Motility and Signaling Systems in Uropathogenic *Escherichia coli*: the Role of Chemotaxis and c-di-GMP Production in Colonization of the Urinary Tract

By

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A dissertation submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

(Microbiology)

at the

UNIVERSITY OF WISCONSIN-MADISON

2012

Date of final oral examination: 06/28/12

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Motility and Signaling Systems in Uropathogenic *Escherichia coli*: the Role of Chemotaxis and c-di-GMP Production in Colonization of the Urinary Tract

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Urinary tract infections are among the most common infections that occur in young, healthy adults. The majority of urinary tract infections are caused by uropathogenic *Escherichia coli* and likely progress by an ascending manner from the urethra, to the bladder, and finally to the kidneys. Motility is a known fitness factor that aids in bacterial survival in the urinary tract, and chemotaxis toward urine may help to efficiently guide the bacteria up the urinary tract before expulsion by micturition. Chemotaxis capillary assays using components of urine as ligands showed that a subset of amino acids acts as the main chemoattractants for uropathogenic *E. coli* strain CFT073. Loss of the chemoreceptors did not result in a loss of fitness in the mouse model of urinary tract infection. However, a true ascending model of urinary tract infection is likely necessary to conclusively determine whether chemotaxis improves the chance of successful colonization of the urinary tract. Increased production of the second messenger c-di-GMP also has an effect on motility by promoting a transition from planktonic, motile cells to sessile biofilm populations. Deletion of *yfiR*, a gene that encodes a periplasmic inhibitor of activity of the diguanylate cyclase YfiN, resulted in attenuation *in vivo*.

Increased production of curli fimbriae and cellulose and decreased motility as a result of increased c-di-GMP levels in the *yfiR* deletion mutant caused attenuation in this strain. Likely the increased metabolic burden contributed to this reduction in fitness. Since a plethora of diguanylate cyclases and phosphodiesterases are encoded in the CFT073 genome, research into other aspects of c-di-GMP-mediated processes might reveal new fitness and colonization factors that were not previously considered in the pathogenesis of uropathogenic *E. coli*.

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Rodney A. Welch, Ph.D.

Acknowledgements

Many people have supported and encouraged me in the pursuit of my graduate degree. First and foremost, I would like to thank my thesis advisor, Rod Welch. You've hung in there with me for seven long years and have displayed incredible patience while I've tried to find my way past various setbacks in my thesis project. I thank for your support during my time in graduate school and during the job search process. I regard myself as lucky to have joined the Welch lab.

I would like to also thank my thesis committee members: Christina Hull, Ned Ruby, Jorge Escalante, and Caitilyn Allen. Everyone has shown unwavering support for me as a young researcher and your insights into and suggestions for the improvement of my research have proved to be invaluable. I appreciate the interest you all have shown in the progress of my projects even outside of committee meetings and your continued support in the pursuit of my future career goals. Thank you all. My thanks also extend to Dr. Ken Bayles, my academic advisor during my undergraduate studies at the University of Idaho. He gave me my first chance to work in a research lab, an experience without which I may not have decided to go (or been accepted to) graduate school.

Thanks to the Welch lab and its members, both past and present, who have made life in the lab enjoyable. Coming into work each day was made easier by this great group of people, especially during the stretches when my research was not going well. Special thanks go to Shai Pellett, our former lab manager, who made sure that the lab ran smoothly and who maintained a sincere interest in the progression of my project even after she had retired. If only Rod had convinced you to stay a little longer. Thank

you as well to the three undergraduates who worked on my various projects- Jeff Gelhausen, Dan Shapiro, and Dan Stevens. I greatly appreciate all of your hard work.

My friends and family have also supported me throughout my time in graduate school. Thanks are due mostly to my parents, who have provided me with encouragement throughout my life that was “disproportionate to my looks and abilities”, to quote Tina Fey. I think that they’re more excited about my graduation than I am. If not for my Dad suggesting that I should try to get an undergraduate research job in a lab, I might not have made it all the way to graduate school. Good job parents, and, despite the good times I’ve had in graduate school, I echo their sentiments- thank god it’s finally over.

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Abbreviations

c-di-GMP- bis-(3'-5')-cylcic dimeric guanosine monophosphate

CFU- colony forming unit

DGC- diguanylate cyclase

ExPEC- extraintestinal *Escherichia coli*

MCP- methyl-accepting chemotaxis protein

OD- optical density

PCR- polymerase chain reaction

PDE- phosphodiesterase

pGpG- 5'-phosphoguanylyl-(3'-5')-guanosine

RDAR- red, dry and rough

UPEC- uropathogenic *Escherichia coli*

UTI- urinary tract infection

Preface

This dissertation is organized into four chapters and four appendices. Chapter 1 provides background information that is necessary to understand the following research chapters. Chapter 2 presents experiments that determined the components of human urine that can act as chemoattractants for uropathogenic *Escherichia coli*. Chapter 3 describes analyses undertaken to determine the role of the *yfiLRNB* locus in c-di-GMP production and persistence *in vivo*. Chapter 4 discusses the results contained in this dissertation and recommends future projects that could extend the current research. Appendix A specifically analyzes a possible mechanism for how *E. coli* CFT073 senses and displays chemotaxis toward D-serine. Appendix B notes problems with reflux in the current mouse model of urinary tract infection. Appendix C describes the motility and *in vivo* phenotypes of a set of mutants that I did not pursued further. Appendix D is a manuscript that contains some of my early work with atypical *Shigella boydii* strains and that was published in *FEMS Microbiology Letters* (2012) 328:20-25.

Chapter 1

Introduction

The sections below provide background information necessary to evaluate the following research chapters in this thesis. An overview of uropathogenic *Escherichia coli* and urinary tract infections is given as is information about the current mouse model of urinary tract infection and the virulence and colonization factors of uropathogenic *E. coli*. These sections are followed by a review of chemotaxis in *E. coli* and of the second messenger cyclic di-GMP. Finally, the research chapters on chemotaxis and c-di-GMP signaling are summarized.

Pathogenic *Escherichia coli*

Escherichia coli is a well-studied gram-negative bacterium and is commonly found in humans as a normal, nonpathogenic member of the intestinal microbiota. However, some *E.coli* strains have acquired virulence factors that shift the lifestyle from commensal/beneficial to pathogenic. These pathogenic strains of *E. coli* are divided into two separate groups that distinguish between strains that cause disease within the digestive tract (diarrheagenic *E. coli*) and those that cause disease outside of the digestive tract (extra-intestinal pathogenic *E. coli* (ExPEC)). The diarrheagenic *E. coli* strains are further divided into five pathotypes- enterohaemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasisve *E. coli* (EIEC), and enteroaggregative *E. coli* (EAEC)- that are distinguished by both the clinical symptoms of the disease that they each cause and by the specialized set of virulence and colonization factors used to infect the host (41). Included in the ExPEC group are the uropathogenic *E. coli* (UPEC) strains that cause urinary tract infections. UPEC

strain CFT073, isolated from a patient with pyelonephritis (infection of the kidneys), is one of the most characterized strains in this group.

Urinary tract infections

Urinary tract infections (UTIs) are among the most common infection of humans and cost more than 1 billion dollars annually for diagnosis and treatment in the United States alone (47, 66). Although men and children are able to contract UTIs, women comprise the majority of UTI patients with about 40-50% of women experiencing a UTI at least once in their lifetimes (24, 25, 47). About a third to half of women will have recurrent UTIs throughout their lifetimes (21, 35). UTIs are believed to be ascending infections in the vast majority of cases that start when the urethra is contaminated with bacteria most likely from a fecal origin. After colonization of the urethra, the bacteria then ascend to the bladder. For the most part, urinary tract infections are uncomplicated cases of cystitis and remain localized in the bladder for a few days before clearance from the urinary tract with the help of antibiotic treatment (7, 64). Occasionally, these bladder infections can spread to the kidneys causing pyelonephritis that can result in more serious complications such as renal scarring and kidney failure. From the kidneys the bacteria can then spread to the bloodstream where they can cause the patient to go into septic shock, potentially ending in death. Uropathogenic *E. coli* account for the majority of community-acquired and hospital-acquired UTIs (7).

The bladder is normally considered a sterile environment, although a recent study suggests that viable but non-culturable organisms may reside continually and

asymptomatically in this location (99). Whether they are commensal or pathogenic, bacteria living in the urinary tract must overcome several hurdles to achieve a measurable degree of colonization. Ascent of the urinary tract for access to the bladder and kidneys is hampered by the turbulent and expulsive flow of urine. Once in the urinary tract, the bacteria face growth inhibition due to limited amounts of free iron. Attachment to the bladder lumen may also be prevented by sloughing of the uroepithelial cells along with any bound bacteria. Those bacteria that stimulate an immune response through signaling by pattern recognition receptors are also susceptible to killing by antimicrobial peptides and invading neutrophils (17, 42, 62, 97).

Virulence and fitness factors of uropathogenic *Escherichia coli*

Several factors involved in the adherence of. *E. coli* to the bladder epithelium and its persistence in the urinary tract are known. Type I fimbriae and Pap fimbriae, although not the only fimbriae present in most UPEC genomes, are the two major fimbriae thought to be responsible for the tight adherence to the umbrella cells of the bladder and the renal cells of the kidneys, respectively (8). Additionally, iron acquisition systems such as the yersiniabactin and heme uptake systems are factors necessary for full fitness in the urinary tract (23). Toxins such as hemolysin and the cytopathic secreted autotransporter toxin (Sat) are involved in UPEC infection process (26, 62, 83). Flagella are also apparently needed for maintenance of infection as nonmotile strains are attenuated within the urinary tract (50, 71, 80, 101). For the most part, the virulence and fitness factors mentioned above contribute to persistence in the urinary

tract but not necessarily to initial colonization. For reasons detailed in the next section, the current mouse model of UTI is unsuitable for analysis of factors needed for ascent of the urinary tract and initial colonization.

Mouse model of urinary tract infection and its limitations

In the early 1980s, a murine model of urinary tract infection was developed by Hagberg *et al.* that, with some modifications, is still used today to study UTIs. Although human urinary pathogens are not natural pathogens in mice, this model was originally chosen because mice express the same glycolipids on the bladder epithelium that are needed for bacterial attachment in human bladders. Mice are also relatively inexpensive compared to other lab animals. The model involves transurethral inoculation of the bladder with a 50 μl inoculum delivered via a small catheter. Due to the non-native nature of UPEC strains with regards to a mouse as a host, inocula typically must contain upwards of 10^7 CFU of bacteria to attain measurable infection levels and consistent results (27, 33, 34, 37). Notably, some mouse strains such as the C3H/HeJ line are more susceptible to urinary tract infection than others for reasons that still remain unclear (32, 87).

While this model has allowed researchers to gain valuable information regarding the virulence factors necessary for attachment to the urinary tract epithelium as well as other factors necessary for full fitness within the urinary tract, the model is insufficient to assess which factors are necessary for initial ascent of the urethra. The inoculum size of 50 μl is delivered directly to the bladder, bypassing the early colonization events that

would take place in the urethra during a natural infection entirely. The lowest inoculum volume reported in the literature is 10 µl delivered to the urethra (33), although given the short distance of the female mouse urethra (~1cm), even this small volume undoubtedly reaches the bladder. Furthermore, as previous reports and Appendix B of this thesis note, the 50 µl inoculum is sufficiently large enough to cause immediate reflux into the kidneys upon infection, thus bypassing the steps in the ascent of the ureters to the kidneys as well. Some labs argue that use of a Harvard pump that delivers the 50 µl inoculum over 30 seconds eliminates the reflux (50). This assertion is doubtful since conflicting results about reflux using this apparatus occur in the literature (37). Despite this disagreement, it remains clear that a true model of ascending urinary tract infection, from colonization of the urethra to ascent up to the kidneys, would provide an improved model for analysis of colonization factors.

Chemotaxis in *Escherichia coli*

Chemotaxis in bacteria is simply defined as the net movement toward an attractant or away from a repellent. First described by Englemann and Pfeffer in the late 19th century and later elaborated on by Julius Adler in the 1960s (1, 2), the chemotaxis field has seen many advances over the past 50 years in our understanding of how *E. coli* senses attractants and repellants and how those signals are transmitted to the flagella to produce movement in the appropriate direction. The core of the chemotaxis machinery is comprised of integral membrane receptors, termed methyl-accepting chemotaxis proteins (MCPs) or chemoreceptors, which usually possess a

periplasmic ligand binding domain and a cytoplasmic signal transduction domain. *E. coli* K-12 has five known chemoreceptors: Tsr, Tar, Trg, Tap, and Aer. Tsr senses serine and pH, Tar senses aspartate and maltose, Trg senses galactose and ribose, Tap senses dipeptides, and Aer senses the oxidative state of the cell (4, 58, 59, 95). Of these five chemoreceptors, *E. coli* CFT073 only encodes *tsr*, *tar*, and *aer* (51). Upon binding of a ligand to its concomitant receptor, a conformational change occurs in the chemoreceptor that inhibits the activity of CheA, a histidine kinase that is docked to the cytoplasmic portion of the chemoreceptor by CheW. CheA phosphorylates the cytoplasmic signal transducer CheY which can then bind to the flagellar motor and reverse the rotation of the flagellar filament, causing the cell to tumble (Fig. 1). When CheY is unphosphorylated, which can be accomplished through the activity of the CheZ phosphatase, rotation of the flagella continues in a counter-clockwise fashion and the cell swims smoothly. In the absence of any attractants or repellants, the cell undergoes varying periods of smooth swimming and tumbling, depending on the ratio of phophorylated and unphosphorylated CheY present. When an attractant is present the number of phosphorylated CheY proteins is decreased, thereby increasing the periods of smooth swimming and eventually biasing the net movement of the bacterium towards the attractant. Additional control of the activity of CheA is mediated by CheB, a methylesterase, and CheR, a methyltransferase, which remove and add methyl groups, respectively, from glutamate residues in the cytoplasmic portion of the chemoreceptors. Methylation of the chemoreceptors increases activity of CheA. CheB, like CheY, is also activated by phosphorylation by active CheA (55, 95).

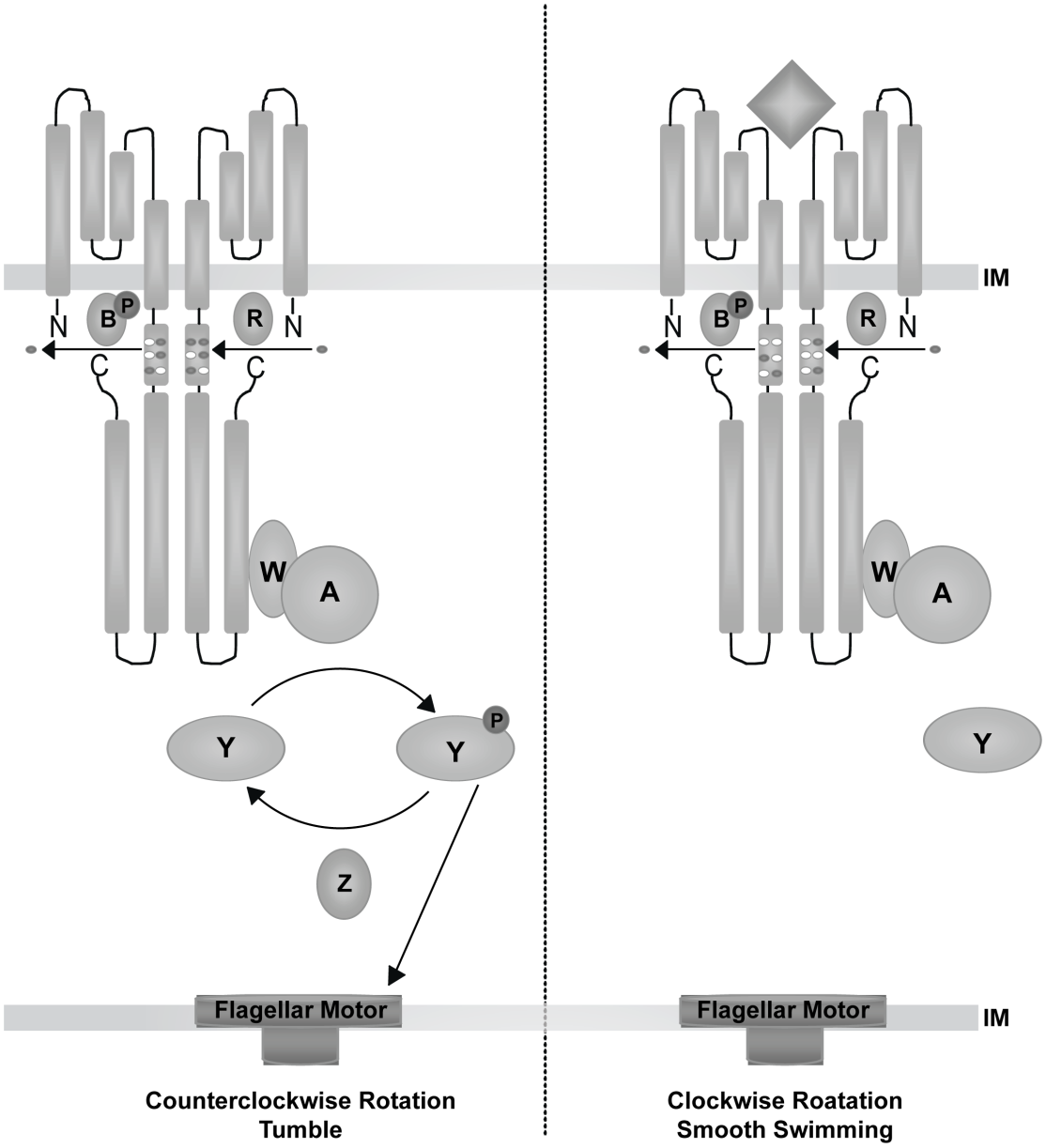

Figure 1: Schematic of the chemotaxis machinery. The membrane spanning chemoreceptors are shown coupled with the core chemotaxis signaling pathway components in the presence and absence of attractant (diamond). Each Che protein is identified by its gene letter and phosphorylated proteins are denoted with attached P-labeled circles. Demethylated and methylated glutamate residues are shown as open

and filled bubbles, respectively. Ultimate results for flagella filament rotation direction and swimming behavior are indicated. IM= inner membrane

The chemoreceptors are associated in large clusters found at the cell poles and smaller clusters arranged along the length of the cell. Within these clusters, chemoreceptor homodimers are arranged in groups of three. Each trimer of chemoreceptor dimers associates with CheW and CheA to form the signaling complex. Organization into these complexes allows the cell increased sensitivity to altered concentrations of ligands since the signal produced by one receptor bound to its chemoeffector is amplified through allosteric interactions between the other chemoreceptor dimers. Adaptation, or a return to pre-stimulus CheA activity levels, is achieved in two ways. CheR methylates the exposed glutamate residues of the chemoreceptors and increases the activity of CheA. Methylation also alters the conformation of the chemoreceptor dimer such that ligand affinity for the receptor is reduced. These changes lead to increased levels of phosphorylated CheY and a return to the state before attractant binding. Active CheA also activates CheB and results in removal of the methyl groups from the chemoreceptor, reducing CheA activity and allowing adaptation to repellants (3, 18, 28, 43, 49, 65, 94).

Chemotaxis in colonization of the host by pathogenic organisms

Various studies show that chemotaxis is used to find preferred niches by a pathogen in the host organism. In *Vibrio cholerae*, the chemotaxis system is used to

navigate to the crypts of the small intestine where the bacteria increase expression of its virulence factors (14, 15, 22). Similarly, chemotaxis is needed for *Campylobacter jejuni* to colonize the subcellular space of the gastric epithelium before initiation of invasion of the host cell can occur (53). Aerotaxis is also used by *Ralstonium solaracerum*, a tomato plant pathogen, for navigation to the plant roots in soil (103, 104). A *cheW* mutant in UPEC strain CFT073 was attenuated in the mouse model of UTI, suggesting that chemotaxis may also be important for fitness in the urinary tract (52). These examples provide evidence that chemotaxis and motility are widely applied by pathogens to gain access to sites within the host where infection can be maintained.

Comparison of the regulation of flagellar genes in *Escherichia coli* strains CFT073 and K-12

Because chemotaxis and flagellar-mediated motility are important for bacterial fitness in the host in general but can also stimulate the immune response (93), UPEC have mechanisms in place to carefully control the expression of these systems. In general, motility in *E. coli* CFT073 is more tightly controlled in comparison to the lab strain of *E. coli* K-12. The master regulator of the flagellar regulon, *flhDC*, in *E. coli* K-12 is expressed at constitutive levels due to an insertion sequence that interrupts the regulator-binding regions (9). The *flhDC* promoter in CFT073 does not contain this insertion sequence and is thus subject to the more stringent control of the ancestral regulatory region. The flagellar regulon consists of three classes of flagellar genes. The genes encoding *flhDC* comprise the class I flagellar genes and their expression is

necessary for transcription of class II genes which include *fliA*. FliA is a sigma factor that is necessary for transcription of class III flagellar genes, which encode the proteins for the chemotaxis system and the flagellin subunit FliC. The *flhDC* promoter responds to multiple regulatory factors and its transcription can be altered in response to various environmental stimuli including temperature, pH, and butyrate concentration. The regulatory factors that act on the *flhDC* promoter include the nucleoid protein H-NS, the RscBA response regulator system, the QseBC quorum-sensing system, and the catabolite repressor protein, CRP (5, 20, 45, 85).

E. coli CFT073 also possesses type I fimbriae whose expression is inversely regulated with flagella expression. The type I fimbriae operon is controlled by the *fimS* switch, an invertible element that is positioned in either the ON or OFF position by specific DNA recombinases. In the ON position, transcription is initiated off the *fim* promoter and procedes through the type I fimbriae genes. While type I fimbriae are being expressed, expression of the flagellar genes is down-regulated. In the OFF position, the *fim* promoter is oriented away from the *fim* operon and expression of flagellar genes is activated (12, 44). One other study reported the repression of the *flhDC* operon by MatA, an activator of the *mat* fimbriae operon that is involved in biofilm formation (54).

As a result of the additional regulation that is absent in many *E. coli* K-12 strains due to the aforementioned insertion sequence in the *flhDC* operon, *E. coli* CFT073 motility is often repressed, resulting in only a small proportion of the population expressing flagellar filaments in broth culture. In contrast, most of the population of an

E. coli K-12 strain containing the insertion element are flagellated in broth culture because *flhDC* is nearly constitutively expressed. Growing the cells in 0.3% swim plates before transferring them to growth in broth culture can enhance the number of flagellated cells in the *E. coli* CFT073 population yet, even with the added manipulation, elevated flagellar expression can remain difficult to obtain. This problem has implications for capillary chemotaxis assays that monitor the chemotactic ability of the bacteria. Because a lower number of bacteria remain flagellated in CFT073 compared to a K-12 strain, a greater number of bacteria are needed for the assay to work. This raises the background values in the assay, and standard-error rates are increased compared to assays performed with K-12 strains. However, reliable, statistically significant results can still be obtained under these conditions. Additional flagellar regulation mechanisms may be present that can cause the bacteria to display different forms of flagellar-based motility, namely swimming and swarming. Different *in vitro* conditions stimulate each motility type, and evaluation of the contribution of each motility phenotype to the infective process in UPEC may provide additional insight into the ways motility contributes to fitness *in vivo*.

Swarming and swimming motility

Swarming motility is defined as the coordinated, multicellular movement of a group of cells across a semisolid surface. Conversely, swimming is performed by independent planktonic cells and is not dependent on association with other bacteria. Swarming motility is observed in a variety of bacterial species including *E. coli* and

Salmonella species and swarmer cells are usually differentiated from independent swimmer cells by an increase in cell length and hyperflagellaton (29, 30). However, *E. coli* does not display the elongated cell morphotype that is seen in other bacterial species. The swarming phenotype is assessed on 0.5% agar plates, although the percentage of agar used can vary between different bacterial species. Swimming motility is assessed in 0.3% agar tryptone plates. Non-swarmer cells form colonies that can spread out by growth of the colony, but swarmer cells display enhanced coverage of the agar surface and tendrils of swarming bacteria can shoot off from the main colony on the plate surface. Under the microscope, rafts of coupled bacteria in the outer edge of the swarming colony move in a swirling pattern that pushes the colony edge forward. Recent studies show that individual bacteria in the swarming colony edge are associated by interactions between the flagella of individual bacteria (13, 91). Swarming motility could be important for colonization of the urinary tract because groups of cells encased in the surfactants that are excreted by swarming cells could be more difficult to expel from the urinary tract by the force of flowing urine than would individual swimmer cells. A recent study in *Pseudomonas aeruginosa* showed that the presence of small amounts of mucin, a glycoprotein often found in the mucus overlaying epithelial surfaces, promoted swarming in *in vitro* plate assays (105). This finding strengthens the possibility that swarming may take place *in vivo* across epithelial cell layers.

Effect of the binding of second messenger c-di-GMP on motility

In addition to the regulatory controls mentioned above, cytoplasmic accumulation of bis-(33'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) can also inhibit both swimming and swarming motility. Inhibition of motility is accomplished by the interaction of c-di-GMP with YcgR, a cytoplasmic protein. When c-di-GMP is bound to the PilZ domain of YcgR, YcgR reverses the rotation of the flagellar motor from clockwise to counterclockwise by binding to components of the motor-stator complex. Reports concluded that YcgR interacts directly with FliG and FliM, components of the flagellar stator, to bias the motor to counterclockwise rotation, which causes smooth swimming (19, 67). Without the ability to tumble and reverse the direction of swimming, the bacteria may become stuck in an unfavorable environment.

Second messenger c-di-GMP metabolism and signaling

c-di-GMP is a small, diffusible molecule that is now recognized as a global second messenger in the same category as cAMP; it primarily controls the transition between motility and sessility. First identified as an activator of cellulose production in *Gluconacetobacter xylinus* (76, 82), studies have since shown that c-di-GMP signaling is common in diverse bacterial species, many of them pathogens, but is absent in eukaryotes and archaea (31, 36, 76, 78). C-di-GMP synthesis and metabolism are primarily regulated by two classes of proteins: diguanylate cyclases (DGCs) that possess the conserved GGDEF (Gly-Gly-Asp-Glu-Phe) domain responsible for cyclizing two molecules of GTP into c-di-GMP and phophosdiesterases (PDEs) that posses the

EAL (Glu-Ala-Leu) domain responsible for linearizing c-di-GMP to 5′-phosphoguanylyl-(3'-5')-guanosine (pGpG). GGDEF and EAL domains are often found in the same protein but can also occur separately (16, 68, 78, 79, 89). Since their discovery GGDEF and EAL domains have been identified in many species of bacteria, although their numbers in each individual species can vary widely. For example, *E. coli* K-12 apparently encodes 19 GGDEF proteins and 16 EAL proteins and *Vibrio cholerae* apparently encodes 31 GGDEF proteins, 22 EAL proteins and 10 GGDEF-EAL proteins; in contrast, *Bacillus subtilis* apparently encodes only 4 GGDEF proteins and 3 EAL proteins (36, 76).

Proteins with either of these domains respond to a diverse set of extra- and intracellular stimuli including the redox state of the cell, starvation consitions, and the levels of oxygen, light, antibiotics, and quorum sensing molecules; thus, they often include domains involved in the sensing of these stimuli (*i.e*. PAS, FAD-associated BLUF, and GAF domains, to name a few)(60). Production of c-di-GMP results in modulation of motility, virulence gene expression, heavy-metal and reactive-oxygen species resistance, fimbrial synthesis, exopolysaccharide production, and biofilm formation. c-di-GMP can modulate the activity of various proteins by binding to allosteric sites, such as PilZ domains, that alter the activity of those proteins (6, 39, 72, 76, 88, 100, 102). c-di-GMP can also feedback to control its own production by binding to I sites on the DGCs (36, 102). In addition,some reports indicate that c-di-GMP may be able to bind directly to riboswitches in the 5’ untranslated region of bacterial mRNA, preventing translation of those messengers (81, 86, 102).

DGCs themselves are regulated at multiple levels. As mentioned above, c-di-GMP can regulate its own production through feedback mechanisms. Additionally, expression of DGCs is regulated at a transcriptional level; for instance, σ^S controls the expression of many DGCs in *E. coli* (84, 96).

Increased c-di-GMP production increases curli fimbriae and cellulose synthesis

Curli fimbriae are found in *E. coli* and other enterobacteria, and are important adhesins involved in biofilm formation, functioning both in initial adhesion and cell-cell interaction. The genes that encode the structural and biosynthetic components of curli fimbriae are found in two divergently transcribed operons. One operon encodes *csgD*, a transcriptional regulator needed for expression of *csgBA*, the genes encoding the structural subunits of the curli fibril, which are encoded on the opposite operon. The operon that encodes *csgD* also encodes the genes *csgEFG,* which are needed for the export and stabilization of the curli fibril. CsgD can also activate transcription of its own operon. The activity of the *csg* promoter is also positively regulated by OmpR, MlrA, and IHF, and repressed by H-NS and CpxR. Expression of the curli operon is temperature sensitive with an optimum at 30°C (10, 11, 38, 84).

Multiple studies indicate that c-di-GMP accumulation is responsible for an increase in curli production resulting from an increase in *csgD* expression (70, 73, 96). Although it has never been experimentally shown, a possible mechanism for this increase in transcription involves the binding of c-di-GMP to CsgD to increase its activity. Such an increased expression and activity also has implications for the

expression of other genes. Microarray and other transcriptional analyses of *csgD* over-expression identified both known and novel genes whose expression is increased or decreased. The genes *cspA*, *cspB* and *cspG,* involved in the cold shock response, and the flagellar gene *fliE* are all repressed, while the transcription of *adrA* and *yedQ,* encoding DGCs, and *yoaD* encoding a putative PDE, are all increased, to name a few (10, 11). AdrA and YedQ dependent production of c-di-GMP have been linked to an increase in the production of cellulose, although these genes and their links to cellulose synthesis are not present in every strain of *E. coli* (39, 61, 74).

Cellulose is also an important player in biofilm formation as it comprises much of the extracellular matrix. The cellulose biosynthetic genes are arranged in two divergently transcribed operons as well (*bcsEFG* and *yhjRQbcsABZC*), although many of the encoded genes have unknown functions. Of these genes, BcsA was identified as the catalytic subunit for cellulose biosynthesis and BcsB was implicated in the regulation of BcsA activity (74, 75, 106). Most likely c-di-GMP binds to the PilZ domain of BcsA to increase its activity (31, 77). A schematic of the downstream effects of c-di-GMP production is provided in Fig. 2.

A simple plate assay can determine whether curli, cellulose, or both structures are being produced by the bacteria. Congo-red dye can bind to both curli fimbriae and cellulose. Bacteria that express both curli and cellulose produce red, dry and rough (RDAR) colonies on Luria-Bertani (LB) agar plates containing the dye but no salt. Expression of curli only produces a deep red colony that is rough but lacks the rugose colony characteristics of a cellulose producing strain. Expression of cellulose only

produces a colony that displays the rugose quality but appears pink due to the reduced binding of the dye. Lack of expression of both curli and cellulose produces a white, smooth colony. Cellulose will also bind calcofluor, which then causes the bacterial colonies to glow under UV light (74). Because c-di-GMP production is closely tied to the expression of the curli and cellulose operons, the development of this method has increased the ease with which potential DGCs and PDEs can be identified.

Effect of c-di-GMP signaling on virulence factor expression

Signalling by c-di-GMP can have diverse effects on the factors needed by bacterial pathogens to mount a successful infection. As previously mentioned, c-di-GMP production decreases the motility of the bacteria (19, 36, 70, 82, 100). Motility is important for many bacterial pathogens to colonize their hosts both by providing navigation to the pathogens preferred niche and by acting as an adhesin. Loss of motility and flagellar genes has resulted in attenuation of pathogens in numerous reports (14, 15, 22, 98, 101, 103). Curli fimbriae are immunogenic and trigger activation of the host coagulation cascade on the bacterial surface (40, 56, 63, 69, 92). Activation of this pathway results in infiltration of immune cells to the infection site with subsequent inflammation.

One of the best-studied examples of c-di-GMP control of virulence factor expression is found in *V. cholerae*. VieA is a response regulator with PDE activity that is necessary for expression of *toxT*, which activates transcription of toxin-coregulated pili and cholera toxin genes. Expression of both of these virulence factors was

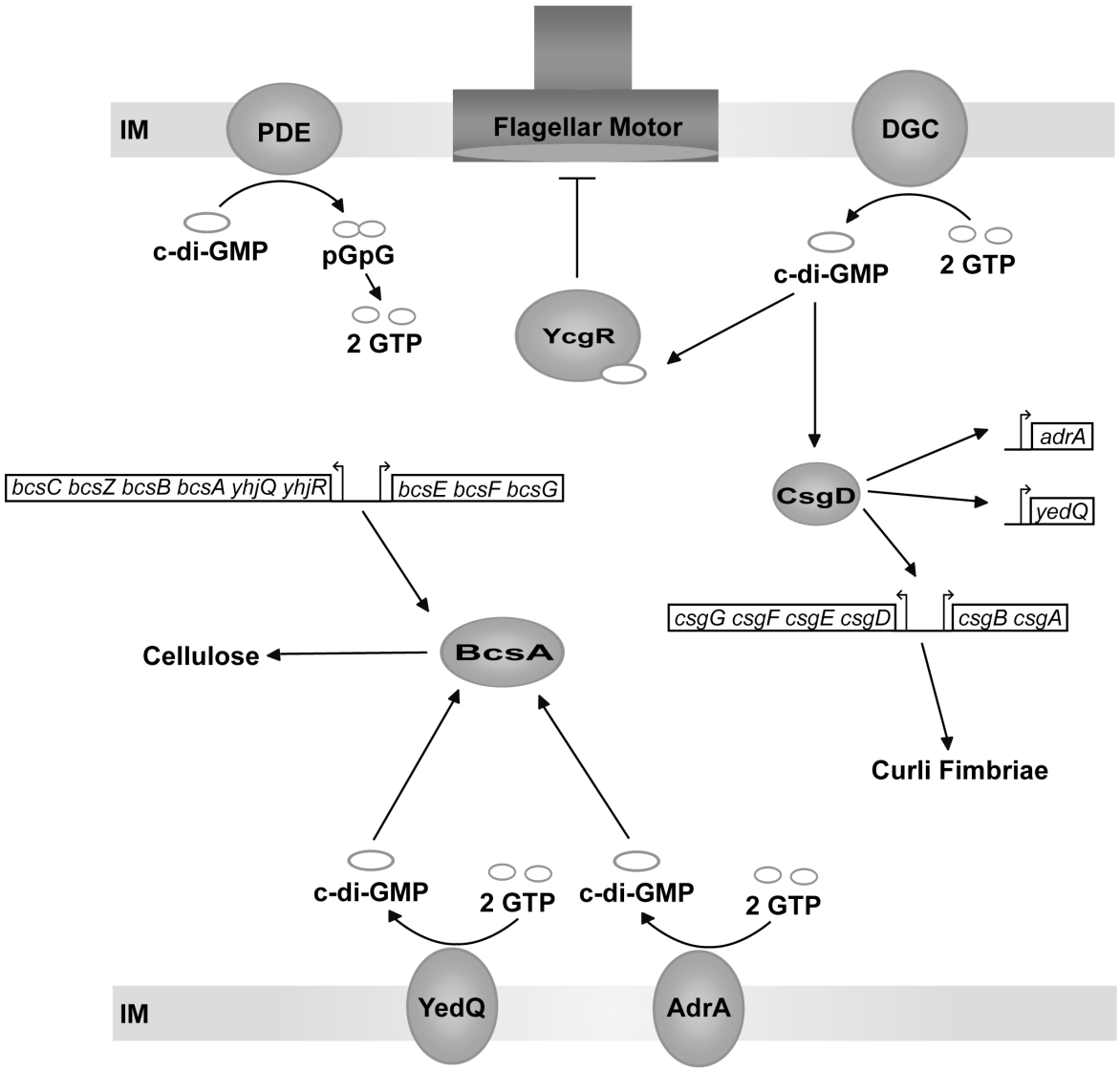

Figure 2: Model of c-di-GMP effects on flagellar function and curli fimbriae and cellulose production. Generic DGCs and PDEs are shown, although YedQ and AdrA are specifically identified because of their known roles in cellulose production. CsgD activation of *yedQ* and *adrA* expression is not present in all *E. coli* strains, and CsgD-independent and YedQ/AdrA-independent pathways for cellulose production do exist. For simplicity, additional regulatory factors involved in control of curli fimbriae and cellulose gene expression are not shown. IM= inner membrane

repressed when the PDE activity of VieA was lost and c-di-GMP accumulated in the cell (88-90). Mutations that cause alterations in c-di-GMP production in *Pseudomonas aeruginosa* also result in attenuation of the organism in animal colonization models, although loss of both DGC and PDE encoding genes can result in this phenotype. Therefore, links between the regulation of c-di-GMP levels and its effect on the virulence of the pathogen may not always be easily deduced (46).

Global analysis of the differential expression of genes in response to high levels of c-di-GMP in *E. coli* K-12 has also been investigated. Genes involved in iron acquisition were down-regulated creating a deficiency that could impair the growth of the bacteria *in vivo*. This increase in c-di-GMP also led to defects in cell wall formation and division (57). Another report showed that loss of a PDE, with the putative concomittant increase in c-di-GMP levels, results in increased sensitivity of *E. coli* K-12 to hydrogen peroxide, *t*-butyl hydroperoxide, and cumene hydroperoxide (48). This could mean that the bacteria may be more susceptible to killing by macrophages and neutrophils *in vivo*. These studies highlight the possible importance of the regulation of c-di-GMP levels during the infectious process.

Research Summary

The primary goal of the research in this thesis was to determine the motility-related factors in uropathogenic *E. coli* strain CFT073 that are important for colonization and persistence in the urinary tract. Chapter 2 describes the components of urine that can act as attractants for *E. coli* CFT073 in the urinary tract, and also details which chemoreceptors are needed for response to each of those attractants. The effect of the

loss of the chemoreceptors on the ability of *E. coli* CFT073 to mount a successful infection is also described. Chapter 3 details the effect that accumulation of c-di-GMP as a result of the loss of the DGC activity inhibitor YfiR has on motility, curli and cellulose production, as well as virulence *in vivo*. Appendix A gives further detail on chemotaxis of *E. coli* CFT073 toward D-serine. Appendix B details the attempts to obtain a true ascending mouse model of urinary tract infection, and the impediment that reflux into the kidneys during infection places on this goal. Appendix C assesses the competitive fitness of various motility mutants. Finally, Appendix D relates the similarity between atypical *Shigella boydii* strains and diarrheagenic strains of *E. coli*.

