

UNIVERSITY OF WISCONSIN-LA CROSSE

Graduate Studies

IN VITRO ANALYSIS OF OMPR REGULATION OF THE *FIMB* AND *FIME* GENES
OF UROPATHOGENIC *ESCHERICHIA COLI*

A Manuscript Style Thesis Submitted in Partial Fulfillment of the Requirements for the
Degree of Master of Science in Biology

Ann E. Rentschler

College of Science and Health
Clinical Microbiology Concentration

December, 2010

IN VITRO ANALYSIS OF OMPR REGULATION OF THE *FIMB* AND *FIME* GEN
OF UROPATHOGENIC *ESCHERICHIA COLI*

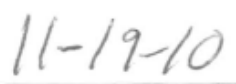
By Ann E. Rentschler

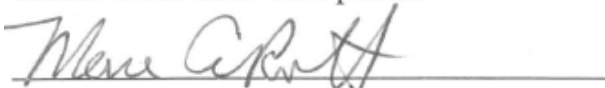
We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the degree Master of Science in Biology, Clinical Microbiology Concentration.

The candidate has completed the oral defense of the thesis.



William Schwan, Ph.D.
Thesis Committee Chairperson


Date

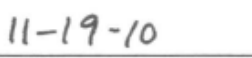


Marc Rott, Ph.D.
Thesis Committee Member


Date



Dean Jobe, MS, RM(AAM)
Thesis Committee Member


Date



Mary E. Stemper, MS, MT(ASCP)
Thesis Committee Member


Date

Thesis accepted



Vijendra K. Agarwal, Ph.D.
Associate Vice Chancellor for Academic Affairs


Date

ABSTRACT

Rentschler, A.E. *In vitro* analysis of OmpR regulation of the *fimB* and *fimE* genes of Uropathogenic *Escherichia coli*. MS in Biology, Clinical Microbiology Concentration. December 2010, 79pp. (W.R. Schwan)

Uropathogenic *Escherichia coli* (UPEC) cause more than 90% of all human urinary tract infections (UTIs). Type 1 pili on the surface of UPEC allow the cells to adhere to and invade bladder epithelial cells. The *fimB* and *fimE* genes encode site-specific recombinases that position an invertible element containing the promoter for the gene of the main structural subunit of the type 1 pili. To sustain a UTI, UPEC must be able to respond to changes in environmental pH and osmolality. The EnvZ/OmpR two-component regulatory system is involved in osmoregulation in *E. coli*. To ascertain if OmpR directly bound to *fimB* or *fimE* gene promoters, DNase I footprinting was performed using OmpR or phosphorylated OmpR (OmpR-P) mixed with the *fimB* and *fimE* promoter regions of UPEC NU149. Both OmpR-P and OmpR bound weakly to one *fimB* promoter. However, neither OmpR nor OmpR-P bound to the *fimE* promoter region. Because there was weak binding to one *fimB* promoter, strain NU149 was grown in different pH and osmolality environments, total RNAs were extracted from each population, and converted to cDNAs. Real-time reverse transcriptase PCR showed no differences in *ompR* transcription among the different growth conditions. Conversely, Western blots showed a significant increase in OmpR protein when the UPEC were grown in a combined acidic pH/high osmolality environment versus a neutral pH/high osmolality environment. Together these results suggest that OmpR may be post-transcriptionally regulated in UPEC grown in an acidic pH/high osmolality environment which may affect *fimB* regulation in UPEC.

ACKNOWLEDGEMENTS

First, I would like to thank my major advisor, Dr. William Schwan, for your guidance throughout this entire project. Thank you for all of the *E. coli* discussions and always encouraging me to think about ‘the big picture’. Thank you to Dr. Marc Rott for your support and encouragement to stay on track with this program. I want to thank Dean Jobe for your willingness to be a sounding board. I am indebted to Dr. Steven Lovrich who, through great kindness, was willing to help me troubleshoot the real-time PCR and Western blotting processes.

To Mary Stemper, words cannot express how much of an impact you have made on my whole graduate career. You truly care about your students and your self-less dedication to help me succeed has been invaluable. Thank you for not only being a wonderful mentor, but a friend as well.

My career as a scientist began working with Dr. Daniel Herman at the University of Wisconsin-Eau Claire. Dr. Herman, I appreciate all of your ‘distance encouragement’ to me to continue along with this program even when times got tough. I respect you tremendously and hope that one day your children will have someone in their respective fields that care about them as much as you have for me.

Thank you to Dr. Linda Kenney at the University of Illinois at Chicago for allowing me the opportunity to be a part of your lab for three weeks and for the generous gift of the primary antibody. To Don Walthers, thank you for your time and expertise in teaching me the art of DNase footprinting. Without you, that part of my thesis would have been nowhere near what it is now. Leslie Morgan and the rest of the Kenney Lab, thank you for making me feel at home in your lab during my short time I spent there.

Last but certainly not least, a tremendous thank you to my mom, Sue, and brother, Thomas, who have supported me through every step of my time at UWL. Mom, even though I do not tell you I appreciate you, I am grateful to all of the sacrifices you have made for me and the mental support you have given to me. Thank you to Casey, your love and support has helped me achieve this Masters degree.

This work was made possible by a UWL Graduate Research, Service, and Educational Leadership grant and an NIH/AREA grant.

DEDICATION

“An education isn't how much you have committed to memory, or even how much you know. It's being able to differentiate between what you do know and what you don't.”

~Anonymous

This thesis and all of the work associated with it is dedicated to my father, Monte, and to my grandfather, Russell J. Hensing, both of whom greatly valued education, but neither had the opportunity to know what I have become.

TABLE OF CONTENTS

	<u>PAGE</u>
ABSTRACT.....	iii
ACKNOWLEDGEMENTS.....	iv
DEDICATION.....	v
TABLE OF CONTENTS.....	vi
LIST OF TABLES.....	viii
LIST OF FIGURES.....	ix
LIST OF APPENDICES.....	xi
INTRODUCTION.....	1
Characteristics and Clinical Significance of <i>Escherichia coli</i>	1
Clinical Significance of Uropathogenic <i>Escherichia coli</i>	1
Diagnosis and Treatment of Urinary Tract Infections.....	3
Physiology of the Human Urinary Tract.....	4
Type 1 Pili.....	5
Genetic Organization of <i>fim</i> Genes in UPEC.....	6
Cofactors and Environmental Conditions that Affect Piliation and Phase Variation.....	10
Response to Increasing Environmental Osmolality by <i>E. coli</i>	12
Mechanisms of Acid Tolerance in <i>E. coli</i>	16

	<u>PAGE</u>
MATERIALS AND METHODS.....	19
Bacterial Strains, Plasmids, and Growth Conditions.....	19
DNase I Footprinting.....	19
Extraction of Total RNAs and Conversion to cDNAs.....	24
Amplification and Detection of <i>ftsZ</i> and <i>ompR</i> Transcripts.....	27
Protein Extraction and Western Blotting.....	28
RESULTS.....	30
Unphosphorylated and Phosphorylated OmpR Bind Weakly to the <i>fimB</i>	
Promoter Region Two.....	30
Both Forms of OmpR Do Not Bind to the <i>fimE</i> Promoter Region.....	30
UPEC <i>ompR</i> Transcription is Constant Despite Growth Conditions.....	33
OmpR Protein Level is Increased When UPEC are Grown in a	
Combined Acidic pH/High Osmolality Medium.....	37
DISCUSSION.....	41
REFERENCES.....	48
APPENDICES.....	59

LIST OF TABLES

<u>TABLE</u>	<u>PAGE</u>
1. Oligonucleotide primers used in this study.....	21
2. Western blot analyses of OmpR protein levels measured by pixelation	
UPEC NU149 grown in four different environments	40

LIST OF FIGURES

<u>FIGURE</u>	<u>PAGE</u>
1. Genetic organization of the <i>E. coli</i> <i>fim</i> gene operon.....	7
2. Schematic representation of the <i>fimB</i> , <i>fimE</i> , and <i>fimA</i> genes including their promoters.....	9
3. Schematic representation of EnvZ, the sensor protein, and OmpR, the response regulator in a UPEC cel.....	14
4. The coding strand of DNA showing the position of two promoter regions of the <i>fimB</i> gene in UPEC strain NU149 including the primer binding sites used for DNase I footprinting.....	20
5. The coding strand of DNA showing the position of the <i>fimE</i> promoter region of UPEC strain NU149 including the primer binding sites used for DNase I footprinting.....	25
6. Schematic representation of phosphorylated OmpR and unphosphorylated OmpR protein DNase I protection of <i>fimB</i> promoter region two.....	31
7. DNase I footprinting analysis of unphosphorylated (OmpR) and phosphorylated OmpR (OmpR-P) at the <i>fimB</i> promoter region two.....	32
8. DNase I footprinting analysis of unphosphorylated and phosphorylated OmpR at the single <i>fimE</i> promoter region.....	34
9. Qualitative determination of <i>ftsZ</i> and <i>ompR</i> mRNA regulation by RT-PCR analysis of strain NU149.....	35

<u>FIGURE</u>	<u>PAGE</u>
10. Quantitative determination of <i>ftsZ</i> and <i>ompR</i> transcript expression by qRT-RT-PCR.	36
11. Comparison of OmpR protein expression in UPEC NU149 cells grown in different environments	39
12. A proposed model for OmpR regulation of the <i>fimB</i> gene when UPEC cells are growing in an acidic/high osmolality environment similar to a kidney.....	46

LIST OF APPENDICES

<u>APPENDIX</u>	<u>PAGE</u>
A. Reagents for DNase I Footprinting.....	59
B. Reagents for RNA Extraction and PCR.....	62
C. Reagents for SDS-PAGE and Western Blotting.....	64
D. Reagents for Media.....	67

INTRODUCTION

Characteristics and Clinical Significance *Escherichia coli*. *Escherichia coli* is the most well studied and understood pathogen in the world today. Of the six known *Escherichia* species, *E. coli* is most frequently isolated from humans. Morphologically, it is a facultative Gram-negative rod, measuring 2 µm by 0.8 µm with peritrichous flagella. *E. coli* is a member of the *Enterobacteriaceae* family and ferments D-glucose (75). *E. coli* is an ubiquitous part of the normal intestinal flora of healthy adult humans, and certain strains can cause serious infections (extraintestinal and intestinal) in both healthy and immunocompromised individuals. The most common clinical symptoms associated with *E. coli* infections include urinary tract infections, bacteremia, meningitis, and diarrheal disease. The particular types of *E. coli* that cause diarrheal diseases can be further classified into six categories: Shiga toxin-producing *E. coli* (also known as enterohaemorrhagic *E. coli*), enterotoxigenic *E. coli*, enteropathogenic *E. coli*, enteroaggregative *E. coli*, enteroinvasive *E. coli*, and diffusely adherent *E. coli*.

Clinical Significance of Uropathogenic *Escherichia coli*. Uropathogenic *Escherichia coli* (UPEC) are the most common cause of urinary tract infections (UTIs). These infections are among the most frequent bacterial infections in adults (29, 52, 89), accounting for more than 90% of uncomplicated UTIs (110). Urinary tract infections can

be asymptomatic, but they typically are characterized by a wide array of symptoms that include painful urination, bacteremia, sepsis, and in extreme cases may cause death (29). These infections can be further categorized as ‘uncomplicated’ or ‘complicated’. UTIs that occur in a normal genitourinary tract with no previous catheterization are considered ‘uncomplicated’. In contrast, ‘complicated’ infections are diagnosed in genitourinary tracts that have structural or functional abnormalities, such as the presence of only one kidney or indwelling catheters (29).

Urinary tract infections are considered an ascending disease. Uropathogens that are part of the fecal flora enter the urinary tract via the urethra and subsequently move up to the bladder which is the site of infection for 95% of all UTIs (4, 90). Cystitis, an inflammation of the bladder, is characterized by dysuria, frequent or urgent urination, and suprapubic pain. In rare instances, UPEC move up the ureters into the kidneys causing pyelonephritis which is characterized by flank pain, nausea, vomiting, fever, chills, and malaise.

Seven million UTIs occur in the United States annually (30, 42), and more than 90% are caused by uropathogenic *E. coli*. The remaining 10% of UTIs are caused by *Staphylococcus saprophyticus*, *Streptococcus agalactiae*, *Proteus mirabilis*, *Klebsiella* species, *Enterococcus faecalis*, and *Corynebacterium urealyticum*. Although everyone is susceptible to UTIs, some populations are more at risk than others. For example groups that are more susceptible include infants, pregnant women, the elderly, patients with spinal cord injuries, catheterized individuals, persons with diabetes, multiple sclerosis, or human immunodeficiency virus /acquired immunodeficiency syndrome, and patients with

underlying urologic abnormalities (29). Males are most susceptible to UTIs during the first few months of life, whereas females remain more prone to UTIs throughout their lifetime. For example, approximately 50 to 60% of all women will have a UTI during their lifetime (31, 32, 59). Additional studies have shown that 21 to 44% of women who presented with a urinary tract infection experienced a second infection within six to twelve months (31, 49, 97). These subsequent infections can either be attributed to the same strain of UPEC or a completely different strain. Uncomplicated urinary tract infections are much less common in men with a prevalence of less than 8 per 10,000 men per year (109). In the United States, deaths due to UPEC infections can range from 5,000 to 10,000 a year, while \$1.6 billion per year is spent to treat and diagnose these infections (29, 42), making UPEC both clinically and financially an important pathogen in the world today.

Diagnosis and Treatment of Urinary Tract Infections. UTIs are diagnosed most commonly by collecting and culturing a clean-catch, midstream urine specimen. Although slightly more invasive, catheterization will allow for collection of bladder urine with less urethral contamination. Urinalysis and rapid leukocyte esterase testing are the most commonly used rapid diagnostics for point of care testing (20). In the microbiology laboratory, an initial Gram stain that demonstrates the presence of at least one Gram-negative rod per oil immersion (1000X) field and greater than 10 white blood cells per field is a presumptive indicator of a urinary tract infection. Culture of a standardized inoculum (calibrated 1 µl loop) onto a 5% sheep blood agar plate is often used to confirm infection. In addition, MacConkey agar is also used to select for Gram-negative bacteria

and can be used in conjunction with a chromID CPS plate that allows the presumptive identification of UPEC. After incubation for at least 24 h at 35-37°C, a colony count of $> 10^5$ colony forming units/ml and a positive spot indole test in conjunction with the patient's history is sufficient for a diagnosis of an *E. coli* urinary tract infection.

Uncomplicated urinary tract infections are generally treated with cotrimoxazole (trimethoprim-sulfamethoxazole), fluoroquinolones (especially ciprofloxacin), fosfomycin, or nitrofurantoin (37). With the exception of fosfomycin (single dose), all of the drugs are typically given for three to seven days to ensure optimal cure rates while reducing the development of antibiotic resistance.

Unfortunately, within the last two decades, there has been an increase in antimicrobial resistance among Gram-negative urinary tract isolates to cotrimoxazole and fluoroquinolones, making treatment more difficult, especially for patients suffering from pyelonephritis (20). Although most human cases of acute pyelonephritis have a good prognosis, they often require prolonged therapy. However, acute pyelonephritis has a mortality rate of 10 to 20% when the patient experiences bacteremia (88). Recently, the use of carbapenem or third generation cephalosporins for 14 days has proven to be effective in greater than 96% pyelonephritic cases (105).

