JOURNAL OF BACTERIOLOGY, Aug. 2010, p. 3944–3950 Vol. 192, No. 15
0021-9193/10/$12.00 doi:10.1128/JB.00304-10

Role of MrkJ, a Phosphodiesterase, in Type 3 Fimbrial Expression and Biofilm Formation in *Klebsiella pneumoniae*[▽]

Jeremiah G. Johnson and Steven Clegg*

Department of Microbiology, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa 52242

Received 17 March 2010/Accepted 18 May 2010

***Klebsiella pneumoniae* is an opportunistic pathogen that has been shown to adhere to human extracellular matrices using the type 3 fimbriae. Introduction of plasmids carrying genes known to alter intracellular cyclic-di-GMP pools in *Vibrio parahaemolyticus* revealed that these genes also altered type 3 fimbrial surface expression in *K. pneumoniae*. Immediately adjacent to the type 3 fimbrial gene cluster is a gene, *mrkJ*, that is related to a family of bacterial genes encoding phosphodiesterases. We identify here a role for MrkJ, a functional phosphodiesterase exhibiting homology to EAL domain-containing proteins, in controlling type 3 fimbria production and biofilm formation in *K. pneumoniae*. Deletion of *mrkJ* resulted in an increase in type 3 fimbria production and biofilm formation as a result of the accumulation of intracellular cyclic-di-GMP. This gene was shown to encode a functional phosphodiesterase via restoration of motility in a *V. parahaemolyticus* strain previously shown to accumulate cyclic-di-GMP and *in vitro* using phosphodiesterase activity assays. The effect of the *mrkJ* mutation on type 3 fimbrial expression was shown to be at the level of *mrkA* gene transcription by using quantitative reverse transcription-PCR. These results reveal a previously unknown role for cyclic-di-GMP in type 3 fimbrial production.**

Klebsiella pneumoniae is an opportunistic pathogen responsible for a variety of nosocomial infections, including urinary and respiratory tract infections. Colonization and subsequent clinical infection of hospitalized patients by these bacteria is frequently associated with insertion of medical devices such as urinary catheters and endotracheal tubes. Both the coating of these devices, *in situ*, by host cell matrix proteins and the disturbance of epithelial surfaces after insertion of these devices provide the conditions that are favorable for bacterial adherence to host cell extracellular matrix proteins (ECMs). Following adherence, the organisms can grow as a biofilm at these sites, and this property, along with the broad spectrum of antibiotic resistance of most *K. pneumoniae* strains, makes treatment of these infections very difficult (12, 13, 17, 32).

Two well-characterized fimbrial types have been described in *K. pneumoniae* and have been implicated in mediating bacterial binding to host surfaces. Both fimbrial types are assembled using the chaperone-usher pathway described by Hultgren and coworkers for P pilus assembly (23, 45). The type 1 fimbriae are closely related to those described in *Escherichia coli* and mediate binding to mannosylated receptors on host cell glycoconjugates. Although genetic regulation of *K. pneumoniae* type 1 fimbrial gene (*fim*) expression has not been described in detail, initial studies indicate that regulation is likely to be related to that described for *E. coli* fimbriae (16, 20, 29, 41). However, the *K. pneumoniae fim* gene cluster possesses a gene, *fimK*, not found in the *E. coli* system that alters surface expression of these organelles (38). Mutants unable to produce FimK are hyperfimbriate and are more able to colonize the murine urinary tract compared to wild-type *K. pneumoniae*. FimK possesses an EAL domain that is characteristic of phosphodiesterases, which in many cases have been shown to modulate the intracellular levels of cyclic diguanylate monophosphate (c-di-GMP), although a direct effect of FimK on the levels of c-di-GMP has yet to be experimentally demonstrated.

The second fimbrial group, type 3 fimbriae, of *K. pneumoniae* has been described by our laboratory and others (1, 14, 18, 35). These fimbriae are encoded by the *mrk* gene cluster and mediate binding *in vitro* to human-derived ECMs prior to biofilm formation on these surfaces (24, 28). The MrkD protein mediates binding to this substrate, whereas the MrkA peptide constitutes the major fimbrial subunit that is polymerized to form the fimbrial shaft (22, 24, 28). MrkD mutants produce nonadhesive fimbriae that facilitate biofilm formation on abiotic surfaces, but the MrkD adhesin is required for mature biofilm production on ECMs (24). Unlike type 1 fimbrial gene expression, the regulation of *mrk* gene expression is poorly understood. The *mrk* gene cluster is not flanked by site specific recombinases that mediate inversion of the *fimS* switch, as seen in the *E. coli fim* system (29, 41). Instead, the *mrk* cluster is adjacent to a three-gene cluster which encodes gene products that exhibit amino acid relatedness to other bacterial proteins involved in c-di-GMP sensing and modulation (Fig. 1). One of these genes, which we have designated *mrkJ*, exhibits homology to EAL domain containing phosphodiesterases (11, 25, 37, 40). Consequently, we investigated the role of MrkJ in type 3 fimbrial gene expression by *K. pneumoniae* and its ability to function as a phosphodiesterase by using *in vitro* assays and in a previously described system used to detect this enzyme activity.

MATERIALS AND METHODS

Strains, plasmids, and DNA manipulations. The strains, plasmids, and oligonucleotides used in the present study are listed in Table 1. Unless otherwise

* Corresponding author. Mailing address: Department of Microbiology, University of Iowa, 3-403 BSB, 51 Newton Rd., Iowa City, IA 52242. Phone: (319) 335-7778. Fax: (319) 335-9006. E-mail: steven-clegg@uiowa.edu.

▽ Published ahead of print on 28 May 2010.

FIG. 1. Organization of the type 3 fimbrial gene cluster. The transcriptional polarity of the *mrk* genes is indicated by the arrowheads. The *mrkA* promoter (P*mrkA*) has previously been mapped, and the location of the P*mrkJ* and P*mrkH* promoters is based upon nucleotide sequence analysis.

stated, all strains were grown in Luria-Bertani (LB) media at 37°C using antibiotics when appropriate at the following concentrations: ampicillin (100 μg/ml), gentamicin (50 μg/ml), kanamycin (100 μg/ml), spectinomycin (100 μg/ml), and tetracycline (25 μg/ml).

Plasmid and chromosomal DNA preparations, restriction enzyme digests, and other enzymatic reactions were performed according to the manufacturers' protocols using commercially available materials.

