ABSTRACT

Title of the Thesis: Regulation of Virulence by BarA-UvrY Two-Component system and LuxS in Extraintestinal Pathogenic *Escherichia coli*.

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Pathogenic *E. coli* cause intestinal or extraintestinal infections in many host species. *E. coli* strains enter the intestinal tract through food and colonize the intestinal epithelium to cause infections. In animals and humans, *E. coli* causes gastroenteritis, neonatal meningitis and urinary tract infections. In birds, *E. coli* causes a complex syndrome called avian colibacillosis.

The orthologs of BarA-UvrY two-component (TCS) system is known to regulate a number of phenotypic traits in gamma proteobacteria, although their role in Extraintestinal pathogenic *Escherichia coli* (ExPEC) virulence is yet to be determined. The *barA* gene is membrane bound sensor kinase protein and the *uvrY* gene encodes the cognate response regulator in *E. coli*. Work in this study has focused how the BarA-UvrY and LuxS system regulates *in vivo* virulence in uropathogenic *E. coli* (UPEC) and avian pathogenic *E. coli* (APEC) during infection.

The main goal of this study is to look at how BarA-UvrY TCS and LuxS regulate virulence in APEC χ7122 and UPEC CFT073. In this study, we studied the role of BarA-UvrY TCS system in regulation of virulence in the aforementioned ExPEC strains using animal model and tissue culture system and the role of LuxS in regulation of virulence determination in ExPEC. Our results indicate that BarA-UvrY regulates multiple virulence properties in APEC χ7122 and UPEC CFT073 and that LuxS regulates partial virulence properties in APEC χ7122 and UPEC CFT073.

REGULATION OF VIRULENCE BY BarA-UvrY TWO-COMPONENT SYSTEM AND LuxS IN EXTRAINTESTINAL PATHOGENIC *ESCHERICHIA COLI*.

By

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Dedication

I would like to dedicate this present work to my father Mr. M. Palaniyandi, my mother Mrs. P. Palaniyayee for their love and support for me.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

APEC	avian pathogenic *E. coli*
cDNA	compliment DNA
CELA	chicken embryo lethality assay
CFU	colony forming unit
CO_2	carbon dioxide
DF1	chicken embryo fibroblast cell line
E. coli	*Escherichia coli*
EHEC	enteroheamorrhagic *E. coli*
EPEC	enteropathogenic *E. coli*
ExPEC	extraintestinal pathogenic *Escherichia coli*
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
h	hour
HD11	chicken macrophage cell line
HK2	human kidney cell line
Hly	hemolysin
HTB-9	human bladder uroepithelial cell line
IL	interleukin
LB	Luria-Bertani broth
LPS	lipopolysaccharide
min	minute
ml	milliliter
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RT	room temperature
SPF	specific pathogen free
SV-HUC-1	human ureter uroepithelial cell line
TCS	two component system
TNF	tumor necrosis factor
UPEC	uropathogenic *E. coli*
UTI	urinary tract infection
vol	volume
wt	weight
µg	microgram
µl	microliter
ºC	degrees Celsius

CHAPTER 1

Introduction

1.1. Background

1.1.1. *Escherichia coli,* a pathogen

E. coli is a gram negative, rod shaped bacterium found in the intestinal tracts of aquatic, avian, warm blooded animals and humans. It is a facultative anaerobe and adapts to its intestinal (anaerobic) and extraintestinal (aerobic or micro aerophilic) habitats. In humans and animals, pathogenic *E. coli* causes both intestinal and extraintestinal infections (1). Extraintestinal pathogenic *E. coli,* such as avian pathogenic *E. coli* (APEC), uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC), causes extraintestinal infections in different hosts (2).

1.1.2. Avian pathogenic *E. coli*

Infection with APEC causes avian colibacillosis, which is characterized by air sacculitis, pericarditis, peritonitis, salpingitis, polyserositis, septicemia, synovitis, osteomyelitis, and yolk sac infection (3). APEC strains are responsible for huge economic losses to the poultry industry due to mortality and carcass condemnations (4). The infection results in loss of over 40 million dollars per year to the U.S. poultry industry (5). APEC strains infect respiratory tract of chickens, turkeys, ducks and other avian species via fecal dust and the predominant isolates belonging

to O1, O2 and O78 serogroups (6, 3, 7).

1.1.3. Uropathogenic *E. coli*

Urinary tract infection (UTI) is considered to be the most common bacterial infectious disease of humans (8). UTI accounts for 7 million office visits resulting in 100,000 hospitalizations and costs approximately $ 1.6 billion per year in the United States alone (9). UTI is one of the most common problems in young women causing morbidity in medical attention and health care costs. UPEC is the main cause of community-acquired UTIs in women (10). UTI is more common among women than man and 40% to 50% of women will experience at least one UTI during their lifetime (9). In healthy individuals up to 90% of uncomplicated UTI is caused by UPEC (11). UTI is most often caused by ascending bacterial infection contaminating the periurethral area from the lower intestinal tract, then colonizing the bladder via the urethra, causing cystitis, and in severe cases, infecting the kidneys via the ureters resulting in pyelonephritis (12). UPEC is distinct from the intestinal pathogenic *E. coli* in that UPEC has to enter urinary tract to establish infection. Extraintestinal *E. coli* isolates possess genes coding for various virulence factors like adhesions (eg. type 1, P, S), iron acquisition system (eg. aerobactin, enterobactin, but not limited to these systems), host defense avoidance mechanisms (eg. capsule, O-specific antigen, oxidative stress response etc) and toxins (eg. cytotoxic necrotizing factor 1, hemolysin etc.) (13, 14).

1.1.4. Two component signal transduction system

Bacteria often utilize signaling systems to respond to the drastic changes in the extracellular environment. The two-component signal (TCS) transduction system forms a major signal-transduction pathway in prokaryotes that involve adaptation to the environmental signals and it regulates gene expression and/or protein function (15). TCS have two conserved proteins, the histidine protein kinase (HK) and the response regulator protein (RR) constituting the phosphotransfer signaling pathway (16). The histidine residue of the conserved HK is autophosphorylated to produce a high energy phosphoryl group. The phosphotransfer from the phospho-histidine of the HK to aspartic acid side chain of the RR is catalyzed by protein dimerization, creating a high energy acyl-phosphate that activates the RR. The level of the phosphorylated RR determines the output response of the TCS (15).

1.1.5. BarA-UvrY TCS in *E. coli*

In several pathogenic gamma proteobacteria, BarA protein (Bacterial Adaptive Response Protein) functions as conserved membrane associated sensor kinase protein (17, 18). Mutations in the *barA* gene, encoding BarA, exhibit sensitivity to oxidative stress due to impairment in catalase expression (19, 20). The cognate response regulator for BarA protein is UvrY protein and orthologs of *uvrY* encoding gene are present in other bacterial species like *gacA* in *Pseudomonas, sirA* in *salmonella, varA* in *vibrio* and *expA* in *Erwinia* (21, 22, 23, 24). Environmental magnesium concentration was a potent stimulus for CsrR-CsrS TCS of group A *Streptococcus* (25) and intestinal short-chain

fatty acids and bile alter gene expression and virulence of BarA-SirA two-component system of *Salmonella* (26, 27).

In *E. coli*, BarA-UvrY Two Component System (TCS) regulate the expression of *csrB* and *csrC* non coding regulatory RNA, which in turn titrates the activity of CsrA global RNA binding protein. Secondary stem loop structure of *csrB* and *csrC* RNA binds to CsrA protein and prevents it from binding to 5' untranslated leader sequences of mRNA. CsrA is known to exert its post-transcriptional regulation for genes involved in carbon metabolism, flagellar biosynthesis and biofilm formation (28, 29, 24, 30).

The orthologs of BarA-UvrY TCS in other gram negative γ-proteobacterial species are BarA-SirA of *Salmonella enterica* (24), ExpS-ExpA of *Erwinia carotovora* (31), VarS-VarA of *Vibrio cholerae* (32), and GacS-GacA of *Pseudomonas* species (31). The disruption of these two component systems in these bacteria leads to reduction in their virulence. **In this study, we investigated the hypothesis that the BarA-UvrY TCS is critical to the virulence of APEC O78:K80:H9 and UPEC CFT073**.

1.1.6. Quorum sensing in *E. coli*

Quorum sensing is a bacterial cell-cell communication via the production of small signaling molecule in a population density-dependent manner. LuxS, the S-ribosylhomocysteine (SRH) cleavage enzyme, has an important metabolic function in the activated methyl cycle, namely the recycling of S-adenosylhomocysteine (SAH) to homocysteine (33). By-products of methyl cycle produce a furanone, called autoinducer-2, which is involved in cell-to-cell communication in a density-dependent way called

quorum sensing (QS) (34). LuxS a highly conserved autoinducer synthase is present in some Gram positive and Gram negative bacterial species (35). This autoinducer is involved in quorum sensing (34).

In enteroheamorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC), *luxS* quorum sensing system activated the transcription locus of enterocyte effacement genes, which are involved in expressing factors and in establishing an infection (36). In EPEC, mutation in the *luxS* gene leads to diminished Type III secretion. The defect in the Type III secretion system attributed to partial reduction in attachment and effacing (AE) lesions on cultured HeLa epithelial cells (37). In a mouse model, mutation in *luxS* gene of *Streptococcus pneumoniae* displayed partial reduction in virulence compared to its wild type (37). In *Vibrio vulnificus*, *luxS* mutation resulted in reduced cytotoxicity activity in cultured HeLa cells and attenuation of lethality in mice. Also deletion of *luxS* in *Neisseria meningitidis* caused defective bacteremia and reduced meningococcal virulence (38). Thus, Quorum sensing has been involved in virulence gene expression (39), biofilm formation (40) and motility (41). **In this present study, we also examined the role of LuxS in the pathogenesis of APEC O78:K80:H9 and UPEC CFT073**.

1.2. Objective

The main goal of this study is to look at how BarA-UvrY two-component system (TCS) and LuxS regulate virulence in APEC χ7122 and UPEC CFT073. To study the role of BarA-UvrY TCS and that of LuxS in regulation of virulence in ExPEC using animal model and tissue culture system were used to understand the regulation of virulence determination in ExPEC.

1.2.1. Avian Pathogenic *Escherichia coli*

Regulation of virulence using *in vivo* method includes infection of specific pathogen free (SPF) eggs with various bacterial strains to study the effect of persistence in various internal organs and its lethality. Regulation of virulence using *in vitro* method includes infection of cultured chicken cells to study attachment, invasion and to study the effect of lipopolysaccharide as a measure of virulence.

1.2.2. Uropathogenic *Escherichia coli*

In our laboratory, it has been shown that BarA-UvrY TCS regulates virulence in murine model. To demonstrate chicken embryo lethality assay as an alternative for this live murine model and to look at the persistence, embryo lethality in infected SPF eggs were determined. Cultured human cells were infected with bacterial cultures to determine attachment, invasion and cytotoxic effects. Additionally the secreted protein profiles, LPS profile of bacterial cultures were determined and how this LPS and bacterial cultures regulate cytokine expression in infected human cells.

Materials and methods

2.1. Strains and plasmids

The bacterial strains, mutant strains and plasmids for complementation of mutation are listed in Table 1. The isogenic *barA, uvrY* and *luxS* mutants of APEC 078:K80:H9 strain χ7122 and UPEC strain CFT073 were constructed using λ Red recombination method as described previously (77).

2.2. Determination of bacterial virulence by chicken embryo lethality assay

To determine the lethality of the *E. coli* strains, a set of fifteen12-day-old embryonated eggs were inoculated with 0.1 ml of 5×10^3 colony forming unit (CFU) of bacteria into the allantoic cavity as described previously by Herren CD *et al*. (42). Bacterial cultures were grown under static condition in LB broth at 37°C for 48 h for two passages to induce type 1 and P type pilus. Cells were washed with phosphate buffered saline (PBS) and then re-suspended in PBS. Bacterial suspensions containing given number of bacteria in 0.1 ml was inoculated into the allantoic cavity by using an 18-gauge needle. The holes were resealed with glue and the eggs were incubated at 37°C in humidified incubator. Candling of embryos was done every 12 h to monitor the viability. Embryos were marked as alive or dead depending upon the integrity of the venous system and movement of embryo. The results were recorded with a score as live, morbid or dead.

Table 1. *E. coli* strains and plasmids used in this study

Bacterial Strain or plasmid	Relevant Genotype	Reference or source
E. coli strains		
DH5α	*luxS* supE44 Δ(ϕ80Δ*lacZ M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen
χ7122	APEC O78:K80:H9 *gyrA*; Nal^r	R. Curtiss III (43)
SM3000	χ7122 *barA*::*kan*	Laboratory collection
SM3001	χ7122 *uvrY*::*cm*	Laboratory collection
SM3003	SM3000 carrying pSM1, Amp^r	Laboratory collection
SM3004	SM3001 carrying pSM2; Amp^r	Laboratory collection
SM3005	χ7122 *luxS::cm*	Laboratory collection
SM3006	SM3005 carrying pSM3, Amp^r	Laboratory collection
E. coli CFT073	Wild type	H. L. Mobley (44)
SM3007	CFT073 *luxS::cm*	Laboratory collection
SM3008	SM3007 carrying pSM4, Amp^r	Laboratory collection
SM3009	CFT073 *barA::cm*	Laboratory collection
SM3010	CFT073 *uvrY::cm*	Laboratory collection
SM3011	CFT073 *csrA::cm*	Laboratory collection
SM3012	SM3009 carrying pSM5, Amp^r	Laboratory collection
SM3013	SM3010 carrying pSM6, Amp^r	Laboratory collection
SM3014	SM3011 carrying pSM7, Amp^r	Laboratory collection
E. coli CFT073	CFT073 *hlyD::kan*	Laboratory collection
E. coli CFT073	CFT073 *hlyD::kan* carrying pSF4000 containing *hlyD*; Cm^r	Laboratory collection

Plasmids		
pBR322	cloning vector, Amp^r	Invitrogen
pSM1	*barA* gene within pBR322, Amp^r	Laboratory collection
pSM2	*uvrY* gene within pBR322, Amp^r	Laboratory collection
pSM3	*luxS* gene within pCR2.1, Amp^r	Laboratory collection
pSM4	*luxS* gene within pBR322, Amp^r	Laboratory collection
pSM5	*barA* gene within pBR322, Amp^r	Laboratory collection
pSM6	*uvrY* gene within pBR322, Amp^r	Laboratory collection
pSM7	*csrA* gene within pBR322, Amp^r	Laboratory collection

2.3. Chicken embryo lethality assay

Chicken embryo lethality assay (CELA) was performed in 12-day-old embryonated eggs as described previously (45). The persistence of the inoculated bacterial cultures in the infected embryos was determined by counting the number of bacteria in the various internal organs. Eight 12-day-old embryonated eggs were inoculated with 0.1 ml of 5 x 10^3 CFU of bacterial cultures in PBS. At 24 and 48 h post inoculation, viability of the embryos were monitored and recorded. At a given period of time, the dead embryos were chosen for dissection. Various internal organs and fluids were collected aseptically. All the samples were weighed, homogenized, and centrifuged in 2 ml microcentrifuge tubes at 1000 x g for 5 min at room temperature. Bacterial load was then determined by plating three serial dilutions of the supernatant in PBS on to LB plates with appropriate antibiotics and incubated at 37°C for 24 h. Bacterial load was determined in log $_{10}$ CFU/ml (fluids) or per mg (tissues or organs) ± standard deviation from four embryos.