Physiology of the Human Urinary Tract. Uropathogenic *E. coli* are able to grow in a myriad of environments, including the human urinary tract. Human urine can support the growth of UPEC, but urine is prone to extreme fluctuations in both pH and osmolality (60). Human urine can have pHs that vary between 5.0 and 8.0 and osmolalities that range from 50 mOsm/kg to 1400 mOsm/kg (92). The urine pH and

osmolality variations depend upon the physiological changes in an individual's diet, physical activity, and health (60, 92).

Besides the aforementioned factors, the anatomy of the human body also plays a part in differences in urine pH and osmolality. For example, kidney urine typically has a lower pH than bladder urine because of the dilution effect in the bladder (92). In order to cause disease, UPEC must overcome natural host defenses that are present in the human urinary tract. These immune mechanisms include oxidizing agents, phagocytic cells, and exfoliation of epithelial cells (51). Phagocytic cells, such as polymorphonuclear leukocytes, are recruited from the bloodstream to neutralize and engulf any foreign bacteria present in the bladder.

Type 1 Pili. *Escherichia coli* has multiple virulence factors that mediate adherence to host cells and tissues, including P pili, S pili, afimbrial adhesins, and type 1 pili. Type 1 pili (fimbriae) are filamentous appendages found on the surface of *E. coli* and other Gram-negative microorganisms (2, 10, 21, 94, 108) and were originally characterized by their ability to produce mannose-sensitive hemagglutination of guinea pig erythrocytes (79, 93), a technique which remains useful for determining the presence of type 1 fimbriae on the surface of UPEC (21, 22, 93).

Adherence to and invasion of uroepithelial cells are important steps for UPEC pathogenesis. By binding to uroepithelial cells, UPEC are able to persist within the urinary tract (77, 91, 103). Bacteria that are unable to attach will be cleared by the flushing of urine in the bladder. Type 1 pili also aid in the invasion of UPEC into the bladder epithelial cells (69), allowing these bacteria to avoid the flow of urine through the

bladder and providing them access to a more nutrient rich environment. In addition, invasion into the uroepithelial cells helps the UPEC bacterial cells to avoid antibiotics and phagocytic cells present within the urine. The expression of type 1 pili allows *E. coli* to avoid the immune system by invading nearby bladder epithelial cells.

After UPEC have invaded the host bladder cells, type 1 pili also assist in the formation of biofilm-like pods called intracellular bacterial communities (IBCs) (90). Interestingly, all of the UPEC bacteria in these IBCs express type 1 pili. Conversely, UPEC without fimbriae are unable to form these complex IBCs, suggesting that type 1 pili are critical for intracellular functions necessary for proper IBC formation (111). These IBCs aid in protecting the bacteria from both innate host defenses and antibiotics (111). IBCs also help the UPEC bacteria to resist detachment with the flushing of urine through the bladder. Eventually, the IBCs fall apart and release more filamentous UPEC into the bladder lumen. Recently, UPEC bacterial cells recovered from women with urinary tract infections, have been shown to produce IBCs when inoculated into mice (35, 90). These findings imply that IBCs may play a part in the recurrence of UTIs in women.

Genetic Organization of *fim* Genes in UPEC. Several genes are involved in the synthesis, assembly, and regulation of type 1 pili in UPEC. Nine different *fim* genes (*fimA-I*) are found within the type 1 pilus operon (96) (FIG. 1). The *fimA* gene encodes a protein that forms the main structural subunit of the type 1 pilus. Each type 1 pilus consists of up to 3000 molecules of FimA and can be 7 nm wide and up to 2 μ m long (36). Uropathogenic *E. coli* can switch reversibly from the fully piliated state (Phase-ON) to the nonpiliated form (Phase-OFF) using a process called phase variation. This process

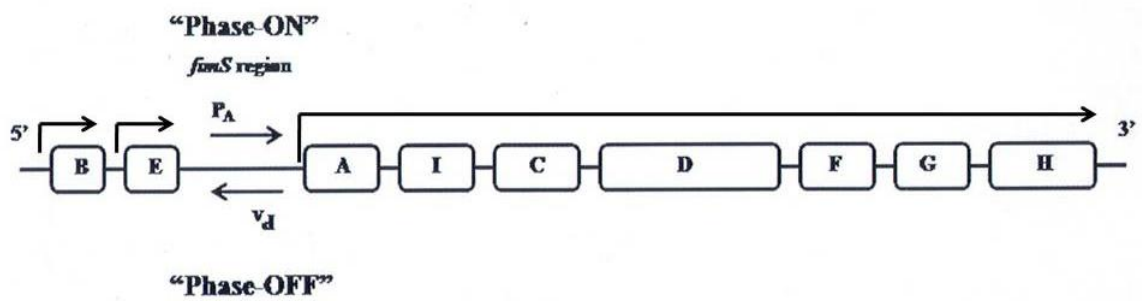


FIG. 1. Genetic organization of the *E. coli* *fim* gene operon. The P_A represents the orientation of the *fimA* promoter.

involves the inversion of a 314-bp region of DNA (called *fimS*) which contains the promoter for *fimA*. The expression of type 1 pili varies from strain to strain and can also differ among individual bacterial cells within the same population. The *fimH* gene product forms the adhesin at the tip of each type 1 pilus and is linked to the pilus rod via FimF and FimG, forming a fibrillum tip (38). FimH is required for invasion of superficial bladder epithelial cells, mediating the contact between UPEC and mannose receptors on host epithelium (1, 57). Type 1 pili are assembled by a chaperone-usher pathway, whereby protein complexes between fimbrial subunits and the FimC chaperone are recruited by the outer membrane protein FimD (the usher) (74). The FimC protein binds the nascently formed FimA, FimG, FimF, and FimH proteins and this complex is transported to the outer membrane for pilus assembly (50). FimC also helps the newly formed proteins resist proteolytic degradation (58). At the outer membrane FimD recognizes FimC, allowing the chaperone proteins to pass through the membrane and assemble into a functional type 1 pilus. Currently, there is no specific function associated with the FimI protein, however, *E. coli fimI* insertion mutants are nonpiliated (107).

The *fimB* and *fimE* genes located upstream of the *fimA* gene code for site-specific recombinases that position the *fimS* invertible element, which controls type 1 pili phase variation (8, 32, 56, 70, 71, 83). The FimB protein mediates inversion of the invertible element in both the Phase-ON and Phase-OFF orientations (70), whereas the FimE protein predominantly rearranges the *fimS* region in the Phase-OFF orientation (83, 102) (FIG. 2). The promoters for both the *fimB* and *fimE* genes have been mapped. To date, only one promoter has been identified for the *fimE* gene (81). In contrast, multiple *fimB*

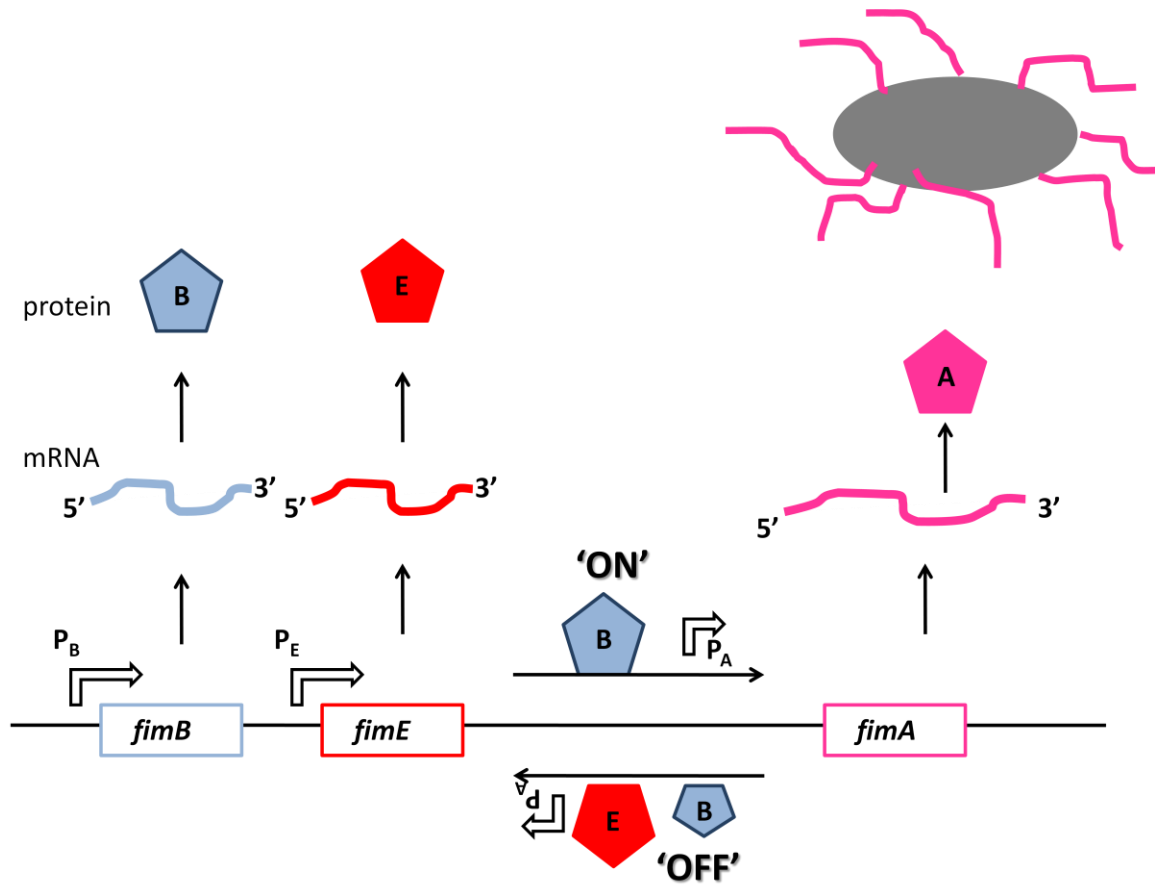


FIG. 2. Schematic representation of the *fimB*, *fimE*, and *fimA* genes including their promoters. The *fimB* and *fimE* genes encode site-specific recombinases that position the invertible element, or *fim* switch. The position of the invertible element determines whether the UPEC cells are type 1 pilated (Phase-ON) or are nonpilated (Phase-OFF). UPEC express type 1 pili when FimA, the main structural subunit of type 1 pili, is transcribed and translated.

promoters have been identified and the number can vary between clinical isolates and laboratory strains (13, 27, 81, 100).

Cofactors and Environmental Conditions That Affect Piliation and Phase

Variation. Phase variation can occur independently of the recombinases with rates estimated to be 10^{-2} to 10^{-3} per cell per generation (10, 23, 76, 104). However, both FimB and FimE recombinases play an integral part in type 1 pilus phase variation. Both recombinases position the invertible element in either the Phase-ON or the Phase-OFF position. Interestingly, when FimB is overexpressed, *fimA* transcription is decreased 10-fold, however the *fimS* element still remains in the ON orientation (19). This suggests that another protein is interacting with the *fim* switch to negate the ON effects by the FimB recombinase. Traditionally, the *fim* switching process has been thought to be a tightly regulated process mediated by the two site-specific recombinases either acting by themselves or in conjunction with other regulatory proteins. Recently, the role of FimB and FimE recombinases has become murkier. A study observing *E. coli* cells in real-time discovered that a random OFF to ON switching occurs at the beginning of the cell cycle, before *fim* gene replication (3). In this scenario, the FimB and FimE recombinases would have not yet been transcribed or translated, so the switching of *fimS* would be independent of the recombinases.

Besides FimB and FimE, other global regulators have been shown to affect piliation in *E. coli*. For example, cAMP (3'-5'-cyclic adenosine monophosphate) bound to CRP (cAMP receptor protein), normally regulates carbohydrate metabolism (23), but also affects phase variation (72). CRP-cAMP acts with DNA gyrase to negatively affect

FimB-mediated recombination, thereby repressing type 1 fimbriation in *E. coli* while simultaneously stimulating *fimA* promoter activity (72). In addition to CRP-cAMP, sialic acid has been shown to affect phase variation by inhibiting FimB expression (27). When DNA gyrase is inhibited, the FimB recombinase has a strong propensity to keep *fimS* in the ON orientation (17). In *E. coli* K-12, mutants that do not express a functional topoisomerase 1 are unable to invert the switch (17). Instead, the switch stays in the previous orientation prior to the topoisomerase mutation. Similarly, integration host factor (18, 26) and leucine-responsive regulatory protein (7, 33) affect phase variation by binding to the *fimS* region and switching the orientation. Finally, the histone-like H-NS protein affects the DNA inversion of the invertible element by directly interacting with *fimS* as well as indirectly by affecting expression of the *fimB* and *fimE* genes (13, 14, 78, 81, 101).

In addition to specific cofactors, certain environmental conditions also play an important role in regulating pilus expression in UPEC. Growth of UPEC in poorly aerated static broth enriches the growth of piliated organisms (62, 80), whereas after several passages on solid agar medium most strains of *E. coli* will become nonpiliated (47). In the human bladder, oxygen is limited and UPEC tend to be more fimbriated. UPEC that express fimbriae adhere not only to each other but also to the bladder uroepithelium. This prevents the bacteria from being dislodged by urine. UPEC express more type 1 pili when grown at 30°C versus 37°C (16). Besides temperature, the concentration of aliphatic amino acids (alanine, isoleucine, leucine, and valine) (34) and

the presence of amino sugars (27) have been shown to affect type 1 pili expression in uropathogenic *E. coli*.

Additionally, the presence of glucose in the medium inhibits type 1 piliation (72), presumably acting through the cAMP-CRP system (24). Lastly, osmolality and pH are two additional environmental cues that affect UPEC type 1 pilus expression. High osmotic growth conditions have been shown to reduce type 1 fimbriae expression (59, 101). Similarly, growth of UPEC cells in an acidic medium results in a greater number of nonpiliated cells (101). However, when the intracellular pH of *E. coli* was decreased following a rapid acid shock, *fimB* gene expression was increased four-fold and then subsided (53).

Response to Increasing Environmental Osmolality by *E. coli*. Two regulatory systems exist that allow *E. coli* to survive in environments with differing osmolalities, including the osmotic differences in the human urinary tract. The *hns* gene encodes a heat-stable nucleoid-structuring protein (H-NS) that functions as a global regulator protein. Two functions have been attributed to H-NS in a bacterial cell: acting as a repressor of genes and aiding in the compaction of the bacterial nucleoid. Recently, it has been shown that the cation composition can affect H-NS binding to DNA and cause conformational changes in the DNA (64). In one instance, H-NS is able to ‘straighten’ out DNA strands (stiffening mode) and coat the length of the DNA (64). By having H-NS proteins bound consecutively on a piece of DNA, this could inhibit RNA polymerase from binding and therefore lead to gene repression.