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Chapter 2

Chemoreceptors of *Escherichia coli* CFT073 Play Redundant Roles in Chemotaxis toward Urine and during Urinary Tract Infection

Submitted to *PLoS ONE*

Authors and contributions:

Erica L. Raterman: Wrote manuscript and designed and performed all experiments.

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Abstract

Community-acquired urinary tract infections (UTIs) are commonly caused by uropathogenic *Escherichia coli* (UPEC). We hypothesize that chemotaxis toward ligands present in urine could direct UPEC into and up the urinary tract. Wild-type *E. coli* CFT073 and chemoreceptor mutants with *tsr*, *tar*, or *aer* deletions were tested for chemotaxis toward human urine in the capillary tube assay. Wild-type CFT073 was attracted toward urine, and Tsr and Tar were the chemoreceptors mainly responsible for mediating this response. The individual components of urine, including L-amino acids, D-amino acids and various organic compounds, were also tested in the capillary assay with wild-type CFT073. Our results indicate that CFT073 is attracted toward glucose and some L- and D-amino acids, but not toward other common compounds found in urine such as urea, creatinine and glucuronic acid. In the murine model of UTI, the presence of any single chemoreceptor is adequate to achieve wild-type colonization levels. Our data suggest that the presence of any strong attractant and its cognizant chemoreceptor is sufficient for colonization of the urinary tract, and that amino acids are the main chemoattractants for *E. coli* strain CFT073 in this niche.

Introduction

Urinary tract infections (UTIs) are some of the most commonly diagnosed and treated infections in the United States, with 40-60% of women experiencing at least one UTI in their lifetime (25, 38). Most community-acquired UTIs are caused by uropathogenic *Escherichia coli* (UPEC)(23). UPEC likely gain access to the urinary tract when the urethra is contaminated with intestinal microorganisms. After colonization of the urethra, the bacteria ascend the urethra to the bladder and, in some cases, continue up the ureters to the kidneys (6). Known UPEC virulence and fitness factors include type I fimbriae, Pap pili, flagella, hemolysin, iron acquisition systems and toxins (16, 22, 23, 47), though factors important for early ascension of the urethra and the ureters and subsequent colonization of the bladder and kidneys remain largely unidentified. Previous reports indicate that motility is important for ascension of the ureters as non-flagellated bacteria were unable to reach the kidneys in the murine model of UTI (26, 28, 43). Because motility is important for a successful infection, chemotaxis may also play a part in the efficient and rapid colonization of the urinary tract by directing the bacteria up the urethra and ureters.

Chemotaxis is defined as the movement toward an attractant or away from a repellant, and the mechanism behind this behavior is well characterized in non-pathogenic *E. coli* strain K-12. The chemotaxis system consists of chemoreceptors, or methyl-accepting chemotaxis proteins, that possess a variable periplasmic ligand binding domain and a conserved cytoplasmic signaling domain. When the periplasmic binding site of the chemoreceptor is occupied, a conformational change occurs that

inhibits the activity of CheA, a histidine kinase that is docked to the chemoreceptor through CheW. CheY is the cytoplasmic protein that is phosphorylated by CheA and shuttles between the chemoreceptor complexes and the flagellar motor to complete transduction of the signal from the chemoreceptor. When it is phosphorylated, CheY binds to a component of the flagellar motor and changes the direction of flagellar rotation to produce a tumble (17, 45). To further increase the magnitude of the response to a ligand, dimers of individual chemoreceptors form teams of mixed trimers that in turn form large signaling clusters capable of amplifying the signal from a few chemoreceptors. Signaling through the chemoreceptors is therefore a cooperative process (39).

E. coli strain K-12 possesses five known chemoreceptors including Tsr (L-serine), Tar (aspartate/maltose), Trg (galactose/ribose), Tap (dipeptides) and Aer (oxygen/redox state) (33, 45). The prototypical UPEC strain CFT073 encodes only three of the five chemoreceptors: *tsr*, *tar* and *aer*. A previous study looking at the prevalence of chemoreceptors across *E. coli* strain groups found that the loss of the Trg and Tap receptors is common among UPEC strains (27), suggesting that the remaining three chemoreceptors are useful for survival in the urinary tract. Loss of *cheW* was also found to attenuate UPEC in the murine model of UTI, indicating that chemotaxis is important for a successful infection (28). However, it is unknown which components of urine act as chemoattractants for *E. coli,* and which chemoreceptors are responsible for detecting those attractants.

In this study, we show that the L-forms of a subset of amino acids act as strong attractants, while other components of urine, including some D-amino acids, caffeine and glucose act as weak to moderate attractants. We also demonstrate that only one chemoreceptor is needed for chemotaxis toward urine as well as for successful infection of the urinary tract in the murine model. Taken together, this evidence indicates that the chemoreceptors of *E. coli* strain CFT073 perform redundant functions in the urinary tract.

Materials and Methods

Bacterial strains and growth conditions

E. coli strain CFT073 was isolated from the blood and urine of a woman admitted to the University of Maryland Medical System with pyelonephritis (34). *E. coli* strain RP437 was a gift from Alan Wolfe. All strains were grown in either Luria Broth (LB) or filter sterilized human female urine from a single volunteer. When growing RP437 in urine, 0.25 mg/mL of L-histidine, L-leucine and L-methionine had to be added to allow for growth. All strains were grown at 37˚ C with shaking at 250 rpm. Urine swim plates were made by mixing 3 parts sterile urine to 1 part sterile agar solution to a final concentration of 0.3% agar. All swim plates were incubated at 37˚ C.

Construction of strains and complements

All gene deletions were performed using the λ-Red recombination system developed by Datsenko and Wanner (15). Plasmids pKD3 and pKD4 were used to

generate the specific λ-Red PCR products for transformation. The antibiotic resistance cassette used to replace the target gene was removed using a Flp recombinase encoded on pCP20, leaving a small, nonpolar scar sequence in place of the deleted gene (15). All gene deletions were verified by PCR and loss of antibiotic resistance on LB agar containing the appropriate antibiotic. Primers used for PCR generation of the λ-Red fragments are listed in Table 1

Allelic repair of the *tsr*, *tar* and *aer* deletion mutants were constructed by transducing each chemoreceptor gene linked to a kanamycin marker outside of the targeted operon into its corresponding deletion mutant using the *E. coli* CFT073 transducing phage EB49 (8). The marker was removed via electroporation of the tranductants with pCP20. Insertion of each gene was verified by PCR and sequencing of the chemoreceptor gene. Primers used for insertion of antibiotic cassettes via λ-Red are listed in Table 1.

Capillary chemotaxis assay

Capillary assays were performed according to Adler's original method (1) with a few modifications. Briefly, strains were grown overnight in urine swim plates (0.3% agar). The following morning, the outer ring of the swim colony was aseptically collected and incubated for a further 3-4 hours in 8 mL urine at 37˚ C with shaking. Bacteria were then harvested and washed 1X in chemotaxis buffer (10^{-2} M phosphate buffer, 10^{-4} M EDTA in ddH_20). Bacteria were resuspended to OD_{600}~0.387 in chemotaxis buffer. Capillary tubes (1mm) were sealed on one end and filled with 0.5 µl

of the attractant solution at the indicated concentrations. Buffer alone was used as a control for random motility and undiluted urine or 500 μg/mL L-aspartate were used as positive controls for all assays. Capillary tubes were then incubated with 100 μl of the bacterial suspension at 30˚ C for 45 minutes. Capillary tubes were washed and the contents were dilution plated. Reported values for each strain and attractant represent combined data from at least three independent assays. Response levels above the buffer control were graphed and analyzed using a two-way ANOVA and Bonferroni post tests with Prism (GraphPad).

Murine model of UTI

Colonization of the urinary tract was determined using the competitive murine model of urinary tract infection as described previously (44). CFT073Δ*lacZYA* was used as the wild-type strain and the chemotaxis mutants had an intact *lacZYA* locus. To select for piliated bacteria, all bacterial strains were grown statically in 3 mL LB at 37˚ C for 2 days. The pellicle formed on the rim of the test tube was then passaged to fresh LB, incubated for 2 more days, and finally passaged again to 40 mL LB for a final 2-day incubation. The broths were adjusted to OD_{600}~0.4 with 1XPBS and the wild-type strain and the mutant strain were mixed equally. The mixed broth was then centrifuged and the cell pellet washed 1X in 1XPBS and resuspended in 500μl 1XPBS. Isofluorane-anesthetized 6-7 week-old female Swiss Webster mice (Harlan, USA) were inoculated via urethral catheterization with 50 μl (10^8 CFU) of the mixed bacterial suspension. Mice were euthanized via CO_2 asphyxiation and the bladder and kidneys were

aseptically harvested and placed in 1XPBS. The organs were homogenized, serially diluted in 1XPBS and plated on MacConkey agar medium (Fisher, USA). Surviving strain ratios were determined by counting white (wild-type) and red (mutant) colonies. Colonization levels were graphed and analyzed using a paired Wilcoxon signed ranked test and Prism (GraphPad). All animal experiments were approved by the UW-Madison Animal Care and Use Committee.

Results

Chemotaxis toward human urine is mainly mediated by Tsr and Tar in *E. coli* CFT073.

While it is known that *E. coli* CFT073 displays chemotaxis toward urine (27), it was unclear if all three chemoreceptors were involved in mediating this attraction. To determine whether one or all chemoreceptors are involved in chemotaxis toward urine, a series of chemoreceptor mutants with all combinations of the genes encoding Tsr, Tar and Aer were deleted from the CFT073 genome via the λ-Red system (15). Each mutant strain was then tested in the capillary assay for chemotaxis toward undiluted human urine, and compared to the wild-type CFT073 response. Because CFT073, unlike K-12, does not flagellate well when grown in broth culture, the bacteria were first grown in swim plates and then transferred to broth culture for all capillary assays. The results showed that Tsr and Tar, even when present alone, are capable of mediating >90% of wild-type chemotaxis toward urine. When Aer is the only chemoreceptor present, however, chemotaxis toward urine is almost completely abolished (<1%) (Fig.

1). Therefore, Tsr and Tar, but not Aer, appear to mediate most of the observed attraction toward urine.

Physiological levels of a subset of L-amino acids are chemoattractants for *E. coli* CFT073.

As was first reported by J. Adler, *E. coli* K-12 is strongly attracted to a variety of L-amino acids (33). All 22 L-amino acids are excreted in human urine, although some are found in higher concentrations than others (9, 20, 44, 50). To determine which of these amino acids could act as attractants for CFT073 during infection, each individual amino acid at its upper physiological concentration found in human urine was used as the attractant for wild-type CFT073 in the capillary assay. As was previously reported by Lane et al. (27), aspartate is an attractant for CFT073. We also identified seven other amino acids that act as strong attractants for CFT073 including alanine, asparagine, cysteine, glutamate, glycine, methionine and serine. Histidine and threonine elicited weak responses that may not be biologically significant, while all other L-amino acids tested elicited no chemotactic response above the buffer control (Table 1). These results concur with the earlier research by J. Adler done in *E. coli* K-12 (33) except for the positive response of CFT073 toward histidine (Table 1). Because the responses at the high concentrations found in urine were low, serine, aspartate and methionine were tested at alternate concentrations that are comparable to the peak concentrations used in Adler's studies (1 mg/mL, 500 μg/mL and 15 mg/mL, respectively) to verify that these amino acids act as attractants at lower concentrations

as well (Table 1). Capillary assay results for K-12 strain RP437 cells prepared in the same way as CFT073 are shown in parentheses in Table 1 to illustrate the similarity of responses to attractants between the two strains.

The chemoreceptor(s) responsible for sensing each individual L-amino acid were investigated using the chemoreceptor knock-out mutants in the capillary assay. These experimental results agree with previously published reports with *E. coli* K-12 with regards to amino acids sensed by Tsr and Tar (33). The *tar* deletion mutant showed decreased chemotaxis toward asparagine, aspartate and glutamate, and loss of either *tsr* or *tar* resulted in a decreased response toward alanine, cysteine, glycine and serine. Additionally, although the results are not statistically significant, the *tsr* mutant did not respond to threonine. Our results indicate that loss of *aer* also lowers the chemotactic response toward alanine and cysteine (Table 3). Allelic repair with the appropriate chemoreceptor gene for each mutant strain restored the altered responses of all of the L-amino acids to wild-type levels (data not shown).

D-amino acids elicit a positive chemotactic response from *E. coli* CFT073.

In addition to L-amino acids, some D-amino acids (D-alanine, D-asparagine, D-phenylalanine, D-proline, D-serine, D-threonine, D-tryptophan, D-tyrosine, and D-valine) are also found in urine, with D-serine and D-alanine present in the highest concentrations (5, 11, 21, 24). These nine D-amino acids were tested at their physiological concentrations with wild-type CFT073 in the capillary assay to determine whether any were attractants. D-asparagine, D-tryptophan and D-valine were the only

D-amino acids tested that did not show any response above the buffer control. Physiological concentrations of D-alanine, D-phenylalanine, D-proline, D-threonine, and D-tyrosine all produced a weak and possibly non-biologically significant response while D-serine produced a moderate response (Table 2). Adler also noted a positive response toward D-serine by K-12 in his report (33).

Because D-serine is present in much higher concentrations in urine than any of the other D-amino acids, the greater effect observed may due to the higher concentration tested and not to a specific preference for D-serine by CFT073. Therefore, all D-amino acids were tested at 300-500 μg/mL to determine whether a concentration increase alone could amplify the chemotactic response toward each D-amino acid. When tested at the higher concentration, the chemotactic response to D-alanine and D-asparagine increased relative to that at their physiological concentration (Table 2). D-phenylalanine, D-proline, D-threonine, D-tryptophan, D-tyrosine, and D-valine did not produce a response above buffer at the higher concentration (data not shown). The results obtained for K-12 strain RP437 toward the D-amino acids again displays the similarity of response to attractants between the two strains (shown in parenthesis in Table 2). These data indicates that D-amino acids, in addition to L-amino acids, are sensed by the chemosensory machinery of CFT073 and may influence the chemotactic behavior of *E. coli in vivo*.

The chemoreceptor(s) mediating the response to the D-amino acids were determined using the chemoreceptor mutants with the amino-acid concentration that elicited the greatest response. Loss of *tsr* diminished the response to D-alanine, D-

phenylalanine, D-proline, D-serine, D-threonine, and D-tyrosine. Loss of *tar* lessened the response toward D-alanine, D-asparagine, D-phenylalanine, D-proline, D-serine, D-threonine, and D-tyrosine, while the loss of *aer* partially diminished chemotaxis toward D-alanine, D-asparagine, D-phenylalanine, D-proline and D-tyrosine (Table 3). The complemented deletion strains were tested in the capillary assay for a return to the wild-type phenotype. Unlike the results for the L-amino acids, the *tsr* complement showed a return to wild-type response levels for only D-alanine and D-serine, and the *tar* complement responded like the wild-type strain to just D-alanine, D-asparagine and D-serine. The *aer* complement did not return the response of any of the D-amino acids tested to wild-type levels (data not shown).

Most non-amino acid components of urine do not act as chemoattractants for *E. coli* CFT073.

A substantial portion of the solutes in human urine consists of organic compounds in addition to amino acids (14, 37, 41, 46). We tested a large subset of the compounds found in the highest abundance in human urine as there were no reports of their study as chemoattractants. Urea, creatine, creatinine, glucuronic acid, uric acid, imidazole, glucose, inositol, urobilin, allantoin, 2'-deoxyadenosine 5'-triphosphate, 2'-deoxyguanosine 5'-triphosphate and caffeine were tested in the capillary assay, using values at the higher range of their physiological concentrations. As reported previously in K-12 (3), glucose elicited a response from wild-type CFT073, but this response was much weaker than observed for K-12 at the tested concentration. Caffeine also

produced a very weak response from wild-type CFT073 that may not be biologically significant (Table 2). No other component tested produced a response above the buffer baseline (data not shown). When caffeine was tested as an attractant with the chemoreceptor knock out mutants, only the *tsr* mutant displayed a decrease in its response (Table 3). However, complementation of the *tsr* mutant did not return the response to caffeine to wild-type levels (data not shown). Because it has been determined that glucose is detected through the interaction of unphosphorylated Enzyme I of the phosphoenolpyruvate-dependent carbohydrate phosphotransferase system with the chemotaxis machinery (2, 3, 9, 32), the chemoreceptor mutants were not tested with glucose as an attractant.

A variety of hormones are also secreted in urine in the ng/mL range, including dopamine, norepinephrine, epinephrine, estrogens and androsterone (35, 36, 40, 51). Another lab has shown that enterohemorrhagic *E. coli* is attracted to norepinephrine and epinephrine (7). To determine whether CFT073 is also attracted to hormones, dopamine, norepinephrine, epinephrine, estradiol, estrone, estriol and androsterone were tested as potential attractants for CFT073 in the capillary assay. Unlike enterohemorrhagic *E. coli*, CFT073 did not respond to epinephrine, norepinephrine or any of the other hormones at their physiological concentrations (data not shown). Therefore, hormones are unlikely to act as attractants for uropathogenic *E. coli* in the urinary tract.

Presence of any single chemoreceptor imparts a fitness advantage *in vivo*.

Studies with *Vibrio cholerae*, *Helicobacter pylori* and *Campylobacter jejuni* reported the necessity for chemotaxis in the colonization of each pathogens' infectious niche (4, 12, 13, 29, 48). To determine whether loss of any chemoreceptor results in attenuation in the urinary tract, female Swiss-Webster mice were co-infected with an equal mix of wild-type CFT073 and a CFT073 chemoreceptor mutant via urethral catheterization. All animals were sacrificed at 48 hours post-infection. The ratios of the wild-type strain to the mutant strain recovered from the bladder and the kidneys revealed that loss of any two chemoreceptors did not affect the ability of the mutant strain to achieve wild-type colonization levels in the urinary tract of mice (Fig. 2 A-C).

Discussion

Initial events in the colonization of the urinary tract by UPEC remain to be characterized in detail. We hypothesize that navigation to and in the urinary tract via chemotaxis are among the first steps to aid UPEC colonization of the urethra and bladder. As previously shown, uropathogenic *E. coli* CFT073 is attracted to undiluted human urine, demonstrating that the attraction of CFT073 to urine may aid the bacteria in gaining access to the urinary tract (27). In support of this hypothesis, other bacterial species use chemotaxis to direct colonization of specific regions of the digestive tract (i.e. *V. cholerae* to the deep crypts of the small intestine and *C. jejuni* to the mucus of the gastric epithelium) (4, 12, 13). Additionally, the plant pathogen *Ralstonia*

solanacearum uses aerotaxis to quickly find the roots of its host plant to initiate colonization (48, 49). Multiple species of bacteria use chemotaxis to direct colonization to their preferred niches, highlighting chemotaxis as an important colonization factor during pathogenesis.

We sought to determine which chemoreceptor(s) is responsible for the attraction of CFT073 to urine. CFT073 deletion mutants missing one of the three chemoreceptors (Δ*tsr*, Δ*tar*, or Δ*aer*) were subsequently tested for chemotaxis toward human urine. Loss of any one chemoreceptor did not have a significant effect on the attraction to urine, indicating that there were redundant ligands shared between the chemoreceptors and/or that there were multiple strong attractants in urine that are sensed by different chemoreceptors. When both Tsr and Tar were absent, the positive response to urine was almost abolished, indicating that these two chemoreceptors are mainly responsible for chemotaxis toward urine. The loss of Aer did not change the response to urine. One explanation for the loss of a response toward urine when only *aer* is present may be that the small number of Aer receptors, which is markedly lower than Tsr and Tar levels (30), is simply too small to direct an efficient chemotactic response without the aid of Tsr and Tar to form the receptor clusters (19). Therefore, urine could still stimulate a response through Aer, but the resulting signal would be too small to produce a chemotactic response that could be detected by the capillary assay. Aer could also be important for sensing oxygen within the urinary tract, but aerotaxis was not tested in this study.

The results obtained in this study with *E. coli* CFT073 mostly concur with those obtained previously with *E. coli* K-12. Just as with K-12, CFT073 is attracted to a variety of L-amino acids including alanine, asparagine, aspartate, cysteine, glutamate, glycine, methionine, serine and threonine. Unlike K-12, CFT073 did positively respond to histidine. The responses obtained in this report were also slightly lower than in Adler's original report, a difference that might be accounted for by growth in urine instead of MOPS glycerol, as well as some slight differences in the purity of the attractants used. However, the experiments using chemoreceptor mutant to determine whether Tsr or Tar mediates the response to each L-amino acid did agree with previous reports (33). Additionally, this study identified Tar as the chemoreceptor most likely to mediate chemotaxis toward L-histidine, and Aer as a partial mediator of chemotaxis toward L-alanine and L-cysteine in *E. coli* CFT073.

In addition to the response to L-amino acids, this study also identified several D-amino acids and two non-amino acid components of urine that produced chemotactic responses above buffer for *E. coli* CFT073 within their physiological ranges. These components include D-alanine, D-phenylalanine, D-proline, D-serine, D-threonine, D-tyrosine, glucose and caffeine with D-serine exhibiting the strongest response. The other D-amino acids on this list, as well as glucose and caffeine, weakly responded even at increased concentrations, although D-asparagine generated a response that surpassed that of D-serine when tested at a concentration that far exceeded its reported concentration range in urine. Because the urine concentrations of each amino acid can vary widely from person to person, the reported range of D-asparagine in human urine

is by no means absolute. Therefore, D-asparagine may act as an attractant in urine for UPEC when its concentration is sufficiently high. Capillary assays using the complemented receptor strains showed that only the responses toward D-alanine, D-asparagine and D-serine could be returned to wild-type levels. This suggests that, from the above group, only these three D-amino acids elicit a true response through the chemoreceptors.

Our results also indicate that the presence of any one chemoreceptor is sufficient for successful colonization of the urinary tract in the mouse model. These results indicate that a certain amount of flexibility is inherent in the use of chemotaxis by the bacteria to colonize the urinary tract, and suggest that any strong attractant present in urine would be sufficient to enhance the efficiency of colonization. However, the direct inoculation of the bladder and potential reflux of bacteria into the kidneys of the mice immediately after inoculation prevents any conclusions that can be made about the necessity of chemotaxis for ascension of the urethra and ureters, and could mask any potential colonization defects. A better model for ascension of the urinary tract is needed to address this issue. Additionally, the chemotaxis system could be important not only for ascension of the urinary tract but also for penetration of the mucus layer to achieve close contact with the bladder epithelium (18, 31). Without the ability to switch rotation of the flagella and the direction of net movement, the bacteria could become stuck delayed in the mucus layer leading to eventual expulsion of the bacteria from the urinary tract by micturition. One final curious observation is that, while the CFT073Δ*tsr*Δ*tar* mutant was attenuated for chemotaxis toward urine *in vitro*, this strain

performed like wild-type CFT073 in the mouse model. Although Aer protein levels appear insufficient to produce a detectable response in the capillary assay, they may be sufficient to produce a chemotactic response *in vivo* that keeps the bacteria competitive during infection.

This study has shown that amino acids, both D and L forms, act as attractants for *E. coli* CFT073 within their physiological concentrations in urine, and that Tsr and Tar are primarily responsible for sensing these attractants. Loss of any one chemoreceptor did not result in a fitness defect *in vivo*, although the current mouse model may be a poor tool to evaluate the contribution of each chemoreceptor in the infectious process. The ability to navigate the urinary tract may result in more efficient colonization, lessening the possibility that the bacteria would be dispelled by urine flow before being able to colonize the bladder. An improved method for monitoring early events in the colonization of the urinary tract is needed to determine whether chemotaxis plays a part in the ascension of the urethra and ureters, resulting in a colonization of the urinary tract.

Acknowledgements.

We thank Dr. Alan Wolfe for kindly providing *E. coli* strain RP437 for use in this study**.** This research was funded by the National Institutes of Health Grant R01 DK063250-06 and by the National Institutes of Health, National Research Service Award AI55397.

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Table 1: Primers used in this study

Gene/Application	Sequence
λ-Red deletions	
tsr deletion	5'- GGCCGAAAATCTTGCATCGGTCCACAGGAAAGAGAAA CCTGTGTAGGCTGGAGCTGCTTC-3'
	5'-AAAACGCCGGATGAAATACTCATCCGGCATCATTACGC ATATGAATATCCTCCTTAG-3'
tsr deletion ck	5'-CGAGAACCGGACCCACCAG-3'
	5'-CCTGGCCTGCGCTGTTCC-3'
tar deletion	5'- CCCATCAGGCGGCAATGACCGCTTTAGTAAATACTCGT GTGTAGGCTGGAGCTGCTTCG-3'
	5'- AATAAAGTTTCCCCCCTCCTTGCCGATAACGAGATCAA CCATATGAATATCCTCCTTAG-3'
tar deletion ck	5'-CCAATGTATCAGCCAGCGTC-3'
	5'-CTACGCCTTGCTGGTGGATC-3'
aer deletion	5'-GGCATTGTGCTCCAACCGCTGGATCCGGCATACCGAT GTGTAGGCTGGAGCTGCTTCG-3'
	5'- GAAGTTAACAACCATATAACCTGCACAGGACGCGAACC ATATGAATATCCTCCTTAG-3'
aer deletion ck	5'-CGATGACAGCCACGGTTACG-3'
	5'-GCTTGCCACTCTACGGCTC-3'
ϕ restorations	
tsr (*tsr-c5431*)	5'-ACGTAATGATGCCGGATGAGTATTTCATCCGGCGTTTT TGTGTAGGCTGGAGCTGCTTCG-3'
	5'-ATATCCCGGCGATTTCACTGTTCCTGGTTTAATAATAAA CCATATGAATATCCTCCTTAG-3'
tar (*argS-flhE*)	5'-AGCGTATGTAATCGCGTTATACGGCAACAGCCGATGTT TGTGTAGGCTGGAGCTGCTTCG-3'
	5'-AATTATCGTTAATGATAAAAAAGCCGATGTCTGCAATAA CCATATGAATATCCTCCTTAG-3'
aer (*aer-ygjL*)	5'-ATATTACGCAACTGGATTAATCGCCGCATCCGCCAGTG TGTGTAGGCTGGAGCTGCTTCG-3'
	5'-GGCGCGTCAAGCGTCGCATCCGGCAATTGCACCGCGC AACCATATGAATATCCTCCTTAG-3'

Table 2. Chemotaxis by *E. coli* CFT073 toward L-amino acids

Component[d]	Reported High Value in Urine (mg/mL)	Concentration Tested (mg/mL)	CFT073 CFU Accumulated ($x10^3$)[a,b,c]	CFT073 Fold Change to Buffer[a]	RP437 CFU Accumulated ($x10^3$)[a,b,c,e]
L-alanine	6.2	5	284±150[c]	10.7±4.8	256±87[c]
L-asparagine	2.9	3	211±130[c]	9.8±5.1	104±27[c]
L-aspartate	1.2	1	62±51	2.7±1.2	86±19[c]
		0.5	209±68[c]	8.1±1.4	192±24[c]
L-cysteine	4.0	1	320±154[c]	15.1±8.6	312±87[c]
L-glutamate	1.7	1.5	157±108[c]	6.3±2.4	130±17[c]
L-glycine	18.0	10	161±87[c]	6.8±2.5	167±43[c]
L-histidine	23.3	20	10±2	1.4±0.2	BB
L-methionine	2.3	2	5±14	1.1±0.4	12±5
		15	58±29	2.9±0.8	60±9
L-serine	7.3	5	15±10	1.6±0.4	135±61[c]
		1	249±42[c]	9.0±1.3	222±35[c]
L-threonine	3.8	0.12	8±3	1.4±0.2	6±1

[a] Values are means ± standard deviations (n=3-6).
[b] Normalized to buffer control.
[c]Statistical significance ($p<0.05$) from buffer control was calculated using a 2-way ANOVA analysis with Bonferroni post-tests on CFU/capillary data.
[d]Responses toward arginine, cystine, glutamine, isoleucine, leucine, lysine, phenylalanine, proline, taurine, tryptophan, tyrosine, and valine were all below the buffer control.
[e]BB= Below buffer

Table 3: Chemotaxis by *E. coli* CFT073 toward D-amino acids, caffeine and glucose

Component	Reported Range in Urine (μg/mL)	Concentration Tested (μg/mL)	CFT073 CFU Accumulated ($x10^3$)[a,b,c]	CFT073 Fold Change to Buffer[a]	RP437 CFU Accumulated ($x10^3$)[a,b,c]
D-alanine	0.68-4.79	500	60±71	2.8±1.8	23±8
		10	2±3	1.0±0.6	
		5	3±6	1.0±0.6	
D-asparagine	0-0.1189	300	92±68	3.9±2.1	178±43[d]
		0.3	BB		
		0.15	BB		
D-phenylalanine	0.02-2.04	4	4±3	1.4±0.3	BB
		2	4±2	1.4±0.4	
D-proline	0.03-1.34	2	5±3	1.5±0.6	BB
		1	3±4	1.2±0.4	
D-serine	3.16-114	200	96±68	5.8±4.6	10±2
		100	17±8	2.8±1.2	
D-threonine	0.03-0.69	1.4	3±1	1.4±0.3	11±14
		0.7	4±5	1.3±0.5	
D-tyrosine	0.008-	5	BB		
	2.51	2.5	3±4	1.3±0.4	BB
Caffeine	1.33-	20	2±3	1.1±0.2	BB
	10.26	5	4±2	1.2±0.1	
D-glucose	30-200	250	3±9	1.4±0.6	
		125	0.3±9	1.3±0.7	

[a] Values are means ± standard deviations (n=3-6).
[b] Normalized to buffer control.
[c] BB= Below buffer
[d] Statistical significance ($p<0.05$) from buffer control was calculated using a 2-way ANOVA analysis with Bonferroni post-tests on CFU/capillary data.

Table 4: Chemotaxis by *E. coli* CFT073 receptor mutants toward identified attractants

Component	Fold Change to Buffer[a]			
	Wild-type	Δ*tsr*	Δ*tar*	Δ*aer*
L-alanine	10.7±4.8	1.2±0.2[b]	1.7±1.0[b]	4.3±4.4[b]
L-asparagine	9.8±5.1	5.9±4.2	2.5±2.1[b]	7.4±5.1
L-aspartate	8.1±1.4	6.7±1.1	0.5±0.2[b]	5.4±1.1
L-cysteine	15.1±8.6	2.8±1.8[b]	2.2±1.5[b]	6.4±4.7[b]
L-glutamate	6.3±2.4	9.7±6.9	0.4±0.2[b]	4.4±4.7
L-glycine	6.8±2.5	1.0±0.1[b]	1.5±0.9	5.0±2.7
L-histidine	1.4±0.2	2.8±2.5	0.7±0.5	2.1±0.8
L-methionine	2.9±0.8	3.3±1.4	0.8±0.2	1.6±0.4
L-serine	9.0±1.3	1.0±0.01[b]	2.7±1.3	6.7±2.2
L-threonine	1.4±0.2	0.6±0.1	1.8±0.7	1.9±0.4
D-alanine	2.8±1.8	0.7±0.1	1.5±0.7	1.6±0.3
D-asparagine	3.9±2.1	8.3±3.7	1.2±0.7	2.0±0.3
D-phenylalanine	1.4±0.3	1.0±0.3	0.7±0.3	1.8±0.2
D-proline	1.5±0.6	0.9±0.2	0.6±0.3	0.9±0.5
D-serine	5.8±4.6	0.9±0.1	1.7±0.9	3.1±1.3
D-threonine	1.4±0.3	0.9±0.2	0.6±0.2	1.0±0.6
D-tyrosine	1.3±0.3	0.8±0.3	0.6±0.2	1.1±0.6
Caffeine	1.1±0.2	.96±0.2	1.1±0.1	1.1±0.1

[a] Values are means ± standard deviations (n=3-6).
[b] Statistical significance ($p<0.05$) from wild-type CFT073 was calculated using a 2-way ANOVA analysis with Bonferroni post-tests using CFU/capillary above buffer data.

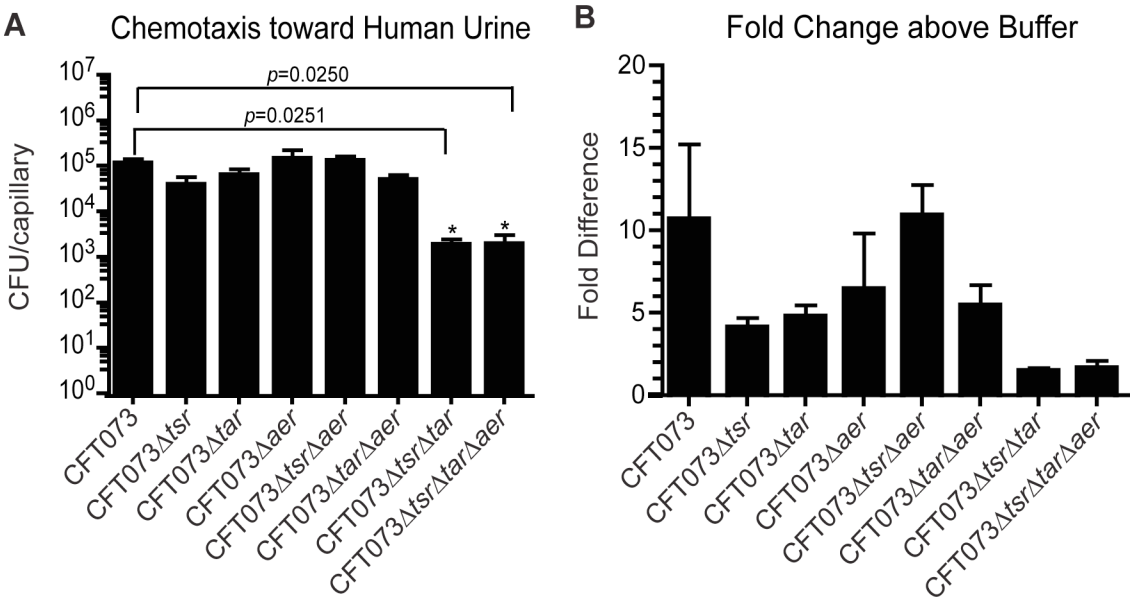

Figure 1: Chemotaxis of wild-type CFT073 and CFT073 chemoreceptor mutants toward undiluted human urine. The colony forming units (CFUs) normalized to the buffer control (A) and the fold increase relative to the buffer control (B) are shown for each strain. Each strain was tested in triplicate, and a nonparametric T-test used to calculate statistical significance between the wild-type and mutant strains.

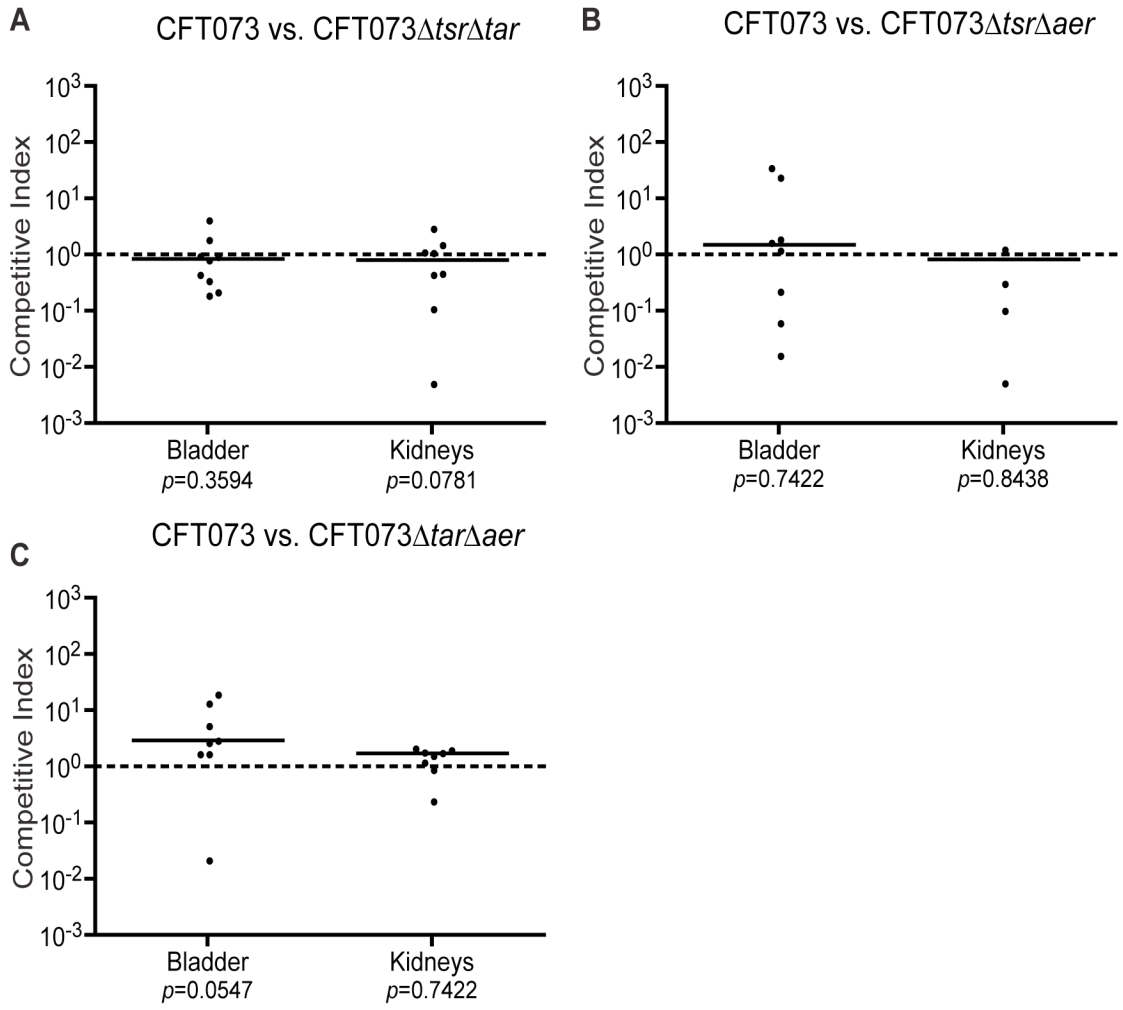

Figure 2: Chemoreceptor mutants compete like the wild-type strain *in vivo*. The results for competitive co-infection with wild-type CFT073 and CFT073ΔtsrΔtar (n=9) (A), CFT073ΔtsrΔaer (n=8) (B), CFT073ΔtarΔaer (n=8) (C) in the mouse model of UTI are shown. The competitive indices were calculated by the following equation: (mutant CFU recovered/wild-type CFU recovered)/(mutant inoculum CFU/wild-type inoculum CFU). The line indicates the median value.