Effect of EAL and GGDEF domain-possessing plasmids on type 3 fimbrial expression. Plasmids LM2449 and LM2796 (kindly supplied by Linda McCarter, University of Iowa) were introduced into *Klebsiella* strains by conjugation using the donor strain *E. coli* S17-1λ*pir* carrying the appropriate plasmid (7). The construction and characterization of LM2449 and LM2796 has been described in detail elsewhere (15). LM2449 is a recombinant plasmid that contains an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible *Vibrio parahaemolyticus scrC* gene and LM2796 contains an IPTG-inducible *V. parahaemolyticus scrABC* gene cluster. The gene product of *scrC* has been shown to possess diguanylate cyclase activity and increase bacterial intracellular pools of c-di-GMP in *V. parahaemolyticus*, while the products of *scrABC* together exhibit phosphodiesterase activity and lead to a net decrease in c-di-GMP concentrations (15). *K. pneumoniae* possessing these plasmids were grown overnight on LB agar containing the appropriate antibiotics with or without 0.5 mM IPTG. Type 3 fimbrial expression of these strains was assayed as previously described by us (18, 21, 39).

Phosphodiesterase activity of MrkJ. Primer pair JGJ132 and JGJ122 was used to amplify the intact *mrkJ* gene from *K. pneumoniae* IApc35. The gene was subcloned into pGEM-T Easy by conventional techniques and subsequently ligated into pACYC184 at the EcoRI-ScaI restriction sites, resulting in inactivation of the plasmid-borne *cat*. The recombinant plasmid bearing only the *mrkJ* gene was used to transform *V. parahaemolyticus* LM6567 (Δ*scrABC*) and *K. pneumoniae* strains. Restoration of the swarming phenotype in *V. parahaemolyticus* was determined as previously described (15). In addition, *in vitro* phosphodiesterase activity assays were performed using partially purified His_6-MrkJ. Briefly, *mrkJ* was amplified from IApc35 genomic DNA using the primers *mrkJ*-

TABLE 1. Strains, primers, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Description or sequence (5′–3′)[a]	Source or reference
Strains		
IApc35	Plasmid cured variant of IA565, type 3 fimbriae$^+$	21
IApc35Δ*mrkJ*	IApc35 *mrkJ* deletion mutant, highly type 3 fimbriate	This study
LM5674	Δ*opaR V. parahaemolyticus*	14
LM6567	Δ*scrABC* mutant of LM5674	14
S17-1λ*pir*	*E. coli* donor strain	6
SM10λ*pir*	*E. coli* donor strain	6
Plasmids		
pACYC184	Tet^r Cam^r cloning vector w/p15A *ori*	ATCC, Manassas, VA
pDS132	Cam^r *sacB* suicide vector	36
pDEST17	Amp^r Gateway compatible His_6 tag expression vector	Invitrogen, Carlsbad, CA
pENTR-D-Topo	Kan^r Gateway entry vector	Invitrogen, Carlsbad, CA
pGEM-T Easy	Amp^r subcloning vector	Promega, Madison, WI
pACYC*mrkJ*	Tet^r Cam^s pACYC184-derived vector with *mrkJ* inserted at EcoRI-ScaI	This study
LM2449	Gen^r broad-host-range vector carrying IPTG-inducible *scrC*	14
LM2796	Gen^r broad-host-range vector carrying IPTG-inducible *scrABC*	14
Oligonucleotides		
JGJ80	CATGTCCAGCATCACCGTTG	
JGJ81	CAAGGAGATCGGCGTTGTCG	
JGJ82	CGACTGCCGACAATAAAGCC	
JGJ83	ACGGCGGTAAAGCCCTGAACA	
JGJ122	CCTGTTCACCTATTACGTTGGC	
JGJ132	CCGGTAAATCAGTAGCGGAT	
JGJ135	CTGCTGCTGTCTACTGACAAC	
JGJ136	CTTAATACGCAGCGTCTGGC	
JGJ137	CTACATCACCATACCGCTGC	
JGJ138	AGGGTAAACGGCTGCGGTTTC	
JGJ143	TCTAGACGTAGCGTACAGCGATATCA	
JGJ144	GCGGCCGCCACCCTGGATAACGCTA	
JGJ145	TCTAGAGGGAATGGAGTGGTTTGCTA	
JGJ146	GCGGCCGCGTACCCTTGCCACAAGC	
*rpoD*qRT-PCRF	CTGACGCGCGACACCAT	
*rpoD*qRT-PCRR	GAACGCGATCCATCATGCT	
*mrkJ*Gateway-F	CACCATGAACACTAAAATATTCGAAG	
*mrkJ*Gateway-R	TTACATGGCAATATCATCGGC	
*mrkA*qRT-PCRF	GAACTGGACCGGCGGTAA	
*mrkA*qRT-PCRR	TCACCCGGGATGATTTTGTT	

[a] Cam^r, chloramphenicol resistance; Gen^r, gentamicin resistance; Tet^r, tetracycline resistance; Amp^r, ampicillin resistance; Kan^r, kanamycin resistance.

Gateway-F and *mrkJ*Gateway-R, cloned into the Gateway vector pENTR-D-Topo (Invitrogen, Carlsbad, CA), and subsequently introduced into the His_6-tagged expression vector pDEST17 (Invitrogen) according to the manufacturer's protocol. MrkJ was batch purified using Ni-NTA resin under native conditions and analyzed by SDS-PAGE. The PilZ domain containing protein, MrkH, was purified in the same manner as a nonphosphodiesterase control. Phosphodiesterase activity assays were performed as previously described with 20 μg of protein in assay buffer (50 mM Tris-HCl, 1 mM $MnCl_2$ [pH 8.5]) supplemented with 5 mM bis(*p*-nitrophenol) phosphate (bis-*p*NPP) (6, 26). Reactions were incubated for ~3 h at 37°C, at which points the release of *p*-nitrophenol was quantified at 410 nm.

Construction of the MrkJ mutant of *K. pneumoniae*. The *mrkJ* and flanking regions (~1 kbp on either side) were amplified from the *K. pneumoniae* IApc35 chromosome by using the primer pairs JGJ143/JGJ144 or JGJ145/JGJ146 and were cloned into the vector pGEM-T Easy. The two regions were ligated together, incorporating an internal XbaI site into which the kanamycin resistance cassette was cloned. Next, the *K. pneumoniae* derived sequences possessing the DNA cassette were cloned into the suicide vector pDS132. pDS132Δ*mrkJ*-kn was introduced into *K. pneumoniae* IApc35 by using the *E. coli* donor strain SM10λ*pir*. After selection for transconjugants and resolution of plasmid sequences from the chromosome, by sucrose counter selection, deletion of *mrkJ* was confirmed by standard PCR techniques.

