

## Transcriptome of Uropathogenic *Escherichia coli* during Urinary Tract Infection

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**A uropathogenic *Escherichia coli* strain CFT073-specific DNA microarray that includes each open reading frame was used to analyze the transcriptome of CFT073 bacteria isolated directly from the urine of infected CBA/J mice. The in vivo expression profiles were compared to that of *E. coli* CFT073 grown statically to exponential phase in rich medium, revealing the strategies this pathogen uses in vivo for colonization, growth, and survival in the urinary tract environment. The most highly expressed genes overall in vivo encoded translational machinery, indicating that the bacteria were in a rapid growth state despite specific nutrient limitations. Expression of type 1 fimbriae, a virulence factor involved in adherence, was highly upregulated in vivo. Five iron acquisition systems were all highly upregulated during urinary tract infection, as were genes responsible for capsular polysaccharide and lipopolysaccharide synthesis, drug resistance, and microcin secretion. Surprisingly, other fimbrial genes, such as *pap* and *foc/sfa*, and genes involved in motility and chemotaxis were downregulated in vivo. *E. coli* CFT073 grown in human urine resulted in the upregulation of iron acquisition, capsule, and microcin secretion genes, thus partially mimicking growth in vivo. On the basis of gene expression levels, the urinary tract appears to be nitrogen and iron limiting, of high osmolarity, and of moderate oxygenation. This study represents the first assessment of any *E. coli* pathotype's transcriptome in vivo and provides specific insights into the mechanisms necessary for urinary tract pathogenesis.**

Urinary tract infections (UTIs) are a serious health concern. Forty to 50% of women experience at least one UTI, leading to an estimated 8 million annual physician visits in the United States alone (39, 46). Uropathogenic *Escherichia coli* (UPEC) is by far the most common etiological agent of all UTIs. UPEC strain CFT073, derived from the clonal group O6:K2:H1 (26), was originally isolated from the blood and urine of a woman diagnosed with acute pyelonephritis (28). It is considered a prototype of the O6 serogroup, one of the most prevalent UPEC clonal lines (23, 24). The virulence of this strain was reproduced in the well-established CBA mouse model of ascending UTI (28). In addition to numerous virulence studies, the genome of *E. coli* CFT073 has recently been sequenced and compared to that of enterohemorrhagic *E. coli* EDL933 and the nonpathogenic laboratory strain *E. coli* MG1655 (42).

Mutations have been introduced into a number of candidate virulence genes in UPEC, leading to attenuated mutants in experimental UTI. These include *fim*, encoding type 1 fimbria (7, 13), *sat*, encoding secreted autotransporter toxin (14), *cnf-1*, encoding cytotoxic necrotizing factor (36), *tonB*, involved in iron transport (40), *proP*, involved in osmoprotectant transport (8), and *degS* (35). Large-scale screens for virulence factors of UPEC have also identified factors that aid UPEC during

growth in urine (38) and have implicated capsule, lipopolysaccharide, iron acquisition systems, and the PhoU regulatory system in virulence (3, 35). Molecular Koch's postulates have been satisfied for type 1 fimbriae, DegS, and TonB (9).

In this report, we have quantified the gene expression for each open reading frame (ORF) of this uropathogen from organisms isolated directly from the urine of experimentally infected CBA/J mice. We have identified multiple virulence and metabolic factors that were upregulated to aid survival in the host. We also defined specific environmental conditions that appear to confront *E. coli* CFT073 during experimental UTI.

### MATERIALS AND METHODS

**Bacterial strains and culture conditions.** For in vitro RNA preparation, UPEC strain CFT073 (28) was grown statically at 37°C in 100 ml of Luria broth (LB) until the optical density at 600 nm (OD<sub>600</sub>) reached 0.65. For RNA preparation from urine, this strain was grown statically at 37°C in 900 ml of pooled human urine until the OD<sub>600</sub> reached 0.135 (mid-exponential phase for growth in urine). Urine was collected from healthy women volunteers aged 20 to 40 who had no history of UTI or antibiotic use in the prior 2 months. Each urine sample was immediately filter sterilized (0.2 μm pore size) and frozen at –80°C for use within 2 weeks. For each experimental replicate, a different pool of urine samples from three to five volunteers was used. The study was approved by the Institutional Review Board of the University of Maryland School of Medicine. LB- and urine-grown cultures were immediately treated with RNA Protect Bacterial Reagent (QIAGEN) to stabilize RNA according to the manufacturer's instructions and was harvested by centrifugation (8 min, 7,500 × g, 25°C). The bacterial pellet was frozen at –20°C until RNA extraction.

**CBA/J mouse model of ascending UTI.** To establish a relevant source of in vivo-grown bacteria, 40 CBA/J mice were transurethrally inoculated as previously described (15, 21) with 5 × 10<sup>9</sup> CFU of *E. coli* CFT073. The infection was monitored daily by pooling urine from 10 mice at random and spiral plating on Luria agar to quantify CFU. Mice were re inoculated after 6 days to maintain

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infection at levels between  $2 \times 10^6$  and  $3 \times 10^7$  CFU/ml of urine, which allowed the collection of sufficient numbers of bacteria needed for RNA extraction and subsequent analysis. Urine was collected for 10 days, with the exception of the first 24 h following the inoculations. On average, 50  $\mu$ l of urine was collected from each mouse every 45 min during an 8-h period. Urine was collected directly into 1.5-ml microcentrifuge tubes containing 650  $\mu$ l of RNA Protect Bacterial Reagent (QIAGEN) and was pooled until the total volume reached approximately 1 ml. The RNA Protect-treated sample remained on ice until all samples had been collected for that day. A crystalline precipitate was allowed to settle before pipetting off the bacteria-containing supernatant into a 15-ml centrifuge tube for collection. Tubes were centrifuged (15 min,  $2,500 \times g$ , 4°C), the supernatant was decanted, and the bacterial pellet was frozen at  $-20^\circ\text{C}$  until RNA extraction.

**RNA isolation and cDNA synthesis.** RNA from both in vitro and in vivo samples was extracted using an RNeasy Mini kit with a 1-h on-column DNase digestion (QIAGEN) according to the RNeasy Mini handbook. Multiple RNA preparations of the same sample were pooled prior to cDNA synthesis. Up to 10  $\mu$ g of RNA was mixed with 750 ng of random hexamers (Invitrogen) for each cDNA synthesis reaction according to a previously described protocol (37). SuperScript II reverse transcriptase (1,500 U; Invitrogen) was added to these reactions, along with First Strand buffer, dithiothreitol, and deoxyribonucleotides at concentrations recommended by the manufacturer (Invitrogen). The reactions 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 RNaseH (Invitrogen) and RNaseA (Ambion) digestion, cDNA was purified with a QIAquick PCR Purification kit (QIAGEN) according to the QIAquick Spin handbook.

