

In Vivo Gene Expression Analysis Identifies Genes Required for Enhanced Colonization of the Mouse Urinary Tract by Uropathogenic *Escherichia coli* Strain CFT073 *dsdA*^{∇†}

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Deletional inactivation of the gene encoding D-serine deaminase, *dsdA*, in uropathogenic *Escherichia coli* strain CFT073 results in a hypermotile strain with a hypercolonization phenotype in the bladder and kidneys of mice in a model of urinary tract infection (UTI). The in vivo gene expression profiles of CFT073 and CFT073 *dsdA* were compared by isolating RNA directly from the urine of mice challenged with each strain individually. Hybridization of cDNAs derived from these samples to CFT073-specific microarrays allowed identification of genes that were up- or down-regulated in the *dsdA* deletion strain during UTI. Up-regulated genes included the known D-serine-responsive gene *dsdX*, suggesting in vivo intracellular accumulation of D-serine by CFT073 *dsdA*. Genes encoding FIC fimbriae, both copies of P fimbriae, hemolysin, OmpF, a dipeptide transporter DppA, a heat shock chaperone IbpB, and clusters of open reading frames with unknown functions were also up-regulated. To determine the role of these genes as well as motility in the hypercolonization phenotype, mutants were constructed in the CFT073 *dsdA* background and tested in competition against the wild type in the murine model of UTI. Strains with deletions of one or both of the two P fimbrial operons, *hlyA*, *fliC*, *ibpB*, c0468, locus c3566 to c3568, or c2485 to c2490 colonized mouse bladders and kidneys at levels indistinguishable from wild type. CFT073 *dsdA* c2398 and CFT073 *dsdA* *focA* maintained a hypercolonization phenotype. A CFT073 *dsdA* *dppA* mutant was attenuated 10- to 50-fold in its colonization ability compared to CFT073. Our results support a role for D-serine catabolism and signaling in global virulence gene regulation of uropathogenic *E. coli*.

Urinary tract infections (UTI) in adult women impose an estimated cost of \$2.4 billion per year in the United States (30). Most women will experience at least one UTI in their lifetimes, resulting in an estimated 6.8 million physician visits, 1.2 million emergency room visits, and nearly a quarter million hospitalizations each year. *Escherichia coli* remains, by far, the primary causative agent of community-acquired UTIs.

The urinary tract is a normally sterile environment, and it poses several challenges to colonization by *E. coli* and other microorganisms. From its natural reservoir in the colon, uropathogenic *E. coli* (UPEC) must colonize the perineum and the periurethral areas, ascend through the urethra to the bladder, and then continue to ascend via the ureter to the kidneys to cause pyelonephritis. The ascent of UPEC is thwarted by the cleansing flow of urine, free iron limitation, exfoliation of host cells to which UPEC attach, and attack by phagocytic cells and inflammatory mediators. A subset of UTIs progresses to septicemia, exposing the microbe to complement lytic factors. UPEC must be able to bind a variety of differentiated cell surfaces during the ascent and colonization process. At the same time, UPEC must obtain nutrients that may be limited,

invade urinary tract epithelial cells, defend against the host response, and contend with other microbe competitors at sites in the colon and perineum. Identification of adhesins, toxins, and other gene products necessary for UPEC infection and disease has been accomplished by the application of a variety of molecular and genetic analyses to prototypic UPEC strain CFT073. Differential fluorescence induction analysis identified promoters up-regulated during murine peritonitis, signature-tagged mutagenesis analysis and direct mutational analysis of candidate genes identified mutants attenuated in murine UTI, and genome sequencing and subsequent analysis revealed genes encoding fimbriae, iron acquisition systems, and potential toxins (7, 46, 58, 65). Of these candidate virulence genes, molecular Koch's postulates are fulfilled for CFT073 type 1 fimbriae; DegS and DegP, important mediators in the regulation of sigma E and the response to extracytoplasmic stress; TonB, the protein which provides the energy for iron transport across the outer membrane; and PhoU, a negative regulator of the Pho regulon (9, 10, 18, 45, 46, 58). Damage to human and murine cells is mediated by uropathogen-associated toxins such as cytotoxic necrotizing factor 1, hemolysin, and the secreted autotransporter toxin, Sat (20, 47). Other UPEC factors implicated in murine UTI include the putative adhesin gene *iha*, capsule production genes, the osmoregulator ProP, and the genes involved in flagellum-based motility and chemotaxis, *fliC* and *cheW* (7, 13, 27, 29, 69).

Global regulation of *E. coli* virulence factors by Lrp (leucine-responsive regulatory protein), cyclic AMP receptor

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protein, H-NS (histone-like nucleoid structuring protein), and Dam methylase has received attention, as have more specific regulators such as CpxARP, RimJ, the site-specific recombinases FimB, FimE, IpuA, and IpbA, and the antitermination factor RfaH (9, 17, 22, 38, 59, 66, 67). However, it is largely unknown what precise environmental signals uropathogens respond to and how those signals subsequently regulate the expression of virulence factors. In order to better understand how UPEC experiences the urinary tract, we, together with the Mobley and Donnenberg laboratories, recently compared the gene expression patterns of wild-type CFT073 isolated from the urine of infected mice to the pattern of expression during log-phase growth in either complex laboratory growth medium or human urine (55). Analyses of *in vivo* and *in vitro* transcriptomes were accomplished using CFT073-specific microarrays. These studies revealed that UPEC responds to the urinary tract as a high osmolarity, low iron, and low nitrogen environment that requires the high-level expression of type 1 fimbriae for colonization. Differential fluorescence induction experiments using CFT073 by Redford et al. revealed that genes within the sigma E regulon are up-regulated *in vivo* (46). Subsequent mutational analyses revealed that, specifically, the sigma E-responsive genes encoding the periplasmic serine proteases DegS and DegP and putative periplasmic chaperone Skp are required by CFT073 for colonization of the murine urinary tract (45, 46). Our results suggest that UPEC must mount a strong response to periplasmic and outer membrane stress during urinary tract infection. Similarly, expression of a major colonization factor, type 1 fimbriae, is responsive to osmolarity and pH (53). Finally, our laboratory proposed that D-serine is a modulator of UPEC pathogenesis (48). D-Serine, the stereoisomer of L-serine, is present in mammalian urine at concentrations ranging from 3.0 to 40 $\mu\text{g ml}^{-1}$, making it one of the most abundant amino acids found in urine (25). Approximately 85% of UPEC isolates from symptomatic infections express D-serine deaminase (DsdA), which specifically degrades D-serine to pyruvate and ammonia (35, 48). We previously demonstrated that, when in competition with wild-type CFT073, a CFT073 *dsdA* strain hypercolonizes the murine urinary tract and is hyperflagellated and hypermotile *in vitro* (48). The significance of D-serine catabolism during human UPEC infection was recently demonstrated by Roos and Klemm, who found that *dsdA* expression for *E. coli* strain 83972 is consistently up-regulated 2.5- to 8-fold in chronically infected humans, compared to growth in defined medium (49). Our current model proposes that the inability to degrade D-serine in a CFT073 *dsdA* mutant results in elevated intracellular D-serine concentrations during murine infection. This state may lead to either direct hypermodulation of a D-serine-dependent regulon or an indirect alteration of the catabolic potential normally experienced by CFT073 during growth and survival in the urinary tract that results in hypercolonization.