Detection of type 3 fimbriae. The production of type 3 fimbriae was detected by using monospecific antiserum as described in detail by our group (9, 38). In addition, samples of bacterial suspensions were bound to a Protran BA85 nitrocellulose membrane (MidSci, St. Louis, MO) and probed with a primary antibody raised against purified, denatured MrkA. A goat anti-rabbit secondary immunoglobulin serum conjugated to alkaline phosphatase (Sigma, St. Louis, MO) was used to detect type 3 fimbria-positive bacteria. Ponceau S staining of immobilized suspensions was performed to normalize protein concentrations bound for each sample.

Biofilm assays. Crystal violet plate assays were performed in 24-well tissue culture plates to determine the effect of the *mrkJ* mutation on biofilm formation compared to its parental strain (19, 30, 34, 48). Wells containing 1 ml of LB broth were inoculated with 10 μl of an overnight culture and subsequently shaken on an orbital shaker (110 rpm) for 20 h at 37°C. After gentle washing to remove planktonic bacteria, biofilms were stained with 1% crystal violet and solubilized using 95% ethanol, followed by measurement at 595 nm as previously described (34, 43).

To eliminate the possibility that differences in biofilm formation were due to differences in growth rates between strains, growth rates of planktonically grown cultures were determined. All strains used in the present study exhibited similar growth rates under these conditions.

Transcriptional analysis of the *mrk* gene cluster. Total RNA was prepared from *K. pneumoniae* strains after growth on LB agar using a modified procedure originally described by Chouikha et al. (8). Briefly, DNA was removed from RNA preparations by using a DNA-free kit (Ambion, Austin, TX) and, prior to cDNA synthesis, RNA preparations were examined for the presence of contaminating DNA by PCR. cDNA was generated by using a SuperScript III reverse transcriptase kit (Invitrogen) as recommended by the manufacturer. The intergenic region spanning *mrkA* to *mrkB* was amplified from the cDNA library by using the primers JGJ135 and JGJ136, that of the *mrkB* to *mrkC* region was amplified using JGJ82 and JGJ83, the *mrkC* to *mrkD* region was amplified using JGJ80 and JGJ81, and finally the *mrkD* to *mrkF* region was amplified using JGJ137 and JGJ138. Amplicons were detected by using conventional electrophoretic techniques through 2% agarose gels.

qRT-PCR of *mrk* gene expression. cDNA isolated from *K. pneumoniae* strains were prepared as described above. A portion (5 μl) of the cDNA was added to a 20-μl quantitative real-time PCR (qRT-PCR) mixture containing 5 μl of the cDNA, 5 μl of the appropriate primer mix containing 2.5 μM concentrations (each) of the primers used to assay for the expression of *rpoD* (*rpoD*qRT-PCRF and *rpoD*qRT-PCRR) or *mrk* genes (*mrkA*qRT-PCRF and *mrkA*qRT-PCRR), and 10 μl of Power SYBR green Master Mix (Applied Biosystems, Foster City, CA). Reactions were performed by using the Applied Biosystems 7300 Real-Time PCR system, and *mrk* transcription was normalized to the *rpoD* concentrations.

RESULTS

Plasmids encoding phosphodiesterase or diguanylate cyclase activity affect type 3 fimbrial production. Plasmids LM2449 and LM2796, encoding a functional diguanylate cyclase and phosphodiesterase, respectively, have previously been used to determine the role of intracellular c-di-GMP concentrations in *V. parahaemolyticus* (15). Consequently, we used these plasmids to transform the parental strain *K. pneumoniae* IApc35 in order to determine whether overexpression of these enzymes altered fimbrial production. Induction of transformants carrying the diguanylate cyclase gene, *scrC*, on LM2449 resulted in a 4-fold increase in surface fimbrial production after induction by IPTG (Table 2). However, the transformants carrying LM2796 bearing the inducible *scrABC* determinants together did not produce detectable levels of type 3 fimbriae postinduction. Similar results were also observed using noninducing culture conditions, although the LM2449 transformants exhibited a 2-fold increase in fimbriation, due to basal-level expression of the plasmid-encoded diguanylate cyclase or phosphodiesterase (Linda McCarter, unpublished data). The products of the *scrABC* genes have previously been shown to decrease intracellular concentrations of c-di-GMP in *V. parahaemolyticus* (15). Therefore, if LM2449 and LM2796 also affect intracellular concentrations of c-di-GMP in *K. pneumoniae*, fimbrial production is significantly affected by the levels of this molecule.

TABLE 2. Type 3 fimbria production and intracellular c-di-GMP concentrations

K. pneumoniae strain(plasmid)	c-di-GMP[a]	Titer with fimbria-specific serum
IApc35	Normal	5,120
IApc35(LM2449) –IPTG	Normal/High	10,240
IApc35(LM2449) +IPTG	High	40,960
IApc35(LM2796) –IPTG	Normal/Low	<40
IApc35(LM2796) +IPTG	Low	<40
IApc35(pACYC184ΔCm^r)	Normal	5,120
IApc35(pACYC*mrkJ*)	Low	<40

[a] Predicted concentrations of intracellular c-di-GMP.

***mrkJ* encodes a functional phosphodiesterase.** Because the predicted amino acid sequence of MrkJ suggests a relatedness to a family of bacterial enzymes exhibiting phosphodiesterase activity we examined whether *mrkJ* produces a functional phosphodiesterase. *V. parahaemolyticus* strain LM6567 has previously been shown to lack this activity and was transformed with the cloned *K. pneumoniae mrkJ* gene. These transformants exhibited a swarming phenotype consistent with restoration of phosphodiesterase activity in this mutant (Fig. 2A). Previous work has demonstrated that the mutant demonstrates this swarming phenotype in the presence of low intracellular levels of c-di-GMP after introduction of a gene encoding phosphodiesterase activity (15). In addition, the phosphodiesterase activity of partially purified MrkJ was determined *in vitro* using the phosphodiesterase-specific substrate bis(*p*NPP). Reactions that contained MrkJ exhibited a significant 2-fold increase in the release of *p*-nitrophenol compared to those containing similarly purified MrkH (Fig. 2B). These results are consistent with MrkJ being a functional phosphodiesterase.

