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Type 3 Fimbriae and Biofilm Formation Are Regulated by the Transcriptional Regulators MrkHI in *Klebsiella pneumoniae*▽

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***Klebsiella pneumoniae* is an opportunistic pathogen which frequently causes hospital-acquired urinary and respiratory tract infections. *K. pneumoniae* may establish these infections *in vivo* following adherence, using the type 3 fimbriae, to indwelling devices coated with extracellular matrix components. Using a colony immunoblot screen, we identified transposon insertion mutants which were deficient for type 3 fimbrial surface production. One of these mutants possessed a transposon insertion within a gene, designated *mrkI*, encoding a putative transcriptional regulator. A site-directed mutant of this gene was constructed and shown to be deficient for fimbrial surface expression under aerobic conditions. MrkI mutants have a significantly decreased ability to form biofilms on both abiotic and extracellular matrix-coated surfaces. This gene was found to be cotranscribed with a gene predicted to encode a PilZ domain-containing protein, designated MrkH. This protein was found to bind cyclic-di-GMP (c-di-GMP) and regulate type 3 fimbrial expression.**

Klebsiella pneumoniae is an opportunistic pathogen which is a significant cause of nosocomially acquired infections, including catheter-associated urinary tract infections (CAUTIs) and ventilator-associated pneumonias (5, 15, 28). *K. pneumoniae* type 3 fimbriae mediate attachment to, and biofilm formation on, extracellular matrix-coated surfaces *in vitro* (2, 13, 16). *In vivo*, indwelling devices rapidly become coated with host material, creating an environment that facilitates infection by type 3 fimbria-producing enterobacteria. In addition, most isolates of *K. pneumoniae* causing nosocomially acquired infections are resistant to multiple antibiotics (22, 23). The ability of *K. pneumoniae* to form biofilms and the antimicrobial resistance of the organism are factors that make infections by these bacteria very difficult to eradicate.

K. pneumoniae possesses several virulence factors which aid in the ability of the organism to persist and thrive within an animal host. One class of virulence factors, those involved in bacterial adherence, includes the type 1 and type 3 fimbrial adhesins. Previously, the type 1 fimbriae in *K. pneumoniae* have been shown to play a role in infectivity by using a murine bladder cystitis model in which a type 1 hyperfimbriate strain more readily forms intracellular bacterial communities (IBCs) within bladder umbrella cells (29). The type 3 fimbriae have previously been characterized by our group and have been shown to mediate bacterial adherence *in vitro* to human extracellular matrix (HECM) components (13). In many strains of *K. pneumoniae*, this fimbrial type is encoded by a chromosomally borne gene cluster previously shown to be comprised of five genes (Fig. 1A). These genes include determinants encoding the major fimbrial subunit (MrkA), a chaperone-usher system (MrkBC, respectively), the fimbrial tip adhesin (MrkD), and an as-yet-uncharacterized structural component (MrkF) (6, 8).

Like many other fimbrial types in enterobacteria, the type 3 fimbriae are assembled by the chaperone/usher pathway (8). However, the regulation of type 3 fimbrial expression in *K. pneumoniae* is poorly understood in comparison to mechanisms of regulation of type 1 and *pap* fimbrial expression in *Escherichia coli* and type 1 fimbrial production in *Salmonella*. Our group recently reported that type 3 fimbrial production is affected by the intracellular concentrations of the second messenger cyclic-di-GMP (c-di-GMP) (14). Mutants of the *K. pneumoniae* phosphodiesterase MrkJ accumulated intracellular c-di-GMP, which resulted in a type 3 hyperfimbriate phenotype that more readily formed biofilms (14). Fimbrial systems often employ complex regulatory circuits, and it is expected that several as-yet-unidentified regulators govern the expression of type 3 fimbriae. Here we describe a screen which identified a mutant within a putative transcriptional regulator, MrkI, that resulted in decreased type 3 fimbrial expression and biofilm formation. Additionally, we have identified a determinant which is cotranscribed with *mrkI* and encodes a PilZ-domain containing protein, MrkH, which was shown to bind c-di-GMP and affect type 3 fimbrial expression.

MATERIALS AND METHODS

Strains, plasmids, and DNA manipulations. The strains and plasmids used in this study are shown in Table 1. To detect the presence of type 3 fimbriae, all strains were grown on either glycerol-Casamino Acids (GCAA) or Luria-Bertani (LB) medium at 37°C unless otherwise stated (6, 7, 11, 16). When necessary, strains were cultured in medium supplemented with antibiotics at the following concentrations: ampicillin (Amp; 100 μg/ml), kanamycin (Kan; 25 μg/ml), spectinomycin (Spec; 100 μg/ml), and tetracycline (Tet; 25 μg/ml).

Plasmid and genomic DNA preparations, restriction enzyme digestions, and PCR procedures were performed by conventional techniques using commercially available material. All manipulations of DNA were performed according to the manufacturers' instructions.