In this study, we aim to identify and explore properties of CFT073 *dsdA* that distinguish this mutant from wild type to determine whether those properties play a role in the UTI hypercolonization phenotype. We compare the *in vivo* gene expression of two strains, the hypercolonizing strain CFT073 *dsdA* and wild-type CFT073. We demonstrate that during colonization of the mouse urinary tract, the two strains have generally similar gene expression patterns in terms of nutrient acquisition, osmolar adaptation, and type 1 fimbriation. How-

ever, compared to the wild type, we observed that the mutant up-regulated well-known UPEC virulence genes encoding P fimbriae, F1C fimbriae, and hemolysin. As predicted, we also saw that CFT073 *dsdA* had elevated expression of the D-serine-dependent gene *dsdX*, which would result from *in vivo* D-serine exposure and its intracellular accumulation in CFT073 *dsdA*. Finally, by deleting up-regulated genes and gene clusters in CFT073 *dsdA*, we show that P fimbriae, hemolysin, a dipeptide transporter, OmpF, a heat shock chaperone, and genes of unknown function are necessary for a CFT073 *dsdA* hypercolonization phenotype.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Urosepsis *E. coli* strain CFT073 was originally isolated from the blood and urine of a woman admitted to the University of Maryland Medical System (33). WAM2615 is a nalidixic acid-resistant derivative of CFT073 in which 445 bp of *dsdA* is deleted (48). WAM2880 is a derivative of CFT073 that has a deletion of *lacZYA* (45). *E. coli* AEAC185 was used as a host for construction of recombinant plasmids (8). UPEC76 is a derivative of CFT073 in which portions of both *pap* operons have been deleted, resulting in CFT073 *papDEFG papE_2F_2G_2* (34). For descriptions of other strains, see Tables 4 and 5 and Fig. 2 and 3. Luria (L) broth and LB agar (Fisher) were used for the propagation of all strains. Carbenicillin, kanamycin, and chloramphenicol were used at concentrations of 250 $\mu\text{g ml}^{-1}$, 50 $\mu\text{g ml}^{-1}$, and 20 $\mu\text{g ml}^{-1}$, respectively.

Scanning electron microscopy. Infected mice were euthanized after 48 h of infection. Approximately 80 μl of Karnovsky's fixative (2% paraformaldehyde, 2.5% glutaraldehyde) was introduced into the bladder of the mouse by catheterization and allowed to fix for 15 min. Subsequently, bladders were removed, bisected, and splayed with dissection pins on small sections of rubber stoppers. Tissues were fixed in Karnovsky's fixative for an additional 2 h at room temperature or overnight at 4°C. Bladders were prepared for scanning electron microscopy using standard methods. Briefly, tissues were washed in sodium phosphate buffer (pH 7.4) and postfixed with 1% osmium tetroxide. Samples were dehydrated in washes containing increasing concentrations of ethanol (30 to 100%), and the last traces of water were removed by washes in increasing concentrations of hexamethyldisilazane (33 to 100% in ethanol). Samples were dried at 65°C for 2 h and mounted to aluminum stubs with carbon adhesive tabs. Stubs were sputter coated with gold-palladium alloy using a SeeVac Auto Conductavac IV sputter coater and viewed on a Hitachi S570 scanning electron microscope.

RNA isolation, cDNA synthesis, and cDNA labeling. Urine-derived RNA samples from CFT073 *dsdA*-colonized mice were obtained essentially as previously described (55). Briefly, 20 CBA/J mice were inoculated with 5×10^9 CFU, and the colonization was allowed to progress for 24 h. Urine was then collected every 0.75 to 1 h directly into 1.5-ml tubes containing 0.650 ml of RNAProtect bacterial reagent (QIAGEN) until the volume measured 1 ml. Two to three of these tubes were obtained during each round of collection. Samples were kept on ice until the bacteria were pelleted from the supernatant by centrifugation at $>20,000 \times g$. The resulting bacterial pellets were stored at -80°C . RNA was isolated from these pellets and DNase treated using a QIAGEN RNeasy kit. Ten micrograms of the resulting pooled RNA was mixed with 750 ng of random hexamers (Invitrogen) and cDNA generated by reverse transcription, as previously described (50). SuperScript II reverse transcriptase (1,500 U; Invitrogen) was added to these reactions, along with First Strand buffer, dithiothreitol, and deoxyribonucleotide at concentrations recommended by the manufacturer (Invitrogen). The reaction mixtures were incubated at 25°C for 10 min, 37°C for 60 min, 42°C for 60 min, and 70°C for 10 min. Following RNase H (Invitrogen) and RNase A (Ambion) digestion for 1 h, cDNA was purified with a QIAquick PCR Purification kit (QIAGEN) according to the QIAquick Spin handbook. Triplicate samples of 3 μg of cDNA were fragmented using RQ1 DNase I (Promega) and then 3' labeled with biotin-N6-ddATP (Perkin-Elmer Life Sciences) using terminal transferase (Roche) as described previously (50).

Microarray hybridization. The *E. coli* CFT073-specific DNA microarray (NimbleGen Systems, Inc.) includes 5,611 open reading frames (ORFs) and stable RNAs from prerelease version 17 of the compiled CFT073 genome sequence (this laboratory; available upon request). Each ORF is represented on the glass slide by 17 unique probe pairs of 24-mer *in situ* synthesized oligonucleotides. Each pair consists of a sequence perfectly matched to the ORF and another adjacent sequence that harbors two mismatched bases for determination of background and cross-hybridization. Labeled cDNA samples were individually

hybridized to the CFT073-specific microarray according to the NimbleGen standard operating procedure. Following washes and labeling with a streptavidin-Cy3 complex according to the NimbleGen procedure, microarrays were scanned at a 5- μ m resolution using a GenePix 4000b scanner.

Microarray data analysis and statistical methods. Data transformations were done simultaneously for all in vivo microarrays, both those produced in this study and the wild-type data from our earlier study (55). Data were extracted using NimbleScan (NimbleGen), and an algorithm (courtesy of Yu Qiu, University of Wisconsin School of Medicine) was applied to obtain a measurement of signal intensity for each ORF as well as a call for a transcript's presence or absence. Data were normalized and converted to estimates of transcript abundance, using the total signal intensity, to allow comparison of individual microarrays (3). A *P* value for each ORF was calculated by a two-tailed Welch's unpaired *t* test comparison of the three microarray replicates for each strain. Relative changes in signal intensity of an ORF between strains were calculated as the following ratio: average CFT073 *dsdA* signal intensity/average wild-type CFT073 signal intensity. Only ORFs with relative changes of at least 2 and a *P* value less than or equal to 0.05 and called present on at least two microarrays were considered significant.

Construction of mutants by λ -Red recombination. Most mutant constructions in CFT073 and WAM2615 were done using the λ -Red recombination system designed by Datsenko and Wanner (14). Oligonucleotides, which were designed to result in precise deletion of the target gene or gene cluster, are listed in Table S1 in the supplemental material. After each gene was replaced with an antibiotic resistance cassette, the cassettes were excised by Flp recombinase, encoded by pCP20, leaving a single Flp recombinase target site, as previously described (14) (Table 1). Excision of the antibiotic resistance cassettes was confirmed by PCR and loss of the antibiotic resistance on the appropriate medium.

Construction of *hlyA::kan* deletion mutant strain of CFT073 *dsdA*. CFT073 *dsdA hlyA::kan* mutant was constructed starting from pWAM1661, which contains the 396 *hlyCABD* operon in which a ~1,550-bp *kan* fragment replaces an 891-bp *NaeI*-*PmeI* fragment within *hlyA*. Using pWAM1661 as a template, oligonucleotides 177 and 279 (see Table S1 in the supplemental material) were used to amplify a 3,285-bp fragment using ExTaq (Roche) according to the manufacturer's protocol. The fragment was gel purified using a QIAGEN gel purification kit. A nested PCR was then performed using the above fragment as a template and oligonucleotides 158 and 163 (see Table S1 in the supplemental material), which amplified a 2,628-bp fragment containing the *hlyA::kan* allele. This fragment was purified using a QIAGEN PCR Cleanup kit and electroporated into CFT073 *dsdA* carrying the λ -Red-expressing plasmid pKD46 (WAM2811). Kanamycin-resistant transformants were patched to sheep red blood cell agar, and nonhemolytic colonies were then screened by PCR using oligonucleotides 177 and 279 to confirm the deletion and *kan* insertion.

Fimbriae expression. The expression of type 1 and P fimbriae was assayed via tests for mannose-sensitive or mannose-resistant hemagglutination (MRHA) of guinea pig erythrocytes (Hema Resource and Supply) or human erythrocytes of type O Rh⁺ or AB Rh⁺. To test for P fimbriae, bacterial strains were grown overnight on LB agar at 37 $^{\circ}$, after which a bacterial sample was transferred to a hemagglutination plate well containing 50 μ l of 3% (vol/vol) human red blood cells (RBCs) and 75 mM D-mannose. Wild-type CFT073 and UPEC76 were used as positive and negative controls, respectively. To assay type 1 fimbrial expression, bacterial strains were grown overnight in LB broth without shaking at 37 $^{\circ}$ C, and a sample was mixed in phosphate-buffered saline (PBS) with 3% (vol/vol) guinea pig RBCs. D-Mannose was added to a final concentration of 50 mM to confirm mannose sensitivity.

