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Transcriptional Responses of Uropathogenic *Escherichia coli* to Increased Environmental Osmolality Caused by Salt or Urea

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Uropathogenic *Escherichia coli* (UPEC) is the most common causative agent of urinary tract infections in humans. The majority of urinary infections develop via ascending route through the urethra, where bacterial cells come in contact with human urine prior to reaching the bladder or kidneys. Since urine contains significant amounts of inorganic ions and urea, it imposes osmotic and denaturing stresses on bacterial cells. In this study, we determined the transcriptional adaptive responses of UPEC strain CFT073 to the presence of 0.3 M NaCl or 0.6 M urea in the growth medium. The cell responses to these two osmolytes were drastically different. Although most of the genes of the osmotically inducible regulon were overexpressed in medium with salt, urea failed to stimulate osmotic stress response. At the same time, UPEC colonization genes encoding type 1 and F1C fimbriae and capsule biosynthesis were transcriptionally induced in the presence of urea but did not respond to increased salt concentration. We speculate that urea can potentially be sensed by uropathogenic bacteria to initiate infection program. In addition, several molecular chaperone genes were overexpressed in the presence of urea, whereas adding NaCl to the medium led to an upregulation of a number of anaerobic metabolism pathways.

Urinary tract infections (UTIs) are one of the most common types of bacterial infections in humans. It is estimated that ca. 50% of women have at least one symptomatic UTI during their lifetime, and many have recurrent episodes (1). UTIs are often classified by the site of infection—in bladder (cystitis), kidney (pyelonephritis), or urine (bacteriuria)—and can be asymptomatic or symptomatic with a range of symptoms from mild irritative voiding to bacteremia, sepsis, or even death. *Escherichia coli* is the primary UTI-causing pathogen; other uropathogenic bacteria include *Staphylococcus*, *Klebsiella*, *Enterobacter*, and *Proteus* (2). Although there is evidence for an intestinal habitat of uropathogenic *E. coli* (UPEC), such *E. coli* strains possess cassettes of virulence and colonization genes allowing them to establish an infection in the human urinary tract (3). These UPEC factors include adhesins such as P, F1C, and type 1 fimbriae that facilitate colonization in the urinary tract; invasins such as invasion plasmid antigen; capsule polysaccharides; and toxins such as hemolysin, cytotoxic necrotizing factor 1, and several autotransporters that induce an inflammatory response and damage tissue cells (4, 5).

Because the majority of UTIs are believed to develop via the ascending route through the urethra (6), UPEC cells are subjected to periodic environmental stress from human urine. Total osmotic pressure in human urine varies from 50 to >1,400 mOsm/kg, with an average in 500- to 600-mOsm/kg range, and with the osmolyte concentration increasing from kidneys to bladder (7, 8). Both urea and inorganic ions are present in urine in significant amounts, and the UTI-causing bacteria must protect themselves against both high osmolality and the denaturing effects of urea (8, 9).

To adapt to a reduction in external water activity, *E. coli* cells accumulate low-molecular-weight solutes that help maintain the proper intracellular osmotic balance (reviewed in references 10 and 11). In enterobacteria these include K^+, glutamate, and trehalose (12). It has been proposed that the elevated uptake of K^+ from the medium is the first response to an external increase in osmotic pressure (13), although not all studies confirm this hypothesis (14). Glutamate is believed to serve as a counter-ion for K^+ (10). Because high K^+ levels interfere with cellular function, further adaptation to osmotic pressure involves a class of compounds called osmoprotectants that can be accumulated to high concentrations inside the cells and thereby alleviate the inhibitory effects of osmotic stress. These include among others glycine betaine, proline, and carnitine (11). The majority of osmoprotectants destabilize the denatured state of proteins (by exerting unfavorable thermodynamic force on peptide backbone only accessible in denatured state) and stabilize the native state (15).

Significantly less is known about responses of *E. coli* cells to urea. Urea can freely penetrate the cell membrane (8) and can destabilize protein conformation by interacting positively with protein backbone (9), an effect opposite to that of many osmoprotectants. Indeed, at higher concentrations urea is used as a denaturing agent in protein analysis studies. In *E. coli*, no transcriptional regulator or receptor is known to specifically respond to urea, and it is not known whether cells can sense its presence in the cytoplasm at all.

Despite the high total osmolyte concentration in urine, it appears that not all osmotically inducible systems are required by many UPEC strains to survive and grow in it. Although deletion of osmoregulatory transporter ProP impaired ability of *E. coli* to colonize mouse bladder in one report (16), other studies showed that known transporters of compatible solutes and osmoprotectants were not necessary for *E. coli* CFT073 to grow in human urine (8,

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17), indicating that other, as-yet-unidentified, osmoprotective system(s) might participate in this strain's osmoadaptation. At the same time, osmotically inducible OmpF was found to be required for efficient colonization of the murine urinary tract by UPEC (18). Other studies have also linked osmotically inducible genes with bacterial pathogenicity in *Listeria monocytogenes*, *Shigella flexneri*, and *Salmonella enterica* serovar Typhimurium (19–21).

Although previous studies looked at UPEC growth in human urine (8, 17, 22) and examined gene expression in UPEC cells isolated from urine and bladder urothelium of experimentally infected mice (23, 24) and from women diagnosed with UTI (25, 26), no studies thus far have explored the relative contributions of inorganic ions and urea to *E. coli* transcriptional responses in urine. We hypothesized that because UPEC encounters urine prior to reaching the primary infection site (bladder, kidneys), sensing the presence of salt or urea in the environment can be a trigger for the expression of the initial wave of virulence and colonization factors. To test this hypothesis, we analyzed genome-wide differences in expression profiles of uropathogenic *E. coli* CFT073 cells grown in media with either salt or urea.

MATERIALS AND METHODS

Growth experiments. A single colony of UPEC strain CFT073 was inoculated into liquid Luria-Bertani (LB) medium and grown aerobically with agitation until reaching the stationary phase. An aliquot was transferred into freshly made K minimal medium (14), and the cells were incubated until the late log phase. At this point, aliquots were used to inoculate individual flasks containing 1/5 volumes of either (i) K medium, (ii) K medium with 0.3 M NaCl, or (iii) K medium with 0.6 M urea. Because urea is unstable during storage (27), urea stock was freshly prepared immediately before use. K medium was chosen because this is a low basal osmolality medium (0.15 osmol/kg H_2O [14]). To impose salt stress, 0.3 M NaCl was added to K medium; this concentration was sufficient to elicit osmotic stress response in our previous study (28). The estimated osmolality of such medium is 0.65 osmol/kg H_2O (14). The urea concentration was chosen so that the osmolality of the K medium with urea is close to that of K medium with NaCl (29). All cultures were incubated in a water bath shaker at 30°C and 200 rpm, and grown until reaching an optical density at 600 nm (OD_{600}) of 0.45. This growth temperature was chosen to match the conditions used in the previous study of commensal *E. coli* transcriptional responses to salt stress (28). We confirmed that bacterial culture growth was similar between 30 and 37°C (doubling time difference of <5 min for cultures grown at 30 and 37°C). The cells were harvested by adding 25 ml of culture from each flask to a 2.5-ml mixture of cold 95% ethanol and 5% phenol to preserve RNA (28). Three individual cultures were run for each growth condition to provide biological replicates for gene expression analyses. Doubling times in the exponential phase of growth were between 50 and 55 min for CFT073 grown in K medium and in K medium plus 0.3 M NaCl and 78 min for CFT073 grown in K medium plus 0.6 M urea.

