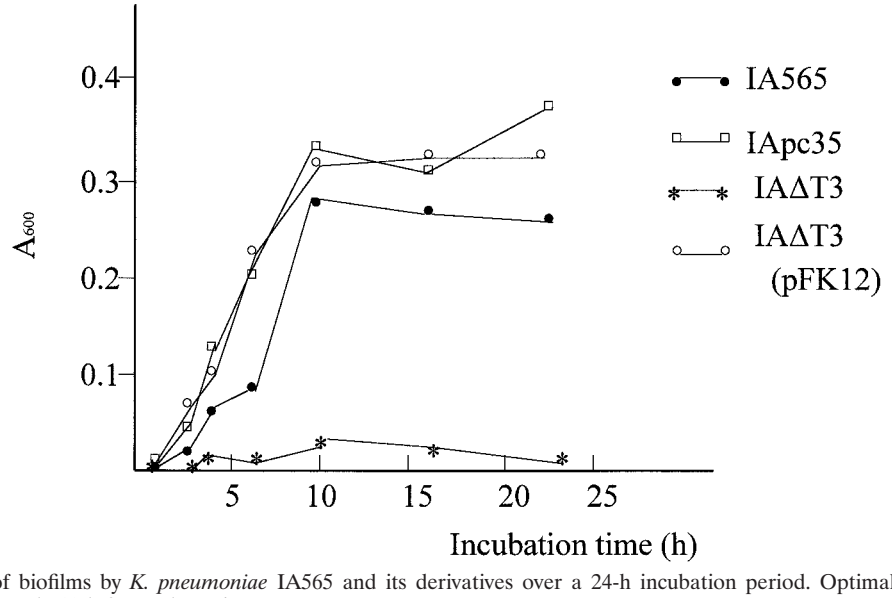

FIG. 3. Formation of biofilms by *K. pneumoniae* IA565 and its derivatives over a 24-h incubation period. Optimal biofilm formation was observed after approximately 10 h for each strain.