Chapter 3

YfiR-dependent Regulation of YfiN Diguanylate Cyclase Activity Mediates Control of Processes Important for Perisitence in the Urinary Tract by *Escherichia coli* CFT073

In preparation for publication

Authors and contributions:

Erica L. Raterman: Wrote manuscript and performed all experiments.

Rodney A. Welch: Supervised all work and contributed to the writing of the manuscript.

Abstract

During urinary tract infections (UTIs) uropathogenic *Escherichia coli* must maintain a delicate balance between sessility and motility to achieve successful infection of both the bladder and kidneys. Previous studies showed that c-di-GMP levels aid in the control of the transition between motile and non-motile states in *Escherichia coli*. A survey of genes purported to be involved in motility identified the *yfiRNB* locus in *E. coli* CFT073, which encodes genes for YfiN, a diguanylate cyclase, and its activity regulators, YfiR and YfiB. Deletion of *yfiR* yielded a mutant that was attenuated in both the bladder and the kidneys when tested in competition with the wild-type strain in the murine model of UTI. A double *yfiRN* mutant was not attenuated in the mouse model, suggesting that YfiR is a repressor of YfiN activity, and unregulated YfiN activity causes a survival defect *in vivo*. Curli fimbriae and cellulose production were increased in the *yfiR* mutant. Additional deletion of *csgD* and *bcsA*, genes necessary for curli fimbriae and cellulose production, respectively, returned colonization levels of the *yfiR* deletion mutant to those of wild type in the mouse model. Peroxide sensitivity assays and iron acquisition assays displayed no significant differences between the *yfiR* mutant and the wild-type strain. These findings indicate that disregulation of c-di-GMP production results in pleiotropic effects that disable *E. coli* in the urinary tract ,and implicate the c-di-GMP regulatory system as an important factor in the persistence of uropathogenic *E. coli in vivo*.

Introduction

Uropathogenic *Escherichia coli* (UPEC) are the most prevalent isolates of patients with urinary tract infections (UTIs). Unlike many other bacterial infections, UTIs are common in young healthy women and are among the most frequently diagnosed infections in the United States (2, 12, 17, 22, 28). The contribution of colonization and virulence factors such as type I fimbriae, iron acquisition systems, and hemolysin to successful infection of the urinary tract by UPEC are well documented (3, 11, 13, 21, 26, 41, 42, 44, 45). However, the contribution of second messengers such as bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) to virulence have thus far not been documented in UPEC. Given the recent advances in the understanding of the downstream effectors of c-di-GMP, interest in the role of c-di-GMP signaling in the virulence of bacterial pathogens has increased.

c-di-GMP is created by diguanylate cyclases (DGCs) from two GTP molecules, and is broken down to 5'-phosphoguanylyl-(3'-5')-guanosine (pGpG) by specific phosphodiesterases (PDEs). Other enzymes are responsible for breaking down pGpG to two GMP molecules. All DGCs possess the GGDEF (Gly-Gly-Asp-Glu-Phe) active site domain, and PDEs are associated with the EAL (Glu-Ala-Leu) domain (40). Various external or internal stimuli are sensed by the DGCs and translated to the concentration of c-di-GMP effector molecules to alter the behavior of the bacteria. Because c-di-GMP effectors are present at all levels of regulation, including transcriptional, translational and post-translational functions, c-di-GMP signaling can produce rapid responses to quickly changing environmental conditions. The most common alterations made by c-

di-GMP signaling in *E. coli* are changes in motility and production of adhesins and exopolysaccharides. In general, increased levels of c-di-GMP decrease motility and increase multicellular community behaviors, while the reverse is true when c-di-GMP levels are low (1, 9, 16, 18, 19, 24, 31, 36, 38, 39, 43, 46).

Groups working with nonpathogenic K-12 strains of *E. coli* have established a link between curli fimbriae production and c-di-GMP levels (7, 19, 33). Curli fimbriae are coiled surface structures that promote both adhesion to surfaces and cell-cell interactions. The curli operon is organized in two divergent operons (*csgDEFG* and *csgBA*), and the transcriptional activator CsgD stimulates expression of *csgBA* as well as its own operon. CsgA is the major subunit of the curli fiber and CsgB is needed for the nucleation of the CsgA oligomer. Increased levels of c-di-GMP lead to increased activity of CsgD (8, 15, 19, 33, 35). Various studies have demonstrated the ability of curli fimbriae to induce an immune response through generation of proinflammatory fibrinopeptides and by activating inducible nitric oxide synthase, a defense mechanism that produces large amounts of nitric oxide (27, 30). Increased bacterial resistance to the antimicrobial peptide LL-37 is also a characteristic of curli fimbriated *E. coli* (20).

Increased activity of CsgD also indirectly affects the production of cellulose. Expression of *adrA* and *yedQ*, two genes that encode DGCs, is activated by CsgD. The subsequent production of c-di-GMP by either or both AdrA and YedQ, depending on the strain of *E. coli*, stimulates increased activity of BcsA, the catalytic subunit of the cellulose biosynthetic machinery. Transcription levels of the cellulose operons (*bcsEFG* and *yhjRQbcsABZC*) are not affected by c-di-GMP levels (1, 8, 19, 34, 37, 47).

Production of cellulose in concert with curli fimbriae dampens the immune response produced by the curli fibers (20). The co-production of these factors also produces a distinctive *in vitro* plate phenotype termed the RDAR morphotype for the red, dry and rough colonies observed on agar plates that contain congo red dye (33). The RDAR phenotype is synonymous with the small colony variant (SCV) phentotype seen in *Pseudomonas aeruginosa* isolates.

This study investigates the links between the DGC YfiN and its activity regulator YfiR that were first identified in a screen for loss of motility in *E. coli* K-12 (14) and the changes observed in curli fimbriae production, cellulose production and motility. The implications of the expression and activity of these systems for the virulence of UPEC strain CFT073 in the urinary tract is also assessed using the mouse model of UTI. Deletion of *yfiR* and the subsequent increased activity of YfiN, lead to increased production of curli fimbriae and cellulose, and a near total loss of motility. We further show that these phenotypes lead to attenuation in the competitive mouse model of UTI. This study is the first report to demonstrate that proper control of c-di-GMP production is essential for UPEC to mount successful infections in the urinary tract.

Materials and Methods

Strains and Media

E. coli strain CFT073 was isolated from a patient with pyelonephritis at the University of Maryland Medical System. Strains were grown in Luria-Bertani broth (LB), MOPS glycerol broth, tryptone broth (2 g tryptone, 1 g NaCl in 200 mL ddH_2O), or filter

sterilized human urine collected from a female volunteer. Curli fimbriae and cellulose expression were determined by growth on modified LB agar plates (10 g tryptone, 5 g yeast extract, 15 g agar per 1 L ddH_2O with or without 40 µg/mL congo red and 20 µg/mL coomassie blue added after autoclaving) or urine agar plates (750 mL sterile human urine mixed with autoclaved 15 g agar in 250 mL ddH_2O with or without 40 µg/mL congo red and 20 µg/mL coomassie blue). Swimming motility was assessed in 0.3% tryptone agar plates (1 g tryptone, 0.5 g NaCl, 0.3 g agar per 100 mL ddH_2O), and swarming motility was assessed on 0.45% swarm agar (0.3 g meat extract, 1 g bacto-peptone, 0.45 g agar per 100 mL ddH_2O with 0.5 g glucose added after autoclaving). Antibiotics were added in the following concentrations as appropriate: 50 µg/mL kanamycin, 20 µg/mL chloramphenicol, 200 µg/mL carbenicillin. 10 mM arabinose was added to the media to induce expression from the pBAD promoter when necessary.

Construction of mutant strains,allelic repair and complementation systems

All primers used for the generation of deletion mutants and cloning are listed in Table 2. All nonpolar gene deletions were performed using the λ-Red recombination system developed by Datsenko and Wanner (10). The PCR products used for transformations in CFT073 were generated off of pKD3 or pKD4 templates using primers specific for the targeted gene. The kanamycin or chloramphenicol-marked gene deletions were then transduced into a fresh CFT073 background using the EB49 transducing phage (4). The antibiotic resistance cassette used to replace the target gene was removed using a Flp recombinase encoded on pCP20 (10). All gene

deletions were verified by PCR and loss of antibiotic resistance on LB agar containing the appropriate antibiotic. The *yfiR* locus was repaired in the *yfiR* deletion mutant by insertion of the kanamycin cassette via λ-Red near, but outside of, the *yfiLRNB* locus, being careful not to disrupt any other loci. The EB49 transducing phage was used to transduce the wild-type *yfiLRNB* locus into the *yfiR* deletion mutant background. Restoration of the locus was confirmed by PCR and sequencing.

Complementation of the *yfiR* deletion strain was obtained by cloning PCR-amplified *yfiR* into the NcoI-PstI restriction sites of pBAD24d. Sequencing of the resulting plasmid confirmed the insertion of *yfiR*. pBAD30-*yhjH* and pBAD30-$yhjH_{E136A}$ were obtained from Ute Römling (40). The *lacZ* transcriptional fusion strains used for the β-galactosidase assays were obtained using the pFUSE system (5). Briefly, approximated 500 bp of the 5' end of the targeted gene was cloned into the XbaI/SmaI sites of pFUSE to link the gene to the plasmid-encoded *lacZYA* locus. The resulting plasmids were used in a tetraparental mating with two other strains carrying the transposase on a suicide plasmid for homologous recombination into a CFT073 Δ*lacZ::Kan* background strain. This method produces a transcriptional fusion of the *lacZYA* operon with the targeted gene in the CFT073 genome. Kanamycin and chloramphenicol were used to select positive strains and inserts were verfied by PCR and sequencing.

Murine model of UTI

Colonization of the urinary tract was determined using the competitive murine model of urinary tract infection as described previously (32). CFT073Δ*lacZYA* was used as the wild-type strain and the chemotaxis mutants had an intact *lacZYA* locus. To select for piliated bacteria, all bacterial strains were grown statically in 3 mL LB at 37˚ C for 2 days. The pellicle formed on the rim of the test tube was then transferred to fresh LB, incubated for 2 more days, and finally passaged again to 40 mL LB for a final 2-day incubation. The broths were adjusted to OD_{600} ~0.4 with 1XPBS and the wild-type strain and the mutant strain were mixed equally. The mixed broth was then pelleted and washed 1X in 1XPBS and resuspended in 500 µl 1XPBS. Isofluorane-anesthetized 6-7 week-old female Swiss Webster mice (Harlan, USA) were inoculated via urethral catheterization with 50 µl (~10^8 CFU) of the mixed bacterial suspension. Mice were euthanized via CO_2 asphyxiation and the bladder and kidneys were aseptically harvested in 1XPBS. The organs were homogenized, serially diluted in 1XPBS, and plated on MacConkey agar medium (Fisher, USA). Surviving strain ratios were determined by counting white (wild-type) and red (mutant) colonies. Colonization levels were graphed and analyzed using a paired Wilcoxon signed ranked test and Prism (GraphPad). Competitive indices were calculated by dividing mutant CFUs by wild-type CFUs from the mice and then dividing this ratio by the ratio of mutant to wild-type in the original inoculum. All animal experiments were approved by the UW-Madison Animal Care and Use Committee.

Preparation and western blotting of cell fractions

Cellular fractionation experiments were performed as previously described. Briefly, 200 mL of LB broth with 50 μg/mL kanamycin, 10 μM IPTG, and 0.2% maltose were inoculated with 2 mL of an overnight broth grown in the same medium at 37°C. The broths were incubated with shaking at 37°C to ~OD_{600} 0.8. Cells were then pelleted at 8,000 rpm and resuspended in 5 mL spheroplasting buffer (0.2 M Tris-HCl, pH 8.0, 1 M sucrose, 1 mM EDTA, 1 mg/mL lysozyme). The samples were incubated at room temperature with shaking for 5 minutes followed by addition of 40 mL of ddH_2O and another 5 minute incubation on ice. Cells were centrifuged at 200,000 xg for 45 minutes at 4°C to separate the periplasmic fraction from the spheroplasts. The spheroplasts were resuspended in 7.5 mL ice cold cell fraction buffer (10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.2 mM DTT) with 50 μl 1 mg/mL DNase. All fractions were stored at -20°C. Protein concentration of each sample was determined using Bradford assays (Biorad, USA)

Before loading on SDS-PAGE gels, each sample was mixed 1:1 with 2X CRACK buffer and boiled for 20 minutes. Samples were run on 15% SDS-PAGE gels at 125 V for ~3 hours. Gels were either coomassie stained or transferred to nitrocellulose membranes at 40 V for 1 hour. Membranes were blocked in 5% milk in 1X TBST for one hour at room temperature before addition of 1:4000 anti-FLAG antibody. Membranes were then incubated overnight at 4°C. Membranes were washed in 1X TBST and probed with 1:20,000 anti-rabbit HRP for 30 minutes at room temperature before being washed again and exposed to ECL reagent (GE Healthcare, USA).

Membranes were exposed to film and processed. Bound antibodies were removed from the membranes by suspension in 100 mL stripping solution (100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris-HCl, pH 6.7) and incubation at 50°C for 1 hour with agitation. The probing and development process was repeated with 1:5,000 anti-maltose binding protein and 1:10,000 anti-rabbit HRP or 1:1,000 anti-RpoS and 1:10,000 anti-mouse HRP to ensure accurate fractionation of periplasmic- and cytoplasmic-associated proteins, respectively.

β-galactosidase assay

All β-galactosidase assays were performed as previously described (2). Briefly, bacterial strains were grown on LB agar plates without salt containing 200 μg/mL carbenicillin and 10 mM arabinose if necessary. Plates were incubated 16-20 hours at 30° C. The bacteria were then collected with cotton swabs and suspended in 3 mL tryptone broth without salt to an OD_{600} between 0.4 and 0.8. 500μl of each broth was mixed with 500 μl Z buffer (60 mM Na_2HPO4, 60 mM NaH_2PO_4, 10 mM KCL, 1 mM $MgSO_4$, β-mercaptoethanol, pH 7.0), 20 μl chloroform, and 10 μl 0.1% SDS and vortexed for 10 seconds. Samples were then incubated at 28° C for 5 minutes, and 200 μl of 4 mg/mL ONPG were then added. The reaction was stopped with 500 μl Na_2CO_3. The OD_{420} and OD_{550} were then recorded and the Miller units were calculated according to the manual.

Congo red binding assay

Congo red binding assays were performed as previously described. Briefly, strains were swabbed on LB agar plates without salt containing 200 μg/mL carbenicillin and 10 mM arabinose if necessary. Plates were incubated 16-20 hours at 30˚ C. The bacteria were collected with cotton swabs and suspended in 5 mL tryptone broth with 40 μg/mL congo red and without NaCl. Samples were taken for dilution plating on LB plates, and broths were separated into two tubes and incubated with shaking at 37° C for 2 hours. The bacteria were pelleted at 8,000 rpm and the OD_{490} was determined for the supernatants. The amount of congo red dye left in the supernatant was determined using a standard curve of known concentrations of the dye.

Congo red plates were used to determine the RDAR morphotype of each strain as previously described. Strains were grown statically in 3 mL LB at 37˚ C for 2 days. The pellicle formed on the rim of the test tube was then transferred to fresh LB, incubated for 2 more days, and finally passaged again to 3 mL LB for a final 2 day incubation. Broths were dilution plated onto the indicated congo red plates and incubated for 5-7 days at 30° C. Colony morphology was then recorded.

Pellicle Assay

Strains were grown for 16 hours at 37° C at 250 rpm in 3 mL of MOPS glycerol broths in glass test tubes. Expression from plasmids was induced with 10 mM arabinose. After growth, broths were removed from the test tubes and the pellicles were stained for 15 minutes with 0.1% crystal violet. Tubes were then washed 3X with 1X PBS. Bound

crystal violet was released by addition of 5 mL of ethanol-acetone solution (80:20, v/v) and the OD 590nm was measured.

Western blot analysis of CsgA expression

Strains were grown overnight at 30°C on LB plates without salt with carbenicillin and arabinose for induction of pBAD-*yfiR* strains. Bacteria were collected using cotton swabs and resuspended in 1 mL 1XPBS. A sample of each broth was taken for plate counts and the remaining sample was prepared according to recommendations from the lab of Dr. Matt Chapman at the University of Michigan, Ann Arbor (personal communication). The bacteria were pelleted at 8,000 rpm, resuspended in 100 µl 99% formic acid, and incubated for 10 minutes on ice. Samples were then evaporated to dryness using a Speedvac. Pellets were resuspended in 200 µl 2X CRACK buffer. The dual precision plus protein standard (Biorad, USA) and the samples were then loaded on 15% SDS-PAGE gels and run at 250 V for 3 hours. Amounts loaded were normalized using the CFUs obtained from the plate counts. Gels were either coomassie stained or transferred to nitrocellulose at 40 V for 1 hour. Nitrocellulose membranes were then blocked with 5% milk in 1X TBST for 1 hour at room temperature. Anti-CsgA antibody (1:7000) (obtained courtesy of the Chapman lab) was added and blots were incubated overnight at 4° C. Blots were then washed 3 times with 1X TBST and probed with anti-rabbit HRP (1:20,000) in 5% milk in 1X TBST for 30 minutes at room temperature. Blots were washed with 1X TBST 3 more times and developed using an ECL kit (GE Healthcare, USA).

Motility assays

Swimming motility was assessed in 0.3% agar tryptone plates. Strains were grown overnight at 37°C in tryptone broth. Broths were adjusted to OD_{600} ~0.1 with fresh tryptone broth before 2 μl was injected in the center of the agar swim plates. Ten microliters of the same broths were placed on the surface of swarm agar plates to assess swarming motility. Swim plates were incubated 12 hours at 30° C, and swarm plates were incubated at 30° C for 2 days. Assays were independently repeated at least three times.

Hydrogen peroxide sensitivity assay

Overnight cultures grown in 3 mL LB were diluted 1:1000 in 1XPBS. Samples were taken for dilution plating to establish CFUs present before addition of H_2O_2. About 0.51 μl of 30% H_2O_2 was added to diluted broths. Samples were incubated statically at 37° C and aliquots were taken every half hour for dilution plating. Diluted broths without H_2O_2 added were used as controls. Two independent assays were performed for each strain.

Iron limitation assay

Growth in iron-limited environments was assessed using LB and MOPS glycerol broths containing either 0, 200, or 400 μM 2,2-dipyridyl, an iron chelator. Strains were grown overnight in LB broths at 37° C with shaking. Broths were diluted with fresh LB to

OD_{600} ~0.1, and 10 µl of each broth was added to 200 µl of the indicated media with the various amounts of 2,2-dipyridyl in a 96 well flat-bottom plate (Corning, USA). Plates were incubated at 37° C with shaking, and the OD_{600} of each well was read and recorded each hour using the Synergy HT multi-mode microplate reader (BioTek, USA). Two independent assays were performed for each strain.

Results

Predicted functions of the YfiLRNB system of *E. coli* CFT073 are similar to the YfiRNB system of *P. aeruginosa*.

A recent report in *P. aeruginosa* identified the YfiRNB system as a regulatory complex that plays a role in the development of the SCV phenotype important for persistence in a subcutaneous catheter model. YfiN was identified as an active membrane-associated DGC and YfiR was identified as a periplasmic repressor of YfiN activity. The authors proposed that YfiB promotes dimerization of YfiR, which in turn relieves repression of YfiN activity. In the analogous operon, *E. coli* encodes an additional gene, *yfiL,*that has no predicted function (Fig. 1A). Like their *P.aeruginosa* counterparts, *E. coli* YfiN has a predicted active site domain with the conserved GGDEF sequence, while YfiR has a predicted signal sequence with a predicted cleavage site between amino acids 22 and 23; this processing prepares the protein for export to the periplasm (Fig. 1B). Export of YfiR to the periplasm in CFT073 was confirmed by cell fractionation of FLAG-tagged YfiR (Fig. 2). YfiN also has a predicted allosteric inhibition

site for feedback inhibition by c-di-GMP (Fig. 1B). However, the sequence identity at both the nucleotide and protein levels between the *P. aeruginosa* and *E. coli* proteins is low, with only 30% and 39% amino acid identity between the two predicted YfiR proteins and YfiN proteins, respectively (Fig. 1B).

A *yfiR* mutant is attenuated *in vivo*.

A *yfiR* mutant in *P. aeruginosa* was attenuated in the acute phase of a skin infection model. To test this phenotype in *E. coli* CFT073, a nonpolar *yfiR* deletion mutant was constructed a tested in competition with the wild-type strain in the murine model of urinary tract infection. As shown in figure 2, the *yfiR* mutant was attenuated in both the bladder ($p=0.0008$) and kidneys ($p=0.004$) by an average of 7.4-fold and 24.8-fold, respectively, at 48 hours post-infection (Fig. 3A). Single deletions of *yfiN* (Fig. 3B), *yfiL* (Fig. 4A), and *yfiB* (Fig. 4B) were not attenuated *in vivo*. Similarly, deletion of *yfiN* from the *yfiR* mutant reversed the competitive defect of the *yfiR* single mutant (Fig. 3C), indicating that unrestricted activity of YfiN c-di-GMP production leads to attenuation in the urinary tract. Allelic repair of the *yfiR* mutation to its wild-type form by transduction also resulted in loss of attenuation *in vivo* (Fig. 3D). Single infections with the *yfiR* mutant and wild-type CFT073 also showed that the *yfiR* mutant is carried at lower loads in the bladder and kidneys than the wild-type strain at 48 hour post-infection (Fig. 4C). Growth curves performed in LB, minimal media, and urine showed no difference in the growth rates between wild-type CFT073 and the *yfiR* mutant (Fig. 5A). The *yfiR* mutant was slightly out-competed by the wild-type strain in competitive LB broth cultures but

competed like the wild-type strain in urine cultures (Fig. 5B). These results indicate that there may be a very slight growth defect in the *yfiR* mutant, but that this defect is unlikely to be the sole reason for the attenuation of the *yfiR* mutant in the urinary tract.

Growth on agar plates induces expression of the *yfiRNB* locus.

To determine the best conditions for expression of *yfiR* and *yfiN*, transcriptional fusions linking *yfiR* and *yfiN* to *lacZYA* were made using the pFUSE system (5). Strains were grown to exponential or late log phase either in tryptone broth cultures or on LB agar plates overnight. β-galactosidase assays were then performed on the strains. No expression was detected in the broth cultures (data not shown), but expression was detected when the strains were grown on the LB agar plates (Fig. 6). This result indicates that expression of the *yfiLRNB* locus may be surface-induced.

Deletion of *yfiR* causes a motility defect.

The detrimental effects of increased levels of c-di-GMP are well documented. To determine whether the mutant strains used in this study also displayed motility defects, they were grown in 0.3% tryptone swim plates and compared to the wild-type strain. A representative image of a swim plate and the average diameters of each strains swim rings are shown in Fig. 7A and B. As expected, the *yfiR* mutant showed a drastic decline in its ability to swim out in the agar. The *yfiN* and *yfiRN* mutants behaved like the wild-type strain. Complementation of the *yfiR* mutant with an arabinose-induced pBAD*yfiR* restored motility to the wild-type level, as did allelic repair of the *yfi* locus with

a CFT073 transducing phage. The swimming defect observed with the *yfiR* deletion strain does not seem to be due to an inability to assembly the flagella because intact flagellar filaments can be observed in electron micrographs of the cells, and motility is seen in live bacteria observed under a light microscope (Fig. 7C).

Deletion of *yfiR* causes increased production of cellulose and curli fimbriae.

In other strains of *E. coli* as well as other species of bacteria an increase in c-di-GMP levels usually results in increase in fimbriation and exopolysaccharide production. *E. coli* strains specifically up-regulate production of curli fimbriae and cellulose. An easy assay to test whether curli fimbriae and cellulose are being produced involves plating the bacteria on LB plates that contain congo red dye, but lack NaCl. After 5-8 days of growth, colonies that express both products will have a deep red, rugose phentotype that is referred to as the RDAR morphotype. Colonies that produce only curli fimbriae will lack the rugose characteristic, but will still be deep red in color and slightly rough. Expression of cellulose only results in a rugose colony that is pink instead of deep red, and loss of expression of both products results in a colony that is white and smooth. All colonies plated for the *yfiR* mutant displayed the RDAR morphotype while the wild-type, *yfiN* and *yfiRN* mutants produced a mixture of both RDAR and smooth white colonies. Restoration of *yfiR* via transduction returned the phenotype of the *yfiR* mutant to a mixture of both RDAR and smooth white colonies (Fig. 8A and B). Over-expression *yfiR* encoded on pBAD in the *yfiR* mutant produced all smooth colonies, although most were slightly rough in the center presumably from loss of arabinose induction as a result of

metabolism (Fig. 11A). Congo red binding assays in liquid culture were also performed as described in the materials and methods section. As expected, the *yfiR* mutant bound more congo red dye per CFU than the other strains, and this increase in bound congo red was mitigated by either transduction of the *yfi* locus to wild type or by plasmid-expressed *yfiR* (Fig. 8C).

To determine whether increased c-di-GMP levels increased *csgD* expression as previously described, the *lacZYA* locus was inserted directly downstream of *csgD* to create a transcriptional fusion using the pFUSE system. Expression levels of *csgD* could then be monitored by β-galactosidase assays. Deletion of *yfiR* resulted in a drastic increase in the Miller units expressed from the *csgD* operon (Fig. 8D), indicating that elevated c-di-GMP production does increase *csgD* expression levels in *E. coli* CFT073. Deletion of both *yfiR* and *yfiN* in the *csgD*::*lacZYA* strain returned *csgD* expression to wild-type levels as did complementation with pBAD*yfiR* in the *yfiR* deletion strain (Fig. 8D). Increased production of the curli fibrils was verified by western blotting using an antibody specific for CsgA, the major curlin subunit (Fig. 8E). Deletion of *adrA*, but not *yedQ*, reduced cellulose production by congo red plating (Fig. 11A). The link between increased *csgD* expression and increased *adrA* expression was also confirmed using an *adrA::lacZYA* fusion in the β-galactosidase assay (Fig. 11B). Expression off of the *bcs* operon was also monitored using the same system to place *lacZYA* downstream of *bcsA*. However, *bcsA* and *bcsE* expression levels in the mutant strains remained unchanged from the wild-type strain (Fig. 12AB). This result is not surprising given that c-di-GMP is thought to bind directly to BcsA, increasing its activity

levels and, thus, elevating cellulose production (24, 33). Because cellulose and curli fimbriae are also components of biofilms, pellicle formation on glass test tubes was analyzed via a crystal violet-staining assay. Deletion of *yfiR* caused increased pellicle formation (Fig. 9AB), indicating that this strain has increased biofilm formation.

Expression of a phosphodiesterase reverses the phenotypes of CFT073Δ*yfiR* to wild type.

The increase in curli fimbriae and cellulose production and pellicle formation in the *yfiR* deletion strain is likely due to increased cytoplasmic levels of c-di-GMP. To test if this is the case, wild-type *yhjH*, which encodes a proven phosphodiesterase (40), was expressed from a plasmid in CFT073Δ*yfiR* in the congo red binding assay, pellicle formation assay, and swimming motility assay. As predicted, expression and, presumably, phosphodiesterase activity of YhjH reduced the amount of congo red dye bound, reduced pellicle formation, and increased swimming motility in comparison to the *yfiR* deletion strain (Fig. 10A-C). Expression of $yhjH_{E136A}$, an active site mutant that cannot degrade c-di-GMP, did not have an effect on any of the noted phenotypes when expressed in the *yfiR* deletion strain, indicating that the phosphodiesterase activity and the resulting reduction in c-di-GMP levels is indeed required for the reversal of the observed phenotypes (Fig. 10 A-C).

Deletion of *yfiR* does not increase sensitivity to hydrogen peroxide or iron limitation.

Past reports have indicated that increased levels of c-di-GMP lead to an increase in sensitivity to peroxide stress and a decrease in the expression of iron acquisition genes. To determine whether the *yfiR* mutant was more sensitive to peroxides than wild-type CFT073, hydrogen peroxide sensitivity assays were performed in both liquid culture and disc-diffusion agar. No differences between the wild-type strain and the *yfiR* mutant were observed in either assay (Fig. 13AB). To determine whether the *yfiR* mutant would have trouble growing in an iron-limited environment like the host, both the wild-type and mutant strains were grown in LB broth with 2,2-dipyridyl, an iron chelator. Again, no differences in growth were observed between the wild-type and *yfiR* mutant strains (Fig. 14).

Deletion of *ycgR* in the *yfiR* mutant partially restores colonization levels in the mouse model.

Motility is a known colonization factor of uropathogenic *E. coli* strains in the urinary tract, and is needed for ascension of the ureters to the kidneys. Multiple studies have recently showed that YcgR can bind c-di-GMP and then interact with the flagellar motor (6, 29). This interaction acts either to inhibit rotation or to keep the flagella turning counterclockwise, causing smooth swimming cells that may become stuck in the agar matrix. To determine whether the swimming defect seen in the *yfiR* mutant causes the attenuated phenotype *in vivo*, *ycgR* was deleted from the *yfiR* mutant and tested in

the competitive mouse model of UTI. Swim plates of this mutant confirmed that motility was partially restored (Fig. 15). As shown in Figure 16A, this partial restoration of motility also resulted in the partial recovery of the *yfiR* mutant to wild-type colonization levels *in vivo*. By itself, a single *ycgR* deletion mutant was not attenuated in the competitive mouse model against wild-type CFT073 (Fig. 17A). Given that the colonization levels were only partially restored, full motility may be needed for full virulence, or another factor may also be partially responsible for the observed *yfiR* mutant attenuation.

Deletion of both *bcsA* and *csgD* in the *yfiR* mutant returns colonization to wild-type levels.

Previous studies have shown that curli fibers produce an immune response by activating the host coagulation system and, thus, promoting an influx of white blood cells (27, 30). However, curli fimbriae can also promote resistance to the host antimicrobial peptide LL-37 (20). Additional mutations in the *yfiR* deletion strain were made to determine whether the overexpression of curli fimbriae, or possibly even the increase in cellulose production, could be responsible for the attenuation of the *yfiR* deletion strain *in vivo*. Deletion of *csgD* or *bcsA* from the *yfiR* mutant does not return colonization to wild-type levels in the competitive UTI model, although perhaps a slight improvement in the mutant's fitness is seen in the kidneys for both double deletion strains (Fig. 16B and C). Single-deletion strains of *csgD* or *bcsA* compete well with the wild-type strain (Fig. 17B and C). Additional deletion of *csgD* from the Δ*yfiR*Δ*ycgR*

double deletion strain did not relieve the attenuated phenotype in the mouse model (Fig. 17D). However, when both *csgD* and *bcsA* are deleted from the *yfiR* mutant, colonization is restored to wild-type levels (Fig. 16D). The motility of these double and triple mutants was determined using tryptone swim plates, on which they remained poorly motile like the *yfiR* single mutant (Fig. 15).

Urine inhibits the RDAR morphotype and *csgD* expression.

In addition to growing the mutant strains on LB congo red plates, we inoculated them on urine agar congo red plates to see whether exposure to urine caused a change in the RDAR phenotype. All of the wild-type CFT073 colonies grown on the urine congo red plates displayed a smooth, white phenotype, indicating that urine represses the expression or activity of the proteins needed for production of cellulose and curli fimbriae (Fig. 18A). Similar results were obtained when the wild-type strain was grown in liquid urine, and then plated on LB congo red plates (Fig. 18B), suggesting that the effect urine has on the regulatory controls for curli fimbriae and cellulose is long-lasting. The change was not permanent, however, because a return of the RDAR phenotype was observed after growth in LB liquid culture followed by growth on LB congo red plates (data not shown). Congo red plates that combined urine and LB were also tested with the wild-type strain as a control to ensure that the loss of the RDAR phenotype was not due to growth inhibition on the urine only plates (Fig. 18A). The presence of urine also affected the phenotype of the *yfiR* mutant on the urine congo red plates, but all colonies were still deep red and somewhat rugose (Fig. 18A), which indicated that curli

fimbriae and cellulose were both still being produced. Growth in liquid urine followed by growth on LB congo red plates produced no differences in the RDAR phenotype for the *yfiR* mutant (Fig. 18B). Because salt is a known inhibitor of the RDAR phenotype, we determined which salts found in urine might be responsible for the all smooth and white phenotype seen in wild-type CFT073. Individual salt components of urine (NaCl, $Ca_3(PO_4)_2$, K_2SO_4, KCl, $KHCO_3$, $MgCO_3$, and $MgSO_4$) at their physiological concentrations were added to LB congo red plates for testing with the wild-type strain. Of the salts tested, only NaCl produced a phenotype similar to that found on the urine congo red plates (Fig. 18A).

To determine whether urine and NaCl affected the expression of *csgD*, the wild-type and *yfiR* mutant *csgD::lacZYA* strains were employed. β-galactosidase assays performed on cells grown on urine plates showed a decrease in expression from the *csg* promoter in the presence of urine compared to LB. However, expression levels in the *yfiR* mutant remained elevated compared to the wild-type strain under all conditions tested (Fig. 18C). Growth on LB-NaCl plates caused no change in expression when compared to growth on LB only plates (Fig. 18C).

Discussion

This study describes the effects that elevated levels of c-di-GMP brought on by loss of inhibition of YfiN by YfiR have on the ability of *E. coli* CFT073 to mount a successful infection in the urinary tract. Results show that loss of YfiR likely relieves inhibition of the activity of the membrane bound DGC YfiN, leading to an increase in

cellular c-di-GMP levels. This increase leads to an elevation in production of both curli fimbriae and cellulose, a reduction in the motility, and ultimately attenuation in the mouse model of UTI. Single deletions of *yfiL*, *yfiB*, or *yfiN* did not attenuate *E. coli* CFT073 *in vivo*. Additional deletion of *yfiN* from the *yfiR* deletion strain, or restoration to the wild-type operon of the *yfiR* deletion strain, brought colonization back to wild-type levels. This indicates that YfiR exerts an inhibitory effect on the activity of YfiN that is important for maintaining control of c-di-GMP levels *in vivo*. Without this control, the bacteria become impaired in the urinary tract, presumably due to disregulation of downstream genes and functions that are sensitive to c-di-GMP signaling.

A study of the homologous *P. aeruginosa* system postulated that YfiR prevents the dimerization of YfiN, a state that is necessary for activity of most DGCs. This same report also indicated that YfiB promotes the dimerization of YfiR, which would presumably inhibit its ability to disrupt YfiN dimerization (23). Overall, though, the function of YfiB and the mechanism by which the YfiRNB system operates is still largely unknown. Further studies would be necessary to determine the hierarchy of the YfiRNB system and the set of external stimuli that may activate or repress YfiN activity. Our work indicates that transcription of the *yfi* locus may be surface induced. If attachment to the host epithelium also induces expression of this locus, the regulation of both *yfiLRNB* transcription and YfiN activity could give insights into the series of events necessary for successful colonization of and persistence in the urinary tract.

The downstream targets of increased c-di-GMP levels in *E. coli* CFT073 are similar to those that have been previously described in other *E. coli* strains (31). The

loss of *yfiR* in CFT073 resulted in decreased motility, increased expression of the *csgD* promoter, and increased production of both curli fimbriae and cellulose. Expression of *yhjH*, which encodes a phosphodiestrase, in the CFT073Δ*yfiR* background returned these phenotypes to the wild-type forms. The phosphodiesterase activity of YhjH was required for this reversal, indicating that increased c-di-GMP levels is indeed the factor driving the over-expression of curli fimbriae and cellulose and the observed decrease in motility in the *yfiR* deletion strain. The motility defect and *in vivo* attenuation of the *yfiR* deletion strain was also partially rescued by additional deletion of *ycgR*. YcgR is capable of interacting with the flagellar motor to either promote smooth swimming or act as a braking mechanism when it is bound to c-di-GMP. Deletion of *ycgR* therefore prevented alteration of flagellar rotation by this mechanism. This observation indicates that motility and the effect of increased c-di-GMP levels on flagellar rotation do impair the ability of CFT073 to successfully infect the urinary tract. The overproduction of curli fimbriae and cellulose also appears to have a detrimental effect on CFT073 *in vivo*. The link between the expression of *csgD* and the activity of the cellulose biosynthetic machinery was established previously in *E. coli* K-12 strains. CsgD activates not only expression of the curli operon, but also expression of *adrA* and *yedQ*, genes that encodes DGCs. The c-di-GMP produced by either AdrA or YedQ would then stimulate increased activity of the cellulose synthetic machinery by binding to components of its core catalytic module (25, 33, 47). Deletion of *adrA*, but not *yedQ*, resulted in a loss of cellulose production on LB congo red plates. Therefore, we

postulate that AdrA expression stimulated by CsgD activity is the likely link involved in CsgD-dependent increases of cellulose production in *E. coli* CFT073.

Curli fibers are known activators of the host coagulase system that lead to increased influx of immune cells and inflammation (30). Another report also implicated the expression of curli fimbriae in the absence of cellulose production as producing an immune stimulant that resulted in increased clearance of uropathogenic *E. coli* strain UTI89 from the urinary tract (20). In contrast to these reports, a Δ*yfiR*Δ*csgD* mutant still displayed attenuation in the competitive mouse model of UTI, even though it was confirmed that curli fimbriae were no longer being produced. Only additional deletion of *bcsA* from the Δ*yfiR*Δ*csgD* mutant rescued the attenuated phenotype. Although the *yfiR* single-deletion mutant did not display any growth defects in various media when grown singly or in competition with the wild-type strain *in vitro*, it is possible that the increased stress of the urinary tract environment attenuated the metabolically over-burdened mutant. The significant amount of cellular resources being consumed for the production of large and unnecessary amounts of curli fimbriae and cellulose may have therefore diverted resources away from the production of other virulence or colonization factors vital for prolonged infection of the urinary tract. Further studies into alterations in the global production of bacterial proteins in the *yfiR* deletion mutant could give insight into whether or not the expression of any other virulence factors were altered.

Given that single deletions of *csgD* or *bcsA* did not attenuate CFT073 *in vivo*, biofilm formation associated with curli or cellulose is likely not a persistence factor involved in colonization in the urinary tract. Additionally, growth in urine decreases

expression of *csgD* and inhibits the RDAR morphology. The salt content of urine appears to be the main suppressor leading to this outcome. Growth at 37° C also inhibits the production of these biofilm components. Taken together, these data indicate that biofilms are not formed by *E. coli* CFT073 *in vivo* and may, instead, be necessary for adaptation and persistence in environments outside of the urinary tract.

