Role of \textit{bax}, \textit{ibpA}, \textit{ibpB} and \textit{cspH} Genes in Protecting CFT073 (Uropathogenic \textit{Escherichia coli}) Against Salt and Urea Stress

Pavani Beesetty
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Role of \textit{bax}, \textit{ibpA}, \textit{ibpB} and \textit{cspH} Genes in Protecting CFT073 (Uropathogenic \textit{Escherichia coli}) Against Salt and Urea Stress

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

PAVANI BEESETTY
B. Pharm, Shadan College of Pharmacy, J.N.T.U, India 2009

2013
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Pavani Beesetty ENTITLED Role of bax, ibpA, ibpB and cspH Genes in Protecting CFT073 (Uropathogenic Escherichia coli) Against Salt and Urea Stress BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Beesetty, Pavani. M.S., Department of Pharmacology and Toxicology, Wright State University, 2013. Role of bax, ibpA, ibpB and cspH genes in protecting CFT073 (Uropathogenic Escherichia coli) against salt and urea stress.

Uropathogenic Escherichia coli (UPEC) are the primary cause of 80 to 90% of uncomplicated urinary tract infections. After entering the urinary tract, uropathogenic E.coli has to tolerate high levels of salt and urea in urine to cause a successful infection. The osmotic stress imposed by urea is different from that by NaCl, as urea can freely move across the cell membrane and is a protein denaturant. Hence, microarray experiments were performed to observe the differential expression of genes in CFT073 (UPEC) due to the presence of 0.3 M NaCl and 0.6 M urea in K medium individually. Based on the results, Bax (a hypothetical protein), IbPB (small heat shock chaperone proteins) and CspH (speculated to be DNA/RNA chaperone protein) were chosen for the current study in which their role in protecting CFT073 against salt or urea stress was investigated. First bax, ibpAB and cspH gene sequences were shown to be well conserved among commensal E.coli MG1655 and 3 different UPEC strains CFT073, UTI89 and E.coli 536. Then GFP transcriptional fusion plasmids were constructed for bax, ibpAB and cspH genes and promoter activity assays were performed in K medium. Results showed that in CFT073, expression of bax was induced significantly in the presence of 0.2 M and 0.3 M NaCl, and expression of ibpAB and cspH genes were induced in the presence of 0.3 M and 0.6 M urea in K medium, though the induction in cspH expression needs further confirmation. It was also shown that RpoS sigma factor does not regulate the expression of these genes during high levels of salt or urea in K medium. To study the
importance of each gene in protecting CFT073 and BW25113 (K-12 strain) against salt or urea stress, growth curve experiments were performed using bax, ibpAB and cspH knockouts of each strain. Results showed that bax deletion reduced the salt tolerance of BW25113 but had no effect on the salt tolerance of CFT073. Deletion of ibpAB operon did not affect the urea tolerance of CFT073 and BW25113. However cspH null mutant of BW25113 showed reduced growth rate in the presence of 0.9 M urea in M9 medium compared to its wild type strain, and further experiments are needed to determine if cspH knockout would produce a similar effect in CFT073. Based on the current study, it can be concluded that even though the expression of bax and ibpAB genes are induced during salt and urea stress respectively, these genes are dispensable for CFT073 to tolerate high levels of salt or urea in K medium and M9 medium.
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ACKNOWLEDGEMENTS

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Last but not the least, I thank all different strains of E.coli that I worked with, especially CFT073 for letting me mutate them and transform them with weird plasmids. I hope the infinite bacteria I killed for science have gone to heaven.
Dedicated to my beloved parents,

Appa Rao Beesetty & Naga Lakshmi Beesetty
INTRODUCTION

Urinary tract infection (UTI): Epidemiology

Infection of urinary tract with microbial pathogens leads to UTI. Urinary tract infections are responsible for 8.27 million office visits, 1.7 million emergency room visits and 366,000 hospitalizations with an annual cost of $3.4 billion in the United States alone during 2000, as shown by the following table (1).

<table>
<thead>
<tr>
<th>Year 2000</th>
<th>Office visits</th>
<th>Emergency room visits</th>
<th>Hospitalizations</th>
<th>Annual cost</th>
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</thead>
<tbody>
<tr>
<td><strong>Women</strong></td>
<td>6.8 million</td>
<td>1.3 million</td>
<td>2,45,000</td>
<td>$2.4 billion</td>
</tr>
<tr>
<td><strong>Men</strong></td>
<td>1.4 million</td>
<td>0.4 million</td>
<td>1,21,000</td>
<td>$1 billion</td>
</tr>
</tbody>
</table>

About 40% to 50% of women are experiencing at least one episode of UTI during their life time, with 1 in every 3 women having their first UTI by the age of 24 (2). Though UTI are acute self-limiting infections, the rate of recurrence is usually high. In children, UTI is an important cause of morbidity effecting 10% of childhood population (3). Though the occurrence of UTI is very low in young males due to a longer urethra, and the release of antibacterial substances and immunoglobulin gamma A (IgA) locally from the prostate gland, the rate is usually high during their early childhood and later stages of life (4, 5).
Classification and Clinical Manifestations of UTI

UTI are mainly classified as Uncomplicated (infection in a healthy individual with a normal genitourinary tract) and Complicated UTI (infection in catheterized, structurally or functionally abnormal genitourinary tract or in immune compromised patients (4).

The 3 main clinical manifestations of UTI include cystitis (infection of bladder with symptoms like dysuria, frequency and urgency of urination, and sometimes suprapubic pain), acute pyelonephritis (infection of kidney with flank pain, fever, nausea, vomiting, sweats and malaise as symptoms) and asymptomatic bacteriuria (patient consistently shows significant quantity of bacteria in the urine but with no symptoms of infection) (2, 4, 6, 7).

Causative agents for UTI

About 80-90% of uncomplicated UTIs are caused by *Escherichia coli*, 10-15% by *Staphylococcus saprophyticus* and 5% by other enterobacteriaceae like *Klebsiella*, *Proteus*, *enterobacter* and *enterococci*. Only 20% of complicated UTI are caused by *E.coli*, other causative agents being *Candida Spp.*, *Staphylococcus aureus*, *Klebsiella* spp., *Enterobacter cloaceae*, *Serratia marcescens*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, Group B streptococci, etc (5, 8). Even a less virulent strain can cause successful infection during a complicated UTI. Thus the range of organisms which can cause complicated UTIs are broader (7-9).
**Uropathogenesis of *E.coli***

*Escherichia coli* are the common inhabitants of the human and animal gut. It maintains a symbiotic relationship with its host and helps in intestinal tract functional maturation, microbial flora stabilization and immune response regulation in the host intestine (10). However some strains of *E. coli* become pathogenic by acquiring virulent factors through horizontal transfer of plasmids, pathogenicity islands, transposons and bacteriophages (11). These pathogenic strains are classified as enteric/diarrheagenic *E. coli* and extraintestinal *E. coli* (ExPEC). Uropathogenic *E. coli* (UPEC) are a highly heterogeneous group of *E. coli* which belong to ExPEC. They reside in the human intestine without producing any disease but can disseminate and colonize the host urinary tract and cause urinary tract infection (11).

UPEC strains are expected to be transmitted from one person to another by sex, food, water or through the hands of hospital personnel (7). Once UPEC enters the intestine it forms a reservoir in the gut which can colonize the periurethral area or vaginal introitus, ascend to the bladder through the urethra and cause bladder infection (12). The exact mechanism of bacterial migration from introitus to bladder is still unknown (13). However sexual intercourse is considered to be an important factor that facilitates this migration (4). Some strains can further ascend to the kidneys, with the help of flagella, producing pyelonephritis (14, 15). The pathogenesis of pyelonephritis is relatively less studied compared to cystitis. The above described path is considered to be the major route for UTI and is called ascending UTI. However some infections can occur in the opposite direction where bacteria enter the kidney via the blood stream. Such infections are called hematogenous UTI (4).
The first line host defense mechanisms to kill bacteria and prevent their adhesion to bladder mucosa include low pH, high salt, urea and organic acids in urine; bulk flow of urine during micturition, mucin layer secreted by bladder transitional cells, Tamm horsfall factor which can bind to type 1 fimbriae of UPEC and defensins which can disrupt cell membrane of UPEC (11). Thus once inside the bladder, the bacterium has to immediately adhere and invade the bladder epithelial cells to protect itself from the above mentioned host defense mechanisms. Uropathogenic *E. coli* have the virulent factors necessary to colonize the bladder successfully and evade the host immune response (4).

UPEC adhere to host uroepithelial cells using adhesins of various types, the 2 main being type 1 fimbriae and P fimbriae. These adhesion molecules not only help in the attachment of UPEC to superficial umbrella cells, but also stimulate the signaling pathways leading to the invasion of bacteria into the urothelial cells (11, 16). After invasion, bacteria enter a highly replicative phase forming a complex intracellular bacterial community (IBC) which shows specific biofilm-like traits. Invasion of urothelial cells and formation of IBC helps UPEC to escape host immune response and multiply in large numbers which are difficult to be cleared completely by polymorphonuclear leukocytes (PMNs) (17).

Attachment of bacteria to the uroepithelial cells through FimH (type 1 fimbriae), and release of bacterial toxins like HylA and CNF1 triggers exfoliation of the entire layer of superficial bladder epithelial cells through a rapid apoptosis-like mechanism. This is considered to be one of the host defense mechanisms to prevent UTI (11, 18). However UPEC already multiplied into an IBC, flux out from the bladder cells to infect underlying transitional epithelium and enter a quiescent intracellular non replicative phase which forms a reservoir for future recurrent infections. This quiescent intracellular reservoir
(QIR) helps UPEC to persist in the urinary tract for months after initial infection and resist systemic antibiotic therapy (19).

Other virulence factors expressed by UPEC include secreting toxins like α-hemolysin (HylA), cytotoxic necrotising factor 1 (CNF1), secreted autotransporter toxin (SAT), etc. These factors help UPEC to not only gain access to host nutrients and iron stores but also to impair host immune response. Some of these toxins and virulence factors like LPS, Type 1 or P fimbriae are recognized by TLR4 (Toll-like receptor 4) of urothelial cells and stimulate the signaling pathway leading to the expression of IL-6 and IL-8 through NF-kB dependent or independent pathways. IL-6 and IL-8 helps in neutrophil recruitment to the site of infection to kill bacteria in the urinary tract (11, 20).

**Importance of known osmoregulatory systems for the survival of UPEC in urine**

Human urine is one of the first line host defense mechanisms against UTI. The physicochemical stress imposed by urine to prevent *E. coli* colonization includes fluctuating pH (5-8) and osmolality (0.04 mol/kg to 1.4 mol/kg), high urea levels (up to 0.5M) and a high concentration of organic acids (21-23). Despite these unfavorable conditions, UPEC can still survive and grow in urine. Growth rate of uropathogenic and non uropathogenic strains (fecal isolates) of *E. coli* are similar in urine. This indicates that the survival of *E. coli* in urine is not a urovirulent trait (24, 25).

The 2 main physicochemical stresses imposed on *E. coli* in urine include osmotic stress due to high concentration of inorganic salts and denaturing stress due to urea.