2.4. Attachment and invasion assays

Chicken embryo fibroblast cells (DF1), human ureter (SV-HUC1) and urinary bladder (HTB-9) uroepithelial cells were obtained from Dr. Siba K. Samal (Virginia Maryland Regional College of Veterinary Medicine (VMRCVM, MD). The cells were cultured according to protocol given by ATCC (Manassas, VA). The chicken embryo fibroblast cells (DF1) were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS)

(Gibco Invitrogen). The human ureter (SV-HUC1) epithelial cells were grown in Ham's F12K medium (Gibco Invitrogen) supplemented with 10% FBS. The human urinary bladder (HTB-9) cells were grown in RPMI 1640 medium (ATCC) supplemented with 10% FBS. All cell lines were grown at 37°C with 5% CO_2 incubation. Infection assays were performed in ~ 70% confluence monolayer by growing cells in 6-well tissue culture plates at 37°C for 2 days. Type 1 pilus formation was induced by growing bacteria in LB media under static condition for 48 h for two passages. P type pilus was induced by growing bacteria on 10 tryptic soya agar plates. The experiments were performed with both bacterial cultures and similar results were observed. DF1 cells were infected with APEC strains and SV-HUC1, HTB-9 cells were infected with CFT073 strains. Adherence assays were performed as described previously (46).

Cultured cells were infected with bacterial suspension with a bacterium: cultured cell ratio of 10:1. Just before the start of infection, medium was removed and fresh medium was added. Tissue culture plates were centrifuged 600 x g for 5 min and then incubated at 37°C for 2 h. After 2 h, 20 µl of 5% Triton X-100 was used to lyse triplicate wells and then plated onto LB agar plates to count the bacterial load (both intra- and extracellular bacteria). Adherent bacteria was calculated from new set of wells washed with PBS for five times, lysed in 1 ml of 0.1% Triton X-100, and plated onto LB agar plates.

To calculate invasion frequencies, a set of wells with adherent cells were washed with PBS for five times and bactericidal antibiotic gentamicin (100 µg/ml), which does not penetrate uroepithelial cells or any eukaryotic cells, was added to the wells to kill

adhered extracellular bacteria and incubated for another 4 h. At the end of incubation, the wells were washed with PBS, lysed with 1 ml of 0.1% Triton X-100, and bacterial load was calculated by plating onto LB agar plates with appropriate antibiotics.

Attachment index was determined as CFU/ml for only adherent bacteria divided by total bacterial inoculum (CFU/ml). Invasion index is determined as number of bacteria surviving gentamicin incubation divided by the total number of bacteria present before gentamicin incubation.

2.5. Chicken macrophage HD11 engulfment and survivability assay

Chicken HD11 macrophage cells (a gift from Dr. Siba K. Samal, VMRCVM, MD) were cultured according to the protocol given by ATCC. The HD-11 cells were grown in RPMI 1640 medium supplemented with 10% FBS. HD11 cells were grown in six-well tissue culture plates at a volume of 3 ml of media at 37°C in 5% CO_2. The APEC bacterial cultures were grown and infectivity assays were performed as above described.

To determine the invasion frequencies, after 2 h initial incubation, an additional set of wells was washed with PBS, and gentamicin (100 μg/ml) was added to wells and further incubated for additional 6 h before enumerating internalized bacteria. Only adherent bacteria were calculated using the formula: $\log_{10}$ CFU/ml of attached and invaded cells – $\log_{10}$ CFU/ml of invaded cells surviving gentamicin treatment.

Attachment index was calculated using the formula: CFU/ml of only adherent bacteria divided by total bacterial inoculum (CFU/ml). Survival index was calculated

using the formula: CFU/ml of bacteria surviving gentamicin treatment divided by CFU/ml of only adherent bacteria.

2.6. Cytotoxicity assay on cultured human kidney cells

The human kidney (HK2) cells were obtained from ATCC and cultured essentially as described by ATCC. The HK2 cells were grown in Keratinocyte-Serum free medium (Gibco Invitrogen) supplemented with 5 ng/ml recombinant epidermal growth factor (Gibco Invitrogen) and 0.05 mg/ml bovine pituitary extract (Gibco Invitrogen). The HK2 cells were grown in 96-well plate and infection assays were performed on ~ 70% monolayer. The HK2 cells were infected with 50 µl of filter sterilized culture supernatants from equal number of bacterial cells grown in LB media under static condition at 37°C for 48 h for two passages with appropriate antibiotics. The cell proliferation WST-1 assay measures the metabolic activity of viable cells. The viable cells convert tetrazolium WST-1 reagent to a water soluble formazan dye (Roche, USA). Cytotoxic effects were analyzed as described by the cell proliferation tetrazolium colorimetric WST-1 assay system (Roche, USA) and the absorbance of water soluble formazan dye was read at 450 nm. The timing of the assay was closely evaluated following a cell proliferation WST-1 assay. After treating the cells with bacterial supernatants for 3 h, 10 µl of WST-1 reagent was added to each well and the cells were incubated for further 180 min at 37°C. Absorbance was read at 450 nm using a PerkinElmer Victor3 multilabel counter.

2.7. Calculation of generation time in artificial urine medium

Simple artificial urine medium was prepared as described previously (47). The pH of the artificial urine medium was adjusted to 6.5 and filter sterilized. The generation time was calculated when the bacterial cells were growing exponentially in the artificial urine medium as per the protocol described by Todar's Online Textbook of Bacteriology http://textbookofbacteriology.net/growth.html (48).

2.8. Hemolysin assay

Hemolysis assay with sheep erythrocytes was performed as described before (49, 50) with slight modifications. The experiments were performed with cells growing in LB medium and artificial urine medium. Supernatants of bacterial cultures harvested at an optical density (O.D 600) of 0.5 were filtered and taken in 2 ml eppendorf vial. Sheep erythrocytes (RBC) were washed and resuspended to 2% (wt/vol) in PBS, 100 µl was added to the eppendorf vial, centrifuge the vials at 8000 rpm for 5 min and incubated at 30.5°C for 7 h. Unlysed cells were pelleted and absorbance of the supernatant was measured at 405 nm.

2.9. Preparation of secreted protein

Secreted supernatant proteins were prepared as described previously (51). Bacterial cells were pelleted by centrifugation and supernatant was filtered using 0.2-µm-pore-size filter. Proteins were precipitated with trichloroacetic acid (25% wt/vol final concentration) on ice for 4 h, washed with acetone and analyzed in Novex 4-20%

Tris-Glycine gel (Invitrogen Corp., Carlsbad, CA). The gel was stained with Coomassie blue and bands of the interest were excised and subjected to mass spectrometry analysis.

2.10. Lipopolysaccharide

Lipopolysaccharide (LPS) extraction was done by a modified phenol-chloroform method using LPS Extraction Kit (Intron Biotechnology, Boca Raton, FL). Bacterial cultures were grown under static condition in Tryptic soya broth at 37°C for 48 h and equal number of cells, as enumerated by colony counts upon plating serial dilution of cultures, was taken for LPS extraction. Cells were centrifuged at 13,000 rpm for 10 min for harvesting. One ml of lysis buffer was added and vortexed vigorously. The 200 µl of chloroform was added, vortexed vigorously for 20 seconds, and incubated at room temperature (RT) for 5 min. Eppendorf vials were centrifuged at 13,000 rpm for 10 min at 4°C. The 400 µl of supernatant was transferred to a new 1.5 ml tube and 800 µl of purification buffer was added and mixed well. Vials were incubated for 10 min at -20°C and centrifuged at 13,000 rpm for 15 min at 4°C. The LPS pellet was washed with 1 ml of 70% ethanol and air dried completely. Finally, LPS was dissolved by boiling in 70 µl of ddH_20 for 1 min. LPS was separated in SDS-PAGE gel as described previously (52). The PAGE gel was stained with silver staining as described by Fastsilver™ (G Biosciences, St. Louis, MO). During the fixation procedure, 0.7 g of periodic acid was added to 100 ml of fixing solution to oxidize the carbohydrate. The pictures were taken using Kodak EDAS 290 camera. Using densitometry assay, the concentration of LPS was calculated by comparing with standard *E. coli* LPS (Sigma, St. Louis, MO).

2.11. Cytokine assay

SV-HUC1 uroepithelial cells were grown to confluence in 6 well plates. The cells were treated with equal amount of purified LPS and incubated for 16 h at 37°C or with whole bacterial cells with multiplicity of infection of 10:1 for 4 h at 37°C. The bacterial cells were grown in LB media under static condition for 48 h at 37°C with appropriate antibiotics. The total RNA was isolated from the tissue culture plates using the TRIzol reagent (Invitrogen, Carlsbad, CA). The RNA was treated with TURBO DNase (Ambion, Austin, TX) and purified using Qiagen RNeasy mini columns (Qiagen, Valencia, CA). For quantitative real-time PCR, first strand cDNA was synthesized from 5 μg of total RNA using Superscript II (Invitrogen, Carlsbad, CA) and 50 ng of random hexamers (Invitrogen, Carlsbad, CA). Internal gene-specific gene primers were used to amplify human TNFα (OSM 375 and OSM 376), IL6 (OSM 373 and OSM 374), IL8 (OSM 411 and OSM 412), GAPDH (OSM 377 and OSM 378) (Table 2). For real-time PCR, the first 10 ng of first-strand cDNA was amplified separately with 10 μM each of gene-specific primer and GAPDH specific primers (OSM 377 and OSM378) in a 25-μl total volume of SYBR green 1 PCR master mix using a PTC-200 Opticon Cycler (Biorad, Hercules, CA). The ΔC_T values between samples were normalized to those for the GAPDH product, as ΔC_T = [C_T of uninfected Cytokine (eg. Mock TNFα) - C_T of infected Cytokine (eg. Infected TNFα)] – [C_T of uninfected GAPDH (mock) – C_T of infected GAPDH]. Because PCR products double with each amplification cycle, the fold difference in the initial concentration of each transcript is determined as $2^{-\Delta\Delta CT}$.

Table 2. Primers used in this study

Primers	Sequence
TNF-α amplification primers	
OSM 375……	5'-AGGCAGTCAGATCATCTTCTCG-3'
OSM 376……	5'-CCTTGAAGAGGACCTGGGAGTA-3'
IL-6 amplification primers	
OSM 373……....	5'-TTCGGTCCAGTTGCCTTCTC-3'
OSM 374……	5'-GTTTTCTGCCAGTGCCTCTTT-3'
IL-8 amplification primers	
OSM 411……	5'-CTCTTGGCAGCCTTCCTGA-3'
OSM 412……	5'-CCTCTGCACCCAGTTTTCCT-3'
GAPDH amplification primers	
OSM 377……	5'-TGGTCTCCTCTGACTTCAACAG-3'
OSM 378……	5'-AGGAGGGGAGATTCAGTGTG-3'

Results and Discussion

3.1. Avian pathogenic *E. coli*

3.1.1. Role of BarA-UvrY TCS in determining virulence in *E. coli* O78:K80:H9 (χ7122)

3.1.1.1. The *barA* and *uvrY* mutants poorly colonize embryonic tissues and fail to persist within the liver and spleen

To investigate the virulence of *barA* or *uvrY* mutant, eight 12-day-old embryos were infected with various strains. At 24 h and 48 h, a set of four embryos was harvested, and the bacterial load was determined in various tissues (Table 3). Although *barA* mutants were able to colonize the chorioallantoic membrane (CAM) and the liver (~3.0 ± 1.4 $\log_{10}$ CFU/mg of tissue), multiply in allantoic fluid (ALF) and amniotic fluid (~2.0 ± 1.0 $\log_{10}$ CFU/ml of fluid), they were unable to persist in the lungs or spleen. The persistence in liver and lungs decreased about10-fold after 48 h of infection. The *uvrY* mutant could initially replicate in ALF and colonize CAM, liver, and lungs, but it failed to persist in these organs, particularly in the liver (~200-fold decrease) after 48 h of incubation. However, complementation of the *uvrY* mutant strain by a plasmid-borne copy of the wild-type *uvrY* gene (p-*uvrY*) (pSM2) restored colonization and persistence. These results, particularly the number of bacteria in the liver and spleen, indicate that UvrY may regulate virulence determinants required for systemic infection in the chicken embryo. Since the initial site of APEC infection is the lungs (air sacculitis), followed by a generalized infection (perihepatitis, pericarditis, or septicemia), our results indicate that a

nonfunctional BarA-UvrY TCS may lead to poor colonization of the lung tissues and limit systemic invasion. Interestingly, unlike wild-type strains, embryos infected with mutants did not exhibit pericardial lesions, a characteristic of cellulitis-derived isolates (53). Our results show the abilities of APEC strain χ7122 to colonize in lungs, invade the internal organs, and disseminate in allantoic and amniotic fluids of a 12-day-old embryos are essentially similar to that shown for the same APEC strain in 3.5-week-old chickens by Mellata et al. (54). Our results, therefore, suggest that 12-day-old SPF chicken embryos could serve as surrogate models for determining virulence.