Type 3 fimbrial production in a *K. pneumoniae* MrkJ mutant. Bacterial suspensions bound to nitrocellulose membranes were probed with polyclonal antibodies specific to the type 3 major fimbrial subunit protein MrkA in order to determine the

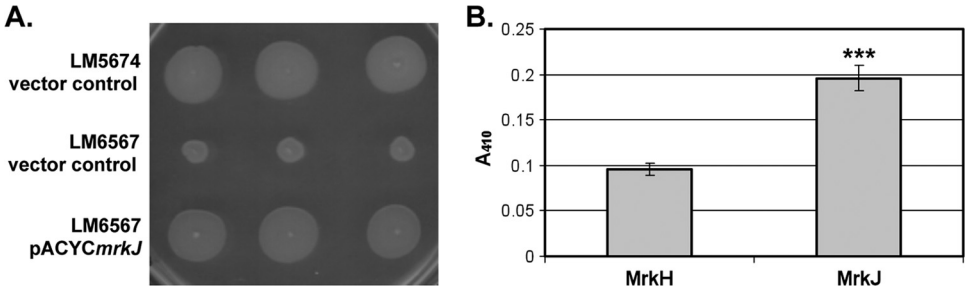

FIG. 2. Phosphodiesterase activity of MrkJ. (A) Swarming phenotypes of *V. parahaemolyticus* strains LM5674 and LM6567 (Δ*scrABC*) transformed with the vector control compared to LM6567 transformed with a plasmid expressing intact MrkJ. (B) *In vitro* phosphodiesterase activity assays comparing the ability of partially purified MrkH and MrkJ to cleave the phosphodiesterase-specific substrate bis(*p*NPP). Release of *p*-nitrophenol was determined at 410 nm (***, $P < 0.001$). Statistically significant differences were determined by using a Student *t* test.

effect of an *mrkJ* mutation on type 3 fimbrial surface expression. Fimbrial production in *K. pneumoniae* IApc35 was detected when ~10^6 cells were immobilized on the nitrocellulose membranes. In contrast, the MrkJ mutant produced detectable amounts of fimbriae when as few as 10^5 bacteria were bound (see Fig. 5A). Therefore, the deletion of *mrkJ* leads to an ~10-fold increase in the expression of surface-associated type 3 fimbriae. Ponceau S staining of these samples revealed that strains IApc35 and IApc35Δ*mrkJ* exhibited similar binding to nitrocellulose, and thus the results are indicative of decreased fimbrial production and not due to differences in bacterial binding to the membranes. The ability of *K. pneumoniae* IApc35 and the MrkJ mutant to react with fimbria-specific antiserum was also determined by serological agglutination assays. Both strains exhibited high titers, with the MrkJ mutant exhibiting a 4-fold increase over that of the parental IApc35 strain. In order to determine whether this increase could be a direct result of c-di-GMP accumulation in the MrkJ mutant, liquid chromatography-tandem mass spectrometry was performed on cultures normalized to the optical density at 600 nm (Mass Spectrometry Facility at Michigan State University). Parental IApc35 cultures exhibited a mean concentration of 0.994 ± 0.035 nM c-di-GMP, whereas MrkJ mutant cultures contained 4.078 ± 0.699 nM c-di-GMP ($P < 0.05$). Therefore, a >4-fold increase in intracellular c-di-GMP concentrations in the MrkJ mutant indicates that this protein is actively involved in the modulation of c-di-GMP levels in *K. pneumoniae*. Transformants bearing the cloned *mrkJ* gene exhibited a nonfimbriate phenotype in these assays and did not react with fimbria-specific antiserum. Both the parental strain and the MrkJ mutant of *K. pneumoniae* bearing the recombinant *mrkJ* gene exhibited no reactivity with immune serum.

Biofilm formation by *K. pneumoniae* IApc35Δ*mrkJ* is increased. The ability of *K. pneumoniae* strains to form biofilms was compared by standard procedures to detect growth on the surfaces of solid substrates (31, 34). The results of these assays are shown in Fig. 3. Compared to the parental strain, the IApc35Δ*mrkJ* mutant is significantly increased in its ability to form a biofilm after 24 h of incubation (Fig. 3A). Transformation of the mutant with the cloned *mrkJ* reduced biofilm-forming capabilities to levels significantly below that of parental IApc35 transformed with the vector control (Fig. 3B). Growth curves of planktonically grown strains indicated that there were no discernible differences in the growth rates between the mutant and its parent.

The *mrkABCDF* gene cluster in *K. pneumoniae* IApc35 is one transcriptional unit. Prior to examining the affect of MrkJ on type 3 fimbrial gene expression, we initially determined whether the *mrk* gene cluster, encoding the structural and assembly components of the type 3 fimbriae, is transcribed as

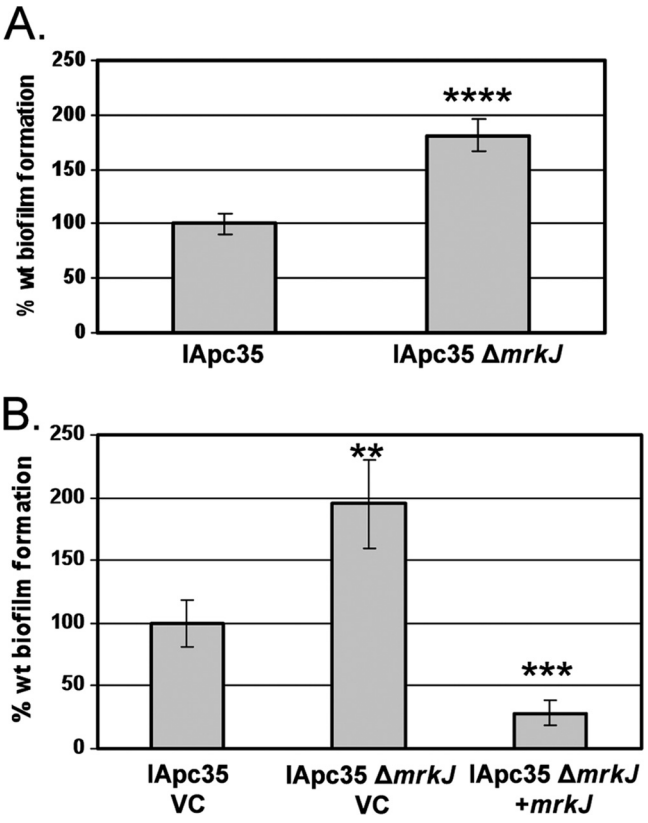

FIG. 3. Biofilm formation phenotypes of *K. pneumoniae* strains. (A) Biofilm formation of *K. pneumoniae* strains IApc35 and IApc35 Δ*mrkJ* (****, $P < 0.0001$). (B) Comparison of *K. pneumoniae* strains IApc35 and IApc35Δ*mrkJ* transformed with the empty vector control (VC) to IApc35Δ*mrkJ* complemented with cloned *mrkJ* (**, $P < 0.01$; ***, $P < 0.001$). Statistically significant differences were determined by using a Student *t* test.