*E. coli* can sense an osmotic upshift in external medium through pressure sensors located in the inner membrane; changes in intracellular water activity, pH and $K^+$; and through
alterations in the protein-ligand or protein-protein interactions inside the cells as a result of increased internal hydrostatic pressure (26). Accumulation of K\(^+\) and synthesis or capture of glutamate to counteract alkalinization effects of K\(^+\) involves the initial response to salt stress especially at low levels (26, 27). Under higher osmotic stress, low molecular weight organic compatible solutes like glycine betaine, carnitine and proline, etc. are preferred over salt accumulation, as they can be synthesized or accumulated at high levels without effecting cellular processes (due to their high solubility and no net charge at physiological pH). These compatible solutes provide osmoprotection by destabilizing the partially denatured proteins and stabilizing their native conformations (26, 28).

The stress imposed by urea is different from the osmotic stress imposed by NaCl or other inorganic salts in urine, because urea can diffuse freely across the cell membrane and is a protein denaturant (22, 29). It was initially believed that osmotic stress induced betain accumulation protects the cell against the toxic effects of urea in urine. However, the lack of betain accumulation did not affect the growth rate of CFT073 in urine (22, 23, 30).

Limited research has been conducted to determine the genes that protect UPEC against high osmotic stress and denaturing stress in urine. A brief summary of the studies till date is discussed in Table 1. Based on these studies, only proP showed an osmoprotective role in HU734 (UPEC) to survive in urine; however it showed mixed results during in vivo colonization of the murine urinary tract. In the case of CFT073 (UPEC), none of the osmosystems studied till date was proved to be important for providing osmoprotection in urine.
Naturally occurring *E. coli* isolates express broad variations in osmotolerance (31). CFT073 and HU734 are UPEC strains with CFT073 being more osmotolerant and urovirulent compared to HU734 (22). Based on Table 1, it can be said that the mechanism used by both strains to grow in urine might be different.

It was recently shown that OmpR is required for the survival of NU134 (UPEC) in urine and to colonize murine urinary tract. OmpR-EnvZ is a two component regulatory system that responds to osmotic stress. EnvZ senses the osmotic shock and gets autophosphorylated which in turn phosphorylates OmpR. The phosphorylated OmpR acts as a transcriptional activator or repressor and alters the expression of various genes involved in osmotic tolerance, acid tolerance, outer-membrane proteins, type 1 pili and virulence factors like IutA (32). However as *E. coli* isolates show broad variations in osmotolerance, the role of OmpR is providing osmoprotection to CFT073 and other UPEC strains in urine needs further confirmation. And the exact gene or the mechanism through which OmpR showed the above osmoprotective property in NU134 needs to be determined.

The osmoprotective property found in urine is greater than predicted based on the concentration of betains in urine (29). In addition, most of the other known osmoprotectants like proline, pipecolate, ectoine, dimethyl glycine, sarcosine carboxymethyl pyridinium, D-carnitine, L-carnitine, butyrobetaine, taurine, betonicin, trigonelline and thiaproline failed to improve the osmotolerance of CFT073 ΔproP ΔproU (33), indicating that there might be an unknown osmoprotectant in urine that provides osmoprotection to UPEC through a mechanism yet to be discovered.
<table>
<thead>
<tr>
<th>System</th>
<th>Function</th>
<th>Importance in survival of UPEC in urine</th>
<th>References</th>
</tr>
</thead>
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<tr>
<td>Kdp and Trk system</td>
<td>K⁺ uptake</td>
<td>No sufficient <em>in vitro</em> and <em>in vivo</em> experiments conducted. But CFT073 and HU734 were able to tolerate upto 0.4 M NaCl in K5 medium containing low K⁺ in micromolar concentrations indicating that these systems might be dispensable under high osmotic conditions in urine.</td>
<td>(22, 26, 33)</td>
</tr>
<tr>
<td>ProP</td>
<td>Uptake of osmoprotectants majorly proline, glycine betaine and ectoine</td>
<td>(+) <em>in vitro</em> cultivation in urine, (-/+)* in vivo* bladder colonization</td>
<td>(22, 23)</td>
</tr>
<tr>
<td>ProU</td>
<td>Uptake of osmoprotectants majorly glycine betaine, proline betaine, carnitine, choline and ectoine</td>
<td>(-) <em>in vitro</em> cultivation in urine, (-) <em>in vivo</em> bladder colonization</td>
<td>(22, 23)</td>
</tr>
<tr>
<td>OtsBA</td>
<td>Trehalose synthesis (regulated by RpoS sigma factor)</td>
<td>RpoS variant in HU734 is defective, leading to insignificant trehalose accumulation under osmotic stress</td>
<td>(22)</td>
</tr>
<tr>
<td>PutPA</td>
<td>Proline uptake</td>
<td>(-) <em>in vitro</em> cultivation in urine, (-) <em>in vivo</em> bladder colonization</td>
<td>(22, 23, 33)</td>
</tr>
<tr>
<td>BetU</td>
<td>Betaine uptake</td>
<td><em>betU</em> was not selected among uropathogens during evolution based on equal distribution of this gene among pathogenic and non-pathogenic <em>E.coli</em> strains. <em>betU</em> is present in HU734 but absent in CFT073.</td>
<td>(33)</td>
</tr>
<tr>
<td>BetTBA</td>
<td>Uptake of Choline and oxidation into glycine betaine</td>
<td>Choline uptake is higher in HU734 compared to CFT073 and was not affected by the lack of <em>proP</em> or <em>proU</em> systems in both the strains. Thus <em>betT</em> might not be responsible for the higher osmotolerance of CFT073 compared to HU734.</td>
<td>(26, 33)</td>
</tr>
</tbody>
</table>

Table 1: Importance of known osmoregulatory systems in UPEC for survival in urine. *In vitro* and *in vivo* experiments are conducted using null mutants of HU734 or CFT073 for that respective gene or system. (+) result indicate growth defect or impaired colonization of murine bladder by the null mutant compared to its wild type and (-) result indicate no difference. For some genes invitro or invivo experiments were not performed. But the information presented in the corresponding reference articles was summarized to predict their potential role in the survival of HU734 or CFT073 in urine.
**Previous work in Dr. Paliy’s lab**

Considering that none of the known osmoregulatory systems are responsible for the survival of uropathogenic *E. coli* in urine, especially in CFT073 (a highly virulent pyelonephritis isolate), microarray experiments were performed to identify genes which are differentially expressed in CFT073 during salt stress (0.3 M NaCl) and urea stress (0.6 M urea) in K medium (osmolality is 0.15 osmol/kg H$_2$O) at 30°C. Osmolality of K medium with 0.3 M NaCl is 0.65 osmol/kg H$_2$O and it led to the over-expression of osmotically inducible regulons like proU, kdpDE, betTIBA etc. most of which are not induced during 0.6 M urea stress. However during the presence of 0.6 M urea in K medium (0.65 osmol/kg H$_2$O), expression of several genes with chaperone activity were induced (34). Based on these results, *bax*, *ibpAB* and *cspH* were chosen for the present study.

[CFT073 was chosen for the microarray experiments because it is a highly virulent pyelonephritis isolate. Its whole genome is sequenced (35) and CFT073 specific DNA microarrays were already created and available (36). K medium was used because it is a low basal osmolality medium and to compare the results with previous study conducted to observe the transcriptional response of commensal *E. coli* to salt stress (34, 37, 38)].

**Bax**

Bax is a hypothetical protein of unknown function. ORF of *bax* is 825 nt long and the protein is made of 274 aa (BioCyc database). The promoter region of *bax* is predicted to be regulated by sigma 70 based on computational analysis (39).
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Log signal</th>
<th>Average expression ratio in NaCl versus control</th>
<th>Average expression ratio in urea versus control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K1</td>
<td>K2</td>
<td>K3</td>
</tr>
<tr>
<td>Bax</td>
<td>6.5</td>
<td>5.7</td>
<td>6.4</td>
</tr>
<tr>
<td>IbpA</td>
<td>8.6</td>
<td>8.5</td>
<td>8.7</td>
</tr>
<tr>
<td>IbpB</td>
<td>5.3</td>
<td>5.2</td>
<td>5.5</td>
</tr>
<tr>
<td>CspH</td>
<td>2.6</td>
<td>4.3</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Table 2: Log signal values of *bax*, *ibpA*, *ibpB* and *cspH* gene expression in microarray experiments (34).

K1, K2, K3: Gene expression in 3 replicate cultures of CFT073 growing in K medium

N1, N2, N3: Gene expression in 3 replicates cultures of CFT073 growing in K medium with 0.3 M NaCl

U1, U2, U3: Gene expression in 3 replicates cultures of CFT073 growing in K medium with 0.6 M urea

Growth conditions used: A single colony of CFT073 strain was inoculated into liquid Luria-Bertani (LB) medium and grown aerobically till saturation. Freshly made K medium was inoculated with an aliquot of above culture and grown till late log phase. Aliquot of this culture was used to inoculate 3 flasks with 1/5th volume of K medium, K medium with 0.3 M NaCl and K medium with 0.6 M urea and grown in water bath shaker at 30°C and 200 RPM till the cultures reached an OD$_{600}$ (optical density at 600 nm) of 0.45. Then cells were harvested and RNA was extracted to perform microarray experiments.
Expression of bax was induced in CFT073 during the salt stress of 0.3 M NaCl in K medium at 30°C with an average expression ratio of 6.6 in NaCl-versus-control (34). Overexpression of bax was also observed as a long term response to 0.3 M NaCl in NCM3722 (E. coli K-12 strain) at 30°C (38) and as an early response (within 9 min) to 0.4 M NaCl in MC4100 (E. coli K-12) at 37°C (40).

Its overexpression was also noticed in acid treated E. coli O157:H7 during in vitro conditions suggesting its involvement in providing acid tolerance to enterohemorrhagic E. coli (41).

Transcription of an alternative sigma factor rpoS was induced shortly after an osmotic upshift (0.4 M NaCl) in MC4100 along with a broad set of genes whose expression depends on RpoS (40). Expression of bax was also induced during those experiments and thus the role of RpoS in regulating the expression of bax was studied in the current project.

IbpA and IbpB

IbpA and IbpB are 16 kDa small heat shock proteins belonging to the ubiquitous family of α-heat shock proteins. The expression of these genes is induced several hundred-fold after heat shock. Extreme heat shock conditions lead to protein aggregation. IbpA and IbpB change the physical property of these protein aggregates so that bichaperone (Hsp70-Hsp100) mediated protein disaggregation and refolding becomes more efficient (42, 43). However E. coli lacking ibpA and ibpB show growth defect, reduced viability or increased protein aggregation only under extreme heat stress of 45°C - 50°C (44-46).
During heat shock conditions, IbpA and IbpB undergo deoligomerization and form IbpA-IbpB heterooligomer (47). Though IbpA and IbpB alone have the property of protein disaggregation, the efficiency of the Hsp100-Hsps70 dependent protein reactivation depends upon the disaggregation of protein aggregates carried out in the presence of both IbpA and IbpB (42, 44, 48) in addition, IbpA is required for targeting IbpB to the aggregated proteins (45).