Table 3. Attributes of APEC strain χ7122 and various isogenic mutants to colonize 12-day-old chicken embryos, invade internal organs, and disseminate in allantoic and amniotic fluids after 24 h and 48 h of infections

Strain	Genotype	Tissue infectivity log10(CFU/ml or mg of tissue) at the following time after infection.		
		Tissue	24h	48h
χ7122	Wt	CAM	2.2 ± 1.2	3.1 ± 1.5
		ALF	2.0 ± 1.2	3.4 ± 1.0
		AMF	2.2 ± 1.2	3.1 ± 1.6
		Liver	3.8 ± 1.9	3.9 ± 1.2
		Spleen	2.0 ± 1.9	3.2 ± 1.8
		Lungs	3.3 ± 1.9	3.8 ± 1.8
SM3000	*barA*	CAM	2.1 ± 1.9	3.2 ± 1.5
		ALF	2.0 ± 0.7	2.0 ± 1.3
		AMF	2.0 ± 1.0	2.1 ± 1.5[a,b]
		Liver	3.1 ± 1.4[a,b]	2.1 ± 1.0
		Spleen	<1 ± 0.1[a,b]	<1 ± 0.1[a,b]
		Lungs	1.8 ± 0.8[a,b]	<1 ± 0.1[a,b]
SM3001	*uvrY*	CAM	2.2 ± 1.2	<1 ± 0.1[a,b]
		ALF	1.8 ± 1.0[a,b]	<1 ± 0.1[a,b]
		AMF	<1 ± 0.1[a,b]	<1 ± 0.1[a,b]
		Liver	3.3 ± 1.4[a,b]	1.5 ± 1.2[a,b]
		Spleen	<1 ± 0.1[a,b]	<1 ± 0.1[a,b]
		Lungs	2.1 ± 1.3[a,b]	<1 ± 0.1[a,b]
SM3004	*uvrY*/p-*uvrY*	CAM	2.7 ± 0.9	3.2 ± 1.3
		ALF	2.1 ± 1.3	2.0 ± 0.9
		AMF	3.6 ± 1.4	3.5 ± 1.0
		Liver	3.5 ± 1.4	3.6 ± 1.5
		Spleen	3.2 ± 1.4	3.5 ± 1.5
		Lungs	3.2 ± 1.3	3.5 ± 1.5

[a] This value is significantly different ($P < 0.05$) from the value for the wild-type strain.

[b] This value is significantly different ($P < 0.05$) from the value for the complemented strain.

3.1.1.2. Mutation in *barA* or *uvrY* reduces adherence and invasion to cultured chicken embryo fibroblasts

To further investigate the decreased embryo lethality in mutant strains of χ7122, we assayed for the possibility of *barA* and *uvrY* might have effects on the initial attachment of bacteria to a cultured chicken embryo fibroblast line, DF-1 (55). For APEC to cause colibacillosis, bacterial cells must be able to invade epithelial cells and move through the host fibroblasts that make up the connective tissue. In vivo, the *uvrY* mutant colonized the chorioallantoic membrane, an epithelium, 100-fold more poorly than the wild-type strain did (Table 3). Since we did not have a transformed chicken epithelial cell line, we assayed the abilities of *barA* and *uvrY* mutant strains and *uvrY*-complemented strain of APEC to adhere to and invade chicken fibroblasts (Table 4). Deletion of either the *barA* or *uvrY* gene in strain χ7122 reduced bacterial attachment to fibroblasts by 100-fold (~2 $\log_{10}$ CFU/ml difference) in comparison with wild type (Table 4). These low adherent phenotypes could be complemented to wild-type χ7122 levels when the respective gene was provided in *trans*. Complementation was best achieved in the *uvrY*/p-*uvrY* complemented strain (Table 4 and Figure 1A to D). About 16% of the adherent APEC could invade DF-1 cells, as indicated by their ability to resist gentamicin treatment after 6 h of initial infection (Table 4). However, a mutation in either *barA* or *uvrY* (especially *uvrY)*, led to almost 100-fold reduction in invasiveness of these mutants that were adhering to DF-1 cells. The invasiveness could be restored to near wild-type levels in the *uvrY*/p-*uvrY* complemented strain. These results indicate that the BarA-UvrY TCS,

either directly or indirectly, regulates a number of bacterial determinants responsible for attachment and invasion of APEC strains. The BarA-SirA TCS has been shown to be required for full virulence in *S. enterica* because of its effects on the type III secretion system (28, 26). This TCS has also been implicated in regulating invasiveness in *Salmonella* by regulating pathogenicity island I genes through the master regulator HilA (26).

Table 4. Mutation in *barA* or *uvrY* reduces attachment and invasion of *E. coli* O78:K80:H9 strain χ7122 to chicken embryo fibroblasts

Relevant genotype	Initial cells[a] (log_{10} CFU / ml)	Attached and invaded after 2h [a] (log_{10} CFU / ml)	Invaded fraction surviving after 4h [a] (log_{10} CFU / ml)	Calculated attached bacteria [a] (log_{10} CFU/ml)	Attachment Index	Invasion Index
wild type	8.6 ± 0.9	7.1 ± 0.9	6.4 ± 0.8	7.1 ± 0.9	2.7×10^{-2}	2.0×10^{-1}
barA	8.6 ± 0.9	6.7 ± 0.8	4.8 ± 0.7	6.6 ± 0.8	1.2×10^{-2}	1.5×10^{-2}
uvrY	8.6 ± 0.9	6.3 ± 0.8	4.0 ± 0.6	6.3 ± 0.8	5.0×10^{-3}	5.0×10^{-3}
uvrY/p-*uvrY*	8.6 ± 0.9	7.2 ± 0.9	6.3 ± 0.8	7.1 ± 0.8	4.5×10^{-2}	1.3×10^{-2}

[a] Results represent mean values ± standard deviations from three independent experiment.

Figure 1. Mutation in *uvrY* reduces the attachment to cultured chicken fibroblasts and macrophages

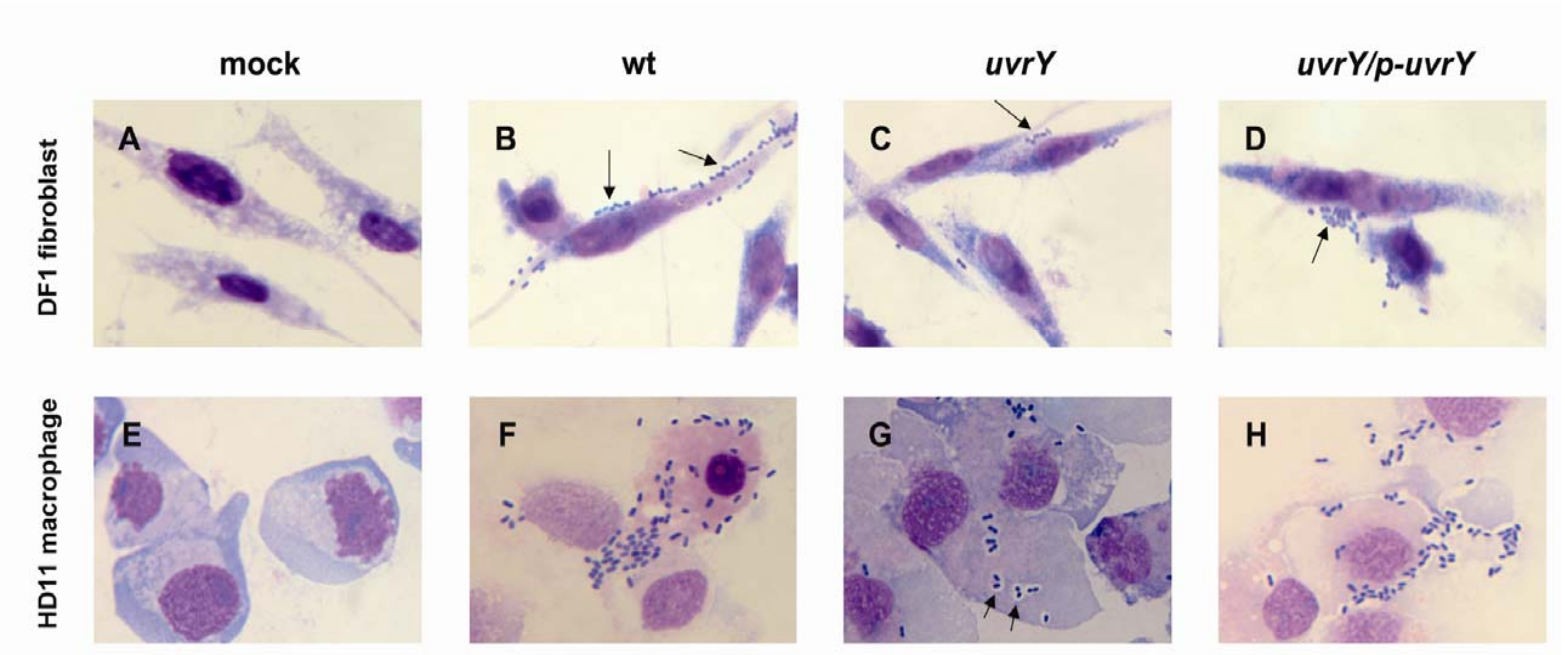


Figure 1. The monolayers of cultured cells over glass coverslips were incubated with bacteria with a multiplicity of infection of 10.

The unattached bacteria were washed, stained with Hema-3 stain and visualized using a Spot RT camera attached to a Olympus

BH-2 microscope with a 100x objective lens. Attachment of *E. coli* χ7122 (wild type [wt]), *uvrY* mutant, and *uvrY* mutant complemented with a plasmid-borne copy of the wild-type *uvrY* gene (p-*uvrY*). (A to D), Attachment to DF-1 chicken fibroblasts. Arrows indicate surface-attached bacteria. (E to H), Attachment to HD11 chicken macrophages. Panels F and H show attached bacteria, and panel G shows bacteria that are mostly engulfed (indicated by arrows).

3.1.1.3. Mutation in *barA* or *uvrY* reduces survival within chicken macrophage

The most common form of APEC infection in poultry is characterized by initial respiratory tract colonization followed by a systemic spread to other parts of the body. Avian air sacs do not have cellular defense mechanisms and depend initially on the influx of heterophils followed by macrophages as a cellular defense (56, 57). APEC strains are known to be associated in vivo with macrophages of the air sacs and lungs, while nonpathogenic strains were observed to lack these attributes (56). Moreover, the pathogenic APEC strains are more resistant to killing by chicken macrophages in vitro than the low-pathogenic strains (54, 58). Therefore, using HD-11 chicken macrophage line, we examined the effects of mutations of *barA* and *uvrY* on bacterial survival within cultured macrophages. Internalized fractions of bacteria surviving within HD-11 macrophages were enumerated by the standard gentamicin protection assay (46). There appeared to be no differences in adhesion (Table 5). However, mutation in *barA* reduced APEC survival within chicken macrophages by 1,000-fold compared to that of the wild type, and the *uvrY* mutant survived but at a level 10^4-fold less than that of the wild type (Table 5). Although there was adherence defect, the difference in survival of internalized bacteria, compared to wild type, is significant ($P < 0.05$). The mutant bacteria appeared to be engulfed quickly by the macrophages, while the wild-type or *uvrY*/p-*uvrY* complemented bacteria appeared to resist engulfment (Figure 1F, H versus G)

Table 5. Mutation in *barA* or *uvrY* reduces survival of *E. coli* O78:K80:H9 (χ7122) in chicken macrophages

Relevant genotype	Initial cells[a] (log_{10} CFU / ml)	Attached and invaded after 2h [a] (log_{10} CFU / ml)	Invaded fraction surviving after 8h [a] (log_{10} CFU / ml)	Calculated attached bacteria [a] (log_{10} CFU/ml)	Attachment Index	Survival Index
wild type	8.4 ± 0.9	7.0 ± 0.8	5.1 ± 0.7	7.0 ± 0.7	4.5×10^{-2}	1.3×10^{-2}
barA	8.9 ± 0.9	7.0 ± 0.8	3.8 ± 0.6	7.0 ± 0.7	1.4×10^{-2}	7.0×10^{-4}
uvrY	8.9 ± 0.9	7.0 ± 0.8	3.4 ± 0.5	7.0 ± 0.5	1.4×10^{-2}	7.0×10^{-4}
uvrY/p-*uvrY*	8.7 ± 0.9	7.1 ± 0.8	5.3 ± 0.7	7.1 ± 0.6	2.2×10^{-2}	1.7×10^{-2}

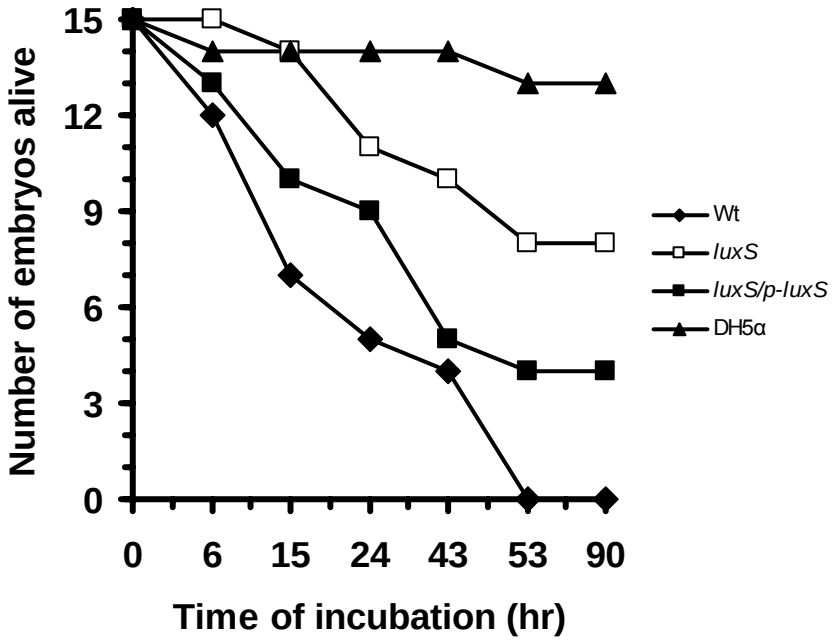
[a] Results represent mean values ± standard deviations from three independent experiments.

3.1.2. Role of LuxS in determining virulence in *E. coli* O78:K80:H9 (χ7122)

3.1.2.1. The mutation in LuxS reduces virulence of *E. coli* O78:K80:H9 (χ7122) in vivo

The virulence of the bacterial strain *E. coli* O78:K80:H9 (χ7122) was determined using chicken embryo lethality assay by the number of embryos killed within a given period of time (Figure 2). A mutation in the *luxS* gene reduced virulence by 47% (7 out of 15), embryos were dead after 53 h post inoculation compared to all embryos dead under similar conditions infected with wild-type strain (Figure 2). The reduction in virulence was significant ($P < 0.05$). Complementation of *luxS* in plasmid increased the virulence by 26% to 73% (Figure 2). The complementation did not restore the virulence to that of wild type, indicating that other factors contribute to the overall virulence. Since the *luxS* mutation revealed only partial reduction in virulence in chicken embryo lethality assay, we performed the complementation assay. The complementation of *luxS* in plasmid could not able to restore the virulence as that of wild type. The loss of plasmid from the embryo due to the absence of antibiotic selection may be contributed to this effect in vivo. However, the reduction in virulence is not similar to that of avirulent strain *E. coli* K-12, indicating that virulence determinants are multifactorial in nature and LuxS is one of the factors contributed for the virulence.

Figure 2. Lethality of APEC χ7122 and its isogenic mutant in 12-day-old embryonated chicken eggs



The lethality was determined by inoculating 0.1 ml of 5 x 10^3 CFU of bacteria into the allantoic cavity of fifteen 12-day-old embryonated chicken eggs.