Sample processing and microarray analysis. Total RNA was extracted from collected *E. coli* cells with a hot phenol-chloroform method (30). Affymetrix *E. coli* Genome v2 arrays that contained probes to 98% of CFT073 genes were used to measure mRNA levels in *E. coli* cells essentially as we did previously (28). Three replicate arrays utilizing mRNA isolated from separate biological cultures were run for each growth condition. The data were normalized in CARMAweb (31) using MAS5-VSN-MAS5-MedianPolish procedure as described previously (32). Specifically, MAS5 algorithm was used for background and PM correction, VSN algorithm for array normalization, and the Median Polish algorithm to obtain expression summaries (i.e., signal values) for each probeset on an array (33). The data were matched with annotation information compiled for all CFT073 genes, as we did previously for *E. coli* K-12 (28). All non-CFT073 gene data were removed. Any gene that was not present in at least one condition or which was always expressed in the bottom 5% of each array signal range was eliminated from further consideration. These procedures produced a data set for 4,688 *E. coli* CFT073 genes. The data were additionally quantile normalized in CARMAweb to obtain similar distribution of expression values across all experiments (33). For both NaCl-versus-basal medium and urea-versus-basal medium comparisons, all nine possible interarray comparisons were carried out, and an average fold change was computed for each gene based on the Tukey's bi-weight algorithm (34). Statistical tests of significance of observed differences were carried out using moderated *t* test with the Benjamini-Hochberg correction for multiple hypothesis testing (35). A list of 418 differentially expressed genes was compiled from the full data set by utilizing the following set of conditions: (i) the gene was reliably expressed in at least one of the three conditions and (ii) there was an at least 3-fold expression difference between at least two conditions coupled with (iii) a *P* value exceeding 99% confidence of statistically significant differential expression. The value of the fold change cutoff was chosen based on the analysis of the distribution of signal log ratios among all comparisons; this fold change matched the average downregulation of flagella gene expression under salt stress.

K-means, hierarchical clustering, and principal component analysis (PCA) of the filtered data set were performed in Genesis as described previously (28). Detailed explanations of these clustering algorithms can be found elsewhere (36). The number of clusters in K-means algorithm (i.e., nine) was estimated by Gap statistic (37) and was confirmed by visually inspecting clustering patterns in K-means clustering runs with 9, 12, and 16 clusters chosen. Since the K-means algorithm is not deterministic (36), we confirmed that the cluster profiles and the total number of genes in each cluster changed little for multiple K-means runs. Each of the K clusters was classified according to the average response of the genes in the cluster to NaCl and urea. Updated version of Genome Images software (38) was used to visualize the microarray data set.

qPCR. Primers for quantitative real-time PCR (qPCR) were designed *de novo* using Primer3 software (39). *In silico* PCR validation was carried out in FastPCR, and the validated primers were pruned against *E. coli* CFT073 genome to ensure that they did not bind anywhere else in the genome. Correct amplicon sizes were confirmed by visualizing qualitative PCRs on agarose gel. Reverse transcription of RNA was performed using the Superscript III reverse transcriptase (Invitrogen). Quantitative real-time PCR was carried out on ABI Prism 7000 sequence detection system using PERFECTa SYBR green qPCR Mastermix (Quanta Biosciences, Inc.) essentially as described previously (28), including mathematical calculations taking into account unequal amplification efficiency for different primer pairs. Three replicate reactions were carried out for each sample/primer combination. As a sample control, the levels of *rrs* genes were measured.

Construction of GFP transcriptional fusion plasmids. CFT073 genomic DNA was used as a template to amplify promoter regions for *bax* (nucleotide positions −394 to +96 relative to the start codon) and *ibpA* (nucleotide positions −389 to +168 relative to the start codon). The amplified PCR products were digested with BamHI and XhoI and cloned into the low-copy-number vector pUA139 (40) linearized with the same restriction enzymes. The vector contained GFPmut2 gene coding for enhanced version of green fluorescent protein (GFP), with a strong ribosome-binding site positioned immediately downstream of the XhoI restriction site. The ligated DNA was transformed into *E. cloni* 10G competent cells (Lucigen) according to the manufacturer's instructions. We confirmed the correct promoter region incorporation by Sanger sequencing. The plasmids were then electroporated into CFT073 strain.

Promoter activity measurements. A fresh single colony of $CFT073_p$*bax*:GFP or $CFT073_p$*ibpA*:GFP was inoculated into 3 ml of LB medium and grown until saturation. As a control, CFT073 cells harboring vector plasmid with GFP gene but no promoter (pUA139) were used. A total of 50 μl of the saturated cultures was used to inoculate 5-ml aliquots of K medium, and the new cultures were incubated overnight at 37°C. Overnight cultures were diluted into 200 μl of fresh

K medium to a final OD_{600} of 0.1 and grown in 96-well plate inside FLUOstar Optima microplate reader (BMG Labtech) at 37°C with shaking (orbital, 7 mm) until the cells reached an OD_{600} of 0.3. The cells were diluted again at a 1:20 ratio into fresh K medium and grown inside microplate reader with absorbance and fluorescence measurements taken every 12 min. When cells reached an OD_{600} of ~0.1, NaCl, urea, or sterile water were added to the appropriate wells, and absorbance/fluorescence measurements were continued. The experiments were also repeated at 30°C and produced similar results. All experiments were done in triplicate and the data were averaged. Acquired data were analyzed in Microsoft Excel. Both fluorescence and optical values were adjusted to remove the effect of the medium; OD values were additionally adjusted to remove the effect of medium evaporation from the plate lid during osmolyte addition.

Supplementary material. Additional figures, tables, and data are available as supplementary material at http://www.wright.edu/~oleg.paliy/Papers/UTI_Osmo/UTI_Osmo.html. The raw microarray data were deposited into NCBI GEO database (record GSE28399).

