Regulation of type 3 fimbrial gene expression in Klebsiella pneumoniae

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REGULATION OF TYPE 3 FIMBRIAL GENE EXPRESSION
IN KLEBSIELLA PNEUMONIAE

by
Jeremiah Gene Johnson

An Abstract
Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Microbiology
in the Graduate College of
The University of Iowa

July 2011

Thesis Supervisor: Professor Steven Clegg
ABSTRACT

*Klebsiella pneumoniae* is an opportunistic, Gram-negative pathogen that is responsible for a variety of infections, including those of the respiratory and urinary tracts, following insertion of medical devices. It is believed that epithelial surfaces are mechanically disrupted through insertion of these devices thereby exposing the underlying extracellular matrix components. This disruption, as well as the *in situ* accumulation of matrix components on the indwelling device, has been proposed to provide a unique colonization niche for *K. pneumoniae* as the type 3 fimbriae of this organism have been shown to adhere to, and subsequently facilitate biofilm formation on, human extracellular matrix coated surfaces.

To identify regulators of type 3 fimbrial expression, a bank of transposon mutants was generated and screened using a colony immunoblotting assay, for isolates that were phenotypically non-fimbriate. One insertion identified was within a gene encoding a putative transcriptional regulator named MrkI. Mutants of *mrkI* were found to be non-fimbriate only under aerobic conditions, and as a result, were deficient for biofilm formation on both biotic and abiotic surfaces. The decrease in type 3 fimbrial production was at the level of fimbrial structural gene (*mrk*) transcription, though purified MrkI could not be shown to specifically bind the *mrk* promoter.

The two genes that flank *mrkI*, *mrkH* and *mrkJ*, encode proteins that contain domains involved in the binding or degradation, respectively, of the second messenger molecule cyclic diguanylate (c-di-GMP). Deletion of *mrkJ* from the chromosome resulted in an increase in type 3 fimbrial surface expression, which led to a concomitant increase in biofilm formation capability. This increase in fimbrial production was found to be due to intracellular accumulation of c-di-GMP as MrkJ was shown to be a functional phosphodiesterase.
Further study found that MrkH binds c-di-GMP and positively regulates type 3 fimbrial expression independently of aerobic growth conditions. The ability of MrkH to induce the production of type 3 fimbriae was also found to be dependent on binding c-di-GMP as evidenced by the inability of a site-directed mutant of MrkH to bind c-di-GMP and restore fimbrial expression in a mutant background. Using an mrk transcriptional reporter, it was found that production of MrkH and MrkI together was required for maximal expression of mrk. This observation, as well as others, alludes to an apparent synergistic effect whereby MrkH and MrkI may interact, forming a novel, c-di-GMP-dependent transcriptional activation complex.

Abstract Approved:  
Thesis Supervisor  

Title and Department  

Date
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CERTIFICATE OF APPROVAL

PH.D. THESIS

This is to certify that the Ph.D. thesis of

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has been approved by the Examining Committee for the thesis requirement for the Doctor of Philosophy degree in Microbiology at the July 2011 graduation.

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To my family.
Be not the slave of your own past. Plunge into the sublime seas, dive deep and swim far, so you shall come back with self-respect, with new power, with an advanced experience that shall explain and overlook the old.

Ralph Waldo Emerson
ABSTRACT

*Klebsiella pneumoniae* is an opportunistic Gram-negative pathogen that is responsible for a variety of infections, most notably those of the respiratory and urinary tracts following insertion of medical devices. During insertion of these devices, it is believed that epithelial surfaces are mechanically disrupted, exposing the underlying extracellular matrix components. This disruption, as well as the *in situ* accumulation of matrix components on the indwelling device, has been proposed to provide a unique colonization niche for *K. pneumoniae* of which the type 3 fimbriae have previously been shown to adhere to, and subsequently facilitate biofilm formation on, human extracellular matrix coated surfaces.

To identify regulators of type 3 fimbrial expression, a bank of transposon mutants was generated and examined for mutants that were phenotypically non-fimbriate by colony immunoblotting. One insertion identified was within a gene encoding a putative transcriptional regulator named *mrkI*. Mutants of *mrkI* were found to be non-fimbriate only under aerobic conditions, and as a result, were deficient for biofilm formation on both biotic and abiotic surfaces. The decrease in type 3 fimbrial production was found to be at the level of fimbrial structural gene (*mrk*) transcription, though purified MrkI could not be shown to specifically bind the *mrk* promoter.

The two genes that flank *mrkI*, *mrkH* and *mrkJ*, are predicted to encode proteins that contain domains involved in the binding or degradation, respectively, of the second messenger molecule cyclic diguanylate (c-di-GMP). Deletion of *mrkJ* from the chromosome resulted in an increase in type 3 fimbrial surface expression, which led to a concomitant increase in biofilm formation. This increase in fimbrial production was found to be due to intracellular accumulation of c-di-GMP as MrkJ was shown to be a functional phosphodiesterase.
Further study found that MrkH binds c-di-GMP and positively regulates type 3 fimbrial expression independently of aerobic growth conditions. The ability of MrkH to induce the production of type 3 fimbriae was also found to be dependent on binding c-di-GMP as evidenced by the inability of a site-directed mutant of MrkH to bind c-di-GMP and restore fimbrial expression in a mutant background. Using an mrk transcriptional reporter, it was found that production of MrkH and MrkI together was required for maximal expression of mrk. This observation, as well as others, alludes to an apparent synergistic effect whereby MrkH and MrkI may interact, forming a novel, c-di-GMP-dependent transcriptional activation complex.
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CHAPTER I
INTRODUCTION

*Klebsiella pneumoniae*

*Klebsiella pneumoniae* is a Gram-negative, non-motile member of the family *Enterobacteriaceae*, which is ubiquitously present in the environment and readily isolated from mammalian mucosal surfaces. In humans, *K. pneumoniae* usually exists as a member of the normal flora, deriving its nourishment from the assimilation of organic substrates, in the nasopharynx and intestinal tract. Carriage rates in the general population vary widely with intestinal isolates being readily cultured from 5-38% of people and a rate of 1-6% for nasopharyngeal isolation (Podschun & Ullmann, 1998). Interestingly, the colonization rates for *Klebsiella* increase dramatically in a hospital setting, most likely due to the use of antibiotics in those facilities.

*K. pneumoniae* is responsible for a variety of infections, many in individuals who are immunocompromised or have a pre-existing underlying disease; from the classical, pneumonias and urinary tract infections, to the emerging, soft tissue infections in those predisposed due to diabetes mellitus and of a Southeast Asian ancestry. The two types of *Klebsiella* infections that predominate are those which result in airway or urinary tract infections, with many following insertion of a medical device, i.e. intubation or catheterization, respectively. *K. pneumoniae* remains a significant cause of ventilator-associated bacterial pneumonias (VABPs) and catheter-associated urinary tract infections (CAUTIs) (Frank *et al.*, 2009; Jones, 2010). Emerging soft tissue infections, originally characterized in liver abscesses, have become increasingly attributed with more invasive infections that have been found to affect the kidneys, the lungs, the parotid gland, and the
brain (Ku et al., 2008). These invasive infections often have poor clinical outcomes with relatively high rates of morbidity and mortality.

In addition to the severity of emerging *K. pneumoniae* infections, there is increasing concern over the spread of *K. pneumoniae* strains that are resistant to all clinically available antibiotics. Initially, nosocomially-acquired *K. pneumoniae* strains were characterized by a resistance to aminoglycoside antibiotics (kanamycin, gentamicin, etc.) (Christensen & Korner, 1972; Curie et al., 1978). Since the early 1980’s, however, *K. pneumoniae* strains have evolved to encode an extended-spectrum β-lactamase, which confers resistance to extended-spectrum cephalosporins, with the hallmark being resistance to ceftazidime (Johnson et al., 1992; Reish et al., 1993; Smith & Chambers, 1995). Due to this particular mechanism conferring resistance to multiple antibiotic classes, treatment of infections caused by these strains has largely relied on the administration of the carbepenem class of antibiotics (imipenem and meropenem) (Podschun & Ullmann, 1998). Over the last several years, multiple strains of *K. pneumoniae* have begun to exhibit increasing resistance to carbepenem antibiotics as well as having acquired novel resistance mechanisms to other classes of antibiotics (Bradford et al., 1997). Currently, there are strains of *K. pneumoniae* that have been isolated in 35 states which exhibit resistance to all available antibiotics.

*K. pneumoniae* possesses a variety of virulence factors, all which appear to be involved in its ability to initially colonize the mucosal surface and persist, despite attempts by the host’s immune system to clear the organism (Figure I.1). These factors include siderophores, lipopolysaccharide (LPS), serum resistance, an abundant capsule, and a variety of fimbrial and non-fimbrial adhesins. Siderophores are required for the
acquisition of iron from the host milieu, as iron within a host is often restricted in an attempt to prevent microbial growth. All strains of *K. pneumoniae* produce the phenolate-type siderophore enterobactin, while few strains are known to produce the hydroxamate-type siderophore aerobactin (Podschun *et al.*, 1992). These two molecules have been postulated to work cooperatively, as enterobactin has been found to sequester its iron from a transferrin source, while aerobactin primarily obtains its iron directly from host cells (Brock *et al.*, 1991). The ability to have access to two sources of iron is thus thought of as an advantage for organisms encoding both siderophores. *K. pneumoniae* LPS O-antigen appears to be involved in dendritic cell activation and in the association of the bacterium with eukaryotic cells (Clements *et al.*, 2008; Evrard *et al.*, 2010). Additionally, LPS has also been implicated in facilitating initial adherence on abiotic surfaces during the early stages of biofilm formation, though these studies are preliminary in nature (Balestrino *et al.*, 2008).

Serum resistance of *K. pneumoniae* has historically been classified as a distinct virulence factor, as little evidence existed as to whether this trait was due to the production of LPS or capsule. Recently, clinical isolates of *K. pneumoniae* deficient for the production of the O side chain of LPS were found to be resistant to serum mediated killing (Alvarez *et al.*, 2000). Examination of these isolates found that mutants that were unable to synthesize capsule exhibited increased C3b deposition on bacterial surfaces, leading to increased complement-mediated and opsonophagocytic killing (Alvarez *et al.*, 2000). Due to this observation, as well as others, it has become widely believed that capsular production in *K. pneumoniae* is the primary virulence factor responsible for conferring serum resistance.
*Klebsiella* produces 77 serologically distinct types of acidic polysaccharide capsules, which are often composed of repeating subunits of four to six sugars, and commonly contain uronic acids. While there has been much research to determine a correlation between capsular serotype and infection, or severity of infections, little consensus has been established. One of the few consistencies is that organisms that produce either the K1 or K2 capsular serotypes are more often associated with human disease. The K2 serotype, specifically, is the most frequent serotype isolated from patients with UTIs, pneumonias, or bacteremias. As this serotype is rarely isolated in nature, it is thus believed to be the predominant serotype of human clinical isolates worldwide (Podschun & Ullmann, 1998). As stated above, the K2 capsule protects the bacterium from not only complement components, in particular C3b, but also from phagocytosis by polymorphonuclear granulocytes. Additional work has also shown that the *K. pneumoniae* capsule exhibits immunomodulatory effects by inhibiting the development and function of macrophages as well as the production of capsular specific antibodies.

Lastly, *K. pneumoniae* encodes a variety of both fimbrial and non-fimbrial adhesins. These include the lesser characterized fimbrial type KPF-28 and non-fimbrial adhesin CF29K, as well as the well characterized type 1 and type 3 fimbriae (Di Martino *et al.*, 1996; Di Martino *et al.*, 1995). Both KPF-28 and CF29K fimbriae, which belong to the same fimbrial family as the K88 adhesin of *E. coli*, have been postulated to be involved in gastrointestinal colonization, as both have been shown to facilitate adherence to Caco-2 tissue culture cells, while CF29K also mediates adherence to Int-407 human intestinal cell lines (Di Martino *et al.*, 1996; Di Martino *et al.*, 1995). The type 1
fimbriae, which exhibit some relatedness to the type 1 fimbriae of *E. coli*, facilitates adherence to the umbrella cells in murine bladders and the formation of biofilm-like intracellular bacterial communities (IBCs) albeit at rates lower than those observed for *E. coli* (Rosen *et al.*, 2008a). This decrease in *K. pneumoniae* IBC formation, relative to that of *E. coli*, is thought to be due to subtle differences in the mannose-binding pocket of the *K. pneumoniae* FimH adhesin from that of the *E. coli* FimH adhesin (Rosen *et al.*, 2008b). The type 3 fimbriae will be described in detail in this thesis.

**Fimbriae and Pathogenesis**

Many bacterial pathogens encode a variety of fimbrial and non-fimbrial adhesins that mediate the adherence of the respective organism to either a host cell or to a soluble, host-derived macromolecule. The concept of adherence as it relates to pathogenesis is important as the association of the bacterium with the host is what, in many cases, allows the bacteria to establish an infection, or maintain contact so that the organism can deliver bacterial effectors, often by a variety of secretion mechanisms. As a result, an array of bacterial adherence factors, including those within both Gram-negative and Gram-positive bacteria have been characterized, as have their roles in bacterial pathogenesis. In Gram-negative bacteria, four families of bacterial adherence factors have been classified. These include the trimeric autotransporter adhesin (TAA), curli, the type IV pili, and the chaperone-usher family of fimbriae (Kline *et al.*, 2009).

The TAA adhesins are a family of distinct adherence factors presently characterized in *Haemophilus influenzae*, *Yersinia enterocolitica*, and *Neisseria meningitidis*. These adhesins are assembled via an autotranslocation pathway where after
being secreted into the periplasm, transporter domains within the adhesin proteins insert into the outer membrane, forming a translocator which facilitates self-transport of passenger domains across the outer membrane (Linke et al., 2006). Once extracellular, these protein domains associate into stable trimers. The *N. meningitidis* TAA adhesin NhA, or *Neisseria* hia/hsf homolog, facilitates adherence to epithelial cells *in vitro* and to the extracellular matrix components laminin and heparan sulphate (Scarselli et al., 2006).

Some enteric bacteria, including some strains of *E. coli* and *Salmonella* spp., express adhesins comprised of amyloid fibers, referred to as curli or thin aggregative fimbriae. These filaments are assembled via a nucleation-dependent pathway where the major subunit protein, CsgA, is initially transported across the outer membrane through a pore comprised of multimeric CsgG (Kline et al., 2009). Once secreted, the CsgA proteins, following interaction with the surface-associated protein, CsgB, polymerize to form the amyloid fibers. These fimbriae appear to be very adherent with no apparent binding specificities, though some evidence exists that these appendages may interact with cellulose in the bacterial matrix (Zogaj et al., 2001).

The type IV pili is a family of fimbriae which is present within the Gram-negative bacteria *Neisseria* spp., *Pseudomonas aeruginosa*, and some strains of enteropathogenic *E. coli*. The neisserial type IV pili are assembled by a distinct pathway whereby pre-pilin subunits are translocated across the inner membrane, when they are cleaved by a peptidase into mature subunits, and subsequently assembled in the periplasm by an as yet unknown mechanism (Carbonnelle et al., 2006; Carbonnelle et al., 2005). Assembled fimbriae are next translocated to the bacterial surface through a secretin pore. While they
have consistently been shown to mediate attachment to epithelial cells, the binding
specificity of the type IV pili is still somewhat unclear and sometimes controversial.
Different cellular receptors have been implicated in Neisseria pilus attachment including
the CD46 cellular receptor on cervical epithelial cells and the I domain of integrins
(Edwards & Apicella, 2005; Gill & Atkinson, 2004). The type IV pili of *P. aeruginosa*
have been found to bind to a variety of cell lines including buccal cells and A549
pneumocytes, likely via specific interactions with the disaccharide moiety GalNAc
beta(1-4)Gal, which is common in both asialo-G(M1) and asialo-G(M2)
glycosphingolipids (Hahn, 1997).

Many of the most well characterized enterobacterial fimbrial types are assembled
via the chaperone-usher pathway. The molecular biology of the assembly of the type 1
fimbriae and Pap-pili (or pyelonephritis-associated pili) in *E. coli* have been described in
detail. In this mechanism of ordered assembly, the structural components of the
appendage are secreted into the periplasm via the SecA/Y pathway. Folding of the pilin
subunit occurs after interaction with the periplasmic chaperone, and is subsequently
presented to the molecular scaffolding protein or usher (Kline *et al.*, 2009). The ushers
have been predicted to exist as twin pores, where one functions as a scaffolding and the
other functions as a translocator, allowing passage of chaperone-subunit complexes to the
outer membrane, where the bound chaperone is replaced by incoming pilin subunits via
donor strand complementation (Remaut *et al.*, 2006; Remaut *et al.*, 2008). The adhesins
of these fimbriae are present at the distal end of the appendage most likely due to the
higher affinity of the chaperone-adhesin complex for the usher compared to the
chaperone-subunit complexes.
The type 1 fimbriae and Pap have distinct binding specificities, which play important roles in the ability of uropathogenic *E. coli* (UPEC) to colonize the human urinary tract. Type 1 fimbriae bind to mannosylated glycoconjugants on the surfaces of many epithelial cell types. This adherence is due to the mannose binding pocket present within the fimbrial tip adhesin, FimH (Schilling *et al.*, 2001). Pap fimbriae bind various globoseries glycosphingolipids including disialosyl galactosyl globoside (DSGG), globotriaosylceramide (Gb3), globotetraosylceramide (Gb4), and sialosyl galactosyl globoside (SGG), with the highest affinity being for SGG (Stapleton *et al.*, 1998). Interestingly, these different binding specificities confer different tissue tropisms that correlate well with each appendage’s role in ascending UTIs, with type 1 aiding in urethra and bladder colonization, and Pap facilitating colonization of the kidneys. Additionally, the type 1 fimbriae have also been shown to be involved in the formation of IBCs in bladder umbrella cells, contributing to the persistence of UPEC by aiding in immune escape and allowing re-colonization after antibiotic therapy (Garofalo *et al.*, 2007; Justice *et al.*, 2004).

**Type 3 fimbriae**

The type 3 fimbriae, originally termed the mannose-resistant hemagglutinin or MR/K HA, were initially identified in *K. pneumoniae* strains by Duguid et al. (Duguid, 1959). The type 3 fimbriae have since been identified in additional members of the *Enterobacteriacea* family including *Serratia* ssp., *Enterobacter* ssp., and most recently, encoded by the conjugative plasmid pOLA52 in olaquindox-resistant *E. coli* isolates (Adegbola & Old, 1982, 1983; Burmollé *et al.*, 2008; Norman *et al.*, 2008). Expression
of the type 3 fimbriae is fairly ubiquitous amongst various clinical and non-clinical isolates of *K. pneumoniae* (Gerlach *et al.*, 1989b). Examination of bacteria isolated from human urinary tract infections found that all strains expressed surface-associated type 3 fimbriae (Tarkkanen *et al.*, 1992). In respiratory isolates, approximately 83% of *K. pneumoniae* isolates possessed the *mrk* genes, while 73% expressed the appendage on their surfaces (Hornick *et al.*, 1991).