Although H-NS has no consensus binding sequence, it preferentially binds to

A-T-rich, curved regions of bacterial DNA that are frequently associated with bacterial promoters (15). In *E. coli*, DNA supercoiling increases as environmental osmolality increases (41). Normally, H-NS represses *fimB* expression (14), but mutations in the *hns* gene affect type 1 pilus expression by causing a higher rate of inversion of *fimS* (54) and causing *fimB* to be constitutively expressed (13, 101). This suggests that H-NS normally functions as a repressor for the *fimB* gene. Environments with a high osmolality may cause H-NS to bind the DNA and repress *fimB* transcription. Without FimB, the *fimS* region would not switch to the Phase-ON state and the UPEC cells would become nonpiliated. In part, this may explain why UPEC tend to be nonpiliated in the human kidneys where the urine would have a higher osmolality.

In addition to H-NS, the EnvZ-OmpR two component global regulatory system is activated when UPEC cells are exposed to osmotic stress (FIG. 3). Two component systems are comprised of a membrane-bound histidine kinase (e.g., EnvZ) and a response regulator (e.g., OmpR). The EnvZ kinase has three main enzymatic activities *in vivo*. First, EnvZ can autophosphorylate itself (autophosphorylation) in the presence of ATP. Secondly, EnvZ can transfer the phosphate groups to OmpR (phosphotransfer). Thirdly, EnvZ acts as a phosphatase and removes phosphates from OmpR proteins in the bacterial cell. By using all of these properties, EnvZ is able to tightly regulate the ratios of phosphorylated versus unphosphorylated OmpR in the bacterial cell. In an environment where both unphosphorylated OmpR and phosphorylated OmpR are present, OmpR has a higher affinity for EnvZ while OmpR-P has a higher affinity for DNA (68).

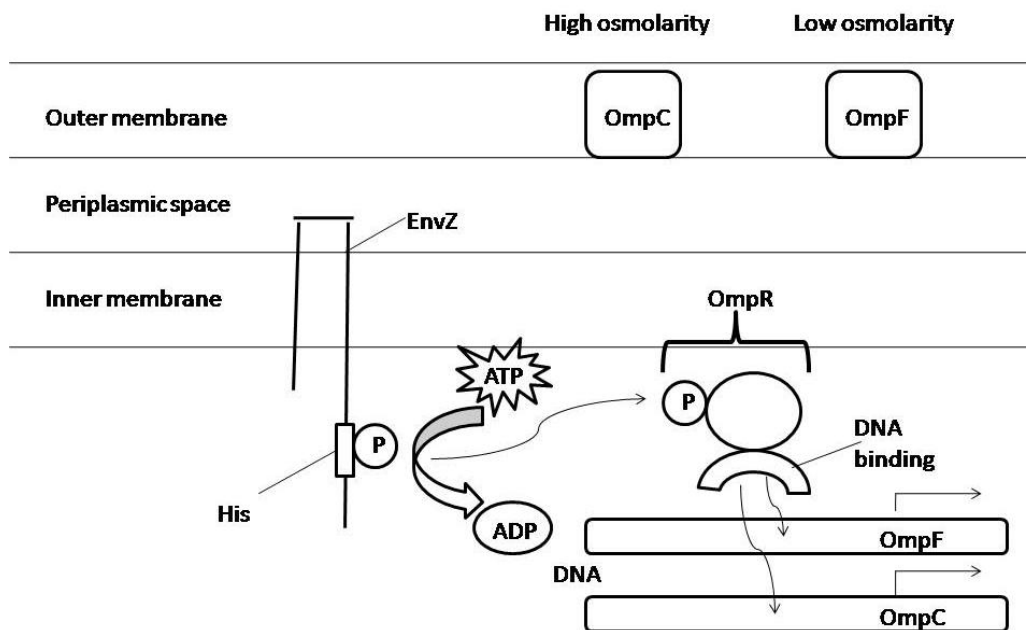


FIG.3. Schematic representation of EnvZ, the sensor protein, and OmpR, the response regulator in a UPEC cell.

An examination of the EnvZ/OmpR regulatory cascade has demonstrated that the EnvZ sensor protein monitors environmental osmotic changes. For example, under hypoosmotic conditions, EnvZ is autophosphorylated by adenosine triphosphate (ATP) at the highly conserved histidine residue 243 located in the bacterial cytoplasm (48). The phosphate is then transferred to aspartate residue 55 on the cytoplasmic OmpR protein. Phosphorylated OmpR (OmpR-P) binds high-affinity sites around a particular promoter and acts as a transcription factor for genes such as *ompF* and *ompC* that encode major porin proteins (12, 55).

Even though *ompR* and *envZ* are co-transcribed as one polycistronic mRNA, the amount of cellular OmpR is approximately 3500 molecules versus only 100 molecules of EnvZ per cell (11), suggesting that translational initiation could be affected. In quantitative Western blot analyses, wild-type *E. coli* grown in nutrient broth with 20% sucrose (high osmolality) had approximately 1.7 times the amount of OmpR protein versus bacteria grown in nutrient broth alone (low osmolality) (11). Yoshida *et al.* suggested that the ratio of OmpR to OmpR-P determines whether gene regulation occurs rather than the overall cellular level of OmpR (112). Differences in the phosphatase/kinase activities of EnvZ directly affect the ratio of OmpR to OmpR-P.

Besides *ompF* and *ompC*, OmpR also regulates 125 additional genes in *E. coli* (82), including several *fim* genes (101). For example, a $\Delta ompR$ mutation in UPEC NU149 caused a significant growth decrease in LB with the addition of 800 mOsm NaCl (98), indicating the expression of the OmpR protein is important for survival of UPEC grown in osmotic stress conditions. Growth of UPEC in a medium with a neutral pH and

NaCl significantly increased *fimE* gene expression, while the *fimA* and *fimB* expression decreased slightly (101). In an *ompR* deletion mutant, derepression of *fimB* gene expression was seen in *E. coli* cells grown in pH 5.5 with added NaCl (101). Further evidence that OmpR regulates *fim* gene expression was demonstrated using an *ompR* mutant strain complemented with an *ompR* gene that had the aspartate changed to an alanine at position 55 in the OmpR protein. The complemented strain did not show a derepression of the *fimB* gene when UPEC was grown under high osmotic conditions (101). Similarly, the *envZ* mutant strain did not show a derepression of *fimB* transcription (101), suggesting that OmpR does not need to be phosphorylated by EnvZ at the usual aspartate to bind to one of the *fimB* promoters. A preliminary gel mobility shift assay showed that purified unphosphorylated OmpR protein bound the *fimB* promoter region in a clinical UPEC strain (W.R. Schwan, personal communication), suggesting OmpR may directly regulate *fimB* gene expression. Taken together, these results suggest that the unphosphorylated OmpR protein or OmpR protein phosphorylated at an amino acid other than aspartate regulates *fimB* gene expression.

Mechanisms of Acid Tolerance in *E. coli*. In addition to osmotic variations, the human urinary tract is prone to fluctuations in pH. Human urine often has an acidic pH (60, 92), a characteristic which aids in the suppression of urinary tract infections. One study showed *fimA* and *fimB* transcription were lower in UPEC grown under acidic conditions (101). Interestingly, OmpR protein expression has been correlated with survival in acidic medium in bacteria other than UPEC. For example, a *Yersinia ompR* deletion mutant grew poorly compared to wild-type cells in pH 4.5 medium (43).

Moreover, OmpR was shown to positively regulate urease gene expression in *Yersinia* (43). Within stationary-phase cultures of *Salmonella*, OmpR protein expression increased approximately four-fold within 30 minutes following a rapid shift from a neutral pH to an acidic pH, while no change in OmpR levels were detected when the bacteria were in pH 8.0 media (6). Conversely, the addition of salt to the medium decreased the OmpR protein levels in both log and stationary phase *Salmonella* cultures (5). These findings suggest that OmpR may help overcome environmental acid shock.

Single environmental variables have uncovered part of the *fim* gene regulatory cascade. However, studies that involve acidic pH combined with high osmolality environments have been relatively limited. In UPEC growing in a combined low pH-high osmolality environment, the *fimB* gene was repressed, causing the *fimS* region to favor the Phase-OFF orientation and a subsequent loss of type 1 pili on the bacterial surface (101). The same study demonstrated that type 1 pilus levels were markedly reduced, and an *ompR* mutant strain grown in pH 5.5 LB with 800 mOsm NaCl exhibited a derepression of *fimB* gene expression compared to the wild-type strain grown in the same conditions (101). This data suggests that in a combined acidic pH and high osmolality environment, OmpR represses *fimB* gene transcription.

Moreover, a low pH-high osmolality environment would be similar to the one found in a human kidney. To date, no studies have been performed to investigate whether OmpR or phosphorylated OmpR protein binds to the *fimB* and *fimE* gene promoters of UPEC. For this study, DNase I footprinting assays were performed to determine the interactions of OmpR/OmpR-P with these promoters. In addition, transcriptional assays

examining *ompR* mRNA or OmpR protein levels have not been studied in UPEC growing *in vitro*. This is the first study that examined changes in OmpR protein levels in a clinical strain of UPEC when grown in different environments and how OmpR may regulate expression of the *fimB* and *fimE* genes of UPEC grown in an acidic/osmotically stressed environment.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions. *Escherichia coli* NU149, a UPEC clinical isolate (95), was used in the traditional reverse transcriptase polymerase chain reaction (RT-PCR), quantitative real-time reverse transcriptase PCR (qRT-RT-PCR) and Western blot analyses. The *E. coli* NU149 was grown in Luria broth (LB) containing 1% glycerol set at pH 5.5, pH 5.5 with 800 mOsm NaCl, pH 7.0, or pH 7.0 with 800 mOsm NaCl (101) (Appendix D) for both the RNA and protein extractions. All cultures were grown shaking at 37°C. Plasmid pWRS2-10 (99) containing the *fimB* and *fimE* genes from UPEC strain J96 was used as the template DNA for DNase I footprinting.

DNase I Footprinting. DNase I footprinting was performed as previously described (28). Footprinting reactions were performed using pWRS2-10 DNA as a template. To generate the 445 base pair fragment that contained *fimB* promoter two region, the primer pair FIMB2 and PEXFIMB (FIG. 4, TABLE 1) was used. Primer FIMB2 was end-labelled with T₄ polynucleotide kinase (NEB) and radiolabelled ATP [γ -³²P]- 3000 Ci/mmol, 5 mCi/ml (NEN Radiochemicals, PerkinElmer) in a thermocycler (BIO-RAD iCycler). The conditions were as follows: 37°C for 15 min followed by 75°C for 10 min. The *fimB* promoter fragment was amplified in a thermocycler (BIO-RAD

5' ATAG **CCACTAAGAAATTACTGCGCTCC** ATGAAATAGCCATTTTG
TGGCAAATGGAGTTGACTAATAATGTCATATGTGAGACGGCTAGT
-35 -10
TGAACGAATATTAAATTTTTGCTGAATTTTTTATGTTGATTTTACTT
+1 (P₂)
GTTACAGAACATATCACATGATATATAGATAAGATTAGTTGCATT
AATGATGAGGGTTATTATTAGATTCGTATCCGATTGATAAATATA
-35
TAAAGGTACATAGCATGCAAGAGCATGGCGTTTGTATGGCAACG
-10 +1 (P₁)
TTATAATAATTAACAGTTGCTACTCCATTTAAGTTCACTCAGAAG
AACTGGTCCACTTACGTTAGTTATTAAGCAAACGTTTCGCTTTTATA
AACATAATCAGGATAAAAATGTTGGATTATTGCTAACCCAGCACA
S/D 3'
GCTAGTGCGCGTCTGTAATTATAAGG**GAAAAACGATGAAGAATA**
5'
AGGCTGATA-3'

FIG. 4. The coding strand of DNA showing the position of two promoter regions of the *fimB* gene in UPEC strain NU149 including the primer binding sites used for DNase I footprinting. The S/D indicates Shine Dalgarno sequence and the ATG indicates the translational start site. The magenta highlighting indicates the FIMB2 primer. The teal highlighting indicates the PEXFIMB primer sequence.

TABLE 1. Oligonucleotide primers used in this study

Primer	Sequence (5'-3')	Description
FIMB2 (forward)	GATTCTAGAAATTACTGCGCTCC	DNase I footprinting
PEXFIMB (reverse)	GCCTTATTCTTCATCGTTTTTCC	DNase I footprinting
FIMEPE4 (forward)	ATACAATGGTTTATGTTCTTCAA	DNase I footprinting
PEXFIME2 (reverse)	TAGGAAACCTCAATCGGTCA	DNase I footprinting
EcFtsZ1(forward)	TAGCGGTATCACCAAAGGACT	RT-PCR
EcFtsZ2 (reverse)	GTGATCAGAGAGTTCACATGC	RT-PCR
OmpR3(forward)	TGCTGACTCGTGAATCTTTCC	RT-PCR
OmpR4 (reverse)	CGTACCGAGGTAAAGTTTGAA	RT-PCR
FtsZfwd (forward)	GAAAGGCGCTGTGCAAGGTAT	qRT-RT-PCR
FtsZrev (reverse)	CGTCTGCAAAGTCCACGTTCATC	qRT-RT-PCR
OmpRfwd (forward)	GCGTCGCTAATGCAGAACAG	qRT-RT-PCR
OmpRrev (reverse)	ACAAGCCATCTTCACCAGGTAAC	qRT-RT-PCR

iCycler) using Platinum Taq (Invitrogen) for 35 cycles: initial denaturation 95°C for 3 min, 95°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec.

To purify the radiolabelled DNA fragment, the PCR product was run through a QIAquick PCR purification kit (Qiagen) following the manufacturer's protocol. Briefly, five volumes of Buffer PB was mixed with the PCR product and added to a QIAquick spin column. Each mixture was centrifuged for 1 min (17,000 x *g*) to bind the DNA to the column. The column was then washed with 0.75 ml Buffer PE and centrifuged for 2 min (17,000 x *g*). Residual ethanol was removed by centrifugation for 2 min in a centrifuge (17,000 x *g*). Clean PCR products were eluted from the column in 100 µl of Buffer EB (10 mM Tris-Cl, pH 8.5) and further purified on a 4.2% (37.5:1) polyacrylamide, 1.25X Tris-acetate-EDTA gel (Appendix A). PCR products were loaded onto the gel and separated by electrophoresis for 1.5 h at 45 watts. Radiolabeled DNA fragments containing the *fimB* promoter region was excised from the gel and eluted overnight in 500 µl diffusion buffer (Appendix A) at 50 °C. To separate the polyacrylamide from the radiolabeled *fimB* promoter region, a spin column with glass wool was used. The eluted DNA was further purified with a QIAquick PCR purification kit (Qiagen) as previously described with the following exception: Qiagen Buffer QG was substituted for Qiagen Buffer PB. Radiolabelled promoter fragments were eluted in 100 µl of Buffer EB. To remove secondary structure in the DNA, 0.5 µl of 5 M NaCl was added to each eluate, incubated for 30 sec at 90°C, and allowed to slowly cool to room temperature to re-anneal the DNA.