CFT073 *pap* complementation. Linear DNA fragments containing each CFT073 *pap* locus were generated using a Triplemaster PCR kit (Eppendorf), using CFT073 genomic DNA as the template and primers 1296 and 1297 for *pap-pheV* and the primers 1300 and 1301 for *pap-pheU* (see Table S1 in the supplemental material). The *pap-pheV* fragment was digested with BamHI and SphI, gel purified, and then ligated with the BamHI-SphI fragment of pACYC184 carrying the origin of replication and the *cat* gene. Similarly, the *pap-pheU* fragment was digested with Sall and BamHI, gel purified, and then ligated with the Sall-BamHI fragment of pACYC184 carrying the origin of replication and the *cat* gene. The ligations were transformed into AE185 and plated to LB-chloramphenicol agar plates. The resulting colonies were screened for MRHA of human RBCs (see above). To confirm proper ligation, plasmids were isolated from MRHA-positive colonies and restricted with NcoI (NEB), and the resulting fragments were separated on a 1% Tris-acetate-EDTA agarose gel. As expected, the pACYC184-*pap-pheV* construct had fragments with apparent sizes of 5,779, 5,020, and 3,395 bp, and the pACYC184-*pap-pheU* had fragments the sizes of 7,155, 5,020, and 2,524 bp. The plasmids pACYC184-*pap-pheV*

TABLE 1. Plasmids used in this study

Plasmid	Description	Source or reference
pWAM1661	Carries a <i>hlyCABD</i> operon with a <i>hlyA::kan</i> deletion	This laboratory
pWAM3455	pACYC184- <i>pap-pheV</i>	This study
pWAM3456	pACYC184- <i>pap-pheU</i>	This study
pKD3	Template for λ -Red <i>cat</i> cassette	14
pKD4	Template for λ -Red <i>kan</i> cassette	14
pKD46	λ -Red recombinase expression	14
pCP20	Encodes FLP recombinase for removal of resistance cassette	14
pSF4000	pACYC184- <i>hlyCABD</i>	64

and pACYC184-*pap-pheU* were designated pWAM3455 and pWAM3456 for the plasmid host strain (Table 1).

Murine model of urinary tract infection. Urinary tract colonization was assayed using the competitive murine model of urinary tract infection essentially as previously described (45, 46). CFT073 *lacZYA* (WAM2880) was used as wild type to allow discrimination of the mutant as pink colonies and WAM2880 as white colonies by differential staining on MacConkey lactose agar medium. This strain colonizes mouse bladders and kidneys indistinguishably from wild type (45). Strains were grown independently in L broth without shaking at 37 $^{\circ}$ C for 7 days, with passage to fresh medium on the second, fourth, and sixth days. Cultures of wild-type or mutant bacteria were concentrated by centrifugation at 7,500 rpm in an Eppendorf 5215D centrifuge for 2 min, resuspended in PBS, and then mixed in a 1:1 ratio. Inocula of 50 μ l, carrying between 1×10^8 to 5×10^8 CFU, were delivered via a catheter to the bladders of isoflurane-anesthetized Swiss Webster mice (Harlan). After 2 days, mice were euthanized by CO₂ asphyxiation, and the bladders and kidneys were excised, homogenized, serially diluted in PBS, and plated to MacConkey lactose agar medium. When tested strains carried plasmids for complementation studies, the medium contained antibiotics at the proper concentrations to select for bacteria that maintained the plasmids. After enumeration, colonization levels were graphed and analyzed using a paired Wilcoxon signed rank test and Prism 4.0c (GraphPad).

Hemolysin expression. Liquid 3-ml LB broth cultures were grown with shaking until an optical density at 600 nm of 0.8 was reached. Bacteria were pelleted, and the supernatants were passed through a 0.45- μ m-pore-size syringe filter. In 96-well plates, filtered supernatants were serially diluted in 0.89% saline–10 mM CaCl₂ and then incubated with a 1% final concentration of sheep RBCs for 1 h. RBCs were pelleted at 4 $^{\circ}$ C, and the *A*₅₄₀ of each reaction mixture was determined with a plate spectrophotometer.

RESULTS

Flagella are required for CFT073 *dsdA* hypercolonization. In this study, we set out to identify CFT073 *dsdA* properties that cause the mouse UTI hypercolonization phenotype. CFT073 *dsdA* exhibits a strong hypercolonization phenotype when in competition with wild type, colonizing the bladder and kidneys at CFU levels eightfold or higher than those of the wild type in all mice. Previously, we found that the CFT073 *dsdA* strain is hyperflagellated and hypermotile compared to wild-type CFT073 (48). A nonmotile CFT073 *dsdA fliC* mutant was constructed and tested in competition with wild-type CFT073 in the murine model of UTI. In competitive challenges of 10 mice, we observed no competitive advantage over wild type by this double mutant in the bladders or kidneys. The median ratios of mutant colonization to wild-type colonization numbers of CFU in bladders and kidneys were 0.13 (*P* = 0.19) and 0.88 (*P* = 0.65), respectively. The loss of hypercolonization behavior in CFT073 *dsdA* by mutation of *fliC* suggests that the CFT073 *dsdA* hypercolonization phenotype is dependent on flagella.

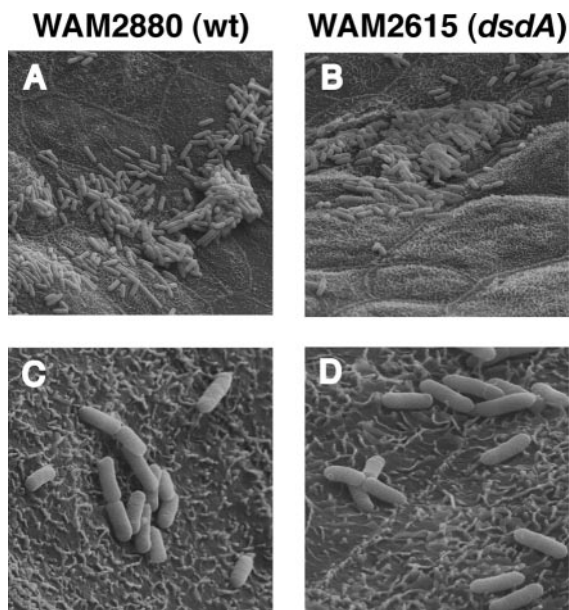


FIG. 1. Electron micrographs of CFT073 and CFT073 *dsdA* during mouse colonization. Shown are scanning electron micrographs of mouse bladders infected with either CFT073 *lacZYA* (WAM2880; wild type) or CFT073 *dsdA* (WAM2615) for 48 h. Panels A and B are the wild-type (wt) and *dsdA* mutant cells, respectively, at a magnification of $\times 3,000$. Panels C and D show the wild-type and *dsdA* mutant cells, respectively, at a magnification of $\times 10,000$.

CFT073 and CFT073 *dsdA* cell morphologies are similar during mouse colonization. We hypothesized that the relative biomass of the wild-type and mutant strains during infection could be similar but with the higher number of CFU for the mutant reflecting either a smaller cell size or the ability to be less aggregated than the wild type. We previously showed by light microscopy that CFT073 *dsdA* grown in human urine has large, swollen cell shapes rather than the typical rod shape seen with CFT073 (48). We used scanning electron microscopy to examine whether the CFT073 *dsdA* hypercolonization phenotype might be due to differences in cell size, morphology, or aggregation in the mouse bladder. Microscopy also permitted us to test the possibility that the mutant possessed differences in the ability to invade bladder epithelial cells to form intracellular bacterial communities or “pods” which Hultgren and colleagues observe with *E. coli* strains UTI89 and NU14 during UTI of C3H mice (5, 37). Presented in Fig. 1 are scanning electron micrographs where it is shown that the CFT073 *dsdA* mutant does not have the aberrant, large swollen-cell morphology in infected bladders of Swiss Webster mice at 48 h postinoculation, which we previously observed when the strain was grown in human urine. The mutant instead appears to be identical to wild-type CFT073 in terms of its cell size and shape and does not appear any more or less aggregated on the epithelial cell surface. In sets of five Swiss Webster mice individually infected with CFT073 or CFT073 *dsdA*, swollen and protruding epithelial structures consistent with what are described as pods by Mulvey et al. were seen on one occasion in only one mouse with each bacterial strain. We also compared the relative in vitro invasion phenotypes of the two strains in gentamicin protection experiments using a human urinary epithelial

cell line, 5637, as previously described (32). No differences between the two strains in the number of CFU of apparent intracellular bacteria were observed (P. Roesch, S. Pellett, and R. Welch, unpublished data).

