ABSTRACT

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Extraintestinal pathogenic strains of *Escherichia coli* cause a wide range of diseases including colibacillosis in chickens and urinary tract infections in humans. Persistent infections in *E. coli* and other gram-negative species are associated with population-dependent physiological processes such as cell-cell signaling and biofilm formation. Such social behaviors require careful coordination and modulation of gene expression in response to environmental cues. Adaptive response of bacteria in new environment is predominantly achieved through a signaling cascade called two-component regulatory systems. The function of the BarA/UvrY two-component regulatory system and its downstream factors in controlling virulence associated processes, specifically regulation of AI-2 based signaling and biofilm formation was investigated.

In *E. coli,* a type of cell-cell signaling termed Quorum Sensing involves release, detection, and response to small molecule called autoinducer (AI-2), synthesis of which is dependent on *luxS* gene products via methyl cycle. The BarA-UvrY and Csr system displayed dual regulation on *luxS* expression at the level of transcription and post-transcription. The uptake of AI-2 by the Lsr transporter is also modulated by the signaling cascade suggested a balance between AI-2 synthesis and uptake in the cell.

The role of transcriptional regulator *uvrY* in biofilm formation in Uropathogenic *Escherichia coli* was also studied. Mutation of *uvrY* reduced expression of *fimA* and *papA,* major fimbrial subunit of Type 1 and Pap pilus respectively. Acidic exopolysaccharide accumulation and the ability to swarm are also being impaired. Finally, *uvrY* mutants demonstrated poor colonization in kidneys and bladders in an ascending model of UTI. Overall, the effect of *uvrY* on biofilm formation seems to be multi-factorial and might play a critical role in adaptation and colonization of UPEC.

The fine tuning of processes associated with cell-cell communication and biofilm formation at the level of transcription and post-transcription by the BarA/UvrY/CsrA signaling cascade indicated that this system might be crucial for quick adaptation, social behavior, colonization and virulence attributes in *Escherichia coli*.

REGULATION OF FACTORS CONTRIBUTING TO VIRULENCE IN ESCHERICHIA COLI

By

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Dedication

I dedicate this dissertation to the memory of my dad, Late Mr. Prabhat Kamal Mitra and to my mother, Mrs. Samira Mitra, for their faith, confidence and unconditional love in my life.

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Chapter I: Background and Literature Review

The ability to adapt under varying environmental conditions and colonize is an important determinant for perpetuation of bacterial species. Adaptation of bacteria requires detection of the signal from the surroundings and appropriate responses that render fitness in a new setting. The integration of diverse signals to appropriate response requires a flow of information from extracellular milieu to the interior of the cell. In bacteria, two-component systems are the major signaling devices for detecting environmental cues and transducing it into the interior of the cell usually via a cascade of phosphorelay. The responses of this signaling cascade enable bacterial adaptation, persistence and virulence of the bacterium by alteration of gene expression [1-4].

Two-component systems respond by alteration of gene expression in diverse physiological processes including osmolarity, metabolism, nutrient acquisition, stress response, pH and expression of virulence factors [5-8]. These signaling systems are required for establishment and maintenance of the infection by a bacterial pathogen. The ability to cooperate and communicate in a community structure in the form of biofilm enables microbes to perform important cellular functions such as that of adaptation and persistence inside host. Interactions among community members are crucial for temporal and coordinated response and are often mediated by a process of population-dependent cell-cell communication known as quorum sensing. The association between Quorum Sensing and Biofilm formation, both in turn regulates virulence, has been demonstrated in a number of bacterial species including *Streptococcus*, *Vibrio*, *Pseudomonas* and *Escherichia coli* [9-11]. Here regulation of social behavior, particularly quorum sensing and biofilm, are explored in *E. coli* using a model two-component regulatory system.

Escherichia coli as commensal and model pathogen

Escherichia coli belong to a major facultative anaerobe commonly found in the intestinal tracts of homeothermic animals including man. *E. coli* colonizes the gastrointestinal tract within few hours after birth. In 1885, *Theodor Escherich* isolated the microbe from fecal flora of normal infants and later on documented them as important commensals in intestinal tract and pathogen in human intestinal and urinary tracts. *E. coli* displays a wide range of strain variation depending on the presence of certain antigens, typically O somatic lipopolysacchides, K capsular and H flagellar antigens [12]. Furthermore, array of adhesins having varying receptor specificity add to this strain diversity [13]. Extensive strain variation makes *E. coli* an ideal model for studying microbial adaptation and host-pathogen interaction [14, 15]. *E. coli* K-12, a prototypic attenuated strain have been commonly used in the laboratory practices [16]. However, this strain lacks virulence factors such as fully functional O-antigen and ability to colonize mammalian intestine[17].

E. coli strains are broadly classified in three groups: commensal strains, intestinal pathogenic (also referred as enteric or diarrheagenic) and extraintestinal pathogenic strains. The commensals are normal residents of the GI tract in birds, mammals and humans. Typically, *E. coli* colonizes the gastrointestinal tract of human neonates within few hours after birth. Commensal *E. coli* usually persist in mucous layer of the mammalian colon where they colonize and thrive making it one of the most abundant facultative anaerobe in the microflora. Commensals are usually beneficial to hosts but they can cause infections in compromised or immunologically challenged patients. In contrast, intestinal and extraintestinal pathogenic species have additional virulence factors such as plasmids, bacteriophages and pathogenicity islands[18-20]. Commonly used name for this group include enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), Shiga toxin-producing *E. coli* or enterohemorrhagic *E.coli* (STEC or EHEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC). The intestinal pathogenic groups are limited in their ability to cause infection only in the intestinal tract. Each pathotypes within intestinal pathogenic strains have unique set of virulence traits resulting in a characteristic syndrome [21, 22]. Strains within each group show distinct phylogenetic relationship and diversity within each group are thought to be result of horizontal gene transfer. Intestinal pathogenic *E. coli* are the leading cause of severe and infant diarrhea in developing countries and remain a major public health problem across the globe resulting two million deaths every year [23, 24].

Extraintestinal Pathogenic Escherichia coli (ExPEC)

Lately, a third group termed as Extraintestinal pathogenic *E. coli* (ExPEC) has been formed based on the presence of specific virulence factors and ability to cause infection outside the intestine including the urinary tract, central nervous system, circulatory and respiratory system [25-27]. Strains which cause extraintestinal disease usually do not cause diarrheagenic disease and vice versa. However, ExPECs are capable of asymptomatically colonizing the intestinal tract in one-fifth of normal human population. ExPECs are distinct both phylogentically and epidemiologically when compared to intestinal and pathogenic strains [28].

ExPECs are increasingly a growing concern as evidenced by being causative agents of a plethora of diseases including urinary tract infections (UTI), neonatal meningitis, intra-abdominal infections, intra-vascular site infection, pneumonia, septicemia, osteomyelitis and other extraintestinal infections resulting huge economic impact on public health and society [29]. ExPECs are the most common gram-negative pathogens that cause extraintestinal infections under clinical settings.

Increasing resistance to antimicrobial agents makes ExPEC associated infections complicated and difficult to treat [30]. Typically ExPEC characteristic virulence factors aid in invasion and colonization leading to infection in extra intestinal sites. Currently, ExPECs are found resistant to many of host's defenses including resistance to bactericidal activity by neutrophils, cationic antimicrobial peptides and complement [31-34].

Virulence factors associated with ExPECs

ExPECs are phylogenetically, epidemiologically, genetically and clinically distinct from commensals and intestinal pathogenic strains [13, 28, 35-37]. The genomes of

ExPECs are larger and much varied than commensals probably due to acquirement of genes through horizontal transfer by mobile genetic elements such as transposons, phages, plasmids and pathogenicity islands (PAI) from diverse related or non related species [38, 39]. The acquired gene pool facilitates better adaptation and infection in extraintestinal sites as compared to commensals.