For the DNase I footprinting reactions, wild-type OmpR protein was purified by fast protein liquid chromatography (FPLC) (gift from Linda Kenney) and phosphorylated using 1 μ l phosphoramidate, 2 μ l 10X phosphorylation buffer (Appendix A), and OmpR protein set at a final concentration of 16 μ M. Each mixture was incubated with shaking at room temperature for 60 min and the reactions halted by freezing on dry ice. For the binding reactions, OmpR-P (200 nM to 1600 nM) was incubated at room temperature for 15 min in 1X binding buffer (Appendix A) with 50,000 cpm of radiolabeled DNA and 1 μ l poly(d[I-C]) (1 μ g/ml) (Roche Diagnostics). To measure radioactivity, 1 μ l of the DNA reaction in a pipette tip was held next to a Geiger counter for 1 min and cpm were read at the 1X setting. RNase-free DNase I (Roche Diagnostics, Mannheim, Germany) (10 U/ μ l) diluted (1:8000) in 1X binding buffer with 0.1 mM CaCl_2 was added to each binding reaction and incubated for 30 sec at room temperature to generate single nicks in the radiolabelled DNA. Reactions were stopped with the addition of 5 μ l of stop dye (Appendix A). DNA protein mixtures were precipitated with 100% ethanol and pelleted by centrifugation at 17,000 x *g* for 20 min. Ethanol was removed and each DNA-OmpR protein pellet was washed with 100 μ l of 100% ethanol, and pelleted by centrifugation at 17,000 x *g* for 5 min. Residual ethanol was aspirated and the DNA-protein pellets were allowed to air-dry for 20 min.

All DNA-OmpR pellets were resuspended in 11 μ l of sequencing grade stop dye (Appendix A), the DNAs were separated by electrophoresis on 8% 7.5M urea-Tris-borate-EDTA polyacrylamide gels (Appendix A), and the gels were developed using a phosphorimaging screen. DNA sequencing ladders for the *fimB* promoter regions were

generated with a Thermo Sequenase cycle sequencing kit (U.S. Biochemicals) using the same labeled primers as were used for the footprinting reactions. Images were acquired with a Molecular Dynamics Storm 860 Phosphorimager using the ImageQuant 5.3 software package.

The same footprinting protocol was followed for the *fimE* promoter region footprinting with the following modifications. To generate a 340 base pair fragment containing the *fimE* promoter region, the primer pair FIMEPE4 and PEXFIME2 (TABLE 1, FIG. 5) was used. The FIMEPE4 primer was end-labelled with T₄ polynucleotide kinase (NEB) and radiolabeled with ATP γ -³²P. The *fimE* promoter fragment was amplified in a thermocycler (BIO-RAD iCycler) using Platinum Taq (Invitrogen) for 35 cycles: initial denaturation 94°C for 3 min, 94°C for 15 sec, 53°C for 30 sec, and 72°C for 30 sec. Phosphorylated OmpR protein concentrations used for the *fimE* promoter region ranged from 400 to 1200 nM and the DNase was diluted 1:1000 in 1X binding buffer.

Extraction of Total RNAs and Conversion to cDNAs. *E. coli* NU149 cells were grown shaking to mid-logarithmic phase (O.D.₆₀₀ 0.5-0.8) at 37°C in pH 5.5 low-osmolality LB medium, pH 5.5 high-osmolality (800 mOsm NaCl) LB medium, pH 7.0 low-osmolality medium, or pH 7.0 high-osmolality (800 mOsm NaCl) LB medium. Bacterial cultures were pelleted by centrifugation (5,000 x *g*) for 10 min at room temperature. Total RNA was extracted from each pellet using TRIzol Reagent (Invitrogen). Briefly, the cells were resuspended in 1 ml Buffer EB (Qiagen) and lysed

5'AGTTAACCATATCCATACAAAATACAATGGTTTATGTTCTTCAA3'
 AATAAATAAACAAAATCATTTCATAAATTTACACATCACTTAAAAT
 CTCCTGTTTCCGCACTTTTTTCTTTATTTTTTAAGCAACTGGAAGTT
 -35 -10
 AATCCACTGCAATCTATTGTTATATTGAATCAAATCAATGAAAAT
 +1 (P₁)
 AGATGTTGTCACATCAGTGATATTTTATTTTTGTATGATATTTAAT
 GTAATTGACTGATAGCCACATCACTCCGTGTGTGGTTATCTTTTAA
 3' 5'
 TCTATTGGGCTAATTTTGACCGATTGAGGTTTCCTATAGGTATTCA
 S/D
 TTCAAATATATCTCAGTTAGGAGTACTACTATTGTGAGTAAACGT
 CGTTATCTTACCGGTAAAGAAGTTCAGGCCATGATGCAGGCGG-3'

FIG. 5. The coding strand of DNA showing the position of the *fimE* promoter region of UPEC strain NU149 including the primer binding sites used for DNase I footprinting. The S/D represents the Shine Dalgarno sequence and GTG represents the translational start site. The green highlighting indicates the FIMEPE4 sequence. The red highlighting indicates the PEXFIME2 primer.

with 50 μ l lysozyme (10 mg/ml) for 15 min at 37°C. TRIzol Reagent (6 ml) was added to the lysed cells, inverted, and incubated for 15 min at room temperature to further dissolve cellular components. Chloroform (1.2 ml) was added and tubes were inverted for 10 sec and then incubated at room temperature for 3 min. RNAs were pelleted by centrifugation at 12,000 x *g* for 15 min at 4°C. Supernatants were removed and the RNAs were precipitated with two volumes of 100% ice-cold ethanol and 1/10 volume of 3 M sodium acetate for two hours at -80°C. RNAs were pelleted by centrifugation (10 min, 10,000 x *g*, 4°C), washed with 70% ethanol made with diethyl-pyrocabonate (DEPC)-treated water (Appendix B), pelleted by centrifugation for 20 min at 10,000 x *g* at 4°C, air-dried, and then resuspended in 100 μ l DEPC-treated water. Residual DNA in the samples was removed with the addition of 1 μ l (10,000 U/ml) of RNase-free DNase (Roche Diagnostics) for 30 min at 37°C. The DNase was heat inactivated for 15 min at 65°C. The quality and concentration of the total RNA was determined spectrophotometrically by reading the OD 260/280 using a BioSpec-1601 spectrophotometer (Shimadzu).

Total RNAs were converted to cDNAs using a SuperScript First-Strand Synthesis Kit (Invitrogen) following the protocol recommended by the manufacturer. Briefly, 5 μ g of RNA was mixed with 1 μ l of random hexamers (50 ng/ μ l), 1 μ l of 10 mM dNTP mix, and DEPC-treated water to a final volume of 10 μ l. The samples were incubated for 5 min at 65°C and chilled on ice for 1 min. Next, 2 μ l of 10X RT buffer (200 mM Tris-HCl pH 8.4 and 500 mM KCl), 4 μ l of 25 mM MgCl₂, 2 μ l of 0.1 M DTT, and 1 μ l of RNaseOUT recombinant ribonuclease inhibitor was added to each sample and incubated at room temperature for 2 min. SuperScript II RT (50 U/ μ l) was added to each tube and

incubated at room temperature for 10 min and then 42°C for 50 min. The reactions were then terminated at 70°C for 15 min and chilled on ice. RNase H (20 U) was added, incubated for 20 min at 37°C, and final cDNA was stored at -20°C.

Amplification and Detection of *ftsZ* and *ompR* Transcripts. Reverse-transcriptase polymerase chain reactions (RT-PCR) were performed using RNA and cDNA from each growth condition. Each 50 µl reaction contained 1 µl template (RNA or cDNA), 10 µl complete Wigler's buffer (Appendix B) (63), 0.6 µl GoTaq Flexi DNA polymerase (5 U /µl, Promega, Madison, WI), 50 pmol of each primer, and PCR-grade water (Sigma). Traditional RT-PCR conditions were as follows: initial denaturation 95°C for 5 min followed by 31 cycles of 95°C for 1 min, 56°C for 1 min, and 72°C for 1 min. The cell-division gene, *ftsZ*, was used to standardize between the samples since *ftsZ* expression was completely independent of the different acidic/osmotic environments (101). The 302-bp *ftsZ* gene product was amplified using the primer pair EcFtsZ1 and EcFtsZ2 (TABLE 1) and the 319-bp *ompR* gene product was amplified using the primer pair OmpR3 and OmpR4 (TABLE 1). RT-PCR products were separated on 1.5% agarose gels and visualized with FOTO/Analyst PC Image Software (FOTODYNE Inc., Hartland, WI).

Real-time reverse-transcriptase PCR (RT-RT-PCR) was performed using a Stratagene platform. One microliter of a 1/10 dilution of cDNA was used in each RT-RT-PCR reaction. A 2X POWER SYBER Green mastermix (Applied Biosystems) was used and products were detected with a FAM setting. A 71-bp fragment of the *ftsZ* gene was amplified using the primer pair FtsZfwd and FtsZrev (TABLE 1). The 96-bp *ompR* gene

segment was amplified using the primer pair OmpRfwd and OmpRrev (TABLE 1). The real-time RT-PCR parameters were as follows: initial denaturation for 10 min at 95°C followed by 38 cycles of 15 sec at 95°C, 1 min at 58°C. Data was analyzed by the $2^{-\Delta\Delta C_T}$ method (65).

Protein Extraction and Western Blotting. *E. coli* strain NU149 was grown shaking to stationary phase (O.D.₆₀₀ equal to 1.7-2.0) at 37°C in the same media that was used for the qRT-RT-PCR experiments. Cells were pelleted by centrifugation at 5,000 x *g* for 10 min at room temperature. Each pellet was resuspended in 3 ml phosphate buffered saline (Appendix C) and disrupted by sonification using a mini-tip on a Sonifier Cell Disruptor model 350 (3 cycles of 45 sec run, 30 sec rest) (Branson Sonic Power Co.). Broken bacterial cells were frozen at -20°C. Total protein concentration from each sample was determined by using a BIORAD protein assay (9) and compared to a bovine serum albumin (Sigma) standard curve.

Ten micrograms of cell sonicate from each growth condition was mixed with an equal volume of sample buffer (Appendix C) and separated by SDS-PAGE on 10-20% Tris-HCl polyacrylamide gels (Bio-Rad). Proteins were separated by electrophoresis at 150 V for 1 h 45 min using 1X running buffer (Appendix C) and stained with Brilliant Blue stain (Appendix C) overnight. Gels were then destained for 2-3 hours to visualize protein bands and to verify that protein concentrations were similar in each lane. A Kaleidoscope Prestained Standard (BIORAD) was used to determine relative protein sizes in each sonicate.

For Western blots, polyacrylamide gels were processed exactly as described for SDS-PAGE gels, but the proteins were transferred to a PVDF (0.45 μm pore size) Immobilon transfer membrane (Millipore) using a Panther semidry electroblotter (Owl Separation Systems) for 1 h at 45 mA. Each membrane was blocked with 5% bovine serum albumin in TBST for 1 h at room temperature with constant agitation. Next, the membrane was washed twice (5 min each) with TBST (Appendix C). A rabbit polyclonal α -OmpR primary antibody (generous gift from Linda Kenney) was diluted in blocking buffer (1:2000) (Appendix C) and rocked for 1 h at room temperature followed by four washes (5-15 min) in TBST. A goat α -rabbit IgG fluorescein conjugated secondary antibody (Millipore) was diluted in blocking buffer (1:500), added to the membrane, and rocked with the membrane for 1 h at room temperature. After the incubation, the membrane was washed one time with TBST for 15 min followed by four (5 min each) washes with TBST. The OmpR protein bands were visualized using a Storm 860 Phosphorimager (Amersham Biosciences). Relative differences between samples were determined using Image Quant 5.2 software based on pixel density. Three separate sonicates were used and a total of seven independent gels were used to determine the means plus standard deviations. The results were averaged and standard deviations were calculated. A student's t-test was used to determine probabilities and the data was considered significant if $p < 0.05$.

RESULTS

Unphosphorylated and Phosphorylated OmpR Bind Weakly to the *fimB*

Promoter Region Two. Previous β -galactosidase assays showed a derepression of the *fimB* gene expression in a UPEC *ompR* mutant grown in a combined acidic pH/high osmolality environment (101) and purified OmpR shifted the mobility of the *fimB* promoter region (W. R. Schwan, personal communication), suggesting OmpR may be directly repressing *fimB* expression. To determine whether OmpR bound directly to a specific *fimB* promoter, DNase I footprinting assays were performed with DNA that was PCR amplified with primers specific for the *fimB* promoter region two. A sequencing ladder of the *fimB* sequence was used to determine exactly where OmpR/OmpR-P bound on the *fimB* promoter region two (data not shown). Unphosphorylated OmpR weakly bound to a 20-bp region (+2 to -18) (FIG.6) and protected three bases of the -10 box, that included a hypersensitive site (FIG. 6 and FIG. 7). In addition a 24-bp region (+2 to -22) of the *fimB* promoter region two was protected by phosphorylated OmpR (FIG. 5), spanning the entire -10 box plus 17 bases downstream of the -10 box.

Both Forms of OmpR Do Not Bind to the *fimE* Promoter Region. The preliminary β -galactosidase assays also suggested that OmpR-P may be regulating the single *fimE* promoter, so DNase I footprinting of the *fimE* promoter region sequence was undertaken using phosphorylated and unphosphorylated OmpR. As the concentration of

5'GATAATAGCCACTAAGAAATTACTGCGCTCCATGAAATAGC
 CATTTTGTGGCAAATGGAGTTGACTAATAATGTCATATGTGAG
 -35
 ACGGCTAGTTGAACGAATATTAAATTTTGCTGAATTTTTTATGTTG
 -10 * +1 (P₂)
 ATTTT**ACTTGTTACAGAACATATCA**CATGATATATAGATAAGATT
 AGTTGCA-3'

FIG. 6. Schematic representation of phosphorylated OmpR and unphosphorylated OmpR protein DNase I protection of *fimB* promoter region two. Yellow highlighting shows OmpR protection of a 20-bp region. The open box indicates protection by OmpR-P of a 24-bp region. The bold bases designate the FIMB2 DNA sequence. The asterisk represents a site of DNase I hypersensitivity seen only with OmpR protection.