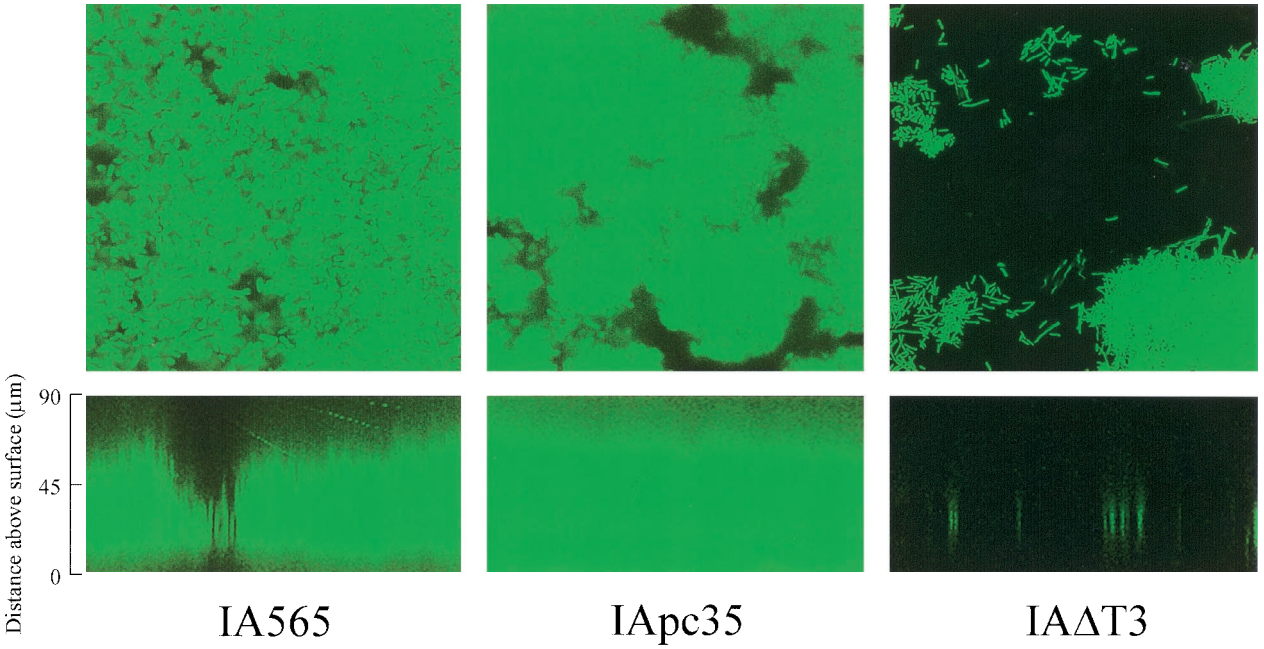

FIG. 4. Epifluorescence and confocal microscopy of *K. pneumoniae* IA565, IApc35, and IAΔT3 biofilms produced by bacteria expressing GFP. (Top) Composite sections obtained by imaging through the *x-y* plane of the biofilms. Images for an 8 μm depth are presented for strains IA565 and IApc35, whereas the depth of image for strain IAΔT3 is 40 μm. (Bottom) Saggital views of a *z* series of bacterial biofilms. The scale indicates the depth of *z*-series sections obtained.

variable isolates of *K. pneumoniae* from a variety of sources for their ability to form biofilms in the microtiter plate assay. The results are shown in Fig. 6 and indicate that surface expression of fimbriae is associated with the increased ability of fimbriate bacteria to grow on the surfaces of these plates. Bacteria were serially passaged at least three times on L-agar or GCAA agar, to produce nonfimbriate phase or fimbriate phase bacteria, respectively, prior to inoculation onto the microtiter plates.

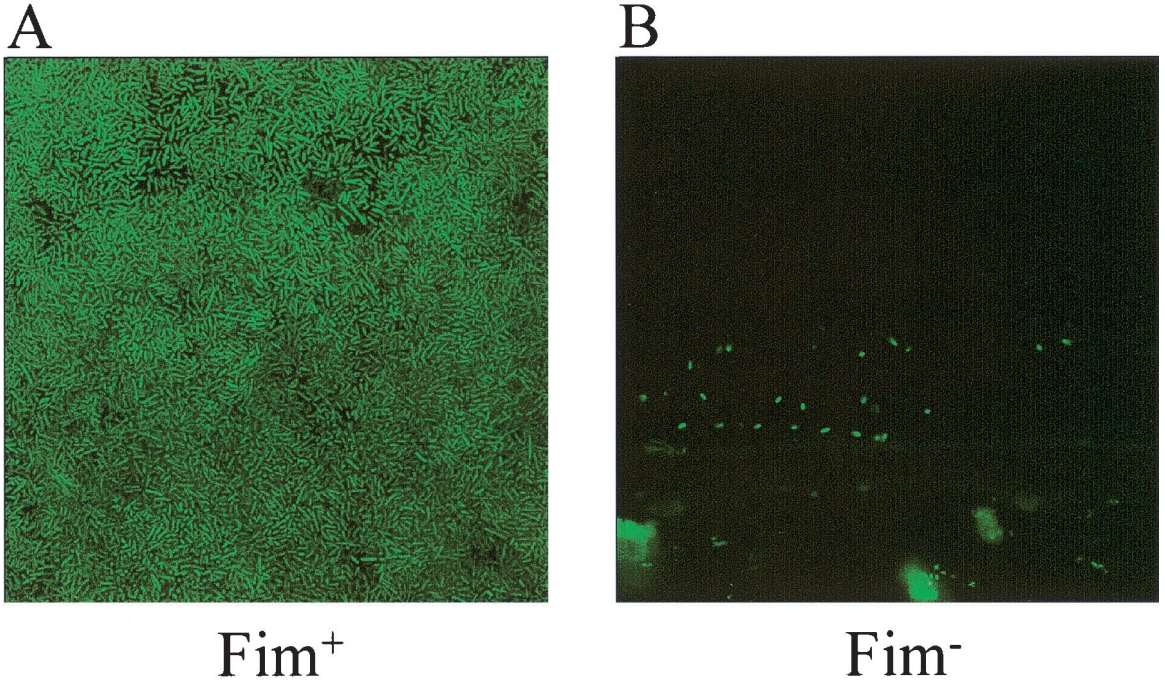

FIG. 5. Scanning confocal laser microscopy of biofilms formed by *K. pneumoniae* 43816 and MBM100. (A) Images of the fimbriate strain were produced by performing a composite analysis of an *x-y* series of images through a 20-μm depth. (B) The nonfimbriate mutant, MBM100, analyzed by identical procedures to those used in panel A.