3.1.2.2. The *luxS* mutant poorly colonizes in embryonic tissues and fails to persist within the liver and spleen

Wild type bacteria χ7122 was able to invade and persist well in internal organs and fluids as tested (Table 6). There was no reduction in the bacterial load by 48 h in wild type bacteria. The infection with mutant *luxS* bacteria showed no significant difference at 24 hr post inoculation (Table 6). However, by 48 h, there was a significant reduction in the bacterial load in amniotic fluid, liver, lung and spleen (~ 200 fold or more) when compared to that of wild type (Table 6). The reduction in bacterial load in the internal organs and fluids can be restored by complementation in plasmid to that of wild type. Mutation in *luxS* had resulted in partial reduction in virulence, which is similar to previous studies reported by Sperandio *et al*., 1999 (36), Stroeher *et al*., 2003 (59) and Sircili *et al*., 2004 (37). The persistence of the mutant bacteria in the internal organ was reduced. Poor colonization of the internal organs, like lung and spleen may limit the systemic spread and reduce generalized infections. Determination of the virulence by enumerating the persistence of bacteria in the internal organs in 12-day-old SPF chicken embryos was similar to the results shown by Herren *et al*. (42)

Table 6. Attributes of APEC strain χ7122 and isogenic mutant to colonize in12-day-old chicken embryos, invade the internal organs, and disseminate in allantoic and amniotic fluids after 24 h and 48 h of infections

Strain	Genotype	Tissue infectivity $\log_{10}$(CFU/ml or mg of tissue) at the following time after infection		
		Tissue	24h	48h
χ7122	Wt	CAM	2.2 ± 1.2	3.1 ± 1.5
		ALF	2.0 ± 1.2	3.4 ± 1.0
		AMF	2.2 ± 1.2	3.1 ± 1.6
		Liver	3.8 ± 1.9	3.9 ± 1.2
		Spleen	2.0 ± 1.9	3.2 ± 1.8
		Lungs	3.3 ± 1.9	3.8 ± 1.8
SM3005	*luxS*$^-$	CAM	2.7 ± 1.2	3.2 ± 0.1
		ALF	2.1 ± 1.0	3.0 ± 0.1
		AMF	3.6 ± 0.1	<1 ± 0.1[a,b]
		Liver	3.5 ± 1.4	1.5 ± 1.2
		Spleen	3.2 ± 0.1	<1 ± 0.1[a,b]
		Lungs	3.2 ± 1.3	<1 ± 0.1[a,b]
SM3006	*luxS*$^-$/ p-*luxS*$^-$	CAM	2.0 ± 0.9	3.2± 1.2
		ALF	2.1 ± 1.0	2.0 ± 0.8
		AMF	3.0 ± 1.2	3.5 ± 1.2
		Liver	3.6 ± 1.2	3.6 ± 1.2
		Spleen	3.0 ± 1.0	3.5 ± 1.4
		Lungs	3.0 ± 1.2	3.5 ± 1.5

[a] This value is significantly different ($P < 0.05$) from the value for the wild-type strain.

[b] This value is significantly different ($P < 0.05$) from the value for the complemented strain.

3.1.2.3. Mutation in *luxS* reduces invasion to cultured chicken embryo fibroblasts

Attachment and invasion abilities of the *luxS* mutant strain to the cultured chicken embryo fibroblasts cells were analyzed (Table 7). There was no significant difference in the attachment of the bacterial cells to the cultured chicken fibroblast cells. However, there was a 10-fold reduction in the invasiveness of the *luxS* mutant strains when compared to that of wild type. Complementation of *luxS* gene in *trans* restored the invasiveness to that of wild type (Table 7). The systemic infection of the bacteria is dependant on its ability to attach and invade the epithelial cells and invade through fibroblast cells. The invasiveness of the mutant *luxS* bacteria was reduced by 10-fold in the cultured chicken embryo fibroblast and this attributed to the decreased embryo lethality and persistence of the mutant bacteria in the internal organs. The adherence of *luxS* mutant bacteria of EPEC had two-fold reduction on cultured HeLa epithelial cells and formed smaller microcolonies than that of wild type and complemented strain (37).

Table 7. Mutation in *luxS* reduces invasion of *E. coli* O78:K80:H9 strain χ7122 to chicken embryo fibroblast cells

Relevant genotype	Initial cells[a] (log_{10} CFU / ml)	Attached and invaded after 2h [a] (log_{10} CFU / ml)	Invaded fraction surviving after 4h [a] (log_{10} CFU / ml)	Calculated attached bacteria [a] (log_{10} CFU/ml)	Attachment Index	Invasion Index
wild type	8.6 ± 0.9	7.0 ± 0.8	6.3 ± 0.8	6.9 ± 0.8	2.0×10^{-2}	2.2×10^{-1}
luxS	8.6 ± 0.9	6.7 ± 0.8	5.0 ± 0.7	6.7 ± 0.8	1.5×10^{-2}	1.5×10^{-2}
luxS/p-*luxS*	8.6 ± 0.9	6.9 ± 0.8	6.0 ± 0.8	6.8 ± 0.8	1.7×10^{-2}	1.6×10^{-1}

[a] Results represent mean values ± standard deviations from three independent experiments.

3.1.2.4. Mutation in *luxS* reduces survival within chicken macrophage

The effects of *luxS* mutation on survival within cultured macrophage were enumerated by standard gentamicin protection assay (Table 8). There was no significant difference in the adherence. However, *luxS* mutation reduced APEC survival by 10-fold when compared to that of wild type within the macrophages (Table 8). Complementation of *luxS* in plasmid restored the survivability to that of wild type. APEC colonizing air sacs resulted in respiratory tract infections like air sacculitis, followed by systemic spread to the other parts of the body. Innate immune defense mechanism within air sacs included migration of heterophils as first line of defense, followed by macrophages (60, 57). The survivability within macrophages depends on the virulence factors of the bacteria to withstand the macrophage killing. The less pathogenic bacteria are more susceptible to killing by chicken macrophages than the pathogenic strains (58). The *luxS* mutant bacteria was survived 10-fold lesser within the cultured chicken macrophage HD11 cells than that of wild type and complemented strain.

Table 8. Mutation in *luxS* reduces survival of *E. coli* O78:K80:H9 χ7122 in chicken macrophage cells

Relevant genotype	Initial cells[a] (log_{10} CFU / ml)	Attached and invaded cells after 2h [a] (log_{10} CFU/ml)	Invaded fraction surviving after 8h [a] (log_{10} CFU/ml)	Calculated attached bacteria[a] (log_{10}CFU/ml)	Attachment index	Survival Index
wild type	8.5 ± 0.9	7.1 ± 0.9	5.3 ± 0.7	7.1 ± 0.9	3.8×10^{-2}	1.5×10^{-2}
luxS	8.7 ± 0.9	7.1 ± 0.9	4.2 ± 0.6	7.1 ± 0.9	2.9×10^{-2}	1.2×10^{-3}
luxS/p-*luxS*	8.7 ± 0.9	7.0 ± 0.8	5.1 ± 0.7	7.0 ± 0.8	2.0×10^{-2}	1.4×10^{-2}

[a] Results represent mean values ± standard deviations from three independent experiments.

3.1.2.5. Mutation in *luxS* alters the lipopolysaccharide profile

Survival from macrophage engulfment or thereafter is attributed to carbohydrates of the outer membrane and is often correlated to LPS. To see whether there was any defect in overall LPS production, LPS was isolated from equal number of cells and resolved in 12 % SDS-PAGE as shown in Figure 3. The LPS profile of the *luxS* mutant exhibited visible differences in the core and O-antigen compared to that of wild type. In the mutant *luxS*, there was a difference in both mobility and quantity of the LPS compared to the wild type. Some bands of the O-antigen were missing and few other bands were less prominent when compared to χ7122 wt LPS (Figure 3). Quantification of the LPS with densitometry was done by comparing with standard *E. coli* LPS (Sigma, St. Louis, MO). We found that from a given number of *E. coli* cells (4×10^9), there was ~ 2-fold reduction in LPS of the *luxS* mutant strain (671 ng/μl) as compared to the wild type (1705 ng/μl). This indicates that the reduced virulence properties can be partially attributed to the over all production of LPS. The change in mobility of the LPS bands also indicates that there is probably change in LPS structure. Therefore, the reduction in virulence is due to the defect in the lipopolysaccharide profile of the bacteria. LPS is a potent endotoxin responsible for gram negative septicemia (61). Additionally, LPS is responsible for the production of variety of proinflammatory cytokines followed by septic shock and disseminated intravascular coagulation of the infected animals (62). Wild-type bacteria had fully functional LPS responsible for cytotoxic and anti-phagocytic effects in chicken embryo model and increased invasion and survivability in cultured chicken cells, while the mutant *luxS* bacteria had a defective LPS resulting in decreased virulence.

Moreover, from equal number of bacterial cells, wild-type bacteria had more concentration of LPS in comparison to that of mutant bacteria. **This study suggests that a disruption of the methyl cycle, ie., recycling methionine, leads to a defect in the assembly and transport of LPS.** This is probably one of the factors modulating the virulence of the APEC strain which is multifactorial in nature.

Figure 3. Detection of equal amount of lipopolysaccharide patterns by silver stain

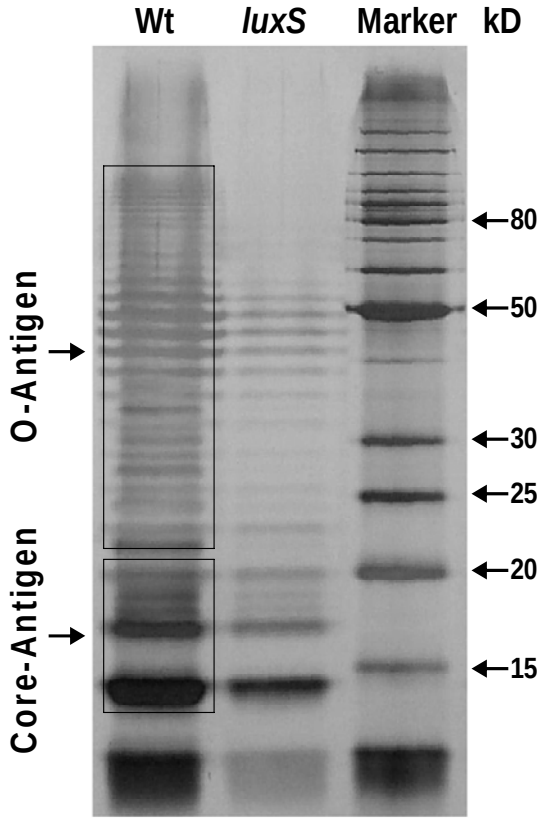


Figure 3. LPS extracted from equal number of cells. LPS resolved in 12% SDS-PAGE gel and stained with silver stain. The upper bands represent the O antigen and the lower bands represent the core LPS antigen.

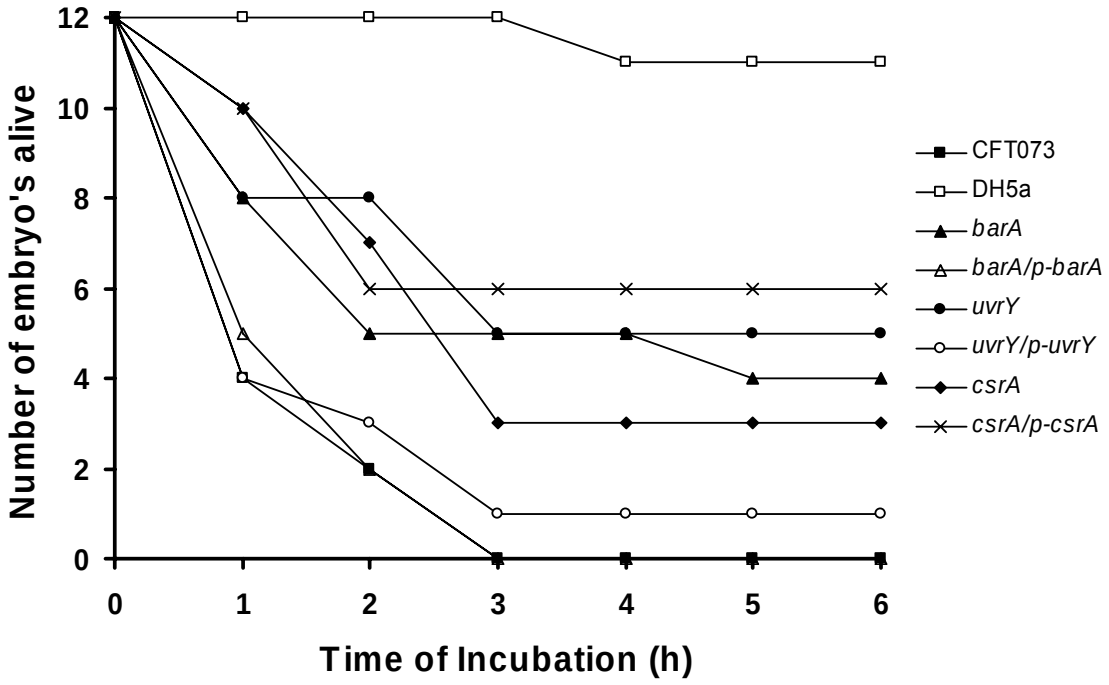
3.2. Uropathogenic *E. coli*

3.2.1. Role of BarA-UvrY TCS in determining virulence in *E. coli* CFT073

3.2.1.1. Mutation in *uvrY* reduces lethality in chicken embryo lethality assay

The mouse UTI assays showed that mutation in *uvrY* reduces the virulence than that of wild type (Dr. Suman Mukhopadhyay, personal communication). We looked for viable alternative for testing virulence using mouse UTI. The virulence of BarA-UvrY TCS of avian pathogenic *E. coli* strain χ7122 was shown using chicken embryo lethality assay (42). Here we demonstrated that the chicken embryo lethality assay could also be used to determine the virulence of these strains and had similar effects to that observed *in vivo* mouse UTI model. The virulence was determined using chicken embryo lethality assay by the number of embryos killed within a given period of time. By 72 h post inoculation, all the embryos infected with wild type strain were dead (100%), whereas mutation in *barA* and *uvrY* genes decreased the virulence by 67% (8 out of 12 embryos were dead), and 58% (7 out of 12 embryos were dead) up to 6 days post inoculation respectively. The reduction in virulence was significant ($P<0.01$) (Figure 4). These virulence could be restored to wild type CFT073 when the respective genes was provided in trans. Complementation was 100% in *barA/*p-*barA* strain and 91% (11 out of 12 embryos dead) in *uvrY/*p-*uvrY* strain. Deletion of *csrA* in strain CFT073 increased the virulence to 75% (9 out of 12 embryos dead). The virulence was decreased to 50% (6 out of 12 embryos dead) in the *csrA/*p-*csrA* strain (Figure 4). Mutation in *hlyD* (hemolysin gene) also reduced the virulence to 58% and complementation of *hlyD* in plasmid restored the effect to that of wild type.