Construction and screening of mini-Tn*5* transposon library. Conjugation of *K. pneumoniae* IApc35 with *E. coli* S17-1 λpir carrying the plasmid pUTminiTn*5*-Kn was performed as previously described by our group (2). Conjugants were selected on M9 minimal medium supplemented with kanamy-

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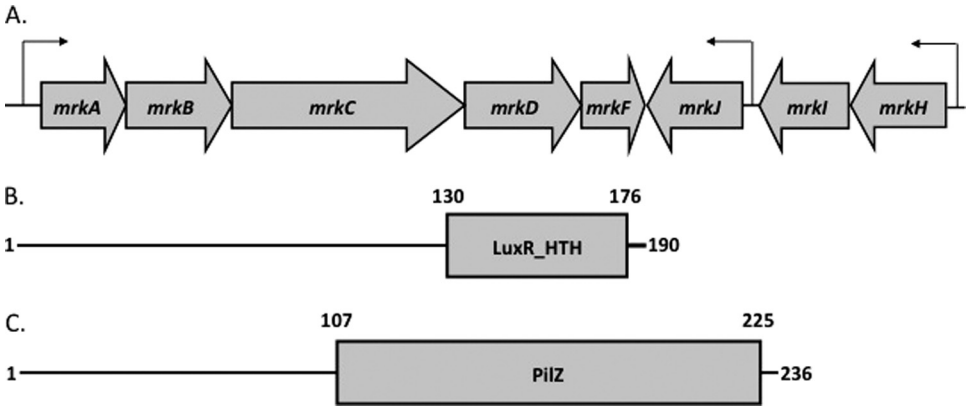

FIG. 1. (A) Genetic organization of the *mrk* gene cluster. Putative promoter regions have been identified by sequence analysis and are indicated by arrows. (B) Predicted domain architecture of MrkI. The site of the mini-Tn*5* insertion is within the predicted LuxR-like DNA binding domain (amino acids 130 to 176) in the C-terminal region of the 190-amino-acid MrkI polypeptide. (C) The location of the predicted PilZ c-di-GMP binding site lies within the C-terminal region (amino acids 107 to 225).

cin to prevent growth of both the donor and recipient strains. Subsequently, appropriate dilutions of bacterial suspensions in phosphate-buffered saline (PBS) were plated on M9 minimal medium and incubated overnight at 37°C. Bacterial colonies were screened for the production of surface-associated type 3 fimbriae using conventional immunoblotting techniques and monospecific fimbrial antiserum at a dilution of 1:40,000 and for subsequent development with goat anti-rabbit serum conjugated to alkaline phosphatase (4, 20). All colonies that did not react with the fimbrial serum were isolated, retested for lack of reactivity with fimbria-specific antiserum, and stored at −80°C. Insertions of the mini-Tn*5* into genes encoding the structural and assembly components of the *mrkABCDF* cluster were identified by standard PCR procedures and not examined further.

Mapping of the mini-Tn*5* insertion site. Genomic DNA was isolated from nonfimbrial mutants, restricted with SphI, and ligated into SphI-digested pACYC184. The nucleotide sequence of the inserted DNA fragment was determined, and *K. pneumoniae*-derived sequences flanking the transposon were identified. Subsequently, the location of these sequences on the *K. pneumoniae* genome was identified using the genome sequence of *K. pneumoniae* MGH

TABLE 1. Plasmids, strains, and oligonucleotides used in this study

Strain/plasmid/oligonucleotide	Description/sequence (5′–3′)[a]	Source/reference
Strains		
IApc35	Plasmid-cured variant of IA565, type 3 fimbria positive	12
IApc35 *mrkI*::Kn^r	Kan^r; IApc35 *mrkI* insertion mutant, type 3 fimbria negative	This study
IApc35 Δ*mrkHI*	IApc35 *mrkHI* deletion mutant, type 3 fimbria negative	This study
BL21 AI	Protein expression strain	Invitrogen (Carlsbad, CA)
NEB 5-α	General *E. coli* cloning strain	NEB (Ipswich, MA)
S17-1 λ*pir*	*E. coli* donor strain	3
SM10 λ*pir*	*E. coli* donor strain	19
Plasmids		
pACYC184ΔCm^r	Tet^r Cam^s; empty vector control for complementation constructs	This study
pACYC*mrkH*	Tet^r; *mrkH* complementation vector	This study
pACYC*mrkI*	Tet^r; *mrkI* complementation vector	This study
pACYC*mrkHI*	Tet^r; *mrkHI* complementation vector	This study
pACYC*mrkH*$_{R113A}$*mrkI*	Tet^r; mutated *mrkHI* complementation vector	This study
pDEST17	Amp^r; 6×His-tagged Gateway expression vector	Invitrogen
pDEST*mrkH*	Amp^r; 6×His-tagged MrkH expression construct	This study
pDEST*mrkH*$_{R113A}$	Amp^r; 6×His-tagged MrkH(R113A) expression construct	This study
pDS132	Cam^r; *sacB* suicide vector	26
pDS132*mrkI*::kn^R	Cam^r Kan^r; construct used to make IApc35 *mrkI*::Kn^r	This study
pDS132Δ*mrkHI*	Cam^r Spec^r; construct used to make IApc35 Δ*mrkHI*	This study
pGEM-T Easy	Amp^r; subcloning vector	Promega (Madison, WI)
pTrc99A	Amp^r; general subcloning vector	Amersham-Pharmacia (Piscataway, NJ)
pTrc99AP$_{mrkA}$-*lacZ*	Amp^r; pTrc99A-based reporter construct	This study
pUTminiTn*5*-Kn	Amp^r Kan^r; mini-Tn*5* delivery plasmid	3
Oligonucleotides		
CNM003	GTGCAGCGTCGCCGCGATCCCGCCTTTCGT TTAGCCCA TGAGCATGACTTTTATTGC	
CNM004	GCAATAAAAGTCATGCTCATGGGCTAAACGAAAGGCG GGATCGCGGCGACGCTGCAC	

[a] Cam, chloramphenicol. Underlining indicates the site of substitution.

78578, available online (http://genome.wustl.edu/genomes). The nucleotide sequences flanking the mini-Tn*5* were determined in mutants and were found not to have resulted in large rearrangements of the DNA during transposition.

