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Virulence Plasmid Harbored by Uropathogenic *Escherichia coli* Functions in Acute Stages of Pathogenesis▽

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Urinary tract infections (UTIs), the majority of which are caused by uropathogenic *Escherichia coli* (UPEC), afflict nearly 60% of women within their lifetimes. Studies in mice and humans have revealed that UPEC strains undergo a complex pathogenesis cycle that involves both the formation of intracellular bacterial communities (IBC) and the colonization of extracellular niches. Despite the commonality of the UPEC pathogenesis cycle, no specific urovirulence genetic profile has been determined; this is likely due to the fluid nature of the UPEC genome as the result of horizontal gene transfer and numerous genes of unknown function. UTI89 has a large extrachromosomal element termed pUTI89 with many characteristics of UPEC pathogenicity islands and that likely arose due to horizontal gene transfer. The pUTI89 plasmid has characteristics of both F plasmids and other known virulence plasmids. We sought to determine whether pUTI89 is important for virulence. Both *in vitro* and *in vivo* assays were used to examine the function of pUTI89 using plasmid-cured UTI89. No differences were observed between UTI89 and plasmid-cured UTI89 based on growth, type 1 pilus expression, or biofilm formation. However, in a mouse model of UTI, a significant decrease in bacterial invasion, CFU and IBC formation of the pUTI89-cured strain was observed at early time points postinfection compared to the wild type. Through directed deletions of specific operons on pUTI89, the *cjr* operon was partially implicated in this observed defect. Our findings implicate pUTI89 in the early aspects of infection.

Urinary tract infections (UTIs) represent, by number, one of the most important bacterial infectious diseases in highly industrialized countries (20). Sixty-percent of all women will have at least one UTI within their lifetime (21, 57, 63). This infection results in nearly 7 million physician office visits and $3.5 billion dollars annually in the United States alone (19). It is thought that acute UTIs develop when bacteria from the fecal flora colonize the vaginal and periurethral mucosa and are subsequently introduced into the bladder by urethral ascension. Women who present with an initial episode of acute UTI have a 25 to 44% chance of developing a second and a 3% chance of experiencing three episodes within 6 months of the initial UTI (20). Recurrence occurs despite appropriate antibiotic treatment and clearance of the initial infection from the urine. A large percentage of recurrent UTI are caused by the same strain of bacteria as the initial infection (65). The high frequency of same-strain recurrences supports the notion that a UPEC reservoir may exist in the affected individual.

Uropathogenic *Escherichia coli* (UPEC) is the leading causative agent of UTI, responsible for up to 85% of community-acquired UTI and 25% of nosocomial UTI (62). Using a well-characterized clinical UPEC isolate, UTI89, in a mouse cystitis model, it has been demonstrated that the pathogenesis of UTI involves intracellular and extracellular components (34, 35, 47). The bladder surface is covered with a urothelium composed of very large superficial umbrella cells which are coated with uroplakins that expose a terminal mannose moiety (77). Type 1 pili and its adhesin, FimH, are required for attachment and invasion, thus playing a critical role in the infection process (47, 50, 76). The FimH protein has a negatively charged pocket that accommodates a mannose unit, namely, those exposed by uroplakins on the bladder surface (28). In addition to mannose, it was shown that FimH binds $\alpha 3\beta 1$ integrin subunits expressed by bladder epithelial cells mediating uptake of UPEC (18). Integrins link extracellular matrix proteins with the actin cytoskeleton and can initiate a signaling cascade resulting in bacterial internalization (46, 47). Invasion of UPEC also involves cellular lipid raft components, including uroplakin 1a, caveolin-1, and Rac-1, and internalized bacteria are retained within compartments resembling fusiform vesicles (6, 17). After invasion, bladder epithelial cells are capable of expelling UPEC, presumably as part of an innate defense (69). Another potent innate defense is the exfoliation of umbrella cells and influx of neutrophils (50). A mechanism used by UPEC to combat these defenses is to escape into the cytoplasm of superficial umbrella cells, where they are capable of rapidly replicating into biofilm-like intracellular bacterial communities (IBCs) comprised of 10^4 to 10^5 bacteria (1, 34). Upon IBC maturation, bacteria detach from the IBC biomass, flux out of the host cell into the bladder lumen, and spread to neighboring cells forming next-generation IBCs (34, 38). Thus, IBC formation represents a mechanism by which invasion of a single bacterium can result in rapid expansion of UPEC numbers in the urinary tract leading to disease (1, 51). Upon resolution of infection, the terminally differentiated umbrella cells are replaced through Bmp4-specific signaling, restoring the impermeable uroplakin barrier (53). In addition, bacteria can invade the transitional epithelium beneath the umbrella cells, estab-

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lish a quiescent intracellular reservoir (QIR) and remain dormant until they reemerge and potentially cause a recurrence (52). Reemergence from a QIR could partially explain why recurrent UTI is often caused by a strain identical to the primary infection (65). Recent work has shown that this is a common pathway among UPEC isolates (22), and evidence of this pathway also has been observed in humans (64). The regulatory mechanisms governing IBC development and dispersal in response to environmental cues are still unknown.

Despite many similarities among UPEC isolates, genomic features that are unique to UPEC have not yet been identified. Many studies have identified UPEC as a diverse class of *E. coli* that have evolved multiple and redundant strategies to colonize the urinary tract (75). These diverse genomes have been acquired through horizontal gene transfer (HGT). HGT generates diversity between bacterial species and, through natural selection, contributes to the evolution of bacterial species (16, 40, 66). In addition to genetic variability, newly acquired DNA can enable adaptation to life in a specialized niche through specific factors. The acquisition of DNA by HGT results in the exchange of large regions of DNA called genomic islands (GIs) between bacteria (31). GIs can be acquired in several ways: (i) inheritance of a plasmid that can remain autonomous or recombine into the chromosome, (ii) integration of a lysogenic phage into the chromosome, and/or (iii) insertion of a linear DNA fragment into the chromosome usually by transposition or recombination (14, 43, 70). Some GIs are referred to as pathogenicity islands (PAIs). PAIs contain large genomic DNA regions often greater than 20 kb that carry at least one virulence gene, are inserted within or near tRNA genes, contain direct repeats and mobility sequences, and can frequently be identified by a differing G+C content relative to the bacterial genome (24). In addition, PAIs often do not represent homogeneous pieces of DNA but can instead be mosaiclike structures generated by a multistep process involving genomic acquisition, loss, and rearrangement (25). Study of UPEC PAIs has revealed that they encode numerous virulence factors, including pili and adhesins, toxins, hemolysins, and iron uptake systems. Interestingly, these same virulence factors can be plasmid or phage encoded on intestinal *E. coli* isolates. Thus, UPEC PAIs could represent former plasmid-derived sequences. In addition, UPEC PAIs carry many cryptic genes, open reading frames (ORFs) of unrelated and even unknown functions, and pseudogenes. A UPEC isolate, UTI89, carries a large extrachromosomal element with many characteristics of UPEC PAIs. This plasmid, pUTI89, has a high proportion of pseudogenes generated by insertion events, ORFs homologous to proteins of nonplasmid origin, and orthologs of enteroinvasive *E. coli* (EIEC) proteins found on a large virulence plasmid (12). These features describe a UPEC PAI that has potentially not yet incorporated into the genome, known as a "PAI precursor" (41). pUTI89 is approximately 114 kb and, like plasmid F, contains the *tra* operon for conjugative transfer, as well as genes associated with plasmid replication and inheritance.

Although UPEC strains can cause disease in the urinary tract, they can also exist within the human intestinal tract as part of the normal microbiota. Thus, UPEC strains are distinct from the commensal *E. coli* residing in the gastrointestinal tract in that they possess virulence factors enabling successful transition to and colonization of the urinary tract (8). Factors required for successful infection in addition to type 1 pili are being extensively investigated (76). While studies have demonstrated that additional adhesin systems, toxins, autotransporters, iron acquisition systems, and other factors (reviewed in reference 75) may be important in establishing infection, there is also an abundance of unknown genes labeled as hypothetical or assigned putative functions that may play a role in virulence (32). Here, we studied the role of pUTI89 in the virulence of UPEC. Regions of pUTI89 were shown to be present in the majority of a panel of clinical isolates, suggesting maintenance and transmission within the community. The effect of curing pUTI89 from strain UTI89 was investigated. We found that pUTI89 is involved in an early feature of *in vivo* infection, even though we could not detect any alterations in growth, type 1 pilus expression and function, or biofilm formation *in vitro* in the absence of pUTI89. A region on pUTI89 encoding the *cjr* operon found in EIEC was implicated in this early defect.

MATERIALS AND METHODS

Bacterial strains. *E. coli* UTI89 is a cystitis-derived isolate of serotype O18:K1:H7 (51). UTI89 was used for all assays within the present study. A panel of clinical isolates was used to identify the presence of pUTI89. This panel has been previously described by Garofalo et al. (22).

Plasmid purification and PCR to assay for pUTI89. Bacterial cultures from 18 clinical isolates and UTI89 were incubated overnight at 37°C under shaking conditions. Plasmid was purified similar to the Kado and Liu method (37). The purified plasmid DNA was then used for PCR analysis. Using the sequence of pUTI89, primers were designed to amplify regions around the plasmid. These eight primer pairs were named A to H (Table 1). A primer pair amplifying *ompA* was used as a control for genomic contamination. Standard PCR protocols were used (33). PCR products were run on a 1% agarose gel, and bands were detected by ethidium bromide staining. A band migrating the correct distance represented a "+" for that region of pUTI89.