Genes ibpA and ibpB belong to an operon, which is expressed by sigma 32 regulated promoter region upstream ibpA ORF. The ORFs of ibpA and ibpB are separated by 112 bp and both the genes have their own ribosome binding sites. ibpA and ibpB share 57.5% protein sequence identity and are believed to have originated from a duplication event. Expression of ibpB is under the control of an additional weak sigma 54 regulated promoter region present upstream ibpB ORF. This promoter region is also heat shock inducible (49, 50).

The bicistronic ibpAB transcript made through sigma 32 promoter is cleaved into monocistronic ibpA and ibpB transcripts by RNase E. RNaseE is also responsible for the further degradation of ibpB transcript leading to the differential expression of ibpA and ibpB. Translation of ibpA is also controlled by an RNA thermometer (structured mRNA region at 5’UTR which inhibits translation by forming a fold that can block Shine-
Dalgarno sequence at low temperature. However, this structure is melted during temperature upshift, allowing the protein translation) (50).

During microarray experiments performed in Dr. Paliy’s lab, expression of *ibpAB* genes was induced during 0.6 M urea stress in CFT073 with an average expression ratio of 4.7 in urea vs control (34). Expression of *ibpAB* genes was also induced in commensal *E. coli* during inflammatory bowel disease (51); and in CFT073 and asymptomatic bacteriuria *E. coli* strains 83972 and VR50 during biofilm growth in urine (52, 53).

Lack of *ibpAB* inhibits the formation of biofilm in *E. coli* (54). The hypercolonization phenotype of CFT073 *dsdA* compared to wild type CFT073 was reduced by *ibpB*. However CFT073 *ibpB* knockout did not show any colonization defects in murine urinary tract (55).

**CspH**

CspH is a small protein made of 70 amino acids and belongs to the CspA family of cold shock proteins. The exact function of CspH is still unknown. CspA family consists of 9 genes (CspA-I), with different functions and only some of them are cold shock inducible. However they all have conserved β-barrel structure and most of them possess the property of binding to ssRNA and ssDNA (but not dsDNA) though a high degree of RNA/DNA substrate specificity is not apparent (56-58).

One of the deleterious effects of cold shock is stabilization of DNA and RNA secondary structures which affects transcription and translation by hindering the moment of RNA polymerase and ribosomes. CspA and its homologues act as RNA chaperones by melting these secondary structures and enabling transcription and translation.
The β-barrel structure of Csp proteins is made of 5 antiparallel β strands with two RNA binding motifs RNP1 and RNP2 on β2 and β3 strands, respectively. Due to electrostatic attraction, RNA approaches the Csp protein and intercalates between the aromatic residues present on the protein surface at RNP1 and RNP2 sites. However due to the overall negative charge on the protein surface (except at the RNP sites), limited intramolecular and intermolecular base pairing occurs with a segment of RNA undergoing charge repulsion. This was the mechanism proposed for the chaperone activity of CspA and its homologues. Systematic studies on CspE amino acid sequence revealed that Phe17, Phe30 and His32 are required for the nucleic acid melting activity with Phe initiating the process and His ending it. When these amino acids were substituted by Arg, the protein retain the property of binding to nucleic acids but lost the ability to melt secondary structure. Interestingly, CspH protein does not have Phe17 and Phe30 in its RNA binding sites. They are substituted with Lysine and Glutamine respectively (59, 60).

Though the β-barrel structure of the CspA family is well conserved in CspH, it shows some unique features like having four aromatic amino acids in its peptide sequence with a high isoelectric point of ~10.72, compared to other CspA homologues found in E. coli which have eight aromatic residues in their amino acid sequence and a low isoelectric point ranging from 5 to 7.5. Thus it is possible that CspH might preferentially bind DNA than RNA (60).

Expression of cold shock genes at lower temperatures is regulated at the level of transcription, mRNA stability and translation, through regulatory elements which include Downstream Box (DB), UP element and Cold Box in the 5’-UTR (59-61). In Salmonella
enterica Serovar Typhimurium, cspH gene is cold shock inducible and is expressed at 37°C. Its cspH mRNA contains a short 23bp 5’-UTR and a well conserved 14-base DB sequence. However as there is only 51.2% homology in the promoter regions of cspH in Salmonella and E.coli, the expression of this gene in E. coli might be different. But amino acid sequences showed a homology of 84.3% indicating that the cspH protein function might be similar in both strains (60, 62). The promoter region of cspH also possesses a consensus sequence for heat shock genes particularly at -10 region though an induction in its expression at high temperatures is not observed (60).

Expression of cspH was induced in CFT073 during 0.6 M urea stress in microarray experiments, with an average expression ratio of 8.4 in urea vs control (34). Expression of cspH was induced 57 fold in asymptomatic bacteriuria E. coli strains 83972 and VR50 during biofilm growth compared to planktonic growth in urine (53). Overexpression of cspH and ibpAB genes was also observed in the antibiotic resistant bacterial persisters which exhibit multidrug tolerance (63).

**RpoS sigma factor**

Alternative sigma factor, RpoS is one of the bacterial transcription initiation factors that directs the RNA polymerase to bind to specific gene promoters. It expected to regulate the expression of about 10% genes in E. coli that are involved in stress response and providing tolerance against conditions like osmotic stress, acid stress, oxidative stress, during stationary phase and non-optimal growth conditions (22, 64).
Overview of the current project

Role of *bax*, *ibpAB* and *cspH* genes in CFT073 to provide protection against salt and urea stress was studied in the current project. First conservation of each gene among 3 different UPEC strains and in MG1655 (K-12 strain) was examined and GFP transcriptional fusion plasmids were constructed to test if the expression of these genes is induced in CFT073 during high levels of salt or urea. Role of RpoS sigma factor in regulating the expression of these genes during salt or urea stress was also investigated. Based on these results, growth curve experiments were performed with *bax*, *ibpAB* and *cspH* knockouts of CFT073 and/or BW25113 (K-12) under salt or urea stress to determine if these strains show any reduction in salt or urea tolerance compared to their respective wild type strains.
HYPOTHESIS AND SPECIFIC AIMS

Hypothesis: In CFT073, expression of \textit{bax} is up regulated under salt stress and deletion of \textit{bax} will reduce the salt tolerance of CFT073 and BW25113 (K-12). Expression of \textit{ibpAB} and \textit{cspH} is induced during urea stress and lack of either of these genes will lead to reduced urea tolerance of CFT073 and/or BW25113.

Specific aim 1: Test if expression of \textit{bax} is induced under salt stress in CFT073 and if lack of \textit{bax} will reduce the salt tolerance of CFT073 and BW25113.

- Aim 1a) Examine the sequence conservation of \textit{bax} and its promoter region in CFT073, UTI89, \textit{E. coli} 536 and K-12 strains.
- Aim 1b) Test if expression of \textit{bax} is induced under salt stress and if RpoS regulates the expression of \textit{bax} in CFT073.
- Aim 1c) Test if deletion of \textit{bax} gene reduces the salt tolerance of BW25113 and CFT073.

Specific aim 2: Test if expression of \textit{ibpAB} operon is induced under urea stress in CFT073 and if lack of \textit{ibpAB} operon will reduce the urea tolerance of CFT073 and BW25113.

- Aim 2a) Examine the sequence conservation of \textit{ibpAB} operon and its promoter region in CFT073, UTI89, \textit{E. coli} 536 and K-12 strains.
- Aim 2b) Test if expression of *ibpAB* operon is induced under urea stress and if RpoS regulates the expression of *ibpAB* operon in CFT073.

- Aim 2c) Test if deletion of *ibpAB* operon reduces the urea tolerance of CFT073 and BW25113.

**Specific aim 3:** To test if expression of *cspH* is induced under urea stress in CFT073 and if lack of *cspH* leads to reduced urea tolerance of BW25113.

- Aim 3a) Examine the sequence conservation of *cspH* and its promoter region in CFT073, UTI89, *E. coli* 536 and K-12 strains.

- Aim 3b) Test if expression of *cspH* is induced under urea stress and if RpoS regulates the expression of *cspH* in CFT073.

- Aim 3c) Test if deletion of *cspH* reduces the urea tolerance of BW25113.
MATERIALS AND METHODS

**Bacterial strains, plasmids and media.** All strains and plasmids used in the current study are listed in Tables 3 & 4. The media used include liquid LB medium (10 g/liter tryptone, 10 g/liter NaCl, and 5 g/liter yeast extract), 1.5% LB agar plates, K medium (1 mM KH$_2$PO$_4$, 1 mM (NH$_4$)$_2$SO$_4$, 0.08 mM MgCl$_2$ · 6H$_2$O, 1.8 µM Fe$_2$SO$_4$ · 7H$_2$O, 0.5% casamino acids, and 10 mM glucose) (37), 1.5% K medium agar plates and M9 medium (47.7 mM Na$_2$HPO$_4$·7H$_2$O, 22 mM KH$_2$PO$_4$, 8.55 mM NaCl, 18.7 mM NH$_4$Cl, 2 mM MgSO$_4$, and 0.1 mM CaCl$_2$ per liter supplemented with 0.2% casamino acids and 0.4% or 22.2 mM glucose) (65, 66). Kanamycin (50 µg/ml), ampicillin (200 µg/ml) and chloramphenicol (35 µg/ml) were added to the medium and LB agar plates when necessary.

**Primer Design.** All the oligonucleotide primers used in the present project are listed in Table 5. They were designed using IDT OligoAnalyzer and FastPCR. Primer specificity was checked using NCBI Primer-BLAST and primers were obtained from Integrated DNA technologies (Coralville, IA).

**Plasmid construction.** Taq 2X Master Mix, T4 DNA ligase and restriction enzymes were obtained from New England Biolabs (Beverly, MA). Purification of plasmid DNA, genomic DNA and PCR products; and gel extractions were performed using spin column kits from Qiagen (Hilden, Germany). For the construction of bax, ibpAB and cspH transcriptional fusion plasmids E. cloni 10G chemically competent cells
(Lucigen, Middleton, WI) was used as the host strain. All the remaining transformations were performed directly into CFT073 using ligated DNA without any intermediate host.

**pBax, pIbpAB, pCspH:** CFT073 genomic DNA was used as a template to amplify \textit{bax} promoter region using Bax-GFP-Fwd and Bax-GFP-Rev primers; \textit{ibpAB} promoter region using IbpAB-GFP-Fwd and IbpAB-GFP-Rev primers; and \textit{cspH} promoter region using CspH-GFP-Fwd and CspH-GFP-Rev primers with the help of Taq 2X Master mix (New England Biolabs). The amplified PCR products were digested with BamHI and XhoI, and cloned into pUA139 vector (67) previously linearized with the same restriction enzymes. The ligated DNA was used to transform \textit{E. coli} 10G chemically competent cells (Lucigen) according to the manufacturer’s instructions. The plasmid DNA was isolated and the inserted promoter regions upstream GFPmut2 were sequenced using Seq-primer through CGR sequencing facility (Wright State University, Dayton, OH).

