Functional Genomic Studies of Uropathogenic Escherichia coli and Host Urothelial Cells when Intracellular Bacterial Communities Are Assembled

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Uropathogenic Escherichia coli (UPEC), the principal cause of urinary tract infection in women, colonizes the gut as well as the genitourinary tract. Studies of mice inoculated with UTI89, a sequenced isolate, have revealed a complex life cycle that includes formation of intracellular bacterial communities (IBCs) in bladder urothelial cells. To understand how UPEC adapts to life in IBCs, we have used GeneChips and/or quantitative reverse transcriptase PCR to study UTI89 recovered from the distal gut of gnotobiotic mice and from IBCs harvested by laser capture microdissection from the bladder urothelium of infected C3H/HeJ female mice. Host responses were characterized in laser capture microdissected urothelial cells that do or do not contain IBCs. The results reveal components of ferric iron acquisition systems in UTI89 that are expressed at significantly higher levels in IBCs compared with the intestine, including the hemin receptor chuA (1,390 ± 188-fold). Localized urothelial responses to IBCs help oppose bacterial salvage of host cell iron (e.g. up-regulation of Tfrc (transferrin receptor) and Lcn2 (lipocalin 2)), facilitate glucose import (e.g. Hk2 (hexokinase 2)), and maintain epithelial structural integrity (e.g. Ivl (involucrin) and Sbsn (suprabasin)). ΔchuA mutants produce significantly smaller IBCs compared with wild type UTI89. This difference was not observed in strains lacking sitA (ABC-type iron/manganese transporter subunit), iroN (socalcin receptor), hlyA (α-hemolysin), or entF (enterobactin synthetase subunit). Together, these studies indicate that heme- and siderophore-associated iron play key roles in IBC development and provide a series of microbial and host biomarkers for comparing UPEC strains isolated from humans.

Acute and recurrent urinary tract infections (UTIs) are prominent health problems in women. In the United States, more than one third of females require antibiotic therapy for this illness by the time they are 24 years old. Over 80% of community-acquired UTIs are caused by uropathogenic Escherichia coli (UPEC). A member of the Proteobacteria division of bacteria, UPEC may evolve within, and adapt to, a number of host habitats. It exists as a member of the human colonic microbiota, a community dominated by two other bacterial divisions, the Firmicutes and the Bacteroidetes, where the density of colonization can exceed 10^{12} organisms/ml of luminal contents (3, 4). UPEC can emerge from the colon and become incorporated into the vaginal and periurethral microbiota before entering the urethra and bladder (reviewed in Ref. 5). Once in the bladder, UPEC attaches to the surface of umbrella cells that form the superficial (lumen-facing) layer of the urothelium; attachment is followed by rapid invasion (6). Attachment and invasion trigger innate immune responses that lead to exfoliation of superficial umbrella cells, thereby ridding the host of bacteria-laden epithelium (6).

UPEC can form intracellular bacterial communities (IBCs) in residual urothelial cells. Umbrella cells typically host a single IBC that expands to contain a population that can exceed 10^9 UPEC. The IBC eventually deconstructs, releasing UPEC into the bladder lumen where they can attach to and invade other urothelial cells (7). If untreated, quiescent intraepithelial reservoirs of UPEC are established that can lead to recurrent infections (8).

Because UPEC must adapt to life in two distinct host habitats (colon and urothelial cell) where there may be intense competition for nutrients, identifying nutrient acquisition strategies that are important for bacterial survival during or following IBC formation may lead to new approaches for the prevention, diagnosis, and/or treatment of acute and recurrent UTI. In the current study, we have examined this issue using the following approach. Gene expression was compared in a recently sequenced cystitis isolate (UTI89) (9) during its growth in vitro under aerobic and anaerobic conditions, in the distal intestines of gnotobiotic mice, and in IBCs harvested from urothelial cells.
using laser capture microdissection (LCM). Host responses to IBC formation in laser-captured urothelial cells were then defined using (i) GeneChips and real-time quantitative RT-PCR, (ii) microanalytic biochemical assays of cellular metabolism, and (iii) multilabel immunohistochemistry. The importance of one prominent aspect of the nutrient acquisition strategy used by this organism that emerged from our analysis was tested through genetic manipulations; comparison of wild type (WT) UTI89 and isogenic mutants strains containing null alleles of five genes that are markedly up-regulated in IBCs compared with the distal intestine established that the ability to acquire ferric iron plays a key role in formation of IBCs.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains**—Two strains of *E. coli*, MG1655 and UTI89, plus five isogenic deletion mutant strains of UTI89 with null alleles of *chuA*, *entF*, *iroN*, *hlyA*, or *sitA* were used in this study. The mutants were constructed according to the method of Datsenko and Wanner (10) using primers described in supplemental Table S1, and pKD4 (10). Amplicons were electroporated into UTI89 harboring pKM208 (11) to remove genes of interest and replace them with the Flp recombinase target-flanked kanamycin-resistance cassette from pKD4. Recombination was confirmed by selection on Luria-Bertani (LB) agar plates supplemented with 25 μg/ml kanamycin and by bacterial colony PCR (see supplemental Table S1 for primers). The Flp recombinase target-flanked cassette was then removed using the ara-inducible Flp recombinase expression plasmid pCP20 (10).