**Quantitative real-time reverse transcription-PCR (qRT-PCR).** Primers designed to amplify *papA\_2* of *E. coli* CFT073 were 5'GTGCCTGCAGAAAATG CAGAT and 5'CCCGTTTTCCACTCGAATCA, and primers for *gapA* were 5'CATCGTTTCCAACGCATCCT and 5'ACCTTCGATGATGCCGAAGTT (forward and reverse primers, respectively). Thirty nanograms of cDNA and 300 nM (final concentration) each primer were mixed with 12.5  $\mu$ l of 2 $\times$  SYBR Green PCR Master Mix (ABI). Assays were performed in triplicate with the ABI Prism model 7900 instrument. All data were normalized to the internal standard *gapA* (encoding glyceraldehyde 3-phosphate dehydrogenase), and melting curve analysis demonstrated that the accumulation of SYBR Green-bound DNA was gene specific. The  $2^{-\Delta\Delta\text{CT}}$  method (25) was used for analysis, and the data were transformed by  $\log_2$  to obtain a fold change difference between growth conditions.

**Microarrays and hybridization.** The *E. coli* CFT073-specific DNA microarray (NimbleGen Systems, Inc.) includes 5,611 ORFs and stable RNAs from version 17 of the compiled CFT073 genome sequence. 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 harbors two mismatched bases for determination of background and cross-hybridization. For each microarray, 3 to 5  $\mu$ g of cDNA was fragmented using RQ1 DNaseI (Promega) partial digest and was then labeled with biotin-N6-ddATP (Perkin-Elmer Life Sciences) using terminal transferase (Roche) as described previously (37). Labeled cDNA samples were individually hybridized in triplicate 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 5- $\mu$ m resolution using a GenePix 4000b scanner.

**Data and statistical analysis.** For LB and in vivo microarrays, data were extracted using NimbleScan (NimbleGen) and an algorithm (courtesy of Yu Qiu, University of Wisconsin School of Medicine) applied to obtain a single measurement of signal intensity for each ORF. Data were normalized and converted to estimates of transcript abundance, using the total signal intensity to allow comparison of individual microarrays (1). A second set of LB-grown microarrays were processed along with the urine-grown microarray, where hybridization and normalization of the microarray were carried out by NimbleGen Systems, Inc. The signal intensity of an ORF was calculated by subtracting the mismatch probe intensity from the perfect match probe intensity for each of the 17 probe pairs, obtaining a mean difference value (excluding values greater than 3.0 standard deviations from the means). These urine- and LB-grown microarrays were normalized with the quantile normalization method and were analyzed with the RMA algorithm (6, 20). For all microarrays, 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 bacterial growth condition. Fold changes of an ORF between growth conditions were calculated by transformation of the following ratio:  $\log_2$  [(average in vivo-grown or urine-grown signal intensity)/(average LB-grown signal intensity)]. Only fold changes of at least  $\pm 2$  and *P*  $\leq 0.05$  were considered significant and are discussed in this report. Thus, ORFs characterized as upregulated (fold change,  $\geq 2$ ; *P*  $\leq 0.05$ ) or downregulated (fold change,  $\leq -2$ ; *P*  $\leq 0.05$ ) during growth in vivo or during growth in urine are relative to growth in LB.

## RESULTS

### Alterations in the transcriptome of *E. coli* CFT073 during UTI.

To further understand the mechanisms UPEC employ in UTIs, we used *E. coli* CFT073-specific DNA microarrays to analyze the transcriptome of this strain grown under both in vivo and in vitro conditions. The source for in vivo-grown bacteria was the urine from infected mice, collected from 1 to 10 days postinoculation for use in RNA isolation. The sources for in vitro-grown RNA were cultures of *E. coli* CFT073 grown statically to mid-exponential phase at 37°C in either LB or filtered human urine. The expression level of each of 5,611 ORFs was determined for each condition. An ORF was considered differentially regulated if the statistically significant change in expression was also greater than twofold. All in vivo- and urine-derived data are described here relative to growth in LB.

Overall, 313 genes were found upregulated (Table 1 lists the 50 most upregulated), and 207 genes were downregulated during growth in vivo. To identify candidate virulence genes specific to *E. coli* CFT073 that may play a role during UTI, all 313 genes upregulated in vivo were evaluated for the absence or presence of a homologue in the *E. coli* K-12 nonpathogenic strain (Table 1). This analysis revealed 45 genes that were not found in *E. coli* K-12 and 41 genes encoding hypothetical proteins that were unable to be confidently categorized. Of these 45 candidate virulence genes, 25 genes previously implicated in virulence encoded iron acquisition, capsule synthesis, or microcin secretion proteins and 13 encoded hypothetical proteins.

By comparison to UPEC CFT073 grown in LB, bacteria coming from the urinary tract have upregulated expression of virulence and metabolic factors in the host (Fig. 1). Not only were a variety of factors upregulated, but these genes were presumably translated at a rapid rate. Thirty-five of the 50 most highly expressed genes in vivo encoded translational machinery (Table 2).

**Adhesins.** Colonization is a major challenge facing UPEC in the urinary tract. Thus, adherence factors that aid in this process are essential to survival. The genome sequence of *E. coli* CFT073 revealed an abundance of fimbrial adhesins relative to levels in both enterohemorrhagic and nonpathogenic laboratory *E. coli* strains. Indeed, as many as 12 fimbrial gene clusters have been predicted or demonstrated (4, 21, 42). Our in vivo microarray data revealed that the *fim* genes encoding type 1 fimbriae were preferentially expressed 12 to 72 times more highly than each of the 11 other fimbrial gene clusters. Additionally, *fimA*, encoding the major structural subunit, was the fourth most highly expressed gene in vivo overall; the first three were related to translation. *fim* genes were the only genes previously implicated as virulence factors among the 50 most highly expressed genes in vivo (Table 2). The 11 remaining fimbrial gene clusters were minimally expressed, if at all.

Not only were type 1 fimbriae highly expressed, but they were also highly upregulated (three- to fivefold) (Fig. 2) compared to levels for the LB culture grown statically, conditions known to enhance type 1 fimbrial production (33). Both of the *pap* gene clusters of strain CFT073 encoding P fimbriae were downregulated two- to fourfold, and the *foc/sfa* gene cluster and *sfaB* recombinase of F1C fimbriae were also downregulated two- to fivefold. The *csq* genes, which encode thin aggregative curli fibers, were downregulated two- to fourfold in vivo.

