PHASE VARIATION IN THE UROPATHOGENIC *ESCHERICHIA COLI* STRAIN CFT073: INSIGHTS INTO A COMPLEX REGULATORY NETWORK

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Eric J. Battaglioli

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The dissertation is approved by the following members of the Final Oral Committee:

Dr. Rodney Welch, Professor and Chair, Medical Microbiology and Immunology
Dr. Joseph Dillard, Associate Professor, Medical Microbiology and Immunology
Dr. Heidi Goodrich-Blair, Professor, Bacteriology
Dr. Richard Gourse, Professor and Chair, Bacteriology
Dr. John-Demian Sauer, Assistant Professor, Medical Microbiology and Immunology

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Eric J. Battaglioli

Under the supervision of Professor Rodney A. Welch

at the University of Wisconsin-Madison

Abstract of Dissertation

Uropathogenic *Escherichia coli* (UPEC) are the most common etiological agents of uncomplicated urinary tract infection (UTI). The study of UPEC in the laboratory is focused on a handful of prototypical clinical isolates, which includes the pyelonephritis causing strain CFT073. The genetic tools available for this pathogenic model strain are limited and this has stymied the quality of genetic research in the field. I sought to develop new tools for the genetic manipulation of CFT073 and other UPEC model strains to improve the quality of the research in the UPEC community, and to make more complicated mutational analyses possible. I isolated four novel generalized transducing bacteriophages for CFT073 from the wastewater treatment plant in Madison, WI. The phages also plaque on UPEC strains 536, UTI89 and NU14 suggesting inter-strain transductions will be possible with further optimization. The bacteriophages have substantially improved the quality and speed at which research on CFT073 is performed. I used this new tool to examine phase variation of CFT073. I identified a novel phase variable element *ipuS* that drives expression of *ipuR* a predicted LuxR-type transcriptional regulator, and *upaE* a predicted

autotransporter. Inversion of *ipuS* is mediated by a set of tyrosine recombinases shared with two other previously characterized invertible elements, *fimS* and *hyxS*. Inversion of *ipuS* to the "ON" state and subsequent expression of *ipuR-upaE* causes a decrease in swimming motility, an increase in adherence to kidney epithelial cells, and a competitive advantage in the kidneys in the murine model of UTI. The identification of a third phase variable element in CFT073 that is catalyzed by a shared set of recombinases with two others suggests the existence of an interconnected recombinase dependent virulence network. Lastly I characterized a novel phenotype for the previously characterized phase variable system *fimS*. The ON state of *fimS* and subsequent production of Type 1 pili causes a decrease in resistance to reactive nitrogen species.

Dedication

Pursuing a Ph.D. is a daunting task. It takes dedication, focus and immense perseverance to complete this scientific training. This is not a journey that I could have completed on my own. As such, I want to dedicate this work to the people that have given me the strength and support I needed to get to this point in my career. First I dedicate this work to my parents John and Michele Battaglioli. They have given me every opportunity to succeeded and have supported me through every challenge I have encountered. Secondly I dedicate this work to my wife Nicole Battaglioli. Her unconditional love and support has been instrumental in keeping me motivated and focused. I could not have completed my degree with out her.

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Preface

This thesis is organized into five chapters and one appendix. Chapter 1 contains an introduction to topics relevant to this thesis. Chapters 2-4 contain manuscripts suitable for publication. As such they are organized into Abstract, Introduction, Methods and Materials, Results, and Discussion sections. Chapter 5 contains a summary of work performed and overall conclusions. Appendix A contains additional experiments.

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List of Abbreviations

5'RACE	5 prime Rapid Amplification of cDNA Ends
BLAST	Basic local alignment search tool
BMM	Bone marrow derived macrophages
bp	Nucleotide base pairs
Carb	Carbenicillin
cDNA	Complementary DNA
CFU	Colony forming units
Cm	Chloramphenicol
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
EMSA	Electrophoretic mobility shift assay
FimB	Tyrosine recombinase linked to *fimS*
FimE	Tyrosine recombinase linked to *fimS*
fimS	Type 1 pili invertible element
FimX	Tyrosine recombinase linked to *hyxS*
FL	Full length
g	Gravity
gDNA	Genomic DNA
Gm	Gentamicin
hpi	Hours post infection
hyxS	*fimX* associated invertible element

IHF	Integration host factor
IpuA	Tyrosine recombinase linked to *ipuS*
IpuB	Tyrosine recombinase linked to *ipuS*
IpuR	Predicted LuxR-type transcriptional regulator
ipuS	*ipuAB* associated invertible element
IR	Inverted repeats
Kan	Kanamycin
kb	Kilobases (10^3 nucleotide base pairs)
kDa	Kilodaltons (10^3 daltons)
L-arg	L-arginine
L-NAME	L-NG-Nitro-L-Arginine Methyl Ester
LB	Luria-Bertani
Lrp	Leucine responsive protein
M	Molar
MBP	Maltose binding protein
MES	2-(*N*-morpholino) ethanesulfonic acid
min	Minutes
ml	Milliters (10^{-3} liters)
mM	Millimolar (10^{-3} molar)
MOI	Multiplicity of infection
NO	Nitric oxide
OD_x	Optical density for light with a wavelength equal to (x) nanometers

ORF	Open reading frame
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PFU	Plaque forming units
pg	Picograms (10^{-15} grams)
pmol	Picomolar (10^{-15} molar)
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
SD	Standard deviation
SEM	Standard error of measurement
T1p	Type 1 pili
Trunc	Truncated
μg	Micrograms (10^{-6} grams)
μl	Microliters (10^{-6} liters)
μm	Micrometer (10^{-6} meters)
UpaE	Predicted autotransporter
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary tract infection
v/v	Volume to volume
w/v	Weight to volume

Chapter 1

Introduction

Epidemiology and Impact of Urinary Tract Infections

Urinary tract infections (UTI) are considered to be the most common bacterial infections in the United States accounting for almost 25% of all reported infections in the clinical setting (1). It has been estimated that 40-50% of women and 12% of men will experience a UTI in their lifetimes and the likelihood of infection increases with age (1, 2). The most common etiological agents of uncomplicated UTI are the uropathogenic *Escherichia coli* (UPEC), which account for 80-85% of community-acquired infections (3). Other less common agents include *Staphylococcus saprophyticus* (5-10% of reported infections), and infrequently Group B *Streptococcus*, *Klebsiella* and *Proteus* are observed (3).

The predicted natural reservoir of UPEC is the colon though they are not pathogenic in this niche. Infection of the urinary tract is mediated by fecal contamination of the periurethral area (2, 4). Bacteria that gain access to the urethra can ascend to the bladder causing an acute bladder infection known as cystitis. If not treated with antibiotics or cleared by the host immune response at this stage, bacteria can ascend through the ureters to the kidneys causing an acute kidney infection known as pyelonephritis (2, 3). Infection of the kidneys can lead to renal scarring and impaired kidney function, and if not treated or cleared by the host at this stage bacteria can escape the kidneys causing septicemia and ultimately death (5).

Clinically, UTI is diagnosed by presentation with increased frequency and painful urination, pyuria, and presence of $\geq 10^5$ CFU/ml of bacteria in a clean catch urine sample (1, 2). Treatment involves prescription of oral antibiotics, typically trimethoprim-sulfamethoxazole, amoxicillin, ciprofloxacin or nitrofurantoin for 3-10 days (3). Holistic remedies such as cranberry juice have also been employed. Cranberries contain

proanthocyanidin, a surfactant that is filtered by the kidneys and inhibits bacterial adhesion in the urinary tract (6). However, the clinical effectiveness of these compounds for acute infection is unclear and as such they are restricted primarily to prophylactic use (6-8). Uncomplicated UTI (infections of otherwise healthy individuals) are typically non-life threatening if treated promptly. However UTI in pregnant women, which is the most common infection experienced by pregnant individuals, can lead to birth defects and death of the fetus (1). In addition to being a potentially life threatening infection, the economic costs associated with treatment of UTI are estimated at $1.6 billion annually in the United States (1). The significant health and economic related concerns of UTI has motivated intense research on understanding the pathogenesis of infection and developing novel treatment strategies.

Host Defense Mechanisms to Infection

The host possesses a suite of defense mechanisms that function to inhibit bacterial growth in the urinary tract (2). The urinary tract is presumed to be a sterile environment in the absence of infection though recent reports using culture independent techniques indicate a resident microbiota may exist (9). These mechanisms include the cleansing flow of urine, low iron levels, induced uroepithelial cell exfoliation, production of antimicrobial peptides, the adhesive pili chelator uromodulin, and rapid influx of professional phagocytes (5, 10-19).

The innate immune response plays a critical role in controlling bacterial growth in the urinary tract. Infecting bacteria stimulate TLRs on uroepithelial cells, inducing IL-8 production and rapid neutrophil recruitment to the bladder lumen (20-22). In addition to

the canonical ligand LPS, TLR4 stimulation is independently induced by the Type 1 pili adhesive subunit FimH and Pap pili allowing for multiple sources of host recognition of UPEC (23-26). The subsequently recruited neutrophils phagocytose infecting bacteria and also secrete nitric oxide (NO) into the bladder raising NO levels 30-50 fold and increased nitrite content in urine can be detected as soon as 6-12 hours post infection (27, 28). NO is a precursor to several different reactive nitrogen species (RNS) including peroxynitrite, S-nitrosothiol, nitrogen dioxide and others (29, 30). These compounds induce DNA damage, generate protein/lipid modifications and inactivate iron sulfur complex containing enzymes, stymieing bacterial growth (29). Little is known about the role of the adaptive immune response to UTI (20).

UPEC Virulence Factors

UPEC possess an assortment of virulence factors to establish and maintain an infection. They encode multiple types of extracellular pili that facilitate both adherence and invasion of the uroepithelium (31-35), redundant iron acquisition systems (36-38), secreted toxins (39, 40) and nitrosative/oxidative stress detoxification systems (41, 42). The genetic diversity among UPEC strains suggests that no one-virulence factor is essential, but rather a functional suite of genes is sufficient to mediate infection (43, 44).

One of the most well studied UPEC virulence factors is the adhesion mediating Type 1 pili (T1p). T1p are polytrichous 1-3μm long hair like projections found on the surface of nearly all UPEC isolates (45). They are composed of 100-1000 copies of the major structural subunit FimA and are capped by FimF, FimG and the terminal adhesive subunit

FimH. Assembly of T1p proceeds by a chaperone usher assembly mechanism. Requisite structural subunits are delivered into the periplasm via a sec-dependent secretion pathway. Once in the periplasm, subunits are captured by the chaperone FimC and delivered to the usher/pili anchor FimD, which secretes the subunits to the exterior of the cell. The various subunits of the completed pili are held together by "donor-strand exchange" using the chaperone as an intermediate. Covalent bond formation is not required to link adjacent subunits together and assembly is an ATP independent process (46-51). The disassembly and potential recycling of T1p remains poorly understood.

T1p mediate attachment and invasion of the bladder epithelium through interactions between the terminal tip residue FimH and mannosylated moieties on host cells. This interaction is a so-called "catch-bond" as an applied tensile force causes an allosteric change in FimH that increases bond strength (52). Deletion of T1p causes a 100 to 1000-fold defect in colonization in the murine model of UTI while over expression leads to a hyper virulent state (33-35, 53-56). FimH is also immunogenic (11, 57) and is independently capable of stimulating TLR4 (26). Additionally, expression of T1p is inversely associated with swimming motility (56). As a result, careful control of T1p production is required for successful colonization of the urinary tract.

Regulation of T1p in *E. coli* K-12 – Phase Variation

Expression of T1p is regulated by phase variation, which is defined as frequent ($>10^{-5}$ inversions/generation) and reversible ON/OFF changes in gene expression (58). T1p phase variation or "switching" occurs by inversion of a 314 bp element, *fimS,* which contains the Fim operon promoter (59, 60). The element is bounded by a pair of 9 bp

inverted repeats (IRs) and in the non-pathogenic laboratory adapted strain *E. coli* K-12 inversion is mediated by the proximally encoded site-specific tyrosine recombinases FimB and FimE (61). The recombinases bind to the IRs in a sequence specific manner, interacting with the repeats themselves and the surrounding DNA sequence (45, 62). Binding specificity is what defines the directionality of each recombinase's catalytic activity. FimE is only capable of binding to the ON state orientation of the switch, which is what restricts its activity to exclusively catalyzing the ON to OFF inversion (62). In addition to the recombinases, the DNA binding proteins IHF, Lrp and H-NS are known to associate with the region and participate in forming and stabilizing the recombinase-DNA complex (63-69). Local DNA secondary structure is also predicted to influence the recombination event. DNA gyrase mutations and chemical treatments that alter DNA supercoiling also influence switching kinetics (61, 70).

Catalysis by the recombinases follows the branch migration model. Recombinase mediated inversion proceeds by two subunits binding to each inverted repeat and bending the DNA to create a tetrameric protein-DNA complex. Two of the recombinase subunits activate to cleave the top strand of the DNA at each repeat. This occurs by nucleophilic attack of the DNA phosphodiester backbone by an active site tyrosine residue. The newly formed free 5'-hydroxyl then attacks the 3'-phosphotyrosine linkage formed on the adjacent strand, resulting in the formation of a Holliday Junction. Branch migration of the cross over region occurs, the second set of recombinases is activated, and the process repeats with the bottom two DNA strands to resolve the junction (71-73). When this reaction occurs between inverted repeats on the same linear DNA template, the result is an inversion of the region between the repeats.

Regulation of T1p in UPEC Strain CFT073

The UPEC strain CFT073 has three additional FimB/FimE like site-specific tyrosine recombinases that are encoded at distant sites in the chromosome with respect to T1p (74). These recombinases are conserved in uropathogens when compared to gut commensal isolates (56). Two of the additional recombinases, IpuA and IpuB are encoded in conjunction with the *dsdCXA* locus, which is responsible for the uptake and deamination of the bacteriostatic amino acid D-serine (75). The third, FimX is encoded next to the betaine locus, a glycine-betaine osmoprotection system. The additional CFT073 recombinases share sequence similarity with the canonical *E. coli* K-12 recombinases FimB and FimE, have the necessary conserved RHRY active site motif, and display catalytic activity at *fimS* during infection in the murine model of UTI (56). Typically, recombinases that mediate inversions are encoded in close proximity to element at which they act (76), however in trans recombinase activity has been widely demonstrated in *Bacteroidies fragilis* (77-80). Characterization of the in trans activity of FimX and IpuA in CFT073 was one of the first reports of distant site activity for tyrosine recombinases in *E. coli* and suggests complex switching networks contribute to UPEC virulence (56).

Another layer of regulation for T1p comes from crosstalk with the other adhesive pili operons. The Pap pili regulator PapB binds to *fimS* inhibiting activity of FimB, which subsequently blocks expression of T1p by keeping the switch OFF (81-83). A similar protein, FocB in the F1C pili operon of the related UPEC strain J96 affects expression of both Pap pili and T1p though the mechanism is not well understood (81). The function of these regulators suggests a mechanism has evolved that prevents more than one pili type

from being expressed at any given time. No such regulator exists in the T1p operon so it remains unclear how the production of other pili is blocked by T1p expression.

The T1p population phase state is known to respond to changes in pH, osmolarity, temperature, nutrient availability, and location within the urinary tract (31, 84-88). Together, the five recombinases and the accessory binding proteins described above modulate the population wide phase state based on changing environmental conditions during colonization of the host and infection of the urinary tract.

Other Phase Variable Systems in CFT073

Recent publications have identified a second switch, *hyxS,* in CFT073 and in the related UPEC strain UTI89 (41). The *hyxS* element is bounded by a set of 16 bp IRs whose sequence shows only limited similarity to the *fimS* IRs. Bidirectional inversion is mediated only by the proximally-encoded recombinase FimX, presumably due to the sequence divergence between the *fimS* and *hyxS* half sites. Orientation of the element controls expression of *hyxR*, a LuxR-type transcriptional regulator. Expression of the regulator decreases resistance to reactive nitrogen species by decreasing expression of *hmpA*, a nitric oxide detoxifying flavohaemoglobin (41, 89). Intracellular macrophage survival is also inhibited by *hyxR* expression, though the effects are *hmpA* independent and the mechanism not well understood (41).

Phase Variation in Other Bacterial Species

Phase variation allows for the generation of preformed subpopulations in alternate phase states, which can be described as planned genetic heterogeneity. This heterogeneity

bypasses the need for transcriptional and translational activation steps in response to rapidly changing environmental conditions and helps insure survival of the population as a whole (90).

Several different bacterial species have evolved phase variation systems and they are regulated by a diverse set of mechanisms. *Neisseria* species use slipped strand mispairing during DNA synthesis to alter promoter spacing and subsequent expression of the virulence genes *fetA*, *opc* and *nadA* (91-93). *Neisseria* also use a RecA based homologous recombination mechanism to induce phase variation of *pilE* (94, 95). Additionally there are reports that the *Neisseria* and *Haemophilus influenzae* methyltransferase *modA* is under phase variable control. ModA controls the expression of a large set of genes by changing DNA methylation patterns. The resulting global change in gene expression is termed a "phasevarion"(96, 97).

Other systems have evolved that function with site-specific recombinases like T1p phase variation. *Mycoplasma agalactiae* displays recombinase mediated phase variation of outer surface proteins which is predicted to aid in the avoidance of the host immune response (98). In *Clostridium difficle,* phase variation of CwpV, a surface protein that is a member of a family of proteins linked to pathogenesis, occurs by recombinase-mediated inversion. This system is unique because instead of the inversion changing orientation of a promoter, the OFF orientation forms a transcriptional attenuator (99). It is the *Bacteroides* though that show the greatest number of recombinase mediated phase variation systems. A suite of recombinases has been identified that act at both proximal and distal locations in the chromosome suggesting a widely interconnected recombinase network (77-80, 100).

The Importance of Good Genetic Practices and Development of New Genetic Tools

One of the complications of studying pathogenic organisms is a general lack of available genetic tools. Many of the tools developed for the genetic manipulation of laboratory-adapted model organisms such as Lambda-red mutagenesis do not function efficiently in pathogenic strains. Extensive optimization of these methods has been required for adoption to pathogenic backgrounds and efficiency is often reduced compared to laboratory-adapted strains (101, 102).

Lambda-red mutagenesis is a useful technique for generating chromosomal gene replacements in the laboratory. The method relies on the brief exogenous expression of the bacteriophage lambda genes *beta*, *exo* and *gam* within a host cell. Expression of these genes induces a hyper-recombinative state and allows for the replacement of up to 100s of kilobases (kb) of the host chromosome with a small PCR generated antibiotic resistance cassette. The cassette contains short regions of homology (20-40 bp) to a targeted destination on the host chromosome (103). Though powerful, a significant limitation of this tool is that the hyper-recombinative state leaves the host cell susceptible to non-tractable secondary site mutations (102, 104). This complication is further compounded in strains that require multiple rounds of mutagenesis. In *E. coli* K-12, the problem is overcome by P1 transduction of the Lambda-red generated mutations into a clean genetic background. However many pathogens have no available transducing phages. In an effort to correct this deficiency investigators have isolated novel transducing phages for *Citrobacter rodentium*, *Bordetella avium*, *Pseudomonas aeruginosa*, and UPEC (105-108).

Until recently, a transducing phage was not available for CFT073 (105). The lack of a phage severely limited the quality of genetic research performed on this strain and as a

result strains with mixed genetic backgrounds have been disseminated to the UPEC community (42). Quality genetic practices are paramount when performing research where the ultimate goal is the treatment of disease. The isolation of novel phages and development of other novel genetic tools is a straightforward task and should be an active area of research for all pathogenic systems lacking such tools.

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Chapter 2

Isolation of generalized transducing bacteriophages for uropathogenic strains of *Escherichia coli*.

Authors and contributors:

Eric Battaglioli: Planned all experiments, organized and analyzed data, and wrote manuscript. Identified the four transducing phages, developed and optimized the transduction protocol, and demonstrated their ability to perform generalized transduction. Analyzed the genome of ΦEB49.

Dr. Gary Baisa: Assisted in development of the bacteriophage discovery protocol, and assisted in sample collection.

Andrew Hryckowian: Demonstrated the ability of ΦEB49 to perform interstrain transductions in UPEC strain 536.

Robin Schroll: Assisted in generating the bacteriophage phylogeny.

Anne Weeks: Assisted in the genomic characterization of the transducing phages

Dr. Rodney A. Welch: Supervised all research, contributed intellectually to the design of the experiments and assisted in review of the manuscript.

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Abstract

The traditional genetic procedure for random or site-specific mutagenesis in *Escherichia coli* K-12 involves mutagenesis, isolation of mutants, and transduction of the mutation into a clean genetic background. The transduction step reduces the likelihood of complications due to secondary mutations. Though well established, this protocol is not tenable for many pathogenic *E. coli* such as uropathogenic strain CFT073 because it is resistant to known K-12 transducing bacteriophages, such as P1. CFT073 mutants generated via a technique such as Lambda-red mutagenesis may contain unknown secondary mutations. Here we describe the isolation and characterization of transducing bacteriophages for CFT073. Seventy-seven phage isolates were acquired from effluent water samples collected from a wastewater treatment plant in Madison, WI. The phages were differentiated by a host-sensitivity-typing scheme with a panel of *E. coli* strains from the ECOR collection and clinical uropathogenic isolates. We found 49 unique phage isolates. These were then examined for their ability to transduce antibiotic resistance gene insertions at multiple loci between different mutant strains of CFT073. We identified 4 different phages capable of CFT073 generalized transduction. These phages also plaque on the model uropathogenic *E. coli* strains 536, UTI89 and NU14. The highest efficiency transducing phage, ΦEB49 was further characterized by DNA sequence analysis revealing a double stranded genome 47,180 bp in length and showing similarity to other sequenced phages. When combined with a technique like Lambda-red mutagenesis, the newly characterized transducing phages provide a significant development in the genetic tools available for the study of uropathogenic *E. coli*.

Introduction

Uropathogenic *E. coli* (UPEC) are the primary cause of urinary tract infections (UTIs) and treatment of these infections is estimated to cost in excess of 1.6 billion dollars annually in the U.S. (1, 2). Over 70% of women will contract a UTI in their lifetime and a significant portion of these women will suffer from recurrent infections (3). Insight into the pathogenesis of UPEC through application of Molecular Koch's postulates has led to a solid understanding of many of the factors needed for colonization of the urinary tract (4-9). These advances in our understanding have come about because UPEC are genetically tractable via homologous recombination methods such as Lambda-red mutagenesis (10-12). This particular technique uses exogenous expression of the phage lambda genes *bet*, *exo*, and *gam* to induce a hyper recombinative state within the host cell that aids the replacement of a chromosomal region with an antibiotic resistance cassette (10, 13). Though Lambda-red is an efficient method to generate mutants, the hyper recombinative state renders the host bacterium susceptible to secondary mutations at unknown sites (12, 14). There is a 10-fold increase in the generation of spontaneous mutants attributable to expression of the Lambda-red genes (12). To overcome this complication in *E. coli* K-12, generalized transduction is used to transfer the Lambda-red mutation into a clean genetic background. However many non-laboratory *E. coli* strains, including the UPEC strain CFT073, the prototypic UPEC strain (5) are resistant to infection by P1, P22 and other characterized transducing phages. This places a significant limitation on the quality of genetics performed with CFT073 because of the potential presence of unknown mutations that may contribute to fitness, colonization or virulence phenotypes.

Several techniques have been used to generate or identify novel transducing phages for other bacterial pathogens. In *Pseudomonas aeruginosa,* temperate phages were isolated from different clinical isolates and spontaneous lytic variants of these phages were isolated that were capable of generalized transduction (15). Other studies isolated phages from the environment capable of generalized transduction in *Bordetella avium*, *Citrobacter rodentium*, *Mycobacterium smegmatis*, *Streptomyces coelicolor* and *Serratia marcescens* (11, 16-20).

Here we report the isolation, purification and physical characterization of a novel CFT073 generalized transducing phage, ΦEB49. The discovery of this phage represents a significant addition to the genetic tools available for UPEC studies.

Methods and Materials

Strains. A list of strains used in this study is provided in Table 1. Kanamycin (Kan) or chloramphenicol (Cm) resistance gene replacement mutations of target genes were generated by the Lambda-red method of homologous recombination (10).