**Growth of E. coli UTI89 and MG1655 in Batch Fermentors**—*E. coli* strains UTI89 or MG1655 were grown separately in the two 1.3-liter vessels of a BioFlo 110 batch fermentor (New Brunswick Scientific) to generate biological duplicate samples. For each strain, anaerobic growth was initiated by inoculating 5 ml of an anaerobic overnight culture into 500 ml of LB medium. Bacteria were then incubated at 37 °C with agitation (100 rpm) and continuous sparging (mixture of 80% N₂ and 20% CO₂ at 0.2 liters/min). Aerobic cultures were also started with a 5-ml aerobic inoculum and incubated in LB at 37 °C at 100 rpm but were sparged with sterile house air at 0.2 liters/min. Aliquots (4 ml) were removed from each vessel at specified time points (see supplemental Fig. S1A) and placed into two volumes of RNAprotect (Qiagen). Bacterial RNA was isolated using the RNeasy midi kit (Qiagen), and contaminating genomic DNA was removed by treatment with RNase-free DNase I (Qiagen) and DNAfree (Ambion).

**Experiments Involving Specified Pathogen-free, Conventionally Raised, and Germ-free Mice**—All manipulations involving mice were performed using protocols approved by the Washington University Animal Studies Committee. C57Bl/6J and C3H/HeJ mice were obtained from the Jackson Laboratory. C3H/HeN mice were obtained from Harlan Sprague-Dawley. Conventionally raised C3H/HeJ and C3H/HeN mice were maintained under a strict 12-h light cycle (lights on at 0600 h) in microisolator cages, in a barrier facility, in a specified pathogen-free state and fed a standard chow diet (Pico Rodent Chow 20; Purina) ad libitum.

Germ-free C57Bl/6J mice were maintained in gnotobiotic isolators (12) under a strict 12-h light cycle (lights on at 0600 h) and given a standard autoclaved chow diet (B&K Universal) ad libitum.

Eight- to 10-week-old germ-free female animals were colonized with a single gavage of UTI89 or MG1655 (10⁵ colony-forming units (CFU) in 100 μl of LB medium/animal). Animals were euthanized 14 days after inoculation, between 1400 and 1600 h. The proximal 90% of the cecum was separated from the rest of the intestine, and bacteria were recovered by extruding cecal contents and by gentle scraping of the mucosa. Recovered cecal contents from each animal were placed immediately into 3 volumes of RNAprotect (Qiagen), and RNA was isolated (RNeasy midi kit; Qiagen).

The distal portions of the ceca of UTI89-colonized mice were fixed for 1 h at room temperature in 1% osmium tetroxide (prepared in 0.1 m PBS), followed by 1% uranyl acetate (in water) for 1 h. Samples were dehydrated in graded ethanol baths followed by immersion in 100% EtOH (3 cycles of 10 min each) and propylene oxide (3 cycles of 10 min). Tissues were then infiltrated overnight in propylene oxide:EMBed 812 (3:1 mixture; Electron Microscopy Sciences), followed by additional washes in 2:1, 1:1, and 1:3 mixtures, and finally in 100% EMBed 812. Sections (75-nm-thick) were prepared and viewed with a JEOL 100C transmission electron microscope.

**Transcriptional Profiling of UTI89 and MG1655 in Vitro and in Vivo**—Biotinylated cDNA targets were prepared from bacterial RNA samples and hybridized to GeneChip *E. coli* Antisense Genome Arrays (Affymetrix) according to the manufacturer’s recommendations. Using the recently completed 5,065,741-bp genome sequence of UTI89 (9) and the previously reported MG1655 sequence (13), an electronic probe mask (14) was applied in MICROARRAY SUITE 5.0 (MAS 5.0) (15) to eliminate hybridization signals from oligonucleotide probes in the MG1655-based GeneChip that did not perfectly match sequences present in both MG1655 and UTI89. 32,216 perfect match oligonucleotides were used to detect expression of 3,481 UTI89 (and MG1655) transcripts after probe masking; profiled genes are listed in supplemental Table S2. After masking, overall fluorescence across all remaining probes was scaled to a target intensity of 500 (MAS 5.0), and a flat-file data base containing expression data from each GeneChip was imported into DNA-CHIP ANALYZER v1.3 (dChip) (16) for normalization, filtering, and sample comparisons.

