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# CLONING AND MUTATIONAL ANALYSIS OF THE *FIMB* PROMOTERS IN UROPATHOGENIC *ESCHERICHIA COLI*

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CLONING AND MUTATIONAL ANALYSIS OF THE *FIMB* PROMOTERS IN UROPATHOGENIC *ESCHERICHIA COLI*

By Brandon M. Reuter

We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the Degree of Master of Science in Biology, Clinical Microbiology Concentration.

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ABSTRACT

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Uropathogenic *Escherichia coli* (UPEC) cause an estimated 7 million urinary tract infections annually in the U.S. Type I pili, expressed by UPEC, are largely responsible for mediating bacterial attachment to host bladder cells. The *fimB* gene encodes a site-specific recombinase that controls the orientation of an invertible DNA segment containing the promoter for the pilus structural gene *fimA*. Three promoters have been mapped for *fimB*, but little is known about their respective function or hierarchy. In this study, *fimB* promoter mutants were created and used to establish a *fimB* promoter hierarchy while substantiating the influence two regulatory proteins, OmpR and GadE, have on type 1 pilus expression and *fimB* transcription. The UPEC strain UTI189 Δ*fimB* was transformed with plasmids containing the *fimB* promoter mutants. Since pH and osmolality are known to affect type 1 pilus expression, transformed Δ*fimB* cells were grown in Luria-Bertani broth pH 5.5 and 7.0 with or without 400 mM NaCl. Variation in surface piliation was determined by hemagglutination while variation in *fimB* transcription was measured by quantitative reverse-transcriptase PCR. Hemagglutination analysis indentified nucleotides that may be important for the interaction of OmpR with *fimB* promoter two, but failed to indentify a region in which GadE interacts with *fimB*. Additional experiments are needed to clarify these findings.

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# INTRODUCTION

**Characteristics and clinical significance of *Escherichia coli*.** *Escherichia coli* has long been used as a model organism for exploring gene regulation in bacteria, and has become the most thoroughly studied organism in the world. *Escherichia coli* is an enteric, gram-negative rod that belongs to the *Enterobacteriaceae* family. *Escherichia coli* is typically motile by peritrichous flagella, although some strains are nonmotile. The bacterium is a facultative anaerobe that can grow on a variety of complete laboratory media. However, *E. coli* can grow in minimal media by utilizing glucose as the sole carbon source to make all macromolecules necessary for growth (58).

Although *E. coli* is a ubiquitous organism that can be readily isolated as bowel flora from healthy individuals, some *E. coli* strains have been widely studied because of their clinical significance as the causative agent of urinary tract infections (UTIs), bacteremias, meningitis, and diarrhea. Over 73,000 diarrheagenic *E. coli* infections are estimated to occur in the United States each year, but a UTI is the most common clinical manifestation of an *E. coli* infection. Uropathogenic *E. coli* (UPEC) cause an estimated 6-7 million UTIs ($2.5 billion in medical expenses) in the United States every year, and is the number one cause of UTIs in humans (89, 136). Although less frequent, *Enterococcus*, *Proteus*, *Klebsiella*, *Pseudomonas*, *Staphylococcus*, and *Serratia* species may also cause UTIs.

Due to anatomical differences such as urethra length and location, the majority of UTIs occur in women, but UTIs in men are rare. A UTI is diagnosed in 50% - 60% of

women in their lifetime while they are only diagnosed in about 20% of men (99). Patients typically contract UTIs when UPEC are introduced into the urethra from the colon. Once introduced, UPEC can ascend to and colonize the bladder or kidneys. As a result, UTIs can present as a localized infection of the urethra (urethritis), bladder (acute cystitis), or kidneys (pyelonephritis). Symptoms of UTIs are diverse and can include acute painful urination, dysuria, urinary frequency and urgency, nocturia, and suprapubic discomfort. Acute cystitis is the most common clinical presentation of a UTI, and is usually effectively treated with an antibiotic like trimethoprim-sulfamethoxazole or a fluoroquinolone, such as ciprofloxacin (135). However, if left untreated, a UTI can eventually lead to renal failure or bacteremia (104).

**Laboratory diagnosis of urinary tract infections.** Rapid and accurate diagnosis of infection is crucial to ensure proper treatment, and to lower the risk of pyelonephritis or sepsis. Diagnosis of a UTI is accomplished by obtaining a midstream clean catch urine sample, or urine from catheterization. The urine can be analyzed using a urinalysis dipstick, microscopy, and culture methods. Dipstick analysis is used to detect the presence of nitrite and leukocyte esterase, which are indicators of bacterial infection. In addition, microscopy can be used for the direct observation of neutrophils and bacteria in urine. During culture analysis, a calibrated 1 µl inoculating loop is used to streak urine onto tryptic soy agar containing 5% sheep red blood cells and MacConkey agar plates. A symptomatic patient can be diagnosed with a UTI when as little as $10^2$ CFU/ml is present in culture while $10^5$ CFU/ml in culture is required for diagnosis in asymptomatic patients (92).

**Characterization, regulation, and factors affecting expression of type 1 pili in uropathogenic *Escherichia coli.*** Pathogenic strains of *E. coli* have well-described virulence factors that allow a particular *E. coli* strain to infect and cause disease within a specific niche in a host (56). However, virtually all pathogenic *E. coli* have iron acquisition proteins, a capsule, hemolysins, host cell effector proteins, and a variety of proteins involved in adherence to the host.

Bacterial adherence to host urethral and bladder cells is widely accepted as a pivotal step in developing a stable UTI (3, 20). To resist elimination by the shear forces caused by urine flow, UPEC has a variety of fimbrial and non-fimbrial adhesins that facilitate attachment of the bacterium to host cells (124). Type 1 pili and P fimbriae are the most common fimbriae expressed by UPEC, however, S-pili and afimbrial adhesins play an important role in the adherence of UPEC to sites other than the bladder (53, 62, 66, 86, 103) .

Type 1 pili are filamentous appendages (3 μm long) that are characterized by their ability to mediate irreversible mannose-sensitive binding that is necessary for UPEC to colonize the bladder (FIG. 1) (2, 20, 94, 107). Several genes (A,C,D,F-I) in the *fim* operon encode the proteins necessary for type 1 pilus expression (FIG. 2A). FimH is the adhesin protein located at the distal end of the pilus that functions as the ligand for mannose-containing glycoprotein receptors (55, 64, 88). FimF and FimG are adaptor proteins that connect the adhesin FimH to the rod of the pilus, but are not directly involved in adhesion (55, 107). The FimA protein comprises the bulk of the pilus structure, but is also not directly involved in adherence. Instead, repeating monomers of

FimA give the pilus its rigid helical structure. Through a spring-like mechanism, this helical structure is most likely responsible for absorbing the physiological shear forces encountered in the bladder (79). The promoter for *fimA* is located on a 314-bp invertible sequence called *fimS*. FimC and FimD function as chaperone and usher proteins, respectively, and are necessary for outer membrane localization of the pilus proteins (54, 60). The function of the *fimI* gene product is largely unknown, except that mutations in *fimI* result in nonpiliated *E. coli* (131). Lastly, FimB and FimE are site-specific recombinases that affect *fimS* positioning (33, 59).

Uropathogenic *E. coli* utilize phase variation to alternate between a piliated and nonpiliated state (2). The inversion of *fimS* (*fim* switching) controls phase variation by controlling expression of the *fim* operon. To catalyze the *fim* switch, FimB and FimE recognize two inverted repeats that flank either side of the invertible region which they use for the recombination event (43). During a *fim* switch, FimE predominately catalyses the inversion from ON to OFF while FimB has a preference for catalyzing the switch from the OFF to ON position, but can also orient *fimS* in the OFF position (59, 76). In addition, FimE has been shown to have a higher binding affinity when the invertible region is oriented in the ON position, thus preferentially inverting it to the OFF position (63).

Environmental conditions also affect expression of type 1 pili (10). For example, *E. coli* tend to be non-piliated when grown on agar while growth in static liquid medium has been shown to strongly promote the growth of type 1 piliated *E. coli* cells (46, 96, 114). Type 1 pilus expression is also influenced by temperature. Gally *et al.* showed that

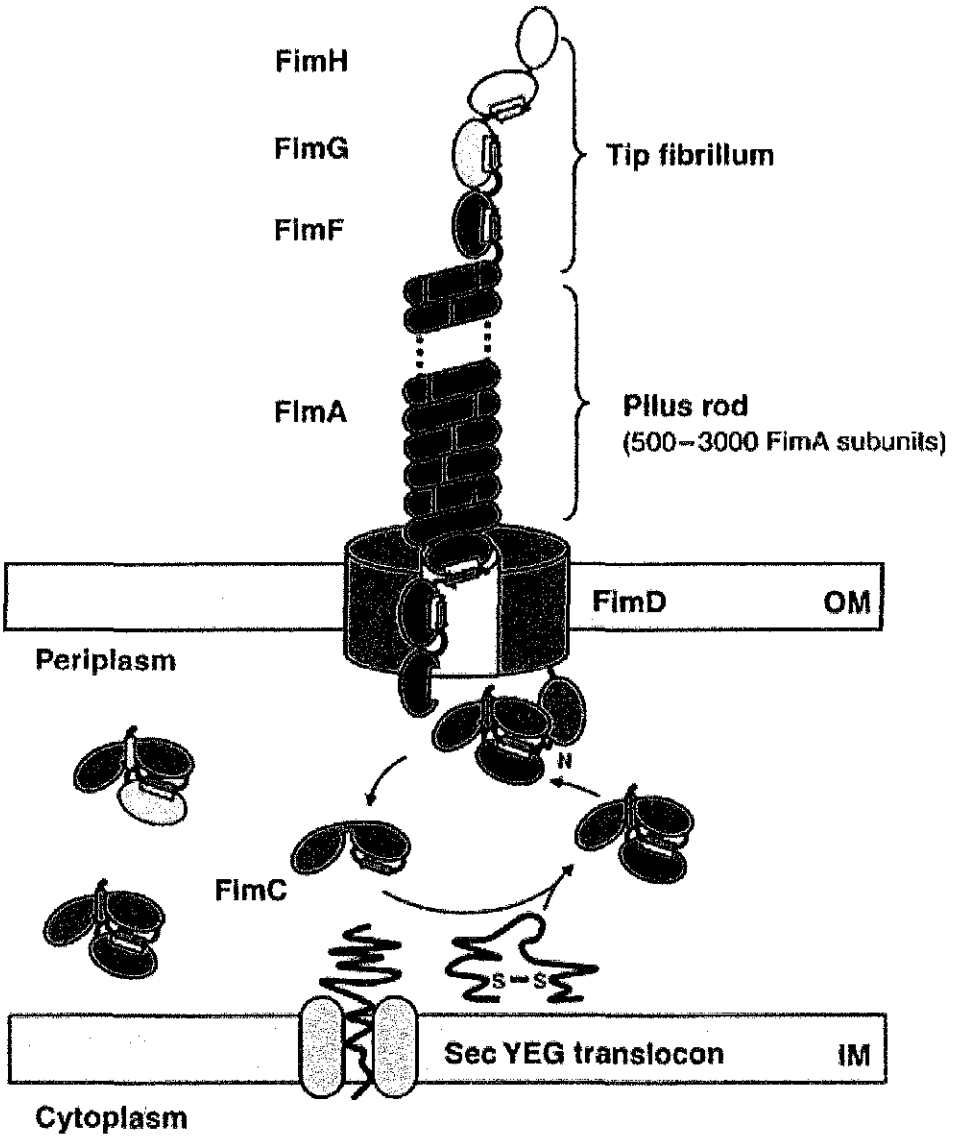


FIG. 1. Schematic of a type 1 pilus and accessory proteins. Figure reproduced with permission from Nishiyama *et al.* (80).