Media and growth conditions. Cultures were grown in Luria-Bertani (LB) broth or on LB agar (Difco, Sparks, MD) at 37^{o}C. Kan (50 μg/ml) and Cm (20 μg/ml) were added when appropriate. Phages were propagated in top agar overlays (L-broth with 0.7% w/v agar) on LB agar plates at room temperature and stored in phage buffer (10 mM tris HCl pH 7.4, 10 mM $MgSO_4$, 0.01% w/v gelatin) at 4^{o}C.

Isolation and enrichment of bacteriophage. Primary effluent samples were collected from the Nine Springs wastewater treatment plant in Madison, WI. A 19 ml

aliquot from these samples was centrifuged at 600 × g for 10 min to remove large particulate matter and sterilized with a 0.45 μm filter (Millipore). The filtered effluent was concentrated to 150 μl by use of a low binding affinity 30 kDa concentration filter (Amicon). A 50 μl aliquot of the concentrate was mixed with 200 μl of an overnight liquid culture of CFT073 and 4 ml top agar, overlayed on L-agar plates and incubated at 37°C overnight. A total of 77 individual plaques were picked from several plates and stored in 1 ml of phage buffer at 4°C.

Titer enrichment. To increase the titer of each phage isolate, 50 μl of the primary phage stock was mixed with 200 μl of overnight liquid CFT073 culture, 4 ml of top agar and overlaid onto L-agar plates. After overnight incubation at room temperature, the top agar was harvested, suspended in 3 ml of phage buffer containing 6.6% (v/v) chloroform, and then shaken vigorously for 2 min. The lysates were centrifuged for 20 min at 600 × g to remove the remnants of soft agar. The supernatants were collected and stored over chloroform at 4°C. Dilution series ranging from 10^{-1}-10^{-12} were generated for each phage stock and 10 μl of each was spotted on a top agar overlay containing 200 μl of overnight CFT073 culture. Plaques were counted to determine the overall titer of the stock. This process was repeated to generate titers ≥ 10^9 PFU/ml.

Phage host-sensitivity typing. In order to eliminate potentially redundant and sibling bacteriophages, each isolate was assayed for its ability to produce plaques on 23 different *E. coli* strains comprising 16 strains from the ECOR collection (21), UPEC strains NU14, J96, CFT073, 536, and UTI89, and 2 clinical isolates from patients presenting with clinical UTI. A host strain plaquing "fingerprint" was generated for each phage. A

hierarchical cluster analysis was performed on the fingerprints and the results visualized by generating a dendrogram using the phylogenetic analysis programs Gene Cluster 3.0 and Java TreeView (22, 23).

Test for genetic transduction. For each unique phage, a high titer lysate (PFU/ml $\geq 10^9$) was prepared by growing the phage on the CFT073 mutant strain WAM3686 *(*Δ*lacZ::Kan*) and harvested as outlined above. A 100 μl aliquot of the high titer sample was mixed with 500 μl of overnight culture of the recipient CFT073 strain WAM2909 (Δ*dsdA::Cm*) and incubated at room temperature for 20 min. Sodium citrate was then added to a final concentration of 50 mM and then incubated at 37°C for 60 min with shaking. The resulting mixtures were pelleted, resuspended in 100 μl of L-broth, plated on L-agar Kan/Cm plates and incubated overnight at 37°C. Putative transductants were streaked on L-agar Kan/Cm plates and examined for presence of both antibiotic resistance gene insertions via PCR with primer pairs flanking the specific insertion sites.

Generalized transduction. High titer lysates were generated for 5 different Kan resistance gene replacement mutations of target genes located around the CFT073 chromosome using the lysate preparation and transduction conditions described above (Table 1). The Kan gene replacement mutations were transduced into the CFT073 strain WAM2909 (*ΔdsdA::Cm*). Putative transductants were confirmed using PCR and primer pairs flanking the specific gene insertion sites.

Multiplicity of infection (MOI) determination. The titer of a high titer lysate was determined as described above in triplicate. Transductions were then preformed at approximate MOIs ranging from $1x10^{-5}$ phage/cell to $1x10^{2}$ phage/cell. Aliquots of the

liquid culture were plated to determine CFU/ml in each transduction and facilitate precise determination of the actual MOI in each reaction. Transductants were counted after overnight growth at 37°C.

Phage DNA isolation. DNA was isolated from 12 ml of high titer (≥10^9 PFU/ml) lysate from each transducing phage. In order to remove contaminating nucleic acids, the lysate was treated with DNase (1 μg/ml) and RNase (10 μg/ml) for 60 min at 37°C with shaking. The resulting samples were passed through a 0.45 μm filter; PEG-8000 (10% w/v) and NaCl (1 M final) was added and incubated at 4°C over night. Samples were then centrifuged at 600 × g for 20 min. The supernatant was removed and the resulting pellet was suspended in 1 ml of phage buffer. The pellet was extracted twice with 1 volume of chloroform. An equal volume of phenol was added to the sample, mixed by inversion and centrifuged at 20,000 × g for 3 min. The aqueous phase was removed and placed in a clean microfuge tube. This step was repeated twice, once with a 1:1 mixture of phenol and chloroform, and again with a 24:1 mixture of chloroform and isoamyl alcohol. Phage DNA was precipitated by adding 1/10 volume of 3 M NaOAc and 1 ml of ice cold 100% ethanol. After incubating on ice for 10 min, the DNA was pelleted by centrifugation for 2 min at 20,000 × g. The DNA pellet was washed twice with 70% ethanol, dried with a Vacufuge Plus (Epenndorf) at 60°C for 15 min and suspended in TE buffer pH 8.0.

Genome sequencing. Purified DNA for ΦEB49 was sequenced using an Illumina GAIIx sequencer. A 36-cycle single direction reaction and *de novo* assembly was performed at the UW Biotechnology Center's Next Generation Sequencing Facility. Remaining

sequence gaps were closed by generating PCR products using primers specific to the regions flanking the gaps and dideoxy-chain termination sequencing.

Electron microscopy. Phage samples were processed by the UW Medical School Electron Microscope Facility with the two-step negative staining method using Nano-W (Nanoprobes Inc.) on pioloform coated Ni thin bar 300 mesh grids. The stained samples were viewed with a Philips CM120 at 80kV and documented using a SIS (Olympus - Soft Imaging Systems) MegaView III digital camera.

Results and Discussion

Phage isolation and typing. In order to identify potential transducing phages for UPEC we collected primary effluent samples from the Nine Springs wastewater treatment plant in Madison, WI, a likely environmental source of *E. coli*-infecting phages. To isolate the phage within the samples, the large particulates and bacteria were removed with centrifugation and subsequent filter sterilization. The filtrate was then concentrated approximately 100-fold and this material was used to infect CFT073. We isolated phage from 77 separate plaques and these were labeled as isolates EB1-EB77.

To avoid processing and testing closely related or sibling phages, a typing scheme was devised based on *E. coli* host range determination. A host-range fingerprint was generated for each phage by testing its ability to plaque on 23 different *E. coli* strains. The strains included 16 evolutionarily divergent members from the ECOR collection (21), 5 laboratory UPEC strains and 2 clinical isolates from patients with diagnosed UTIs (Table 1). An evolutionarily broad spectrum was chosen to decrease the likelihood of redundant phage types. A cluster analysis was performed on the fingerprint data to identify phages

with identical host strain patterns using the program Gene Cluster 3.0 and a dendrogram was constructed to visualize the clustering using the program Java TreeView (22, 23) (Figure. 1). In addition to unique isolates, the clustering analysis identified 15 phage clusters, each with identical infection patterns. For these groups, a single representative was chosen at random, and the remaining members excluded from further evaluation. The final group consisted of 49 apparently unique phage isolates.

Identification of UPEC transducing phages. Each of the 49 phage isolates was examined for its ability to transduce a single antibiotic resistance marker. The phages were individually grown on the CFT073 donor strain WAM3686 *(*Δ*lacZ::Kan*) and high titer lysates were incubated in the presence of the recipient CFT073 strain WAM2909 (Δ*dsdA::Cm*). Any phages capable of packaging and transducing the Δ*lacZ::Kan* insertion into the recipient strain would result in a double resistant Δ*lacZ::Kan,* Δ*dsdA::Cm* strain. No colonies were found on plates inoculated with 100 μl of the high titer (>10^9 PFU/ml) lysates indicating the lysates were bacteria free. Of the 49 phages tested, ΦEB5, ΦEB32, ΦEB47, and ΦEB49 were able to transduce the Kan marker into WAM2909 and the proper integration of both antibiotic cassettes was confirmed by PCR (Figure. 2).

To determine if the 4 phages were capable of generalized transduction, a series of strains with Kan gene replacement mutations of target genes positioned throughout the CFT073 chromosome were selected from our laboratory stocks and we attempted to transduce these different markers into WAM2909. With the exception of ΦEB5, each phage was capable of packaging and transducing the different mutations (Table 2). Additionally, ΦEB49 consistently generated the greatest number of transductants. An empirical

examination of transduction efficiency with no further optimization of the reaction conditions indicated the following order of transduction efficiency from highest to lowest: ΦEB49 >>> ΦEB47 > ΦEB32 > ΦEB5 (data not shown).

UPEC Host range. The plaquing patterns used for the isolate typing doubled as host range determinations for the 4 transducing phages. Of particular interest is the ability to plaque on prototrophic *E. coli* K-12 strain MG1655 and classic model UPEC strains (Table 3). None of the four CFT073 transducing phages were able to plaque on *E. coli* K-12, though each was able to plaque on at least one other well-studied UPEC strain including UTI89, NU14 and 536. Their ability to infect other UPEC strains suggests they could be used to transduce mutations between strains, and interstrain transductions were successful between CFT073 and 536 with ΦEB49 (data not shown). Further optimization of transduction conditions in order to move genetic markers among different strains is the subject of further work in our laboratory.

Phage morphology. In order to further characterize the 4 CFT073 transducing phages, their morphology was determined by transmission electron microscopy (Figure 3). ΦEB47, ΦEB32, and ΦEB5 are structurally similar, sharing a rounded head and flexible S-shaped non-contractile tails characteristic of the *Siphoviridae* family. The spiral tail morphology could be an artifact of EM sample preparation, however similar structures were observed on a previously characterized phage, CVX-5, isolated from a patient with colitis (24). ΦEB49 has an icosahedral head and straight tail stalk classifying it with the *Myoviridae*. Individual tails and heads were often observed suggesting either mechanical shearing during preparation or incomplete assembly. All 4 phage have heads

approximately 50 nm in diameter suggesting they have similar genome sizes which was later confirmed by pulse field gel electrophoresis (data not shown).

Transduction optimization. Due to its enhanced transducing efficiency, ΦEB49 was selected for further optimization and characterization. The protocol developed to search for putative transducers (MOI approximately equal to 1), yielded on average 10^1-10^2 transductants per reaction with ΦEB49 (data not shown). Though this efficiency was above our limit of detection, we explored a range of MOIs to further increase transduction efficiency and MOIs ranging between 10^{-5}-10^2 phages/cell were evaluated. We could not significantly increase the efficiency by varying the MOI, but we did identify a suitable range. A MOI between 0.1-3 is optimal for ΦEB49 transduction of CFT073 (data not shown).

ΦEB49 temperature dependent lysis and possible lysogen formation. Addition of high titer phage stocks to liquid cultures of CFT073 did not cause complete lysis of the bacterial suspension. Additionally, lytic activity of ΦEB49 is inhibited during growth at 37^{o}C in solid and liquid media (data not shown). Temperature dependence on selection of a lytic vs. lysogenic pathways has been characterized in phage lambda and *Listeria* phages where colder temperatures block lysis (25, 26). However, ΦEB49 shows the opposite effect, increasing lytic activity at lower temperatures. The temperature dependence may be a result of a defect in phage adsorption or shift in preference to a lysogenic phase thought it is not clear if ΦEB49 forms lysogens.

The lack of complete lysis and temperature effects suggested the possibility that ΦEB49 is capable of lysogeny. To examine if transduced strains were free of ΦEB49, the supernatants of liquid cultures from primary, secondary and tertiary re-isolations of

ΦEB49 transductants were examined for the ability to produce plaques on CFT073. The primary isolates of transduced strains were found to produce plaques by plating on soft agar overlays but plaques could not be generated in secondary and tertiary isolations (data not shown). In addition, southern hybridizations performed on isolated genomic DNA from secondary and tertiary isolates using probes specific to ΦEB49 were negative, indicating that infectious phage and stable lysogens are not evident past the second passage of the transducant (data not shown).

ΦEB49 genome sequence. The ΦEB49 genome was sequenced in order to further characterize its genetic potential and relatedness to previously described bacteriophages. ΦEB49 DNA was sequenced using an Illumina GAIIx sequencer, generating 3.1 x 10^7 36-bp reads. A *de novo* assembly of the raw sequence data produced 7 contigs ranging from 16,000× to 22,000× coverage and totaling 42 kb. Pulse field gel electrophoresis of the uncut DNA suggested a genome size of 48 kb, so the gap regions were examined to identify the missing sequence information. The contigs were first oriented relative to one another based on apparent sequence similarity to the phage JK06 (GenBank: DQ121662) and adjacent contigs were joined with PCR using primers specific to the ends of the gap regions. An additional 5 kb of sequence was found in the various gaps resulting in a complete genome of 47,180 bp (Genbank: JF770475). The sequencing and PCR data suggest the ΦEB49 genome is circularly permutated and restriction digestion indicates it is composed of double stranded DNA (data not shown). The completed sequence was annotated with DNA Master (http://cobamide2.bio.pitt.edu/), which predicted the location of 74 putative ORFs and 2 tRNAs (Figure 4). BLAST searches of the predicted ORFs showed sequence

homology to the enteric bacteriophages JK06 and Rtp (27) (Table S1). Predicted ORFs in ΦEB49 were identified with high sequence identity to tail fiber structural and assembly components (70.4%-99.5% identical), a prohead protease (96.7% identical), a putative portal protein (98.5% identical) and several hypothetical ORFs from JK06, Rtp and other sequenced phages. Other predicted ORFs showed limited sequence identity to 4 putative endonucleases (59.1%-63.6% identical), and a putative DNA primase (74.9% identical). Additionally, 8 predicted ORFs appear specific to ΦEB49 showing no significant similarity to any of the queried GenBank sequences. CFT073 is known to contain components of remnant phages (28). However, the ΦEB49 genome shares no significant similarity with the published CFT073 genome sequence suggesting that ΦEB49 did not originate from the CFT073 isolate.

Up until our work reported here, the genetic tools available for pathogenic *E. coli* limited the relative quality of the genetics performed in CFT073 and other UPEC strains. We successfully identified a novel generalized transducing phage for the model UPEC strain CFT073. ΦEB49 is a generalized transducing phage capable of high efficiency transduction among CFT073 strains and greatly reduces the complications of unknown secondary site mutations generated when performing Lambda-red mutagenesis. With this phage available, we feel the standard of genetic analyses in CFT073 has been raised significantly.

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Table 1. Strains used

Strain	Description	Source or reference
WAM2267	WT CFT073	Gift from Dr. Harry Mobley
WAM2625	WT *E. coli* K-12	Gift from Dr. Fred Blattner
WAM2909	CFT073 *ΔdsdA::Cm*	Our Laboratory
WAM3686	CFT073 *ΔlacZ::Kan*	Our Laboratory
WAM3403	CFT073 *Δlrp::Kan*	Our Laboratory
WAM4248	CFT073 *ΔcycA::Kan*	Our Laboratory
WAM4227	CFT073 *ΔmppA::Kan*	Our Laboratory
WAM3847	CFT073 *Δaer::Kan*	Our Laboratory
WAM3432	CFT073 *ΔphoA::Kan*	Our Laboratory
WAM2039	UPEC strain 536	Gift from Dr. Werner Goebel
WAM1218	UPEC strain J96	Gift from Dr. Barbara Minshew
WAM2645	UPEC strain NU14	Gift from Dr. Scott Hultgren
WAM4054	UPEC strain UTI89	Gift from Dr. Scott Hultgren
WAM3244	*E. coli* K1 cystitis clinical isolate	(29)
WAM3229	*E. coli* K1 cystitis clinical isolate	(29)
ECOR 2	A:OH:H32	(21)
ECOR 4	A:79:NM	(21)
ECOR 14	A:OM:HN	(21)
ECOR 21	A:O121:HN	(21)
ECOR 27	B1:O104:NM	(21)
ECOR 29	B1:O150:H21	(21)
ECOR 35	D:O1:NM	(21)
ECOR 36	D:79:H25	(21)
ECOR 41	D:O7:NM	(21)
ECOR 47	D:OM:H18	(21)
ECOR 51	B2:O25:HN	(21)
ECOR 56	B2:O6:H1	(21)
ECOR 58	B1:O112:H8	(21)
ECOR 62	B2:O2:NM	(21)
ECOR 64	B2:O75:NM	(21)
ECOR 71	B1:O78:NM	(21)

Table 2. Test for generalized transduction[a]

Mutation	CFT073 Gene Location	ΦEB5	ΦEB32	ΦEB47	ΦEB49
lacZ	448924	+	+	+	+
phoA	475723	-	+	+	+
lrp	986723	+	+	+	+
mppA	1636492	+	+	+	+
aer	3658151	+	+	+	+
cycA	5046982	+	+	+	+

[a]Ability to perform generalized transduction shown as "+" for yes, and "-" for no

Table 3. UPEC host range[a]

Strain	ΦEB5	ΦEB32	ΦEB47	ΦEB49
CFT073 (WAM2267)	+	+	+	+
UTI89 (WAM4054)	+	+	+	-
NU14 (WAM2645)	-	+	+	-
J96 (WAM1218)	-	-	-	-
536 (WAM2039)	-	-	+	+
MG1655 (WAM2625)	-	-	-	-

[a]Plaquing ability shown as "+" for plaques observed and "-" for no plaques observed

Table S1. Summary of BLAST hits for ΦEB49

ORF Number	Strand/Coordinates	Hit Number	Definition	E Value	Similarity
1	661 - 879	1	hypothetical protein JK_48 Enterobacteria phage JK06	4.70E-15	83.3
2	876 - 1115	1	hypothetical protein JK_49 Enterobacteria phage JK06	4.30E-36	94.9
2	876 - 1115	2	hypothetical protein rtp73 Enterobacteria phage RTP	5.70E-36	94.9
3	1116 - 1268	1	hypothetical protein rtp72 Enterobacteria phage RTP	1.30E-10	83
4	1338 - 1727	1	hypothetical protein JK_50 Enterobacteria phage JK06	1.50E-31	75.2
4	1338 - 1727	2	hypothetical protein rtp69 Enterobacteria phage RTP	3.60E-31	76.9
4	1338 - 1727	3	gp73 Enterobacteria phage TLS	1.40E-14	59.2
4	1338 - 1727	4	hypothetical protein T1p07 Enterobacteria phage T1	3.20E-11	67.6
5	1805 - 3388	1	hypothetical protein rtp67 Enterobacteria phage RTP	0.00E+00	97.2
5	1805 - 3388	2	YfiJ Enterobacteria phage TLS	0.00E+00	87.8
5	1805 - 3388	3	hypothetical protein JK_52 Enterobacteria phage JK06	0.00E+00	99.1
5	1805 - 3388	4	hypothetical protein T1p09 Enterobacteria phage T1	0.00E+00	75.1
5	1805 - 3388	5	hypothetical protein JK_55 Enterobacteria phage JK06	0.00E+00	98.8
5	1805 - 3388	6	hypothetical protein VIP0048 Salmonella phage E1	0.00E+00	61
5	1805 - 3388	7	hypothetical protein NE0232 Nitrosomonas europaea ATCC 19718	6.70E-16	47.5
5	1805 - 3388	8	hypothetical protein JK_46 Enterobacteria phage JK06	7.90E-11	74.2
5	1805 - 3388	9	hypothetical protein HRM2_17350 Desulfobacterium autotrophicum HRM2	9.60E-11	45.2
6	3688 - 3924	1	YdbL Enterobacteria phage JK06	5.60E-32	89.7
6	3688 - 3924	2	hypothetical protein T1p10 Enterobacteria phage T1	8.50E-17	69.2
6	3688 - 3924	3	gp70 Enterobacteria phage TLS	1.90E-16	70.9
6	3688 - 3924	4	hypothetical protein rtp66 Enterobacteria phage RTP	4.30E-16	72.7

Table S1. Continued

ORF Number	Strand/Coordinates	Hit Number	Definition	E Value	Similarity
7	complement (3938 - 4342)	1	hypothetical protein JK_57 Enterobacteria phage JK06	0.00E+00	94
7	complement (3938 - 4342)	2	hypothetical protein rtp65 Enterobacteria phage RTP	2.40E-13	65.8
8	complement (4324 - 4806)	1	hypothetical phage-related lysozyme Enterobacteria phage JK06	0.00E+00	88.8
8	complement (4324 - 4806)	2	LysN Enterobacteria phage TLS	6.90E-41	67.9
8	complement (4324 - 4806)	3	putative endolysin Enterobacteria phage RTP	1.20E-36	70.3
8	complement (4324 - 4806)	4	endolysin Enterobacteria phage A5	3.20E-33	73
8	complement (4324 - 4806)	5	endolysin Enterobacteria phage T1	9.10E-33	65.4
8	complement (4324 - 4806)	6	lysozyme Providencia rustigianii DSM 4541	1.30E-32	64.5
8	complement (4324 - 4806)	7	phage lysozyme Salmonella enterica subsp. enterica serovar Virchow str. SL491	5.00E-31	62.7
8	complement (4324 - 4806)	8	putative lysozyme; DLP12 prophage Escherichia fergusonii ATCC 35469	1.90E-30	62
8	complement (4324 - 4806)	9	hypothetical protein PROVALCAL_01194 Providencia alcalifaciens DSM 30120	6.30E-30	62.7
8	complement (4324 - 4806)	10	hypothetical protein ESA_02747 Cronobacter sakazakii ATCC BAA-894	9.70E-30	62.9
9	complement (4806 - 4973)	1	HolN Enterobacteria phage TLS	4.40E-05	64.8
10	complement (5136 - 5309)	1	hypothetical protein rtp62 Enterobacteria phage RTP	1.20E-17	78.9
12	complement (5576 - 6703)	1	hypothetical protein rtp61 Enterobacteria phage RTP	0.00E+00	85.1
12	complement (5576 - 6703)	2	hypothetical protein T1p16 Enterobacteria phage T1	0.00E+00	81.4
12	complement (5576 - 6703)	3	gp66 Enterobacteria phage TLS	0.00E+00	74.3
12	complement (5576 - 6703)	4	conserved hypothetical protein Escherichia coli MS 21-1	0.00E+00	73
12	complement (5576 - 6703)	5	hypothetical protein JK_64 Enterobacteria phage JK06	0.00E+00	98.4
12	complement (5576 - 6703)	6	hypothetical protein JK_63 Enterobacteria phage JK06	0.00E+00	98.8

Table S1. Continued

ORF Number	Strand/Coordinates	Hit Number	Definition	E Value	Similarity
12	complement (5576 - 6703)	7	hypothetical protein Escherichia phage K1G	0.00E+00	61.5
12	complement (5576 - 6703)	8	hypothetical protein Escherichia phage K1ind1	0.00E+00	61.5
14	complement (7062 - 7319)	1	hypothetical protein T1p17 Enterobacteria phage T1	1.30E-09	63.9
15	complement (7316 - 7501)	1	hypothetical protein JK_65 Enterobacteria phage JK06	3.00E-21	98
16	complement (7552 - 7677)	1	hypothetical protein rtp58 Enterobacteria phage RTP	1.30E-11	92.7
17	complement (7674 - 7874)	1	hypothetical protein JK_67 Enterobacteria phage JK06	1.60E-24	95.4
18	complement (7871 - 8089)	1	hypothetical protein rtp56 Enterobacteria phage RTP	5.70E-21	82.4
19	complement (8094 - 8417)	1	hypothetical protein VDA_002080 Photobacterium damselae subsp. damselae CIP 102761	5.02	67.6
20	complement (8484 - 8873)	1	hypothetical protein JK_69 Enterobacteria phage JK06	5.50E-44	98.9
20	complement (8484 - 8873)	2	gp59 Enterobacteria phage TLS	2.00E-28	68
20	complement (8484 - 8873)	3	hypothetical protein T1p21 Enterobacteria phage T1	5.00E-25	71.5
20	complement (8484 - 8873)	4	hypothetical protein rtp54 Enterobacteria phage RTP	1.20E-19	61.4
20	complement (8484 - 8873)	5	hypothetical protein Phage PY100	1.70E-13	58.5
20	complement (8484 - 8873)	6	VRR-NUC domain protein Capnocytophaga ochracea DSM 7271	2.60E-11	57
21	complement (8873 - 10816)	1	hypothetical protein yejH Enterobacteria phage JK06	0.00E+00	96.2
21	complement (8873 - 10816)	2	HelA Enterobacteria phage TLS	0.00E+00	73.6
21	complement (8873 - 10816)	3	putative ATP-dependent helicase Enterobacteria phage T1	0.00E+00	72.8
21	complement (8873 - 10816)	4	putative ATP-dependent helicase Enterobacteria phage RTP	0.00E+00	68.8
21	complement (8873 - 10816)	5	putative ATP-dependent helicase Salmonella phage E1	0.00E+00	61