**GeneChip Analysis of Urothelial Responses to IBC Formation**—Cultures of WT or mutant UTI89 strains were grown statically in LB for 48 h at 37 °C, recovered by centrifugation, washed once, and adjusted to 2 x 10⁸ CFU/ml with sterile PBS. Female 8- to 10-week-old conventionally raised C3H/HeJ mice were inoculated by transurethral catheterization with 50 μl of this bacterial suspension (6). Animals were euthanized 24 h later and their bladders were immediately embedded in optimal cutting temperature (O.C.T.) compound (Sakura Finetek) and frozen in Cryocool II (Richard-Allen Scientific). 7-μm-thick cryosections were cut, placed on Superfrost/Plus slides (Fisher Scientific), and stained with eosin Y in preparation for LCM (17).
Four distinct populations of urothelial cells were harvested using a PixCell IIe system (laser spot diameter, 7.5 μm) and CapSure HS LCM caps (Arcturus): (i) from uninfected animals; (ii) from mock-infected mice (24 h after “inoculation” with sterile PBS), (iii) IBC-distal urothelium from infected hosts (n = 5 animals/pool) (located >10 cell diameters from any IBCs in the section plane), and (iv) IBC-proximal (<10 cell diameters from any IBCs in the section plane). RNA was isolated from populations (i) to (iv) using the PicoPure kit (Arcturus) with on-column DNase-I treatment (Qiagen). RNA integrity was evaluated using RNA 6000 Pico LabChips with a 2100 Bioanalyzer (Agilent Technologies).

Biological duplicate 1-ng aliquots of pooled intact RNA from each of the four populations of laser-captured urothelial cells were each amplified and labeled with the RiboAmp HS RNA Amplification kit (Arcturus) and the BioArray High Yield RNA Transcript Labeling kit (ENZO Biochem). The eight resulting biotinylated cRNA targets were each used to probe MOE430A mouse GeneChips (Affymetrix). Following hybridization, overall fluorescence across each GeneChip was scaled to a target intensity of 1500 and analyzed using MAS 5.0 (Affymetrix) and dChip (16).

Real-time Quantitative Reverse Transcriptase PCR—RNA was isolated from LCM IBCs using a protocol developed by the manufacturer of the RNA amplification kit (Arcturus) with on-column DNase I (Qiagen). qRT-PCR assays of RNAs isolated from UTI89 IBCs or LCM urothelial cells were performed in 25-μl reactions containing random hexamer-primed cDNA (synthesized from DNase-treated RNA samples), 1× SYBR Green Master Mix buffer (Applied Biosystems), 900 nM gene-specific primers (supplemental Table S3), and 0.25 units of uracil DNA glycosylase (Invitrogen). A melting curve was performed for each primer pair to identify a temperature where only amplicon, and not primer dimers, accounted for SYBR Green Master Mix buffer (Applied Biosystems), 900 nM gene-specific primers (supplemental Table S3), and 0.25 units of uracil DNA glycosylase (Invitrogen). A melting curve was performed for each primer pair to identify a temperature where only amplicon, and not primer dimers, accounted for SYBR Green-bound fluorescence. Assays were performed in triplicate using an Mx3000P qPCR apparatus (Stratagene). Bacterial qRT-PCR assays were normalized to 16 S rRNA and urothelial qRT-PCR assays to 18 S rRNA using the ΔΔC_{t} analysis method (18).

Comparing IBC Sizes Obtained with WT UTI89 versus Isogenic ΔchuA, ΔentF, ΔhlyA, ΔiroN, and ΔsitA Mutants—A single, freshly picked bacterial colony (from LB-agar plates) was introduced into LB medium, and the culture was grown to an OD of 0.4 units/ml. Each culture was then diluted 1:100 in 25 ml of fresh LB and grown statically for 16 h. Bacteria were recovered by centrifugation and adjusted to ~2 × 10^{8} CFU/ml of PBS. Groups of five C3H/HeJ mouse bladders were inoculated (transurethrally) with 10^{7} CFU of either WT, ΔchuA, ΔentF, ΔhlyA, ΔiroN, or ΔsitA UTI89. Six hours after inoculation, animals were killed, and bladders were removed, embedded in O.C.T. compound, frozen in blocks, and sectioned. Every tenth 4-μm section was stained with either 1% aqueous toluidine blue (Carolina Biological) or hematoxylin and eosin and viewed under a Zeiss Axiovert 200M inverted microscope. 100–400 bladder sections were examined for each of the bacterial strains assayed. Cross-sectional areas of all IBCs were calculated using AxioVision software (Zeiss). IBC sizes obtained from each isogenic mutant were compared with those obtained from WT UTI89 by using a two-tailed, heteroscedastic Student’s t test. Average IBC cross-sectional area for each strain is plotted with S.E. (n = the number of IBC cross-sectional areas measured for a given strain).