The genetic determinants encoding the type 3 fimbriae were originally identified and sequenced from the clinical *K. pneumoniae* isolate IA565. In this strain, a large plasmid was found to carry a copy of the fimbrial gene cluster (Gerlach *et al.*, 1989a). The type 3 fimbrial gene cluster contains five genes which encode for structural components of the fimbrial appendage (Figure I.2). These genes, termed *mrk* for the mannose resistant *Klebsiella*-like hemagglutination associated with the type 3 fimbriae, are *mrkABCD*. The *mrkA* gene encodes the major fimbrial subunit (MrkA), while *mrkD* encodes the fimbrial tip adhesin (MrkD) and *mrkF*, a minor fimbrial subunit (MrkF). These structural components are predicted to assemble the type 3 fimbria via the chaperone-usher pathway, using the periplasmic chaperone MrkB and the outer membrane scaffolding protein, or usher, MrkC. Using this model, all proteins are predicted to be secreted into the periplasm by the general secretory pathway, followed by the insertion of MrkC into the outer membrane. The fimbrial adhesin MrkD, associated with the chaperone MrkB, is first presented to the usher. Subsequently, the major subunit protein MrkA is polymerized behind the adhesin, forming the length of the fimbrial shaft. While little data exists as to the role of MrkF in fimbrial structure, recent work has shown MrkF to be distributed along the fimbrial shaft, interspersed within the MrkA polymer
(Huang et al., 2009). Since the initial identification of the plasmid encoded mrk in IA565, several strains of *K. pneumoniae* have undergone genomic sequencing and all possess a chromosomal copy of the mrk gene cluster. Indeed the carriage of the mrk gene cluster in *K. pneumoniae* isolates is invariably associated with the bacterial chromosome, whereas relatively few strains also possess an additional plasmid-borne copy of the gene cluster.

Type 3 fimbriae have been consistently shown to play an important role, *in vitro*, in the ability of *K. pneumoniae* to initially adhere to and form multicellular communities, called biofilms, on both abiotic and biotic substrates. Initially, the type 3 fimbriae were found to mediate adherence to respiratory tract tissue at both the basolateral surface of tracheal cells and the basement membrane regions of bronchial epithelia (Hornick et al., 1992). Adherence to the basement membrane has been mimicked, *in vitro*, by coating surfaces with purified human extracellular matrix material (HECM) or by exposing secreted HECM components by physical disruption of immortalized human bronchial epithelial (HBE) cell monolayers (Jagnow & Clegg, 2003). The components within the HECM that *K. pneumoniae* was found to specifically adhere to were two different species of collagen, collagen IV and collagen V, via the type 3 fimbrial tip adhesin, MrkD. (Jagnow & Clegg, 2003; Sebghati et al., 1998). Following adherence, subsequent biofilm formation on HECM-coated surfaces involves interactions between the MrkA subunits comprising the fimbrial shaft (Langstraat et al., 2001). While little data exists as to the exact role of the type 3 fimbria in human disease, the ubiquity of type 3 fimbrial expression on *K. pneumoniae* isolates and the presence of the HECM substrate on indwelling devices following intubation or catheterization, either through physical
disruption of the epithelial surface or by *in situ* accumulation of HECM on the inserted device, may be alluding to a specific niche that *K. pneumoniae* exploits for establishing infections in patients.

**Regulation of Fimbrial Production**

Fimbrial types that are assembled via the chaperone-usher pathway are often not constitutively expressed, but rather are subject to a variety of distinct regulatory schemes, with different fimbrial types being expressed under different environmental conditions or during different phases of growth. This certainly makes sense from a bacterial perspective, due to the relatively high metabolic costs required to produce functional appendages, only producing adherence factors under conditions when they are likely needed. Some common regulatory mechanisms of chaperone-usher fimbriae have emerged, particularly among *Enterobacteriacea* family members. These include regulation of fimbriae by inversion of DNA sequences which often contain the promoter regions of fimbrial genes, the methylation-state of the fimbrial promoters, the intracellular concentrations of the second messenger c-di-GMP, and transcriptional regulation by DNA-binding proteins not affiliated with the above mechanisms (Figure I.3).

The most well characterized fimbrial type that is regulated by an invertible DNA element, is the type 1 fimbriae (*fim*) of *E. coli*. Regulation of the production of type 1 fimbriae involves the inversion of a DNA element called the *fim* switch (*fimS*). This DNA fragment is flanked by two 9-base pair inverted repeats and lies immediately upstream of *fimA*, which encodes the major fimbrial subunit protein and contains the
promoter which drives transcription of the fimbrial gene cluster (Corcoran & Dorman, 2009; Holden et al., 2007). In what is referred to as the ‘ON’ orientation, \( \text{fimS} \) is oriented to promote \( \text{fim} \) transcription, whereas in the ‘OFF’ orientation, \( \text{fimS} \) is oriented so that the \( \text{fim} \) promoter is directed away from the fimbrial gene cluster. Inversion of this DNA element is mediated by two recombinases, FimBE, with FimB mediating inversion of the \( \text{fim} \) promoter in both directions and FimE primarily inverting \( \text{fimS} \) from an ‘ON’ to ‘OFF’ orientation (Aberg et al., 2008; Adiciptaningrum et al., 2009). In addition to FimBE, inversion of \( \text{fimS} \) is also mediated by the nucleoproteins leucine-responsive regulatory protein (Lrp), integration host factor (IHF), and the histone-like nucleoid-structuring (H-NS) protein. These proteins have distinct binding sites within the \( \text{fim} \) promoter region with Lrp and IHF both maintaining a phase ‘ON’ bias and H-NS promoting a phase ‘OFF’ bias (Blomfield et al., 1993; Corcoran & Dorman, 2009; Gally et al., 1994; Holden et al., 2007).

Regulation of fimbrial production due to the methylation status of DNA has been most well characterized for the Pap fimbriae of \( \text{E. coli} \). As mentioned above, this fimbrial type has been identified in a majority of UPEC strains and has been implicated in the ability of these organisms to form upper urinary tract infections. Methylation of DNA occurs at two GATC sites upstream of the \( \text{papBA} \) promoter, of which \( \text{papA} \) encodes the major fimbrial subunit protein, and is regulated by the intracellular concentrations of the global regulator Lrp and the fimbria-specific regulator PapI. When concentrations of Lrp are high and PapI are low, it has been proposed that Lrp efficiently binds to the region of the GATC site closest (proximal) to the \( \text{papBA} \) promoter, inhibiting \( \text{pap} \) transcription (Peterson & Reich, 2006). Lrp binding at this site can only occur when the
site is un-methylated therefore Lrp competes for binding at this site with DNA adenine methylase (Dam). After a round of cell division and DNA replication, when concentrations of Lrp are low and PapI concentrations are relatively high, Lrp binds to the hemimethylated distal GATC site blocking methylation. This allows for Dam mediated methylation of the proximal GATC and subsequent transcription of the pap operon (Peterson & Reich, 2008). Interestingly, this model of regulation partially explains the fimbrial coordination between the type 1 fimbrial system and that of the Pap-fimbriae, where concentrations of Lrp, influenced by growth phase, have inverse effects on those fimbrial types.

Recently, fimbrial production in some bacteria has been shown to be regulated by intracellular levels of the second messenger molecule c-di-GMP. *P. aeruginosa* is an opportunistic bacterium which causes both acute and chronic biofilm-associated infections. Biofilm formation in this organism requires a variety of surface organelles including the chaperone-usher pathway (*cup*) fimbriae. Encoded on the *P. aeruginosa* genome are four distinct *cup* fimbrial genes, termed *cupA-D*. Recently, a novel variant of a two-component system, containing the sensor kinase RocS1, the response regulator RocA1, and the RocA1 antagonist RocR were found to regulate both *cupB* and *cupC* transcription (Kulasekara et al., 2005). RocR is predicted to contain two protein domains, a phosphoryl-receiver domain and a phosphodiesterase domain (EAL), which in many cases functions in the degradation of c-di-GMP. RocS1 interacts with the receiver domain of RocR and initiate its function as a repressor, likely through the depletion of intracellular pools of c-di-GMP (Kulasekara et al., 2005; Mikkelsen et al., 2009). Subsequently, both *cupA* and *cupD* expression are affected by the intracellular
concentrations of c-di-GMP, which is altered by the regulator, PvrR (Meissner et al., 2007; Mikkelsen et al., 2009). Like RocR, PvrR contains both a phosphoryl-receiver domain and an EAL phosphodiesterase domain. PvrR is phosphorylated by the sensor kinase PvrS. Once activated, like RocR, it is also expected to mediate localized depletion of c-di-GMP, ultimately leading to decreased production of both CupA and CupD fimbriae. Additionally, PA1120 and RcsB are also positive regulators of CupA and CupD, respectively. The protein PA1120 contains a diguanylate cyclase domain (GGDEF), which is responsible for the production of c-di-GMP. Mutation of this gene in P. aeruginosa results in decreased intracellular concentrations of c-di-GMP and decreased production of CupA fimbriae (Meissner et al., 2007).

In addition to the mechanisms described above, there are fimbrial types which are regulated by DNA-binding proteins that are not yet associated with either invertible DNA elements, DNA methylation, or intracellular concentrations of c-di-GMP. One of the most well characterized regulatory systems is that controlling type 1 fimbrial production in Salmonella enterica serovar Typhimurium (S. Typhimurium). Three regulatory genes, fimW, fimY, and fimZ, all control the expression of the major fimbrial subunit gene, fimA (Saini et al., 2009; Tinker & Clegg, 2000; Tinker et al., 2001; Yeh et al., 1995). Both FimZ and FimY are positive regulators of fimbrial production while FimW negatively regulates type 1 fimbrial expression. Of the three proteins, only FimZ has been experimentally shown to bind the promoter region of fimA, though FimW is known to interact with FimZ by bacterial two-hybrid assays (Tinker & Clegg, 2000; Yeh et al., 2002). FimY’s function has yet to be determined, as it possesses little relatedness to previously characterized protein domains. In addition to the regulatory schemes
described for invertible DNA elements and DNA methylation, type 1 fimbrial production in *S. Typhimurium* is also controlled by the levels of intracellular Lrp, most likely through positive effects on FimZ expression (McFarland *et al.*, 2008).

**Cyclic-di-GMP Signalling**

Bis-(3’-5’)-cyclic dimeric guanosine monophosphate (c-di-GMP) was originally identified as a factor that activates the membrane-bound cellulose synthase of *Gluconacetobacter xylinus* (Ross *et al.*, 1987). Since, it has emerged as a vastly important molecule that controls the switching from motile, planktonic bacteria to sessile, biofilm-associated bacteria. Of equal importance, c-di-GMP controls virulence in a variety of pathogens including *P. aeruginosa*, *S. Typhimurium*, and *Vibrio cholerae* (Tamayo *et al.*, 2007).

The principle of c-di-GMP signaling begins with the second messenger control module, which consists of enzymes required for the generation and degradation of the molecule, effector proteins involved in binding c-di-GMP, and target components that are ultimately the output of the control module (Figure I.4). While the components are similar to cyclic AMP (cAMP) signalling in *E. coli*, where only a single protein for either the generation or degradation of the molecule exists and possesses only a single effector (CRP), c-di-GMP differs greatly due to the multiplicity of all components and an inherent increase in complexity (Hengge, 2009).

As previously mentioned, c-di-GMP is generated by diguanylate cyclases (DGC). DGCs often exist as dimers with two opposing subunits, each containing a GGDEF motif. The active site of the enzyme, site A, is present at the interface of the two GGDEF
containing subunits, with each subunit binding one molecule of GTP (Schirmer & Jenal, 2009). Dimerization of the GGDEF domains is required for the generation of c-di-GMP due to the antiparallel arrangement of the GTP needed to mediate intermolecular phosphodiester bond formation of symmetrical c-di-GMP (Paul et al., 2007). The structure of the DGC PleD, in complex with a GTP analogue, allowed for the designation of specific functions to residues within the DGC active site that are consistent with the function of conserved residues in adenylate cyclases and DNA polymerases (Wassmann et al., 2007). An additional feature of DGCs is the presence of a c-di-GMP binding RxxD motif (I site), which is responsible for restricting the generation of c-di-GMP by product feedback inhibition, preventing the depletion of intracellular pools of guanosine triphosphate (GTP) and the accumulation of c-di-GMP (Christen et al., 2006).

The degradation of c-di-GMP is the responsibility of specific phosphodiesterases (PDE) that contain either an EAL or an HD-GYP domain. A majority of EAL domain-containing PDEs exist as monomers and catalyze the hydrolysis of an ester bond within c-di-GMP, using either Mg$^{2+}$ or Mn$^{2+}$, generating 5'-phosphoguanylyl-(3'→5')-guanosine (pGpG) (Christen et al., 2005; Schmidt et al., 2005). The divalent cation is situated at the bottom of the c-di-GMP binding pocket and is coordinated by four conserved residues, including the glutamate in the EAL motif, and a phosphate oxygen within c-di-GMP (Schirmer & Jenal, 2009). Recently, it was found that a second divalent cation is coordinated within this region, in the EAL domain-containing protein BlrP1 from *K. pneumoniae*, though its relevance to c-di-GMP degradation is unknown (Barends et al., 2009). Once the macrocycle of c-di-GMP has been linearized, non-specific phosphodiesterases within the cell degrade the resulting pGpG further, into two
molecules of guanosine monophosphate (GMP). HD-GYP phosphodiesterases are poorly understood, relative to the unrelated EAL PDEs, but it is known that they are members of the HD superfamily of metal-dependent hydrolases, and are able to catalyze the degradation of c-di-GMP to GMP without the aid of non-specific cellular PDEs (Galperin et al., 1999; Ryan et al., 2006).

In addition to the levels of expression and the specific activity of DGCs and PDEs on intracellular concentrations of c-di-GMP, most GGDEF, EAL, and HD-GYP domains are fused to N-terminal signal input domains. These input domains include those which perceive oxygen and redox conditions (PAS), light (BLUF), small ligands and protein-protein interactions (GAF), starvation signals, antibiotics, and bacterial signaling molecules (Hengge, 2009). The GGDEF, EAL, and HD-GYP domains can also serve as an output for a variety of two-component signal transduction networks due to the presence of N-terminal phosphoryl-receiver (REC) domains, much like RocR mentioned above. While it appears that the majority of sensory domains activate DGC activity through dimerization of GGDEF domains, no such constant has been observed for the PDEs. It has been suggested in some cases that upon stimulation, the sensor domain facilitates EAL domain dimerization, much like with DGCs, while in other cases it has been postulated that activation of the sensor leads to relatively large rearrangements in the quaternary structure of the PDE, exposing the active site to c-di-GMP.

The activity of the DGCs and PDEs are often only responsible for affecting the intracellular concentrations of c-di-GMP. In order for c-di-GMP to exert its function, it must bind to and allosterically change the structure and output of an effector. Several c-di-GMP effectors have been identified in recent years including the transcriptional factor
FleQ, which functions as a repressor that is inactivated by bound c-di-GMP, and PelD, which binds c-di-GMP using a binding motif that resembles the I site in some DGCs.

There are also the riboswitches, which bind c-di-GMP at a GEMM (RNA element occurring in genes for the environment, membranes, and motility) site within the 5’-untranslated region in some mRNAs, and the PilZ domain, which is the best studied c-di-GMP binding protein (Schirmer & Jenal, 2009). The PilZ family of proteins represents the same c-di-GMP binding domain that is present within the G. xylinus cellulose synthase, for which c-di-GMP signaling was first described, and is named for a protein in P. aeruginosa that controls type IV pili biogenesis. Recently, PilZ domain-containing proteins have been found to regulate a variety of bacterial processes including motility, exopolysaccharide production, and virulence (McCarthy et al., 2008; Pratt et al., 2007; Ryjenkov et al., 2006). Nuclear magnetic resonance (NMR) imagining of the PilZ domain-containing proteins PA4608 from P. aeruginosa and PlzD from V. cholerae found that the PilZ domain exists as a six-stranded antiparallel β-barrel fold (Schirmer & Jenal, 2009). It was further shown that c-di-GMP bound to the β-barrel surface and to conserved arginine residues within its signature motif, resulting in significant conformational changes (Benach et al., 2007). While it is likely that these conformational changes enable the PilZ domain-containing protein to interact with target proteins, this mechanism remains to be determined.

In the following chapters I describe the identification of a transposon mutant that has an insertion in mrkI, which encodes a transcriptional regulator. Mutants of mrkI are unable to produce surface-associated type 3 fimbriae, which results in an inability to form biofilms on both abiotic and HECM-coated surfaces. Interestingly, mrkI is flanked by the
genes \textit{mrkH} and \textit{mrkJ}, which encode for proteins that are involved in c-di-GMP sensing/binding or metabolism, respectively. Mutants of MrkH which cannot bind c-di-GMP are unable to induce type fimbrial gene transcription while MrkJ mutants accumulate intracellular c-di-GMP, leading to a concomitant increase in type 3 fimbrial production. These results are the first to describe the direct relationship between concentrations of intracellular c-di-GMP and type 3 fimbrial gene expression. Lastly, I describe the initial observations that indicate that MrkH and MrkI may form a c-di-GMP-dependent transcriptional activation complex as both proteins are required for maximal type 3 fimbrial gene expression.
Figure I.1. Schematic representation of *Klebsiella pneumoniae* pathogenicity factors (Podschun & Ullmann, 1998).
Figure 1.2. *Klebsiella pneumoniae* type 3 fimbrial gene cluster. The major fimbrial subunit protein is encoded by *mrkA*, *mrkF* encodes for a minor fimbrial subunit, and *mrkD* the fimbrial tip adhesin. The fimbrial chaperone and the outer membrane scaffolding protein/usher are encoded by *mrkB* and *mrkC*, respectively.
**Figure 1.3.** Examples of fimbrial gene regulation involving invertible DNA elements, DNA methylation and DNA/protein as well as protein/protein interactions. (A) The expression of the *E. coli* type 1 fimbrial gene cluster (*fim*) is dependent on the orientation of the *fimS* invertible DNA fragment flanked by two inverted repeat sequences (IR). Recombination at these sites is mediated by the fimbrial specific recombinases FimB and FimE. In addition, the global regulators IHF and Lrp facilitate *fim* gene transcription whereas H-NS binding inhibits gene expression depending on the orientation of the fragment. (B) *E. coli* *pap* gene expression is dependent upon the methylation status of two GATC sites upstream of the *pap* operon. Lrp and Dam compete for binding at these sites and the *pap*-encoded protein PapI is a positive regulator of gene expression. (C) Expression of the *Salmonella* type 1 fimbrial gene cluster (*fim*) is controlled by two *fim*-encoded positive activators (FimZ and FimY) and one negative regulator (FimW). FimZ is a DNA-binding protein and also interacts with FimW. The Fim regulators also control expression of the three regulatory genes via feedback loops. In addition, Lrp binds to the promoter region of *fimZ* to affect its expression.
Figure I.4. The c-di-GMP signalling module. Intracellular c-di-GMP is generated from two GTP molecules by diguanylate cyclases, containing GGDEF motifs, and is degraded to linear diguanylate by phosphodiesterases containing either EAL or HD-GYP domains. c-di-GMP is subsequently bound by a variety of effectors, including PilZ domain-containing proteins, and act on targets affecting motility, virulence, and biofilm formation (Schirmer & Jenal, 2009).
CHAPTER II
IDENTIFICATION OF THE TYPE 3 FIMBRIAL REGULATOR, MRKI

Introduction

Bacteria express a variety of adhesins, which mediate initial attachment of the organisms to an array of cell types and soluble macromolecules, including cell receptors, mannosylated glycoproteins, and human extracellular matrix components (Edwards & Apicella, 2005; Gill & Atkinson, 2004; Scarselli et al., 2006; Schilling et al., 2001). Following initial adherence, many of these adhesins further promote the establishment of multicellular biofilms, a bacterial lifestyle that is inherently more resistant to components of the immune system, antibiotics, and dessication, than individual, planktonic cells (Drenkard, 2003; Kristian et al., 2008; Pruthi et al., 2003). Many acute and chronic infections, including those of burn wounds, the urinary tract, and cystic fibrosis, are caused by or result from biofilm-associated bacteria (Guiton et al., 2010; Hoiby et al., 2010; Kennedy et al., 2010).