Identification of genes differentially expressed by CFT073 or CFT073 *dsdA* during experimental UTI. To identify additional differences that might explain the hypercolonization phenotype, we compared the gene expression profiles of wild-type CFT073 and CFT073 *dsdA* during infection of the mouse urinary tract. RNA was isolated from the urine of CBA/J mice colonized with CFT073 *dsdA*. The isolated RNA was then subjected to microarray analysis and compared to a similar data set obtained from the urine of mice colonized by wild-type CFT073 (55). We hypothesized that we would see differential recovery of transcripts encoding known and putative virulence factors from the two populations of infected mice. Hereafter, we refer to this differential recovery of transcripts from mice infected with the wild-type CFT073 or CFT073 *dsdA* strain as differential gene expression, with the term up-regulated or down-regulated referring to higher or lower abundance, respectively, in the CFT073 *dsdA* sample, relative to the wild-type sample.

The gene expression profiles of the wild-type and CFT073 *dsdA* strains during infection were broadly similar. A comparison of the expression data for the in vivo derived samples reveals a Pearson correlation coefficient (r^2) value of 0.7583, where perfect correlation is represented by an r^2 of 1.0. In our earlier study, the r^2 value in the comparison of the gene expression of wild-type CFT073 RNA isolated from LB- and urine-derived samples was much lower, at an r^2 of 0.4059 (55). The two strains share 46 genes in their respective top 50 expressed genes, 82 genes in the top 100, and 416 genes in the top 500, further confirming the similarity of gene expression (data not shown). These highly expressed genes included the translation machinery and type 1 fimbriae, as reported earlier (55). In our previous report on CFT073 *dsdA*, the in vitro expression of type 1 fimbriae was similar for the two strains (48). The microarray results presented here supported the observation that the in vivo transcription of the *fim* operon is unchanged between the two strains. Despite the overall similarity in gene transcription, we identified clear expression differences in a subset of genes, with 44 genes up-regulated and 41 genes down-regulated (Tables 2 and 3). First, the D-serine-responsive genes *dsdX* and the remaining fragment of *dsdA* were up-regulated. McFall and colleagues hypothesized that DsdX is a D-serine-specific transporter, which we recently confirmed (6, 40). We had previously hypothesized that a CFT073 *dsdA* mutant would accumulate intracellular D-serine, which would drive expression of *dsdXA* through the transcriptional regulator DsdC. The increased expression of these known D-serine-responsive genes in CFT073 *dsdA* supports this hypothesis (48).

Along with the *dsdXA*, known virulence genes encoding P fimbriae, hemolysin, and F1C fimbriae were up-regulated in the *dsdA* mutant, as were a number of genes of known and unknown function. We sought to determine if the members of this class of up-regulated genes were necessary for the hypercolonization of this mutant in a competitive infection with wild-type CFT073. Focusing on highly up-regulated genes and loci, uropathogen-specific sequences, and known virulence factors, we made mutations in genes and gene clusters in the *dsdA*

TABLE 2. Genes up-regulated relative to wild type in CFT073 *dsdA* during infection

Locus	Gene	Product	Relative change (<i>n</i> -fold)	<i>P</i> value
c2486		COG2120; hypothetical proteins; LmbE homologs	20.12	0.0037
c2490		hypothetical protein <i>yaiO</i>	19.66	0.0132
c0468		COG1140; putative transferase; LpxD family	15.48	0.0051
c2900	<i>dsdX</i>	D-Serine permease	11.14	0.0002
c2489		COG1140; putative transferase; LpxD family	9.95	0.0005
c3568		Hypothetical protein	8.47	0.0063
c3592	<i>papA</i>	Major P pilin subunit	8.40	0.0001
c2487		Pseudogene	8.06	0.0015
c0704	<i>citF</i>	Citrate lyase alpha chain	8.00	0.0020
c3566		COG4117; putative membrane anchoring protein	7.01	0.0011
c2488		Pseudogene	6.31	0.0000
c1239	<i>focA</i>	F1C major fimbrial subunit precursor	6.25	0.0009
c0459	<i>lacZ</i>	Beta-galactosidase	6.21	0.0018
c3569	<i>hlyC</i>	Hemolysin C	5.72	0.0073
c3570	<i>hlyA</i>	Hemolysin A	5.22	0.0243
c2541		Hypothetical protein	4.01	0.0283
c1244	<i>focG</i>	F1C minor fimbrial subunit protein G	3.81	0.0212
c2398		Hypothetical protein	3.42	0.0023
c0728	<i>ybeB</i>	Hypothetical protein	3.38	0.0130
c1071	<i>ompF</i>	Outer membrane protein F precursor	3.12	0.0005
c2283	<i>yecN</i>	COG3788; hypothetical protein	3.02	0.0063
c0399	<i>yagU</i>	Hypothetical protein YagU	2.78	0.0038
c0074	<i>araA</i>	L-Arabinose isomerase	2.77	0.0286
c0457	<i>lacA</i>	Galactoside <i>O</i> -acetyltransferase	2.53	0.0397
c4606	<i>ibpB</i>	16-kDa heat shock protein B	2.53	0.0082
c3591	<i>papH</i>	PapH protein	2.46	0.0202
c0707		Pseudogene	2.35	0.0045
c2901	<i>dsdA</i>	D-Serine dehydratase	2.35	0.0482
c4799		Pseudogene	2.34	0.0227
c2564		Hypothetical protein; O6 polymerase Nissle?	2.34	0.0058
c2563		COG0463; WcaA family of glycosyltransferases	2.26	0.0062
c0243		Pseudogene	2.25	0.0187
c4361	<i>dppA</i>	Periplasmic dipeptide transport protein	2.24	0.0027
c0579	<i>ybaJ</i>	Hypothetical protein YbaJ	2.19	0.0260
c4967		Pseudogene	2.17	0.0214
c2232	<i>yobF</i>	Hypothetical protein YobF	2.14	0.0007
c4830		Putative shikimate transporter	2.12	0.0336
c4038		Pseudogene	2.11	0.0265
c2566		Polysaccharide synthase/flippase	2.09	0.0089
c2231	<i>cspC</i>	Cold shock-like protein CspC (CSP-C)	2.07	0.0290
c3203		Hypothetical protein	2.05	0.0044
c4798		Pseudogene	2.04	0.0200
c2291	<i>argS</i>	Arginyl-tRNA synthetase	2.03	0.0421
c4228	<i>yhhW</i>	Protein YhhW	2.02	0.0321

background by λ -Red-mediated recombination (14). Each mutant was then tested in competition with the wild type in the murine model of UTI to determine whether the deleted gene or locus was required for the hypercolonization phenotype (Table 4). We decided that because of the large number of candidate genes that arose from our genome-wide screen, complementation constructs would not be made for each mutant. This was based on the difficulties presented by the unknown transcriptional organization of some of the genes, the “hit-or-miss” difficulties in using plasmid complementation constructs in the mouse UTI model as described below, and, lastly, a desire to limit the number of mice used. We expect that for the significant genes identified below, detailed genetic analyses and appropriate complementation studies using Tn7-based chromosomal insertions will be performed by us or other interested investigators.