Multilabel Immunohistochemistry—Cryosections (7-μm-thick) were prepared from bladders embedded in O.C.T. compound and then incubated in PBS (three cycles, 5 min each at 25 °C) and blocking buffer (1% bovine serum albumin/0.1% Triton X-100 in PBS, 1 h at 25 °C). One of the following primary antibodies was then added (overnight at 4 °C): (i) goat anti-E. coli (diluted 1:500 in blocking buffer; United States Biological, Inc.), (ii) rabbit anti-mouse involucrin (1:200; Covance), or (iii) fluorescein isothiocyanate-conjugated rat monoclonal antibody to mouse transferrin receptor (1:200; U.S. Biological). Following three washes in PBS, antigen-antibody complexes were visualized directly (in the case of the fluorescein isothiocyanate-labeled monoclonal antibody) or with Alexa Fluor-conjugated secondary antibodies to rabbit or goat Ig (1:500; Molecular Probes). Nuclei were stained with bis-benzimide (Hoechst 33258). Sections were examined and photographed using a Zeiss Axiosvert 200M inverted microscope.

Hexokinase Assays—C3H/HeJ mouse bladders were inoculated with UTI89 as described above or mock-infected with sterile PBS (n = 3 animals/group). Animals were killed 24 h later. Bladders were immediately embedded in a 10:1 mixture of O.C.T. compound and gum tragacanth (Sigma-Aldrich) and then then flash-frozen in liquid nitrogen. Cryosections (7-μm-thick) were prepared from each bladder, placed on Superfrost Plus microscope slides, and freeze-dried (17). Cell lysates were generated from bladder epithelial and mucosal cells harvested from freeze-dried sections (250–500 ng of protein as determined by Quantigold; Diversified Biotech) by adding 1 μl of a solution containing 20 mM sodium phosphate buffer, pH 7.0, 5 mM β-mercaptoethanol, 25% glycerol plus 0.5% Triton and incubating the mixture for 1 h at 20 °C. Hexokinase activity was then assayed using the “oil well” method (19). Specifically, 0.2 μl of the lysate (50–100 ng of protein) was added to 1 μl of enzyme reagent containing 100 mM Trizma HCl, pH 8.1, 5 mM MgCl_{2}, 1 mM glucose, 5 mM ATP, 0.1 mM NADP^{+}, 0.02% bovine serum albumin, and 0.3 units/ml Leuconostoc mesenteroides glucose-6-phosphate dehydrogenase (specific activity, 250 units/mg protein; Calbiochem). The mixture was incubated for 1 h at 20 °C, and the reaction was terminated by adding 1 μl of 0.15 M NaOH (80 °C, 20 min). A 1-μl aliquot was then transferred to 100 μl of NADP cycling reagent and subjected to 2000 cycles of amplification (19). Glucose 6-phosphate standards (20–40 μM) were included throughout all steps.

RESULTS AND DISCUSSION

Comparison of UPEC Transcription during Growth in Vitro under Aerobic versus Anaerobic Conditions and in the Distal Intestines of Gnotobiotic Mice—We recently determined the complete DNA sequence of UTI89 (9): its 5,065,741-bp genome contains 5,066 predicted protein-coding genes, 3,481 of which are shared with MG1655, the prototypic laboratory strain of E. coli originally isolated from human feces whose metabolic
networks and signaling pathways have been extensively characterized (ecocyc.org) (20).

We identified 32,216 oligonucleotide probes on the MG1655-based Affymetrix E. coli Antisense Genome Array whose sequences perfectly matched those in 3,481 UTI89 genes. Using these GeneChips, we performed genome-wide transcriptional profiling of both strains during aerobic and anaerobic growth, from early log to stationary phase, in batch fermentors containing LB medium (n = 2 independent experiments/strain; see supplemental Fig. S1A for time points sampled). Following hybridization, all signals from oligonucleotide probes that did not perfectly match sequences in both genomes were masked electronically.3 The resulting data sets were highly congruent for both strains: differences in the responses of UTI89 in the gut as prelude to comparing expression of selected genes in IBCs versus the distal intestine. To do so, we colonized germ-free adult female C57Bl/6J mice with a single gavage of 10^8 CFU of UTI89 or the reference control MG1655 strain (see “Experimental Procedures”). Animals (n = 5/group) were maintained on an autoclaved, standard polysaccharide-rich chow diet where glucose, arabinose, xylose, and galactose are the principal neutral sugars (mole ratio 10:8:5:1) (21). All animals were killed 14 days after inoculation with bacteria, a period that encompasses several cycles of turnover of the underlying, perpetually renewing intestinal epithelium. Both strains efficiently colonized all mice. Both strains reached peak densities in the cecum (supplemental Fig. S2A). This sac-like structure separates the distal small intestine from the proximal colon and is normally home to a diverse population of bacteria defined as having higher expression in UTI89: they include genes involved in carbohydrate metabolism, stress responses, and various aspects of iron metabolism (supplemental Fig. S5C). The latter group contained feoA, which participates in ferrous iron transport (24), plus tonB and exbB, which encode components of the TonB energy transduction complex that couples transport of iron-siderophore complexes across the outer membrane with the proton motive force across the cytoplasmic membrane.