This study not only emphasizes the importance of regulation of curli fimbriae, cellulose production, and motility in the colonization of UPEC in the urinary tract but also highlights c-di-GMP production as a key regulator of bacterial processes during infection. The abundance of DGCs (~19) and PDEs (~17) in *E. coli* alone suggests that control of c-di-GMP production and its downstream effects are likely important at a number of times in different environments. Further studies into the distinct or perhaps redundant roles played by the various DGCs and PDEs would illuminate the steps in the fine-tuned control that the bacteria have over their expression of both colonization and virulence factors to mount successful infections in the host.

Acknowledgements

This research was funded by the National Institutes of Health Grant R01 DK063250-06 and by the National Institutes of Health, National Research Service Award AI55397.

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Table 1: Primers used in this study

Gene/Application	Sequence
λ-Red Deletions	
yfiR deletion	5'-CGATGCATTTCGAGCGAAGATGGTGAGGATCCCTGA ATGTGTGTAGGCTGGAGCTGCTTC-3'
	5'-ATTAAGAGAATTATCGTTATCCATCATTTTTCTTCCGT GCCATATCAATATCCTCCTTAG-3'
yfiR deletion ck	5'-GCCAACTGCATGAAGTATGGC-3'
	5'-CTGCAGTTGAAAATTGGCCTTGC-3'
yfiN deletion	5'-CAACCCGGATGTCTTAATGCTCGCACGGAAGAAAAAT GTGTGTAGGCTGGAGCTGCTTC-3'
	5'-GTGCTAGCAGGTGCTTTATCATATCGATATATCCTTGT TACATATGAATATCCTCCTTAG-3'
yfiB deletion	5'-GCCGAAAAGCTGGTGAGATAACAAGGATATATCGATA TGTGTGTAGGCTGG AGCTGCTTC-3'
	5'-AATTGGCTGGCCCTTTTTTTGTCTGTTTGCTCGTGTTT TACATATGAATATCCTCCTTAG-3'
yfiL deletion	5'-AAATTTACACCAGCTTTAAAAACAATCCGCTATGCTTT GTGTGTAGGCTGGAGCTGCTTC-3'
	5'-TGAGTAAAATCTCATGCTTAATTGATTTGGGGGGTAAT TACATATGAATATCCTCCTTAG-3'
yfi locus	5'-CTCAGCGAGGATGACGTAACG-3'
deletion ck	5'-CGTGAGCGTACTGGTAAGGC-3'
csgD deletion	5'-TCAGGTGTGCGATCAATAAAAAAAGCGGGGTTTCATC ATGGTGTAGGCTGGAGCTGCTTC-3'
	5'-TGCCGCCACAATCCAGCGTAAATAACGTTTCATGGCT TTACATATGAATATCCTCCTTAG-3'
csgD deletion ck	5'-CACACAGCAGTGCAACATCTGTCA-3'
	5'-GCCGCCACAATCCAGCGTAAATAA-3'
csgA deletion	5'-CCATTCGACTTTTAAATCAATCCGATGGGGGTTTTAC ATGGTGTAGGCTGGAGCTGCTTC-3'
	5'-AGGGCTTGCGCCCTGTTTCTTTCATACTGATGATGTA TTACATATGAATATCCTCCTTAG-3'
csgA deletion ck	5'-GCAAATGGCTATTCGCGTGACACA-3'
	5'-GGCTTGCGCCCTGTTTCTTTCATA-3'
bcsA deletion	5'-CGGGCTGAAAACGCCAGTCGGGAGTGCATCATGAGT ATCTGTGTAGGCTGGAGCTGCTTC-3'
	5'-GTTTCGCTCCGCTCCGGGCGGCGCGGAATAAACGAT ACAACATATGAATAT CCTCCTTAG-3'
bcsA deletion ck	5'-GCTGAGTTTGTGCGATCACTCG-3'
	5'-CTGGCTCAGCATTGATCAGTGG-3'
yedQ deletion	5'-AAACTCGCACGCCGCCTGGGGCCTGGTCATATCGTT AATTGT GTAGGCTGGAGCTGCTTC-3'
	5'-GCTGGCGTGAAGGGCTGGACCATTTTTTCTCCGCCC

	GTTACATATGAATATCCTCCTTAG -3’
yedQ deletion ck	5’-GGATCACATCCGGCCTGG-3’
	5’-CCCAACTGGATTATCGCGACC-3’
adrA deletion	5’-AGCCCCGTCTCTATAATTTGGGAAAATTGTTTCTGAA TGTGTGTAGGCTGGAGCTGCTTC-3’
	5’-GAAAAACTCAGTAAATCCTGATGACTTTTGCCGGACG TCACATATGAATATCCTCCTTAG-3’
adrA deletion ck	5’-GCAACAACAGCGTATGACCTCC-3’
	5’-CGGTGTGAGCGGTTCTTCG-3’
ycgR deletion	5’-AACCAAAGACAGTTTGTCAGTCAGGAGTTTTTCCGCG TGTGTGTAGGCTGGAGCTGCTTC-3’
	5’-ATCTACAAACTTGAGCAGGCACTGGACGCGATGTAAA TCACATATGAATATCCTCCTTAG-3’
ycgR deletion ck	5’-CCTCGTTTCGATGAATCGGACG-3’
	5’-CTGGTGGTGTCATACGCTAACG-3’
fliC deletion	5’-GGCGTTGCCGTCAGTCTCAGTTAATCAGGTTACGGC GATGTGTAGGCTGGAGCTGCTTC-3’
	5’-AAAACGTAATCAACGACTTGCAATATAGGATAACGAA TCCATATGAATATCCTCCTTAG-3’
fliC deletion ck	5’-CGCAATGGGTCTGGCTGTG-3’
	5’-GAGTGATGGTCAGCGTCTGGTTGC-3’
lacZ deletion	5’-GTTGGTCTGGTGTCAAAAATAATAATAACCTGCGGCC GCATCTAG-3’
	5’-ACGCGAAATACGGGCAGACATAGCCTGCCCATTCCG GGGATCCGT-3’
lacZ deletion ck	5’-GCAGACCAAACAGCGGTTGG-3’
	5’-TTGAATTATGGCCCACACCAGTG-3’
yfiR ϕ restoration (*pheA-tyrA)*	5’-GAATGACAATCGCCAGTAATAATCCAGTGCCGGATGA TTTGTGTAGGCTGGAGCTGCTTC-3’
	5’-CTGTTGATCCAACCTGATGAAAAAGTGCCGGATGATG TGACATATGAATATCCTCCTTAG-3’
pBAD24d Cloning	
yfiR-NcoI	5’-GTGAGGCCATGGGAATGCGTTTTTCTCACCGACTG-3’
yfiR-PstI	5’-AGAGAACTGCAGTTATCCATCATTTTTCTTCCGTGCGA G-3’
Insertion site ck	5’-GGACCAAAGCCATGACAAAAACG-3’
	5’-CTACTCAGGAGAGCGTTCACC-3'
pFUSE Cloning	
csgD	5’-AACGTTCCCGGGCTTTATCGCCTGAGGTTATCGTTTGC-3’
	5’-TTCTTTTCTAGAGACAGCTCTCTTGCAGCACC-3’
bcsA	5’-GAAAGTCCCGGGCCACATATCATTGCCGTCCTGC-3’

	5’-TGAAACTCTAGAGTGGATTGTGCTGGTGCTCG-3’
bcsE	5’-GATATCCCCGGGGGTCATCATGATGAGCGCTCC-3’
	5’-AAGATATCTAGACCTTGCTGTCAATGACCCAGC-3’
adrA	5’-AAATCCCCCGGGCTTTTGCCGGACGTCAGG-3’
	5’-ATCGTCTCTAGACCTCTGCTGTTTGGCTGG-3’
yfiR	5’-TAAGAGCCCGGGCGTTATCCATCATTTTTCTTCCGTGC-3’
	5’-GATGGTTCTAGACCCTGAATGCGTTTTTCTCACC-3’
yfiN	5’-CATATCCCCGGGTCCTTGTTATCTCACCAGCTTTTCG-3’
	5’-CGTTTCTCTAGAGCATCAACACGTTGATGAACAATTCC-3’
yfiB	5’-CTGTTTCCCGGGGTTTTAAGGGGTTGTAATCACCACTGC-3’
	5’-GATAAATCTAGAGCTAGCACCCCTGATTTTCACC-3’

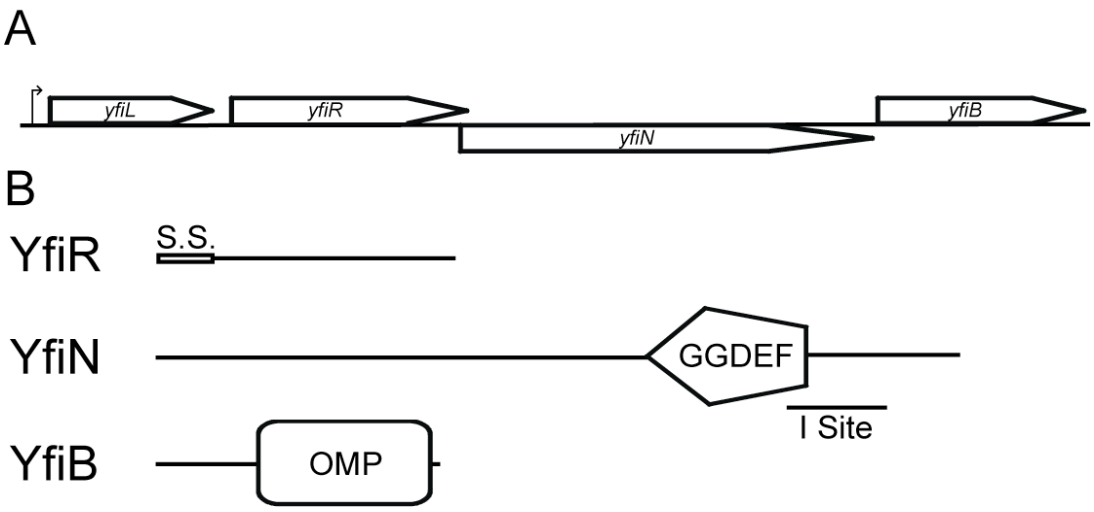


Figure 1: Organization of the *yfiLRNB* locus and predicted protein functions. (A) The *yfiLRNB* locus consists of four genes that are expressed off a single predicted promoter. (B) YfiR is a predicted periplasmic protein of unknown function with a signal sequence (S.S.), and YfiN is a predicted membrane-bound diguanylate cyclase with an intact GGDEF active site and an allosteric inhibition site (I site). YfiL and YfiB are predicted lioproteins of unknown function.

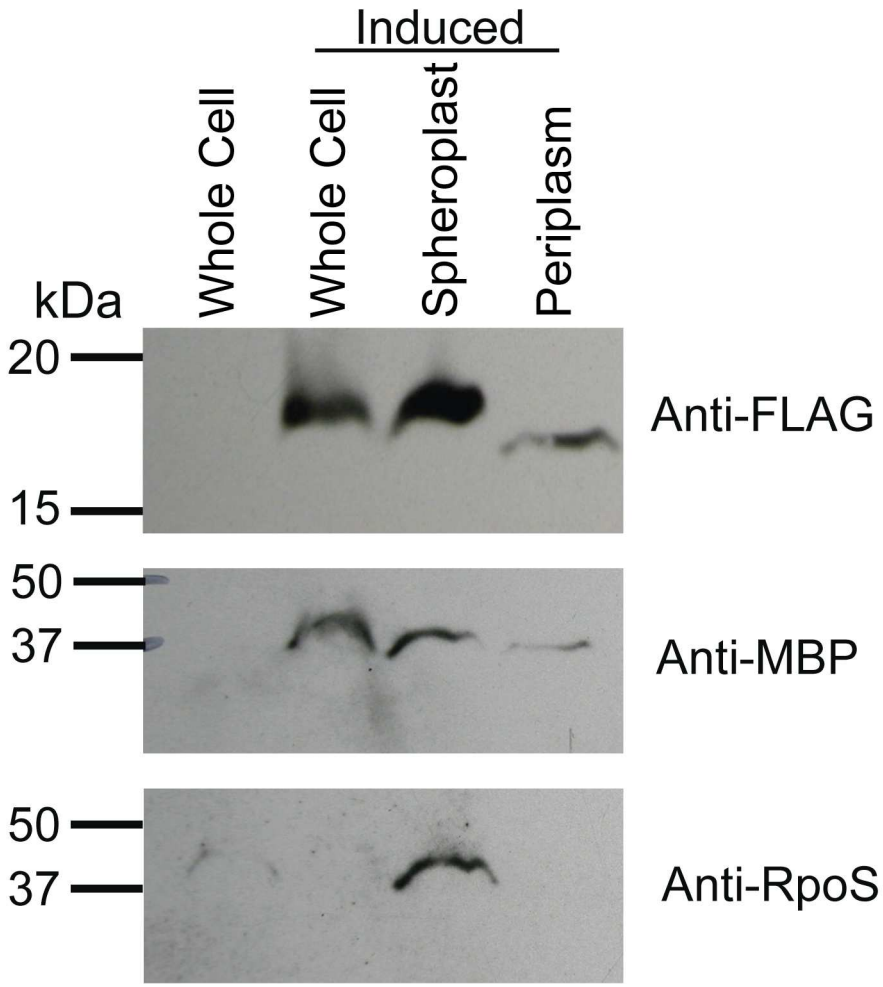


Figure 2: YfiR localizes to the periplasm. Wild-type CFT073 producing YfiR with an C-terminus FLAG tag via IPTG-mediated induction of expression of *yfiR*-FLAG off of the pJMP69 plasmid was spheroplasted and separated into their inner membrane/cytoplasmic and periplasmic fractions by centrifugation. Western blots of the SDS-PAGE gel-separated fractions were probed with antibodies against either the FLAG tag, periplasmic maltose-binding protein (MBP), or cytoplasmic RpoS.

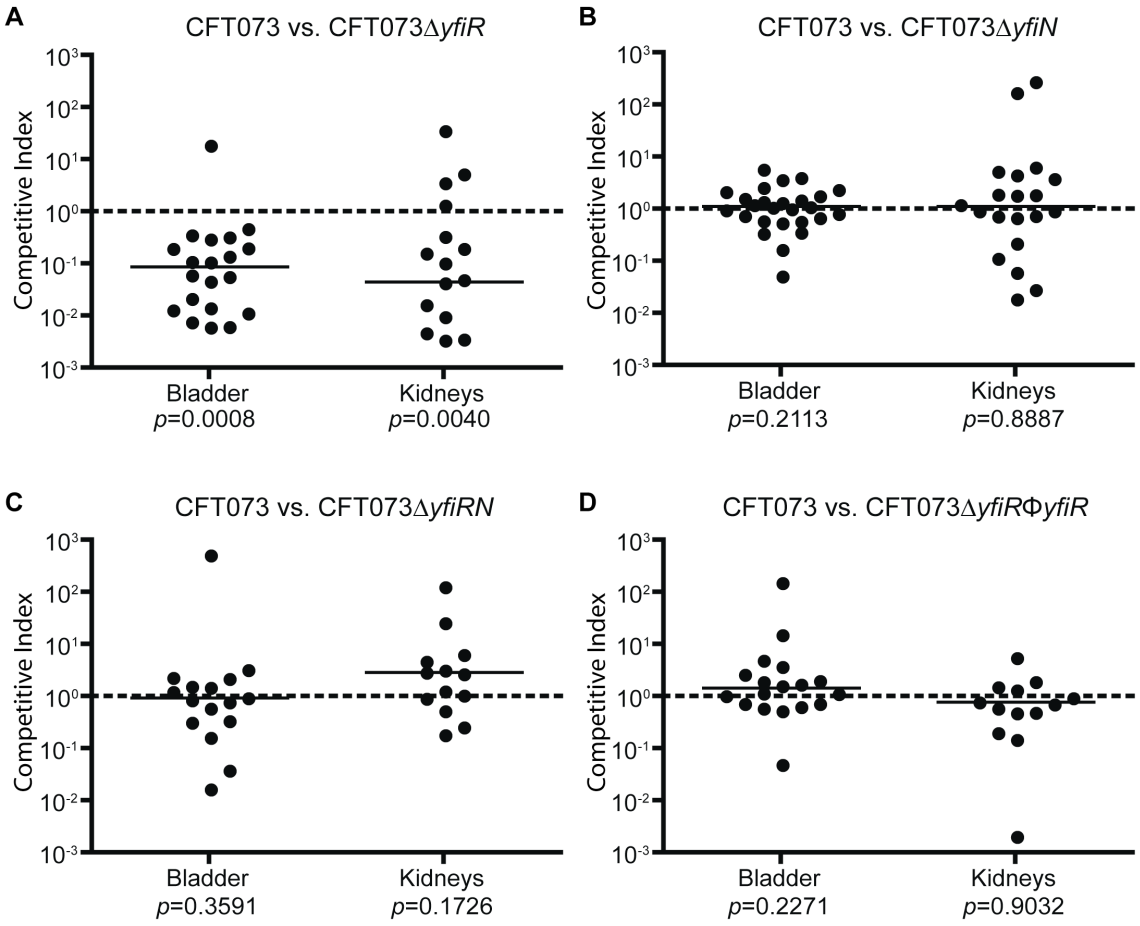


Figure 3: Deletion of *yfiR* causes attenuation in the mouse model of competitive UTI. Results for co-infection of female Swiss Webster mice with CFT073Δ*yfiR* (n=21) (A), CFT073Δ*yfiN* (n=26) (B), CFT073Δ*yfiRN* (n=16) (C), or the CFT073Δ*yfiR* phage restoration mutant (n=18) (D) and CFT073Δ*lacZYA* using the mouse model of UTI. The competitive indices were calculated by the following equation: (*yfi* mutant CFU recovered/wild-type CFU recovered)/(mutant inoculum CFU/wild-type inoculum CFU). The line indicates the median value.

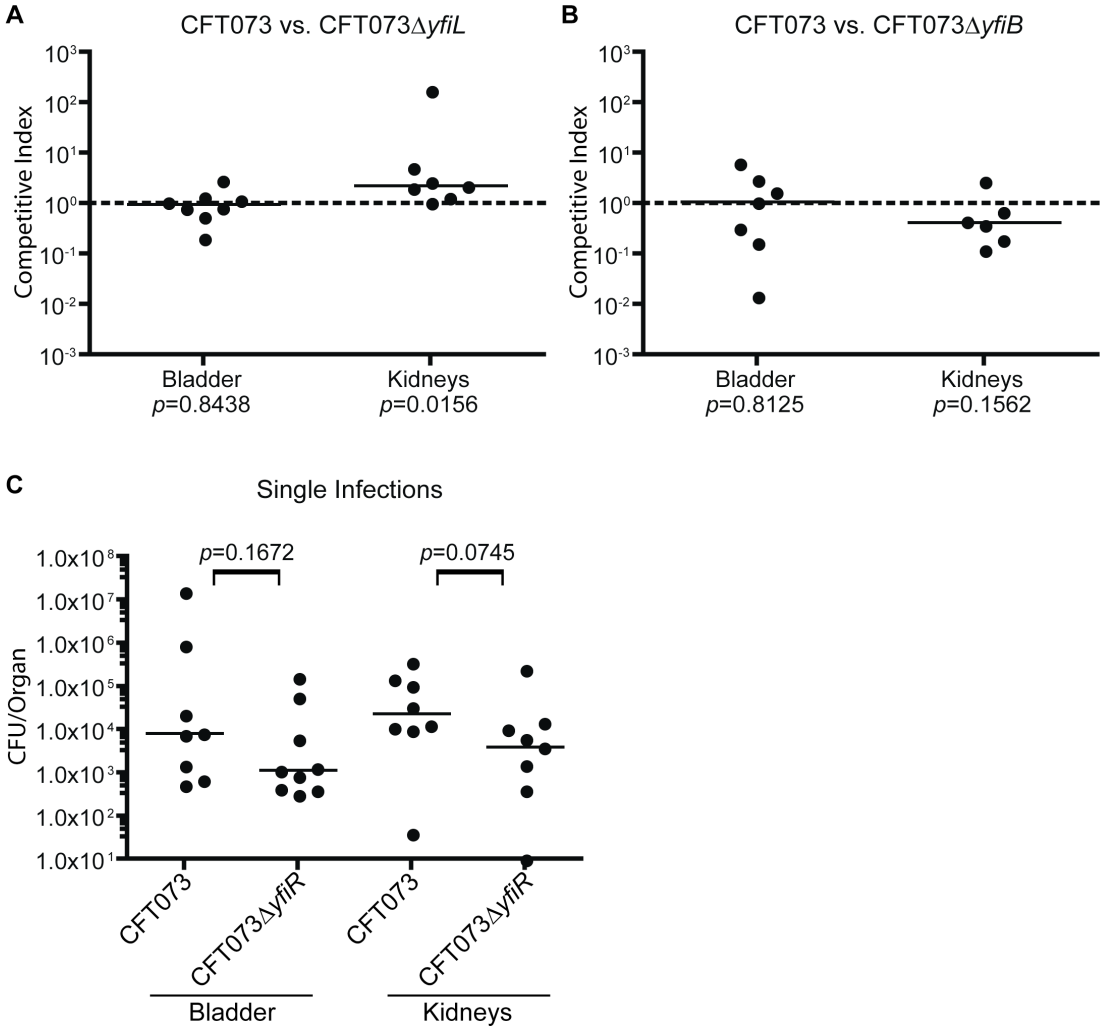


Figure 4: Deletion of *yfiL* or *yfiB* produces no *in vivo* defect and CFT073Δ*yfiR* is carried at lower loads than wild-type CFT073 in single infections. Results for co-infection of female Swiss Webster mice with CFT073Δ*yfiL* (n=8) (A) and CFT073Δ*yfiB* (n=7) (B) and CFT073Δ*lacZYA* using the mouse model of UTI. The competitive indices were calculated by the following equation: (*yfi* mutant CFU recovered/wild-type CFU recovered)/(mutant inoculum CFU/wild-type inoculum CFU). (C) Single infections of

wild-type CFT073 (n=8) and CFT073Δ*yfiR* (n=9) in female Swiss Webster mice. The line indicates the median value.

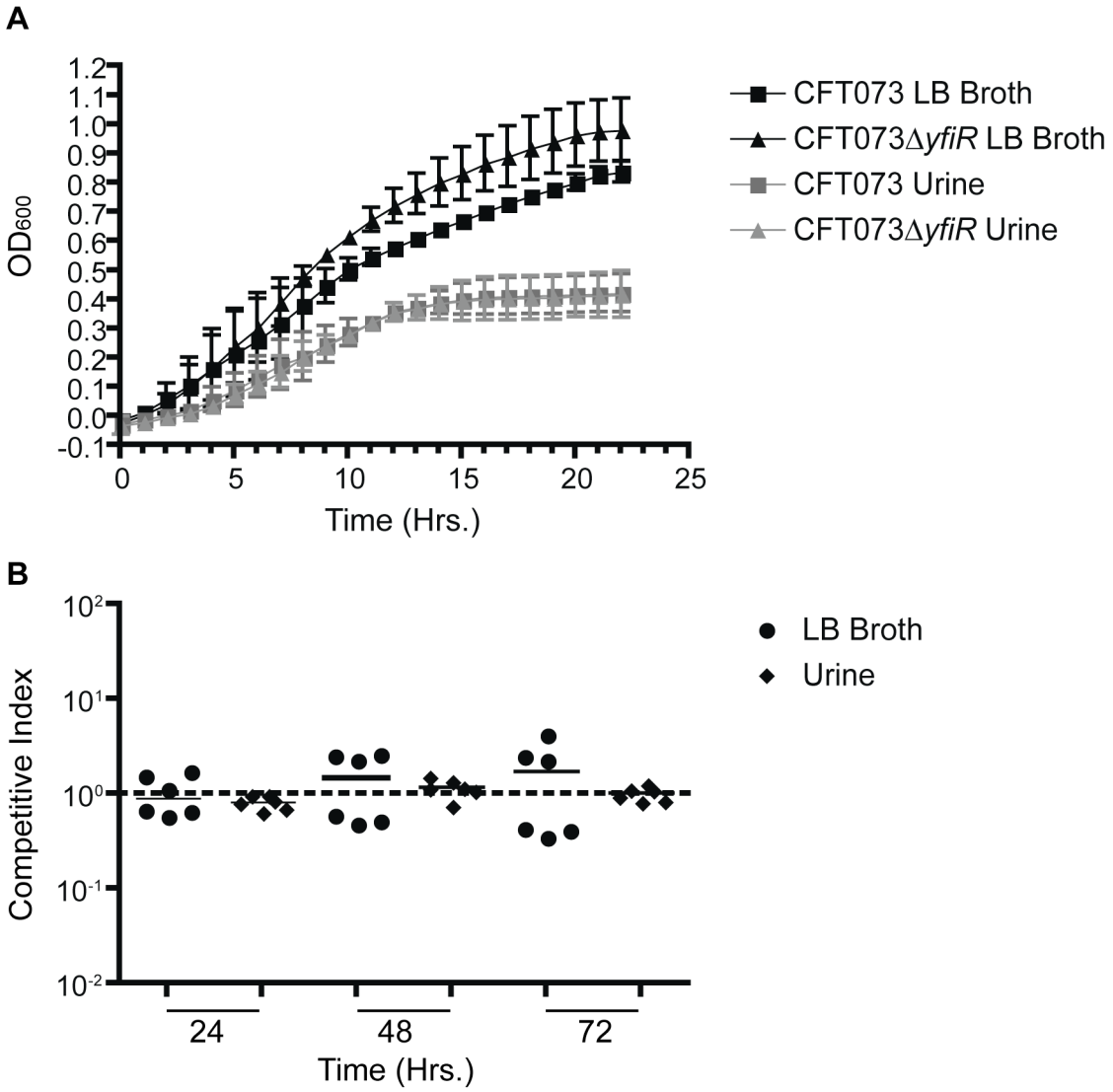


Figure 5: Deletion of *yfiR* does not cause a significant *in vitro* growth defect. (A) CFT073Δ*yfiR* grew much like wild-type CFT073 in both LB and urine in single-strain broths. (B) Competitve growth broths with wild-type CFT073 and CFT073Δ*yfiR* showed no differences in nutrient acquisition by either strain when grown in LB and urine. The line indicates the median.

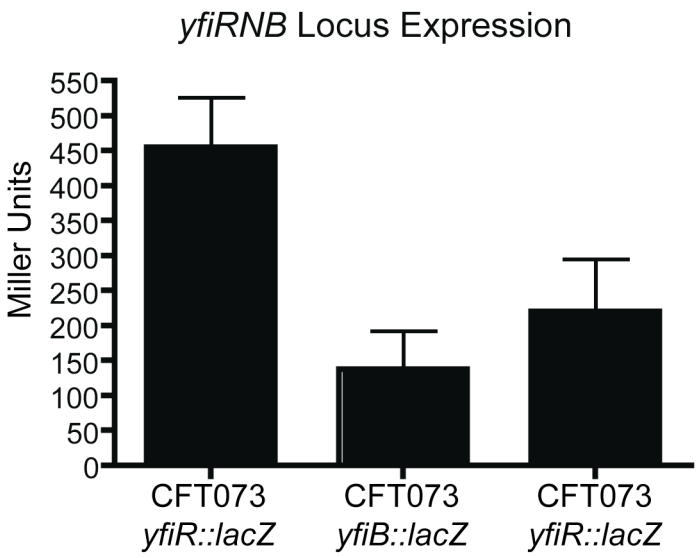
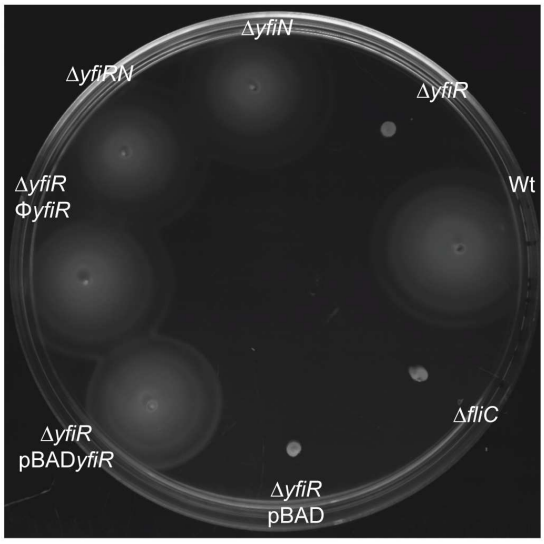


Figure 6: The *yfi* locus is expressed on agar plates. β-galactosidase assays using the CFT073 *yfiR::lacZ*, CFT073 *yfiB::lacZ*, and *yfiN::lacZ* transcriptional fusions displayed locus expression when grown on LB agar plates without salt at 30° C.

A



B

Strain	Avg. Diameter (mm) ± SEM
CFT073	41.4±4.5
CFT073Δ*yfiR*	3.0±0.5
CFT073Δ*yfiN*	36.9±5.6
CFT073Δ*yfiRN*	36.2±5.6
CFT073Δ*yfiR*Φ*yfiR*	36.6±4.7
CFT073Δ*yfiR* pBAD*yfiR*	33.2±5.0
CFT073Δ*yfiR* pBAD	3.8±0.2
CFT073Δ*fliC*	3.2±0.6

C

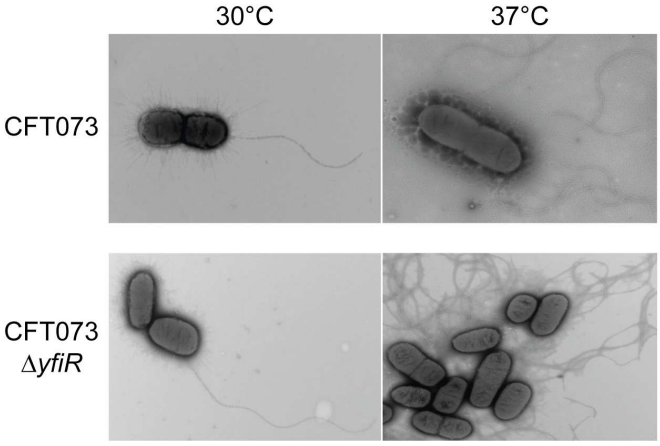


Figure 7: Motility phenotypes of mutants in the *yfiLRNB* locus. (A) Swim phenotype in 0.3% tryptone agar plates with 10 mM arabinose grown at 30° C. (B) Average diameters of swim rings of strains grown in 0.3% tryptone agar plates at 30° C. (C) Transmission electron micrographs of wild-type CFT073 and CFT073Δ*yfiR* grown in 0.3% tryptone agar plates at 30°C and 37° C.

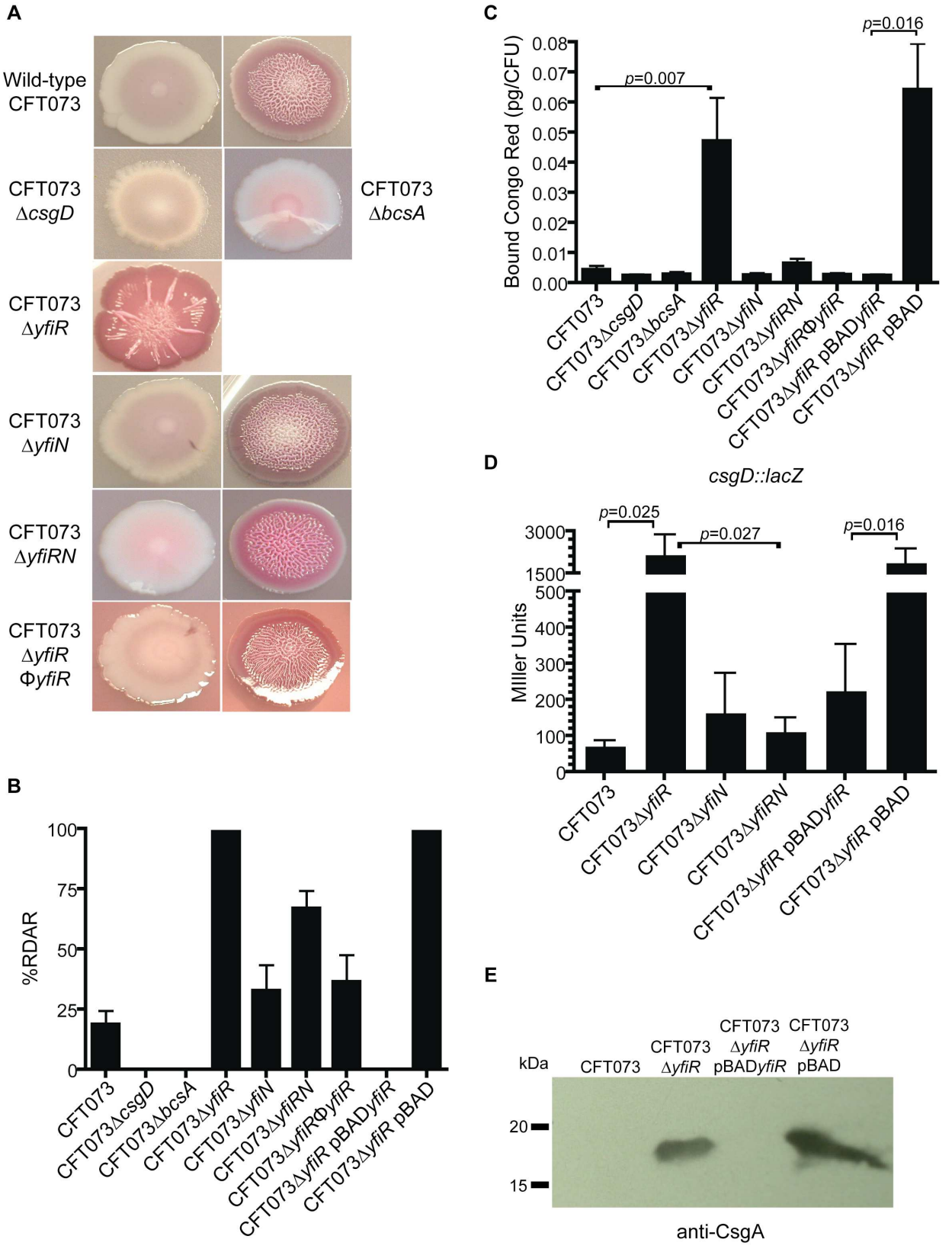

A
Wild-type CFT073
CFT073 ΔcsgD
CFT073 ΔbcsA
CFT073 ΔyfiR
CFT073 ΔyfiN
CFT073 ΔyfiRN
CFT073 ΔyfiR ΦyfiR
B
%RDAR
100
75
50
25
0
CFT073
CFT073ΔcsgD
CFT073ΔbcsA
CFT073ΔyfiR
CFT073ΔyfiN
CFT073ΔyfiRN
CFT073ΔyfiRΦyfiR
CFT073ΔyfiR pBADyfiR
CFT073ΔyfiR pBAD
C
Bound Congo Red (pg/CFU)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
p=0.007
p=0.016
CFT073
CFT073ΔcsgD
CFT073ΔbcsA
CFT073ΔyfiR
CFT073ΔyfiN
CFT073ΔyfiRN
CFT073ΔyfiRΦyfiR
CFT073ΔyfiR pBADyfiR
CFT073ΔyfiR pBAD
csgD::lacZ
D
Miller Units
3000
1500
500
400
300
200
100
0
p=0.025
p=0.027
p=0.016
CFT073
CFT073ΔyfiR
CFT073ΔyfiN
CFT073ΔyfiRN
CFT073ΔyfiR pBADyfiR
CFT073ΔyfiR pBAD
E
kDa
CFT073
CFT073 ΔyfiR
CFT073 ΔyfiR pBADyfiR
CFT073 ΔyfiR pBAD
20
15
anti-CsgA

Figure 8: Curli fimbriae and cellulose production are increased in CFT073Δ*yfiR*. (A) Phenotypes of *yfiLRNB* locus mutants grown on LB congo red plates at 30°C. All colony phenotypes that were observed are pictured. (B) Percentage of mutant colonies from the LB congo red plates displaying the RDAR phenotype. (C) Congo red binding assay in liquid broth. The amount of congo red dye bound is presented as pg of bound dye per CFU. P values were calculated using the student's T-test. (D) β-galactosidase assays performed on wild-type CFT073 and CFT073Δ*yfiR* strains with the *csgD::lacZYA* transcriptional fusion. P values were calculated using the student's T-test. (E) Western blot of acid-treated, whole-cell preparations grown on LB no-salt plates at 30°C. A rabbit anti-CsgA antibody was used to detect CsgA monomers.

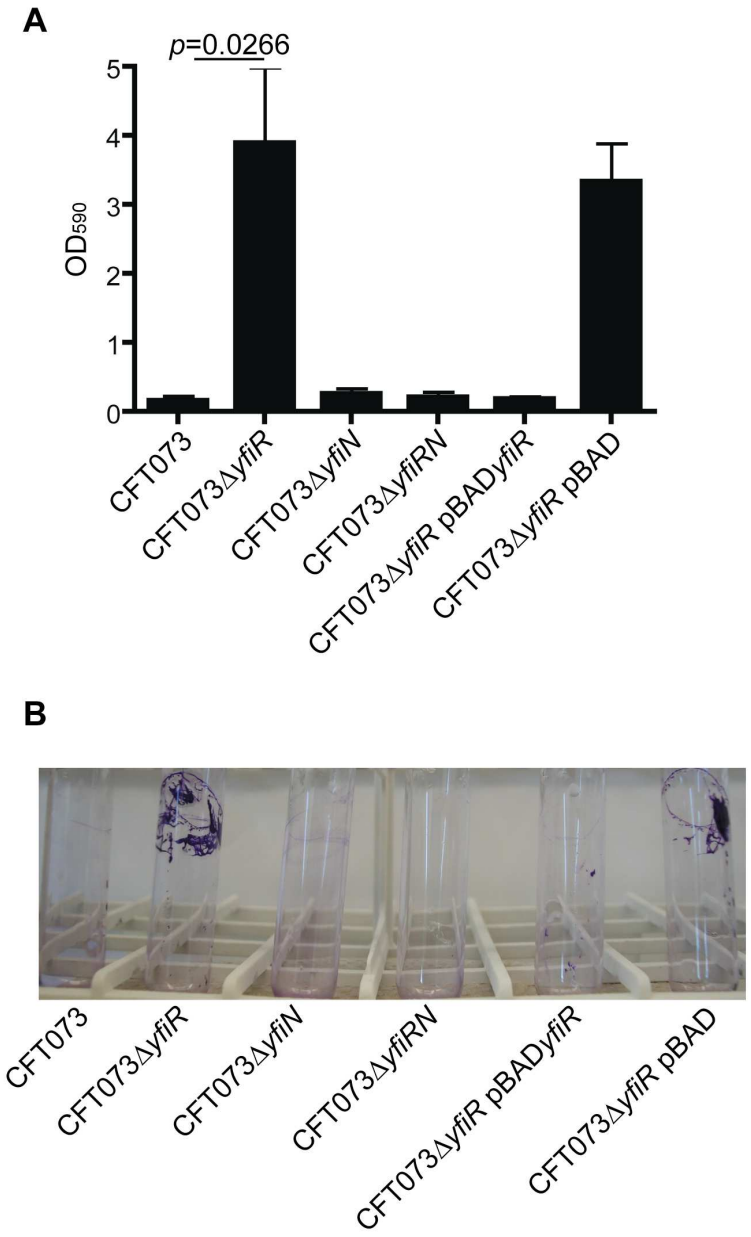


Figure 9: Pellicle formation is increased in CFT073Δ*yfiR*. (A) Optical density of crystal violet stain bound and released from pellicles formed by each strain after growth in MOPS glycerol broths for 16 hours at 37° C. Expression of *yfiR* from pBAD plasmids was induced with 10 mM arabinose. P values were calculated using the student's T-test. (B) Observed pellicles of each strain dyed with crystal violet.