RESULTS

Analysis of mRNA expression in uropathogenic *E. coli* cells. The goal of the present study was to delineate the gene expression changes used by UPEC in response to the challenge with high concentrations of urea or inorganic ions. *In vitro* growth conditions were chosen to use low-osmolality K medium as a control and to use similar osmolal concentrations of NaCl (0.3 M) and urea (0.6 M) that closely matched the average concentrations of these compounds in human urine (8, 41). UPEC cells grew well in all three conditions used with slower growth observed in medium with urea (the doubling time in K medium and in K medium with NaCl was 50 to 55 min; in K medium with urea it was 78 min). The cells were harvested in the exponential phase of growth after being adapted to each condition for at least three generations, and the gene expression profiles in each environment were profiled and compared.

We used Affymetrix *E. coli* Genome v2 arrays to obtain expression profiles for all samples. Figure 1 shows a genome image view of the average expression comparisons between NaCl-grown cells and controls and between urea-grown cells and controls. The overall expression profiles of CFT073 cells were unique to each growth condition but were similar among biological replicates, as is evident from the PCA plot in Fig. 2. The normalized expression data were exported, filtered to compile a list of 418 differentially expressed genes, and then used for statistical clustering (30, 42). Because we used three biological replicates for each growth condition, nine pairwise comparisons were computed for NaCl-versus-control and for urea-versus-control expression evaluations. K-means clustering (42) was used as a primary method to partition the complete data set. Figure 3 shows a distribution of the differentially expressed genes in nine clusters using K-means algorithm. We confirmed the general partitioning of the genes into K-means clusters by visualizing the positioning of the genes in the PCA space and by running hierarchical clustering of the data set (see Fig. S2 and S3 [http://www.wright.edu/~oleg.paliy/Papers/UTI_Osmo/UTI_Osmo.html]).

Overall, application of osmotic stress with NaCl led to a significantly higher transcriptional network remodeling (318 genes differentially expressed) compared to the observed CFT073 response to urea presence in the medium (119 genes differentially expressed; note that some genes responded to both NaCl and urea). The largest set of genes (160 total) displayed an increased expression under salt stress and no response to the urea presence; a total of 59 genes were upregulated in K medium with urea, but not with NaCl (see Fig. 3).

CFT073 transcriptional responses to salt stress. Four clusters of differentially expressed genes (clusters 3, 4, 8, and 9; 160 genes in all, see Fig. 3) contained members that were overexpressed under increased salt concentration but showed no or little response to urea presence. Most of the known genes of *E. coli* osmotically induced regulon were present in these clusters (Table 1). These included gene members of the glycine betaine and proline transporter *proVWX* (*proU* locus [c3230-c3232], an average expression ratio in NaCl versus control of 110.4, and an average expression ratio in urea versus control of 1.4 [subsequent data are presented parenthetically as "locus, NaCl-versus-control ratio, urea-versus-control ratio"]), an osmotically induced two-component sensor-response system *kdpDE* (c0780-c0779, 2.6, 1.0), a betaine biosynthesis cluster *betIBA-betT* (c0433-c0431 and c0434, 1.9, 2.4), osmotically inducible gene *osmE* (c2138, 3.5, 0.8), and the putative gene *bax* (c4390, 6.6, 2.4). Among these, only *bax* and betaine biosynthesis genes were also overexpressed in urea. Trehalose biosynthesis genes *otsBA* (c2311-c2310, 2.0, 1.1) and proline transporter *proP* (c5116, 2.4, 1.5) were expressed at higher levels in salt only but were not included in the 418 gene list since their expression was below the threshold. The outer membrane proteins OmpC and OmpF were expressed similarly in all three conditions. The known potassium transporter genes *kdpABC* (c0783-c0781, 7.0, 0.4) were partitioned into cluster 1 as their gene expression was downregulated in the presence of urea. Expectedly, constitutively expressed potassium influx and efflux transporters Trk and Kef, as well as osmolality sensor system EnvZ-OmpR, were not regulated by either salt or urea. The YiaMNO transporter, recently implicated in maintaining K-12 strain fitness in high-salt medium (43), also showed no differential expression. A large proportion of the genes in the four salt-responsive clusters were of unknown or putative function.

Surprisingly, a number of genes in the salt response clusters were those participating in *E. coli* anaerobic metabolism. These included nitrate reductase encoding *narK* (c1684, 3.2, 1.0) and *narG* (c1685, 3.1, 0.7), nitrite reductase encoding *nirD* (c4142, 25.5, 0.4), formate-dependent nitrite reductase encoding *nrfA* (c5066, 5.0, 1.0), fumarate reductase encoding *frdABCD* (c5342-c5239, 5.2, 0.5), fumarate uptake transporter encoding *dcuC* (c0712, 3.5, 0.8), anaerobic dimethyl sulfoxide reductase encoding *dmsA* (c1031, 6.9, 0.8), and anaerobic glycerol-3-phosphate dehydrogenase encoding *glpA* (c2782, 7.3, 0.9). Although several of these anaerobiosis associated genes (*narK*, *narG*, *dcuC*, and *dmsA*) contain binding sites for anaerobic transcriptional regulator Fnr, a consistent induction of the complete Fnr regulon was not observed (44). All cultures were grown aerobically in flasks with vigorous shaking, and RNAs were stabilized within 30 s after the stoppage of cultivation. Thus, the observed upregulation of these anaerobic metabolism genes is unlikely to be associated with the lack of oxygen in the culture. Indeed, a similar high expression level of the aerobic cytochrome *o* oxidase *cyoAB* genes was measured in all conditions. However, since uropathogenic *E. coli* is likely to encounter osmotic stress in the urinary tract, cross-regulation of anaerobic gene expression by osmotic stress can serve as an adaptation mechanism to the reduced oxygen content in the urethra and bladder lumen (45). In a study by Hagan et al. (25), among eight bacteriuria strains isolated directly from the urine of