Many fimbrial systems involved in biofilm formation are governed by complex regulatory circuits, with various inputs that either promote or repress fimbrial expression. These regulatory mechanisms include those which involve invertible promoter DNA elements, the methylation of sites within fimbrial promoter regions, the intracellular concentrations of c-di-GMP, and/or classical signal transduction. While the extracellular signal, which induces expression of a particular fimbrial system, is in many case unknown, the production of many fimbrial types is influenced by growth phase and various environmental conditions. One of the best examples of differential expression of a fimbrial system is that of the type 1 fimbriae in uropathogenic E. coli. As mentioned
previously, type 1 fimbrial expression in *E. coli* is dependent upon inversion of a DNA element, which contains the structural gene promoter, and is mediated by the recombinases FimBE (Holden et al., 2007). Inversion of this DNA element from a phase ‘OFF’ to ‘ON’ orientation is influenced by osmolarity, pH, oxygen concentrations, and growth phase (Dove et al., 1997; Lane et al., 2009; Schwan et al., 2002; Snyder et al., 2004). Complex regulation of such a metabolically expensive process is understandable as constitutive expression often inhibits growth. As such, it is expected that like many of its counterparts, the type 3 fimbriae in *K. pneumoniae* would be subject to similarly complex regulatory schemes.

*K. pneumoniae* utilizes type 3 fimbrial appendages to form biofilms on abiotic and biotic surfaces (Hornick et al., 1992; Jagnow & Clegg, 2003; Lavender et al., 2005). Despite the apparent importance of the type 3 fimbriae in biofilm formation and its proposed role in the ability of *K. pneumoniae* to establish infections in human hosts, very little information exists as to the regulatory elements that may control type 3 fimbrial production. In fact, most work regarding the type 3 fimbriae has focused on the molecular characterization of the appendages and their frequency in clinical isolates (Chen et al., 2011; Ong et al., 2010). To date, only three gene products have been suggested to regulate type 3 fimbrial production. The first, MrkE, was identified by our group, as it lies immediately upstream of the type 3 fimbrial gene cluster carried on a plasmid in strain IA565, that was originally used to sequence the type 3 genetic determinants (Gerlach et al., 1989a). MrkE is predicted to contain a CheY-like phosphoryl-receiver domain and a LytTR DNA-binding domain and is adjacent to what is believed to be its cognate histidine kinase, MrkG. The second regulator identified,
OxyR, was found to regulate both the type 1 and type 3 fimbriae (Hennequin & Forestier, 2009). OxyR is thought to regulate both fimbrial systems non-specifically due to its apparent pleiotropic effects under conditions of oxidative stress, but was found to be involved in colonization of intestinal cell lines, in vitro, and the murine gastrointestinal tract in vivo. The third protein, PecS, has not been experimentally shown to alter type 3 fimbrial production in K. pneumoniae, but lies close to the type 3 fimbrial gene cluster and exhibits relatedness to a regulator of virulence factors in Erwinia ssp. (Struve et al., 2009).

Due to the lack of identification of type 3 fimbria-specific regulators in K. pneumoniae, in this chapter I describe the identification of a previously unknown regulator of type 3 fimbrial production by colony immunoblotting a library of K. pneumoniae IApc35 transposon mutants. One of these mutants, which I further characterized, contained a transposon insertion within a putative transcriptional regulator which has been named MrkI. An insertion mutant of mrkI is defective for expression of surface-associated type 3 fimbriae. The type 3 fimbrial mutant phenotype results in decreased biofilm formation on both abiotic and biotic surfaces. Following characterization of the type 3 fimbrial gene cluster as operonic, the effect of the mrkI mutation was found to be at the level of mrk structural gene transcription. Purified MrkI was unable to specifically bind the mrkA promoter region.


**Materials and Methods**

**Strains, plasmids and DNA manipulations.** The strains, plasmids, and oligonucleotides used in this study are shown in Table II.1. To detect the presence of type 3 fimbriae all strains were grown on either glycerol-casamino acids (GCAA) media or L-B media at 37°C unless otherwise stated (Gerlach *et al*., 1988, 1989b; Hornick *et al*., 1992; Langstraat *et al*., 2001). When necessary, strains were cultured in media supplemented with antibiotics at the following concentrations: ampicillin (100 µg/ml), kanamycin (25 µg/ml), spectinomycin (100 µg/ml), and tetracycline (25 µg/ml).

Plasmid and genomic DNA preparation, restriction enzyme digestions, and PCR procedures were performed by conventional techniques using commercially available materials. All manipulation of DNA was performed according to the manufacturers’ instructions.

**Construction and screening of mini-Tn5 transposon library.** Conjugation of *K. pneumoniae* IApc35 with *E. coli* S17-1 λpir carrying the plasmid pUTminiTn5-Kn was performed as previously described by our group (Boddicker *et al*., 2006). Conjugants were selected on M9 minimal media supplemented with kanamycin to prevent growth of both the donor and recipient strains. Subsequently, appropriate dilutions of bacterial suspensions in PBS were plated on M9 minimal media 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 subsequent development with goat anti-rabbit serum conjugated to alkaline phosphatase (Dickson, 2008; Moravek *et al*., 2004). All colonies that did not
react with the fimbrial serum were isolated, retested for lack of reactivity with fimbriae specific antiserum and stored at -80°C. Mini-Tn5 insertions in the structural and assembly components of the mrkABCDF cluster were identified by standard PCR procedures and not examined further.

**Mapping of mini-Tn5 insertion site.** Genomic DNA was isolated from non-fimbrial 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 available on-line genome sequence of *K. pneumoniae* MGH 78578 (http://genome.wustl.edu/genomes). The nucleotide sequences flanking the mini-Tn5 were determined in mutants and were found not to have resulted in large rearrangements of the DNA during transposition.

**Construction of a site-directed mrkI insertion mutant.** Approximately one kb regions of DNA flanking and encompassing the mrkI gene were cloned, using plasmid pairs JGJ119/JGJ120 and JGJ122/JGJ147, into the vector pGEM-T Easy. Fragments were ligated together, incorporating an internal XbaI restriction site, into which either a kanamycin (for strain IApc35) or spectinomycin (for strain 43816) resistance determinant carried on a DNA cassette, was introduced. The interrupted mrkI gene and accompanying flanking region fragments were excised from pGEM-T Easy using SacI and SphI, and ligated into the suicide vector pDS132. The resulting plasmids, pDS132mrkI::knR and pDS132mrkI::specR, were transformed into the permissive host *E.*
coli SM10 λpir and subsequently introduced into the *K. pneumoniae* strains IApc35 and 43816, respectively, via conjugation. Transconjugants were selected on either LB-Kan/Amp or LB-Spec/Amp plates, followed by counter-selection on 5% sucrose plates (Link *et al.*, 1997; Philippe *et al.*, 2004). Identification of *mrkI* insertion mutants was performed using standard PCR techniques. For complementation studies, intact *mrkI* was cloned from the IApc35 chromosome as an *EcoRI/Scal* fragment using primer set JGJ117/JGJ118, and subcloned into pGEM-T Easy. Following digestion, *mrkI* was ligated into *EcoRI/Scal* cut pACYC184, producing plasmid pACYC*mrkI*.

**Detection of type 3 fimbriae.** Surface production of fimbrial appendages was detected using monospecific fimbrial antiserum as described elsewhere by our group (Hornick *et al.*, 1992). 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 on either 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 (Rosen *et al.*, 2008a).

**Biofilm formation assays.** The ability of *K. pneumoniae* strains to form biofilms on solid surfaces was determined as previously described (Johnson & Clegg, 2010; O'Toole & Kolter, 1998; O'Toole *et al.*, 1999). Biofilm formation of *K. pneumoniae* 43816 *mrkI::spec*<sup>R</sup> on HECM was also examined using confocal microscopy and once flow-
through biofilm chambers as previously described by our group (Boddicker et al., 2006; Jagnow & Clegg, 2003).

**Transcriptional analysis of the mrk gene cluster.** Total RNA was prepared from *K. pneumoniae* strains following growth on L-B agar using a modified procedure originally described by Chouikha and colleagues (Chouikha et al., 2008). Briefly, DNA was removed from RNA preparations using the DNA-free Kit (Ambion, Austin, TX), and prior to cDNA synthesis, RNA preps were examined for the presence of contaminating DNA by PCR. cDNA was generated using the SuperScript III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. The intergenic region spanning mrkA-B was amplified from the cDNA library using primers JGJ135 and JGJ136, that of the mrkB-C region using JGJ82 and JGJ83, the mrkC-D region using JGJ80 and JGJ81 and finally the mrkD-F region using primers JGJ137 and JGJ138. Amplicons were detected using conventional electrophoretic techniques through 2% agarose gels.

**Transcription of mrkA.** Total bacterial RNA was isolated from aerobically grown agar cultures as described above. The level of mrkA transcription was determined using qRT-PCR as described by our group (Johnson & Clegg, 2010). In addition, following construction of a mrkA promoter-reporter fusion plasmid, the ability of MrkI to affect mrkA transcription was determined. This fusion was constructed by cloning an 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. The resulting plasmid was introduced into E. coli NEB 5-α possessing either the cloned mrkl gene or an empty vector. The expression of β-galactosidase was assayed in triplicate at 37° C.

**Purification of MrkI and EMSAs.** The DNA fragment possessing the intact mrkl gene was amplified from the chromosome of strain IApc35 and subsequently subcloned into the vector pGEM-T Easy as a BamHI and PstI-tailed fragment. Following digestion with BamHI and PstI, the DNA fragment was ligated into the expression vector pMAL-c4x (New England Biolabs, Ipswich, MA) and the gene product was produced as a maltose-binding protein fusion (MBP-MrkI). Confirmation of biological activity of the MBP-MrkI fusion was confirmed by complementation of IApc35 mrkl::knR and testing for fimbrial production. Purification of MBP-MrkI using amylose resin was performed following induction of protein expression at 30° C according to the manufacturer’s protocol (New England Biolabs). Analysis of MBP-MrkI purity was performed using 12% SDS-PAGE and Coomassie Blue staining.

DNA binding assays were performed in 1x DNA binding buffer (10 mM Tris pH 7.5, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, 100 μg/ml BSA, 25 μg/ml poly[dIdC]) containing 0.25 nM of specific and non-specific [32P]-labeled DNA fragments and approximately 50 nM of MBP-MrkI (Brutinel *et al.*, 2008). Reactions were incubated for 15 min at room temperature and then examined on a 5% native glycine acrylamide gel. DNA fragment mobility was examined by phosphoimagery.
Results

**Immunoblotting of a mini-Tn5 transposon bank of insertion mutants in *K. pneumoniae IApc35***. Colony immunoblotting of type 3 fimbriate *K. pneumoniae IApc35* and the non-fimbriate derivative, *K. pneumoniae IAΔT3*, is shown in Figure II.1 and demonstrates the specificity of the serum raised against type 3 fimbriae. More than 21,000 insertion mutants were screened for their ability to produce surface-associated type 3 fimbriae. Of these, 11 (0.05%) mutants failed to consistently react with fimbrial specific antiserum even after growth on GCAA agar which favors the phenotypic expression of these fimbriae (Gerlach *et al.*, 1989b). Following mapping of the insertion site of the mini-*Tn5*, three of the mutants were shown to have the transposon inserted into genes that are part of the previously described *mrk* gene cluster (Figure II.2). Further analysis found that three of the eight remaining mutants were siblings of the *mrkI* insertion event discussed below and that the remaining five were illegitimate mutants.

*K. pneumoniae IApc35 MrkI 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 possible transcriptional regulator and annotated as KPN_03273 on the *K. pneumoniae MGH 78578* genome (Accession number: CP000647). The predicted size of this gene is 573 base pairs 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 in its C-terminal region spanning amino acids 130-176 (Figure II.3B). The precise site of
insertion of the mini-Tn5 was within the predicted DNA binding region encoding amino acid 151. This insertion was in a gene I have named *mrkI*, which is located between *mrkH* and *mrkJ*. The *mrkHIJ* genes are located adjacent to the previously characterized *mrk* gene cluster and exhibit opposite transcriptional polarity to these genes. The intergenic region between *mrkJ* and *mrkF* is 34 nucleotides and the genetic organization of these determinants is shown in Figure II.3A.

Both the *K. pneumoniae* IApc35 *mrkI::Tn5* and the site-directed defined mutant strain IApc35 *mrkI::kn*R do not produce surface-associated fimbriae following growth under aerobic conditions (Table II.2). Interestingly, the MrkI mutants do express fimbriae when grown anaerobically or microaerophilically as either agar or static broth cultures, respectively (Table II.2). Transformants of MrkI mutants carrying a plasmid-encoded intact *mrkI* gene are hyperfimbriate when grown under aerobic conditions. Electron microscopy confirmed the absence of type 3 fimbriae on MrkI mutants and many fimbriae on the surface of complemented strains (Figure II.4).

**MrkI mutants are decreased for biofilm formation on both abiotic and HECM coated surfaces.** Using crystal violet plate assays, it was shown that *K. pneumoniae* IApc35 *mrkI::kn*R is decreased in its ability to form biofilms on an abiotic surface compared to the parental strain. Using these assays, an average $A_{595}$ of 7.12 ± 1.52 was determined for the parental strain IApc35, whereas the MrkI mutant yielded an average $A_{595}$ of 2.33 ± 0.54, representing a more than 3-fold decrease in biofilm-associated bacteria in these assays (Figure II.5A). Complementation of the MrkI mutant with plasmid-borne *mrkI* restored biofilm formation to levels indistinguishable from those of
the parental strain carrying the empty vector plasmid (Figure II.5B). Strain IApc35

mrkI::knR transformed with the empty vector alone also exhibited a significant (more than
two-fold) decrease in biofilm formation compared to the parental strain (Figure II.5B).

In addition to differences in biofilm formation on abiotic surfaces, we also
examined the effects of a mrkI mutation on biofilm formation on HECM coated surfaces.
For these experiments, a defined MrkI mutant of K. pneumoniae 43816 was constructed.
The parental strain has previously been shown by our group to form robust biofilms on
HECM mediated by the presence of type 3 fimbriae (Jagnow & Clegg, 2003; Lavender et
al., 2005). Biofilms formed by 43816 mrkI::specR were observed to be thinner and
sparser throughout the chamber than wild-type 43816 (Figure II.6A & 6B).

Reintroducing mrkI into 43816 mrkI::specR restored the ability to form mature biofilms
on an HECM coated surface when compared to the mutant transformed with the vector
control plasmid (Figure II.6C & 6D).

The mrkABCDF gene cluster in K. pneumoniae IApc35 is one transcriptional unit.
Prior to examining the affect of MrkI on type 3 fimbrial gene expression, I initially
determined whether the mrk gene cluster, encoding the structural and assembly
components of the type 3 fimbriae are transcribed as a single unit in K. pneumoniae
IApc35 by detecting mRNA from intergenic regions of the gene cluster. Representative
results are summarized in Figure II.7. Figure II.7 demonstrates that PCR amplicons of
the predictable sizes could be generated, following RT-PCR, for the intergenic regions
between mrkAB, mrkBC, mrkCD, and mrkDF using RNA isolated from strain IApc35.
The results using 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*. Similar results demonstrating monocistronic units have been shown for other bacterial fimbrial gene clusters (Baumler *et al.*, 1996a, b; Baumler *et al.*, 1996c; Blomfield, 2001; Collinson *et al.*, 1996). 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 I examined whether the MrkI mutant is altered in *mrkA* gene expression.

**The mrkI mutation affects structural gene transcription.** Using qRT-PCR of total RNA extracted from aerobically grown agar cultures, it was found that IApc35 *mrkI::kn* is significantly reduced in *mrkA* expression. The transcription of *mrkA* in the MrkI mutant was reduced more than 30-fold compared to the parental strain (Figure II.8). This result is consistent with the inability of the IApc35 *mrkI::kn* mutant to produce detectable amounts of surface-associated fimbria.

**Purified MrkI does not directly bind to the mrkA promoter.** The ability to purify full length MrkI with a 6x-His tag proved unsuccessful due to the insolubility of MrkI when produced as a His-tagged protein. Expression of MrkI using a vector in which the protein is fused to a maltose binding protein allowed for the purification of small amounts of this fusion protein. Electrophoretic mobility shift assays (EMSAs) of MBP-MrkI binding to a DNA fragment containing the promoter region of *mrkA*, were unable to demonstrate specific binding of this protein to the DNA target (data not shown). Transformation of the plasmid encoding the fusion protein into IApc35 *mrkI::kn* indicated that the MBP-
MrkI product maintained its function since such transformants were fully fimbriate (data not shown). The inability of MrkI to directly activate the mrkA promoter was also demonstrated using a \( P_{mrkA-lacZ} \) reporter fusion. Introduction of a plasmid carrying the cloned functional \( mrkI \) into an \( E. coli \) host also possessing the plasmid-borne \( mrkA-lacZ \) fusion resulted in no increase in \( mrkA-lacZ \) activity. However, the \( mrkA \) reporter fusion expresses high levels of \( \beta \)-galactosidase in a \( lac \) deletion strain of \( K. pneumoniae \) (data not shown) (Cali et al., 1989).