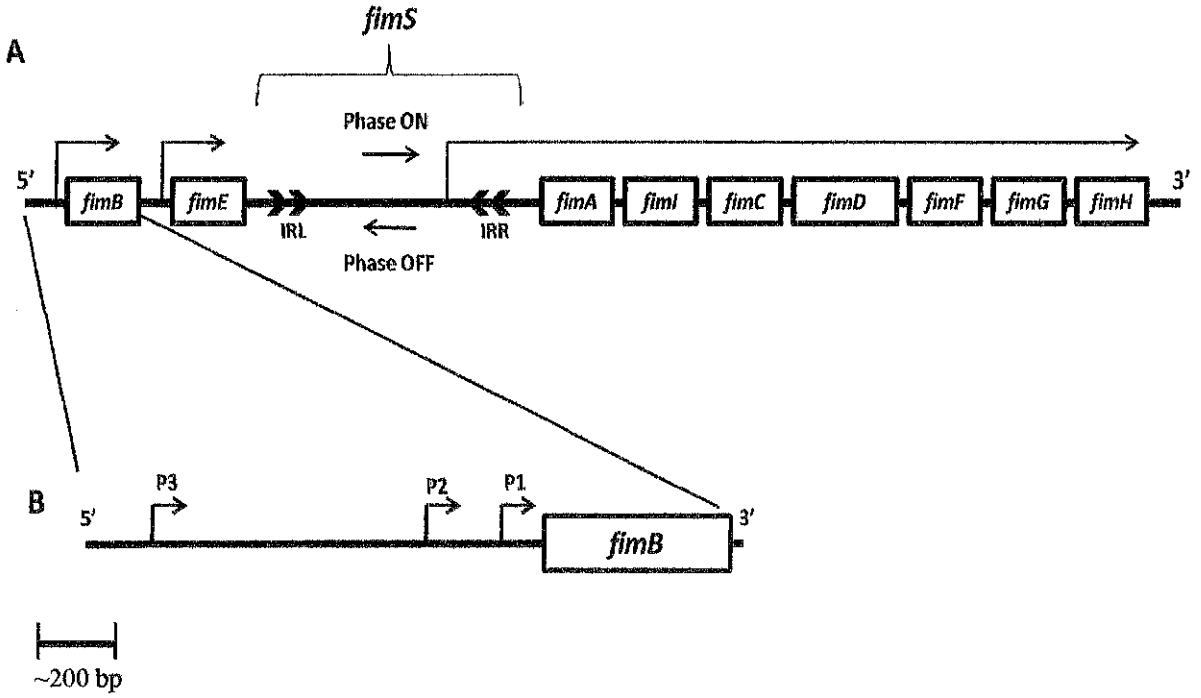


FIG. 2. A. The *fim* operon, which encodes proteins required for type 1 pilus expression. The 314-bp invertible element, which contains the promoter for *fimA*, is named *fimS*. Within the *fimS* region is the inverted repeat left (IRL) and right (IRR) which are used by FimB and FimE recombinases to catalyze the *fim* switch. **B.** *fimB* promoter region enlarged to show all three promoters and relative position.

that FimB-promoted inversion was greatest at temperatures between 37 and 41°C, while FimE-promoted inversion decreased at the same temperatures (35). In contrast, Dorman *et al.* showed that transcription of the *fim* operon increased significantly at 30°C when compared to transcription at 37°C (24).

Osmolality and pH also influence the transcription and expression of *fimB* and *fimE* (117). Growth of *E. coli* in a neutral pH-low osmolality environment favors the expression of type 1 pili. However, growth in an acidic pH-high osmolality environment leads to a reduction in *fimB* transcription and subsequent lower levels of type 1 pili.

Although FimB and FimE are major players in controlling phase variation, several other proteins have been shown to affect type 1 pilus expression both by stimulating *fimS* inversion directly or indirectly by altering expression of *fimB* or *fimE* (113). Leucine response regulatory protein (Lrp) and integration host factor (IHF) bind with high affinity to *fimS* (34). One study showed that a mutation in the *lrp* gene resulted in a 100-fold decrease in the frequency of *fim* switching, but expression of *fimB* and *fimE* was reduced only slightly (12). The amino acids leucine, isoleucine, valine, and alanine interact with Lrp and alter its binding to *fimS* to stimulate *fim* switching (41, 106, 119). In addition, several studies have shown that IHF is required for *fim* switching (25, 28). Both Lrp and IHF form loops in the DNA which places the inverted repeats within the *fimS* in a conformation more favorable for recombination, and subsequent phase variation (11, 34).

The histone-like protein H-NS is a global regulator involved in bacterial chromatin condensation (9, 23, 127). However, H-NS directly affects *fim* switching by binding to *fimS*, and indirectly by suppressing both *fimB* and *fimE* transcription (23, 97,

117). More recently, the SlyA protein was shown to activate *fimB* expression by displacing H-NS, thereby negating the inhibitory effects of H-NS (78). Other global regulators such as cAMP, RcsB, RpoS, ppGpp, NanR, and NagC have also been implicated in the regulation of phase variation primarily by controlling expression of the *fimB* and *fimE* recombinase genes (1, 26, 85, 116, 126).

**Physiological Conditions and Host Defenses in the Human Urinary Tract**. In order to colonize the bladder, UPEC must overcome a variety of physiological obstacles. Low nutrient (glucose and amino acids) and iron content, high urea concentration, variable pH (4.5 to 8.0 depending on metabolism and diet) and osmolality (0.038 to 1.4 mol/kg) are all physiological barriers which UPEC must overcome in order to infect and successfully colonize the urinary tract (73, 105).

Innate host immunity also acts to inhibit UPEC from colonizing the urinary tract. Shear forces caused by urine flow provide a mechanical barrier which will remove unattached bacteria. However, the abundance of mannose-containing receptors on uroepithelial cell surfaces in the lower urinary tract provide many locations for type 1 pilus-mediated adherence and subsequent colonization (19). However, apoptosis of the superficial epithelium may be triggered by type 1 pili binding and more specifically by the recognition of FimH by toll-like receptor four (5, 61). This interaction can cause the exfoliation of infected or damaged uroepithelial cells which is a means to remove attached bacteria (32).

UPEC can further resist elimination by invading bladder epithelial cells and host phagocytic cells (77, 120). Localized cytoskeletal changes of host bladder cells, at

contact points between FimH and host cells, appear to envelope the bacterium inducing internalization (32, 74, 88). After internalization, UPEC form biofilm-like pods on the luminal surface of the bladder that are tightly packed with intracellular dividing bacterial cells. This biofilm-like formation is mediated by type 1 pili, and mutating *fimH* completely abolishes the ability to form these pods (4). Once inside host cells, UPEC are protected from antibiotics, shear forces, and host immune cells. Intracellular environments also provide UPEC access to nutrient-rich conditions. Internalized bacteria are able to persist even when a patient is no longer considered bacteriuric, and UPEC reemergence from these pods is a major source of recurrent UTIs. Reemergence is also a means by which UPEC invades other epithelial cells, thus establishing a persistent intracellular infection (87).

Expression of type 1 pili by UPEC during a bladder infection is advantageous due to the abundance of mannose-containing receptors in the bladder epithelium (95, 134). However, as UPEC ascend from the bladder to the kidneys, production of type 1 pili is reduced over time (in mice) since they are not necessary for colonization of the kidneys, and there is a lack of mannose containing receptors in the kidneys (103, 112, 130, 133).

Osmolality and pH affect phase variation by having an effect on *fimB* and *fimE* transcription (117). In environmental conditions similar to those found in the human kidney (low pH and high osmolality) (73), UPEC tend to exist in the nonpiliated state because of preferential transcription of *fimE*, which catalyzes the promoter of *fimA* from the ON to OFF orientation. This discovery led to the idea that pH and osmolality somehow repress expression of *fimB* while simultaneously activating expression of *fimE*.

Three promoters (P1-P3) were mapped for *fimB* by primer extension, but not much is known of their respective functions in regulating *fimB* transcription (FIG. 2B) (115). Strong products were produced from primer extension originating 148-bp (P1) and 288-bp (P2) upstream of *fimB*, while a weak product was produced about 900-bp upstream (P3). Since both pH and osmolarity have been shown to influence *fimB* transcription, the different *fimB* promoters are thought to be preferentially used depending on the environmental growth condition (117). If this is the case, then each promoter might have unique regulatory factors acting upon it during growth in different pH and osmotic growth conditions. Several of the factors thought to regulate *fimB* transcription play a prominent role as central transcriptional regulators in their native systems. These systems will now be discussed.

**Response to changes in osmolality.** *Escherichia coli* are able to survive in very diverse environments, including the human kidneys. Since osmolytes from blood are concentrated in the kidneys before either being replaced to the blood or sent to the bladder for removal, osmolality can reach as high as 1400 mOsm inside human kidneys and 3000 mOsm in murine kidneys (105). UPEC survival in hyperosmotic environments is possible due to the OmpC and OmpF proteins. These proteins form pores in the outer membrane which allow polar molecules (less than 600 Da) to passively diffuse across the membrane (83). It is hypothesized that OmpC, which has a smaller pore diameter and slower flow rate than OmpF, is expressed in high osmotic conditions as a means to limit

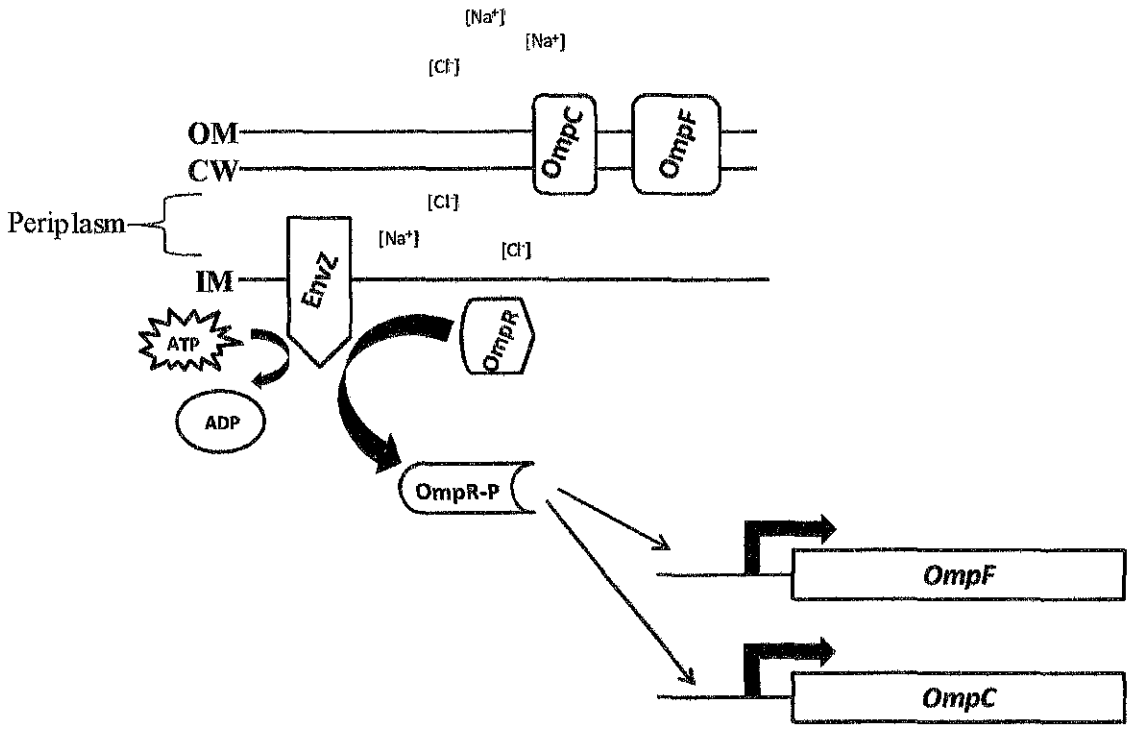


FIG. 3. Schematic representation of the EnvZ/OmpR regulatory system. OM: outer membrane, CW: cell wall, IM: inner membrane.

the diffusion of solutes across the bacterial membrane (82, 90, 132), while ompF is expressed in lower osmotic conditions.