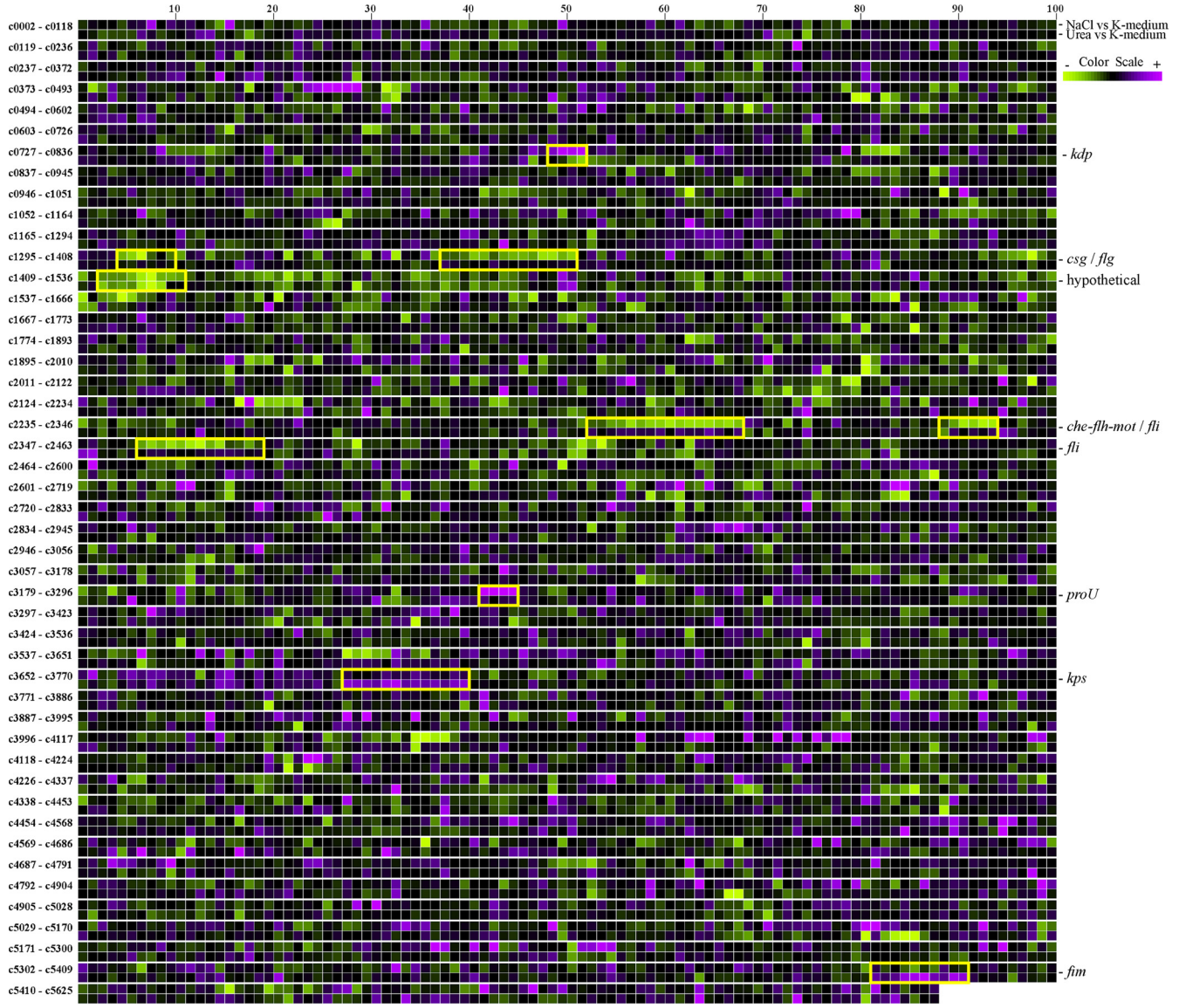

FIG 1 Genome image view of gene expression comparisons. All CFT073 genes that produced robust signal were arranged in chromosomal order and were colored according to their $\log_2$-transformed expression ratios in each comparison using a green-to-purple color scheme. The genes were arranged in 100 genes per row, the c number ranges for each row are shown on the left of the image. In each row, the upper half corresponds to the K medium plus 0.3 M NaCl versus K medium alone comparison, whereas the bottom half represents the K medium plus 0.6 M urea versus K medium alone comparison. The weighted mean of nine corresponding pairwise comparisons among individual experiments was used to represent the average gene expression ratio in each case. Examples of gene clusters that were up- or downregulated in these comparisons are highlighted in the image by rectangles, and the identities of the highlighted genes are shown on the right.

UPEC-infected women, five expressed at least one of the anaerobic metabolism genes, indicating that those strains have likely experienced anaerobiosis. Similarly, an asymptomatic bacteriuria (ABU) strain downregulated *cyoABCD* genes and upregulated many *nar* and *nir* genes during bladder colonization (26).

Genes partitioned into cluster 6 were downregulated in NaCl-containing K medium but did not respond to urea presence. A large group of these genes represented motility and chemotaxis regulon (33 genes in all). Flagellar genes were shown previously to be downregulated by osmotic stress (28) and were also found to be downregulated or not expressed in several *in vivo* UPEC transcription studies (Table 1) (24, 25). In addition, UPEC hemolysin (*hlyCABD*, c3569-c3570 and c3573-c3574, 0.3, 1.4), spermidine-putrescine transporter precursor (*potF*, c0987, 0.3, 0.6), TolQR cell envelope complex proteins (*tolQR*, c0817-c0818, 0.2, 1.2), and four proteins of purine and pyrimidine biosynthesis (*purEK*, c0637-c0636, 0.2, 1.1; *pyrC*, c1329, 0.3, 0.7; *pyrD*, c1081, 0.3, 0.7) showed low mRNA expression in medium with salt. A significant number of genes of unknown or putative function were also present in this cluster.

CFT073 transcriptional responses to urea. Two K-means clusters (clusters 5 and 7) included genes upregulated by urea, whereas cluster 2 contained genes that decreased their expression with urea presence. None of these genes responded consistently to

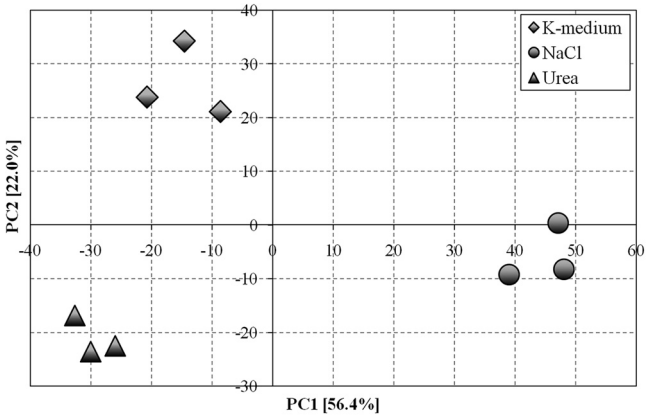

FIG 2 Principal component analysis of the microarray experiments. Principal component 1 values for each experiment are plotted on the *x* axis, and principal component 2 values are plotted on the *y* axis. The percent data set variability explained by each shown principal component is indicated in brackets.