Construction of defined site-directed mutants. Approximately 1-kb regions of DNA flanking the *mrkI* and the *mrkHI* genes were cloned into the vector pGEM-T Easy. Fragments were ligated together, incorporating an internal XbaI restriction site, into which a kanamycin resistance determinant was introduced for the construction of the MrkI mutant only. *K. pneumoniae*-derived DNA was excised from the pGEM-T Easy recombinant plasmids using SacI and SphI. These fragments were ligated into either the suicide vector pDS132 (for *mrkI*) or pDS132-spec^R (for *mrkHI*). The resulting plasmids, pDS132*mrkI*::kn^R and pDS132Δ*mrkHI*, were transformed into the permissive host *E. coli* SM10 λ*pir* and subsequently introduced into *K. pneumoniae* IApc35 via conjugation. Transconjugants were selected on either LB-Kan/Amp or LB-Spec/Amp plates, followed by counterselection on 5% sucrose plates (17, 26). Characterization of *mrkI* insertion or *mrkHI* deletion mutants was performed using standard PCR techniques.

Detection of type 3 fimbriae. Surface production of fimbrial appendages was detected using monospecific fimbrial antiserum as described elsewhere by our group (11, 14). Aerobic cultures were grown at 37°C overnight on either LB agar or as 25-ml LB cultures grown in a 125-ml flask shaken at 220 rpm. Anaerobic and microaerophilic cultures were grown either on LB agar in anaerobic Bio-Bag type A environmental chambers (Becton-Dickinson, Sparks, MD) or as static LB broth cultures, respectively. When necessary, fimbriae were observed by transmission electron microscopy using formaldehyde-fixed bacteria stained with uranyl acetate as previously described (29).

Transcription of *mrk*. Expression of *mrk* genes in *K. pneumoniae* strains grown under aerobic or anaerobic conditions was determined by quantitative reverse transcription-PCR (qRT-PCR) as previously described (14). Comparison of gene expression between strains grown aerobically and anaerobically was done following cDNA synthesis from equal concentrations of total cellular RNA. Also, the cloned *mrk* genes in the *K. pneumoniae* IApc35 Δ*mrkHI* mutant were assayed for *mrkA* expression under aerobic conditions using qRT-PCR.

In addition, the ability of the cloned *mrkH*, *mrkI*, and *mrkHI* genes and their derivatives to affect expression of *mrkA* was determined using a plasmid-borne reporter fusion, pTrc99AP$_{mrkA}$-*lacZ*, in an *E. coli* host. This fusion was constructed by cloning a XbaI/HindIII-tailed 444-bp fragment of DNA immediately upstream of *mrkA*, and possessing the promoter region, into those respective sites in pTrc99A containing a promoterless *lacZ* gene.

Biofilm formation assays. The ability of *K. pneumoniae* strains to form biofilms on solid surfaces was determined as previously described (14, 24, 25).

Mutation of the MrkH c-di-GMP binding site. Arginine-113 of MrkH was mutated to alanine using overlapping oligonucleotides. Using primers CNM003 and CNM004 which contained the desired mutation and pACYC*mrkHI* as template, the FailSafe PCR enzyme mix (Epicentre, Madison, WI) was used with 18 cycles of the following reaction: 95°C for 30 s, 55°C for 1 min, and 68°C for 5 min. The resulting plasmid PCR product was digested with DpnI for 1 h at 37°C and then transformed into chemically competent *E. coli* DH5α (Invitrogen, Carlsbad, CA). The appropriate mutation in the resulting plasmid and the absence of any additional mutations in pACYC*mrkH*$_{R113A}$*mrkI* were verified by DNA sequencing.

Purification of MrkH and MrkH(R113A). The *K. pneumoniae* IApc35 *mrkH* and *mrkH*(*R113A*) genes were amplified from pACYC*mrkHI* and pACYC*mrkH*$_{R113A}$*mrkI*, respectively, by standard PCR procedures and cloned into the Gateway vector pENTR/D-Topo (Invitrogen). These genes were subsequently integrated into the Gateway-compatible destination vector pDEST17 to construct genes encoding His-tagged fusion proteins and introduced into the expression strain BL21-AI. *E. coli* BL21-AI transformants carrying either pDEST17*mrkH* or pDEST17*mrkH*$_{R113A}$ were used to prepare native MrkH or MrkH(R113A), respectively, by Ni-nitrilotriacetic acid (NTA) affinity chromatography by following the manufacturer's instructions (Qiagen, Valencia, CA). Successful purification of both MrkH and MrkH(R113A) was assessed by 12% SDS-PAGE and Western blotting using anti-6×His antibody (Qiagen). Additionally, MrkI was purified as a maltose binding protein (MBP)-fusion gene product (MBP-MrkI) using a commercially available system (NEB, Ipswich, MA).

Binding of c-di-GMP to MrkH. Generation of ^{32}P-labeled c-di-GMP was performed as previously described (9, 10). The c-di-GMP binding assay was based on that described by Hickman and Harwood (9). A 20-μl mixture of 0.2 mM protein and 2.0 μM [^{32}P]c-di-GMP in binding buffer (40 mM Tris [pH 7.8], 10 mM magnesium acetate, 50 mM KCl) was incubated on ice for 25 min. The reaction mixtures were then brought to a 100-μl volume with binding buffer and immediately loaded onto a slot blot apparatus (PR 600 slot blot; Hoefer Scientific) containing a 0.2-μM nitrocellulose membrane (0.45 mM Protran BA85; Whatman), followed by a wash using 1.0 ml cold binding buffer. The membrane was removed and scanned on a phosphorimager (Packard Instant Imager; Packard Instrument Company) to measure radioactive counts of membrane-bound [^{32}P]c-di-GMP. For the competition assay, a 10-fold excess (20 μM) of cold c-di-GMP was added to the reaction mixture (Biolog, Bremen, Germany). In additional experiments, an equal amount of [α-^{32}P]GTP was substituted for [^{32}P]c-di-GMP to further assess MrkH binding specificity. Additionally, MBP-MrkI was examined for the ability to bind [^{32}P]c-di-GMP. Reaction mixtures containing purified LacZα or protein buffer alone were used as controls.