**pAL139-CspH:** The copy number of \textit{cspH} promoter fusion plasmid was increased from \(~5\) to 50-70 copies per cell by replacing pSC101 origin of replication in pCspH plasmid to CoIE1 origin from pZE21 plasmid (68). Plasmids, pZE21 and pCspH were digested with BglII and AvrII. The DNA fragment containing \textit{cspH} promoter region and GFP from pCspH plasmid was gel extracted and ligated to the gel extracted DNA fragment containing CoIE1 origin and kanamycin resistance marker from pZE21 plasmid.

**pAL139-IbpAB:** Promoter region of \textit{ibpAB} is amplified from CFT073 genomic DNA using IbpAB-GFP-Fwd and IbpAB-GFP-Rev primers and digested with XhoI and BamHI. Plasmid pAL139-CspH is also digested with BamHI and XhoI and the pAL139
vector was gel extracted. The digested \textit{ibpAB} promoter region was cloned into the gel extracted pAL139 vector to produce pAL139-\textit{IbpAB} plasmid.

\textbf{pAL139 vector:} 5’ phosphorylated primers, pAL139-Fwd and pAL139-Rev each at a final concentration of 10 ng/µl are added to 50 µl of 1X T4 DNA ligase buffer. The sample was kept at 95°C for 2 min and cooled to 25°C over a period of 45 min for the oligos to anneal. The annealed oligos form a 24 bp insert with BamHI and XhoI sticky sites on opposite ends, which was subsequently cloned into BamHI and XhoI digested pCspH-HC plasmid (after separating the \textit{cspH} promoter region through gel extraction) using T4 DNA ligase.

pDOC-Bax: Bax-Null-Fwd and Bax-Null-Rev primers were used to amplify kanamycin resistance cassette from BW25113 \textit{Δbax} genomic DNA using Taq 2X Master Mix. The amplified PCR product contained the following regions:

| EcoRI site – H1 – GACCGGTCAATTGGCTGGAG – Flp1 site – Kanamycin cassette – Flp 2 site – GGAACTAAGGAGGATATT - H2 - NheI site |
| H1: 35 nt of sequence homologous to the \textit{bax} start codon and 32 nt upstream to it. |
| H2: 52 nt of sequence homologous to the 21 nt of \textit{bax} C-terminal region, \textit{bax} stop codon and 28 nt downstream to it. |

The PCR product was digested with EcoRI and NheI and cloned into pDOC-C linearized with EcoRI and NheI restriction enzymes.

pDOC-IbpAB: IbpAB-Null-Fwd and IbpAB-Null-Rev primers were used to amplify kanamycin resistance cassette from pKD13 plasmid (69) using Taq 2X Master Mix. The amplified PCR product contained the following regions:
EcoRI site – H1 – GACCGGTCAATTGGCTGGAG – Flp1 site – Kanamycin cassette – 
Flp 2 site – GGAACAAAGGGGATATT - H2 - NheI site

H1: 28 nt of sequence homologous to the ibpA start codon and 25 nt upstream to it.
H2: 28 nt of sequence homologous to the 3 nt of ibpB C-terminal region of, ibpB stop 
codon and 22 nt downstream to it. The PCR product was cloned into pDOC-C vector to 
construct pDOC-IbpAB plasmids using EcoRI and NheI restriction sites.

**Electroporation.** All strains were transformed through electroporation during the 
current project except *E. cloni* 10G chemically competent cells.

**Preparation of electrocompetent cells:** A single fresh colony of the desired strain 
was inoculated in 50 ml LB medium in a 500 ml flask and grown with vigorous aeration 
overnight at 37°C. 100 ml of LB in 500 ml flask was inoculated with 0.1 ml of the 
overnight grown culture and grown at room temperature with vigorous aeration till the 
culture reached an OD$_{600}$ of 0.4 – 0.6 OD. The culture was kept on ice for 25 minutes 
and the cells were harvested by centrifugation at 2600 x g for 15 minutes. Cell pellet was 
washed twice with ice cold 10% glycerol and resuspended in 1 ml of ice cold 10% 
glycerol. The 25 µl aliquots were made for the resuspended cells, snap frozen and stored 
at -80°C.

**Electroporation:** CFT073, DH5α and BW25113 were transformed with ligated 
DNA or plasmid sample through electroporation using Cell-Porator and Voltage booster 
according to the manufacturer’s instructions (GIBCO/BRL). Briefly 1.25 µl of the ligated 
DNA or plasmid sample was added to 25 µl of electrocompetent cells and suspended 
between the electrode bosses of 0.15 cm micro-electroporation chamber. An electric 
pulse was applied with a voltage of 2.4 kV and pulse length of 6 milliseconds. 1 ml of
SOC medium was added to the cells and incubated at 37°C with gentle shaking for 1 hour. 50 - 200 µl of culture was plated on LB agar plate with appropriate antibiotic to select for transformants.

**Construction of null mutants.** Gene doctoring method (70) was used to construct null mutants of CFT073 and BW25113. Figure 3.1 describes the construction of CFT073 Δbax using gene doctoring method. Briefly, a sucrose sensitive colony of CFT073 or BW25113 containing pACBSCE and pDOC-Bax or pDOC-IbpAB plasmids was grown in 1 ml LB medium supplemented with kanamycin (50 µg/ml), ampicillin (200 µg/ml), chloramphenicol (35 µg/ml) and glucose (0.5%) for 2 hours at 37°C. The cells were harvested and resuspended in 1 ml of fresh LB medium containing 0.5% L-arabinose and grown with vigorous aeration at 37°C for 5 hours. Dilutions of the culture were plated on LB agar plate containing kanamycin (50 µg/ml), 6% sucrose and no NaCl. The isolated colonies were checked for the loss of pACBSCE and pDOC-Bax / pDOC-IbpAB plasmid by patching onto LB plates containing chloramphenicol (35 µg/ml) and ampicillin (200 µg/ml) respectively. Nine colonies, which were sensitive to ampicillin and chloramphenicol but resistant to sucrose and kanamycin, were further screened through colony PCR. Colony PCR was performed using a forward primer (Bax-GFP-Fwd, IbpAB-GFP-Fwd, CspH-GFP-Fwd) which binds upstream of the target region in CFT073 or BW25113 genome and the reverse primer (K1) that binds inside the kanamycin cassette. The colony which gave desired PCR product was further tested through additional colony PCR reactions with 3 different primer pairs as described below to confirm the knockout.
1. Forward primer which binds inside the kanamycin cassette (K2) and reverse primer (Bax-ORF-Rev, IbpAB-ORF-Rev) which binds downstream of the target region in CFT073 or BW25113 genome.

2. Forward (Bax-GFP-Fwd, IbpAB-GFP-Fwd) and reverse primers (Bax-ORF-Rev, IbpAB-ORF-Rev) bind upstream and downstream of the target region respectively in CFT073 or BW25113 genome.

3. Forward (Bax-RT-Fwd, IbpA-RT-Rev, CspH-RT-Fwd) and reverse primer (Bax-qPCR-Rev, IbpA-qPCR-Rev, CspH-RT-Rev) bind inside the bax, ibpA or cspH open reading frames and amplify a small region of 74-82 bp.

Colony PCR with 2\textsuperscript{nd} and 3\textsuperscript{rd} primer pairs was performed to confirm that the cells did not retain the knocked out gene as a result of partial duplication in bacterial genome (71, 72).

During the construction of ibpAB null mutants of CFT073 and BW25113, pACBSR plasmid (73) was used instead of pACBSCE plasmid, with a procedure being described above. Plasmid pACBSR is similar to pACBSCE plasmid but with no SceI restriction enzyme site. Thus the plasmid was retained in the final recombinant, which was cured or removed through electroporation (74, 75).

**Colony-PCR.** A colony was picked using a sterile pipet tip and suspended in 20 µl of DNase/RNase-Free water. The colony suspension was kept at 100°C for 10 minutes, then on ice for 2 min and centrifuged at maximum speed (18000 x g) for 5 minutes. 3 µl of the supernatant was added to 10 µl of Taq 2X Master Mix, 1 µl of forward primer (10 µM, 1 µl of reverse primer (10 µM) and the volume was made upto 20 µl with sterile water. PCR was performed using the appropriate annealing temperature and elongation time depending upon the melting temperature of the primers and PCR product size.
**Promoter activity assay.** A fresh single colony was inoculated into 3 ml of LB medium and grown till saturation. 50 µl of the saturated culture was used to inoculate 5 ml of K medium and incubated over night at 37°C. The culture was diluted in 200 µl of fresh K medium to a final OD$_{600}$ of 0.1 and grown in microplate reader (FLUOstar OPTIMA) with shaking (orbital, 7 mm) till the cells reached an OD$_{600}$ of 0.3. The cells were diluted at 1:20 ratio in fresh K medium to a total volume of 200 µl and grown in the microplate reader with absorbance (OD$_{600}$) and fluorescence (Ex. 485 nm, Em. 530 nm) measurements taken every 12 minutes. When the cells reached an OD$_{600}$ of ~0.1, NaCl, urea or sterile water were added to the appropriate wells, and absorbance and fluorescence measurements were continued for the next 6-8 hours. Measurements were blank corrected and graphs were plotted using Microsoft Excel. LB or K medium was supplemented with kanamycin (50 µg/ml) when necessary. For each condition, experiments were performed in triplicate.

**Growth curve experiments.** The protocol used for the growth curve experiments was similar to that described for promoter activity assays, except for taking only absorbance measurements (OD$_{600}$) while the cells were growing in the microplate reader. For each condition, experiments were performed in triplicate.