Important ExPEC specific virulence factors include adhesins such as Type 1 fimbriae or P fimbriae, factors that evade defense mechanisms such as capsules, lipopolysaccharides, toxins including hemolysins, and factors to acquire nutrient availability such as siderophores [40]. ExPECs were defined as isolates of *E. coli* having at least two virulence markers from a list of *papA*, *papC*, *sfa/foc*, *afa/dr*, *kpsMTII* and *iutA*. Other ExPEC associated virulence markers include *papGIII*, *fimH, hly, K1, ireA* etc. Among the ExPECs, uropathogenic *E. coli* (UPEC) and avian pathogenic *E. coli* (APEC) cause significant morbidity and or mortality in humans and poultry respectively.

Uropathogenic Escherichia coli (UPEC)

UPEC is the leading cause of urinary tract infections in the United States. Every year in the United States alone, UPEC associated UTI results in 6-8 billion cases of uncomplicated cystitis with a healthcare cost of $1 billion, 250,000 cases of uncomplicated pyelonephritis with a direct cost of $175 million, and 250,000 to 525,000 cases of catheter associated UTI healthcare cost of which is $170-350 million dollar [30]. UPEC is one of the well characterized pathogen in UTI and often used as a model species for studying host-pathogen interaction [14].

In contrast to commensal strains, UPEC possess large regions of DNA termed "Pathogenicity islands" (PAI) consisting of clustered genes encoding virulence associated factors [41-44]. The virulence attributes of UPEC include adhesins, toxins, lipopolysaccharides, capsule, proteases and iron acquisition systems [45-48]. Adhesins are the key components mediating attachment with biotic and abiotic surfaces often marked with biofilm formation [49]. Biofilms formed on abiotic surfaces by UPEC such as that on the surfaces of medical implants and urinary catheters result in chronic recurrent infections presumably due to increasing antibiotic resistance. Within hosts, adhesins initiate biofilm formation which plays an important role in protection from hosts innate immune responses and persistence of UPEC [50-52]. Several adhesins including outer membrane proteins, curli, and pili or fimbriae are important for mediating attachment [53, 54]. Among the pili, Type 1 and Pap Pilus are critically important for pathogenesis of UPEC in the UTI [55-58]. Other fimbriae such as F1C, M, S and Dr/Afa also contribute to colonization [47, 59-61]. The pilus shows diversity in terms of structure and tissue specificity. Type 1 pilus is short and stubby whereas the Pap pilus is long and flexible [62]. Type 1 pilus is essential for mediating cystitis and shows tropism for mannose specific receptors on the bladder epithelium, Pap pilus, on the other hand, have predisposition towards digalactoside receptors on the kidney epithelium [55, 56, 63, 64]. Type 1 pilus have been demonstrated to be continually expressed in strains that cause cystitis whereas pap pilus is more predominantly expressed in pyelonephritic strains [65]. Flagellar motility may further promote ascension in the urinary tract [66, 67].

Avian Pathogenic Escherichia coli (APEC)

Avian Pathogenic *Escherichia coli* (APEC) is found in the intestinal microflora of healthy birds and usually affects chickens, turkeys, ducks and other avian species [68]. APEC is responsible for infections in extraintestinal sites, particularly to respiratory tract and systemic infections. APEC is the leading cause of avian colibacillosis, a disease characterized by air sacculitis, pericarditis, peritonitis, salpingitis, polyserositis, septicemia, synovitis, osteomyelitis and yolk sac infection [69, 70]. Fecal contamination on egg surface often leads to yolk sac infection resulting death of embryo or within few weeks after hatching of eggs.

On the other hand, in the US, cellulitis caused by APEC is the second leading cause of condemnation of broiler chickens and results in an estimated loss of $40 million every year. Diseases caused by APEC are often a secondary outcome of environmental and host predisposing conditions. Previous infections with viruses such as Newcastle Disease virus (NDV) or infectious bronchitis virus (IBV) and few other agents affecting respiratory tract increases the chance of occurrence of APEC infections, presumably due to loss of cilia in the epithelial layer. Commonly APEC isolates belong to O1, O2 and O78 serogroups. Like UPEC, APEC also posses certain pathogenicity islands encoding virulence genes such as *pap* and *ireA* [71, 72]. However unlike UPEC, APEC harbors one or more plasmids associated with virulence genes such as iron acquisition, toxin production and antimicrobial resistance [73, 74]. Such plasmids have been demonstrated to be lethal in embryos and have the ability to cause urinary tract infection in mice [75]. Other important virulence determinants of APEC include Type 1 fimbriae, curli, K1 capsule, hydrogen peroxide resistance*,* LPS, temperature sensitive hemmaglutinin and serum survival [76-80].

Biofilms and colonization

Historically, microorganisms have been categorized as planktonic or sessile cells. While planktonic cells are considered important for rapid propagation and moving into new territories, the sessile cells in contrast, are thought to be important for perseverance. It is believed that in nature bacteria often remain associated in the form of a sessile community known as biofilms enabling a unicellular existence in a multicellular community. Biofilms may be defined as surface-attached microorganisms enclosed in a matrix [81-83]. The self synthesized microbial matrix termed as extracellular polymeric substances (EPS) contains polysaccharides, proteins and nucleic acids [84]. In nature, EPS is highly hydrated allowing free flow of nutrients and metabolites mimicking primitive circulatory system. EPS serves as a guard against environmental changes, antibiotics and chemical agents and plays a crucial role for formation and maintenance of biofilm architecture [85]. The composition of extracellular matrix is varied among species. Both non-pathogenic and pathogenic species are capable of forming biofilms [86, 87].

Biofilms have a significant impact on human or animal health, environmental and industrial settings. Biofilms contribute up to 80% of chronic inflammatory diseases including urinary tract infections (UTI), cystic fibrosis, otitis media, colitis, conjunctivitis, endocarditis, peridontitis, and prostatitis [86, 88]. Presence of biofilms in indwelling medical devices (such as urinary catheters) and other devices in healthcare settings have often resulted in increase in nosocomial infections [89-92]. Biofilm associated microorganisms have been considered responsible for many yet, undiagnosed infections in humans. Biofilms are highly resistant to antibiotics and immune responses which make them difficult for treatment [91, 93, 94]. Secreted catalase helps in preventing ingress of hydrogen peroxide, while the matrix prevents antibodies to enter inside biofilms. Even phagocytes have been demonstrated to be unsuccessful in removal of biofilms. Additionally, periodical shedding of individual bacteria from the biofilm into the surrounding tissues cause certain infections to recur [95]. Advantages of persistence in biofilms include protection from environmental stresses (such as chemicals, UV, antibiotics), prevention from dehydration, horizontal gene transfer, exchange of nutrients and ease of communication within the community [96].

Biofilms could form on diverse environments including inorganic surfaces such as soil, minerals, and metals as well as on organic surfaces such as tissues. In nature, mixed species of biofilms can be frequently observed, but single species of biofilms are also seen in medical and device associated infections. Molecular genetics studies of single species biofilms have aided in understanding that biofilm formation is a multi-step process, requires cellular communication and expression of genes in biofilm associated bacteria is quite different as compared to planktonic cells. Studies have demonstrated that biofilms are typically formed in high shear environment in both natural and artificial systems.

The multi-stages of biofilm formation include initiation by attachment to a substrate, maturation into a microcolony, maintenance of biofilm architecture and dissolution. The process of initiation seems to be triggered by environmental signals such as nutrient availability [97]. Bacterial adhesion is facilitated by several adhesins and proteinaceous appendages that facilitate attachment by binding to cell surface receptors [51, 98]. This step is a crucial step for both native and pathogenic species for colonization. Typically repulsion between bacterial and tissue cell surface prevents attachment and hence, hairy appendages, termed fimbriae or pili are usually located at the distal end of the bacterial surface to facilitate adhesion. The term “Pili” and “Fimbria” refers to non-flagellar bacterial filaments, have been often used interchangeably even though they have different connotation. “Pili” is often used for transmission of genetic material during conjugation whereas “Fimbria” is more commonly used for appendages of attachment. Pili are proteinaceous appendages having a thickness of 2-7nm in width and extending from 0.2 to 20μm outward from the bacterial surface. The formation of pili involves helical assembly of multiple subunits of pilin protein which constitute the thick long proximal shaft. The thin distal part encodes a tip adhesin protein promoting attachment to various surfaces while conferring binding specificity and tissue tropism in pathogens. The longer shaft is presumed to distance the adhesin from the bacterial surface to facilitate the adhesion [99, 100].