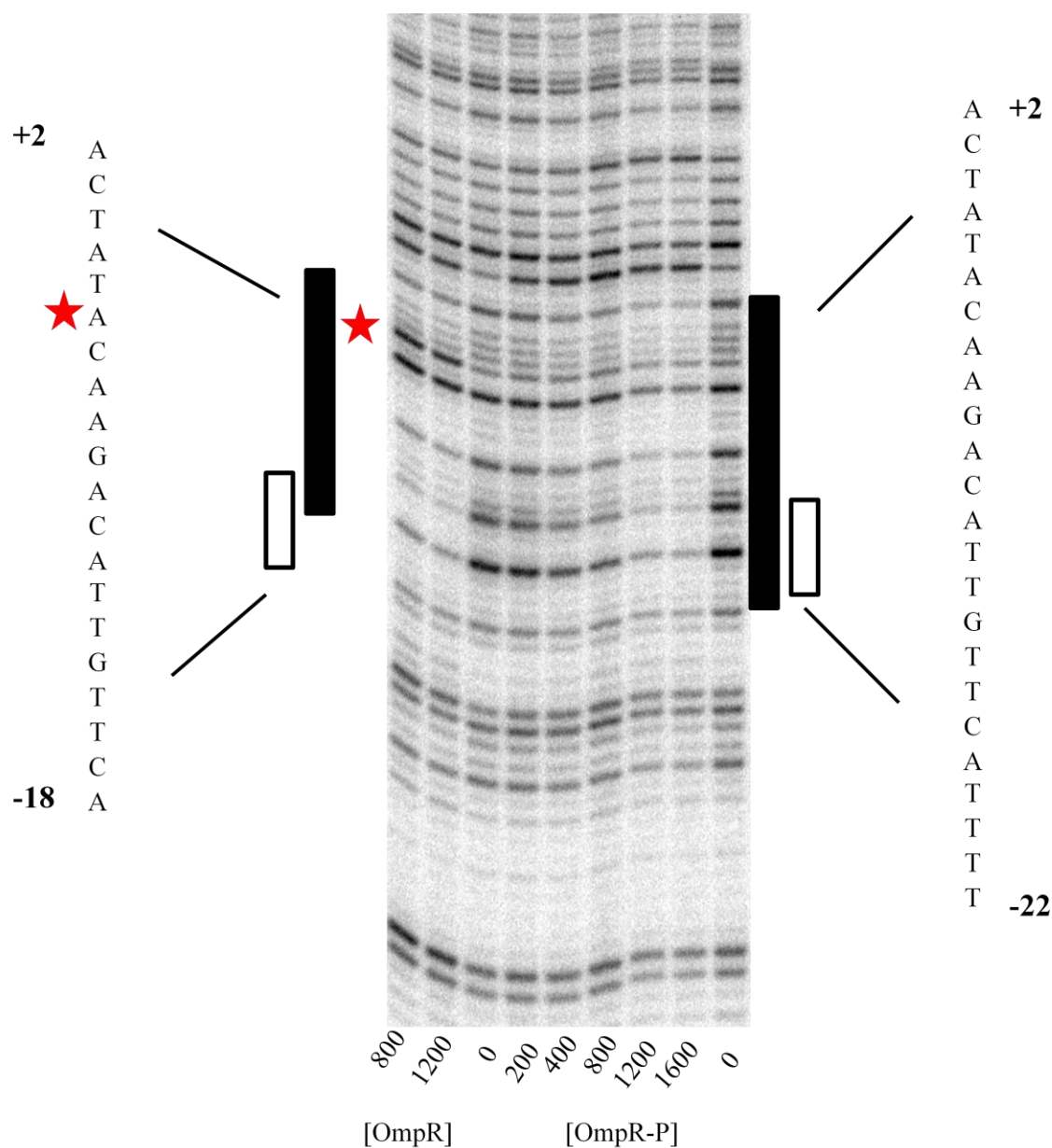


FIG. 7. DNase I footprinting analysis of unphosphorylated (OmpR) and phosphorylated OmpR (OmpR-P) at the *fimB* promoter region two. The concentration (nM) of OmpR and OmpR-P used in each lane is indicated at the bottom. The lanes with '0' indicate the cleavage pattern by DNase I in the absence of OmpR/OmpR-P. The coordinates indicate positions relative to the *fimB* promoter two site. Regions of protection are indicated by black bars for OmpR (+2 to -18) and OmpR-P (+2 to -22) and a hypersensitive site is indicated by a star. White bars represent the -10 box.

phosphorylated or unphosphorylated OmpR was increased from 400 to 1200 nM, progressively more non-specific binding occurred along the DNA of the *fimE* promoter region (FIG. 8). Because there was no specific binding by either OmpR or OmpR-P, these results suggest that OmpR does not directly bind to the *fimE* promoter region.

UPEC *ompR* Transcription is Constant Despite Growth Conditions. Since both OmpR and phosphorylated OmpR bound weakly to one of the *fimB* promoters, we investigated whether the environmental growth conditions may affect *ompR* transcription, which could in turn influence *fimB* regulation. To determine the level of *ompR* transcription, total RNAs were extracted from UPEC NU149 grown in four different media [pH 5.5 (low osmolality), pH 5.5 + 800 mOsm NaCl (high osmolality), pH 7.0 (low osmolality), and pH 7.0 + 800 mOsm NaCl (high osmolality)] and converted to cDNAs. Using reverse-transcriptase PCR (RT-PCR), no discernable difference in *ompR* transcription was observed for the UPEC grown in the four different conditions (FIG. 9).

Since traditional PCR lacks sensitivity, quantitative real-time reverse transcriptase PCR (qRT-RT-PCR) was performed to ascertain differences in *ompR* transcription compared to an internal control, *ftsZ*. After using the $2^{-\Delta\Delta C_T}$ method (65) to normalize the *ompR* transcripts to the *ftsZ* housekeeping gene, no significant difference in *ompR* transcription was found in either logarithmic or stationary phase UPEC cultures grown in the various media (FIG. 10).

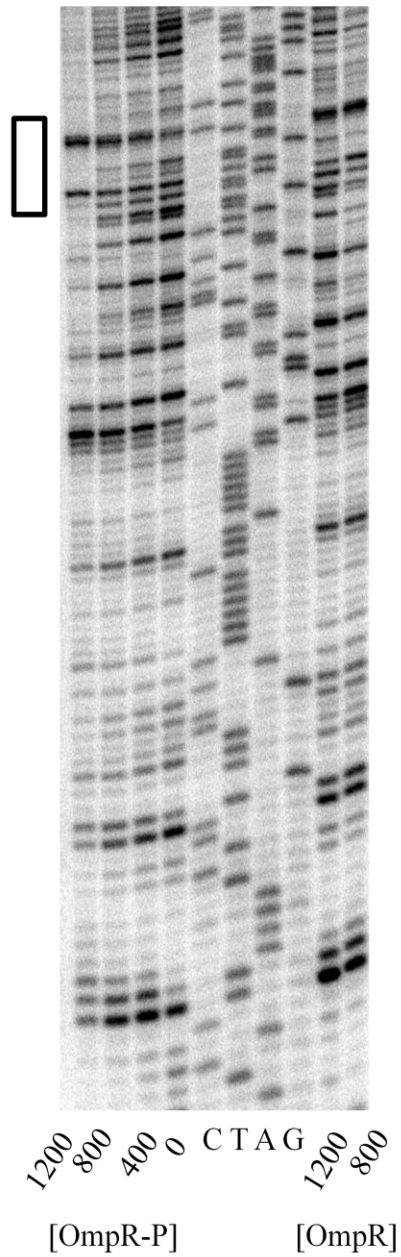


FIG. 8. DNase I footprinting analysis of unphosphorylated and phosphorylated OmpR at the single *fimE* promoter region. The concentration of OmpR or OmpR-P (in nM) used in each lane is indicated at the bottom. The -35 box of the *fimE* promoter region is shown to the left as a white bar. A sequence ladder of the *fimE* promoter region is shown in the middle.

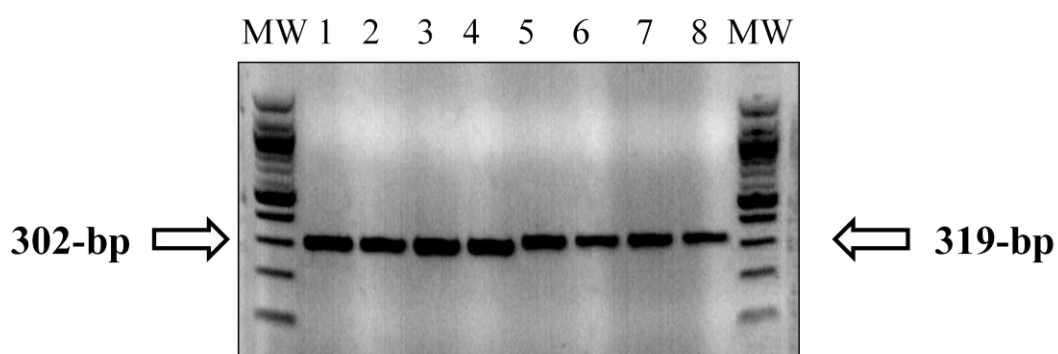
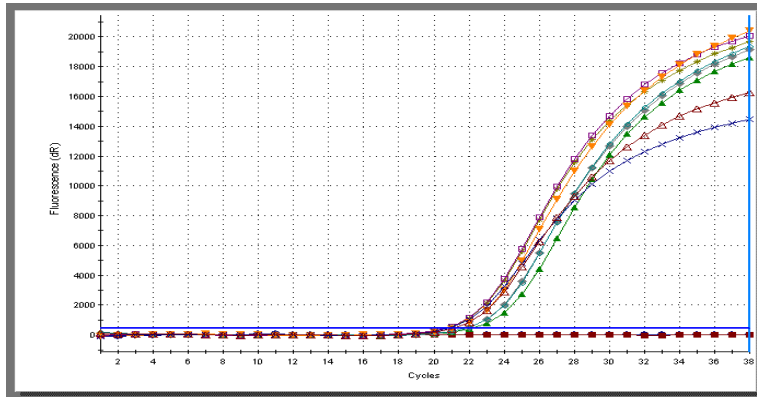


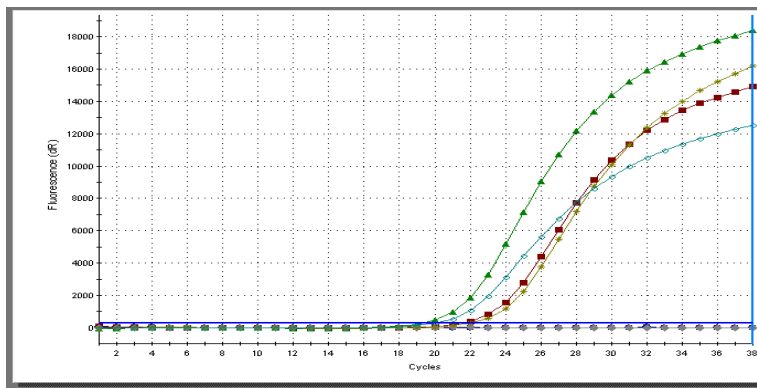
FIG. 9. Qualitative determination of *ftsZ* and *ompR* mRNA regulation by RT-PCR analysis of strain NU149. Cells were grown in pH 5.5 (lanes 1 and 5), pH 5.5 + 800 mOsm NaCl (lanes 2 and 6), pH 7.0 (lanes 3 and 7), and pH 7.0 + 800 mOsm NaCl (lanes 4 and 8). The MW lanes represent a 100-bp ladder. The EcFtsZ1-EcFtsZ2 primer pair was used to amplify cDNAs targeting *ftsZ* (302-bp product). The OmpR3-OmpR4 primer pair was used to amplify a 319-bp *ompR* fragment. All products were electrophoresed on 1.5% agarose gels.

A.



Mean Ct = 21.33

B.



Mean Ct = 20.77

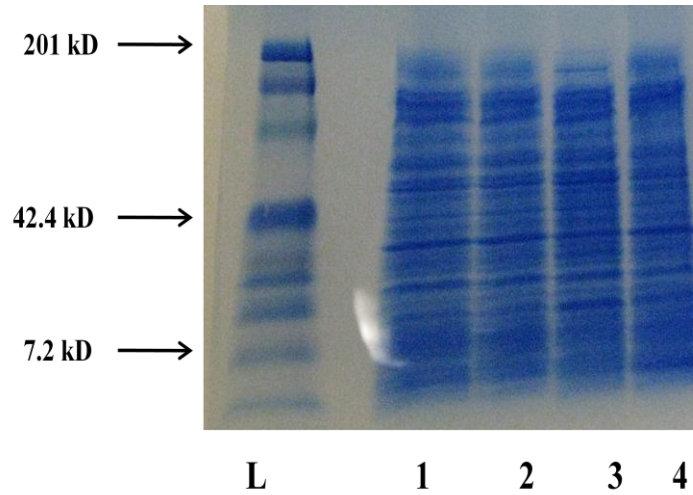
FIG. 10. Quantitative determination of *ftsZ* and *ompR* transcript expression by qRT-RT-PCR. UPEC NU149 cells were grown to A.) mid-logarithmic or B.) stationary-phase growth in four different media. A.) Negative controls are indicated by red closed squares (*ftsZ*) and blue closed circles (*ompR*). Amplification curves of *ftsZ* are orange triangles (pH 5.5 LB), blue x's (pH 5.5 + 800 mOsm NaCl LB), purple squares (pH 7.0 LB), and gray diamonds (pH 7.0 + 800 mOsm NaCl LB). Amplification of *ompR* transcripts are light blue diamonds (pH 5.5), red triangles (pH 5.5 + NaCl), green asterisk (pH 7.0), and green triangle (pH 7.0 + NaCl). B.) Gray triangles (pH 7.0 + 800 mOsm NaCl) and filled blue circles (pH 5.5 + 800 mOsm NaCl) indicate negative controls. Green triangles (pH 7.0 + 800 mOsm NaCl) and yellow asterisks (pH 5.5 + 800 mOsm NaCl) indicate amplification of the *ftsZ* gene. Open blue circles (pH 7.0 + 800 mOsm NaCl) and red squares (pH 5.5 + 800 mOsm NaCl) indicate amplification of the *ompR* gene. The mean of the crossing thresholds are shown to the left of the amplification plots.

OmpR Protein Level is Increased When UPEC are Grown in a Combined Acidic pH/ High Osmolality Medium. Although *ompR* transcription did not differ among UPEC NU149 grown in the four growth conditions, post-transcriptional regulation could affect OmpR protein levels, which could then have an effect on *fimB* transcription. To determine whether there was a difference in OmpR protein levels from cultures from each growth condition, SDS-PAGE and Western blot analyses were performed on stationary phase UPEC NU149 cell lysates. First, 10 µg of total protein was added in each lane and shown to be equivalent for each growth condition cell lysate by performing SDS-PAGE followed by Coomassie Blue stain (FIG. 11A).

To determine if OmpR protein levels varied when grown in different environments, strain NU149 cell lysates were separated by SDS-PAGE and probed with polyclonal rabbit anti-OmpR antibody. Purified histidine-tagged OmpR protein reacted well with the antibody (FIG. 11B). OmpR protein concentration, regardless of osmolality, was greater when the bacterial cells were grown in an acidic medium versus a neutral medium (FIG. 10B). However, there was no significant difference in OmpR protein expression when cells were grown in pH 5.5 (low osmolality) medium versus pH 5.5 with 800 mOsm NaCl (high osmolality) ($p < 0.67$). When NaCl was added to the broth medium, there was a significant (2.13-fold) increase in OmpR protein expression in pH 5.5 plus 800 mOsm NaCl medium (36,600 pixels) compared to pH 7.0 plus 800 mOsm NaCl medium (17,164 pixels) ($p < 0.017$) (TABLE 2). In addition, there was a significant (1.95-fold) increase in OmpR protein expression when NU149 cells grown in pH 5.5 medium (33,475 pixels) were compared to the same strain grown in the pH 7.0 plus 800

mOsm NaCl medium (17,164 pixels) ($p < 0.00042$) (TABLE 2). Although OmpR expression in strain NU149 grown in pH 5.5 medium was slightly higher than the OmpR protein levels in UPEC cells grown in pH 7.0, the difference was not significant ($p < 0.19$). However, OmpR protein expression from cells grown in a pH 7.0 (27,740 pixels) environment was significantly higher (1.6-fold) compared to UPEC cells grown in pH 7.0 with 800 mOsm NaCl (17,164 pixels) ($p < 0.014$) (TABLE 2). These results demonstrate that UPEC grown *in vitro* in an acidic/high osmolality environment express more OmpR protein than cells grown in a neutral pH/high osmolality environment.