UTI89 curing. Using the phage lambda red recombinase system (13), UP062 and UP063 encoding the stability operon were deleted from pUTI89 to make UTI89/pUTI89ΔUP062-63, creating a kanamycin-resistant UTI89. This mutant of UTI89 was then cured of pUTI89 by ethidium bromide treatment. Curing was done essentially as described by Bastarrachea and Willetts (4). Briefly, UTI89/pUTI89ΔUP062-63 was grown overnight in LB plus 50 μg of kanamycin/ml. Approximately 10^2 to 10^4 cells of the overnight culture/ml were inoculated into flasks containing LB plus ethidium bromide (100, 90, 80, 70, 60, 50, 40, 30, 20, and 10 μg/ml), followed by shaking at 37°C for 16 h. Using the highest concentration of ethidium bromide that sustained growth (based on visual turbidity), the culture was diluted, plated onto LB agar plates, and grown overnight at 37°C. Single colonies were picked from the LB agar plate and patched onto LB agar plates containing 50 μg of kanamycin/ml. Colonies sensitive to kanamycin were grown overnight in LB and subjected to plasmid purification and PCR for pUTI89 as described above. Cultures with no positive reactions in the PCR assay were considered to be cured of pUTI89.

Growth curves. Bacteria were grown in LB at 37°C shaking overnight. Cultures were diluted 1:1,000 into either LB or M9 minimal medium plus 10 μg of niacin/ml, and 200 μl was inoculated into a 96-well plate in triplicate. A microplate reader running the SoftmaxPro program was used to read at 37°C every 20 min for 10 h with shaking at an optical density at 600 nm (OD_{600}). The data from each read was averaged and graphed to obtain the growth curve.

Immunoblot assay for FimH and FimA. For immunoblot assays of FimH and FimA expression, bacteria were grown statically in LB at 37°C for 24 h to obtain optimal type 1 pilus expression. Equivalent numbers of cells (OD_{600} = 1.0) from each isolate were suspended in sodium dodecyl sulfate (SDS) sample buffer, 1 M HCl was added dropwise until a pH indicator (bromophenol blue) turned yellow, and the solution was heated at 95°C for 5 min. The samples were then neutralized with NaOH, analyzed by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose, followed by immunoblotting with primary antibodies to either FimH or FimA (raised in rabbits using purified FimH or FimA protein, respectively) and secondary antibody alkaline phosphate-conjugated anti-rabbit whole-molecule immunoglobulin G. Specific FimH or FimA proteins were visu-

TABLE 1. Primers for plasmid PCR

Gene	Primer sequence (5′–3′)	Primer name	Size of product (bp)	Primer coordinate position[a]
scsD	CATACGCTGGACGGGGAAAC	A-forward	143	+4384
	GACGCTCTCCCCTTCCGACT	A-reverse		–4527
senB	GCAGATTCGCGTTTTGAGCA	B-forward	302	+25705
	CGGATCTTTCAACGGGATGG	B-reverse		–26006
guaA	CCCGTAGTGGGGGTGTTGAG	C-forward	375	+49081
	GCCCCTGCCACCTACCTTCT	C-reverse		–49456
Transposase	GCTTCGGGAACGCTGTAACG	D-forward	414	+60645
	AGAAGGCTGCGGTGCTGAAG	D-reverse		–61059
ycfA	CGCCTGGTGGTGAAGGAAAG	E-forward	236	+64115
	GACCACCTCCCGCAGAACAC	E-reverse		–64351
UP100, *UP101*	TGGGGGCTGAAAACCAGAGA	F-forward	531	+75326
	ACCGAAGGCACGAACTGCAT	F-reverse		–75857
traU	TTCCTTCTCGCCGGTCATGT	G-forward	111	+89649
	CCAGCGAGAGCGGGAAAATA	G-reverse		–89760
traI	GCGATGCGGTCAGTGTTCTG	H-forward	190	+106531
	GGACAGCCGTTCATCCTGCT	H-reverse		–106721

[a] GenBank accession no. NC_007941.

alized on nitrocellulose membranes by using the BCIP/NBT liquid substrate system (Sigma). Reactions were analyzed for intensity using ImageJ software (http://rsb.info.nih.gov/ij/).

HA assay. Bacteria were grown for 24 h (as described in the immunoblot assay) to induce expression of type 1 pili. Hemagglutination (HA) assays with guinea pig erythrocytes were performed using well-established protocols (27). Briefly, equivalent numbers of cells (OD_{600} = 1.0) from each isolate were suspended in phosphate-buffered saline (PBS) or in PBS plus 2% mannose and added in twofold dilutions to V-bottom 96-well plates (Costar). Guinea pig erythrocytes were resuspended in PBS to an OD_{640} of 1.9 and added to the 96-well plate. The plate was incubated at 4°C overnight. The HA titer was scored by the last well with visible agglutination. An HA titer of 1 equals agglutination at a 1:2 dilution, whereas an HA titer of 9 equals agglutination at a 1:512 dilution, indicating a high level of type 1 pilus function.

Biofilm assay. Bacteria grown overnight in LB were diluted 1:1,000 into LB, M9 minimal medium plus 10 μg of niacin/ml, or YESCA broth and then added to 96-well polyvinyl chloride microtiter plates. LB and M9 plus 10 μg of niacin/ml biofilms were grown at room temperature for 48 h, and YESCA biofilms were grown at 30°C for 48 h. Plates were rinsed and stained with crystal violet and quantified as described previously (56).

Mouse infections. Eight-week-old C3H/HeN (NCI) female mice were anesthetized by inhalation of isoflurane and infected via transurethral catheterization with a 50-μl suspension of ~10^7 CFU of a given isolate. Bacteria for inoculation were grown 24 h at 37°C statically. At the indicated times postinfection, mice were sacrificed by cervical dislocation under anesthesia, and the bladders were immediately harvested and processed as described below. All mouse infection studies were approved by the Animal Studies Committee of Washington University.

Tissue bacterial titer determination. To enumerate the bacteria present, bladders were aseptically harvested at 6, 24, or 48 h or 1 or 2 weeks postinfection; homogenized in PBS; serially diluted; and plated onto LB agar plates. The CFUs were enumerated after 16 h of growth at 37°C.

Gentamicin protection assay. To enumerate the bacteria present in the intracellular versus extracellular compartments, bladders were aseptically harvested at 1 and 6 h postinfection. The bladders were then bisected twice and washed three times in 500 μl of PBS each. The wash fractions were pooled, lightly spun at 500 rpm for 5 min to pellet exfoliated bladder cells, serially diluted, and plated onto LB agar to obtain the luminal fraction. The bladders were treated with 100 μg of gentamicin/ml for 90 min at 37°C. After treatment, the bladders were washed twice with PBS to eliminate residual gentamicin, homogenized in 1 ml of PBS, serially diluted, and plated onto LB agar to obtain the intracellular fraction. The CFUs were enumerated after 16 h of growth at 37°C.

Confocal scanning laser microscopy. To visualize bacterial behavior within the bladder, bladders were aseptically harvested at 6 h postinfection. Bladders were bisected and pinned open revealing the luminal surface. The splayed bladders were then incubated for 20 min at room temperature with (i) Alexa Fluor 488-conjugated wheat germ agglutinin (WGA; 1:1,000 in PBS; Molecular Probes) to stain the bladder luminal surface and (ii) nucleic acid dye SYTO61 red (1:1,000 in PBS; Molecular Probes) to stain bacteria and bladder epithelial cell nuclei. Trolox (10 μM; Fluka) was added to the splayed bladders to reduce photobleaching (67). Bladders were examined with a Zeiss LSM 510 Meta laser scanning inverted confocal microscope using a 63× oil immersion objective lens. WGA and SYTO61 were excited at 488 and 633 nm, respectively.

Enumeration of IBCs. To accurately count the number of IBCs, bladders were aseptically harvested at 6 h postinfection, bisected, splayed on silicone plates, and fixed in 2% paraformaldehyde. IBCs, readily discernible as punctate violet spots, were quantified by LacZ staining of whole bladders (36).

Statistical analysis. Observed differences in bacterial titers and IBC numbers between isolates were analyzed for significance by using the nonparametric Mann-Whitney U test (Prizm; GraphPad Software).

Microarray. UTI89 and ΔpUTI89 were inoculated into LB broth and grown for 24 h at 37°C statically. After 24 h of growth, bacteria were diluted 1:1,000 into fresh LB and grown for an additional 24 h at 37°C statically. RNA was isolated by using an RNeasy minikit (Qiagen). Contaminating DNA was removed from all RNA samples by treating with DNase I for 1 h at 37°C. RNA samples were converted to cDNA, biotinylated, and hybridized to gene chip arrays as described for prokaryotic sample and array processing (Affymetrix). The custom array was designed using ORFs of *E. coli* UTI89, CFT073, and K-12 strains and enterohemorrhagic *E. coli* (EHEC) strains (available sequences at the time of design), resulting in 20,462 sequences. The Affymetrix GeneChip is named UTI89-01a520299F and contains 98.7% (4,977 of 5,044) of the UTI89 chromosome genes and 77.9% (113 of 145) of the pUTI89 plasmid genes. The data were analyzed according to recommendations from analysis of the Golden Spike data set (58), using multi-mgMOS (see reference 14 in reference 58) for summarization, all genes for the post-summarization Loess normalization, and the probability of positive log ratio (PPLR; see reference 13 in reference 58) to identify differentially expressed genes. A cutoff of 0.95 was used as a threshold for the PPLR test.

