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Signature-Tagged Mutagenesis of *Klebsiella pneumoniae* To Identify Genes That Influence Biofilm Formation on Extracellular Matrix Material

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Klebsiella pneumoniae **causes urinary tract infections, respiratory tract infections, and septicemia in susceptible individuals. Strains of *Klebsiella* frequently produce extended-spectrum beta-lactamases, and infections with these strains can lead to relatively high mortality rates (approximately 15%). Other virulence factors include production of an antiphagocytic capsule and outer membrane lipopolysaccharide (LPS), which mediates serum resistance, as well as fimbriae on the surface of the bacteria. Type 1 fimbriae mediate adherence to many types of epithelial cells and may facilitate adherence of the bacteria to the bladder epithelium. Type 3 fimbriae can bind in vitro to the extracellular matrix of urinary and respiratory tissues, suggesting that they mediate binding to damaged epithelial surfaces. In addition, type 3 fimbriae are required for biofilm formation by *Klebsiella pneumoniae* on plastics and human extracellular matrix; thus, they may facilitate the formation of treatment-resistant biofilm on indwelling plastic devices, such as catheters and endotracheal tubing. The presence of these devices may cause tissue damage, allowing *Klebsiella* to grow as a biofilm on exposed tissue basement membrane components. Though in vivo biofilm growth may be an important step in the infection process, little is known about the genetic factors required for biofilm formation by *Klebsiella pneumoniae*. Thus, we performed signature-tagged mutagenesis to identify factors produced by *K. pneumoniae* strain 43816 that are required for biofilm formation. We identified mutations in the *cps* capsule gene cluster, previously unidentified transcriptional regulators, fimbrial, and sugar phosphotransferase homologues, as well as genetic loci of unknown function, that affect biofilm formation.**

Klebsiella pneumoniae, a gram-negative opportunistic pathogen, is the causative agent of approximately 14 to 20% of hospital-acquired urinary tract infections, respiratory tract infections, and septicemias (for a review, see reference 46). Respiratory infections are particularly severe, as they involve extensive tissue destruction and can result in mortality rates as high as 50% (11). Individuals most at risk for *K. pneumoniae* infections are infants, the elderly, and those with compromised defense mechanisms. Patients that suffer from *Klebsiella* infections are often initially colonized in their gastrointestinal tract, and organisms may spread among patients via the hands of hospital workers (40, 46, 47, 55). Those patients receiving antibiotic treatment commonly become colonized in the gastrointestinal tract by antibiotic-resistant *K. pneumoniae* isolates following selection for resistant strains (55, 65). These resistant strains of *Klebsiella* produce extended-spectrum beta-lactamases and are of particular concern as infections lead to relatively high mortality rates (about 15%) due to treatment failure and subsequent septicemia (27). Virulence factors that are associated with *Klebsiella* pathogenesis include the production of an antiphagocytic capsule as well as lipopolysaccharide (LPS) that contributes to serum resistance (39, 44, 45, 56, 57, 66), siderophores to capture host iron (44, 49), and type 1 and type 3 fimbriae that allow the bacteria to bind to host structures (3, 6, 26, 62, 63, 67).

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Biofilms are organized communities of bacteria living on surface environments, and growth as a biofilm is linked to virulence and colonization for a variety of bacterial pathogens, including *Pseudomonas aeruginosa*, which causes chronic, life-threatening respiratory infections in cystic fibrosis patients (10, 21). Recent studies suggest that biofilm formation may also be an important virulence factor for *K. pneumoniae* (28). It has been demonstrated in vitro that bacteria growing within biofilms are more resistant to antibiotic treatment than bacteria growing planktonically (reviewed in reference 37). Within a host, biofilm growth is thought to enhance resistance to antibiotic therapies, as well as host defense mechanisms (21). *K. pneumoniae* is able to form a robust biofilm on plastic surfaces as well as surfaces coated with human extracellular matrix (HECM) and host-derived proteins (15, 28, 34). This has clinical significance, since *Klebsiella* infections are often associated with the presence of indwelling plastic devices that, over time, become coated with matrix proteins (17). Therefore, *Klebsiella* is likely to form biofilms on indwelling plastic devices, such as catheters and endotracheal tubing, and subsequently colonize human tissue in *K. pneumoniae* infections. However, little is known about the genetic requirements for biofilm formation of *K. pneumoniae* growing on these substrates. In the studies described below, we used signature-tagged mutagenesis (STM) to isolate mutants deficient in biofilm formation on HECM.

Previously, our laboratory constructed a library of Tn*5* transposon mutants in *Klebsiella pneumoniae* to identify genes necessary for biofilm formation using the microtiter plate assay developed by O'Toole and Kolter (43). These studies demonstrated that type 3 fimbriae are necessary for biofilm formation

and that the type 3 fimbrial adhesin, MrkD, is required for biofilm growth on HECM (28). However, certain types of biofilm-deficient mutants are unlikely to be identified in the microtiter biofilm assay since the bacteria are grown statically in microtiter wells, a closed system not representative of bacterial growth on HECM in vivo. In the present study, we screened pools of unique signature-tagged *K. pneumoniae* mutants using a flowthrough continuous culture system to identify additional genetic loci needed for biofilm formation on HECM in a dynamic environment. Mutations in several genetic loci were identified that caused defects in biofilm formation, including the capsule gene cluster (*cps*), previously unidentified transcriptional regulators, fimbrial genes, sugar phosphotransferase (PTS) systems, and an open reading frame (ORF) of unknown function.