A.



B.

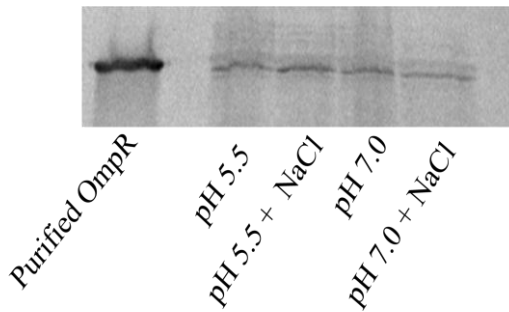


FIG. 11. Comparison of OmpR protein expression in UPEC NU149 cells grown in different environments. A.) An SDS-PAGE gel of protein extracts from NU149 grown cells grown in pH 5.5 LB (lane 1), pH 5.5 + 800 mOsm NaCl LB (lane 2), pH 7.0 LB (lane 3), pH 7.0 + 800 mOsm NaCl LB (lane 4). A Kaleidoscope protein standard (L) was used as a reference. B.) An immunoblot of the same protein extracts from NU149 cells grown in four different environments probed with polyclonal rabbit anti-OmpR antibody. Purified OmpR protein (molecular weight 23.9 kD) is shown on the far left.

TABLE 2. Western blot analyses of OmpR protein levels measured by pixelation in UPEC NU149 grown in four different environments

Growth Condition	Pixelation ^a
pH 5.5 LB	33,475 $\pm$ 7,264
pH 5.5 + 800 mOsm NaCl LB	36,600 $\pm$ 17,774
pH 7.0 LB	27,740 $\pm$ 8,214
pH 7.0 + 800 mOsm NaCl LB	17,164 $\pm$ 5,201

^a Average pixelation $\pm$ standard deviation based on 7 independent Western blots.

DISCUSSION

Type 1 piliated UPEC cells attach to host bladder epithelial cells. However, when UPEC ascend into the kidneys they become nonpiliated (95). The ability of UPEC to change reversibly between being piliated and nonpiliated depends on the orientation of the *fim* switch. This *fim* switch is mediated by two site-specific recombinases, FimB and FimE. The orientation of the *fim* switch has been shown to change in response to changing medium pH and osmolality (101). *E. coli* survive these differing pH/osmotically stressed environments by utilizing the EnvZ/OmpR system. Previous studies have shown OmpR is essential for UPEC growth within the murine urinary tract (98) and an *ompR* mutant showed derepression of the *fimB* gene in an acidic/high osmolality growth condition compared to wild-type (101). Because of this indirect link to OmpR, the purpose of this study was to determine if OmpR bound to and potentially regulated either *fim* recombinase gene. Using DNase I footprinting, this study is the first to show any degree of OmpR binding to a *fim* promoter region.

Previously, other *E. coli* genes have been shown to be regulated by OmpR (82). Several studies demonstrated OmpR/OmpR-P binding upstream of the -35 and -10 boxes of the *ompF* and *ompC* promoter regions (40, 66, 106). OmpR binding to a 20-bp region in the *ompF* promoter region has been previously observed (39, 44), and a loose

consensus sequence containing an AC at positions 1,2 and 11,12 with a central GXXXC sequence has been proposed for OmpR (40).

Our results show OmpR and OmpR-P protected 20-bp and 24-bp sites at the *fimB* promoter two, respectively. Both of the protected regions spanned the -10 box of the *fimB* two promoter. Within these protected regions, a putative OmpR binding motif, 5'-ACTTGTTACAGAAC-3', sequence with an AC at positions 1, 2 as well as an internal GTTAC sequence is found (FIG. 6). It is possible that even though the weak OmpR/OmpR-P binding within the *fimB* promoter two region did not exactly match the proposed high affinity sequence for *ompF* and *ompC*, it may allow low affinity OmpR binding and repression of the *fimB* gene.

Previous studies by Cai *et al.* have indicated that at low osmolality only 3.5% (120 nM or 70 OmpR-P molecules/cell) of total OmpR protein (2024 molecules of OmpR per cell) would be sufficient for promoting *ompF* transcription (11). In a high osmolality medium, 10% (580 nM or 350 OmpR-P molecules/cell) of total OmpR molecules in a cell (3500 molecules of OmpR per cell) would allow repression of the *ompF* gene and stimulation of the *ompC* gene (11). Our *fimB* footprint showed protection at 1600 nM (FIG. 7), which equates to 933 OmpR molecules in a cell, or 27% of total OmpR protein present in a high osmolality environment, demonstrating that our results are well within the physiological range OmpR protein present within *E. coli* growing in a high osmolality environment. Whether OmpR binds to either of the two remaining *fimB* promoters remains unknown and will serve as an area of further exploration.

Conversely, neither form of OmpR bound to the single *fimE* promoter region. A previous group created *E. coli envZ/ompR* deletion mutants and observed 125 other genes to have been affected by this change compared to the wild-type (82), suggesting that the EnvZ/OmpR system regulates a wide variety of genes. Cross-regulation between the histidine kinase, ArcB, and the EnvZ/OmpR system has been demonstrated (67). It was hypothesized that under anaerobic conditions ArcB together with OmpR could regulate the porin genes. It is possible that OmpR indirectly regulates *fimE* expression by acting through an intermediary protein, similar to ArcB, which in turn acts on *fimE*. Our results demonstrate non-specific binding with 1600 nM OmpR-P (FIG. 8), but no binding at lesser concentrations that suggest the DNA was saturated with OmpR protein. This phenomenon can happen when so much protein is added to the DNA, which at some point, because of the protein's DNA-binding nature, will bind to DNA, but will not be considered physiologically relevant. Conversely, this 'OmpR stampede' was not seen with any of the protection sites on the *fimB* footprint. Another explanation for the lack of protection of the *fimE* promoter region by OmpR, is that there is no correlation between *fimE* and OmpR.

Since OmpR bound weakly to the *fimB* promoter region two, we investigated whether there might be a transcriptional or translational change that could in turn affect the levels of OmpR when UPEC is grown in different environments. Our hypothesis was that in an acidic/high osmolality environment there may be a greater concentration of OmpR present. This increase in OmpR protein could lead to low affinity binding to the *fimB* promoter and directly regulate *fimB* transcription. However, the RT-PCR and RT-

RT-PCR analyses of *ompR* showed no difference in *ompR* transcript levels in strain NU149 grown in the four growth conditions we have used in a previous study (101). Thus, transcriptional changes in *ompR* were not responsible for *fimB* regulation.

A prior study showed *envZ* and *ompR* are co-transcribed, but 35 times more OmpR protein is present compared to EnvZ in a single *E. coli* cell (11). This difference between the *ompR* and *envZ* genes was presumably because of differences in translational initiation (11). Based on this previous study and our transcriptional assays, we hypothesized that OmpR could be regulated by a similar mechanism. The Western blots indicated OmpR protein expression was overall greater when UPEC cells were grown in acidic conditions versus a neutral pH, however the increase was not statistically significant. Even more interesting was a two-fold increase in OmpR protein when the UPEC cells were grown in an acidic/high osmolality medium versus UPEC grown in a neutral pH/high osmolality medium. These results suggest a possible role for OmpR in acidic/high osmolality environments such as the human kidneys.

Prior studies have indicated that OmpR protein expression in *Salmonella* and enterohaemorrhagic *E. coli* increased after acid shock or growth in an acidic environment compared to a neutral pH environment (6, 45). These prior studies provide evidence that OmpR protein is important for survival in low pH environments. Previously, wild-type *E. coli* grown in a high osmolality environment (added sucrose) had a 1.7-fold increase in OmpR protein as compared with the same cells grown in a low osmolality medium (11), so there is precedence for OmpR protein level changes which in turn could affect *E. coli* gene regulation.

From this work, we have demonstrated that there appears to be more OmpR available in an environment that is similar to the murine urinary tract (acidic/high osmolality) to repress *fimB* transcription. A previous study demonstrated that in an acidic/high osmolality environment UPEC cells expressed more *fimE* than *fimB* transcripts causing the invertible element to be in primarily in the Phase-OFF orientation (101). Moreover, high osmolality conditions (eg. murine or human kidneys) have been shown to promote phosphorylation of OmpR (55) and OmpR-P binds with greater affinity to DNA (68). Greater concentrations of OmpR and OmpR-P in UPEC growing in kidney urine would allow OmpR-P to directly bind first to the higher affinity sites on the *fimB* promoter region. The excess of OmpR or OmpR-P subsequently would bind to lower affinity sites on *fimB* similar to the binding affinity that leads to the regulation of the *ompF* gene (44, 86, 87). With more OmpR and OmpR-P binding to the *fimB* promoter region, *fimB* transcription would be repressed, thereby increasing the cellular ratio of FimE to FimB (FIG. 12). In turn, FimE would bind to the invertible element and switch it to the Phase-OFF orientation resulting in nonpiliated bacteria in the kidneys observed in previous studies (46, 95).

Due to the fact that type 1 pili are highly immunogenic (73, 84) and the synthesis of pili is a vast energy expenditure, it is logical that type 1 pili production may be tightly regulated. Thus, the ability to phase-vary type 1 pilus expression serves UPEC well when residing in the urinary tract. In the bladder, the urine is less acidic and has a lower level of osmolytes as compared to kidney urine (92). In murine and human urine, sodium and chloride molecules represent the second and third most common osmolytes (92). In

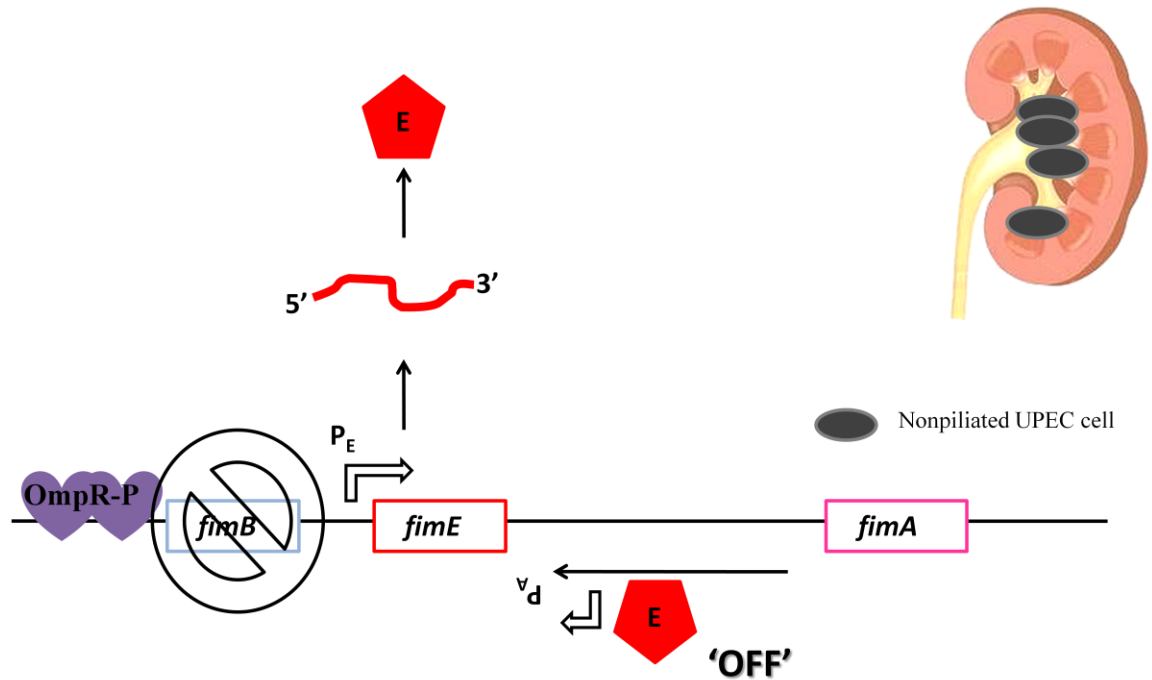


FIG. 12. A proposed model for OmpR regulation of the *fimB* gene when UPEC cells are growing in an acidic/high osmolality environment similar to a kidney. In this model, OmpR-P would bind at least one promoter region of *fimB* repress the levels of FimB recombinase being made. While the OmpR repression may not completely negate *fimB* transcription, it would still be enough to increase the cellular concentration of FimE in the cell. FimE protein would bind to the invertible element and lock it in the Phase-OFF orientation resulting in non-piliated UPEC cells residing in the kidneys.

addition, the bladder contains many more mannose-containing receptors making it easier for type 1 piliated UPEC cells to bind to bladder epithelium during a bladder infection. When UPEC ascend into the kidneys, they enter a more acidic/high osmolality environment with less mannose-containing receptors. In this environment, host antibody specific for type 1 pili on UPEC cells can bind and activate host phagocytes (85). Therefore, energy conservation (few mannose-containing receptors, few type 1 pili) and being able to avoid engulfment by phagocytic cells (with the presence of capsule) make it advantageous for UPEC cells to be nonpiliated in the kidneys.

REFERENCES

1. **Abraham, S. N., D. Sun, J. B. Dale, and E. H. Beachey.** 1988. Conservation of the D-mannose-adhesion protein among type 1 fimbriated members of the family *Enterobacteriaceae*. *Nature*. **336**:682-684.
2. **Abraham, S. N., J. P. Babu, C. S. Gainpapa, D. L. Hasty, W. A. Simpson, and E. H. Beachey.** 1985. Protection against *Escherichia coli*-induced urinary tract infections with hybridoma antibodies directed against type 1 fimbriae or complementary D-mannose receptors. *Infect. Immun.* **48**:625-628.
3. **Adiciptaningrum, A. M., I. C. Blomfield, and S. J. Tans.** 2009. Direct observation of type 1 fimbrial switching. *EMBO Reports*. **10**:527-532.
4. **Bacheller, C. D, and J. M. Bernstein.** 1997. Urinary tract infections. *Med. Clin. N. Am.* **81**:719-730.
5. **Bang, I. S., J. P. Audia, Y. K. Park, and J. W. Foster.** 2002. Autoinduction of the OmpR response regulator by acid shock and control of the *Salmonella enterica* acid tolerance response. *Mol. Microbiol.* **44**:1235-1250.
6. **Bang, I. S., B. H. Kim, J. W. Foster, and Y. K. Park.** 2000. OmpR regulates the stationary-phase acid tolerance response of *Salmonella enterica* serovar *Typhimurium*. *J. Bacteriol.* **182**:2245-2252.
7. **Blomfield, I. C., P. J. Calie, K. J. Eberhardt, M. S. McClain, and B. I. Eisenstein.** 1993. Lrp stimulates phase variation of type 1 fimbriation in *Escherichia coli* K-12. *J. Bacteriol.* **175**:27-36.
8. **Blomfield, I. C., M. S. McClain, J. A. Princ, P. J. Calie, and B. I. Eisenstein.** 1991. Type 1 fimbriation and *fimE* mutants of *Escherichia coli* K-12. *J. Bacteriol.* **173**:5298-5307.
9. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248-252.