RESULTS

Immunoblotting of a mini-Tn*5* transposon bank of insertion mutants in *K. pneumoniae* IApc35. More than 21,000 insertion mutants were screened for their ability to produce surface-associated type 3 fimbriae. Of these, 11 (0.05%) mutants consistently failed to react with fimbria-specific antiserum, even after growth on GCAA agar, which favors the phenotypic expression of these fimbriae (7). Following mapping of the insertion site of the mini-Tn*5*, three of the mutants were shown to have the transposon inserted into genes that are part of the previously described *mrk* gene cluster. Therefore, eight mutations in genes that do not encode either structural or assembly components of the type 3 fimbrial system were isolated. The insertion sites of these mutants are currently being identified, and one of these is described below.

***K. pneumoniae* IApc35 MrkI and MrkHI mutants do not produce surface-associated fimbriae.** Of the eight mutants isolated that possess the transposon in a gene distinct from the *mrkABCDF* cluster, one of these was further characterized. The site of insertion in this mutant was found to be in a gene encoding a putative transcriptional regulator and annotated KPN_03273 on the *K. pneumoniae* MGH 78578 genome (GenBank accession number CP000647). The predicted size of this gene is 573 bp, encoding a gene product of 190 amino acids. BLAST analyses of this gene product suggested that it belongs to a family of regulators characterized by a LuxR-like DNA binding domain spanning amino acids 130 to 176 in its C-terminal region (Fig. 1B). The precise site of insertion of the mini-Tn*5* was within the predicted DNA binding region encoding amino acid 151. We previously named this gene *mrkI*, which is located between *mrkH* and *mrkJ*, though the *K. pneumoniae* MGH 78578 genome lacks the correct annotation for *mrkH* (14). The *mrkHIJ* genes are located adjacent to the previously characterized *mrk* gene cluster and exhibit opposite transcriptional polarity to these genes (Fig. 1A). Using intergenic RT-PCR, it was found that *mrkH*, *mrkI*, and *mrkJ* are cotranscribed (data not shown). *mrkH* is predicted to encode a protein containing a PilZ c-di-GMP binding domain at its C terminus and an N terminus that exhibits little homology to currently characterized domains (Fig. 1C).

MrkI and MrkHI mutants of *K. pneumoniae* IApc35 were constructed by conventional techniques. Neither of the *K. pneumoniae* IApc35 *mrkI*::Kn^r and IApc35 Δ*mrkHI* mutants produce surface-associated type 3 fimbriae following growth under aerobic conditions (Table 2). Interestingly, the MrkI mutant does express type 3 fimbriae when grown anaerobically or microaerophilically as either agar or static broth cultures, respectively, while the Δ*mrkHI* mutant remains nonfimbriate under either condition (Table 2). The mutants can be complemented to restore fimbrial production by transformation with

TABLE 2. Type 3 fimbrial production of *Klebsiella* strains

Culture condition	Serum titer[a]				
	IApc35	IApc35 *mrkI*::Kn^r	IApc35 *mrkI*::Kn^r plus *mrkI*	IApc35 Δ*mrkHI*	IApc35 Δ*mrkHI* plus *mrkHI*
Aerobic (shaken flask)	5,120	<40	40,960	<40	40,960
Aerobic (agar grown)	5,120	<40	40,960	<40	40,960
Microaerophilic (static tube)	40,960	20,480	ND	<40	40,960
Anaerobic (agar grown)	5,120	10,240	ND	<40	40,960

[a] The serum titer represents the reciprocal of the anti-MrkA serum dilution needed to produce visible agglutination. The lowest dilution of serum used was 1:40. ND, not determined.

the cloned genes (Table 2). Electron microscopy confirmed the absence of fimbriae on the MrkI and MrkHI mutants and many fimbriae on the surfaces of complemented strains (Fig. 2). Interestingly, in a Δ*mrkHI* background, introduction of a plasmid solely expressing *mrkI* was unable to complement fimbrial expression, while a plasmid expressing only *mrkH* was able to restore type 3 fimbriation (Table 3). Also, overexpression of *mrkH* in an *mrkI* mutant background was able to restore fimbrial expression despite the absence of *mrkI* (Table 3).

MrkI and MrkHI mutants are affected in *mrkA* gene transcription. Using qRT-PCR analysis of RNA extracted from aerobically grown agar cultures, it was found that both IApc35 *mrkI*::Kn^r and IApc35 Δ*mrkHI* have significantly reduced levels of *mrkA* transcription. Levels of the *mrkA* transcript in the IApc35 *mrkI*::Kn^r strain were approximately 20-fold lower than those in IApc35. Similarly, using RNA extracted from IApc35 Δ*mrkHI*, a significant decrease in *mrkA* expression was observed, with a 33-fold reduction in transcription compared to that of the parental strain (Fig. 3A). Since MrkI mutants assemble surface-associated fimbriae when grown anaerobically, *mrkA* expression under these conditions was determined. *K. pneumoniae* IApc35 *mrkI*::Kn^r grown anaerobically exhibited levels of *mrkA* expression that were indistinguishable from those of the parental IApc35 strain. *mrkA* transcription levels in the IApc35 Δ*mrkHI* mutant were significantly lower than those of the parental strain and were reduced by approximately 2,000-fold (Fig. 3B). Also, we examined a possible autoregulatory role of MrkI on *mrkHI* transcription and observed no decrease in gene expression in the MrkI mutant (data not shown).