MATERIALS AND METHODS

Construction and biofilm screening of a *K. pneumoniae* STM library. A library of signature-tagged mutants was created by conjugation of 47 separate *E. coli* S17 λ *pir* (pUT/mini-Tn*5*Km2) strains with *Klebsiella pneumoniae* 43816, a clinical isolate that is virulent in the murine model of acute airway infection (4, 35). Individual transconjugants were selected on M9 minimal medium supplemented with kanamycin (25 μg/ml) and were subsequently used to create pools containing 47 uniquely tagged *Klebsiella* Tn*5* mutants. To inoculate biofilms, each strain within a pool was grown individually in the wells of a microtiter dish in glycerol-Casamino Acids agar (GCAA) medium overnight (22). The contents of each well were then mixed, the optical density at 600 nm (OD_{600}) was adjusted to 0.4 to 0.6 ($\sim 8 \times 10^8$ CFU/ml), and 3 ml of bacterial suspension was used to inoculate the biofilm chamber. Part of the inoculum was adjusted to an OD_{600} of 1.2 (2.0×10^9 CFU/ml) and saved as a source of DNA template representing the input pool in the STM procedure. Biofilm chambers consisted of T75 tissue culture flasks standing on their sides and were modified with in-flow and out-flow ports located 1/2 inch from the base of the flask. The flowthrough surface was coated with HECM (BD Biosciences, Franklin Lakes, NJ) at an optimal coating concentration of 0.109 mg ml^{-1} as previously described (53). The HECM was diluted in carbonate buffer and incubated overnight at 4°C in the biofilm chamber. Subsequently, the chambers were rinsed with phosphate-buffered saline (PBS), the bacterial inoculum was added, and this mixture was incubated for 45 min without shaking at 37°C. Next, diluted (25%) GCAA medium containing 25 μg/ml kanamycin was passed through the biofilm chambers, and the flow was maintained for 72 h at 37°C at a rate of 528 μl/min. Following incubation, the medium was removed and the biofilm was harvested from the surface, resuspended in PBS by vortex mixing, and adjusted to an OD_{600} of 1.2.

DNA isolated from equal numbers (2×10^9 CFU/ml) of the inoculum and the harvested biofilm was used as a template to PCR amplify each variable region contained within the strains present in each pool. Primers P2 (5′ TACCTACA ACCTCAAGCTT) and P4 (5′ TACCCATTCTAACCAAGC) were used to amplify the signature tag using puReTaq Ready-To-Go PCR beads (Amersham, Piscataway, NJ) according to the manufacturer's directions. The 80-bp PCR product was isolated following electrophoresis through a low-melting-temperature agarose gel (1.6% [wt/vol]) and boiled for 2 min. Subsequently, 3 μl of the boiled DNA and agarose gel solution was used as template for a second PCR performed in the same manner, except that digoxigen (DIG) probe synthesis mix (Roche, Palo Alto, CA) was added to each sample as recommended by the manufacturer. DIG-labeled PCR products were hybridized to positively charged nylon membranes that had been loaded, and cross-linked by UV light, with each of the 47 unique signature-tagged plasmids. Hybridization membranes possessing DNA from the inoculum and the mature biofilm were developed using anti-DIG–alkaline phosphatase Fab fragments and nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) tablets. Hybridization signals that were visibly missing or lighter on the biofilm membrane compared to the input membrane allowed the identification of putative biofilm-deficient strains within particular pools.

Competitive index assays. Mixed biofilms using wild-type and mutant strains were used to generate a biofilm competitive index (CI). The biofilm inoculum was a 1:1 ratio of *K. pneumoniae* 43816 plus individual STM strains grown overnight in GCAA medium. Biofilm cultures were grown as described above, with the exception that the diluted GCAA flow medium did not contain kanamycin. After 72 h, the biofilm was resuspended in PBS, vigorously vortexed, and plated on LB or LB-kanamycin to determine the percentage of the biofilm that consisted of the wild-type strain compared to the mutant. For planktonic competitive index assays, 5 ml of diluted GCAA medium was inoculated with 10 μl each of the LB overnight cultures of strain 43816 and individual STM strains and grown with shaking at 37°C for 72 h. Dilutions of the mixtures were then plated on LB or LB-kanamycin agar to determine the percentage of wild-type and mutant strains in the culture. The ratio of wild type to mutant within the inoculum (input) for both the biofilm and planktonic growth conditions was also determined by plating appropriate dilutions. The CI value was determined using the equation CI = (no. of mutant bacteria in output/no. of wild-type bacteria in output)/(no. of mutant bacteria in input/no. of wild-type bacteria in input).

TABLE 1. Primers used for arbitrary PCR

Primer	Sequence
Tn*903-3*	5′TGAATGTTCCGTTGCGCTGCCC3′
Tn*903-4*	5′GCAGTTTCATTTGATGCTCGAT3′
Arb1	5′GGCCACGCGTCGACTAGTAC-N_{10}-GATAT3′
Arb6	5′GGCCACGCGTCGACTAGTAC-N_{10}-ACGCC3′
Arb5	5′GGCCACGCGTCGACTAGTAC-N_{10}-CAAGG3′
Arb9	5′GGCCACGCGTCGACTAGTAC-N_{10}-CGACG3′
Arb2	5′GGCCACGCGTCGACTAGTAC3′

Single-inoculum biofilm assays. All mutants demonstrating reduced growth in the presence of the wild-type strain as described above were also examined using the biofilm HECM-coated chambers inoculated only with the appropriate mutant. The monitoring of green fluorescent protein-labeled bacteria in the chambers over a 48-h incubation period was performed by scanning confocal laser microscopy as previously described by our group (28). The ability of each strain to form a biofilm on HECM was compared to that of the wild-type, parental strain 43816 by procedures previously described by us (34).