***hlyA* is necessary for hypercolonization of the bladder.** We observed statistically significant fivefold up-regulation of *hlyCA*

and a trend toward up-regulation of *hlyB* (Table 2). The full-length *hlyCABD* transcript is known to be quickly degraded to a more stable *hlyCA* transcript, which may account for difficulty in assaying the full-length *hlyCABD* by microarrays (62). CFT073 *dsdA hlyA::kan* did not hypercolonize (Table 4). This suggested that the expression of the hemolysin toxin is necessary for the increased colonization phenotype of CFT073 *dsdA*. To confirm the role of hemolysin expression in CFT073 *dsdA* hypercolonization, a plasmid carrying the full *E. coli* J96 *hlyCABD* operon (pSF4000) was introduced into both wild-type CFT073 and CFT073 *dsdA hlyA::kan*, and the resulting strains were tested in competition in the murine model UTI. It is unlikely that the allelic differences between the CFT073 and J96 hemolysins results in significant differences in toxicity. CFT073 *dsdA hlyA::kan* carrying pSF4000 significantly out-competed the CFT073 carrying pSF4000 in bladder colonization, and there was a trend toward complementation of hypercolonization of the kidney (Fig. 2). Similar results were

TABLE 3. Genes down-regulated relative to wild type in CFT073 *dsdA* during infection

Locus	Gene	Product	Relative change (<i>n</i> -fold)	<i>P</i> value
c3104		Hypothetical protein	-11.82	0.0214
c0696	<i>ybdQ</i>	Universal stress protein G, UspG	-6.35	0.0328
c4309		Hypothetical protein	-5.26	0.0021
c1223		Pseudogene	-4.89	0.0151
c2138	<i>osmE</i>	Osmotically inducible lipoprotein E	-4.43	0.0143
c4938		Hypothetical protein	-3.69	0.0409
c3986	<i>yhcM</i>	Hypothetical protein; predicted ATPase	-3.63	0.0372
c2839		Hypothetical protein	-3.61	0.0166
c1905		Hypothetical protein	-3.59	0.0407
c0813		Hypothetical protein	-3.53	0.0461
c3343		Hypothetical protein	-3.50	0.0109
c4088		Hypothetical protein	-3.34	0.0325
c3103	<i>yfiD</i>	COG1882; putative formate acetyltransferase	-3.30	0.0103
c2225		Hypothetical protein	-3.21	0.0100
c0556	<i>hupB</i>	DNA-binding protein HU-beta, NS1 (HU-1)	-2.98	0.0151
c4942		Hypothetical protein	-2.90	0.0071
c2325	<i>pgsA</i>	Phosphatidylglycerophosphate synthetase	-2.83	0.0329
c1040		Hypothetical protein	-2.76	0.0142
c4064		Hypothetical protein	-2.76	0.0168
c4081		Hypothetical protein	-2.72	0.0477
c2072	<i>lpp</i>	Murein lipoprotein	-2.67	0.0386
c3306		<i>rpoS</i> pseudogene	-2.64	0.0009
c2722	<i>rplY</i>	50S ribosomal subunit protein L25	-2.55	0.0333
c5138	<i>lysU</i>	Lysine tRNA synthetase; heat inducible	-2.50	0.0040
c4067		Hypothetical protein	-2.49	0.0445
c3942	<i>rpmA</i>	50S ribosomal subunit protein L27	-2.48	0.0289
c4957	<i>hupA</i>	DNA-binding protein HU-alpha (HU-2)	-2.47	0.0147
c4587	<i>yicN</i>	Hypothetical protein	-2.35	0.0069
c1406		Hypothetical protein	-2.33	0.0098
c3232	<i>proX</i>	Transport system for glycine betaine and proline	-2.31	0.0049
c3253	<i>recA</i>	DNA recombinase A	-2.27	0.0048
c3127	<i>rplS</i>	50S ribosomal subunit protein L19	-2.25	0.0268
c0764		Putative pyridoxine phosphate biosynthetic protein	-2.22	0.0002
c0018		Hypothetical protein	-2.21	0.0127
c1796	<i>tpx</i>	Thiol peroxidase	-2.18	0.0163
c2385	<i>yedU</i>	Chaperone protein HchA	-2.16	0.0070
c0464	<i>yaiM</i>	COG0627 putative esterase	-2.13	0.0401
c3944		Hypothetical protein	-2.09	0.0280
c4661		Hypothetical protein	-2.03	0.0292
c1907	<i>fdnI</i>	Formate dehydrogenase N; gamma subunit	-2.03	0.0395
c0864		Hypothetical protein	-2.01	0.0488

obtained when the CFT073 *dsdA* strain carried only the parental vector plasmid, pACYC184, in competition with CFT073 *dsdA* *hlyA::kan*(pSF4000) (data not shown). These data confirmed the necessity of hemolysin expression for bladder hypercolonization by CFT073 *dsdA*.

Each CFT073 *pap* determinant is needed for hypercolonization. CFT073 contains two P fimbrial operons, present on genomic islands near the tRNA genes *pheU* and *pheV*. Gene expression analysis identified statistically significant up-regulation in the *pap-pheV* operon genes *papA*, *papD*, and *papH*, as well as the *pap-pheU* gene *papA_2* (Table 2). The double *pap* deletion strain CFT073 *dsdA pap-pheV pap-pheU* and the two single deletion strains CFT073 *dsdA pap-pheV* and CFT073 *dsdA pap-pheU* were unable to hypercolonize (Table 4). This result suggests that these two *pap* operons are not redundant and that both are required for hypercolonization of the mouse urinary tract by CFT073 *dsdA*. CFT073 *dsdA* and both single *pap* deletion strains remained positive for MRHA of human type O and AB RBCs, whereas CFT073 *dsdA pap-pheV pap-*

pheU was negative, indicating that each CFT073 *pap* operon is functional (data not shown).

Recombinant plasmids carrying either the *pap-pheV* or *pap-pheU* loci were constructed as pACYC184-based episomes, pWAM3455, and pWAM3456. These constructs were found to be sufficient to render an *E. coli* K-12-based cloning strain AAEC185 MRHA positive. These plasmids were individually transformed into CFT073 *dsdA pap-pheV pap-pheU*. The resulting strains were also MRHA positive (data not shown). Therefore, the plasmids were able to complement the *pap* hemagglutination phenotype.

Both wild-type CFT073 and CFT073 *dsdA pap-pheV* strains were transformed with pWAM3455, resulting in strains with presumably similar copy numbers of *pap-pheV*. These two strains were tested in the competitive murine UTI model. No difference in colonization levels was observed for the two strains (Fig. 3). The plasmid, while able to restore P fimbriation in a fully *pap* null strain, did not restore hypercolonization to the CFT073 *dsdA pap-pheV* mutant with respect to the wild type carrying the same

TABLE 4. Relative recovery at 48 h of 18 mutants of CFT073 *dsdA* and wild type after competitive infection of the urinary tract

WAM strain ^a	Deleted gene(s)	<i>n</i> ^b	Relative recovery in: ^c			
			Bladder		Kidney	
			Ratio	<i>P</i>	Ratio	<i>P</i>
2615 ^d	N/A	15	68	<0.01	110	<0.01
3196	<i>c2398</i>	10	11	<0.01	13	0.03
3183	<i>focA</i>	7	24	0.03	2200	0.02
3215 ^e	<i>pap-pheV</i> , <i>pap-pheU</i>	13	3.1	0.17	0.38	0.41
3206	<i>pap-pheV</i>	14	1.8	0.71	2.9	1.0
3205	<i>pap-pheU</i>	10	3.3	0.43	2.9	0.65
3187	<i>hlyA::kan</i>	15	1.7	0.52	0.66	0.22
3275	<i>c3566</i>	10	0.80	0.43	0.41	0.32
3276	<i>c3568</i>	10	3.7	0.38	2.2	0.85
3277	<i>c3566-68</i>	13	1.5	0.95	1.2	0.41
3221	<i>c2485-c2490</i>	7	1.1	0.94	0.14	0.46
3752	<i>ibpB</i>	12	1.6	0.57	1.7	0.41
3750	<i>c0468</i>	10	0.44	0.08	0.64	0.57
3754	<i>dppA</i>	8	0.02	0.02	0.07	0.03
3761	<i>ompF</i>	8	3.1	<0.01	1.4	0.64

^a Unless otherwise noted, all strains are derivatives of WAM2615, which is a nalidixic acid-resistant derivative of CFT073 in which 445 bp of *dsdA* are deleted (48).

^b Number of mice analyzed.

^c Data shown are the ratio of mutant versus wild-type CFU (median). *P* values were determined by a Wilcoxon signed rank test.