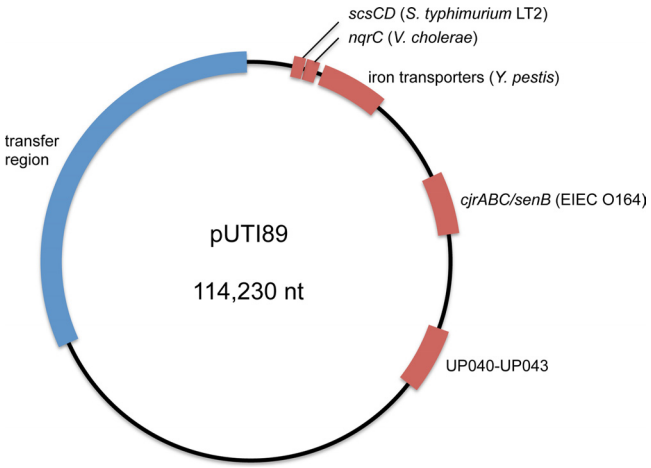


FIG. 1. pUTI89 diagram. pUTI89 can be divided into two major portions. One half contains genes involved in conjugative DNA transfer (in blue), and the other half contains genes present on plasmids from pathogenic *E. coli* and other pathogenic bacteria, as well as genes encoded chromosomally in other bacteria (in red). Indicated next to each region is the gene name, organism it is also found in, and/or the operon function.

RESULTS

UTI89 contains a plasmid with characteristics of a PAI precursor. Many *E. coli* strains possess extrachromosomal genetic elements carrying genes encoding for a DNA transfer apparatus. These elements include the conjugative plasmids F and R100. Pathogenic *E. coli* often possess plasmids, such as pO157 in EHEC isolates, that contain genes encoding for putative virulence factors such as toxins, type III secretion systems, and/or adhesive organelles (9, 44). Many studies have demonstrated the involvement of these plasmids in EHEC pathogenesis (5, 39, 71, 72); however, the role of UPEC plasmids has not been well studied.

UTI89 contains an epichromosomal genetic element of 114,230 nucleotides (12). This plasmid can be divided into two major portions based on its homology with other *E. coli* plasmids. One half of the plasmid contains genes involved in plasmid replication and conjugative DNA transfer similar to those on Plasmid F, an archetypical conjugative plasmid. A large number of ORFs on the other half of pUTI89 show homology (>70% at the amino acid level) to genes present on plasmids carried by pathogenic strains of *E. coli* (Fig. 1). Three pUTI89 ORFs were identified to be orthologs of EIEC O164 *cjrA*, *cjrB*, and *cjrC* genes (68). Expression of these genes in EIEC appears to be negatively regulated by iron and positively regulated by temperature via VirB. A putative iron regulation *fur* box is located upstream of pUTI89 *cjrA* (nucleotides 21010 to 21028) similar to what is seen with their orthologs in EIEC O164 plasmid. UTI89 CjrB is homologous to the TonB protein present in many Gram-negative bacteria, which is thought to be involved in energy transduction between the inner and outer membrane (60). CjrB appears to be an EIEC-specific TonB protein homolog, since *E. coli tonB* strains without *cjrB* were not sensitive to colicin Js.

Likewise, UTI89 CjrC shows homology to putative outer membrane siderophore receptors. Similar to the EIEC O164 large virulence plasmid, pUTI89 possesses the *senB* gene, encoding an enterotoxin, downstream of and partially overlapping the *cjrC* gene. In the EIEC strain, EI34, the SenB gene product was demonstrated to account for at least 50% of the enterotoxic activity (55).

Six pUTI89 ORFs also showed high identity to genes located on the UTI89 chromosome. Four of these were either transposases or hypothetical proteins of unknown function. Two other ORFs, UP042 and UP043, are homologous to genes predicted to possess membrane transport functions. UP042 exhibited 100% amino acid identity to a gene encoding a component of a putative ABC transporter that is also found in UPEC strains F11 and UMN026. UP043 was 95% identical at the amino acid level to an outer membrane heme receptor encoded by the UTI89 chromosome. Similar to their counterparts on the plasmid, the two homologs to UP042 and UP043 in the UTI89 genome (UTI89_C1129 and UTI89_C1130) were also adjacent to each other on the chromosome. Two transposases adjacent to one another on the plasmid, UP040 and UP041, also had homologs (UTI89_C2257 and UTI89_C2258) on the chromosome. However, whereas these two sets of ORFs were located near each other on the plasmid, their homologous sets were over 1 Mb apart on the chromosome. It is unclear whether these genes were acquired separately or as a result of a gene duplication event.

Several ORFs within pUTI89 are homologous to sequences encoding proteins of nonplasmid origin in the database. These were *scsCD* (*Salmonella enterica* serovar Typhimurium LT2), *nqrC* (sodium-translocating NADH dehydrogenase [ubiquinone] gamma subunit [*V. cholerae*]), and iron transporters (putative membrane protein and putative periplasmic protein [*Y. pestis*]). Acquisition of these genes likely occurred through evolution of the plasmid, suggesting that pUTI89 is a hot spot for recombination and genetic exchange. Furthermore, many pseudogenes were present on the plasmid as the result of gene disruption by insertion and deletion (12). The presence of gene homologs from pathogenic bacteria and the fluid nature of the plasmid genome argue that pUTI89 may be a PAI precursor and thus play an important role in UTI pathogenesis.

pUTI89 in UPEC clinical isolates. We sought to determine whether pUTI89 and/or its genes were common among UPEC isolates since strong selection to maintain the plasmid by human strains suggests importance in pathogenesis. We have previously characterized a panel of UPEC clinical isolates (22). This panel of 18 isolates was isolated from women and encompasses all clinical syndromes of UTI and thus is thought to be a representative pool of UPEC strains (22). Extrachromosomal DNA was purified from each of these strains and PCR amplification of eight regions on pUTI89 was used to assess its presence in the clinical isolates (Table 2). Of the 18 isolates, 6 (33%) had all eight regions of pUTI89, suggesting the presence of the entire plasmid, and 5 (28%) had no evidence of pUTI89. Genomic DNA was purified from the five isolates that had no positive PCRs for pUTI89 to determine whether these regions were present on the chromosome. All PCRs were still negative, suggesting these isolates also did not have plasmid genes on their chromosome. Of the 13 isolates that had evidence of pUTI89 (72%), 100% of them had the regions containing *ycfA* and *UP100* and *UP101*, all of which are hypothetical proteins. This would suggest that these proteins are an important char-

TABLE 2. Evidence of pUTI89 in a panel of clinical isolates[a]

Strain	Presence (+) or absence (–) of gene (primer pair used)							
	scsD (A)	*senB* (B)	*guaA* (C)	Transposase (D)	*ycfA* (E)	*UP100*/*UP101* (F)	*traU* (G)	*traI* (H)
ASB1	+	+	+	+	+	+	+	+
ASB2	+	+	+	+	+	+	+	+
ASB3	+	+	+	+	+	+	+	+
ASB4	+	+	+	+	+	+	+	+
ASB5	+	+	+	+	+	+	+	+
acute1	–	–	–	–	+	+	+	–
acute2	–	–	–	–	–	–	–	–
acute3	–	–	–	–	–	–	–	–
acute4	–	–	–	–	–	–	–	–
rUTI1	–	–	–	–	–	–	–	–
rUTI2	–	–	–	–	+	+	–	–
rUTI3	–	+	–	–	+	+	–	–
rUTI4	+	+	+	+	+	+	+	+
rUTI5	–	–	–	–	+	+	+	+
pyelo1	–	–	+	+	+	+	+	–
pyelo2	–	–	–	–	–	–	–	–
pyelo3	–	+	–	+	+	+	+	+
pyelo4	–	–	–	–	+	+	+	–
UTI89	+	+	+	+	+	+	+	+

[a] Gene descriptions and percent prevalences: *scsD*, secreted copper-sensitivity suppressor D, 33.3%; *senB*, enterotoxin TieB protein, 44.4%; *guaA*, hypothetical protein, 38.9%; transposase, transposase for insertion sequence element IS*21*, 44.4%; *ycfA*, hypothetical protein, 72.2%; *UP100*/*UP101*, hypothetical proteins, 72.2%; *traU*, F pilus assembly protein, 61.1%; *traI*, DNA helicase I, 44.4%. Primer pairs A to H are described and defined in Table 1.

acteristic of pUTI89 since they are most commonly maintained in human UPEC isolates. Furthermore, the prevalence of pUTI89 regions within this panel of clinical isolates (72%) suggests its involvement in pathogenesis within the urinary tract. It is possible that the regions identified within the clinical isolates were a reflection of small amounts of genomic contamination in the plasmid preparation and would thus represent chromosomal genes. However, that these regions are still maintained in the population is evidence of their importance.

Deletion of pUTI89. We sought to eliminate pUTI89 from UTI89 and subsequently assess the effect of the loss of the plasmid on pathogenesis as well as in a panel of *in vitro* assays. Traditional ways of eliminating a plasmid from its host involve intercalating dyes, DNA gyrase inhibitors, rifampin, and SDS, whereas more advanced ways use transposon-based elimination (30) and SacB curing (29). pUTI89 did not respond to any of the above methods and was thus not successfully eliminated from UTI89 using any of these approaches. We noted that pUTI89 contains an *stb* locus with 63% homology to StbA of R plasmid NR1 (49). In NR1, StbA is involved in the partition of plasmid molecules to daughter cells during cell division and is therefore essential for stable plasmid inheritance. Mutations that inactivate *stb* function result in plasmid instability, so that plasmid-free cells are segregated at a rate consistent with random distribution of plasmid copies at cell division (48). We therefore deleted the *stbAB* genes from pUTI89 using the λ red recombinase system (13). After deletion, pUTI89 was not spontaneously lost so UTI89/pUTI89ΔstbAB was then subjected to curing by an intercalating dye, ethidium bromide. At a concentration of 90 μg of ethidium bromide/ml, the plasmid was eliminated from UTI89; this was confirmed by selective plating and PCR (Fig. 2). Thus, it required both gene manipulation and a traditional curing method to delete pUTI89 from UTI89.