Identification of Tn*5* insertion sites. Transposon insertion sites were determined by arbitrary primed PCR as previously described (14) using a variety of arbitrary primers (Table 1). Briefly, a primary PCR was performed using 10 ng of purified genomic DNA from each mutant, with 0.5 μM arbitrary primer (Arb1, Arb6, Arb5, or Arb9) and 0.2 μM transposon-specific primer (Tn*903-3* or Tn*903-4*, which amplify from opposite ends of the transposon). In the primary PCR, the first six cycles used 30°C annealing (30 s) and 72°C elongation (1.5 min) temperatures. The next 30 cycles used 45°C annealing (30 s) and 72°C elongation (2 min) conditions. Subsequently, 10% of the primary PCR product was used as template in a secondary PCR that used 0.2 μM transposon-specific primers and Arb2 primer with 30 cycles of 52°C annealing (30 s) and 72°C elongation (2 min). After electrophoresis through 1% agarose, DNA fragments from mutants (which were not present in the wild-type strain) were extracted from the gel and purified for sequencing.

Detection of type 3 or type 1 fimbriae. To detect type 3 fimbriae on the mutant strains, serum agglutination assays were performed with monospecific antisera raised against purified fimbriae, as previously described (53). The presence of the MrkD adhesin on type 3 fimbriae was detected by the hemagglutinating activity of *K. pneumoniae* strains with tanned human erythrocytes (18). We tested for the presence of type 1 fimbriae by determining the hemagglutinating activity of the bacteria with guinea pig red blood cells, as previously described (23).

Reactivity with K2-specific antiserum. The Ouchterlony double immunodiffusion assay was performed to determine the antigenic specificity of capsule (41). Capsular polysaccharide was isolated by a small-scale capsule preparation method, as previously described (60). Capsule material (10 μl) isolated from approximately equal numbers (10^9) of CFU of each strain was placed into wells made in 1% Noble agar, while 10 μl of undiluted K2 antiserum (Statens Serum Institute, Copenhagen, Denmark) was added to the central well. Capsular polysaccharide from the non-K2 isolate, *K. pneumoniae* IA565 (28), was used to demonstrate the specificity of the antiserum. The gels were incubated in a humidified chamber at 37°C. Lines of precipitation were assessed 4 to 5 days later to determine the reaction of capsular antigens with the antiserum.

To perform inhibition enzyme-linked immunosorbent assay (ELISA) assays, a 96-well microtiter plate was coated overnight at 4°C with 200 μl of 10^7 CFU/ml of *K. pneumoniae* 43816 grown on 5% sucrose agar and resuspended in carbonate buffer. The next day, coated wells were blocked with 1% bovine serum albumin (BSA) in PBS for 2 h and washed with PBS-Tween 20 (0.05% [vol/vol]). Dilutions of K2 antiserum in PBS-Tween 20 were incubated in microcentrifuge tubes with 10^8 CFU/ml of either 43816 or the mutant strain 17-B5 (grown on sucrose agar and resuspended in PBS-Tween 20) for 2 h at 37°C. This mixture (100 μl) was added to the coated wells of the microtiter plate and incubated at 37°C for

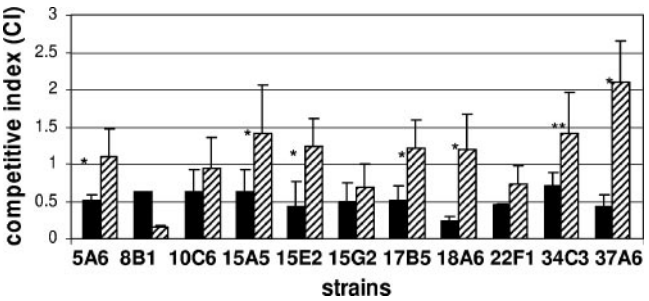

FIG. 1. Mixed competitive index biofilm assays with the wild-type 43816 and biofilm-deficient strains identified by STM screening. Solid bars represent 72-h biofilm CI, and hatched bars represent 72-h planktonic CI for each strain. Data represent the mean ± standard deviation of CI results from two or more independent experiments for the biofilm assays and three or more experiments for the planktonic assays. *, $P < 0.05$ as determined by a one-tailed Student's t test; **, $P < 0.1$.

2 h. The wells were washed with PBS-Tween 20, and 200 μl of a 1:30,000 dilution of goat anti-rabbit immunoglobulin G (IgG)-alkaline phosphatase conjugate (Sigma-Aldrich, St Louis, Mo) was added to the wells and incubated at 37°C for 1 h. The wells were washed, and 100 μl of *p*-nitrophenyl phosphate solution was added. The enzyme/substrate reaction was allowed to develop for 45 min, and the OD_{405} was determined using an ELISA microplate reader. Concentrations of bacteria used in this assay were determined by plate dilution counts prior to coating of the microtiter plate and incubation with antiserum.

Microtiter plate biofilm assay. Biofilms formed in microtiter plates were assessed as described by O'Toole and Kolter (43), with the exception that the plates were coated with HECM as reported elsewhere by our group (43, 54).

Mouse infectivity assays. Five- to 6-week-old female BALB/c mice were infected intranasally with 10^3 or 10^8 CFU of *K. pneumoniae* suspended in 50 μl of PBS. All infectivity assays were performed as previously described by our group (35).