Recovery of IBCs by Laser Capture Microdissection—We next determined how UTI89 gene expression in IBCs compared with that in its cecal habitat. C3H/HeJ mice, deficient in Toll-like receptor 4 signaling, were chosen for this analysis for several reasons: (i) they have been used extensively to characterize the temporal and cellular features of the UTI89 IBC life cycle (7, 25); (ii) they exhibit increased numbers of IBCs that we could score; and (iii) the reduced influx of neutrophils in the vicinity of IBCs (26) makes it feasible to harvest highly purified populations of IBCs, with or without host urothelial cells, using LCM. LCM of bladder cryosections was an attractive method because it offered a way of retrieving IBCs directly from their intracellular environment while still preserving their transcriptional profile.

Bladders were isolated from mice killed 24 h after transurethral inoculation with 10^8 CFU of UTI89, frozen immediately in O.C.T. compound, and subjected to cryosectioning. Careful microdissection, using a laser spot diameter of 7.5 μm, allowed us to excise the IBC from its host urothelial cell, as shown in Fig. 1, A–D. Each bladder cryosection yielded, on average, 5–10 mid-late stage IBCs. The average amount of RNA recovered from each microdissected IBC section was ~20 pg, of which >50% was bacterial as judged by the ratio of 16 to 18 S rRNA (Fig. 1H).

qRT-PCR was used to quantify the levels of various UTI89 transcripts in (i) RNA isolated from laser-captured IBCs (RNA pooled from ~20–30 IBC sections/mouse; 4–5 mouse bladders/qRT-PCR assay), (ii) RNA isolated from gnotobiotic mice (pooled from 5 ceca/assay), and (iii) RNA prepared from bacteria that had been harvested from batch fermentors at mid-log and stationary phases of growth in LB medium under aerobic and anaerobic conditions (n = 2 independent cultures/condition). All RNA preparations were assayed in triplicate and the results expressed relative to the levels in stationary phase anaerobic cultures.

Genes Involved in Iron Acquisition Are Expressed at Markedly Higher Levels in IBCs Compared with the Cecum—Host defense against infection frequently involves limiting the availability of iron to bacteria, especially on mucosal surfaces (reviewed in Ref. 27). Genes involved in iron acquisition are important virulence factors in UPEC (28–30). However, the relationship between iron acquisition/utilization and IBC formation had not been delineated. Intriguingly, by comparing the UTI89 genome with the genomes of six other E. coli strains (MG1655, CFT073 (an isolate from a patient with pyelonephritis), plus four enteropathogens), we recently identified 29 genes under positive selection in the two sequenced UPEC strains, including several involved in iron scavenging (e.g. entD, entF, and fluA) (9).

Under aerobic conditions, iron exists primarily in the oxidized ferric form (Fe^{3+}). The low solubility of ferric iron at physiological pH requires that specialized mechanisms be deployed to obtain sufficient quantities of iron to meet the needs of bacteria (31). These mechanisms include synthesis and excretion of low molecular weight, iron-chelating compounds.
known as siderophores (32). Siderophores have been shown to be important for the intracellular growth of pathogens such as Legionella pneumophila (33). In addition, when Snyder et al. (30) examined the transcriptome of the CFT073 UPEC strain, collected from the urine of mice with UTI, they observed increased expression of several genes involved in iron and siderophore biosynthesis compared with growth in LB medium.

To define the relative aerobicity experienced by UTI89 in IBCs versus the cecum, we characterized expression of the fumarate-nitrate reductase regulon. Our GeneChip analysis revealed that transcriptional changes in components of this regulon at a given phase of growth under aerobic conditions revealed that transcriptional changes in components of the fumarate-nitrate reductase regulon. Our GeneChip analysis revealed that transcriptional changes in components of this regulon under aerobic conditions revealed that transcriptional changes in components of this regulon. Our GeneChip analysis revealed that transcriptional changes in components of this regulon under aerobic conditions revealed that transcriptional changes in components of this regulon. Our GeneChip analysis revealed that transcriptional changes in components of this regulon under aerobic conditions revealed that transcriptional changes in components of this regulon.