Table S1. Continued

ORF Number	Strand/Coordinates	Hit Number	Definition	E Value	Similarity
21	complement (8873 - 10816)	6	hypothetical helicase-related protein Enterobacteria phage JK06	0.00E+00	98.3
21	complement (8873 - 10816)	8	hypothetical protein VIBHAR_02656 Vibrio harveyi ATCC BAA-1116	1.00E-40	48.8
21	complement (8873 - 10816)	9	helicase-related protein Vibrio mimicus MB-451	1.10E-40	49.8
21	complement (8873 - 10816)	10	helicase-related protein Vibrio mimicus VM573	1.40E-40	49.8
22	10910 - 11383	1	hypothetical transcriptional regulator Enterobacteria phage JK06	0.00E+00	95.8
22	10910 - 11383	2	putative transcriptional regulator Enterobacteria phage RTP	0.00E+00	95.8
22	10910 - 11383	3	gp57 Enterobacteria phage TLS	2.40E-32	71
22	10910 - 11383	4	hypothetical protein T1p23 Enterobacteria phage T1	3.60E-32	72.2
23	11478 - 12377	1	putative DNA primase Enterobacteria phage T1	0.00E+00	74.9
23	11478 - 12377	2	PriA Enterobacteria phage TLS	0.00E+00	69
23	11478 - 12377	3	hypothetical alpha replication protein Enterobacteria phage JK06	0.00E+00	65
23	11478 - 12377	4	putative DNA primase Enterobacteria phage RTP	0.00E+00	67.2
23	11478 - 12377	5	putative DNA primase Salmonella phage E1	3.50E-32	53.5
24	12476 - 15469	1	hypothetical tail fiber Enterobacteria phage JK06	1.10E-42	85.6
24	12476 - 15469	2	putative tail fiber Enterobacteria phage RTP	2.20E-32	77
24	12476 - 15469	3	putative tail fiber Enterobacteria phage T1	4.90E-25	70.1
24	12476 - 15469	4	Stf Enterobacteria phage TLS	1.50E-23	68.5
24	12476 - 15469	5	conserved domain protein Escherichia coli MS 21-1	5.40E-13	65.5
24	12476 - 15469	6	hypothetical protein ykris0001_43420 Yersinia kristensenii ATCC 33638	1.90E-11	58.6
24	12476 - 15469	7	hypothetical protein PLES_22831 Pseudomonas aeruginosa LESB58	6.30E-11	59.8

Table S1. Continued

ORF Number	Strand/Coordinates	Hit Number	Definition	E Value	Similarity
25	complement (15497 - 15850)	1	gene 26 protein Enterobacteria phage Sf6	6.30E-12	54.4
25	complement (15497 - 15850)	2	hypothetical protein Rleg2_4631 Rhizobium leguminosarum bv. trifolii WSM2304	7.60E-11	66.1
25	complement (15497 - 15850)	3	hypothetical protein Rleg2_1131 Rhizobium leguminosarum bv. trifolii WSM2304	8.70E-11	49.2
26	complement (15909 - 16370)	1	hypothetical protein JK_77 Enterobacteria phage JK06	0.00E+00	93.6
26	complement (15909 - 16370)	2	putative single-stranded DNA binding protein Enterobacteria phage RTP	2.30E-37	84
26	complement (15909 - 16370)	3	Ssb Enterobacteria phage TLS	1.70E-21	70.2
26	complement (15909 - 16370)	4	hypothetical protein T1p27 Enterobacteria phage T1	2.60E-19	72.4
27	complement (16418 - 17065)	1	hypothetical protein JK_79 Enterobacteria phage JK06	0.00E+00	100
27	complement (16418 - 17065)	2	putative recombination protein Enterobacteria phage RTP	0.00E+00	71.3
27	complement (16418 - 17065)	3	putative recombination protein Enterobacteria phage T1	0.00E+00	69.8
27	complement (16418 - 17065)	4	RecT Enterobacteria phage TLS	1.20E-43	63.2
27	complement (16418 - 17065)	5	hypothetical protein JK_78 Enterobacteria phage JK06	3.00E-34	91
27	complement (16418 - 17065)	6	putative recombination protein Salmonella phage E1	1.00E-33	63.7
27	complement (16418 - 17065)	7	hypothetical protein mma_2226 Janthinobacterium sp. Marseille	5.00E-24	58.8
27	complement (16418 - 17065)	8	ERF family protein Delftia acidovorans SPH-1	6.40E-21	61.8
27	complement (16418 - 17065)	9	recombination protein phage associated Bacillus thuringiensis str. Al Hakam	1.70E-19	60.5
27	complement (16418 - 17065)	10	ERF family protein Desulfovibrio vulgaris subsp. vulgaris DP4	5.20E-18	56.9
28	complement (17121 - 18089)	1	putative exodeoxyribonuclease VIII RecE Enterobacteria phage RTP	0.00E+00	75.5
28	complement (17121 - 18089)	2	RecE Enterobacteria phage TLS	0.00E+00	62.6
28	complement (17121 - 18089)	3	exodeoxyribonuclease VIII Enterobacteria phage T1	0.00E+00	62.8

Table S1. Continued

ORF Number	Strand/Coordinates	Hit Number	Definition	E Value	Similarity
28	complement (17121 - 18089)	4	putative exodeoxyribonuclease Salmonella phage E1	0.00E+00	62.8
28	complement (17121 - 18089)	5	hypothetical protein JK_80 Enterobacteria phage JK06	1.40E-45	89.3
28	complement (17121 - 18089)	6	hypothetical protein JK_81 Enterobacteria phage JK06	1.70E-41	97.7
28	complement (17121 - 18089)	7	exodeoxyribonuclease 8 Simonsiella muelleri ATCC 29453	9.70E-13	50.4
30	complement (18365 - 18580)	1	hypothetical protein EcHS_A0274 Escherichia coli HS	8.70E-08	61.5
31	complement (18655 - 19284)	1	hypothetical protein JK_82 Enterobacteria phage JK06	2.30E-40	74.1
31	complement (18655 - 19284)	2	Phage regulatory protein Rha-like protein Pectobacterium wasabiae WPP163	1.70E-12	64.1
31	complement (18655 - 19284)	3	hypothetical protein KPK_0786 Klebsiella pneumoniae 342	7.20E-11	63.5
32	19813 - 20043	1	putative phage lipoprotein Enterobacteria phage RTP	2.40E-25	85.3
33	20043 - 21005	1	hypothetical protein JK_1 Enterobacteria phage JK06	0.00E+00	77.8
33	20043 - 21005	2	conserved hypothetical protein Salmonella enterica subsp. enterica serovar Schwarzengrund str. SL480	0.00E+00	64.9
33	20043 - 21005	3	conserved hypothetical protein Salmonella enterica subsp. enterica serovar Virchow str. SL491	0.00E+00	64.6
33	20043 - 21005	4	hypothetical protein rtp44 Enterobacteria phage RTP	0.00E+00	62.7
33	20043 - 21005	5	gp23 Sodalis phage SO-1	0.00E+00	59.8
33	20043 - 21005	6	hypothetical protein ECL_01716 Enterobacter cloacae subsp. cloacae ATCC 13047	2.00E-26	50.6
33	20043 - 21005	7	hypothetical protein PY54p23 Yersinia phage PY54	8.50E-11	46.3
34	complement (21032 - 24421)	1	tail fiber Enterobacteria phage JK06	0.00E+00	98.5
34	complement (21032 - 24421)	2	putative tail fiber protein Enterobacteria phage RTP	0.00E+00	89.8
34	complement (21032 - 24421)	3	TspJ Enterobacteria phage TLS	0.00E+00	75

Table S1. Continued

ORF Number	Strand/Coordinates	Hit Number	Definition	E Value	Similarity
34	complement (21032 - 24421)	4	putative tail fiber protein Enterobacteria phage T1	0.00E+00	72.1
34	complement (21032 - 24421)	5	gp21 Enterobacteria phage N15	0.00E+00	63.3
34	complement (21032 - 24421)	6	fibronectin type III domain-containing protein Enterobacter sp. 638	0.00E+00	64
34	complement (21032 - 24421)	7	Protein of unknown function DUF1983 Pantoea sp. At-9b	0.00E+00	62.4
34	complement (21032 - 24421)	8	hypothetical protein ESA_01044 Cronobacter sakazakii ATCC BAA-894	0.00E+00	63.5
34	complement (21032 - 24421)	9	hypothetical protein Ctu_17620 Cronobacter turicensis z3032	0.00E+00	63.4
34	complement (21032 - 24421)	10	Phage host specificity protein Yersinia kristensenii ATCC 33638	0.00E+00	60.8
35	complement (24502 - 25068)	1	hypothetical tail assembly protein I Enterobacteria phage JK06	0.00E+00	99.5
35	complement (24502 - 25068)	2	putative tail assembly protein Enterobacteria phage RTP	0.00E+00	82.4
35	complement (24502 - 25068)	3	gp49 Enterobacteria phage TLS	2.40E-37	63.6
35	complement (24502 - 25068)	4	putative tail assembly protein Enterobacteria phage T1	6.70E-34	60.8
35	complement (24502 - 25068)	5	lambda tail assembly I Shewanella baltica OS223	5.00E-22	53.2
35	complement (24502 - 25068)	6	bacteriophage lambda tail assembly I Shewanella sp. W3-18-1	1.20E-21	55.4
35	complement (24502 - 25068)	7	bacteriophage lambda tail assembly I Shewanella sp. W3-18-1	4.90E-21	64.6
35	complement (24502 - 25068)	8	prophage LambdaSo tail assembly protein I Shewanella oneidensis MR-1	9.90E-21	55.9
35	complement (24502 - 25068)	9	bacteriophage lambda tail assembly I Pseudomonas syringae pv. syringae B728a	7.30E-20	63
35	complement (24502 - 25068)	10	bacteriophage lambda tail assembly I Pseudomonas syringae pv. syringae 642	1.20E-19	61.7
36	complement (25049 - 25795)	1	hypothetical GP19 Enterobacteria phage JK06	0.00E+00	94.8
36	complement (25049 - 25795)	2	putative minor tail protein Enterobacteria phage RTP	0.00E+00	85.3

Table S1. Continued

ORF Number	Strand/Coordinates	Hit Number	Definition	E Value	Similarity
36	complement (25049 - 25795)	3	gp48 Enterobacteria phage TLS	0.00E+00	74.3
36	complement (25049 - 25795)	4	putative minor tail protein Enterobacteria phage T1	0.00E+00	73.6
36	complement (25049 - 25795)	5	Predicted metal-dependent protease of the PAD1/JAB1 superfamily Enterobacter cloacae subsp. cloacae NCTC 9394	0.00E+00	65
36	complement (25049 - 25795)	6	hypothetical protein ESA_01042 Cronobacter sakazakii ATCC BAA-894	0.00E+00	61.9
36	complement (25049 - 25795)	8	Gp19 Enterobacteria phage HK97	0.00E+00	64.8
36	complement (25049 - 25795)	9	hypothetical protein Ctu_17600 Cronobacter turicensis z3032	0.00E+00	61.5
36	complement (25049 - 25795)	10	gp19 Enterobacteria phage HK022	0.00E+00	63.4
37	complement (25855 - 26220)	1	PREDICTED protein R52.2-like Danio rerio	1.98	38.4
38	complement (26295 - 27059)	1	hypothetical protein JK_7 Enterobacteria phage JK06	0.00E+00	96.9
38	complement (26295 - 27059)	2	putative minor tail protein Enterobacteria phage RTP	0.00E+00	90.8
38	complement (26295 - 27059)	3	putative minor tail protein Enterobacteria phage T1	0.00E+00	80.6
38	complement (26295 - 27059)	4	thmL Enterobacteria phage TLS	0.00E+00	78.4
38	complement (26295 - 27059)	5	gp18 Citrobacter sp. 30_2	0.00E+00	61
38	complement (26295 - 27059)	6	putative phage protein phage tail protein Xenorhabdus bovienii SS-2004	0.00E+00	61.6
38	complement (26295 - 27059)	7	gp18 Enterobacter cloacae subsp. cloacae ATCC 13047	0.00E+00	61
38	complement (26295 - 27059)	8	phage tail protein Yersinia pestis KIM 10	0.00E+00	59.8
38	complement (26295 - 27059)	9	gp18 Enterobacteria phage N15	0.00E+00	60.6
38	complement (26295 - 27059)	10	phage minor tail protein L Yersinia pseudotuberculosis YPIII	0.00E+00	58.9
39	complement (27100 - 27453)	1	putative minor tail protein Enterobacteria phage JK06	2.80E-45	90.5
39	complement (27100 - 27453)	2	putative minor tail protein Enterobacteria phage RTP	1.20E-37	87.1

Table S1. Continued

ORF Number	Strand/Coordinates	Hit Number	Definition	E Value	Similarity
39	complement (27100 - 27453)	3	putative minor tail protein Enterobacteria phage T1	3.70E-21	67.5
39	complement (27100 - 27453)	4	gp46 Enterobacteria phage TLS	8.80E-18	60.5
40	complement (27450 - 27929)	1	putative HNH endonuclease Enterobacteria phage RTP	7.00E-38	63.6
40	complement (27450 - 27929)	2	EndY Enterobacteria phage TLS	2.80E-34	63.1
40	complement (27450 - 27929)	3	EndZ Enterobacteria phage TLS	6.90E-33	59.8
40	complement (27450 - 27929)	4	HNH endonuclease Shigella flexneri 2002017	3.20E-30	58.3
40	complement (27450 - 27929)	7	endonuclease Escherichia coli B088	3.40E-27	60.8
40	complement (27450 - 27929)	8	putative HNH-endonuclease Enterobacteria phage RTP	2.10E-25	58.4
40	complement (27450 - 27929)	9	putative endonuclease Enterobacteria phage T1	1.60E-23	56.7
40	complement (27450 - 27929)	10	HNH endonuclease Nitrobacter hamburgensis X14	1.80E-23	63.2
41	complement (28001 - 30979)	1	putative tail length tape measure protein precursor Enterobacteria phage JK06	0.00E+00	88.3
41	complement (28001 - 30979)	2	TtpM Enterobacteria phage TLS	0.00E+00	66.5
41	complement (28001 - 30979)	3	putative tail tape measure protein Enterobacteria phage T1	0.00E+00	65.4
41	complement (28001 - 30979)	4	putative tail tape-measure protein Enterobacteria phage RTP	0.00E+00	82.9
41	complement (28001 - 30979)	5	minor tail protein H Escherichia coli O157 H7 str. EC4501	0.00E+00	62.2
41	complement (28001 - 30979)	6	phage tail tape measure protein lambda family Enterobacter cloacae subsp. cloacae NCTC 9394	0.00E+00	55.2
41	complement (28001 - 30979)	7	Minor tail protein H Escherichia coli O55 H7 str. CB9615	0.00E+00	61.5
41	complement (28001 - 30979)	8	phage tail tape measure protein Proteus mirabilis ATCC 29906	0.00E+00	56.3
41	complement (28001 - 30979)	9	putative tail length tape measure protein Escherichia coli O111 H- str. 11128	0.00E+00	62.7
41	complement (28001 - 30979)	10	minor tail protein H Escherichia coli O157 H7 str. EC508	0.00E+00	62.5

Table S1. Continued

ORF Number	Strand/Coordinates	Hit Number	Definition	E Value	Similarity
42	complement (31014 - 31268)	1	hypothetical protein T1P39 Enterobacteria phage JK06	3.10E-31	97.6
42	complement (31014 - 31268)	2	hypothetical protein rtp36 Enterobacteria phage RTP	6.70E-18	83.5
42	complement (31014 - 31268)	3	TfmS Enterobacteria phage TLS	2.40E-14	70.8
42	complement (31014 - 31268)	4	TfmB Enterobacteria phage TLS	3.10E-14	70.8
43	Complement (31331 - 31645	1	hypothetical protein JK_16 Enterobacteria phage JK06	2.20E-43	99
43	complement (31331 - 31645)	2	hypothetical protein rtp35 Enterobacteria phage RTP	2.10E-23	73.8
43	complement (31331 - 31645)	3	TfmA Enterobacteria phage TLS	1.00E-20	73.8
43	complement (31331 - 31645)	4	TfmS Enterobacteria phage TLS	1.10E-20	73.8
43	complement (31331 - 31645)	5	hypothetical protein T1p40 Enterobacteria phage T1	2.70E-20	73.1
44	complement (31718 - 32272)	1	hypothetical protein JK_17 Enterobacteria phage JK06	0.00E+00	92.9
44	complement (31718 - 32272)	2	putative major tail protein Enterobacteria phage RTP	0.00E+00	83.2
44	complement (31718 - 32272)	3	putative major tail protein Enterobacteria phage T1	0.00E+00	79.2
44	complement (31718 - 32272)	4	TsbA Enterobacteria phage TLS	0.00E+00	78.5
44	complement (31718 - 32272)	5	Phage tail protein. Enterobacter cloacae subsp. cloacae NCTC 9394	3.70E-33	64.8
44	complement (31718 - 32272)	6	major tail protein Proteus mirabilis ATCC 29906	1.10E-31	61.7
44	complement (31718 - 32272)	7	phage protein Proteus mirabilis HI4320	1.20E-31	61.7
44	complement (31718 - 32272)	8	hypothetical protein y2199 Yersinia pestis KIM 10	1.40E-30	63.5
44	complement (31718 - 32272)	9	hypothetical protein PROVALCAL_02309 Providencia alcalifaciens DSM 30120	1.50E-29	61.7
44	complement (31718 - 32272)	10	hypothetical protein YE2316 Yersinia enterocolitica subsp. enterocolitica 8081	7.00E-28	62.9
45	complement (32390 - 32872)	1	putative HNH endonuclease Enterobacteria phage RTP	8.50E-21	59.1

Table S1. Continued

ORF Number	Strand/Coordinates	Hit Number	Definition	E Value	Similarity
45	complement (32390 - 32872)	2	putative endonuclease protein Salmonella phage E1	1.30E-19	59.8
45	complement (32390 - 32872)	3	EndY Enterobacteria phage TLS	2.80E-19	57.4
45	complement (32390 - 32872)	4	EndZ Enterobacteria phage TLS	4.50E-19	57.7
45	complement (32390 - 32872)	5	endonuclease Escherichia coli B088	1.40E-16	58.3
45	complement (32390 - 32872)	6	predicted endonuclease Escherichia coli O127 H6 str. E2348/69	1.50E-16	58.3
45	complement (32390 - 32872)	7	putative HNH-endonuclease Enterobacteria phage RTP	2.20E-16	55.2
45	complement (32390 - 32872)	8	HNH endonuclease Shigella flexneri 2002017	4.10E-16	58.3
46	complement (32943 - 33329)	1	hypothetical protein JK_18 Enterobacteria phage JK06	0.00E+00	94.5
46	complement (32943 - 33329)	2	hypothetical protein rtp32 Enterobacteria phage RTP	3.10E-40	76
46	complement (32943 - 33329)	3	gp41 Enterobacteria phage TLS	2.20E-28	64
46	complement (32943 - 33329)	4	hypothetical protein T1p42 Enterobacteria phage T1	1.40E-24	64.2
47	complement (33331 - 33759)	1	phage-related hypothetical protein Enterobacteria phage JK06	0.00E+00	96.5
47	complement (33331 - 33759)	2	conserved phage-related protein Enterobacteria phage RTP	1.10E-42	73.9
47	complement (33331 - 33759)	3	gp40 Enterobacteria phage TLS	2.70E-25	60.4
47	complement (33331 - 33759)	4	hypothetical protein T1p43 Enterobacteria phage T1	2.00E-20	57
47	complement (33331 - 33759)	5	hypothetical protein BPKS7gp02 Salmonella phage KS5	3.00E-11	52.7
47	complement (33331 - 33759)	6	putative tail protein Escherichia phage K1ind1	5.50E-11	57.1
47	complement (33331 - 33759)	7	putative tail protein Escherichia phage K1ind3	7.80E-11	56.3
47	complement (33331 - 33759)	8	putative tail protein Escherichia phage K1G	7.90E-11	56.3
47	complement (33331 - 33759)	9	putative tail protein Escherichia phage K1H	8.10E-11	56.3
47	complement (33331 - 33759)	10	hypothetical protein SPSV3_gp44 Salmonella phage SETP3	9.20E-11	51.9
48	complement (33752 - 34120)	1	hypothetical protein JK_22 Enterobacteria phage JK06	7.00E-45	96.8

Table S1. Continued

ORF Number	Strand/Coordinates	Hit Number	Definition	E Value	Similarity
48	complement (33752 - 34120)	2	conserved phage-related protein Enterobacteria phage RTP	5.10E-30	69.9
48	complement (33752 - 34120)	3	gp39 Enterobacteria phage TLS	3.70E-22	63.4
48	complement (33752 - 34120)	4	hypothetical protein T1p44 Enterobacteria phage T1	8.10E-11	51.2
49	complement (34117 - 34518)	1	hypothetical protein JK_23 Enterobacteria phage JK06	0.00E+00	95.2
49	complement (34117 - 34518)	2	hypothetical protein rtp29 Enterobacteria phage RTP	5.30E-22	66.2
49	complement (34117 - 34518)	3	gp38 Enterobacteria phage TLS	4.40E-18	61.9
50	complement (34562 - 34807)	1	hypothetical protein rtp28 Enterobacteria phage RTP	2.50E-37	98.8
50	complement (34562 - 34807)	2	hypothetical protein JK_24 Enterobacteria phage JK06	9.10E-11	70.4
51	complement (34853 - 35377)	1	EndZ Enterobacteria phage TLS	6.10E-25	68.2
51	complement (34853 - 35377)	2	Putative HNH endonuclease Enterobacteria phage Felix 01	2.10E-23	61
51	complement (34853 - 35377)	3	hypothetical protein yberc0001_14950 uncultured organism MedDCM-OCT-S09-C20	1.30E-20	57.5
51	complement (34853 - 35377)	4	predicted HNH endonuclease Escherichia coli O127 H6 str. E2348/69	2.30E-20	61.1
51	complement (34853 - 35377)	5	Pathogenesis-related transcriptional factor and ERF protein Hyphomicrobium denitrificans ATCC 51888	2.60E-20	57.2
51	complement (34853 - 35377)	6	P42.1 Burkholderia glumae BGR1	3.80E-20	58.6
51	complement (34853 - 35377)	7	AP2 domain protein Escherichia coli MS 196-1	6.10E-20	60.4
51	complement (34853 - 35377)	8	endonuclease of the HNH family with predicted DNA-binding module in the C-terminus Xanthomonas phage Xp10	1.80E-19	63
51	complement (34853 - 35377)	9	hypothetical protein YE2335 Yersinia enterocolitica subsp. enterocolitica 8081	1.90E-19	60.1
51	complement (34853 - 35377)	10	p42.1 Xanthomonas phage Xop411	1.20E-18	64.4
52	complement (35448 - 36389)	1	hypothetical protein rtp27 Enterobacteria phage RTP	0.00E+00	90.4