^d Data are from an analysis of WAM 2615, CFT073 *dsdA*, in Roesch et al. (48) and are included here for reference purposes.

^e WAM3215 is a derivative of WAM3206.

vector plasmid used for complementation. Failure of some plasmid-based complementation constructs during experimental mouse UTIs has been observed by others (M.S. Donnenberg and H. L. Mobley, personal communications).

Genes for an outer membrane, a periplasmic, and a cytoplasmic protein contribute to hypercolonization. *ompF*, *dppA* and *ibpB*, which, respectively, encode an outer membrane

porin, a periplasmic dipeptide transporter, and a small cytoplasmic heat shock chaperone were all up-regulated in vivo in CFT073 *dsdA* (Table 2). All three genes are ubiquitous among *E. coli* strains. Mutations in any of these three genes in the CFT073 *dsdA* background resulted in loss, to various degrees, of the hypercolonization phenotype (Table 4). The CFT073 *dsdA ompF* mutant had clearly lost the ability to hypercolonize the kidney; however, in the bladder it still hypercolonizes to a significant degree. Interestingly, the CFT073 *dsdA dppA* double mutant was attenuated 10- to 50-fold in its ability to colonize mouse bladders and kidneys compared to the wild type.

Potential sulfur metabolism genes are required for hypercolonization. Genes *c3566*, *c3567*, and *c3568*, which are located 5' of the hemolysin operon, were up-regulated seven- to nine-fold in CFT073 *dsdA* (Table 2). Based on BLAST searches, these genes are predicted to encode a thiosulfate reductase cytochrome *b* subunit, a sulfite-oxidase, and a small protein of unknown function, respectively. Nearly identical matches to these genes in the genome database are found only in uropathogenic *E. coli* strains 536 and UTI89, where they are located at the exact location relative to the hemolysin operon, but the genetic islands encoding these genes are located at different tRNA loci. Three mutants with deletions within this region in CFT073 were constructed. Based on liquid hemolysis assays, CFT073 *dsdA* carrying locus *c3566* to *c3568* (*c3566-c3568*) and CFT073 *dsdA c3566* display reduced hemolytic activity relative to wild type, but CFT073 *dsdA c3568* displays hemolytic activity levels similar to wild type (data not shown). Deletion of the entire *c3566-c3568* locus, *c3566* only, or *c3568* only from CFT073 *dsdA* results in a loss of the hypercolonization phenotype (Table 4). The loss of hypercolonization of CFT073 *dsdA c3568*, which does not display a reduction in hemolytic activity, suggests that alterations in this region may affect CFT073 *dsdA* hypercolonization independent from their

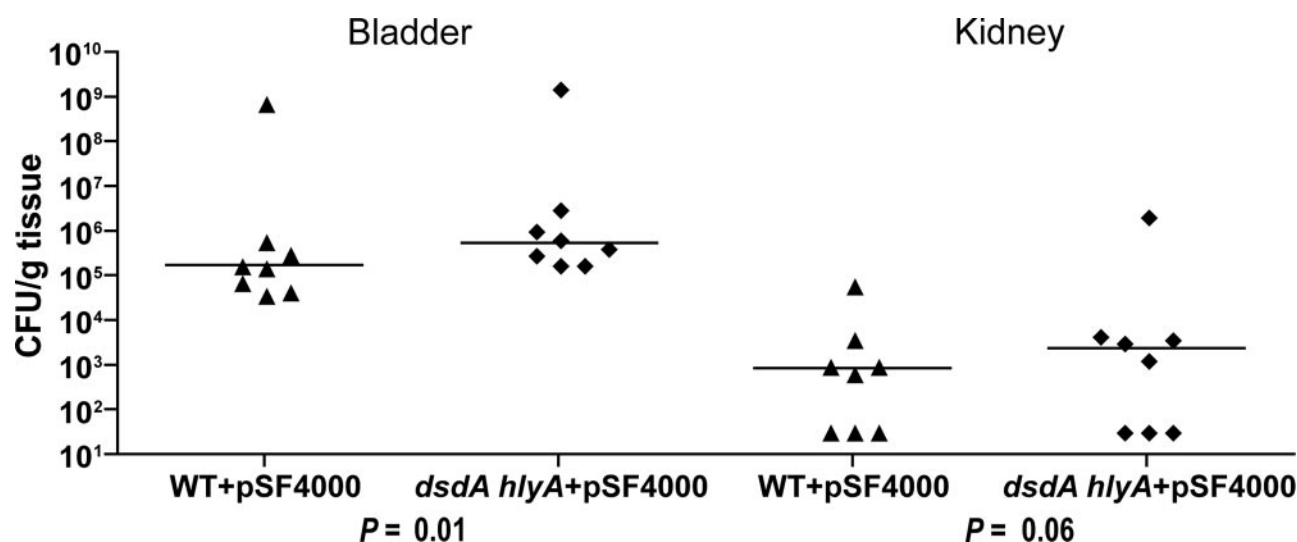


FIG. 2. Challenges of mice with wild type (WT) and CFT073 *dsdA hlyA::kan* with an *hlyCABD* complementing plasmid pSF4000. Mice were transurethrally inoculated with a 1:1 ratio of CFT073 *lacZYA* (wild type; triangle) and CFT073 *dsdA hlyA::kan* (diamond), each carrying pSF4000, which carries the J96 hemolysin operon. Each data point represents the number of CFU/g of tissue for the designated strain in each mouse organ. Horizontal bars represent the median value of CFU/g of tissue for a particular bacterial strain. The median ratios of complemented mutant CFU to complemented wild-type CFU in bladder and kidney are 3.6 and 4, respectively.

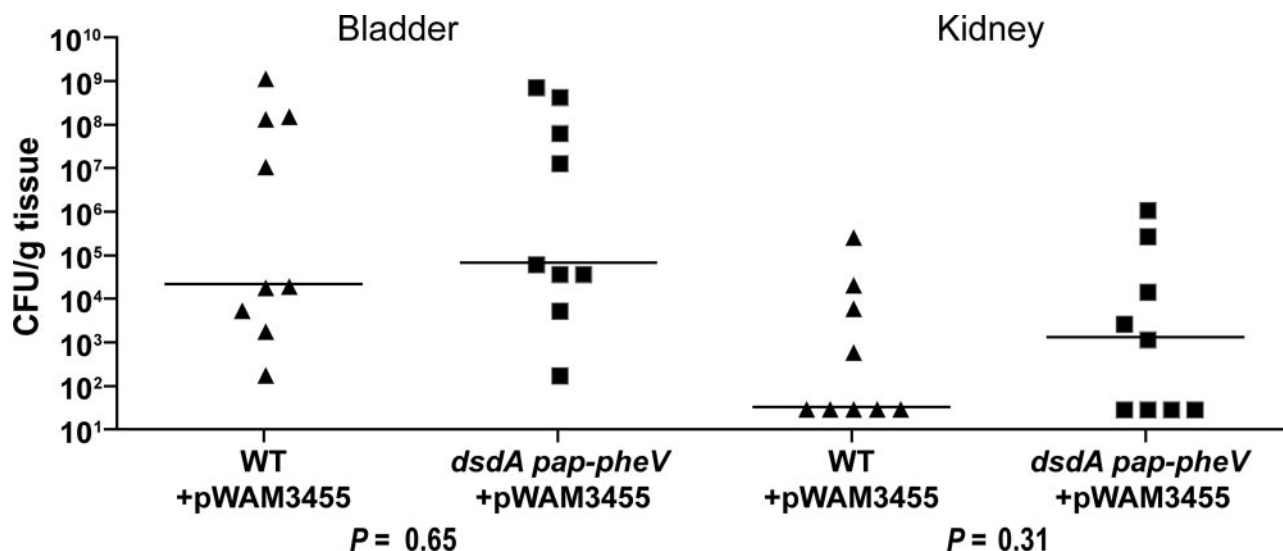


FIG. 3. Challenges of mice with CFT073 *lacZYA* and CFT073 *dsdA pap-pheV*, each carrying pWAM3455. Mice were transurethrally inoculated with a 1:1 ratio of CFT073 *lacZYA* (wild type; triangle) and CFT073 *dsdA pap-pheV* (square) carrying pWAM3455, encoding the entire *pap-pheV* operon. Each data point represents the number of CFU/g of tissue for the designated strain in each mouse organ. Horizontal bars represent the median value of CFU/g of tissue for a particular bacterial strain. The median ratios of complemented mutant CFU to complemented wild-type CFU in bladder and kidney are 2.8 and 13, respectively.

possible effect on transcription of the hemolysin operon, which in the case of CFT073 remains uncharacterized.