NaCl (Fig. 3). Among the genes overexpressed by urea addition were capsule biosynthesis genes (*kpsEDBCS*, c3687-c3691, and *kpsMT*, c3696-c3695, 1.3, 2.7), type 1 fimbriae encoding gene cluster (*fimB-fimE-fimAICDFGH*, c5391-c5397 and c5399-c5400, 0.7, 3.8), two outer membrane porin-encoding *nmpC* genes (c2348 and c1560, 1.3, 5.7), molecular chaperone encoding *cspH* (c1122, 1.3, 8.4), *cspF* (c3185, 1.0, 7.4), *ibpA* (c4607, 1.1, 2.7), *ibpB* (c4606, 1.4, 8.2), and *stpA* (c3218, 1.3, 4.5), and two genes involved in drug resistance (*yojI*, c2752, codes for putative ABC transporter, 0.4, 3.2; *ydhE*, c2057, codes for a multidrug transporter, 0.7, 3.3), as well as a number of genes (15 in all) of putative function.

Genes that were downregulated in the presence of urea included those participating in acid stress and in sulfur metabolism. Among the former were periplasmic stress chaperone genes *hdeB*, *hdeA*, and *hdeD* (c3420, c4321, and c4322, 1.0, 0.3). Among the latter were transcriptional regulator *cbl* (c2447, 0.6, 0.3), sulfate adenyl transferase/kinase genes *cysDNC* (c3319-3317, 1.2, 0.4), taurine transport and degradation system *tauABCD* (c0472-c0473 and c0475-c0476, 0.9, 0.1), and sulfate-binding protein *sbp* (c4869, 1.3, 0.1). It is not immediately clear why sulfur utilization operons were downregulated in urea containing medium—all three media were supplied with the same amount of sodium sulfate, and no other source of sulfur was available to *E. coli* cells. However, the observed downregulation of these genes is consistent with urea adversely affecting the activity of sulfur starvation transcriptional regulator CysB (30).

CFT073 genes responding both to NaCl and urea. A total of 31 genes in cluster 1 displayed increased expression in the presence of NaCl but lower expression compared to controls in the presence of urea. No general theme was evident from consideration of the biological functions of these genes, but a few interesting examples were found. The outer membrane porin protein Slp (*slp*, c4304, 5.3, 0.3) participates in acid resistance that is consistent with the downregulation of some acid resistance genes in urea presence. At the same time, this gene is strongly induced under anaerobic growth conditions (46), and its upregulation in the medium with 0.3 M NaCl is consistent with that for a group of other anaerobically induced genes (see above). In addition to *slp*, three other anaerobically induced genes—*ansB* (c3543, 12.2, 0.2), *nirB* (c4141, 10.4, 0.1), and *ynfE* (c1977, 8.4, 0.4)—were in this cluster. Three sulfur metabolism genes were also in this cluster: *cysB* (c1742, 1.8, 0.4), *ynfK* (c1984, 1.6, 0.2), and *metE* (c4751, 7.3, 0.4), their lower expression in the urea presence was consistent with the behavior of other sulfur utilization genes discussed above.

Expression of UPEC virulence and colonization genes. Table 1 lists major types of CFT073 virulence and colonization genes and their expression in medium with high salt or urea. Three operons encoding UPEC colonization factors were found to respond to the presence of urea in the medium—these code for capsular biosynthesis (*kpsEDCS*, c3687-c3688 and c3690-c3691; kpsTM, c3698-c3697), type 1 pili (*fimAICDFGH*, c5391-c5397 and c5399-c5400), and F1C fimbriae (*focA-sfaD-focCDFGH*, c1239-c1245). All three factors participate in the initial stages of UTI process (5). None of the CFT073 operons of iron acquisition proteins was found to be upregulated by either salt or urea. Flagella and the chemotaxis regulon, as well as the curli operon, were transcriptionally downregulated under salt stress (28) but not in K medium with urea. All four genes of the hemolysin synthesis and export system were downregulated by NaCl but not urea. Somewhat surprisingly, all hemolysin genes were expressed at a moderate level in basal K medium (34th percentile of mRNA expression among all CFT073 genes on average).

Comparison of CFT073 expression in minimal medium under osmotic and denaturing stresses to gene expression of UPEC *in vivo* and *in vitro*. A number of reports are available that profiled genome-wide gene expression of uropathogenic *E. coli in vivo* or during *in vitro* growth in urine. We have considered in detail mRNA levels of UPEC virulence and osmotically inducible genes observed in four studies: in UPEC strains isolated from human urine of women diagnosed with urinary tract bacteriuria (25), in CFT073 cells isolated from urine of experimentally infected mice (24) and grown in human urine (22), and in asymptomatic bacteriuria strain 83972 during human urinary tract colonization (26). The available data are consistent with the model that the expression of UPEC genes during urinary infection can be attributed at least partially to the presence of urea and salt in the environment (Table 1). Previous microarray data revealed the expression of some of the osmotic stress genes (*proVWX* and *proP*) by UPEC during UTI, increased expression of capsule biosynthesis genes during infection, and downregulation of the motility, chemotaxis, and curli gene clusters in these conditions. There was some variation among previous transcriptomic studies regarding the expression of UPEC fimbriae during UTI infection or during growth in urine (see Table 1). For example, there seems to be a difference in the expression of UPEC type 1 fimbriae between cells isolated from humans (do not express) and cells isolated from murine UTI model (upregulation of type 1 fimbriae expression) (24, 25). Expression levels of the P and F1C fimbriae were high during CFT073 growth in human urine but were low in UPEC cells isolated from human and mouse urinary tract. Our data are consistent with a recently proposed hypothesis (25) that fimbriae expression is needed in the initial stages of infection development (first contact with urine and initial bladder colonization) but is reduced once the cells attach and/or penetrate bladder epithelium. Whereas most of the iron acquisition systems were usually expressed *in vivo* and during *in vitro* growth in urine (23, 24), these systems were turned off in all of our experimental conditions (see Table 1), most likely because the medium contained sufficient trace amounts of accessible iron.