Table S1. Continued

ORF Number	Strand/Coordinates	Hit Number	Definition	E Value	Similarity
52	complement (35448 - 36389)	2	hypothetical protein JK_25 Enterobacteria phage JK06	0.00E+00	86.1
52	complement (35448 - 36389)	3	hypothetical protein T1p47 Enterobacteria phage T1	0.00E+00	67.3
52	complement (35448 - 36389)	4	HsbB Enterobacteria phage TLS	0.00E+00	65.3
52	complement (35448 - 36389)	5	hypothetical protein Phage PY100	6.60E-27	47.1
52	complement (35448 - 36389)	6	hypothetical protein Bpet0981 Bordetella petrii DSM 12804	3.10E-26	50
52	complement (35448 - 36389)	7	hypothetical protein GCWU000324_01019 Kingella oralis ATCC 51147	7.20E-25	48.5
52	complement (35448 - 36389)	8	hypothetical protein CGSHi6P18H1_09335 Haemophilus influenzae 6P18H1	2.30E-24	48.7
52	complement (35448 - 36389)	9	hypothetical protein NTHI1532 Haemophilus influenzae 86-028NP	5.10E-24	48.7
52	complement (35448 - 36389)	10	conserved hypothetical protein Haemophilus influenzae NT127	5.10E-24	48.7
53	complement (36482 - 37171)	1	hypothetical protein PA2G_00830 Pseudomonas aeruginosa 2192	2.49	46.5
54	complement (37268 - 37789)	1	hypothetical protein rtp26 Enterobacteria phage RTP	4.00E-42	76.1
54	complement (37268 - 37789)	2	hypothetical protein T1p49 Enterobacteria phage T1	8.90E-15	62.9
54	complement (37268 - 37789)	3	hypothetical protein T1p48 Enterobacteria phage T1	9.80E-12	59.8
54	complement (37268 - 37789)	4	HtjA Enterobacteria phage TLS	4.40E-11	56
55	complement (37802 - 38887)	1	possible prohead protease Enterobacteria phage RTP	0.00E+00	96.7
55	complement (37802 - 38887)	2	hypothetical protein JK_31 Enterobacteria phage JK06	0.00E+00	80.7
55	complement (37802 - 38887)	3	HspA Enterobacteria phage TLS	0.00E+00	68.8
55	complement (37802 - 38887)	4	putative major head subunit precursor Enterobacteria phage T1	0.00E+00	66.2
55	complement (37802 - 38887)	5	hypothetical protein GCWU000324_01017 Kingella oralis ATCC 51147	1.20E-37	68.5
55	complement (37802 - 38887)	6	hypothetical protein Bpet0979 Bordetella petrii DSM 12804	1.30E-36	56.6

Table S1. Continued

ORF Number	Strand/Coordinates	Hit Number	Definition	E Value	Similarity
55	complement (37802 - 38887)	7	hypothetical protein NTHI1530 Haemophilus influenzae 86-028NP	3.90E-31	53.7
55	complement (37802 - 38887)	8	hypothetical protein CGSHi22121_00917 Haemophilus influenzae 22.1-21	6.70E-31	52.8
55	complement (37802 - 38887)	9	hypothetical protein SG1554 Sodalis glossinidius str. 'morsitans'	2.40E-30	60.5
55	complement (37802 - 38887)	10	hypothetical protein CGSHi6P18H1_09325 Haemophilus influenzae 6P18H1	2.70E-30	52.8
56	complement (38880 - 40133)	1	putative portal protein Enterobacteria phage RTP	0.00E+00	98.5
56	complement (38880 - 40133)	2	PorT Enterobacteria phage TLS	0.00E+00	70.8
56	complement (38880 - 40133)	3	putative portal protein Enterobacteria phage T1	0.00E+00	72.8
56	complement (38880 - 40133)	4	putative bacteriophage protein Enterobacteria phage JK06	0.00E+00	81.6
56	complement (38880 - 40133)	5	hypothetical protein JK_33 Enterobacteria phage JK06	0.00E+00	90.5
56	complement (38880 - 40133)	6	hypothetical protein GCWU000324_01015 Kingella oralis ATCC 51147	3.10E-29	48.3
56	complement (38880 - 40133)	7	hypothetical protein NTHI1528 Haemophilus influenzae 86-028NP	1.10E-25	49.6
56	complement (38880 - 40133)	8	conserved hypothetical protein Haemophilus influenzae NT127	1.20E-25	49.6
56	complement (38880 - 40133)	9	hypothetical protein CGSHi22121_00907 Haemophilus influenzae 22.1-21	6.00E-25	49.4
56	complement (38880 - 40133)	10	hypothetical protein Bpet0977 Bordetella petrii DSM 12804	9.60E-25	50.9
57	complement (40316 - 40522)	1	hypothetical minor outer capsid protein Enterobacteria phage JK06	6.76	50
58	complement (40592 - 42154)	1	putative terminase large subunit Enterobacteria phage RTP	0.00E+00	88.1
58	complement (40592 - 42154)	2	putative terminase large subunit Enterobacteria phage T1	0.00E+00	73.8
58	complement (40592 - 42154)	3	TerL Enterobacteria phage TLS	0.00E+00	71

Table S1. Continued

ORF Number	Strand/Coordinates	Hit Number	Definition	E Value	Similarity
58	complement (40592 - 42154)	4	hypothetical protein ECL_03565 Enterobacter cloacae subsp. cloacae ATCC 13047	0.00E+00	61.7
58	complement (40592 - 42154)	5	phage uncharacterized protein Yersinia pseudotuberculosis IP 31758	0.00E+00	59.5
58	complement (40592 - 42154)	6	hypothetical protein KPK_4143 Klebsiella pneumoniae 342	0.00E+00	60.4
58	complement (40592 - 42154)	7	Phage uncharacterized protein Yersinia frederiksenii ATCC 33641	0.00E+00	59.7
58	complement (40592 - 42154)	8	hypothetical protein Bpse38_36064 Burkholderia thailandensis MSMB43	0.00E+00	59.2
58	complement (40592 - 42154)	9	Phage uncharacterized protein Burkholderia dolosa AUO158	0.00E+00	59.4
58	complement (40592 - 42154)	10	putative phage relative terminase Enterobacteria phage JK06	0.00E+00	98.8
59	complement (42154 - 42642)	3	putative endonuclease protein Salmonella phage E1	5.90E-29	68.4
59	complement (42154 - 42642)	4	EndY Enterobacteria phage TLS	1.10E-27	65.6
59	complement (42154 - 42642)	5	putative HNH endonuclease Enterobacteria phage T1	3.90E-27	69.3
59	complement (42154 - 42642)	6	HNH endonuclease Shigella flexneri 2002017	5.20E-27	61.3
59	complement (42154 - 42642)	7	HNH endonuclease Shigella sp. D9	5.30E-26	60.7
59	complement (42154 - 42642)	8	endonuclease Escherichia coli B088	9.70E-26	62.6
59	complement (42154 - 42642)	9	putative endonuclease Enterobacteria phage T1	1.30E-25	67.3
59	complement (42154 - 42642)	10	putative HNH-endonuclease Enterobacteria phage RTP	2.80E-24	56.9
60	complement (42708 - 43223)	1	putative terminase small subunit Enterobacteria phage RTP	0.00E+00	81.6
60	complement (42708 - 43223)	2	putative terminase small subunit Enterobacteria phage T1	4.70E-43	71.6
60	complement (42708 - 43223)	3	packaging protein gp3 Enterobacteria phage JK06	2.50E-40	93.1
60	complement (42708 - 43223)	4	TerS Enterobacteria phage TLS	1.00E-39	70.8
60	complement (42708 - 43223)	5	RecName Full=DNA-packaging protein gp3; AltName Full=Terminase small subunit	9.30E-11	52.9

Table S1. Continued

ORF Number	Strand/Coordinates	Hit Number	Definition	E Value	Similarity
60	complement (42708 - 43223)	6	Gp3 Salmonella phage SETP1	9.40E-11	52.9
60	complement (42708 - 43223)	7	Terminase small subunit Salmonella phage epsilon34	9.80E-11	52.9
65	complement (44237 - 44476)	1	hypothetical protein rtp15 Enterobacteria phage RTP	4.60E-14	70.5
65	complement (44237 - 44476)	2	hypothetical protein T1p59 Enterobacteria phage T1	6.80E-11	69.4
66	complement (44478 - 44669)	1	hypothetical protein rtp14 Enterobacteria phage RTP	0.15	55.3
67	complement (44672 - 44821)	1	hypothetical protein T1p04 Enterobacteria phage T1	4.70E-05	73.8
68	complement (44803 - 44937)	1	hypothetical protein JK_42 Enterobacteria phage JK06	1.50E-06	84.4
71	complement (45289 - 45540)	1	hypothetical protein rtp5 Enterobacteria phage RTP	8.50E-26	86.3
71	complement (45289 - 45540)	2	hypothetical protein JK_43 Enterobacteria phage JK06	1.80E-22	83.8
71	complement (45289 - 45540)	3	hypothetical protein SPSV3_gp48 Salmonella phage SETP3	6.90E-16	70.3
74	complement (45795 - 46034)	1	hypothetical protein JK_44 Enterobacteria phage JK06	1.50E-18	74
75	complement (46106 - 46648)	1	hypothetical protein JK_46 Enterobacteria phage JK06	1.30E-30	65.2
75	complement (46106 - 46648)	2	hypothetical protein JK_55 Enterobacteria phage JK06	7.80E-23	87.1
75	complement (46106 - 46648)	3	hypothetical protein rtp67 Enterobacteria phage RTP	2.90E-22	85.7
75	complement (46106 - 46648)	3	hypothetical protein rtp67 Enterobacteria phage RTP	1.30E-07	66.7
75	complement (46106 - 46648)	4	YfiJ Enterobacteria phage TLS	4.30E-13	70.7
76	complement (46648 - 47163)	1	hypothetical protein rtp1 Enterobacteria phage RTP	3.60E-39	70.2
76	complement (46648 - 47163)	2	hypothetical protein JK_47 Enterobacteria phage JK06	5.30E-20	56.5

Figure 1. Dendrogram of the 77 phage isolates generated from a hierarchical cluster analysis of the plaque fingerprints of each phage on 23 different *E. coli* strains. Clusters represent groups of phage with identical host plaque patterns. The starred isolates are capable of generalized transduction.

Figure 1.

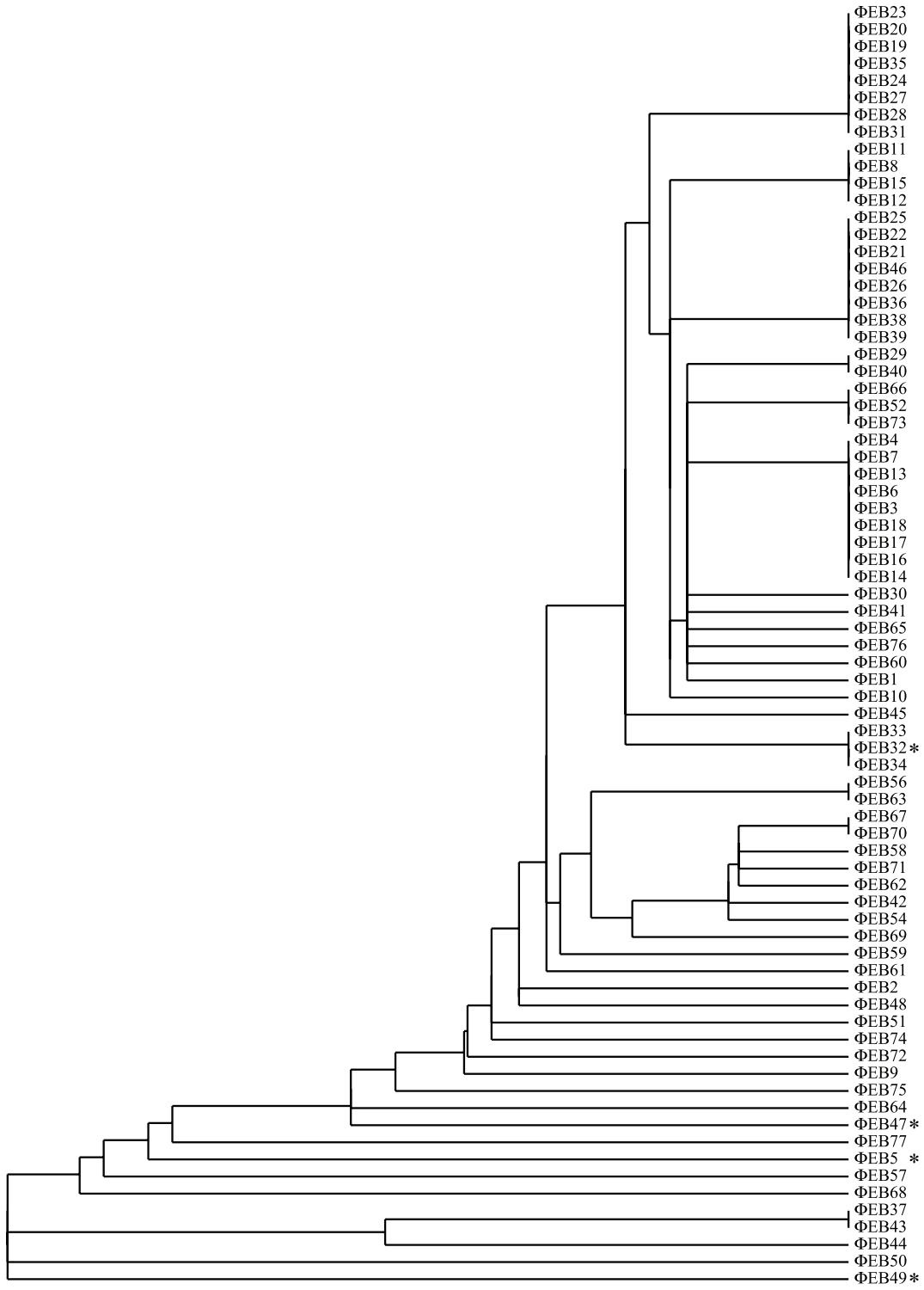

ΦEB23
ΦEB20
ΦEB19
ΦEB35
ΦEB24
ΦEB27
ΦEB28
ΦEB31
ΦEB11
ΦEB8
ΦEB15
ΦEB12
ΦEB25
ΦEB22
ΦEB21
ΦEB46
ΦEB26
ΦEB36
ΦEB38
ΦEB39
ΦEB29
ΦEB40
ΦEB66
ΦEB52
ΦEB73
ΦEB4
ΦEB7
ΦEB13
ΦEB6
ΦEB3
ΦEB18
ΦEB17
ΦEB16
ΦEB14
ΦEB30
ΦEB41
ΦEB65
ΦEB76
ΦEB60
ΦEB1
ΦEB10
ΦEB45
ΦEB33
ΦEB32*
ΦEB34
ΦEB56
ΦEB63
ΦEB67
ΦEB70
ΦEB58
ΦEB71
ΦEB62
ΦEB42
ΦEB54
ΦEB69
ΦEB59
ΦEB61
ΦEB2
ΦEB48
ΦEB51
ΦEB74
ΦEB72
ΦEB9
ΦEB75
ΦEB64
ΦEB47*
ΦEB77
ΦEB5 *
ΦEB57
ΦEB68
ΦEB37
ΦEB43
ΦEB44
ΦEB50
ΦEB49*

Figure 2. Colony PCR with *dsdA* (A) and *lacZ* (B) specific primers on transductants from each phage in duplicate. WAM3686 (*ΔdsdA::Kan*) and WAM2909 (WT *dsdA*) and WAM2909 (*ΔlacZ::Cm*) were included as controls.

Figure 2.

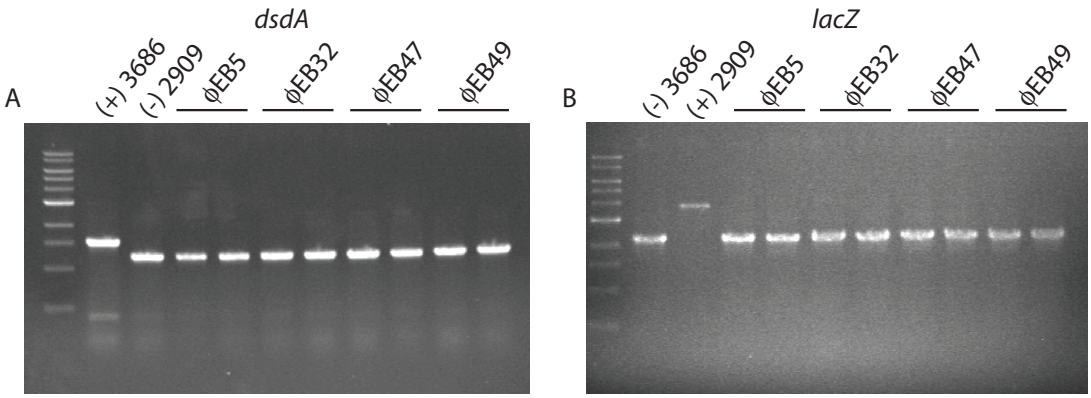

A
dsdA
(+) 3686
(-) 2909
ϕEB5
ϕEB32
ϕEB47
ϕEB49
B
lacZ
(-) 3686
(+) 2909
ϕEB5
ϕEB32
ϕEB47
ϕEB49

Figure 3. Electron micrographs of the 4 confirmed transducing phages. (A) ΦEB49, (B) ΦEB5, (C) ΦEB32, (D) ΦEB47.

Figure 3.

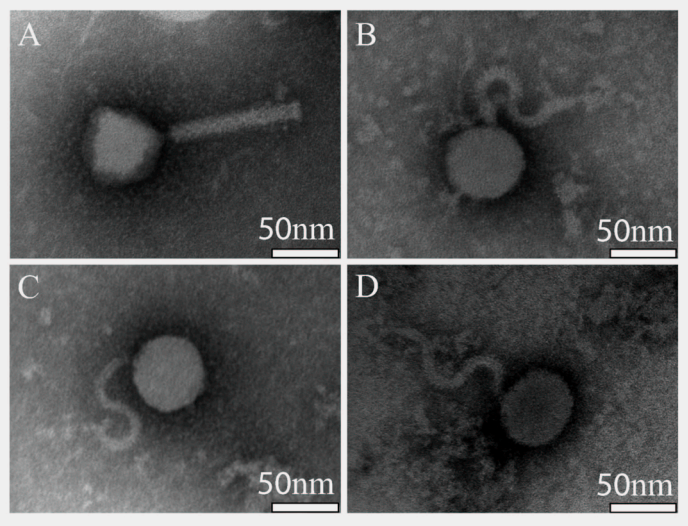
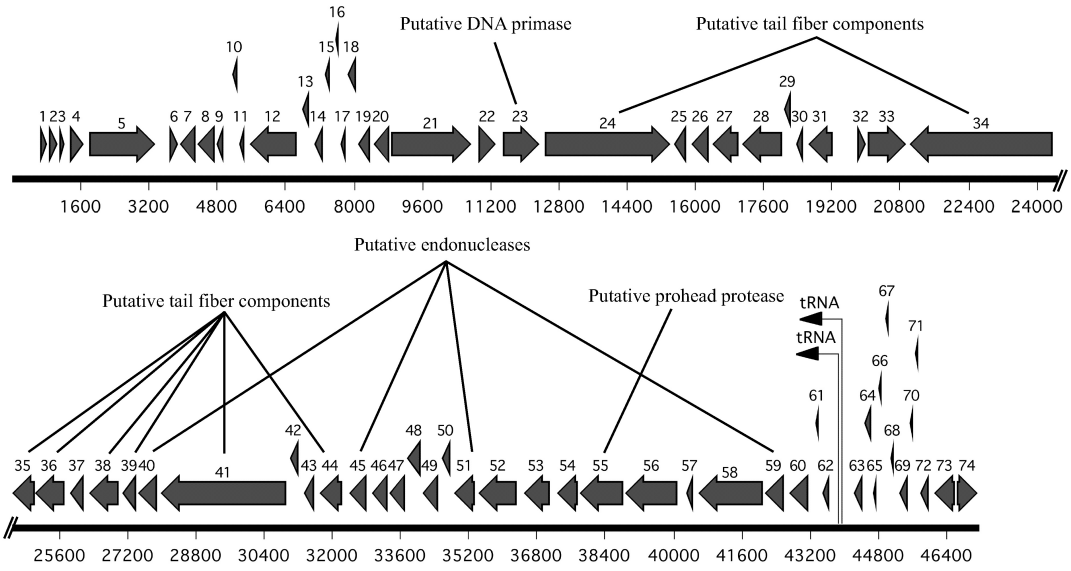


Figure 4. Annotated map of the 47,180 bp ΦEB49 genome. The computer based genome annotation software DNAMaster (http://cobamide2.bio.pitt.edu/) predicted the location of 74 ORFs and 2 tRNAs.

Figure 4.


Putative DNA primase
Putative tail fiber components
1600
3200
4800
6400
8000
9600
11200
12800
14400
16000
17600
19200
20800
22400
24000
Putative endonucleases
Putative tail fiber components
Putative prohead protease
tRNA
tRNA
25600
27200
28800
30400
32000
33600
35200
36800
38400
40000
41600
43200
44800
46400

Chapter 3

ipuS, a novel third phase variable element in the uropathogenic *Escherichia coli* strain CFT073 contributes to infection and reveals a complex recombinase mediated virulence network.

Authors and Contributors:

Eric Battaglioli: Wrote manuscript, planned and performed all experiments, and analyzed data except as indicated below.

Michelle Rothenhoefer: Provided technical assistance with mouse infection assays.

Kevin Schwartz: Planned and performed kidney epithelial cell adhesion assays for the phase lock mutant strains.

Rodney Welch: Supervised all research and provided intellectual guidance for the design and execution of the experiments.

This chapter is being prepared for submission to Infection and Immunity.

Abstract

Uropathogenic *Escherichia coli* (UPEC) are the most common etiological agent of uncomplicated urinary tract infection (UTI). Over 50% of women will experience a UTI in their lifetime and treatment of these common infections constitutes a significant burden on the US health care system. UPEC possess a suite of virulence factors that are carefully regulated to facilitate survival within the urinary tract. Phase variation by DNA rearrangement is one mechanism used to regulate virulence genes in the UPEC strain CFT073. These systems function by associated tyrosine recombinases inverting promoter-containing elements, and re-orientation of the promoter turns ON or OFF expression of target genes. CFT073 has five tyrosine site-specific recombinases that function at its two known inversion systems, *fimS* and *hyxS*. Three of the five recombinases are encoded in close proximity to the two elements, which is typical of inversion systems. The other two recombinases, IpuA and IpuB are encoded distantly from both sites. Because of the spatial separation, it was predicted that an additional invertible element was located proximal to *ipuA* and *ipuB*. Here we show the identification and characterization of a third invertible element in CFT073, *ipuS*. *ipuS* inversion is catalyzed by at least 4 of the 5 recombinases in CFT073. Orientation of the element determines expression of a two-gene operon upstream of *ipuA,* which contains *ipuR* and *upaE*, a predicted LuxR-type regulator and predicted secreted autotransporter respectively. The *ipuS* ON state causes a defect in swimming motility, an increase in adherence to human kidney epithelial cells, and has a competitive advantage over the *ipuS* OFF state in the kidneys in a murine model of UTI. The identification of a third invertible element with virulence effects that is regulated by a

shared set of recombinases outlines the existence of a phase variable network that simultaneously regulates all three sites to facilitate infection.

Introduction

Urinary tract infections (UTIs) are one of the most common infections diagnosed in clinics and hospitals in the United States. Nearly 50% of women will experience a UTI in their lifetime with annual treatment costs exceeding $1.6 billion annually (1, 2). The most common etiological agent of uncomplicated UTIs are uropathogenic *Escherichia coli* (UPEC) which account for 80-90% of reported infections (3). The predicted reservoir of UPEC is the colon, and infection arises from fecal contamination of the urethra. Once the bacteria gain access to the urinary tract, they face a variety of host defenses including shedding of uroepithelial cells, low iron levels, rapid recruitment of professional phagocytes, and the cleansing flow of urine (4-10). UPEC possess many specialized virulence factors that help it establish and maintain an infection. These include secreted toxins, multiple iron acquisition systems, a polysaccharide capsule, effective reactive nitrogen species detoxification systems and adhesive pili (11-18).

Type 1 pili are polytrichous hair like projections expressed on the surface of nearly all UPEC isolates (19). They mediate attachment and invasion of the bladder epithelium, are a key component of the “stick or swim” lifestyle choice and are critical to establish and maintain an infection in the murine model of UTI (11, 12, 20, 21). Expression of Type 1 pili is phase variable as a result of a promoter rearrangement of the invertible element, or “switch”, *fimS* (22, 23). In the non-pathogenic laboratory strain *E. coli* K-12, inversion of *fimS* is catalyzed by the tyrosine site-specific recombinases FimB and FimE (24). In

addition to the recombinases, the DNA binding proteins IHF, LRP and H-NS interact with *fimS* to facilitate formation of the appropriate 3D conformation necessary for recombination. Regulation of the recombinases and the DNA binding proteins leads to changes in the population phase state (25-28). Additionally, crosstalk with genes from Pap pili and specific environmental conditions including pH, osmolarity, temperature and metabolite availability are known to create phase biases (29-34).