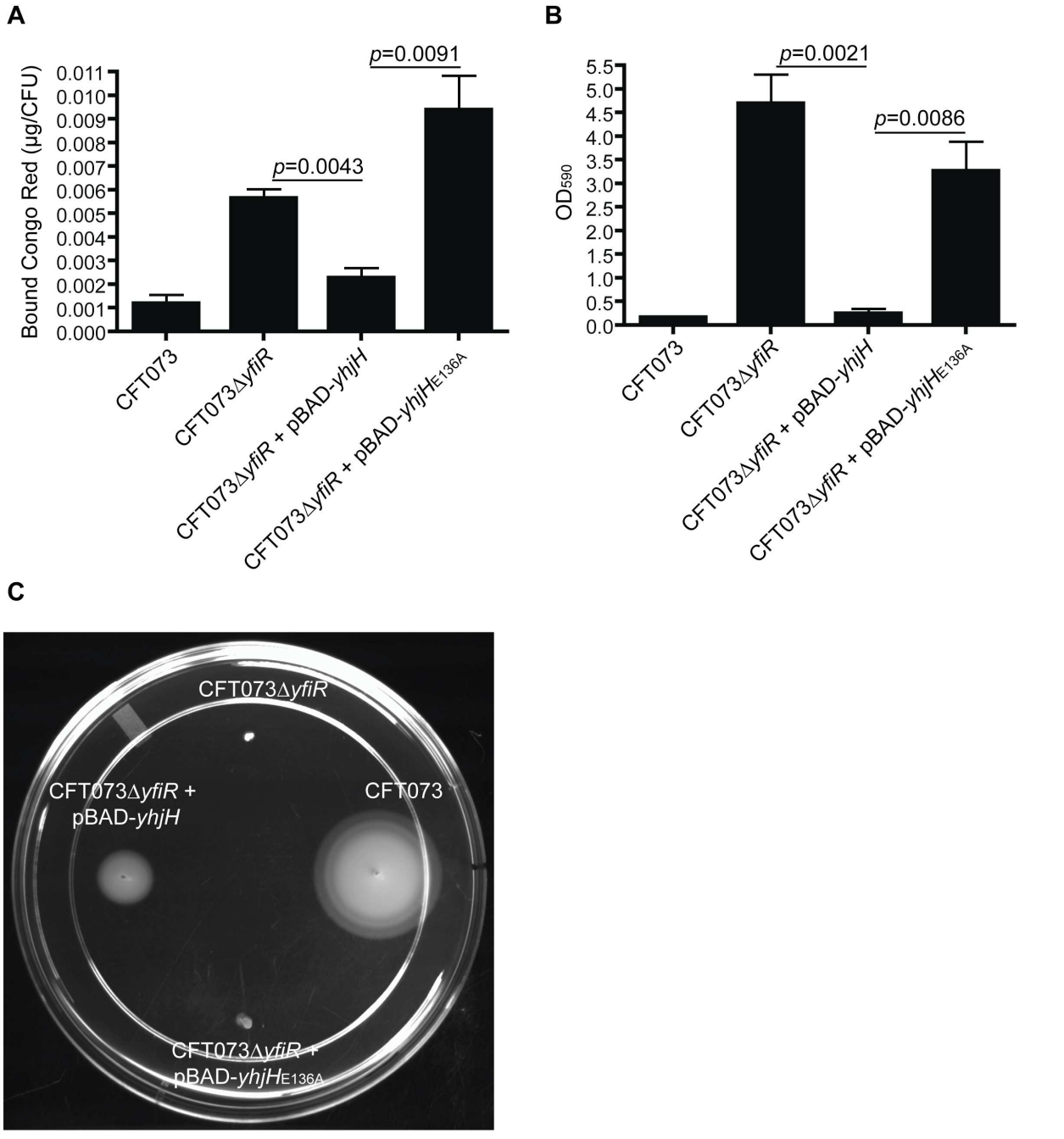


Figure 10: Expression of *yhjH* reverts the CFT073Δ*yfiR* phenotypes to the wild-type forms. (A) Congo red binding assay in liquid broth. Expression of *yhjH* from pBAD plasmids was induced with 10 mM arabinose. P values were calculated using the student's T-test. (B) Optical density of crystal violet stain bound and released from

pellicles formed by each strain after growth in MOPS glycerol broths for 16 hours at 37° C. Expression of *yhjH* from pBAD plasmids was induced with 10 mM arabinose. P values were calculated using the student's T-test. (C) Swim phenotype in 0.3% tryptone agar plates with 10 mM arabinose grown at 30° C.

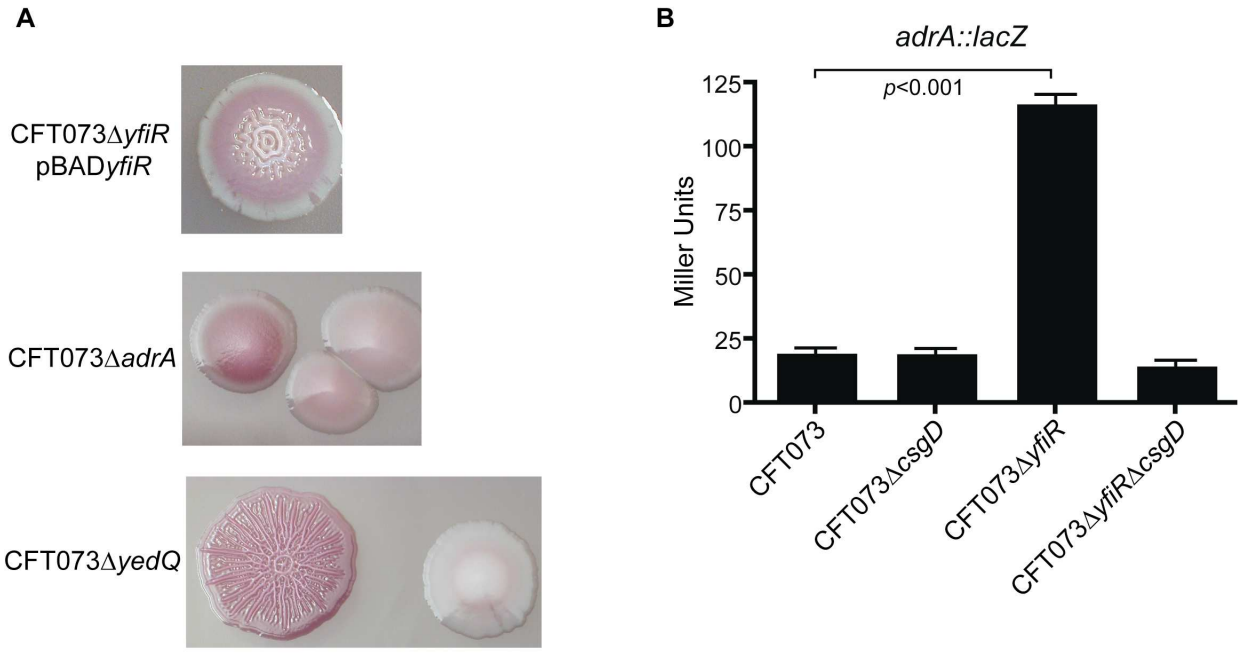


Figure 11: Loss of *adrA* reduces cellulose production. (A) Strains grown on LB congo red plates at 30° C. All colony phenotypes observed for each strain are pictured. (B) β-galactosidase assays with *adrA::lacZYA* fusion strains. The *p* value was calculated using a nonparametric student's T test.

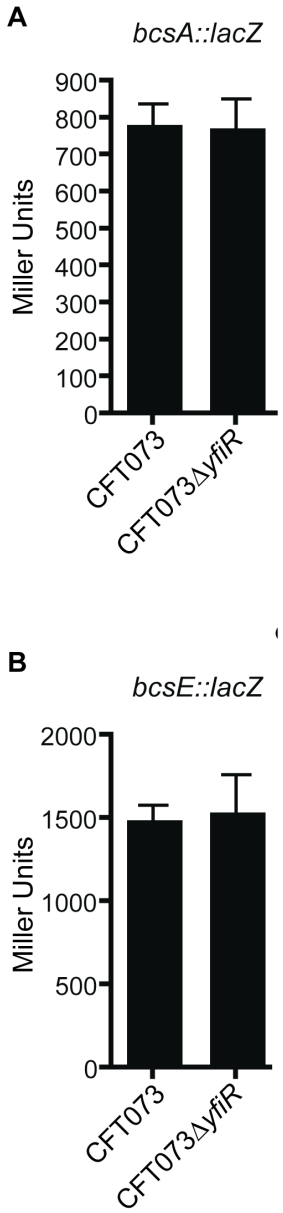


Figure 12: Loss of *yfiR* has no effect on *bcsA* or *bcsE* expression. β-galactosidase assays performed on wild-type CFT073 and CFT073Δ*yfiR* strains with the *bcsA::lacZYA* (A) or *bcsE::lacZYA* (B) transcriptional fusion.

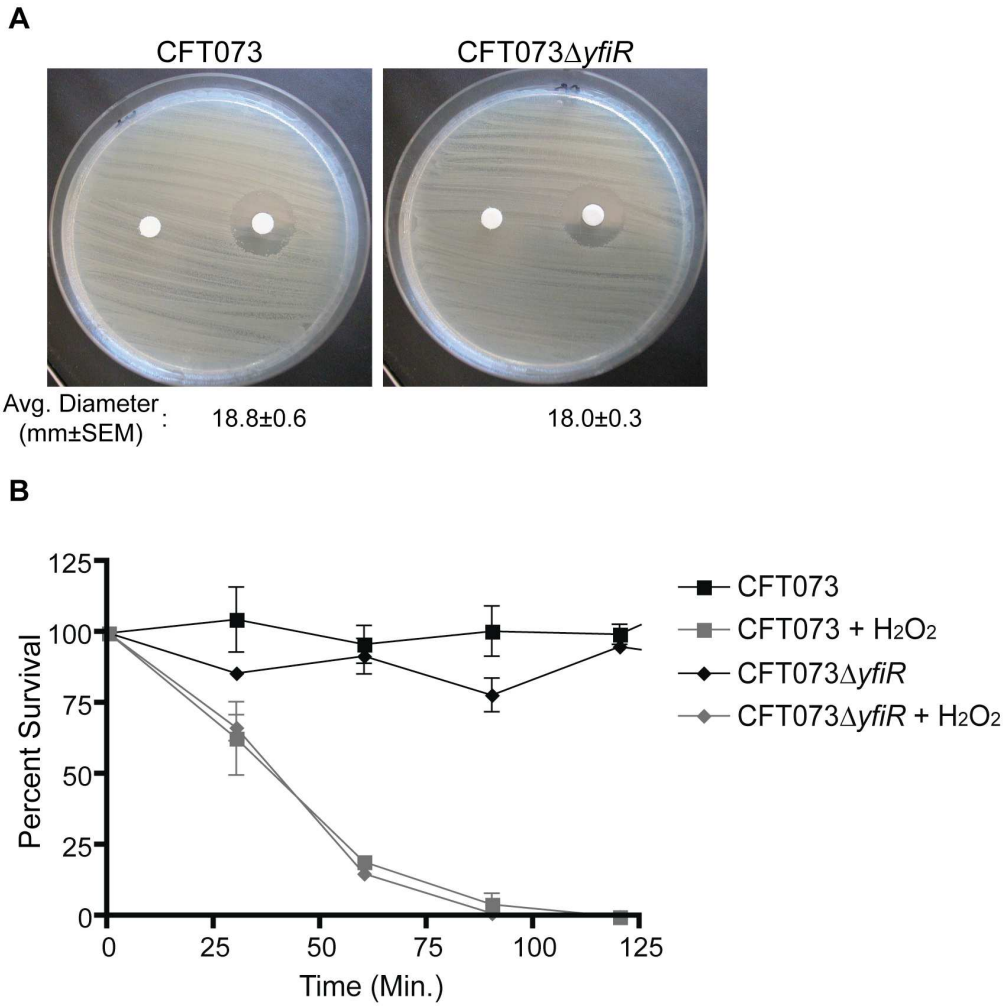


Figure 13: Deletion of *yfiR* does not increase sensitivity to hydrogen peroxide.

(A) Disc diffusion assays with wild-type CFT073 and CFT073Δ*yfiR* grown aerobically at 37° C. 1X PBS was spotted on the left-side disc and 300 mM H_2O_2 was spotted on the right-side disc. The average diameters of the zones of inhibition are given below the plate pictures. (B) Liquid broths assays performed with wild-type CFT073 and CFT073Δ*yfiR*. Diluted cultures were exposed to either PBS or 300 mM H_2O_2. Percent survival was calculated by dividing the CFUs at each time point by the CFUs obtained from the pre-exposure diluted broths.

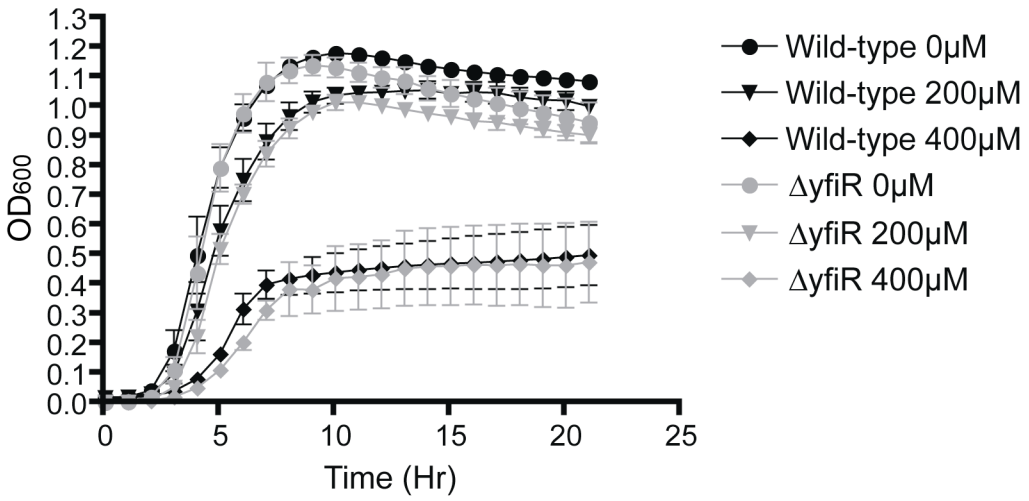


Figure 14: Deletion of *yfiR* does not cause a defect in growth in an iron-limited environment. Wild-type CFT073 and CFT073Δ*yfiR* were grown in LB at 37°C with two different concentrations of the iron chelator 2,2-dipyridyl added to the growth medium.

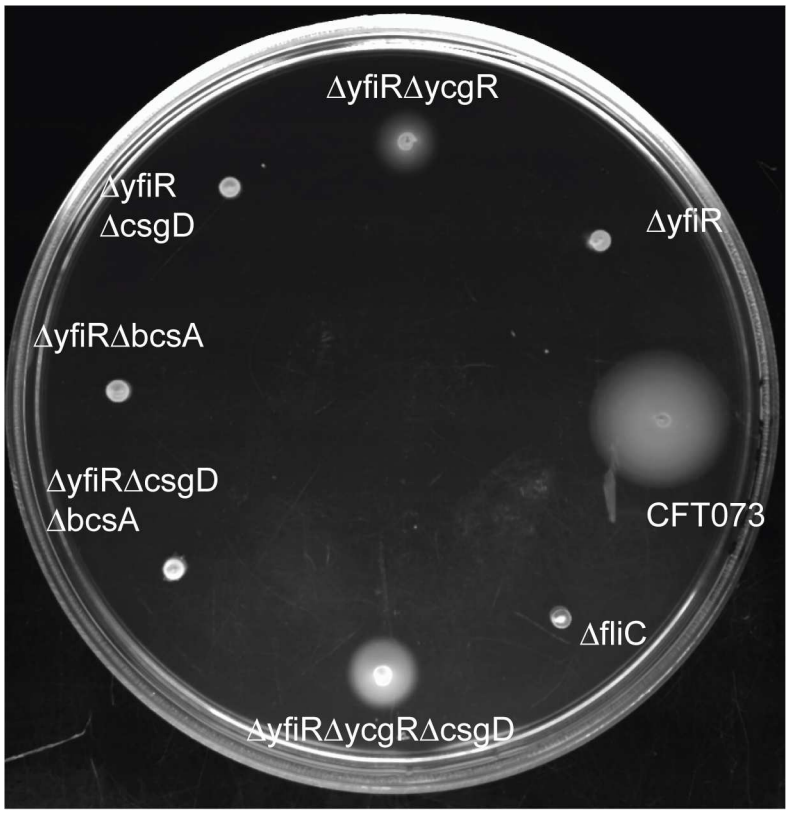


Figure 15: Deletion of *ycgR* from the *yfiR* single-deletion mutant partially restores motility. Strains were grown in 0.3% agar tryptone swim plates at 30° C.

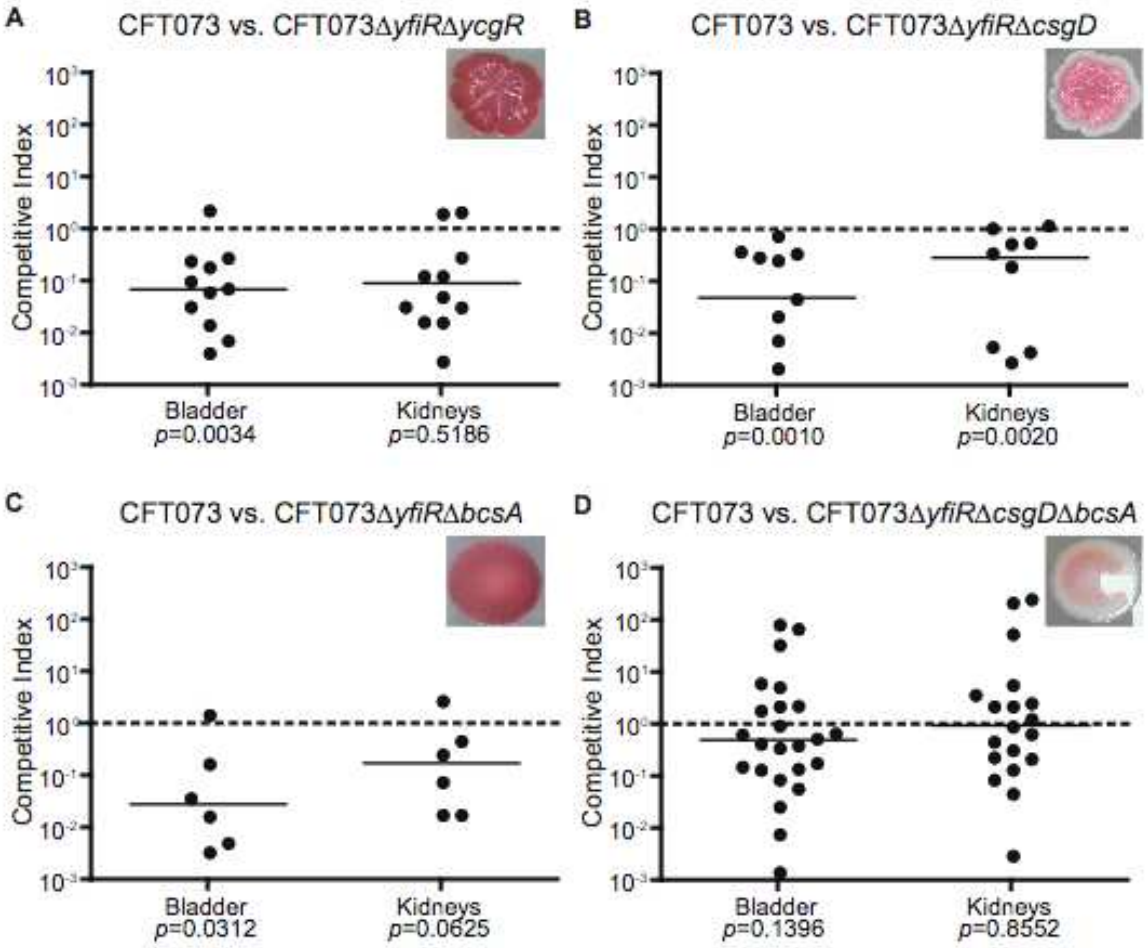


Figure 16: Deletion of both *bcsA* and *csgD* from the *yfiR* deletion mutant restores fitness to wild-type CFT073 levels in the mouse model of competitive UTI. Results for co-infection of female Swiss Webster mice with CFT073Δ*yfiR*Δ*ycgR* (n=14) (A), CFT073Δ*yfiR*Δ*csgD* (n=11) (B), CFT073Δ*yfiR*Δ*bcsA* (n=7) (C), or CFT073Δ*yfiR*Δ*csgD*Δ*bcsA* (n=24) (D) and CFT073Δ*lacZYA* using the mouse model of UTI. The insets show the phenotype of each mutant on congo red plates grown at 30° C. The competitive indices were calculated by the following equation: (*yfi* mutant CFU recovered/wild-type CFU recovered)/(mutant inoculum CFU/wild-type inoculum CFU). The line indicates the median value.

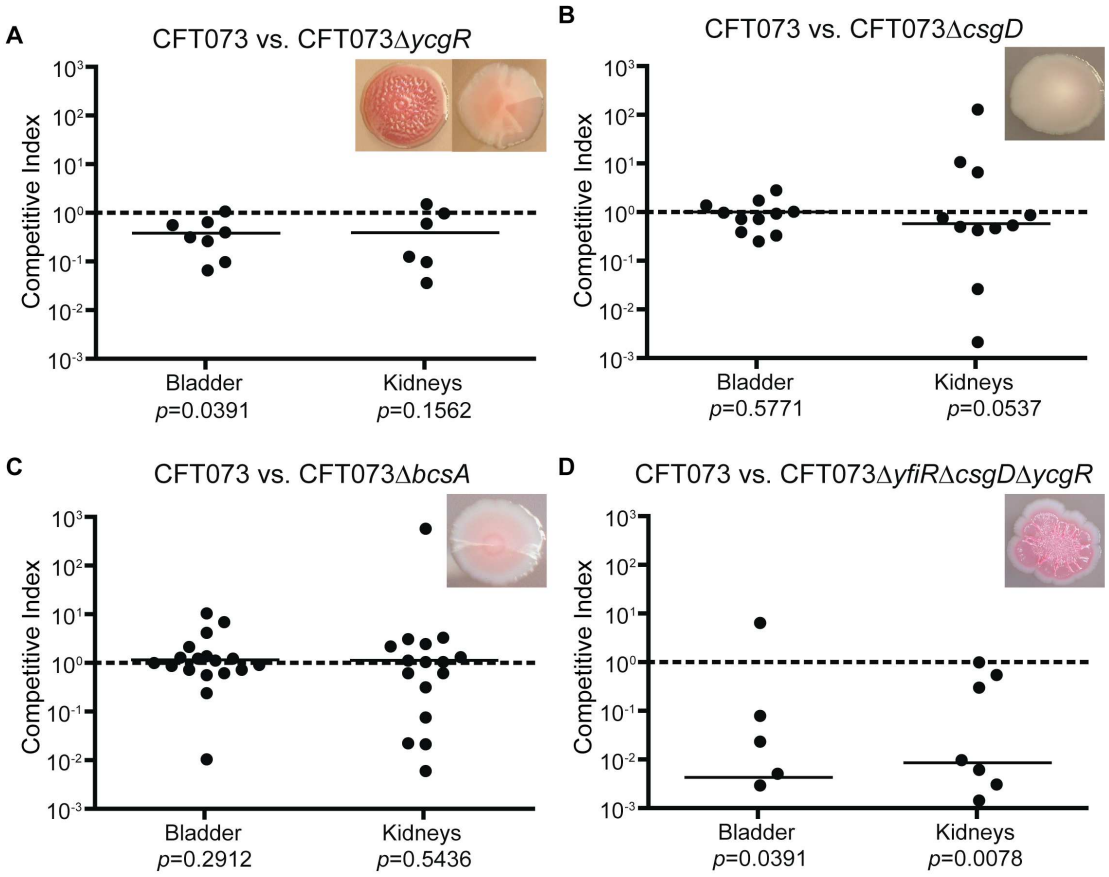


Figure 17: Deletion of *bcsA*, *csgD*, or *ycgR* from wild-type CFT073 does not affect fitness in the mouse model of competitive UTI. Results for co-infection of female Swiss Webster mice with CFT073Δ*ycgR* (n=8) (A), CFT073Δ*csgD* (n=11) (B), CFT073Δ*bcsA* (n=18) (C), or CFT073Δ*yfiR*Δ*csgD*Δ*ycgR* (n=8) (D) and CFT073Δ*lacZYA* using the mouse model of UTI. The insets show the phenotype of each mutant on congo red plates grown at 30° C. The competitive indices were calculated by the following equation: (*yfi* mutant CFU recovered/wild-type CFU recovered)/(mutant inoculum CFU/wild-type inoculum CFU). The line indicates the median value.

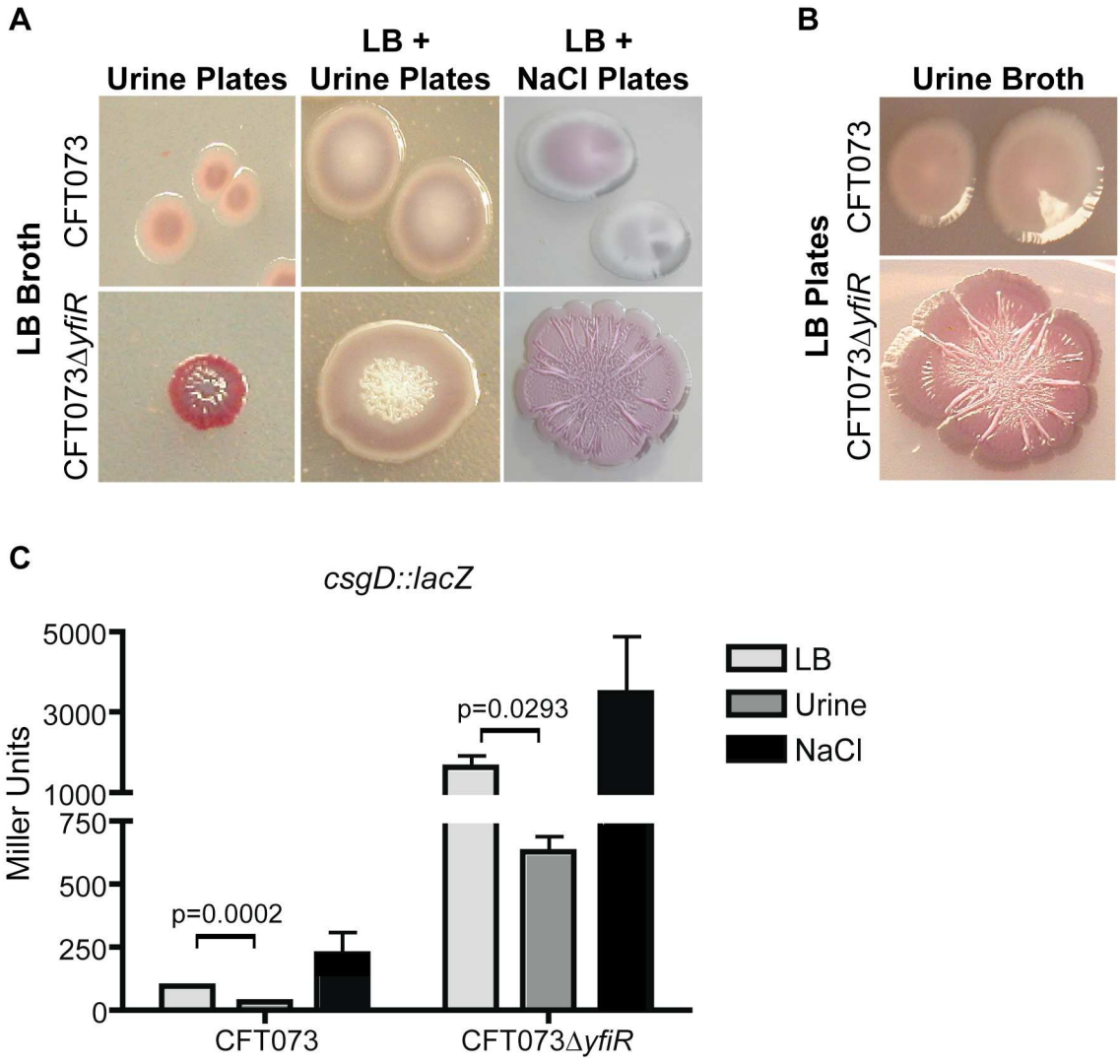


Figure 18: Urine inhibits the RDAR phenotype in CFT073. (A) Phenotypes of wild-type CFT073 or CFT073Δ*yfiR* grown in LB broth before plating on urine, LB + urine, or 8 mg/mL NaCl congo red plates. (B) Phenotypes of wild-type CFT073 or CFT073Δ*yfiR* grown in urine broths before plating on LB congo red plates. All colony phenotypes are pictured for each strain grown under each condition. (C) β-galactosidase assays performed on wild-type CFT073 and CFT073Δ*yfiR* strains with the *csgD::lacZYA* transcriptional fusion. P values were calculated using the student's T-test.

Chapter 4

Discussion and Future Directions

The preceding chapters in this thesis discussed the importance of motility and signaling systems on the persistence of uropathogenic *E. coli* (UPEC) strain CFT073 in the urinary tract during infection. Chapter 2 identified the most likely candidates to act as attractants for CFT073 in the urinary tract, and provided evidence for the functional redundancy of the chemoreceptors in the chemotactic response toward urine. Chapter 3 detailed the downstream effects of the over-production of c-di-GMP on motility, curli fimbriae and cellulose production, and the consequences of this disregulation for the persistence of the bacteria *in vivo*. This chapter will further discuss the implications of chemotaxis, c-di-GMP signaling, and motility in general on the ability of uropathogenic *E. coli* to colonize and persist in the host. Complications with the use of the mouse model to detect colonization factors will also be discussed, and suggestions for further improvement of the model will be made. Suggestions for future investigation of the role of c-di-GMP production in the pathogenic process will also be considered.

Signaling systems are important for adaptation to the host environment

A single bacterium may encounter many different environments throughout its life cycle and must be able to adapt to changing conditions to improve its chances of survival. UPEC, specifically, must be able to survive the transition from the relatively nutritionally rich environment of the host intestine to the nutritionally poor but nitrogen rich environment of the urinary tract (2). Besides the metabolic concerns, life in the urinary tract is also more challenging due to the near constant expulsive force of discharged urine, and an influx of antimicrobial peptides and neutrophils in response to

the presence of the pathogenic bacteria. While uropathogens reside in the intestinal tract they are not faced with these defenses (26). To deal with this change in environmental conditions, the bacteria often employ a variety of two-component systems and other sensory systems that can trigger the expression of the set of genes and processes most able to deal with the added stresses encountered in the urinary tract. I discuss below the possible roles that the chemotaxis sensory system and the regulation of c-di-GMP production by a periplasmic repressor of diguanylate cyclase activity may play in the virulence of UPEC strain CFT073 in the urinary tract.

Amino acids are the main attractants found in urine for *E. coli* CFT073

Results in Chapter 2 of this thesis demonstrated that amino acids act as the main attractants for *E. coli* CFT073 in human urine. This finding is perhaps not surprising given that amino acids and small peptides are also the major nutrients used by UPEC in the urinary tract. In general, *E. coli* tend to display chemotaxis towards compounds that they can also metabolize, mainly amino acids, peptides, and sugars (1, 25). Because sugars are largely absent within the urinary tract, amino acids are left as the main easily metabolized substances in urine. The absence of sugars in urine may also explain the common loss of *trg*, the gene encoding the chemoreceptor responsible for the chemotactic response to ribose, in UPEC strains, including CFT073 (21). The absence of Trg may also indicate that chemotaxis is more important for the colonization of the urinary tract then for the time spent in the intestinal tract in UPEC strains, perhaps because sugars are the main compounds that are metabolized by *E. coli* in the digestive

tract (2). The gene encoding the Tap chemoreceptor, which is responsible for sensing dipeptides, is also absent from many/most UPEC strains (21), although an explanation for its absence is less clear. Small peptides are present in the urine, and UPEC strains should be capable of transporting and metabolizing them (2). However, the smaller amino acids may take less energy to transport and metabolize. This perhaps lessens the incentive for UPEC to maintain a chemoreceptor that is not highly expressed even when present in the genome and whose ligand is most likely not the best energy source for the bacteria.

Hormones present in the urine were also potential candidates as chemoattractants because epinephrine and norepinephrine are both chemoattractants for enterohemorrhagic *E. coli* strain O157:H7 (4). However, neither epinephrine nor norepinephrine elicited a chemotactic response from CFT073 in the analysis included in this thesis. Other hormones in urine such as estrogen derivatives, androsterone, and dopamine also did not act as attractants at their physiological concentrations. Therefore, hormones would likely not be stimulants involved in the ascension of the urinary tract by UPEC. One more avenue in the analysis of hormones as chemoattractants may be worth further study, though: while pregnant women experience simple cases of cystitis at about the same rate as the rest of the female population, they are at increased risk for development of pyelonephritis (32, 38). Progesterone, a hormone produced during pregnancy, was not included in the analyses of chemoattractants in this thesis but might act as an attractant when present in high concentrations in urine. However, progesterone also induces the relaxation of smooth

muscle, including the ureters. As a result, peristalsis of the ureters decreases, which, along with the increased dilation in the renal pelvis and ureters, may allow increased reflux of bacteria from the bladder into the kidneys (8, 15). Perhaps a combination of increased attraction and chemotaxis on the part of the bacteria, and decreased physical barriers to ascension of the urinary tract, play roles in the increased number of cases of pyleonephritis in pregnant women. The direct effects of progesterone on other bacterial processes and gene expression, if there are indeed any effects, would also be interesting to investigate. In addition, glycosuria and aminoaciduria are also increased in pregnant women (15), raising the possibility that concentrations of established growth substrates and chemoattractants are increased in pregnant women and enhance ascension of the ureters to the kidneys via increased chemotactic behavior of the bacteria.

As shown in Chapter 2 of this thesis, *E. coli* CFT073 chemoreceptor mutants did not show a decrease in competitive fitness compared to the wild-type strain. However, these results may not indicate that the chemotaxis system is not needed to establish a successful infection. The current mouse model of urinary tract infection can inform us which bacterial factors may be needed for fitness and persistence in the urinary tract, but the model is inadequate to assess factors that are needed for ascension of the urinary tract. Under the typical inoculation protocol, bacteria can be found in the kidneys of the infected mice as a result of reflux of the bacterial inoculum up the ureters immediately upon infection. Any steps, be they from the urethra to the bladder or the bladder to the kidneys, needed for colonization are bypassed in this model and, thus, it

is difficult to draw any conclusions about the contribution of any chemoreceptor (or chemotaxis in general) in the ascension of the urinary tract. The following section discusses this topic more thoroughly and introduces steps that could be taken to improve the model.

Limitations of the mouse model of UTI and suggestions for improvement

The current murine model of urinary tract infection is a good tool for identifying bacterial factors needed for persistence in the urinary tract; however, it is lacking in its usefulness for discerning which factors are needed for initial colonization. Early studies in the development of the mouse model of UTI noted that reflux of bacteria into the kidneys occurred during inoculation with as little as 10 μl of the bacterial inoculum (10, 12, 16, 17). Experiments performed in Appendix B of this thesis showed reflux into the kidneys with as little as 2 μl of inoculum in certain mouse strains. This reflux bypasses the need for active ascension of the ureters to gain access to the kidneys, making determination of bacterial ascension factors difficult.

