Host-specific induction of *Escherichia coli* fitness genes during human urinary tract infection

Sargurunathan Subashchandrabose^a, Tracy H. Hazen^b, Ariel R. Brumbaugh^a, Stephanie D. Himpel^a, Sara N. Smith^a, Robert D. Ernst^c, David A. Rasko^b, and Harry L. T. Mobley^{a,1}

^aDepartment of Microbiology and Immunology and ^cUniversity Health Service, University of Michigan Medical School, Ann Arbor, MI 48109; and ^bDepartment of Microbiology and Immunology, Institute for Genome Sciences, School of Medicine, University of Maryland, Baltimore, MD 21201

Edited by Scott J. Hultgren, Washington University School of Medicine in St. Louis, St. Louis, MO, and approved November 18, 2014 (received for review August 18, 2014)

Uropathogenic *Escherichia coli* (UPEC) is the predominant etiological agent of uncomplicated urinary tract infection (UTI), manifested by inflammation of the urinary bladder, in humans and is a major global public health concern. Molecular pathogenesis of UPEC has been primarily examined using murine models of UTI. Translational research to develop novel therapeutics against this major pathogen, which is becoming increasingly antibiotic resistant, requires a thorough understanding of mechanisms involved in pathogenesis during human UTIs. Total RNA-sequencing (RNA-seq) and comparative transcriptional analysis of UTI samples to the UPEC isolates cultured in human urine and laboratory medium were used to identify novel fitness genes that were specifically expressed during human infection. Evidence for UPEC genes involved in ion transport, including copper efflux, nickel and potassium import systems, as key fitness factors in uropathogenesis were generated using an experimental model of UTI. Translational application of this study was investigated by targeting Cus, a bacterial copper efflux system. Copper supplementation in drinking water reduces *E. coli* colonization in the urinary bladder of mice. Additionally, our results suggest that anaerobic processes in UPEC are involved in promoting fitness during UTI in humans. In summary, RNA-seq was used to establish the transcriptional signature in UPEC during naturally occurring, community acquired UTI in women and multiple novel fitness genes used by UPEC during human infection were identified. The repertoire of UPEC genes involved in UTI presented here will facilitate further translational studies to develop innovative strategies against UTI caused by UPEC.

uropathogenic *E. coli* | UPEC | UTI | RNA-seq | metal transport

Urinary tract infection (UTI) caused by uropathogenic *Escherichia coli* (UPEC) is a global public health concern, especially for women (1). In the United States alone, 7–11 million cases of UTI are reported annually resulting in expenditures of \$3.5 billion (2, 3). UPEC is the predominant cause (75–95% of cases) of UTIs in otherwise healthy individuals (1). UTIs caused by UPEC are one of the most common indications for antibiotic prescription and rapid increase in antibiotic resistance in UPEC (4) requires development of next-generation therapeutic agents (5). A targeted approach that selectively diminishes *in vivo* fitness of pathogens has been proposed as a promising alternative to treatment with conventional antibiotics (6). The molecular mechanisms of UPEC pathogenesis have been extensively investigated, primarily using murine models (7–9). Identification of next-generation therapeutics against UPEC, however, requires a thorough knowledge of the fitness and virulence mechanisms involved in the pathogenesis of community-acquired UTIs in humans.

Transcriptional profiling, a powerful functional genomic tool, can be used to elucidate host–pathogen interaction during infection (10, 11). UPEC represents a heterogeneous group of bacterial strains (12, 13) and DNA microarrays, based on the sequence of a pyelonephritis strain CFT073, were used in a previous study to determine UPEC genes highly expressed during human infection (11). A clinic-to-laboratory approach was used in this study to identify novel human UTI-specific genes expressed

by UPEC during naturally occurring infection in women. Total RNA sequencing (RNA-seq) was used to overcome the limitations of hybridization-based technology (14) and probe the transcriptome of UPEC in urine from patients with acute uncomplicated UTI. We have identified UPEC fitness genes that are not only highly expressed during human UTIs, but are also required for optimal colonization in a mouse model of UTI. This study demonstrates the importance of bacterial copper efflux systems during UTI and offers evidence for the protective role of copper against UPEC colonization in a mouse model. Previous studies have revealed the importance of oxidative stress resistance mechanisms in UPEC in the pathogenesis of UTIs. Evidence for the role of UPEC genes involved in adaptation to low-oxygen conditions during UTIs is presented here. These novel fitness genes represent candidates for evaluation as targets for development of therapeutics and prophylactics against UPEC.

Results

Identification of Differentially Expressed UPEC Genes During UTI.

Women presenting at the University of Michigan Health Service Clinic with symptoms of cystitis were invited to participate in this study. Informed consent was obtained from all voluntary participants (ages 18–50; median = 21 y). A total of 42 (48.8%) urine samples collected from 86 participants were culture-positive for uropathogens and 38 (90.5%) urine samples contained *E. coli* ($\geq 10^5$ CFU/mL). Additionally, 7.7% and 15.3% of these UPEC isolates were resistant to ciprofloxacin and trimethoprim/sulfamethoxazole, respectively, two first-line antimicrobial agents currently used in the treatment of UTI (4). *Citrobacter freundii*, *Citrobacter koseri*,

Significance

Escherichia coli is the most common cause of urinary tract infections (UTI) in humans. This bacterium is a major global public health concern because it is becoming resistant to currently available antibiotics. Therefore, it is imperative to develop new treatment and prevention strategies against this bacterium. However, the processes that promote survival of this bacterium within the human urinary tract during UTI are not clearly understood. Here we identify *E. coli* genes that promote survival within the urinary tract during naturally occurring UTI in women. Genes identified in this study represent targets for development of new therapies against UTI caused by *E. coli*.