**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 (Hornick et al., 1995; Jagnow & Clegg, 2003; Langstraat et al., 2001; Schurtz et al., 1994). It has previously been 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 mediate biofilm formation on abiotic surfaces (Johnson & Clegg, 2010). Consequently, the production of type 3 fimbriae could lead to initiation of biofilm formation on inserted devices such as catheters at short times after insertion and also after these devices become coated *in situ* with host factors. The genetic regulation of \( mrk \) gene expression is poorly understood, but like other enterobacterial fimbrial systems, is likely to involve complex regulatory circuits.
To identify regulatory elements of the type 3 fimbrial operon, I constructed a mini\textit{Tn5-Kn} transposon library in \textit{K. pneumoniae} IApc35. This strain is a plasmid-cured derivative of the clinical isolate \textit{K. pneumoniae} IA565, and produces high levels of type 3 fimbriae and forms robust biofilms on abiotic surfaces (Hornick \textit{et al.}, 1995). It possesses only one chromosomally-borne copy of the \textit{mrk} gene cluster. One non-fimbriate mutant from this library possessed a transposon insertion within a gene encoding a putative transcriptional regulator which I have named \textit{mrkI}. 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, \textit{mrkI} is located between two genes: the first which I have named \textit{mrkH}, is predicted to encode a protein which contains a C-terminal c-di-GMP binding domain (PilZ) (see Chapter IV), and the second, \textit{mrkJ}, is a gene which is predicted to produce a functional phosphodiesterase which modulates the intracellular levels of c-di-GMP within \textit{K. pneumoniae} (see Chapter III) (Johnson \& Clegg, 2010). A defined MrkI mutant of strain IApc35 was constructed and, like the original transposon mutant, was unable to assemble type 3 fimbriae. Interestingly, I also demonstrated that both MrkI mutants were non-fimbriate 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 those observed for the parental strains. These results suggest that under anaerobic conditions type 3 fimbrial expression is independent of MrkI and that regulation of type 3 fimbrial production may be subject to
an alternate regulatory pathway. Work is currently underway to identify those factors which promote anaerobic expression of the type 3 fimbria.

Biofilm formation in vitro on both abiotic and HECM coated surfaces is highly dependent upon type 3 fimbrial production. Therefore the decrease in type 3 fimbrial expression seen in MrkI mutants resulted in a significant decrease in biofilm formation on a solid surface. To further investigate the role of MrkI in biofilm formation on biotic surfaces and in virulence, we constructed an MrkI mutant of the murine hypervirulent strain *K. pneumoniae* 43816. The parental strain adheres specifically to components of the HECM and also causes an acute, lethal infection following intranasal inoculation of mice. The 43816 MrkI mutant exhibited the same non-fimbrial phenotype as the mutant of strain IApc35 and fimbrial expression could be restored by complementing with mrkI. When the MrkI mutant of 43816 was examined for biofilm formation on an HECM coated surface, it was found that this strain is unable to form robust biofilms on this substrate. This decreased biofilm phenotype, when compared to the wild-type strain, was characterized by sparse patches of growth in biofilm chambers and an overall reduction in thickness in these areas. The growth rates of both strains grown as planktonic cultures were identical.

The MrkI mutant of strain 43816 was not altered in virulence and resulted in 100% morbidity and mortality in acutely infected animals (see Chapter IV). Our results indicate that the ability to produce type 3 fimbriae plays no role in this infection model that involves rapid invasion and dissemination following infection of healthy animals. This type of infection is dissimilar to human infections that are most frequently associated with insertion of medical devices such as urinary catheters into compromised
hosts. Indeed, most clinical isolates of *K. pneumoniae* are avirulent in the murine airway infection model. Our results also indicate that MrkI does not decrease the expression of virulence genes necessary for lethality in the murine respiratory infection model. This is consistent with the observation that MrkI mutants remain phenotypically capsulate (see Chapter IV).

It was found that the *mrkl* mutation in strain IApc35 resulted in a significant decrease in *mrkA* transcription. This decrease explains the reduction in surface-associated fimbria in the mutant and lends support to the annotation of MrkI as a putative transcriptional regulator. However, binding of purified MrkI to the *mrkA* promoter region could not be demonstrated. There may be several reasons for this observation. First, the *mrkA* promoter is not the target DNA for MrkI binding but its effect on *mrkA* transcription is indirectly mediated by altering gene expression in an as yet unidentified *mrk* gene. I also attempted to demonstrate MrkI binding to the promoter region of *mrkJ* due to the affect of an *mrkJ* deletion on type 3 fimbrial expression (see Chapter III), but did not observe any binding. Second, MrkI may bind to the *mrkA* promoter only in the presence of additional proteins or co-factors. It was interesting to observe that the cloned and functional *mrkl* gene could not increase activity of an *mrkA*-reporter fusion in an *E. coli* host. This suggests that the role of MrkI in facilitating *mrkA* transcription may require an ancillary *K. pneumoniae* specific co-regulator. MrkH could be such a co-regulator and we are currently investigating this possibility.

It is becoming increasingly clear that the genetic regulation of fimbrial genes assembled by the chaperone-usher pathway is subject to a complex regulatory circuit involving different families of DNA binding proteins (Blomfield, 2001; Mikkelsen *et al.*, 2002).
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 multi-tiered regulatory networks that govern other fimbrial systems, it is possible that MrkI regulates 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 underway to further investigate the role of MrkI as a fimbrial regulator, and also to indentify whether MrkI regulates expression of non-fimbrial genes.
Table II.1. Bacterial strains, plasmids, and oligonucleotides used in this study.

<table>
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<tr>
<th>Strain</th>
<th>Description/Sequence (5’-3’</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>IApc35</td>
<td>Plasmid cured variant of IA565, type 3 fimbriae+</td>
<td>(Hornick <em>et al.</em>, 1995)</td>
</tr>
<tr>
<td>IApc35 <em>mrkI::kn</em></td>
<td>KanR; IApc35 <em>mrkI</em> insertion mutant, type 3 fimbriae+</td>
<td>This study</td>
</tr>
<tr>
<td>43816</td>
<td>Murine virulent, HECM binding proficient, type 3 fimbriae+</td>
<td>ATCC (Manassas, VA)</td>
</tr>
<tr>
<td>43816 <em>mrkI::spec</em></td>
<td>SpecR; 43816 <em>mrkI</em> insertion mutant, type 3 fimbriae+</td>
<td>This study</td>
</tr>
<tr>
<td>NEB 5-α</td>
<td>General <em>E. coli</em> cloning strain</td>
<td>NEB (Ipswich, MA)</td>
</tr>
<tr>
<td>S17-1 <em>λpir</em></td>
<td><em>E. coli</em> donor strain</td>
<td>(de Lorenzo &amp; Timmis, 1994)</td>
</tr>
<tr>
<td>SM10 <em>λpir</em></td>
<td><em>E. coli</em> donor strain</td>
<td>(Miller &amp; Mekalanos, 1988)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description/Sequence (5’-3’</th>
<th>Source</th>
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<tr>
<td>pACYC184ΔCmR</td>
<td>TetR, CamS; empty vector control for <em>mrkI</em> complementation construct</td>
<td>This study</td>
</tr>
<tr>
<td>pACYCmrkI</td>
<td>TetR; <em>mrkI</em> complementation vector</td>
<td>This study</td>
</tr>
<tr>
<td>pDS132</td>
<td>CamR; <em>sacB</em> suicide vector</td>
<td>(Philippe <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td>pDS132 <em>mrkI::kn</em></td>
<td>CamR, KanR; construct used to make IApc35 <em>mrkI::kn</em></td>
<td>This study</td>
</tr>
<tr>
<td>pDS132 <em>mrkI::spec</em></td>
<td>CamR, SpecR; construct used to make 43816 <em>mrkI::spec</em></td>
<td>This study</td>
</tr>
<tr>
<td>pGEM-T Easy</td>
<td>AmpR; TA subcloning vector</td>
<td>Promega (Madison, WI)</td>
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<tr>
<td>pMal-c4x</td>
<td>AmpR; maltose binding protein expression vector</td>
<td>NEB (Ipswich, MA)</td>
</tr>
<tr>
<td>pMalMrkI</td>
<td>AmpR; MBP-MrkI fusion expression vector</td>
<td>This study</td>
</tr>
<tr>
<td>pTrc99A</td>
<td>AmpR; General subcloning vector</td>
<td>Amersham-Pharmacia (Piscataway, NJ)</td>
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<tr>
<td>pTrcP*mrkA-lacZ</td>
<td>AmpR; pTrc99A-based reporter construct</td>
<td>This study</td>
</tr>
<tr>
<td>pUTminiTn5-Kn</td>
<td>AmpR, KanR; mini-Tn5 delivery plasmid</td>
<td>(de Lorenzo &amp; Timmis, 1994)</td>
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Table II.1. continued.

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<th>Oligonucleotides</th>
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<tr>
<td>JGJ80</td>
<td>CATGTCAGCATTACCAGAGTCCACTGGAACCTGGAGTCTCG</td>
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<td>JGJ81</td>
<td>CAAGGAGATCGGCGTTGTCG</td>
</tr>
<tr>
<td>JGJ82</td>
<td>CGAAGTGCGAATGAAAGCC</td>
</tr>
<tr>
<td>JGJ83</td>
<td>AGGGCGGTAAACCCCTGAACCA</td>
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<tr>
<td>JGJ117</td>
<td>GAATTCTGACTGGTACCTGCCTTACCTAGAAGGCG</td>
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<tr>
<td>JGJ118</td>
<td>AGTAGAAGTACCTTTTGCCATAGGACCTGGGAG</td>
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<td>JGJ119</td>
<td>GACCATCTGACTGGCACCCCTGTGTAAGCC</td>
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<td>JGJ120</td>
<td>TCTAGAGTACGTTTTTGCCATAGACCTGACCGG</td>
</tr>
<tr>
<td>JGJ122</td>
<td>CCTGTTGACCTATTACGTGGC</td>
</tr>
<tr>
<td>JGJ135</td>
<td>CTGCTGCTGACCTACTGACCA</td>
</tr>
<tr>
<td>JGJ136</td>
<td>CTCAATACGCACGCATCTGGCC</td>
</tr>
<tr>
<td>JGJ137</td>
<td>CATACATCACATACCCGCTGCG</td>
</tr>
<tr>
<td>JGJ138</td>
<td>AGGGTAAAACGGCTGCGGCTGTTG</td>
</tr>
<tr>
<td>JGJ147</td>
<td>TCTAGAGAGGATGACAGTCTCGTCGAGACTGCG</td>
</tr>
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</table>
Table II.2. Type 3 fimbrial production of *Klebsiella* strains.

| Culture conditions    | IApC35 | IApC35
mrkI::Tn5 | IApC35
mrkI::knR | IApC35
mrkI::knR | IApC35
mrkI::knR |
<table>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic (Shaken flask)</td>
<td>5,120¹</td>
<td>&lt;40</td>
<td>40,960</td>
<td>&lt;40</td>
<td>40,960</td>
</tr>
<tr>
<td>Aerobic (Agar grown)</td>
<td>5,120</td>
<td>&lt;40</td>
<td>40,960</td>
<td>&lt;40</td>
<td>40,960</td>
</tr>
<tr>
<td>Microaerophilic (Static tube)</td>
<td>40,960</td>
<td>20,480</td>
<td>ND²</td>
<td>20,480</td>
<td>ND</td>
</tr>
<tr>
<td>Anaerobic (Agar grown)</td>
<td>5,120</td>
<td>5,120</td>
<td>ND</td>
<td>10,240</td>
<td>ND</td>
</tr>
</tbody>
</table>

¹Serum titer represents the reciprocal of anti-MrkA serum dilution needed to produce visible agglutination. Lowest dilution of serum used was 1:40.

²Not determined
Figure II.1. Colony immunoblot of type 3 fimbriate wild-type IApc35 and the non-fimbriate mutant, IAΔT3.
Figure II.2. PCR of non-type 3 fimbriate transposon mutants with oligonucleotides that amplify across either *mrkAB* or *mrkC*, to determine whether insertions or re-arrangements occurred within genes encoding for type 3 structural proteins. Isolate #138 is the original *mrkI::Tn5* mutant.
Figure II.3. (A) Genetic organization of the mrk gene cluster. Putative promoter regions of mrk genes were identified by sequence analysis and are indicated by the arrows. The bold arrow head indicates the site of insertion of the mini-Tn5 transposon. (B) The precise location of the mini-Tn5 insertion is within a LuxR-like DNA binding domain (amino acids 130-176) in the C-terminal region of the 190-amino acid MrkI polypeptide.
Figure II.4. Fimbrial production by *K. pneumoniae* strains. (A) *K. pneumoniae* IApc35, (B) MrkI mutant and (C) MrkI mutant transformed with cloned *mrkI* gene. The lower panels are magnifications of the squares in the upper panels and demonstrate the presence of type 3 fimbriae in the parental strain and transformant.
Figure II.5. Biofilm phenotypes of \textit{K. pneumoniae} strains. (A) Biofilm formation of parental IAp35 and the MrkI mutant on an abiotic surface. (B) Restoration of parental levels of biofilm formation on an abiotic surface in mutants transformed with plasmid-encoded \textit{mrkI}. Both the parental strain and MrkI mutant were also transformed with an empty cloning vector (VC). Statistical significance was determined using a Student’s t-test. (p-value: *** < 0.001, **** < 0.0001)
**Figure II.6.** HECM biofilm formation of *K. pneumoniae* strains. (A) Wild-type 43816 and (B) the 43816 MrkI mutant biofilms on an HECM-coated surface. (C) The 43816 MrkI mutant transformed with vector control compared to (D) the MrkI mutant carrying the cloned *mrkI* gene. All strains expressing plasmid-encoded GFP.
Figure II.7. Single transcriptional unit of the mrk gene cluster. RT-PCR analysis of the intergenic regions of *mrkA*-*F* 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 are those predicted from the location of the primers used.
Figure II.8. qRT-PCR of mrkA encoding the major fimbrial subunit transcription in *K. pneumoniae* strains. *mrkA* transcription in the mutant is shown as relative decrease to transcription in the parental strain. Statistical significance was determined using Student’s t-test. (p-value: *** < 0.001)
CHAPTER III
ROLE OF THE CYCLIC-DI-GMP PHOSPHODIESTERASE, MRKJ, IN
TYPE 3 FIMBRIAL REGULATION

Introduction

In recent years, several bacterial processes have been found to be modulated by
the intracellular concentrations of the second messenger c-di-GMP. This cyclic
nucleotide is synthesized from two molecules of GTP by GGDEF domain-containing
diguanylate cyclases and is degraded by EAL domain-containing phosphodiesterases into
two molecules of GMP. The intracellular concentrations of c-di-GMP are sensed by a
variety of effector proteins, including those which contain c-di-GMP binding PilZ
domains. Once bound by c-di-GMP, these effectors are believed to interact with, in most
cases, unidentified target proteins that often modulate gene expression either at the
transcriptional or post-translation level.

Two fimbrial adhesins, the type IV pilus and the Cup fimbriae (both from *P.
aeruginosa*), are regulated by the intracellular concentrations of c-di-GMP. Production
of the type IV pili was initially found to be regulated by the GGDEF-EAL domain
containing protein, FimX. Originally thought to have a catalytically active EAL domain,
and thus regulating type IV expression by modulation of intracellular concentrations of c-
di-GMP, subsequent work found that the EAL domain of FimX binds c-di-GMP and
causes long-range conformational changes in FimX, resulting in unipolar localization of
the protein (Kazmierczak *et al.*, 2006; Qi *et al.*, 2011). Exactly how FimX governs type
IV pilus expression is still unknown. As previously mentioned, the Cup fimbriae (CupA-
D), are also regulated by the intracellular concentrations of c-di-GMP. The CupAD and
CupBC fimbriae are regulated by the REC and EAL domain-containing proteins PvrR and RocR, respectively (Kulasekara et al., 2005; Meissner et al., 2007; Mikkelsen et al., 2009). Both of these regulatory proteins are activated via phosphorylation by their respective cognate sensor kinases, PvrS for PvrR, and RocS1 for RocR. Once phosphorylated, the EAL domains become enzymatically active and begin reducing intracellular c-di-GMP concentrations. This reduction in c-di-GMP results in decreased expression of the respective fimbrial types. The environmental signal that stimulates the kinases as well as how the c-di-GMP concentrations are communicated to each fimbrial system, remains unknown.

Due to the observation that MrkI was unable to bind the promoter region of mrkA, \textit{in vitro}, I proposed that the genes flanking mrkI may be alluding to a different model of regulation other than MrkI’s direct interaction with the mrkA promoter. As previously mentioned, mrkI lies downstream of mrkH, a gene which is predicted to encode for a protein that contains a PilZ c-di-GMP binding domain, and downstream of mrkJ, which is predicted to produce a c-di-GMP specific EAL phosphodiesterase (Figure III.1). Due to the proximity of mrkI to these two genes, I hypothesized that intracellular concentrations of c-di-GMP affect surface expression of the type 3 fimbria in \textit{K. pneumoniae}.

Here, I describe the initial observation that the accumulation or depletion of the second messenger c-di-GMP has profound effects on the production of type 3 fimbriae. Expression of either a \textit{Vibrio parahaemolyticus} DGC or PDE resulted in the overproduction or absence of surface-associated type 3 fimbriae, respectively. Following deletion of mrkJ, \textit{K. pneumoniae} IApc35 was found to be hyperfimbriate, which led to an increased ability of this mutant to form biofilms. MrkJ was further found to be a
functional phosphodiesterase and the mrkJ mutant exhibited increased intracellular concentrations of c-di-GMP. This represents only the second chaperone-usher fimbrial family member, and the first among the Enterobacteriaceae, that has been found to be regulated by intracellular c-di-GMP concentrations.

Materials and Methods

Strains, plasmids, and DNA manipulations. The strains, plasmids, and oligonucleotides used in this study are listed in Table III.1. Unless otherwise stated, all strains were grown in Luria-Bertani (L-B) 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 Dr. Linda McCarter, University of Iowa, IA, USA) were introduced into Klebsiella strains by conjugation using the donor strain E. coli S17-1λpir carrying the appropriate plasmid (Boddicker et al., 2006). The construction and characterization of LM2449 and LM2796 has been described in detail elsewhere (Ferreira et al., 2008). LM2449 is a recombinant plasmid that contains an isopropyl β-D-1-thiogalactopyranoside (IPTG) –inducible V. parahaemolyticus scrC gene and LM2796 contains an IPTG-inducible V.
parahaemolyticus scrABC gene cluster. ScrC possesses 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 (Ferreira et al., 2008). K. pneumoniae possessing these plasmids were grown overnight on L-B agar containing the appropriate antibiotics with or without 0.5 mM of IPTG. Type 3 fimbrial expression of these strains was assayed as previously described by us (Gerlach et al., 1989b; Hornick et al., 1991; Sebghati et al., 1998).