Expression of *mrk* genes is increased following anaerobic growth. Quantitative RT-PCR analysis using RNA extracted from *K. pneumoniae* IApc35 cultures grown anaerobically indicated increased *mrkA*, *mrkH*, and *mrkI* expression compared to that of cultures incubated aerobically. Increased expression levels of approximately 285-, 77-, and 91-fold were observed for *mrkA*, *mrkH*, and *mrkI*, respectively (Table 4).

MrkI and MrkHI mutants have a decreased ability to form biofilms on an abiotic surface. Using crystal violet plate assays, it was shown that *K. pneumoniae* IApc35 *mrkI*::Kn^r has a decreased ability to form a biofilm on plastic surfaces compared to that of the parental strain. When the cloned *mrkI* gene was reintroduced into the IApc35 *mrkI*::Kn^r strain, full restoration of biofilm formation was observed, as shown in Fig. 4A. Also, using these assays, the IApc35 *mrkI*::Kn^r mutant transformed with an empty vector had a significantly decreased ability to form biofilms, whereas the parental IApc35 strain carrying the same plasmid is a biofilm producer. Likewise, as shown in Fig. 4B, the *mrkHI* deletion mutant exhibited a significantly reduced (approximately 7-fold) ability to form a biofilm compared to that exhibited by the parental strain. Restoration of biofilm formation was achieved by complementation with the cloned *mrkHI* determinants, and such transformants also exhibited an increased ability to form biofilms compared to that exhibited by the parental strain (approximately 2-fold). Transformants of the MrkHI mutant possessing an empty cloning vector did not demonstrate biofilm formation.

Mutation of a conserved PilZ residue results in the inability to induce type 3 fimbria production. Alignment of the PilZ domain in MrkH with other PilZ domain-containing proteins revealed complete conservation of five residues which have previously been shown to be important in the ability of the PilZ domain to bind c-di-GMP (27) (Fig. 5A). Replacement of the

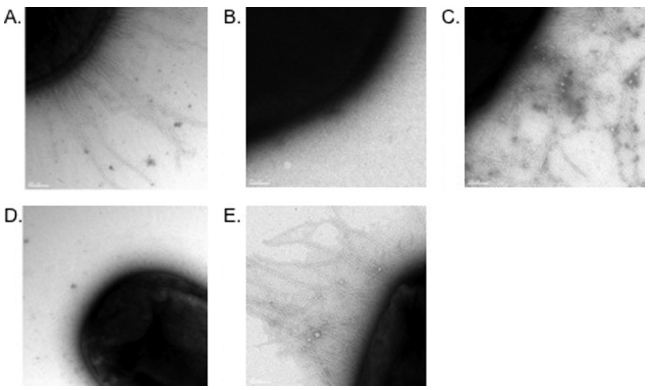

FIG. 2. Fimbrial production by *K. pneumoniae* strains. (A) *K. pneumoniae* IApc35; (B) MrkI mutant; (C) MrkI mutant transformed with the cloned *mrkI* gene; (D) MrkHI mutant; (E) complemented MrkHI mutant carrying cloned *mrkHI*.

TABLE 3. Phenotypic complementation analyses

Strain (plasmid)	Serum titer
IApc35 (pACYCΔCm^r)	5,120
IApc35 *mrkI*::Kn^r (pACYCΔCm^r)	<40
IApc35 *mrkI*::Kn^r (pACYC*mrkH*)	40,960
IApc35 *mrkI*::Kn^r (pACYC*mrkI*)	40,960
IApc35 *mrkI*::Kn^r (pACYC*mrkHI*)	40,960
IApc35 Δ*mrkHI* (pACYCΔCm^r)	<40
IApc35 Δ*mrkHI* (pACYC*mrkH*)	40,960
IApc35 Δ*mrkHI* (pACYC*mrkI*)	<40
IApc35 Δ*mrkHI* (pACYC*mrkHI*)	40,960

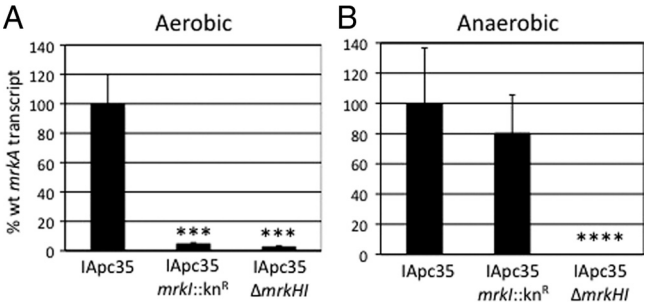

FIG. 3. qRT-PCR of *mrkA* encoding the major fimbrial subunit in *K. pneumoniae* strains. Aerobic (A) and anaerobic (B) *mrkA* transcription in both IApc35 *mrkI*::Kn^r and IApc35 Δ*mrkHI* are shown as the relative decreases in transcription compared to that shown by the parental strain. Statistical significance was determined using Student's *t* test (***, *P* value < 0.001; ****, *P* value < 0.0001).

conserved arginine-113 with alanine on a plasmid-borne copy of *mrkH* was performed and confirmed by nucleotide sequencing. Introduction of this plasmid, pACYC*mrkH*$_{R113A}$*mrkI*, into IApc35 Δ*mrkHI* did not restore type 3 fimbrial expression compared to that observed using the parental pACYC*mrkHI* plasmid (Fig. 5B). This significant reduction in type 3 fimbrial production was further investigated using the reporter plasmid pTrc99AP$_{mrkA}$-*lacZ* in an *E. coli* background. When the parental plasmid pACYC*mrkHI* was introduced into this *E. coli* strain, an increase in β-galactosidase production was observed compared to that observed in the strain carrying the empty vector (pACYC184ΔCm^r), as shown in Fig. 5C. In contrast, when pACYC*mrkH*$_{R113A}$*mrkI* transformants were assayed, a significant decrease in β-galactosidase activity was observed compared to that of the parental plasmid (Fig. 5C).