Deletion of pUTI89 has no *in vitro* effects. The effect of curing UTI89 of pUTI89 on growth under different conditions, pilus expression, and biofilm formation was first evaluated in a series of *in vitro* analyses. UTI89 and ΔpUTI89 grew equally well in both rich media (LB) and minimal medium (M9 plus 10 μg of niacin/ml), suggesting that, under these conditions, the plasmid is not essential for bacterial growth (Fig. 3A). Type 1 pili are an essential virulence factor to UTI89 *in vivo* (76); thus, we investigated whether the expression of the *fim* operon was altered by removal of pUTI89. An immunoblot with anti-FimH and anti-FimA antisera showed no difference in expression levels of either protein in UTI89 versus ΔpUTI89 bacteria (Fig. 3B). Since expression does not necessarily confirm function, the pili were tested for their ability to agglutinate guinea pig red blood cells. HA titers were analyzed in the presence or absence of mannose to confirm that agglutination was a specific measurement of type 1 pilus function. Both strains had equal mannose-resistant and mannose-sensitive HA titers, suggesting that pUTI89 is not involved in type 1 pilus expression or function (Fig. 3D). Previous work has shown that UTI89 forms type 1 pilus-dependent biofilms during growth in LB and curli-dependent biofilms during growth in YESCA (11). Fur-

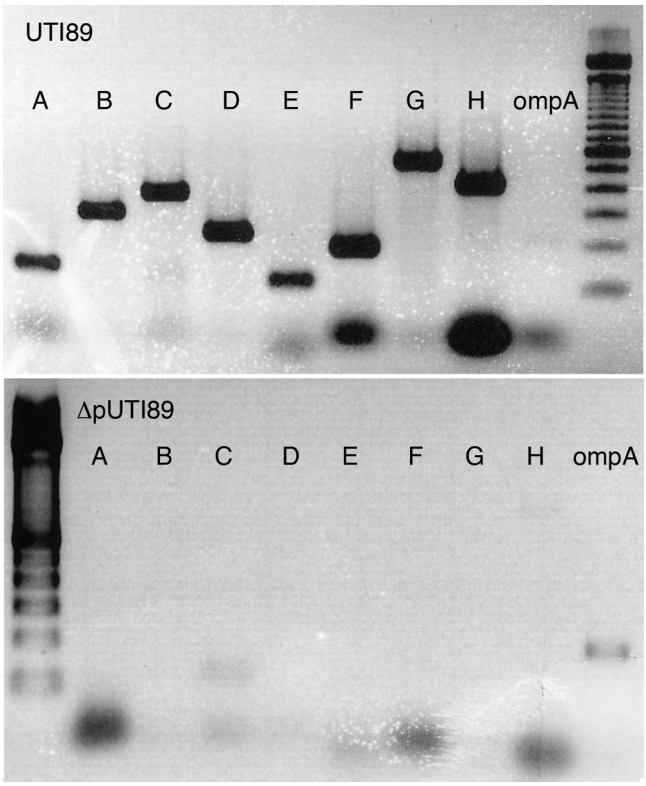
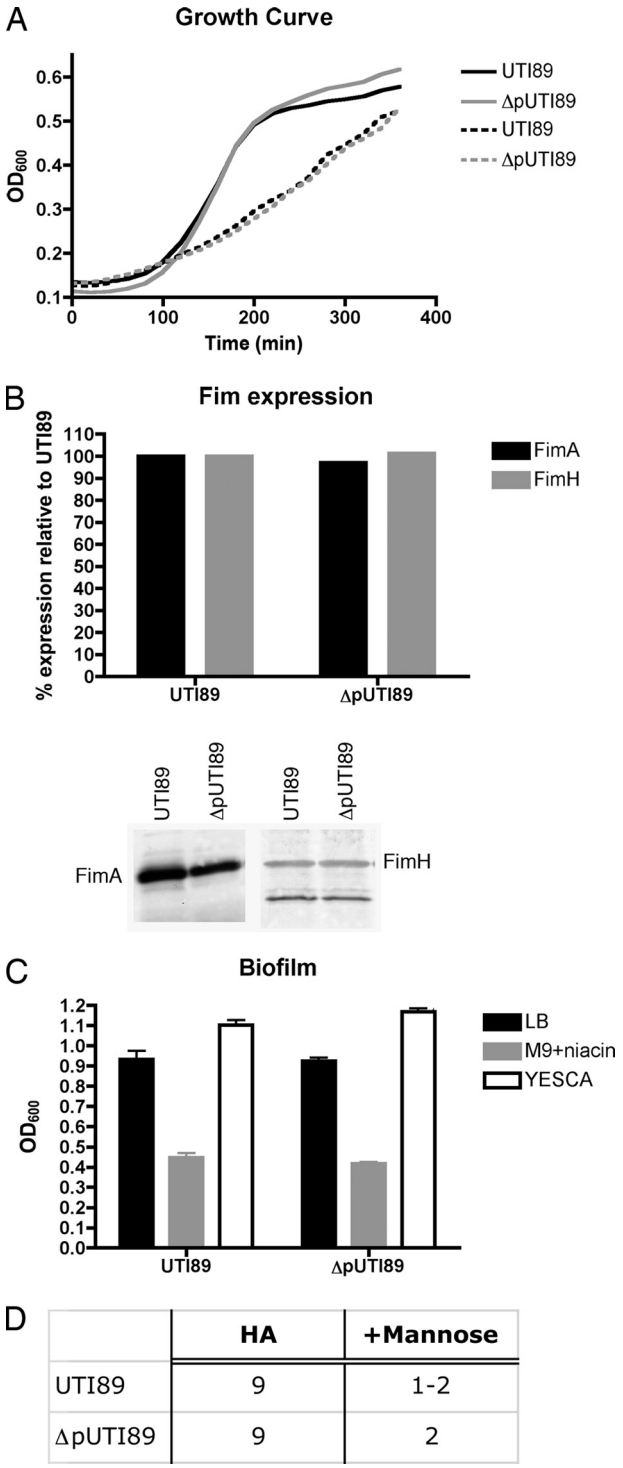


FIG. 2. Confirmation of pUTI89 curing by PCR. pUTI89 was cured from UTI89 by ethidium bromide treatment. Plasmid purification, followed by PCR with primer pairs A to H, was used to identify plasmidless strains. UTI89 with intact pUTI89 (top panel) reveals a banding pattern as observed with very little genomic contamination, as evidenced by the ompA band. PCR of the plasmid-cured strain (bottom panel) revealed no positive PCR confirming the elimination of pUTI89.



D

	HA	+Mannose
UTI89	9	1-2
ΔpUTI89	9	2

FIG. 3. *In vitro* characteristics of pUTI89. Plasmid-cured UTI89 behaved identically to UTI89. (A) Growth of UTI89 and ΔpUTI89 were equivalent in LB broth (solid line) and M9 plus 10-μg/ml niacin broth (dashed line). (B) Type 1 pili expression based on FimH and FimA immunoblots was equivalent in both strains. Densitometry analysis was used to quantify the bands observed (ImageJ software). (C) Biofilm formations under type 1-dependent and curli-dependent conditions were indistinguishable in UTI89 versus ΔpUTI89. (D) Both UTI89 and ΔpUTI89 exhibited equal mannose-sensitive HA titers, indicating equal expression levels and functionality of type 1 pili.

thermore, F plasmids have been shown to promote biofilm formation in a K-12 genetic background during growth in minimal media (23). Thus, the ability of UTI89 and ΔpUTI89 to form these various types of biofilms *in vitro* was evaluated. In both rich medium (LB) and minimal medium (M9 plus 10 μg of niacin/ml), ΔpUTI89 formed biofilms identical to wild-type UTI89 (Fig. 3C). Biofilm formation during growth in YESCA medium at 30°C was also equivalent between the two strains (Fig. 3C). Thus, pUTI89 has no effect on general growth, pilus expression or function, or curli expression or function.

pUTI89 is important for early infection. We sought to determine whether the loss of pUTI89 could have affected the virulence of UPEC in a way that was not measurable by the *in vitro* tests. Thus, *in vivo* fitness was assessed in the UTI mouse model. In this infection model, UTI89 as well as multiple clinical isolates proceed through an IBC pathway during the acute stages of infection (1, 22, 34). Thus, C3H/HeN mice were inoculated with 10^7 of either UTI89 or ΔpUTI89, and the bacterial burden was evaluated at different times postinfection in order to determine whether the plasmid played a role in infection. At 6 h postinfection the bacterial load of ΔpUTI89-infected bladders was significantly lower ($P < 0.0001$) than that of UTI89-infected bladders (Fig. 4A). To minimize the possibility that a secondary mutation contributed to the attenuation, additional clones of ΔpUTI89 were constructed independently, and they were also found to have a significant defect in titers at 6 h postinfection (Fig. 4B). Nevertheless, by 24 h postinfection, ΔpUTI89 titers were comparable to UTI89 and remained that way through the entirety of the time course. These data suggest that genes on pUTI89 may be involved in the acute stages of infection.

pUTI89 is important for intracellular infection. Due to the significant defect during early infection of the ΔpUTI89 strain despite equivalent *in vitro* type 1 pili expression, we assessed the bacteria's ability to bind to and invade the urothelium *in vivo*. Bladders from 1 and 6 h infected C3H/HeN mice were removed, and both the intracellular and the luminal populations were enumerated. Luminal bacteria were defined as the bacteria that were washed from the bladder after removal. Bladders were then treated with gentamicin to kill any residual extracellular bacteria, followed by rinsing. Homogenates of the treated bladders were then plated to enumerate surviving intracellular bacteria. At both 1 and 6 h postinfection, significant decreases in both the luminal and the intracellular populations of ΔpUTI89 were observed relative to UTI89 (Fig. 5). These results suggested that pUTI89 may be involved in enhancing binding, invasion, extracellular survival, and/or aiding in immune evasion. In order to gain more insight into these results, we investigated whether we could visually observe a diminished invasion or reduced extracellular colonization by confocal microscopy. At 6 h postinfection, UTI89- or ΔpUTI89-infected bladders were removed, splayed, and stained. Examination by confocal microscopy revealed that ΔpUTI89-infected bladders had fewer IBCs (Fig. 6) and little to no observable extracellular bacteria compared to its wild-type-infected counterpart. UTI89-infected bladders had numerous extracellular bacteria, many in filamentous morphology. In an effort to more accurately enumerate IBCs, we performed LacZ staining on UTI89- and ΔpUTI89-infected bladders. This quantification revealed a significant defect in the number of IBCs observed