Among the pili, Type 1 pilus is commonly present in almost all species and isolates of *Enterobacteriaceae* including the Uropathogenic *E. coli* (UPEC) and considered as a virulence factor in ascending model of UTI [101]. The biogenesis of the Type 1 pilus takes place by a conserved chaperone usher pathway which is involved in assembly of thirty other adhesive organelles in gram negative species including the P-type fimbriae. In this pathway, the assembly of the fimbriae relies on a periplasmic chaperone, and an outer membrane usher. The chaperone helps stabilizing and folding of fimbrial subunits and a lack of it leads to aggregation of the subunits and subsequent degradation by the protease. The usher facilitates the assimilation of fimbrial subunits into the growing pilus shaft [102, 103].

In *E. coli*, Type 1 pilus and flagellar motility is necessary for biofilm maturation [49, 104]. Type 1 pilus is encoded by *fim* (*fimA-fimH*) gene cluster consisting of eleven genes including *fimA*, encoding the major pilus subunit, *fimC* encoding periplasmic chaperone, *fimD* encoding outer membrane usher and *fimH* encoding the tip adhesin. In *E. coli* variants of FimH have been detected which prefers a particular sugar moiety on cell surface over others for adhesion; for e.g., Fim H variants in commensal isolates of *E. coli* preferentially binds to mono-mannose residues whereas pathogenic species including uropathogenic ones attach with higher affinity to trimannose moiety, as typically found in the urinary tract [105]. Such interaction mediates internalization in bladder cells, leading to persistence and chronic urinary tract infections. Type 1 pilus facilitates attachment and subsequent colonization by binding to mannose containing receptors on the eukaryotic cell surface [61]. Both Type 1 and P pilus has been used successfully as a vaccine candidate [101, 106, 107].

Cell-cell communication

Another aspect of cooperative behavior in bacteria is demonstrated very well in a recently investigated physiological process dubbed as "Quorum Sensing"[108]. Quorum Sensing (QS) refers to the ability of bacteria to coordinate activities in a population-dependent manner by utilization of small molecules termed autoinducers [109-112]. The accumulation of autoinducers in the external environment increases with cell density and on achieving a critical threshold concentration, signaling transduction cascade activation leads to alteration in gene expression. Such induced or repression of genes could include virulence, antibiotic production, motility, metabolism, chemotaxis, and biofilm formation [113-117]. Coordination of bacterial gene expression is thought to be crucial for a protection of bacterial community from immune responses as well as successful colonization in the new or harsh environment inside host.

The phenomenon of Quorum sensing was originally identified in *Vibrio fisheri* [118, 119]. The initial observation was the ability of the bacteria to produce light only at high-cell density led to the characterization of autoinducer, N-acyl homoserine lactone. The symbiotic association between *V. fisheri* and fishes and squids has gained considerable interest in which the bacteria thrive in nutrient-rich light organs of marine animals and produce light in a population-dependent manner. The animals, in turn, use the light as a predatory device avoiding being preyed or catch a prey. The phenomenon of bioluminescence has been observed only in symbiotic state of the bacteria even though the bacteria are able to exist between free living and in symbiotic association with the host. In the free-living state, the autoinducer diffuse into the environment and the signal gets lost in the surroundings, whereas in a confined environment of the light organ of the squid the signal accumulates and flows back into the cell. Light production in *V. fisheri* takes place in a population dependent manner through regulation of *luxCDABE* operon which encodes luciferase enzyme complex. Two regulatory proteins are involved in this circuit. LuxI protein, the autoinducer synthase synthesize the autoinducer molecule, acylated homoserine lactone (HSL), accumulation of which in extracellular environment increases directly with cell density. Upon entering inside the cell, the autoinducer gets bound and activates LuxR, a response regulator. The activated response regulator, LuxR in turn binds to "lux" box a sequence to the upstream of the QS regulatory genes, recruits RNA polymerase and activates luciferase operon inducing bioluminescence [120, 121]. Additionally, mutations in *lux* genes in *Vibrio fischeri* reduce the ability to colonize and persist in the hosts [122]. A transcriptional regulator, GacA is also required for symbiotic association between the bacteria and the host [5].

In contrast, *Vibrio harveyi* utilizes two signaling molecules, termed as AI-1, and AI-2 synthesized by LuxN and LuxQ respectively. The signaling system utilizes three sensor kinases which autophosphorylates at low cell densities and phosphate is sequentially relayed to LuxO, a transcriptional regulator via LuxU, a Phosphotransferase protein. Phosphorylated LuxO, in turn, activates transcription of small RNAs (sRNA) which in association with Hfq destabilize the transcript encoding the $LuxR_{VH}$, a transcriptional regulator. This results in repression of luciferase operon and no light production. At high cell densities, kinase change to phosphates and the flow of phosphates reverses, resulting in dephosphorylation of LuxO and collapse of small RNA synthesis and enhanced transcription of LuxR, which in turn increases light production [123].

Quorum sensing plays a key role in both the early and later stages of biofilm development. Autoinducer such as acylated homoserine lactones (AHL), which senses bacterial cell density, frequently plays a role in microcolony formation whereas cross-species bacterial communication signal Autoinducer 2 (AI-2) influences thickness and biomass. This mode of communication is particularly important as biofilms in nature are often present in a group of mixed species. Thus, agents targeting such steps in community signaling could be an important step in controlling biofilm related infections [93, 124-126].

Interestingly, the social behavior of quorum sensing and biofilm formation seems to be interdependent [9, 127]. While QS may be a key contributor of biofilm formation, high cell densities during biofilm development may be crucial in achieving “quorum”. Inhibition of Quorum Sensing offers a novel strategy for controlling biofilm related infections because of reduced risk of developing antibiotic resistance [128]. These two-processes may be mutually dependent or temporal, depends on the environment and are crucial for efficient adaptation of the bacteria. Environmental adaptation in bacteria, on the other hand, relies on a signaling cascade called the “Two-component system”

Two-component system

Bacteria live in an environment where conditions change frequently. Such conditions include a wide range of environmental cues such as change in pH, oxygen deficiency, temperature fluctuations, nutrient limitation, chemical signals. Survival of microbes in any environment relies on adaptive responses that enhance persistence during unfavorable conditions. Adaptive behaviors such as the ability to carefully utilize energy sources like carbon and nitrogen, a capacity to establish communication among members and resist toxic effects of the metabolic processes are critical for persistence of microbes. Adaptive responses necessitate monitoring and detection of environmental signals, transduction of that information within the cell and elucidation of appropriate responses usually by alteration of gene expression. The response could take place at the level of transcription or translation initiation.

Adaptation of bacteria to new environment in bacteria is largely mediated by a sophisticated signaling system termed as the “Two-Component System” (TCS). Two component systems are wide spread signal transduction devices that enable bacteria to detect, respond and adapt to environmental stimuli mostly through changes in gene expression [1]. More than four thousand TCSs have been detected in 145 completed prokaryotic genomes. Such systems were also detected in lower eukaryotes, yeasts, fungi, yeasts, protozoa and in plants but not in *C. elegans*, Drosophila, mouse and human. The number of TCSs in bacteria seems to be directly correlated with increasing genome size and range of adaptation needed to persist in varying environments.