RESULTS

STM biofilm screening and mutant identification. A total of 1,175 mutants were screened, and 55 (4.6%) did not grow well within the biofilm, as evidenced by decreased detection of hybridization signals from harvested biofilms compared to the inoculum. To confirm that the strains identified in the STM screen were deficient in biofilm formation compared to strain 43816, we examined each mutant individually in a mixed biofilm competitive assay. CI values equal to 1.0 would indicate that both strains grew equally well, while a CI of >1.0 indicates greater numbers of the mutant compared to wild type, and a CI of <1.0 indicates that the mutant did not grow as well as the wild type. Using this competitive assay, we found that 11 of the 55 strains identified by STM were not able to compete well with the wild-type strain in the CI assay (CI, <1) (Fig. 1). For most of the strains, there were approximately twofold fewer mutant bacteria present than wild type. However, for the STM mutant 18-A6 there was a greater than threefold decrease in numbers compared to the wild-type strain. To eliminate the possibility that these strains had growth defects that were not specific for growth in a biofilm, we performed planktonic CI assays. A total of 8 of the 11 mutants were able to grow as well as or better than the wild-type strain (CI, ≥1) during planktonic growth for 72 h, while three strains (10C6, 15G2, and 22F1) did not grow as well as the wild-type strain (Fig. 1). These three mutants were not further investigated. Therefore, a total of 8 (0.7%) of the initial 1,175 mutant strains that were screened were confirmed to be defective specifically for biofilm growth.

To identify the genes responsible for the observed defects in biofilm growth, we determined, by arbitrary primed PCR, the site of the transposon insertions in these mutants. Subsequently, these sequences flanking this site were used to conduct BLAST searches against the sequenced *Klebsiella pneumoniae* genome of strain MGH78578 (http://genome.wustl.edu), as well as open reading frame and BLAST searches on the NCBI database. The results are summarized in Table 2. For mutant strain 5-A6 (GenBank accession no. DQ526025), the flanking region at the site of insertion was identical to a region within the *K. pneumoniae* MGH78578 genome sequence, representing a conserved sucrose/glucose phosphotransferase system IIB domain, most similar to a IIB PTS protein from *Y. pestis*. The sequence analysis of the mutant 15-A5 (GenBank accession no. DQ526027) indicated no homology to any region of the *K. pneumoniae* MGH78578 genome, suggesting that the transposon insertion site in *K. pneumoniae* 15-A5 is not represented in the genome of strain MGH78578. An analysis of the region into which the transposon had inserted in mutant 15-A5 revealed that the transposon is within an open reading frame that encodes a putative cellobiose-specific transferase enzyme. The nucleotide sequence obtained for the mutant 10-C6 (GenBank accession no. DQ526026) exhibited 92% similarity to a region on the *K. pneumoniae* MGH78578 genome and suggested that the mutation disrupts an ORF encoding a conserved LuxR helix-turn-helix DNA binding domain. Nucleotide sequences adjacent to the Tn*5* insertion site in strain 15-E2 (GenBank accession no.

TABLE 2. Characteristics of the mutants examined in this study

Strain	Mutation site	Predicted protein function	% Lethality (no. of animals)[a]	Mean no. of days to death[b]
5-A6	Phosphotransferase EIIB homologue	Sugar transport	100 (3)	3.6 ± 1.5
10-C6	LuxR homologue	Transcriptional regulation	62.5 (8)	3 ± 0.7
15-A5	Phosphotransferase EIIC homologue	Sugar transport	100 (3)	2.7 ± 1.2
15-E2	*Y. pseudotuberculosis* YPTB1848 homologue	Unknown	100 (3)	2.7 ± 1.2
17-B5	K2 capsule ORF12	Capsule synthesis	0 (8)	None
18-A6	LysR homologue	Transcriptional regulation	66.6 (9)	3.5 ± 2.3
34-C3	CRP homologue	Transcriptional regulation	62.5 (8)	2.8 ± 0.8
37-A6	FimA homologue	Fimbrial synthesis	75 (8)	4.2 ± 1.3

[a] Percent lethality after nasal inoculation of mice with 10^{-3} CFU was assessed in an initial experiment with three mice. If any mice survived, a second experiment was conducted with five or six more mice. Data are the combined results from these experiments.

[b] Mean ± standard deviation number of days to death was calculated only for mice that died during the experiment.

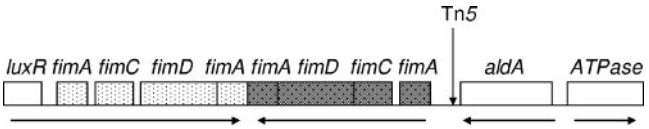

FIG. 2. Genetic map of ORFs found in the *K. pneumoniae* genome adjacent to the 37-A6 Tn*5* insertion site. Horizontal arrows indicate direction of transcription. ORF names are based on domain homologies determined by pBLAST sequence analysis. The Tn*5* mutation is denoted by a vertical arrow and is located at bp 3,290,052, as determined by analysis of the genome sequence of strain MGH78578.