FIGURE 1. LCM of UTI89 IBCs and host urothelial cell populations from bladder cryosections. Sections were stained with eosin Y to outline cellular architecture prior to LCM. A, arrows point to IBCs formed 24 h after inoculation of UTI89 into mouse bladders. Dashed lines outline IBC-proximal and IBC-distal urothelial cell populations. B and C, bladder section with IBCs before (B) and after (C) LCM, D, captured IBCs affixed to the ethylene vinyl acetate plastic film of an LCM cap prior to RNA isolation. E–G, LCM of IBC-proximal urothelial cells. Urothelial cells are shown in a cryosection before (E) and after (F) capture. L, bladder lumen. G, captured urothelial cells on the LCM cap. H, RNA isolated from LCM IBCs and urothelial cells. The isolated IBC RNA preparation is >50% bacterial based on the ratio of UTI89 16 S rRNA to mouse 18 S rRNA. Scale bars, 40 μm.

Under anaerobic conditions iron is more soluble and found predominantly in its ferrous form (Fe^{2+}) (36). We observed that the ferrous iron transport protein *feoA* is expressed at slightly higher levels by UTI89 in the cecum compared with IBCs (Fig. 2A).

Our qRT-PCR analysis revealed that transcripts encoding components of the TonB energy transduction complex TonB and ExbB, are expressed at significantly, albeit modestly, higher levels in IBCs compared with the cecum (2.8 ± 1.0-fold in the case of ExbB and 2.0 ± 0.2-fold in the case of TonB; *p* < 0.01; Fig. 2, B and C). *E. coli* responds to iron deprivation by synthesizing and excreting the cyclic tricarboxylate siderophore enterobactin (Ent). Extracellular iron-bound Ent is transported into the cytoplasm, where Fe^{3+} is reduced and released from Ent (37). qRT-PCR assays disclosed that the levels of mRNAs encoding FepA (a TonB-dependent active transporter that recognizes extracellular ferric enterobactin and translocates it into the periplasm) and EntE (enterobactin synthetase) are 94 ± 21-fold and 232 ± 30-fold higher, respectively, in IBCs than in the cecum (*p* < 0.001; Fig. 2, D and E). Consistent with this observation, *entF*, another component of enterobactin synthetase, is also up-regulated in IBCs (167 ± 43-fold; *p* < 0.001; Fig. 2F). *iroB*, which is involved in salmochelin siderophore glucosylation, is up-regulated 45 ± 3-fold in IBCs compared with cecal-based UTI89 (Fig. 2G). *iroN*, which encodes the salmochelin siderophore receptor, is up-regulated 234 ± 28-fold (Fig. 2H).

The UTI89 proteome also includes enzymes required to produce a siderophore system initially identified in *Yersinia pestis*. One of these genes, *ybtS*, is 587 ± 72-fold more highly expressed in IBCs than the cecum (Fig. 2I).

ChuA, a 69-kDa outer membrane protein that shares features of TonB-dependent outer membrane iron-transport proteins, is present in many pathogenic strains of *E. coli* (38). ChuA is the hemin receptor and is sufficient for bacterial utilization of heme, or hemoglobin, as sources of iron. Heme is the most abundant source of iron in mammalian hosts. Most heme is found in the cytoplasm and mitochondria of cells in the form of hemoproteins (reviewed in Refs. 39 and 40). Heme biosynthesis is critical for the intracellular growth of the pathogenic bacterium *Neisseria gonorrhoeae* (41).

Heme stores within host epithelia may be accessed by pathogenic *E. coli* through production of cytotoxins, e.g. cytotoxic necrotizing factor (cnf1) and/or α-hemolysin (hlyA) in UPEC (42). *hlyA* is 22 ± 2-fold more highly expressed by UTI89 in IBCs than in the cecum (Fig. 2F), whereas *chuA* is expressed at 1,390 ± 188-fold higher levels (*p* < 0.001, Fig. 2K). Moreover, levels of *chuA* mRNA are >16-fold higher in laser-captured IBCs than in log-phase cells harvested during aerobic growth in LB broth (*p* < 0.001). These results suggest that this gene plays a major role in bacterial iron metabolism within urothelial cells (see below).

SitA, a putative periplasmic protein found in enteroinvasive strains of *E. coli*, is part of an ABC-type ferric iron transport system (43). Like *chuA*, expression of *sitA* is significantly higher in IBCs (104 ± 14-fold) than in the cecum (*p* < 0.001, Fig. 2L).

Together, these findings reveal that deployment of systems for acquiring siderophore- and heme-associated ferric iron is a feature that distinguishes communities of UTI89 in IBCs from...
communities of UTI89 in the distal intestines of gnotobiotic mice. We subsequently defined the effects of IBC formation on urothelial iron metabolism as well as other aspects of host cell biology.