Author contributions: S.S. and H.L.T.M. designed research; S.S., A.R.B., S.D.H., and S.N.S. performed research; S.S., T.H.H., R.D.E., and D.A.R. contributed new reagents/analytic tools; S.S., T.H.H., and D.A.R. analyzed data; and S.S. and H.L.T.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: Genome alignment files (BAM) and corresponding genome sequences (FASTA) for HM26, HM27, HM46, HM65, and HM69 have been archived in the NCBI short read archive (accession no. SRP041701).

¹To whom correspondence should be addressed. Email: hmobley@umich.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1415959112/-DCSupplemental.

Fig. 1. Uropathogenic *E. coli* colonization in a mouse model of UTI. CBA/J mice ($n = 10$) were transurethraly inoculated with UPEC isolates, HM26, HM27, HM65, HM46, and HM69 belonging to sequence types 1057, 28, 694, 259, and 260, respectively. Urine and tissue bacterial loads at 48 hpi reveal that these isolates, belonging to phylogroups B2 (purple) or D (green), are capable of colonizing a mouse model of ascending UTI. Bars indicate median bacterial burden and dotted line indicates limit of detection.

Enterobacter aerogenes, and *Proteus mirabilis* were other uropathogens isolated in this study, but not characterized further. Because participants were prescribed antibacterial agents upon clinical examination, only initial urine samples were collected. Total RNA was isolated from de-identified UTI urine samples and corresponding clinical isolates cultured in urine from healthy volunteers or laboratory medium.

E. coli isolates HM26, HM27, HM46, HM65, and HM69 were chosen for RNA-seq because they yielded high quantities of quality RNA. These isolates were also found to successfully colonize the urinary tract of CBA/J mice during experimental UTI (Fig. 1) at levels comparable to UPEC strain CFT073. Genomes of these five UPEC isolates were sequenced and phylogenomic analysis demonstrated that these isolates belong to two different *E. coli* phylogroups (B2 and D) (12). BLAST score ratio (BSR) analysis was used to probe the degree of conservation of protein encoding sequences (15). Conservation (BSR values ≥ 0.8 indicate protein encoding genes that are highly conserved, values ≥ 0.4 but < 0.8 indicate similar but divergent, and values < 0.4 indicate a sequence is absent) of each predicted protein from UPEC strain CFT073 to UPEC strains 536, F11, UMN026, UTI89, HM26, HM27, HM46, HM65, and HM69, reveals that UPEC are genetically heterogeneous (Fig. S1) and is consistent with previous findings (12, 13).

RNA-seq reads (~ 7 – 38 million) from human UTI samples mapped to the UPEC genomes with the exception of HM26 (~ 0.4 million). A summary of RNA-sequencing and genome alignment results is presented in Table 1. Because only one UTI sample was available from each patient, a stringent cutoff of fourfold change in expression was used to delineate differences in transcript abundance between UTI, urine, and lysogeny broth (LB).

Iron uptake gene transcript levels were used to validate the results of RNA-seq analyses. Previous studies have demonstrated that urine is an iron-limited milieu (16), and iron uptake genes in UPEC are highly expressed in the RNA samples isolated from UPEC collected from UTI urine samples and culture in urine from healthy volunteers (11). RNA-seq results are consistent with these findings; iron uptake genes were among highly expressed genes in UTI compared with LB (Fig. S24 and Dataset S1). Levels of iron uptake gene transcripts were not higher in UTI compared with urine (Fig. S24) because urine itself is a documented iron-limited environment (16). Next, RNA-seq results were independently confirmed using qRT-PCR on select genes, not involved in iron uptake, in HM17, HM26, HM27, and HM46 (Fig. S2B). After successful validation of RNA-seq, we sought to identify UPEC transcripts that are abundant in UTI samples, compared with culture in urine of healthy volunteers and in laboratory medium (Fig. 24). UPEC transcripts that were $>$ fourfold higher in UTI samples, compared with urine or LB, are delineated as UTI-specific genes (Fig. 2B), in contrast to urine-specific genes whose transcripts were found in high levels in urine, compared with LB (Fig. 2).

UPEC Genes Expressed During Human Infection Contribute to Fitness During UTI. Selective expression of a gene implicates its involvement in adaptation, but not necessarily its role in survival within a particular niche. In this report, genes that contribute to wild-type level of survival of UPEC during UTI in a mouse model of infection are referred to as fitness genes. A subset of UTI-specific genes with $>$ fourfold change in transcript levels in at least three UTI samples (Dataset S1) and regulated at transcriptional level was selected for further investigation (Table S1). Isogenic mutants lacking select genes, including *cus* (copper efflux), *eut* (ethanolamine uptake), *fdhF* (formate dehydrogenase H), *kdp* (potassium import system), and *nik* (nickel import) (Table S1), in UPEC strain CFT073 (17) were constructed using lambda red recombineering (18) (Fig. S3). Relative fitness was assessed in a mouse model of ascending UTI (19) and competitive indices were calculated based on tissue bacterial load (Table 2).

A copper efflux system, *Cus*, is involved in resistance to Cu toxicity in *E. coli* (20). Transcripts of cognate two-component regulatory system (*cusRS*) and structural genes (*cusCFBA*) comprising the *Cus* system were found at higher levels during UTI compared with urine in RNA-seq (Fig. 3A and Dataset S1) and quantitative RT-PCR (qRT-PCR) (Fig. S4). Urine from UTI patients contains higher quantities of Cu compared with urine from healthy subjects (Fig. S4). A *cus* mutant displayed enhanced susceptibility to Cu toxicity under anaerobic conditions (Fig. 3B). Coinfection experiments revealed that the *Cus* system is required for optimal fitness of UPEC during colonization in a murine model of UTI (Table 2). Transcripts of other copper detoxification genes, *copA* and *cueO* and their positive transcriptional regulator *cueR*, were also found at higher levels during UTI (Fig. 3A), compared with culture in urine.