Expression of *ompC* and *ompF* is regulated by the two-component regulatory system OmpR /EnvZ. In this system, EnvZ acts as a transmembrane sensor protein and OmpR acts as a cytoplasmic signal transduction protein that positively regulates the expression of *ompC* and *ompF* and greatly contributes to the survival of UPEC in the murine urinary tract (FIG. 3)(82, 112). The DNA binding domain of OmpR binds with high and low affinity to sites contained within the promoters of *ompC* and *ompF*, while

the transcription initiating domain is sensitive to phosphorylation by EnvZ (50, 52, 67, 72, 93, 128). Although environmental sensing by EnvZ is not understood, EnvZ is autophosphorylated (EnvZ-P) in high osmotic conditions when ATP is present. Once phosphorylated, EnvZ-P transphosphorylates OmpR (OmpR-P) (22, 30, 48, 49, 57). Phosphorylation causes a conformational change, which allows OmpR-P to directly interact with the α subunit of RNA polymerase and initiate transcription of *ompC* and *ompF* (57). Under low osmolality conditions, low levels of OmpR-P interact with high affinity sites on the *ompF* promoter and subsequently stimulate its expression. Conversely, a high level of OmpR-P, present in hyperosmotic conditions, allows the protein to interact with low affinity sites on the *ompC* and *ompF* promoters. The net effect of this interaction is the suppression of *ompF* and transcription of *ompC* (108, 122, 123, 138).

The EnzV/OmpR regulatory system has primarily been used to better understand *E. coli* response to changes in osmolality, but there is evidence suggesting that OmpR has a regulatory role in *fim* gene expression. A previous study showed an increase in *fimB* expression in a UPEC *ompR* mutant especially when grown in an acidic, hyperosmotic environment (117). In contrast, the effects of OmpR on *fimE* expression were not significant. DNAse I footprinting further substantiated the role OmpR has in *fimB* regulation by showing that OmpR interacts with *fimB* P2 (100). The identification of this putative binding site will allow further examination of the effects that OmpR has on *fimB* expression.

**Acid tolerance in *Escherichia coli.*** Besides survival in a wide range of osmolalities, *E. coli* can also survive extreme pH fluctuations. Independent of external conditions, *E. coli* maintain an internal pH of about 7.6 (91, 139). Homeostasis is mediated by at least five acid response (AR) systems (31, 68, 69, 101). System one is induced in stationary phase independent of environmental pH, but provides protection from a pH as low as 2.5 when grown in minimal media. The protective mechanism for this system is not well described, but it is repressed by glucose and requires the use of the cAMP receptor protein (CAP) and the alternative sigma factor RpoS. Systems two through four are induced under acidic conditions by their respective, exogenous amino acids, and make use of decarboxylases and their cognate antiporters to maintain near neutral internal pH of the cell. System two (AR 2) is induced by glutamate in stationary phase, and requires a glutamate decarboxylase and a glutamate:γ-aminobutyric acid (GABA) antiporter. System three is induced by arginine under anaerobic, acidic conditions and requires arginine decarboxylase (AdiA) and the arginine:agamatine antiporter (AdiC) (36, 51). Induction of system four occurs under anaerobic, acidic conditions by lysine and requires the lysine decarboxylase (CadA) and a lysine:cadaverine antiporter. A fifth mechanism is induced under log phase growth, but is not well characterized.

When using the AR 2 system (also known as the GAD system), *E. coli* can survive a pH 2 challenge when exogenous glutamate is present. System two requires the antiporter GadC and two inducible glutamate decarboxylases, GadA and GadB. The antiporter is responsible for transporting glutamate into the cell while transporting the

product of glutamate decarboxylation, GABA, out of the cell (16, 42, 125). This method of AR provides the greatest protection against cellular acidification. The decarboxylation of glutamate consumes one intracellular hydrogen, while the transportation of GABA out of the cell inverts the *E. coli* membrane potential from negative outside/positive inside to positive outside/negative inside (102). The consumption of internal hydrogen directly increases the internal pH of the cells while the charge inversion repels external protons and disrupts the proton motive force which further contributes to the maintenance of a neutral internal pH.

GadE has been identified as the central transcriptional activator of *gadA/BC*, and provides the primary means of *gadA/BC* activation (47, 71). GadE binds to a 20-bp sequence (GAD box) located 63-bp upstream of the transcriptional start site of both *gadA* and the *gadBC* operon, and is necessary for expression of these genes under all conditions (15, 16, 17, 18).

GadE is a known activator of the genes involved in glutamate-dependent acid resistance in *E. coli*, but also controls the expression of numerous other genes (75). In stationary phase, during low pH challenges, an increase of GadE and subsequent increase in GadA and GadB are observed. Since low pH-high osmolality have a synergistic effect on the UPEC piliation, it is hypothesized that GadE might have some pH-dependent control over *fimB* transcription. In one study, a *gadE* knock-out mutant (in a K-12 background) was transformed with the plasmid pJB5A, which contains the promoter regions of *fimB* ligated to a promoterless *lacZ* gene. The level of *fimB* transcription was then measured by the amount of β-galactosidase activity. Deleting *gadE* led to an

increase of *fimB* transcription in a pH 5.5 medium, providing evidence that GadE may have some pH-dependent influence on *fimB* transcription (W.R. Schwan unpublished results).

**Objectives of this study.** In the current study, mutations were introduced to the TATA box and TSS of *fimB* P1 and P2. Other mutations were created in *fimB* P2 promoter as an attempt to alter the binding affinities of GadE and OmpR to *fimB* P2. These mutations were created in order to:

1. Establish a *fimB* P1 and P2 hierarchy.
2. Better understand the regulatory role GadE and OmpR have in *fimB* transcription and type 1 pilus expression.

## MATERIALS AND METHODS

**Plasmids, bacterial strains, and antibiotics.** All plasmids used in this study are described in Table 1. Plasmid pWRS4-1 (114) contains the *fimB* gene with all three promoters from the clinical isolate J96 (45) and a kanamycin resistance gene. The single copy plasmid pPP2-6 contains a single copy origin of replication (mini-F replicon), a multiple cloning site, and a chloramphenicol (Cm) resistance gene (81, 117, 129). A gentamicin (Gm) resistance gene was taken from the plasmid pUCGM and inserted in the plasmid pPP2-6 (118). All antibiotics were purchased from Sigma-Aldrich (St. Louis, MO) and were used in the following concentrations: 40 μg/ml kanamycin, 12.5 μg/ml chloramphenicol, and either 10 or 14 μg/ml gentamicin. The bacterial strains used in this study are shown in Table 2.

**Construction of plasmid pPPBMR1**. The *fimB* promoter mutants described below were created in the multi-copy number plasmid, pWRS4-1. Since there is only one copy of *fimB* per *E. coli* chromosome, physiological relevancy was established by cloning the *fimB* promoter mutants into the single copy number plasmid, pPP2-6. The restriction sites used for cloning inactivated the Cm resistance gene within pPP2-6. To create a new, selectable plasmid the pPPBMR1 plasmid was made by inserting a Gm resistance gene from pUCGM into the pPP2-6 plasmid. To isolate the Gm resistance gene, pUCGM was first digested with *Bam*HI [New England Biolabs (NEB), Beverly, MA] and then the Gm

TABLE 1. Plasmids used in this study.

| Plasmid | Characteristics | Antibiotic Resistance | Copy Number | Reference or Source |
|---|---|---|---|---|
| pWRS4-1 | pHSS22 + *fimB* from *E. coli* J96 | Km | High copy | 114 |
| pPP2-6 | pPR274 + MCS[a] | Cm | Single copy | 117 |
| pUCGM | Gm acetyltransferase-3-1 | Gm | High copy | 118 |
| pPPBMR1 | pPP2-6 + $Gm^r$ | Gm | Single copy | This study |
| pBMR41 | pPPBMR1 + *fimB* from *E. coli* J96 | Gm | Single copy | This study |
| pBMR42 | pPPBMR1 + P1TATA box mutation | Gm | Single copy | This study |
| pBMR43 | pPPBMR1 + P1TSS mutation | Gm | Single copy | This study |
| pBMR44 | pPPBMR1 + P2TATA box mutation | Gm | Single copy | This study |
| pBMR45 | pPPBMR1 + P2TSS mutation | Gm | Single copy | This study |
| pBMR46 | pPPBMR1 + P2 $1^{st}$ AC mutation | Gm | Single copy | This study |
| pBMR47 | pPPBMR1 + P2 $2^{nd}$AC mutation | Gm | Single copy | This study |
| pBMR48 | pPPBMR1 + P2 $3^{rd}$AC mutation | Gm | Single copy | This study |
| pBMR49 | pPPBMR1 + OmpR higher affinity mutation | Gm | Single copy | This study |
| pBMR50 | pPPBMR1 + GAD Box mutation | Gm | Single copy | This study |

[a] Multiple cloning site.

TABLE 2. Bacteria used in this study.

| Strain | Description | Reference or Source |
|---|---|---|
| *E. coli* UTI189 | Virulent cystitis UPEC isolate | Clinical Isolate |
| *E. coli* UTI189 Δ*fimB* | *E. coli* UTI189 containing a deletion of the *fimB* gene | 39 |
| *E. coli* DH5α | F– Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1 | Invitrogen |
| *E. coli* XL-1 Blue | *rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac* [F′ *pro*AB *lac*$I^q$ZΔM15 Tn10 ($Tet^r$)] | Agilent Technologies |

resistance DNA fragment was separated from the pUCGM backbone using gel electrophoresis. The Gm resistance DNA fragment was cut from the gel, spun through a glass wool-filled column, and washed using an Amicon Ultra centrifugal filter (Millipore, Billerica, MA). The Gm resistance gene was then ligated to *Bam*HI-digested, alkaline phosphatase-treated pPP2-6 using $T_4$ ligase (NEB). *Escherichia coli* DH5α cells were transformed with the ligation mixture, and plated on Luria-Bertani (LB) agar containing 10 μg/ml gentamicin. pPPBMR1 plasmid DNA was extracted using a QIAprep Spin Miniprep kit (Qiagen, Germanton, MD) and then compared to a supercoiled DNA ladder (Invitrogen, Carlsbad, CA) and purified pPP2-6 DNA using gel electrophoresis.