**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 (ΔscrABC) and K. pneumoniae strains. Restoration of the swarming phenotype in V. parahaemolyticus was determined as previously described (Ferreira et al., 2008). In addition, in vitro phosphodiesterase activity assays were performed using partially purified 6xHis-MrkJ. Briefly, mrkJ was amplified from IApc35 genomic DNA using primers mrkJGateway-F and mrkJGateway-R, cloned into the Gateway vector pENTR-D-Topo (Invitrogen, Carlsbad, CA), and subsequently introduced into the 6xHis-tagged expression vector pDEST17 (Invitrogen) using 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 non-phosphodiesterase control. Phosphodiesterase activity assays were performed as previously described using 20 µg of protein in assay buffer (50 mM Tris-HCl, 1 mM MnCl₂, pH 8.5) supplemented with 5 mM bis(p-nitrophenol) phosphate (bis-pNPP) (Bobrov et al., 2005; Kuchma et al., 2007). Reactions were incubated for approximately 3 hours at 37° C when the release of p-nitrophenol was quantified at 410 nm.

Construction of the MrkJ mutant of K. pneumoniae. The mrkJ flanking regions (approximately 1kbp on either side) were amplified from the K. pneumoniae IApc35 chromosome using the primer pairs JGJ143 and JGJ144, or JGJ145 and 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. pDS132ΔmrkJ-kn was introduced into K. pneumoniae IApc35 using the E. coli donor strain SM10λpir. Following 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 using monospecific antiserum as described in detail by our group (Clegg et al., 1994; Rosen et al., 2008a). 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 fimbriae-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 (Hinsa & O'Toole, 2006; Merritt et al., 2007; O'Toole, 2003; Toutain et
al., 2007). Wells containing one ml of L-B broth were inoculated with 10µl of an
overnight culture and subsequently shaken on an orbital shaker (110 rpm) for 20 hours at
37°C. Following 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 (O'Toole, 2003; Sturgill et al., 2004).

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 this study exhibited similar growth rates under these
conditions.

**Quantitative Real-Time PCR (qRT-PCR) of *mrk* gene expression.** cDNA isolated
from *K. pneumoniae* strains were prepared as previously described (see Chapter II). A 20
µl qRT-PCR reaction consisted of 5 µl of cDNA, 5 µl of appropriate primer mix
containing 2.5 µM each of the primers used to assay for expression of *rpoD* (*rpoDqRT-
PCR* and *rpoDqRT-PCRR*) or *mrk* genes (*mrkAqRT-PCR* and *mrkAqRT-PCRR*) and
10 µl of Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA).
Reactions were performed using the Applied Biosystems 7300 Real-Time PCR System and *mrk* transcription was normalized to *rpoD* concentrations.

**Results**

**Plasmids encoding phosphodiesterase or diguanylate cyclase activity affect type 3 fimbrial production.** The plasmids LM2449 and LM2796, encoding a functional diguanylate cyclase and phosphodiesterase respectively, have previously been used to determine the role of intracellular cyclic-di-GMP concentrations in *V. parahaemolyticus* (Ferreira *et al.*, 2008). Consequently, I used these plasmids to transform the parental strain *K. pneumoniae* IApc35 to determine whether over-expression of these enzymes altered fimbrial production. IPTG Induction of transformants carrying the diguanylate cyclase gene, *scrC*, on LM2449 resulted in a four-fold increase in surface fimbrial production (Table III.2). However, the transformants carrying LM2796 bearing the inducible *scrABC* determinants together did not produce detectable levels of type 3 fimbriae post-induction. Similar results were also observed using non-inducing culture conditions, albeit the LM2449 transformants exhibited a two-fold increase in fimbriation, due to basal-level expression of the plasmid-encoded diguanylate cyclase or phosphodiesterase (personal communication, Linda McCarter). The products of the *scrABC* genes have previously been shown to decrease intracellular concentrations of c-di-GMP in *V. parahaemolyticus* (Ferreira *et al.*, 2008). 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.
**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 I 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 (Figure III.2A). Previous work has demonstrated that the mutant exhibits this swarming phenotype in the presence of low intracellular levels of c-di-GMP following introduction of a gene encoding phosphodiesterase activity (Ferreira *et al.*, 2008). In addition, the phosphodiesterase activity of partially purified MrkJ was determined *in vitro* using the phosphodiesterase specific substrate bis(pNPP). Those reactions which contained MrkJ exhibited a significant two-fold increase in the release of *p*-nitrophenol compared to those containing similarly purified MrkH (Figure III.2B). These results are consistent with MrkJ being a functional phosphodiesterase.

**Type 3 fimbrial production in a *K. pneumoniae* MrkJ mutant.** To determine the effect of an *mrkJ* mutation on type 3 fimbrial surface expression bacterial suspensions bound to nitrocellulose membranes were probed with polyclonal antibodies specific to the type 3 major fimbrial subunit protein MrkA. Fimbrial production in *K. pneumoniae* IApc35 was detected when approximately $10^6$ 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 (Figure III.4A). Therefore, the deletion of *mrkJ* leads
to an approximately 10-fold increase in expression of surface associated type 3 fimbriae. Ponceau S staining of these samples revealed that IApc35 and IApc35 ΔmrkJ 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 four-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, LC-MS/MS was performed on cultures normalized by OD A$_{600}$ (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-value<0.05). Therefore, a greater than four-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 non-fimbriate phenotype in these assays and did not react with fimbria-specific antiserum. Both the parental strain and MrkJ mutant of *K. pneumoniae* bearing the recombinant mrkJ gene exhibited no reactivity with immune serum.

**Biofilm formation by *K. pneumoniae* IApc35 ΔmrkJ 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 (Merritt *et al.*, 2005; O’Toole, 2003). The results of these assays are shown in Figure III.3. Compared to the parental strain, the
IApC35 ΔmrkJ mutant is significantly increased in its ability to form a biofilm after 24 hours incubation (Figure III.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 (Figure III.3B). Growth curves of planktonically grown strains indicated that there were no discernible differences in the growth rates between the mutant and its parent.

**Transcription of mrkA is increased in the absence of MrkJ.** Quantitative RT-PCR was used to determine the level of mrkA gene expression in *K. pneumoniae* IApC35 ΔmrkJ compared to the parental strain following growth under conditions for optimal type 3 fimbriae formation. As shown in Figure III.4B, the expression of mrkA is significantly increased (6-7-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 (Hornick *et al.*, 1995; Kukkonen *et al.*, 1993; Tarkkanen *et al.*, 1990). However, MrkD mutants that produce fimbriae without ECM binding activity still form biofilms on abiotic surfaces (Hornick *et al.*, 1995; Jagnow & Clegg, 2003; Langstraat *et al.*, 2001). 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 transcription of *fimA* (Gally *et al.*, 1996; Sohanpal *et al.*, 2001). In *Salmonella*, however, *fimA* is regulated by at least two activators (*fimZ* and *fimY*) and one repressor (*fimW*) with FimZ being a DNA-binding protein (Tinker & Clegg, 2000; Tinker *et al.*, 2001; Yeh *et al.*, 2002). 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 (Baumler *et al.*, 1996c; Blomfield, 2001; Nicholson & Low, 2000). 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 phosphodiesterase and diguanylate cyclase activities (Kazmierczak *et al.*, 2000).
Klepuestos et al., 2005; Meissner et al., 2007). Immediately adjacent to the *K.
pneumoniae mrk* gene cluster are three genes that I have designated *mrkH, I* and *J*. The predicted amino acid sequence of MrkJ exhibits relatedness to bacterial phosphodiesterases possessing EAL domains (Cotter & Stibitz, 2007; Kim & McCarter, 2007; Simm et al., 2007). Therefore, I investigated whether this gene product exhibited phosphodiesterase activity and also played a role in type 3 fimbrial expression in *K.
pneumoniae*. In addition, I 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*.

To examine the effect of c-di-GMP in type 3 fimbrial production I 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 (Hornick et al., 1995). 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 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* (Ferreira et al., 2008). 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 *mrkA-F* 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 (see Chapter IV) 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 (see Chapter II). 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 using 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 of *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 \textit{mrkJ} gene. These transformants were completely non-fimbriate in the presence of multiple copies of \textit{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 spectrometry. Introduction of the \textit{K. pneumoniae} derived \textit{mrkJ} determinant into a \textit{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(pNPP). 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 IV pilus production in \textit{P. aeruginosa} is inhibited by low intracellular concentrations of c-di-GMP (Cotter & Stibitz, 2007; Starkey \textit{et al.}, 2009). Type IV pili are assembled by a pathway distinct from the chaperone/usher pathway used by many enterobacteria and employed for type 3 fimbria production by \textit{K. pneumoniae}. Additionally, two fimbrial types assembled by the chaperone/usher pathway are also regulated by gene products that are predicted to contain phosphodiesterase domains. These include the Sfa fimbriae from \textit{E. coli}, which is regulated by the putative phosphodiesterase SfaY, and the
*K. pneumoniae* type 1 fimbriae, which was shown to be regulated by the EAL domain-containing protein FimK (Rosen *et al.*, 2008a; Sjostrom *et al.*, 2009). However, neither SfaY or FimK, have been shown to be functional phosphodiesterases, nor have they been found to alter intracellular concentrations of c-di-GMP. These observations may indicate that c-di-GMP is an important molecule that influences fimbrial production regardless of pilus type and assembly machinery. This is likely evidenced by the observation that the *K. pneumoniae* MGH78578 genome is predicted to encode for several proteins which may be involved in the turnover of c-di-GMP (Figure III.5).

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. We have previously reported that some strains of *K. pneumoniae* possess additional regulatory factors involved in type 3 fimbria production (Clegg *et al.*, 1994). 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.
Table III.1. Bacterial strains, plasmids, and oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description/ Sequence (5′-3′)</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>IApC35</td>
<td>Plasmid cured variant of IAp565, type 3 fimbriae^</td>
<td>(Hornick et al., 1995)</td>
</tr>
<tr>
<td>IApC35 ΔmrkJ</td>
<td>IApC35 mrkJ deletion mutant, highly type 3 fimbriate</td>
<td>This study</td>
</tr>
<tr>
<td>LM5674</td>
<td>ΔopaR V. parahaemolyticus</td>
<td>(Ferreira et al., 2008)</td>
</tr>
<tr>
<td>LM6567</td>
<td>ΔscrABC mutant of LM5674</td>
<td>(Ferreira et al., 2008)</td>
</tr>
<tr>
<td>S17-1pir</td>
<td>E. coli donor strain</td>
<td>(de Lorenzo &amp; Timmis, 1994)</td>
</tr>
<tr>
<td>SM10pir</td>
<td>E. coli donor strain</td>
<td>(Miller &amp; Mekalanos, 1988)</td>
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<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Source</th>
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<tbody>
<tr>
<td>pACYC184</td>
<td>ATCC, Manassas, VA</td>
</tr>
<tr>
<td>pDS132</td>
<td>(Philippe et al., 2004)</td>
</tr>
<tr>
<td>pDEST17</td>
<td>Invitrogen, Carlsbad, CA</td>
</tr>
<tr>
<td>pENTR-D-Topo</td>
<td>Invitrogen, Promega, Madison, WI</td>
</tr>
<tr>
<td>pGEM-T Easy</td>
<td>Gateway compatible 6xHis-tag expression vector</td>
</tr>
<tr>
<td>pACYCmrkJ</td>
<td>Tet^R, Cam^R sub-cloning vector</td>
</tr>
<tr>
<td>LM2449</td>
<td>Gen^R broad-host range vector carrying IPTG-inducible scrC</td>
</tr>
<tr>
<td>LM2796</td>
<td>Gen^R broad-host range vector carrying IPTG-inducible scrABC</td>
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<td>JGJ146</td>
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<td>mrkJGateway-F</td>
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<td>mrkJGateway-R</td>
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Table III.2. Type 3 fimbriae production and intracellular c-di-GMP concentrations

<table>
<thead>
<tr>
<th>Strain(plasmid)</th>
<th>c-di-GMP&lt;sup&gt;1/&lt;/sup&gt;</th>
<th>Titer with fimbria-specific serum</th>
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<tr>
<td><em>K. pneumoniae</em> IAp35</td>
<td>Normal</td>
<td>5120</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> IAp35(LM2449) -IPTG&lt;sup&gt;2/&lt;/sup&gt;</td>
<td>Normal/High</td>
<td>10,240</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> IAp35(LM2449) +IPTG</td>
<td>High</td>
<td>40,960</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> IAp35(LM2796) -IPTG</td>
<td>Normal/Low</td>
<td>&lt;40</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> IAp35(LM2796) +IPTG</td>
<td>Low</td>
<td>&lt;40</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> IAp35(pACYC184ΔCm&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Normal</td>
<td>5120</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> IAp35(pACYCmrkJ)</td>
<td>Low</td>
<td>&lt;40</td>
</tr>
</tbody>
</table>

<sup>1/</sup> Predicted concentrations of intracellular c-di-GMP

<sup>2/</sup> -IPTG, uninduced plasmids are leaky and produce appropriate gene products (Ferreira et al., 2008)
Figure III.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.
Figure III.2. Phosphodiesterase activity of MrkJ. (A) Swarming phenotypes of *Vibrio parahaemolyticus* strains LM5674 and LM6567 (ΔscrABC) 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(pNPP). Release of *p*-nitrophenol was determined at 410 nm (***: *p*-value<0.001). Statistically significant differences were determined using a Student’s T-test.
Figure III.3. Biofilm formation phenotypes of *K. pneumoniae* strains. (A) Biofilm formation of *K. pneumoniae* strains IApc35 and IApc35 ΔmrkJ (****: p-value<0.0001). (B) Comparison of *K. pneumoniae* strains IApc35 and IApc35 ΔmrkJ transformed with the empty vector control (VC), to IApc35 ΔmrkJ complemented with cloned *mrkJ* (**: p-value<0.01, ***: p-value<0.001). Statistically significant differences were determined using a Student’s T-test.
**Figure III.4.** Analysis of fimbrial expression in IApc35 and IApc35 ΔmrkJ. (A) Cell suspension immunoblots with MrkA specific antibody. Numbers represent the colony forming units bound to the nitrocellulose membrane. (B) qRT-PCR analysis of *mrkA* transcript levels in *Klebsiella* strains IApc35 and IApc35 ΔmrkJ (**: p-value<0.01). Statistically significant differences were determined using a Student’s T-test.
Figure III.5. Domain architectures of predicted proteins encoded on the *K. pneumoniae* MGH 78578 chromosome which are proposed to either facilitate the turnover of cyclic-di-GMP (GGDEF and EAL) or the binding of the molecule (PilZ). Loci and corresponding architectures were obtained from the MiST2 database.
Cyclic-di-GMP Turnover

KPN_00268

KPN_00425

KPN_00782

KPN_00899

KPN_01010

KPN_01159

KPN_01163

KPN_01172
Figure III.5. continued
Figure III.5. continued
Figure III.5. continued
CHAPTER IV
CHARACTERIZATION OF THE CYCLIC-DI-GMP BINDING PROTEIN, MRKH, AND ITS ROLE IN TYPE 3 FIMBRIAL REGULATION

Introduction

Using a bioinformatics approach, proteins containing PilZ domains were the first c-di-GMP receptors identified (Amikam & Galperin, 2006). Interestingly, it was further found that many of the PilZ domain-containing proteins also had a phylogenetic distribution similar to GGDEF and EAL domain-containing proteins, indicating that these domains have evolved in parallel to one another. Also, many of these PilZ domain-containing proteins maintain various fused regulatory domains, including phosphoryl-receiver and PAS domains, and catalytic regions, including GGDEF and EAL domains. While there are many cases where PilZ domains have been found to bind c-di-GMP, there have been very few examples of how the binding of c-di-GMP to this domain affects downstream signaling.

As c-di-GMP concentrations have been found to influence the switching of bacteria from sessile to motile cultures, and vice versa, it is not surprising that several adherence mechanisms appear to be regulated by intracellular concentrations of c-di-GMP. These adhesins include the previously mentioned Cup fimbriae and type IV pili of P. aeruginosa, and may include the type 1 and Sfa fimbriae of K. pneumoniae and E. coli, respectively. Of these appendages, the best characterized in terms of c-di-GMP dependent signaling are the type IV pili, with most of the work having focused on the PilZ, FimX, and PilB proteins. PilZ, for which the PilZ domain was originally named, was initially identified as being necessary for type IV pilus biogenesis in P. aeruginosa.
Though many homologous domains also bind c-di-GMP, PilZ has not been shown to bind the cyclic nucleotide itself. FimX, previously mentioned in Chapter II, contains both REC and PAS signaling domains, and also GGDEF and EAL enzymatic domains, of which both appear enzymatically inactive. PilB is a hexameric ring localized to the inner membrane and facilitates type IV pilus polymerization via ATPase activity. Recently, it was shown that in *Xanthomonas campestris*, PilZ interacts directly with PilB and that a FimX homolog interacts with the PilB-bound PilZ protein. It is believed that c-di-GMP dependent regulation of type IV biogenesis is mediated through these interactions as c-di-GMP was found to bind to the non-catalytic EAL domain of FimX (Guzzo et al., 2009). While the exact mechanism remains unknown, it was recently shown using *P. aeruginosa* FimX that c-di-GMP binding to the EAL domain results in long-range conformational changes that affect the cellular localization of the protein (Qi et al., 2011). Taken together these results appear to indicate that c-di-GMP dependent signaling may often entail complex protein-protein interactions that are not only facilitated by proper molecular conformations, but also by cellular localization of the effectors and targets.

Due to the previous observation that MrkJ affects type 3 fimbrial expression via modulation of intracellular concentrations of c-di-GMP, the presence of a gene encoding the PilZ domain-containing protein MrkH in the *mrkJ* locus led me to hypothesize that MrkH is responsible for sensing the intracellular concentrations of c-di-GMP and regulating type 3 fimbrial expression, either directly or indirectly. Here I report the observation that *mrkJ* appear to be co-transcribed. The deletion of both *mrkHI* resulted in a non-fimbriate phenotype, much like the *mrkI* mutant described in Chapter I, but this
phenotype was found to be independent of aerobic growth. Additionally, the mrkHI mutant of the murine virulent *K. pneumoniae* strain 43816 was found to be avirulent in an intranasal murine model of infection. This attenuation is likely due to decreased capsular expression seen in the 43816 mrkHI mutant, as this strain also exhibits hypersensitivity to human serum components. Further, mutation of the putative c-di-GMP binding pocket in *mrkH* resulted in a gene product that was unable to restore fimbrial expression in an *mrkHI* mutant. Also, purified MrkH was proficient at binding radio-labeled c-di-GMP whereas the mutant mentioned above was unable to bind this molecule. These results indicate that MrkH is involved in regulating type 3 fimbrial expression in a c-di-GMP dependent manner in *K. pneumoniae*.

**Materials and Methods**

**Strains, plasmids and DNA manipulations.** The strains and plasmids used in this study are shown in Table IV.1. To detect the presence of type 3 fimbriae all strains were grown on either glycerol-casamino acids (GCAA) media or LB media at 37°C unless otherwise stated (Gerlach *et al.*, 1988, 1989b; Hornick *et al.*, 1992; Langstraat *et al.*, 2001). When necessary, strains were cultured in media supplemented with antibiotics at the following concentrations: ampicillin (100 µg/ml), kanamycin (25 µg/ml), spectinomycin (100 µg/ml), and tetracycline (25 µg/ml).