Figure 4. Lethality of UPEC CFT073 and its isogenic mutant in 12-day-old embryonated chicken eggs



■ = CFT073, □ = DH5αK12, ▲ = *barA*, Δ = *barA*/p-*barA*, ● = *uvrY* ○ = *uvrY*/p-*uvrY*, ◊ = *csrA*, x = *csrA*/p-*csrA*. The lethality was determined by inoculating 0.1 ml of 5 x 10^3 CFU of bacteria into the allantoic cavity of twelve 12-day-old embryonated chicken eggs.

3.2.1.2. Mutation in *uvrY* reduces the invasion to cultured ureter uroepithelial cells

The functional roles of *barA* and *uvrY* genes in regulating attachment and invasion in cultured uroepithelial cells was investigated by using standard gentamicin protection assays (46). Attachment and invasion abilities of mutant *barA* and *uvrY* bacteria compared to that of wild type were unaltered in cultured urinary bladder (HTB-9) epithelial cells (Table 10 and Figure 5). Mutation in *uvrY* reduced the invasion abilities by two logs (~ 100-fold) in cultured ureter (SV-HUC1) epithelial cells (Table 9 and Figure 5). The attachment of the bacteria was unaltered and there was no significant difference. Expression of *uvrY* in trans in plasmid pBR322 restored the invasion abilities in cultured ureter (SV-HUC1) cells similar to that of wild type. However, deletion of *barA* had no effect on the attachment and invasion in cultured ureter (SV-HUC 1 cells) (Table 9). This indicates that as a transcriptional regulator, UvrY protein has a larger role in determining virulence and an *uvrY* mutant is less virulent than a *barA* mutant. Deletion of *csrA* which controls carbon metabolism, flagellum biosynthesis (29, 24, 30) resulted in unaltered invasiveness like that of wild-type, while the *csrA*/p-*csrA* complemented strain reduced the invasiveness by one log (~ 10-fold) (Table 9).

Table 9. Mutation in *uvrY* reduces invasion of *E. coli* CFT073 strain to cultured Ureter uroepithelial cells

Genotype	Initial cells[a] (log10 CFU / ml)	Attached and invaded after 2h[a] (log10 CFU / ml)	Invaded fraction surviving after 4h[a] (log 10 CFU / ml)	Calculated attached bacteria[a] (log 10 CFU/ml)	Attachment Index	Invasion Index
wild type	8.4 ± 0.9	7.4 ± 0.8	3.1 ± 0.5	7.5 ± 0.8	7.7×10^{-2}	6.8×10^{-5}
barA	8.3 ± 0.9	7.2 ± 0.9	3.0 ± 0.5	7.2 ± 0.9	8.3×10^{-2}	6.6×10^{-5}
barA/p-barA	8.9 ± 0.9	7.7 ± 0.9	3.3 ± 0.5	7.7 ± 0.9	6.3×10^{-2}	4.7×10^{-5}
uvrY	8.6 ± 0.9	6.8 ± 0.8	0.7 ± 0.1	6.8 ± 0.8	1.7×10^{-2}	7.1×10^{-7}
uvrY/p-*uvrY*	8.8 ± 0.9	7.6 ± 0.9	2.9 ± 0.5	7.6 ± 0.9	6.3×10^{-2}	1.9×10^{-5}
csrA	7.7 ± 0.9	6.6 ± 0.8	2.2 ± 0.3	6.6 ± 0.8	8.9×10^{-2}	3.6×10^{-5}
csrA/p-*csrA*	7.7 ± 0.9	6.6 ± 0.8	0.7 ± 0.1	6.6 ± 0.7	7.8×10^{-2}	1.3×10^{-6}

[a] Results represent mean values ± standard deviations from three independent experiments.

Table 10. Attachment and invasion of *E. coli* CFT073 strain is unaltered in cultured Urinary bladder uroepithelial cells

Genotype	Initial cells[a] (log10 CFU / ml)	Attached and invaded after 2h[a] (log10 CFU / ml)	Invaded fraction surviving after 4h[a] (log 10 CFU / ml)	Calculated attached bacteria[a] (log 10 CFU/ml)	Attachment Index	Invasion Index
wild type	8.8 ± 0.9	7.1 ± 0.8	3.6 ± 0.6	7.1 ± 0.8	1.41×10^{-2}	4.4×10^{-4}
barA	8.7 ± 0.9	6.7 ± 0.8	3.3 ± 0.5	6.7 ± 0.8	1.02×10^{-2}	4.5×10^{-4}
barA/p-barA	8.6 ± 0.9	6.7 ± 0.8	3.3 ± 0.5	6.7 ± 0.8	1.36×10^{-2}	3.6×10^{-4}
uvrY	8.7 ± 0.9	6.7 ± 0.8	3.5 ± 0.5	6.7 ± 0.8	1.32×10^{-2}	3.9×10^{-4}
uvrY/p-*uvrY*	8.5 ± 0.9	7.4 ± 0.9	5.5 ± 0.7	7.4 ± 0.9	6.9×10^{-2}	1.4×10^{-2}
csrA	7.4 ± 0.9	6.3 ± 0.8	2.9 ± 0.5	6.3 ± 0.8	8.7×10^{-2}	3.9×10^{-3}
csrA/p-*csrA*	7.3 ± 0.9	5.0 ± 0.7	2.2 ± 0.3	5.0 ± 0.7	4.9×10^{-3}	1.6×10^{-3}

[a] Results represent mean values ± standard deviations from three independent experiments.

Figure 5. Mutation in *uvrY* reduces the attachment to cultured human ureter epithelial cells

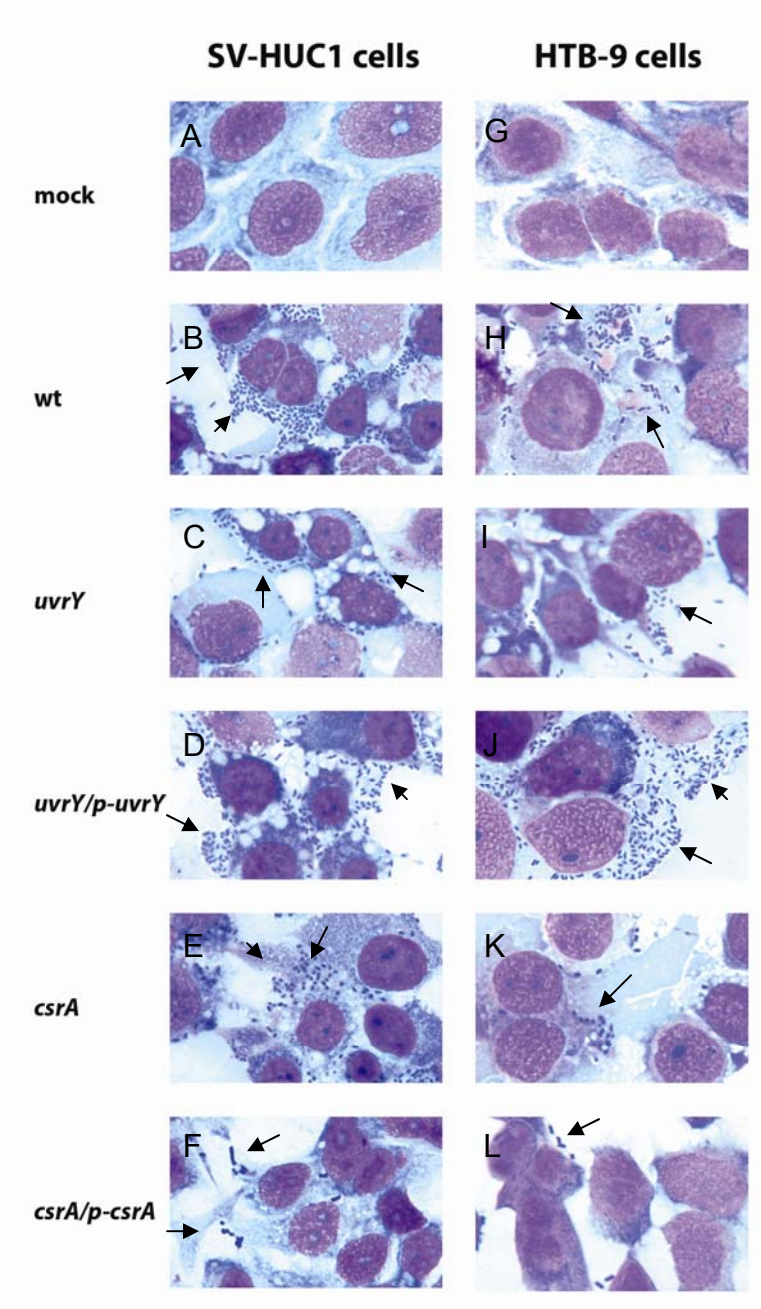


Figure 5. The monolayer of cultured cells over glass coverslips were incubated with

bacteria (grown under static conditions) with a multiplicity of infection of 10. The unattached bacteria were washed, stained with Hema-3 stain, and visualized using a Spot RT camera attached to an Olympus BH-2 microscope with a 100x objective lens. Attachment of *E. coli* CFT073, *uvrY* mutant, *uvrY* mutant complemented with a plasmid-borne copy of the wild-type *uvrY* gene (p-*uvrY*), *csrA* mutant and *csrA* mutant complemented with a plasmid-borne copy of the wild-type *csrA* gene (p-*csrA*). Attached bacteria indicated by arrows. (A to F), Attachment to human urinary bladder (HTB-9) cells. (G to L), Attachment to human ureter SV-HUC1 cells.

3.2.1.3. Mutation in *uvrY* reduces the cytotoxic effect to cultured human kidney cells

Mutation in *uvrY* reduced the cytotoxic effects of the bacterial supernatants on cultured human kidney HK-2 cells. The timing of the assay was closely evaluated (data not given), and suitable time point for reading the assay was calculated and found to be 180 min after the addition of WST-1 reagent to the infected cells (Table 11 and Figure 6). The growth was inhibited and 21% of the treated cells were alive in wild type, while deletion of *barA*, *uvrY* and *hlyD* reduced the cytotoxic effects and 38%, 68% and 35% of the cells with relative to the control. The reduction in the cytotoxicity was significant for *uvrY* ($p \leq 0.001$) and *barA*, *hlyD* ($p \leq 0.01$) mutant bacteria compared to the wild type (Table 11). Complementation of the genes in plasmid restored the effects to that of wild type. This experiment also demonstrated that *uvrY* mutant is less virulent than *barA* mutant. However *csrA* mutant is more cytotoxic compared to wild type as only 13% of treated cells were alive.

Figure 6. Mutation in *uvrY* reduces the cytotoxic effect to cultured human kidney cells

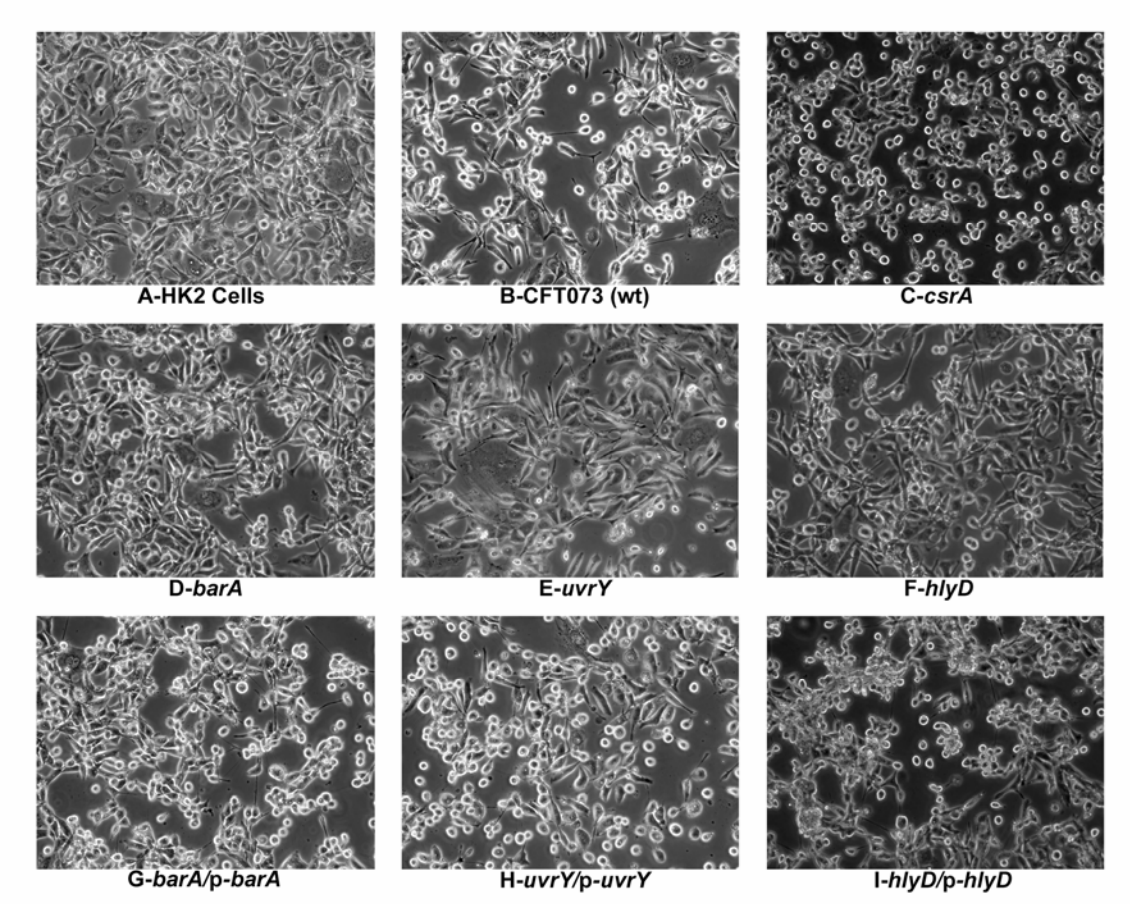


Figure 6. Phase-contrast photomicrographs of human kidney cells (HK-2 cells) (A), 6 hours after treating cells with supernatants of

CFT073 wild type (B), *csrA* (C), *barA* (D), *uvrY* (E) and *hlyD* (F), *barA*/p-*barA* (G), *uvrY*/p-*uvrY* (H) and *csrA*/p-*csrA* (I). Note the rounding and detachment of cells in the treated monolayer.