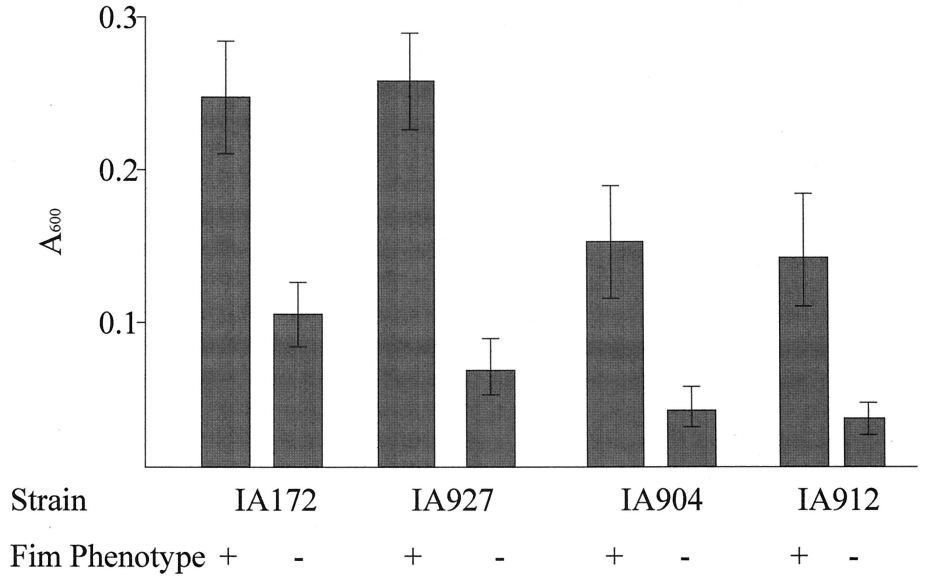

FIG. 6. Biofilm formation, using the microtiter plate assay, by *K. pneumoniae* isolates exhibiting phenotypic fimbrial phase variation. Biofilm formation was determined after 8 h of incubation following inoculation of the plates using the appropriate phase variant. The strains were obtained from the University of Iowa Hospitals and Clinics microbiology laboratory or the Iowa Sate Hygienic Laboratory. The sites of isolation for the following are as indicated: IA172, sputum; IA927, water; IA904, blood; IA912, tissue biopsy specimen. Bars represent means ± standard errors of the means (error bars) from three experiments.

DISCUSSION

The construction of a transposon bank of *K. pneumoniae* mutants indicated that one of the isolated mutants that was impaired in its ability to form biofilms, in vitro, on polyvinyl chloride surfaces is phenotypically nonfimbriate. This mutant also demonstrated a reduced capacity to grow on the surface of polystyrene microtiter plates (data not shown). The microtiter plate assay has been used to detect biofilm mutants in several bacterial species and provides a relatively rapid and accurate determination of the ability of bacteria to form biofilms (22, 23). The mutant was not impaired in its ability to grow in vitro as a planktonic culture since it exhibited growth rates identical to those of the parental strain. Although the bacteria were consistently nonfimbriate, even after growth favoring the expression of type 3 fimbriae in *Klebsiella* species (12), the mutation did not map to the site of the *mrk* gene cluster. The complete genomic sequence of a *K. pneumoniae* isolate is presently available, and an analysis of the nucleotide sequence flanking the site of the insertion indicated that the Tn*5* had inserted into the chromosome at a site most closely related to an *E. coli* gene, *yadH*, of unknown function. Because the function of YadH in *E. coli* is unknown, it is unclear how the Tn*5* insertion into the *K. pneumoniae* at this site eliminates type 3 fimbrial expression. However, transformation of this mutant with a plasmid possessing the complete *mrk* gene cluster resulted in the restoration of both fimbrial expression and biofilm formation. Therefore, the phenotypic expression of type 3 fimbriae in *K. pneumoniae* correlates with the ability of bacteria to adhere to and grow on the surface of the microtiter plates.

We have previously described the construction and characterization of *K. pneumoniae* mutants that either produce a nonadhesive fimbrial appendage or are nonfimbriate (27). In order to confirm the observations using the nonfimbriate Tn*5* mutant generated by transposon mutagenesis, we examined these strains for their ability to form biofilms in vitro. The nonfimbriate mutant of *K. pneumoniae* was impaired in its growth on the plastic surface of the microtiter plates compared to the parental strain. This inability could be overcome by transformation with a plasmid allowing fimbrial expression. Interestingly, the expression of a functional MrkD adhesin was not necessary for biofilm formation since *K. pneumoniae* IApc35 (Fim^+ Adh^-) grows as a biofilm. In fact, this mutant demonstrates a more rapid biofilm formation than the parental strain. However, we have previously shown that the nonadhesive mutant is strongly fimbriate, and, therefore, the increased efficiency of biofilm formation is most likely associated with increased fimbriation. As previously reported by our group, the MrkD adhesin is necessary for collagen binding. Consequently, using three different fimbrial mutants the lack of fimbrial appendages on the bacterial surface correlated with poor biofilm formation in the microtiter assay.