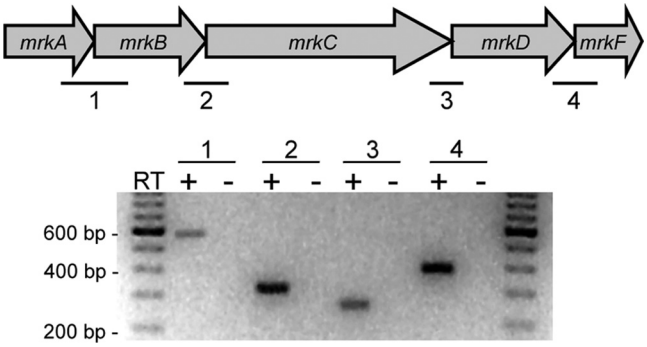

FIG. 4. Single transcriptional unit of the *mrk* gene cluster. RT-PCR analysis of the intergenic regions of the *mrkABCDF* operon was performed using primers described in the text to detect mRNA at these regions. The location of the transcripts are indicated by the numbered lines. Electrophoretic analysis was performed on reaction mixtures possessing (+) or lacking (−) reverse transcriptase (RT). The sizes of the cDNA amplicons were predicted from the locations of the primers used.

a single unit in *K. pneumoniae* IApc35 by detecting mRNA from intergenic regions of the gene cluster. Representative results are summarized in Fig. 4. Figure 4 demonstrates that PCR amplicons of the predictable sizes could be generated, after RT-PCR, for the intergenic *mrkAB*, *mrkBC*, *mrkCD*, and *mrkDF* regions by using RNA isolated from strain IApc35. The results with all combinations of primers indicate that the gene cluster is arranged as a single transcriptional unit. We have previously mapped the transcription initiation site of *mrkA* (1). Similar results demonstrating monocistronic units have been shown for other bacterial fimbrial gene clusters (2–5, 10). Since *mrkA* encodes the major fimbrial subunit and this family of genes is the most common target of fimbrial gene regulators in fimbriae assembled by the chaperone/usher pathway, we examined whether the MrkJ mutant is altered in *mrkA* gene expression.

Transcription of *mrkA* is increased in the absence of MrkJ. qRT-PCR was used to determine the level of *mrkA* gene expression in *K. pneumoniae* IApc35Δ*mrkJ* compared to the parental strain after growth under conditions for optimal type 3 fimbria formation. As shown in Fig. 5, the expression of *mrkA* is significantly increased (6- to 7-fold) in the mutant. These data are consistent with the increased level of surface fimbrial production by the mutant.

DISCUSSION

K. pneumoniae type 3 fimbriae play a vital role in the development of biofilms, *in vitro*, on human ECMs. We have previously proposed that collagen molecules represent a class of receptors recognized by the fimbrial adhesin MrkD (22, 27, 44). However, MrkD mutants that produce fimbriae without ECM binding activity still form biofilms on abiotic surfaces (22, 24, 28). Therefore, the MrkA and MrkD proteins may both play significant roles in forming biofilms on medically inserted devices. *K. pneumoniae* is an opportunistic pathogen frequently associated with infections in hospitalized patients with indwelling urinary catheters or endotracheal tubes leading to nosocomially acquired urinary and respiratory tract infections, respectively. The insertion of these devices also results in localized damage to epithelial surfaces, leading to the exposure of basement membranes and formation of another ecological niche where the type 3 fimbriae could play a role in the infective process. The ability of bacteria to produce type 3 fimbriae therefore is likely to play an important role in the initial stages of the infective process and the subsequent development of growth in compromised individuals.

The genetic regulation of fimbriae assembled by the chaperone-usher assembly pathway has been examined in *E. coli* and *Salmonella enterica* serovars. In these cases it has been demonstrated that the mechanism of gene regulation is diverse. For example, the gene encoding *E. coli* type 1 pili is regulated by the *fimS* invertible switch that, depending upon its orientation, controls the transcription of *fimA* (16, 41). In *Salmonella* spp., however, *fimA* is regulated by at least two activators (*fimZ* and *fimY*) and one repressor (*fimW*), with FimZ being a DNA-binding protein (46, 47, 49). The *E. coli* P pili and *Salmonella* Pef pili are also regulated by the production of DNA-binding proteins that are distinct from those associated with the type 1 fimbriae (2, 5, 33). Consequently, it appears that the production of enterobacterial fimbriae has evolved to utilize diverse regulatory cascades.

In some groups of bacteria, pilus production has been shown to be influenced by the intracellular concentration of the second messenger molecule c-di-GMP, which is mediated by

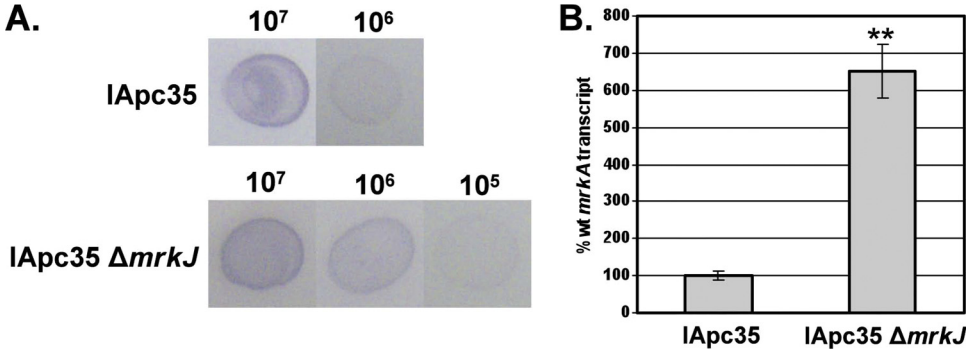

FIG. 5. Analysis of fimbrial expression in strains IApc35 and IApc35Δ*mrkJ*. (A) Cell suspension immunoblots with MrkA specific antibody. Numbers represent the CFU bound to the nitrocellulose membrane. (B) qRT-PCR analysis of *mrkA* transcript levels in *Klebsiella* strains IApc35 and IApc35Δ*mrkJ* (**, $P < 0.01$). Statistically significant differences were determined by using a Student *t* test.