TABLE 1. Top 50 genes upregulated in vivo during UTI

Gene	Function	Fold change <sup>a</sup>	P value
<i>rspA</i>	Repression of stationary-phase gene sigma-S	6.021	0.0059
<i>sitA<sup>b</sup></i>	Iron transport system SitA protein	5.634	<0.0001
<i>c1903</i>	Unknown	5.556	0.0018
<i>rspB</i>	Repression of stationary-phase gene sigma-S	5.532	<0.0001
<i>entE</i>	Enterobactin synthetase component E (enterochelin)	5.482	0.0032
<i>chuW<sup>b</sup></i>	Putative coproporphyrinogen III oxidase; hemin utilization	5.417	0.0016
<i>yhjX</i>	Putative drug resistance protein YhjX	5.416	0.0312
<i>iucA<sup>b</sup></i>	Aerobactin protein IucA	5.166	0.0025
<i>proV</i>	Glycine betaine/L-proline transport ATP-binding protein ProV	5.150	0.0025
<i>entA</i>	Enterobactin system dehydrogenase	5.115	0.0113
<i>entF</i>	Enterobactin synthetase component F	5.012	0.0134
<i>uxuB</i>	D-mannonate oxidoreductase	4.982	0.0049
<i>srlD</i>	Sorbitol-6-phosphate 2-dehydrogenase	4.912	0.0055
<i>uxuA</i>	Mannonate dehydratase	4.740	0.0026
<i>tyrA</i>	T protein	4.643	0.0002
<i>fdnI</i>	Formate dehydrogenase, nitrate inducible	4.623	0.0066
<i>deaD</i>	Cold-shock DEAD-box protein A	4.614	0.0024
<i>nirD</i>	Nitrite reductase [NAD(P)H] small subunit	4.601	0.0022
<i>yeaR</i>	Hypothetical protein YeaR	4.579	0.0018
<i>lpxB</i>	Lipid-A-disaccharide synthase	4.570	0.0006
<i>fimC</i>	Type 1 fimbriae; chaperone protein FimC precursor	4.553	<0.0001
<i>c1905<sup>b</sup></i>	Hypothetical protein	4.552	0.0081
<i>c5174<sup>b</sup></i>	Putative iron-regulated outer membrane virulence protein	4.539	0.0024
<i>c1220</i>	Phospho-2-dehydro-3-deoxyheptonate aldolase, Trp sensitive	4.529	0.0013
<i>yhfI</i>	Hypothetical oxidoreductase YdfI	4.523	0.0065
<i>iroN<sup>b</sup></i>	Siderophore receptor IroN	4.491	0.0207
<i>dut</i>	Deoxyuridine 5'-triphosphate nucleotidohydrolase	4.488	0.0163
<i>chuS<sup>b</sup></i>	Putative heme/hemoglobin transport protein; hemin utilization	4.473	0.0128
<i>iroB<sup>b</sup></i>	Putative glucosyltransferase; siderophore system	4.466	0.0056
<i>iucD<sup>b</sup></i>	Aerobactin protein IucD	4.433	0.0009
<i>sitB<sup>b</sup></i>	Iron transport system SitB protein	4.404	0.0020
<i>yncE</i>	Hypothetical protein YncE precursor	4.341	0.0058
<i>rplI</i>	50S ribosomal protein L9	4.318	0.0122
<i>c4088</i>	Hypothetical protein	4.271	0.0142
<i>yoaG</i>	Hypothetical protein YoaG	4.268	<0.0001
<i>c3610<sup>b</sup></i>	Putative receptor	4.242	0.0113
<i>c0672</i>	Conserved hypothetical protein	4.234	0.0030
<i>c2203</i>	Hypothetical protein	4.218	0.0001
<i>kpsE<sup>b</sup></i>	Capsule biosynthesis KpsE protein	4.127	0.0126
<i>entC</i>	Isochorismate synthase EntC, enterobactin system	4.108	0.0011
<i>srlE</i>	PTS system, glucitol/sorbitol-specific IIBC component	4.096	0.0002
<i>mpA</i>	Ribonuclease P protein component	4.094	0.0122
<i>fimF</i>	Type 1 fimbriae; FimF protein precursor	4.084	0.0001
<i>gidA</i>	Glucose inhibited division protein A	4.052	0.0021
<i>marR</i>	Multiple antibiotic resistance protein MarR	4.038	0.0002
<i>hmpA</i>	Flavo-hemoprotein (hemoglobin-like protein)	3.993	0.0148
<i>glnP</i>	Glutamine transport system permease protein GlnP	3.992	0.0016
<i>c4090</i>	Hypothetical protein	3.966	0.0225
<i>srlB</i>	PTS system, glucitol/sorbitol-specific IIA component	3.956	0.0012
<i>chuT<sup>b</sup></i>	Putative periplasmic binding protein; hemin utilization	3.956	0.0270

<sup>a</sup> Fold change refers to growth in vivo relative to growth in LB.

<sup>b</sup> This gene was not present in *E. coli* K-12.

The *flu* gene, encoding antigen 43 and implicated in autoaggregation and biofilm formation, was also downregulated two-fold in vivo (Fig. 2). Besides type 1 fimbriae, only one other adhesion-related gene (*ydeS*, encoding a hypothetical fimbria-like structural protein) was upregulated during UTI.

**Iron acquisition systems.** Iron is essential for growth of *E. coli*, and five iron uptake and assimilation systems were upregulated during bacterial growth in the urinary tract. Siderophore systems were upregulated in vivo two- to fivefold, providing evidence that the urinary tract is an iron-limiting environment (31). These systems include *ent* genes encoding enterobactin, *iuc* genes encoding aerobactin, and *iro* genes encoding an *ent*-like system. Additionally, *iutA*, *exbD*, *fhuA*, *fhuC*, and the *fep* genes, all involved in the transport of these ferrisiderophore complexes; the *chu* genes, required for hemin utilization; and *sit* genes, encoding an iron transport system, were upregulated two- to fivefold (Fig. 3). The expression of *tonB*, necessary for iron and siderophore uptake, remained unchanged between in vivo and LB growth.

**Motility.** *E. coli* CFT073 is motile, as is characteristic of the species. There has been speculation that flagella assist UPEC in the ascent from the bladder to the kidneys through the ureters (29). The role of flagella in colonization is unknown, but epidemiological data indicate that nonmotile strains may be overrepresented among UTI isolates (10, 16). Our in vivo microarray data demonstrated that several genes involved in flagellation were downregulated in comparison to these genes during LB growth (Table 3), including *flgL* and *flgK*, class III genes encoding hook-filament flagellar structural components, and *cheW*, encoding a positive regulator of chemotaxis. Importantly, the *fliC* gene, encoding flagellin, was downregulated fivefold. Although not meeting statistical criteria, all 11 remaining class III flagellar and chemotaxis genes demonstrated a trend toward downregulation in vivo. Overall, these data do not support a primary role for motility during infection of the urinary tract.