In addition to these methods of regulation, the UPEC strain CFT073 has three additional FimB/FimE-like tyrosine recombinases, FimX, IpuA and IpuB, which are conserved in uropathogens (20). Typically, the site-specific recombinases that mediate inversion of phase switches are encoded proximal to the switch suggesting the existence of other switches local to the three additional recombinases (35). Inversion of *fimS* in CFT073 can be catalyzed by FimX and IpuA despite being located distantly from *fimS* in the CFT073 chromosome (20). Recently, a second phase variable element, *hyxS*, was characterized proximal to *fimX* in CFT073 and the related UPEC strain UTI89 (36). Inversion of *hyxS* regulates *hyxR*, a LuxR-type regulator. Only FimX is capable of catalyzing inversion of this switch, and *hyxS*-dependent expression of *hyxR* affects resistance to reactive nitrogen species and intracellular macrophage survival (36).

With known switches next to *fimB*, *fimE* and *fimX*, we sought to determine if a phase switch existed proximal to *ipuA* and *ipuB*. Here we report the identification of a third invertible switch in CFT073, *ipuS*. The switch is bounded by a set of 7 bp-inverted repeats and the recombination half sites share sequence similarity with the *fimS* and *hyxS* invertible elements. 5'RACE analysis indicates the presence of a putative transcription start site in the element and 4 of the 5 recombinases (FimB excluded) are independently

capable of catalyzing inversion. An *ipuS* phase lock ON strain shows a defect in swimming motility, an increase in adherence to kidney epithelial cells and a colonization advantage in the kidneys when compared to the *ipuS* lock OFF strain. The identification of a third switch that relies on a shared set of recombinases with two other virulence-regulating switches reveals a complex inter-regulated virulence network.

Methods and Materials

Bacterial strains, cell lines, plasmids and culture conditions. All of the strains, cell lines and plasmids used in this study are listed in Table 1. In frame deletion mutants of the uropathogenic *Escherichia coli* strain CFT073 were generated using a modification of the Lambda-red method of homologous recombination to include phage transduction of the marker into a clean genetic background by ΦEB49 prior to removal of the cassette via pCP20 (37, 38). *lacZ* transcriptional fusions were generated using methods described previously with the suicide vector pFUSE (39). All strains were cultivated in Luria Bertani (LB) broth, LB agar, or on MacConkey's lactose medium unless otherwise indicated. Antibiotic selection (kanamycin [50 μg/ml], chloramphenicol [20 μg/ml], carbenicillin [250 μg/ml] was used where appropriate.

The kidney epithelial cell line A-498 (ATCC HTB-44) was grown in RPMI 1640 with L-glutamine (Mediatech, Inc., Manassas, VA) supplemented with 20% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 10 mM HEPES, and 1 mM sodium pyruvate (Mediatech, Inc.). Cells were grown at 37°C with 5% CO_2 and utilized at less than 10 passages.

***ipuS* switch state analysis.** The *ipuS* region was amplified by PCR using GoTaq Green Master mix (Promega, Madison, WI) from 0.5 μl of over night LB broth cultures using the forward primer 5'- GTGGCGATGGGAAGGAAACG-3' and reverse primer 5'-AAAACCCCGCCAACGCATACTC-3'. Thermocycling conditions were 94^{o}C for 2 min, 25 cycles of 94^{o}C for 30 sec, 57^{o}C for 30 sec, 72^{o}C for 1 min 30 sec, and 72^{o}C for 7 min. The resulting approx. 1289 bp product was purified using a Qiaquick PCR purification kit (Qiagen, Valencia, CA) and digested with PacI (NEB, Ipswitch, MA). Digested fragments were electrophoresed through 2% agarose gel and stained with ethidum bromide. Sizes of the restriction products correspond to the state of the switch (407 bp and 882 bp – phase ON; 186 bp and 1103 bp, phase OFF).

Recombinase complementation plasmid construction. Each CFT073 recombinase was cloned into either pACYC177 or pACYC184. Constructs built within the pACYC177 backbone are constitutively expressed from the plasmid encoded Kan^{r} promoter. Constructs built within pACYC184 are expressed from the native promoter of the cloned insert. All clones were confirmed by PCR and Sanger dideoxy chain termination sequencing of the inserts.

5'RACE of *ipuS* element. A putative *ipuS* transcription start site was identified using 5' Rapid Amplification of cDNA Ends (5'RACE) (Invitrogen, Grand Island, NY). Gene specific nested primers were designed according the manufacturers instructions. RNA was extracted from 1 ml of log phase (OD_{600}=0.5) culture of WAM5064 and WAM5065 using Trizol reagent (Invitrogen, Grand Island, NY). Contaminating DNA was removed by on column DNase treatment and Pure Link RNA spin column purification (Invitrogen, Grand

Island, NY) and the resulting purified RNA samples were stored in nuclease-free water at -20°C. Aliquots of the isolated RNA were processed using the 5'RACE Kit and gene specific primers (Invitrogen, Grand Island, NY) according to manufacturers instructions. The resulting PCR products were sequenced using Sanger dideoxy chain termination sequencing to identify putative transcription start sites.

Murine model of UTI. Six-week-old female CBA/J (Harlan Laboratories, Indianapolis, IN) mice were used for all infections and were performed as described previously (40). For competitive infections WAM5146, a *lacZYA* mutant variant of WAM5065, was used to facilitate generation of competitive indices with MacConkey's lactose medium. Previous experiments indicate that *lacZYA* activity has no influence on colonization in the murine model of UTI (41). Bars on presented data indicate the medians of the non-Gaussian distributed data sets. Wilcoxin-Signed Rank Tests were performed with the data analysis software package Prism (Graphpad Software, La Jolla, CA) to determine statistical significance and reported when $p<0.05$.

Swimming motility assay. Strains were grown in LB broth with appropriate selection over night at 37°C with shaking. The overnight cultures were normalized to OD_{600}=0.5 and 1 μl was inoculated directly into the center of a Petri plate containing 20 ml of Adler's Motility Medium (0.3% agar, 0.5% NaCl, 1.0% tryptone) (N=7). Plates were incubated lid side up at room temperature for approx. 21 hours. The diameter of the zone of swimming was measured twice at perpendicular angles for each plate and the averages were plotted. The data was analyzed using 1-way ANOVA with the data analysis software

package Prism (Graphpad Software, La Jolla, CA) to determine statistically significant differences between strains and reported when $p<0.05$.

Kidney epithelial cell adherence assay. A-498 cells were seeded into 12-well plates at 2.5 x 10^5 cells/well and grown to near confluence. Monolayers were washed two times with assay medium (serum and antibiotic free culture medium) and pre-incubated for 20 min at 4°C in 1 ml assay medium. Triplicate wells were inoculated with bacteria (MOI=10) and were settled onto host cells by centrifugation at 500 x g for 5 min. After 1hr incubation at 4°C, monolayers were washed three times with HBSS (Hyclone, Logan, UT), incubated for 5 min at 37°C in 500 µl 0.025% trypsin/0.03% EDTA in HBSS, lysed with 0.1% Triton X-100 in ddH_2O and plated on LB-agar plates. Adherence was calculated as the ratio of the number of bacteria recovered to the number of bacteria inoculated into each well and expressed as percent adherence.

Results

Identification of a novel phase variable element, *ipuS*. The recent identification of a second switch in CFT073 proximal to the tyrosine recombinase encoding gene *fimX* prompted us to explore the region surrounding *ipuA* and *ipuB* for a third switch (36). Previous studies using extensive PCR and sequence analysis designed to detect inversions were performed on the region downstream of *ipuB* in the *dsdCXA* locus and no switches were identified (data not shown) so we focused on the region upstream of *ipuA*. Immediately upstream of *ipuA* is a two-gene operon containing *ipuR* and *upaE*, a predicted LuxR-type regulator and predicted autotransporter respectively. Separating *ipuA* from the

operon is a 317 bp intergenic spacer containing no predicted open reading frames (Figure 1A). The size of this spacer is consistent with other promoter inversion systems so a *lacZ* transcriptional fusion was generated with *ipuR* (WAM5009) to detect putative inversion events. When plated on MacConkey's lactose medium, the reporter strain displays a mix of red and white colonies, the majority white (data not shown). The intergenic spacer predicted to contain an invertible element was amplified by PCR from a red and white colony and sequenced by Sanger dideoxy chain termination. The sequencing revealed the presence of a 260 bp invertible element, *ipuS* (Figure 1B). The element is bounded by a pair of 7 bp-inverted repeats with the distal inverted repeat located within the annotated coding sequence of *ipuA* (Figure 1B). In the OFF state, defined as no expression from the *ipuR-lacZ* transcriptional fusion, the full-length form of IpuA is produced. Upon inversion to the ON state, defined as expression of the *ipuR-lacZ* fusion, a truncation of the *ipuA* coding sequence occurs. The truncation removes 11 amino acids from the C-terminus of IpuA and generates a K-L substitution of the terminal amino acid (Figure 1C). None of the four predicted required RHRY active site residues are altered by the truncation, suggesting the truncated form may still be catalytically active (Figure 1C).

***ipuS* half site analysis.** The *ipuS* invertible element is defined by a pair of 7 bp-inverted repeats (Figure 2A). *ipuS* has the shortest inverted repeats of the three described elements in CFT073 with *fimS* and *hyxS* having 9 and 16 bp repeats respectively (23, 36). In addition to the core repeat sequence, 5 bp surrounding the core can participate in base pairing and the sequence of these residues is similar to the respective required regions of *fimS* (19). The predicted hairpin structure generated during recombination illustrates these potential base-pairing interactions (Figure 2B).

The inverted repeats and surrounding sequence of *ipuS* were compared to the same respective regions of *hyxS* and *fimS*. For consistency, the OFF state of each element was used for the comparisons. A high degree of sequence similarity is shown between *ipuS* and the other two switches, particularly *fimS* (Figure 2C). Lines connecting bases indicate sequence identity, inverted repeat sequence is in bold and the grey boxes indicate potential base pairing interactions (Figure 2C). This analysis was also performed for the ON state of the elements and yielded similar results (data not shown). Previous reports comparing the half sites of *hyxS* and *fimS* show only limited similarity (36).

Recombinase activity at *ipuS.* The activity of tyrosine recombinases at *fimS* is known to be sequence specific. Similarity among the inverted repeats suggests that many if not all of the five recombinases would have activity at *ipuS*. To test this, we deleted the five recombinases by Lambda-red mutagenesis and ΦEB49 phage transduction to lock the orientation of all three switches. In the case of *ipuA*, only 404 bp from the 5' end was deleted. The truncation removes one of the predicted active site residues but preserves the distal inverted repeat allowing for inversion via exogenous expression of the recombinases. A five-way recombinase mutant strain created with the *ipuA* 404 bp deletion is phase locked suggesting the deletion creates a non-functional gene product. Strains were created with all four possible combinations of *ipuS* and *fimS* phase states for later analysis. For simplicity, *hyxS* was left OFF in all strains examined (Table 1).

Each recombinase including the full length (FL) and truncated (Trunc) forms of IpuA was provided in trans on multi-copy expression constructs in both the *ipuS* ON and OFF locked backgrounds. All the recombinase complementation plasmids were constructed in

pACYC177 and constitutively expressed except for IpuA-FL and IpuA-Trunc. The IpuA plasmids were constructed in pACYC184 with the native *ipuA* promoter driving expression. In a previous publication we showed that constitutive expression of *ipuA* from pACYC177 causes cell morphology defects and expression from its native promoter is required (20).

The ability of a single recombinase to switch the orientation from a starting locked state was assayed by PCR amplification of the switch and asymmetric restriction digestion of the product by PacI. With the exception of FimB, all the recombinases are independently capable of catalyzing inversion in both directions, including the truncated form of IpuA (Figure 3). FimB shows no detectable catalytic activity under the conditions tested. However the same pACYC177::*fimB* construct is capable of inverting *fimS* suggesting the lack of activity is not due to complications with exogenous expression (20). For completeness, it was confirmed that only FimX is capable of inverting *hyxS* in CFT073 and IpuA-Trunc has the same activity at *fimS* as IpuA-FL (data not shown).

Identification of a putative promoter in *ipuS.* The *ipuA* transcription start site was mapped by primer extension to a position 27 bp upstream of the ATG start codon and most closely resembles a sigma-70 dependent promoter (unpublished results). We postulated that the invertible element could control expression of *ipuR-upaE* by containing a promoter, or by blocking read through of the native *ipuA* promoter. To test this, we subjected the *ipuS* region to 5'RACE. The assay was performed on cDNA generated from both *ipuS* phase lock ON and OFF strains. Only the ON strain generated a product and subsequent sequencing revealed the location of a putative transcriptional start site in *ipuS* (Figure 4A). We analyzed the region immediately upstream of the predicted transcriptional

start site and identified a putative promoter most closely resembling the *rpoE* consensus sequence (Figure 4B).

***ipuS* ON cells show a defect in swimming motility.** The ON state of the switch results in expression of the predicted LuxR-type regulator *ipuR* and the predicted autotransporter *upaE*. Due to the switch's conservation in UPEC and the known links between LuxR-type regulators/autotransporters in pathogenesis (17, 18, 42-46), we predicted orientation of the element would affect virulence. We began testing for an *ipuS* specific affect on known virulence phenotypes. Swimming motility was examined because of the adhesive properties of autotransporters and the reciprocal relationship known to exist between adhesion and motility. Overnight liquid cultures were used to inoculate Adler's Motility Medium plates and diameters of the swimming zones were measured after ~21 hrs of growth at room temperature. In a *fimS* OFF (motile) background, the *ipuS* ON state decreases swimming motility. The same trend is observed in the *fimS* ON background, however the non-motile nature of *fimS* ON cells made it difficult to discern the *ipuS* effects (Figure 5).

***ipuS* orientation affects colonization of the kidneys in a murine model of UTI and adherence to human kidney epithelial cells.** We then determined if the in vitro *ipuS* swimming phenotype resulted in any differences in colonization in the murine model of UTI. We competed *ipuS* locked ON and OFF strains against one another to address *ipuS* specific effects on colonization. A *ΔlacZYA* mutant variant of the *ipuS* lock OFF strain (WAM5146) was used to facilitate generation of competitive indices using MacConkey's lactose medium. Previous experiments indicate that a *ΔlacZYA* mutant of CFT073 competes

equally against WT (40). 50 μl inoculums containing an equal ratio of the *ipuS* lock ON and OFF strains totaling 10^8 CFU were transurethrally delivered into the bladder of 6-week-old female CBA/J mice. The infections were allowed to progress for 24, 48 or 72 hours. The animals were sacrificed at the appropriate time point and their bladders and kidneys were excised, homogenized and plated on MacConkey's lactose medium. Ratios of ON/OFF bacteria at sacrifice were normalized to the input ratio to generate relative competitive indices (RCI). An RCI=1 indicates the strains compete equally against one another, an RCI<1 indicates the *ipuS* OFF state has an advantage and an RCI>1 indicates the *ipuS* ON state has an advantage. The *fimS* lock ON variants of the *ipuS* ON/OFF strains were selected for the competition because the *fimS* lock OFF strains did not yield consistent infections (data not shown). At 72 hpi, a 5-fold advantage ($p<0.05$) for the *ipuS* ON state is observed in the kidneys (Figure 6A). No difference is seen in the bladder at 72 hpi, or in the bladder and kidneys at 24 hpi and 48 hpi (data not shown). We also performed competition experiments with a *ΔipuR, ΔupaE* double mutant vs. WT and determined RCIs at 24, 48 and 72 hpi. No difference in colonization of the bladder or kidneys was observed at any of these time points (data not shown).

The locked strains that were used in the competitive infection assays are virtually non-motile due to the constitutive expression of Type 1 pili. The lack of motility suggests that the in vitro swimming motility defect of the *ipuS* ON/*fimS* OFF strain (WAM5088) was not the cause of the *ipuS*-dependent kidney colonization advantage. A defect in swimming motility can be attributed to the production of adhesion promoting-structures (20). We predicted that the swimming motility defect of the *ipuS* ON state is an indirect effect of the

production of a kidney specific adhesion moiety. To test for kidney adherence we assayed the four *ipuS*/*fimS* phase locked strains for their ability to adhere to human kidney epithelial cells. The strains were allowed to adhere to confluent monolayers of A-498 cells (MOI=10) on ice for 1 hr. Non-adherent bacteria were removed with media washes and the remaining bacteria were enumerated by lysing the kidney cells with triton and plating on LB-agar medium. The number of adherent bacteria was normalized to the input and expressed as percent adherence (Figure 6B). Locking *ipuS* ON in a *fimS* OFF background increases adherence to kidney epithelial cells ($p<0.05$). Type 1 pili also promote kidney adherence, however locking both switches on does not cause a synergistic increase in adhesion (Figure 6B).

Discussion

Phase variation is defined as frequent and reversible ON/OFF changes in gene expression (47). It has been shown to occur by several different molecular mechanisms and to contribute to virulence in pathogenic *Escherichia coli*, *Neisseria meningiditis*, *Mycoplasma agalactiae*, *Listeria monocytogenes* and others (48-51). It is an advantageous form of gene regulation for pathogens as it helps a population cope with sudden changes in environmental conditions during infection (52). Having a preformed subset of the population in alternative phase states circumvents the need for transcriptional and translational activation steps. CFT073 has two known phase variable elements (20, 36), and this report presents the identification of a third, *ipuS* (Figure 1).

When comparing the sequences of the half sites, *ipuS* appears to be an intermediate between *fimS* and *hyxS* (Figure 2). This suggests that the non-proximal recombinases FimB,

FimE and FimX would have activity at *ipuS* and indeed it was shown that FimE and FimX are catalytically active at *ipuS* (Figure 3). Only limited sequence similarity was reported between *fimS* and *hyxS* in UTI89, which may account for why only the proximally encoded FimX is able to function at *hyxS* in both CFT073 and UTI89 (36).

The five recombinases display varying abilities for inverting the *ipuS* element, which indicates potential directional biases (Figure 3). The directional bias for FimB/FimE at *fimS* has been characterized extensively in *E. coli* K-12 and is due to DNA binding specificity of the recombinases at the inverted repeats and surrounding sequence (19, 53-56). FimE is unable to bind to the *fimS* half sites in the OFF orientation, which restricts its activity to catalyzing the ON to OFF inversion. By mutating the regions outside the inverted repeats to resemble the ON or OFF state, the specificity can be reversed (54). It is possible that the patterns of recombinase activity at *ipuS* are due to differences in the recombinase's ability to bind to the template in the ON/OFF state.

The 5'RACE analysis identified the 5' end of an mRNA transcript within *ipuS* that could be a transcriptional start site in the *ipuS* element (Figure 4). The predicted start site is part of the full-length *ipuA* coding region. By sequence gazing for conserved promoter motifs proximal to the putative start site we were able to identify a putative *rpoE* dependent promoter. RpoE, or σ^E, is the alternative sigma factor that responds to cell-envelope stress and plays a critical role in UPEC pathogenesis (41, 57). An *rpoE* promoter was also predicted for IpuA (58). This suggests a mechanism where IpuA expression could be induced by σ^E, which drives inversion of *ipuS* and subsequent transcription of *ipuR*/*upaE* through a putative *ipuS* σ^E dependent promoter. Conditions known to induce σ^E

were examined for their ability to enrich for the ON state of the switch but no effect was observed (data not shown). Identifying a condition that selects for the ON state of *ipuS* is an active area of pursuit in the laboratory.

The orientation of *ipuS* may directly influence *fimS* or *hyxS* orientation but such effects were masked by the need to lock all three switches in this analysis. Other investigators have generated *fimS* locked strains by mutating the sequence of the inverted repeats (51). Using this approach would facilitate locking *ipuS* orientation while permitting inversion of the other two elements helping to identify *ipuS* effects at *fimS* and *hyxS*. However, the five recombinases recognize the inverted repeats in a sequence specific manner (54-56) so manipulating the local sequence may inherently change recombinase-binding affinity. In the context of a complete network, where multiple sites are competing for limiting quantities of the recombinases, changing the half sites could perturb the orientation of the other switches by altering recombinase availability.

ipuR is a predicted LuxR-type transcriptional regulator. LuxR-type regulators, are two domain proteins that contain an autoinducer and DNA binding domain. They have been implicated in virulence, biofilm production, and motility of *Vibrio* spp., several classes of pathogenic *E. coli*, and *Mycobacterium tuberculosis* (36, 43, 44, 59). The regulon sizes of these proteins are highly variable. Some regulate one or a few specific targets while others have much broader effects (43). It is possible that the effects of *ipuS* in murine and tissue culture models are due to IpuR-mediated regulation of a kidney specific adhesion moiety. CFT073 produces a variety of extracellular adhesion-promoting structures including Type 1, Pap and F1C pili as well as multiple adhesive autotransporters (17, 34, 60-63). Pap pili are kidney specific adhesive structures that bind to sialosyl galactosyl globoside (SGG) and

disialosyl galactosyl globoside (DSGG), which are found in high abundance on kidney epithelial cells (64). The regulation of Pap pili is complex, including phase variation mechanisms and cross talk with other pili operons (33, 34, 60, 65). It is possible that IpuR participates in this regulation leading to the observed kidney specific phenotypes (Figure 6).

UpaE is a predicted autotransporter. These are large multi-domain proteins that typically function as secreted toxins or adhesion moieties. They are transported to the periplasm via a sec-dependent pathway and then self transport through the outer membrane using their beta-barrel pore domain. Once surface exposed, they can remain anchored to the outer membrane or be cleaved either by self-proteolytic domains or by association with other surface exposed proteases and released into the extracellular space (66). CFT073 has a number of different characterized autotransporters that function as adhesion factors and secreted toxins (17, 18, 45, 62, 63, 67). UpaE could be a kidney specific adhesion factor and experiments designed to test that hypothesis are underway.

To assess the role of *ipuS* in virulence in the murine model of UTI we infected female mice transurethrally into the bladder. A significant complication with the murine model of UTI is the bottleneck that occurs immediately after inoculation. The animals void their bladders upon recovering from anesthesia, resulting in the loss of 10^4 – 10^5 CFU of the initial 10^8 CFU inoculums (unpublished results). Additionally, the competition experiments were performed with *fimS* lock ON cells to facilitate consistent animal infections (Figure 6A). These strains are non-motile and unable ascend the urinary tract via swimming motility during infection. Any bacteria present in the kidneys when infecting with these strains likely originated from reflux during inoculation (68). It is difficult to quantify both

the heterogeneity and total number of cells that are deposited by reflux, which in addition to the rapid loss of much of the inoculums may account for the high degree of variability observed in the kidneys at 72 hpi (Figure 6A).

Most tyrosine recombinases that function at invertible elements are encoded in close proximity to the sites at which they act (35). However, in depth analysis of *Bacteriodes fragilis* has revealed extensive networks of switches and recombinases that function at local and distant sites in the chromosome (69-73). One such enzyme, Mpi, can catalyze inversion of 13 elements located throughout the *B. fragilis* chromosome (73). This inversion network controls the expression of surface architecture components and is predicted to function as a mechanism for global surface remodeling in response to changing environmental conditions (70, 73). The identification of a third switch in CFT073, demonstrated recombinase cross reactivity among the invertible elements, and known environmental stimuli that influence inversion of *fimS* (29, 30, 74, 75) suggests the existence of a similar network (Figure 3) (20, 36).

During colonization of a human host CFT073 must transition from the gut, to the periurethral area to the urinary tract. In these niches CFT073 encounters diverse environmental conditions and interacts with a wide variety of host microbiota. Once in the urinary tract, it faces components of the host immune response, the cleansing flow of urine, low iron levels and periods of intracellular growth (7, 16, 76). The characterization of *ipuS* and the shared function of the five recombinases among the three CFT073 phase switches outline a complex inter-regulated virulence network in *E. coli*. It reveals the existence of a complex system that simultaneously regulates the orientation of three different elements,

selecting for 1 of 8 possible phase states to suit changing conditions during colonization and infection.