DQ526028) were also related to a sequence within *K. pneumoniae* MGH78578. Further analysis indicated that this region possesses nucleotide sequences similar to those reported to encode a protein of unknown function from *Yersinia pseudotuberculosis*. For the mutant 17-B5 (GenBank accession no. DQ526021), sequence analysis indicated that the site of transposon insertion was into ORF12 of the *K. pneumoniae* K2 capsule gene cluster (2). For the mutant 18-A6 (GenBank accession no. 526022), little relatedness to the *K. pneumoniae* MGH78578 sequences was observed. However, BLAST analysis of the obtained sequence from this mutant revealed the presence of an ORF encoding a gene product with a LysR substrate binding domain, suggesting that this mutation may interrupt an ORF that encodes a transcriptional regulator. For strain 34-C3 (GenBank accession no. DQ526023), the arbitrary PCR sequence analysis demonstrated that the transposon disrupts an ORF, also found on *K. pneumoniae* MGH78578, that contains a cyclic AMP receptor protein (CRP) catabolite gene activator domain and is related to a putative inner membrane protein found in *Salmonella enterica* serovars Typhimurium and Typhi. Finally, for the STM mutant 37-A6 (GenBank accession no. DQ526024), the transposon insertion is located 508 bp upstream of a gene cluster possessing determinants related to *fimA*, *fimC*, and *fimD* of the type 1 fimbrial gene cluster and 194 bp downstream of an ORF predicted to encode aldehyde dehydrogenase (Fig. 2).

Biofilm formation on HECM following single inoculation. In order to demonstrate the ability of each mutant to grow as a biofilm on the surface of the HECM and in the absence of the competing parental strain, each mutant was observed for biofilm growth when used as a single inoculum in the chambers. Each strain was observed for biofilm growth following 16, 24, and 48 h of incubation at 37°C. Mutants 15-A5, 18-A6, 17-B5, 37-A6, and 10-C6 demonstrated significantly less growth on HECM after 48 h of incubation, with only patches of limited growth detected in the chambers. Three of the mutants (15-E2, 5-A6, and 34-C3) exhibited confluent growth on the coated chambers similar to that observed for the parental strain. However, the biofilm formed by each of these strains was consistently thinner than that of the wild-type strain and was characterized by the presence of isolated stacks of bacterial growth in the HCEM.

Characterization of specific biofilm-defective mutants. The sites of insertion of the transposon in four of the eight STM mutants exhibiting reduced ability to compete with the wild-type strain during growth on HECM indicated insertion into genes that control gene expression. The remaining four mutants demonstrated insertion sites that could result in the alteration of membrane-bound protein systems or changes in surface-associated structures. These four mutants were further examined phenotypically.

Mutant 37-A6. The site of the transposon insertion in this mutant is close to a putative fimbrial gene cluster. Since fimbriae have been linked to the ability of *K. pneumoniae* to form biofilms (15, 34), and because mutant 37-A6 may have an effect on fimbrial expression, this strain was tested for the presence of type 1 and type 3 fimbriae. Unlike all the other STM mutants, strain 37-A6 produced detectably lower levels of the MrkD adhesin and type 3 fimbrial antigen as determined by hemagglutination and serum agglutination assays, respectively. Using monospecific anti-type 3 fimbrial serum, the serological titer of mutant 37-A6 was five times lower than that of the wild-type strain. Consistent with this decrease in reactivity with the fimbrial antiserum, 37-A6 also demonstrated a 10-fold decrease in red cell agglutinating activity compared to strain 43816 when the hemagglutination activities of bacterial suspensions were compared by standard techniques (18, 53). All of the other strains produced similar amounts of type 3 fimbriae to those detected on *K. pneumoniae* 43816. None of the strains investigated express type 1 fimbriae following subculture in static liquid medium, including the parental *K. pneumoniae* isolate.

Mutant 17-B5. *Klebsiella pneumoniae* strains produce copious exopolysaccharide in the form of various capsular antigenic serotypes (8, 12). Specific serotypes—for example, those producing the K2 antigen—are frequently isolated from human infections (8, 12). Also, bacterial exopolysaccharides have been implicated as important components of biofilm architecture (13, 61). We examined each of our biofilm-deficient mutant strains for the production of the K2 capsular antigen following growth on sucrose-supplemented media to enhance capsule production. Each of the mutants grew as mucoid colonies, similar to the wild-type parent, except for strain 17-B5. The site of the transposon insertion in this mutant is within ORF12 of the K2 gene cluster, and it had a flatter, less mucoid colony morphology compared to the wild type (Fig. 3A). To determine if this mutant was altered in capsular expression, we isolated capsular material from 17-B5 and *K. pneumoniae* 43816, as well as the non-K2 capsulate strain *K. pneumoniae* IA565 (54). Double-immunodiffusion assays using K2 antiserum and capsular antigens from 17-B5 and 43816 resulted in bands of precipitation with no spurs, suggesting identity with no significant modification of antigen in the mutant strain. No precipitation reaction was observed for IA565 capsule. In order to determine if there were quantitative differences in the amount of capsule produced by 17-B5, we performed a competitive inhibition ELISA using soluble antigen to inhibit reactivity of antiserum with a solid-phase antigen (Fig. 3B). Dilutions of K2 antiserum were incubated with 10^8 CFU/ml of strain 17-B5 or 43816 and subsequently incubated in the wells of a microtiter plate coated with *K. pneumoniae* 43816. At high dilutions of serum (1:640 and above), there was little difference in the ability of soluble antigen (17-B5 or 43816) to inhibit reactivity with K2-positive bacteria immobilized on the microtiter plate, indicating that both strains, 17-B5 and 43816, reacted with limiting concentrations of antisera. However, at high concentrations of serum, the mutant strain 17-B5 exhibited less ability to inhibit serum reaction with solid-phase an-