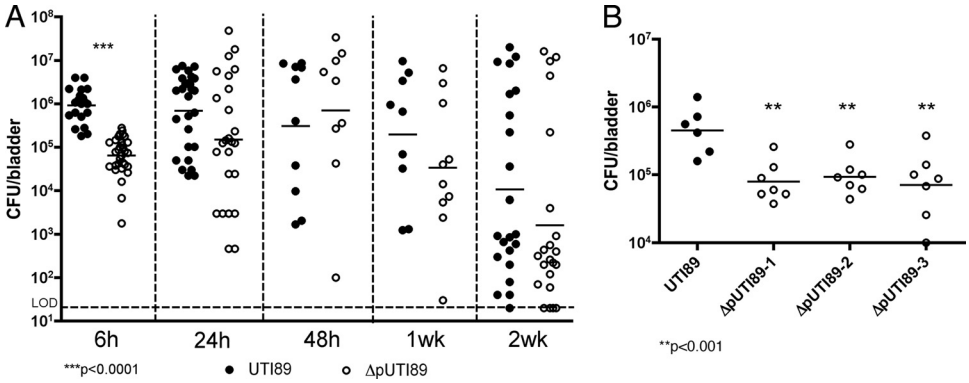


FIG. 4. *In vivo* time course of UTI89 and ΔpUTI89. (A) ΔpUTI89 tested in our mouse model of UTI revealed a significant decrease in bladder colonization at 6 h postinfection compared to wild-type UIT89 ($P < 0.0001$ [Mann-Whitney]). However, by 24 h postinfection and beyond, colonization was equivalent in both strains. (B) Additional clones of ΔpUTI89 confirmed that the defect was due to the loss of pUTI89 and not additional mutations within the genome ($P < 0.001$ by Mann-Whitney).

between the two isolates (Fig. 6). These data are consistent with the results from the gentamicin protection assay and identifies pUTI89 as a potential mediator of binding and invasion, extracellular survival, and/or host evasion at the early stage of infection.

Identification of virulence factors on pUTI89. Deletion of pUTI89 alters UTI89's behavior *in vivo*. In an attempt to decipher which region of pUTI89 is responsible for this observed phenotype, we eliminated regions of the plasmid and examined their effects in the mouse UTI model. Regions were selected for deletion based on their annotation and/or their upregulation upon growth in urine relative to growth in LB (data not shown). Table 3 specifies the gene, size, and predicted function of the regions chosen for deletion. A total of ~23.5 kb was deleted from pUTI89, resulting in nearly one-fourth of the plasmid. Each of the constructed deletions was individually inoculated into the mouse, and the bacterial load was evaluated at 6 h postinfection. Of the seven deletions made, two had a significant decrease on bacterial load relative to UTI89 at 6 h postinfection (Fig. 7). These mutants were ΔUP028-30 and ΔUP062-63. ΔUP062-63 has a 1.4-kb region deleted encoding for the *stbA* and *stbB* genes of the stability operon. These genes are involved in maintenance of the plasmid. The reduction in bacterial titers upon deletion of *stbAB* presumably reflects the plasmid instability and is consistent with the results seen with the ΔpUTI89 mutant (Fig. 3); when pUTI89 is not successfully maintained *in vivo*, the bacterial infection is reduced. ΔUP028-30 has a 4.7-kb region deleted encoding for *cjrB*, *cjrC*, and *senB*. The *cjr* operon (for colicin Js receptor) encodes for proteins that have been described to be involved in colicin Js uptake (68). However, their function in UTI89 is unknown. ΔUP028-30, however, was not as severely attenuated as ΔpUTI89, which suggests that other genes on pUTI89 may also be important during UTI pathogenesis. This study shows that pUTI89 is used to acquire important virulence factors for the early stage of infection. Further work will be required to understand the role of these operons in early infection. Studies are currently under way to examine the expression patterns of the *cjr* and *sen* loci and their impact on UTI pathogenesis.

DISCUSSION

Virulence in *Enterobacteriaceae* has been associated with the acquisition of large plasmids that confer distinguishing pheno-

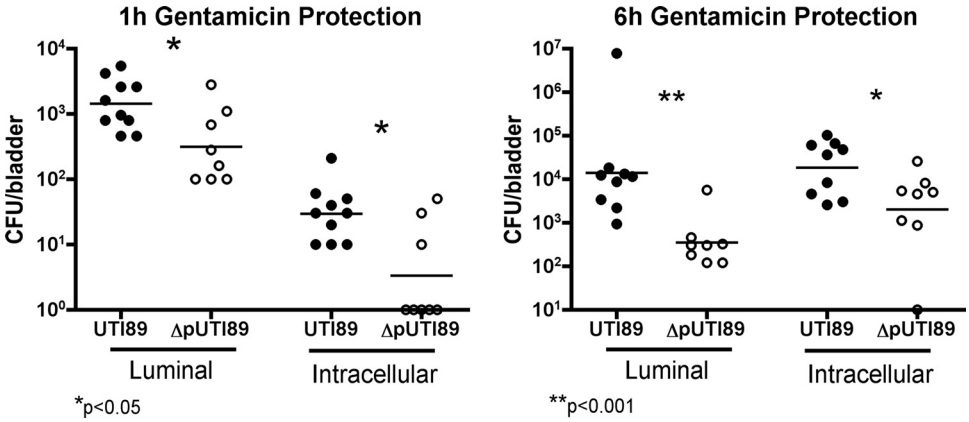


FIG. 5. Colonization and invasion of UTI89 versus ΔpUTI89. Gentamicin protection assays performed at 1 and 6 h revealed a significant decrease in both the extracellular (luminal) and intracellular population for ΔpUTI89. A more significant decrease in luminal colonization with ΔpUTI89 was observed at 6 h postinfection ($P < 0.001$ [Mann-Whitney]).

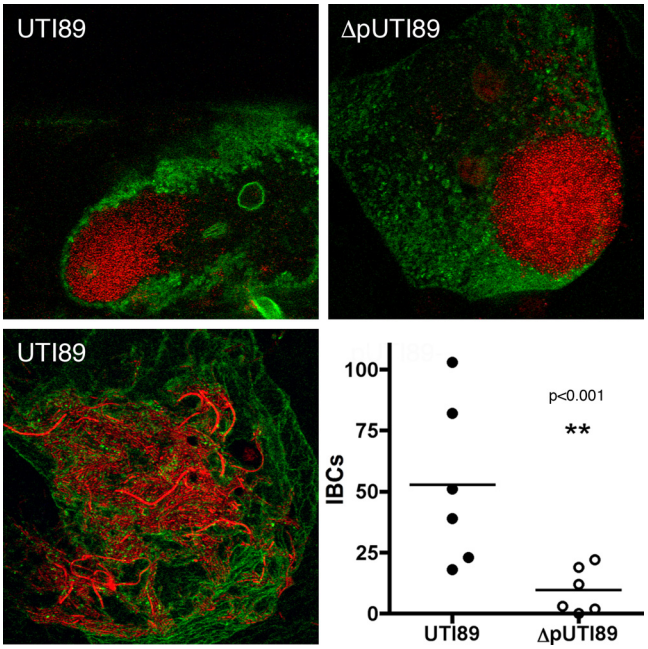


FIG. 6. Confocal microscopy and LacZ staining for IBCs. Mice were infected with UTI89 or ΔpUTI89 and bladders were harvested at 6 h postinfection, splayed, and stained with WGA (bladder surface in green) and SYTO61 (bacteria in red). Confocal microscopy revealed IBC formation in bladders infected with either isolate. However, extensive filamentation was only observed in wild-type UTI89. IBCs were quantitated by LacZ staining revealing a significant defect ($P < 0.001$ [Mann-Whitney]) in the number of IBCs observed in ΔpUTI89-infected bladders.

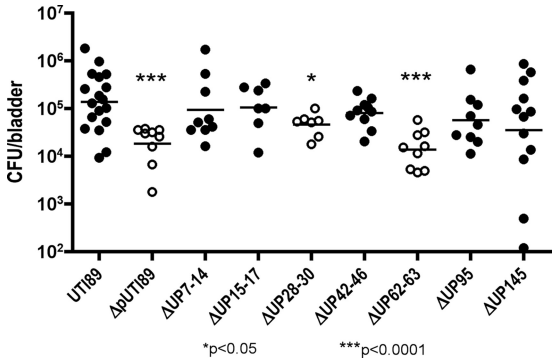


FIG. 7. Identification of virulence factors on pUTI89. Regions on pUTI89 were deleted by using the lambda red recombinase system. Deletions encompassing UP028-30 and UP062-63 (○) resulted in a significant reduction in bacterial load at 6 h postinfection ($P < 0.05$ and $P < 0.0001$ [Mann-Whitney], respectively). UP062-63 encodes the stability operon, and UP028-30 encodes the *cjr* operon.

typic pathogenic traits. These virulence plasmids are generally low copy number, are approximately 60 to 200 kb in size, and are usually similar to F plasmid or R100 plasmid (74). Virulence plasmids in species of enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli*, and enteroaggregative *E. coli* are large F-like plasmids that typically encode different fimbrial adhesins that facilitate characteristic modes of colonization of intestinal epithelia (54). The presence of these F-like virulence plasmids in UPEC has been largely unstudied. Generally, it is thought that virulence genes involved in UPEC colonization are encoded on the chromosome instead of a virulence plasmid. However, a UPEC isolate, UTI89, carries a large F-like plasmid, pUTI89, similar to typical *Enterobacteriaceae* plasmids, in which approximately half of the plasmid encodes genes of unknown function and/or virulence-associated genes. It was recently discovered that a plasmid, pEC14_114, which is nearly identical to pUTI89, seems to be widely present in cystitis-causing UPEC strains, suggesting that it might contribute to UPEC virulence and/or fitness (15). We investigated the role of pUTI89 as a virulence plasmid and discovered that pUTI89 conferred a fitness advantage upon UTI89 in the acute stages of infection.