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Table 1. Strains and plasmids used in this study

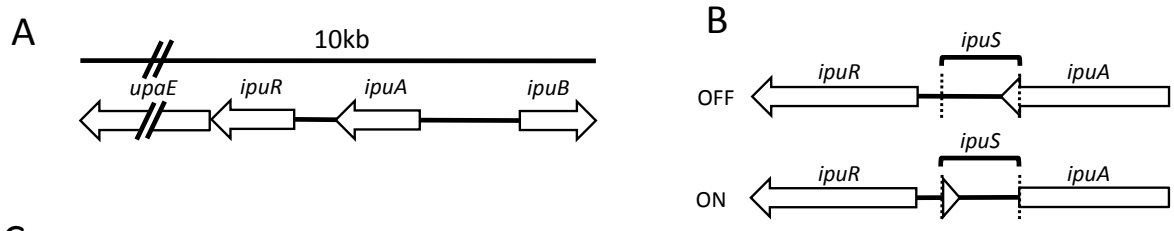
Strain or Plasmid	Description	Source or reference
Bacterial Strains		
WAM2266	CFT073 Nal; urosepsis isolate	(15)
WAM5009	CFT073 with pFUSE *ipuR* txn fusion	This study
WAM5063	CFT073 NaI *fimBE, fimX, ipuA, ipuB* (*fimS* OFF, *ipuS* OFF)	This study
WAM5064	CFT073 NaI *fimBE, fimX, ipuA, ipuB* (*fimS* ON, *ipuS* ON)	This study
WAM5065	CFT073 NaI *fimBE, fimX, ipuA, ipuB* (*fimS* ON, *ipuS* OFF)	This study
WAM5088	CFT073 NaI *fimBE, fimX, ipuA, ipuB* (*fimS* OFF, *ipuS* ON)	This study
WAM5144	WAM5063 *lacZYA*	This study
WAM5145	WAM5064 *lacZYA*	This study
WAM5146	WAM5065 *lacZYA*	This study
WAM5147	WAM5088 *lacZYA*	This study
WAM5070	WAM 5064 with pACYC184	This study
WAM5079	WAM 5064 with pACYC177	This study
WAM5071	WAM 5064 with pWAM2801	This study
WAM5081	WAM 5064 with pWAM2957	This study
WAM5072	WAM 5064 with pWAM2961	This study
WAM5080	WAM 5064 with pWAM2775	This study
WAM5082	WAM 5064 with pWAM3579	This study
WAM5073	WAM 5064 with pWAM5073	This study
WAM5074	WAM 5065 with pACYC184	This study
WAM5083	WAM 5065 with pACYC177	This study
WAM5075	WAM 5065 with pWAM2801	This study
WAM5085	WAM 5065 with pWAM2957	This study
WAM5076	WAM 5065 with pWAM2961	This study
WAM5084	WAM 5065 with pWAM2775	This study
WAM5077	WAM 5065 with pWAM3759	This study
WAM5078	WAM 5065 with pWAM5073	This study
Plasmids		
pKD46	Lambda-Red recombinase helper plasmid	(38)
pCP20	FLP helper plasmid	(38)
pKD4	kan^+ template	(38)
p2779	pFUSE *lacZYA* transcriptional fusion suicide vector	(39)
pACYC177	Cloning plasmid with constitutively active promoter	New England Biolabs
pACYC184	Cloning plasmid with expression driven by the insert promoter	New England Biolabs
pWAM2801	pACYC177, *fimB*; Kan^r	(20)

Table 1. Continued

Strain or Plasmid	Description	Source or reference
pWAM2957	pACYC177, *fimE*; Kan^r	(20)
pWAM2775	pACYC177, *ipuB*; Kan^r	(20)
pWAM2961	pACYC177, *ipuA-FL*; Kan^r	(20)
pWAM3579	pACYC184, *ipuA-FL*; Cm^r	(20)
pWAM5073	pACYC184, *ipuA-Trunc;* Cm^r	This study
Cell Lines		
A-498	Human kidney epithelial carcinoma cells	ATCC

Figure 1. Identification of the *ipuS* invertible element. (A) Schematic representation of the context of *ipuS.* (B) DNA rearrangement as a result of *ipuS* inversion. The ON/OFF state is defined by expression of an *ipuR-lacZ* reporter fusion in the pictured orientation. Location of the inverted repeats is indicated by the dotted lines. (C) Inversion to the ON state results in an 11 amino acid truncation of *ipuA* and a K-F substitution of the truncated forms terminal amino acid. Conserved RHRY active site residues are indicated in bold.

Figure 1.



C

```
IpuA-WT       1 MQNRKFLTHHEINLLLQSVKQKSCSSRDVCMILLAYFHGLRVSELLSLQL  50
IpuA-Trunc    1 MQNRKFLTHHEINLLLQSVKQKSCSSRDVCMILLAYFHGLRVSELLSLQL  50

IpuA-WT      51 SDLELTTEKIYIQRIKNGFSTVHPLQKEEVIAITNWLNERNSLNVKHFND 100
IpuA-Trunc   51 SDLELTTEKIYIQRIKNGFSTVHPLQKEEVIAITNWLNERNSLNVKHFND 100

IpuA-WT     101 NPWLFVSRTGKPLSRQRFYNIVSAAGKNAGLNIKVHPHMLRHACGYSLAD 150
IpuA-Trunc  101 NPWLFVSRTGKPLSRQRFYNIVSAAGKNAGLNIKVHPHMLRHACGYSLAD 150

IpuA-WT     151 NGVDTRLIQDYLGHRNIRHTVIYTASNSMRFEKMWGIGDAKKQHFDPKCK 200
IpuA-Trunc  151 NGVDTRLIQDYLGHRNIRHTVIYTASNSMRFEKMWGIGDAKKQHFDPF*   199

IpuA-WT     201 PNLCLEILV* 210
IpuA-Trunc  200            199
```

Figure 2. *ipuS* invertible element characterization. (A) The invertible element is 260 bp and is defined by a pair of 7 bp-inverted repeats shown in bold with a grey background. (B) Predicted DNA hairpin created during recombination. The connecting lines indicate base pair interactions among core inverted repeat and local surrounding sequence. (C) Comparison of the *ipuS* vs. *fimS* inverted repeat sequences (top panel) and *ipuS* vs. *hyxS* inverted repeat sequences (bottom panel). Proximal and distal half sites are shown on the left and right respectively. Inverted repeat sequence and surrounding sequences that can participate in base paring during recombination are boxed in grey and connecting bars indicate sequence identity. Residues that are part of the core-inverted repeat of each switch are shown in bold.

Figure 2.

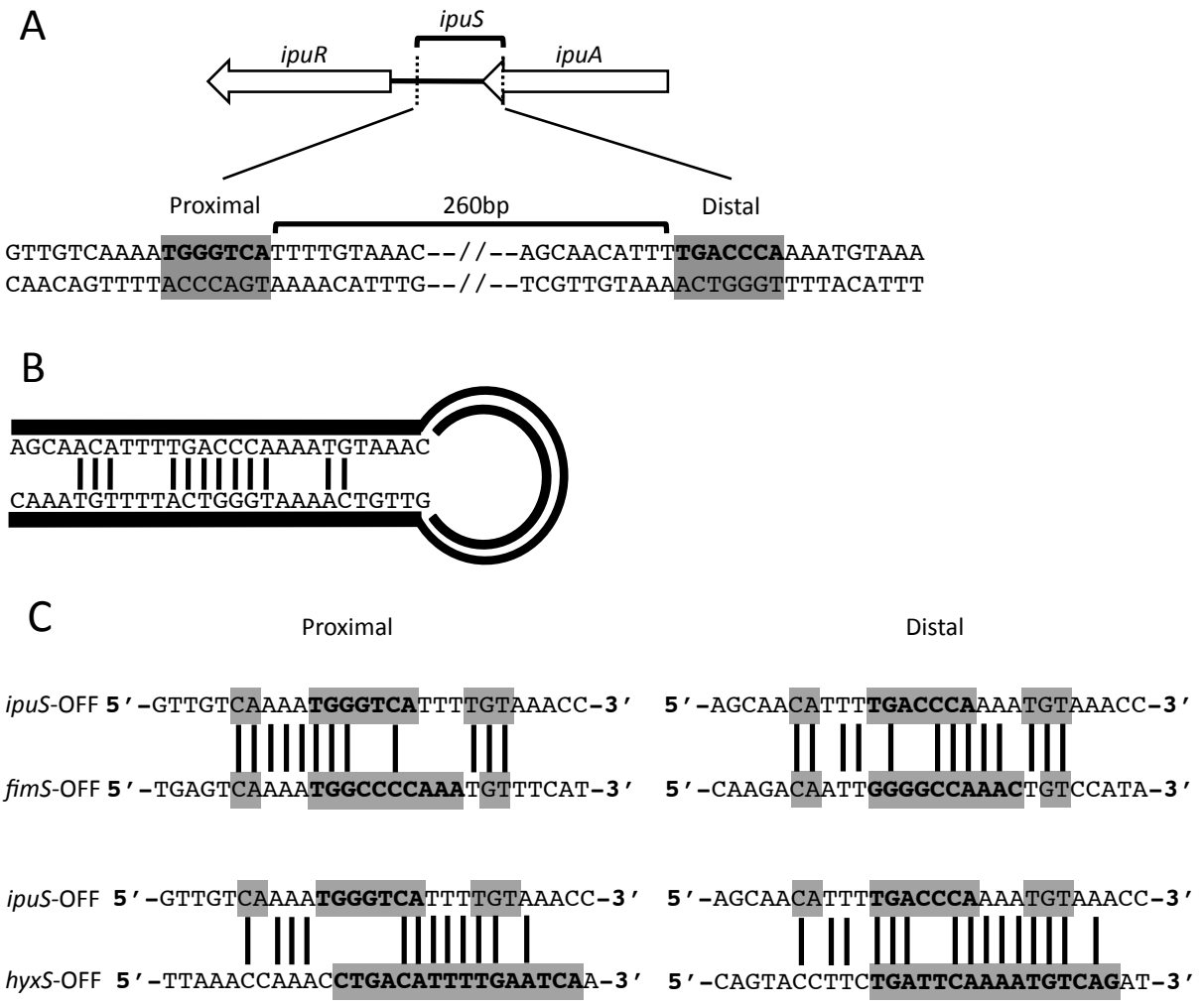

A
ipuS
ipuR
ipuA
Proximal
260bp
Distal
GTTGTCAAAATGGGTCATTTTGTAAAC--//--AGCAACATTTTGACCCAAAATGTAAA
CAACAGTTTTACCCAGTAAAACATTTG--//--TCGTTGTAAAACTGGGTTTTACATTT
B
AGCAACATTTTGACCCAAAATGTAAAC
CAAATGTTTTACTGGGTAAAACTGTTG
C
Proximal
Distal
ipuS-OFF 5'-GTTGTCAAAATGGGTCATTTTGTAAACC-3'
5'-AGCAACATTTTGACCCAAAATGTAAACC-3'
fimS-OFF 5'-TGAGTCAAAATGGCCCCAAATGTTTCAT-3'
5'-CAAGACAATTGGGGCCAAACTGTCCATA-3'
ipuS-OFF 5'-GTTGTCAAAATGGGTCATTTTGTAAACC-3'
5'-AGCAACATTTTGACCCAAAATGTAAACC-3'
hyxS-OFF 5'-TTAAACCAAACCTGACATTTTGAATCAA-3'
5'-CAGTACCTTCTGATTCAAAATGTCAGAT-3'

Figure 3. Assessment of the ability of each recombinase to catalyze inversion of *ipuS* in vitro. Ethidium bromide stained electrophoretic gels of PacI digested PCR products are shown. Lanes 1 and 2 contain digested PCR products from CFT073 *ipuS* phase lock ON (WAM5064) and OFF (WAM5065) strains generated by 5-way recombinase deletion. Lanes 3 and 4 contain digested PCR products from vector only controls (WAM5070, WAM5079, WAM5074 and WAM5083). Lanes 5-10 contain PCR products from the locked OFF strain WAM5065 (top panel) or locked ON strain WAM5064 (bottom panel) after transforming each with a recombinant plasmid containing the indicated recombinase. Both the full length and truncated forms of *ipuA* were tested for activity. The OFF orientation restriction fragments are 186 bp/1103 bp and the ON orientation restriction fragments are 407 bp/882 bp.

Figure 3.

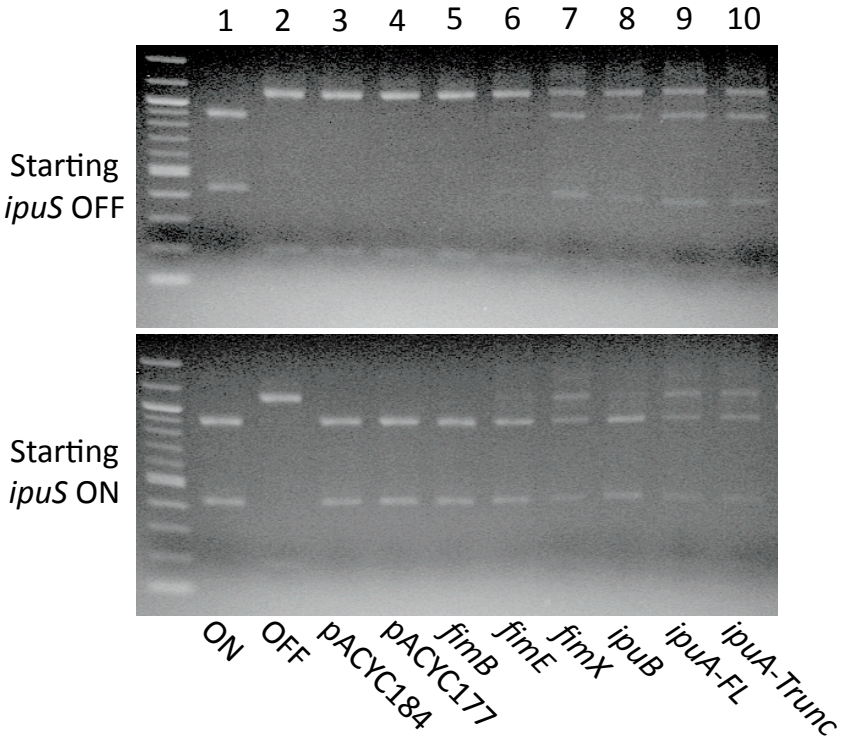
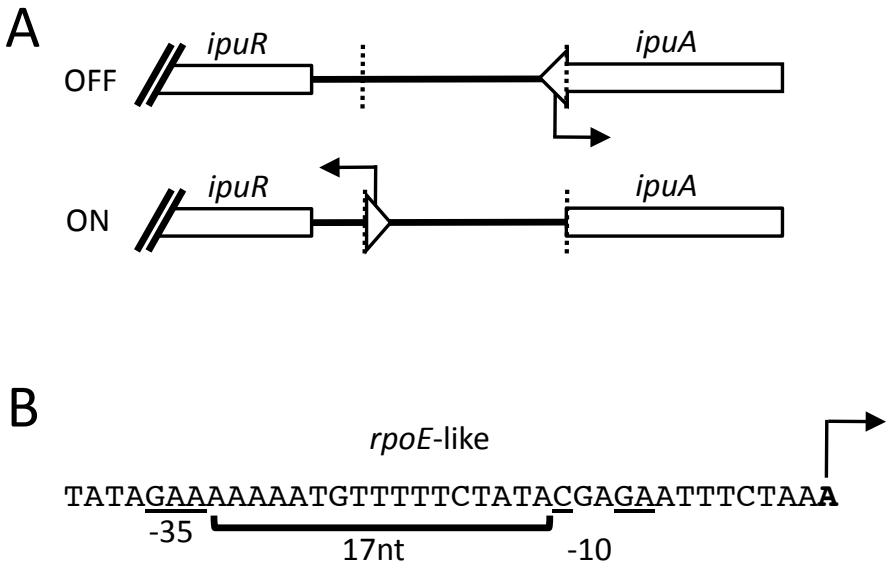

1 2 3 4 5 6 7 8 9 10
Starting *ipuS* OFF
Starting *ipuS* ON
ON
OFF
pACYC184
pACYC177
fimB
fimE
fimX
ipuB
ipuA-FL
ipuA-Trunc

Figure 4. Putative *ipuS* promoter mapping by 5'RACE. (A) A putative *ipuS* transcriptional start site was identified by 5'RACE and is located within the full-length *ipuA* coding sequence. The arrow indicates its location and directionality. The inverted repeats are indicated by the dotted lines. (B) A predicted promoter was identified in proximity to the predicted transcription start site that most closely matches a consensus *rpoE* promoter. Locations of the predicted -35 and -10 regions and spacer lengths are indicated. Nucleotides that match consensus are underlined.

Figure 4.



Consensus σE promoter: GAA-16nt-YCTGA

Figure 5. Swimming motility of phase locked strains. 1 μl of liquid culture normalized to OD_{600} = 0.5 of each of the strains: WT (WAM2266), *fimS* OFF/*ipuS* ON (WAM5088), *fimS* OFF/*ipuS* OFF (WAM5063), *fimS* ON/*ipuS* ON (WAM 5064), and *fimS* ON/*ipuS* OFF (WAM5065) were inoculated in the center of 7 Petri plates containing Adler's motility medium. Values represent diameter of the swimming zone after ~21 hrs of room temperature incubation.

Figure 5.

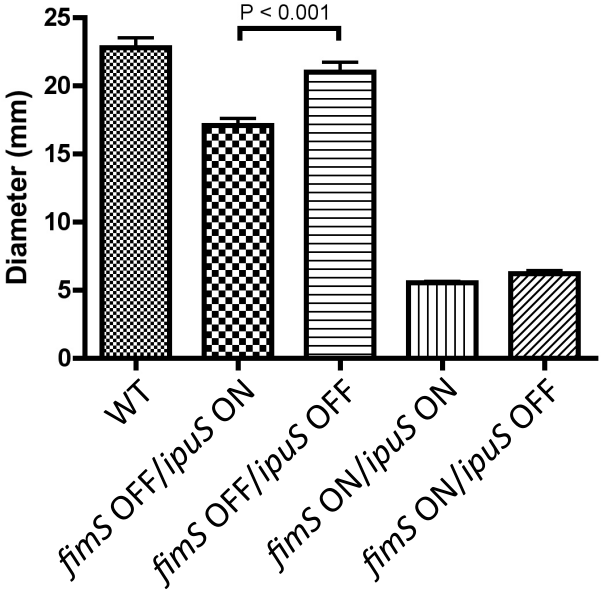
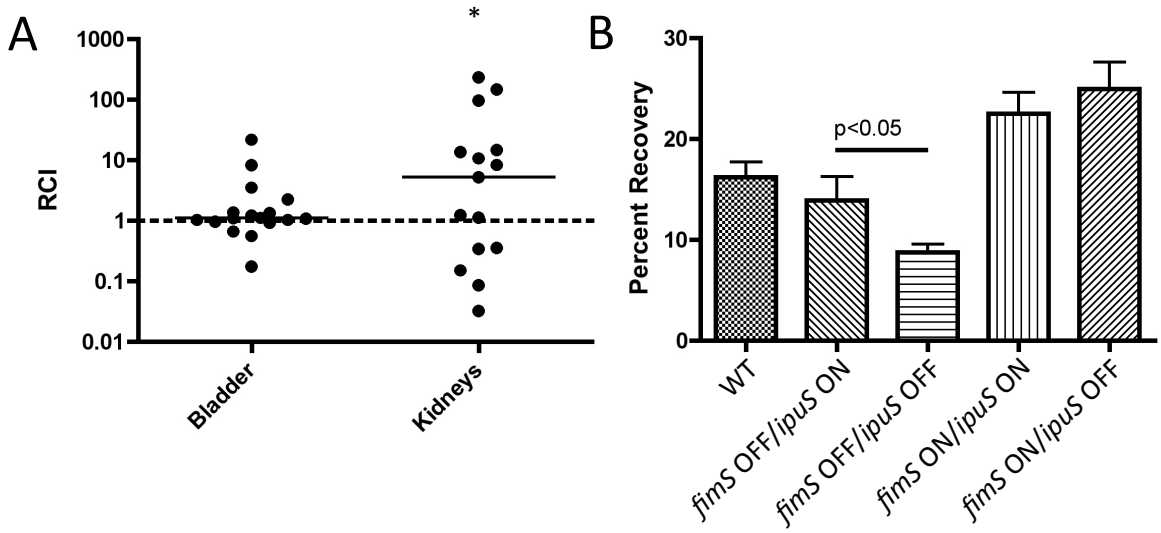

P < 0.001
Diameter (mm)
25
20
15
10
5
0
WT
fimS OFF/ipuS ON
fimS OFF/ipuS OFF
fimS ON/ipuS ON
fimS ON/ipuS OFF

Figure 6. In vivo effects of *ipuS.* (A) 72 hr competitive infection of *fimS* ON/*ipuS* ON (WAM5064) vs. *fimS* ON/*ipuS* OFF, *lacZYA* (WAM5146). Relative competitive indices were calculated from bladder and kidney homogenates at 72 hpi with bars representing the median. *fimS* OFF/*ipuS* ON (WAM5064) has a 5-fold advantage in the kidneys ($p<0.05$) at 72 hpi. (B) Adherence to A-498 human kidney epithelial cells. WT (WAM2266), *fimS* OFF/*ipuS* ON (WAM5088), *fimS* OFF/*ipuS* OFF (WAM5063), *fimS* ON/*ipuS* ON (WAM 5064), and *fimS* ON/*ipuS* OFF (WAM5065) were allowed to adhere to monolayers of A-498 cells then unbound bacteria were removed by washing. In a *fimS* OFF background, turning *ipuS* ON causes a 5% increase in adherence (*fimS* OFF/*ipuS* ON vs. *fimS* OFF/*ipuS* OFF) ($p<0.05$).

Figure 6.


A
1000
100
10
1
0.1
0.01
RCI
*
Bladder
Kidneys
B
30
20
10
0
Percent Recovery
p<0.05
WT
fimS OFF/ipuS ON
fimS OFF/ipuS OFF
fimS ON/ipuS ON
fimS ON/ipuS OFF

Chapter 4

Phase variation of uropathogenic *Escherichia coli* strain CFT073 and its contribution to macrophage uptake and reactive nitrogen resistance

Authors and Contributors:

Eric Battaglioli: Wrote manuscript, planned and performed all experiments, and analyzed data except as indicated below.

John-Demian Sauer: Supervised macrophage uptake experiments by providing technical assistance and intellectual guidance.

Rodney Welch: Supervised all research and provided intellectual guidance for the design and execution of the experiments.

This chapter is being prepared for submission to Infection and Immunity

Abstract

Uropathogenic *Escherichia coli* (UPEC) are the most common etiological agent of uncomplicated urinary tract infection (UTI). UPEC contain a suite of virulence factors to facilitate infection and their regulation is critical for successful colonization of the host. Phase variation by epigenetic promoter inversion is one method that is used to modulate expression of UPEC virulence genes. The UPEC strain CFT073 contains three known invertible elements that modulate virulence, *fimS, ipuS* and *hyxS*. *fimS* orientation controls expression of Type 1 pili, a critical UPEC virulence factor. *hyxS* orientation controls the expression of *hyxR*, a LuxR-type regulator that affects resistance to reactive nitrogen species (RNS) and intracellular macrophage survival. *ipuS* orientation affects adherence to kidney epithelial cells and colonization of the kidneys in a murine model of UTI. Inversion of these elements is mediated by five tyrosine recombinases: FimB, FimE, FimX, IpuA and IpuB. Here we demonstrate that *fimS*-locked OFF CFT073 is more resistant to reactive nitrogen species (RNS) and has a defect in macrophage uptake when compared with either WT CFT073 or *fimS*-locked ON CFT073. Together our results suggest that phase variation of *fimS* provides a mechanism to avoid macrophage uptake while simultaneously aiding resistance to RNS-mediated killing.

Introduction

Urinary tract infections are one of the most common infections experienced in US hospitals and clinics. Over 50% of women will experience a UTI in their life time (1). The most common etiological agent of infection are the uropathogenic *Escherichia coli* (UPEC) which account for 80-85% of reported infections (2). Uncomplicated infections (infections

in otherwise healthy individuals) are typically non-life threatening in the developed world due to readily available antibiotic treatment (3). However, untreated infections can lead to renal scarring and permanently impaired kidney function. Additionally, UTI in pregnant women, which is one of the most common infections for this demographic, can be fatal for the fetus (1). Associated treatment costs of these infections in the US exceed $1.6 billion annually (1). The significant health and economic costs of these infections has motivated intense research on UPEC pathogenesis to better understand infection and develop novel treatment strategies.