**Capsule and LPS.** Capsular polysaccharide and lipopolysaccharide (LPS) O side chains may be involved in UPEC virulence, perhaps by conferring resistance to serum and phagocytosis (18). These microarray data confirmed the importance of extracellular polysaccharides. The genes *kpsE*, *kpsD*, *kpsC*, and *kpsS*, involved in the transport of polysaccharide to the cell surface, were upregulated two- to fourfold during infection. *lpxA* and *lpxB*, both involved in lipid A biosynthesis, were

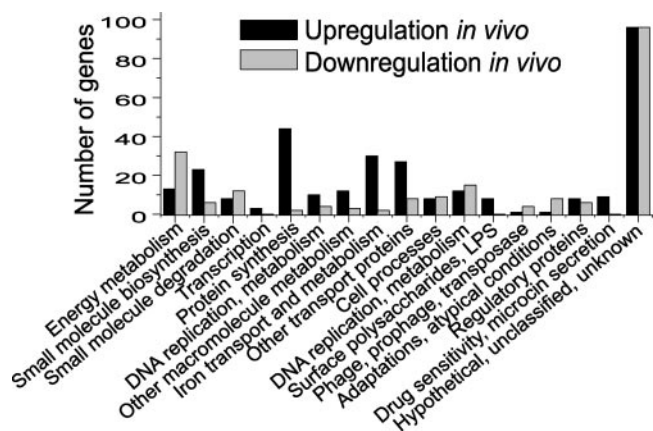


FIG. 1. Functional categories of differentially expressed *E. coli* CFT073 genes. The number of genes that are significantly upregulated (black bars) or downregulated (grey bars) during growth in vivo (growth during UTI) relative to growth in static LB culture are categorized based on function.

TABLE 2. Top 50 genes expressed by *E. coli* CFT073 in vivo during UTI

Gene	Function	Signal intensity	Fold change <sup>a</sup>	P value	Among top 50 in urine <sup>c</sup>	Among top 50 in LB <sup>c</sup>
<i>tufB</i>	Protein chain elongation factor EF-Tu	34.27	1.02	0.015	+	+
<i>rpsK</i>	30S ribosomal subunit protein S11	33.99	1.15	<0.001	+	+
<i>rplE</i>	50S ribosomal subunit protein L5	32.91	1.73	0.004	+	
<i>fimA<sup>b</sup></i>	<b>Major type 1 fimbrial subunit; fimbrin/pilin</b>	<b>32.75</b>	<b>2.97</b>	<b>0.009</b>		
<i>rpsS</i>	<b>30S ribosomal subunit protein S19</b>	<b>31.58</b>	<b>2.31</b>	<b>0.003</b>		
<i>rplX</i>	50S ribosomal subunit protein L24	31.27	1.71	<0.001	+	
<i>rpsE</i>	30S ribosomal subunit protein S5	29.86	1.93	<0.001	+	
<i>rpsN</i>	30S ribosomal subunit protein S14	29.28	1.65	0.006	+	
<i>rplR</i>	50S ribosomal subunit protein L18	29.01	1.87	<0.001	+	
<i>rplJ</i>	<b>50S ribosomal subunit protein L10</b>	<b>28.11</b>	<b>2.24</b>	<b>0.013</b>		
<i>rpmD</i>	<b>50S ribosomal subunit protein L30</b>	<b>27.97</b>	<b>2.39</b>	<b>0.004</b>	+	
<i>tufA</i>	Protein chain elongation factor EF-Tu	27.53	0.94	0.039	+	
<i>rplD</i>	<b>50S ribosomal subunit protein L4</b>	<b>27.18</b>	<b>2.75</b>	<b>0.006</b>		
<i>rplC</i>	<b>50S ribosomal subunit protein L3</b>	<b>27.00</b>	<b>2.88</b>	<b>0.003</b>		
<i>ompA</i>	Outer membrane protein A (II*)	26.05	-1.23	0.016	+	+
<i>rplO</i>	<b>50S ribosomal subunit protein L15</b>	<b>25.60</b>	<b>2.07</b>	<b>0.008</b>	+	
<i>rpsD</i>	30S ribosomal subunit protein S4	25.49	0.97	0.028	+	
<i>rplF</i>	<b>50S ribosomal subunit protein L6</b>	<b>25.42</b>	<b>2.17</b>	<b>0.012</b>	+	
<i>rplL</i>	<b>50S ribosomal subunit protein L7/L12</b>	<b>25.11</b>	<b>2.04</b>	<b>0.038</b>		
<i>prlA</i>	Putative ATPase subunit of translocase	24.74	1.98	0.011	+	
<i>rpsJ</i>	<b>30S ribosomal subunit protein S10</b>	<b>24.11</b>	<b>2.59</b>	<b>&lt;0.001</b>		
<i>rplW</i>	<b>50S ribosomal subunit protein L23</b>	<b>23.92</b>	<b>2.49</b>	<b>0.018</b>		
<i>rpsM</i>	30S ribosomal subunit protein S13	23.91	1.04	0.029	+	
<i>rpsH</i>	30S ribosomal subunit protein S8	23.27	1.96	0.013	+	
<i>rpsC</i>	<b>30S ribosomal subunit protein S3</b>	<b>22.35</b>	<b>2.02</b>	<b>0.009</b>		
<i>gapA</i>	Glyceraldehyde-3-phosphate dehydrogenase A	22.31	0.04	0.178	+	+
<i>fusA</i>	GTP-binding protein chain elongation factor EF-G	21.96	1.76	0.002	+	
<i>rplB</i>	<b>50S ribosomal subunit protein L2</b>	<b>21.83</b>	<b>2.30</b>	<b>0.007</b>	+	
<i>rplP</i>	50S ribosomal subunit protein L16	21.09	1.72	0.018		
<i>rplV</i>	50S ribosomal subunit protein L22	20.59	1.71	0.010		
<i>rpoA</i>	RNA polymerase, alpha subunit	20.33	1.28	0.007	+	
<i>ptsH</i>	PTS system protein HPr	18.67	0.53	<0.001		
<i>rpsG</i>	30S ribosomal subunit protein S7	18.45	1.44	0.001	+	
<i>fimI</i>	<b>Type 1 fimbrial protein of unknown function</b>	<b>17.97</b>	<b>3.65</b>	<b>0.007</b>		
<i>rpsL</i>	30S ribosomal subunit protein S12	17.71	1.28	0.008	+	
<i>rplN</i>	50S ribosomal subunit protein L14	17.40	1.70	0.006	+	
<i>ompC</i>	Outer membrane protein C (porin)	17.27	-0.84	0.002	+	+
<i>rplK</i>	<b>50S ribosomal subunit protein L11</b>	<b>16.78</b>	<b>2.09</b>	<b>&lt;0.001</b>		
<i>c1041</i>	Unknown	16.08	-1.01	0.016		+
<i>rpsR</i>	<b>30S ribosomal subunit protein S18</b>	<b>15.99</b>	<b>2.66</b>	<b>&lt;0.001</b>		
<i>rpsB</i>	<b>30S ribosomal subunit protein S2</b>	<b>15.75</b>	<b>2.20</b>	<b>0.001</b>		
<i>uxuA</i>	Mannanase hydrolase	<b>15.60</b>	<b>4.74</b>	<b>0.003</b>		
<i>rpsA</i>	<b>30S ribosomal subunit protein S1</b>	<b>15.51</b>	<b>2.16</b>	<b>&lt;0.001</b>		
<i>c4086</i>	<b>Hypothetical protein</b>	<b>15.50</b>	<b>3.57</b>	<b>0.017</b>		
<i>ahpC</i>	Alkyl hydroperoxide reductase, C22 subunit	15.13	0.89	0.005		
<i>rplA</i>	<b>50S ribosomal subunit protein L1</b>	<b>14.91</b>	<b>2.74</b>	<b>0.020</b>		
<i>infC</i>	Protein chain initiation factor IF-3	14.46	-0.09	0.408	+	+
<i>c0287</i>	Hypothetical protein	14.46	1.28	0.036		
<i>aceE</i>	Pyruvate dehydrogenase (decarboxylase)	14.14	1.05	0.042		
<i>acpP</i>	Acyl carrier protein	13.85	0.09	0.579		