**Streak plate experiments:** A single fresh colony of the desired strain was picked with a sterile loop and streaked on K medium 1.5% agar plates containing no urea, 0.3 M urea or 0.6 M urea. The plates were incubated at room temperature, as urea degrades at higher temperatures producing toxic products (76). Digital and fluorescence images were taken after 48 and 96 hours of incubation to determine the growth and fluorescence of the cells.
Construction of *E. coli* knockout strains using gene doctoring method

CFT073 Genome

Figure 3.1: Gene doctoring method to construct *bax* null mutant of CFT073. CFT073 wild type strain is transformed with pDOC-Bax plasmid (with kanamycin resistance cassette flanked by 35 and 52 nt of *bax* upstream and downstream homologous regions respectively) and pACBSCE plasmid. The culture was treated with 0.5% L-arabinose in the exponential phase to induce the expression of I-SceI restriction enzyme and λ-Red gene products. I-SceI restriction enzyme liberates the DNA fragment containing kanamycin resistance cassette and *bax* flanking regions from the pDOC-Bax plasmid, that later recombines with CFT073 genome using λ-Red system. An aliquot of the culture was plated on LB kanamycin plate supplemented with 6% sucrose and incubated overnight at 30°C. The colonies that grew were tested for ampicillin and chloramphenicol sensitivity and screened by colony-PCR for the true recombinant.
<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>F⁻ endA1 hsdR17 (rK−, mK−) supE44 λ- thi-1 recA1 gyrA96 relA1 Δ(argF- lacZYA) U169 y80d lacZD15</td>
<td>Lab strain</td>
</tr>
<tr>
<td><em>E. coli</em> 10G</td>
<td>F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) endA1 recA1 Δ80dlacZΔM15 ΔlacX74 araD139 Δ(ara,leu)7697 galU galK rpsL (StrR) nupGΔ tonA</td>
<td>Commercial (Lucigen)</td>
</tr>
<tr>
<td>CFT073</td>
<td>Wild-type pyelonephritis isolate</td>
<td>(77)</td>
</tr>
<tr>
<td>CFT073 ΔrpoS</td>
<td>CFT073 ΔrpoS2062</td>
<td>(22)</td>
</tr>
<tr>
<td>CFT073 Δbax</td>
<td>CFT073 bax::kan, Kan↑</td>
<td>This study</td>
</tr>
<tr>
<td>CFT073 ΔibpAB</td>
<td>CFT073 ibpAB::kan, Kan↑</td>
<td>This study</td>
</tr>
<tr>
<td>BW25113</td>
<td>Δ(araD-araB)567, ΔlacZ4787(::rmB-3), lambda-, rph-1, Δ(rhaD-rhaB)568, hsdR514</td>
<td>Keio strain (80)</td>
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<tr>
<td>BW25113 Δbax</td>
<td>BW25113 bax::kan, Kan↑</td>
<td>Keio strain (80)</td>
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<tr>
<td>BW25113 ΔibpAB</td>
<td>BW25113 ibpAB::kan, Kan↑</td>
<td>This study</td>
</tr>
<tr>
<td>BW25113 ΔcspH</td>
<td>BW25113 cspH::kan, Kan↑</td>
<td>Keio strain (80)</td>
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Table 3: Strains used
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<tr>
<th>Plasmids</th>
<th>Description</th>
<th>Reference or source</th>
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<td>pUA139</td>
<td>GFP transcriptional fusion plasmid, promoter less, pSC101 ori, Kan&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(67)</td>
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<td>pBax</td>
<td>GFP transcriptional fusion plasmid for bax promoter, pSC101 ori, Kan&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pIbpAB</td>
<td>GFP transcriptional fusion plasmid for ibpAB operon promoter, pSC101 ori, Kan&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<td>pCspH</td>
<td>GFP transcriptional fusion plasmid for cspH promoter, pSC101 ori, Kan&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>pZE21</td>
<td>GFPaav reporter plasmid, ColE1 ori, Kan&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>This study</td>
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<td>pAL139-CspH</td>
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<td>This study</td>
</tr>
<tr>
<td>pAL139-IbpAB</td>
<td>GFP transcriptional fusion plasmid for ibpAB operon promoter, ColE1 ori, Kan&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<td>pBax-ORF</td>
<td>Encodes bax gene under the control of its native promoter, pSC101 ori, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<td>pIbpAB-ORF</td>
<td>Encodes ibpAB operon under the control of its native promoter, pSC101 ori, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>pDOC-Bax</td>
<td>Kanamycin cassette flanked by bax upstream and downstream homologous regions, SacB, I-SceI site, pMBI ori , oriT, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>pDOC-IbpAB</td>
<td>Kanamycin cassette flanked by ibpA upstream and ibpB downstream homologous regions, SacB, I-SceI site, pMBI ori , oriT, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pACBSR</td>
<td>λ-Red recombinase, endonuclease I-SceI, Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(73)</td>
</tr>
<tr>
<td>pACBSCE</td>
<td>λ-Red recombinase, endonuclease I-SceI, I-SceI site, Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(70)</td>
</tr>
<tr>
<td>pDOC-C</td>
<td>Cloning plasmid for Gene doctoring system, pMBI ori , oriT, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(70)</td>
</tr>
<tr>
<td>pKD13</td>
<td>Template plasmid used to construct pDOC-IbpAB, FRT-flanked Kan&lt;sup&gt;r&lt;/sup&gt;, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(69)</td>
</tr>
</tbody>
</table>

Table 4: Plasmids used
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
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<tbody>
<tr>
<td>Bax-GFP-Fwd</td>
<td>ACTCGTGGATCCTTTATTAGAACCCGGAGAACGTC</td>
</tr>
<tr>
<td>Bax-GFP-Rev</td>
<td>ATCAGCCTCGAGTTATTATGTTGCTGCTTCGCC</td>
</tr>
<tr>
<td>IbpAB-GFP-Fwd</td>
<td>ATCGTTGGATCCTTTATTAGGTATGGGACATCAGCT</td>
</tr>
<tr>
<td>IbpAB-GFP-Rev</td>
<td>GCTCGCGGATCCTTTATTAGGTATGGGACATCAGCT</td>
</tr>
<tr>
<td>CspH-GFP-Fwd</td>
<td>GCTCGCGGATCCTTTATTAGGTATGGGACATCAGCT</td>
</tr>
<tr>
<td>CspH-GFP-Rev</td>
<td>GCTATTTCTCAGTTATTCGTAATTCTGTGATAAGCAC</td>
</tr>
<tr>
<td>Seq-primer</td>
<td>CACCTTACCCCTCTCCACGACAG</td>
</tr>
<tr>
<td>Seq-GFP</td>
<td>TACTCAGGAGAGCGTTCACC</td>
</tr>
<tr>
<td>Fwd-pAL139 (5' phosphorylated)</td>
<td>GATCCGATCTCTAGAATTCAGCAGTCTTC</td>
</tr>
<tr>
<td>Rev-pAL139 (5' phosphorylated)</td>
<td>TCGAGAAGTGAAGCTAATTAGAGATCG</td>
</tr>
<tr>
<td>Bax-ORF-Rev</td>
<td>TACATCTCTAGACGCTACGTAACTCAGCAC</td>
</tr>
<tr>
<td>IbpAB-ORF-Rev</td>
<td>ATACATCTCTAGACGCTACGTAATTAGAGATCG</td>
</tr>
<tr>
<td>Bax-Null-Fwd</td>
<td>ATGTTAGAATTCGTTGGAATGATTAAATCCGACC</td>
</tr>
<tr>
<td>Bax-Null-Rev</td>
<td>GACTATCGTACGCGTACAGTCCGATAAGGCTCA</td>
</tr>
<tr>
<td>IbpA-Null-Fwd</td>
<td>ACTTATGAATTCTCAGCTTTACAGGAGCTATTGATTA TGATCCGCGGGATCCGAGGCTTCAC</td>
</tr>
<tr>
<td>IbpB-Null-Rev</td>
<td>GACTATGCTAGCGCGGCGCGAAGAGAATGCTAGTTA GCTGTAGCAGAGGCAGGTGCTGCTCG</td>
</tr>
<tr>
<td>K1 (69)</td>
<td>CAGTCATAGCCGAATAGCCT</td>
</tr>
<tr>
<td>K2 (69)</td>
<td>CGGTGCCCTGAATGAACTGCGC</td>
</tr>
<tr>
<td>Bax-RT-Fwd</td>
<td>CAATACGGCACCAGGCTTACT</td>
</tr>
<tr>
<td>Bax-RT-Rev</td>
<td>TGTGGCAGTCCATTCCTGTGATC</td>
</tr>
<tr>
<td>IbpA-RT-Fwd</td>
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<tr>
<td>IbpA-RT-Rev</td>
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<tr>
<td>CspH-RT-Fwd</td>
<td>TCCGACGGTCGTAAGAAGTTC</td>
</tr>
<tr>
<td>CspH-RT-Rev</td>
<td>CGCGTAAATCCTGGTATAAGCAC</td>
</tr>
</tbody>
</table>

*Table 5: Primers used.* *Restriction sites are underlined.*
RESULTS

Aim 1:

Conservation of *bax* and its promoter region in K-12 and 3 different UPEC strains

Presence of *bax* was identified in 3 different UPEC strains, CFT073 (pyelonephritis isolate) (77), UTI89 (cystitis isolate) (78) and *E. coli* 536 (pyelonephritis isolate) (79) and in commensal *E. coli* MG1655 (K-12 strain) using BioCyc database as shown in the Figure 4.1.

(CFT073, accession NC_004431.1; UTI89, accession NC_007946.1; *E. coli* 536, accession NC_008253.1; MG1655, accession U00096.2)

Figure 4.1: Image showing *bax* and its upstream and downstream regions in MG1655, *E. coli* 536, CFT073 and UTI89 genomes. The image was generated using Multi-Genome Browser in BioCyc database (Image not to scale).
Bax is a hypothetical protein, whose expression and function have not been studied in detail. Though sigma 70 regulated promoter region has been identified for bax with transcriptional start sites located at -57 nt and -44 nt upstream to the start codon of bax ORF, it was based on computational analysis with no experimental evidence (39). Between bax and malS there is an ORF for a hypothetical protein, the existence of which is questionable. Hence for the present study, the intergenic region between bax and malS along with 50 - 100 bp upstream and downstream to it was chosen to contain the bax promoter region (67).

Before designing primers to amplify bax promoter region, nucleotide sequences containing bax ORF and 400 nt upstream to it were collected for MG1655, CFT073, E. coli 536 and UTI89 strains from BioCyc database and multialigned using ClustalW. The results (Figure 4.1.2) showed pairwise scores ranging from 98-100%. The 1-2% single nucleotide changes in the bax ORF among these 4 strains were silent mutations with no change in the protein sequence, indicating that bax is a well conserved gene and its expression and function might be similar among these 4 strains.

**Expression of bax is induced under salt stress and is not regulated by RpoS sigma factor in CFT073**

The GFP transcriptional fusion plasmid for bax promoter region was constructed by amplifying -394 nt to +96 nt relative to the bax start codon from CFT073 genomic DNA and cloning into pUA139 vector, upstream from the strong ribosome binding site of GFPmut2. The plasmid was transformed into CFT073 and promoter activity assays were
performed as described in the Methods section. CFT073 transformed with pUA139 plasmid (promoter less GFP) was used as a negative control during these experiments.

To determine if the expression of bax is induced during salt stress, CFT073 strain containing pBax plasmid was grown in a microplate reader untill exponential phase and 0.2 M and 0.3 M NaCl were added. The growth was continued in the microplate reader with absorbance and fluorescence measurements taken every 12 minutes. The measurements were blank corrected and graphs of RFU, OD$_{600}$ and RFU/ OD$_{600}$ against time were plotted. Results (Figure 4.3) suggest that the expression of GFP (regulated by bax promoter) was induced significantly in CFT073 due to the addition of 0.2 M and 0.3 M NaCl to the K medium and the level of induction was proportional to the amount of NaCl added to the medium.