A prototypic two-component system consists of a membrane-located sensor histidine kinase (HK) and a cognate response regulator (RR). Upon reception of the environmental signal/s, the sensor kinase transduces the information to the response regulator via a cascade of phospho-transfer reactions. The activated regulator then elucidates appropriate responses to make the organism acclimatize in the new environment usually involving gene regulation expression at the level of transcription. Direct interactions of response regulators with proteins were also reported [2, 129].

Initial studies have demonstrated that two proteins EnvZ, a membrane protein and OmpR, a cytoplasmic regulator control outer membrane protein genes *ompF* and *ompC* in response to osmolarity changes in the environment. This study demonstrated that information must be transmitted inside the cell via a membrane protein which must be able to sense environmental cues [130-132]. It was found that there are conserved amino acid sequences of OmpR and EnvZ in a set of *E. coli* proteins that responds to environmental cues which are then divided into two groups, one group having a conserved sequence of 240 amino acids while the other group shares 120 amino acid residues in common [133]. Furthermore, one group demonstrated unique ability to undergo autophosphorylaylation at a conserved histidine residue and referred as “transmitters”, whereas the later group can receive a phosphate group at a conserved aspartate residue from the former group and called “receivers” [134-137]. Appropriately, the transmitter and receiver group of proteins are subsequently referred as “sensor histidine kinase (HK)” and “response regulators (RR)” and together they constitute “two-component regulatory system” which are environmental detection devices facilitating adaptation in a new environment by altering modulate gene expression [1, 138]. Interestingly, few systems employ an additional histidine domain called “phosphotransfer domain (Hpt)” which serves as an intermediate during transfer of phosphoryl from or to aspartate residue in RR. Since then, several such systems have been detected in numerous bacterial species, indicating the importance of such regulatory systems [139-143].

Bacterial pathogens produce virulence factors such as adherence factors, capsules, enzymes, and toxins in order to overcome the host’s defense and cause successful colonization. Virulence factors are expressed temporally through various stages of infection and carefully controlled. Many pathogenic bacteria require motility for a successful colonization either in initial phase and/or for maintenance of infection. Successful colonization of a pathogen also needs coordination among members of community to express virulence in a population-dependent manner utilizing Quorum Sensing. The importance of TCS in regulation of virulence has become apparent over the years as several TCS are implicated in physiological processes associated with virulence including motility, adhesion, colonization, toxin expression, cell-cell communication and bacterial adaptation inside host [5, 8, 144-146]. These processes are carefully synchronized for initiation, persistence and adaptation of bacteria inside the host. However, on a cautious note, the phenotypes associated with attenuation of virulence could be due to interference with metabolic requirements of the cell. Some examples are listed in Table 1. One such two-component system, the BarA-UvrY TCS in *Escherichia coli* regulates diverse physiological processes including oxidative stress, sigmaS expression, biofilm formation, carbon metabolism and virulence.

Figure 1. Schematic of two-component regulatory systems. The arrows indicate the direction of phosphorelay. The classical sensor transfers the phosphate group from the histidine residue to the aspartate residue of the response regulator. The unorthodox sensor kinases have additional receiver and histidinephosphotransfer domain. The phosphorelay cascade in this case follows His→Asp→ His →Asp

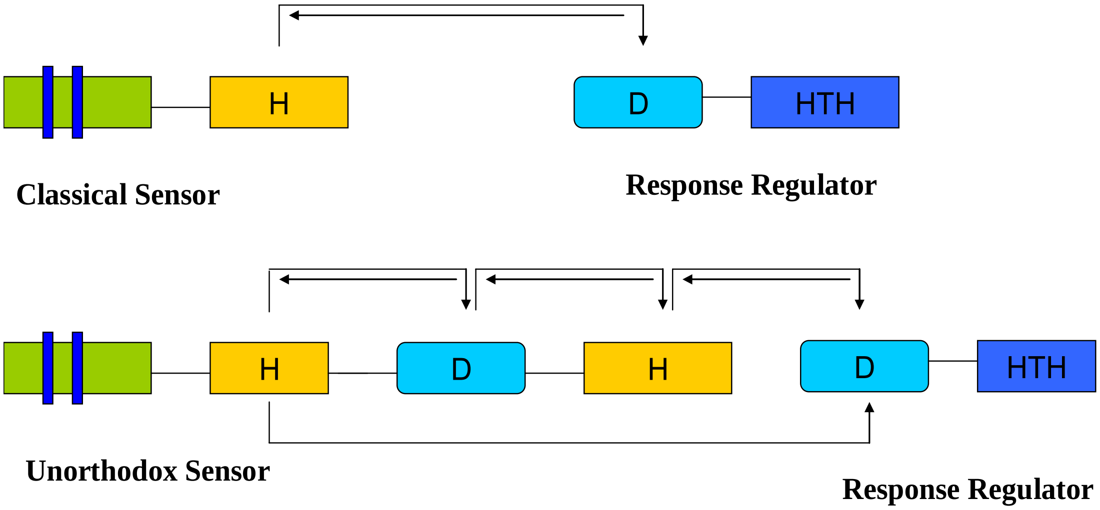


Table 1. Virulence phenotypes associated with two-component regulatory system in diverse bacterial species.

Organism	TCS	Phenotypes
Salmonella enterica	PhoP-PhoQ	LPS modification [147]
	BarA-SirA	TTSS, Invasion [148, 149]
Bordatella Pertusis	BvgA-BvgS	Toxin [150]
Vibrio cholerae	ArcA-ArcB	VF *toxT* [151]
	VarS-VarA	VF *hapR* [152]
Vibrio fischeri	GacS-GacA	Bioluminescence [5]
Shigella flexneri	OmpR-EnvZ	Invasion [153]
Pseudomonas aeruginosa	GacS-GacA	AHL, biofilm [154]
	RocA1-RocS1	Fimbriae, Biofilm [155]
Neisseria gonorrhea	PilA-PilB	Pili synthesis [156]
Helicobacter pylori	FlgR-FlgS	Flagella [157]
Staphlylococcus aureus	AgrA-AgrC	Regulatory RNA III [158]
Erwinia cartovora	ExpS-ExpA	Enzymes [159]
Serratia marcescens	PigW-PigQ	Prodiogsin [160]
Legionella pneumophilles	LetS-LetA	Cytotoxicity [161]
Escherichia coli	BarA-UvrY	Biofilm formation [162]

Chapter II: The BarA/UvrY TCS - A model two-component system

Adaptation of *E. coli* to new milieu requires several two-component systems which plays a crucial role for survival in a dynamically fluctuating environment. Sequencing of the entire *Escherichia coli* genome have aided in determining 29 Histidine Kinase and 32 Response Regulator genes [163]. The BarA-UvrY TCS in *Escherichia coli* is pleiotropic and have been linked with several physiological processes including biofilm formation, oxidative stress, sigmaS expression, and efficient adaptation in carbon utilization [162, 164, 165]. The *barA* and *uvrY* gene is located at 62 and 42 minutes of the *Escherichia coli* chromosome, unlike many two-component pair which are located next to one another. The BarA-UvrY two-component system and its orthologues are highly conserved in γ-division of proteobacteria. Orthologues of this system in *Pseudomonas* (GacS-GacA), *Salmonella* (BarA-SirA), *Erwinia* (ExpA-ExpS) and *Vibrio* (VarS-VarA) have been shown to be strongly associated with virulence of the respective bacteria (Table 1).

BarA - The Sensor Kinase

The *barA* (**b**acterial **a**daptive **r**esponse) gene (also called airS) encodes a 102kD membrane associated protein having both the sensor kinase and the response regulatory domains. Out of 29HK detected so far, only 5 sensor kinases are hybrid sensor kinases including BarA, ArcB, EvgS, RcsC and TorS. BarA is a member of "tripartite" or "hybrid" kinases in *E. coli* with characteristic three domains: a regular transmitter domain with a conserved histidine residue (H1), a central receiver domain with a conserved aspartate residue (D1) and C terminal Phosphotransfer domain (HPt). The Phosphorelay in this TCS is presumed to act in His-Asp-His-Asp fashion from the Sensor kinase to the Response Regulator. Such multistep phosphorelay might offer reversible flow of phosphoryl group providing tighter control or incorporate various signals at an intermediary step or facilitate cross talk between two or more signaling cascades.