UTI89 was cured of the pUTI89 plasmid, creating the resulting strain, ΔpUTI89. No differences were observed between ΔpUTI89 and UTI89 in growth kinetics, type 1 pilus formation, or biofilm formation *in vitro*. However, we hypothesized that genes present on pUTI89 that encode hypothetical proteins or other virulence-associated factors could provide a fitness advantage *in vivo* that would not necessarily have been detected in our limited *in vitro* analysis. *Chlamydia trachomatis* contains a plasmid in which no observable phenotype has been identified *in vitro*; however, in the absence of the plasmid, *C. trachomatis* was significantly attenuated and failed to accumulate glycogen *in vivo* (10). The defect was partially attributed to plasmid-dependent regulation of multiple chromosomal genes. Plasmid control of chromosomally encoded genes has also been observed in *Yersinia pestis*, EPEC and *Bacillus anthracis* (3, 7, 59). Thus, we performed a microarray analysis after growth in static LB in order to compare gene expression of UTI89 versus ΔpUTI89. A total of 76 plasmid genes were differentially detected in the presence of pUTI89, including the genes within the *tra* operon, arguing that these genes are expressed *in vitro* under these conditions. In addition, altered regulation of 14 chromosomal genes was observed in the ab-

TABLE 3. Directed deletions of pUTI89 regions

Gene annotation	Size (kb)	Gene name	Function
UP007-UP014	8.4	*scsC*, *scsD*	Secreted copper-sensitivity suppressor, *Yersinia* export
UP015-UP017	1.6		ABC transporter, ATP-binding protein, putative thioredoxin-family protein
UP028-UP030	4.7	*cjrB*, *cjrC*, *senB*	Colicin Js sensitivity protein and outer membrane receptor, enterotoxin
UP042-UP046	6.8		Hemin receptor, serine-threonine protein kinase
UP062-UP063	1.4	*stbA*, *stbB*	Stable plasmid inheritance proteins
UP095	0.4	*psiB*	Regulator of SOS induction
UP145	0.2	*hmo*	Putative regulator

TABLE 4. Chromosomal genes demonstrating a ≥2-fold transcript differential via microarray analysis

Gene[a]	ORF	Fold change[b]
Hypothetical	UTI89_C1048	–458.7
Hypothetical	UTI89_C3298	–140.7
Hypothetical	UTI89_C1896	–130.8
Hypothetical	UTI89_C3859	–69.5
Hypothetical YfaQ precursor	UTI89_C2508	–43.3
Hypothetical	UTI89_C3244	–23.2
Conserved protein YdjA	UTI89_C1960	–14.5
Hypothetical	UTI89_C4976	–4.7
Hypothetical	UTI89_C1130	–3.0
Outer membrane heme/hemoglobin receptor	UTI89_C1129	–2.6
Lambdoid prophage Qin tail fiber assembly proteinlike protein	UTI89_C0564	–2.4
Hypothetical	UTI89_C0954	2.5
Bacteriophage ST64T antitermination protein gp23	UTI89_C2663	2.8
Hypothetical	UTI89_C1727	6.9

[a] Gene transcripts passing all statistical and quality tests performed as described in Materials and Methods.
[b] Change in ΔpUTI89 signal compared to UTI89 signal.

sence of pUTI89, suggesting that pUTI89 influences the regulation of multiple chromosomal genes. However, the majority of these genes are hypothetical and will require further analysis (Table 4).

We have demonstrated that pUTI89 provides a fitness advantage to UTI89 in the acute stages of infection. We observed a significant reduction in luminal colonization, invasion and IBC formation from 1 to 6 h postinfection (Fig. 5 and 6). At the inoculation sizes used in the present study (10^7 bacteria/bladder) no significant differences in CFU per bladder were seen at later time points. Thus, under these conditions, ΔpUTI89 was attenuated in the acute stages but was later able to reach a colonization threshold that allowed it to establish an infection in the bladder. However, under more severe selection pressures, difficult to recapitulate in the laboratory, such as a much smaller inoculum size or ascension from the fecal flora to the urinary tract, fitness advantages in the acute stages would most likely impact the long-term outcome of disease.

Selective deletions of operons on pUTI89 revealed that the *cjr* operon may be playing a role in infection. The operon contains three proteins; however, it was shown that only CjrB and CjrC are essential for colicin Js sensitivity. The CjrC protein of UTI89 is 99% identical to that of EIEC O164. However, it was shown that despite the similarity of CjrBC proteins to gene products of other strains, no bacterial strains other than EIEC and *Shigella* were found to be sensitive to colicin Js. Future experiments to test whether UTI89 is sensitive to colicin Js will identify whether these proteins perform a similar function in UTI89. CjrC is homologous to a putative outer member siderophore receptor, and a *fur* box is located upstream of this operon, suggesting that it is regulated by iron. Iron is an essential cofactor of many enzymes and bacteria require iron concentrations at around 10^{-7} to 10^{-5} M to achieve optimal growth. The level of free iron is estimated to be very low in the environment and biological fluids (10^{-18} M) (2). Bacteria have evolved strategies to acquire iron sequestered by the host in order to survive in specific niches and consequently cause infections. Thus, iron acquisition for the survival of UPEC within the urinary tract is critical (26, 61, 73, 78). UPEC typically contains three to five iron acquisition systems, whereas fecal/commensal isolates contain two to three iron-related operons, suggesting that the redundancy in these systems may provide a competitive advantage to UPEC within the urinary tract (42). In addition, strong preferential expression of yersiniabactin and salmochelin have been observed among urinary strains compared to fecal strains from same patient studies (26). Reigstad et al. showed that in the absence of *chuA* there is a significant decrease in IBC size but not an elimination of IBCs (61). Given our results, we propose that the *cjr* operon is important for iron acquisition; however, it is not essential due to the redundancy of iron acquisition systems within UTI89. Work is currently under way to elucidate the role of Cjr proteins in the early stages of infection.

Strong selection for UTI89 to maintain pUTI89 provides a compelling argument for its importance in pathogenesis. Large plasmids impose a significant metabolic burden on the bacteria and, in the absence of selection, may be lost from the host in spite of plasmid maintenance systems that are assumed to prevent loss (74). pUTI89 has two prominent features: the presence of the *tra* operon involved in DNA transfer and the presence of virulence-associated genes homologous to those found on other pathogenic plasmids, in other organisms. Plasmid transfer via conjugation is an important mechanism for DNA exchange or dissemination and accelerates the evolution of bacterial pathotypes (24). The presence of the *tra* operon allows pUTI89 to be maintained and disseminated to other UPEC strains and *Enterobacteriaceae*. In addition, the large virulence plasmid can serve as a genetic hot spot where extrachromosomal DNA can be freely acquired without disrupting essential genes. The plasmid, pUTI89, may facilitate the free exchange of DNA between the symbiotic gut bacteria. The presence of the *cjr* operon on pUTI89, as well as on a virulence plasmid, in EIEC supports this hypothesis. Presumably, genes, such as *cjr*, that increase fitness in the bladder may be selected upon introduction of the bacteria into the urinary tract. This is supported by our observation that 44% of our tested isolates demonstrated the presence of this operon either within a plasmid or on the chromosome.

The presence of pUTI89-like plasmids is widespread in UPEC strains (15) and could serve multiple roles, allowing for efficient incorporation of foreign DNA without disruption of essential genes. Plasmid stability genes ensure newly acquired genes are replicated without the strong selective pressure of a bladder environment, whereas a functional *tra* operon allows for conjugation and dissemination of these genes to other UPEC strains and *Enterobacteriaceae*. All of these activities provide UPEC with a broad arsenal of proteins, some of which are necessary for colonization of the bladder. Due to the lack of constant selective pressure, UPEC strains have evolved multiple strategies for colonization of the bladder, resulting in the diverse genomes of UPEC strains seen today. The pUTI89 plasmid, with maintenance and stability machinery, may have facilitated the acquisition and incorporation of extrachromosomal DNA without disrupting essential cellular pathways and provided a mechanism that selected for genes that increased fitness in the bladder. This constant evolution has allowed

UPEC to acquire multiple and redundant mechanisms to adapt and thrive in the urinary tract environment, explaining why, despite the well-studied nature of UPEC, especially in regard to virulence factors and colonization strategies, no single feature accurately defines an isolate as UPEC (45). Instead, UPEC contains a mosaic genome that is the result of genomic acquisition, loss, and rearrangement.

It remains unclear as to when, evolutionarily, pUTI89 was acquired and to what extent it helped shape the landscape of the UTI89 genome by acting as a midway point for virulence gene incorporation into the genome. We discovered that the presence of pUTI89 increases the ability of UTI89 to invade and colonize the bladder. Further investigations will examine how less virulent strains without pUTI89 are influenced by its addition. Examining a gain of function in less-virulent strains could help elucidate at what stage(s) pUTI89 virulence genes function. In addition, characterizing plasmids and genes from different UPEC will facilitate our understanding of the mechanism of virulence and the evolution of UPEC and aid in the design of efficacious strategies to fight UTI.