10. **Brinton, C. C., Jr.** 1959. Non-flagellar appendages of bacteria. *Nature (London)* **183**:782-786.
11. **Cai, S. J. and M. Inouye.** 2002. EnvZ-OmpR interaction and osmoregulation in *Escherichia coli*. *J. Biol. Chem.* **277**:24155-24161.
12. **Delgado, J., S. Forst, S. Harlocker, and M. Inouye.** 1993. Identification of a phosphorylation site and functional analysis of conserved aspartic residues of OmpR, a transcriptional activator for *ompF* and *ompC* in *Escherichia coli*. *Mol. Microbiol.* **10**:1037-1047.
13. **Donato, G. M., and T. H. Kawula.** 1999. Phenotypic analysis of random *hns* mutations differentiate DNA-binding activity from properties of *fimA* promoter inversion modulation and bacterial motility. *J. Bacteriol.* **181**:941-948.
14. **Donato, G. M., M. J. Lelivelt, and T. H. Kawula.** 1997. Promoter-specific repression of *fimB* expression by the *Escherichia coli* nucleoid-associated protein H-NS. *J. Bacteriol.* **179**:6618-6625.
15. **Dorman, C. J.** 2004. H-NS: A universal regulator for a dynamic genome. *Nat. Rev. Microbiol.* **2**:391-400.
16. **Dorman, C. J., and N. N. Bhriain.** 1992. Thermal regulation of *fimA*, the gene coding form the type 1 fimbrial subunit protein. *FEMS Micobiol. Lett.* **99**:125-130.
17. **Dorman, C. J., and C. P. Corcoran.** 2009. Bacterial DNA topology and infectious disease. *Nucleic Acids Res.* **37**:672-678.
18. **Dorman, C. J., and C. F. Higgins.** 1987. Fimbrial phase variation in *Escherichia coli*: dependence on integration host factor and homologies with other site-specific recombinases. *J. Bacteriol.* **169**:3840-3843.
19. **Dove, S.L., and C. J. Dorman.** 1996. Multicopy *fimB* gene expression in *Escherichia coli*: binding to inverted repeats *in vivo*, effect on *fimA* gene transcription and inversion. *Mol. Microbiol.* **21**:1161-1173.
20. **Drekonja, D. M., and J. R. Johnson.** 2008. Urinary tract infections. *Prim. Care.* **35**:345-367.
21. **Duguid, J. P., and D. C. Old.** 1980. Adhesive properties of *Enterobacteriaceae*, p. 185-217. *In* E. H. Beachey (ed.), *Bacterial adherence. Receptors and recognition*, series B. vol. 6. Chapman and Hall, London.

22. **Duguid, J. P., E. S. Anderson, and I. Campbell.** 1966. Fimbrial and adhesive properties in salmonellae. *J. Pathol. Bacteriol.* **92**:107-138.
23. **Eisenstein, B. I.** 1981. Phase variation of type 1 fimbriae in *Escherichia coli* is under transcriptional control. *Science* **214**:337-339.
24. **Eisenstein, B. I., and D. C. Dodd.** 1982. Pseudocatabolite repression of type 1 fimbriae of *Escherichia coli*. *J. Bacteriol.* **151**:1560-1567.
25. **Eisenstein, B. I., E. H. Beachey, and S. S. Solomon.** 1981. Divergent effects of cyclic adenosine 3'5'-monophosphate on formation of type 1 fimbriae in different K-12 strains of *Escherichia coli*. *J. Bacteriol.* **145**:620-623.
26. **Eisenstein, B. I., D. S. Sweet, V. Vaughn, and D. I. Freidman.** 1987. Integration host factor is required for the DNA inversion that controls phase variation in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA.* **84**:6506-6510.
27. **El-Labany, S., B. K. Sohanpal, M. Lahooti, R. Ackerman, and I. C. Blomfield.** 2003. Distant cis-active sequences and sialic acid control the expression of *fimB* in *Escherichia coli* K-12. *Mol. Microbiol.* **49**:1109-1118.
28. **Feng, X., Walthers, D., Oropeza, R., and L. J. Kenney.** 2004. The response regulator SsrB activates transcription and binds to a region overlapping OmpR binding sites at *Salmonella* pathogenicity island 2. *Mol. Microbiol.* **54**:823-835.
29. **Foxman, B.** 2003. Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. *Dis. Mon.* **49**:53-70.
30. **Foxman, B., R. Barlow, H. D'Acry, B. Gillespie, and J.D. Sobel.** 2000. Urinary tract infection: self reported incidence and associated costs. *Ann. Epidemiol.* **10**:509-515.
31. **Foxman, B., B. Gillespie, J. Koopman, L. Zhang, K. Palin, P. Tallman, J. V. Marsh, S. Spear, J. D. Sobel, M. J. Marty, and C. F. Marrs.** 2000. Risk factors for second urinary tract infection among college women. *Am. J. Epidemiol.* **151**:1194-1205.
32. **Freitag, C. S., J. M. Abraham, J. R. Clements, and B. I. Eisenstein.** 1985. Genetic analysis of the phase variation control of expression of type 1 fimbriae in *Escherichia coli*. *J. Bacteriol.* **162**:688-675.
33. **Gally, D. L., T. J. Rucker, and I. C. Blomfield.** 1994. The leucine-responsive regulatory protein binds to the *fim* switch to control phase variation of type 1 fimbrial expression in *Escherichia coli* K-12. *J. Bacteriol.* **176**:5665-5672.

34. **Gally, D. L., J. A. Bogan, B. I. Eisenstein, and I. C. Blomfield.** 1993. Environmental regulation of the *fim* switch controlling type 1 fimbrial phase variation in *Escherichia coli* K-12: effects of temperature and media. *J. Bacteriol.* **175**:6186-6193.
35. **Garofalo, C. K., T. M. Hooten, S. M. Martin, W. E. Stamm, J. J. Palermo, J. I. Gordon, and S. J. Hultgren.** 2007. *Escherichia coli* from urine of female patients with urinary tract infections is competent for intracellular bacterial community formation. *Infect. Immun.* **75**:52-60.
36. **Gossert, A. D., P. Bettendorff, C. Puorger, M. Vetsch, T. Herrmann, R. Glockshuber, and K. Wüthrich.** 2008. NMR structure of the *Escherichia coli* type 1 pilus subunit FimH and its interactions with other pilus subunits. *J. Mol. Biol.* **375**:752-763.
37. **Guay, D. R. P.** 2008. Contemporary management of uncomplicated urinary tract infections. *Drugs.* **68**:1169-1205.
38. **Hahn, E., P. Wild, U. Hermanns, P. Sebbel, R. Glockshuber, M. Häner, N. Taschner, P. Burkhard, U. Aebi, and S. A. Müller.** 2002. Exploring the 3D molecular architecture of *Escherichia coli* type 1 pili. *J. Mol. Biol.* **323**:845-857.
39. **Harlocker, S. L., L. Bergstrom, and M. Inouye.** 1995. Tandem binding of six OmpR proteins to the *ompF* upstream regulatory sequence of *Escherichia coli*. *J. Biol. Chem.* **270**:26849-26856.
40. **Head, C. G., A. Tardy, and L. J. Kenney.** 1998. Relative binding affinities of OmpR and OmpR-phosphate at the *ompF* and *ompC* regulatory sites. *J. Mol. Biol.* **281**:857-870.
41. **Higgins, C. F., C. J. Dorman, D. A. Stirling, L. Waddell, I. R. Booth, G. May, and E. Bremer.** 1988. A physiological role for DNA supercoiling in the osmotic regulation of gene expression in *S. typhimurium* and *E. coli*. *Cell.* **52**:569-584.
42. **Hooton, T. M., and W. E. Stamm.** 1997. Diagnosis and treatment of uncomplicated urinary tract infection. *Infect. Dis. Clin. North Amer.* **11**:551-581.
43. **Hu, Y., P. Lu, Y. Wang, L. Ding, S. Atkinson, and S. Chen.** 2009. OmpR positively regulates urease expression to enhance acid survival of *Yersinia pseudotuberculosis*. *Microbiol.* **155**:2522-2531.
44. **Huang, K. and M. M. Igo.** 1996. Identification of the bases in the *ompF* regulatory region, which interact with the transcription factor OmpR. *J. Mol. Biol.* **262**:615-628.

45. **Huang, Y. J., T. Y. Tsai, and T. M. Pan.** 2007. Physiological response and protein expression under acid stress of *Escherichia coli* O157:H7 TWC01 isolated from Taiwan. *J. Agric. Food Chem.* **55**:7182-7191.
46. **Hultgren, S. J., T. N. Porter, A. J. Schaeffer, and J. L. Duncan.** 1985. Role of type 1 pili and effects of phase variation on lower urinary tract infections produced by *Escherichia coli*. *Infect. Immun.* **50**:370-377.
47. **Hultgren, S. J., W. R. Schwan, A. J. Schaeffer, and J. L. Duncan.** 1986. Regulation of production of type 1 pili among urinary tract isolates of *Escherichia coli*. *Infect. Immun.* **54**:613-620.
48. **Igo, M. M., and T. J. Silhavy.** 1988. EnvZ, a transmembrane environmental sensor of *E. coli* K-12 is phosphorylated *in vitro*. *J. Bacteriol.* **170**:5971-5973.
49. **Ikaheimo, R., A. Siitonen, T. Heiskanen, U. Karkkainen, P. Kuosmanen, P. Lipponen, P. H. Makela.** 1996. Recurrence of urinary tract infection in a primary care setting: analysis of a 1-year follow-up of 179 women. *Clin. Infect. Dis.* **22**:91-99.
50. **Jones, C. H., J. S. Pinkner, A. V. Nicholes, L. N. Slonim, S. N. Abraham, and S. J. Hultgren.** 1993. FimC is a periplasmic PapD-like chaperone that directly assembly of type 1 pili in bacteria. *Proc. Natl. Acad. Sci.* **90**:8397-8401.
51. **Justice, S. S., D. A. Hunstad, P. C. Seed, S. J. Hultgren.** 2006. Filamentation by *Escherichia coli* subverts innate defenses during urinary tract infections. *Proc. Natl. Acad. Sci.* **103**:19884-19889.
52. **Kahlmeter, G.** 2003. An international survey of the antimicrobial susceptibility of pathogens from uncomplicated urinary tract infections: the ECO.SENS Project. *J. Antimicrob. Chemother.* **51**:69-76.
53. **Kannan, G., J. C. Wilks, D. M. Fitzgerald, B. D. Jones, S. S. BonDurant, and J. L. Slonczewski.** 2008. Rapid acid treatment of *Escherichia coli*: transcriptomic response and recovery. *BMC Microbiol.* **8**:37-50.
54. **Kawula, T. H., and P. E. Orndorff.** 1991. Rapid site-specific DNA inversion in *Escherichia coli* mutants lacking the histone-like protein H-NS. *J. Bacteriol.* **173**:4116-4123.
55. **Kenney, L. J., M. D. Bauer, and T. J. Silhavy.** 1995. Phosphorylation-dependent conformational changes in OmpR, an osmoregulatory DNA-binding protein of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **92**:8866-8870.

56. **Klemm, P.** 1986. Two regulatory *fim* genes, *fimB* and *fimE*, control the phase variation of type 1 fimbriae in *Escherichia coli*. EMBO J. **5**:1389-1393.
57. **Krogfelt, K. A., H. Bergmans, and P. Klemm.** 1990. Direct evidence that the FimH protein is the mannose-specific adhesion of *Escherichia coli* type 1 fimbriae. Infect. Immun. **58**:1995-1998.
58. **Kuehn, M. J., S. Normark, and S. J. Hultgren.** 1991. Immunoglobulin-like PapD chaperone caps and uncaps interactive surfaces of nascently translocated pilus subunits. Proc. Natl. Acad. Sci. **88**:10586-10590.
59. **Kunin, C. M.** 1994. Urinary tract infections in females. Clin. Infect. Dis. **18**:1-10.
60. **Kunin, C. M., and S. T. Chambers.** 1989. Osmoprotective properties for bacteria of renal papilla and urine: role of betaines as osmoprotectant molecules, p. 327-332. In E. Kass and C Svanborg Eden (ed.), Host-parasite interactions in urinary tract infections. University of Chicago Press, Chicago, IL.
61. **Kunin, C. M., T. H. Hua, R. L. Guerrant, and L. O. Bakaletz.** 1994. Effect of salicylate, bismuth, osmolytes, and tetracycline resistance on expression of fimbriae by *Escherichia coli*. Infect. Immun. **62**:2178-2186.
62. **Lane, M.C., X. Li, M. M. Pearson, A. N. Simms, and H. L. T. Mobley.** 2009. Oxygen-limiting conditions enrich for fimbriate cells of uropathogenic *Proteus mirabilis* and *Escherichia coli*. J. Bacteriol. **191**:1382-1392.
63. **Lisitsyn, N., N. Lisitsyn, and M. Wigler.** 1993. Cloning the differences between two complex genomes. Science. **259**:946-951.
64. **Liu, Y., H. Chen, L. J. Kenney, and J. Yan.** 2010. A divalent switch drives H-NS/DNA-binding conformations between stiffening and bridging modes. Genes Dev. **24**:339-344.
65. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods. **25**:402-408.
66. **Maeda, S. Y. Ozawa, T. Mizuno, and S. Mizushima.** 1988. Stereospecific positioning of the cis-acting sequence with respect to the canonical promoter is required for activation of the *ompC* gene by a positive regulator, OmpR, in *Escherichia coli*. 202:433-441.

67. **Matsubara, M., S. I. Kitaoka, S. I. Takeda, and T. Mizuno.** 2000. Tuning of the porin expression under anaerobic growth conditions by his-to-Asp cross-phosphorelay through both the EnvZ-osmosensor and ArcB-anaerosensor in *Escherichia coli*. *Genes Cells* **5**:555-569.
68. **Mattison, K. and L.J. Kenney.** 2002. Phosphorylation alters the interaction of the response regulator OmpR with its sensor kinase EnvZ. *J. Biol. Chem.* **277**:11143-11148.
69. **Martinez, J. J., M. A. Mulvey, J. D. Schilling, J. S. Pinkner, and S. J. Hultgren.** 2000. Type 1 pilus-mediated bacterial invasion of bladder epithelial cells. *EMBO J.* **19**:2803-2812.
70. **McClain, M. S., I. C. Blomfield, and B. I. Eisenstein.** 1991. Roles of *fimB* and *fimE* in site-specific DNA inversion associated with phase variation of type 1 fimbriae in *Escherichia coli*. *J. Bacteriol.* **173**:5308-5314.
71. **McClain, M. S., I. C. Blomfield, K. J. Eberhardt, and B. I. Eisenstein.** 1993. Inversion-independent phase variation of type 1 fimbriae in *Escherichia coli*. *J. Bacteriol.* **175**:4335-4344.
72. **Müller, C. M., A. Åberg, J. Straseviciene, L. Emödy, B. E. Uhlin, and C. Balsalobre.** 2009. Type 1 fimbriae, a colonization factor of uropathogenic *Escherichia coli*, are controlled by the metabolic sensor CRP-cAMP. *PLOS. Pathog.* **5**(2):e1000303.
73. **Mulvey, M. A., J. D. Schilling, J. J. Martinez, and S. J. Hultgren.** 2000. Bad bugs and beleaguered bladders: interplay between uropathogenic *Escherichia coli* and innate host defense. *PNAS.* **97**:8829-8835.
74. **Munera, D., C. Palomino, and L.A. Fernández.** 2008. Specific residues in the N-terminal domain of FimH stimulate type 1 fimbriae assembly in *Escherichia coli* following the initial binding of the adhesion to FimD usher. *Mol. Microbiol.* **69**:911-925.
75. **Nataro, J. P., C. A. Bopp, P. I. Fields, J. B. Kaper, and N. A. Strockbine.** 2007. *Escherichia*, *Shigella*, and *Salmonella*. p. 670-687. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of clinical microbiology*, 9th ed., vol. 1. ASM Press, Washington D. C.
76. **Nowicki, B., M. Rhen, V. Vaisanen-Rhen, A. Pere, and T. K. Korhonen.** 1984. Immunofluorescence study of fimbrial phase variation in *Escherichia coli* KS71. *J. Bacteriol.* **160**:691-695.