Because *cus* genes are specifically expressed human infection and a *cus* mutant exhibits compromised fitness in a mouse model of UTI, we hypothesized that copper supplementation might minimize colonization by UPEC. Copper was added to drinking water for 8 d before infection and mice were inoculated with UPEC CFT073. Mice in the copper supplemented group were colonized by lower levels of UPEC (Fig. 3C), indicating that copper supplementation can ameliorate colonization of UPEC. Serum chemistry did not reveal an increase in liver enzyme or copper levels, but copper content of urine was elevated in mice on supplemental copper, compared with the control group (Fig. S4).

Transcripts of high-affinity nickel uptake genes, *nikABCDEF*, were found at high levels during human UTI (Fig. 4A). Transcription of *nik* genes are repressed by NikR in the presence of

Table 1. Summary of RNA-seq reads and alignment

Isolate	Condition	Reads		
		Total	Mapped*	% Mapped
HM26	HUTI	22750524	430632	1.89
	LB	73227334	53796646	73.47
	UR	64421626	38235680	59.35
HM27	HUTI	72913406	11965706	16.41
	LB	69738674	51498928	73.85
	UR	102494326	61908242	60.4
HM46	HUTI	67767992	6972582	10.29
	LB	80819130	52305182	64.72
	UR	87677548	53898016	61.47
HM65	HUTI	79243432	37893778	47.82
	LB	75942540	52393964	68.99
	UR	79179256	49011996	61.9
HM69	HUTI	79210084	25070852	31.65
	LB	150290446	54236110	36.09
	UR	70072174	44453412	63.44

HUTI, human urinary tract infection; LB, lysogeny broth; and UR, human urine.

*Number of reads mapped to native genomes.

Fig. 2. Identification of human UTI-specific genes. Transcriptomes of each UPEC isolate from patient urine samples (UTI), cultured in urine from healthy volunteers (UR), or lysogeny broth (LB) were compared to identify human UTI-specific genes. (A) Three-way comparison was used to delineate human UTI-specific genes from urine-specific genes that are expressed in response to growth in urine. (B) Difference in transcript abundance from RNA-seq analyses of representative UTI-specific genes is depicted here. Iron uptake gene (*fepA*) is highly expressed during both UTI and culture in urine from healthy volunteers, compared with LB. Other genes depicted here are selectively expressed only during human UTIs, indicating their potential involvement in human UTI. *nikA*, nickel import; *cusC* and *cusR*, copper efflux; *kdpA*, potassium import; *fdhF*, formate dehydrogenase; *hyaA*, hydrogenase 1 small subunit; *c3734*, hydrogenase 2 small subunit; *wcaL*, colanic acid biosynthesis; *cysP*, thiosulfate import; *tauA*, taurine import; and *ssuE*, alkane sulfonate import.

excess nickel and positively regulated by Fnr, a transcriptional regulator governing transition to anaerobiosis (21). Nickel is a cofactor for hydrogenases in the formate hydrogen lyase (FHL) complex and FHL is involved in growth under anaerobic conditions (21). Transcript of the FHL-associated *fdhF* was also found at high levels during UTI (Fig. 4A). Transcripts of a transcriptional regulator of FHL genes (*fhlA*) were also found at high levels during UTI (Fig. 4A). Mutants lacking either *nik* or *fdhF* genes were both compromised in fitness in the mouse model of UTI (Table 2). Collectively, these findings suggest that UPEC experiences oxygen depletion resulting in an increased need for nickel during UTI.

Potassium import is mediated by three uptake systems in *E. coli* (22). Transcripts of two-component regulatory system (*kdpDE*) and structural components (*kdpABC*) comprising the inducible potassium import system, Kdp, were found at higher levels during human UTI (Fig. 4B). However, *trk* and *kup*, other potassium uptake system genes, were not differentially regulated in response to growth within the human urinary tract (Dataset S1). Additionally, the *kdp* mutant exhibits reduced fitness during colonization of murine urinary bladder and kidney, suggesting an increased demand for potassium during UTI (Table 2). K and Ni concentration in the urine samples from UTI patients, however, was not statistically different from that of control (Fig. S4).

Ethanolamine is a major component of phospholipids in the host cell membranes (23). Genes involved in regulation, uptake, and metabolism of ethanolamine were highly expressed in HM26, HM27, and HM46 (Fig. S5A and Dataset S1). Transcripts of all genes in this cluster were abundant during UTI and the positive transcriptional regulator of the ethanolamine gene cluster, encoded by *eutR*, is depicted as an example in Fig. S5A. A mutant lacking the entire ethanolamine gene cluster (~15 Kb) exhibits a fitness defect in the urinary bladder, indicating that the ability to metabolize ethanolamine might promote fitness during UTI (Table 2).

UPEC Genes That Might Not Contribute to in Vivo Fitness. Transcripts of colanic acid biosynthesis (24), phosphonate utilization, sulfate/thiosulfate uptake, taurine uptake, and alkanesulfonate uptake (25)

genes were highly expressed during human UTIs (Fig. 2B, Fig. S5, and Dataset S1). Growth of *cys*, *tau*, and *ssu* mutants lacking sulfate/thiosulfate, taurine, and alkanesulfonate uptake systems, respectively, and *cysB*, a cysteine auxotroph, in a sulfur-limited medium (26) confirmed the loss of function phenotype in these mutants (Fig. S6). Coinfection experiments in a mouse model of UTI, however, did not reveal a measurable loss of fitness for these mutants (Table 2), indicating that these genes do not affect fitness, at least in the mouse model under our experimental conditions.