In order to visualize the two-dimensional structure of biofilms, epifluorescence and confocal scanning laser microscopy were used. These techniques have previously shown the formation of pillar- and mushroom-like structures by *P. aeruginosa* during biofilm growth (7). Type 3 fimbriate strains of *K. pneumoniae* also produced these structures, consistent with their ability to grow as biofilms. The formation of these structures was associated with bacteria that express fimbriae but do not require the production of a fimbria-associated adhesin. Consequently, in the flowthrough chambers, *K. pneumoniae* IA565 (Fim^+ Adh^+) and IApc35 (Fim^+ Adh^-) clearly formed bacterial pillars, whereas *K. pneumoniae* IAΔT3 did not. Similarly, the fimbriate strain 43816 formed projecting colonies from the chamber surfaces, whereas a nonfimbriate strain did not. The absence of fimbriae on the bacterial surface did not

prevent the growth of *Klebsiella* on the surfaces of the chambers, because large mats of bacterial growth could be observed using the nonfimbriate strains. However, this growth differed from that of fimbriate strains in two ways. First, the mats of bacterial growth were only observed following prolonged incubation, usually more than 4 or 5 days depending upon the strain, compared to fimbriate bacteria. Second, outgrowth of bacterial pillars from the chamber walls was never observed with nonfimbriate strains that consistently grew as flat mats of cells.

Using four additional *K. pneumoniae* isolates from diverse sources, the increased ability to form biofilms in the microtiter plate assay was consistently associated with the surface expression of type 3 fimbriae. Nonfimbriate-phase bacteria were always reduced in biofilm production compared to fimbriate-phase organisms. The incubation of these *Klebsiella* isolates under conditions favoring the growth of nonfimbriate bacteria may also result in the phenotypic variation of additional undefined traits. However, these results along with those using the genetically defined fimbriate strains are indicative that the type 3 fimbriae play a role in the early and mature development of biofilms in vitro.

The role of type IV pili in facilitating biofilm formation has been demonstrated in *P. aeruginosa* (22). Here we present evidence that the type 3 fimbriae, members of the chaperone-usher family of fimbriae (15), play a role in biofilm formation. The adhesin, MrkD, associated with these fimbriae has been shown to mediate binding, in vitro, to eucaryotic tissues (11, 28, 30). However, the MrkD protein does not appear to be necessary for rapid and efficient biofilm formation, although this fimbria-associated polypeptide is required for binding to ECMPs. The major structural component of type 3 fimbriae (MrkA) is a hydrophobic protein (3), and this hydrophobicity may facilitate bacterial interactions leading to efficient growth as a biofilm. Consequently, the type 3 fimbriae could provide two functions for the bacteria, one enabling a specific receptor-ligand interaction with host cells and tissues and the other facilitating outgrowth of the bacteria as an efficient biofilm. A multifunctional role of bacterial fimbriae has previously been suggested by Korhonen and his group (16). Type 3 fimbriae are produced by many members of the *Enterobacteriaceae* that are associated with opportunistic infections (1, 19, 20). The ability to form biofilms on the surfaces of implants and catheters could be critical to the survival of these bacteria in vivo. Also, growth of these bacteria in nonclinical environments may frequently necessitate biofilm formation.

Several investigations have indicated that biofilm formation by bacteria requires numerous signals and the production of multiple gene products, including flagella, pili, and capsules (23, 31). Since *Klebsiella* strains are invariably nonflagellate, these appendages will play no role in biofilm formation in these bacteria. However, *Klebsiella* isolates are frequently mucoid, and the formation of the typical pillar- and mushroom-like structures associated with these forms of growth is most likely to be the result of a complex genetic network. Currently, we are investigating the nature of those Tn*5* mutants that are impaired in biofilm formation but exhibit normal levels of fimbriation. The investigation of biofilm formation by the multiple-antibiotic-resistant enterobacteria such as *K. pneumoniae* could provide the development of mechanisms to prevent their growth in the clinical environment.

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