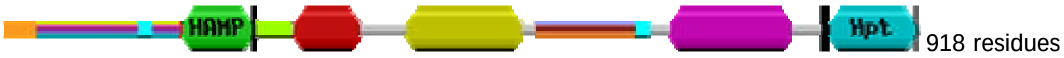
BarA has been initially identified to phenotypically suppress the effect of a deletion mutation of *envZ* gene, which has been shown to regulate expression of outer membrane proteins with OmpR [166]. GacS of *Psedomonas syringae* pv. s*yringae*, orthlogue of BarA, contributes to lesion formation in plants [167, 168] while BvgS in *Bordatella* spp. regulates siderophore production [169]. Environmental signals to which BarA responds remain unclear, however the system seems to be activated upon reaching an optimal pH [170]. In *Salmonella,* intestinal short chain fatty acids have an effect on the virulence of BarA/SirA TCS [171]. Attachment of P-pilus to human red blood cells induces transcription of *barA* in UPEC which in turn upregulates the expression of iron acquisition system [172].

BarA plays a role in bacterial adaptive response, particularly in regulation of oxidative stress response by enhancing catalase production through transcriptional activation of the *rpoS* gene [173, 174]. RpoS, the alternative sigma factor of *E. coli*, is also involved in regulating gene expression in response to pH changes and changes in osmolarity [175]. BarA may have a significant influence on these processes, as it is one of the transcriptional regulators of *rpoS*. It is yet to be seen whether this process is interdependent or not. A domain analysis was performed to further understand the potential role of the kinase.

Domain Analysis of BarA

A domain analysis of BarA was performed using Simple Modular Architecture Research Tool (SMART)

Figure 2. Domain Organization of BarA.



HAMP 180-249

HisKA 292-357

HATPase_c 404-519

Response_reg 668-789

Hpt 828-912

SMART analysis of BarA shows 5 domains:

1. **HAMP** – This domain is known as the HAMP domain for histidine kinases, adenlyl cyclases, methyl binding proteins and phosphatases. Commonly found in bacterial sensor and chemotaxis proteins as well as in eukaryotic Histidine kinases. The bacterial proteins are usually integral membrane proteins and part of a two-component signal transduction pathway.

2. **HisKA** –. The Histidine kinase A (phosphoacceptor) N-terminal domain is a dimerisation and phosphoacceptor domain of histidine kinases. It has been found in bacterial sensor protein/Histidine kinases.

3. **HTPase C** – This family includes several ATP binding proteins – Histidine kinase, DNA gyrase B, topoisomerases, heat shock protein HSP90, phytochrome-like ATPases and DNA mismatch repair proteins.

4. **REC** – CheY homologous receiver domain regulates the clockwise rotation of *E. coli* flagellar motors. This domain contains a phosphoacceptor site that is phosphorylated by histidine kinase homologues.

5. **HPT** – The Histidine Phosphotransferase domain contains active Histidine residues that mediate phosphotransfer reactions. This domain is detected only in eubacteria.

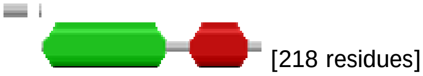
UvrY - The Response Regulator

UvrY, a 218 amino acid protein belongs to FixJ protein family was identified as the cognate response regulator of BarA in *E. coli* [176]. It has an N-terminal phosphoacceptor domain with a conserved aspartic acid residue at position 54 followed by a LuxR type helix-turn-helix DNA binding domain in the C-terminal region. The name *uvrY* derives its name due to close proximity on a biscistronic mRNA with *uvrC,* which is involved in DNA repair, but *uvrY* seems to have little or no role in UV-induced DNA damage repair [177].

Mutation in *uvrY* leads to a hydrogen peroxide sensitive phenotype due to reduced expression of catalase in *E. coli*. UvrY also have a role in biofilm formation. Interestingly, UvrY is critcal for switching between glycolysis and gluconeogenesis pathway for efficient adaptation which is presumably important for infection. In *Salmonella*, SirA regulates virulence and directly binds to genes for *hilA*, *hilC* and *csrB* promoters [178, 179]. Mutation of *gacA*, in *Pseudomonas* and *varA* in *Vibrio* demonstrated reduced levels of autoiducers, defective in social behavior and virulence attributes in animal models [152, 180-183]. Salmonella ortholog *sirA* have been demonstrated to be activated by cya/crp regulation [184]. In *Pseudomonas* and *Erwinia* species *uvrY* othrologue, *gacA* controls quorum sensing, secondary metabolism and phytopathogenesis. Increased expression of *sdiA,* which encodes a LuxR protein and involved in cell division, led to a significant increase in *uvrY* transcription. In *Photorhabdus luminescens,* UvrY have been shown to regulate several virulence associated traits including quorum sensing, motility, bioluminescence and oxidative stress [165]. UvrY and its orthologues in control the expression of small RNA that is predicted to be present in γ-proteobacteria [180, 185].

Domain Analysis of UvrY

Figure 3. Domain organization of UvrY.



Response_reg 2-123

GerE 147-204

SMART analysis indicated two important domains of the Response Regulator UvrY:

1. **REC** – CheY homologous receiver domain regulates the clockwise rotation of *E. coli* flagellar motors. This domain contains a phosphoacceptor site that is phosphorylated by Histidine kinase homologues.

2. **HTH LuxR** – The lux regulon which activates the bioluminescence operon. They are a class of regulators which when bound to autoinducer “(AHL) gets activated. The Helix turn helix DNA binding domain of these proteins is located in the C-terminal section of the sequence. The many bacterial transcription regulation proteins which bind DNA through a 'helix-turn-helix' motif can be classified into subfamilies on the basis of sequence similarities. One of these subfamilies which includes proteins with sizes ranging from 74 (gerE) to 901 amino acids (malT), can be further subdivided into two classes on the basis of the mechanism by which they are activated. The first is a class of regulators which belong to a two-component sensory transduction system where the protein is activated by its

phosphorylation, generally on an aspartate residue, by a transmembrane kinase. The members belong to this class include bvgA, comA, dctR; degU, evgA, fimZ, fixJ, gacA, glpR, narL, narP, nodW, rcsB and uhpA. The second is a class of regulators which is activated when bound to autoinducer molecules such as N-(3-oxohexanoyl)-L-homoserine lactone (OHHL). Members belong to this class are carR, echR, esaR, expR, lasR, luxR, phzR, rhlR, traR and yenR. The 'helix-turn-helix' DNA-binding motif of these proteins is located in the C-terminal section of the sequence.

Integration of the BarA/UvrY/Csr System

Recently there are increasing numbers of studies demonstrating importance of post-transcriptional regulation by small noncoding RNA in adaptation and virulence [186]. Apart from transcription control, translation initiation is important for efficient adaptation and expression of virulence of bacteria [187].