Table 11. Mutation in *uvrY* reduces cytotoxic effects on cultured human kidney cells

Genotype	Percentage of survival [a] (relative to control)
Control	100
CFT073 wild type	21 ± 3
hlyD	35 ± 2*
hlyD/p-*hlyD*	21 ± 2
barA	38 ± 3*
barA/p-*barA*	22 ± 2
uvrY	68 ± 2**
uvrY/p-*uvrY*	22 ± 3
csrA	13 ± 4
csrA/p-*csrA*	36 ± 3

[a] Data obtained from three independent experiments with 6 replicates per condition. (*, $p \leq 0.01$; **, $p \leq 0.001$, relative to wild type).

3.2.1.4. Mutation in *uvrY* causes growth defect in artificial urine medium

Mutation in *uvrY* reduced the virulence in the mouse model, chicken embryo lethality assay, cytotoxicity of the HK-2 cells and also reduced the invasion abilities in SV-HUC1 cells. Consequently, we examined the virulence factors that are responsible for the reduction in the virulence. We tried to identify virulence factors in the bacterial culture supernatant, differences in lipopolysaccharide and for growth defects in the artificial urine medium. The bacteria causing urinary tract infection has to grow in human urine. Urine is a variable fluid and its composition varies between individuals and in the same individual over time (63). The composition of the urine varies by fluid intake, exercise, ambient temperature and diet. The pH can vary from 4.5 to 8 (64). To avoid the variations found in the human urine over time, a simple artificial urine medium having mid-point values for each component is used to determine the growth properties of the bacteria. The bacteria were grown in filter sterilized artificial urine medium (pH 6.5). When compared with the wild type, the generation time of the *uvrY,* and *barA* mutant bacteria was increased by 62 and 29 min respectively in the exponential phase (Table 12). However, all the bacterial strains reached almost the same OD in the stationary phase (data not given). Deletion of *hlyD* and *csrA* also increased the generation time by 93 and 54 min, respectively. Complementation of the genes in plasmid restored the generation time almost to that of wild type. The growth defect of the mutant bacteria observed during the exponential phase may result in the decreased fitness in the urine medium, resulting in decreased virulence.

Table 12. Mutation in *barA* or *uvrY* increases the generation time during exponential phase and decreases hemolysis of sheep erythrocytes in coculture with bacterial culture supernatant grown in artificial urine medium

Genotype	Generation time (min)[a]	Hemolysis of sheep erythrocytes (OD 405)[a]
CFT073 wild type	102.24 ± 2.31	2.7 ± 0.19
hlyD	195.6 ± 3.34*	0.57 ± 0.09
hlyD/p-*hlyD*	128.32 ± 1.62	10.3 ± 0.28
barA	135.05 ± 2.15*	0.53 ± 0.12***
barA/p-*barA*	115.16 ± 3.42	1.85 ± 0.10
uvrY	164.31 ± 3.42*	0.35 ± 0.14***
uvrY/p-*uvrY*	122.48 ± 3.42	3.6 ± 0.18
csrA	156.53 ± 3.42*	3.9 ± 0.28**
csrA/p-*csrA*	125.44 ± 3.42	0.39 ± 0.22***

[a]Data obtained from mean values ± standard deviations from three different experiments. (*, $p \leq 0.1$; **, $p \leq 0.01$; ***, $p \leq 0.001$ compared to wild type).

3.2.1.5. Mutation in *uvrY* alters secreted protein Hemolysin

Since the *uvrY* mutant exhibited decreased virulence in mouse model, chicken embryo killing and cytotoxicity to cultured HK-2 cells, we compared the secreted protein profile in culture supernatant to identify the virulence determinants of various mutants to that of wild type bacteria. The protein profiles of these filtered bacterial culture supernatant were identified by resolution via 4-20% Tris-Glycine gradient SDS-PAGE gel (Figure 7). The bands of the interest were excised and subjected to mass spectrometric analysis. One band of approximately 110 kDa was found to be Hemolysin by protein BLAST analysis. Hemolysin is a cytotoxic protein. Decrease in the hemolytic activity of *uvrY* mutant is correlated with its decreased virulence on cultured HK- cells, chicken embryo killing and in mouse UTI. The UPEC bacteria express its virulence factors and colonize the bladder, causing cystitis or in kidney resulting in pyelonephritis. The exotoxin Hly is one of the virulence factors associated with pathogenesis and the production of hemolysin protein contributes to the virulence of extra-intestinal pathogenic *E. coli* infections (65, 49). The BarA-UvrY family of TCSs is known to regulate virulence in different bacterial species (24, 31, 32, 42). The 110 kDa hemolysin protein (HlyA) (66, 67) was down regulated in the mutant compared to wild type (Figure 7). We investigated the role of the activity of hemolysin protein obtained from filtered bacterial culture supernatant grown in artificial urine medium against sheep erythrocytes at 30.5°C for 7 h. The hemolytic activity of *uvrY* and *barA* mutants when compared to wild type bacteria was considerably different ($p < 0.01$). There was more than 5 and 7-fold decrease in hemolytic activity in the mutants *barA* and *uvrY* respectively (Table 12).

Complementation in plasmid restored the hemolytic activity similar to wild type. The decreased exotoxin HlyA activity contributed to the reduction in the virulence of mutant bacteria. However deletion of *csrA* resulted in moderate increase in hemolytic activity and it's complementation in plasmid decreased the hemolytic activity. Thus, it appears that hemolysin expression is regulated by BarA-UvrY TCS. The hemolytic activities of cultured bacterial supernatant contribute to the reduced cytotoxic effects against cultured human kidney HK-2 cells and reduced virulence in chicken embryo killing.

Figure 7. Mutation in *uvrY* or *csrA* alters secreted protein Hemolysin

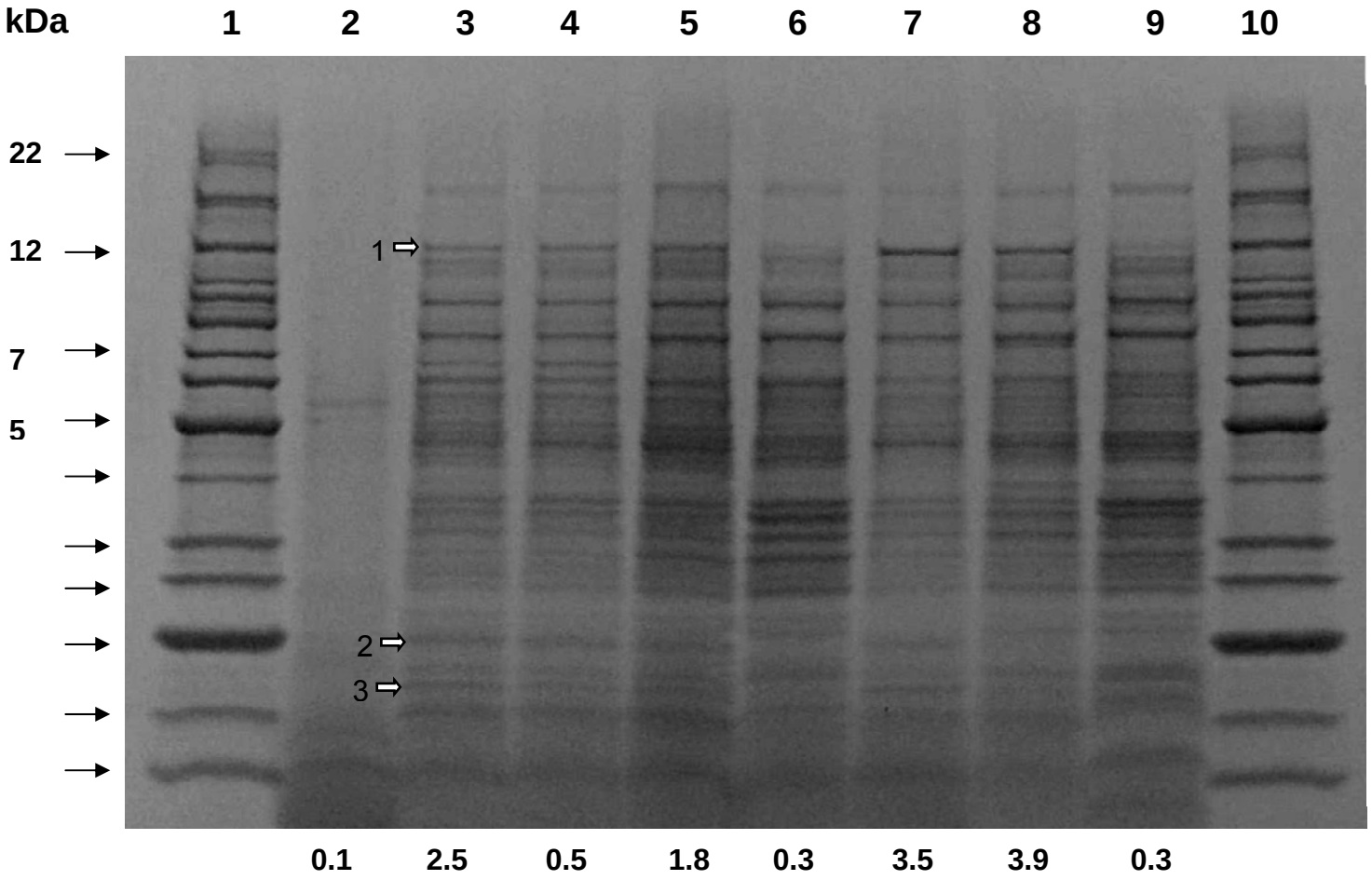


Figure 7. Protein samples were separated by 4 to 20% SDS-PAGE and stained with Coomassie blue. Lanes: 1. Protein marker, 2. LB media, 3. CFT073 wt, 4. *barA*, 5. *barA/*p-*barA*, 6. *uvrY*, 7. *uvrY*/p-*uvrY*, 8. *csrA*, 9. *csrA/*p-*csrA*, 10. Protein marker.

MS analysis result.

Hemolysin A [Escherichia coli CFT073] (indicated by arrow mark 1)

Score = 40.8 bits (94), Expect = 0.017, Identities = 21/21 (100%), Positives = 21/21 (100%), Gaps = 0/21 (0%)

Query 1 KNLTETDNLYSVEELIGTTRA 21

KNLTETDNLYSVEELIGTTRA

Sbjct 708 KNLTETDNLYSVEELIGTTRA 728

Table 13. Secreted proteins, analyzed by mass spectrometry, that are differentially expressed by the CFT073 strains

Protein band identification number	MS protein BLAST analysis
1	Hemolysin A *Escherichia coli* CFT073
2	Pilin type F7 1 precursor *Escherichia coli,* major pilus subunit
3	Adhesion protein PapA *Escherichia coli*

3.2.1.6. Mutation of either *uvrY* or *csrA* alters Lipopolysaccharide profile

We investigated the role of LPS in pathogenesis. LPS is a potent endotoxin responsible for gram negative septicemia (61) and responsible for the production of variety of proinflammatory cytokines followed by septic shock and disseminated intravascular coagulation of the infected animals (62). LPS was isolated from equal number of cells and resolved in 12 % SDS-PAGE as shown in the Figure 8. The LPS profile of the *uvrY* mutant exhibited visible differences in the core and O-antigen compared to that of wild type. In the mutant *uvrY*, there was a difference in both mobility of bands and quantity of the LPS compared to the wild type. Some bands of the O-antigen were missing and few other bands were less prominent when compared to CFT073 wt LPS (Figure 8). Complementation restored the effect to wild type. Deletion of *csrA* resulted one high molecular weight O-antigen band became less prominent and few low molecular weight O-antigen bands were more prominent. The *csrA*/p-*csrA* complemented strain reduces the LPS expression. Quantification of the LPS with densitometry assay was done by comparing with standard *E. coli* LPS. We found that from a given number of *E. coli* cells (4×10^9), there was partial reduction in LPS of the *uvrY* mutant strain (723 ng/µl) as compared to the wild type (853 ng/µl). The *csrA* mutant had the highest concentration of LPS (2080 ng/µl) and *csrA*/p-*csrA* complemented strain leads to reduced expression of LPS (643 ng/µl). Thus LPS production is probably regulated at the post transcription level. This partial reduction in the LPS may also contribute to reduction in virulence.

Figure 8. Detection of lipopolysaccharide patterns by silver stain

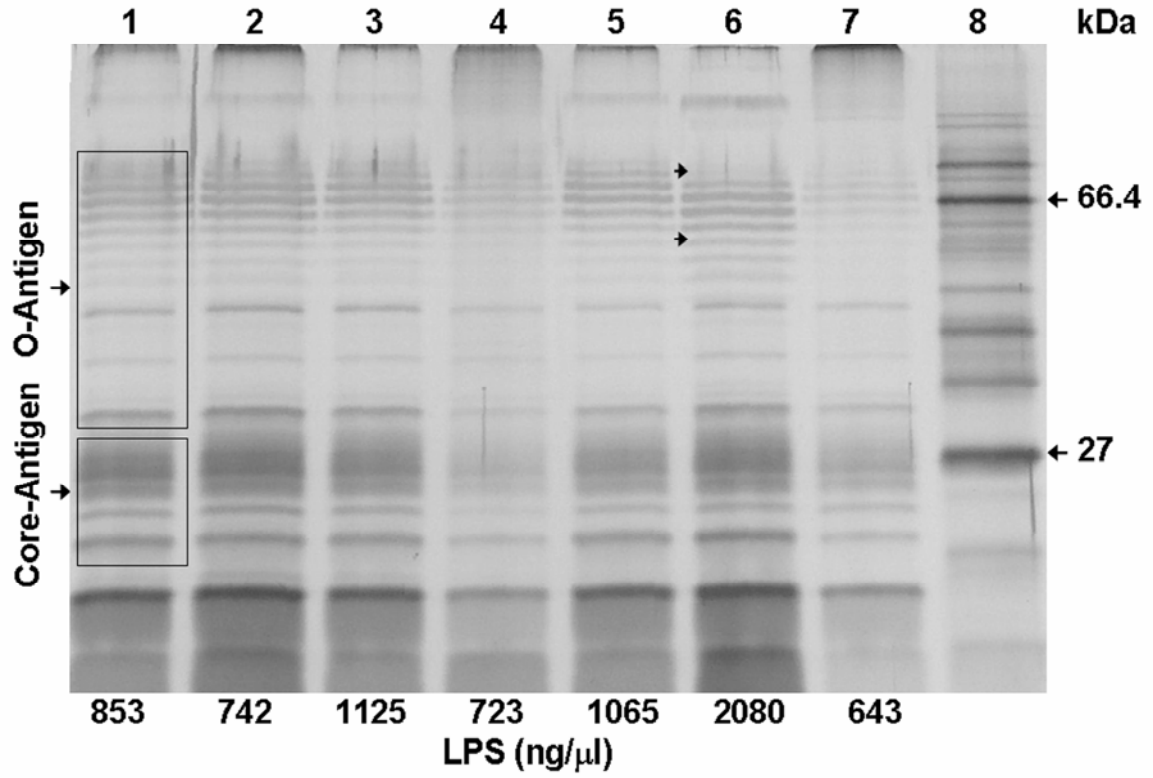


Figure 8. LPS extracted from equal number of cells. LPS resolved in 12% SDS-PAGE gel and stained with silver stain. The upper bands represent the O antigen and the lower bands represent the core LPS antigen. Lanes: 1. CFT073 wt, 2. *barA*, 3. *barA*/p-*barA*, 4. *uvrY*, 5. *uvrY*/p-*uvrY*, 6. *csrA*, 7. *csrA*/p-*csrA*, 8. Protein marker.