Known UPEC Virulence Genes Highly Expressed During UTI. Transcripts of some previously established virulence genes were abundant during UTI (Fig. 5 and Dataset S1). Transcripts of type 1 fimbrial tip adhesin (*fimH*), capsule export (*kpsD*), negative regulator of phosphate uptake (*phoU*), transcriptional antiterminator (*rfaH*), GMP synthase (*guaA*), and periplasmic dipeptide-binding protein (*dppA*) were selectively expressed during UTI but not as a milieu-specific response to culture in urine (Fig. 5A and C). Expression of a GMP synthase gene during UTI suggests a surge in GMP demand during infection (Fig. 5A). Consistent with previous findings (11, 27), flagellin gene (*fliC*) was down-regulated under all conditions tested. Because of the differences in osmolarity between urine and LB, periplasmic osmoprotectant (glycine betaine) binding protein (*proV*) was up-regulated both during UTI and culture in urine compared with LB (Fig. 5). Other known virulence genes, such as those encoding P fimbriae, hemolysin A, and succinate dehydrogenase, did not meet the threshold of fourfold change in transcript level (Dataset S1).

Discussion

Although pathogenesis of bacterial UTIs, especially UPEC, has been well studied, little is known about the roles of known and putative fitness factors and global transcriptome during uncomplicated UTI in humans. Here, RNA-seq, a sequencing-based transcriptional profiling technique, was used to detect bacterial fitness determinants in urine from female patients with naturally occurring uncomplicated UTIs. RNA-seq has been used to measure pathogen transcript levels in experimental models of *Vibrio cholerae* infection (28) and *Campylobacter jejuni* colonization (29). Previously, DNA microarrays were used to define transcriptional profiles of UPEC during human infection (11). However, intrinsic limitations of hybridization-based technology, inherent genetic heterogeneity in UPEC, and variable amount of host-nucleic acid in the samples precluded direct comparison of transcript levels.

Recently, RNA-seq was applied to probe gene expression in UPEC in an older patient population (>60 y), who are more likely to suffer from other comorbidities (30), whereas our samples

Table 2. Role of UTI-specific genes in fitness

Mutant	Function	Competitive indices	
		Bladder	Kidney
<i>cus</i>	Copper resistance	0.04	0.39
<i>cys</i>	Thiosulfate uptake	1.83	1.83
<i>eutR</i>	Ethanolamine uptake and metabolism	0.049	0.44
<i>fdhF</i>	Formate dehydrogenase	0.42	0.42
<i>kdp</i>	Potassium uptake	0.33	0.26
<i>nik</i>	Nickel uptake	0.29	0.43
<i>phnR</i>	Phosphonate uptake and metabolism	1.47	2.64
<i>tau</i>	Taurine uptake	2.18	1.33
<i>wcaLM</i>	Colanic acid biosynthesis	2.23	2.23

Statistically significant fitness defect ($P < 0.05$, Wilcoxon signed rank test) is denoted in boldface.

Fig. 3. UPEC copper efflux system genes are involved in UTI. (A) Fold-change in levels of *Cus* copper detoxification system genes during UTI compared with culture in urine from healthy volunteers was determined by RNA-seq. Genes with greater than fourfold change in transcript levels, indicated by dotted lines, were considered as highly expressed. Structural (*cusCFBA*) and regulatory genes (*cusRS*) involved in the *Cus* system are found at higher levels during UTI, compared with urine. Transcripts of periplasmic copper oxidase *cueO*, but not copper exporter *copA* and transcriptional regulator *cueR*, are found at higher levels during UTI. (B) The *Cus* system is required for copper resistance in UPEC. Wild-type and *cus* mutant were cultured in the presence of copper under anaerobic conditions. Optical densities of stationary phase cultures reveal that loss of the *Cus* system impairs resistance to copper. (C) Copper supplementation mitigates UPEC CFT073 colonization in the urinary tract. Each symbol corresponds to data from a mouse and bars indicate median bacterial load. Bacterial burden in control and copper-supplemented groups ($n = 20$) were compared by Mann-Whitney test and $P < 0.05$ was considered as a statistically significant difference. The dotted line indicates limit of detection. Bars represent mean $\pm$ SEM for five human UTI isolates (A) or three independent experiments (B).

represent uncomplicated UTI from otherwise healthy young women (median age = 21 y). RNA-seq reads were mapped to a consortium of known *E. coli* genes (30) and we used the native genomes of these isolates to determine transcriptional signature during UTI. Additionally, *E. coli* isolates from phylogroups A and B1 encompassing fecal commensal strains were included, along with isolates from B2 and D phylogroups, which comprise known UPEC strains (30). In this study, we report identification of genes that are not only selectively expressed within urinary tract during UTI but are also involved in fitness in a mouse model of UTI.

Isolates cultured from UTI urine samples have been demonstrated to represent the dominant strain (31) and the dominant strain from each patient sample was used for RNA isolation in vitro. Voided urine from patients with UTI contains UPEC in various lifestyles, including planktonic (rod and filamentous morphotypes), sessile (adherent to exfoliated epithelial cells), and intracellular communities that are associated with the pathogenesis of cystitis (32). Because planktonic bacteria are the predominant morphotype, it is likely that UTI RNA samples are enriched in transcripts from planktonic cells. Differences in proportion of other morphotypes could account for heterogeneity in gene expression between patient samples, at least in part. UPEC in voided urine represents the closest surrogate to UPEC thriving in the bladder that can be obtained from humans and was used to probe the transcriptional state of UPEC during UTIs.