**Construction of *fimB* promoter mutants**. In order to gain a better understanding of the significance the individual *fimB* promoters have on *fimB* expression, a QuikChange Multi Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA) was used to introduce base pair substitutions (Table 3) into the following 9 sequences upstream of *fimB*: four different sites within the OmpR binding site in P2 (FIG. 4.), the transcriptional start sites (TSS) and the TATA boxes of the P1 and P2 promoters (FIG. 4) that were mapped by Schwan *et al.*, as well as a suspected GAD box (FIG. 5) (115). Plasmid DNA from pWRS4-1 was used as the template for each mutagenesis, and reactions were performed as specified by the manufacturer. After the mutagenesis reactions, *E. coli* XL1-Blue cells were transformed with the mutated plasmids, and transformants were selected for on LB agar containing kanamycin. Plasmids were extracted using a QIAprep Spin Miniprep kit (Qiagen), compared to a supercoiled DNA ladder (Invitrogen) using gel electrophoresis, and sequenced by ETON Biosciences (San Diego, CA).

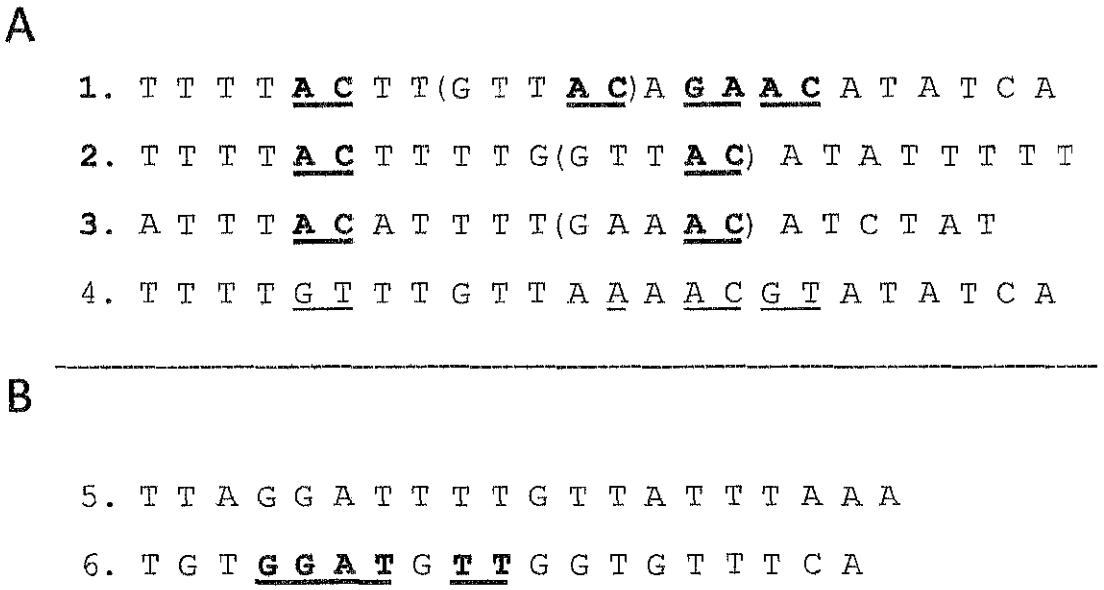


FIG. 4. Comparative analysis of OmpR (A) and GadE (B) consensus sequences in *E. coli*. Underlined bases represent proposed regions of importance between the different OmpR binding sequences, and regions that were targets for site-directed mutagenesis. **1.** OmpR binding sequence associated with *fimB* P2 (100). **2 and 3.** The proposed OmpR consensus sequences found in the promoter regions of *ompF* and *ompC*, respectively (93). Sequences in parentheses indicate a conserved GXXXC motif found in OmpR high affinity sites (41). **4.** Underlined sequences indicate the resulting mutated nucleotides. **5.** The GadE consensus sequence, located 63-bp upstream from the transcriptional start site of *gadABC* (15). **6.** A proposed GAD box located 374-bp upstream from the transcriptional start site of *fimB* P2 and identified for the purposes of this study. Underlined nucleotides indicate mutagenized sequence.

**Proposed GAD Box**

AAGCCGATCTGGAGAGGCTTGTGGATGTTGGTGTTTCAGGATGATGTTTCACTTAGT

TTGTTTGCCGTATCGCCCGGCGAATGGCTGTGATTGAGGAAGGTTTAAGTCGTAGTG

ACCCAAAGCTATATTTACCAACGAATGTAGATGAAAAAATCATCTCGTGCGTTCCCC

CATATCTCCAGGATAAAAAGGAATGTAACAATCTCATGCGTAAGCTGACGAATCAGC

AGCAGGAATAATCGCTAGGGACCTAAGAATTAGCATGATAATAGCCACTAAGAAATT

AACTGCGCTCCATGAAATAGCCATTTTGTGGCAATGGAGTTGACTAATAATGTCATA

-35 -10

TGTGAGACGGCTAGTTGAACGAATATTAAATTTTGCTGAATTTTTTATGTTGA**TTTT**

+1 (P2)

**ACTTGTTACAGAACATATCA**CATGATATATAGATAAGATTAGTTGCATTAATGATGA

GGGTTATTATTAGATTCGTATCCGATTGATAAATATATAAAGGTACATAGCATGCAA

-35 -10 +1 (P1)

GAGCATGGCGTTTGTATGGCAACGTTATAATAATTAACAGTTGCTACTCCATTTAAG

TTCACTCAGAAGAACTGGTCCACTTACGTTAGTTATTAAGCAAACGTTCGCTTTTAT

AAACATAATCAGGATAAAAATGTTGGATTATTGCTAACCCAGCACAGCTAGTGCGCG

TCTGTAATTATAAGGGAAAAACG**ATG**AAG . . .

FIG. 5 . Sequence of the *fimB* P1 and P2 promoter regions identified by Schwan *et al* (115). Bolded sequence in P2 was identified by Rentschler as the OmpR binding region (100).

Once the mutations in the *fimB* promoters were confirmed, they were inserted into the pPPBMR1 plasmid using *Not*I and *Sal*I (NEB) restriction sites. Each mutated *fimB* promoter sequence was separated from the pWRS4-1 backbone by gel electrophoresis. The resulting 2.5-kb *fimB* fragments were cut out of the gels, spun through a glass wool-filled column, and washed three times with TE buffer using an Amicon Ultra centrifugal filter (Millipore). The purified *fimB* fragments were then ligated to *Not*I/*Sal*I (NEB) digested pPPBMR1 DNA using $T_4$ DNA ligase (NEB). *Escherichia coli* DH5α cells were transformed with these ligation mixtures and plated on LB agar containing 10 μg/ml gentamicin. Plasmid DNA was extracted from several transformants using the QIAprep Spin Miniprep kit (Qiagen) and compared to a supercoiled DNA ladder and purified pPPBMR1 DNA using gel electrophoresis. The presence of the 2.5-kb *fimB* fragment in pPPBMR1 was confirmed by separating *Not*I/*Sal*I digested plasmids on a 0.8% agarose gel using electrophoresis.

The plasmids containing the mutated *fimB* promoters (Table 1) were extracted from *E. coli* DH5α cells and used to transform the clinical *E. coli* strain UTI189 Δ*fimB* cells (39). *E. coli* UTI189 Δ*fimB* cells were mixed with plasmid DNA, electroporated (1.8 kV, time constant 4.5), and plated on LB agar containing 14 μg/ml gentamicin after a one hour recovery in 500 μl SOC medium (37°C, 200 rpm) (appendix A). In order to confirm the presence of the plasmids in *E. coli* UTI189 Δ*fimB* cells, plasmid DNAs were extracted using the QIAprep Spin Miniprep kit and then compared to a supercoiled DNA ladder using gel electrophoresis.

**Hemagglutination assays.** *Escherichia coli* UTI189 Δ*fimB* cells containing the mutant plasmids were serially passaged in LB broth for three days (allowing them to grow statically at 37°C during each passage) in order to maximize type 1 pilus expression (46). After several passages in LB broth, cells were grown statically in separate tubes containing LB broth pH 5.5 and 7.0 with and without 400 mM NaCl. Since type 1 pili are known to agglutinate guinea pig erythrocytes (GPEs) (109), changes in type 1 pilus expression were measured by incubating UTI189 Δ*fimB* cells with 1% GPEs (Hardy Diagnostics, Santa Maria, CA) in a hemagglutination (HA) assay (27, 46). After growth in a specific condition, bacteria were pelleted and suspended in 100 µl phosphate buffered saline (PBS) before being serially, two-fold diluted in 50 µl PBS using a 96-well plate. The bacteria were then incubated with 50 µl of 1% GPEs overnight at 4°C. HA results were recorded as the reciprocal of the last bacterial dilution where HA had occurred. Wild-type *E. coli* UTI189, *E. coli* UTI189 Δ*fimB*, and *E. coli* UTI189 Δ*fimB* containing wild-type *fimB* ligated to pPPBMR1 (pBMR41) were used as controls in the HA assays.

**Quantitative reverse transcriptase PCR (qRT-PCR).** *E. coli* UTI189 Δ*fimB* cells containing mutant plasmids, *E. coli* UTI189, *E. coli* UTI189 Δ*fimB*, and *E. coli* UTI189 Δ*fimB* containing wild-type *fimB* ligated to pPPBMR1 (pBMR41) were grown in LB broth pH 5.5 and pH 7.0 with or without 400 mM NaCl. After static overnight growth at 37°C, total RNAs were extracted. Briefly, cells from a 20 ml culture were pelleted at 10,000 x *g* for 10 minutes, and then suspended in 1 ml PBS (appendix B). An equal volume of TRIzol reagent (Invitrogen) was added to each suspension and incubated at

37°C for 20 minutes. After incubation, an equal volume of chloroform was mixed with each suspension, then pelleted by centrifugation at 12,000 x *g* for 10 minutes at room temp. Each aqueous layer was then extracted once with UltraPure buffer-saturated phenol (Invitrogen) and twice with chloroform. The RNAs were precipitated by adding 1/10 volume of 3 M sodium acetate and two volumes of 100% ethanol and incubated overnight at -20°C. RNAs were pelleted by centrifugation at 12,000 x *g* for 15 minutes. Each RNA pellet was washed (12,000 x *g*) with 70% ethanol (appendix C) and allowed to dry completely before being suspended in 50 µl of 0.1% DEPC- treated water (appendix C). The quality and concentration of the RNA preparations were determined by taking $OD_{260/280}$ readings using a Bio Spec-1601 spectrophotometer (Shimadzu, Columbia, MD). Contaminating chromosomal DNA was removed by incubating RNA preparations with two units of RNase-free DNase (NEB) at 37°C for two hours. After incubation, the DNase was heat inactivated at 75°C for 10 minutes.

cDNAs were synthesized from the RNA preparations using a Superscript First-Strand Synthesis kit (Invitrogen). All cDNA synthesis reactions were performed as directed in the product manual. Random hexamers (100 ng), 1 µl dNTPs (10 mM), and 3 µg total RNA from each strain grown in a specific condition were mixed and diluted to a final volume of 10 µl using 0.1% DEPC- treated water. Each mixture was then incubated at 65°C for five minutes and immediately chilled on ice for two minutes. Next, 9 µl of a reaction mix (described in manual) was added to each reaction and incubated at room temperature for two minutes. After reverse transcriptase was added, tubes were

incubated at 25°C for 10 minutes. The reverse transcription reactions followed at 42°C for 50 minutes, and were terminated at 70°C for 15 minutes.