phosphodiesterase and diguanylate cyclase activities (10, 39). Immediately adjacent to the *K. pneumoniae mrk* gene cluster are three genes that we have designated *mrkH*, *mrkI*, and *mrkJ*. The predicted amino acid sequence of MrkJ exhibits relatedness to bacterial phosphodiesterases possessing EAL domains (11, 25, 40). Therefore, we investigated whether this gene product exhibited phosphodiesterase activity and also played a role in type 3 fimbrial expression in *K. pneumoniae*. In addition, we examined whether gene products that have previously been shown to affect intracellular c-di-GMP concentrations in *V. parahaemolyticus* could influence fimbria production in *Klebsiella*.

In order to examine the effect of c-di-GMP in type 3 fimbrial production, we first introduced the plasmids LM2449 and LM2796 into *K. pneumoniae* IApc35. This strain has previously been described by our group and is a plasmid cured variant of the clinical isolate *K. pneumoniae* IA565 (22). It possesses only one chromosomally borne copy of the *mrk* gene cluster, produces type 3 fimbrial filaments, and forms biofilms on abiotic surfaces. Induction of the diguanylate cyclase encoding *scrC* gene on plasmid LM2449 resulted in increased expression of surface associated type 3 fimbriae, as determined by reactivity with a specific antiserum. ScrC has previously been shown to be responsible for increasing intracellular concentrations of c-di-GMP in *V. parahaemolyticus* due to diguanylate cyclase activity. Inversely, the induction of the three-gene cluster *scrABC* on LM2796 led to undetectable levels of type 3 fimbrial production by strain IApc35. This is most likely due to the phosphodiesterase activity exhibited by the *scrABC* genes previously reported to decrease intracellular c-di-GMP pools in *V. parahaemolyticus* (15). These results taken together suggest that the expression of the *K. pneumoniae* type 3 fimbrial operon may be responsive to differing levels of intracellular c-di-GMP. However, these transformants are likely to possess relatively high or low levels of c-di-GMP due to the copy number of the cloned heterologous genes in the *K. pneumoniae* host. Nucleotide sequencing of the region adjacent to the *mrk* gene cluster of *K. pneumoniae* has revealed a three-gene cluster convergently transcribed to the *mrkABCDF* operon and includes one gene (*mrkJ*) that exhibits amino acid relatedness to other genes known to modulate intracellular levels of c-di-GMP by phosphodiesterase activity. Of the other two genes the *mrkH* gene product is predicted to contain a PilZ c-di-GMP binding domain and the *mrkI* gene is predicted to encode a transcriptional regulator containing an uncharacterized N-terminal region and a C-terminal LuxR-like DNA-binding domain. MrkH and MrkI are currently under investigation. To address the role that MrkJ plays in type 3 fimbrial expression, a site-directed deletion mutant of *mrkJ* was constructed.

Increased fimbrial production by the MrkJ mutant compared to the parental strain was observed with fimbria-specific antiserum. The parental strain is a strongly fimbriate organism and the increase in fimbrial production by the mutant is detectable but may be limited by the number of fimbriae that can be polymerized in these strains. However, the mutant consistently exhibited increased ability to form biofilms on abiotic surfaces which is consistent with increased fimbria production. Also, transcriptional analysis showed that the *mrkJ* mutation led to a significant increase in *mrkA* gene expression. It is possible that MrkJ limits the availability of free c-di-GMP, which would normally be bound by a protein possessing a PilZ domain such as that found on MrkH. If MrkJ does indeed possess phosphodiesterase activity, increased expression of MrkJ should result in decreased levels of c-di-GMP, leading to poor fimbrial production. This is the phenotype observed in the MrkJ mutant transformed with the cloned and functional (see below) *mrkJ* gene. These transformants were completely nonfimbriate in the presence of multiple copies of *mrkJ*, which inhibited fimbrial assembly and production in an otherwise strongly fimbriate strain. Analysis of the intracellular concentration of c-di-GMP in an MrkJ deletion mutant revealed that the absence of MrkJ results in a net accumulation of c-di-GMP in the cellular compartment as determined by mass spectroscopy. Introduction of the *K. pneumoniae* derived *mrkJ* determinant into a *V. parahaemolyticus* strain known to accumulate c-di-GMP, and as a result exhibits decreased motility, resulted in restoration of swarming motility back to that seen in the parental strain. Also, the partially purified MrkJ protein possesses the ability to cleave the phosphodiesterase-specific substrate bis(*p*NPP). Taken together, these results, along with those described above, indicate that MrkJ is a functional phosphodiesterase that mediates a decrease in the intracellular concentrations of c-di-GMP, leading to decreased fimbrial production.

Recently, it has been suggested that type 4 pilus production in *Pseudomonas aeruginosa* is inhibited by low intracellular concentrations of c-di-GMP (11, 42). Type 4 pili are assembled by a pathway distinct from the chaperone/usher pathway used by many enterobacteria and used for type 3 fimbria production by *K. pneumoniae*. However, c-di-GMP may be an important molecule influencing fimbrial production regardless of pilus type and assembly machinery.

As for other fimbrial systems, it is likely that the regulation of type 3 fimbrial production in *K. pneumoniae* involves a complex and multifactorial regulon in which *mrkJ* is an important component. Currently, we are continuing to define this regulon and are investigating the role of *mrkHI* in this system. We have previously reported that some strains of *K. pneumoniae* possess additional regulatory factors involved in type 3 fimbria production (9). Since these organelles clearly play an important role in biofilm formation, the regulation of type 3 fimbrial expression plays a contributing role to the virulence of these bacteria.

ACKNOWLEDGMENTS

This study was supported by a grant from the National Institutes of Health to S.C. (R01 AI050011).

We thank Linda McCarter for supplying plasmids LM2449 and LM2796 and *Vibrio* strains and for helpful comments on the manuscript. We also thank Theresa Ho for her assistance with the qRT-PCR protocol. Determinations of the intracellular c-di-GMP concentrations were performed by the Mass Spectrometry Facility at Michigan State University.