3.2.1.7. Mutation in *uvrY* down regulates inflammatory cytokines

LPS is an integral part of outer membrane of UPEC. Bacterial infections or LPS triggers mucosal cytokine production (68, 69, 70). The mucosal cytokines are derived in part from epithelial cells. Uroepithelial cells secrete interleukin-6 (IL-6) and IL-8 when stimulated by UPEC (69, 71). During gram negative septicemia, these proinflammatory cytokines, like tumor necrosis factor (TNF) and IL-6, activates the inflammatory cascade and trigger the systemic infection (72). So we assessed the role of cytokine responses of human ureter uroepithelial cell line SV-HUC1 cells to whole bacterial cells and LPS by quantitative real time PCR (Table 14). Both intact bacterial cells and LPS stimulated the mRNA expression of proinflammatory cytokines, including TNF-α, IL-6 and IL-8. The expression levels of TNF-α, IL-6 and IL-8 were higher in cells stimulated with intact bacterial cells than in cells stimulated with LPS. The wild type bacteria and its LPS resulted in highest expression of both cytokine and chemokine, while the cells treated with *uvrY* mutant bacteria has lowered TNF-α, IL-6, and IL-8 expression by ~ 4, 5 and 9-fold. The cells treated with purified LPS from *uvrY* mutant also down-regulated the cytokines TNF-α and IL-6 by ~ 3-fold. The *uvrY*/p-*uvrY* complemented strain restored the phenotype like that of wild type. Although the *csrA* mutant had the highest level of LPS (2080 ng/μl) from equal number of cells, cells treated with equal amount of LPS (10ng/μl) did not stimulated the expression of cytokines like that of wild type (Table 14). This may explain that there is a difference in the LPS pattern (Figure 8) which accounts for the lowered expression of the cytokines. It indicates that *uvrY* and *csrA* may affect LPS assembly and transport. Thus mutation in *uvrY* affects assembly and mutation in

csrA may affect synthesis and probably transport. Also, stimulating with *csrA* mutant bacteria strain had similar effect on expression of cytokines. Our result suggests that *uvrY* mutant is less virulent than all other strains and BarA-UvrY TCS may be a global regulator of UPEC virulence.

Table 14. Mutation in *uvrY* gene in CFT073 strain leads to lower cytokine and chemokine expression as detected by quantitative real-time PCR

Genotype	Fold change in mRNA levels. SV-HUC1 ureter cells treated with					
	Purified LPS (10 ng)			Whole bacterial cells		
	TNFα	IL6	IL8	TNFα	IL6	IL8
CFT073 Wild type	3.7±0.3↑	5.5±0.4↑	2.2±0.3↑	8.7±0.6↑	17.4±0.9↑	12.8±0.5↑
uvrY	1.0±0.6↓*	2.0±0.1↓*	1.6±0.4↓*	2.1±0.3↑**	3.6±0.1↑**	1.4±0.4↓**
uvrY/p-*uvrY*	2.9±0.3↑	4.9±0.3↑	2.1±0.2↑	8.2±0.9↑	17.8±0.5↑	12.1±0.7↑
csrA	1.8±0.2↑*	3.6±0.2↑*	1.5±0.1↑	3.6±0.6↑*	6.1±0.4↑*	4.3±0.4↑**
csrA/p-*csrA*	1.1±0.4↑	1.8±0.7↑	1.0±0.2↑	2.0±0.2↑	3.2±0.3↑	1.5±0.7↑

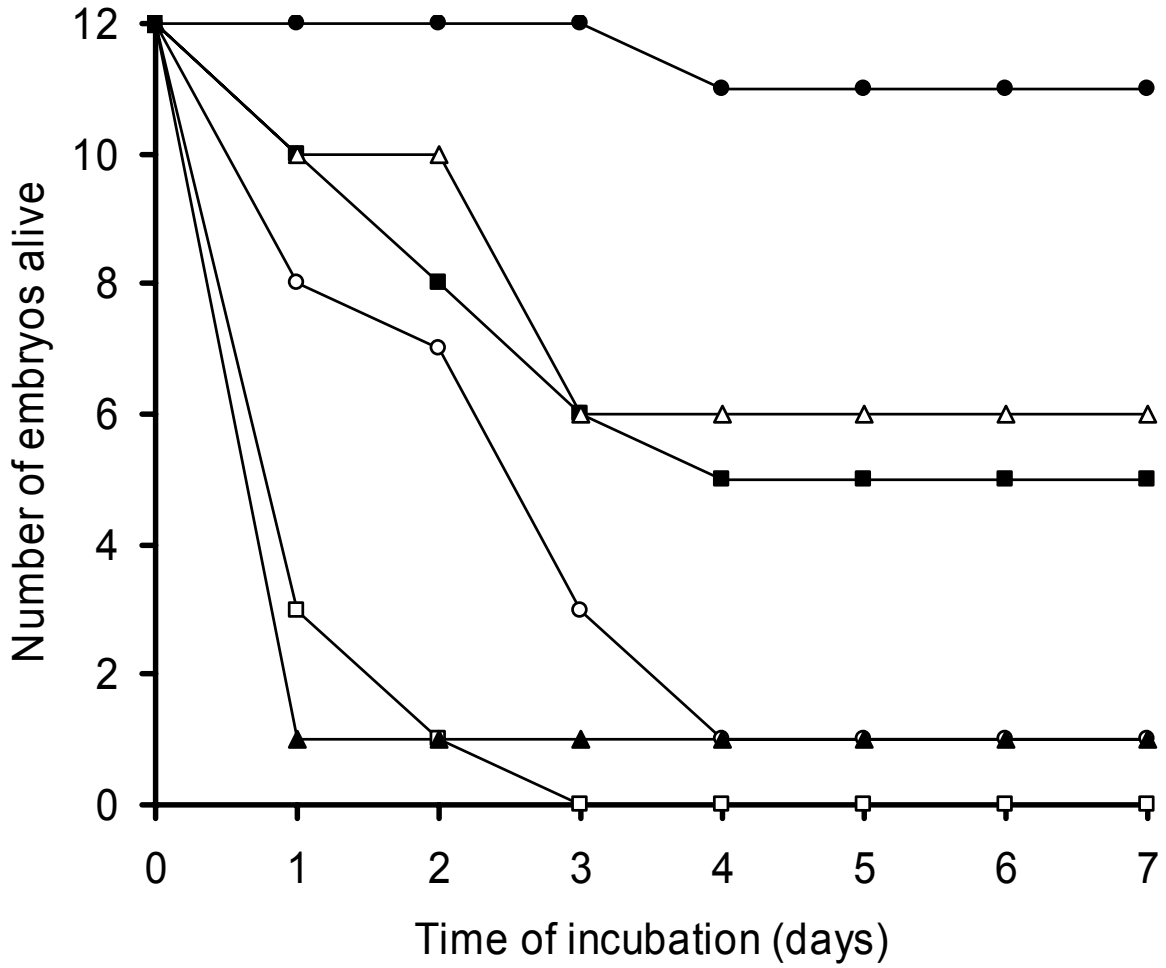
The values are mean ± SD of the mean for two independent experiments with triplicate samples. The downward arrow indicates down regulation compared to the wild type. (*, $p \leq 0.1$; **, $p \leq 0.01$compared to wild type).

3.2.2. Role of LuxS in determining virulence in *E. coli* CFT073

3.2.2.1. Mutation in *luxS* reduces lethality in chicken embryo lethality assay

In our laboratory the mouse UTI assays showed that mutation in *luxS* reduced the virulence than that of wild type. We looked for viable alternate for mouse UTI to determine the virulence determinants. The virulence of BarA-UvrY TCS of avian pathogenic *E. coli* strain χ7122 was shown using chicken embryo lethality assay (42). Here we tried to demonstrate the chicken embryo lethality assay as a surrogate model for in vivo mouse UTI. The virulence was determined using chicken embryo lethality assay by the number of embryos killed within a given period of time. By 72 h post inoculation, all the embryos infected with wild type strain were dead, whereas mutation in *luxS* reduced virulence by 58% (7 out of 12 embryos were dead) after 7 days post inoculation. The reduction in virulence was significant ($P<0.01$) (Figure 9). Complementation of *luxS* in plasmid restored the virulence to 91%. Mutation in *hlyD* (hemolysin gene) also reduced the virulence to 50% and complementation of *hlyD* in plasmid restored the effect to that of wild type (Figure 9).

Figure 9. Chicken embryo lethality assay of UPEC CFT073 and its mutant in 12-day-old embryonated eggs



□ = CFT073, ■ = *luxS*, ◊ = *luxS*/p-*luxS*, Δ = *hlyD*, ▲ = *hlyD*/p-*hlyD* ● = DH5αK-12.

The lethality was determined by inoculating 0.1 ml of 5 x 10^3 CFU of bacteria into the allantoic cavity of twelve 12-day-old embryonated chicken eggs.

3.2.2.2. Mutation in *luxS* reduces the invasion to cultured ureter uroepithelial cells

The role of *luxS* in mediating attachment and invasion in cultured uroepithelial cells was investigated by using standard gentamicin protection assays (46). Attachment and invasion abilities of mutant *luxS* bacteria compared to that of wild type were unaltered in cultured urinary bladder (HTB-9) epithelial cells (Table 15). However, mutation in *luxS* reduced the invasion abilities by one log (~ 10 fold) in cultured ureter (SV-HUC1) epithelial cells (Table 16). The attachment of the bacteria was unaltered and there was no significant difference. Expression of *luxS* in trans in plasmid pBR322 restored the invasion abilities in cultured ureter (SV-HUC1) cells as that of wild type.

3.2.2.3. Mutation in *luxS* reduces the cytotoxic effect to cultured human kidney cells

Mutation in *luxS* reduces the cytotoxic effects of the bacterial supernatants on cultured human kidney HK-2 cells. The suitable time point for reading the assay was closely evaluated (data not given), and found to be 180 minutes after the addition of WST-1 reagent into the infected cells (Table 17 and Figure 10). The growth was inhibited 80 % in wild type, while mutation in *luxS* and *hlyD* reduced the cytotoxic effects; the growth was inhibited 65 % and 62 % respectively relative to the control. The reduction in the cytotoxicity was significant for both *luxS* ($P<0.05$) and *hlyD* ($P \leq 0.01$) mutant compared to the wild type (Table 17). Complementation of the genes in plasmid restored the effects to that of wild type.

Table 15. Attachment and invasion of *E. coli* CFT073 strain to cultured Urinary bladder uroepithelial cells

Relevant genotype	Initial cells [a] (log_{10} CFU ml^{-1})	Attached and invaded after 2h[a] (log_{10} CFU ml^{-1})	Invaded fraction surviving after 4h[a] (log_{10} CFU ml^{-1})	Calculated attached bacteria[a] (log_{10} CFU ml^{-1})	Attachment Index	Invasion Index
Wild type	8.8 ± 0.9	7.0 ± 0.8	3.6 ± 0.5	7.0 ± 0.8	1.4×10^{-2}	4.4×10^{-4}
luxS	8.7 ± 0.9	7.0 ± 0.8	3.2 ± 0.5	7.0 ± 0.8	2.0×10^{-2}	1.7×10^{-4}
luxS/p-*luxS*	7.9 ± 0.9	6.8± 0.8	3.7 ± 0.5	6.8 ± 0.8	7.5×10^{-2}	9.0×10^{-4}

[a] Results represent mean values ± standard deviations from three independent experiments.

Table 16. Mutation in *luxS* reduces invasion of *E. coli* CFT073 strain to cultured Ureter uroepithelial cells

Relevant genotype	Initial cells [a] (log_{10} CFU ml^{-1})	Attached and invaded after 2h [a] (log_{10} CFU ml^{-1})	Invaded fraction surviving after 4h [a] (log_{10} CFU ml^{-1})	Calculated attached bacteria [a] (log_{10} CFU ml^{-1})	Attachment Index	Invasion Index
Wild type	8.6 ± 0.9	7.4 ± 0.8	3.3 ± 0.5	7.5 ± 0.9	7.5×10^{-2}	6.7×10^{-5}
luxS	8.7 ± 0.9	7.4 ± 0.9	2.1 ± 0.3	7.4 ± 0.9	4.6×10^{-2}	5.6×10^{-6}
luxS/p-*luxS*	8.3 ± 0.9	7.1 ± 0.8	3.8 ± 0.6	7.1 ± 0.8	6.0×10^{-2}	5.0×10^{-4}

[a] Results represent mean values ± standard deviations from three independent experiments.