Mammalian hosts actively sequester iron and an intense competition ensues between host and pathogen for iron during infection (33). Previous studies have demonstrated that UPEC

iron uptake genes are highly expressed during colonization (34) and UTIs in human (11) and murine hosts (27, 35, 36). Additionally, vaccination with iron uptake receptor proteins FyuA, Hma, IreA, and IutA protected mice against experimental UTI (37, 38). Hematuria (39) and massive urothelial exfoliation (40) are hallmarks of cystitis. UPEC, endowed with hemolysin and cytotoxic necrotizing factor (41), can potentially access iron and heme released from damaged cells. Our results indicate that urine from healthy volunteers is iron-limited compared with urine from UTI patients. However, bioavailability of Fe in urine from patients with UTI might be limited because of sequestration (33) and is still low enough to derepress Fur regulon genes.

Copper-mediated killing is a key innate immune mechanism used to hamper bacterial infection (42). *E. coli* uses three major mechanisms involving CopA, CueO, and Cus proteins to mitigate Cu toxicity (20). An additional Cu resistance mechanism was identified in UPEC, where yersiniabactin, a siderophore, binds and sequesters Cu (43) and Cu-yersiniabactin complexes exhibit superoxide dismutase activity (44). UPEC uses yersiniabactin to protect not only against copper but also against superoxide. A *cueO* mutant, defective in periplasmic multicopper oxidase, colonized a mouse model of UTI better than the wild-type strain (45). *cus* genes are expressed during human UTI, Cu levels are elevated in urine during UTI, and a *cus* mutant exhibits reduced fitness in a mouse model, indicating that *Cus* proteins are critical for wild-type level of fitness during UTI. *CopA* and *CueO* transcripts are not as strongly differentially expressed as *Cus* transcripts, suggesting a potential hierarchy in copper detoxification systems during UTI. Our results indicate that copper supplementation indeed reduces, but does not eliminate, UPEC colonization in a mouse model of UTI. Effects of lower inocula and follow-up on infected mice for a longer time remain to be tested.

Research on nickel in bacterial pathogenesis has focused on its role as a cofactor for urease activity. Mutants lacking nickel uptake genes in *Helicobacter pylori* (46) and *Staphylococcus aureus* (47) are attenuated in animal models of infection; this attenuation is attributed primarily to reduced urease activity. *P. mirabilis* mutants lacking urease genes are severely attenuated in a mouse model of UTI (48). UPEC are usually urease-negative; therefore, up-regulation of nickel uptake genes in conjunction with the FHL complex genes (*fdhF* and *fhIA*) and anaerobic

Fig. 4. Abundance of nickel and potassium uptake system transcripts during human UTI. Fold-change in levels of select UPEC nickel and potassium uptake system transcripts in UTI compared with culture in urine from healthy volunteers was determined by RNA-seq. Genes with greater than fourfold change in transcript levels, indicated by dotted lines, were considered as highly expressed. (A) Structural (*nikABCDE*) and regulatory (*nikR*) genes involved in nickel uptake, formate hydrogenase (*fdhF*), and transcriptional regulator of formate hydrogen lyase complex (*fhIA*) are selectively expressed during human UTIs. (B) Inducible potassium import (*kdpABC*) and the corresponding two-component regulatory system (*kdpDE*) transcripts in UPEC are found in high levels during UTI. Bars represent mean $\pm$ SEM for five human UTI isolates.

Fig. 5. Expression of previously established UPEC virulence factors during human UTI. Fold-change in abundance of select UPEC virulence genes: type 1 fimbrial tip adhesin (*fimH*), flagellin (*fliC*), capsule assembly (*kpsD*), phosphate transport system protein (*phoU*), transcriptional anti-terminator (*rfaH*), osmoprotection system (*proV*), GMP synthase (*guaA*), and dipeptide importer (*dppA*). Transcripts with greater than fourfold change in abundance in RNA-seq analyses, indicated by dotted lines, were considered as differentially expressed. Flagellin gene is down-regulated under all three comparisons. (A) *fimH*, *kpsD*, *phoU*, *rfaH*, *guaA*, and *dppA* were expressed during human UTI. (B) *fimH*, *kpsD*, *phoU*, *rfaH*, *proV*, and *dppA* genes are up-regulated in Ur compared with LB. (C) The osmoprotectant system gene *proV* is induced not only during UTI but also during growth in urine. Bars represent mean $\pm$ SEM for five human UTI isolates (A, B, and C). Ur, urine from healthy volunteers.

Cu resistance system (Cus) suggests that UPEC might encounter oxygen depletion within the urinary bladder during UTI.

UPEC genes involved in regulating oxidative stress resistance, *hfq* (49), *oxyR* (50), and *rpoS* (51), are involved in fitness and colonization in murine models of UTI. Here, we present evidence for the role of UPEC genes involved in adaptation to low oxygen milieu during acute UTI. Recently, neutrophils were demonstrated to cause localized oxygen depletion during intestinal inflammation (52). Because massive influx of neutrophils is a hallmark of UTI (39), it is likely that infected urinary bladder is oxygen-depleted and UPEC up-regulates genes required for optimal survival under low oxygen conditions as an adaptation strategy. This study introduces a new dimension to the current model of pathogenesis, where adaptation to a low oxygen environment is critical for successful colonization of the bladder during active UTI. Because chemical modulators of hypoxia-inducible factors, host-cell transcriptional regulators involved in adaptation to hypoxia, are available (53), the role of hypoxia-inducible factors and hypoxia in UTI could be tested.