In the current project, the role of RpoS in regulating the expression of bax during osmotic upshift was studied by transforming rpoS null mutant of CFT073 (kind gift from Dr. Janet Wood, University of Guelph, Canada) with pBax plasmid and performing the promoter activity assays as described above. Results (Figure 4.4) showed that the expression of GFP from pBax plasmid in CFT073 wild type and ΔrpoS null mutant was similar with no difference in the level of GFP expression before and after the addition of 0.3 M NaCl to the K medium. These indicate that the expression of bax during salt stress is not regulated by RpoS sigma factor in CFT073.
Figure 4.2: The multialignment results of bax ORF and 400 nt upstream of it in CFT073, MG1655, UTI89 and E. coli 536 genomes. The multialignment results were represented with the sequence of CFT073; nucleotides that differed in at least 2 of the 4 strains a indicated as uncolored bases. The forward and reverse primers (Bax-GFP-Fwd and Bax-GFP-Rev) used to amplify bax promoter region from CFT073 genome are underlined. ORF of bax is shown as a white text in the above sequence from 401 nt to 1225 nt. Image was created using ClustalW multialignment results and Jalview.
Figure 4.3: Expression of GFP from *bax* promoter in pBax plasmid during salt stress in K medium. CFT073 strain containing pBax plasmid or pUA139 vector plasmid (negative control) were grown in microplate reader in K medium till exponential phase and 0.2 M NaCl, 0.3 M NaCl or equal volumes of sterile water were added. The absorbance (OD$_{600}$) and fluorescence measurements were taken every 12 minutes before and after the addition of NaCl and graphs of RFU, OD$_{600}$ and RFU/ OD$_{600}$ against time were plotted. (A) RFU/ OD$_{600}$, (B) RFU and (C) OD$_{600}$ measurements. Each data point represent mean ± SEM of 3 independent experiments from one culture.
Figure 4.4: Effect of RpoS on bax promoter activity during salt stress in CFT073. CFT073 and CFT073 ΔrpoS strains transformed with pBax plasmid were grown in microplate reader till exponential phase in K medium and 0.3 M NaCl or equal volumes of sterile water were added. CFT073 (pUA139 vector) was used as a negative control and treated similar to other cultures. The absorbance and fluorescence measurements were taken every 12 minutes before and after the addition of NaCl and graphs were plotted. (A) RFU/ OD$_{600}$, (B) RFU and (C) OD$_{600}$ measurements. Each data point represent mean ± SEM of 3 independent experiments from one culture.
Deletion of *bax* reduced the salt tolerance of BW25113 but had no effect on CFT073

In the current project, the role of *bax* in providing salt tolerance was compared in CFT073 and BW25113 (K-12 strain) by using *bax* null mutant of each strain. CFT073 null mutant of *bax* was constructed using gene doctoring method (Lee et al. 2009) as described in methods section. BW25113 Δ*bax* and BW25113 Δ*cspH*, Keio strains (80) were obtained as a kind gift from Dr. Gyaneshwar Prasad (University of Wisconsin, Milwaukee). Colony PCR was performed using at least 2 different primers pairs (K1, K2, Bax-GFP-Fwd, Bax-ORF-Rev, Bax-RT-Fwd and Bax-RT-Rev) to confirm the *bax* knockout in CFT073 (Figure 4.5) and BW25113 (Figure 4.6) as described in the Methods section.

It was noticed that BW25113 shows a growth defect in K medium. Therefore growth curve experiments to study the salt tolerance of CFT073 and BW25113 *bax* null mutants were performed in M9 minimal medium which has been previously used to study osmotic systems in BW25113. The osmolality of M9 medium used in the current project would be around 240 mosmol/kg H$_2$O (66, 81, 82). Results (Figure 4.7) showed that BW25113 Δ*bax* has reduced salt tolerance compared to its wild type strain in the presence of 0.6 M NaCl in M9 medium. However CFT073 wild type and its *bax* null mutant showed similar salt tolerance in M9 medium with 0.6 M NaCl.

The growth curve experiments were repeated in K medium for CFT073 and CFT073 Δ*bax* using 0.6 M NaCl. But once again no difference was observed in the salt tolerance of CFT073 wild type and its *bax* null mutant (Figure 4.8).
Figure 4.5: Confirmation of bax knockout in CFT073 by colony PCR. Colony PCR was performed for CFT073 Δbax and CFT073 WT strains, using 4 different primers pairs (K1, K2, Bax-GFP-Fwd, Bax-ORF-Rev, Bax-RT-Fwd and Bax-RT-Rev) as shown by the graphic images above the electrophoresis gel images. Lane M shows DNA ladder, Lane 1 shows the colony PCR reaction results of CFT073 WT and Lane 2 shows the colony PCR reaction results of CFT073 Δbax (Graphic images are not to scale).
Figure 4.6: Confirmation of bax knockout in BW25113 (Keio strain) by colony PCR. Colony PCR was performed for BW25113 Δbax and BW25113 WT strains using 2 different primer pairs (K1, Bax-GFP-Fwd, Bax-RT-Fwd, Bax-RT-Rev) as shown by the graphic images above the electrophoresis gel images. **Lane M** shows DNA ladder, **Lane 1** shows the colony PCR reaction results of BW25113 wild type and **Lane 2** shows the colony PCR reaction results of BW25113 Δbax (Graphic images are not to scale).
Figure 4.7: Effect of \textit{bax} knockout on the growth rate of CFT073 and BW25113 during salt stress in M9 medium. 0.6 M NaCl or equal volumes of sterile water were added to exponentially growing wild type and the \textit{bax} knockout strains of BW25113 (A) and CFT073 (B) in M9 medium. The absorbance measurements (OD$_{600}$) were taken every 12 minutes before and after the addition of NaCl and graphs were plotted. Error bars represent mean ± SEM among 3 replicates of each culture.
Figure 4.8: Effect of bax knockout on the growth rate of CFT073 during salt stress in K medium. NaCl (0.6 M) or equal volumes of sterile water were added to exponentially growing wild type and bax null mutant of CFT073 in K medium. The absorbance measurements (OD$_{600}$) were taken every 12 minutes before and after the addition of NaCl and graphs were plotted. Each data point represent mean ± SEM of 3 independent experiments from one culture.
Aim 2:

Conservation of *ibpAB* operon in K-12 and 3 different UPEC strains (CFT073, UTI89 and *E. coli* 536)

Operon, *ibpAB* is present in MG1655 (K-12), CFT073, UTI89 and *E. coli* 536, which was identified using BioCyc database as shown by the Figure 5.1. There is a difference in the annotation of start codon for *ibpA* and *ibpB* ORF among these 4 strains in BioCyc database. For CFT073 and UTI89 *ibpA* ORF starts from TTG codon and for MG1655 and *E. coli* 536, it starts from ATG codon (Figure 5.2). The distance between these 2 codons is only 3 bp. For *ibpB*, the ORF starts with first ATG start codon for CFT073 and second ATG start codon for the remaining 3 strains. The distance between these 2 ATG start codons is 3 bp (Figure 5.2).

**Figure 5.1:** Image showing *ibpAB* and its upstream and downstream regions in MG1655, *E. coli* 536, CFT073 and UTI89 genomes. The image is generated using Multi-Genome Browser in BioCyc database (Image not to scale).
To test the sequence conservation of *ibpAB* operon among these 4 strains, the nucleotide sequence of *ibpA* and *ibpB* open reading frames along with the intergenic region between them plus 400 - 406 nt upstream to the start codon of *ibpA* was collected from BioCyc database for CFT073, UTI89, *E. coli* 536 and MG1655 strains, and multialigned using ClustalW. The results showed pairwise score of 99% (Figure 5.2). The 1% single nucleotide changes were silent mutations with no change in the IbpA and IbpB protein sequences. Based on the multialignment results, it is clearly evident that the *ibpAB* operon is well conserved among the 3 UPEC and K-12 strain indicating a high possibility of similar expression and function among the 4 strains.

Because *ibpAB* operon and its expression is well studied, the region -383 nt to +174 nt relative to the start codon of *ibpA* ORF in CFT073 genome was amplified using IbpAB-GFP-Fwd and IbpAB-GFP-Rev primers and cloned into pUA139 vector upstream to the ribosome binding site of GFPmut2. The resulting plasmid is named pIbpAB plasmid. The amplified region contains the sigma 32 regulated promoter region of *ibpAB* operon, which includes -10 and -35 regions, and transcriptional start site located -90 nt relative to the start codon of *ibpA* ORF in CFT073.
Figure 5.2: The multialignment results of *ibpA* and *ibpB* ORFs, intergenic region and 400-406 nt upstream to the start codon of *ibpA* in CFT073, MG1655, UTI89 and *E. coli* 536 genomes. The multialignment results were represented with the sequence of CFT073; nucleotides that differed in at least 2 of the 4 strains are indicated as uncolored bases. The forward and reverse primers (IbpAB-GFP-Fwd and IbpAB-GFP-Rev) used to amplify *ibpAB* promoter region from CFT073 genome are also indicated with red underline. Sequence in white text from 401 nt - 820 nt is *ibpA* ORF and 926 nt - 1360 nt is *ibpB* ORF. The 2 start codons for *ibpA* and *ibpB* ORFs are underlined in green.
Expression of *ibpAB* is induced during urea stress and is not regulated by RpoS sigma factor in CFT073

To determine *ibpAB* promoter activity during urea stress, CFT073 strain was transformed with pIbpAB plasmid and promoter activity assays were performed as described in the Methods section using 0.3 M and 0.6 M urea. CFT073 with pUA139 plasmid was used as a negative control and treated similar to test cultures.

Results (Figure 5.3) indicate that *ibpAB* promoter activity in CFT073 was induced due to the presence of 0.3 M and 0.6 M urea in K medium. The induction in the promoter activity was higher in the presence of 0.6 M urea compared to 0.3 M urea. Hence, it can be concluded that the expression of *ibpAB* operon is induced due to the presence of urea in K medium and the rate of induction depends upon the extent of urea stress.

To test if expression of *ibpAB* operon is regulated by RpoS sigma factor, CFT073 Δ*rpoS* strain was transformed with pIbpAB plasmid and promoter activity assay was repeated. Results (Figure 5.4) showed no significant difference in the expression of GFP from pIbpAB plasmid in CFT073 wild type and *rpoS* null mutant in the absence or presence of urea stress suggesting that the expression of *ibpAB* is not regulated by RpoS sigma factor.
Figure 5.3: Expression of GFP from *ibpAB* promoter in pIbpAB plasmid during urea stress in K medium. CFT073 strains containing pIbpAB plasmid or pUA139 vector plasmid (negative control) were grown in microplate reader till exponential phase in K medium and 0.3 M urea, 0.6 M urea or equal volumes of sterile water were added. The absorbance (OD$_{600}$) and fluorescence measurements were taken every 12 minutes before and after the addition of urea and graphs were plotted. (A) RFU/OD$_{600}$, (B) RFU and (C) OD$_{600}$ measurements. Each data point represent mean ± SEM of 3 independent experiments from one culture.
Figure 5.4: Effect of RpoS on ibpAB promoter activity during urea stress in CFT073. CFT073 and CFT073 ΔrpoS strain with pIbpAB plasmid were grown in microplate reader till exponential phase in K medium and 0.3 M urea, 0.6 M urea or equal volumes of sterile water were added. CFT073 (pUA139 vector) was used as a negative control and treated similar to other cultures. The absorbance (OD_{600}) and fluorescence measurements were taken every 12 minutes before and after the addition of urea and graphs were plotted. (A) RFU/OD_{600}, (B) RFU and (C) OD_{600} measurements. Each data point represent mean ± SEM of 3 independent experiments from one culture.
Lack of *ibpAB* operon did not affect the urea tolerance of BW25113 and CFT073.