A locus of unknown function is necessary for hypercolonization. The gene cluster c2485-c2490 contained the most highly up-regulated genes found in this study. c2486 and c2490 were up-regulated 20-fold (Table 2). Two members of the cluster, c2485 and c2489, are predicted to encode activities associated with lipopolysaccharide (LPS) synthesis, and other members of the cluster had predicted functions involving the recognition or modification of sugar molecules. c2485 to c2487 are ORFs with homologs in all sequenced strains of *E. coli*. However, the predicted proteins are most similar to, and in most cases identical to, predicted proteins in sequenced UPEC strains 536 and UTI89. The LPS structures of CFT073, CFT073 *dsdA*, and CFT073 *dsdA* c2485-c2490 were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and LPS-specific stains, but no differences in species, sizes, or relative amounts were observed (data not shown). We observed no competitive advantage over wild type by CFT073 *dsdA* c2485-c2490 in either the bladder or the kidney (Table 4). c0468, a paralog of c2489, was also up-regulated (Table 2). CFT073 *dsdA* c0468 was also unable to hypercolonize in competition with the wild-type strain (Table 4).

Genes not contributing to hypercolonization. Only two of the 14 constructed CFT073 *dsdA* double mutants, CFT073 *dsdA focA* and CFT073 *dsdA* c2398, continued to hypercolonize the mouse urinary tract, although perhaps to a lesser degree than the CFT073 *dsdA* strain (Table 4). The F1C fimbrial operon of CFT073 is encoded by a locus with component genes that have similarity to the component genes of both S and F1C fimbriae, encoded by *sfa* and *foc* genes, respectively (44). Among sequenced bacteria, c2398 in its entire length is seen in only *E. coli* strains CFT073 and 536 (4, 23). It contains a C-terminal Toll/interleukin-1 (IL-1)

receptor (TIR) domain similar in amino acid sequence to Toll and IL-1 receptors.

Do genes up-regulated in CFT073 *dsdA* affect wild-type CFT073 colonization? We felt it was important to identify which genes up-regulated in the CFT073 *dsdA* background play a role in mouse urinary tract colonization on the parental CFT073 background alone. A second set of mutants of the up-regulated genes was constructed and tested in the mouse model (Table 5). CFT073 strains with deletions of *fliC*, *hlyA*, and the two *pap* operons were analyzed previously, so they were not tested here (29, 34; P. Redford and R. Welch, unpublished results). Previous reports showed that CFT073 *fliC* is attenuated, while CFT073 *hlyA::kan* and CFT073 *papDEFG papE_2F_2G_2* colonize the urinary tract at 48 h at levels indistinguishable from the wild-type strain. We observed no

TABLE 5. Relative recovery at 48 h of 8 mutants of CFT073 and wild type after competitive infection of the urinary tract

WAM strain ^a	Deleted gene (s)	n ^b	Relative recovery in: ^c			
			Bladder		Kidney	
			Ratio	P	Ratio	P
3760	<i>ompF</i>	8	0.99	0.84	0.75	0.75
3749	<i>c0468</i>	8	0.88	0.31	1.1	1.0
3753	<i>dppA</i>	8	1.3	0.15	1.1	1.0
3188	<i>c2398</i>	8	0.69	0.20	0.72	0.47
3272	<i>c3566</i>	7	1.2	0.29	0.85	1.0
3273	<i>c3568</i>	8	0.44	0.04	0.62	0.55
3220	<i>c2485-c2490</i>	6	0.92	0.68	0.82	1.0
3751	<i>ibpB</i>	5	1.2	0.44	0.91	0.63

^a All strains are derivatives of CFT073.

^b Number of mice analyzed.

^c Data shown are the ratio of mutant versus wild-type CFU (median). P values were determined by a Wilcoxon signed rank test.

significant colonization defects in CFT073 mutants for *ibpB*, *dppA*, c2485-c2490, c0468, c3566, c3568, or *ompF* (Table 5). This suggests that the detrimental effect on colonization by the deletion of these genes, *hlyA*, and the *pap* operons is restricted to CFT073 *dsdA*.

DISCUSSION

In this study, we demonstrate the first transcriptome-wide comparison of the urinary tract colonization experience of two isogenic UPEC strains. Using urosepsis *E. coli* strain CFT073-specific microarrays and the established murine model of UTI, we demonstrate a link between colonization phenotype and gene expression differences. Further, we verified the predictive value of this comparative analysis by constructing mutations in up-regulated genes in the hypercolonizing CFT073 *dsdA* genetic background to screen for loss of the hypercolonizing phenotype. We found that the hypercolonization phenotype of CFT073 *dsdA* was abolished by the deletion of any one of several of the up-regulated genes. However, the alteration of colonization by deleting these genes was restricted to CFT073 *dsdA*, because when mutants for these genes were tested in the wild-type background, we observed no change in colonization phenotype. CFT073 *dsdA* uses virulence genes for hypercolonization that have been long suspected or proven to be important for human UTIs (70).

Many virulence factors are secreted or associated with the cell envelope. In the comparison of gene expression of CFT073 *dsdA* to wild type, many genes whose products affect synthesis of, or are part of, the cell envelope showed positive or negative expression changes. We observed significant up-regulation of genes encoding P and F1C fimbriae, OmpF, a potential LPS modification system (c2485-c2489), hemolysin, IbpB, and DppA. We also observed further evidence for the remodeling of the cell envelope with the down-regulation of genes *lpp*, *pgsA*, *tpx*, and *proX* encoding the major outer membrane lipoprotein, phosphatidylglycerol synthase, a periplasmic thiol-dependent peroxidase, and an osmoprotectant transporter, respectively (11, 21, 56). Additionally, the gene expression of *osmE*, *ybdQ*, and *proX* increases after osmotic shock, suggesting a role in the outer membrane stress response (15, 19, 61). Mutations affecting F1C fimbriae in CFT073 *dsdA* did not alter the hypercolonization phenotype, but mutations in c2485-c2490, *hlyA*, *ibpB*, and *ompF* did change the colonization phenotype to be indistinguishable from wild-type CFT073.

The loss of hypercolonization for the CFT073 *dsdA fliC* mutant supports, in part, the observations made by others about the positive role of motility in *E. coli* uropathogenesis (29, 69). Although we originally observed that CFT073 *dsdA* was hyperflagellated and hypermotile in vitro compared to the wild type, surprisingly, we did not observe increased in vivo transcription of genes related to flagellar motility or chemotaxis. We hypothesize that there is posttranscriptional control of hypermotility in the CFT073 *dsdA* background, a regulatory mechanism suggested by a recent report from the Hughes laboratory with *Salmonella* (51). We also previously observed in the CFT073 type 1 *fim* phase-locked "off" state that there is up-regulation of *pap* and *foc* fimbrial transcription and this occurs without an increase in flagellar gene expression (54). We later demonstrated that there are reciprocal type 1 and

motility phenotypes that occur with the *fim* on and off phase states (9). Taken together, these observations indicate that type 1, P, and F1C fimbriae, along with motility, can be regulated in some coordinated fashion through a mixture of transcriptional and posttranscriptional mechanisms.

The most highly up-regulated genes, the cluster c2485-c2490, may encode an LPS modification system. Deleting the entire gene cluster from CFT073 *dsdA* resulted in a loss of the hypercolonization phenotype, which suggests that products of this operon are important for colonization. c2486 is predicted to encode an *N*-acetylglucosamine deacetylase (57, 60). The putative gene c2489 is predicted to encode a nucleoside-diphosphate-sugar pyrophosphorylase (COG1208), an enzymatic activity associated with LPS synthesis. A paralogous gene c0468 was also up-regulated 15-fold, and deletion of this gene also resulted in a loss of the hypercolonization phenotype, suggesting this activity is important. c2485 belongs to a group of predicted phosphoadenosine phosphosulfate sulfotransferases (COG3969) (52). These genes may represent an LPS modification system, but we did not observe changes in LPS structure, as reflected by migration of isolated LPS on sodium dodecyl sulfate-polyacrylamide gels, in these mutants when grown in vitro. Any potential structural modifications may be small in size, limited to a small subset of LPS molecules, or present only during infection. Interestingly, this gene cluster is directly adjacent to a gene cluster which produces a newly recognized peptide-polyketide cytotoxin (41). Further work will be needed to establish the biochemical functions of these operons and whether their products interact.