KdpE, a regulator of the Kdp potassium import system, also affects multiple virulence genes in enterohemorrhagic *E. coli* (54). Kdp genes are up-regulated during UTI and are required for wild-type level of fitness during experimental UTI. It is plausible that in addition to potassium import, indirect regulation of nonpotassium uptake genes by KdpE might also contribute to in vivo fitness in UPEC. *Salmonella* mutants defective in ethanolamine uptake and metabolism exhibit reduced fitness within intestines, especially during inflammation when there is substantial release of ethanolamine from host cell membranes (23). Enterohemorrhagic *E. coli* uses ethanolamine not only as a nitrogen source but also as a signal to regulate expression of virulence genes (55). Here, we demonstrate the role of ethanolamine uptake and metabolism genes in the survival of a bacterial pathogen during UTI.

Some of the transcripts found at high levels during human UTI, not surprisingly, do not appear to be involved in fitness in a mouse model of UTI. Because it is feasible to test the fitness of isogenic mutants only in a laboratory animal model of UTI, host-specific changes in transcript levels between humans and mice might account for this discrepancy. Although a measurable loss of fitness could not be demonstrated in our experiments, these genes might be involved in fitness in other niches or at different time points during infection.

Type 1 fimbria is a canonical virulence factor that mediates adhesion of UPEC to uroplakins, mannosylated host epithelial cell-surface receptors (40). Based on absolute expression, type 1 fimbrial genes were previously reported to be poorly expressed during human UTI (11), in contrast to a mouse model of UTI, where they are among the most highly expressed genes (27). Hagan et al. (11), also note that the high degree of variation observed in type 1 fimbrial genes might have interfered with accurate quantification of their expression using DNA microarrays. RNA-seq overcomes this limitation and here, relative expression was used to quantify differences in transcript levels. In our patient samples, the phase-variable type 1 fimbrial genes were up-regulated in some strains, but not expressed in others. These findings are based on the analysis of total bacterial RNA and further studies on planktonic, sessile, and intracellular communities during UTI are required to understand the heterogeneity in expression of type 1 fimbria.

In summary, we establish a repertoire of fitness genes in UPEC, a major human pathogen, during active UTI. Using independent lines of investigation, we have identified novel fitness factors and offer new insights into the conditions encountered by UPEC within the human urinary tract during acute uncomplicated UTI in women. A logical extension of this study is to identify and develop small molecules that selectively target these UTI-specific fitness mechanisms for treatment of uncomplicated UTIs.

Materials and Methods

Ethics Statement. All procedures involving human samples were performed in accordance to the protocol (HUM00029910) approved by the Institutional Review Board at the University of Michigan. This protocol is compliant with the guidelines established by the National Institutes of Health for research using samples derived from human subjects. Additional information on UTI urine sample collection is provided in *SI Materials and Methods*. Mouse infection experiments were conducted according to the protocol (08999-3) approved by the University Committee on Use and Care of Animals at the University of Michigan. This protocol is in complete compliance with the guidelines for humane use and care of laboratory animals established by the National Institutes of Health.

Bacterial Strains and Culture Conditions. Bacterial strains and isolates used in this study are listed in *Table S1*. Detailed methods are presented in *SI Materials and Methods*. Oligonucleotides used in this study are listed in *Table S2*.

RNA Isolation. Cell pellets, obtained from centrifugation of human UTI urine samples, were stored at -80°C until RNA extraction. Cell pellets were treated with Proteinase K (0.06 mAU/ μL) and total RNA was extracted using RNeasy mini kit (Qiagen). Turbo DNase (Ambion) was used to remove contaminating bacterial and human DNA. RNA integrity was assessed using a Bioanalyzer (Agilent). For LB and human urine samples, RNAprotect was added to cultures in midexponential phase and RNA isolated from three biological replicates was mixed in equal quantity and treated as a single sample. Bacterial RNA content of HM26, HM27, HM46, HM65, and HM69 was enhanced by selective depletion of human RNA using the MICROBEnrich kit (Ambion).

RNA Sequencing. The depleted RNA was used to generate sequencing libraries using the Ovation Prokaryotic RNA-Seq system (NuGen) and the Encore next-generation sequencing library system (NuGen). The libraries were sequenced using an Illumina HiSeq2000 by the Genome Resource Center at the Institute for Genome Sciences, University of Maryland, Baltimore, MD. Bioinformatic analyses and qRT-PCR are described in *SI Materials and Methods*.

BLAST Score Ratio. BSR analysis was performed as previously described (15) and detailed methods are presented in *SI Materials and Methods*.

Mouse Model of Ascending UTI. Five- to 6-wk-old ($n = 10$), female CBA/J mice (Harlan Laboratories) were transurethrally inoculated with 10^8 CFU of UPEC isolate. Mice were killed 48 h postinoculation (hpi); urine, homogenates of bladder and kidneys were plated using Autoplate 4000 (Spiral Biotech), and CFU counts were determined using Q-count (Spiral Biotech). A description of copper supplementation experiments in mice is provided in *SI Materials and Methods*.

Competitive Indices. Coinfections with wild-type (CFT073) and isogenic mutant strains were conducted as described previously (19). Briefly, CBA/J mice ($n = 10$) were inoculated transurethrally with 10^8 CFU comprising wild-type and mutant strain in a 1:1 ratio. Mice were killed at 48 hpi; bladders and kidneys were collected, homogenized, and plated on plain and selective plates to enumerate wild-type and mutant CFUs. Competitive indices were determined as the ratio of mutant to wild-type in an organ to the ratio of mutant to wild-type in the inoculum. Results were analyzed by Wilcoxon-

signed rank test and $P < 0.05$ was considered as a statistically significant difference.

Determination of Metal Levels. Metal content in human urine samples were determined using an inductively coupled plasma mass spectrophotometer, as described in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank the staff at the University Health Service Clinical Laboratory for help with sample collection; members of the H.L.T.M. laboratory, especially Christopher Alteri, Cody Springman, Courtney Luterbach, and Michael Engstrom, for insightful discussions; and A. Shetty at the Institute for Genome Sciences for technical assistance with informatics. This work was supported in part by DK094777 from the National Institute of Diabetes and Digestive and Kidney Diseases (to H.L.T.M.); a Research Scholars Fellowship from the American Urological Association-North Central Section, administered by the Urology Care Foundation (to S.S.); and funds from the State of Maryland (T.H.H. and D.A.R.).