MrkH binds c-di-GMP. To determine whether MrkH is capable of binding, c-di-GMP filter binding assays were used as previously described (9). Purification of 6×His-tagged MrkH and MrkH(R113A) was performed, and protein purity was determined by Western blot analysis. Equimolar amounts of MrkH proteins immobilized on nitrocellulose were used in the c-di-GMP binding assays. Bound [^{32}P]c-di-GMP was determined by phosphorimaging, and those results are shown in Fig. 6. MrkH bound radiolabeled c-di-GMP, whereas the MrkH(R113A) protein did not (Fig. 6A). Inhibition of MrkH-mediated binding of labeled c-di-GMP was achieved by competition with an unlabeled nucleotide (Fig. 6B). Additionally, binding assays were also performed using [α-^{32}P]GTP, but neither MrkH nor MrkH(R113A) bound this nucleotide (data not shown).

TABLE 4. Aerobic and anaerobic transcriptions of *mrk* genes in parental IApc35

Transcript (condition)	Fold change (± SD)
mrkA (aerobic)	1.0 (± 0.1)
mrkA (anaerobic)	284.78 (± 47.75)[a]
mrkH (aerobic)	1.0 (± 0.15)
mrkH (anaerobic)	76.93 (± 24.45)[a]
mrkI (aerobic)	1.0 (±0.15)
mrkI (anaerobic)	90.65 (± 12.34)[a]

[a] *P* value < 0.0001.

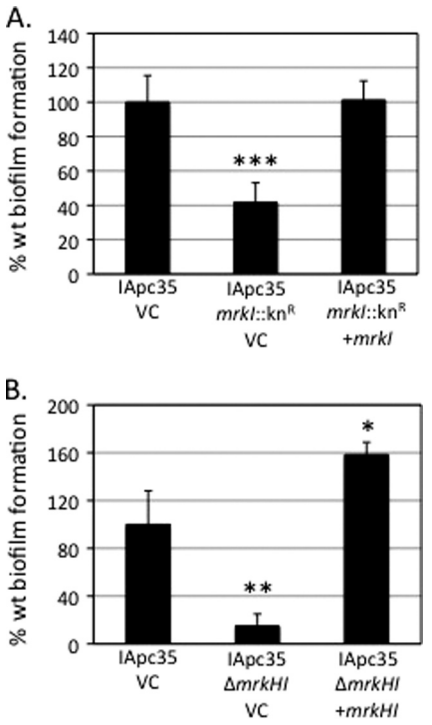

FIG. 4. Biofilm phenotypes of *K. pneumoniae* strains. (A) Biofilm formation of parental IApc35 and the MrkI mutant carrying the empty vector (VC) compared to that of the complemented MrkI mutant on an abiotic surface. (B) Decreased biofilm formation of the IApc35 MrkHI mutant compared to those of parental IApc35 and the MrkHI mutant complemented with plasmid-borne *mrkHI*. Statistical significance was determined using Student's *t* test (*, *P* value < 0.05; **, *P* value < 0.01; ***, *P* value < 0.001).

MrkH and MrkHI activate the *mrkA* promoter. To examine whether MrkH, MrkI, or MrkHI were sufficient to induce transcription from the *mrkA* promoter, β-galactosidase assays were used. Plasmids comprised of the same vector backbone, carrying either *mrkH* or *mrkI* alone or *mrkHI* together, were introduced into *E. coli* NEB 5-α transformed with a P$_{mrkA}$-*lacZ* reporter fusion. The strain carrying both the reporter fusion and *mrkH* alone was found to exhibit a significant increase (approximately 114-fold) in transcriptional activity from the *mrkA* promoter compared to that exhibited by a transformant possessing the cloning vector alone. When *mrkI* alone was introduced into the strain carrying the reporter plasmid, no increase in β-galactosidase production was seen compared to that exhibited by transformants without *mrkI*. When a plasmid carrying both *mrkH* and *mrkI* was transformed into the reporter strain, a significant increase in *mrkA* transcription, compared to that exhibited by the strain carrying *mrkH* alone, was observed (approximately 8-fold) (Fig. 7).

Similarly qRT-PCR analysis of *K. pneumoniae* IApc35 Δ*mrkHI* transformed with the *mrkI*, *mrkH*, and *mrkHI* genes also indicated that MrkH but not MrkI could affect *mrkA* expression. The MrkHI mutant transformed with *mrkI* alone did not exhibit any increase in *mrkA* expression compared to that exhibited by mutants transformed with the empty vector. However, transformation with a plasmid bearing the *mrkHI*

A.