**Localized Urothelial Responses to IBCs**—Mouse MOE430A GeneChips were used to initially compare gene expression in LCM populations of “IBC-proximal” bladder urothelium (defined as being <10 cell diameters from any IBC in the plane of a bladder cryosection) with expression in “IBC-distal” urothelium (>10 cell diameters from any IBC in the section plane) (Fig. 1, A and E–G). The two cell populations were harvested from cryosections prepared from the same female mice used for qRT-PCR analysis of bacterial gene expression in isolated IBCs (n = 5,000 urothelial cells microdissected/cell population/mouse; 5 mice/experiment; 2 biological replicates). Two control urothelial populations were also analyzed, those harvested by LCM from uninfected mouse bladders and those harvested from mice killed 24 h after “mock infection” with sterile PBS.

One-nanogram aliquots of each RNA preparation were independently amplified to generate sufficient quantities of target cDNA for hybridization to the mouse GeneChips (one GeneChip/amplified target). Fifty-eight genes (supplemental Fig. S6, A and B) satisfied our selection criteria for significantly altered
expression in IBC-proximal urothelial cells (see “Experimental Procedures” for a description of the selection criteria, which include a ≥2-fold difference in the average level of expression in IBC-proximal versus the average level of expression in IBC-distal, mock-infected, and uninfected populations).

When mammalian cells have sufficient levels of intracellular iron, import of transferrin-iron complexes is reduced by increasing the degradation rate of transferrin receptor (Tfrc) mRNA; this quickly leads to a decrease in the level of transferrin receptor. When levels of intracellular iron fall, Tfrc mRNA is stabilized and synthesis of Tfrc protein increases (reviewed in Ref. 44). Siderophores, secreted by bacteria under iron-limiting conditions, are able to scavenge iron from a number of mammalian iron-binding proteins, including transferrin (e.g. Ref. 45).

qRT-PCR verified our GeneChip result that levels of Tfrc mRNA are increased in IBC-proximal compared with IBC-distal urothelial cell populations (1.8 ± 0.2-fold; p < 0.01; Fig. 3A) and 4.6 ± 0.4-fold in IBC-proximal versus uninfected urothelial cells. Follow-up multilabel immunohistochemical studies established that Tfrc was readily detectable in urothelial cells surrounding IBCs (Fig. 3B). Increased expression of Tfrc in IBC-proximal cells could function to help host cells satisfy their requirements for iron in the face of competition with UPEC in IBCs.

Another gene affected by IBC formation in the urothelium helps oppose bacterial salvage of host cell iron. Lipocalin 2 (Lcn2) is a secreted protein that binds to enterobactin (46). Lcn2−/− mice have increased susceptibility to bacterial infection, and Lcn2-deficient neutrophils have reduced bactericidal activity. Because the microbicidal activity of WT neutrophils is reduced by adding exogenous iron, Berger et al. (47) have postulated that Lcn2 functions as a key part of the innate immune response to bacteria by limiting iron availability. High levels of expression of the human ortholog of Lcn2 (NGAL) have been documented in areas of inflammation of the colonic epithelium (48).

LCM-qRT-PCR revealed that Lcn2 mRNA is up-regulated 138 ± 16-fold in IBC-proximal urothelium compared with uninfected cells and 2.1 ± 0.2-fold in IBC-proximal versus IBC-distal cells from the same infected bladders (p < 0.001; Fig. 3A). Expression of the receptor for Lcn2, Lrp2/Megalin, is only modestly up-regulated (<3-fold in the proximal urothelium of infected mice compared with uninfected) (data not shown).

In Average IBC Size—Given these findings, we performed direct genetic tests of the contributions of five genes implicated in iron acquisition on IBC formation by UTI89: chuA (most markedly up-regulated gene in IBCs compared with the cecum among all genes surveyed), sitA (highly up-regulated in IBCs compared with all other conditions), hlyA (a common virulence factor in UPEC isolates), iroN (highly up-regulated in IBCs compared with other conditions), and entF (identified as a gene undergoing positive selection in UPEC strains and highly up-regulated in IBCs).

None of the mutants exhibited growth defects in LB medium compared with WT UTI89 (data not shown). Isogenic WT and mutant strains were then cultured independently, and each was inoculated into female C3H/HeN mice (n = 5 animals/experiment; 10⁷ CFU/bladder). The Toll-like receptor 4 signaling-competent C3H/HeN inbred strain was used for these experiments so that we could investigate IBC formation in the context of a normal innate immune response. Animals were euthanized 6 h after inoculation, the time point when their bladders generally contain numerous mid-stage IBCs (7). 4-μm-thick serial cryosections were prepared from each mouse bladder; every tenth section was stained with toluidine blue and used to measure the cross-sectional areas of IBCs (n = 100–400 bladder sections scored/bacterial strain) (Fig. 4, A and B). Remarkably, compared with WT, only the ΔchuA mutant exhibited a significant reduction in IBC size (WT UTI89 = 482 ± 43-μm²; ΔchuA = 230 ± 25-μm²; p < 0.001; n = 2 independent experiments; Fig. 4C).