Plasmid and genomic DNA preparations, restriction enzyme digestions, and PCR procedures were performed by conventional techniques using commercially available material. All manipulation of DNA was performed according to the manufacturers’ instructions.
**Construction of site-directed mutants.** Approximately one 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 either a kanamycin (for strain IApc35) or spectinomycin (for strain 43816) resistance determinant was introduced for only the *mrkI* mutant. The deletion construct for *mrkHI*, and the interrupted *mrkI* gene and accompanying flanking region fragments, were excised from pGEM-T Easy using *Sacl* and *SphI*. These fragments were ligated into either the suicide vector pDS132 (for *mrkI*) or pDS132-spec\textsuperscript{R} (for *mrkHI*). The resulting plasmids, pDS132*mrkI::kn*\textsuperscript{R}, pDS132*mrkI::spec*\textsuperscript{R}, and pDS132Δ*mrkHI*, were transformed into the permissive host *E. coli* SM10 λpir and subsequently introduced into *K. pneumoniae* strains IApc35 and 43816 via conjugation. Transconjugants were selected on either LB-Kan/Amp or LB-Spec/Amp plates, followed by counter-selection on 5% sucrose plates (Link *et al.*, 1997; Philippe *et al.*, 2004). Identification 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 (Hornick *et al.*, 1992; Johnson & Clegg, 2010). 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 on either 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 (Rosen et al., 2008a).

**Transcription of mrk.** Expression of the *mrk* genes in *K. pneumoniae* strains grown under aerobic or anaerobic conditions was determined by quantitative RT-PCR (qRT-PCR) as previously described (Johnson & Clegg, 2010). 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 ΔmrkHI 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<sub>mrkA</sub>-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* IApc35 and its derivatives to form biofilms on solid surfaces was determined as previously described (Johnson & Clegg, 2010; O'Toole & Kolter, 1998; O'Toole et al., 1999). Biofilm formation of both *K. pneumoniae* 43816 *mrkI::spec<sup>R</sup> and 43816 Δ*mrkHI* on HECM was also examined using confocal microscopy and once flow-through biofilm chambers as previously described by our group (Boddicker et al., 2006; Jagnow & Clegg, 2003).
**Virulence assays.** Murine virulence via intranasal inoculation was determined for the parental strain *K. pneumoniae* 43816, the 43816 *mrkI::specR* mutant, and 43816 ΔmrkHI (Lavender et al., 2005). Briefly, approximately $10^3$ cfu suspended in 50µl of sterile PBS was used to intranasally infect BALB/c mice. With 43816 ΔmrkHI, increasing numbers of bacteria ($10^4$, $10^5$, and $10^6$) were used to inoculate mice. In addition, the virulence of the above strains was also determined by an intraperitoneal infection route using $10^3$ cfu. All animal studies were approved by the University of Iowa Institutional Animal Care and Use Committee.

**Determine capsular expression.** Capsular expression in 43816 ΔmrkHI was determined using two phenotypic assays. First, capsular expression in wild-type 43816 and the mutants, 43816 *mrkI::specR* and 43816 ΔmrkHI, was examined using LB-5% sucrose agar grown cultures incubated overnight at room temperature. Also, expression of capsule was determined using Congo red plates and incubating strains at 37°C for 48 hours.

**Serum sensitivity assays.** Bacterial suspensions of $2\times10^6$ cfu/ml in PBS were prepared from aerobically grown LB broth cultures. Approximately 25 µl (~$5\times10^4$ cfu) of bacterial suspension was added to 75 µl of pooled human serum and incubated at 37°C. Bacterial viability was determined at time zero and at every hour up to 4 hours of incubation. Inactivation of human serum was achieved by incubating at 56°C for 30 minutes (Sahly et al., 2004).
**Mutation of MrkH c-di-GMP binding site.** Arginine-113 of MrkH was substituted to alanine using overlapping oligonucleotides. Using primers CNM003 and CNM004 which contained the desired mutation and pACYCmrkHI as template, the FailSafe PCR Enzyme Mix (Epicentre) was used with 18 cycles of the following reaction: 95°C for 30 seconds, 55°C for 1 minute, 68°C for 5 minutes. The resulting plasmid DNA was digested with DpnI for 1 hour at 37°C and then transformed into chemically competent DH5α E. coli (Invitrogen, Carlsbad, CA). Mutations in the resulting plasmid, pACYCmrkHR113AmrkI, were verified by DNA sequencing.

**Purification of MrkH and MrkH\textsubscript{R113A}.** The \textit{mrkH} and \textit{mrkH\textsubscript{R113A}} alleles were amplified from pACYCmrkHI or pACYCmrkHR113AmrkI, 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 (Invitrogen) and introduced into the expression strain BL21-AI (Invitrogen). Both BL21-AI strains carrying either pDEST17mrkH or pDEST17mrkHR113A were used to produce native MrkH or MrkH\textsubscript{R113A}, respectively, by Ni-NTA affinity chromatography per the manufacturer’s instructions (Qiagen, Valencia, CA). Successful purification of both MrkH and MrkH\textsubscript{R113A} was assessed by 12% SDS-PAGE and Western blotting using anti-6x His antibody (Qiagen). Additionally, MrkI was purified as an MBP-fusion protein per the manufacturer’s protocol (NEB, Ipswich, MA).

**Binding of c-di-GMP to MrkH.** Generation of [\textsuperscript{32}P]-labeled c-di-GMP was performed as previously described (Hickman & Harwood, 2008; Hickman \textit{et al}., 2005). The c-di-
GMP binding assay was based on that of Hickman & Harwood, as follows (Hickman & Harwood, 2008). 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 minutes. The reactions were then brought to a 100 µl volume with binding buffer and immediately loaded onto a slot blot apparatus (PR600 SlotBlot, Hoeffer Scientific) containing a 0.2 µM nitrocellulose membrane (Protran BA85, 0.45 mM, Whatman), followed by a wash with 1.0 ml cold binding buffer. The membrane was removed and scanned on a phosphoimager (Packard Instant Imager, Packard Instrument Company) to measure radioactive counts of membrane-bound [\(^{32}\)P]c-di-GMP.

For the competition assay, a ten-fold excess (20 µM) of cold c-di-GMP was added to the reaction (Biolog, Bremen, Germany). An equal amount of [\(\alpha-{^{32}}\)P]GTP was substituted for [\(^{32}\)P]c-di-GMP to further determine MrkH binding specificity. Additionally, MBP-MrkI was examined for the ability to bind [\(^{32}\)P]c-di-GMP. Reactions containing similarly purified LacZa or protein buffer alone were used as negative controls.

Results

*K. pneumoniae* IApc35 MrkI and MrkHI mutants do not produce surface-associated fimbriae. The *mrkI* mutant isolated in Chapter II possessed a transposon insertion within the C-terminal LuxR-like DNA binding domain, spanning amino acids 130-176 (Figure IV.1B). The gene, *mrkI*, is located between *mrkH* and *mrkJ*, though the *K. pneumoniae* MGH 78578 genome lacks the correct annotation for *mrkH*. The *mrkHIJ* genes are located adjacent to the previously characterized *mrk* gene cluster and exhibit opposite transcriptional polarity to these genes (Figure IV.1A). Using intergenic RT-
PCR it was found that \textit{mrkH}, \textit{mrkI}, and \textit{mrkJ} are co-transcribed (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 (Figure IV.1C).

Both the \textit{K. pneumoniae} IAp35 \textit{mrkI}::\textit{kn}R and IAp35 \textit{ΔmrkHI} mutants do not produce surface-associated type 3 fimbriae following growth under aerobic conditions (Table IV.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 \textit{ΔmrkHI} mutant remains non-fimbriate under either condition (Table IV.2). Electron microscopy confirmed the absence of fimbriae on the MrkI and MrkHI mutants and many fimbriae on the surface of complemented strains (Figure IV.2). Interestingly, in a \textit{ΔmrkHI} background, introduction of a plasmid solely expressing \textit{mrkI} was unable to complement fimbrial expression while a plasmid only expressing \textit{mrkH} was able to restore type 3 fimbriation (Table IV.3). In addition, overexpressing \textit{mrkH} in an \textit{mrkI} background was also able to restore fimbrial expression despite the absence of \textit{mrkI} (Table IV.3).

The \textit{mrkI} and \textit{mrkHI} mutations affect \textit{mrkA} transcription. Using qRT-PCR of total RNA extracted from aerobically grown agar cultures, it was found that both IAp35 \textit{mrkI}::\textit{kn}R and IAp35 \textit{ΔmrkHI} are significantly reduced in \textit{mrkA} expression. Levels of \textit{mrkA} transcript in the IAp35 \textit{mrkI}::\textit{kn}R strain were approximately 20-fold lower than those in IAp35, with values only 5.04\% ± 0.42\% of the parental strain (100\% ± 20.16\%). Similarly, with IAp35 \textit{ΔmrkHI}, significant decreases in \textit{mrkA} transcript were
seen with an approximately 33-fold reduction or 3.02% ± 0.39% of parental IApc35 mrkA transcript levels (Figure IV.3). Since MrkI mutants assemble surface-associated fimbriae when grown anaerobically, mrkA expression under these conditions was determined. *K. pneumoniae* IApc35 mrkI::knR grown anaerobically exhibited levels of mrkA expression that were indistinguishable from the parental IApc35 strain. mrkA transcription in the IApc35 ΔmrkHI mutant were significantly lower than those of the parental strain and were reduced approximately 2,000-fold (Figure IV.3). 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 are 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 cultures incubated aerobically. Increased expression of approximately 285-, 77- and 91-fold respectively were observed for mrkA, mrkH and mrkI (Table IV.4).

**MrkI and MrkHI mutants have a decreased ability to form biofilms on both abiotic and HECM coated surfaces.** Using crystal violet plate assays, it was shown that *K. pneumoniae* IApc35 mrkI::knR has a decreased ability to form a biofilm on an abiotic surface compared to the parental strain. Using these assays, the IApc35 mrkI::knR mutant transformed with an empty vector control had a significantly decreased ability to form biofilms on an abiotic surface compared to the parental IApc35 strain carrying the same plasmid, indicating no affect of the cloning vector alone on biofilm formation. The
IApc35 mrkI::knR mutant exhibited a more than two-fold decrease, yielding OD A_{595} values that were only 41.6% ± 11.41% of parental values (100% ± 15.45%). When mrkI was re-introduced into the IApc35 mrkI::knR strain, full restoration of biofilm formation with OD A_{595} values 101.21% ± 11.12% of parental absorbances (Figure IV.4A). Likewise, when IApc35 ΔmrkHI carrying the empty vector control was examined for deficiencies in biofilm formation using the same assay, it was found to have significantly reduced OD A_{595} values that were 15.03% ± 10.01% of parental absorbances (100% ± 28.1%). Restoration of biofilm formation was observed when mrkHI was re-introduced into IApc35 ΔmrkHI with values significantly higher than those seen with the parental strain (158.42% ± 10.35%) (Figure IV.4B).

In addition to differences in biofilm formation on abiotic surfaces, I also examined the effects of an mrkHI mutation on biofilm formation on HECM coated surfaces. For these experiments, a defined MrkHI mutant of K. pneumoniae 43816 was constructed. The parental strain has previously been shown by our group to form robust biofilms on HECM mediated by the presence of type 3 fimbriae (Jagnow & Clegg, 2003; Lavender et al., 2005). Biofilms formed by 43816 ΔmrkHI were observed to be thinner and sparser throughout the chamber than wild-type 43816 (Figure IV.5).

**Absence of MrkH affects murine virulence.** To examine the effects of mrkI and mrkHI mutations on K. pneumoniae virulence, an intranasal murine infection model was used (Lavender et al., 2005). Groups of five BALB/C mice were infected by intranasal inoculation of 10^3 bacteria using strains 43816, 43816 mrkI::specR, and 43816 ΔmrkHI. Approximately 18 hrs post-infection, mice infected with either 43816 or 43816
*mrkI::spec*<sup>R</sup> were moribund and were euthanized (Table IV.5). This experiment was repeated and the same results were found. Interestingly, mice infected with 43816 Δ*mrkHI* survived challenge with inoculation of up to 10<sup>6</sup> cfu (Table IV.5). These results were also seen using an intraperitoneal route of infection (data not shown).

**MrkHI mutants exhibit decreased capsule production and increased serum sensitivity.** As capsule production has previously been shown to be important in a murine model of *K. pneumoniae* infection, capsular expression in 43816, 43816 *mrkI::spec*<sup>R</sup>, and 43816 Δ*mrkHI* was determined using Congo Red agar and LB-5% sucrose agar. As expected, 43816 exhibited a capsulate phenotype of white, mucoid colonies after 48 hrs of growth at 37° C on Congo Red agar. Interestingly, the 43816 *mrkI::spec*<sup>R</sup> mutant had a similar white, mucoid phenotype while the 43816 Δ*mrkHI* mutant grew as red, non-mucoid colonies under the same growth conditions (Figure IV.6A). 43816 and 43816 *mrkI::spec*<sup>R</sup> colonies grown overnight on LB-5% sucrose exhibited a hypermucoid phenotype, due to the presence of the disaccharide in the media, while 43816 Δ*mrkHI* colonies exhibited a substantial reduction in the mucoid phenotype (Figure IV.6B).

Since capsule production is important for serum resistance in *K. pneumoniae*, serum sensitivity of 43816, 43816 *mrkI::spec*<sup>R</sup>, and 43816 Δ*mrkHI* was determined as previously described (Sahly *et al.*, 2004). Following incubation at 37°C, both 43816 and 43816 *mrkI::spec*<sup>R</sup> were found to be serum resistant with no significant changes in numbers of viable bacteria relative to the starting inoculum over the four hour time-course. The 43816 Δ*mrkHI* mutant exhibited almost immediate serum sensitivity with
very few viable bacteria being present after only one hour of incubation and none being detected at subsequent sampling (2-4hrs) (Figure IV.7). These results are representative of duplicate experiments. Heat-inactivated human serum was found to not affect the viability of 43816 ΔmrkHI cells.

**Mutation of a conserved PilZ residue of MrkH results in the inability to induce type 3 fimbriae production.** Alignment of the PilZ domain in MrkH 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 (Pratt et al., 2007) (Figure IV.8A). Substitution of a conserved arginine-113 to alanine was successful as determined by sequencing. Introduction of plasmid pACYCmrkH<sub>R113A</sub>mrkI into IApc35 ΔmrkHI did not restore full production of type 3 fimbriae, with serum titers of 320 in these transformants. Transformants possessing the parental pACYCmrkHI plasmid exhibited titers of 40,960 (Figure IV.8B). The titers of pACYCmrkH<sub>R113A</sub>mrkI transformants indicated only slightly higher fimbrial production than that observed using the IApc35 ΔmrkHI mutant alone (<40). This inability to induce type 3 fimbrial production was further investigated using the reporter plasmid pTrc99AP<sub>mrkA-lacZ</sub> in an mrk-negative E. coli background. When the plasmid pACYCmrkHI was introduced into this strain, significant increases, relative to a similar strain carrying the empty vector control plasmid pACYC184ΔCm<sup>R</sup>, of β-galactosidase were observed with values averaging 945.99 ± 47.3 Miller Units compared to 53.01 ± 1.29, respectively. In contrast, when pACYCmrkH<sub>R113A</sub>mrkI was assayed in the same manner a significant decrease, compared to the parental plasmid, in β-galactosidase activity was observed (123.05 ± 1.19 Miller Units) (Figure IV.8C).
**MrkH binds c-di-GMP.** To determine whether MrkH is capable of binding c-di-GMP filter binding assays were used as previously described (Hickman & Harwood, 2008). Purification of 6xHis-tagged MrkH from induced cells containing pDESTmrkH was successful as determined by Western blot analysis. Similarly, purification of MrkH$_{R113A}$ from transformants possessing pDESTmrkH$_{R113A}$mrkI was also successful. The MrkH protein immobilized on nitrocellulose was proficient at binding $[^{32}\text{P}]$c-di-GMP as analyzed by phosphoimagery with total counts (t.c.) of approximately 63,000 (Figure IV.9A). This was significantly higher than both the LacZa control (~2,000 t.c.) and the MrkH$_{R113A}$ mutant protein (~2,600 t.c.). These results are representative of duplicate experiments.

To confirm that binding was c-di-GMP specific, competition assays with non-radiolabeled c-di-GMP and GTP were performed. In the presence of unlabelled c-di-GMP, binding of the labeled compound could be competitively inhibited (Figure IV.9B). Inversely, the addition of non-radiolabeled GTP did not compete with binding of radiolabeled c-di-GMP to MrkH (data not shown). As indicated above, the MrkH$_{R113A}$ protein bound relatively little $[^{32}\text{P}]$c-di-GMP without competitor (~400 t.c.) and even less with the addition non-radiolabeled c-di-GMP (~200 t.c.) (Figure IV.9B). These values are representative of duplicate experiments. Additionally, binding assays were also performed using $[\alpha-^{32}\text{P}]$GTP, of which neither MrkH nor MrkH$_{R113A}$ were able to bind this nucleotide (data not shown). Also, purified MBP-MrkI was used in the binding assays and was unable to bind $[^{32}\text{P}]$c-di-GMP (data not shown).
**MpkH and MpkHI activate the mrkA promoter.** To examine whether MpkH, MpkI, or MpkHI 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 an *E. coli* NEB 5-α transformed with a P<sub>mrkA</sub>-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 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 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 the strain caring mrkH alone, was observed (approximately 8-fold) (Figure IV.10).
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. The fimbrial tip adhesin, MrkD, has previously been shown to specifically interact with collagen components within the extracellular matrix, facilitating initial adherence to HECM coated surfaces (Hornick et al., 1995; Jagnow & Clegg, 2003; Langstraat et al., 2001; Schurtz et al., 1994). Following attachment, subsequent biofilm formation is mediated by interactions between the type 3 fimbrial shafts comprised of the polymerized MrkA subunit protein. The production of the type 3 fimbriae, and their ability to bind host-derived matrix components, has been proposed to aid in the colonization of disrupted human mucosal surfaces and indwelling medical devices, which become coated, *in situ*, with matrix components. Additionally, fimbriate bacteria that possess no functional MrkD adhesin, though unable to form biofilms on HECM coated surfaces, are able to form biofilms on abiotic surfaces (Hornick et al., 1995; Johnson & Clegg, 2010; Langstraat et al., 2001). As many other enterobacterial fimbrial systems have been found to be governed by complex regulatory circuits, it is believed that the type 3 fimbriae are subject to similar levels of regulation.