Appendix B of this thesis details the attempts made to obtain a reliable mouse model of ascending urinary tract infection that would eliminate the possibility of reflux into the kidneys. Ideally, the inoculum would be swabbed on the entrance of the urethra, avoiding penetration of the bladder altogether, and eliminating the chance for reflux. Unfortunately, mice exposed to the bacteria in this manner never developed a detectable urinary tract infection. Even placement of a 2 μl inoculum in the urethra distal to the sphincter, and repeated inoculation of the same mouse, failed to produce

reliable infection of the urinary tract. The 2 µl inoculum had to be placed proximal to the sphincter and directly into bladder to achieve consistent infections; however, this inoculation method also resulted in reflux into the kidneys in C3H/HeJ mice. The same infection method was tried with Swiss Webster mice that are less likely to experience reflux during the inoculation procedure, but all of these mice quickly cleared the infection. Some of these obstacles may be overcome by alteration of the inoculation process and of the bacterial strain used for the infections. Although repeated inoculation distal to the urethral sphincter did not provide improved results, the inoculation step was only repeated over the course of a few hours. Expansion of the time frame to a few days or weeks of repeated inoculation using this procedure may result in an eventual increase in the number of bacteria that stably reside in the urethra. Once they have reached a sufficient colonization level in the urethra, the bacteria may eventually progress to colonize the bladder and kidneys. This method would be time-consuming and would also make the analysis of the ability of mutants to ascend the urinary tract more difficult. Careful determination of how many inoculations over a given time period result in successful infections in the majority of mice with the wild-type strain would have to be undertaken. Cutoffs and statistical analyses of the number of mice with ascending UTIs using mutant bacterial strains could then be employed to assess defects in ascension. This method also has the disadvantage of most likely requiring more mice per experiment to make accurate conclusions. Some of the negatives of this proposed model could be mitigated with a UPEC strain enhanced for colonization of the urinary tract. A recent report found that UPEC exposed to acidified sodium nitrite or

cadaverine before inoculation displayed increased colonization in the mouse model (5). Isolation of a UPEC strain from a naturally infected mouse or conditioning of the human pathogenic strain to the mouse urinary tract by repeatedly infecting new mice with the expelled bacteria from another infected mouse may also result in a strain of UPEC that is better suited for study in the mouse model of UTI. Any of these methods could produce a strain that could be capable of establishing an ascending infection with fewer or even one inoculation event. Development of a true ascending mouse model of UTI would mark a major advancement in the study of UPEC colonization factors *in vivo*.

c-di-GMP production has an effect on the persistence of UPEC in the urinary tract

Although the mouse model of UTI is not a true ascending model of infection, motility has still arisen as an important fitness factor during infection by UPEC (20, 22, 40). An initial project aimed at deciphering the roles that swimming motility (single planktonic cells moving through liquid) and swarming motility (population movement across a surface) using motility mutants originally identified in two motility screens (9, 13) eventually led to the study of the *yfiLRNB* locus, the subject of Chapter 3 of this thesis. As is presented in Appendix C, the other mutants identified by the motility screens either did not display the expected motility phenotypes in CFT073 or were not attenuated in the mouse model of UTI and were therefore not pursued. The lack of the ability to separate the two forms of motility in the mouse model prohibited any meaningful conclusions from being made about the contributions of each motility type to fitness in the urinary tract. Perhaps further insight into either the differences in gene

expression or the identification of a reliable genetic or phenotypic marker for swarming motility will allow future studies to differentiate between swimming motility and swarming motility *in vivo*.

As mentioned above, preliminary experiments on a mutant in the *yfiLRNB* locus looked promising enough that further evaluation of this locus was undertaken. Deletion of *yfiR* resulted in attenuated swimming and swarming motility, and also caused attenuation in the mouse model of UTI when competed against the wild-type strain. The locus is comprised of genes encoding a diguanylate cyclase, YfiN, and a predicted periplasmic protein, YfiR. YfiL and YfiB are both predicted to be outer membrane proteins with unknown functions. A similar system in *Pseudomonas aeruginosa* with the same gene names verifies YfiN as an active diguanylate cyclase and YfiR as the periplasmic inhibitor of YfiN activity in that organism (23). Although the sequence identities between the *E. coli* CFT073 genes and the *P. aeruginosa* genes are low (~30% identity), the same relative phenotypes are obtained when *yfiR* is deleted from the genome of each organism, suggesting that despite the sequence dissimilarity the proteins appear to perform the same function in each organism. As was shown in the *P. aeruginosa* mutant, removal of YfiR inhibition likely results in both increased activity of YfiN and cellular increases in levels of c-di-GMP, a bacterial second messenger, in *E. coli* CFT073. Ultimately, production of two biofilm components, curli fimbriae and cellulose, were increased via the curli operon regulator CsgD and the diguanylate cyclase AdrA-dependent pathways that were previously discovered (18, 29, 41).

In vivo studies showed that further deletion of *csgD* and *bcsA*, encoding the catalytic unit of the cellulose biosynthetic machinery, from the *yfiR* deletion mutant diminished production of curli fimbriae and cellulose and restored competition to wild-type levels in the mouse model of UTI. Because the *yfiR* deletion mutant did not display increased sensitivity to either hydrogen peroxide stress or iron limitation when compared to the wild-type strain, the attenuation displayed *in vivo* is likely due to the inability of a metabolically over-burdened cell to successfully compete for nutrients with the wild-type strain and not due to increased susceptibility to these host defenses. The added stress of producing an excess of curli fimbriae and cellulose in a nutrient-limited environment while simultaneously trying to defend against the host immune response likely contributed to a decreased *in vivo* growth rate and eventual loss of competitive parity with the wild-type strain. However, the increase in surface-exposed curli fibers on the *yfiR* mutant surface may facilitate binding of host anti-microbial peptides to the mutant bacteria. This effect would inhibit attachment of the mutant bacteria to the host epithelium and lead to eventual expulsion by the flow of urine from the urinary tract. The *P. aeruginosa yfiR* mutant was also out-competed by the wild-type strain in an acute subcutaneous infection model but surprisingly persisted longer than the wild-type strain in a chronic model of subcutaneous infection. Perhaps a chronic model of UTI would reveal increased persistence of the *yfiR* mutant over the wild-type strain *in vivo,* as well.

Overall, the study of the *yfiLRNB* operon illustrated the tight control that UPEC likely have over the production of c-di-GMP as well as the downstream processes

affected by intracellular levels of this ubiquitous second messenger. Loss of motility as a result of high c-di-GMP levels may decrease fitness of the bacteria *in vivo*. Production of curli fimbriae and cellulose also likely needs to be tightly controlled so as to conserve resources. Curli fimbriae can also be immunogenic in the right context (19, 27), giving another reason to regulate their expression *in vivo*. Curiously, curli fimbriae and cellulose are not well expressed *in vitro* until temperatures are dropped to 30° C or lower, and their expression is inhibited at 37° C (29, 33). These data suggest that curli fimbriae and cellulose production *in vivo* would likely be expressed at low levels, if at all. Deletion of the genes necessary for the synthesis of these extracellular factors did not attenuate CFT073 *in vivo* in the two-day infection model, which also suggests that the role of biofilm formation during urinary tract infections is minimal. *Vibrio cholerae* also decrease the expression of genes needed for biofilm formation *in vivo* while increasing the expression of genes that promote a motile lifestyle or enhance virulence. Biofilm formation is instead presumably important for persistence in the estuary environment (6, 7, 34-37). Like *V. cholerae*, expression of biofilm genes may be more important for persistence in the environment after the UPEC have been expelled from the urinary tract.

Future directions for the study of c-di-GMP signaling networks in *E. coli* CFT073

Many interesting avenues for the further study of the regulation of c-di-GMP production and its effects on downstream processes exist. Of the 19 diguanylate cyclases (DGCs) and 16 phosphodiesterases (PDEs) predicted to be encoded by *E. coli*

K-12, *E. coli* CFT073 shares 17 and 14 of these enzymes, respectively. One of the most perplexing aspects of c-di-GMP signaling is why the cell encodes so many of the same type of proteins. Some researchers postulate that certain DGCs and PDEs are expressed only under certain conditions, giving the bacteria a finer degree of control of the downstream processes in a greater number of possible environments. Others theorize that c-di-GMP is locally produced and protein bound, limiting the range of their diffusion, and thus infuence, in the cell. Therefore, different DGCS and PDEs and the c-di-GMP levels that they control have effects only on a subset of possible c-di-GMP effectors. This specificity would allow the cell to more precisely control the intended target, whether that involves modulation of flagellar rotation and motility, biofilm formation, or expression of virulence factors. In line with this theory, not all DGCs and PDEs exhibit the same effects on the bacteria that express them (3, 11, 14, 28, 30, 33, 35, 39).

So far little is known either about the stimuli that DGCs and PDEs respond to or, outside of the curli fimbriae and cellulose, about the factors that are affected by c-di-GMP levels. Analysis of possible stimulants and inhibitors in both the expression and activity of each DGC and PDE could clarify whether a specific set of DGCs or PDEs is needed for survival in or outside of the host. Alternative pathways affected by c-di-GMP levels could also give insight into the factors needed for adaptation to various environments and stresses. In addition to the effects on the bacteria themselves, c-di-GMP can also stimulate an inflammatory response through the host innate immune system (24, 31). Determination of the levels of c-di-GMP being produced by UPEC

during infection in the mouse model might therefore not only give new information about the lifestyle maintained by the bacteria *in vivo* but may also indicate whether part of the inflammatory response to infections in the urinary tract might be mediated by sensing of bacterial c-di-GMP.

A systematic evaluation of the contribution of each DGC and PDE to the regulation of the known c-di-GMP-controlled pathways and functions, as well as to the fitness and infectivity of UPEC *in vivo,* might highlight the factors that are important for colonization, fitness, and virulence in the urinary tract. Microarray and proteomic analysis as well as phenotypic arrays of the DGC and PDE mutants and other proteins that may be encoded in the same operon may highlight novel regulatory networks in UPEC that could inform both how the bacteria sense and respond to the urinary tract environment.

Conclusion

The studies contained in this thesis originally sought to determine the role of chemotaxis and swimming and swarming motility in the colonization of the urinary tract by *E. coli* strain CFT073. Although lack of a true ascending mouse model of UTI precluded any conclusions that could be made concerning the initial colonization steps in the urinary tract, chemotaxis studies showed that amino acids are likely the main chemoattractants for CFT073 in human urine. Examination of various swimming and swarming mutants also expanded the scope of these projects to include signaling by c-di-GMP. Experiments performed with mutants in the *yfiLRNB* locus, which encodes a

diguanylate cyclase involved in the synthesis of c-di-GMP, highlighted the importance of controling of the production of this second messenger in order to achieve optimal infection levels in the urinary tract. This research into c-di-GMP signaling has opened new lines of investigation into global signaling networks that could potentially inform new lines of study into how UPEC coordinate expression and activity of various factors needed for adaptation to both *in vivo* and *ex vivo* environments.

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Appendix A

Escherichia coli strain CFT073 displays chemotaxis toward physiological concentrations of D-serine present in urine

Authors and contributions:

Erica L. Raterman: Wrote manuscript and designed and performed all experiments not noted below.

Jeff Gelhausen: Performed chemotaxis experiments for CFT073 and MG1655 chemoreceptor reciprocol crosses.

Shahaireen Pellett: Helped develop the chemotaxis assay.

Daniel Shapiro: Performed PCR screen and southerns for *dscO* in clinical isolates.

Rodney A. Welch: Supervised all work and contributed to the writing of the manuscript.

Abstract

Community-acquired urinary tract infections (UTIs) are commonly caused by uropathogenic *Escherichia coli* (UPEC). We hypothesize that chemotaxis toward a ligand present in urine could direct UPEC into and up the urinary tract. Compared to other amino acids, D-serine is found in uncommonly high concentrations in the urine of mammals and is a candidate chemotactic attractant for UPEC. We found that UPEC strain CFT073 chemotaxed toward D-serine but not L-serine at concentrations typically found in human urine. In contrast, *E. coli* strain MG1655 chemotaxed toward L-serine but not D-serine. Deletion of *tsr*, the known MG1655 L-serine chemoreceptor, eliminated the positive CFT073 chemotactic response to D-serine. Exchanges of *tsr* alleles revealed that expression of tsr_{CFT073} in the MG1655Δ*tsr* background did not result in chemotaxis toward D-serine. Further mutational analyses revealed that *dscO*, a gene linked to *tsr* in UPEC genomes but missing in MG1655, encodes a putative periplasmic binding protein that is needed for CFT073 chemotaxis toward D-serine when grown in minimal medium. However, deletion of *dscO* did not inhibit chemotaxis of CFT073 toward D-serine when grown in rich medium nor could expression of *dscO* in MG1655 produce chemotaxis toward D-serine. Attempts at purification of DscO were unsuccessful and it remains to be shown through biochemical methods if DscO can bind D-serine and interact with Tsr.

Introduction

Urinary tract infections (UTIs) are some of the most commonly diagnosed and treated infections in the United States with 40-60% of women will experience at least one UTI in their lifetime (23, 30). Approximately 80-90% of community-acquired UTIs are caused by uropathogenic *Escherichia coli* (UPEC)(11, 12). UPEC likely gain access to the urinary tract when the urethra is contaminated with intestinal microorganisms. After colonization of the urethra, the bacteria ascend the urethra to the bladder and, in some cases, continue up the ureters to the kidneys (2). Known UPEC virulence factors include type I fimbriae, Pap pili, flagella, hemolysin, iron acquisition systems, and toxins (8, 20, 21, 35), though factors important for early ascension of the urethra and the ureters and subsequent colonization of the bladder and kidneys remain largely unidentified. Previous reports indicate that motility is important for ascension of the ureters as non-flagellated bacteria were unable to reach the kidneys in the murine model of UTI (24, 26, 32). Since motility is important for a successful infection, chemotaxis may also play a part in the efficient and rapid colonization of the urinary tract by directing the bacteria up the urethra and ureters.

Chemotaxis is defined as the movement toward an attractant or away from a repellant. The chemotaxis machinery has been well characterized in non-pathogenic *E. coli* strain K-12. The chemotaxis system consists of chemoreceptors, or methyl-accepting chemotaxis proteins, that possess a variable periplasmic ligand binding domain and a conserved cytoplasmic signaling domain. Cytoplasmic components of the chemotaxis system sense the occupation state of the chemoreceptor and, based on

their phosphorylation state, change the direction of flagellar rotation to produce tumbling or smooth swimming (9, 34).

E. coli strain K-12 possesses five known chemoreceptors including Tsr (L-serine), Tar (aspartate/maltose), Trg (glactose/ribose), Tap (dipeptides), and Aer (oxygen/redox state). Each chemoreceptor is specific for the indicated set of ligands (28, 34). The prototypical UPEC strain CFT073 encodes only three of the five chemoreceptors: *tsr*, *tar*, and *aer*. A previous study looking at the prevalence of chemoreceptors across *E. coli* strain groups found that the loss of the Trg and Tap receptors is common among UPEC strains (25), suggesting that only these three chemoreceptors are useful for survival in the urinary tract. Loss of *cheW* was also found to attenuate UPEC in the murine model of UTI, indicating that chemotaxis is important for a successful infection (26).

Our previous work demonstrated that D-serine serves as a signal for up-expression of virulence and colonization genes of UPEC strain CFT073 (14, 33). This study examines if D-serine is a possible attractant for UPEC strain CFT073. We show that CFT073 chemotaxes toward D-serine *in vitro* and that a putative periplasmic binding protein, DscO (D-serine chemotaxis), is necessary to produce this response. Furthermore, we show that deletion of all three chemoreceptors causes a fitness defect in the murine model of UTI. This work highlights the fitness advantage gained by the presence of the chemotaxis system as well as the possible gain in colonization efficiency imparted by expedited ascension of the urinary tract.

Materials and Methods

Bacterial strains and growth conditions

E. coli strain CFT073 was isolated from the blood and urine of a woman admitted to the University of Maryland Medical System with pyelonephritis (29). *E. coli* strain MG1655 was obtained from Ian Blomfield. Clinical isolates tested in the PCR screen were obtained from Ann Stapleton at the University of Washington. All strains were grown in either Luria Broth (LB) or 1XMOPS glycerol minimal medium with 50µg/mL kanamycin, 20µg/mL chloramphenicl, or 200µg/mL carbenicillin as appropriate. All strains were grown at 37˚C and 250rpm.

Construction of strains

All gene deletions were performed using the λ-Red recombination system developed by Datsenko and Wanner (7). The primers used for each deletion are listed in Table 1. The antibiotic resistance cassette used to replace the target gene was removed using a Flp recombinase encoded on pCP20 (7). All gene deletions were verified by PCR and loss of antibiotic resistance on LB agar containing the appropriate antibiotic.

Complementation of *tsr* in E. coli strains CFT073 and MG1655 were performed by inserting each strains *tsr* allele under the control of the native promoter into the PstI-HincII site of pACYC177 (New England Biolabs, USA). Complementation of the *dscO* deletion strain was performed by inserting the promoterless *dscO* gene into the NcoI-HindIII restriction sites of pBAD24d (Invitrogen, USA). The vector for expression and

purification of DscO was constructed by cloning promoterless *dscO* into the BamHI-HindIII restriction sites of the pMAL-c2x vector (New England Biolabs, USA). The sequence for a C-terminal histidine tag was included in the reverse primer. All constructs were verified by sequencing.

The *dscO*::*lacZYA* strain used for the ß-galactosidase experiments was constructed by inserting the pFUSE *lacZYA* vector downstream of *c5433* in the genome of a CFT073 *lacZ* deletion strain to create a transcriptional fusion, as previously described (3). The insertion event was verified by PCR.

The MG1655Δ*tsr* + Tn7::tsr_{CFT073} strain was constructed by mating the CFT073 *tsr* region carried on a Tn7 plasmid into the MG1655Δ*tsr* background. The same plasmid used to complement the *dscO* deletion strain was electroporated into the MG1655Δ*tsr* + Tn7::tsr_{CFT073} strain to produce a MG1655 background strain expressing *dscO*.

Capillary chemotaxis assay

Capillary assays were performed according to Adler's original method (1) with a few modifications. Briefly, strains were grown aerobically overnight in 1xMOPS glycerol with the appropriate antibiotic at 37˚C. Bacteria were harvested at OD_{600}~0.4 (approx. 10^7 CFUs) and washed 1X in chemotaxis buffer (10^{-2} phosphate buffer, 10^{-4} EDTA). Bacteria were resuspended to OD_{600}~0.387 in chemotaxis buffer. Capillary tubes (1mm) were sealed on one end and filled with 0.5ul attractant solution (L-serine or D-serine, 31-500µg/mL). Capillary tubes were then incubated with 100µl of the bacterial

suspension at 30˚C for 30 minutes. Capillary tubes were washed and the contents were dilution plated. Graphs for each strain represent combined data from at least three independent assays, unless otherwise noted. Response levels above the buffer control were graphed and analyzed using a two-way ANOVA and Bonferroni post tests with Prism (GraphPad).

PCR analysis

PCR analysis of the region downstream of *tsr* was performed using the primers listed in SI Table 1. Reactions were run using GoTaq Master Mix (Promega, USA) and the appropriate primers with cells taken either from an LB plate for the UTI and fecal isolates or a freezer stock for the DEC isolates.

Purification of DscO

Purification was performed according to the NEB pMAL expression manual (New England Biosciences, USA). Briefly, a DH5α cloning strain carrying the pMAL-c4--*dscO* plasmid with C-terimus histidine tag was grown in 6 L of LB broth with 200 μg/mL carbinecillin and 1% glucose at 15°C. At OD_{600}~0.4, the *dscO* expression was induced by the addition of 0.3 mM IPTG and growth was continued for a further 12 hours. Cells were then pelleted at 4,000 x g and resuspended in 300 mL column buffer (20 mM Tris-HCl, 200 mM NaCl, 1mM EDTA). Cell suspensions were stored at -20°C overnight and then lysed by sonication. Cell debris was pelleted by centrifugation at 9,000 x g and the supernatant was diluted 1:5 in column buffer. The diluted crude cell extract was run

over an amylose column (New England Biolabs, USA) and the bound MBP-DscO fusion protein was eluted with 10 mM maltose. The eluted protein was further purified by binding the fusion protein on a nickel column (Fisher Scientific, USA) to remove any MBP degradation fragments. Protein concentration and purity were determined by Bradford assay (Biorad, USA) and separation on a 10% SDS-PAGE gel followed by coomassie staining. Protein was also transferred to nitrocellulose and probed with 1:5000 α-penta His-tag (Qiagen, USA) and 1:5000 α-MBP (New England Biolabs, USA) antibodies to confirm the presence of the fusion protein.

Southern analysis

Southerns were performed by digesting genomic DNA isolated using a genomic DNA preparation kit (Promega, USA) with PstI (New England Biolabs, USA). Reactions were run on a 0.8% gel and transferred to a Hybond-N+ nylon membrane (Amersham Biosciences, USA). After UV-crosslinking, the blot was probed overnight at 60°C using a PCR product spanning the entire putative transport locus. The probe was labeled with $\propto$-^{32}P using the Prime-It random primer labeling kit (Stratagene). Blots were visualized using the Typhoon Imager 8600 phosphorimager.

β-galactosidase assays

All β-galactosidase assays were performed as previously described (2). Briefly, bacterial strains were grown in 1XMOPS glycerol with the appropriate antibiotic and were grown either aerobically or anaerobically at 37˚C. Anaerobic media was prepared

by pumping nitrogen gas over media aliquoted in test tubes. The test tubes were then sealed and autoclaved. Broths were inoculated with bacteria, antibiotics, and IPTG by syringe after the tubes had cooled. Broths were placed on ice for 20 minutes after the OD_{600} reached 0.3-0.7. 500µl of each broth was mixed with 500µl Z buffer and 20µl chloroform and 10µl 0.1% SDS was added. Samples were then incubated at 28˚C for 5 minutes and then 200µl 4mg/mL ONPG was added. The reaction was stopped with 500µl Na_2CO_3. The OD_{420} and OD_{550} were then recorded and the Miller units were calculated according to the manual.

Murine model of UTI

Colonization of the urinary tract was determined using the competitive murine model of urinary tract infection as described previously (31). CFT073Δ*lacZYA* was used as the wild-type strain and CFT073Δ*tsr*Δ*tar*Δ*aer* was used as the mutant strain. Bacterial strains were grown statically in 3mL LB at 37˚C. Strains were passaged to fresh LB every two days for 6 days with the final passage into 40mL LB. After 2 days of growth, the broths were adjusted to OD_{600}~0.4 and the wild-type strain and the mutant strain were mixed equally. The mixed broth was then washed 1X in 1XPBS and resuspended in 500µl 1XPBS. Isofluorane-anesthetized 6-7 week-old female Swiss Webster mice (Harlan, USA) were inoculated via urethral catheterization with 50µl (10^8 CFU) of the mixed bacterial suspension. Mice were euthanized via CO_2 asphyxiation and the bladder and kidneys were aseptically harvested and placed in 1XPBS. The organs were homogenized, serially diluted in 1XPBS, and plated on MacConkey agar

medium (Fisher, USA). Surviving strain ratios were determined by counting white (wild-type) and red (mutant) colonies. Colonization levels were graphed an analyzed using a paired Wilcoxon signed ranked test and Prism (GraphPad). All animal experiments were approved by the UW-Madison Animal Care and Use Committee.

Results

Tsr mediates *E. coli* strain CFT073 chemotaxis toward D-serine.

Human urine consists of amino acids, peptides, urea, and salts (5). In particular, D-serine is found in high concentrations in the urine (4-115 µg/mL)(S. Pellett and R. A. Welch, unpublished data) and brain tissue of mammals (6, 13). Though a variety of D-amino acids are found in urine, D-serine is unusual in that its D- to L-serine molar ratio is much higher than the D- to L-enantiomer ratios of other amino acids. D-serine even accounts for more than half of the total serine in the urine of some individuals (6, 18). We hypthesize that D-serine is used as a specific signal for UPEC to ascend the urinary tract. To test if uropathogenic *E. coli* strain CFT073 and K-12 substrain MG1655 could chemotax toward L-serine or D-serine, the capillary chemotaxis assay described by Julius Adler was performed using various concentrations of L-serine and D-serine (31-500 ug/mL) with buffer alone serving as a baseline control for random motility. Within the concentration range tested, MG1655 chemotaxed toward L-serine but did not chemotax toward D-serine (Fig. 1B), while CFT073 chemotaxed toward D-serine but not L-serine (Fig. 1A). *E. coli* chemotaxis toward L-serine is well documented in *E. coli* K-12 substrain RP437 and is mediated by the Tsr chemoreceptor (16, 17, 28). A

CFT073Δ*tsr* deletion strain did not chemotax toward D-serine or L-serine, confirming that Tsr mediates the chemotactic response toward both forms of serine (Fig. 1D). Chemotaxis toward D-serine was reported in the early literature on chemotaxis in *E. coli* strain RP437 (28). However, in our lab, RP437 did not chemotax toward D-serine, but did display strong chemotaxis toward L-serine (Fig. 1C). The discrepancy could be attributed to the purity of D-serine used in the previous study because contamination with L-serine could have produced the apparent response to D-serine.

Single amino acid changes in the periplasmic binding domain of $Tsr_{K\text{-}12}$ lessen or even abolish chemotaxis toward L-serine (17, 27). Therefore, a single or a few amino acid substitutions in Tsr_{CFT073} could account for the difference seen between CFT073 and MG1655 chemotaxis toward D and L forms of serine. When the Tsr amino acid sequences were aligned, three amino acid differences between the two strains were found in the periplasmic binding domain (Fig. 2A). To determine if these differences could account for the difference in attraction to D-serine and L-serine, a MG1655Δ*tsr* strain expressing tsr_{CFT073} and a CFT073Δ*tsr* strain expressing tsr_{MG1655} were tested in the capillary chemotaxis assay. CFT073 complemented with tsr_{MG1655} responded positively to L-serine, but not to D-serine (Fig. 2B). However, MG1655 complemented with tsr_{CFT073} did not respond D-serine but still chemotaxed toward L-serine, albeit to a lesser degree than the wild-type MG1655 strain (Fig. 2C). Each *tsr* deletion mutant behaved like its wild-type parent when complemented with its own allele of *tsr* (Fig. 2D,E). These results suggest that an additional component present in CFT073 but not in MG1655 is required for chemotaxis toward D-serine.

A putative periplasmic binding protein, DscO, may be necessary for chemotaxis toward D-serine.

Occasionally a periplasmic binding protein is necessary to mediate ligand binding to chemoreceptors. For example, MalE, a maltose periplasmic binding protein, works together with the Tar chemoreceptor to produce a chemotactic response to maltose. MalE is a component of the maltose transport system that binds maltose to form a closed complex. In the closed complex, MalE can either dock at the maltose transport machinery or it can bind with Tar to produce a chemotactic signal (4, 15, 22).

Examination of the MG1655 and CFT073 genomes revealed a potential transport system containing *dscO*, a putative periplasmic binding protein (locus tag c5433 and c5434), immediately downstream of *tsr* in the CFT073 genome but not in the MG1655 genome. The MG1655 genome instead has *yjjL*, a predicted transporter of the Major Facilitator Superfamily, in the place of the putative transport locus (Fig. 3A). To determine if the loss of *dscO* resulted in altered chemotaxis to D-serine, the gene was deleted from the CFT073 genome via the λ-Red system (7). Absence of *dscO* resulted in the loss of chemotaxis toward D-serine in the capillary chemotaxis assay (Fig. 3B). Complementation of the deletion mutant with *dscO* under the control of an arabinose-inducible promoter resulted in the return of chemotaxis toward D-serine and a loss of chemotaxis toward L-serine (Fig. 3C). However, sequencing of the complementing plasmid exposed a frameshift mutation that would result in an additional 15 amino acids being added to the C-terminus of DscO. Despite this alteration, the protein seems to be

a functional mutant that can result in a successful complementation. If Tsr_{CFT073} and DscO are the only components needed to produce a chemotactic response to D-serine, the expression of these two genes in the MG1655Δ*tsr* background should impart the ability to chemotax toward D-serine on this strain. However, an attempt to complement strain MG1655Δ*tsr* with tsr_{CFT073} inserted at the Tn7::*att* site and plasmid-encoded *dscO* did not result in chemotaxis toward D-serine or L-serine (Fig. 3D). MG1655Δ*tsr* complemented with only tsr_{CFT073} at the Tn7::*att* site regained chemotaxis toward L-serine (Fig. 3E), suggesting that recombinant *dscO* in multiple copies had a deleterious effect on the ability of the strain to be attracted toward serine in general. Additionally, although CFT073 shows a DscO-dependent response toward D-serine when the strain is grown in minimal medium, deletion of *dscO* does not seem to have an effect on chemotaxis toward D-serine by CFT073 when grown in rich medium (Fig. 3F). To determine if DscO does bind D-serine, an attempt to purify DscO using the pMAL expression system was made. Unfortunately, the fusion MBP-DscO protein could only be obtained in small quantities insufficient to undertake isothermal calorimetry experiments to determine binding affinity of DscO for D-serine (Fig. 4).

A protein BLAST search for *dscO* across all sequenced and annotated strains of *E. coli* revealed that *dscO* is most often present in extraintestinal pathogenic *E. coli* (ExPEC) strains over the nonpathogenic or diarrheagenic strains of *E. coli* ($p=0.0073$ and $p=0.00003$, respectively, for ExPEC comparison to nonpathogenic and diarrheagenic strains, Fisher's exact test). Additionally, *dscO* occurs in the same genomic context (i.e. downstream of *tsr*) in every strain observed (Table 2). To further

this epidemiological survey, *E. coli* stool isolates from both healthy patients and patients with diarrhea and urine isolates from patients with UTIs were analyzed by PCR for *dscO*. The analysis revealed that 42.4% of UTI isolates and 40.0% of normal fecal isolates possessed *dscO* with most isolates demonstrating the same genomic organization as CFT073. Of the diarrheagenic *E. coli* (DEC) strains, only 6.67% of the tested isolates possessed the locus (Table 2), showing again that UPEC strains are more likely to encode *dscO* (p=0.0013, Fisher's exact test). Southern blots confirmed the absence of the locus in strains that tested negative by PCR (Fig. 5). Most strains encoded *tsr*, as expected from previous reports (25).

To examine conditions where *dscO* would be expressed, promoterless *lacZYA* genes were inserted downstream of *dscO* via pFUSE (3) in a CFT073Δ*lacZYA* background strain to produce a transcriptional fusion reporter. β-galactosidase assays were then performed under various inducing conditions. Malate, succinate, and fumarate were included since the putative transport system bears sequence similarity to the tripartite ATP-independent periplasmic (TRAP) transport system responsible for the transport of those substrates (10, 19). These molecules as well as D-serine and L-serine did not induce *dscO* expression (Fig. 6A), nor did growth under anaerobic conditions (Fig. 6B). Growth at 30˚C or 37˚C did not result in significant increase in the gene expression of *dscO* (Fig. 6C). The combined data suggest that *dscO* is apparently expressed constitutively at very low levels.

Previous studies on the role of chemotaxis in the urinary tract by CFT073 have shown that at least one chemoreceptor is required for full fitness of the bacteria *in vivo*.

Similarly, the CFT073 *dscO* deletion mutant was co-infected with wild-type CFT073 in the competitive mouse model of UTI to see if loss of this putative periplasmic binding protein would result in attenuation of the mutant strain. Although the results of three combined experiments show that there may be a slight trend toward attenuation (Fig. 7), the results are not statistically significant (bladder p=0.09, kidney p=0.30). This result suggests that DscO is not critical for successful infection of the urinary tract.

Discussion

Initial events in the colonization of the urinary tract by UPEC remain relatively undetermined. Navigation up the urinary tract via chemotaxis could be the first step to aid UPEC entry into the urethra and bladder. A UPEC-specific ligand present in urine but found minimally in the digestive tract could provide the needed stimulus for UPEC fecal contaminants occupying the urethra to initiate movement up the urinary tract. The ability to chemotax toward a urine-specific attractant could also give UPEC an advantage over other fecal organisms with a limited or different chemotactic repertoire. D-serine is found in high concentrations in the urine but, by comparison, is present in the digestive tract in trace amounts (13). Although various UPEC strains, including CFT073, chemotax towards the same attractants that K-12 strains chemotax towards, no UPEC-specific attractants capable of acting as a ligand for UPEC when tested within their physiological concentrations were previously identified. Therefore, D-serine, while not the sole possible attractant for UPEC, is a comparatively unique signal that could

stimulate ascension of the urinary tract by UPEC when present in its physiological concentration.

In this study, we reported that chemotaxis of uropathogenic *E.coli* strain CFT073 toward D-serine is mediated by the Tsr chemoreceptor and a putative periplasmic binding protein, DscO, when grown in minimal medium but not rich medium. The range in which CFT073 responds to D-serine is within the physiological range found in urine (4-114µg/mL). Chemotaxis toward D-serine could not be established in MG1655 in the presence of tsr_{CFT073} and *dscO*. However, this inability could be attributed to copy number effects that could disrupt the Tsr-DscO interaction. Attempts to purify DscO for further biochemical analysis were unsuccessful, leaving the true function of DscO uncertain.

Uropathogenic and nonpathogenic fecal isolates were more likely to encode *dscO* than DEC isolates. Although the fecal isolates were isolated from healthy patients, the ability of these isolates to cause disease in the urinary tract remains undetermined. Urinary tract infections are hypothesized to originate from fecal contamination of the urethra, leaving open the possibility that the fecal strains possessing *dscO* could be capable of causing UTIs. However, as has been shown in this study, the presence of *dscO* is not necessary for successful infection of the urinary tract.

This study provides D-serine as one possible attractant in urine that may stimulate ascension of the urinary tract. The ability to navigate the urinary tract may result in more efficient colonization, lessening the possibility that the bacteria would be

dispelled by urine before being able to gain a foothold in the bladder. Further biochemical studies are needed to determine if *dscO* is capable of binding D-serine. Without this data, the actual function of DscO, especially when grown in rich medium, remains in doubt.

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Table 1: Primer table

Gene/Application	Sequence
tsr deletion	5'-GGCCGAAAATCTTGCATCGGTCCA CAGGAAAGAGAAACCTGTGTAGGCTGGAGCTGCTTC-3'
	5'-AAAACGCCGGATGAAATACTCATCCGGCATCATTA CGCATATGAATATCCTCCTTAG-3'
(MG1655)	5'-CCGATAACATTTTGCTTATCGGGCATTTTCATGGCGA CATATGAATATCCTCCTT-3'
tsr deletion check	5'-CGAGAACCGGACCCACCAG-3'
	5'-CCTGGCCTGCGCTGTTCC-3'
(MG1655)	5'-CACTGCGTGGCTGTATCTGG-3'
aer deletion	5'-GGCATTGTGCTCCAACCGCTGGATCCGGCATACC GATGTGTAGGCTGGAGCTGCTTCG-3'
	5'-GAAGTTAACAACCATATAACCTGCACAGGACGCGAA CCATATGAATATCCTCCTTAG-3'
aer deletion check	5'-CGATGACAGCCACGGTTACG-3'
	5'-GCTTGCCACTCTACGGCTC-3'
tar deletion	5'-CCCATCAGGCGGCAATGACCGCTTTAGTAAATAC TCGTGTGTAGGCTGGAGCTGCTTCG-3'
	5'-AATAAAGTTTCCCCCCTCCTTGCCGATAACGAGATCA ACCATATGAATATCCTCCTTAG-3'
tar deletion check	5'-CCAATGTATCAGCCAGCGTC-3'
	5'-CTACGCCTTGCTGGTGGATC-3'
dscO deletion	5'-GCCGTTACTGGGCAGACCTATTAAACAAAAAAAG TAATGTGTGTAGGCTGGAGCTGCTTC-3'
	5'-CCTAAGAGAGATGAGGCAGGACGCTGAGCATTAATG CTTACATATGAATATCCTCCTTAG-3'
dscO deletion check	5'-CCACCACTCCACCTAACATCAC-3'
	5'-CTCCCGACAACAATGTTGGAG-3'
Clone *tsr* into pACYC177 (CFT073)	5'-TTGACTACTGCAGGATGTTGCCAGAGTTACGCGC-3' 5'-TACTTCTGTCAACCGTTAAAACGTTTCCCAGTTCT CCT C-3'
(MG1655)	5'-TACTTCTGTCAACGGCGATTAAAATGTTTCCCAGT TCTCC-3'
Clone *dscO* into pBAD24	5'-ACAAAACCATGGATGGCTGAAATAACACTTGCGCT C-3'
	5'-GAGCATAAGCTTTTATTGCAGTTGCTGTTGAATGGTT TCG-3'
Clone *dscO* into pMAL	5'- GGAGTAAGGATCCATGAGCAAAATAATAGCGATGCT AATTACTGTCAG-3'
	5'- GAGATGAAGCTTTTAGTGGTGATGGTGATGGTGTTG CAGTTGCTGTTGAATGGTTTCGTA-3'
Clone *dscO* into pFUSE	5'-AACCCATCTAGACCGTTGACGATCTCGCAGG-3' 5'-CTGAGCCCCGGGGCTTATTGCAGTTGCTGTTGAATG

	G-3’
dscO PCR screen	5’-TTATTGCAGTTGCTGTTGAATGGTTTCG-3’
	5’-ATGAGCAAAATAATAGCGATGCTAATTACTGTC-3’
tsr PCR screen	5’-GGCCGAAAATCTTGCATCGG-3’
	5’-CGTTAAAACGTTTCCCAGTTCTCCTC-3’
DEC strain *tsr* region	5’-CCTTGATGCCACCCTTGC-3’
PCR	5’-CACCGATGATTCCGAGAATCACC-3’

Table 2: Prevalence of *dscO* in *E. coli*

		tsr	*dscO*	CFT073 context
Screened	UTI (n=33)	87.9%	42.4%*	42.4%
	Fecal (n=15)	93.3%	40.0%*	40.0%
	DEC (n=30)	90.0%	6.7%	6.7%
Sequenced	ExPEC (n=9)	100%	77.8%†‡	77.8%
	Non-Path (n=13)	92.3%	15.4%†	15.4%
	DEC (n=28)	100%	3.6%‡	3.6%

Fisher's exact test: *p=0.0013, †p=0.0073, ‡p=0.00003

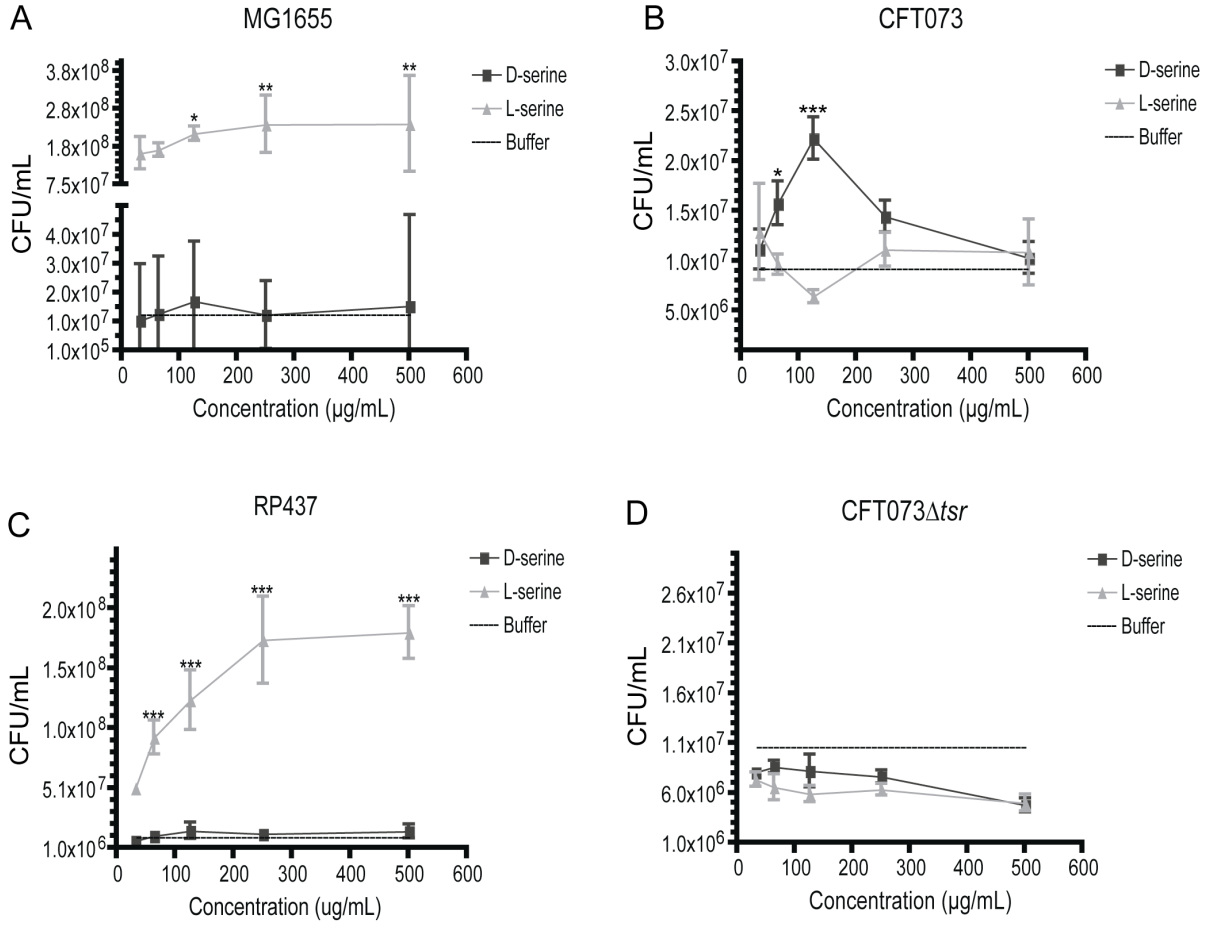

Figure 1: Chemotaxis of CFT073 and MG1655 toward D-serine and L-serine.