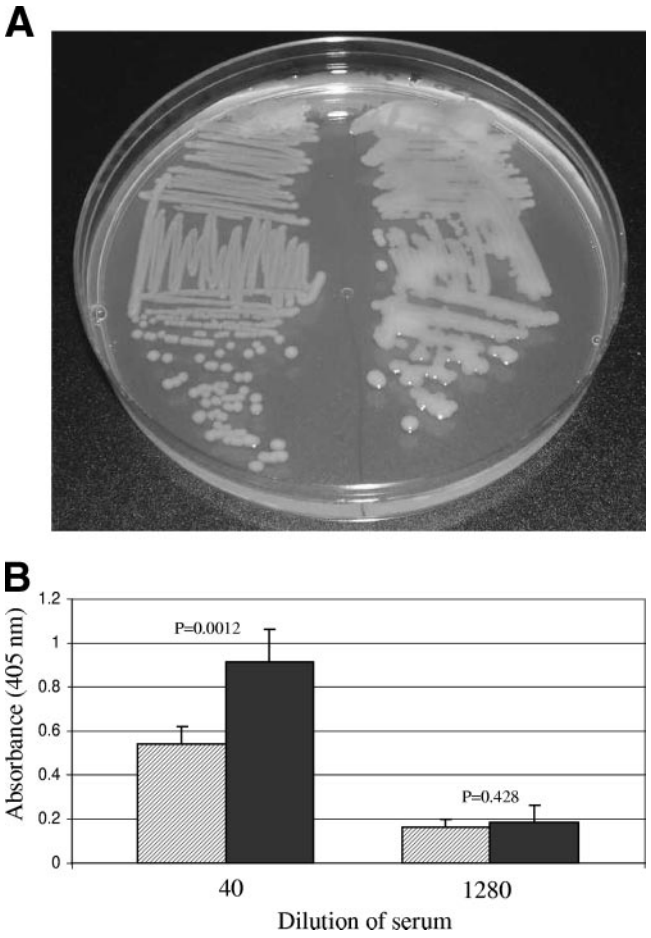

FIG. 3. Less capsular antigen is produced by strain 17-B5 than wild-type 43816. (A) 17-B5 and 43816 streaked on a 5% sucrose agar plate. (B) Quantitative ELISA. Solid bars represent values for wild-type 43816, and hatched bars represent strain 17-B5 preincubated with 1:40 or 1:1,280 dilutions of K2 antiserum. Data shown are the mean ± standard deviation of six samples from two separate experiments performed in triplicate. *P* values were determined by Student's *t* test.

tigen compared to *K. pneumoniae* 43816. This indicates that the STM mutant produces less capsule than the parental strain. These results are shown in Fig. 3.

Mutants 5-A6 and 15-A5. The location of the transposon insertions in mutants 5-A6 and 15-A5 indicated that the mutations are in determinants demonstrating relatedness to glucose/sucrose and cellobiose phosphotransferase enzymes IIC and IIB, respectively. However, both strains exhibited similar growth rates to the wild-type parental strain when grown in minimal medium supplemented with sucrose, glucose, or cellobiose (data not shown). Also, both strains could be shown to ferment the appropriate carbohydrates following growth in bromcresol purple broth (Becton Dickinson, Sparks, MD). Therefore, strains 5-A6 and 15-A5 are able to transport and metabolize glucose, sucrose, and cellobiose.

Mouse virulence of biofilm-deficient mutants. We have previously demonstrated that many strains of *K. pneumoniae* isolated from human infections are not virulent in the murine model of acute airway infection (35). However, *K. pneumoniae* 43816 is a highly virulent strain in the mouse, and an infective

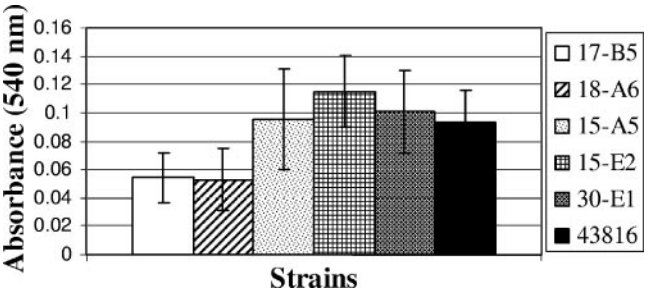

FIG. 4. Microtiter plate biofilm assay of strains identified in the STM one-flowthrough biofilm screening. Bacteria were allowed to grow for 9 h within microtiter wells coated with human extracellular matrix. Data presented are the mean ± standard deviation of six samples from two separate experiments performed in triplicate.

dose of as little as 10^3 CFU results in 100% mortality within 3 to 5 days of intranasal inoculation (35). To determine if STM mutations resulting in the biofilm deficiency on HECM also caused decreased virulence in vivo, we inoculated mice intranasally with 10^3 CFU of each mutant (Table 2). The mutants 5-A6, 15-A5, and 15-E2 exhibited identical lethality to *K. pneumoniae* 43816, causing a fatal infection in all animals within 3 to 5 days. For strains 10-C6, 18-A6, 34-C3, and 37-A6, intranasal infection did not result in a lethal infection for all animals inoculated but the mortality rates of infected animals were as follows. Strains 10-C6 and 34-C3 exhibited a 62.5% mortality rate, strain 18-A6 exhibited a 66% mortality rate, and strain 37-A6 was responsible for a 75% mortality rate in infected animals. The STM mutant 17-B5 was avirulent following inoculation with 10^3 organisms. However, following intranasal inoculation with 10^7 bacteria, all animals were lethally infected. We have previously shown that a completely avirulent mutant of *K. pneumoniae* 43816 is not responsible for a lethal infection in mice following intranasal inoculation with 10^7 bacteria (35).