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REFERENCES

1. **Anderson, G. G., J. J. Palermo, J. D. Schilling, R. Roth, J. Heuser, and S. J. Hultgren.** 2003. Intracellular bacterial biofilm-like pods in urinary tract infections. Science **301:**105–107.
2. **Andrews, S. C., A. K. Robinson, and F. Rodriguez-Quinones.** 2003. Bacterial iron homeostasis. FEMS Microbiol. Rev. **27:**215–237.
3. **Bai, G., E. Smith, A. Golubov, J. Pata, and K. A. McDonough.** 2007. Differential gene regulation in *Yersinia pestis* versus *Yersinia* pseudotuberculosis: effects of hypoxia and potential role of a plasmid regulator. Adv. Exp. Med. Biol. **603:**131–144.
4. **Bastarrachea, F., and N. S. Willetts.** 1968. The elimination by acridine orange of F30 from recombination-deficient strains of *Escherichia coli* K12. Genetics **59:**153–166.
5. **Bauer, M. E., and R. A. Welch.** 1996. Characterization of an RTX toxin from enterohemorrhagic *Escherichia coli* O157:H7. Infect. Immun. **64:**167–175.
6. **Bishop, B. L., M. J. Duncan, J. Song, G. Li, D. Zaas, and S. N. Abraham.** 2007. Cyclic AMP-regulated exocytosis of *Escherichia coli* from infected bladder epithelial cells. Nat. Med. **13:**625–630.
7. **Bourgogne, A., M. Drysdale, S. G. Hilsenbeck, S. N. Peterson, and T. M. Koehler.** 2003. Global effects of virulence gene regulators in a *Bacillus anthracis* strain with both virulence plasmids. Infect. Immun. **71:**2736–2743.
8. **Brzuszkiewicz, E., H. Bruggemann, H. Liesegang, M. Emmerth, T. Olschlager, G. Nagy, K. Albermann, C. Wagner, C. Buchrieser, L. Emody, G. Gottschalk, J. Hacker, and U. Dobrindt.** 2006. How to become a uropathogen: comparative genomic analysis of extraintestinal pathogenic *Escherichia coli* strains. Proc. Natl. Acad. Sci. U. S. A. **103:**12879–12884.
9. **Burland, V., Y. Shao, N. T. Perna, G. Plunkett, H. J. Sofia, and F. R. Blattner.** 1998. The complete DNA sequence and analysis of the large virulence plasmid of *Escherichia coli* O157:H7. Nucleic Acids Res. **26:**4196–4204.
10. **Carlson, J. H., W. M. Whitmire, D. D. Crane, L. Wicke, K. Virtaneva, D. E. Sturdevant, J. J. Kupko III, S. F. Porcella, N. Martinez-Orengo, R. A. Heinzen, L. Kari, and H. D. Caldwell.** 2008. The *Chlamydia trachomatis* plasmid is a transcriptional regulator of chromosomal genes and a virulence factor. Infect. Immun. **76:**2273–2283.
11. **Cegelski, L., J. S. Pinkner, N. D. Hammer, C. K. Cusumano, C. S. Hung, E. Chorell, V. Aberg, J. N. Walker, P. C. Seed, F. Almqvist, M. R. Chapman, and S. J. Hultgren.** 2009. Small-molecule inhibitors target *Escherichia coli* amyloid biogenesis and biofilm formation. Nat. Chem. Biol. **5:**913–919.
12. **Chen, S. L., C. S. Hung, J. Xu, C. S. Reigstad, V. Magrini, A. Sabo, D. Blasiar, T. Bieri, R. R. Meyer, P. Ozersky, J. R. Armstrong, R. S. Fulton, J. P. Latreille, J. Spieth, T. M. Hooton, E. R. Mardis, S. J. Hultgren, and J. I. Gordon.** 2006. Identification of genes subject to positive selection in uropathogenic strains of *Escherichia coli*: a comparative genomics approach. Proc. Natl. Acad. Sci. U. S. A. **103:**5977–5982.
13. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U. S. A. **97:**6640–6645.
14. **Davison, J.** 1999. Genetic exchange between bacteria in the environment. Plasmid **42:**73–91.
15. **Debroy, C., M. S. Sidhu, U. Sarker, B. M. Jayarao, A. L. Stell, N. P. Bell, and T. J. Johnson.** 2010. Complete sequence of pEC14_114, a highly conserved IncFIB/FIIA plasmid associated with uropathogenic *Escherichia coli* cystitis strains, and its prevalence among extraintestinal pathogenic isolates from humans and animals. Plasmid **63:**53–60.
16. **Dobrindt, U., F. Agerer, K. Michaelis, A. Janka, C. Buchrieser, M. Samuelson, C. Svanborg, G. Gottschalk, H. Karch, and J. Hacker.** 2003. Analysis of genome plasticity in pathogenic and commensal *Escherichia coli* isolates by use of DNA arrays. J. Bacteriol. **185:**1831–1840.
17. **Duncan, M. J., G. Li, J. S. Shin, J. L. Carson, and S. N. Abraham.** 2004. Bacterial penetration of bladder epithelium through lipid rafts. J. Biol. Chem. **279:**18944–18951.
18. **Eto, D. S., T. A. Jones, J. L. Sundsbak, and M. A. Mulvey.** 2007. Integrin-mediated host cell invasion by type 1-piliated uropathogenic *Escherichia coli*. PLoS Pathog. **3:**e100.
19. **Foxman, B.** 2002. Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. Am. J. Med. **113**(Suppl. 1A)**:**5S–13S.
20. **Foxman, B.** 2003. Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. Dis. Mon. **49:**53–70.
21. **Foxman, B., R. Barlow, H. D'Arcy, B. Gillespie, and J. D. Sobel.** 2000. Urinary tract infection: self-reported incidence and associated costs. Ann. Epidemiol. **10:**509–515.
22. **Garofalo, C. K., T. M. Hooton, S. M. Martin, W. E. Stamm, J. J. Palermo, J. I. Gordon, and S. J. Hultgren.** 2007. *Escherichia coli* from urine of female patients with urinary tract infections is competent for intracellular bacterial community formation. Infect. Immun. **75:**52–60.
23. **Ghigo, J. M.** 2001. Natural conjugative plasmids induce bacterial biofilm development. Nature **412:**442–445.
24. **Hacker, J., G. Blum-Oehler, I. Muhldorfer, and H. Tschape.** 1997. Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. Mol. Microbiol. **23:**1089–1097.
25. **Hacker, J., and J. B. Kaper.** 2000. Pathogenicity islands and the evolution of microbes. Annu. Rev. Microbiol. **54:**641–679.
26. **Henderson, J. P., J. R. Crowley, J. S. Pinkner, J. N. Walker, P. Tsukayama, W. E. Stamm, T. M. Hooton, and S. J. Hultgren.** 2009. Quantitative metabolomics reveals an epigenetic blueprint for iron acquisition in uropathogenic *Escherichia coli*. PLoS Pathog. **5:**e1000305.
27. **Hultgren, S. J., J. L. Duncan, A. J. Schaeffer, and S. K. Amundsen.** 1990. Mannose-sensitive haemagglutination in the absence of piliation in *Escherichia coli*. Mol. Microbiol. **4:**1311–1318.
28. **Hung, C. S., J. Bouckaert, D. Hung, J. Pinkner, C. Widberg, A. DeFusco, C. G. Auguste, R. Strouse, S. Langermann, G. Waksman, and S. J. Hultgren.** 2002. Structural basis of tropism of *Escherichia coli* to the bladder during urinary tract infection. Mol. Microbiol. **44:**903–915.
29. **Hynes, M. F., J. Quandt, M. P. O'Connell, and A. Puhler.** 1989. Direct selection for curing and deletion of Rhizobium plasmids using transposons carrying the *Bacillus subtilis sacB* gene. Gene **78:**111–120.
30. **Imre, A., F. Olasz, J. Kiss, and B. Nagy.** 2006. A novel transposon-based method for elimination of large bacterial plasmids. Plasmid **55:**235–241.
31. **Jain, R., M. C. Rivera, J. E. Moore, and J. A. Lake.** 2003. Horizontal gene transfer accelerates genome innovation and evolution. Mol. Biol. Evol. **20:**1598–1602.
32. **Johnson, J. R.** 1991. Virulence factors in *Escherichia coli* urinary tract infection. Clin. Microbiol. Rev. **4:**80–128.
33. **Johnson, J. R., and A. L. Stell.** 2000. Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. J. Infect. Dis. **181:**261–272.
34. **Justice, S. S., C. Hung, J. A. Theriot, D. A. Fletcher, G. G. Anderson, M. J. Footer, and S. J. Hultgren.** 2004. Differentiation and developmental pathways of uropathogenic *Escherichia coli* in urinary tract pathogenesis. Proc. Natl. Acad. Sci. U. S. A. **101:**1333–1338.
35. **Justice, S. S., D. A. Hunstad, P. C. Seed, and S. J. Hultgren.** 2006. Filamentation by *Escherichia coli* subverts innate defenses during urinary tract infection. Proc. Natl. Acad. Sci. U. S. A. **103:**19884–19889.
36. **Justice, S. S., S. R. Lauer, S. J. Hultgren, and D. A. Hunstad.** 2006. Maturation of intracellular *Escherichia coli* communities requires SurA. Infect. Immun. **74:**4793–4800.
37. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.
38. **Lane, M. C., A. N. Simms, and H. L. Mobley.** 2007. complex interplay between type 1 fimbrial expression and flagellum-mediated motility of uropathogenic *Escherichia coli*. J. Bacteriol. **189:**5523–5533.
39. **Lathem, W. W., T. E. Grys, S. E. Witowski, A. G. Torres, J. B. Kaper, P. I. Tarr, and R. A. Welch.** 2002. StcE, a metalloprotease secreted by *Escherichia coli* O157:H7, specifically cleaves C1 esterase inhibitor. Mol. Microbiol. **45:**277–288.