Expression of stress response proteins. We considered whether

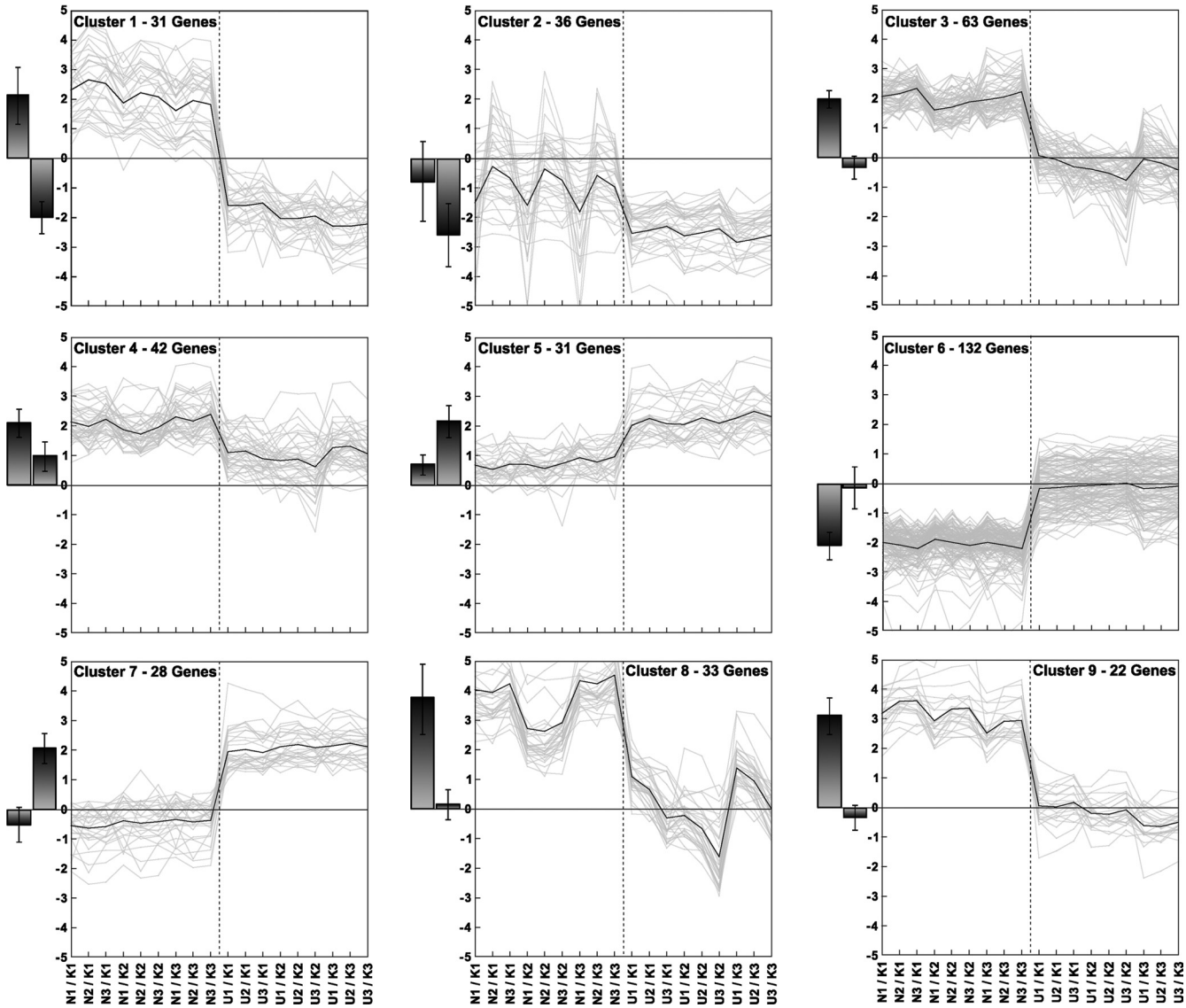

FIG 3 K-means clustering of the complete data set. Each box shows a transcriptional response of genes partitioned into a separate cluster. Cluster numbers and the total number of genes in each cluster are shown at the top of each cluster. The 18 pairwise comparisons are indicated on the *x* axis: "K" represents control K medium, "N" represents K medium with 0.3 M NaCl, and "U" represents K medium with 0.6 M urea. The dotted line separates N-versus-K and U-versus-K comparison sets. The $\log_2$-transformed expression ratios are indicated on the *y* axis. The gray lines in each cluster represent the data for individual genes; the bold black line designates the centroid. The average expression difference for N-versus-K and U-versus-K comparisons for all genes in each cluster is indicated on the left of each cluster box, and error bars show the standard deviations of the values.

any of the known stress response genes were upregulated in the presence of either salt or urea. Genes constituting heat shock, envelope stress, oxidative stress, and *rpoS* general stress response regulons were analyzed. Although sigma factors *rpoS* (c3307, 2.3, 1.4), *rpoH* (c4254, 2.2, 1.5), and *rpoE* (c3097, 2.2, 1.2) and the oxidative stress transcriptional factor *soxS* (c5053, 2.9, 0.5) were all expressed higher in NaCl-supplemented medium, this did not lead to consistent changes in the expression of corresponding regulons, likely because most of these regulons are controlled at the regulator activity level rather than by regulator abundance (47). Heat shock regulon genes *ibpAB* (c4607 and c4606, 1.3, 4.7) and *pphA* (c2247, 1.4, 4.4), as well as *cspH* and *cspF* genes (c1122 and c3185, 1.1, 7.9), were all upregulated in medium with urea compared to control K medium. IbpA and IbpB are small heat shock chaperone proteins that bind to aggregated proteins and inclusion bodies within cells (48). The transcription of these genes was induced upon biofilm formation (49), which might indicate their more general role in dealing with different environmental conditions. Gene *ibpB* was also found to be expressed highly in CFT073 cells isolated from mouse urine (18). CspF and CspH are cold-shock-like proteins identified by their homology to the CspA protein family (50). Their role is likely to be that of nucleic acid chaperones since they both have RNA/DNA-binding domains. Finally, PphA (= PrpA) is a protein phosphatase that is induced by heat shock and is thought to participate in the misfolded protein signaling pathway (51). Interestingly, *cspH*, *ibpB*, and *pphA* were among the most highly upregulated genes in ABU strains during static growth in human urine (52).

TABLE 1 Expression of UPEC virulence and osmotically inducible genes[a]