In silico metabolic reconstructions of the UTI89 genome identified all pathway components required for heme biosynthesis in E. coli (data not shown). Although our GeneChip analysis of localized host urothelial responses did not identify significant differences in the expression of any heme-containing or heme-related proteins in IBC-associated urothelium, our
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...some functional redundancy may exist between the sid...and/or has an effect on the kinetics of IBC formation, and (ii) ferric iron is important in determining the ultimate size of IBCs...ized barrier around IBCs, we performed qRT-PCR assays of...tofore unappreciated host response that may provide a local...

FIGURE 4. Genetic evidence that ChuA affects the size of IBCs. Toluidine blue-stained bladder cross-sections depict an IBC formed by WT UTI89 (A) and an IBC formed by an isogenic mutant lacking the hemin receptor ChuA (B). Panels A and B show representative examples of IBCs formed by WT and ΔchuA UTI89 strains, respectively. Dashed lines denote the margins of an IBC in each section. C, average cross-sectional areas of IBCs formed by WT UTI89 and five isogenic mutants, ΔchuA, ΔentF, ΔiroN, ΔhlyA, and ΔsitA (n = 5 bladders assayed/bacterial strain/experiment; two independent experiments for comparison of WT to ΔchuA; ***, p < 0.001 by two-tailed Student’s t test). Scale bars, 20 μm.

...results indicate that (i) chuA-mediated access to heme-derived ferric iron is important in determining the ultimate size of IBCs and/or has an effect on the kinetics of IBC formation, and (ii) some functional redundancy may exist between the siderophore systems deployed by intracellular UTI89.

Other Features of IBC-Urothelial Cell Interactions—Like chuA, expression of α-hemolysin (hlyA) is regulated by iron (49). Subtoxic levels of hemolysin induce intracellular calcium oscillations in renal epithelial cells (50). Interestingly, calcium is known to modulate expression of involucrin and other structural components of the cornified envelope in keratinocytes (51) and in cultured epithelial cells (52).

Our GeneChip studies revealed that involucrin, which is cross-linked by transglutaminases to keratin, desmoplakin, periplakin, and envoplakin in keratinocytes (reviewed in Ref. 53), is prominently expressed in IBC-proximal cells. Suprabasin, a cross-linked structural protein that is restricted to suprabasal layers of stratified epithelia in the stomach, tongue, and epidermis (54), is also expressed in IBC-proximal urothelial cells. Significantly increased expression of involucrin and suprabasin in IBC-proximal compared with IBC-distal urothelial cells was verified by qRT-PCR (Fig. 3A). Multilabel immunohistochemical studies confirmed expression of involucrin in IBC-proximal urothelial cells (Fig. 3C).

Because these findings raise the possibility of a link between augmented expression of an iron-regulated UTI89 gene, hlyA, in IBCs and expression of urothelial proteins involved in a here-tofore unappreciated host response that may provide a localized barrier around IBCs, we performed qRT-PCR assays of involucrin and suprabasin mRNA in LCM IBC proximal and distal urothelial cell populations harvested from C3H/HeN mice 6 h after infection with WT or the isogenic ΔhlyA mutant. The results disclosed that bacteria lacking hemolysin did not have a significantly different effect, compared with WT, on the ratio of expression of these host genes in proximal versus distal urothelial cells, or in IBC proximal urothelial cells versus urothelium harvested from uninfected controls (data not shown).

The urothelium may play an important role in defining the size, stability, and lifespan of IBCs through other mechanisms. Our GeneChip and qRT-PCR assays disclosed that IBC formation elicits antimicrobial responses (lysozyme) as well as induction complement factors C3 and B (Fig. 3A). Concomitant induction of Cds5/Daf-1 may limit complement-mediated damage to host cells (Daf-1 is also up-regulated in the small intestine of gnotobiotic mice colonized with the prominent human gut symbiont Bacteroides thetaiotaomicron) (55).

Finally, the GeneChip study revealed that the two mRNAs encoding genes involved with glucose import, hexokinase (HK2) and solute carrier family 2, member 1 (also known as glucose transporter, type 1 (Glut1)), are up-regulated in IBC-proximal urothelium. In the case of hexokinase, this elevated level of expression was validated by qRT-PCR (supplemental Fig. S7A) and by microanalytic biochemical assays of enzyme activity in populations of microdissected bladder cells (supplemental Fig. S7B). These changes suggest that urothelial cells hosting IBCs require more glucose to sustain their needs in the face of competition with large UPEC communities.

In summary, the functional genomic analysis of laser-captured IBCs and their surrounding host cells described in this study represents a step toward elucidating the molecular mechanisms responsible for UPEC pathogenesis. Studies in mice have shown that clinical isolates from patients produce IBCs with different morphotypes (56). The analytic approaches described above set the stage for correlating these morphotypes with expression of specified bacterial genes, as well as host genes, in urothelial cells that have been exfoliated into the urine of patients with UTI.

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