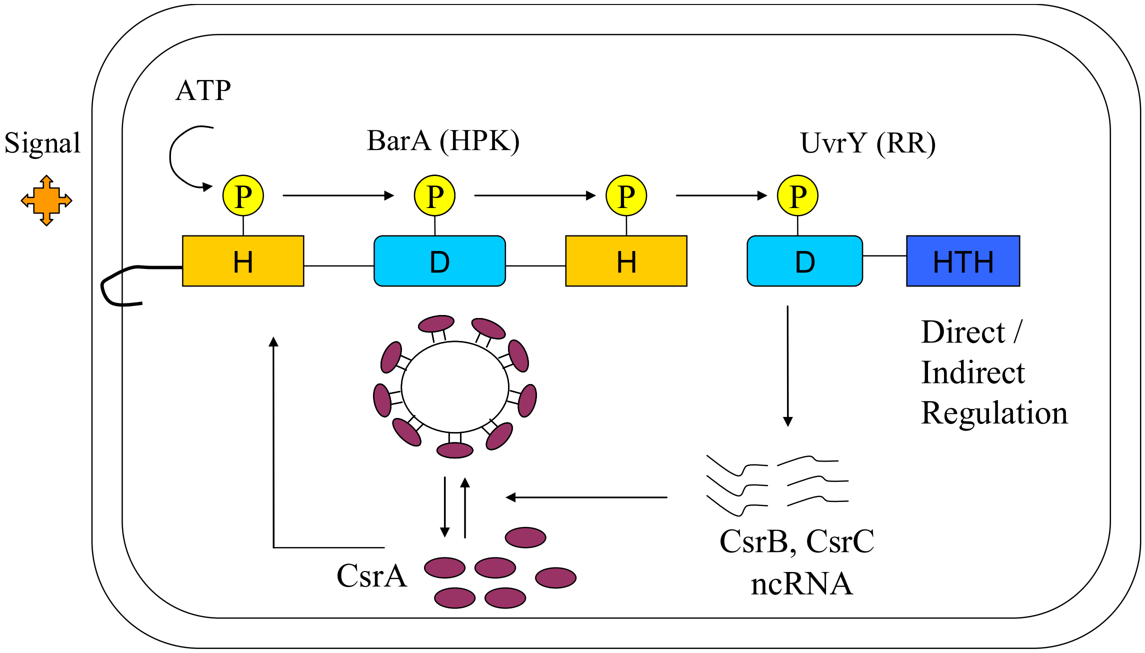
Presently, two classes of small RNA are known to influence the rate of translation initiation by different mechanisms [188]. The first class of small RNA’s act by base pairing at the 5’end of the transcript, which could either stimulate or interfere with ribosome loading of various target mRNA. Hfq, the RNA chaperone facilitates the base-pairing in gram-negative bacterial species [189].

On the other hand, another group of small RNA displays high affinity for a RNA-binding protein, which control translational initiation and message stability of the transcripts. The RNA binding protein is designated as CsrA or RsmA in various gram-negative bacterial species. The acronym Csr stands for carbon storage regulator in *E. coli*, *Salmonella* and *Vibrio* species and Rsm for regulator of secondary metabolism in *Pseudomonas* and *Erwinia* species.

Part of the downstream effect of the BarA-UvrY TCS in *E. coli* is mediated via Carbon Storage Regulatory system (Csr). In this circuit, UvrY enhances transcription of two noncoding RNA's called CsrB and CsrC. These small RNAs in turn bind and titrates the activity of global RNA binding protein, CsrA [190, 191]. In an auto-regulatory loop, CsrA also regulates this TCS and controls its own expression (Figure 4) [192]. The control of CsrA could be both positive and negative for various target transcripts. CsrA could interfere with translation of target mRNA by binding at or near shine-dalgarno sequences thus occluding ribosome loading while accelerating message decay. On the other hand, CsrA could also stabilize and increase translation of target mRNA. CsrB and CsrC RNA's contain several imperfect sequences that serve as multiple binding sites (upto 22 in CsrB) for CsrA protein. An important feature of these putative binding sites for CsrA is presence of a conserved GGA sequence in the stem loop regions of various target RNA's. Few direct regulatory interaction of CsrA have been recognized, *glg* operon which encodes genes in glycogen biosynthesis, *pgaA* transcripts that encodes a polysaccharide adhesin involved in biofilm formation, *cstA* that encodes a peptide transporter and *hfq*, that assists base pairing of transcripts [193-196].

CsrA was initially identified as global regulator of glycogen biosynthesis, where a transposon mutagenesis in *csrA* increased accumulation of glycogen as compared to the parent strain [197]. Since then, CsrA homologues are detected in more than hundred species including proteobacteria, even some species having more than one CsrA homologue. Structural studies indicated that CsrA acts as a dimer consisting of five β strands and one α helix per monomer. The binding of CsrA with CsrB and CsrC is coopertaive. CsrA plays a major role in central carbon metabolism, motility and biofilm formation in *E.coli* [192, 198, 199]. The BarA-UvrY TCS balances the carbon flux and switches between metabolic pathways by the use of the Csr system in *E. coli* [164].

Figure 4. Schematic representation of the BarA/UvrY/Csr System.
BarA, the hybrid sensor kinase undergoes autophosphorylation upon reception of signal in an ATP-dependent manner and phosphate is subsequently relayed to a conserved aspartate residue in the response regulator, UvrY presumably via His→Asp→His→Asp phosphorelay cascade. UvrY also upregulate the expression of small non coding RNAs, CsrB and CsrC which in turn, titrates the activity of the global regulatory protein, CsrA by binding to it. CsrA also regulates BarA/UvrY TCS in an autoregulatory feedback loop. Part of the effect of the BarA/UvrY TCS is direct whereas part of it is indirect via Csr system.



Significance, rationale and approach of the study

The major objective of this work is to further understand the role of the BarA-UvrY signaling cascade in adaptation and virulence. Adaptation of bacteria in a new environment requires careful coordination among members of a community. Such synchronized behaviors in microorganisms are carefully controlled in response to multiple environmental cues. Two aspect of such social behavior are studied here:

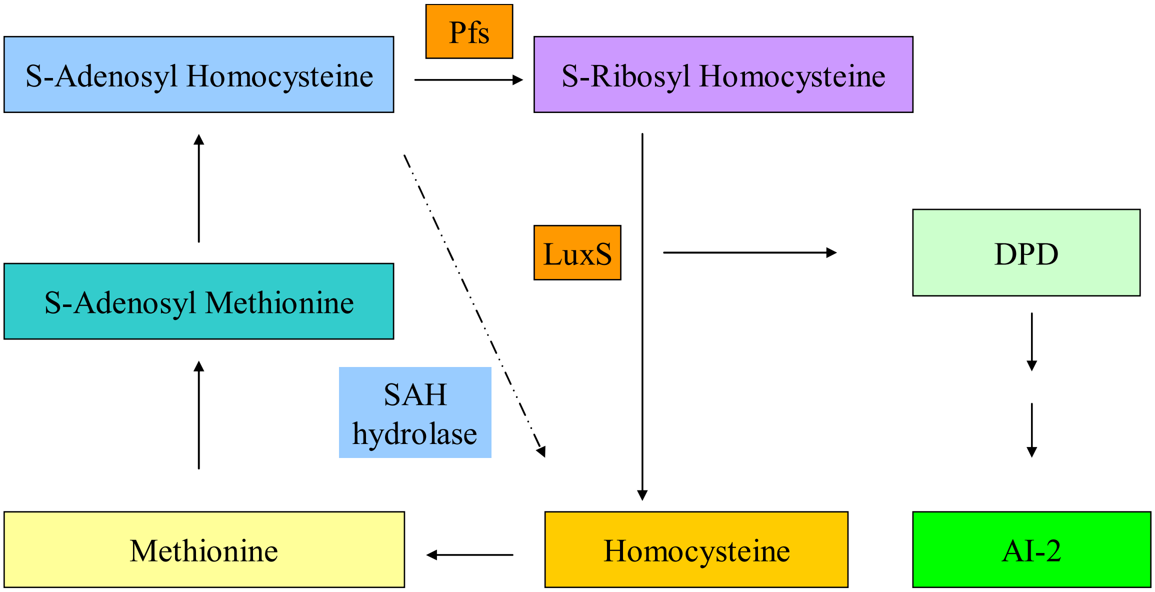
a) Population dependent gene regulation termed Quorum Sensing

b) Complex community structure interaction through the formation of Biofilms

Objective 1: To determine the role of the BarA-UvrY two-component system in regulation of quorum sensing in *E. coli*

Bacteria employ cell-cell communication to assess environmental cues and adapt accordingly to different niches for attachment and colonization. Population dependent adaptation or quorum sensing in gram-negative bacterial species employs three kinds of signaling molecules. These small signaling molecules are acyl homoserine lactone (AHL) called autoinducer-1 (AI-1) and a furanone called autoinducer-2 (AI-2) and AI-3. The production of AI-2 is dependent on the *luxS* gene encoding the AI-2 synthase. Importantly A1-2 is synthesized as a by product of activated methyl cycle. In. *E. coli* S-adenosyl Methionine (SAM), a methyl donor to DNA, RNA and proteins donates methyl group to various substrates generating S-adenosyl homocysteine (SAH). SAH is broken down to homocysteine by two gene products, *pfs* and *luxS*. Pfs, a nucleosidase, breaks down SAH into S-ribosyl homocysteine (SRH) which further undergoes breakdown by the enzyme LuxS, generating homocysteine which goes back to the cycle. One of the by product of this last reaction catalyzed by LuxS is a compound called DPD (4, 5-dihydroxypentanedione) which spontaneously undergoes cyclization and forms AI-2 (Figure 5). Both SAH and SRH are toxic to the cell and thus both Pfs and LuxS play a role in detoxification. *E. coli* is not known to produce AI-1 as it does not have the AI-1 synthase. It has been suggested that AI-2 may represent a universal signal molecule, used for intra- as well as interspecies communication.