Gene(s)	Function role[b]	This study[c]		Human UTI[d]	Mouse UTI[e]	Human ABU[f]	Human urine[g]
		NaCl	Urea				
proVWX	Import of GB, proline	**110.6**	1.4	**Expressed**	**35.5** (*proV*)	–	–
proP	Import of GB, proline	**2.4**	1.4	**Expressed**	**~4.0**	–	–
betABIT	GB synthesis	**2.0**	**2.4**	Absent	–	–	–
kdpABCD	K^+ import	**7.0**	1.0	Absent	–	–	–
otsAB	Trehalose synthesis	**2.0**	1.1	Absent	–	–	–
kps genes	Capsule b/s	1.4	**4.0**	**Expressed**	**17.5** (*kpsE*)	**0.2–0.4**	–
fim genes	Type 1 fimbria b/s	0.7	**3.8**	Absent	**9.0**	**0.3**†	**Down**
pap genes	P fimbria b/s	1.6	1.0	Absent	**0.4***	1.9†	**Up**
foc genes	F1C fimbria b/s	1.9	**2.1**	Absent	**0.1***	1.3†	**Up**
chu genes	Heme utilization	1.1	1.2	**Expressed**	**10.0**	**16.3**	**Up**
ent/fep genes	Enterobactin iron acq.	1.1	1.2	**Expressed**	**5.8**	**12.1**	**Up**
iro genes	Salmochelin iron acq.	0.9	1.1	Absent	**8.0**	**19.9**	**Up**
sit genes	Iron transport	1.3	1.0	**Expressed**	**14.3**	**28.8**	**Up**
iuc genes	Aerobactin iron acq.	1.7	1.0	**Expressed**	**10.3**	**37.7**	**Up**
hly genes	Hemolysin b/s	**0.3**	1.4	Absent	–	**<0.1**	–
csg genes	Curli b/s	**0.3**	0.9	Absent	**0.3**	–	–
Motility and chemotaxis regulon		**0.3**	1.4	Absent	**0.2**	–	–

[a] Numbers correspond to the fold change difference in each comparison; a value of 1 corresponds to no difference. Fold changes higher than 2 or lower than 0.5 are highlighted in boldface. –, No data were available or provided. "Down" and "Up" represent reported significant down- and upregulation of the gene, respectively. Absent, not expressed; Expressed, the gene signal was significantly higher than the background.
[b] GB, glycine betaine; b/s, biosynthesis; acq., acquisition.
[c] Comparisons between cells grown in K medium with 0.3 M NaCl versus control K medium and between cells cultured in K medium with 0.6 M urea versus control.
[d] Expression in UPEC strains isolated from human urine of women diagnosed with urinary tract bacteriuria (25). Expression was estimated from the complete data set based on the authors' definition of gene expression above background. The gene cluster was considered expressed if at least half of the profiled eight strains had at least half of the cluster genes robustly expressed. Note that for the *proVWX* operon the expression was inconsistent among genes; data for *proX* were used.
[e] Expression in CFT073 cells isolated from urine samples of experimentally infected mice compared to that in cells grown statically in LB medium (24). The numbers shown are geometric means for all genes in the cluster as estimated from the tables, figures, and the text of the manuscript. *, Genes highly expressed by CFT073 while grown *in vitro* in human urine.
[f] Gene expression in asymptomatic bacteriuria *E. coli* strain 83972 during human urinary tract colonization compared to cell growth in morpholinepropanesulfonic acid (MOPS) medium (26). The numbers shown are geometric means for all genes in each cluster based on Fig. 3, except for *hly* and *kps*, which were taken from the text of the manuscript. †, The *pap* and *foc* genes do not produce functional fimbriae, and *fimEAIC* are not present in ABU strain 83972.
[g] Gene expression in CFT073 cells grown in human urine (22). Up- and downregulation was estimated from the text.

Similarity of transcriptional responses of K-12 and UPEC to osmotic stress. We have recently carried out a study of transcriptional cross-regulation between continuous osmotic and heat stresses in *E. coli* K-12 (28). As a part of the study, the gene expression profile of *E. coli* K-12 cells adapted to growth in K medium containing 0.3 M NaCl was determined. Upon comparing CFT073 and K-12 responses to osmotic stress caused by salt, known osmotically inducible genes were similarly overexpressed by both strains. These included *kdpABC*, *osmC*, *osmE*, *proVWX*, and the putative gene *bax*. A number of oxidative stress protection genes were induced in K-12 under salt stress. Several of these were also upregulated by CFT073 under osmotic stress including *osmC* (c1916, 2.1, 1.1), *soxS* (c5053, 2.9, 0.5), and *fpr* (c4876, 2.9, 0.5). In addition, other genes of the oxidative stress regulon including *dps* (c0898, 2.0, 1.5), *grxB* (c1331, 3.0, 1.0), and *gor* (c4299, 2.3, 1.6) were overexpressed under salt stress by the UPEC strain. Motility and chemotaxis regulon was downregulated under salt stress by both K-12 and CFT073, with latter showing more drastic response, likely explained by the fact that CFT073 is a more motile strain (O. Paliy, unpublished data).

Confirmation of microarray data with quantitative PCR. We have used quantitative real-time PCR to validate microarray findings. Primers were developed to genes that were found to be differentially expressed in at least one of the conditions. As evident in Table 2, the qPCR results matched microarray data well, with only a slight discrepancy observed for *proW* mRNA level in urea-supplemented medium.

Measurements of promoter activity for *bax* and *ibpA*. Because our microarray experiments have profiled transcriptional long-term stress responses of UPEC cells to the addition of salt or urea to the environment, we sought to also assess the induction of several responsive genes in real time. We chose *bax* as an example of a NaCl-induced gene because it was found in multiple transcriptomic studies to be elevated under salt stress; *ibpA* was chosen

TABLE 2 Confirmation of microarray results with qPCR[a]

Gene	Microarray data[b]		qPCR data[c]	
	NaCl	Urea	NaCl	Urea
proW	61.0	0.8	537.1 ± 323.1	2.4 ± 1.6
nrfA	6.4	1.2	12.4 ± 9.4	1.5 ± 1.7
tauA	0.6	0.02	1.3 ± 0.9	0.02 ± 0.01
kpsE	0.9	4.2	1.4 ± 0.8	8.9 ± 5.0
fimF	0.4	3.3	0.4 ± 0.2	4.7 ± 2.5

[a] NaCl, mRNA expression ratio between K medium plus 0.3 M NaCl and basal K medium; urea, mRNA expression ratio between K medium plus 0.6 M urea and basal K medium.
[b] Microarray data are indicated as an array signal ratio for each chosen experimental comparison.
[c] qPCR data are shown as arithmetic means of the mRNA ratios ± SEM.