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          ★  ★                          ★★ ★
PlzD    134 QLRKEPRFELNLAGKVLFDEHRGDCELR-DLSRSGCRF  170
PlzE    128 AIRSHERYKVAIPAEVSEDNKNYAGTLV-DFSISGCGV  164
PA4608    5 HDRFH-RIAFDADSEILQGERRWEVLLH-DVSLHGILV   40
MrkH    111 DPRFRLRHEHDFYCRGRHKNGENYLFDIKDISDGGCAL  148
MrkH⌘   111 DPAFRLRHEHDFYCRGRHKNGENYLFDIKDISDGGCAL  148
```

B.

Strain (plasmid)	Serum titer
IApc35 (VC)	5,120
IApc35 Δ*mrkHI* (VC)	<40
IApc35 Δ*mrkHI* (pACYC*mrkHI*)	40,960
IApc35 Δ*mrkHI* (pACYC$mrkH_{R113A}mrkI$)	320

C.

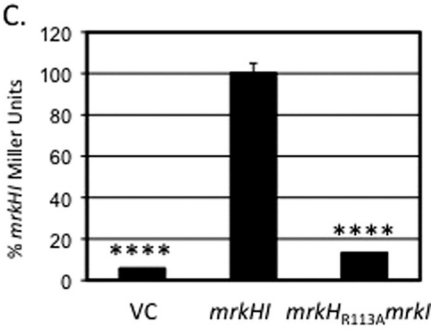

FIG. 5. Analysis of the R113A mutation in MrkH. (A) Alignment of previously characterized PilZ domain-containing proteins with MrkH. Conserved residues shown to affect c-di-GMP binding are indicated with stars. The alignment of the R113A mutant is also indicated (⌘). (B) Effect of the R113A mutant on type 3 fimbria production. Values are reciprocals of serum titers needed to cause visible agglutination. (C) Use of a P_{mrkA}-*lacZ* fusion to examine the ability of MrkHI to induce expression in *E. coli* transformants. Statistical significance was determined using Student's *t* test (****, *P* value < 0.0001).

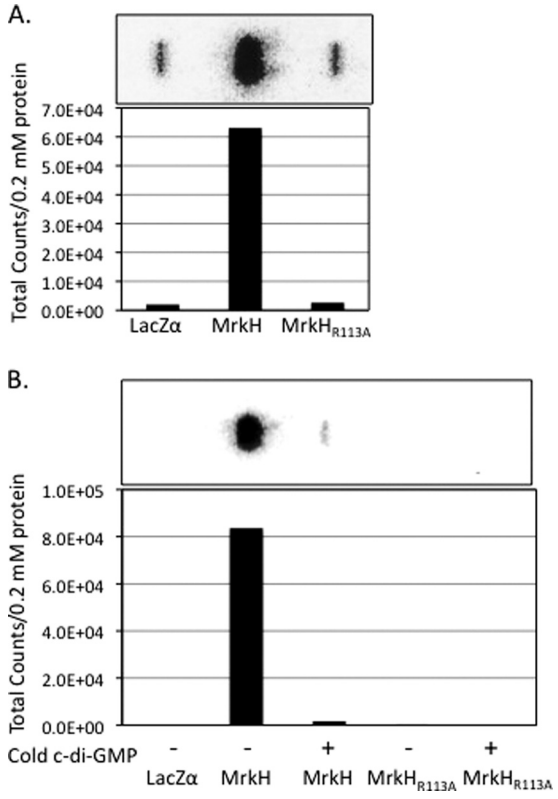

FIG. 6. Ability of MrkH to bind [^{32}P]c-di-GMP. (A) Filter binding assay using purified LacZα, MrkH, and MrkH(R113A) proteins as targets for binding. Graph represents total specific counts detected from assays represented above the graph. (B) Filter binding assays of binding reactions with (+) or without (−) the addition of unlabeled c-di-GMP.

genes resulted in a 36-fold increase in *mrkA* transcription compared to that seen with MrkH alone ($P < 0.001$).

DISCUSSION

K. pneumoniae type 3 fimbriae play an important role in the ability of the bacteria to bind to, and subsequently form biofilms on, HECM-coated surfaces. Both the fimbrial adhesin (MrkD) and the polymerized fimbrial shaft protein (MrkA) play important roles in this function (12, 13, 16, 30). We have previously proposed that MrkD facilitates the adherence of the organism to specific collagen molecules that form part of the HECM. However, fimbriate bacteria that possess no functional adhesin also form biofilms on abiotic surfaces (12, 14, 16). Consequently, the production of type 3 fimbriae could lead to the initiation of biofilm formation on inserted devices such as catheters shortly after insertion and also after these devices become coated *in situ* with host factors. The genetic regulation

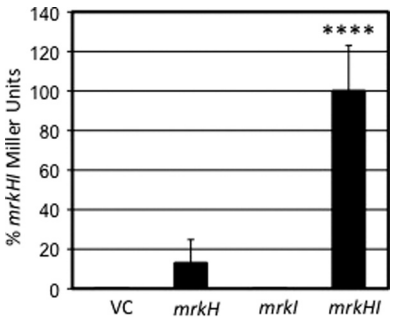

FIG. 7. Ability of cloned *mrkH*, *mrkI*, and *mrkHI* to induce transcription of a P_{mrkA}-*lacZ* reporter in *E. coli* compared to that of a vector control (VC). Statistical significance was determined using Student's *t* test (****, *P* value < 0.0001).

of *mrk* gene expression is poorly understood but, like other enterobacterial fimbrial systems, is likely to involve complex regulatory circuits.

In order to identify regulatory elements of the type 3 fimbrial operon, we constructed a mini-Tn*5* transposon library in *K. pneumoniae* IApc35. This strain is a plasmid-cured derivative of the clinical isolate *K. pneumoniae* IA565, produces high levels of type 3 fimbriae, and forms robust biofilms on abiotic surfaces (12). It possesses only one chromosomally borne copy of the *mrk* gene cluster. One nonfimbriate mutant from this library possessed a transposon insertion within a gene encoding a putative transcriptional regulator, which we have previously termed *mrkI* (14). This gene is predicted to encode, by comparison to families of functional proteins, a protein which contains only one identifiable domain, a LuxR-like DNA binding domain in its C-terminal region. The N-terminal region of MrkI exhibits little relatedness to any characterized protein domains and therefore has no readily identifiable receiver domain. Interestingly, *mrkI* is located between two genes, as follows: the first, which we have named *mrkH*, is predicted to encode a protein which contains a C-terminal c-di-GMP binding domain (PilZ), and the second, *mrkJ*, is a gene which we have previously shown to produce a functional phosphodiesterase which modulates the intracellular levels of c-di-GMP within *K. pneumoniae* (14). A defined MrkI mutant of strain IApc35 was constructed and, like the original transposon mutant, was found to be unable to assemble type 3 fimbriae. Also, we found that *mrkI* is cotranscribed with *mrkH*. This is consistent with the observation that the only promoter identified by sequence analysis, which is likely to drive *mrkI* transcription, lies upstream of *mrkH*. In addition to *mrkHI* cotranscription, we also found that *mrkJ* transcription can also occur from the *mrkH* promoter, though the *mrkI* and *mrkHI* mutations were not found to significantly alter the levels of *mrkJ* transcription (data not shown). Therefore, it is possible that transcription of *mrkJ* can also occur from a promoter immediately upstream of it. Deletion of *mrkHI*, like the single *mrkI* mutation, resulted in the decreased ability of *K. pneumoniae* to produce surface-associated type 3 fimbriae. Repeated attempts were made to construct an *mrkH* deletion mutant but proved unsuccessful. The precise reason for this is unclear but suggests that such a mutation may be lethal, even though deletion of *mrkHI* together and reintroduction of *mrkI* alone is not.