A striking finding is that unlike other mutants in the CFT073 *dsdA* background, the CFT073 *dsdA dppA* double mutant was attenuated 10- to 50-fold compared to the wild type for colonization of the bladders and kidneys. *dppA* encodes a periplasmic protein that binds dipeptides and delivers them to a cognate ABC-type transporter encoded by *dppBC*. DppA is also proposed to play a role in chemotaxis by delivering dipeptides to Tap (1, 2). This interaction is unlikely to take place with CFT073, which does not encode Tap and lacks a dipeptide chemotaxis phenotype (28). The CFT073 *dppA* mutant does not show a loss in colonization ability compared to wild type. We hypothesize that D-serine and dipeptides may be extremely important nutrient sources for UPEC in the urinary tract where sugars like glucose, maltose, and lactose are rare. The inability to utilize one or the other substrates may not be detrimental to colonization, but the loss of both catabolic abilities results in significant attenuation relative to wild type.

Although diminished in degree, CFT073 *dsdA ompF* still significantly hypercolonizes the bladder but not the kidney. OmpF and OmpC are the most abundant outer membrane proteins in *E. coli*, and *ompF* is subject to positive evolutionary selection in UPEC (12). While normally regulated with *ompC* by EnvZ/OmpR, *ompF* can also be up-regulated in response to high osmolarity (68). The need for *ompF* for CFT073 *dsdA* hypercolonization of the kidney suggests that osmoregulation needed for colonization of the kidney is different than that for the bladder.

There were two up-regulated genes identified in our array screen, c2398 and *focA*, which surprised us because their respective mutations failed to cause a loss of the hypercolonization phenotype in the CFT073 *dsdA* mutant background. The

degree of hypercolonization in the bladder and kidney by CFT073 *dsdA* c2398 appeared less intense, but the significance of this reduction in degree of hypercolonization is unknown. c2398 is predicted to encode a protein with a TIR domain present in its C-terminal half. The TIR domain is a feature shared among the Toll-like and IL-1 receptors that are key to immune activation and regulation. Besides CFT073, this gene is present in only one other sequenced *E. coli* strain, UPEC 536 (23). *Salmonella enterica* serovar Enteritidis carries a gene encoding a TIR-like protein that suppresses NF- κ B signaling and contributes to murine pathogenesis (39). UPEC have been observed to suppress NF- κ B signaling (26). We hypothesized that this suppression might be mediated by c2398 or a similar factor, potentially modulating the innate immune response to gain an advantage in colonization. If the UPEC c2398 product is immunomodulatory, its effect is not apparent during murine CFT073 bladder and kidney colonization at 48 h postinoculation.

F1C fimbriae have been implicated as potential urovirulence factors by epidemiological association to UPEC and their ability to mediate bacterial binding to glycolipids on kidney cell surfaces. Recently, expression of this operon was observed in a type 1 and P fimbria-deficient CFT073 strain and was associated with an auto-aggregation phenotype (54). In this study, we noted statistically significant up-regulation of some of the genes encoding F1C fimbriae, *sfaB*, *focA*, and *focG*. Additionally, *sfaD* was up-regulated nonsignificantly. CFT073 *dsdA* with a deletion of either *focA* or the full F1C determinant maintained a hypercolonization phenotype. There seemed to be a less intense hypercolonization phenotype in the bladder and a more intense hypercolonization phenotype in the kidney. As discussed for *focA*, the significance of these differences in intensity of hypercolonization is unknown. We therefore concluded that the CFT073 *dsdA* hypercolonization phenotype in mice is not fully dependent on F1C fimbriae. The possibility exists that the up-regulation of the F1C fimbriae in UPEC is relevant in a different host.

This study revealed a necessity for both P fimbrial operons, *pap-pheV* and *pap-pheU*, in the CFT073 *dsdA* hypercolonization phenotype. Deleting either or both of the two *pap* loci in the CFT073 *dsdA* mutant resulted in an inability of that strain to hypercolonize in the murine model of UTI. This is the first demonstration in the CFT073 strain of a role for P fimbriae in murine UTI. P fimbrial expression has been shown to aid *E. coli* colonization of the human and murine urinary tracts (42, 70). Previous work with CFT073 found no difference in colonization when portions of the two loci were deleted in the wild-type background or in a strain lacking type 1 fimbriae (34, 54).

Highly virulent UPEC strains often contain two copies of *pap* operons. The advantage, if any, of possessing multiple operons is unclear. This study demonstrates a nonredundancy in the two P fimbriae-encoding loci in strain CFT073, *pap-pheV* and *pap-pheU*. Each locus encodes a nearly identical PapGII-type adhesin (65). Additionally, the two operons contain different *papIB* intergenic regions and PapA alleles, with sequence identity to either PapA serotype F7(1) or F7(2) (16). The functional consequences of these differences are unknown. The two loci may produce fimbriae that have different specificities or structural properties, or perhaps each locus is

required under distinct spatial or temporal conditions and thus responds to different environmental signals. It is known that one *pap* operon can affect the expression of a second *pap* or other fimbrial operon present in a strain (24, 31). It may be that such cross talk is necessary for function of the two CFT073 *pap* operons during mouse UTI. This may serve to explain the observation that the two *pap* operons in CFT073 are not redundant for CFT073 *dsdA* hypercolonization.

Why are the *pap* operons required for CFT073 *dsdA* hypercolonization? The simplest answer is that they act as adhesins, providing enhanced persistence in the urinary tract. However, it has been hypothesized that P fimbriae act to deliver hemolysin and other toxins, such as Sat, cytotoxic necrotizing factor 1, and endotoxin, directly to host cells (34). O'Hanley et al. demonstrated a synergistic effect of P fimbriae and hemolysin in causing mortality and kidney damage in a murine UTI model using fecal *E. coli* strains carrying cosmid clones derived from a uropathogenic isolate (43). The need for both P fimbriae and hemolysin in the present study supports this synergistic association. We demonstrated a necessity for hemolysin in the hypercolonization phenotype of CFT073 *dsdA*. Hemolysin was one of the earliest identified virulence factors in septicemic and uropathogenic *E. coli* (63). No effect by hemolysin on *E. coli* colonization of the urinary tract has been previously reported. Although hemolysin is classically considered an extracellular toxin, its up-regulation in the CFT073 *dsdA* mutant appeared to only affect colonization by the CFT073 *dsdA* mutant and not wild-type CFT073 present in the same tissue. This suggests that hemolysin is not diffused throughout the lumen of the bladder and kidney. Our results suggest that the CFT073 *dsdA* hypercolonization is a multilocus-dependent phenotype, perhaps where P fimbriae aid delivery of the hemolysin to specific cell types, enabling greater growth, access to a special niche, or survival of cellular host defenses.

Finally, this study demonstrated that a CFT073 *dsdA* strain accumulates D-serine during UTI. We have previously proposed that this accumulation affects gene expression, and in this study we have demonstrated an association between D-serine accumulation and virulence gene expression during UTI and verified that a subset of the expressed genes is required for the *dsdA* hypercolonization phenotype. The mechanism by which D-serine accumulation might cause these gene expression differences is unclear. Deletion of *dsdA* from CFT073 resulted in the increased expression in vivo of a regulon-like set of UPEC-relevant virulence genes, particularly hemolysin and F1C and P fimbrial genes. Interestingly, deletion of *hms* from UPEC strain 536 also results in increased expression of hemolysin, P-related fimbriae, and S fimbriae (36). We hypothesize that D-serine accumulation in CFT073 *dsdA* antagonizes H-NS repression of virulence genes, which results in a hypercolonization phenotype in the murine model of UTI.

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