REFERENCES

1. **Allen, B. L., G. F. Gerlach, and S. Clegg.** 1991. Nucleotide sequence and functions of *mrk* determinants necessary for expression of type 3 fimbriae in *Klebsiella pneumoniae*. J. Bacteriol. **173:**916–920.
2. **Baumler, A. J., R. M. Tsolis, F. A. Bowe, J. G. Kusters, S. Hoffmann, and F. Heffron.** 1996. The *pef* fimbrial operon of *Salmonella typhimurium* mediates adhesion to murine small intestine and is necessary for fluid accumulation in the infant mouse. Infect. Immun. **64:**61–68.
3. **Baumler, A. J., R. M. Tsolis, and F. Heffron.** 1996. Contribution of fimbrial

operons to attachment to and invasion of epithelial cell lines by *Salmonella typhimurium*. Infect. Immun. **64:**1862–1865.
4. **Baumler, A. J., R. M. Tsolis, and F. Heffron.** 1996. The *lpf* fimbrial operon mediates adhesion of *Salmonella typhimurium* to murine Peyer's patches. Proc. Natl. Acad. Sci. U. S. A. **93:**279–283.
5. **Blomfield, I. C.** 2001. The regulation of pap and type 1 fimbriation in *Escherichia coli*. Adv. Microb. Physiol. **45:**1–49.
6. **Bobrov, A. G., O. Kirillina, and R. D. Perry.** 2005. The phosphodiesterase activity of the HmsP EAL domain is required for negative regulation of biofilm formation in *Yersinia pestis*. FEMS Microbiol. Lett. **247:**123–130.
7. **Boddicker, J. D., R. A. Anderson, J. Jagnow, and S. Clegg.** 2006. Signature-tagged mutagenesis of *Klebsiella pneumoniae* to identify genes that influence biofilm formation on extracellular matrix material. Infect. Immun. **74:**4590–4597.
8. **Chouikha, I., A. Bree, M. Moulin-Schouleur, P. Gilot, and P. Germon.** 2008. Differential expression of *iutA* and *ibeA* in the early stages of infection by extra-intestinal pathogenic *Escherichia coli*. Microbes Infect. **10:**432–438.
9. **Clegg, S., T. K. Korhonen, D. B. Hornick, and A. M. Tarkkanen.** 1994. Type 3 fimbriae of *Enterobacteriaceae*, p. 97–103. *In* P. Klemm (ed.), Fimbriae: adhesion, genetics, biogenesis, and vaccines. CRC Press, Inc., Boca Raton, FL.
10. **Collinson, S. K., S. C. Clouthier, J. L. Doran, P. A. Banser, and W. W. Kay.** 1996. *Salmonella enteritidis agfBAC* operon encoding thin, aggregative fimbriae. J. Bacteriol. **178:**662–667.
11. **Cotter, P. A., and S. Stibitz.** 2007. c-di-GMP-mediated regulation of virulence and biofilm formation. Curr. Opin. Microbiol. **10:**17–23.
12. **Craven, D. E., and K. A. Steger.** 1996. Nosocomial pneumonia in mechanically ventilated adult patients: epidemiology and prevention in 1996. Semin. Respir. Infect. **11:**32–53.
13. **Craven, D. E., K. A. Steger, and L. R. Hirschhorn.** 1996. Nosocomial colonization and infection in persons infected with human immunodeficiency virus. Infect. Control Hosp. Epidemiol. **17:**304–318.
14. **Di Martino, P., N. Cafferini, B. Joly, and A. Darfeuille-Michaud.** 2003. *Klebsiella pneumoniae* type 3 pili facilitate adherence and biofilm formation on abiotic surfaces. Res. Microbiol. **154:**9–16.
15. **Ferreira, R. B., L. C. Antunes, E. P. Greenberg, and L. L. McCarter.** 2008. *Vibrio parahaemolyticus* ScrC modulates cyclic dimeric GMP regulation of gene expression relevant to growth on surfaces. J. Bacteriol. **190:**851–860.
16. **Gally, D. L., J. Leathart, and I. C. Blomfield.** 1996. Interaction of FimB and FimE with the *fim* switch that controls the phase variation of type 1 fimbriae in *Escherichia coli* K-12. Mol. Microbiol. **21:**725–738.
17. **Gastmeier, P., F. Schwab, S. Barwolff, H. Ruden, and H. Grundmann.** 2006. Correlation between the genetic diversity of nosocomial pathogens and their survival time in intensive care units. J. Hosp. Infect. **62:**181–186.
18. **Gerlach, G. F., B. L. Allen, and S. Clegg.** 1989. Type 3 fimbriae among enterobacteria and the ability of spermidine to inhibit MR/K hemagglutination. Infect. Immun. **57:**219–224.
19. **Hinsa, S. M., and G. A. O'Toole.** 2006. Biofilm formation by *Pseudomonas fluorescens* WCS365: a role for LapD. Microbiology **152:**1375–1383.
20. **Holden, N., I. C. Blomfield, B. E. Uhlin, M. Totsika, D. H. Kulasekara, and D. L. Gally.** 2007. Comparative analysis of FimB and FimE recombinase activity. Microbiology **153:**4138–4149.
21. **Hornick, D. B., B. L. Allen, M. A. Horn, and S. Clegg.** 1991. Fimbrial types among respiratory isolates belonging to the family *Enterobacteriaceae*. J. Clin. Microbiol. **29:**1795–1800.
22. **Hornick, D. B., J. Thommandru, W. Smits, and S. Clegg.** 1995. Adherence properties of an *mrkD*-negative mutant of *Klebsiella pneumoniae*. Infect. Immun. **63:**2026–2032.
23. **Hultgren, S. J., S. Normark, and S. N. Abraham.** 1991. Chaperone-assisted assembly and molecular architecture of adhesive pili. Annu. Rev. Microbiol. **45:**383–415.
24. **Jagnow, J., and S. Clegg.** 2003. *Klebsiella pneumoniae* MrkD-mediated biofilm formation on extracellular matrix- and collagen-coated surfaces. Microbiology **149:**2397–2405.
25. **Kim, Y. K., and L. L. McCarter.** 2007. ScrG, a GGDEF-EAL protein, participates in regulating swarming and sticking in *Vibrio parahaemolyticus*. J. Bacteriol. **189:**4094–4107.