Figure 5. A1-2 is formed as a by product of activated methyl cycle. LuxS converts S-ribosyl homocysteine to homocysteine generating AI-2 as a by product. In eukaryotes, an enzyme termed SAH hydrolase converts S-adenosyl homocysteine to homocysteine bypassing the pathway.



The role of BarA-UvrY and its downstream regulators in *luxS* based quorum sensing was demonstrtated by using a single copy chromosomal *luxS::lacZ* transcriptional fusion. The corresponding AI-2 levels were measured using a modified *Vibrio harveyi* reporter. The involvement of CsrA was shown by transcript stability assay following addition of rifampicin, computational prediction of putative binding sites of *luxS* transcripts and direct regulatory interaction of CsrA with *luxS* transcripts. Furthermore, the regulation of *lsr* transport was assessed by reporter activity and real time RT-PCR. The involvelemt of *hfq* and crp-cAMP was also assessed. This work is detailed in Chapter 3.

Objective 2: To identify candidate genes involved in biofilm formation by the BarA-UvrY two-component system in Uropathogenic *Escherichia coli*.

Uropathogenic *Escherichia coli* (UPEC) are the leading cause of Urinary tract infections in US resulting loss of productivity and financial burden on society. UPEC is also the leading cause of nosocomial infections due to formation of biofilms on the abiotic catheter surfaces. The virulence of UPEC depends on several surface structures which facilitates adhesion and biofilm formation eventually leading to persistent infections in the urinary tract. Two adhesins, Type 1 pilus and Pap Pilus are crucial for efficient colonization in the urinary bladder and kidneys respectively. Other factors, such as extracellular polymeric substances and flagellar motility play an important role in biofilm formation and virulence. Part of the downstream effect of the Bar/UvrY TCS in biofilm formation is regulated by the Csr System.

The role of the BarA-UvrY TCS in UPEC biofilm formation was initially tested on abiotic surfaces (Polystyrene and PVC surface). Both Type 1 and Pap pilus expression was monitored in *uvrY* and *csrA* mutant to identify potential downstream regulation. Transcript stability assy after addition of rifampicin was performed to ascertain potential role of CsrA. Type 1 pilus undergoes phase variation and switches between ON (fimbriated) and OFF (afimbriated) phase. The role of *uvrY* in Type 1 phase inversion was determined by an inverse PCR method. Potential role in acidic exopolysachharide accumulation was measured by Ruthenium Red staining. The ability to swarm in soft agar was further tested. Finally, mutants were tested for an ability to colonize in the bladder, kidneys and urinary tract in an ascending model of UTI in mice. This work is summarized in Chapter 4.

Objective 3: To identify novel candidates affected by the BarA-UvrY genes in Uropathogenic *Escherichia coli* that can be employed for detection of toxicity.

UPEC genome has unique 1600 Open Reading Frames whch are not found in commensals. Adaptive stress responses in *Escherichia coli* are largely mediated by several two-component systems. Bacterial biosensors have utilized stress response for detection of toxicity. The BarA-UvrY TCS is pleiotropic and regulates diverse physiological processes including stress response through, stationery phase sigma factor, *rpoS* . Transcription profiling of the TCS was performed to identify potential other genes that might be utilized as toxicity sensors. The potential of this two-component system as bacterial biosensor is reviewed in Chapter 5.

Finally, work from Chapter 4 has contributed to a study where we showed mutation of the BarA-UvrY TCS in Avian Pathogenic *Escherichia coli* displayed reduced virulence in chicken embryo model and poor attachement in chicken fibroblasts and macrophage. Mutation in BarA/UvrY TCS also demonstrated a reduction in mannose resistant haemmagluttination (Table 3). Downregulation of both Type 1 and Pap pilus, reduced exopolysaccharide production, and increased susceptibility to oxidative stress have been attributed for attenuation in virulence (Table 4) [200].

Chapter III: Regulation of AI-2 based signaling by the BarA/UvrY/Csr system in *Escherichia coli*

Abstract

In *Escherichia coli* the BarA/UvrY/Csr system works in concert affecting physiological processes including carbon metabolism, biofilm formation and motility. Here, we report that the signaling pathway regulates *luxS* dependent AI-2 signaling system by evaluating a single copy transcriptional *luxS::lacZ* reporter expression, transcript levels and direct regulatory interactions. The BarA/UvrY and Csr system displayed opposite regulation on *luxS*, the enzyme involved in synthesis of AI-2, indicated a potential dual regulation at the level of transcription and post-transcription. The uptake of AI-2 by the *lsr* (*luxS* regulated) transporter is also modulated by the signaling cascade suggested a possible dynamics of AI-2 synthesis and uptake in the cell.

Introduction

Alteration of gene expression in bacteria is critical for survival and persistence in a changing environment. Perception of signal and appropriate response is indispensable for the fitness of bacterial species. Two-component regulatory systems in bacteria are signaling cascades critical for adaptation in a new milieu and regulate gene expression usually at the level of transcription [1, 2]. A two-component system consists of a membrane-bound sensor histidine kinase and a cytoplasmic reposne regulator which interacts one another by a phosphorelay cascade. Several orthologues of this TCS were detected in other γ proteobacteria such as the BarA-SirA of *Salmonella*, VarS-VarA of *Vibrio*, GacS-GacA of *Pseudomonas*, ExpS-ExpA of *Erwinia* species, all of which were involved in virulence of the respective bacteria [148, 149, 183] . Part of the downstream effect of such TCS is often mediated via a global regulatory RNA binding protein known as Csr (Carbon storage regulator in *Escherichia*, *Salmonella* and *Vibrio* species) or Rsm (Repressor of secondary metabolites in *Pseudomonas* and *Erwina* species). The response regulator UvrY/GacA controls expression of few non-coding RNA which in turn, binds to CsrA/Rsm regulator and titrates its activity.

The BarA/UvrY/Csr signaling cascade in *Escherichia coli* is involved in adaptive response of diverse physiological process including carbon metabolism, motility, biofilm formation and virulence. The BarA-UvrY TCS is involved in switching between metabolic pathways and balances carbon flow via CsrA activity in *E. coli* [164]. The downstream effect of this TCS is mediated via Csr (Carbon Storage Regulator) system whereby *uvrY* positively regulate expression of two non-coding RNA, CsrB and CsrC which in turn sequesters CsrA, the global regulatory protein, by binding to it. The consensus sequence for CsrA binding seems to be conserved GGA motif usually present in the stem loop or hairpin or linear region of the transcripts.

Adaptation signals called autoinducers are involved in gene expression in a population-dependent manner. In *E. coli,* the synthesis of Autoinducer 2 (AI-2) is obtained as a by product of of *luxS* gene products via methyl-cycle. Interestingly, a conserved LuxR-type domain commonly associated with Quorum Sensing is present in UvrY.