<sup>a</sup> Fold change refers to growth in vivo relative to growth in LB.

<sup>b</sup> Genes statistically upregulated in vivo at least twofold are in boldface.

<sup>c</sup> A plus sign indicates this gene was also present among the 50 most expressed genes in human urine or LB.

upregulated three- to fivefold. *nanA*, encoding an enzyme involved in capsular sialic acid catabolism, and *wzzE*, encoding a putative transport protein involved in LPS biosynthesis, were also upregulated in vivo.

**Antibiotic resistance.** Genes that confer antibiotic resistance were found upregulated during bacterial growth in vivo. These include *marAB* (two- to three fold), encoding multiple antibiotic resistance proteins, *yhjX* (fivefold), encoding a putative drug resistance protein, and *emrA* (twofold), encoding a multi-drug resistance secretion protein. Microcins are secreted antibiotic peptides that may be used to compete with surrounding bacteria. *mchCDEF*, encoding microcin H47, was upregulated two- to fourfold in the urinary tract.

***E. coli* CFT073 transcriptome as a probe for nutrient levels and conditions within the urinary tract.** *E. coli* senses its environment and modulates gene expression to best utilize available resources. By examining the transcriptome of *E. coli* CFT073 isolated from the urine of infected mice, we established that these bacteria can act as sensitive bioprobes to reveal the composition of this environment.

**Oxygen level.** *E. coli*, a facultative anaerobe, can adapt to growth in various redox environments. Based on the pattern of gene expression of *E. coli* CFT073, the mouse urinary tract is more oxygen rich than the LB static culture. Many genes indicative of anaerobiosis were downregulated under in vivo conditions. Most interestingly, *frdABCD* (encoding fumarate

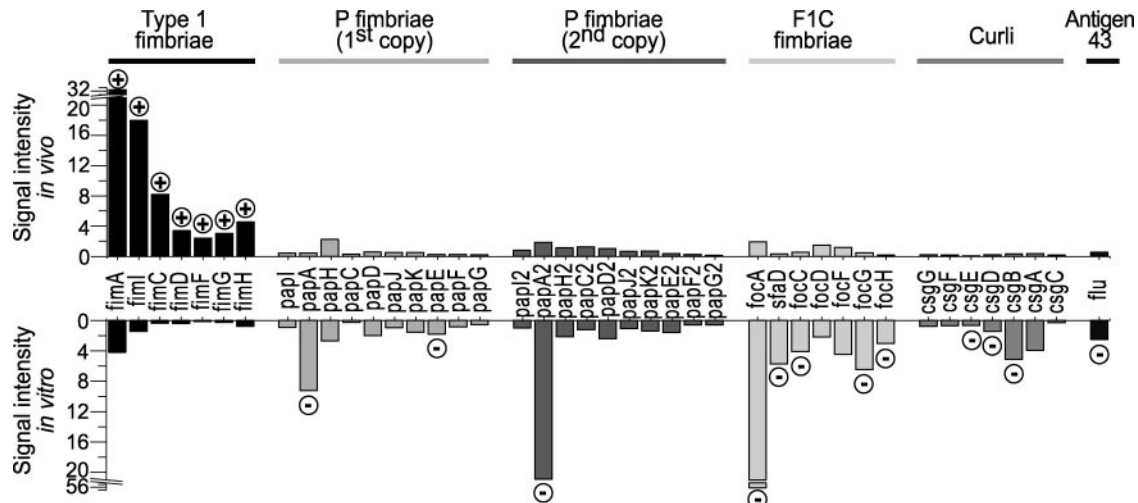


FIG. 2. Expression of adhesins in *E. coli* CFT073. The signal intensity, corresponding to the relative expression of a gene, is shown for selected adhesin genes in vivo (growth during UTI) or in vitro (growth in static LB culture). Genes upregulated (encircled plus signs) or downregulated (encircled minus signs) in vivo relative to in vitro are indicated.

reductase), *glpABC* (encoding *sn*-glycerol-3-phosphate dehydrogenase), and *aspA* (encoding aspartase), all Fnr-regulated genes downregulated during growth in the urinary tract, were all found to be upregulated in *Vibrio cholerae* during in vivo growth in the intestinal rabbit ileal loop model (45). *tdcABC* (encoding genes for anaerobic threonine usage), *tdcE* (encoding formate acetyltransferase), and Fnr-regulated genes *ansB* (encoding L-asparaginase II) and *hypC* (affects hydrogenase activity) were also downregulated during growth in the urinary tract. Accordingly, *cyoAB* and *cyoE*, cytochrome *o* genes indicative of aerobic growth, were upregulated twofold in vivo. On the other hand, the *fdnGHI* operon encoding anaerobic formate dehydrogenase-N was upregulated under in vivo conditions, thus implying oxygen limitation. *arcA* and *fnr*, the key regulators of respiration and indicators of oxygenation, remained unchanged between in vivo and LB growth. Thus, bacteria were growing neither strictly anaerobically nor strictly aerobically. The urinary tract is likely a combination of these conditions, as bacteria isolated from urine may have been growing in different niches of the urinary tract with different levels of oxygenation.