Once the cDNAs were synthesized, qPCR assays were used to quantify the number of *fimB* transcripts found in *E. coli* cells grown in the different conditions. A LightCycler FastStart DNA Master $^{PLUS}$ SYBR Green I master mix (Roche, Indianapolis, IN.) was used as described in the product manual. The FimB5 (40.3 pmol/reaction) and FimB6 (51 pmol/reaction) primers were used to detect *fimB* cDNAs (Table 3), whereas the primers EcFtsZ1 (50.4 pmol/reaction) and EcFtsZ2 (50.6 pmol/reaction) were used as a control to detect *ftsZ* cDNAs. The qPCR assays were performed with the LightCycler 1.5 platform (Roche) and the LightCycler 4.0 software using the following parameters: initial denaturation for 5 min at 95°C followed by 45 cycles of 95°C for 30 sec, 57°C for 30 sec, and 72°C for 30 sec. Variations in transcription were calculated using the formula noted by Livak *et al.* (70).

TABLE 3. Primers used in this study and their purpose.

| Primer Name | Sequence (5'-3') | Purpose |
|---|---|---|
| FimBP1TATF | GCATGGCGTTTGTATGGCAACGGGCGCCGAATTAACAGTTGCTACTCCATTTAAG | P1 TATA box mutagenesis |
| FimBP1TATR | CTTAAATGGAGTAGCAACTGTTAATTCGGCGCCCGTTGCCATACAAACGCCATGC | |
| L2.M4F | TAACAGTTGCGGGTCCATTTAAG | P1 TSS mutagenesis |
| L2.M4R | CTTAAATGGACCCGCAACTGTTA | |
| FimBP2TATF | GCTGAATTTTTTATGTTGATGCGCGCTGTTACAGAACATATCACATG | P2 TATA box mutagenesis |
| FimBP2TATR | CATGTGATATGTTCTGTAACAGCGCGCATCAACATAAAAAATTCAGC | |
| L2.M5F | CTTGTTACAGAACATAGACCATGATATATAG | P2 TSS mutagenesis |
| L2.M5R | CTATATATCATGGTCTATGTTCTGTAACAAG | |
| FimP2ACGT1F | GCTGAATTTTTTATGTTGATTTTGTTTGTTACAGAACATATCACATG | OmpR-1$^{st}$AC mutagenesis |
| FimP2ACGT1R | CATGTGATATGTTCTGTAACAAACAAAATCAACATAAAAAATTCAGC | |
| L1.M2F | ACTTGTTXXAGAACATATCACATGA[b] | OmpR-2$^{nd}$AC mutagenesis |
| L1.M2R | TCATGTGATATGTTCTXXAACAAGT | |
| L1.M3F | TGTTGATTTTACTTGTTACAGAXXATATCA | OmpR-3$^{rd}$AC mutagenesis |
| L1.M3R | TGATATXXTCTGTAACAAGTAAAATCAACA | |
| L1.M4F | GATTTTACTTGTTACAACACATATCACATG | OmpR higher affinity mutagenesis |
| L1.M4R | CATGTGATATGTGTTGTAACAAGTAAAATC | |
| FimBP3GADF | CGATCTGGAGAGGCTTGTAACCGACGGTGTTTCAGGATGATGTTTCAC | Mutagenesis of the proposed Gad Box |
| FimBP3GADR | GTGAAACATCATCCTGAAACACCGTCGGTTACAAGCCTCTCCAGATCG | |
| FimB5 | AACGCACCCGCTATTGAACA | qRT-PCR *fimB* detection |
| FimB6 | CTCTATCCCAGATGCCGTAAA | |
| EcFtsZ1 | TAGCGGTATCACCAAAGGACT | qRT-PCR *ftsz* detection |
| EcFtsZ2 | GTGATCAGAGAGTTCACATGC | |

[a] Underlined portions of primers indicate where mutations were introduced.
[b] Degenerate oligonucleotide. Nucleotides at postion x are randomly a G or T. Exact sequence change was determined by sequencing.

## RESULTS

**Phenotypic effects of the *fimB* promoter mutations on the expression of type 1 pili.** Primer extension experiments performed by Schwan *et al.* revealed three promoters for the *fimB* gene (115). Two of these promoters, P1 and P2, were confirmed by another group (23). To further characterize the *fimB* P1 and P2 promoters, mutations were created using site-directed mutagenesis. The mutated *fimB* promoters were transferred to the single copy number plasmid pPP2-6, and then the recombinant *fimB* plasmids were transformed into *E. coli* UTI189 Δ*fimB* cells. Wild-type *E. coli* UTI189 cells, *E. coli* UTI189 Δ*fimB*, and a representative from each *E. coli* UTI189 Δ*fimB* transformed with a *fimB* plasmid construct were grown in LB pH 5.5 and pH 7.0 with (+) or without 400 mM NaCl before the phenotypic effects the mutations had on type 1 pilus expression were measured by HA (FIG. 6).

Wild-type UTI189 cells grown at pH 5.5 and pH 7.0 showed the highest level of type 1 pili expression, resulting in HA titers of 2048 (Table 4). However, wild-type cells grown at pH 5.5+ and pH 7.0+ resulted in HA titers of 768 and 1024, respectively. As expected, HA titers of the UTI189 Δ*fimB* mutant were markedly lower (Table 4). UTI189 Δ*fimB* cells grown in LB pH 7.0 expressed an HA titer of 512 (four-fold less type 1 pilus expression than wild-type cells grown at the same condition). When UTI189 Δ*fimB* cells

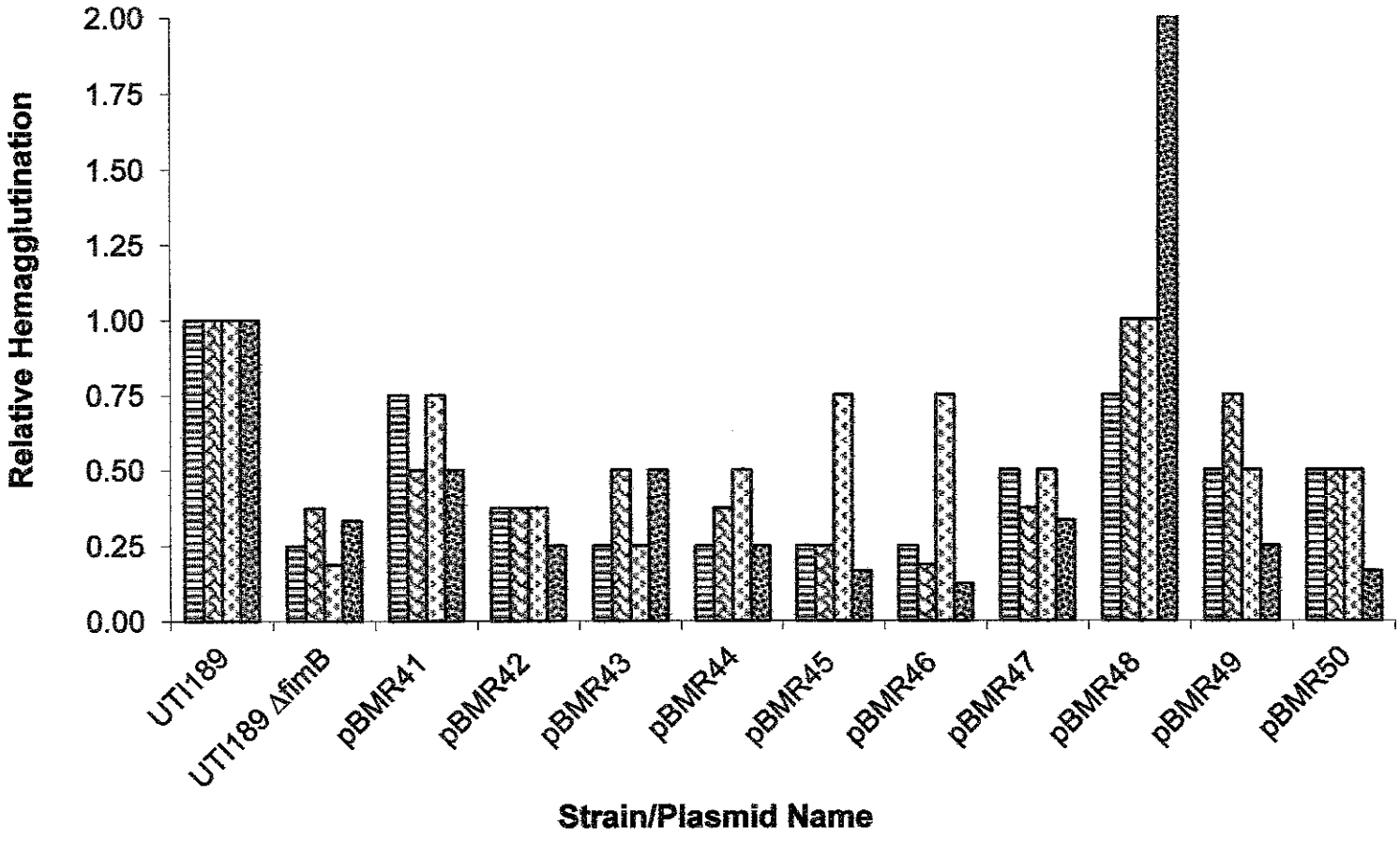


FIG. 6. Hemagglutination titers relative to wild-type *E. coli* UTI189. Data points indicate relative hemagglutination titers of cell populations grown in LB broth pH 7.0 (straight lines), 7.0+ (slanted lines), 5.5 (chevrons), and 5.5+ (dots), respectively.

TABLE 4. Hemagglutination titers of *E. coli* UTI189 Δ*fimB* cells after being transformed with plasmids containing mutations in the *fimB* P1 and P2 TATA box or TSS.

| Construct | Plasmid Description | pH | | | |
|---|---|---|---|---|---|
| | | 7.0 | 7.0+[a] | 5.5 | 5.5+ |
| *E. coli* UTI189 | | 2048[b] | 1024 | 2048 | 768 |
| *E. coli* UTI189 Δ*fimB* | | 512 | 384 | 384 | 256 |
| pBMR41 | Wild-Type | 1536 | 512 | 1536 | 384 |
| pBMR42 | P1TATA | 768 | 384 | 768 | 192 |
| pBMR43 | P1TSS | 512 | 512 | 512 | 384 |
| pBMR44 | P2TATA | 512 | 384 | 1024 | 192 |
| pBMR45 | P2TSS | 512 | 256 | 1536 | 128 |

[a] + indicates LB medium with 400 mM NaCl.
[b] HA titers were reported as an average of three separate runs.

were grown in LB pH 5.5 and pH 7.0+, an HA titer of 384 was observed. The lowest HA titer (256) was observed when UTI189 Δ*fimB* cells were grown in LB pH 5.5+. Complementing the Δ*fimB* mutation in *E. coli* UTI189 with a single, wild-type copy of *fimB* (pBMR41) restored the HA phenotype to near wild-type levels when cells were grown in LB pH 5.5 and 7.0, while growth in LB plus 400 mM NaCl seemed to reduce the ability of pBMR41 to complement the Δ*fimB* mutation.

After complementation of the Δ*fimB* mutation by pBMR41 was established, the *fimB* promoter mutant constructs were examined in strain UTI189 Δ*fimB*. Δ*fimB*/pBMR42 cells, containing a mutated *fimB* P1 TATA box, showed an HA titer of 768 when grown in LB without added salt and resulted in a slight (no more than two-

fold) increase in the expression of type 1 pili compared to the *ΔfimB* strain (Table 4). This same mutation failed to complement the Δ*fimB* mutation when cells were grown in the presence of 400 mM NaCl. A mutated *fimB* P1 TSS (pBMR43) was unable to complement the Δ*fimB* mutation under any of the environmental conditions tested (Table 4).