To identify regulatory elements of the type 3 fimbrial operon, I previously constructed a mini-*Tn5* transposon library in *K. pneumoniae* IApc35. One non-fimbriate mutant from this library possessed a transposon insertion within a gene encoding a putative transcriptional regulator, which I have previously termed *mrkl* (see Chapter II). This gene is predicted to encode a protein with only a C-terminal LuxR-like DNA binding domain and an N-terminal region that exhibits little relatedness to any
characterized protein domains. Interestingly, \textit{mrkI} is located between \textit{mrkH}, which is predicted to encode a protein which contains a C-terminal c-di-GMP binding domain (PilZ), and \textit{mrkJ}, which I have previously shown to produce a functional phosphodiesterase which modulates the intracellular levels of c-di-GMP within \textit{K. pneumoniae} (see Chapter III). Due to the proximity of \textit{mrkH} to \textit{mrkI} and the presence of a predicted c-di-GMP binding domain within MrkH, I determined whether MrkH plays a role in type 3 fimbrial gene regulation and whether it is responsible for the c-di-GMP dependent production of the fimbria observed in Chapter III. Initially, I found that \textit{mrkH} is co-transcribed with \textit{mrkI}. This is consistent with the observation that the only promoter identified by sequence analysis, which is likely to drive \textit{mrkI} transcription, lies upstream of \textit{mrkH}. In addition to \textit{mrkHI} co-transcription I also found that \textit{mrkJ} transcription can also occur from the \textit{mrkH} promoter, though the \textit{mrkI} and \textit{mrkHI} mutations were not found to significantly alter the levels of \textit{mrkJ} transcription (data not shown). Therefore it is possible that transcription of \textit{mrkJ} can also occur from a promoter immediately upstream of it. Deletion of both \textit{mrkHI}, like the single \textit{mrkJ} mutation, resulted in the decreased ability of \textit{K. pneumoniae} to produce surface-associated type 3 fimbriae. Repeated attempts were made to construct an \textit{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 \textit{mrkHI} together and re-introduction of \textit{mrkJ} alone is not.

Interestingly, I also demonstrated that the MrkI mutant was non-fimbriate 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 non-fimbriate 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 that facilitates fimbriae production independently of MrkI. Consequently, during anaerobic growth, MrkH and MrkI are likely to facilitate increased mrkA expression resulting in a strongly fimbriate phenotype. In the absence of MrkI however, the increased MrkH production under anaerobic conditions, may enable MrkH to interact with an orphan activator to facilitate mrkA transcription. Also, it is possible that K. pneumoniae, in response to varying environmental conditions, produces different regulators that 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 have the more general function of sensing the intracellular concentrations of c-di-GMP. This is supported by the observation that unlike many organisms where 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 many members of this family only contain the PilZ domain containing 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.
Biofilm formation *in vitro* on both abiotic and HECM coated surfaces is highly dependent upon type 3 fimbrial production. Therefore, the decrease in type 3 fimbrial expression seen in MrkI and MrkHI mutants resulted in a significant decrease in biofilm formation on a solid surface. To further investigate the role of MrkH and MrkI in biofilm formation on biotic surfaces and in virulence, I constructed both MrkI and MrkHI mutants of the murine hypervirulent strain *K. pneumoniae* 43816. The parental strain is able to adhere specifically to components of the HECM and also causes an acute, lethal infection following intranasal inoculation of mice. The 43816 MrkI mutant exhibited the same non-fimbrial phenotype as the mutant of strain IApc35 and fimbrial expression could be restored by complementing with *mrkI*. When the MrkI mutant of 43816 was examined for biofilm formation on an HECM coated surface, it was found that this strain is unable to form robust biofilms on this substrate. This decreased biofilm phenotype, when compared to the wild-type strain, was characterized by sparse patches of growth in biofilm chambers and an overall reduction in thickness in these areas. Likewise, the MrkHI mutant of 43816 was also non-fimbriate and unable to form biofilms on HECM components. The growth rates of both strains grown as planktonic cultures were identical to those of wild-type 43816.

The MrkI mutant of strain 43816 was not altered in virulence and resulted in 100% morbidity and mortality in acutely infected animals. Interestingly, the MrkHI mutant of 43816 was completely attenuated at intranasal inoculums up to $10^6$ cfu, further indicating a regulatory role separate from that of MrkI. Previous results have indicated that the ability to produce type 3 fimbriae plays no role in the murine infection model as it involves rapid invasion and dissemination following infection of healthy animals. This
type of infection is dissimilar to human infections that are most frequently associated with insertion of medical devices such as urinary catheters into compromised hosts. Indeed, most clinical isolates of *K. pneumoniae* are avirulent in the murine airway infection model. These results indicate that MrkI does not decrease the expression of virulence genes necessary for lethality in the murine respiratory infection model unlike MrkH. This may mean that MrkI is a specific regulator of type 3 fimbrial expression under aerobic conditions as opposed to the more widespread role of MrkH in *K. pneumoniae* colonization and virulence.

Since capsule production is important in the murine model of *K. pneumoniae* infection, I next examined both the 43816 MrkI and MrkHI mutants for deficiencies in capsule production. Consistent with the results stated above, the MrkI mutant exhibited a capsular phenotype similar to wild-type 43816, while the MrkHI mutant is decreased in capsular expression compared to wild-type. Once again, this may allude to a regulatory network where MrkH is acting as an adaptor for a regulator, distinct from MrkI, which is responsible for capsule production. In addition, I also determined whether there were differences between the MrkI and MrkHI mutants in terms of serum resistance. Using pooled human serum, the MrkI mutant remains resistant to serum components at levels similar to wild-type 43816. In contrast, the MrkHI mutant exhibited a marked susceptibility to serum components as viable cell numbers quickly declined, eventually becoming undetectable. While it is tempting to speculate on a direct relationship between MrkH, virulence, capsular expression, and serum resistance, an absolute causation cannot be inferred. Still, it is interesting to note that because of the nature of the murine infection model, i.e. rapid dissemination into the bloodstream, it is likely that the first
host defenses \textit{K. pneumoniae} encounters are those present within the blood. As such, if MrkH is found to directly regulate capsular expression, it would be possible to more soundly implicate MrkH’s role in virulence.

The ability of MrkH and MrkI to facilitate transcription of \textit{mrkA} in an \textit{E. coli} transformant that possesses no \textit{mrk} genes of its own, and the DNA binding domain present in MrkI, led me to speculate that MrkI binds the promoter region of \textit{mrkA}. However, I was not able to demonstrate binding \textit{in vitro} using gel mobility shift assays (data not shown). Even in the presence of MrkH, c-di-GMP, and using various experimental conditions it was not possible to observe MrkI bound to the \textit{mrkA} promoter. However, it is possible that MrkI binds to this region and we are unable to replicate, \textit{in vitro}, the conditions for binding \textit{in vivo}. Additional bacterial factors may be required for this activity. The results in \textit{E. coli} transformants are consistent with the observation that MrkH and MrkI affect transcription, as detected by qRT-PCR in \textit{K. pneumoniae}. The presence of MrkH alone facilitates detectable levels of \textit{mrkA} transcription in \textit{E. coli}, but this is significantly lower than that observed when both MrkH and MrkI are present. The MrkI mutant of \textit{K. pneumoniae} would be predicted to produce MrkH, but this mutant is phenotypically non-fimbriate and exhibits no \textit{mrkA} transcription. The level of \textit{mrkA} transcription in \textit{E. coli} possessing only MrkH could be due to the relatively high concentrations of MrkH produced by the cloned gene. Since analysis of the \textit{K. pneumoniae} genome indicates that MrkH is the only PilZ-possessing protein that is not involved in cellulose metabolism it is possible that MrkH acts as a c-di-GMP signaling adaptor for many systems that are regulated by the intracellular concentrations of c-di-GMP. Consequently, c-di-GMP’s 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. I do not think that MrkH and MrkI indirectly regulate mrkA transcription by affecting mrkJ gene expression and thus altering the levels of intracellular c-di-GMP due to the phosphodiesterase activity of MrkJ since no mrkJ homolog is found in *E. coli* (Johnson & Clegg, 2010).

The genetic regulation of fimbrial systems belonging to the chaperone-usher family of adhesins is often subject to complex regulatory schemes. As a member of the chaperone-usher family, it is likely that the type 3 fimbrial system is regulated by multiple gene products. Here I have identified two gene products which regulate type 3 fimbrial gene expression, but it remains likely that other regulators, either upstream or downstream of MrkHI, exist. Regardless, the location of the mrkHIJ gene cluster immediately adjacent to the mrkABCDF structural genes may be alluding to an evolutionary selection for these two genes clusters. Currently, we are trying to determine the exact mechanism by which MrkH and MrkI regulate type 3 gene expression and identifying the gene products within *K. pneumoniae* modulate the intracellular pool of c-di-GMP.
### Table IV.1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>IApc35</td>
<td>Plasmid cured variant of IA565, type 3 fimbriae⁺</td>
<td>(Hornick et al., 1995)</td>
</tr>
<tr>
<td>IApc35 mrkI::kn&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;, IApc35 mrkI insertion mutant, type 3 fimbriae⁻</td>
<td>This study</td>
</tr>
<tr>
<td>IApc35 ΔmrkHI</td>
<td>IApc35 mrkHI deletion mutant, type 3 fimbriae⁻</td>
<td>This study</td>
</tr>
<tr>
<td>43816</td>
<td>Murine virulent, HECM binding proficient, type 3 fimbriae⁺</td>
<td>ATCC (Manassas, VA)</td>
</tr>
<tr>
<td>43816 mrkI::spec&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Spec&lt;sup&gt;R&lt;/sup&gt;, 43816 mrkI insertion mutant, type 3 fimbriae⁻</td>
<td>This study</td>
</tr>
<tr>
<td>43816 ΔmrkHI</td>
<td>43816 mrkHI deletion mutant, type 3 fimbriae⁻</td>
<td>This study</td>
</tr>
<tr>
<td>BL21 AI</td>
<td>Protein expression strain</td>
<td>Invitrogen (Carlsbad, CA)</td>
</tr>
<tr>
<td>NEB 5-α</td>
<td>General <em>E. coli</em> cloning strain</td>
<td>NEB (Ipswich, MA)</td>
</tr>
<tr>
<td>SM10 λpir</td>
<td><em>E. coli</em> donor strain</td>
<td>(Miller &amp; Mekalanos, 1988)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pACYC184ΔCm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Tet&lt;sup&gt;R&lt;/sup&gt;, Cam&lt;sup&gt;S&lt;/sup&gt;; empty vector control for complementation constructs</td>
<td>This study</td>
</tr>
<tr>
<td>pACYCmrkH</td>
<td>Tet&lt;sup&gt;R&lt;/sup&gt;, mrkH complementation vector</td>
<td>This study</td>
</tr>
<tr>
<td>pACYCmrkI</td>
<td>Tet&lt;sup&gt;R&lt;/sup&gt;, mrkI complementation vector</td>
<td>This study</td>
</tr>
<tr>
<td>pACYCmrkHI</td>
<td>Tet&lt;sup&gt;R&lt;/sup&gt;, mrkHI complementation vector</td>
<td>This study</td>
</tr>
<tr>
<td>pACYCmrkH&lt;sub&gt;R113A&lt;/sub&gt;mrkI</td>
<td>Tet&lt;sup&gt;R&lt;/sup&gt;; mutated mrkHI complementation vector</td>
<td>This study</td>
</tr>
<tr>
<td>pDEST17</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;; 6x-His tag Gateway expression vector</td>
<td>Invitrogen</td>
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<tr>
<td>pDESTmrkH</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;; 6x-His tag MrkH expression construct</td>
<td>This study</td>
</tr>
<tr>
<td>pDESTmrkH&lt;sub&gt;R113A&lt;/sub&gt;</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;; 6x-His tag MrkH&lt;sub&gt;R113A&lt;/sub&gt; expression construct</td>
<td>This study</td>
</tr>
<tr>
<td>pDS132</td>
<td>Cam&lt;sup&gt;R&lt;/sup&gt;; sacB suicide vector</td>
<td>(Philippe et al., 2004)</td>
</tr>
<tr>
<td>pDS132ΔmrkHI</td>
<td>Cam&lt;sup&gt;R&lt;/sup&gt;, Spec&lt;sup&gt;R&lt;/sup&gt;; construct used to make 43816 ΔmrkHI</td>
<td>This study</td>
</tr>
<tr>
<td>pGEM-T Easy</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;; subcloning vector</td>
<td>Promega (Madison, WI)</td>
</tr>
<tr>
<td>pTrc99AP&lt;sub&gt;mrkA-lacZ&lt;/sub&gt;</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;; pTrc99A-based reporter construct</td>
<td>This study</td>
</tr>
</tbody>
</table>
Table IV.2. Type 3 fimbrial surface expression in *K. pneumoniae* strains

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>IApc35</th>
<th>IApc35&lt;sup&gt;mrkI::kn&lt;/sup&gt;</th>
<th>IApc35&lt;sup&gt;mrkI::kn +mrkI&lt;/sup&gt;</th>
<th>IApc35&lt;sup&gt;ΔmrkHI&lt;/sup&gt;</th>
<th>IApc35&lt;sup&gt;ΔmrkHI +mrkHI&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic (Shaken flask)</td>
<td>5,120&lt;sup&gt;1&lt;/sup&gt;</td>
<td>&lt;40</td>
<td>40,960</td>
<td>&lt;40</td>
<td>40,960</td>
</tr>
<tr>
<td>Aerobic (Agar grown)</td>
<td>5,120</td>
<td>&lt;40</td>
<td>40,960</td>
<td>&lt;40</td>
<td>40,960</td>
</tr>
<tr>
<td>Microaerophilic (Static tube)</td>
<td>40,960</td>
<td>20,480</td>
<td>ND&lt;sup&gt;2&lt;/sup&gt;</td>
<td>&lt;40</td>
<td>40,960</td>
</tr>
<tr>
<td>Anaerobic (Agar grown)</td>
<td>5,120</td>
<td>10,240</td>
<td>ND</td>
<td>&lt;40</td>
<td>40,960</td>
</tr>
</tbody>
</table>

<sup>1</sup>Serum titer represents the reciprocal of anti-MrkA serum dilution needed to produce visible agglutination. Lowest dilution of serum used was 1:40.

<sup>2</sup>Not determined.
**Table IV.3.** Complementation analysis of *K. pneumoniae* strains

<table>
<thead>
<tr>
<th>Strain (plasmid)</th>
<th>Serum titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>IApC35 (pACYCΔCm&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>5,120</td>
</tr>
<tr>
<td>IApC35 mrkI::kn&lt;sup&gt;R&lt;/sup&gt; (pACYCΔCm&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>&lt;40</td>
</tr>
<tr>
<td>IApC35 mrkI::kn&lt;sup&gt;R&lt;/sup&gt; (pACYCmrkH)</td>
<td>40,960</td>
</tr>
<tr>
<td>IApC35 mrkI::kn&lt;sup&gt;R&lt;/sup&gt; (pACYCmrkI)</td>
<td>40.960</td>
</tr>
<tr>
<td>IApC35 mrkI::kn&lt;sup&gt;R&lt;/sup&gt; (pACYCmrkHI)</td>
<td>40,960</td>
</tr>
<tr>
<td>IApC35 ΔmrkHI (pACYCΔCm&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>&lt;40</td>
</tr>
<tr>
<td>IApC35 ΔmrkHI (pACYCmrkH)</td>
<td>40,960</td>
</tr>
<tr>
<td>IApC35 ΔmrkHI (pACYCmrkI)</td>
<td>&lt;40</td>
</tr>
<tr>
<td>IApC35 ΔmrkHI (pACYCmrkHI)</td>
<td>40,960</td>
</tr>
</tbody>
</table>

<sup>1</sup>Serum titer represents the reciprocal of anti-MrkA serum dilution needed to produce visible agglutination. Lowest dilution of serum used was 1:40.
Table IV.4. Aerobic and anaerobic expression of mrk genes

<table>
<thead>
<tr>
<th>Transcript (condition)</th>
<th>Fold-change (± St. Dev.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mrkA (aerobic)</td>
<td>1.0 (± 0.1)</td>
</tr>
<tr>
<td>mrkA (anaerobic)</td>
<td>284.78 (± 47.75)****</td>
</tr>
<tr>
<td>mrkH (aerobic)</td>
<td>1.0 (± 0.15)</td>
</tr>
<tr>
<td>mrkH (anaerobic)</td>
<td>76.93 (± 24.45)****</td>
</tr>
<tr>
<td>mrkI (aerobic)</td>
<td>1.0 (± 0.15)</td>
</tr>
<tr>
<td>mrkI (anaerobic)</td>
<td>90.65 (± 12.34)****</td>
</tr>
</tbody>
</table>

****: p-value < 0.0001
Table IV.5. Murine virulence of *K. pneumoniae* strains

<table>
<thead>
<tr>
<th>Strain (cfu)</th>
<th>Survived/Inoculated (% survival)</th>
</tr>
</thead>
<tbody>
<tr>
<td>43816 (10^3)</td>
<td>0/10 (0%)</td>
</tr>
<tr>
<td>43816 <em>mrkI</em>:spec&lt;sup&gt;R&lt;/sup&gt; (10^3)</td>
<td>0/10 (0%)</td>
</tr>
<tr>
<td>43816 Δ<em>mrkHI</em> (10^3)</td>
<td>10/10 (100%)</td>
</tr>
<tr>
<td>43816 Δ<em>mrkHI</em> (10^4)</td>
<td>5/5 (100%)</td>
</tr>
<tr>
<td>43816 Δ<em>mrkHI</em> (10^5)</td>
<td>5/5 (100%)</td>
</tr>
<tr>
<td>43816 Δ<em>mrkHI</em> (10^6)</td>
<td>5/5 (100%)</td>
</tr>
</tbody>
</table>
Figure IV.1. (A) Genetic organization of the mrk gene cluster. Putative promoter regions have been identified by sequence analysis and are indicated by arrows. (B) The predicted domain architecture of MrkI. The point of mini-\textit{Tn5} insertion is within the predicted LuxR-like DNA binding domain (amino acids 130-176) in the C-terminal region of the 190-amino acid MrkI polypeptide. (C) Predicted domains within the MrkH polypeptide. The putative PilZ c-di-GMP binding site lies within the C-terminal region (amino acid 107-225).
**Figure IV.2.** Fimbrial production by *K. pneumoniae* strains. (A) *K. pneumoniae* IApe35, (B) MrkI mutant and (C) MrkI mutant transformed with cloned *mrkI* gene. Also, (D) the MrkHI mutant and (E) the complemented MrkHI mutant carrying cloned *mrkHI*.
Figure IV.3. qRT-PCR of *mrkA* encoding the major fimbrial subunit in *K. pneumoniae* strains. Aerobic and anaerobic *mrkA* transcription in both IAp35 *mrkI::kn*R and IAp35 Δ*mrkHI* are shown as the relative decrease in transcription compared to the parental strain. Statistical significance was determined using a Student’s t-test. (p-value: *** < 0.001, **** < 0.0001)
**Figure IV.4.** Biofilm phenotypes of *K. pneumoniae* strains. (A) Biofilm formation of parental IApc35 and the MrkI mutant carrying the empty vector control (VC) compared to the complemented MrkI mutant on an abiotic surface. (B) Decreased biofilm formation of the IApc35 MrkHI mutant compared to parental IApc35 and MrkHI mutant complemented with plasmid-borne *mrkHI*. Statistical significance was determined using a Student’s t-test. (p-value: * < 0.05, ** < 0.01, *** < 0.001)
**Figure IV.5.** HECM biofilm phenotypes of both wild-type *K. pneumoniae* 43816 and the 43816 Δ*mrkHI* mutant.
Figure IV.6. Capsular phenotypes of wild-type 43816, the 43816 MrkI mutant, and the 43816 MrkHI mutant (A) on Congo Red agar plates and (B) on 5% sucrose plates.
Figure IV.7. Serum resistances of wild-type 43816, the MrkI mutant, and the MrkHI mutant after incubation at 37° C for 4 hrs. Bacterial viability is represented as a ratio of the number of viable cells at each time point relative to the starting inoculum.
Figure IV.8. Analysis of 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 asterisks (★). The resulting R113A alignment is also indicated (⌘). (B) Ability of $mrkH_{R113A}$ cloned with $mrkl$ to restore type 3 fimbrial expression compared to parental IApc35, the MrkHI mutant, and the MrkHI mutant complemented. Values are reciprocals of serum titer needed to cause visible agglutination. (C) Use of a $P_{mrkA-lacZ}$ to examine ability of MrkHI to induce expression of the reporter in an $E. coli$ background compared to an empty vector control and the MrkH$R_{113A}$ mutant. Statistical significance was determined using a Student’s t-test. (p-value: **** < 0.0001)
**Figure IV.9.** Ability of MrkH to bind $[^{32}\text{P}]c$-di-GMP. (A) Filter binding assay of LacZα control, MrkH, and MrkH$_{R113A}$ proteins bound with $[^{32}\text{P}]c$-di-GMP and immobilized on nitrocellulose. Graph represents total counts detected from spots directly above. (B) Filter binding assays of above reactions with (+) or without (-) the addition of pure c-di-GMP.
Figure IV.10. 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 a Student’s t-test. (p-value: **** < 0.0001)
CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

*Klebsiella pneumoniae* is an opportunistic pathogen, the isolation of which appears to be on the rise in long-term care facilities and hospitals. While *K. pneumoniae* has historically caused pneumonias, urinary tract infections, and neonatal septicemias, it is becoming more frequently associated with a variety of soft tissue infections, including wound infections. Further underscoring the importance and seriousness of these types of infections is the emergence of multi-drug resistant *K. pneumoniae* strains that exhibit resilience against every clinically available antibiotic. Not surprisingly, these types of infections often carry relatively high rates of morbidity and mortality compared to susceptible strains.