- Foxman B (2010) The epidemiology of urinary tract infection. *Nat Rev Urol* 7(12):653–660.
- Litwin MS, et al.; Urologic Diseases in America Project (2005) Urologic diseases in America Project: Analytical methods and principal findings. *J Urol* 173(3):933–937.
- Litwin MS, Saigal CS, Beerbohm EM (2005) The burden of urologic diseases in America. *J Urol* 173(4):1065–1066.
- Gupta K, et al.; Infectious Diseases Society of America; European Society for Microbiology and Infectious Diseases (2011) International clinical practice guidelines for the treatment of acute uncomplicated cystitis and pyelonephritis in women: A 2010 update by the Infectious Diseases Society of America and the European Society for Microbiology and Infectious Diseases. *Clin Infect Dis* 52(5):e103–e120.
- Barber AE, Norton JP, Spivak AM, Mulvey MA (2013) Urinary tract infections: Current and emerging management strategies. *Clin Infect Dis* 57(5):719–724.
- Rasko DA, Sperandio V (2010) Anti-virulence strategies to combat bacteria-mediated disease. *Nat Rev Drug Discov* 9(2):117–128.
- Sivick KE, Mobley HL (2010) Waging war against uropathogenic *Escherichia coli*: Winning back the urinary tract. *Infect Immun* 78(2):568–585.
- Alteri CJ, Mobley HL (2012) *Escherichia coli* physiology and metabolism dictates adaptation to diverse host microenvironments. *Curr Opin Microbiol* 15(1):3–9.
- Hannan TJ, et al. (2012) Host-pathogen checkpoints and population bottlenecks in persistent and intracellular uropathogenic *Escherichia coli* bladder infection. *FEMS Microbiol Rev* 36(3):616–648.
- Merrell DS, et al. (2002) Host-induced epidemic spread of the cholera bacterium. *Nature* 417(6889):642–645.
- Hagan EC, Lloyd AL, Rasko DA, Faerber GJ, Mobley HL (2010) *Escherichia coli* global gene expression in urine from women with urinary tract infection. *PLoS Pathog* 6(11):e1001187.
- Subashchandrabose S, Hazen TH, Rasko DA, Mobley HL (2013) Draft genome sequences of five recent human uropathogenic *Escherichia coli* isolates. *Pathog Dis* 69(1):66–70.
- Lloyd AL, Rasko DA, Mobley HL (2007) Defining genomic islands and uropathogen-specific genes in uropathogenic *Escherichia coli*. *J Bacteriol* 189(9):3532–3546.
- Croucher NJ, Thomson NR (2010) Studying bacterial transcriptomes using RNA-seq. *Curr Opin Microbiol* 13(5):619–624.
- Rasko DA, Myers GS, Ravel J (2005) Visualization of comparative genomic analyses by BLAST score ratio. *BMC Bioinformatics* 6:2.
- Alteri CJ, Mobley HL (2007) Quantitative profile of the uropathogenic *Escherichia coli* outer membrane proteome during growth in human urine. *Infect Immun* 75(6):2679–2688.
- Mobley HL, et al. (1990) Pyelonephritogenic *Escherichia coli* and killing of cultured human renal proximal tubular epithelial cells: Role of hemolysin in some strains. *Infect Immun* 58(5):1281–1289.
- Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97(12):6640–6645.
- Subashchandrabose S, Smith SN, Spurbeck RR, Kole MM, Mobley HL (2013) Genome-wide detection of fitness genes in uropathogenic *Escherichia coli* during systemic infection. *PLoS Pathog* 9(12):e1003788.
- Outten FW, Huffman DL, Hale JA, O'Halloran TV (2001) The independent cue and cus systems confer copper tolerance during aerobic and anaerobic growth in *Escherichia coli*. *J Biol Chem* 276(33):30670–30677.
- Rowe JL, Starnes GL, Chivers PT (2005) Complex transcriptional control links NikABCDE-dependent nickel transport with hydrogenase expression in *Escherichia coli*. *J Bacteriol* 187(18):6317–6323.
- Laermann V, Čudić E, Kipschull K, Zimmann P, Altendorf K (2013) The sensor kinase KdpD of *Escherichia coli* senses external K⁺. *Mol Microbiol* 88(6):1194–1204.
- Thiennimitr P, et al. (2011) Intestinal inflammation allows *Salmonella* to use ethanolamine to compete with the microbiota. *Proc Natl Acad Sci USA* 108(42):17480–17485.
- Mijalovic H, Cooke NM, Moran GP, Rogers TR, Smith SG (2014) Response of extra-intestinal pathogenic *Escherichia coli* to human serum reveals a protective role for Rcs-regulated exopolysaccharide colanic acid. *Infect Immun* 82(1):298–305.
- van der Ploeg JR, Eichhorn E, Leisinger T (2001) Sulfonate-sulfur metabolism and its regulation in *Escherichia coli*. *Arch Microbiol* 176(1-2):1–8.
- Uriá-Nickelsen MR, Leadbetter ER, Godchaux WV, 3rd (1993) Sulphonate utilization by enteric bacteria. *J Gen Microbiol* 139(2):203–208.
- Snyder JA, et al. (2004) Transcriptome of uropathogenic *Escherichia coli* during urinary tract infection. *Infect Immun* 72(11):6373–6381.
- Mandlik A, et al. (2011) RNA-Seq-based monitoring of infection-linked changes in *Vibrio cholerae* gene expression. *Cell Host Microbe* 10(2):165–174.