A mutation in the *fimB* P2 TATA box (pBMR44) failed to complement the Δ*fimB* mutation under all conditions tested except LB pH 5.5 (Table 4). When grown at pH 5.5, the *fimB* P2 TATA box mutation was able to slightly restore HA compared to Δ*fimB* cells, resulting in a three-fold increase in type 1 pilus expression. In addition, complementation of the Δ*fimB* mutation occurred with the *fimB* P2 TSS plasmid (pBMR45) when the cells were grown at pH 5.5 as evidence by a four-fold increase in HA titer (Table 4). In contrast, when these Δ*fimB*/pBMR45 cells were grown in LB pH 5.5+, the HA titer dropped two-fold compared to the Δ*fimB* mutant. Complementation of the Δ*fimB* mutation did not occur using the pBMR45 plasmid when Δ*fimB*/pBMR45 cells were grown at pH 7.0 with or without 400 mM NaCl.

Recently, DNase I footprinting analysis showed that OmpR interacts with the *fimB* P2 promoter (100). OmpR is the central transcriptional regulator of *ompC* and *ompF* in response to changes in osmolarity (82), and has been shown to affect type 1 pilus expression (117). Several studies have identified both the consensus sequences and the nucleotides important for OmpR binding within the *ompC* and *ompF* promoters (41, 44, 93, 98). Analyses of the OmpR consensus sequences revealed that two sets of AC nucleotides separated by one helical turn of DNA (10-bp) are crucial for OmpR binding

(FIG. 3). The OmpR binding site associated with *fimB* P2 is similar to the OmpR consensus sequence associated with the *ompF* and *ompC* promoters, but does not exactly match it (100).

In order to substantiate the effect OmpR has on repression of *fimB* transcription, the OmpR binding site associated with *fimB* P2 was mutated in an attempt to yield either lower affinity (pBMR46, pBMR47, pBMR48) or higher affinity (pBMR49) binding sites. The greatest effect of mutating the first AC site was observed after Δ*fimB*/pBMR46 cells were grown in pH 5.5 (Table 5). These cells expressed four-fold more type 1 pili than the Δ*fimB* mutant grown in the same condition. However, when the same strain was grown in pH 5.5+ and 7.0+, three-fold and two-fold decreases in type 1 pili expression were observed, respectively. No complementation was observed when Δ*fimB*/pBMR46 cells were grown in LB broth pH 7.0.

Next, the effect of the second AC mutation (pBMR47) on type 1 pilus expression was analyzed (Table 5). The Δ*fimB* mutation was not complemented by pBMR47 when Δ*fimB*/pBMR47 cells were grown in the presence of 400 mM NaCl. However, respective two-fold and three-fold increases in type 1 pilus expression were observed when Δ*fimB*/pBMR47 cells were grown in LB broth pH 5.5 and 7.0.

Complementation in every growth condition tested occurred when UTI189 Δ*fimB* cells were transformed with the plasmid containing the third AC mutation (pBMR48)(Table 5). In each condition tested, type 1 pilus expression was near wild-type levels. Interestingly, pBMR48 cells grown in LB pH 5.5+, expressed two-fold more type 1 pili than wild-type cells and five-fold more than Δ*fimB* cells with no plasmid.

TABLE 5. Hemagglutination titers of *E. coli* UTI189 Δ*fimB* cells after being transformed with plasmids containing mutations in the OmpR binding region that is associated with *fimB* promoter two.

| Construct | Plasmid Description | pH | | | |
|---|---|---|---|---|---|
| | | 7.0 | 7.0+[a] | 5.5 | 5.5+ |
| *E. coli* UTI189 | | 2048[b] | 1024 | 2048 | 768 |
| *E. coli* UTI189 Δ*fimB* | | 512 | 384 | 384 | 256 |
| pBMR41 | Wild-Type | 1536 | 512 | 1536 | 384 |
| pBMR46 | P2ACGT1 | 512 | 192 | 1536 | 96 |
| pBMR47 | P2ACAA2 | 1024 | 384 | 1024 | 256 |
| pBMR48 | P2ACGT3 | 1536 | 1024 | 2048 | 1536 |
| pBMR49 | P2GAAC | 1024 | 768 | 1024 | 192 |

[a] + indicates LB medium with 400 mM NaCl.
[b] HA titers were reported as an average of three separate runs.

The previously described changes to the OmpR binding sites in *fimB* P2 were to disrupt OmpR binding to the OmpR binding site tied to P2. Conversely, the fourth OmpR binding mutant, pBMR49, was created to more closely mimic the OmpR consensus sequence associated with *ompC* and *ompF* and possibly increase the affinity of OmpR for *fimB* P2 (FIG. 3). Δ*fimB*/pBMR49 cells expressed two-fold more type 1 pili when grown in LB pH 7.0 with and without 400 mM NaCl, and three-fold more when grown in LB pH 5.5 than Δ*fimB* cells grown under the same, respective conditions (Table 5). There was no complementation by pBMR49 when Δ*fimB*/pBMR49 cells were grown in LB broth pH 5.5+.

Since both high osmolarity (OmpR) and pH affect type 1 pilus expression, the regulatory effects of GadE were also examined. GadE is the central transcriptional activator of genes involved in glutamate-dependent acid resistance in *E. coli*, but also controls the expression of numerous other genes (47, 71, 75). In UPEC strain NU149, HA titering showed that a *gadE* deletion resulted in a decrease in type 1 pilus expression when cells were grown at pH 5.5 (Schwan unpublished results).

A DNA sequence located approximately 375-bp upstream from the *fimB* P2 TSS (FIGS. 3 and 4) was identified in this study that shows 65% similarity with the GAD box associated with *gadA/BC* (15). Mutations introduced in the proposed *fimB*-associated GAD box resulted in a sequence with only 35% similarity to the known GAD box and is represented in the pBMR50 plasmid. The pBMR50 plasmid was able to partially restore HA in Δ*fimB* cells under all growth conditions tested except pH 5.5+ (Table 6). At pH 5.5+ Δ*fimB*/pBMR50 cells showed a two-fold decrease in HA compared to Δ*fimB* cells with no plasmid. While pBMR50 was able to increase the HA titers of Δ*fimB* cells under most conditions, HA titers were still lower than HA titers observed for Δ*fimB*/pBMR41 cells grown in LB pH 5.5 and pH 7.0. The HA titers were the same for Δ*fimB*/pBMR50 and Δ*fimB*/pBMR41 cells grown in LB pH 7.0+.

**Effects of the *fimB* promoter mutations on *fimB* transcription.** The HA titers demonstrated how *fimB* promoter mutations phenotypically altered type 1 pilus expression, but did not show the mechanism of *fimB* regulation. To measure the relative level of *fimB* transcription associated with the *fimB* promoter mutations, qRT-PCR was used. *Escherichia coli* UTI189, *E. coli* UTI189 Δ*fimB*, and *E. coli* UTI189 Δ*fimB* cells

TABLE 6. Hemagglutination titers of *E. coli* UTI189 Δ*fimB* cells after being transformed with a plasmid containing a mutation in the proposed GAD box that is associated with *fimB*.

| Construct | Plasmid Description | pH | | | |
|---|---|---|---|---|---|
| | | 7.0 | 7.0+[a] | 5.5 | 5.5+ |
| *E. coli* UTI189 | | 2048[b] | 1024 | 2048 | 768 |
| *E. coli* UTI189 Δ*fimB* | | 512 | 384 | 384 | 256 |
| pBMR41 | Wild-Type | 1536 | 512 | 1536 | 384 |
| pBMR50 | *fimB* GAD box | 1024 | 512 | 1024 | 128 |

[a] + indicates LB medium with 400 mM NaCl.

[b] HA titers were reported as an average of three separate runs.

were transformed with pBMR41, pBMR42, pBMR43, pBMR47, and pBMR48 plasmids (Table 1) were chosen for qRT-PCR analysis based on their significance to the experiment and HA titers (Tables 4-6). *Escherichia coli* UTI189 Δ*fimB* cells, containing these mutant plasmids, were grown in LB broth pH 5.5 and 7.0 with and without 400 mM NaCl prior to RNA extraction. No contaminating DNA was detected in the RNA extracts using traditional PCR (data not shown).

To establish the background level of *fimB* transcription in *E. coli* UTI189 Δ*fimB*, cells were grown in the different environmental conditions and analyzed by qRT-PCR. When compared to wild-type populations, Δ*fimB* cells contained between $10^3$ and $10^6$ fewer *fimB* transcripts depending on growth condition. Cells containing pBMR41 demonstrated higher levels of *fimB* than wild-type UTI189 cells in almost all

environmental conditions: a 5.69-fold increase at pH 7.0, a 19.8-fold increase at pH 7.0+, and a 8800-fold increase at pH 5.5. However, wild-type UTI189 cells grown at pH 5.5+ produced nearly three times more *fimB* transcripts than Δ*fimB*/pBMR41 cells.

All of the *fimB* mutant plasmids were able to complement the Δ*fimB* mutation, but *fimB* transcript levels fell conspicuously short of those found in Δ*fimB*/pBMR41 cells (Table 7). Of the mutant plasmids examined, mutations in either the P1 TATA box or the TSS seemed to complement the Δ*fimB* mutation best by exceeding the *fimB* transcript level found in Δ*fimB*/pBMR41 cells grown at pH 7.0 (6.45-fold increase and 3.05-fold increase, respectively). Also at pH 7, Δ*fimB*/pBMR47 cells had nearly 100-fold fewer *fimB* transcripts and Δ*fimB*/pBMR48 cells had about 10-fold fewer *fimB* transcripts than Δ*fimB*/pBMR41 cells.

The Δ*fimB* cells with either pBMR42 or pBMR48 produced nearly the same level of *fimB* transcripts as Δ*fimB*/pBMR41 cells when grown in LB broth pH 5.5+. In this growth condition, the pBMR43 and pBMR48 plasmids showed the best complementation of the Δ*fimB* mutation compared to any other condition tested (Table 7). In LB pH 5.5, cells containing pBMR42, pBMR43, pBMR47, or pBMR48 had at least a 1000-fold decrease in the level of *fimB* transcripts when compared to cells containing pBMR41 grown in the same condition. The Δ*fimB* cells containing pBMR42, pBMR43, pBMR47, or pBMR48 grown in LB broth pH 7.0+ had at least a 100-fold decrease in the level of *fimB* transcripts when compared to Δ*fimB*/pBMR41 cells grown in the same condition.

TABLE 7. Quantitative reverse transcriptase PCR results. *E. coli* UTI189 Δ*fimB* cells, containing mutant *fimB* P1 and P2 plasmids, were grown in different environmental conditions before *fimB* transcription was determined.

| pH[a] | Sample Name | Fold Difference[b] |
|---|---|---|
| 7.0 | pBMR42 | 6.45 |
| | pBMR43 | 3.05 |
| | pBMR47 | 0.085 |
| | pBMR48 | 0.210 |
| 7.0+ | pBMR42 | 0.027 |
| | pBMR43 | 0.043 |
| | pBMR47 | 0.010 |
| | pBMR48 | 0.018 |
| 5.5 | pBMR42 | 0.005 |
| | pBMR43 | 0.007 |
| | pBMR47 | 0.002 |
| | pBMR48 | 0.006 |
| 5.5+ | pBMR42 | 1.16 |
| | pBMR43 | 0.129 |
| | pBMR47 | 0.267 |
| | pBMR48 | 0.980 |

[a] + indicates addition of 400 mM NaCl.