UPEC encounter a variety of host defense mechanisms en route to establishing and maintaining an infection. The cleansing flow of urine, exfoliation of uroepithelial cells, and production of reactive nitrogen species (RNS) are all mechanisms that are employed by the host to combat infection (4). A rapid influx of nitric oxide (NO)-producing neutrophils is one of the first host immune responses. This increases the amount of NO in the bladder 30-50 fold and increased nitrite levels are detected as early as 6-12 hours post infection (5, 6). The secreted NO is processed into a variety of reactive nitrogen species that induce DNA damage, create protein/lipid modifications and inactivate iron-sulfur complex containing enzymes (7). Similar compounds are produced by macrophages which are also an active component of the innate immune response to UTI (4).

Recently an invertible switch, *hyxS*, was described in the UPEC strain UTI89 that regulates resistance to RNS and intracellular macrophage survival (8). Inversion of *hyxS* is catalyzed by the linked recombinase FimX and subsequent orientation of the element drives expression of *hyxR* a LuxR-type transcriptional regulator. HyxR inhibits expression of *hmpA*, a RNS detoxifying enzyme leading to decreased resistance to RNS (8). FimX is also

capable of inverting the Type 1 pili invertible element *fimS* and the *ipuS* invertible element (unpublished data) (9). Due to the FimX link among the three switches, we sought to determine if orientation of *fimS* and *ipuS* also contributes to reactive nitrogen resistance and intracellular macrophage survival independent of *hyxS*. Here we demonstrate a novel activity for *fimS* in RNS resistance in the related UPEC isolate CFT073. The *fimS* ON state results in a significant decrease in resistance to RNS and an increase in macrophage uptake. The *fimS* OFF state results in a modest increase in resistance to RNS and a decrease in macrophage uptake. Taken together, this suggests a *fimS* mediated mechanism for the avoidance of the host immune response by inhibiting uptake while simultaneously increasing resistance to secreted RNS.

Methods and Materials

Bacterial strains, plasmids, cell lines, and culture conditions. All of the strains and plasmids used in this study are listed in Table 1. In frame deletion mutants of *Escherichia coli* CFT073 were generated using a modification of Lambda-red method of homologous recombination to include phage transduction of the marker into a clean genetic background with ΦEB49 prior to removal of the cassette via pCP20 (10, 11). Bacteria were prepared by inoculating single colonies from Luria Bertani (LB)-agar plates into LB broth and grown at 37^{o}C over night with shaking. Antibiotic selection (kanamycin [50 μg/ml], chloramphenicol [20 μg/ml], carbenicillin [250 μg/ml] was used where appropriate.

Bone marrow derived macrophages (BMMs) were prepared from murine bone marrow as described previously (12, 13). Cells were prepared and analyzed in macrophage media (RPMI + 20% fetal bovine serum v/v, 10% colony stimulation factor v/v, 1% L-Glutamine v/v, 1% sodium pyruvate v/v, 0.1% beta-mercaptoethanol v/v) and gentimicin (200 μg/ml or 20 μg/ml) was added when indicated. BMMs were treated with 1 mM L-Arg (Sigma Aldrich, St. Louis, MO), or 1 mM L-NAME (Sigma Aldrich, St. Louis, MO) from 1 hr prior to inoculation through the duration of the infection where indicated.

Reactive nitrogen species resistance assays. Bacteria were prepared as described above. The resulting cultures were diluted to OD_{600}=0.8 in LB broth and 10 μl was inoculated in duplicate into 24 well plates containing 500 μl of LB-MES (100 mM) pH 5.0 or 500 μl of LB-MES (100 mM) pH5.0 + 3 mM sodium nitrite. Sodium nitrite was dissolved in water, filter sterilized with a 0.45 μm filter and added to the appropriate wells immediately before inoculating with bacteria. The plates were then incubated at 37°C with shaking in a Synergy HT automated plate reader (Biotek, Winooski, VT) and OD_{600} absorbance readings taken every 30 min for 40 hours. Growth curves were generated from the OD_{600} readings for each of the strains tested and the data were analyzed with the biostatistics software package Prism (Graphpad, La Jolla, CA). Data represent the averages from at least 3 biological replicates with error bars indicating standard error of measurement (SEM).

Macrophage uptake assays. Bacteria were prepared as described above. BMMs ($5x10^5$) were infected with bacteria (MOI=10) in 24-well plates, centrifuged for 5 min at 500 x g to facilitate contact with the BMMs, and allowed to infect for 30 min at 37°C with 5% CO_2. After 30 min, the media was removed and replaced with fresh media containing

200 μg/ml gentamicin and incubated as above for another 30 min. The media was removed, the macrophages washed 3 times with an equal volume of 1X PBS and fresh media was added to the wells containing 20 μg/ml gentamicin. At 1 and 24 hours post infection (hpi) cells were washed 3 times with an equal volume of PBS, lysed in 200 μl of 0.1% Triton and 100 μl was plated using a Autoplate Spiral Plating System (Advanced Instruments, Norwood, MA). Colonies were counted with a QCount Automated Colony Counter (Advanced Instruments, Norwood, MA) and intracellular bacterial loads determined in CFU/ml. Fold killing was calculated as the ratio of bacteria at 1 hpi/24 hpi. Aliquots of the inoculums were diluted and plated on LB-agar plates to ensure consistent MOIs.

Results

***fimS* orientation contributes to reactive nitrogen species resistance.** Recent reports have identified a new invertible element in CFT073, *hyxS*, which regulates resistance to reactive nitrogen species and intracellular macrophage survival (8). Inversion of this element is catalyzed by the linked recombinase FimX. Due to the linked activity of FimX at *hyxS, ipuS,* and *fimS* we examined the contribution of the *ipuS* and *fimS* phase states to reactive nitrogen resistance and intracellular macrophage survival. We created *ipuS*/*fimS* phase locked strains in all four possible combinations (Table 1) and determined their ability to grow in the presence of reactive nitrogen species (RNS). The *hyxS* switch was locked OFF (RNS resistant state) in all strains tested (data not shown). RNS were generated within the growth medium by adding sodium nitrite (3 mM final concentration) to LB-MES pH 5.0. The acid environment processes the sodium nitrite into RNS during

growth to produce a consistent RNS mediated stress. Strains that are able to process the RNS more rapidly display a reduced lag before entering logarithmic phase growth. Compared to WT, locking *fimS* OFF increases resistance to RNS, while locking *fimS* ON decreases resistance to RNS. With *fimS* locked OFF, a slight increase in resistance is observed when *ipuS* is also locked OFF (Figure 1).

***fimS* orientation affects macrophage uptake but not intracellular macrophage survival.** Type 1 pili facilitate contact with and invasion of a variety of host cells including macrophages (14, 15). To determine if CFT073 shows the same Type 1 pili dependent effects on macrophage uptake and survival as other strains of *E. coli*, we infected mouse bone marrow-derived macrophages with the phase locked strains. Extracellular bacteria were eliminated with gentamicin treatment and intracellular populations were enumerated at 1 and 24 hpi. Locking *fimS* ON or OFF results in an increase and decrease in macrophage uptake respectively when compared to WT at 1 hpi (Figure 2A). The *ipuS* state had no effect on uptake. At 24 hpi, a 1-1.5log reduction in bacterial loads were observed for all four strains (Figure 2A). Fold killing ratios were determined and indicate that while different numbers of bacteria invade based on *fimS* orientation they are eliminated at similar rates (Fig. 2B). Treatment of the macrophages with 1 mM L-arg (increased RNS stress) or 1 mM L-NAME (decreased RNS stress) during infection had no affect on intracellular survival of any of the phase locked strains (data not shown).

Discussion

The innate immune response is a key component of the host defense against UPEC and consists of a rapid neutrophil and macrophage influx during the early stages of

infection (4, 16). These immune cells produce nitric oxide that can be processed into a variety of reactive nitrogen species (RNS) including peroxinitrite, S-nitrosothiol, nitrogen dioxide and others (7, 17). UPEC have robust systems for coping with this stress which make them more resistant to RNS than non-pathogenic lab adapted *E. coli* strains (18). These systems rely on the activity of the envelope stress response sigma factor RpoE, HmpA (a NO detoxifying enzyme) and production of polyamines like cadaverine (19). Recent reports indicate that phase variation contributes to this process in UTI89 and CFT073 via the inversion of *hyxS* (8).

fimS orientation regulates expression of Type 1 pili (20). Type 1 pili are a critical virulence factor for UPEC pathogenesis. They bind to mannose residues on host cells and mediate attachment and invasion of the bladder epithelium (21-24). Pleiotropic links have been established between Type 1 pili expression, swimming motility and production of Pap and F1C pili (9, 25-27). *fimS* orientation and subsequent Type 1 expression may have broad effects on global gene expression so it is not surprising that *fimS* orientation can also be linked to the regulation of RNS detoxifying systems. The mechanism for how *fimS* orientation affects RNS sensitivity remains unclear. Transcriptomic analyses evaluating global differences in gene expression of the *fimS* ON/OFF strains grown under RNS stress conditions are underway to begin to characterize this mechanism.

In addition to promoting adhesion to the uroepithelial cells, Type 1 pili enhance adhesion to macrophages, which results in increased phagocytosis (Figure 2) (14, 15). However, despite the increased entry, *fimS* orientation and subsequent Type 1 pili production has little affect on intracellular macrophage survival of CFT073 and other *E. coli* strains even under high RNS stress inducing conditions (data not shown) (28). The

environment within macrophage phagosomes is not the only location in the urinary tract where *E. coli* encounters RNS. An additional source of nitrosative stress is in solution within the bladder lumen where neutrophils secrete RNS in response to infection (5, 6). Experiments examining the sensitivity of the phase locked strains to neutrophil mediated killing are underway.

Bidirectional inversion of *hyxS* is catalyzed only by the linked recombinase FimX and over expression of FimX is required to observe the ON state (8). The *hyxS* ON state leads to decreased resistance to RNS and intracellular macrophage survival via activity of the LuxR-type regulator HyxR on *hmpA* expression. Additionally, FimX is capable of catalyzing the OFF to ON inversion of *fimS* and data reported here shows decreased RNS resistance for the *fimS* ON state (Figure 1). *fimS* locked ON cells display increased invasion of uroepithelial cells, which is predicted to be a mechanism for avoiding the host immune response (23, 29). Taken together, these data indicate that expression of *fimX* could result in simultaneous selection of the ON state of both switches. In this configuration, the cells will display increased sensitivity to RNS, but the expression of Type 1 pili will increase their ability to invade resident epithelial cells to avoid RNS exposure in the bladder lumen. Conversely, decreased *fimX* expression will select for the double OFF configuration. In this state the cells are more resistant to RNS and highly motile due to the inverse relationship between Type 1 expression and motility (9). This would allow for cells to escape from their intracellular “hideouts”, swim through the RNS storm in the bladder lumen to continue propagating through the urinary tract. Cells could then turn both switches ON to invade a nearby epithelial cell and “weather the storm” till their next traversing event (Figure 3).

Specific phenotypes are associated with all three switches in CFT073 (8, 23, 24, 29, 30). These previous studies combined with the data provided here suggest that the simultaneous selection of a particular combination of phase states may be advantageous at different times during infection. The effects of a combination of phase states may be more important than the characterized affects of each switch in isolation. A better understanding of the global effects of each of the 8 possible recombinase mediated phase states in CFT073 and how the orientation of one switch is linked to the other two is needed to fully understand the pathogenesis of UPEC.

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Table 1. Strains and plasmids used in this study

Strains or Plasmids	Description	Source or reference
Bacterial Strains		
WAM2266	CFT073 Nal; urosepsis isolate	(31)
WAM5063	CFT073 NaI *fimBE, fimX, ipuA, ipuB* (*fimS* OFF, *ipuS* OFF)	This study
WAM5064	CFT073 NaI *fimBE, fimX, ipuA, ipuB* (*fimS* ON, *ipuS* ON)	This study
WAM5065	CFT073 NaI *fimBE, fimX, ipuA, ipuB* (*fimS* ON, *ipuS* OFF)	This study
WAM5088	CFT073 NaI *fimBE, fimX, ipuA, ipuB* (*fimS* OFF, *ipuS* ON)	This study
Plasmids		
pKD46	Lambda-Red recombinase helper plasmid	(11)
pCP20	FLP helper plasmid	(11)
pKD4	kan^+ template	(11)

Figure 1. *ipuS* and *fimS* orientation contributes to reactive nitrogen resistance. WT (WAM2266) and the phase locked strains *fimS* OFF/*ipuS* ON (WAM5088), *fimS* OFF/*ipuS* OFF (WAM5063), *fimS* ON/*ipuS* ON (WAM 5064), and *fimS* ON/*ipuS* OFF (WAM5065) were grown in LB-MES pH5.0 with and without 3 mM sodium nitrite. (A) All 5 strains grow equally well in the LB-MES pH 5.0 control medium. (B) Locking *fimS* ON causes a decrease in resistance to RNS (*fimS* ON/*ipuS* ON (WAM 5064) - magenta, *fimS* ON/*ipuS* OFF (WAM5065) – blue compared to WT (WAM2266) – orange). Locking *fimS* OFF causes an increase in resistance to RNS (*fimS* OFF/*ipuS* ON (WAM5088)– green, *fimS* OFF/*ipuS* OFF (WAM5063)– black) compared to WT (WAM2266 – orange). Locking *ipuS* OFF in a *fimS* OFF background (*fimS* OFF/*ipuS* OFF (WAM5063)– black) causes a further increase in resistance compared to the respective *ipuS* lock ON strain (*fimS* OFF/*ipuS* ON (WAM5088)– green).

Figure 1.

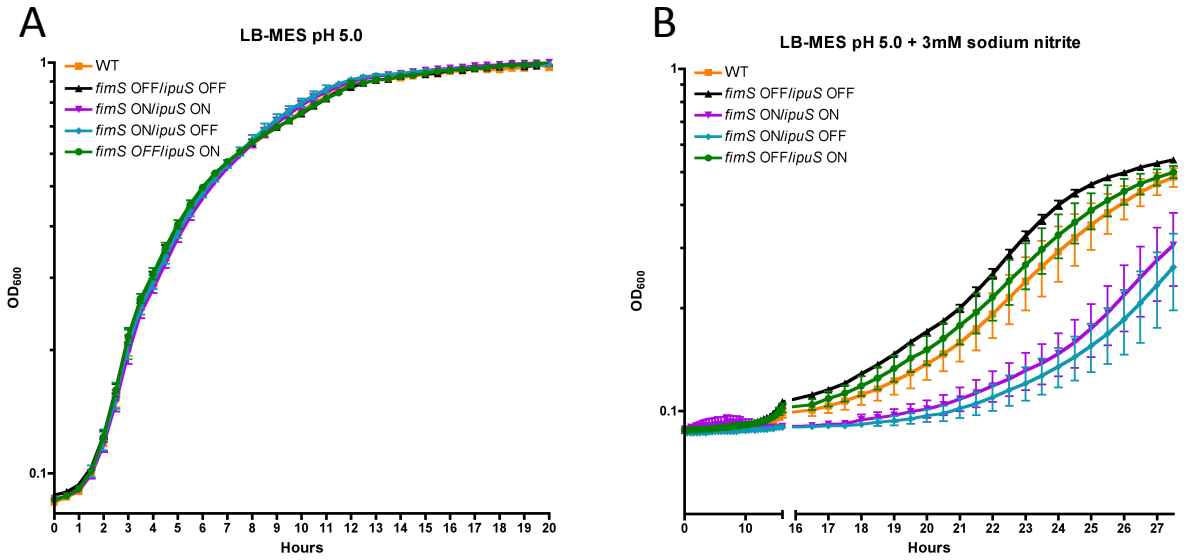

A
LB-MES pH 5.0
WT
fimS OFF/ipuS OFF
fimS ON/ipuS ON
fimS ON/ipuS OFF
fimS OFF/ipuS ON
OD600
Hours
B
LB-MES pH 5.0 + 3mM sodium nitrite
WT
fimS OFF/ipuS OFF
fimS ON/ipuS ON
fimS ON/ipuS OFF
fimS OFF/ipuS ON
OD600
Hours

Figure 2. Macrophage infections with the phase locked strains. (A) WT (WAM2266) and the phase locked strains *fimS* OFF/*ipuS* OFF (WAM5063), *fimS* OFF/*ipuS* ON (WAM5088), *fimS* ON/*ipuS* OFF (WAM5065), and *fimS* ON/*ipuS* ON (WAM 5064) were allowed to infect mouse bone marrow derived macrophages for 30 min. Extracellular bacteria were eliminated with Gm treatment and intracellular macrophage burdens determined at 1 and 24 hpi. The *fimS* ON state results in an increase in macrophage uptake at 1 hpi. (B) Fold killing ratios were extrapolated from the 1 hpi/24 hpi intracellular burdens. Despite the differences in uptake, all 5 strains were eliminated from the macrophages at similar rates.

Figure 2.

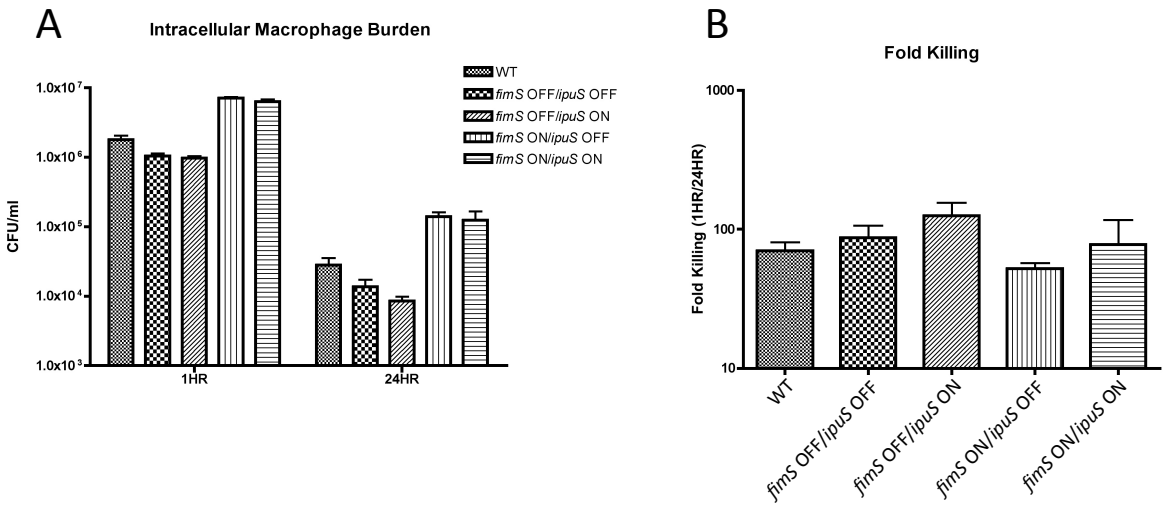
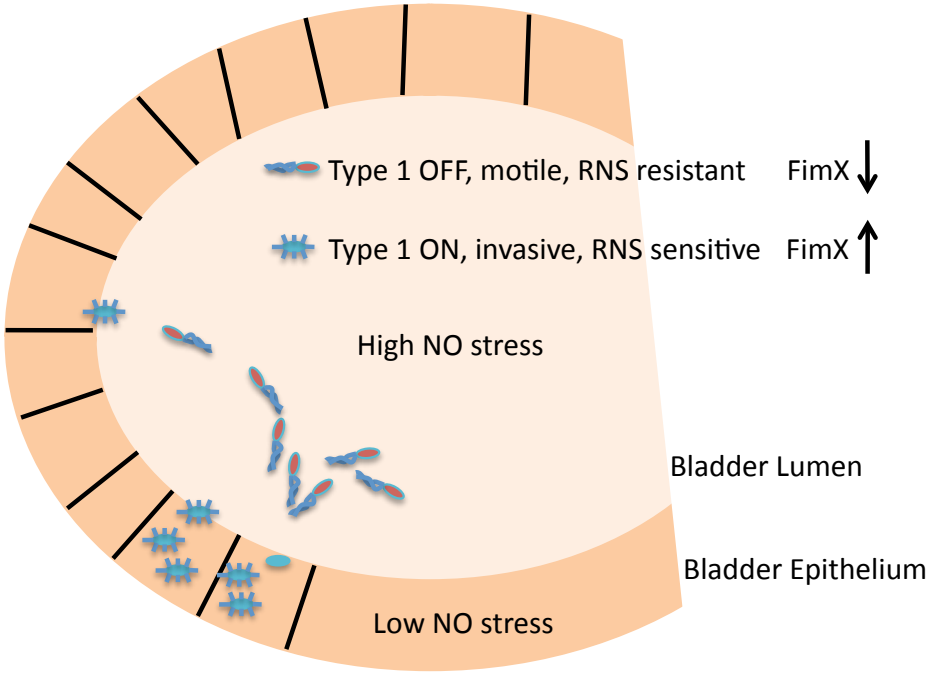

A
Intracellular Macrophage Burden
WT
fimS OFF/ipuS OFF
fimS OFF/ipuS ON
fimS ON/ipuS OFF
fimS ON/ipuS ON
CFU/ml
1.0x10 7
1.0x10 6
1.0x10 5
1.0x10 4
1.0x10 3
1HR
24HR
B
Fold Killing
Fold Killing (1HR/24HR)
1000
100
10
WT
fimS OFF/ipuS OFF
fimS OFF/ipuS ON
fimS ON/ipuS OFF
fimS ON/ipuS ON

Figure 3. Putative mechanism for FimX mediated inversion of *hyxS* and *fimS* in conferring survival in the bladder. Expression of *fimX* could result in simultaneous selection of the ON state of both switches. In this configuration, the cells will display increased sensitivity to RNS, and increase ability to invade resident epithelial cells to avoid RNS exposure in the bladder lumen. Conversely, decreased *fimX* expression will select for the double OFF configuration. In this state the cells are more resistant to RNS and motile due to the inverse relationship between Type 1 expression and motility (9). This would allow for cells to escape from their intracellular "hideouts", swim through the RNS storm in the bladder lumen to continue propagating through the urinary tract.

Figure 3.


Type 1 OFF, motile, RNS resistant
FimX
Type 1 ON, invasive, RNS sensitive
FimX
High NO stress
Bladder Lumen
Bladder Epithelium
Low NO stress

Chapter 5

Summary and Conclusions

Chapter 2 describes the discovery and characterization of four novel generalized transducing bacteriophages for CFT073 and other UPEC strains. A total of 77 phage isolates capable of forming plaques on CFT073 were collected from the wastewater treatment plant in Madison, WI. The phages were typed based on their ability to plaque on a panel of diverse *E. coli* isolates and redundant phages eliminated from subsequent analysis. Of the unique phages, ΦEB5, ΦEB32, ΦEB47 and ΦEB49 are capable of generalized transduction in CFT073. Empirical evidence indicates that ΦEB49 is the highest efficiency transducer. The four phages were further characterized by electron microscopy indicating that ΦEB5, ΦEB32, ΦEB47 are members of the *Siphoviridae* and ΦEB49 a member of the *Myoviridae*. The genome of ΦEB49 was sequenced and annotated revealing 74 putative open reading frames and two tRNAs. The predicted identities of the open reading frames are consistent with those of other phage genomes.

Chapter 3 describes the identification and characterization of a novel phase variation system in CFT073, *ipuS*. Strains were created with *lacZ* transcriptional fusions to the predicted LuxR-type regulator *ipuR* and this strain revealed that expression of *ipuR* is phase variable. PCR amplification and sequencing of the predicted variable region identified a 260 bp invertible element or "switch", *ipuS*, which is bounded by 7 bp inverted repeats (IRs). The distal inverted repeat is located within the predicted IpuA coding sequence. Inversion of the element results in the truncation of 11 amino acids from the C-terminus of IpuA and a K-L substitution of the terminal amino acid. The inverted repeats share sequence homology with the two other known switches in CFT073, *fimS* and *hyxS* (1, 2).

Locked switch state strains were generated by deleting all five recombinases and were used to determine activity of the five recombinases at *ipuS*. Each recombinase was provided in trans on multi-copy expression constructs to the *ipuS* lock ON and OFF strains to determine catalytic ability. The switch orientation was determined using PCR amplification of *ipuS* and asymmetric restriction digestion of the resulting product with PacI. Four of the five recombinases (FimB excluded) display catalytic activity at *ipuS*.