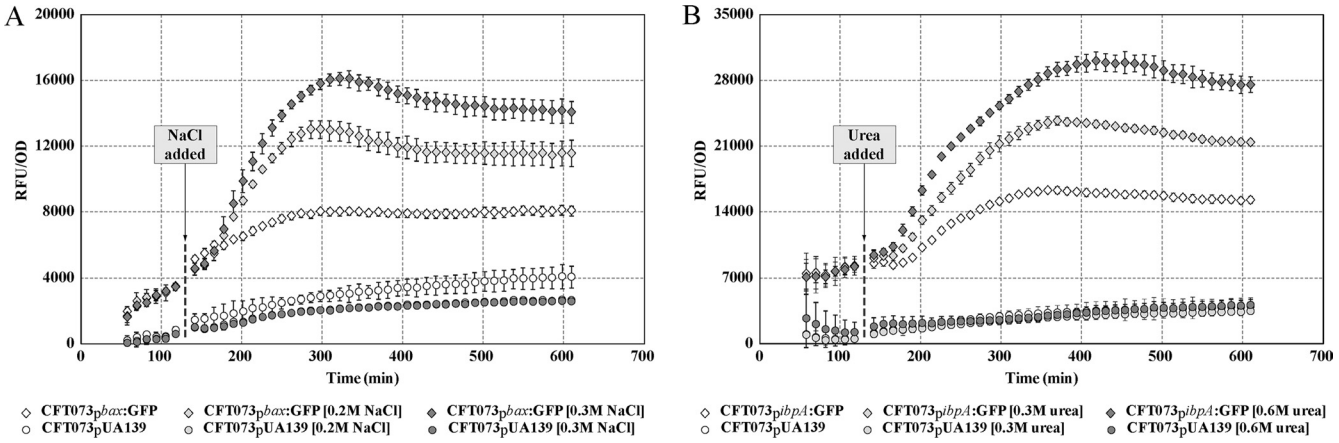

FIG 4 Promoter activity of *bax* and *ibpA* under salt and urea shock. The figure displays fluorescent output of cultures subjected to either salt (A) or urea shock (B). The *y* axis represents OD-adjusted average fluorescent output of each culture, and the *x* axis displays the time of the measurements. RFU, relative fluorescence units; OD, optical density. CFT073 cells harboring vector plasmid (pUA139) were used as a control. Arrows indicate the times when salt or urea were added to each well; equivalent volumes of sterile water were added to control cultures. Error bars represent the standard deviations of the values among three replicate runs.

as an urea-induced gene because of its role as a molecular chaperone (see above). The promoter regions of each gene were fused to the GFP gene and fluorescence of *E. coli* cells was measured as shown in Fig. 4. The addition of NaCl to the growth medium led to a significant increase in culture fluorescence for CFT073$_p$*bax*:GFP cells but not for CFT073 cells carrying vector plasmid. Similarly, *ibpA* promoter elevated its activity upon addition of urea. A dose-dependent effect was evident for the induction of both promoters. These results confirm rapid induction of transcription for *bax* and *ibpA* genes upon addition of salt and urea, respectively.

DISCUSSION

This study has explored the restructuring of gene expression networks in uropathogenic *E. coli* cells subjected to continuous growth in medium with high salt or urea. The conditions were chosen to simulate the effects of NaCl and urea that UPEC cells experience during colonization of human urinary tract. Our data demonstrate that *E. coli* responds differently to the presence of these osmolytes in the medium. Whereas most of the known osmoresponsive genes were induced in the medium with 0.3 M NaCl, there was no change in their expression when 0.6 M urea was added to the base medium. Since Na^+ and Cl^- ions cannot freely diffuse through cytoplasmic membrane, their addition to the growth medium creates a water activity difference between the cytoplasm and extracellular environment (53). Such hyperosmotic environment triggers upregulation of osmotic gene regulon (11). On the other hand, urea is able to permeate through the cell membrane and thus no difference in intracellular versus extracellular osmolality is created by its addition to the medium. Thus, osmotic stress regulon is not activated (8, 53).

Urea is able to destabilize the native three-dimensional structure of proteins by forming stable complexes with protein backbone (9). This can potentially lead to significant protein denaturation and aggregation inside cells, although it is unlikely to be fatal at the typical urea concentrations in human urine, and different *E. coli* strains are able to grow in human urine for at least several generations (16, 54). Despite the failure of urea to stimulate glycine betaine (GB) uptake, betaine accumulation invoked by osmotic stress was shown to attenuate the growth-inhibitory effects of high urea concentrations (8) and, because human urine contains a variety of betaines, it promotes cell tolerance to urea presence (55). In agreement with these previous findings, we detected no upregulation of GB uptake systems *proVWX* and *proP* in medium with urea, but the *betIBA-betT* gene cluster coding for the synthesis of GB from choline was overexpressed in such medium. In addition, we determined that at least five genes encoding proteins with chaperone function were significantly upregulated in urea presence, which is consistent with the hypothesis of *E. coli* cells experiencing environmental stress in medium with urea. We confirmed the rapid induction of one of these genes, *ibpA*, upon urea addition in promoter activity assay. These genes can be considered potential targets for future UTI therapies, since their disruption might result in a decreased survival of UPEC cells inside the urinary tract.

The original objective of the present study was to determine whether the presence of urea or salt in the environment can serve as a trigger for the expression of UPEC infection program. Indeed, we observed that operons coding for capsule biosynthesis and the expression of type 1 and F1C fimbriae were induced by 0.6 M urea. Salt stress was not found to consistently induce any known UPEC virulence or colonization factors. Similar upregulation of pili and capsule biosynthesis genes was observed in *E. coli* cells grown in medium with sucrose, another membrane-permeable solute (56). The identified pattern of colonization gene expression led us to a hypothesis that it is the presence of urea in the environment that initiates the UPEC infection program and the expression of the first line of UPEC colonization factors. Among these, type 1 fimbriae are used by UPEC cells to attach to bladder epithelium, whereas capsule provides protection against host defenses (4, 5). It is unlikely that UPEC can encounter urea in sufficient concentrations in any of its other usual environments (intestine, soil). It can thus be argued that expression of these colonization factors observed in UPEC cells *in vivo* (see Table 1) can be caused by urea penetrating through the cellular membrane into cytoplasm. Once *E. coli* cells attach to epithelial cell surfaces, other transcriptional responses elicit a switch in virulence gene expression.

To the best of our knowledge, mechanisms of how urea causes changes in gene expression are not known. One potential mechanism can involve modification of transcriptional factor activity through selective destabilization of proteins with more easily accessible protein backbones. Further structural studies will be required to delineate this potential induction mechanism in detail.

We also observed a significant downregulation of the motility, chemotaxis, and curli operons in CFT073 cells grown in medium with NaCl, which is consistent with previous reports for other bacteria (28, 57). Elegant studies from Harry Mobley's group show that flagellar expression is downregulated in UPEC in the urinary tract (24, 25, 54, 58, 59). Flagellar expression was reduced in UPEC cells during the establishment of initial infection and was also decreased in response to expression of type 1 and P fimbriae. Although the expression of flagella did not influence bladder colonization in mice, the *fliC* mutant was significantly attenuated in kidneys (60), suggesting that bacterial movement is important for UPEC to traverse from bladder to kidneys. Because kidney urine usually has a lower osmolyte concentration compared to urine in the bladder (41), it is plausible that flagellar synthesis and function in UPEC is osmotically controlled.

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