[b] The number of *fimB* transcripts detected in a particular cell population relative to (divided by) the number of *fimB* transcripts detected in *E. coli* UTI189 Δ*fimB* cell populations transformed with pBMR41.

## DISCUSSION

Uropathogenic E. coli use type 1 pili to attach to uroepithelial cells within the host bladder. UPEC can alter expression of type 1 pili by switching the fimS invertible element to be either phase ON (piliated) or phase OFF (nonpiliated). The orientation of *fimS* is responsive to environmental cues, such as pH and osmolarity, through a variety of proteins (113). These proteins affect phase variation directly by interacting with *fimS*, or indirectly by influencing the transcription of two genes encoding the site-specific recombinases, FimB and FimE, that affect *fimS* orientation (33). While FimE orients *fimS* in the phase OFF position, FimB can position the *fimS* element in both positions (59, 76). Several proteins interact with the *fimB* promoters that can influence transcription of *fimB* and subsequent type 1 pilus expression. Three promoters have been mapped for *fimB* (115), but not much is known about their respective functions or hierarchy. The primary objective of this study was to establish a promoter hierarchy in *fimB* while substantiating the influence two regulatory proteins, OmpR and GadE, have on *fimB* transcription and type 1 pilus expression.

To establish a hierarchy for *fimB* regulation, the entire *fimB* open reading frame and 478-bp upstream (coding strand positions 4905222-4906354) were deleted from the UPEC strain UTI189 before being transformed with a library of plasmids which contained either a wild-type *fimB* gene or the *fimB* gene with a promoter mutation (Table 1) (39). During HA analysis, background HA was observed in UTI189 Δ*fimB* that could have been a result of non specific HA. It is also possible that type 1 pilus expression was

initiated independent of FimB. The site-specific recombinase, HbiF, called FimX in *E. coli* UTI189 (GenBank accession number ABE05839), can initiate OFF to ON inversion of *fimS* (39, 137). Other recombinases (ex. IpuA, IpuB, IpbA) can also alter *fimS* orientation, and have been implicated as regulators of type 1 pilus expression that contribute to UPEC pathogenesis (14). These recombinases could have contributed to background HA in the absence of *fimB*.

The pBMR41 control plasmid, containing a wild-type copy of *fimB* and its promoters, complemented the Δ*fimB* mutation in all environmental conditions tested when measured by HA, thus corroborating previous work that demonstrated FimB supports expression of type 1 pili (Table 4) (59, 76). Moreover, qRT-PCR analysis demonstrated *fimB* transcription in Δ*fimB*/pBMR41 cells surpassed *fimB* transcription levels found in wild-type UTI189 cells grown in pH 7.0, pH 7.0+ and pH 5.5 media, but not in a pH 5.5+ environment. In LB broth pH 5.5+, the number of *fimB* transcripts in Δ*fimB*/pBMR41 cells was lower than in wild-type UTI189 cells grown in the same condition, but pBMR41 was still able to complement the Δ*fimB* mutation. For these reasons, transcriptional differences found during qRT-PCR analysis were reported relative to Δ*fimB*/pBMR41 cells (Table 7).

The -10 sequence (TATAAT) within the *fimB* P1 exactly resembles the consensus sequence recognized by the $\sigma^{70}$ RNA polymerase cofactor (40) and could suggest transcription of *fimB* under normal, unstressed growth conditions originates from P1

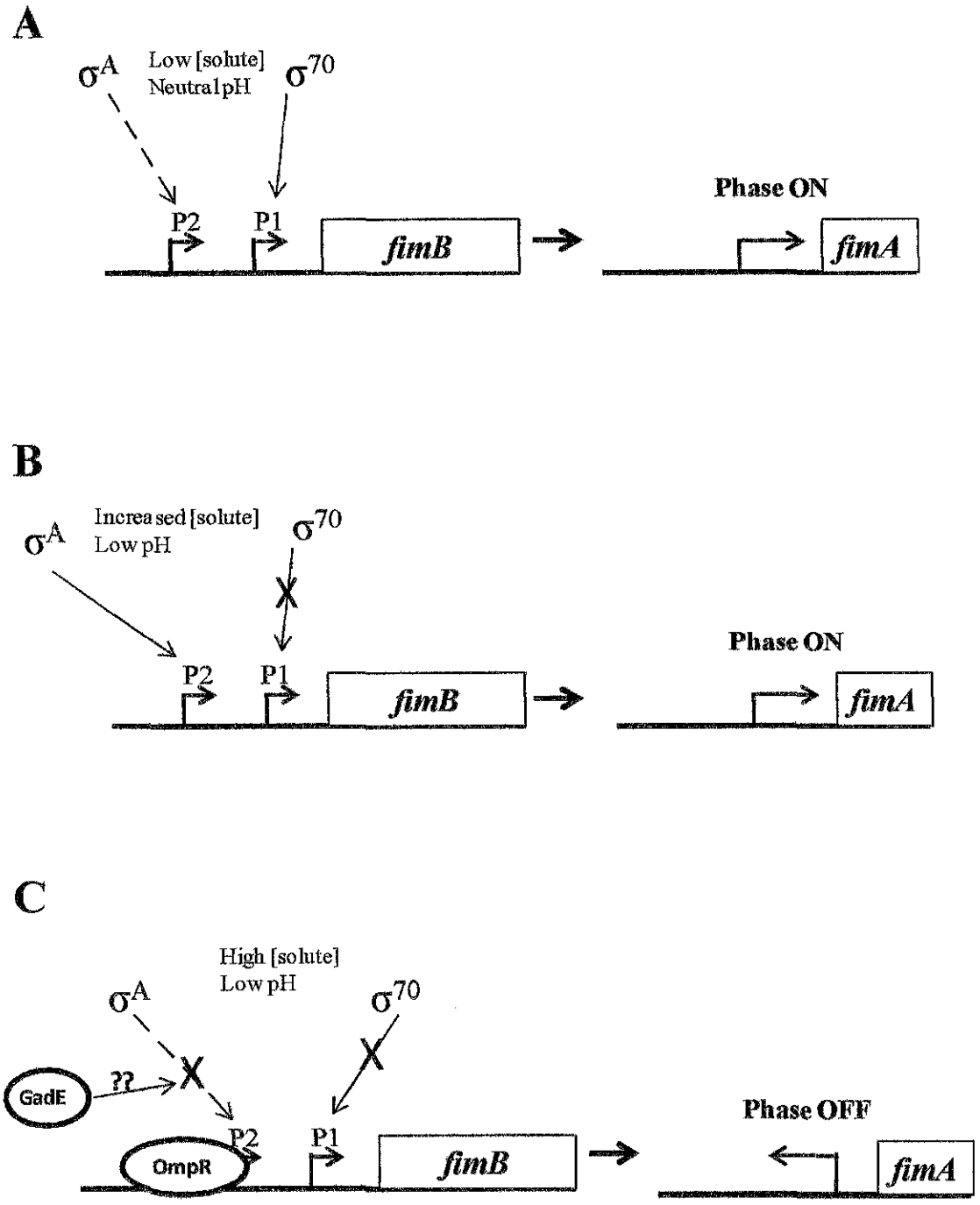


FIG. 7. Model of *fimB* transcription regulation in a non stressed (**A**), slightly stressed (**B**), and stressed environment (**C**). $\sigma^A$ represents an alternative RNA polymerase sigma factor.

(FIGs 5 and 7), while P2 may serve as a stress-regulated promoter. Promoter two could serve as a binding site for osmotic or acidic-specific RNA polymerase sigma factors or regulatory proteins like OmpR and GadE. Recent work has identified an OmpR binding site associated with *fimB* P2 (100), while the current study located a GAD box-like sequence upstream of *fimB* P2 (FIGs 4 and 5). Recruiting different sigma factors that initiate transcription of genes during growth in a stressful environment is well described during *Pseudomonas aeruginosa* and *E. coli* responses to heat-shock (7, 29, 109). During heat-shock, the alternative sigma factor RpoH is transcribed at its promoter three by another alternative sigma factor, RpoE. Using this system as a model, it is reasonable to suggest that the different *fimB* promoters are used based on the environmental conditions in which an *E. coli* cell is growing.

In support of the hypothesis that P1 is the non-stress condition promoter, HA titering showed that a P1 TATA box mutant (pBMR42) slightly complemented the Δ*fimB* mutation during growth at pH 7.0, but was unable to complement the Δ*fimB* mutation during growth at pH 5.5+ and pH 7.0+ (Table 4). However, Δ*fimB*/pBMR42 cells grown in LB broth pH 5.5 had a two-fold increase in HA titer compared to Δ*fimB* cells without a plasmid. Similarly, the P1 TSS mutant construct (pBMR43) failed to complement the Δ*fimB* mutation in any of the growth conditions using HA titering.

Further analysis using qRT-PCR showed the level of *fimB* transcripts in Δ*fimB*/pBMR42 and Δ*fimB*/pBMR43 cells were nearly 6.5 times and 3 times greater, respectively, than those found in Δ*fimB*/pBMR41 cells grown at pH 7.0 (Table 7). Unfortunately, qRT-PCR was performed only once, but needs to be repeated to confirm these results and establish

statistical significance. If this data is real, it would suggest that mutating the P1 TATA box or the TSS may increase expression of *fimB* at pH 7.0. While a myriad of factors can compensate for slight differences in the -10 sequence of a promoter (13), the entire hexamer of the TATA box was altered in pBMR42 (Table 3), and so the qRT-PCR results were mostly likely inaccurate.

A decrease in HA titer and *fimB* transcription was observed for both Δ*fimB*/pBMR42 and Δ*fimB*/pBMR43 cells grown in LB broth pH 5.5 and pH 7.0 when compared to Δ*fimB*/pBMR41 cells, but the decrease in transcription was not proportional to the decrease in HA titer. At most a three-fold decrease in HA titer was observed, but a 30- to 200-fold decrease in *fimB* transcription was observed, depending on growth condition (Tables 4-6 and 7). However, the level of *fimB* transcription was proportional to the HA titer for the Δ*fimB*/pBMR42 and Δ*fimB*/pBMR41 cells grown at pH 5.5+.

A HA titer measures the relative amount of type 1 pili present on the surface of *E. coli* cells. Thus, mutations that affect the *fimB* promoters should in turn cause changes in type 1 pilus expression that can be measured by HA. However, in cases where *fimB* transcription was disproportionately lower than the HA titer, it is possible that type 1 pilus expression was initiated by alternative recombinases previously discussed. It is also possible that post-transcriptional regulation of the *fim* operon contributed to the disparity between the HA titers and the level of *fimB* transcription.

We hypothesize that *fimB* P1 is primarily used for transcription of *fimB* in non-stressed environments, while *fimB* P2 is primarily used for *fimB* transcription in hyperosmotic and acidic environments (FIG. 7). Based on this model, we expected that

the P2 TATA Box (pBMR44) and TSS (pBMR45) mutants would not be able to complement the Δ*fimB* mutation during growth in an acidic or hyperosmotic environment. If P2 is a pH-regulated promoter, it is perplexing why both pBMR44 and pBMR45 were able to complement the Δ*fimB* mutation when cells were grown at pH 5.5 (Table 4). Since a wild-type copy of P1 is present in all P2 mutant constructs, a wild-type (pBMR41) HA titer was expected for any cell population that contained a P2 mutant construct when grown at pH 7.0. However, pBMR44 and pBMR45 failed to complement the Δ*fimB* mutation at pH 7.0. The HA data may be indicating that both P1 and P2 are needed for wild-type level expression of type 1 pili at certain growth conditions and may confound the establishment of a promoter hierarchy.