Interestingly, we also demonstrated that the MrkI mutant was nonfimbriate only when cultured under aerobic conditions. When these strains were grown anaerobically on agar or microaerophilically as deep static broth cultures, the mutants exhibited fimbrial titers equivalent to or higher than those observed for the parental strains grown aerobically. The MrkHI mutant, in contrast, was consistently nonfimbriate under both aerobic and anaerobic conditions. It is possible that the MrkI mutant is fimbriate when grown anaerobically due to increased expression of *mrkH* under these conditions, which facilitates fimbria production independently of MrkI. Consequently, during anaerobic growth, MrkH and MrkI are likely to facilitate increased *mrkA* expression, resulting in a strongly fimbriate phenotype. However, in the absence of MrkI, the increased MrkH production, compared to that exhibited by bacteria grown aerobically, may enable MrkH to interact with an orphan activator to facilitate *mrkA* transcription. Also, it is possible that *K. pneumoniae*, in response to different environmental conditions, produces different regulators that can interact with MrkH to modulate surface expression of type 3 fimbriae. Currently, we are investigating the interaction between MrkH and MrkI in the presence and absence of c-di-GMP. However, MrkH may act as a protein with a more general function of sensing the intracellular concentrations of c-di-GMP. This is supported by the observation that unlike many organisms in which c-di-GMP serves a regulatory role, all sequenced *K. pneumoniae* genomes (*K. pneumoniae* MGH78578, *K. pneumoniae* NTUH-K2044, and *K. pneumoniae* 342) possess only one PilZ domain-containing protein (MrkH) that is not predicted to act as a cellulose synthase. This is not unique within the *Enterobacteriaceae*, as it appears that many members of this family contain only the PilZ domain carrying protein YcgR. However, the previously described N-terminal YcgR domain of these proteins exhibits no relatedness to that of MrkH. Currently, no other c-di-GMP binding proteins have been characterized in *K. pneumoniae*, so it is possible that c-di-GMP sensing is a major function of MrkH.

The ability of MrkH and MrkI to facilitate transcription of *mrkA* in an *E. coli* transformant that possesses no *mrk* genes of its own and the DNA binding domain present in MrkI led us to speculate that MrkI binds the promoter region of *mrkA*. However, we were not able to demonstrate binding *in vitro* using gel mobility shift assays (data not shown). The fact that MrkA can be made in the absence of MrkI under specific growth conditions makes it less surprising that it was not possible to demonstrate this interaction. However, it is possible that MrkI binds to this region, and we were unable to replicate *in vitro* the conditions for binding *in vivo*. Additional bacterial factors may be required for this activity. The results shown by *E. coli* transformants are consistent with the observation that MrkH and MrkI affect transcription, as detected by qRT-PCR in *K. pneumoniae*. The presence of MrkH alone facilitates detectable levels of *mrkA* transcription in both *E. coli* and *K. pneumoniae* transformants, but this is significantly lower than that observed when both MrkH and MrkI are present. The MrkI mutant of *K. pneumoniae* is able to produce MrkH, but this mutant is phenotypically nonfimbriate and exhibits no *mrkA* transcription. The level of *mrkA* transcription in *E. coli* transformants possessing only MrkH could simply be due to the relatively high concentrations of MrkH produced by the cloned gene. Since analysis of the *K. pneumoniae* genome indicates that the only PilZ-possessing protein in these bacteria that is not involved in cellulose metabolism is MrkH, it is possible that this protein acts as a c-di-GMP signaling adaptor for many systems that are regulated by the intracellular concentrations of the molecule. Consequently, its effect on gene transcription may depend on the intracellular concentrations of MrkH. In the absence of MrkI, high levels of MrkH may more weakly interact, directly or indirectly, with additional regulators. Further studies will be required to investigate this hypothesis.

It is becoming increasingly clear that the genetic regulation of fimbrial genes encoding appendages assembled by the chaperone-usher pathway is subject to a complex regulatory circuit involving different families of DNA binding proteins (1, 18, 21). The type 3 fimbrial system, a fimbrial type commonly observed to be produced by enterobacteria associated with nosocomially acquired infections, is also likely to be regulated

by multiple gene products. The identification of these regulatory factors will facilitate an understanding of type 3 fimbria production and its role in host cell interaction. Due to the multitiered regulatory networks that govern other fimbrial systems, it is possible that MrkH and MrkI regulate type 3 fimbrial expression by acting upstream of a primary regulator. However, the location of *mrkHIJ* immediately adjacent to *mrkABCDF* may indicate an evolutionary selection for these two gene clusters. Currently, efforts are under way to further investigate the role of both MrkH and MrkI as fimbrial regulators and also to determine whether MrkI regulates expression of nonfimbrial genes.

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