77. **Ofek, I., and E. H. Beachey.** 1978. Mannose binding and epithelial cell adherence of *Escherichia coli*. *Infect. Immun.* **22**:247-254.
78. **O’Gara, J. P., and C. J. Dorman.** 2000. Effects of local transcription and H-NS on inversion of the *fim* switch of *Escherichia coli*. *Mol. Microbiol.* **181**:941-948.
79. **Old, D. C.** 1972. Inhibition of the interaction between fimbrial hemagglutinins and erythrocytes by D-mannose and other carbohydrates. *J. Gen. Microbiol.* **71**:149-157.
80. **Old, D. C., and J.P. Duguid.** 1970. Selective outgrowth of fimbriae bacteria in static liquid medium. *J. Bacteriol.* **103**:447-456.
81. **Olsen, P. B., and P. Klemm.** 1994. Localization of promoters in the *fim* gene cluster and the effect of H-NS on the transcription of *fimB* and *fimE*. *FEMS Microbiol. Lett.* **116**:95-100.
82. **Oshima, T. H., Aiba, Y. Masuda, S. Kanaya, M. Sugiura, B. L. Wanner, H. Mori, and T. Mizuno.** 2002. Transcriptome analysis of all two-component regulatory system mutants of *Escherichia coli* K-12. *Mol. Microbiol.* **46**:281-291.
83. **Pallesen, L., O. Madsen, and P. Klemm.** 1989. Regulation of the phase switch controlling expression of type 1 fimbriae in *Escherichia coli*. *Mol. Microbiol.* **3**:925-931.
84. **Peerayeh. S. N., N. Nooritalab, M. Moazeni, and M. Sattari.** 2007. Influence of type 1 piliation on chemotaxis and intracellular killing of uropathogenic *E. coli* by human polymorphonuclear leukocytes. *Pak. J. Biol. Sci.* **10**:2599-2602.
85. **Perry, A., I. Ofek, and F. J. Silverblatt.** 1983. Enhancement of mannose-mediated stimulation of human granulocytes by type 1 fimbriae aggregated with antibodies on *Escherichia coli* surfaces. *Infect. Immun.* **39**:1334-1345.
86. **Qin, L., T. Yoshida, and M. Inouye.** 2001. The critical role of DNA in the equilibrium between OmpR and phosphorylated OmpR mediated by EnvZ in *Escherichia coli*. *Proc. Natl. Acad. Sci.* **98**:908-913.
87. **Rampersaud, A., S. L. Harlocker, and M. Inouye.** 1994. The OmpR protein of *Escherichia coli* binds to sites in the *ompF* promoter region in a hierarchical manner determined by its degree of phosphorylation. *J. Biol. Chem.* **269**:12559-12566.
88. **Roberts, F. J., I. W. Geere, and A. Coldman.** 1991. A three-year study of positive blood cultures, with emphasis on prognosis. *Rev. Infect. Dis.* **13**:34-46.

89. **Ronald, A.** 2003. The etiology of urinary tract infection: traditional and emerging pathogens. *Dis. Mon.* **49**:71-82.
90. **Rosen, D. A., T. M. Hooten, W. E. Stamm, P. A. Humphrey, and S. J. Hultgren.** 2007. Detection of intracellular bacterial communities in human urinary tract infection. *PLoS Medicine*. **4**:1949-1958.
91. **Rosenstein, I. J., D. Grady, J. M. T. Hamilton-Miller, and W. Brumfitt.** 1985. Relationship between adhesion of *Escherichia coli* to uro-epithelial cells and the pathogenesis of urinary infection: problems in methodology and analysis. *J. Med. Microbiol.* **20**:335-344.
92. **Ross, D. L., and A. E. Neely.** 1983. Renal function. p. 97-112. *In* Textbook of urinalysis and body fluids. Appleton Century Crofts, Norwalk, VA.
93. **Salit, I. E., and E. C. Gotschlich.** 1977. Hemagglutination by purified type 1 *Escherichia coli* pili. *J. Exp. Med.* **146**:1169-1181.
94. **Schaeffer, A. J., S. K. Amundsen, and L. M. Schmidt.** 1979. Adherence of *Escherichia coli* to human urinary tract epithelial cells. *Infect. Immun.* **24**:753-759.
95. **Schaeffer, A. J., W. R. Schwan, S. J. Hultgren, and J. L. Duncan.** 1987. Relationship of type 1 pilus expression in *Escherichia coli* to ascending urinary tract infections in mice. *Infect. Immun.* **55**:373-380.
96. **Schilling, J. D., M. A. Mulvey, and S. J. Hultgren.** 2001. Structure and function of *Escherichia coli* type 1 pili: new insight into the pathogenesis of urinary tract infections. *J. Infect. Dis.* **183**(Suppl 1):S36-40.
97. **Scholes D., T. M. Hooten, P. L. Roberts, A. E. Stapleton, K. Gupta, and W. E. Stamm.** 2000. Risk factors for recurrent urinary tract infection in young women. *J. Infect. Dis.* **182**:1177-1182.
98. **Schwan, W. R.** 2009. Survival of uropathogenic *Escherichia coli* in the murine urinary tract is dependent on OmpR. *Microbiology*. **155**:1832-1839.
99. **Schwan, W. R., H. S. Seifert, and J. L. Duncan.** 1992. Growth conditions mediate differential transcription of *fim* genes involved in phase variation of type 1 pili. *J. Bacteriol.* **174**:2367-2375.
100. **Schwan, W. R., H. S. Seifert, and J. L. Duncan.** 1994. Analysis of *fimB* promoter region involved in type 1 pilus phase variation in *Escherichia coli*. *Mol.Gen. Genet.* **242**:623-629.

101. **Schwan, W. R., J. L. Lee, F. A. Lenard, B. T. Matthews, and M. T. Beck.** 2002. Osmolarity and pH growth conditions regulate *fim* gene transcription and type 1 pilus expression in uropathogenic *Escherichia coli*. *Infect. Immun.* **70**:1391-1402.
102. **Stentebjerg-Olesen, B. T., Chakraborty, and P. Klemm.** 2000. FimE-catalyzed off-to-on inversion of the type 1 fimbrial phase switch and insertion sequence recruitment in and *Escherichia coli* K-12 *fimB* strain. *FEMS Microbiol. Lett.* **182**:319-325.
103. **Svanborg Eden, C., U. Jodal, L. A. Hanson, U. Lindberg, and A. S. Akerland.** 1976. Variable adherence to normal human urinary tract epithelial cells of *Escherichia coli* strains associated with various forms of urinary-tract infection. *Lancet* ii:490-492.
104. **Swaney, L. M., Y. P. Liu, C. M. To, C. C. To, K. Ippen-Ihler, and C. C. Brinton Jr.** 1977. Isolation and characterization of *Escherichia coli* phase variants and mutants deficient in type 1 pilus production. *J. Bacteriol.* **130**:495-505.
105. **Takahashi, S., Y. Kurimura, K. Takeyama, K. Hashimoto, S. Miyamoto, K. Ichihara, M. Igarashi, J. Hashimoto, R. Furuya, H. Hotta, K. Uchida, N. Miyao, M. Yanase, Y. Takagi, H. Tachiki, K. Taguchi, and T. Tsukamoto.** 2009. Efficacy of treatment with carbapenems and third-generation cephalosporins for patients with febrile complicated pyelonephritis. *J. Infect. Chemother.* **15**:390-395.
106. **Tsung, K., R. E. Brissette, and M. Inouye.** 1989. Identification of the DNA-binding domain of the OmpR protein required for transcriptional activation of the *ompF* and *ompC* genes of *Escherichia coli* by *in vivo* DNA footprinting. *J. Biol. Chem.* **264**:10104-10109.
107. **Valenski, M. L., S. L. Harris, P. A. Spears, J. R. Horton, and P. E. Orndorff.** 2003. The product of the *fimI* gene is necessary for *Escherichia coli* type 1 pilus biosynthesis. **185**:5007-5011.
108. **van den Bosch, J. F., V. Verboom-Sohmer, P. Postma, J. de Graaff, and D. M. MacLaren.** 1980. Mannose-sensitive and mannose-resistant adherence to human uroepithelial cells and urinary virulence of *Escherichia coli*. *Infect. Immun.* **29**:226-233.
109. **Vorland, L. H., K. Carlson, and O. D. D. Aalen.** 1985. An epidemiological survey of urinary tract infections among outpatients Northern Norway. *Scand. J. Infect. Dis.* **17**:277.

110. **Warren, J. W.** 1996. Urinary tract infections: molecular pathogenesis and clinical management. 1st ed, Washington, D. C. ed. J. W. Warren. ASM Press.
111. **Wright, K. J., P. C. Seed, and S. J. Hultgren.** 2007. Development of intracellular bacterial communities of uropathogenic *Escherichia coli* depends on type 1 pili. *Cell. Microbiol.* **9**:2230-2241.
112. **Yoshida, T., L. Qin, and M. Inouye.** 2002. Formation of the stoichiometric complex of EnvZ, a histidine kinase, with its response regulator, OmpR. *Mol. Microbiol.* **46**:1273-1282.

APPENDIX A
REAGENTS FOR DNASE I FOOTPRINTING

Appendix A. Reagents for DNase I Footprinting

a.) 5X Binding Buffer

300 mM potassium glutamate
200 mM HEPES, pH 7.4
5 mM dithiothreitol
2 mM MgCl₂
0.25 % NP-40

b.) Stop buffer (per reaction)

3.25 µl 3M sodium acetate, pH 5.2
1.25 µl 0.5M EDTA
0.5 µl glycogen (5 µg/ml stock)

c.) Diffusion Buffer

10 mM magnesium acetate
1 mM EDTA pH 8.0
0.5 M ammonium acetate
0.1% SDS

d.) 10X Phosphorylation Buffer

500 mM TrisHCl pH 7.6
500 mM KCl,
200 mM MgCl₂

e.) Sequencing Grade Loading Dye

20 mM EDTA
95% formamide
0.05% xylene cyanol
0.05% bromphenol blue

f.) 1.25X TBE/urea stock solution

141.0 g urea
62.5 ml 5X TBE
H₂O to 250 ml
*filter sterilize

g.) 4.2% (37.5:1) polyacrylamide 1.25X TAE gel

30 ml 1.25X TAE

5 ml 37.5:1 BIORAD acrylamide

*polymerize with 200 µl 10% ammonium persulfate and 35 µl TEMED

h.) 8% 7.5M urea-Tris-borate-EDTA polyacrylamide gel

10 ml 40% 19:1 BIORAD acrylamide/bisacrylamide solution

40 ml 1.25X TBE/urea solution

*polymerize with 300 µl 10% ammonium persulfate and 50 µl TEMED

APPENDIX B

REAGENTS FOR RNA EXTRACTION AND PCR

Appendix B. Reagents for RNA Extraction and PCR

a.) 0.1% DEPC-treated H₂O

300 ml Milli-Q H₂O

300 µl diethylpyrocarbonate

*incubate shaking overnight at 37°C, autoclave the next day

b.) 70% Ethanol in 0.1% DEPC-treated H₂O

140 ml 100% biotech-grade ethanol

60 ml 0.1% DEPC-treated H₂O

c.) Incomplete Wigler's Buffer

26.08 ml sterile Milli-Q H₂O

16.75 ml 1M TrisHCl pH 8.8

4 ml 1M (NH₄)₂SO₄

0.5 ml 1M MgCl₂

*aliquot 946.3 ml into 1.5 ml Sarstedt Tubes, store at -20°C

d.) Complete Wigler's Buffer

to one tube of the above buffer add:

50 µl bovine serum albumin (10 mg/ml)

3.47 µl β-mercaptoethanol

APPENDIX C
REAGENTS FOR SDS-PAGE AND WESTERN BLOTTING

Appendix C. Reagents for SDS-PAGE and Western Blotting

a.) 10X Running Buffer

15 g Tris base

72 g Glycine

5 g SDS

*bring volume to 500 ml using Milli-Q H₂O, filter sterilize, and store at 4°C

b.) Sample buffer

2.9 ml Milli-Q H₂O

1.0 ml 0.5M TrisHCl (pH6.8)

2.0 ml glycerol

1.6 ml 10% SDS

0.1 ml 1% bromphenol blue

8 µl 2.5M dithiothreitol

c.) Brilliant Blue Stain

200 ml Milli-Q H₂O

50 ml isopropyl alcohol

20 ml glacial acetic acid

0.5 g Brilliant Blue R G-250

*let the first two ingredients dissolve in each other for 30 min, then add H₂O and acid

d.) SDS-PAGE Destain

270 ml Milli-Q H₂O

100 ml methanol

30 ml glacial acetic acid

e.) Transfer Buffer

200 ml Methanol

14.4 g Glycine

3.05 g Tris

*bring volume to 1 L using Milli-Q H₂O

f.) TBST

3.15 g Tris-HCl

8.76 g NaCl

0.5 ml Tween-20

*bring volume to 1 L, pH to 7.4, filter sterilize, and store at 4 °C

g.) Blocking Buffer

5% Bovine serum albumin in TBST

*made fresh daily

h.) Phosphate Buffered Saline

9.00 g sodium chloride

1.15 g sodium phosphate, dibasic

0.23 g sodium phosphate, monobasic

* bring volume to 1 L using Milli-Q H₂O and adjust the pH to 7.2

APPENDIX D
REAGENTS FOR MEDIA

Appendix D. Reagents for Media

a.) Luria Bertani (LB) broth pH 5.5 (Schwan *et al.* 2002)

34.5 ml 1M NaH₂PO₄
1.5 ml 1M Na₂HPO₄
3.6 ml glycerol
7.2 g LB base (Invitrogen)
Milli-Q H₂O to 360 ml
*pH to 5.5 and autoclave 25 min

b.) LB broth pH 5.5 + 1 M NaCl

recipe is exactly the same as above except add 21.03 g NaCl

c.) LB broth pH 7.0

20.77 ml 1M Na₂HPO₄
15.23 ml 1M NaH₂PO₄
7.2 g LB base (Invitrogen)
Milli-Q H₂O to 360 ml
*pH to 7.0 and autoclave 25 min

d.) LB broth pH 7.0 + 1 M NaCl

recipe is exactly the same as above except add 21.03 g NaCl