Capillary chemotaxis assay results for (A) CFT073, (B) MG1655, (C) RP437 and (D) CFT073Δ*tsr*. *, $p<0.05$; ***, $p<0.001$. Bars indicate standard error (s.e.m.).

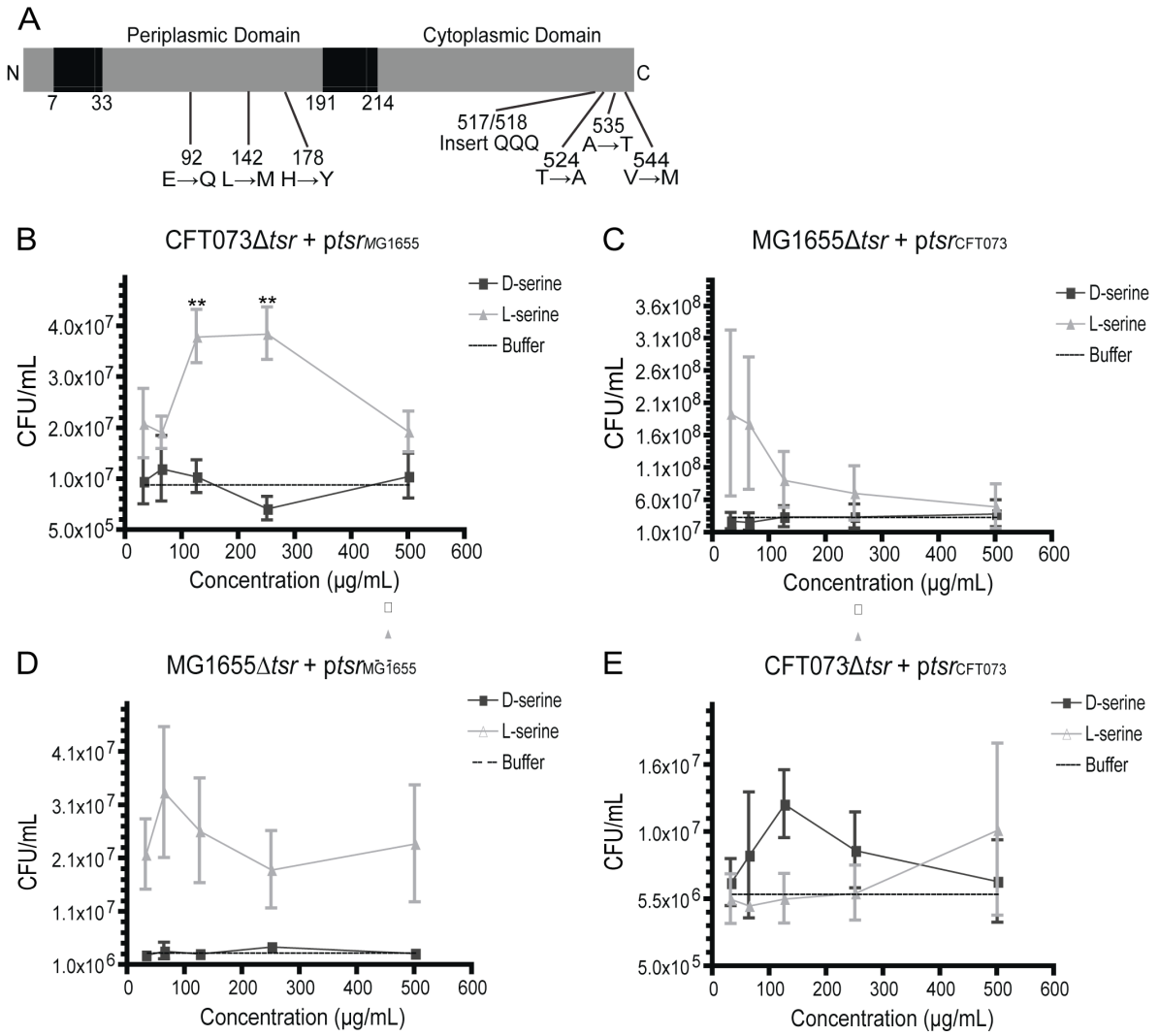

Figure 2: Chemotaxis of MG1655Δ*tsr*+p*tsr*$_{CFT073}$, CFT073Δ*tsr*+p*tsr*$_{MG1655}$, MG1655Δ*tsr*+ p*tsr*$_{MG1655}$ and CFT073Δ*tsr*+p*tsr*$_{CFT073}$ toward D-serine and L-serine. (A) Amino acid sequence differences between MG1655 Tsr (left) and CFT073 Tsr (right). Capillary chemotaxis assay results for (B) CFT073Δ*tsr*+p*tsr*$_{MG1655}$, (C) MG1655Δ*tsr*+p*tsr*$_{CFT073}$, (D) MG1655Δ*tsr*+p*tsr*$_{MG1655}$ and (E) CFT073Δ*tsr*+p*tsr*$_{CFT073}$. The *tsr* alleles were cloned into pACYC177 under the control of their native promoters.

The result shown for MG1655Δ*tsr*+Tn7::tsr_{CFT073} represents a single experiment. *, p<0.05; **, p<0.01; ***, p<0.001. Bars indicate standard error (s.e.m.).

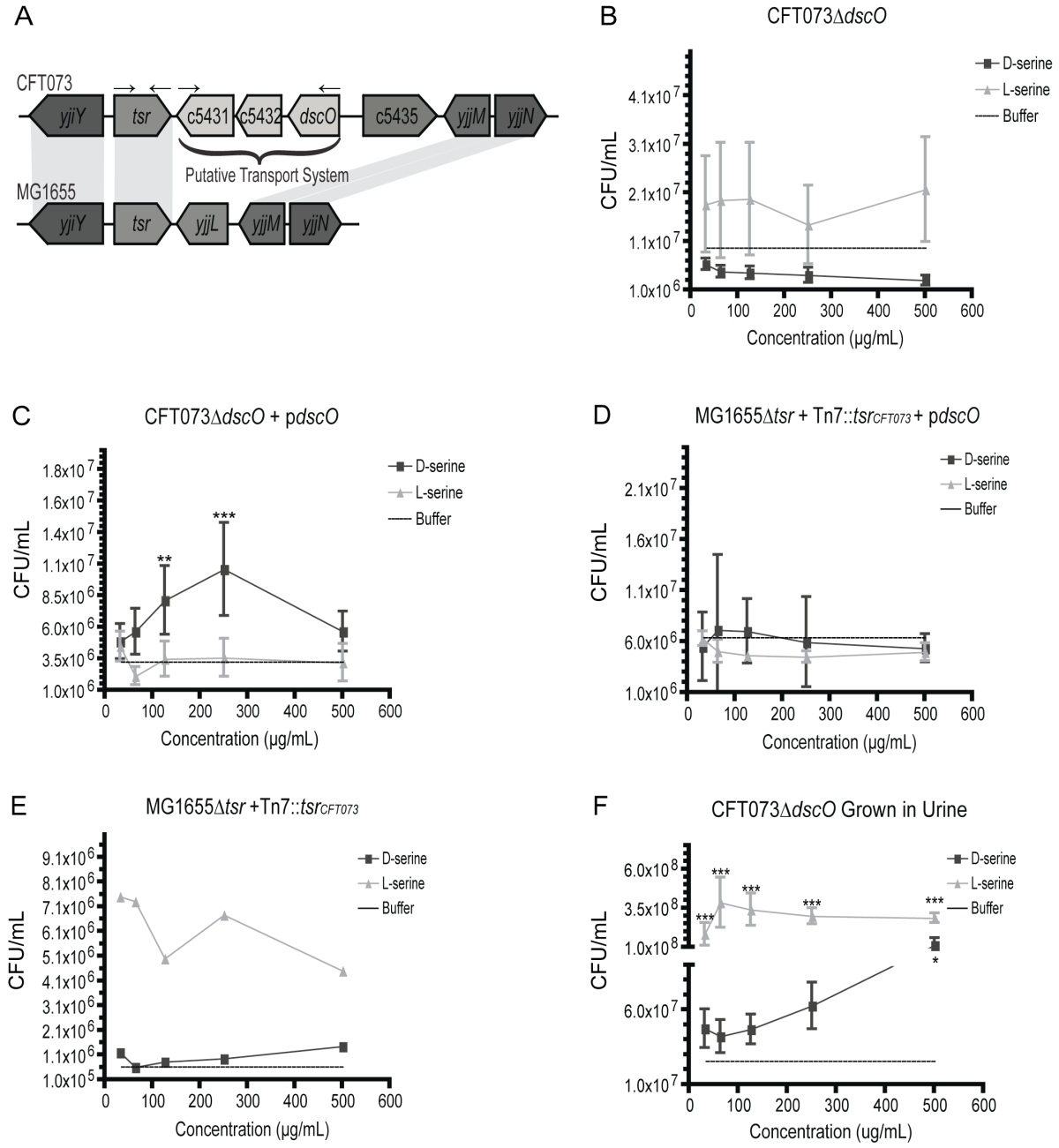

Figure 3: Effect of *dscO* on chemotaxis toward D-serine and L-serine. (A) Genomic comparison of the region downstream of *tsr* in MG1655 and CFT073. Arrows

above genes show the placement of primers for the PCR screen reported in Table 1. Capillary chemotaxis assay results for (B) CFT073Δ*dscO*, (C) CFT073Δ*dscO*+pBAD24*dscO*, (D) MG1655Δ*tsr att*::Tn7-tsr_{CFT073}+pBAD24*dscO* (E) MG1655Δ*tsr att*::Tn7-tsr_{CFT073} and (F) CFT073Δ*dscO* grown in rich medium. Expression of *dscO* for the complementing strain was induced with 10μM arabinose and all strains were grown in MOPS minimal media unless otherwise noted. *, $p<0.05$; **, $p<0.01$. Bars indicate standard error (s.e.m.).

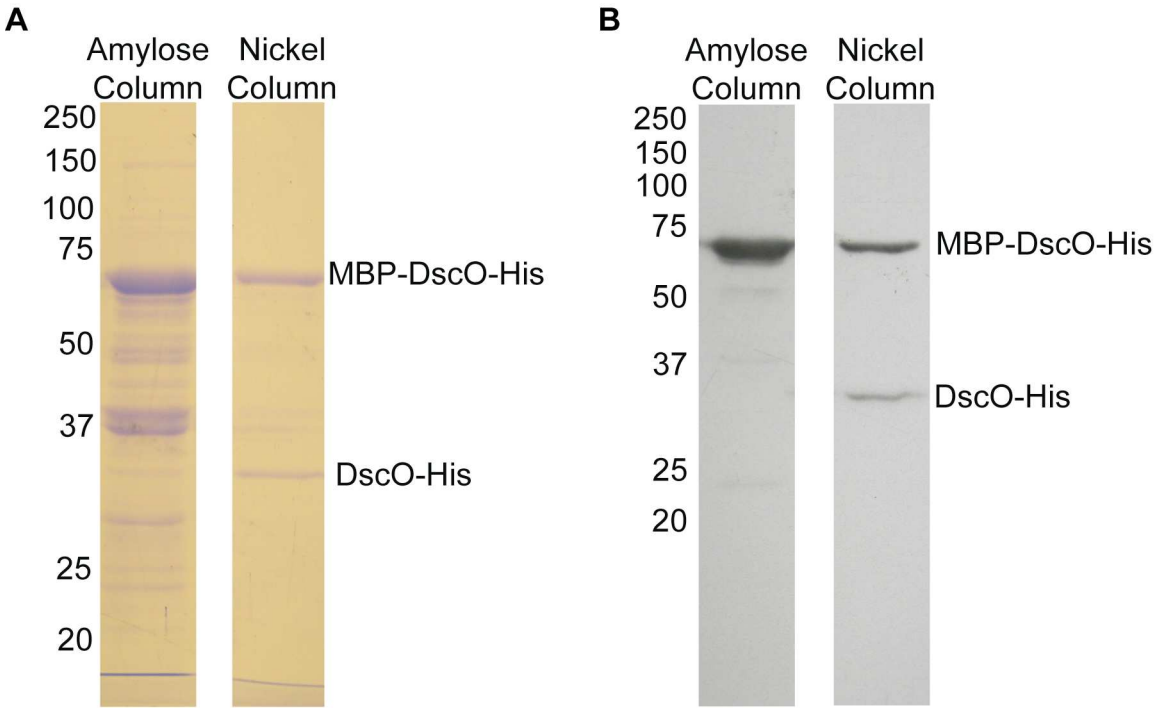

Figure 4: Attempted purification of MBP-DscO. (A) Coomassie-stained SDS-PAGE gel of purified protein from amylose and nickel columns. (B) Immunoblot of purified protein (~1mg total purified) from amylose and nickel columns using α-penta His tag antibodies.

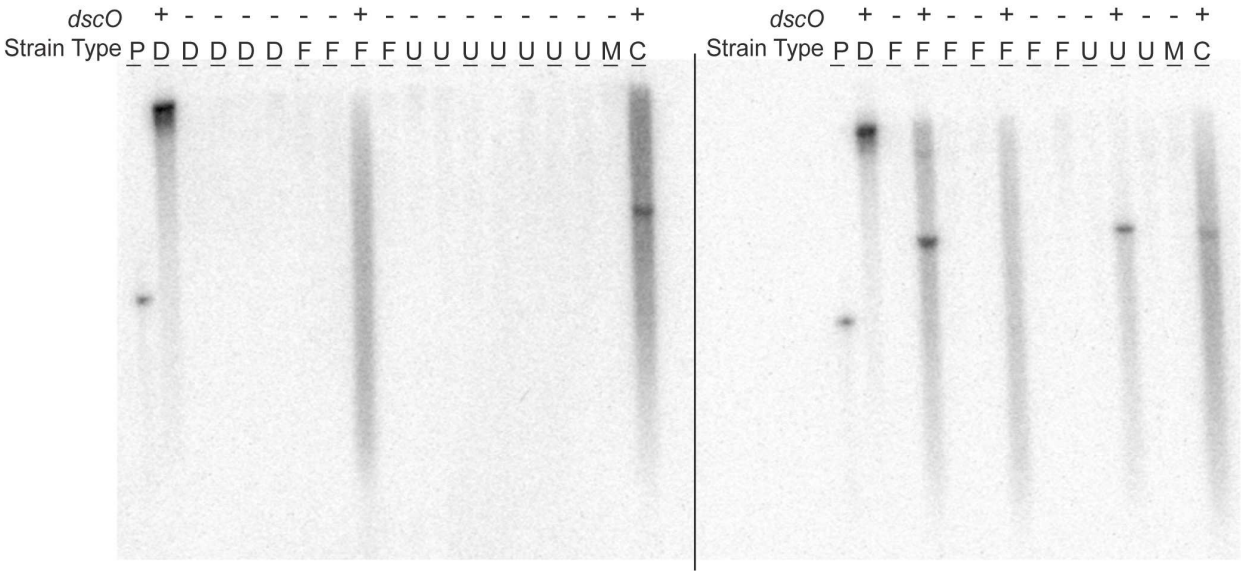

Figure 5: Southern blot analysis of strains tested in the *dscO* PCR screen. Blots were probed with the *dscO* region of the CFT073 genome, including the entire putative transport system. Each lane contains the digested genomic DNA of an individual *E. coli* isolate. Results of the PCR screen for *dscO* and the strain origin are indicated above each lane. PCR positive strains were included for comparison. A representative set of strains was used for the DEC isolates. P=probe, D=DEC strain, F=fecal strain, U=UTI strain, M=MG1655, and C=CFT073.

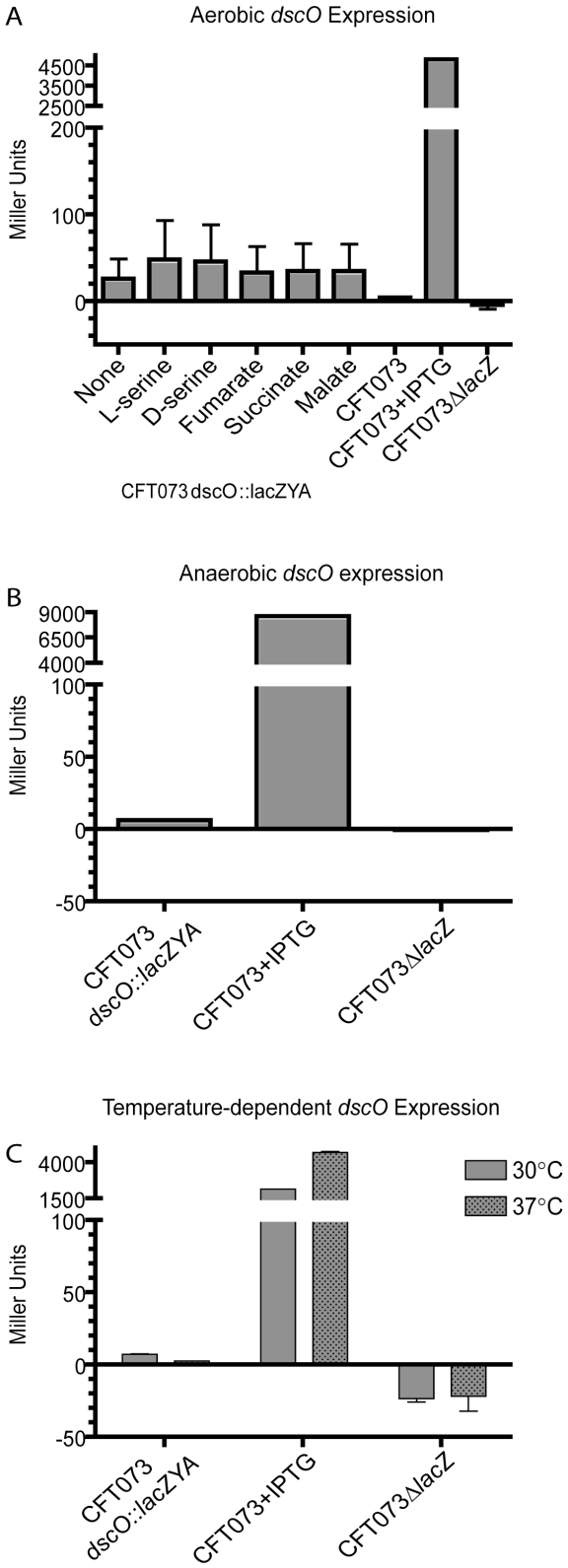
A
Aerobic dscO Expression
4500
3500
2500
200
100
0
Miller Units
None
L-serine
D-serine
Fumarate
Succinate
Malate
CFT073
CFT073+IPTG
CFT073ΔlacZ
CFT073 dscO::lacZYA
B
Anaerobic dscO expression
9000
6500
4000
100
50
0
-50
Miller Units
CFT073 dscO::lacZYA
CFT073+IPTG
CFT073ΔlacZ
Temperature-dependent dscO Expression
C
4000
1500
100
50
0
-50
Miller Units
30°C
37°C
CFT073 dscO::lacZYA
CFT073+IPTG
CFT073ΔlacZ

Figure 6: Expression of *dscO* under varying conditions. ß-galactosidase assay results for *dscO* expression under (A) aerobic (n=3), (B) anaerobic (n=3), (C) and different temperatures (n=1). Bars indicate standard error (s.e.m.).

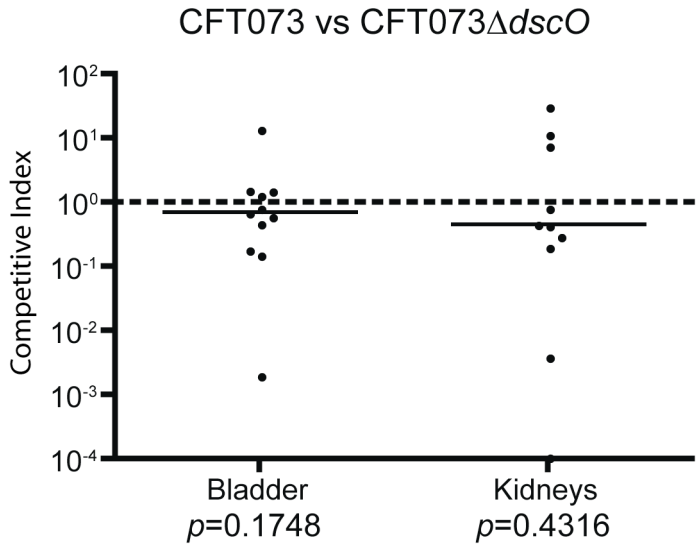

Figure 7: Coinfection of female Swiss Webster mice with wild-type CFT073 and CFT073Δ*dscO*. The competitive indices were calculated by the following equation: (*yfi* mutant CFU recovered/wild-type CFU recovered)/(mutant inoculum CFU/wild-type inoculum CFU). The line indicates the median value.

Appendix B

Reflux in the Mouse Model of Urinary Tract Infection

In preparation for publication

Authors and contributions:

Erica L. Raterman: Wrote manuscript and performed all experiments.

Rodney A. Welch: Supervised all work and helped in writing of the manuscript

Abstract

The mouse model of urinary tract infection has endured controversy over whether it is an accurate model for ascending urinary tract infections. This report provides additional evidence that the kidneys are infected during inoculation via reflux, making this model suitable only for reliable prediction of persistence but not ascension factors.

The mouse model of urinary tract infection was first developed in 1983 by Hagberg *et al.* (1). Over the following two decades, several reports emerged showing the immediate infection of the kidneys, presumably due to reflux up the ureters as a result of the pressure administered during inoculation. Although the model was still deemed suitable for the determination of virulence factors such as fimbriae for attachment to epithelial cells, the model did not constitute a true model of ascending urinary tract infection due to this immediate infection of the entire urinary tract. Subsequently, several reports gave different inoculation volumes (10 µl, 25 µl, and 50 µl) that purportedly would cause limited to no immediate reflux into the kidneys, thus allowing assessment of factors needed for ascension from the bladder to the kidneys (2-5). However, these reports disagreed on both the volume of inoculation that would produce reflux and the method of inoculation. One report alleged that administration of a 50 µl inoculum by a Harvard pump to control flow rate would limit reflux (5, 6), yet a second report found that, even when using the Harvard pump, the inoculum volume had to be reduced to 25 µl to avoid reflux (3). A third report found that the inoculum had to be reduced to 10 µl to avoid reflux when administering the inoculum by hand, and had to be placed in the urethra, not the bladder (2). Other factors such as mouse strain, mouse weight, and euthanasia method also were also noted as variables that could affect reflux during and subsequent to inoculation (3).

With no clear consensus on which method would reliably prevent reflux, we sought to determine whether a small volume of only 2 µl (~2 x 10^7 CFU) administered

into the urethra instead of the bladder of 6-7 week old female mice could be used to simulate an ascending urinary tract infection. Attempts were made to place the inoculum in the urethra distal to the urethral sphincter; however, the infection did not progress to the bladder and kidneys even after two weeks of monitoring. Therefore, the 2 μl inoculum was instead placed proximal to the sphincter, resulting in direct inoculation of the bladder. Immediately after inoculation, mice were euthanized by CO_2 asphyxiation and the spleen, kidneys, and bladder were aspectically harvested in that order. Organs were homogenized and dilution plated on MacConkey's agar for incubation overnight at 37ºC. This procedure was used with three different mouse strains (C3H/HeJ (Jackson Labs), Swiss Webster (Harlan), and CBA/J (Harlan)), and results showed that bacteria could be detected in the bladder and kidneys of all three strains, although fewer bacteria were present in mice inoculated with 50 μl (~$5x10^7$ CFU) of inoculum (Fig. 1 A-C). All mice with bacteria present in the spleen were removed from the analysis and the peritonea of all mice were sterilized before extraction of the organs to ensure that no contaminating bacteria were present. Because bacteria in the bloodstream may not always be detected in the spleen, two other experiments were performed to rule out the possibility that bacteria had gained access to the bloodstream during the trauma of inoculation, and thereby reached the kidneys via a venous route instead of by reflux up the ureters. Firstly, the mice were asphyxiated prior to inoculation to remove blood flow as a possible carrier of the bacteria to the kidneys. One ureter was then ligated in two places by tying a knot with silk sutures to differentiate between bacteria in the kidney acquired through reflux or through some

other means. As shown in Figure 2A, inoculation post-mortem did eliminate bacteria in the kidneys with the 2 μl inoculum, but not with the 50 μl inoculum in the non-ligated kidney. Finally, live mice with one ureter surgically ligated in two places were purchased from Jackson Labs (USA) and inoculated with either 2 μl or 50 μl of inoculum. Bacteria were found only in the non-ligated kidney (Fig. B) of the surgically altered mice. Together these results indicate that reflux via the ureters is indeed the route that the bacteria gain access to the kidneys at the time of inoculation.

Our data indicate that as little as 2 μl of a bacteria inoculum is sufficient to reach the kidneys during inoculation. We also, for the first time, have shown that the bacteria reach the kidneys via reflux up the ureters, as was previously assumed. Although bacteria are not found in the kidneys in all animals tested, enough of the mice had bacteria in the kidneys to make this method an unreliable model for assessing bacterial factors needed for ascension of the urinary tract during infection.

Acknowledgements

This research was funded by the National Institutes of Health Grant R01 DK063250-06 and by the National Institutes of Health, National Research Service Award AI55397.

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alpha Gal(1-4) beta Gal binding in virulence of a wild-type strain. Mol. Microbiol. **10:**143-155.

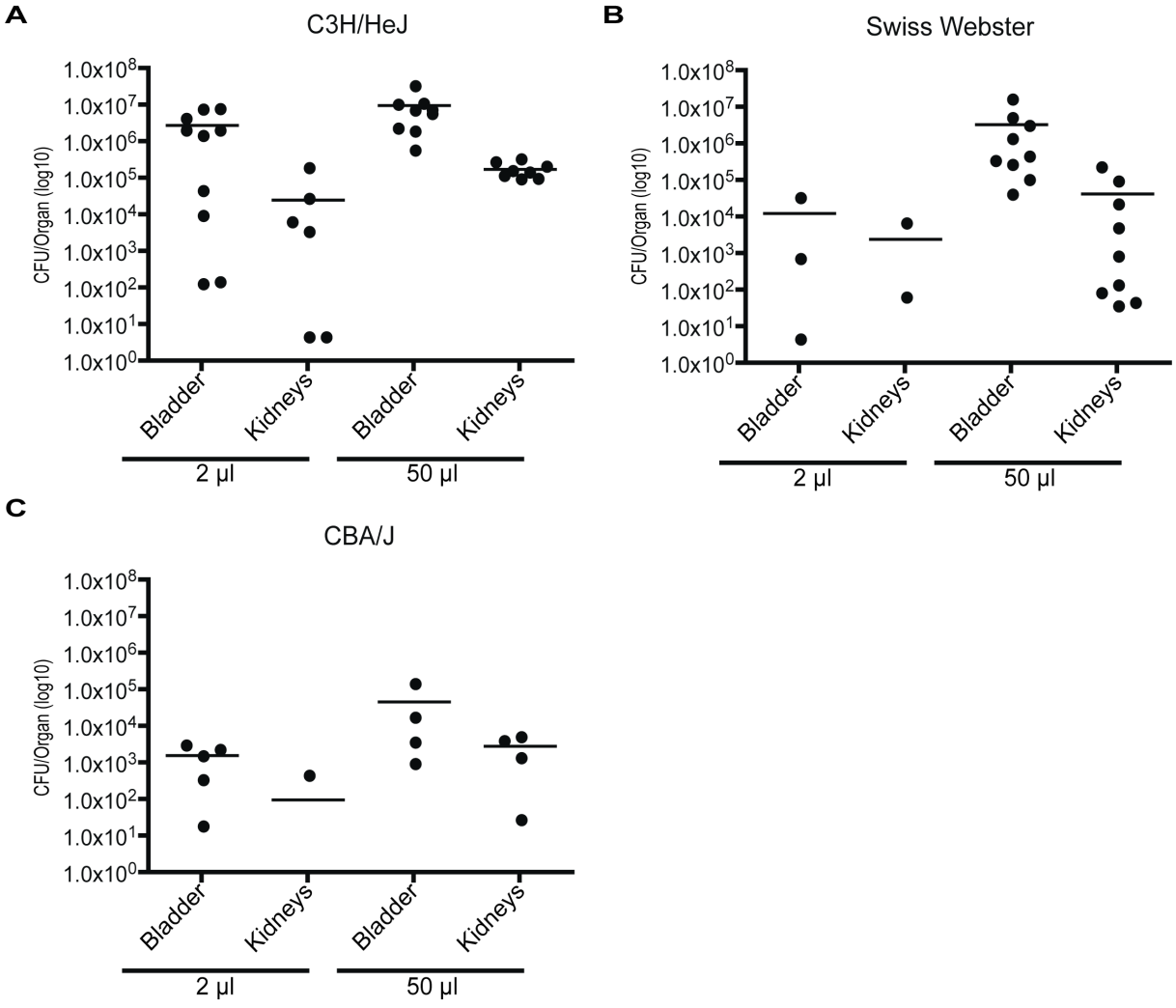

Figure 1: Colony counts in the bladder and kidneys 5 minutes after inoculation. Results for the CFUs found in each organ in C3H/HeJ mice (n=10, n=9) (A), Swiss Webster mice (n=3, n=9) (B), and CBA/J mice (n=5, n=4) (C) for both 2 µl and 50 µl inoculation volumes are shown. The line indicates the median value.

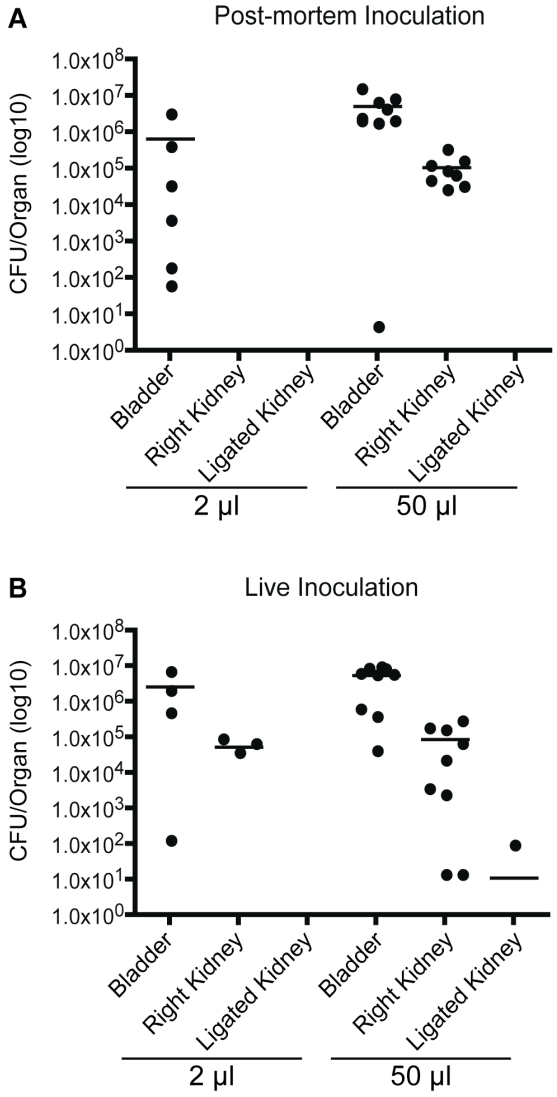

Figure 2: Surgical ligation of the left ureter blocks reflux into the kidneys. Bladder and kidney CFUs recovered 5 minutes after inoculation for mice infected post-mortem (A) and live (B) are shown. The line indicates the median value.

Appendix C

In vitro and *in vivo* phenotypes for swim and swarm mutants not used in manuscripts

Appendix C summarizes data obtained for other identified swimming or swarming mutants that will not be published.

One of the original projects of this thesis sought to determine whether swimming motility and/or swarming motility was important for colonization and persistence in the urinary tract. To differentiate between the two motility types *in vivo*, a series of mutants that could either swim but not swarm, or could swarm but not swim, were sought. Two screens for motility performed in *E. coli* K-12 identified a number of mutants that were defective for either swimming motility or swarming motility (3, 5). Other papers studying *Salmonella* motility also identified promising motility mutants (1, 7). A subset of these mutants was selected for study in CFT073 motility based on their predicted phenotypes of swim$^+$/swarm$^-$ or swim$^-$/swarm$^+$. Care was also taken to select mutants that would not interfere with core metabolic processes. The final set of genes selected to be deleted from the CFT073 genome included *yhjH* (predicted phosphodiesterase), *yehP* (unknown function), *fliR* (flagellar biosynthesis protein), *fliL*(flagellar basal body-associated protein), *flhE* (flagellar protein), and *yojN* (previously called *rcsD* in *E. coli* K-12) (predicted sensor-like histidine kinase) (primers for deletion of each gene via λ-Red are listed in Table 1). The expected motility phenotypes from the previous reports and the experimentally derived motility phenotypes in CFT073 are summarized in Table 2. While mutants that were defective for swarming motility but not swimming motility were

found, no mutants resulted in a non-swimmer that could still swarm (Fig. 1). Swarming motility phenotype was confirmed by microscopic examination of the swarm front movement. As a result, the project overall was not pursued. However, a few mutants were selected for testing in the competitive mouse model of urinary tract infection. The *in vivo* results for the *yojN*, *yhjH*, and *flhE* deletion mutants in competition with the wild-type CFT073 strain are shown in Figure 2. The *yhjH* (Fig. 2C) and *flhE* (Fig. 2B) mutants both colonized as well as the wild-type strain, although the *yhjH* did yield a slightly attenuated phenotype in the bladder that was statistically significant. The *yojN* mutant was also attenuated in the bladder and kidneys (Fig. 2A). However, this phenotype is likely due to more than just a defect in motility as YojN is part of the Rcs signaling system whose regulon has multiple effects on bacterial virulence factor expression (2, 4, 6). Despite the complexity involved, further study with this mutant could yield potential insights into how *E. coli* CFT073 regulates expression of its virulence and colonization factors in response to the urinary tract environment.

Materials and Methods

Construction of mutant strains

All primers used for the generation of deletion mutants and cloning are listed in Table 2. All nonpolar gene deletions were performed using the λ-Red recombination system developed by Datsenko and Wanner . The PCR products used for transformations in CFT073 were generated off of pKD3 or pKD4 templates using primers specific for the targeted gene. The kanamycin or chloramphenicol-marked

gene deletions were then transduced into a fresh CFT073 background using the EB49 transducing phage. The antibiotic resistance cassette used to replace the target gene was removed using a Flp recombinase encoded on pCP20. All gene deletions were verified by PCR and loss of antibiotic resistance on LB agar containing the appropriate antibiotic.

Murine model of UTI

Colonization of the urinary tract was determined using the competitive murine model of urinary tract infection as described previously. CFT073Δ*lacZYA* was used as the wild-type strain and the chemotaxis mutants had an intact *lacZYA* locus. To select for piliated bacteria, all bacterial strains were grown statically in 3 mL LB at 37˚C for 2 days. The pellicle formed on the rim of the test tube was then transferred to fresh LB, incubated for 2 more days, and finally passaged again to 40 mL LB for a final 2 day incubation. The broths were adjusted to OD_{600}~0.4 with 1XPBS and the wild-type strain and the mutant strain were mixed equally. The mixed broth was then pelleted and washed 1X in 1XPBS and resuspended in 500 µl 1XPBS. Isofluorane-anesthetized 6-7 week-old female Swiss Webster mice (Harlan, USA) were inoculated via urethral catheterization with 50 µl (~10^8 CFU) of the mixed bacterial suspension. Mice were euthanized via CO_2 asphyxiation and the bladder and kidneys were aseptically harvested in 1XPBS. The organs were homogenized, serially diluted in 1XPBS, and plated on MacConkey agar medium (Fisher, USA). Surviving strain ratios were determined by counting white (wild-type) and red (mutant) colonies. Colonization levels

were graphed and analyzed using a paired Wilcoxon signed ranked test and Prism (GraphPad). Competitive indices were calculated by dividing mutant CFUs by wild-type CFUs from the mice and then dividing this ratio by the ratio of mutant to wild-type in the original inoculum. All animal experiments were approved by the UW-Madison Animal Care and Use Committee.

Motility Assays

Swimming motility was assessed in 0.3% agar tryptone plates. Strains were grown overnight at 37°C in tryptone broth. Broths were adjusted to OD600~0.1 with fresh tryptone broth before 2 μl was injected in the center of the agar swim plates. 10 μl of the same broths were placed on the surface of swarm agar plates to assess swarming motility. Swim plates were incubated 12 hours at 30°C, and swarm plates were incubated at 30°C for 2 days. Assays were independently repeated at least three times.