Figure 10. Mutation in *luxS* reduces the cytotoxic effect to cultured human kidney cells

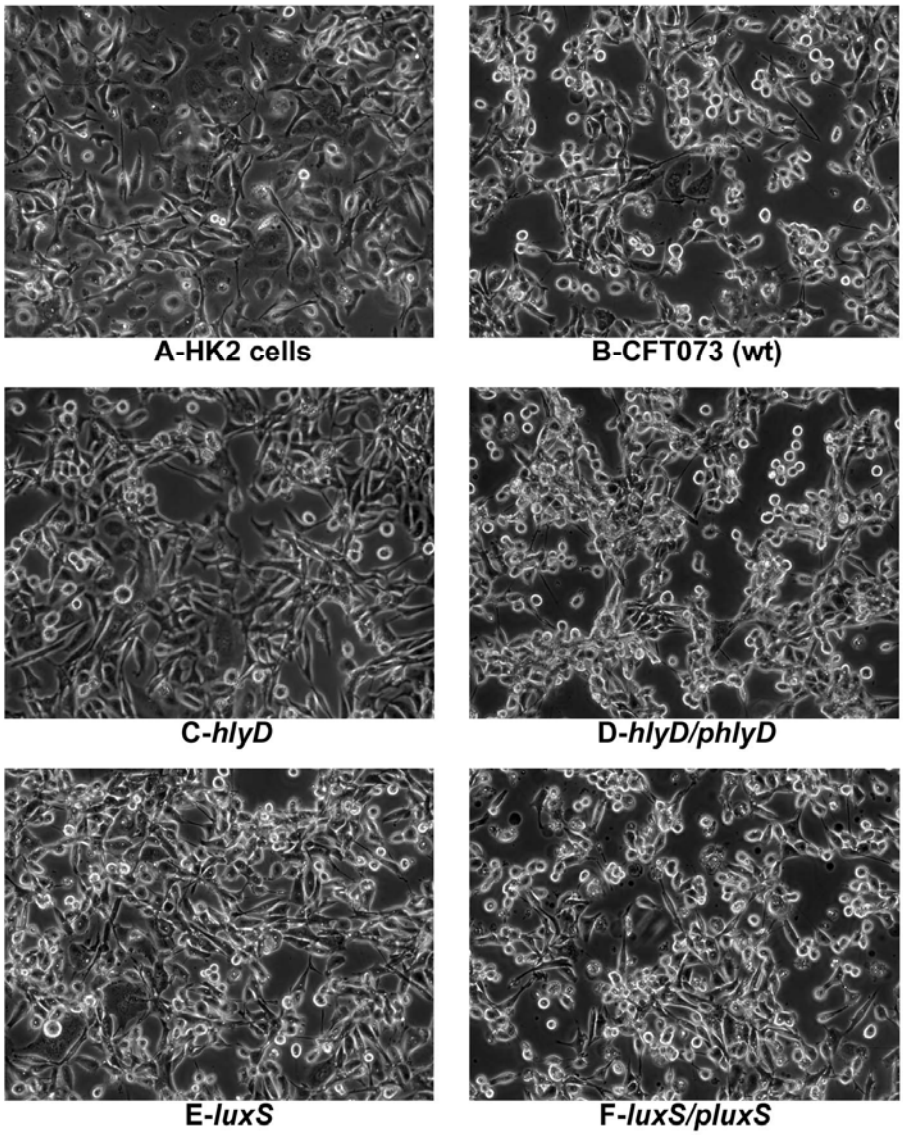


Figure 10. Phase-contrast photomicrographs of human kidney control (HK-2 cells) (A), 6 hours after treating cells with supernatants of CFT073 wild type (B), *hlyD* (C), *hlyD*/p-*hlyD* (D), *luxS* (E) and *luxS*/p-*luxS* (F). Note the rounding and detachment of cells in the treated monolayer.

Table 17. Mutation in *luxS* reduces cytotoxic effects on cultured human kidney cells

Genotype	Percentage of survival (relative to control)
Control	100
CFT073 wild type	20 ± 2
hlyD	38 ± 3**
hlyD/p-*hlyD*	24 ± 3
luxS	36 ± 2*
luxS/p-*luxS*	23 ± 2

Data obtained from three independent experiments with 6 replicates per condition. (*, $p < 0.05$; **, $p \leq 0.01$ relative to wild type).

3.2.2.4. Mutation in *luxS* causes growth defect in artificial urine medium

Mutation in *luxS* reduced the virulence in the mouse model, chicken embryo lethality assay, cytotoxicity of the HK-2 cells and reduced the invasion abilities in SV-HUC1 cells. Consequently, we examined for the virulence factors that are responsible for the reduction in the virulence. We examined for differences in virulence factors in the bacterial culture supernatant, differences in lipopolysaccharide and for growth defects in the artificial urine medium. The bacterial pathogen causing urinary tract infection has to grow in human urine. Urine is a variable fluid and its composition varies between individuals and in the same individual over time (63). The composition of the urine varies by fluid intake, exercise, ambient temperature and diet. The pH can vary from 4.5 to 8 (64). To avoid the variations found in the human urine over time, a simple artificial urine medium having mid-point values for each component is used to determine the growth properties of the bacteria. The bacteria were grown in filter sterilized artificial urine medium (pH 6.5). When compared with the wild type the generation time of the *luxS* mutant bacteria was increased in the exponential phase (Table 18). However, all the bacterial strains reached almost the same OD in the stationary phase (data not given). The growth defect of the mutant bacteria observed during the exponential phase may result in the decreased fitness in the urine medium, resulting in decreased virulence.

3.2.2.5. Mutation in *luxS* alters the secreted protein Hemolysin and Pap

Since the *luxS* mutant showed decreased virulence in mouse model, chicken embryo killing and cytotoxicity to cultured HK-2 cells, we compared the secreted protein profile in culture supernatant to identify the virulence determinants of *luxS* mutant to that of wild type bacteria. The protein profiles of these filtered bacterial culture supernatant were identified in 4-20 % Tris-Glycine gradient SDS-PAGE gel. The bands of the interest were excised and subjected to mass spectrometric analysis. Two bands of approximately 110 kDa and 16.5 kDa were found to be Hemolysin and Pap protein by protein BLAST analysis. Hemolysin is a cytotoxic protein and decrease in the hemolytic activity of *luxS* mutant was correlated with its decreased virulence on cultured HK- cells, chicken embryo killing and in mouse UTI. The UPEC bacteria express its virulence factors and colonize in the bladder, causing cystitis or in the kidney resulting in pyelonephritis. The exotoxin Hly is one of the virulence factors associated with pathogenesis and the attachment organelle Pap pili helps in colonizing the uroepithelial tissues (73, 74). The production of hemolysin protein contributes to the virulence of extra-intestinal pathogenic *E. coli* infections (65, 49). Mutation in *luxS* resulted in partial reduction in virulence as reported by Sperandio *et al*., (36), Stroeher *et al*., (59) and Sircili *et al*., (37). Both 110 kDa hemolysin protein (HlyA) (66, 67) and 16.5 kDa PapA protein (75) were down regulated in the mutant compared to wild type. We investigated the role of the activity of hemolysin protein obtained from filtered bacterial culture supernatant grown in artificial urine medium against sheep erythrocytes at 30.5°C for 7 h. The hemolytic activity of *luxS* mutant and wild type bacteria was considerably

different ($p < 0.01$). There was more than 3-fold decrease in hemolysis activity in the mutant *luxS* (Table 18). Complementation of *luxS* in plasmid restored the hemolytic activity similar to wild type. The decreased exotoxin HlyA activity contributed to the reduction in the virulence of mutant bacteria. The hemolytic activity of the cultured bacterial supernatant contributes to the reduced cytotoxic effects against cultured human kidney HK-2 cells. P pilus is one of the critical factors in causing urinary tract infections (76). Although the attachment of the *luxS* mutant bacteria to the cultured uroepithelial tissue was not affected, its invasion was reduced in cultured SV-HUC1 ureter cells. This reduction may be due to the down regulation of the Pap protein, as detected in SDS-PAGE gel (Figure 11).

Table 18. Mutation in *luxS* increases the generation time during exponential phase and decreases hemolysis of sheep erythrocytes in coculture with bacterial culture supernatant grown in artificial urine medium

Genotype	Generation time (min) [a]	Hemolysis of sheep erythrocytes (OD 405) [a]
CFT073 wild type	101.06 ± 3.01	2.6 ± 0.14
hlyD	198.6 ± 4.14	0.55 ± 0.08
hlyD/p-*hlyD*	130.84 ± 2.32	10.6 ± 0.18
luxS	154.55 ± 2.15*	0.7 ± 0.12**
luxS/p-*luxS*	124.77 ± 3.42	2.4 ± 0.10

[a]Data obtained from mean values ± standard deviations from three different experiments. (*, $p < 0.001$; **, $p < 0.01$ compared to the wild type).

Figure 11. Protein profiles of wild type and *luxS* mutant bacteria

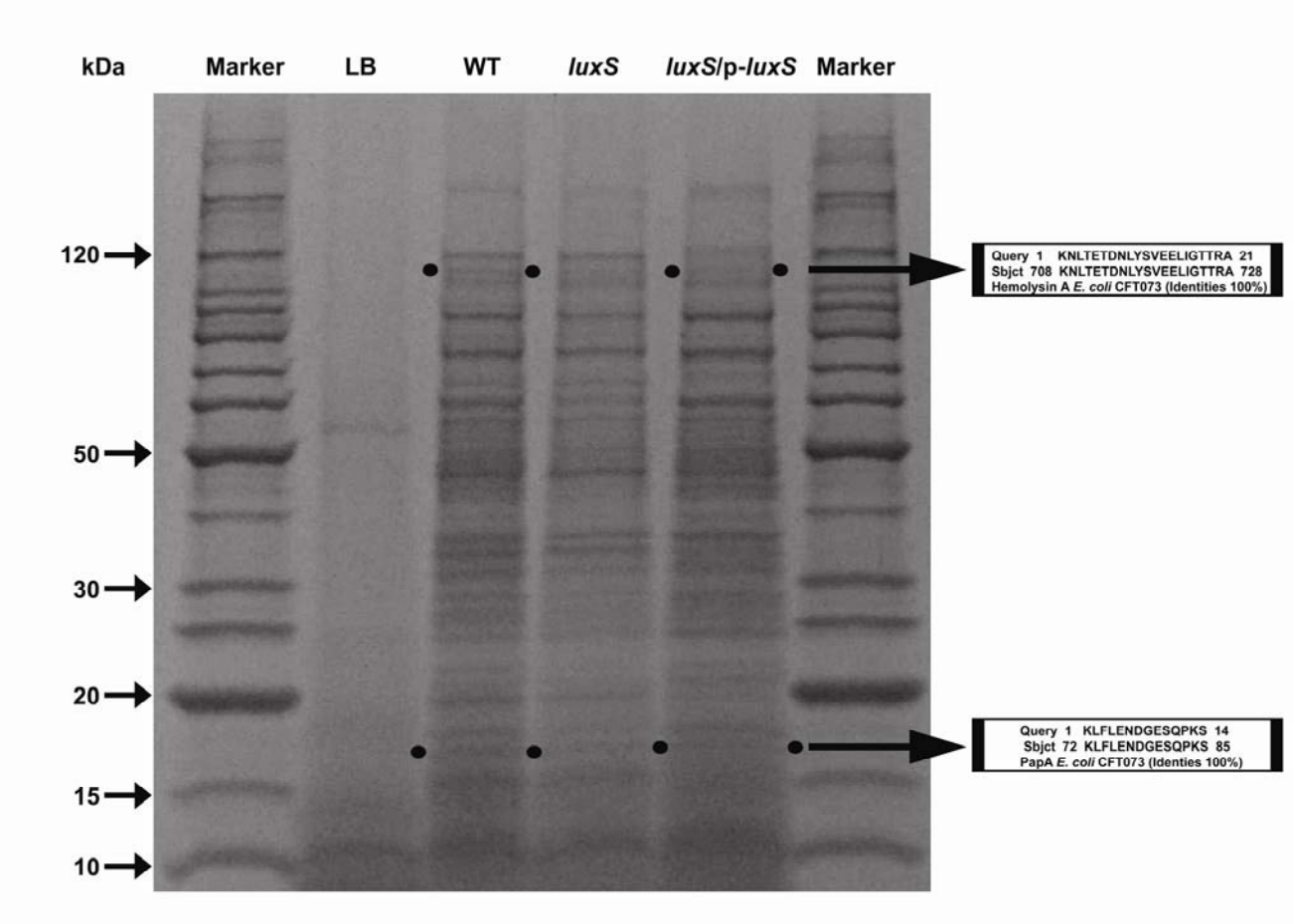


Figure 11. Protein samples were separated by 4 to 20% SDS-PAGE and stained with Coomassie blue and protein bands of interest analyzed by MS analysis.

3.2.2.6. **Mutation in *luxS* alters Lipopolysaccharide profile**

We investigated the role of lipopolysaccharide in pathogenesis. LPS is a potent endotoxin responsible for gram negative septicemia (61) and responsible for the production of variety of proinflammatory cytokines followed by septic shock and disseminated intravascular coagulation of the infected animals (62). Quantification of the LPS with densitometry assay was done by comparing with standard *E. coli* LPS. We found that from a given number of *E. coli* cells (4×10^9) there was partial reduction in LPS of the *luxS* mutant strain (605 ng/μl) as compared to the wild type (758 ng/μl). This partial reduction in the LPS may also contribute to reduction in virulence. Thus, our results showed that LuxS may contribute to the virulence determinants of the UPEC bacteria and the virulence determinants are multifactorial in nature.

Summary

Using a chicken embryo lethality assay, we have shown that BarA-UvrY TCS regulates virulence factors in APEC serotype O78:K80:H9. A combination of virulence determinants, such as the abilities to adhere, invade, and survive within antigen-presenting cells, such as macrophages are compromised in mutants lacking either *barA* and *uvrY* genes. The ability to persist within macrophages provides a survival advantage to APEC strains by potentiating efficient replication while abrogating elimination by the host immune responses. This was evident in our chicken embryo lethality assay, where isogenic mutant strains were rapidly eliminated from the livers and spleens of the infected embryos, while the wild-type APEC strain persisted within tissues, causing mortality. Our results also indicate that 12-day-old SPF chicken embryos can be used as a model to determine the initial virulence properties of APEC strains conveniently, since the mortality and colonization results of the wild-type strain are similar to those of 3.5-week-old chickens (54). Our results, therefore, suggest that BarA-UvrY TCS may be a global regulator of APEC virulence.

In this study, mutation of *luxS* in APEC serotype O78:K80:H9 leads to partial reduction in the virulence of the bacteria. The virulence of the bacteria in chicken embryo lethality assay was compromised and the abilities to invade and persist in the internal organs like liver and spleen are reduced in the *luxS* mutant bacteria. The adherence to cultured chicken embryo fibroblast and chicken macrophage cells was unaltered while the ability to invade these cells was reduced. The disruption of the

LuxS quorum sensing signaling system leads to the defect in assembly and transport of the LPS contributing to the reduced virulence of the APEC strain.

In Uropathogenic *E. coli* CFT073, mutating either *barA* or *uvrY* reduces virulence in chicken embryo lethality assay. A number of virulence determining factors like ability to invade ureter uroepithelial cells, cytotoxicity to cultured human kidney cells and ability to grow in artificial urine medium during exponential phase are compromised in *barA* or *uvrY* mutant bacteria. The contributing factors for reduction in the virulence was due to the downregulation of secreted exotoxin hemolysin, alteration in LPS pattern and downregulation of proinflammatory cytokines TNF-α, IL-6 and chemokine IL-8 in mutant bacteria lacking *uvrY* gene. Our results suggest that mutation in transcriptional activator UvrY leads to reduction in virulence and BarA-UvrY TCS is a global regulator of virulence in UPEC CFT073.

The mutation of *luxS* in UPEC CFT073 leads to partial reduction in virulence in chicken embryo lethality assay, reduces invasion ability to ureter uroepithelial cells, and decreases cytotoxic effect to human kidney cells. The mutant *luxS* bacteria had a growth defect in the artificial urine medium leading to decreased fitness. The exoprotein like hemolysin and adhesion protein PapA in the supernatant was reduced. The reduced quantity of LPS also contributes to the reduced virulence. Thus, our results indicate that the *luxS* mutation reduces the virulence in UPEC CT073.

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