Microtiter biofilm assay. Our laboratory and others have previously used the microtiter plate biofilm assay designed by O'Toole and Kolter to identify strains with defects in biofilm formation (28, 35, 43). We wanted to determine if the one-flowthrough STM biofilm screening facilitated identification of mutants that would not have been isolated using the microtiter plate assay. Therefore, the ability of the STM mutants to form an intact biofilm on HECM-coated microtiter plates was investigated using four of the mutants isolated from the biofilm chamber (Fig. 4). In this assay, we also used the STM mutant 30-E1 since it was not identified as a biofilm-deficient strain in the STM screening. Two mutants, 17-B5 and 18-A6, had a noticeable defect in biofilm formation following growth on HECM-coated plates. The strains 15-A5 and 15-E2 did not have a demonstrable defect in biofilm formation in the microtiter plate and, therefore, would not have been identified in this type of assay. As expected, the STM mutant 30-E1 that was not deficient in forming a biofilm in the chambers also was capable of forming a biofilm in the microtiter plate assay.

DISCUSSION

The procedure of STM has most often been used for in vivo screening to identify genes that are necessary for growth in an

animal model (50). In this study, we employed STM to identify genes in *K. pneumoniae* that are necessary for biofilm formation on HECM in an in vitro continuous culture biofilm system. We have previously demonstrated that clinical isolates of *K. pneumoniae* causing infections in humans attach to extracellular components of basement membranes (28, 54). Use of the continuous culture flowthrough system in a chamber coated with HECM results in extensive biofilm formation by *K. pneumoniae* (28). Growth of *K. pneumoniae* on denuded epithelial surfaces during human infection occurs in an open system; therefore, we decided to use STM in the biofilm chambers to identify mutants impaired in growth on HECM. Also, it is likely that biofilm growth in a continuous culture flowthrough system may require expression of genes not required in static culture. In fact, a screen of several STM mutants using the microtiter plate biofilm assay indicated that some of these mutants would not have been identified by this assay. Therefore, the use of STM in the biofilm chambers allowed identification of mutants that would not have been identified by the microtiter plate assay.

Analysis of the nucleotide sequences around the transposon insertion sites for three of the mutant strains (15-A5, 15-E2, and 18-A6) indicated less than 60% homology to the nucleotide sequence of strain MGH78578 that has been used to determine the genome sequence of *K. pneumoniae*, suggesting that these regions in *K. pneumoniae* 43816 are divergent or absent in MGH78578. Previous subtractive hybridization studies comparing the sequences of strains of *K. pneumoniae* have also revealed that many nucleotide sequences specific to clinical strains are not present in strain MGH78578 (33). Indeed, hybridization analyses suggested that the distribution of these sequences among other pathogenic strains of *K. pneumoniae* was quite heterologous (33). Consequently, the identification of the mutations isolated in our study is based upon either relatedness to determinants present on the chromosome of the sequenced MGH78578 strain or similarity to genes present in other bacteria.

We identified mutations in three different groups of genes that resulted in biofilm deficiency: transcriptional regulators (LuxR, LysR, and CRP homologues), sugar phosphotransferases (EIIB and EIIC homologues), and genes involved in the synthesis of extracellular structures (fimbriae and capsule). LysR-type transcriptional regulators affect a very diverse group of genes and functions and have been associated with the regulation of genes involved in biofilm formation by *Vibrio cholerae* (52). In this case, activation of signal transduction proteins by the LysR-type regulator AlsR was suggested to decrease biofilm formation (30). Often LysR regulators are adjacent to and inversely transcribed from the genes they regulate (52). Further investigation of the sequence surrounding the transposon insertion site in mutant 18-A6 may provide information about the function of this LysR homologue in biofilm formation. However, the site of insertion in strain 18-A6 is in a region not present in MGH78578, and, therefore, this region of DNA will have to be characterized further.

LuxR-type transcriptional regulators are often involved in the regulation of genes involved in quorum sensing. They have been implicated in regulation of biofilm formation for a variety of organisms, and members of this regulator family typically respond to cell density by binding an autoinducer molecule and affecting several different phenotypes, including biofilm formation (20). The LuxR homologue identified in this study contains a LuxR-like DNA binding domain, but does not appear to have an autoinducer binding region, suggesting that it may not be involved in quorum sensing.

The mutation in strain 34-C3 interrupts an ORF that contains a CRP activation domain and is related to a putative *Salmonella* inner membrane protein. In enteric bacteria, CRP mediates catabolite repression, and in *K. pneumonia*e it has been shown to mediate catabolite repression of citrate fermentation genes (5, 64). A recent study demonstrated that a mutation in CRP resulted in decreased detachment of *Shewanella oneidensis* MR-1 biofilms under oxygen-depleted conditions (64). It is unclear precisely what role the CRP homologue plays in biofilm formation, and it may be involved in transcriptional regulation of a biofilm-specific gene.

It is interesting that two additional mutations isolated in our studies were found in determinants proposed to encode phosphotransferase components EIIC and EIIB that were most closely related to systems that transport glucose/sucrose and cellobiose, respectively. These mutations did not affect the ability of the bacteria to grow in and utilize glucose, sucrose, or cellobiose. However, other bacterial phosphotransferase systems exist within *Klebsiella* for the transport of glucose, sucrose, and cellobiose (25, 31, 58). Future work will be aimed at determining if the mutations in these genes affect transport of other specific sugars. It is unclear whether a specific defect in sugar transport is responsible for the effect on biofilm formation. Since sugar transport is linked to catabolite repression and transcription, mutation of these phosphotransferase systems may affect a regulatory cascade that is important for transcription of genes necessary for optimal biofilm growth. Of note, however, various phosphotransferase systems have been linked with *Streptococcus gordonii* biofilm formation and infection of damaged heart valves (29). Also, an in vivo expression technology screen conducted with *Klebsiella pneumoniae* strain CG43 indicated that *ptfA*, encoding a fructose phosphotransferase, was positively regulated during murine intraperitoneal infection (32).