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Table 1: Primers used in this study

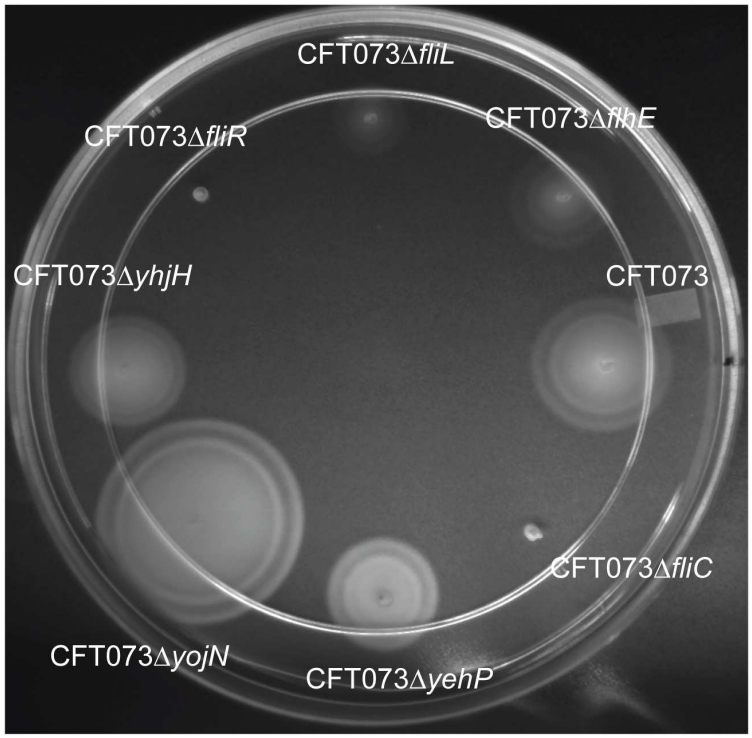
Gene/Application	Sequence
flhE deletion	5’-CATATCCGTATGACGGCGACGATTGGCGGCAAATAATG TGTGTAGGCTGGAGCTGCTTC-3’
	5’-CCGATGTTATTGCAGACATCGGCTTTTTTATCATTACAT ATGAATATCCTCCTTAG-3’
flhE deletion ck	5’-CACGCCGCATGTGATGTG-3’
	5’-CGATACACCGCTGGAACG-3’
yojN deletion	5’-ATCACTTCGCGAAGTTTTAACAGGTCATAAACACGAAT GTGTGTAGGCTGGAGCTGCTTC-3’
	5’-GCTACAGCAAGCTCTTGACATAACTGTCAATGTCGCTG ATCATATGAATATCCTCCTTAG-3’
yojN deletion ck	5’-CTGCTACCAGCAGAGCTGG-3’
	5’-GAGAGCCTTCGGCAAATCGG-3’
yehP deletion	5’-CATCGCTGCAACACTGGGGAGTTTTCCATGTCTGAACT GTGTGTAGGCTGGAGCTGCTTC-3’
	5’-TTCCGGACGTAGTGAATTCATGACTGAAGATTCTCCGC AACATATGAATATCCTCCTTAG-3’
yehP deletion ck	5’-CCATCAACTGGCCTGTCAGC-3’
	5’-GCAGGTGCACTGAGCCTC-3’
yhjH deletion	5’-TACACCTGGCAGCCGATCTATCAAACATGCGGGCGGT TATGTGTAGGCTGGAGCTGCTTC-3’
	5’-CCTAAAGATAGTCCAGCCAGGCGGAAAATGAGGCAGC TTACATATGAATATCCTCCTTAG-3’
yhjH deletion ck	5’-GCGAACGGACGATTCAACTCC-3’
	5’-GAAACCGCGCTTAACGTCC-3’
fliR deletion	5’-CACTAACCTGCCGTATATCATCGGGTAGCCCGTACAAT GTGTGTAGGCTGGAGCTGCTTC-3’
	5’-GGATAATCCTTAGGGTAGCATGATAAACGTTACGGAAT TACATATGAATATCCTCCTTAG-3’
fliR deletion ck	5’-GATGAAAGTCGCGCTGGC-3’
	5’-CACCTAATGCGCCACTCTCG-3’
fliL deletion	5’-CGATAAGCAGTAGCGACACAGGAAGACCGCAACACAT GTGTGTAGGCTGGAGCTGCTTCG-3’
	5’-TTCAGCTTGAGAAAGAATACTATCGCCCATGTCGTTAA TGAATATCCTCCTTAG-3’
fliL deletion ck	5’-GCGGCGTTGATATTTTCGCC-3’
	5’-CAGCGCCTGCAAACGTTC-3’
fliC deletion	5’-GGCGTTGCCGTCAGTCTCAGTTAATCAGGTTACGGCGA TGTGTAGGCTGGAGCTGCTTC-3’
	5’-AAAACGTAATCAACGACTTGCAATATAGGATAACGAATC CATATGAATATCCTCCTTAG-3’

fliC deletion ck	5’-CGCAATGGGTCTGGCTGTG-3’ 5’-GAGTGATGGTCAGCGTCTGGTTGC-3’

Table 2: Expected and experimental motility phenotypes of mutant strains

Strain	**Expected Result**		**Experimental Result**	
	Swim	Swarm	Swim	Swarm
CFT073	++	++	++	++
CFT073Δ*flhE*	++	-	+	-
CFT073Δ*yhjH*	+	++	++	-
CFT073Δ*yojN*	+	++	+++	+++
CFT073Δ*fliR*	++	-	-	-
CFT073Δ*fliL*	+	-	++	+
CFT073Δ*yehP*	++	-	++	++

A


CFT073ΔfliL
CFT073ΔfliR
CFT073ΔflhE
CFT073ΔyhjH
CFT073
CFT073ΔfliC
CFT073ΔyojN
CFT073ΔyehP

B

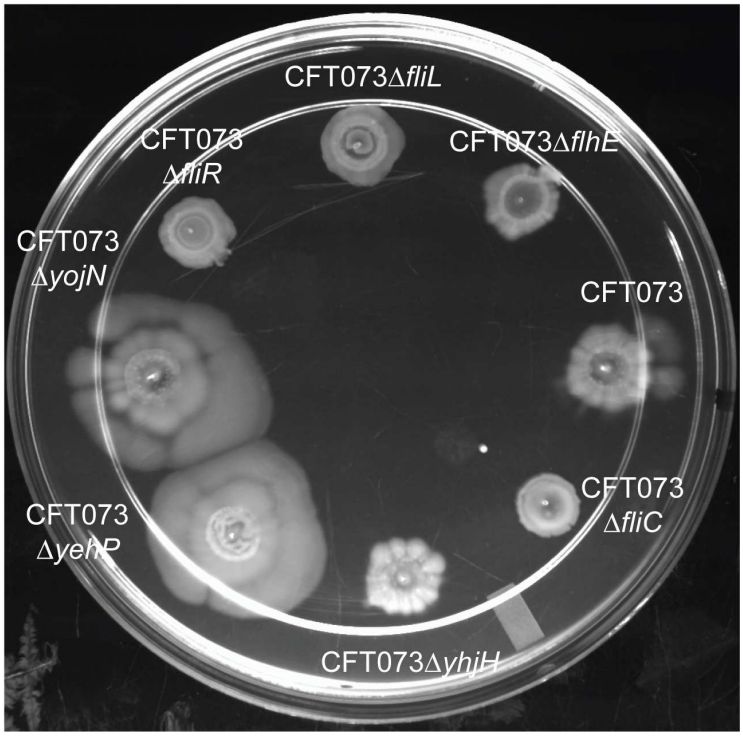

CFT073ΔfliL
CFT073 ΔfliR
CFT073ΔflhE
CFT073 ΔyojN
CFT073
CFT073 ΔfliC
CFT073 ΔyehP
CFT073ΔyhjH

Figure 1: Swim and swarm phenotypes of predicted motility mutants in *E. coli* CFT073. Strains were inoculated either in 0.3% tryptone agar swim plates (A) or on 0.45% agar swarm plates (B) and incubated at 30° C for either 12 hours or 2 days, respectively. The wild-type CFT073 strain is shown on the left and the motility mutant is shown on the right for the swarm plates.

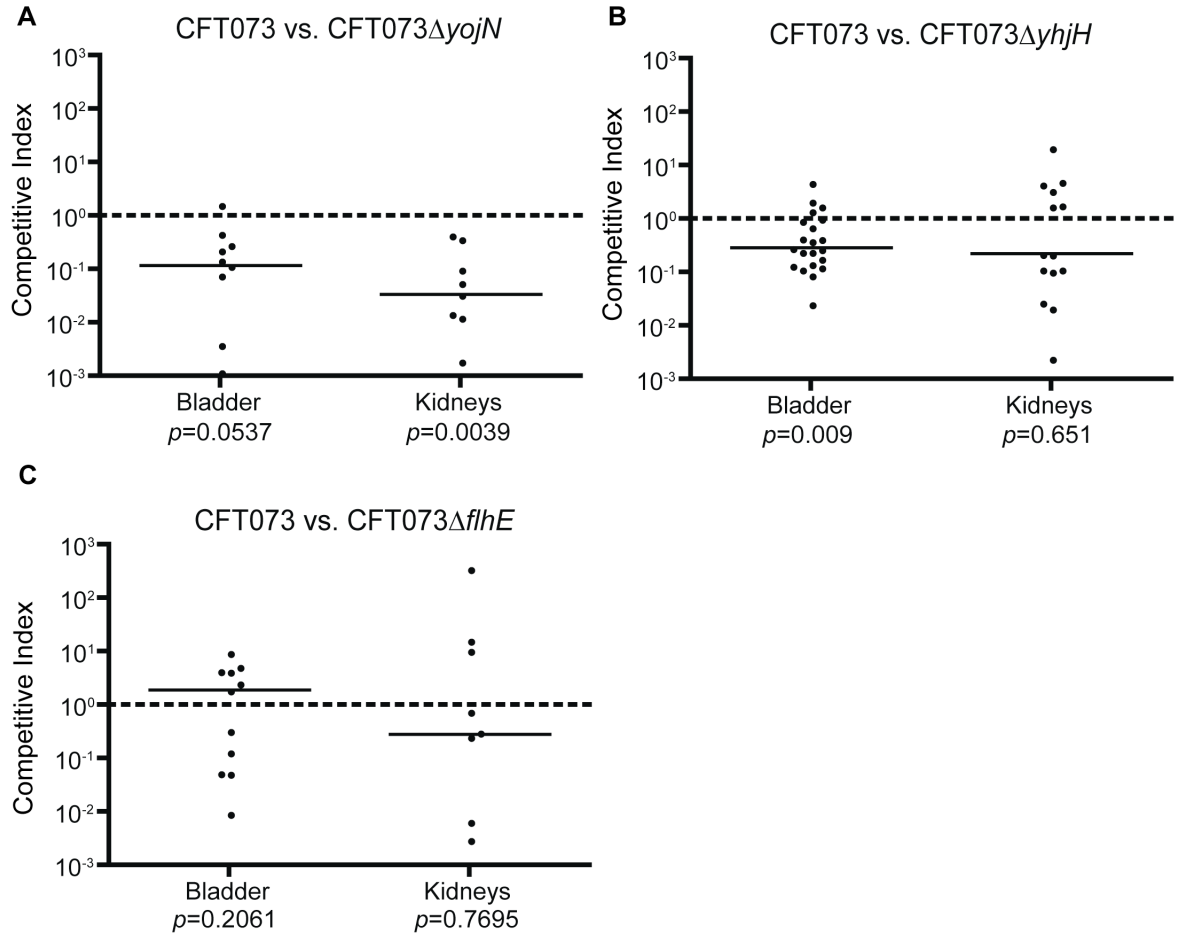


Figure 2: Coinfection of female Swiss Webster mice with wild-type CFT073 and the motility mutants. Results for the CFT073Δ*yojN* (n=12) (A), CFT073Δ*yhjH* (n=21) (B), and CFT073Δ*flhE* (n=13) (C) are shown. The competitive indices were calculated by the following equation: (*yfi* mutant CFU recovered/wild-type CFU recovered)/(mutant inoculum CFU/wild-type inoculum CFU). The line indicates the median value.

Appendix D

Atypical *Shigella boydii* 13 encodes virulence factors seen in attaching and effacing *Escherichia coli*

Published in *FEMS Microbiology Letters* (2012) 328: 20-5.

Authors and contributions:

Laura L. Walters: Wrote manuscript and performed all experiments except as noted below.

Erica L. Raterman: Revised manuscript and performed some of the PCR and southern screens for *stcE*.

Thomas E. Grys: Initially developed project.

Rodney A. Welch: Supervised all work and contributed to the writing of the manuscript.

Abstract

Enterohemorrhagic *E. coli* (EHEC) is a foodborne pathogen that causes watery diarrhea and hemorrhagic colitis. In this study, we identified StcE, a secreted zinc metalloprotease that contributes to intimate adherence of EHEC to host cells, in culture supernatants of atypical *Shigella boydii* 13 (*Shigella* B13) strains. Further examination of the *Shigella* B13 strains revealed that this cluster of pathogens does not invade but forms pedestals on HEp-2 cells similar to EHEC and enteropathogenic *E. coli*. This study also demonstrates that atypical *Shigella* B13 strains are more closely related to attaching and effacing *E. coli* and that their evolution recapitulates the progression from ancestral *E. coli* to EHEC.

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) cause diarrheal disease that ranges from watery diarrhea to hemorrhagic colitis. Virulence factors of EHEC include the chromosomally-encoded Shiga toxin and the locus of enterocyte effacement (LEE). LEE is a 35 kb pathogenicity island that confers the attaching and effacing phenotype to both EHEC and enteropathogenic *E. coli* (EPEC), wherein intimate adherence of the bacteria to host cells induces formation of actin-rich pedestals beneath the bacteria. The majority of the clinical EHEC disease in United States is caused by serotype O157:H7 (Manning *et al*., 2007), which carries a 92 kb virulence plasmid, pO157, that encodes many potential virulence factors, including *stcE* (Burland *et al*., 1998).

The *stcE* gene is encoded on the large virulence plasmids of *E. coli* O157:H7, O157:H-, ON:H7, and O55:H7 (Lathem *et al*., 2003). In all cases, *stcE* is found linked to *etpD*, which encodes the subunit of the type II secretion apparatus responsible for the secretion of StcE protein (Lathem *et al*., 2002). StcE is a 96 kDa zinc metalloprotease that cleaves specific O-linked glycoproteins and contributes to the intimate adherence of *E. coli* O157:H7 to HEp-2 cell surfaces (Grys *et al*., 2005). Evidence supports a role for StcE in EHEC disease in clearing the mucus that forms a protective barrier over the colonic epithelium. Following colonization, intimate adherence, and pedestal formation by EHEC, the clinical syndrome progresses from watery diarrhea to hemorrhagic colitis. At this stage, StcE plays an anti-inflammatory role by localizing the human complement regulator, C1 esterase inhibitor (C1-INH), to cell surfaces, decreasing the complement-mediated lysis of both bacteria and host cells (Lathem *et al*., 2004; Grys *et al*., 2006).

Shigella, another enteropathogen, is indistinguishable from *E. coli* by DNA-DNA hybridization techniques, with the exception of *Shigella boydii* 13 (*Shigella* B13) (Pupo *et al*., 2000). *Shigella* B13 is more closely related to *Escherichia albertii* than the *E. coli-Shigella* group, and lacks the large virulence plasmid, (pINV), that confers the invasion phenotype in all other *Shigella*. Hyma et al. demonstrated that *Shigella* B13 and *E. albertii* strains carry *eae*, a marker for LEE (Hyma *et al*., 2005). A small subset of analyzed *Shigella* B13 strains encoding *eae* were more related to the *E. coli-Shigella* group and labeled atypical *Shigella* B13. Many of these strains also carried markers for the pO157 plasmid, such as *ehxA* and *toxB*, suggesting that atypical *Shigella* B13 may be similar to EHEC and, thus, may encode *stcE*. This study describes the identification of *stcE* in atypical *Shigella* B13 strains and the genetic and phenotypic profile of this unique cluster of *Shigella*.

Materials and Methods

Bacterial culture, DNA extraction and PCR amplification

The *S. boydii* 7 and 13 and *E. albertii* strains used in this study are listed in table 2 and were provided by Thomas Whittam. *E. coli* O157:H7 EDL933 and *E. coli* O127:H6 E2348/69 were provided by Alison O'Brien. *E. coli* K12 MG1655 and *S. flexneri* 5a M90T were provided from Fred Blattner. Internal fragments of *Shigella* (Venkatesan *et al*., 2001) and *E. coli* (Burland *et al*., 1998) genes were amplified using the primers shown in table 1. Strains stored at –80°C in Luria-Bertani (LB) medium with 50% glycerol were directly inoculated into PCR reactions with GoTaq polymerase (Promega).

The *stcE* gene was sequenced from PCR products amplified with primers IR ApaI 5' 1 and *etpD* 3' 1803 (table 1) and TripleMaster polymerase (Eppendorf) from plasmid DNA extracted from the atypical *Shigella* B13 strains using a Maxi Prep Kit (Qiagen). The nucleotide sequence for the *stcE* gene from the atypical *Shigella* B13 strains 3556-77, 3557-77, 3052-94, and 3053-94 have been submitted to GenBank under accession numbers EU159265, EU159266, EU159267, EU159268, respectively. For Southern blot analysis, plasmid DNA isolated from the atypical *Shigella* B13 strains was electrophoresed on a 0.6% agarose gel. Gel and *stcE* probe preparation and hybridization were performed as previously described (Lathem *et al*., 2003).

StcE activity assay

To examine secretion of StcE, strains were grown in 25 ml Lennox L broth overnight at 37°C with aeration and cells removed by centrifugation. Three mls of culture supernatant were precipitated with 10% trichloroacetic acid on ice for ≥1 hour. To measure StcE activity, 12 ml of culture supernatant were incubated with 0.5 μg C1-INH protein (CompTech) overnight at room temperature prior to TCA precipitation. Precipitated protein was separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with polyclonal anti-rStcE' antisera (Grys *et al*., 2005) or anti-C1-INH IgG (Cedarlane Laboratories).

Invasion assay

The gentamicin protection assay was used to determine the invasion phenotypes of the atypical *Shigella* B13 strains (Elsinghorst *et al*., 1994). A colony of each strain grown overnight on LB agar was inoculated into 2 ml of LB broth and incubated statically overnight at 37°C. Overnight culture (40 μl) was diluted into a total volume of 1 ml of HEp-2 media (EMEM, 1 mM sodium pyruvate, 10% FBS) prior to the addition to a monolayer of HEp-2 cells in a 24-well tissue culture plate (MOI of 14-95) and incubated at 37°C in 5% CO_2 for 2 hours. Monolayers were washed with Dulbecco's PBS (D-PBS) and fresh media containing 100 μg/ml gentamicin added for an additional 2 hours. The monolayers were washed with D-PBS and lysed with 1 ml 0.1% Triton X-100 per well. Suspensions were serially diluted and plated onto LB agar. Results are presented as the average percent of inoculum recovered after gentamicin treatment and are representative of duplicate samples in three independent experiments. Statistical analysis was preformed using a one-way ANOVA with a Tukey's post hoc test.

Pedestal formation assay

To determine the ability of atypical *Shigella* B13 strains to form pedestals, HEp-2 cells were seeded onto 8-well microscope slides (Nalge Nunc International) 48 hours prior to infection so that cells would reach 50-80% confluency. Overnight bacterial cultures (10 μl of $2.5x10^8$ to $9.0x10^8$ CFUs/ml) grown as for the invasion assay were diluted into a total volume of 250 μl with HEp-2 media and added to each well of washed HEp-2 cells. The mixtures were incubated at 37°C in 5% CO_2 for a total of 6-7

hours with a media exchange after 3 hours. Wells were washed with D-PBS and the cells fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton X-100. Bacterial cells were stained with 1:200 goat anti-lipid A (Abcam), followed by 1:200 anti-goat-Alexa 488 and HEp-2 cells stained with 1:100 phalloidin-Alexa 594 (Invitrogen). Preparations were mounted with Prolong Gold (Invitrogen) and analyzed by epifluorescence microscopy (Carl Zeiss MicroImaging Inc.).

Results

PCR screen for presence of *stcE* in atypical *S. boydii* and *E. albertii* strains.

We set out to identify *stcE* in other bacterial species recently found to carry *eae*, the gene that encodes the bacterial adhesin (intimin) required for pedestal formation. A PCR screen of numerous *S. boydii* and *E. albertii* strains showed that an internal fragment of *stcE* can be PCR amplified from only a subset of the *S. boydii* strains known as atypical *S. boydii* 13 (table 2). Atypical *Shigella* B13 strains 3557-77, 3556-77, 3052-94, and 3053-94, which form a distinct phylogenetic cluster, were all positive for the *stcE* gene. Atypical *Shigella* B13 strain 5216-70, which is phylogenetically clustered with enteroinvasive *E. coli* strains, was negative for the *stcE* gene. The presence or absence of the *stcE* gene in all strains was confirmed by Southern blot (data not shown). Analysis of isolated plasmid DNA by Southern blot demonstrated that *stcE* was encoded on the large plasmid of the four atypical *Shigella* B13 strains (data not shown). Sequence analysis of the 2.7 kb *stcE* gene showed only three synonymous substitutions shared among the atypical *Shigella* B13 strains and a Q727L substitution

in strain 3556-77 compared to the EHEC EDL933 allele (data not shown). Six substitutions within 220 nucleotides of the intergenic region upstream of the predicted *stcE* promoter are present in the plasmids of all four atypical *Shigella* B13 strains compared to pO157.

Activity of StcE in atypical *S. boydii* B13 strains.

To determine if the StcE protein was expressed and secreted by the atypical *Shigella* B13 strains, TCA-precipitated supernatants of overnight cultures were analyzed by immunoblot. StcE protein was identified in supernatants from strains 3556-77, 3052-94, and 3053-94, but not from 3557-77 or 5216-70 (table 2). StcE activity in culture supernatants was assayed for C1-INH proteolysis by immunoblots, and detected with all atypical *Shigella* B13 strains except 3557-77 and 5216-70 (Fig. 1, table 2).

Atypical *Shigella* B13 strains encode other EHEC virulence factors.

To determine if the atypical *Shigella* B13 plasmid encoding *stcE* is similar to the large invasion plasmid of *Shigella* (pINV), several pINV-encoded virulence factors were sought by PCR amplification (table 2). None of the pINV-encoded virulence factors could be amplified from the atypical *Shigella* B13 strains. PCR analysis using primers specific for pO157-encoded genes resulted in amplification of *etpD*, but not *katP*. The gene, *traC*, which is an F plasmid gene that is also encoded on the large virulence plasmid of *E. coli* O157:H-, pSFO157, did not PCR amplify from any of the atypical *Shigella* B13 strains tested.

The presence of additional *E. coli*-specific chromosomally-encoded genes was determined by colony PCR (table 2). The LEE-encoded regulator (Ler) is a global virulence regulator that has been shown to positively regulate the expression of LEE (Mellies *et al*., 1999), *stcE*, and the *etp* operon in *E. coli* O157:H7 (Lathem *et al*., 2002). PCR analysis of the atypical *Shigella* B13 strains identified the *ler* gene in the four atypical *Shigella* B13 strains encoding *eae* and *stcE*. An additional LEE-encoded gene, *espA*, encodes a subunit of the type III secretion system unique to EPEC and EHEC and is encoded by the atypical *Shigella* B13 strains encoding *eae* and *stcE*. PCR analysis of *cadA*, which encodes lysine decarboxylase and is universally absent in *Shigella* but present in most *E. coli* strains (Day *et al*., 2001), revealed that none of the atypical *Shigella* B13 strains encoded *cadA*.

Some atypical *Shigella* B13 strains invade and form pedestals on HEp-2 cells like *E. coli*.

The abilities of the atypical *Shigella* B13 strains to invade HEp-2 cells were determined. Strains 3556-77 and 3557-77 showed invasion levels below the level of detection, whereas strains 3052-94 and 3053-94 showed relative levels of invasion more similar to *E. coli* than *S. flexneri* strains (Fig. 2A). The presence of the LEE operon and *stcE* suggested that the atypical *Shigella* B13 strains might form pedestals on host cells. We tested this hypothesis by infecting HEp-2 cells and observing for co-localization of bacteria with actin bundles on the surface of cells. Pedestal formation on

HEp-2 cells could be detected for atypical *Shigella* B13 strains 3556-77, 3052-94, and 3053-94, but not 3557-77 (Fig. 2B).

Discussion

In this study, we discovered the *stcE* gene in the atypical *Shigella* B13 cluster. The relatively low incidence of three nucleotide substitutions within the 2.7 kb *stcE* gene compared to the six nucleotide substitutions within 220 nucleotides of the upstream intergenic region suggests selection for the preservation of StcE function. The acquisition of the large plasmid carrying *stcE* and the *etp* operon, in combination with the LEE element encoded on the chromosome, may provide a selective advantage by increasing the level of intimate adherence to host cells. A role of StcE in intimate adherence is further supported by the observation that a lack of extracellular StcE coincides with absence of pedestal formation by strain 3557-77.

The current model of *Shigella* evolution proposes that multiple ancestral *E. coli* clones acquired the pINV *Shigella* invasion plasmid, leading to selection for the loss of traits such as motility and lysine decarboxylation (Pupo *et al*., 2000). In contrast, the atypical *Shigella* B13 strains show loss of *E. coli* traits in the apparent absence of pINV selective forces. Furthermore, strains 3556-77 and 3557-77 display metabolic phenotypes intermediate between Shigella and *E. coli*, and atypical Shigella B13 DNA is more similar to *E.coli* than other Shigella B13 strains based on DNA-DNA hybridization assays (Brenner *et al*., 1982). These atypical *Shigella* B13 strains also form a distinct phylogenetic cluster and possess intermediate chromosomal genotypes between *E. coli*

and *Shigella* groups (Hyma *et al.*, 2005). As was previously suggested by Hyma *et al.*, these data indicate that the atypical *Shigella* B13 strains were misclassified as *Shigella* and that they actually represent a lineage that evolved from ancestral forms of Shigella and attaching and effacing *E. coli*. The data presented here strengthen this argument by showing the acquisition of LEE and a pO157-like plasmid encoding *stcE* which we suggest recapitulates the model of EHEC evolution, described as the step-wise acquisition of the LEE element, followed by pO157 and then the Shiga toxin phage (Reid *et al.*, 2000). We therefore propose to reclassify the atypical Shigella B13 strains as an *E. coli* group that, through convergent evolution or horizontal transfer of virulence genes on an ancestral background that shared both *E. coli* and Shigella characteristics, has evolved to closely resemble pathotypes of *E. coli* that form attaching and effacing lesions.

Sequelogs of *stcE*, historically named *tagA*, have been functionally characterized in other pathogens, including *Vibrio cholerae* and *Aeromonas hydrophila* (Szabady *et al.*, 2010 and Pillai *et al.*, 2006). Like StcE, *V. cholerae* TagA is a secreted mucinase and contributes to colonization of the intestinal epithelium (Szabady *et al.*, 2010). The *A. hydrophilia* TagA exhibits an additional StcE function by cleaving and localizing C1-INH the surface of bacterium, increasing the serum resistance of the bacterium *in vitro*. An isogenic deletion mutant of *tagA* decreased the mortality of mice compared to wild-type *A. hydrophila* in a mouse model of peritonitis (Pillai *et al.*, 2006). Thus, StcE-like metalloproteases play a role in the virulence phenotypes of *A. hydrophila*, *V. cholerae* and *E. coli* O157:H7. In this study, we identified *stcE* as a possible virulence factor in

atypical *Shigella* B13 strains, and further characterized this unique cluster of attaching and effacing pathogens.

Acknowledgements

We would like to thank Thomas Whittam, Alison O'Brien, and Fred Blattner for bacterial strains, Nancy Strockbine for information regarding the atypical *Shigella* B13 strains, Jay Bangs for use of his epifluorescence microscope, and Rose Szabady and Becca Moritz for insightful discussions regarding the project and critical reading of the manuscript. This work was supported by NIH grant RO1 AI051735.

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Table 1. Primers used in this study.

Primer Name	**Sequence**
stcE 5’ 693	5’-CCGCTCCGGTGAACTGGAGAATA-3’
stcE 3’ 1841	5’-CCTTATCTGCGGAGGCTGTAGGG-3’
etpD SacI 5’ 1	5’-CCGAGCTCCGTGTTCACTACAGTAATTTTG-3’
etpD XbaI 3’ 1929	5’-CCTCTAGATTACATCTCCTGCGCATAAA-3’
katP 5’ 16	5’-CTTCCTGTTCTGATTCTTCTGG-3’
katP 3’ 2141	5’-AACTTATTTCTCGCATCATCC-3’
ler 5’ 38	5’-CACATACAACAAGTCCATACATTCAGC-3’
ler 3’ 378	5’-CAGCGGTATTATTTCTTCTTCAGTGTCC-3’
espA 5’ 81	5’-GTCGAAGGATGAGGTGGTTAAGCTA-3’
espA 3’ 535	5’-ATTGCACATCAGAACGTGCACTCG-3’
cadA 5’ 455	5’-ACATGGGTGGTACTGCATTCCAGA-3’
cadA 3’ 1603	5’-ACAGCAGGTTATACGGACCGGTTT-3’
ipaB 5’ 1	5’-ATGCATAATGTAAGCACCACAACC-3’
ipaB 3’ 1743	5’-TCAAGCAGTAGTTTGTTGCAAAAT-3’
ipaD 5’ 1	5’-ATGAATATAACAACTCTGACTAAT-3’
ipaD 3’ 999	5’-TCAGAAATGGAGAAAAAGTTTATC-3’
virK 5’ 188	5’-TTCTGGCAATACAACCCACGTTGC-3’
virK 3’ 915	5’-TGCATCCAAAGAGCGGATAGCAGT-3’
icsA 5’ 348	5’-AGGTCATGGTGGTGCTGGTGATAA-3’

icsA 3’ 2002	5’-CTGCAATTTCCAGCCGGTCAGTTT-3’
ipaH7.8 5’ 500	5’-ACAGGCTGACAACATTACCCGACT-3’
ipaH7.8 3’ 1624	5’-TCTGCTGTTCAGTCTCACGCATCA-3’
mxiM 5’ 27	5’-TGCTCTGCAGCAAAGATTAAATAGTGAAGA-3’
mxiM 3’ 407	5’-TACCATGTCGAATCATCTGCCTCTCTC-3’
traC 5’ 318	5’-TGGTGACAGGATTGAATACGGGCT-3’
traC 3’ 1732	5’-GCAACAGCAGACCTTCATGCACTT-3’
IR ApaI 5’ 1	5’-AAGGGCCCCTCTGAGGTGTCTGTTAAACCCGTGG-3’
etpD 3’ 1803	5’-CGACTGCACCTGTTCCTGATTA-3’

Table 2. Prevalence of *E. coli* and *Shigella* specific genes detected by PCR and secreted StcE antigen and C1 cleavage activity detected by immunoblot.

Strain[a]	Species	*E. coli* O157:H7 plasmid		*E. coli* O157:H7 chromosome		*S. flexneri* 5a plasmid		StcE Sec.	StcE Act.
		stcE/ etpD	*katP*	*ler/ espA*	*cadA*	*ipaBD/ icsA/mxiM*	*virK/ ipaH7.8*		
EDL 933	*E. coli* O157:H7	+	+	+	+	-	-	+	+
M90T	*S. flexneri* 5a	-	-	-	-	+	+	-	-
5216-70	Atypical *S. boydii* 13	-	-	-	-	-	+	-	-
3556-77	Atypical *S. boydii* 13	+	-	+	-	-	-	+	+
3557-77	Atypical *S. boydii* 13	+	-	+	-	-	-	-	-
3052-94	Atypical *S. boydii* 13	+	-	+	-	-	-	+	+
3053-94	Atypical *S. boydii* 13	+	-	+	-	-	-	+	+

[a] *stcE* was not detected in the following additional strains tested: *S. boydii* 13 3103-99, *S. boydii* 13 ATCC 12032, *S. boydii* 13 2045-54, *S. boydii* 13 K-694, *S. boydii* 7 K-1, *E. albertii* 9194, *E. albertii* 10790, *E. albertii* 10457, *E. albertii* 12502, *E. albertii* 19982.

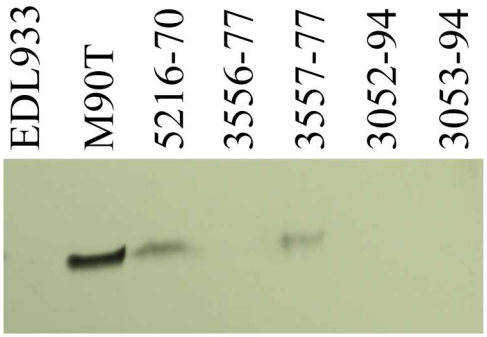

Figure 1: StcE activity of atypical *Shigella* B13 strains. Immunoblot of overnight culture supernatants of atypical *Shigella* B13 strains 5216-70, 3556-77, 3557-77, 3052-94, and 3053-94 incubated overnight at room temperature with purified C1-INH and probed with anti-C1-INH IgG. *E. coli* O157:H7 EDL933 (EHEC) and *S. flexneri* 5a M90T were used as positive and negative controls for C1-INH cleavage, respectively.

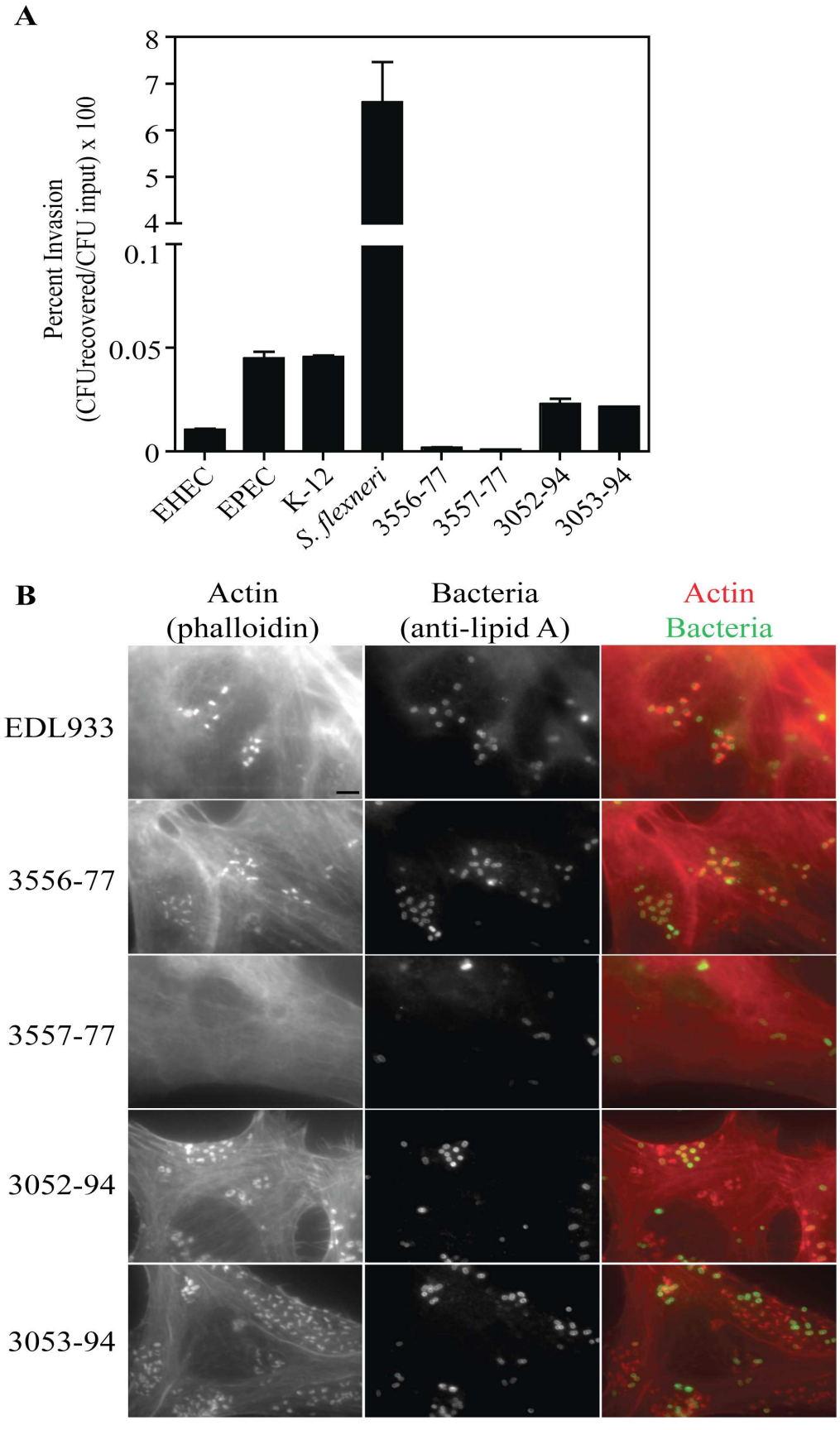
A
Percent Invasion
(CFUrecovered/CFU input) x 100
8
7
6
5
4
0.1
0.05
0
EHEC
EPEC
K-12
S. flexneri
3556-77
3557-77
3052-94
3053-94
B
Actin
(phalloidin)
Bacteria
(anti-lipid A)
Actin
Bacteria
EDL933
3556-77
3557-77
3052-94
3053-94

Figure 2: Invasion and pedestal formation of atypical *Shigella* B13 strains. *A*, Percent HEp-2 invasion by *E. coli* O157:H7 EDL933 (EHEC), *E. coli* O127:H6 E2348/69 (EPEC), *E. coli* K12 MG1655, *S. flexneri* 5a M90T, and the atypical *Shigella* B13 strains 3556-77, 3557-77, 3052-94, and 3053-94 as determined by the gentamicin protection assay with duplicate samples in three independent experiments. Statistical analysis was preformed using a one-way ANOVA with a Tukey's post hoc test. *S. flexneri* invasion is significantly different from all other strains ($P<0.001$). Invasion was not significantly different among all other strains ($P>0.05$). *B*, Pedestal formation of the atypical *Shigella* B13 strains and *E. coli* O157:H7 EDL933. Infected HEp-2 cells were fixed and permeabilized and were stained with anti-lipid A antibody, followed by anti-goat-Alexa 488 and phalloidin-Alexa 594 antibodies. The scale bar located in the lower right corner of the EDL933 actin field represents 5 μm and is applicable to all micrographs.