Phenotypic analyses were performed to describe the function of the *ipuS* phase switch. The *ipuS* ON state shows a decrease in swimming motility compared to the *ipuS* OFF state when examined in a motile (*fimS* OFF) background. The *ipuS* ON state also shows an increase in adherence to kidney epithelial cells. When the *ipuS* ON and OFF strains are used in competition experiments in the murine model of UTI, the *ipuS* ON strains show a 5-fold advantage in colonization of the kidneys at 72 hours post infection (hpi). The mechanism that describes the effect of *ipuS* orientation on these phenotypes remains unclear.

Chapter 4 describes the effects of *fimS* orientation on reactive nitrogen species (RNS) resistance and intracellular macrophage survival. A previous study showed that *hyxS* orientation contributes to both of these phenotypes (2). FimX is the only recombinase capable of inverting *hyxS* and it also has activity at *ipuS* and *fimS*. RNS challenge experiments were performed with the phase lock strains described in Chapter 3 to determine if *ipuS* and *fimS* also contribute to RNS resistance and intracellular macrophage survival.

The phase locked strains were subjected to growth in liquid medium that spontaneously generates RNS and growth curves were generated. Strains that are able to detoxify the RNS in the medium more rapidly show a decreased lag before entering

logarithmic phase growth. The *fimS* lock OFF strains show an increase in resistance to RNS relative to the *fimS* lock ON strains. The *ipuS* state had only a modest effect on RNS resistance, slightly increasing the resistance of the *fimS* lock OFF strain.

The ability of the locked strains to survive within macrophages was examined by infecting mouse bone marrow derived macrophages and examining intracellular bacterial loads at 1 and 24 hpi. The *fimS* lock ON strains show an increase in macrophage uptake at 1 hpi. However, no difference in intracellular survival is observed as both *fimS* ON and OFF strains are eliminated at similar rates. The *ipuS* state had no impact on uptake or survivability.

The importance of good genetic practices

Prior to the work presented here, few genetic tools were available for the study of UPEC. The majority of the tools designed for the genetic manipulation of *E. coli* are optimized for non-pathogenic laboratory adapted strains (3). As a result they typically function at a reduced efficiency in pathogens, if they function at all (4, 5). Lambda-red mutagenesis is a powerful tool for generating site-specific mutations in *E. coli*. This technique has been adapted for use in UPEC, however the technology renders the host cell susceptible to non-tractable secondary site mutations (4, 6). This issue is further compounded in strains that require multiple Lambda-red mutations in the same background. In *E. coli* K-12, secondary site mutations are eliminated by phage transduction of the mutation into a clean genetic background. However, prior to the work described here, no such tools were available for UPEC. As a result, strains with secondary site mutations have been disseminated to the UPEC community, stymieing progress of the field (7).

Investigators studying other organisms with similar limitations have outlined the process of identifying novel transducing phages (8-12). The identification and characterization of a novel UPEC specific generalized transducing phage presented here has raised the standard for the quality of genetic research in the field. It allows for the generation of more complex mutations that are not easily created by Lambda-red. The phage also provides the flexibility to move mutations to other genetic backgrounds quickly and with a limited risk of incorporating non-tractable mutations. Without this tool, the complex and multiple mutant strains needed for the work presented in this thesis could not have been constructed.

Is *ipuS* part of a larger phase variable network that facilitates infection?

Previous reports have described two distinct switches in CFT073, *fimS* and *hyxS* (2, 13). I have described the existence of a third switch, *ipuS*. The *fimS* switch regulates expression of the adherence promoting extracellular structures Type 1 pili. (14). The *hyxS* switch regulates expression of *hyxR*, a LuxR-type regulator that modulates resistance to reactive nitrogen species and intracellular macrophage survival (2). The work presented here shows the *ipuS* switch is involved in swimming motility and adherence to the kidney epithelium. However, the mechanism underlying this effect remains unclear.

Inversion of these three elements is catalyzed by a shared set of recombinases, FimB, FimE, IpuA, IpuB and FimX. The recombinases and their respective linked switches are encoded at three distantly spaced locations in the chromosome, which is atypical of traditional tyrosine recombinase systems (1, 15). Additionally, is predicted that the recombinases are expressed at low levels predominantly due to their absence in

microarray analyses (16). With three distant binding sites competing for a limited amount of each recombinase, the inversion of one switch may influence the orientation of other two. I predict that this may result in a widely interconnected switching network, where recombinase availability creates a link among the three elements.

Extensive investigation of *fimS* in *E. coli* K-12 and UPEC strains shows that many different environmental conditions regulate *fimS* phase variation. Osmolarity, pH, nutrient availability, temperature, cross talk with other pili operons and location within the urinary tract can alter the population wide *fimS* phase state (17-22). With a potential link between *fimS* phase variation and the other two elements, by extension I postulate that these environmental signals could also alter the state of *hyxS* and *ipuS*. The resulting network could respond to these signals and vary all three elements simultaneously. The result being to select for 1 of the 8 possible phase state combinations that suits the conditions experienced at a given time.

Examples of such regulatory networks exist in other organisms. *Bacteroides fragilis* has an extensive network of switches and recombinases that function at local and distant sites (23-27). One recombinase, Mpi, catalyzes inversion of 13 elements located throughout the chromosome (27). This inversion network controls the expression of surface architecture components and is predicted to function as a mechanism for global surface remodeling in response to changing environmental conditions (24, 27). In *Neisseria* spp. and *Haemophilus influenzae*, phase variation of DNA methyl transferases has global affects on gene expression by altering chromosome wide DNA methylation patterns (28, 29).

The existence of such networks suggests that rather than relying on one or a few essential virulence factors, UPEC strains have evolved mechanisms to regulate broad arrays

of genes concurrently to facilitate infection. This is further supported by the genetic diversity among UPEC strains (30-34). There are few canonical virulence factors shared by all prototypical laboratory UPEC isolates. A functional suite of virulence genes is sufficient rather than any one in particular. Phase variable networks like that described for CFT073 may provide a mechanism to sense a wide variety of signals and coordinate the regulation of many loci. The ability to sense and respond to a broad array of conditions is a useful trait for uropathogens as they occupy a variety of niches including the gut, the periurethral area, and many distinct locations within the urinary tract (21, 35).

Are Type 1 pili a novel factor in reactive nitrogen species resistance?

UPEC face a variety of host defense mechanisms that they must overcome to establish and maintain an infection. One of the most robust host responses is a rapid neutrophil influx that occurs early in infection. This arises from bacterial stimulation of TLR4 and TLR5 on host epithelial cells, leading to production of IL-8 and subsequent recruitment of circulating neutrophils (36-38). Once in the bladder lumen, these professional immune cells secrete nitric oxide (NO), raising NO levels by 30-50 fold in the bladder lumen (39, 40). Nitric oxide is processed into a variety of compounds that inhibit bacteria growth by inducing DNA damage, causing protein and lipid modifications, and inactivating iron-sulfur center enzymes (41). For bacteria to thrive in this hostile environment they must be able to cope with the highly reactive NO derived compounds. The primary mechanism employed by UPEC to deal with this stress is based on the activity of the flavohemoglobin HmpA. HmpA is an oxygen binding protein that oxidizes the highly reactive NO to inert NO_3^- blocking generation of peroxinitrite, S-nitrosothiols, nitrogen

dioxide and other reactive products (41-45). In CFT073, HmpA expression is regulated by the LuxR-type transcriptional regulator HyxR, whose expression is controlled by recombinase mediated phase variation of the invertible element *hyxS* (2).

Phase variation of *hyxS* is dependent on the proximally encoded tyrosine recombinase FimX. This protein is also able to catalyze the OFF to ON inversion of *fimS* the phase variable element of the Type 1 pili locus (1). The OFF state of both *fimS* and *hyxS* independently increase reactive nitrogen resistance and conversely the ON states of both switches independently decrease reactive nitrogen resistance. The reliance on a shared set of recombinases may link the orientation of *fimS* and *hyxS*, so the discovery that *fimS* orientation contributes to similar phenotypes as *hyxS* is not surprising. However, it was unlikely that RNS resistance would be predicted to be a phenotype of *fimS* given its extensively characterized role in regulating the adhesion promoting Type 1 pili.

The mechanism for how *fimS* modulates RNS resistance remains unclear. The analysis presented in Chapter 4 indicate that the effects are independent of HyxR activity, suggesting a direct effect of *fimS* on some component of the RNS resistance pathway. It is possible that *fimS* induced Type 1 pili production silences *hmpA* transcription as is observed with HyxR. It is also possible that the effect is tied to the production of polyamines, which has been linked to RNS resistance (46, 47).

Clear pleiotropic effects have been demonstrated for Type 1 pili expression. Swimming motility is inversely associated with pili production and this reciprocal relationship is not restricted to *E. coli* (1). Similar phenotypes exist in *Vibrio cholerae*, *Salmonella enterica* serovar Typhimirium, *Bordetella pertussis* and *Proteus mirabilis* (48-51). Phase variation of a single locus has also been linked to global changes in gene

expression in *Neisseria* spp. and *Haemophilus influenzae* (28, 29). While no prototypical global regulators are known to be a part of the Type 1 pili locus, this illustrates the potential for pleiotropic effects from phase variable elements. This also suggests that "-omic" style approaches should be a regular component of the characterization of invertible elements to fully understand the implications of phase state determination.

A complex network: multi-site phase variation models and perspectives

Of the 5 recombinases, FimX is the most versatile with detectible catalytic activity at all three loci. IpuB and FimB are the most specific with detectible catalytic activity only at the switches most proximal to them, *ipuS* and *fimS* respectively. FimE and IpuA have detectible activity at both *ipuS* and *fimS*. Conditions that elicit changes in the expression of *ipuA*, *fimE* and *fimX* may have multi site effects that could coordinate the orientation of two to three elements simultaneously. Increased expression of *fimE* could select for the *fimS* OFF/*ipuS* ON state, which could be a mechanism to facilitate the transition from the bladder to the kidneys. The *fimS* OFF/*ipuS* ON state turns off Type 1 expression and increases adherence to kidney epithelial cells via *ipuS*. Increased expression of *fimX* could select for the *fimS* ON/*hyxS* ON/*ipuS* ON states. The triple ON combination should render cells motile and RNS resistant, which would be a useful combination for traversing an RNS rich aqueous environment like the bladder lumen during infection (39, 40).

Further complexity is added when considering that multiple recombinases may be differentially expressed at a given time and this could create competition for binding sites. The ability to vary multiple sites by changing the expression of one or a few recombinases could allow UPEC to coordinate the expression of a diverse set of genes in response to

changing conditions. Additionally, the potential for five different recombinases to contribute to multi-site selection may diversify the conditions that can be sensed by the network. Understanding the dynamics of recombinase expression in vivo would be a valuable step in characterizing multi-site phase state selection.

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Appendix A

Recombinase contribution to phase variation in an otherwise complete network

Results and Discussion

Previous studies have outlined the phase variation of Type 1 pili in the UPEC strain CFT073 (1). In this analysis, the five site-specific tyrosine recombinases that facilitate phase variation of *fimS* were deleted to create Type 1 lock ON and OFF strains. Each recombinase was then provided in trans on a multi copy expression vector to test for catalytic activity at the switch using PCR amplification and restriction digestion. The report showed that four of the five recombinases had independent catalytic activity at *fimS*. With at least four of the recombinases able to catalyze inversion, it was hypothesized that complex interactions and competition would occur in a complete network. However, the dynamics of these interactions were not elucidated by the single activity studies.

To evaluate the role of each recombinase in an otherwise complete network, we deleted each recombinase individually and quantified the resulting population phase state using qPCR. Primers were designed that are specific to either the ON or OFF state and used to PCR amplify genomic DNA (gDNA) template isolated from overnight growth liquid culture. An ON and OFF reaction was performed for each gDNA sample. The copy number of the ON/OFF template in the mixed gDNA samples was then back calculated based on standard curves generated from pure ON or OFF PCR product template. The *ipuA* and *ibpA* mutants show no significant departure from WT levels of population heterogeneity (Figure 1A). Deleting *fimB* results in a nearly phase locked OFF population and deleting *fimE* results in a nearly phase lock ON population (Figure 1A). This was expected as FimB is known to be the primary recombinase for performing the OFF to ON inversion and FimE is the primary recombinase for performing the ON to OFF inversion (2-4). Interestingly, the *ipuB* mutant also results in a nearly phase locked ON population (Figure 1A). Both mutations

were complemented by providing the respective recombinase in trans on multi copy expression vectors (Figure 1B).

No catalytic activity could be detected for IpuB at *fimS* when it is the only recombinase provided in trans to phase locked strains (1). The same *ipuB* construct is capable of inverting the related element *ipuS*, suggesting that the lack of observed activity at *fimS* is not due to complications with exogenous expression (data not shown). This suggests that though IpuB is not capable of catalyzing inversion at *fimS*, it may participate in inversion by excluding other catalytic members from interacting with the switch via competition for binding sites. Removing that competition in the context of a complete network thus results in a significant departure from WT population heterogeneity under the conditions tested (Figure 1).

Functional copies of both *fimE* and *ipuB* are necessary to maintain WT population heterogeneity when under the control of their native promoters. To evaluate their ability to cross complement one another we generated an *ipuB*, *fimE* double mutant and provided *ipuB*, *fimE* or both in trans. At sufficient levels of expression, either recombinase is capable of returning the population to WT levels of heterogeneity (Figure 2). This provides further evidence that while *fimE* and *ipuB* have the same function at *fimS*, to maintain the OFF state, they do so with distinct mechanisms. Having two recombinases with differing mechanisms may provide diversity in the regulation of the Type 1 phase state, allowing it to be regulated more precisely during infection.

Further experiments are necessary to fully characterize the mechanism of IpuB in maintaining the OFF state of the switch. A valuable first step will be to show direct interaction of IpuB with the *fimS* half sites and the ability of IpuB to compete with other

recombinases for binding using electrophoretic mobility shift assays (EMSAs). Due to their insolubility, the recombinases must be generated as maltose binding protein (MBP) fusions. Using these techniques, direct sequence specific binding for FimB and FimE at *fimS* has been demonstrated and similar analyses can be performed for IpuB (3-5). The recombinases are similar in size so competition experiments will require an IpuB specific antibody or an additional affinity tag engineered into the IpuB-MBP fusion protein and shift westerns performed (6, 7).

Phase variation of Type 1 pili is known to respond to a variety of environmental conditions, DNA secondary structure changes, interactions with other pili operons and the ability of accessory proteins to interact with the switch (8-19). In *E. coli* K-12, where the majority of the work on *fimS* phase variation has taken place, there are only two recombinases capable of inverting the switch, FimB and FimE (2). FimE is unable to interact with the switch when it is in the OFF orientation, so competition between the two recombinases for binding is minimal and restricted to the ON state (3). The data reported here outlines an additional UPEC specific layer of complexity to *fimS* phase variation in CFT073 where non-catalytic recombinases contribute to maintaining population phase state heterogeneity.

Methods and Materials

Bacterial strains, plasmids and growth conditions. All of the strains and plasmids used in this study are listed in Table 1. In frame deletion mutants of *E. coli* CFT073 were generated using a modification of Lambda-red method of homologous recombination to include phage transduction of the marker into a clean genetic background

with ΦEB49 prior to removal of the cassette via pCP20. Bacteria were prepared by inoculating single colonies from Luria Bertani (LB)-agar plates into LB broth and grown at 37°C over night with shaking. Antibiotic selection (kanamycin [50 μg/ml], chloramphenicol [20 μg/ml], carbenicillin [250 μg/ml] was used where appropriate.

Population heterogeneity analysis. The population wide Type 1 phase state was calculated by qPCR. Each strain was grown over night in LB broth and gDNA was extracted from 1 ml of the resulting culture using a Wizard gDNA Purification kit (Promega, Madison, WI). qPCR primers were designed with a common anchored external reverse and an ON or OFF specific internal forward primers with the following sequences: ON FWD 5'-ACATATAGCGGAGGTGATGTGAA-3', OFF FWD 5'- CGACGCAACTTCCTCATTCT-3', Common REV 5'- GACATGGGCAGTCGTTCTGT-3'. ON reactions were prepared using BioRad SYBR Green Supermix (BioRad, Hercules, CA) and 10 pmol of ON FWD and Common REV primer. OFF reactions were prepared similarly but 8 pmol of Common REV primer was used. Cycling conditions consisted of: 95°C for 3 min, and 40 cycles of 95°C for 30 sec, 61.9°C for 30 sec, 72°C for 30 sec using a BioRad iCycler (BioRad, Hercules, CA). Between 50-80 pg of gDNA template was used and both ON and OFF reactions were performed in triplicate for each sample. Standard curves were generated for both ON and OFF reactions using 10 fold serial dilutions of PCR production template ranging from 10^0-10^5 copies. Ct values from experimental samples were compared to the standard curves to back calculate the total number of ON and OFF template copies in a given sample. Data presented are the averages of at least three biological replicates each done in triplicate with error bars indicating 1-SD from the mean.

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Table 1. Strains and plasmids used in this study

Strain or Plasmid	Description	Source or reference
Bacterial Strains		
WAM2267	CFT073; urosepsis isolate	(20)
WAM4568	WAM2267 *rpoS*+, *lacZ*	This study
WAM4569	WAM2267 *rpoS*+, *lacZ fimB*	This study
WAM4570	WAM2267 *rpoS*+, *lacZ, ipuB*	This study
WAM4571	WAM2267 *rpoS*+, *lacZ, ipuA*	This study
WAM4572	WAM2267 *rpoS*+, *lacZ, ibpA*	This study
WAM4573	WAM2267 *rpoS*+, *lacZ, fimE*	This study
WAM4707	WAM4573 w/ pWAM2957	This study
WAM4717	WAM4570 w/ pWAM2775	This study
WAM4710	WAM2267 *rpoS*+, *lacZ, ipuB, fimE*	This study
WAM4711	WAM2267 *rpoS*+, *lacZ, ipuB, fimE* w/ pACYC177	This study
WAM4712	WAM2267 *rpoS*+, *lacZ, ipuB, fimE* w/ pACYC184	This study
WAM4713	WAM2267 *rpoS*+, *lacZ, ipuB, fimE* w/ pWAM2957	This study
WAM4714	WAM2267 *rpoS*+, *lacZ, ipuB, fimE* w/ pWAM3580	This study
WAM4715	WAM2267 *rpoS*+, *lacZ, ipuB, fimE* w/ pWAM2957 and pWAM3580	This study
Plasmids		
pKD46	Lambda-Red recombinase helper plasmid	(21)
pCP20	FLP helper plasmid	(21)
pKD4	kan^+ template	(21)
pACYC177	Cloning plasmid with constitutively active promoter	New England Biolabs
pACYC184	Cloning plasmid with expression driven by the insert promoter	New England Biolabs
pWAM2957	pACYC177, *fimE*; Kan^r	(1)
pWAM3580	pACYC184, *ipuB*; Cm^r	(1)

Figure 1. Effect of single recombinase deletion in the context of an otherwise complete network on the Type 1 population phase state. The population phase state from shaking overnight liquid cultures was determined for WT (WAM4568) and each single recombinase mutant (WAM4569 – *fimB*, WAM4570 – *ipuB*, WAM4571 – *ipuA*, WAM4572 – *ibpA*, and WAM4573 – *fimE*) by qPCR analysis. Deleing *fimB* generates a virtually phase locked OFF population. Deleting either *ipuB* or *fimE* generates a virtually phase locked ON population. Deleting either *ipuA* or *ibpA* has no significant impact on population composition relative to WT under the conditions tested.

Figure 1.

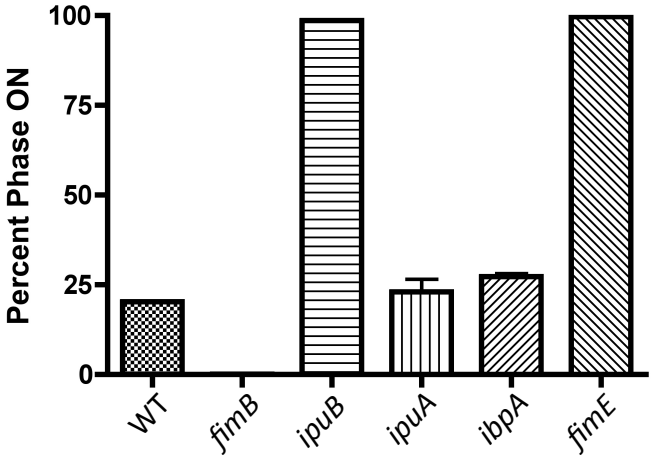
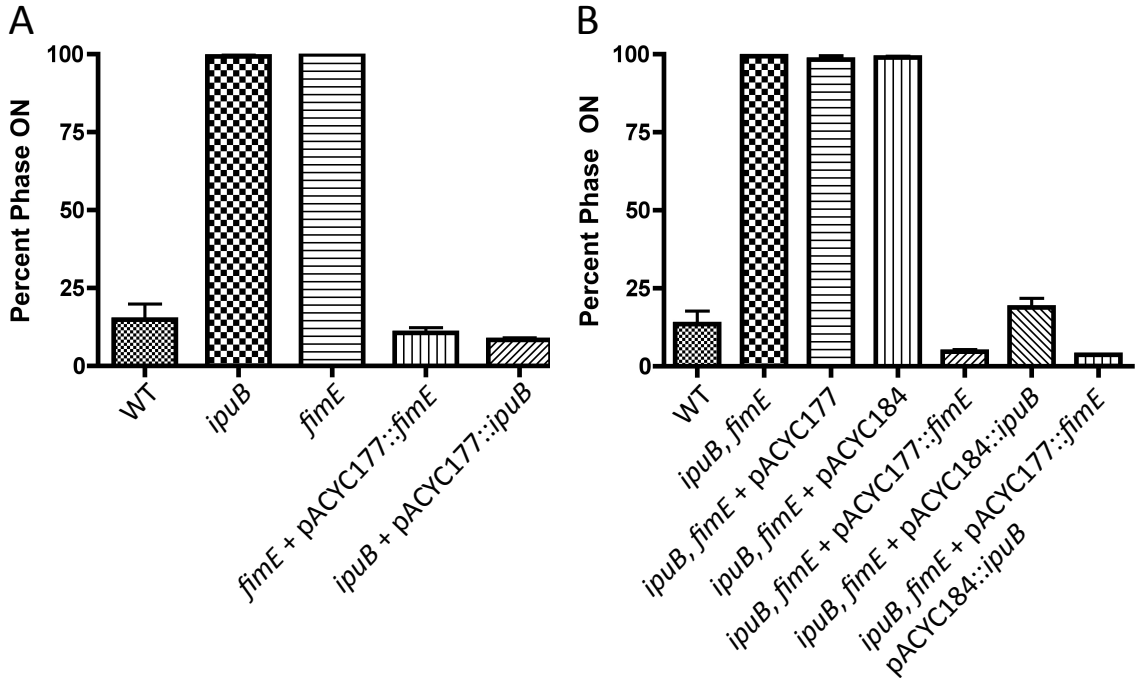


Figure 2. Complementation of the phase locked phenotypes by providing recombinases in trans. (A) Providing either *fimE* (WAM4707) or *ipuB* (WAM4717) back in trans to the respective single mutant (WAM4570 – *ipuB*, WAM4573 – *fimE*) is sufficient to restore WT (WAM4568) population heterogeneity as measured by qPCR. (B) Providing either *ipuB* (WAM4714) or *fimE* (WAM4713) in trans is sufficient to restore WT (WAM4568) population heterogeneity to the *fimE*, *ipuB* double mutant (WAM4710). Vector only addition has no affect on population phase state (WAM4711, WAM4712). Addition of both plasmids results in ON state levels consistent with pACYC177::*fimE* alone (WAM4715).

Figure 2.


A
Percent Phase ON
100
75
50
25
0
WT
ipuB
fimE
fimE + pACYC177::fimE
ipuB + pACYC177::ipuB
B
Percent Phase ON
100
75
50
25
0
WT
ipuB, fimE
ipuB, fimE + pACYC177
ipuB, fimE + pACYC184
ipuB, fimE + pACYC177::fimE
ipuB, fimE + pACYC184::ipuB
ipuB, fimE + pACYC177::fimE
pACYC184::ipuB