26. **Kuchma, S. L., K. M. Brothers, J. H. Merritt, N. T. Liberati, F. M. Ausubel, and G. A. O'Toole.** 2007. BifA, a cyclic-Di-GMP phosphodiesterase, inversely regulates biofilm formation and swarming motility by *Pseudomonas aeruginosa* PA14. J. Bacteriol. **189:**8165–8178.
27. **Kukkonen, M., T. Raunio, R. Virkola, K. Lahteenmaki, P. H. Makela, P. Klemm, S. Clegg, and T. K. Korhonen.** 1993. Basement membrane carbohydrate as a target for bacterial adhesion: binding of type I fimbriae of *Salmonella enterica* and *Escherichia coli* to laminin. Mol. Microbiol. **7:**229–237.
28. **Langstraat, J., M. Bohse, and S. Clegg.** 2001. Type 3 fimbrial shaft (MrkA) of *Klebsiella pneumoniae*, but not the fimbrial adhesin (MrkD), facilitates biofilm formation. Infect. Immun. **69:**5805–5812.
29. **McClain, M. S., I. C. Blomfield, and B. I. Eisenstein.** 1991. Roles of *fimB* and *fimE* in site-specific DNA inversion associated with phase variation of type 1 fimbriae in *Escherichia coli*. J. Bacteriol. **173:**5308–5314.
30. **Merritt, J. H., K. M. Brothers, S. L. Kuchma, and G. A. O'Toole.** 2007. SadC reciprocally influences biofilm formation and swarming motility via modulation of exopolysaccharide production and flagellar function. J. Bacteriol. **189:**8154–8164.
31. **Merritt, J. H., D. E. Kadouri, and G. A. O'Toole.** 2005. Growing and analyzing static biofilms. Curr. Protoc. Microbiol. **Chapter 1:**Unit 1B-1.
32. **Monstein, H. J., M. Tarnberg, and L. E. Nilsson.** 2009. Molecular identification of CTX-M and blaOXY/K1 β-lactamase genes in *Enterobacteriaceae* by sequencing of universal M13-sequence tagged PCR-amplicons. BMC Infect. Dis. **9:**7.
33. **Nicholson, B., and D. Low.** 2000. DNA methylation-dependent regulation of *pef* expression in *Salmonella typhimurium*. Mol. Microbiol. **35:**728–742.
34. **O'Toole, G. A.** 2003. To build a biofilm. J. Bacteriol. **185:**2687–2689.
35. **Ong, C. L., G. C. Ulett, A. N. Mabbett, S. A. Beatson, R. I. Webb, W. Monaghan, G. R. Nimmo, D. F. Looke, A. G. McEwan, and M. A. Schembri.** 2008. Identification of type 3 fimbriae in uropathogenic *Escherichia coli* reveals a role in biofilm formation. J. Bacteriol. **190:**1054–1063.
36. **Philippe, N., J. P. Alcaraz, E. Coursange, J. Geiselmann, and D. Schneider.** 2004. Improvement of pCVD442, a suicide plasmid for gene allele exchange in bacteria. Plasmid **51:**246–255.
37. **Romling, U., and D. Amikam.** 2006. Cyclic di-GMP as a second messenger. Curr. Opin. Microbiol. **9:**218–228.
38. **Rosen, D. A., J. S. Pinkner, J. M. Jones, J. N. Walker, S. Clegg, and S. J. Hultgren.** 2008. Utilization of an intracellular bacterial community pathway in *Klebsiella pneumoniae* urinary tract infection and the effects of FimK on type 1 pilus expression. Infect. Immun. **76:**3337–3345.
39. **Sebghati, T. A., T. K. Korhonen, D. B. Hornick, and S. Clegg.** 1998. Characterization of the type 3 fimbrial adhesins of *Klebsiella* strains. Infect. Immun. **66:**2887–2894.
40. **Simm, R., A. Lusch, A. Kader, M. Andersson, and U. Romling.** 2007. Role of EAL-containing proteins in multicellular behavior of *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **189:**3613–3623.
41. **Sohanpal, B. K., H. D. Kulasekara, A. Bonnen, and I. C. Blomfield.** 2001. Orientational control of *fimE* expression in *Escherichia coli*. Mol. Microbiol. **42:**483–494.
42. **Starkey, M., J. H. Hickman, L. Ma, N. Zhang, S. De Long, A. Hinz, S. Palacios, C. Manoil, M. J. Kirisits, T. D. Starner, D. J. Wozniak, C. S. Harwood, and M. R. Parsek.** 2009. *Pseudomonas aeruginosa* rugose small-colony variants have adaptations that likely promote persistence in the cystic fibrosis lung. J. Bacteriol. **191:**3492–3503.
43. **Sturgill, G., C. M. Toutain, J. Komperda, G. A. O'Toole, and P. N. Rather.** 2004. Role of CysE in production of an extracellular signaling molecule in *Providencia stuartii* and *Escherichia coli*: loss of CysE enhances biofilm formation in *Escherichia coli*. J. Bacteriol. **186:**7610–7617.
44. **Tarkkanen, A. M., B. L. Allen, B. Westerlund, H. Holthofer, P. Kuusela, L. Risteli, S. Clegg, and T. K. Korhonen.** 1990. Type V collagen as the target for type-3 fimbriae, enterobacterial adherence organelles. Mol. Microbiol. **4:**1353–1361.
45. **Thanassi, D. G., and S. J. Hultgren.** 2000. Assembly of complex organelles: pilus biogenesis in gram-negative bacteria as a model system. Methods **20:**111–126.
46. **Tinker, J. K., and S. Clegg.** 2000. Characterization of FimY as a coactivator of type 1 fimbrial expression in *Salmonella enterica* serovar Typhimurium. Infect. Immun. **68:**3305–3313.
47. **Tinker, J. K., L. S. Hancox, and S. Clegg.** 2001. FimW is a negative regulator affecting type 1 fimbrial expression in *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **183:**435–442.
48. **Toutain, C. M., N. C. Caizza, M. E. Zegans, and G. A. O'Toole.** 2007. Roles for flagellar stators in biofilm formation by *Pseudomonas aeruginosa*. Res. Microbiol. **158:**471–477.
49. **Yeh, K. S., J. K. Tinker, and S. Clegg.** 2002. FimZ binds the *Salmonella typhimurium fimA* promoter region and may regulate its own expression with FimY. Microbiol. Immunol. **46:**1–10.