**Nitrogen limitation.** Although nitrogen is abundant in urine (e.g., urea is present at ~0.5 M [11]), it is a limiting resource in the urinary tract for *E. coli*, which typically lacks the urease enzyme required to catalyze the hydrolysis of urea to ammonia and CO<sub>2</sub>. Nitrogen limitation induces nitrogen-regulated (Ntr) genes, such as *glnA*, which encodes glutamine synthetase. While growing in the urinary tract, *glnA* was upregulated fourfold. To provide an exogenous nitrogen source, glutamine importers *glnP* and *glnQ* were also upregulated threefold. *gadA* and *gadB* (encoding glutamate decarboxylases), for the conversion of L-glutamate to  $\gamma$ -aminobutyrate, and *ybaS* (encoding a probable glutaminase), for the degradation of glutamine, were downregulated 7- to 10-fold, perhaps as a coordinated response to maximize the amount of ammonia assimilated. These data indicate that *E. coli* CFT073 faces nitrogen limitation in the urinary tract.

**Iron limitation.** *E. coli* CFT073 faces iron limitation in the urinary tract as indicated by the uniform upregulation of iron acquisition systems as described above.

**High osmolality and osmotic stress.** The cytosolic concentration of osmoprotectants is elevated under high osmolality,

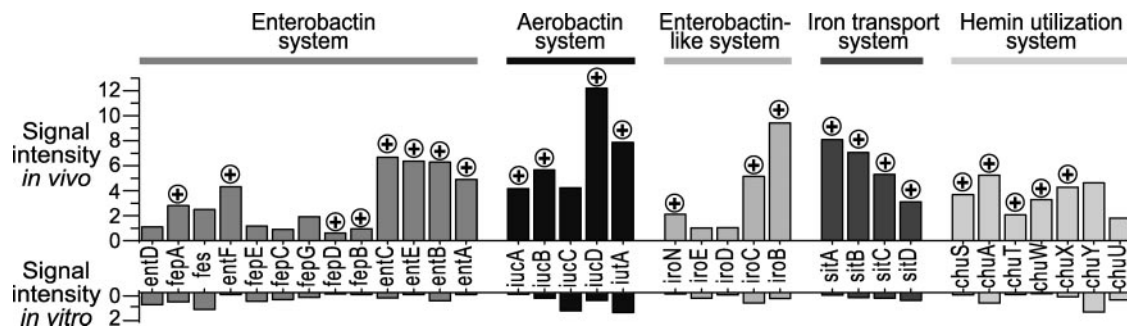


FIG. 3. Expression of iron acquisition systems in *E. coli* CFT073. The signal intensity, corresponding to the relative expression of a gene, is shown for selected iron acquisition systems in vivo (growth during UTI) or in vitro (growth in static LB culture). Genes upregulated (encircled plus signs) in vivo relative to the same genes in vitro are indicated.

TABLE 3. Expression of *E. coli* CFT073 class III genes of motility and chemotaxis

Gene	Function	Fold change <sup>a</sup>	P value
<i>flgL</i> <sup>b</sup>	<b>Flagellar biosynthesis; hook-filament junction protein</b>	<b>-3.19</b>	<b>0.054</b>
<i>flgK</i>	<b>Flagellar biosynthesis, hook-filament junction protein 1</b>	<b>-2.36</b>	<b>0.004</b>
<i>fliD</i>	Flagellar biosynthesis; filament capping protein; enables filament assembly	-1.31	0.018
<i>fliS</i>	Flagellar biosynthesis; repressor of class 3a and 3b operons (RflA activity)	-2.53	0.206
<i>fliT</i>	Flagellar biosynthesis; repressor of class 3a and 3b operons (RflA activity)	-1.04	0.441
<i>fliC</i>	<b>Flagellar biosynthesis; flagellin, filament structural protein</b>	<b>-4.65</b>	<b>0.006</b>
<i>tar</i>	Methyl-accepting chemotaxis protein II, aspartate sensor receptor	-3.88	0.060
<i>cheZ</i>	Chemotactic response; CheY protein phosphatase	-3.49	0.107
<i>cheY</i>	Transmits chemoreceptor signals to flagellar motor components	-3.73	0.146
<i>cheB</i>	Response regulator for chemotaxis (CheA sensor); protein methylesterase	-0.89	0.397
<i>cheR</i>	Response regulator for chemotaxis; protein glutamate methyltransferase	-0.35	0.678
<i>cheW</i>	<b>Positive regulator of CheA protein activity</b>	<b>-3.35</b>	<b>0.012</b>
<i>cheA</i>	Sensory transducer kinase between signal receptors and CheB and CheY	-2.03	0.117
<i>motB</i>	Enables flagellar motor rotation	-1.36	0.186
<i>motA</i>	Proton conductor component of motor	-0.14	0.903

<sup>a</sup> Fold change refers to growth in vivo relative to growth in LB.

<sup>b</sup> Genes statistically downregulated in vivo at least two-fold are in boldface.

as measured here with the upregulation of *pro* gene expression of *E. coli* CFT073 in the urinary tract. *proP* and *proVWX*, encoding systems for the transport of osmoprotectants proline and glycine betaine, were upregulated two- to fivefold in vivo.

**Carbon utilization and starvation response.** *E. coli* CFT073 utilized different sources of carbon depending on whether growth was in vivo or in LB. While in the urinary tract, metabolism shifted so that hexuronates and hexanates could be exploited to support growth. *uxuA* and *uxuB*, encoding mannonate dehydratase and oxidoreductase, and *uxaA* and *uxaB*, encoding altronate hydrolase and oxidoreductase, respectively, were upregulated two- to fivefold. Genes encoding glucitol and fructose uptake and catabolism were upregulated three- to fivefold. The genes encoding the uptake and catabolism of glycerol, *sn*-glycerol-3-phosphate, and trehalose were downregulated three- to sevenfold in vivo. Carbon sources that were used equally during in vivo and LB growth conditions include glucose, mannose, and mannitol. Genes encoding carbon starvation proteins were not consistently up- or downregulated. Thus, *E. coli* CFT073 was not limited for carbon sources during acute infection of the urinary tract.

**Conditions unchanged between in vivo and LB growth.** Groups of genes that were not differentially expressed between in vivo and LB growth conditions provide insight into the similarity between these environments. There was no measurable change in genes that normally respond to pH, oxidative stress, DNA damage, heat or cold shock, cell density dependence, sulfur availability, or phosphate availability (data not shown).

**Verification of microarray.** qRT-PCR was used to independently verify the levels of transcript for an example gene, *papA\_2*, which was found downregulated during growth in vivo 3.5-fold by microarray analysis. *gapA*, encoding glyceraldehyde 3-phosphate dehydrogenase, was used as the normalizing internal standard. Microarray analysis demonstrated that *gapA* expression remained unchanged between in vivo and in vitro growth, thus confirming the validity of this standard. qRT-PCR analysis demonstrated a greater transcript level sensitivity than microarray analysis, as *papA\_2* was downregulated 6.24-fold  $\pm$  0.15-fold during growth in vivo relative to growth in LB.