To determine the importance of *ibpAB* in providing protection against urea stress in *E. coli*, *ibpAB* null mutants of BW25113 and CFT073 were constructed using gene doctoring method (Lee et al. 2009) as described in the Methods section. The null mutants were confirmed through colony PCR using 4 different primer pairs (K1, K2, IbpAB-GFP-Fwd, IbpAB-ORF-Rev, IbpA-RT-Fwd and IbpA-RT-Rev) (Figure 5.5).

Because BW25113 showed reduced growth rate in K medium, growth curve experiments were performed in M9 medium using 0.9 M urea. Results (Figure 5.6) showed no difference in the growth rate of *ibpAB* null mutants compared to their respective wild type strains in the absence and presence of 0.9 M urea in M9 medium. Similar results were obtained when the experiment was repeated for CFT073 WT and its *ibpAB* null mutant in K medium (Figure 5.7).
Figure 5.5: Confirmation of *ibpAB* knockout in CFT073 and BW25113 by colony PCR. Colony PCR was performed for WT and *ibpAB* knockout strains of CFT073 and BW25113 using 4 different primers pairs (K1, K2, IbpAB-GFP-Fwd, IbpAB-ORF-Rev, IbpAB-RT-Fwd and IbpAB-RT-Rev) as shown by the graphic images above the electrophoresis gel images. Lane M shows DNA ladder, Lane 1 shows the colony PCR reaction results of CFT073 WT, Lane 2 for CFT073 Δ*ibpAB*, Lane 3 for BW25113 WT and Lane 4 for BW25113 Δ*ibpAB* (Graphic images are not to scale).
Figure 5.6: Effect of *ibpAB* knockout on the growth rate of CFT073 and BW25113 under urea stress in M9 medium. Urea (0.9 M) or equal volume of sterile water were added to exponentially growing wild type and *ibpAB* knockout strains of BW25113 (A) and CFT073 (B) in M9 medium. The absorbance (OD₆₀₀) measurements were taken every 12 minutes before and after the addition of urea and graphs were plotted. Each data point represent mean ± SEM of 3 independent experiments from one culture.
Figure 5.7: Effect of *ibpAB* knockout on the growth rate of CFT073 during urea stress in K medium. Urea (0.9 M) or equal volume of sterile water were added to exponentially growing wild type and the *ibpAB* null mutant of CFT073 in K medium. The absorbance (OD$_{600}$) measurements were taken every 12 minutes before and after the addition of urea and graphs were plotted. Each data point represent mean ± SEM of 3 independent experiments from one culture.
Aim 3:

Conservation of *cspH* gene in K-12 and 3 different UPEC strains (CFT073, UTI89 and *E. coli* 536)

Presence of *cspH* in MG1655 (K-12) and in UPEC strains CFT073, UTI89 and *E. coli* 536 was identified using BioCyc database (Figure 6.1). The expression and function of *cspH* has not been studied in detail. The presence of sigma 32 (heat shock sigma factor), sigma 24 (the extracytoplasmic / extreme heat stress sigma factor) and sigma 70 (housekeeping sigma factor) transcription initiation factor binding sites have been identified upstream of *cspH* ORF. However, experimental evidence is lacking to confirm that the expression of *cspH* is under the regulation of these sigma factors.

Figure 6.1: Image showing *cspH* and its upstream and downstream regions in MG1655, *E. coli* 536, CFT073 and UTI89 genomes. The image is produced using Multi-Genome Browser in BioCyc database. (Image not to scale)
To test the sequence conservation of \textit{cspH} among CFT073, UTI89, \textit{E. coli} 536 and K-12 strains, \textit{cspH} ORF and 400 nt upstream to the start codon of \textit{cspH} ORF was collected for these 4 strains from BioCyc database and multialigned using ClustalW. The results showed a pairwise score of 98-100% (Figure 6.2). There is no difference in the protein sequence among the 4 strains indicating that the 2% difference in nucleotide sequences were silent mutations. Thus \textit{cspH} is well conserved among these 4 strain and it’s expression and function might be similar among them.

**Figure 6.2: The multialignment results of \textit{cspH} ORF and 400 nt upstream to it in CFT073, MG1655, UTI89 and \textit{E. coli} 536 genomes.** The multialignment results were represented with the sequence of CFT073; with nucleotides that differed in at least 2 of the 4 strains as uncolored bases. The forward and reverse primers (CspH-GFP-Fwd and CspH-GFP-Rev) used to amplify \textit{cspH} promoter region from CFT073 genome are also indicated with red underline. Part of the sequence in white text from 401 nt – 613 nt is \textit{cspH} ORF. The 14 bp downstream box, which is located +13 nt relative to the start codon of \textit{cspH} ORF is indicated with blue underline. Image is created using ClustalW multialignment results and Jalview.
Primers were designed to amplify -390 nt to +150 nt relative to the start codon of cspH ORF from CFT073 genome. This region was expected to capture all the transcription regulator binding sites which regulate the expression of cspH along with DB or downstream box (ATGACAGGAATTGT) located +13 nt relative to the start codon of cspH ORF. PCR was performed to amplify cspH promoter region using these primers (CspH-GFP-Fwd and CspH-GFP-Rev) with CFT073 genomic DNA as a template and cloned into pUA139 vector.

**Expression of cspH is induced during urea stress and is not regulated by RpoS sigma factor in CFT073**

Promoter activity assays were performed to test the activity of cspH promoter during urea stress in CFT073. CFT073 (pCspH) and CFT073 with no plasmid (negative control) were grown till exponential phase in K medium in microplate reader followed by which, 0.3 M urea, 0.6 M urea or equal volumes of sterile water were added. Absorbance and fluorescence measurements were taken every 12 min before and after the addition of urea and graphs were plotted. The results (Figure 6.3) showed no difference in the expression of GFP from CFT073 (pCspH) and negative control in the absence or presence of urea. Changing experimental conditions like reducing the temperature to 30°C, increasing the gain of fluorescence measurements and using black 96 well plate instead of clear plate yielded no positive results. The cspH promoter region and GFPmut2 sequence in pCspH plasmid were sequenced using Seq-primer and Seq-GFP primers respectively to make sure that there were no mutations in these regions.
While troubleshooting, it was noticed that though expression of *cspH* is induced 8.4 fold in CFT073 as a stress response to 0.6 M urea in microarray experiments (34), the actual signal values for *cspH* gene expression in K medium without or with urea are very low especially when compared to that of *bax* and *ibpAB* (Table 2). Thus the reason for not being able to detect any fluorescence from CFT073 (pCspH) cultures compared to negative control can be due to the weak activity of *cspH* promoter region.

To solve the issue, the copy number of pCspH plasmid was increased from ~5 to 50-70 copies per cell. This was done by replacing pSC101 origin of replication in pCspH plasmid to ColE1 origin of replication (from pZE21-GFPaav plasmid). The constructed plasmid was named pAL139-CspH. To construct pAL139 vector plasmid, which can be used as a negative control, *cspH* promoter upstream GFPmut2 in pAL139-CspH plasmid was replaced by a 24 bp DNA sequence as described in the methods section.

CFT073 and CFT073 ΔrpoS strains were transformed with pAL139-CspH or pAL139 vector plasmid and promoter activity assays were repeated using 0.3 M or 0.6 M urea in K medium (Figure 6.4). Once again, a significant difference in the expression of GFP was not seen in strains containing pAL139-CspH plasmid compared to negative control, in the absence or presence of urea. Repeating the experiment in a black 96 well plate reduced the background fluorescence but did not yield expected results (Figure 6.5).

It was noticed that the LB plates with streaks of CFT073 (pCspH) show fluorescence slightly higher than CFT073 with pAL139 vector plasmid when observed under UV transilluminator. Therefore streak plate experiments were performed to test the promoter activity of *cspH* during urea stress. Before performing the experiments, pAL139-IbpAB
plasmid was constructed as described in methods section to use as a positive control. A single colony of CFT073 (pAL139-CspH), CFT073 ΔrpoS (pAL139-CspH), CFT073 (pAL139) - negative control and CFT073 (pAL139-IbpAB) - positive control were streaked uniformly on each side of the K medium 1.5% agar plates according to the plate layout (Figure 6.6). A single colony was used for each streak and the streaks were made on 3 replicates of K medium agar plates, 3 replicates of K medium agar plates with 0.3 M urea and 3 replicates of K medium agar plates with 0.6 M urea. The plates were incubated at room temperature because urea degrades producing toxic products at higher temperatures. The digital and fluorescence images were taken for the plates after 48 and 96 hours of incubation at room temperature. Results showed that there was an increased fluorescence in cells with pAL139-CspH plasmid compared to negative control in K medium plates with 0.3 M and 0.6 M urea. The increase in fluorescence was higher in 0.6 M urea compared to 0.3 M urea which was further higher than in K medium with no urea. The expression of GFP was similar in CFT073 WT and CFT073 ΔrpoS strains containing pAL139-CspH in the absence or presence of urea, indicating that the expression of cspH might not be regulated by RpoS sigma factor during urea stress. However the results of this experiment cannot be used to make definitive conclusions because the growth rate differed significantly among the plates with different concentrations of urea. Though replicate plates showed similar growth, it was noticed that CFT073 grew better on plates with 0.3 M urea compared to plates with no urea. Interestingly, on 0.6 M urea plates, CFT073 with pAL139-CspH plasmid showed reduced growth rate but the positive and negative controls grew normally.
Based on the results of streak plate experiments, it can be concluded that expression of cspH might be induced during urea stress but not at high levels and this expression might not be regulated by RpoS sigma factor. However these results need further confirmation.
Figure 6.3: Expression of GFP from cspH promoter in pCspH plasmid during urea stress in K medium. CFT073 strains containing pCspH plasmid and CFT073 with no plasmid (negative control) were grown in microplate reader till exponential phase in K medium and 0.3 M urea, 0.6 M urea or equal volumes of sterile water were added. The absorbance (OD$_{600}$) and fluorescence measurements were taken every 12 minutes before and after the addition of urea and graphs were plotted. (A) RFU/OD$_{600}$, (B) RFU and (C) OD$_{600}$ measurements. Each data point represent mean ± SEM of 3 independent experiments from one culture.
Figure 6.4: Promoter activity of cspH during urea stress in CFT073 wild type and CFT073 ΔrpoS measured using pAL139-CspH plasmid in K medium. CFT073 and CFT073 ΔrpoS strains with pAL139-CspH plasmid were grown in microplate reader till exponential phase in K medium and 0.3 M urea, 0.6 M urea or equal volumes of sterile water were added. CFT073 (pAL139 vector) was used as a negative control and treated similar to other cultures. The absorbance (OD_{600}) and fluorescence measurements were taken every 12 minutes before and after the addition of urea and graphs were plotted. (A) RFU/ OD_{600}, (B) RFU and (C) OD_{600} measurements. Each data point represent mean ± SEM of 3 independent experiments from one culture.
Figure 6.5: Promoter activity of \( cspH \) during urea stress in CFT073 wild type and CFT073 \( \Delta rpoS \) measured using pAL139-CspH plasmid in Costar black 96 well plate. CFT073 and CFT073 \( \Delta rpoS \) strains with pAL139-CspH plasmid were grown in microplate reader using black 96 well plate till exponential phase in K medium and 0.3 M urea, 0.6 M urea or equal volumes of sterile water were added. CFT073 (pAL139 vector) was used as a negative control and treated similar to other cultures. The \( \text{OD}_{600} \) and fluorescence measurements were taken every 12 minutes before and after the addition of urea and graphs were plotted. (A) RFU/ \( \text{OD}_{600} \), (B) RFU and (C) \( \text{OD}_{600} \) measurements. Each data point represent mean ± SEM of 3 independent experiments from one culture.
Figure 6.6: Growth and fluorescence images of cspH streak plate experiment plates after 48 hours incubation at room temperature. K medium agar plates containing 0 M, 0.3 M or 0.6 M urea were streaked uniformly according to the plate layout with a fresh colony of CFT073 ΔrpoS (pAL139-CspH), CFT073 (pAL139-CspH), CFT073 (pUA139) - negative control and CFT073 (pAL130-IbpAB) - positive control. The plates were incubated at room temperature for 48 hours and digital images were taken to show growth and florescence images were taken to show cspH promoter activity. There were 3 replicates for each condition but the images of only one replicate are shown.
Figure 6.7: Growth and fluorescence images of cspH streak plate experiment plates after 96 hours incubation at room temperature. K medium agar plates containing 0 M, 0.3 M or 0.6 M urea were streaked uniformly according to the plate layout with a fresh colony of CFT073 ΔrpoS (pAL139-CspH), CFT073 (pAL139-CspH), CFT073 (pUA139) - negative control and CFT073 (pAL130-IbpAB) - positive control. The plates were incubated at room temperature for 96 hours and digital images were taken to show growth and florescence images were taken to show cspH promoter activity. There were 3 replicates for each condition but the images of only one replicate are shown.
Deletion of cspH reduced the urea tolerance of BW25113 in M9 medium