We also identified a mutant with an insertion immediately upstream of a putative fimbrial gene cluster possessing determinants related to *fimA*, *fimC*, and *fimD*, which encode the main structural subunit, chaperone, and usher, respectively, for type 1 fimbriae in *Escherichia coli* and *K. pneumoniae* (19). However, we were not able to detect phenotypic expression of type 1 fimbriae in the mutant or in the wild-type strain after growth in vitro. The genes in this operon are related to *fim* genes, but are not identical, and this cluster most likely represents an uncharacterized fimbrial gene cluster of *K. pneumoniae*. Phenotypic expression of different fimbrial types by *K. pneumoniae* has previously been described, but only the type 1 (*fim*) and type 3 (*mrk*) fimbrial determinants have been defined genetically (1, 7, 22–24, 48). This mutant did demonstrate a decreased expression of type 3 fimbriae, even though the transposon insertion is not located close to the *mrk* gene cluster. Mutations in regions in close proximity to one fimbrial gene cluster have been shown to affect fimbrial expression in heterologous fimbrial gene clusters in *S. enterica* serovar Enteritidis (9). Therefore, it is possible that the insertion site of mutant 37-A6 may influence type 3 fimbrial expression without being

located within the *mrk* gene cluster. The mechanism by which such a mutation can exert its affect is currently unknown.

Strains of *Klebsiella pneumoniae* express antigenically diverse exopolysaccharide capsular serotypes, and a few capsular serotypes are associated with human infections, including the K2 capsule expressed by strain 43816 (8). The STM mutant 17-B5 contains a mutation within a determinant designated ORF12 of the K2 capsular gene cluster. Transposon insertion mutations in ORF12 were previously reported to abolish expression of capsule by *K. pneumoniae* strain Chedid (2, 51). ORF12 falls within an operon containing 15 genes, and the precise function of many of these genes is unknown. ORF12 is upstream of three genes whose products are predicted to function as an acetyltransferase, glycosyltransferase, and 6-phosphogluconate dehydrogenase, respectively (2). Thus, it is unclear if the effect on biofilm formation caused by the mutation in 17-B5 is due to disruption of ORF12 or contiguous genes downstream of ORF12. The mutant 17-B5 appears morphologically distinct from the wild-type strain, as its colonies are flatter and less mucoidy. This could be due to an effect on the composition of the capsule, the amount that is produced, or both. The antigenicity of the capsule produced by this mutant was not significantly altered from that produced by the parental strain, although relatively minor changes in capsule composition would not have been detected in our studies. However, a quantitative ELISA indicated that less capsular antigen is produced by strain 17-B5 than the wild-type strain. This decrease in capsule production would account for the difference in colony phenotype between the two strains. Also, such a decrease in capsule production is likely to be responsible for the observed decrease in biofilm formation. It has previously been shown that the *K. pneumoniae* K2 capsule is associated with virulence in murine infectivity studies and that the amount of capsule produced can alter the virulence of *K. pneumoniae* (16, 42). It is interesting that the STM mutant 17-B5 is severely attenuated in its virulence following intranasal inoculation of mice.

Although *K. pneumoniae* 43816 is a highly virulent strain following intranasal inoculation of mice, many clinical isolates of *K. pneumoniae* implicated in human infection are not virulent using the murine model of airway infection. Consequently, mouse lethality following airway inoculation is a good model for acute infections of healthy hosts. However, the majority of human infections due to *K. pneumoniae* are associated with compromised individuals with disrupted epithelial surfaces. In this study, we have identified eight *K. pneumoniae* derivatives with mutations in genes necessary for optimum biofilm growth on HECM in a one-flowthrough biofilm system. When examined for their ability to cause infection in vivo in a healthy host, five of the eight demonstrated less virulence. Planktonic CI assays and growth curves (data not shown) indicate that these strains do not exhibit differences in growth rate relative to the wild-type strain. Clearly, both the in vitro biofilm assay and the in vivo acute airway infection model are both likely to detect factors mediating the pathogenesis of *K. pneumoniae*. In some instances, these may be shared attributes. In other cases, bacterial products related to host-specificity and health may be identified by only one of these procedures.

STM of *K. pneumoniae* has previously been used to identify genes that play a role in facilitating colonization and growth within the intestines, airways, and spleens of mice (36, 38, 59). These studies identified genes encoding a variety of functionally different proteins, and a small number of these were subsequently confirmed to play a role in infection. Our studies focused upon identifying genes that specifically are involved in infection of human-derived material and may possibly play a role in mediating the pathogenesis of *K. pneumoniae* growing on disturbed epithelial surfaces or surfaces coated with extracellular matrices. Each of these different selection procedures is likely to contribute to a more complete understanding of the *K. pneumoniae* infections and the pathogenesis of this organism. The function, at the molecular level, on biofilm formation on HECM of each of these mutants is currently being investigated. However, it is clear from our studies that some genes required for growth on this substrate have not previously been identified using other selection procedures. Therefore, growth of *K. pneumoniae* on HECM, a phenomenon that is clinically important, may require expression of a distinct group of genes.

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