Despite these concerns, very little information exists as to the mechanisms behind *K. pneumoniae* virulence and even less regarding the regulation of these factors. Several constraints have contributed to limiting information about this organism. First, the genetic tools that have been used in other members of the *Enterobactericeae* frequently do not work in *K. pneumoniae*. For example, mutagenesis techniques using linear DNA transformations, suicide DNA vectors, and the TargeTron mutagenesis system, have not proved to be consistently successful in *K. pneumoniae*. Secondly, due to the inherent antibiotic resistance of *K. pneumoniae* to ampicillin and chloramphenicol, very few commercially available DNA vectors are available for gene cloning without first introducing different or additional antibiotic resistance cassettes into those vectors. Third, there appears to be a high degree of genome plasticity in *K. pneumoniae*, which
may not only decrease the efficiency of homologous recombination in the organism, but may also result in occasional illegitimate recombination events.

To identify genetic regulators of type 3 fimbrial production in *K. pneumoniae*, I initially began using a variety of transposon-based transcriptional reporters. For example, as *K. pneumoniae* possesses two copies of the β-galactosidase gene (*lacZ*), and no defined *lac* mutants exist, I utilized transposons that contained either the luciferase (*lux*), the green fluorescent protein (GFP), or the β-glucuronidase (GUS) reporters. As none of these reporters proved to be productive (for a variety of reasons), I approached the goal of identifying regulators using a colony immunoblot procedure that would identify transposon mutants that were phenotypically non-type 3 fimbriate. This screen had several benefits including high resolution, being able to easily identify fimbriate from non-fimbriate colonies, and rapidity, allowing me to screen large numbers of mutants. Using this technique, I identified eleven non-fimbriate mutants. Using standard PCR analyses to screen for insertions in structural genes, I isolated three such mutants and these were eliminated from further study. Therefore, the results from this screen indicated that 0.03% of mutants screened may be mutations in fimbrial regulators. Based on the complex regulatory schemes, involving multiple proteins, controlling other fimbrial systems, it is likely that I did not isolate mutations in all the genes involved in fimbrial regulation. However, from the 8 insertion mutants of interest arising from this screen, one of them was used to develop a model for the role of c-di-GMP in type 3 fimbria production.

One of the identified mutants contained a transposon which had inserted within a gene, which I have named *mrkl*, that is predicted to encode a putative transcriptional
regulator. This protein is predicted to possess a C-terminal LuxR-like DNA binding domain and an undefined N-terminal region. Both the transposon mutant and a site-directed insertion mutant were unable to assemble type 3 fimbriae on their surface, which resulted in decreased ability of the mutants to form biofilms on both biotic and abiotic surfaces. Interestingly, these fimbrial phenotypes were found only when the strains were grown aerobically as either agar or broth cultures, but once grown anaerobically, these strains became strongly type 3 fimbriate regardless of the mutations. While I have shown that mrkH expression is increased under anaerobic conditions, and this may explain the oxygen-dependent phenotype of the MrkI mutant, it may also suggest that under anaerobic conditions, type 3 fimbrial production is controlled by a regulatory pathway independent of MrkI. Based on this observation it would be interesting in future studies to perform the original screen that identified MrkI, but following growth of the mutants under anaerobic conditions. This may address the question of which regulators are able to control mrk gene expression in an MrkI-independent manner under anaerobic conditions. Such studies would contribute not only to a greater understanding of how this fimbrial system is regulated, but also how *K. pneumoniae* may respond to anaerobic environments within the human host.

Further study of the MrkI mutants indicated that the effect of the mutations on fimbrial production was at the level of *mrkA* transcription, as determined by qRT-PCR. Since this observation supported the initial annotation of MrkI as a transcriptional regulator, I investigated whether MrkI exerted its effect directly by interacting with the *mrkA* promoter. Partial purification of soluble MrkI was only achieved as a maltose-binding protein (MBP) fusion. Difficulties in purifying proteins due to insolubility is a
characteristic that is not uncommon among proteins which contain LuxR-like DNA binding domains, with many of those proteins requiring co-expression of their cognate signaling molecules to make purification possible. Regardless, due to MrkI having an undefined N-terminal domain, it is not apparent what signaling molecules, if any, MrkI may require for purification. Unfortunately, the MBP-MrkI fusion protein, while found to adequately restore fimbrial expression in an MrkI mutant, was unable to bind to the promoter region of mrkA in vitro. Also, MrkI was unable to activate a functional mrkA promoter-lacZ fusion in an E. coli background which lacks any endogenous mrk genes. There are several possibilities why I was unable to observe MrkI binding. First, the in vitro conditions I tested may have been unfavorable for MrkI binding, for a variety of reasons. Perhaps MrkI binds a novel signaling molecule, or possesses a novel binding domain for an already established signaling molecule, that is still not apparent. Interaction with the signaling molecule may not only be important for MrkI to take on a DNA-binding proficient conformation, but may also allow for purification without a cumbersome soluble domain. Also, though a variety of established DNA-binding reaction buffers were used for the in vitro studies, it is possible that MrkI may require additional, undefined conditions for binding. MrkI may also require an accessory protein to maintain a proper conformation that was not present in my experimental conditions. To a limited extent this was addressed by using lysates from a K pneumoniae MrkI-overexpressing strain in the DNA binding assays, but I was still unable to observe specific binding to the mrkA promoter. Lastly, there still remains the possibility that the target site of MrkI is not the mrkA promoter.
Due to the proximity of \textit{mrkI} to genes (\textit{mrkH} and \textit{mrkJ}) that are predicted to encode proteins involved in c-di-GMP signaling, I investigated whether the intracellular concentrations of c-di-GMP affect the expression of surface-associated type 3 fimbriae. Initially, it was possible to show that increases in c-di-GMP increased expression of type 3 fimbriae, and vice versa, using defined and functional diguanylate cyclases and phosphodiesterases from \textit{V. parahaemolyticus}. Subsequently, I was able to demonstrate that deletion of the MrkJ phosphodiesterase resulted in a hyperfimbriate phenotype due to the intracellular accumulation of c-di-GMP. Not surprisingly, this increase in type 3 fimbrial surface production also resulted in a hyperbiofilm formation phenotype. Inversely, when overexpressing MrkJ, surface-associated fimbriae cannot be detected. These results are consistent with the central c-di-GMP signaling model in which increases in intracellular c-di-GMP are believed to promote a biofilm/sessile lifestyle, whereas lower concentrations of the molecule often induce biofilm dispersal and/or motility. The type 3 fimbrial system represents only the second chaperone/usher fimbrial family member, the first being the Cup fimbriae of \textit{P. aeruginosa}, that is regulated by intracellular c-di-GMP concentrations. The \textit{K. pneumoniae} MGH78578 genome contains several genes that are predicted to encode proteins that may alter the concentrations of this molecule, including 12 EAL domain-containing proteins, 11 proteins which contain the GGDEF domain, and 5 which contain both an EAL and a GGDEF domain. Additionally, this sequenced strain was found to contain several plasmids, on which reside genes encoding 3 EAL and 2 GGDEF domain-containing proteins. There is a high degree of variability among these genes with some predicted to be stand-alone cytoplasmic proteins, some which have transmembrane domains, and others with
different sensory domains, most notably BLUF, PAS, GAF, and MASE domains. This observation alludes to, like many organisms that utilize c-di-GMP signaling, a complex regulatory network where several signals are likely to be responsible for decreasing or increasing the intracellular pools of c-di-GMP. Perhaps most importantly, it remains to identify which other modular signaling components influence type 3 fimbrial production, in particular whether certain diguanylate cyclases increase pools of c-di-GMP specific for fimbria gene regulation.

The observation that c-di-GMP influences type 3 fimbrial expression, led me to modify the hypothesized role of MrkI in type 3 fimbrial expression. It is possible that as a positive regulator of type 3 production, MrkI may serve a role whereby its expression leads to increases in intracellular c-di-GMP. This could be due to direct expression of one or more diguanylate cyclases or by repressing the expression of phosphodiesterases and specifically MrkJ. First, I determined the concentrations of c-di-GMP in the *K. pneumoniae* MrkI mutant as well as strains overexpressing MrkI. If MrkI is responsible for increasing the intracellular pools of c-di-GMP it would be predicted that the MrkI mutant would have had decreased levels of the molecule while strains overexpressing MrkI would have accumulated c-di-GMP. Due to the very low levels of c-di-GMP in *K. pneumoniae* (compared to strains such as *P. aeruginosa*), it was technically very challenging to detect significant differences in the MrkI mutant compared to the parental strain. Regardless, if MrkI does affect c-di-GMP concentrations, I would have expected the MrkI overexpressing strains to make significantly more c-di-GMP, but they did not.

As a corollary, I also examined the expression of *mrkJ* in the MrkI mutant by qRT-PCR and did not find significant differences in *mrkJ* transcription between the MrkI mutant
and parental IApC35. If MrkI functioned as a repressor of \textit{mrkJ} expression, I would have expected that \textit{mrkJ} gene expression would have been increased in the MrkI mutant. However, I observed that \textit{mrkJ} expression was slightly decreased in an MrkI mutant. This, as mentioned below, is most likely due to polar effects since transcription of \textit{mrkJ} may occur from a common promoter upstream of \textit{mrkH}.

Finally, I examined whether MrkH serves as an effector protein for c-di-GMP signaling to the type 3 fimbrial gene cluster. RT-PCR analyses indicated that the \textit{mrkHIJ} genes are operonic even though sequence analysis of the region upstream of \textit{mrkJ} predicts a separate promoter for this gene and that insertional mutations of \textit{mrkI} do not result in drastically lower levels of \textit{mrkJ} transcript. It is possible that \textit{mrkJ} transcription uses two promoters under, as yet undefined, different conditions. Multiple attempts were made to create an in-frame deletion mutant of MrkH by homologous recombination, but these were never successful. It is possible that single \textit{mrkH} mutations are lethal for the bacteria even though re-introduction of \textit{mrkI} into the \textit{mrkHI} mutant (essentially an \textit{mrkH} mutant) was not. The nature of this complication remains to be determined.

The MrkHI mutant exhibited many similar phenotypes to the MrkI mutant. These included a decreased ability to produce surface-associated fimbriae, which, like the MrkI mutant, resulted in an inability to form biofilms on both abiotic and biotic surfaces. Also, the MrkHI mutant was found to exert this effect at the level of structural gene (\textit{mrkABCDF}) transcription. However, several different phenotypes between MrkI and MrkHI mutants are also apparent. Using complementation analysis, I found that overexpression of MrkI in the MrkHI mutant was unable to restore fimbrial expression in this strain, indicating that MrkH itself is necessary for type 3 fimbrial production. Also, I
found that unlike the MrkI mutant, where fimbrial expression is only affected under aerobic conditions, the MrkHI mutant is unable to produce fimbriae under both aerobic and anaerobic conditions. This proved to be an interesting observation as it may indicate that fimbrial expression may be under a modular regulation scheme much like the central c-di-GMP paradigm. For example, MrkH could be serving as a general effector protein for many systems whereas MrkI is only utilized or present as a target under aerobic conditions. Further supporting this hypothesis is the observation that there is only one gene present in the *K. pneumoniae* genome that is predicted to encode a c-di-GMP binding protein that is not implicated in cellulose synthesis. This is not uncommon among other members of *Enterobacteriacea* as many are only predicted to encode the c-di-GMP binding protein YcgR. Interestingly, both YcgR and MrkH contain the PilZ c-di-GMP binding domain, but MrkH lacks homology to the N-terminal domain of YcgR. Absence of this domain may signify that MrkH is involved in c-di-GMP mediated signaling interactions that are unique to *K. pneumoniae*.

In one strain of *K. pneumoniae*, additional phenotypes have been attributed to only the MrkHI mutant that distinguish it from the MrkI mutant. The MrkHI mutant of the murine virulent strain *K. pneumoniae* 43816 was found to be avirulent in a mouse model of infection, was found to have decreased capsular expression, and exhibited sensitivity to pooled human serum. Unfortunately, complementation by the cloned *mrkHI* genes in this mutant was not observed. It is unclear why this is the case since the cloned genes could restore fimbrial production in the MrkHI mutant of *K. pneumoniae* IApC35 (a murine avirulent strain). The reason for this remains unclear since both mutants were constructed in an identical manner. However, the murine virulent strain
43816 is not typical of most human isolates in being a hypervirulent and invasive strain in mice. The strain is genetically different to IApc35 and this, along with the reported high degree of plasticity in \textit{K. pneumoniae} strains, may explain those observed differences.

Purified MrkH was found to specifically bind c-di-GMP \textit{in vitro}. Additionally, a site-directed substitution of a conserved amino acid within the putative c-di-GMP binding pocket resulted in a protein that was unable to bind c-di-GMP. This derivative of MrkH, in combination with wild-type MrkI, was unable to complement fimbrial expression in the IApc35 MrkHI mutant. This observation not only confirms the importance of MrkH in fimbrial expression, but also indicates that c-di-GMP binding is required for MrkH to function properly in type 3 regulation. Wild-type MrkH and this mutated protein were also used to investigate induction of the \textit{mrkA} promoter-lacZ reporter fusion plasmid in \textit{E. coli}. Interestingly, MrkH alone activated this fusion whereas the mutated MrkH was unable to activate the transcriptional reporter. This result might suggest that MrkH may directly interact with the \textit{mrkA} promoter regardless of the absence of a predicted DNA binding domain since \textit{E. coli} lacks all the \textit{mrk} genes including \textit{mrkI}. I used purified MrkH in DNA binding assays to determine if MrkH could bind \textit{in vitro} to the \textit{mrkA} promoter region. However, even in the presence of exogenous c-di-GMP, no binding was detected. Possibly in an \textit{E. coli} background MrkH may interact with a host protein, in the absence of MrkI, to activate \textit{mrkA}.

From the data presented in this thesis, it is possible that MrkH and MrkI may be interacting partners. For example, MrkH and MrkI may interact in a c-di-GMP dependent manner and subsequently become proficient at binding the \textit{mrkA} promoter.
Supporting this is the additional observation that introduction of a plasmid encoding MrkHI results in increased expression of the mrkA-lacZ fusion over that seen with MrkH or MrkI alone. As indicated above, the ability of MrkH alone to activate the mrkA-lacZ fusion may be indicative of a non-preferred interacting partner present in the *E. coli* background. Examination of the *E. coli* K-12 genome revealed 8 genes predicted to encode proteins with domain architectures similar to MrkI (undefined N-terminal domain and a C-terminal LuxR/GerE-like helix-turn-helix DNA binding domain). Conversely, MrkI alone is unable to activate the mrkA-lacZ reporter and thus does not appear to interact with the *E. coli* YcgR protein that is the closest functional homolog to MrkH.

Future studies will focus on examining the interaction of MrkH and MrkI using various “pull-down” assays to detect interaction in the bacterial cells.

In conclusion, I have identified regulators of type 3 fimbrial gene expression involved in either the direct modulation or sensing of intracellular concentrations of the second messenger c-di-GMP. Work within the field of c-di-GMP-based signaling is ever evolving and often increasing in complexity. Current investigations are aimed at understanding not only the environmental signals that feed into the modulation of intracellular concentrations of the molecule, but also how a molecule which appears to be so ubiquitous within the cellular compartment is able to affect only one particular process. My investigations have provided much of the initial characterization of this particular type of signaling in *K. pneumoniae*, but a great amount of work remains to be done. Interestingly, my studies have implicated an environmental component of c-di-GMP signaling in *K. pneumoniae* (oxygen tension), but also has postulated a novel mechanism of c-di-GMP mediated transcriptional regulation. Namely, the interaction of
two proteins, one of which binds c-di-GMP, to act as a transcriptional activation complex of fimbrial genes (Figure V.1).
Figure V.1. Model of proposed MrkHI transcriptional activation complex. In the top panel, the overabundance of the MrkJ phosphodiesterase leads to a net decreased in intracellular concentrations of c-di-GMP. Under these low levels, MrkH is unable to bind the c-di-GMP, preventing formation of the MrkHI complex. In a high c-di-GMP environment it is proposed that concentrations of MrkJ are low, and that the levels of c-di-GMP are such that MrkH can find and bind c-di-GMP, and promote interactions between MrkH and MrkI. This interaction is suspected to be essential for the formation of a transcriptional activation complex that binds the mrkA promoter region.
REFERENCES