Because expression of cspH was induced at low levels even during the presence of 0.6 M urea, we were skeptical if cspH knockout of CFT073 would show any growth defect due to urea stress. So the growth curve experiments were performed using only BW25113 wild type and its ΔcspH knockout (Keio strain). Knockout of cspH in BW25113 ΔcspH (Keio strain) was confirmed though colony PCR as shown in Figure 6.8. Urea at 0.3 M, 0.6 M or 0.9 M concentrations or equal volumes of sterile water were added to exponentially growing wild type and cspH null mutant of BW25113 in M9 medium. The absorbance measurements were taken at 12 min interval before and after the addition of urea and graphs were plotted. Results (Figure 6.9) showed that there is no difference in the growth rate of BW25113 and BW25113 ΔcspH in M9 medium without or with 0.3 or 0.6 M urea. However at 0.9 M urea concentration, cspH null mutant showed slightly reduced growth rate compared to the wild type BW25113. However further experiments are needed to determine if this effect can be reverted by cspH expression plasmid.
Figure 6.8: Confirmation of cspH knockout in BW25113 (Keio strain) by colony PCR. Colony PCR was performed for BW25113 ΔcspH and BW25113 WT using 2 different primer pairs (K1, CspH-GFP-Fwd, CspH-RT-Fwd, CspH-RT-Rev) as shown by the graphic images above the electrophoresis gel images. Lane M shows DNA ladder, Lane 1 shows the colony PCR reaction results of BW25113 WT and Lane 2 shows the colony PCR reaction results of BW25113 ΔcspH (Graphic images are not to scale).
Figure 6.9: Effect of *cspH* knockout on the growth rate of BW25113 during urea stress in M9 medium. Urea at 0.3 M, 0.6 M or 0.9 M concentrations or equal volumes of sterile water were added to exponentially growing wild type and the *cspH* null mutant of BW25113. The absorbance (OD$_{600}$) measurements were taken every 12 minutes before and after the addition of urea and graphs were plotted. Each data point represent mean ± SEM of 3 independent experiments from one culture.
DISCUSSION AND CONCLUSIONS

Even though most UTIs can be treated effectively with antibiotics, a higher rate of its recurrence and the rapid increase in the development of antibiotic-resistant UPEC strains call for an alternative strategy to treat UTIs. Significant research is being conducted to better understand the pathogenesis of urinary tract infections. However, the mechanism behind how UPEC can tolerate high salt and urea levels in urine is still not clearly known and is currently under investigation. Because UPEC strains are exposed to urine during initial stages of infections, determining the genes or the mechanisms used to survive these conditions would be valuable for the development of future UTI therapies. In this regard, our lab conducted microarray experiments (34) to determine the differential expression of genes in CFT073 during salt and urea stress. Based on the microarray results, 2 genes ($bax$ and $cspH$) and 1 operon ($ibpA$ and $ibpB$) were chosen for the current study.

In the current project, the sequence conservation of the 4 genes ($bax$, $ibpA$, $ibpB$ and $cspH$) among 3 different UPEC strains CFT073, UTI89 and E. coli 536 and commensal E. coli K-12 strain MG1655 was identified. This information will be useful to predict the significance of these genes, as mutations or loss of important genes is a rare event during the development of pathogenic strains. The results showed that all the 4 genes were well conserved among these four strains.
Because none of the known osmoregulatory systems were proven to be important for the survival of *E. coli* in urine, it was speculated that *E. coli* uses an unknown mechanism to survive in urine, which is yet to be discovered. On this note, *bax* is one of the interesting genes to study, and encodes a hypothetical protein shown to be significantly induced during salt stress in multiple studies. In the current project, using GFP promoter activity assays, we demonstrated that expression of *bax* is induced significantly in CFT073 due to the presence of 0.2 M and 0.3 M NaCl in K medium. This took us to our next step, which is to determine if *bax* protects *E. coli* against salt stress. To accomplish this aim, *bax* knockouts of BW25113 (K-12 strain) and CFT073 were used. In BW25113, lack of *bax* caused reduced growth rate under salt stress compared to the wild type strain. In CFT073, deletion of *bax* did not affect the salt tolerance of this strain. It is possible that an alternative mechanism exists in CFT073 which can compensate for the lack of *bax* during salt stress. However further experiments are needed to confirm that the reduced salt tolerance observed in BW25113 Δ*bax* is due to the lack of *bax*.

Two other genes that were upregulated under urea stress in CFT073 during microarray experiments were *ibpA* and *ibpB*. Because urea is a protein denaturant, an induced expression of several molecular chaperon genes including *ibpAB* operon was noticed in CFT073 due to the presence of 0.6 M urea in K medium (34). In the present project this was confirmed by constructing GFP transcriptional fusion plasmids and performing promoter activity assays. It was already shown that *ibpB* is not required for CFT073 to colonize murine urinary tract (55). Therefore CFT073 lacking both *ibpA* and *ibpB*, was studied in the current project. Growth curve experiments were performed using *ibpAB* null mutants of BW25113 and CFT073 in the absence or presence of 0.9 M urea in M9.
medium and K medium and it was noticed that lack of *ibpAB* had no effect on the urea tolerance of these strains. Thus *ibpAB* is dispensable in CFT073 and BW25113 strains to survive under urea stress in K medium and M9 medium.

Another gene that showed significantly induced expression in CFT073 due to presence of 0.6 M urea in K medium was CspH, which is speculated to be a DNA/RNA chaperone. Since urea is a nucleic acid denaturant at higher concentrations (83), it was expected that *cspH* might protect *E. coli* against denaturation effects of urea by acting as a DNA/RNA chaperone. First, we wanted to prove that the expression of *cspH* was induced due to the presence of urea by performing promoter activity assays using GFP transcriptional fusion plasmid of *cspH*. Since *cspH* has a weak promoter, the expression of GFP from the GFP transcriptional fusion plasmid of *cspH* was undetectable by the microplate reader. Hence, streak plate experiments were performed, which showed that expression of *cspH* was induced due to 0.3 M and 0.6 M urea stress in CFT073, but as CFT073 showed inconsistent growth on plates with different concentrations of urea, these results need further confirmation.

Considering the issues faced while performing promoter activity assays of *cspH*, we decided to test if BW25113 Δ*cspH* (Keio strain) would show reduced urea tolerance compared to wild type CFT073 before constructing CFT073 Δ*cspH* strain. The results showed that in the presence of 0.9 M urea, BW25113 Δ*cspH* shows reduced growth rate compared to the wild type BW25113.

RpoS is a stress responsive alternative sigma factor which was shown to influence the expression of virulent genes in *Salmonella* and enterohaemorrhagic *E. coli* (22). The role
of RpoS sigma factor in regulating the expression of \textit{bax}, \textit{ibpAB} and \textit{cspH} genes under high salt or urea levels was investigated in the current project and it was shown that RpoS does not regulate the expression of these genes. However, regulation of \textit{cspH} by RpoS sigma factor needs further confirmation.

Future experiments that need to be performed to determine the importance of \textit{bax}, \textit{ibpAB} and \textit{cspH} genes in the uropathogenesis of \textit{E. coli} are as follows:

(i) Construction of CFT073 $\Delta$cspH strain and \textit{cspH} expression plasmid to test if the reduced urea tolerance observed in BW25113 $\Delta$cspH can be reproduced in CFT073 and if this effect can be compensated by \textit{cspH} expression plasmid.

(ii) Confirm that the reduced salt tolerance of BW25113 $\Delta$bax compared to the wild type BW25113 was due to the lack of \textit{bax} expression, by observing if this effect can be reverted with \textit{bax} expression plasmid.

(iii) Perform \textit{in vitro} growth curve experiments of \textit{bax}, \textit{ibpAB} and \textit{cspH} knockouts of CFT073 in urine

(iv) Perform \textit{In vivo} colonization experiments of \textit{bax}, \textit{ibpAB} and \textit{cspH} knockout strains of CFT073 in murine urinary tract

Though \textit{bax} and \textit{ibpAB} knockouts of CFT073 did not show reduced growth rate during salt and urea stress respectively, in K medium and M9 medium compared to their respective wild type strains, it would be worth testing if these knockouts strains show reduced growth rate in urine or reduced colonization of murine urinary tract. These experiments will help to fully assess their role in the uropathogenesis of \textit{E.coli}. 

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CFT073 is exposed to more severe stress in urine due to the presence of both salt as well as urea at high levels, unlike in the current project in which CFT073 was exposed to either high concentrations of salt or urea. Bax, IbpAB and CspH proteins were speculated or proved to have other functions: for example Bax might have a potential role in providing acid tolerance to enterohemorrhagic *E. coli* (41), proven importance of IbpAB in the formation of *E. coli* biofilms (54), induced expression of *ibpAB* and *cspH* genes in asymptomatic bacteriuria strains during biofilm growth in urine (52, 53). Therefore it could be possible that *bax*, *ibpAB* and *cspH* null mutants of CFT073 might show reduced growth rate in urine and show defective colonization of murine urinary tract by protecting CFT073 against acidic pH in urine (*bax*) or playing a role in the formation of intracellular bacterial community (*ibpAB* and *cspH*), in addition to their potential role in protecting CFT073 against high levels of salt and urea in urine.
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