40. **Lawrence, J. G.** 1999. Gene transfer, speciation, and the evolution of bacterial genomes. Curr. Opin. Microbiol. **2:**519–523.
41. **Lee, C. A.** 1996. Pathogenicity islands and the evolution of bacterial pathogens. Infect. Agents Dis. **5:**1–7.
42. **Lloyd, A. L., D. A. Rasko, and H. L. Mobley.** 2007. Defining genomic islands and uropathogen-specific genes in uropathogenic *Escherichia coli*. J. Bacteriol. **189:**3532–3546.
43. **Maiden, M. C.** 1998. Horizontal genetic exchange, evolution, and spread of antibiotic resistance in bacteria. Clin. Infect. Dis. **27**(Suppl. 1)**:**S12–20.
44. **Makino, K., K. Ishii, T. Yasunaga, M. Hattori, K. Yokoyama, C. H. Yutsudo, Y. Kubota, Y. Yamaichi, T. Iida, K. Yamamoto, T. Honda, C. G. Han, E. Ohtsubo, M. Kasamatsu, T. Hayashi, S. Kuhara, and H. Shinagawa.** 1998. Complete nucleotide sequences of 93-kb and 3.3-kb plasmids of an enterohemorrhagic *Escherichia coli* O157:H7 derived from Sakai outbreak. DNA Res. **5:**1–9.
45. **Marrs, C. F., L. Zhang, P. Tallman, S. D. Manning, P. Somsel, P. Raz, R. Colodner, M. E. Jantunen, A. Siitonen, H. Saxen, and B. Foxman.** 2002. Variations in 10 putative uropathogen virulence genes among urinary, faecal and peri-urethral *Escherichia coli*. J. Med. Microbiol. **51:**138–142.
46. **Martinez, J. J., and S. J. Hultgren.** 2002. Requirement of Rho-family GTPases in the invasion of type 1-piliated uropathogenic *Escherichia coli*. Cell Microbiol. **4:**19–28.
47. **Martinez, J. J., M. A. Mulvey, J. D. Schilling, J. S. Pinkner, and S. J. Hultgren.** 2000. Type 1 pilus-mediated bacterial invasion of bladder epithelial cells. EMBO J. **19:**2803–2812.
48. **Miki, T., A. M. Easton, and R. H. Rownd.** 1980. Cloning of replication, incompatibility, and stability functions of R plasmid NR1. J. Bacteriol. **141:** 87–99.
49. **Min, Y. N., A. Tabuchi, D. D. Womble, and R. H. Rownd.** 1991. Transcription of the stability operon of IncFII plasmid NR1. J. Bacteriol. **173:**2378–2384.
50. **Mulvey, M. A., Y. S. Lopez-Boado, C. L. Wilson, R. Roth, W. C. Parks, J. Heuser, and S. J. Hultgren.** 1998. Induction and evasion of host defenses by type 1-piliated uropathogenic *Escherichia coli*. Science **282:**1494–1497.
51. **Mulvey, M. A., J. D. Schilling, and S. J. Hultgren.** 2001. Establishment of a persistent *Escherichia coli* reservoir during the acute phase of a bladder infection. Infect. Immun. **69:**4572–4579.
52. **Mysorekar, I. U., and S. J. Hultgren.** 2006. Mechanisms of uropathogenic *Escherichia coli* persistence and eradication from the urinary tract. Proc. Natl. Acad. Sci. U. S. A. **103:**14170–14175.
53. **Mysorekar, I. U., M. Isaacson-Schmid, J. N. Walker, J. C. Mills, and S. J. Hultgren.** 2009. Bone morphogenetic protein 4 signaling regulates epithelial renewal in the urinary tract in response to uropathogenic infection. Cell Host Microbe **5:**463–475.
54. **Nataro, J. P., and J. B. Kaper.** 1998. Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev. **11:**142–201.
55. **Nataro, J. P., J. Seriwatana, A. Fasano, D. R. Maneval, L. D. Guers, F. Noriega, F. Dubovsky, M. M. Levine, and J. G. Morris, Jr.** 1995. Identification and cloning of a novel plasmid-encoded enterotoxin of enteroinvasive *Escherichia coli* and *Shigella* strains. Infect. Immun. **63:**4721–4728.
56. **O'Toole, G. A., and R. Kolter.** 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signaling pathways: a genetic analysis. Mol. Microbiol. **28:**449–461.
57. **Patton, J. P., D. B. Nash, and E. Abrutyn.** 1991. Urinary tract infection: economic considerations. Med. Clin. N. Am. **75:**495–513.
58. **Pearson, R. D.** 2008. A comprehensive re-analysis of the Golden Spike data: toward a benchmark for differential expression methods. BMC Bioinformatics **9:**164.
59. **Porter, M. E., P. Mitchell, A. J. Roe, A. Free, D. G. Smith, and D. L. Gally.** 2004. Direct and indirect transcriptional activation of virulence genes by an AraC-like protein, PerA from enteropathogenic *Escherichia coli*. Mol. Microbiol. **54:**1117–1133.
60. **Postle, K.** 1993. TonB protein and energy transduction between membranes. J. Bioenerg. Biomembr. **25:**591–601.
61. **Reigstad, C. S., S. J. Hultgren, and J. I. Gordon.** 2007. Functional genomic studies of uropathogenic *Escherichia coli* and host urothelial cells when intracellular bacterial communities are assembled. J. Biol. Chem. **282:**21259–21267.
62. **Ronald, A. R., L. E. Nicolle, E. Stamm, J. Krieger, J. Warren, A. Schaeffer, K. G. Naber, T. M. Hooton, J. Johnson, S. Chambers, and V. Andriole.** 2001. Urinary tract infection in adults: research priorities and strategies. Int. J. Antimicrob. Agents **17:**343–348.
63. **Ronald, A. R., and A. L. Pattullo.** 1991. The natural history of urinary infection in adults. Med. Clin. N. Am. **75:**299–312.
64. **Rosen, D. A., T. M. Hooton, W. E. Stamm, P. A. Humphrey, and S. J. Hultgren.** 2007. Detection of intracellular bacterial communities in human urinary tract infection. PLoS Med. **4:**e329.
65. **Russo, T. A., A. Stapleton, S. Wenderoth, T. M. Hooton, and W. E. Stamm.** 1995. Chromosomal restriction fragment length polymorphism analysis of *Escherichia coli* strains causing recurrent urinary tract infections in young women. J. Infect. Dis. **172:**440–445.
66. **Saunders, N. J., D. W. Hood, and E. R. Moxon.** 1999. Bacterial evolution: bacteria play pass the gene. Curr. Biol. **9:**R180–R183.
67. **Scheenen, W. J., L. R. Makings, L. R. Gross, T. Pozzan, and R. Y. Tsien.** 1996. Photodegradation of indo-1 and its effect on apparent Ca^{2+} concentrations. Chem. Biol. **3:**765–774.
68. **Smajs, D., and G. M. Weinstock.** 2001. The iron- and temperature-regulated *cjrBC* genes of *Shigella* and enteroinvasive *Escherichia coli* strains code for colicin Js uptake. J. Bacteriol. **183:**3958–3966.
69. **Song, J., B. L. Bishop, G. Li, M. J. Duncan, and S. N. Abraham.** 2007. TLR4-initiated and cAMP-mediated abrogation of bacterial invasion of the bladder. Cell Host Microbe **1:**287–298.
70. **Sowers, K. R., and H. J. Schreier.** 1999. Gene transfer systems for the *Archaea*. Trends Microbiol. **7:**212–219.
71. **Stevens, M. P., A. J. Roe, I. Vlisidou, P. M. van Diemen, R. M. La Ragione, A. Best, M. J. Woodward, D. L. Gally, and T. S. Wallis.** 2004. Mutation of *toxB* and a truncated version of the *efa-1* gene in *Escherichia coli* O157:H7 influences the expression and secretion of locus of enterocyte effacement-encoded proteins but not intestinal colonization in calves or sheep. Infect. Immun. **72:**5402–5411.
72. **Tatsuno, I., M. Horie, H. Abe, T. Miki, K. Makino, H. Shinagawa, H. Taguchi, S. Kamiya, T. Hayashi, and C. Sasakawa.** 2001. toxB gene on pO157 of enterohemorrhagic *Escherichia coli* O157:H7 is required for full epithelial cell adherence phenotype. Infect. Immun. **69:**6660–6669.
73. **Torres, A. G., P. Redford, R. A. Welch, and S. M. Payne.** 2001. TonB-dependent systems of uropathogenic *Escherichia coli*: aerobactin and heme transport and TonB are required for virulence in the mouse. Infect. Immun. **69:**6179–6185.
74. **Venkatesan, M. M., and V. Burland.** 2004. Genome-scale analysis of virulence plasmids: the contribution of plasmid-borne virulence genes to enterobacterial pathogenesis, p. 395–411. *In* B. E. Funnell and G. J. Phillips (ed.), Plasmid biology. ASM Press, Washington, DC.
75. **Wiles, T. J., R. R. Kulesus, and M. A. Mulvey.** 2008. Origins and virulence mechanisms of uropathogenic *Escherichia coli*. Exp. Mol. Pathol. **85:**11–19.
76. **Wright, K. J., P. C. Seed, and S. J. Hultgren.** 2007. Development of intracellular bacterial communities of uropathogenic *Escherichia coli* depends on type 1 pili. Cell Microbiol. **9:**2230–2241.
77. **Wu, X. R., T. T. Sun, and J. J. Medina.** 1996. In vitro binding of type 1-fimbriated *Escherichia coli* to uroplakins Ia and Ib: relation to urinary tract infections. Proc. Natl. Acad. Sci. U. S. A. **93:**9630–9635.
78. **Zhao, L., S. Gao, H. Huan, X. Xu, X. Zhu, W. Yang, Q. Gao, and X. Liu.** 2009. Comparison of virulence factors and expression of specific genes between uropathogenic *Escherichia coli* and avian pathogenic *E. coli* in a murine urinary tract infection model and a chicken challenge model. Microbiology **155:**1634–1644.

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