To verify the biological reproducibility of our in vivo mi-

croarray analysis, the results presented here were compared to those of an analogous microarray of an *E. coli* CFT073 *dsdA* (D-serine deaminase) mutant isolated from the urine of infected CBA/J mice. The upregulation of specific genes encoding type 1 fimbriae, several iron acquisition systems, capsular polysaccharide, and microcin during the in vivo growth of *E. coli* CFT073 *dsdA* demonstrated the biological reproducibility of our major findings (our unpublished data). Pearson's correlation coefficient ( $r^2$ ) between wild-type *E. coli* CFT073 grown in vivo and CFT073 *dsdA* grown in vivo was 0.7583. By comparison, the correlation coefficient between wild-type *E. coli* CFT073 grown in vivo and grown in vitro in LB was 0.4059, suggesting that the wild type and *dsdA* mutant have similar overall gene expression patterns when grown in vivo.

**Alterations in the transcriptome of *E. coli* CFT073 during growth in human urine.** *E. coli* CFT073 grown to mid-exponential phase in filtered human urine partially mimics growth in the urinary tract. The transcriptome of *E. coli* CFT073 grown in vitro in human urine, relative to growth in LB, demonstrated the upregulation of genes encoding two iron acquisition systems (*iuc* and *iro* gene clusters), capsular sialic acid catabolism genes (*nanA* and *nanT*), and a microcin secretion gene (*mchB*). Overall, 54 genes were found upregulated (Table 4 lists the 50 most upregulated) and 88 genes were found downregulated during growth in human urine. Many of the most highly expressed genes in vivo are also among the most highly expressed in human urine, including *ompA*, *ompC*, and many ribosomal protein genes (Table 2). However, in sharp contrast to growth in vivo, *papA\_2* (encoding the major P fimbriae subunit), *focA* (encoding the major F1C fimbriae subunit), and *fliC* (encoding flagellin) were highly expressed in human urine (data not shown).

## DISCUSSION

This study represents the first quantification of the genome-wide transcriptional profile of the most common human uropathogen, *E. coli*, during colonization of the urinary tract. These measurements, taken with bacteria collected from the urine of infected mice, allow us to assess the conditions in the urinary tract

TABLE 4. Top 50 genes upregulated during in vitro growth in human urine

Gene	Function	Fold change <sup>a</sup>	P value	Also upregulated in vivo <sup>b</sup>
<i>yeiC</i>	Hypothetical sugar kinase YeiC	6.646	0.0022	+
<i>rbsD</i>	High affinity ribose transport protein	4.987	0.0021	
<i>artJ</i>	Arginine-binding periplasmic protein 2 precursor	4.730	0.0195	+
<i>c0336</i>	PTS system, mannitol (cryptic)-specific IIA component	4.721	0.0032	
<i>ompF</i>	Outer membrane protein F precursor	4.686	0.0048	
<i>iucD</i>	Aerobactin protein IucD	4.587	0.0047	+
<i>c0335</i>	Hypothetical protein	4.513	0.0032	+
<i>nanA</i>	N-acetylneuraminase lyase subunit	4.225	0.0374	+
<i>leuC</i>	3-Isopropylmalate dehydratase large subunit	4.180	0.0219	
<i>iroB</i>	Siderophore glucosyltransferase	4.144	0.0138	+
<i>leuB</i>	3-Isopropylmalate dehydrogenase	4.141	0.0232	
<i>dppA</i>	Periplasmic dipeptide transport protein precursor	4.124	0.0131	+
<i>c0088</i>	Hypothetical protein	4.030	0.0377	
<i>uxuA</i>	Mannonate dehydratase	3.967	0.0170	+
<i>c0334</i>	Putative integral membrane protein	3.889	0.0079	
<i>ihvC</i>	Ketol-acid reductoisomerase	3.887	0.0343	
<i>yncE</i>	Hypothetical protein YncE precursor	3.849	0.0225	+
<i>ybiW</i>	Putative formate acetyltransferase 3	3.728	0.0491	
<i>yeaR</i>	Hypothetical protein YeaR	3.709	0.0202	+
<i>argC</i>	N-acetyl-gamma-glutamyl-phosphate reductase	3.682	0.0267	
<i>ihvE</i>	Branched-chain amino acid aminotransferase	3.594	0.0274	+
<i>narK</i>	Nitrite extrusion protein 1	3.568	0.0422	
<i>ybeJ</i>	Glutamate/aspartate periplasmic binding protein precursor	3.370	0.0262	+
<i>argB</i>	Acetylglutamate kinase	3.363	0.0320	
<i>ygbJ</i>	Hypothetical oxidoreductase YgbJ	3.263	0.0257	
<i>nanT</i>	Putative sialic acid transporter	3.194	0.0241	
<i>fdnH</i>	Formate dehydrogenase-N beta subunit	3.155	0.0427	+
<i>galE</i>	UDP-glucose 4-epimerase	3.039	0.0235	
<i>narG</i>	Respiratory nitrate reductase 1 alpha chain	3.014	0.0287	
<i>leuL</i>	Leu operon leader peptide	3.003	0.0167	
<i>fdnI</i>	Formate dehydrogenase-N gamma subunit	2.953	0.0207	+
<i>mgIA</i>	Galactoside transport ATP-binding protein	2.925	0.0226	
<i>galK</i>	Galactokinase	2.895	0.0455	
<i>hmpA</i>	Flavo-hemoprotein (hemoglobin-like protein)	2.843	0.0302	+
<i>iutA</i>	Aerobactin protein IutA	2.796	0.0349	+
<i>serA</i>	D-3-phosphoglycerate dehydrogenase	2.793	0.0444	
<i>ytfE</i>	Hypothetical protein YtfE	2.753	0.0395	+
<i>c3835</i>	Hypothetical protein	2.582	0.0401	
<i>livG</i>	High-affinity branched-chain amino acid transport ATP-binding protein	2.479	0.0493	
<i>ihvH</i>	Acetolactate synthase isozyme III small subunit	2.443	0.0166	
<i>uxaC</i>	Uronate isomerase	2.436	0.0392	
<i>livJ</i>	Leu/Ile/Val-binding protein precursor	2.416	0.0291	
<i>hisJ</i>	Histidine-binding periplasmic protein precursor	2.385	0.0117	
<i>yjiY</i>	Hypothetical protein YjiY	2.278	0.0479	
<i>mchB</i>	Microcin H47 secretion protein	2.228	0.0462	
<i>asnB</i>	Asparagine synthetase B (glutamine hydrolyzing)	2.204	0.0395	
<i>cyoD</i>	Cytochrome <i>o</i> ubiquinol oxidase protein	2.184	0.0458	
<i>narI</i>	Respiratory nitrate reductase 1 gamma chain	2.176	0.0278	
<i>cyoE</i>	Protoheme IX farnesyltransferase	2.176	0.0370	+
<i>argE</i>	Acetylmethionine deacetylase	2.088	0.0199	

<sup>a</sup> Fold change refers to growth in human urine relative to growth in LB.