- Taveirne ME, Theriot CM, Livny J, DiRita VJ (2013) The complete *Campylobacter jejuni* transcriptome during colonization of a natural host determined by RNAseq. *PLoS one* 8(8):e73586.
- Bielecki P, et al. (2014) In vivo mRNA profiling of uropathogenic *Escherichia coli* from diverse phylogroups reveals common and group-specific gene expression profiles. *MBio* 5(4):e01075-14.
- Willner D, et al. (2014) Single clinical isolates from acute uncomplicated urinary tract infections are representative of dominant in situ populations. *MBio* 5(2):e01064–e13.
- Rosen DA, Hooton TM, Stamm WE, Humphrey PA, Hultgren SJ (2007) Detection of intracellular bacterial communities in human urinary tract infection. *PLoS Med* 4(12):e329.
- Cassat JE, Skaar EP (2013) Iron in infection and immunity. *Cell Host Microbe* 13(5):509–519.
- Roos V, Klemm P (2006) Global gene expression profiling of the asymptomatic bacteriuria *Escherichia coli* strain 83972 in the human urinary tract. *Infect Immun* 74(6):3565–3575.
- Hagan EC, Mobley HL (2007) Uropathogenic *Escherichia coli* outer membrane antigens expressed during urinary tract infection. *Infect Immun* 75(8):3941–3949.
- Reigstad CS, Hultgren SJ, Gordon JI (2007) Functional genomic studies of uropathogenic *Escherichia coli* and host urothelial cells when intracellular bacterial communities are assembled. *J Biol Chem* 282(29):21259–21267.
- Brumbaugh AR, Smith SN, Mobley HL (2013) Immunization with the yersiniabactin receptor, FyuA, protects against pyelonephritis in a murine model of urinary tract infection. *Infect Immun* 81(9):3309–3316.
- Alteri CJ, Hagan EC, Sivick KE, Smith SN, Mobley HL (2009) Mucosal immunization with iron receptor antigens protects against urinary tract infection. *PLoS Pathog* 5(9):e1000586.
- Foxman B (2014) Urinary tract infection syndromes: Occurrence, recurrence, bacteriology, risk factors, and disease burden. *Infect Dis Clin North Am* 28(1):1–13.
- Mulvey MA, et al. (1998) Induction and evasion of host defenses by type 1-piliated uropathogenic *Escherichia coli*. *Science* 282(5393):1494–1497.
- Wiles TJ, Kulesus RR, Mulvey MA (2008) Origins and virulence mechanisms of uropathogenic *Escherichia coli*. *Exp Mol Pathol* 85(1):11–19.
- White C, Lee J, Kambe T, Fritsche K, Petris MJ (2009) A role for the ATP7A copper-transporting ATPase in macrophage bactericidal activity. *J Biol Chem* 284(49):33949–33956.
- Chaturvedi KS, Hung CS, Crowley JR, Stapleton AE, Henderson JP (2012) The siderophore yersiniabactin binds copper to protect pathogens during infection. *Nat Chem Biol* 8(8):731–736.
- Chaturvedi KS, et al. (2014) Cupric yersiniabactin is a virulence-associated superoxide dismutase mimic. *ACS Chem Biol* 9(2):551–561.
- Tree JJ, et al. (2008) Trade-off between iron uptake and protection against oxidative stress: deletion of *cueO* promotes uropathogenic *Escherichia coli* virulence in a mouse model of urinary tract infection. *J Bacteriol* 190(20):6909–6912.
- Nolan KJ, et al. (2002) In vivo behavior of a *Helicobacter pylori* SS1 nixA mutant with reduced urease activity. *Infect Immun* 70(2):685–691.
- Hiron A, et al. (2010) A nickel ABC-transporter of *Staphylococcus aureus* is involved in urinary tract infection. *Mol Microbiol* 77(5):1246–1260.
- Johnson DE, et al. (1993) Contribution of *Proteus mirabilis* urease to persistence, urolithiasis, and acute pyelonephritis in a mouse model of ascending urinary tract infection. *Infect Immun* 61(7):2748–2754.
- Kulesus RR, Diaz-Perez K, Slechte ES, Eto DS, Mulvey MA (2008) Impact of the RNA chaperone Hfq on the fitness and virulence potential of uropathogenic *Escherichia coli*. *Infect Immun* 76(7):3019–3026.
- Johnson JR, Clabots C, Rosen H (2006) Effect of inactivation of the global oxidative stress regulator *oxyR* on the colonization ability of *Escherichia coli* O1:K1:H7 in a mouse model of ascending urinary tract infection. *Infect Immun* 74(1):461–468.
- Hryckowian AJ, Welch RA (2013) RpoS contributes to phagocyte oxidase-mediated stress resistance during urinary tract infection by *Escherichia coli* CFT073. *MBio* 4(1):e00023-13.
- Campbell EL, et al. (2014) Transmigrating neutrophils shape the mucosal microenvironment through localized oxygen depletion to influence resolution of inflammation. *Immunity* 40(1):66–77.
- Semenza GL (2012) Hypoxia-inducible factors in physiology and medicine. *Cell* 148(3):399–408.
- Njoroge JW, Nguyen Y, Curtis MM, Moreira CG, Sperandio V (2012) Virulence meets metabolism: Cra and KdpE gene regulation in enterohemorrhagic *Escherichia coli*. *MBio* 3(5):e00280-12.
- Kendall MM, Gruber CC, Parker CT, Sperandio V (2012) Ethanolamine controls expression of genes encoding components involved in interkingdom signaling and virulence in enterohemorrhagic *Escherichia coli* O157:H7. *MBio* 3(3):e00050-12.