To better assess the condition-specific use of promoters in *fimB*, we examined the binding capabilities of OmpR to P2. OmpR interacts with several regions upstream of the *ompC* and *ompF* promoters with different affinities, which has made discovery of a definitive consensus sequence elusive. However, a proposed consensus sequence shows an AC conformation at positions 1/2 and 11/12 along with a central GXXXC motif (FIG. 3) (41). Several characteristics associated with the proposed OmpR consensus sequence are also found in the OmpR binding sequence found in *fimB* P2 identified by Rentschler (100).

In an effort to reduce the affinity of OmpR for *fimB* P2, three different promoter mutations were introduced (FIG. 3). Since OmpR has been shown to repress *fimB* transcription (110, 117) in hyperosmotic conditions, we expected the OmpR lower affinity mutations would allow greater transcription of *fimB* and thus greater type 1 pilus

expression. Contrary to what was expected, mutating the first AC site (pBMR46) caused a decrease in HA titer, relative to Δ*fimB*/pBMR41 cells, in cells grown in all conditions except pH 5.5. If the first AC site in the proposed consensus sequence is the most critical for OmpR binding to *ompC* and *ompF* promoters as previously suggested (98), then a mutation in this region should have led to decreased OmpR binding and a subsequent higher HA titer in cells grown in a hyperosmotic environment.

The second AC site is located within a GXXXC motif that was thought to be important for OmpR interaction with *fimB* P2. However, this GXXXC sequence was 'out of frame' in comparison to the proposed consensus sequences in the *ompC* and *ompF* promoters (FIG. 3). Compared to Δ*fimB*/pBMR41 cells, mutating the second AC site (pBMR47) resulted in a decrease in HA titer (Table 5) and a notable decrease in *fimB* transcription (Table 7) in populations grown in all conditions tested. In LB broth pH 5.5+ and pH 7.0+, pBMR47 failed to complement the Δ*fimB* mutation using HA analysis. Of the three AC sites of interest, the second AC site showed the least amount of similarity with the proposed consensus sequence of OmpR and may only slightly contribute to the affinity of OmpR to *fimB* P2. Rather than decreasing the affinity of OmpR for *fimB* P2, the mutation in pBMR47 could have increased the affinity of OmpR for P2 since this plasmid failed to complement the Δ*fimB* mutation when NaCl was added.

A third AC mutation (pBMR48) resulted in the most notable change in the HA titer when compared to Δ*fimB*/pBMR41 cells. Complementation with this mutated plasmid restored the HA titer of Δ*fimB* cells to true wild-type (UTI189) levels at pH 7.0, pH 7.0+, and pH 5.5. In addition, the HA titer increased two-fold in Δ*fimB*/pBMR48 cells

at pH 5.5+. However, qRT-PCR analysis showed the level of *fimB* transcripts were much lower in Δ*fimB*/pBMR48 cells than in Δ*fimB*/pBMR41 cells in almost all conditions (Table 7). The PCR data was most likely a result of experimental error since it does not fit with the HA titer observations in this study or a previous study that showed an increased expression of *fimB* and type 1 pili in an *ompR* mutant (117).

The previously discussed mutations in the *fimB* P2 OmpR binding site were attempts to create lower affinity binding sites. A fourth OmpR binding mutant was made by changing a GA site in *fimB* P2 to an AC site that corresponds to the AC nucleotides found at position 11/12 in the proposed OmpR consensus sequence (FIG. 3). If this mutation did result in a higher affinity site for OmpR, then a decrease in HA titer, due to repression of *fimB* transcription, would be expected relative to Δ*fimB*/pBMR41 cells when Δ*fimB*/pBMR49 cells were grown in hyperosmotic conditions. Indeed, a two-fold decrease in HA titer was observed when Δ*fimB*/pBMR49 cells were grown in pH 5.5+, but an increase in HA titer was observed at pH 7.0+ (Table 5).

Hyperosmolarity and acidity seem to synergistically repress type 1 pili expression since the lowest HA titers were observed at pH 5.5+ except in Δ*fimB*/pBMR48 cells. The synergistic repression of type 1 pili expression observed in this as well as a prior study could be due in part to the dual repression of *fimB* transcription by OmpR and the positive transcriptional regulator of the GAD acid resistance system, GadE (117). If GadE is a negative regulator of *fimB*, then a mutation in the GAD box-like sequence upstream of *fimB* P2 (pBMR50), should have resulted in an HA titer greater than Δ*fimB*/pBMR41 cells grown in LB broth pH 5.5 and pH 5.5+. While plasmid pBMR50

was able to restore HA titers of Δ*fimB* cells to near Δ*fimB*/pBMR41 levels in most growth conditions, up to a three-fold decrease in HA was observed at pH 5.5 and pH 5.5+, respectively, when compared to Δ*fimB*/pBMR41 cells. qRT-PCR analysis was not performed on cells containing the pBMR50 plasmid. Since HA titers do not provide strong evidence that this site is indeed a GAD box, analysis of *fimB* transcription levels could be more informative in determining the significance of this site.

An operator site for NanR, a sialic acid-response regulator, and NagC, a GlcNAc-6P-responsive protein, has been identified approximately 600-bp upstream of the *fimB* translational start site (126). In the current study, only the region of DNA between *fimB* P3 and P1 was analyzed for the presence of a GAD box, and so a true GAD box could have been missed further upstream. Some evidence indicates that GadE can only bind to the GAD box associated with *gadA* as a heterodimer with RcsB, a protein belonging to the RcsCDB signal transduction system of the *Enterobacteriaceae* family (18). Furthermore, RcsB alone has been shown to influence *fimB* transcription, *fimS* orientation, and type 1 pilus expression (116). The interaction of GadE with RcsB has not been explored in regard to *fimB*, but may play a role in *fimB* regulation.

If a GAD box is not present in the *fimB* promoter region, it may be because GadE does not directly interact with the promoters of *fimB*, and could explain why mutating the GAD box-like sequence near *fimB* P2 did not cause the HA titer of Δ*fimB*/pBMR50 cells to be greater than the HA titer of Δ*fimB*/pBMR41 cells, as was expected. In its native system, GadE directly initiates transcription of *gadABC* and several other genes related to acid resistance, but could indirectly regulate *fimB* transcription using an intermediate

protein that directly interacts with the *fimB* promoters (75). The increase in *fimB* transcription in a *gadE* mutant previously observed (W.R. Schwan, unpublished results) could be a result of indirect regulation by GadE. There is also the possibility that GadE not only represses *fimB*, but also *fimE*. Since FimE orients *fimS* from ON to OFF, GadE repression of *fimE* could account for the decrease in type 1 pilus expression in a *gadE* mutant previously observed in a pH 5.5 medium (W.R. Schwan, unpublished results).

In this study, a *fimB* promoter hierarchy was not clearly established since HA data seemed to suggest that both wild-type P1 and wild-type P2 are needed for wild-type levels of type 1 pilus expression in all growth conditions tested. However, we were able to identify a set of nucleotides that could potentially be important for interaction of OmpR with *fimB* P2 supporting the idea that P2 is the promoter used to transcribe *fimB* in stressful environments, and should be investigated further. We also noted that, overall, HA increased markedly at pH 5.5 in Δ*fimB* cells containing any P2 mutant plasmid. Though the implication of this is unclear, this observation may suggest that *fimB* transcription is induced at pH 5.5 from P2 without the synergistic repression effect observed in cells grown at pH 5.5+.

Type 1 pilus expression is most likely regulated by a complicated network of proteins and environmental signals that extend beyond the scope of this study. From an evolutionary perspective, it makes sense that many *E. coli* proteins have dual roles as transcription initiators or repressors depending on the environment. Proteins like GadE and OmpR are required for or greatly contribute to growth in low pH and high osmolality, but could also act to remove the cell from stressed conditions since growth in

such conditions is taxing. Though type 1 pili may irreversibly attach a cell to a potentially hazardous environment, they are also necessary for UPEC to attach to the human bladder where they can grow very well. UPEC must constantly balance environmental risk signals with the advantage of living in the human bladder. Previous work has shown that the host cell products can both repress and stimulate type 1 pilus expression, and that UPEC may receive a positive feedback signal that originates from type 1 pilus interaction with mannose receptors on the surface of the host bladder that maintain type 1 pilus production (124, W.R. Schwan personal communication).

As bacteria become more resistant to conventional antibiotics, it is increasingly important to find new targets that can be used to treat bacterial infections. Type 1 pili have a prominent role in UPEC virulence and can serve as potential targets for future treatments. One study has identified novel compounds called mannosides that bind FimH up to 200,000 times stronger than the mannose containing receptors FimH normally binds (38). Further examination of these mannosides led to an orally administered, mannose-derived compound that rapidly reduced the number of UPEC cells in mice experiencing a UTI and prevented new infections (21). Mannosides are also effective at removing and preventing UPEC biofilms *in vitro* as well as increasing the efficacy of trimethoprim/sulfamethoxazole treatments (37). Furthermore, type 1 pili are immunogenic and are targeted by host phagocytic cells (8, 84, 119, 121). This observation led to vaccination experiments using the FimH fimbrial tip adhesin have shown positive results (6, 64, 65, 84). Understanding type 1 pilus expression and

regulation may reveal other drug targets as well as contribute to our understanding of UPEC pathogenesis.

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# APPENDIX A

# REAGENTS FOR MEDIA

## Appendix A. Reagents for Media

**1. LB broth pH 7.0**

28.8 ml $Na_2HPO_4$
21.2 ml $NaH_2PO_4$
5 ml glycerol
444 ml Milli-Q H2O
10 g LB broth base
-pH to 7.0
-add 29.22 g NaCl to make 1M NaCl LB
-Autoclave

**2. LB broth pH 5.5**

2.1 ml $Na_2HPO_4$
48.9 ml $NaH_2PO_4$
5 ml glycerol
444 ml Milli-Q $H_2O$
10 g LB broth base
-pH to 5.5
-add 29.22 g NaCl to make 1 M NaCl LB
-Autoclave

**3. SOC (recovery medium)**

20 g Tryptone
5 g Yeast Extract
2 ml 5M NaCl
2.5 ml 1M KCl
10 ml 1M $MgCl_2$
10 ml 1M $MgSO_4$
20 ml 1M glucose
956 ml Milli-Q $H_2O$
-Autoclave

# APPENDIX B

# REAGENTS FOR HEMAGGLUTINATION ASSAYS

## Appendix B. Reagents for Hemagglutination Assays

### 1. Phosphate Buffered Saline

0.23g $NaH_2PO_4$
1.15g $Na_2HPO_4$
9.00g NaCl
1 L Milli-Q $H_2O$
-pH to 7.2
-Autoclave

# APPENDIX C

# REAGENTS FOR RNA EXTRACTION

## Appendix C. Reagents for RNA extraction

**1. 0.1% DEPC-Treated Water**

300 ml Milli-Q $H_2O$
300 µl diethylpyrocarbonate
-Incubate shaking overnight at 37°C
-Autoclave

**2. 70% Ethanol**

70 ml 100% ethanol
30 ml 0.1 % DEPC-Treated Water