<sup>b</sup> A plus sign indicates this gene was also upregulated during growth in vivo relative to growth in LB.

as seen from the point of view of the pathogen. We describe the first use of microarray technology, based on the sequence of a urinary tract pathogen, to globally examine the induction of specific virulence genes and the coordinate regulation among different virulence and metabolic gene clusters during a UTI. Expression patterns were verified by an example gene by qRT-PCR and by a comparison of major findings to those of in vivo microarray analysis of an *E. coli* CFT073 *dsdA* mutant. In addition, this microarray analysis represents the first assessment of the transcriptome of any *E. coli* pathotype in vivo.

The transcriptional analysis of *E. coli* CFT073 during colo-

nization of the murine urinary tract was feasible because the urinary tract is naturally sterile and because the amount of host nucleic acid contamination from exfoliated epithelial cells (12, 30) was insignificant. This allowed us to collect the urine from experimentally infected mice and directly extract CFT073-specific RNA. We experienced limited complications in isolating ample RNA, provided a sufficient volume of urine was collected from infected animals. Previous studies demonstrated that individual CBA/J mice are consistently and relatively uniformly infected with *E. coli* CFT073 (13). Additionally, urine from 20 mice individually cultured at the conclusion of these

microarray experiments demonstrated that all mice remained infected (data not shown). Urine was sampled and pooled over a range of time (1 to 10 days) from a large collection of infected mice ( $n = 40$ ). Bacteria expelled in the urine likely represent an appropriate sample of the bacteria that had recently infected the kidneys, ureters, bladder, and urethra. Therefore, we assume that the RNA used for this *in vivo* microarray analysis provides a representation of the responses of a uropathogen to a summation of these environmental situations encountered in the urinary tract.

Urine was not collected within the first 24 h of the experimental UTI. We desired to exclude noncolonizing bacteria that were present in the inoculum that may have washed out following transurethral challenge. Instead we focused on a bacterial population that had colonized and adapted to the murine urinary tract. Admittedly, this analysis likely excluded the transient transcription of genes encoding colonization factors and other virulence determinants required for the transition from the *in vitro* LB culture to growth in the urinary tract. Additionally, any gene transiently expressed during the ongoing urine collection would be diluted out in the process of pooling urine samples. Thus, our analysis emphasizes genes that are efficiently expressed throughout space and time during UTI.

Microarray data presented here are internally consistent and compatible with previous studies. There is coordinated regulation of genes within an operon, and transcript levels reflect the expected abundance. For example, the entire *fimAICDFGH* gene cluster of type 1 fimbriae is upregulated *in vivo* compared to that in LB growth (Fig. 2), and as expected there is significantly more *fimA* (major structural subunit gene) transcript than *fimI*, more *fimI* than *fimC*, more *fimC* than *fimD*, and more *fimD* than *fimF*. This is consistent with the prediction of a transcript-stabilizing stem-loop structure at the end of *fimA* (34) as well as *in vitro* expression studies of other chaperone-usher fimbrial gene clusters (2). Genes within iron acquisition operons are also uniformly upregulated (Fig. 3), consistent with a previous study of *E. coli* CFT073 that observed the induction of three siderophore systems during murine peritonitis (35).

We affirm the importance of several known virulence genes by reporting the upregulation of these genes *in vivo*, such as those encoding type 1 fimbriae, siderophores, capsule, drug resistance, and microcin. We have also identified 13 new candidate virulence genes, encoding hypothetical proteins, which were upregulated during *in vivo* growth and were simultaneously not found in the nonpathogenic *E. coli* K-12 strain. Additionally, these analyses provide valuable information on the expression of surface proteins *in vivo* for the purpose of vaccine-directed research.

Several other microarray analyses found that genes encoding ribosomal proteins were highly expressed when cells were exponentially growing in rich media (5, 41, 45). This suggests that the high level of *in vivo* expression of genes involved in translation indicates a rapid rate of exponential growth of pyelonephritogenic *E. coli* CFT073 in the urinary tract. This is somewhat surprising, given that urine is an incomplete and relatively poor growth medium *in vitro* (19, 38). Although bacteria may acquire some nutrients via contact with the urinary tract epithelium, our experiments indicate that nitrogen and iron remain limiting. In addition, bacteria are expending additional energy responding to osmotic stress and producing virulence

and metabolic factors specific for survival in the host. Despite these specific nutrient deprivations and energy expenditures, rapid growth is sustained in the urinary tract. This is in contrast to observations of *V. cholerae* during growth in the intestine, where fewer genes for protein synthesis tended to be found among the 300 most highly expressed genes compared to that of growth in LB (45).

Microarray analysis demonstrates a strikingly high level of type 1 fimbrial expression during growth *in vivo*, as *fimA* expression was the fourth highest gene expressed overall. In addition, type 1 fimbrial genes are highly upregulated compared to that of growth in LB. These data support the findings of studies using signature-tagged mutagenesis and phase-locked mutants (3, 13). Type 1 fimbrial expression *in vivo* contrasts sharply with the near lack of expression of the 11 other fimbrial types previously demonstrated (4, 21) or predicted by the *E. coli* CFT073 genome (42). Coordinate regulation among fimbrial operons has been described previously and may provide an explanation for this observation (17, 44). P fimbriae, argued as being virulence factors during UTI (43), are found downregulated here *in vivo* despite the presence of P-fimbrial Gal-Gal receptors in mice (22, 32). Our microarray experiments suggest that a better way to study the importance of other adherence factors may be use of a Fim-deficient UPEC mutant.

The signals necessary for transcriptional alterations in UPEC during transition from growth in the intestine to growth in the urinary tract remain undisclosed. However, this microarray analysis of bacteria responding to the urinary tract can be compared to the two other true *in vivo* microarray analyses, where *V. cholerae* was isolated from rice-water stools of infected patients (5, 27). By using bacteria as bioprobes for their respective environments, differences in these niches are revealed by patterns of bacterial gene expression. For example, the urinary tract is nitrogen and iron limiting for *E. coli*, of moderate oxygenation, and of higher osmolarity and pH than the anaerobic, nitrogen-rich gastrointestinal tract.

Differences in gene expression between growth *in vivo* and growth *in vitro* in human urine may be partially due to species-specific differences in urine composition. This use of human urine as a growth medium thus examines the important relationship between *in vivo* murine studies and human infection. This work also suggests that *in vitro* growth of UPEC in human urine, rather than in LB, is a useful tool to more closely imitate growth conditions encountered during UTI. The upregulation of important virulence factors, including iron acquisition systems, capsule, and microcin, was observed. However, the most highly expressed virulence factor during UTI, type 1 fimbriae, was not induced in urine. The examination of gene expression *in vivo* by using the experimental murine model of ascending UTI thus remains essential.

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