In this study, we showed that the mutation of the BarA-UvrY TCS reduced expression of a merodiploid reporter strain *luxS-lacZ* whereas loss of *csrA,* displayed a concurrent increase in expression of the reporter in a growth phase dependent manner. AI-2 levels were correspondingly synchronized with reporter activity specifically from mid-log to entry of stationery phase. The uptake of AI-2 takes place by an ATP-dependent transporter *lsr* (luxS regulated), expression of which is modulated by this signaling cascade in an opposite manner as compared to AI-2 levels and reporter activity, suggesting a balance of carbon flow at the entry of stationery phase. Direct regulatory interactions of CsrA with *luxS* transcripts furthermore confirmed the post transcriptional control of CsrA. Loss of *hfq* also reduced the exprerssion of reporter activity suggest association of small RNA in this regulation. These findings suggest a complex interplay of BarA/UvrY/Csr signaling pathway in a crucial pathway for adaptation by population dependent gene expression in *E. coli*.

Materials and Methods

Strains, plasmids and phages

The bacterial strains, plasmids, and bacteriophages used in this study are listed in Table 6 and 7.

Chemicals and Reagents

Most of the chemicals were bought from Fisher Scientific (Pittsburg, PA) and Difco (Sparks, MD). Antibiotics were bought from Sigma (St. Louis, MO), restriction enzymes, ligases, from NewEngland Biolabs (Beverly, MA), Taq Polymerase, Hifi Taq, and Pfu Polymerase, nucleotides for PCR from Invitrogen (Carlsbad, CA), Tgo Polymerase and CyberGreen RT-PCR kit from Roche Applied Sciences (Indianapolis, IN), plasmid DNA, PCR purification, gel extraction, RNA purification kits from Qiagen (Valancia, CA), DNAse, RNAse, and RNAse-free water from Ambion (Austin, TX). Oligonucleotide primers were purchased from Invitrogen. Radioactive nucleotides were purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Media and growth condition

All media was prepared as described in Miller [201]. Luria Bertani medium was used for routine cultures (10 gl^{-1} tryptone, 5 gl^{-1} yeast extract, 10 gl^{-1} NaCl, pH 7.0) and Tryptone Broth (10gl^{-1} tryptone, 5gl^{-1} NaCl, pH 7.0) was used for growing strains harboring λ fusions. Selection of phage λ lysates and platings were done in R medium (10 gl^{-1} tryptone, 1 gl^{-1} yeast extract, 5 gl^{-1} NaCl, $2x10^{-3}$ M $CaCl_2$ and 0.1% glucose). M9 minimal media (Na_2HPO_4 6 gl^{-1} , K_2HPO_4 was used for glucose induction assays supplemented with 0.1% casamino acids as a C-source for cultures that were grown in the absence of glucose. M63 medium was used to select for *rel*$^+$ transductants when transducing *barA* mutation from MC4100 into MG1655Δ*lac* strain [162]. *V. harveyi* strains were grown in AB medium (17.5 gl^{-1} NaCl, 12.3 gl^{-1} $MgSO_4$, 2 gl^{-1} casaminoacids, pH 7.5) supplemented with $1x10^{-2}$ potassium phosphate (pH 7.0), 1x 10^{-3} M L-arginine, 1% glycerol [202]. Plates were supplemented with 50μg ml^{-1} of 5-bromo-4-chloro-3-indolyl-β-D- galactopyranoside (X-gal) for visualization of β-galactosidase activity. The following antibiotics were added as required at the given concentration: ampicillin 100 μg ml^{-1}, chloramphenicaol 20 μg ml^{-1}, kamamycin 50 μg ml^{-1}, streptomycin 50 μg ml^{-1}, and tetracycline 10 μg ml^{-1}. Overnight cultures, starting from a single colony, were grown in test tubes with 5 ml of medium shaken on a rotary drum at required temperature. For proper growth, all experimental cultures were grown in baffled flasks with 1/5 volume of media at 150 r.p.m in shaking water bath set at appropriate temperature (37°C or 30°C). Growth was monitored using a Shimadzu UV-1601 spectrophotometer at 600 nm (OD_{600}). For gene expression experiments, overnight cultures were diluted 1:100 and serially subcultured two times to an OD_{600} of 0.3, before inoculation into pre-warmed fresh media to an initial OD_{600} 0.05.

Recombinant DNA techniques

Standard molecular techniques were used for transformation, elctroporation, restriction enzyme digestion, gel electrophoresis, PCR amplification, Northern and Southern Blot analysis. All amplifications for cloning were done using Tgo Polymerase (Roche, Indianapolis, IN) and other amplifications were done using Taq or Pfx Polymerase (Invitrogen, Carlsbad, CA) from chromosomal DNA prepared from MG1655 strain using Wizard Kit (Promega, Madison, WI). All clones were confirmed by sequencing.

The *barA* gene was cloned by amplifying the 3.2-kb *barA* locus from MG1655 with OSM 5’CCCGAATTCATA GCATACGCCAAAATGAGGACAG3’ and OSM 5’CCCGATATCATA ACTCGACAAGACATCCATTA 3’ with a 5’EcoR1-3’EcoRV restriction site. The resultant product was cloned into pCR2.1 using DNA Topoisomerase meditated ligation using the TOPO-TA cloning system (Invitrogen). A 3.2-kb EcoR1-EcoRV fragment was subcloned within the EcoR1-EcoRV sites of pBR322. The *barA* ORF is in the direction of the *tet* gene of the vector. The *uvrY* gene was amplified with additional 178-bp 5’ sequence just before the divergent *yecF* promoter using primers OSM64 5’-CCCGAATTCATAATTTCATCGTAGGGCTTACTGTGA -3’ and OSM74 5’-CCCCTGCAGATGCACGCCTGGCTGGTTAC - 3’. The amplified product was cloned using TOPO-TA cloning method into vector pCR2.1 (Invitrogen). Few clones were sequenced to confirm intact amplification. A 700-bp BamH1-EcoRV fragment was cloned within the BamH1-EcoRV site of pBR322, with the open reading frame of the *uvrY* gene oriented in the same direction as the *tet* gene in the vector. The *luxS* gene (denoted as *ygaG*, b2687) was similarly amplified using OSM34 5’-GTGAAGCTTGTTTACTGACTAGAT - 3’ and OSM35 5’-GTGTCTAGAAAAACACGCCTGACAG - 3’ and cloned into pCR2.1, p*lux*S14. A 700 bp EcoR1 fragment was clone into pBR322 where the *luxS* ORF is in the same direction as the *tet* gene.

Genetic techniques

P1*vir* transductions were performed as described by Miller [201]. For transduction of the *barA::kan* and *barA::lacZ* from MC4100 into MG1655, special precaution was taken not to select for *relA* mutation, which is only 1.4 kb from the *barA* locus as selecting transductants that could grow on M63 supplemented medium [192].

Construction of chromosomal insertional mutants

The *uvrY* and the *luxS* genes in MG1655 were disrupted using Datsenko & Wanner method. The *uvrY* gene was knocked with a chloramphenicaol cassette from plasmid pKD3, using linear amplified DNA with 36 bp flanking region of *uvrY* gene using the primers OSM-43 5’-TGGTGCCGCCAGGGATACGACGCATTCTGGAAGTTGCATATGAATATCCTCCTTAGT -3’ and OSM-44 5’-CATTTGTTGAGCGATGTCAGAAGCAATGTAACGCTGACCGTGTAGGCTGGAGCTGCTTC -3’. The *luxS* gene was similarly knockout with a kanamycin cassette with 36-bp flanking region with primers OSM-49 5’-TGCGCTTCTGCGTGCCGAACAAAGAAGTGATGCCAGTTGCATATGAATATCCTCCTTAGT -3’, OSM-50 5’-CACGCTGCTCATCTGGCTGTACCAATCAGACTCATATACTGTGTAGGCTGGAGCTGCTTCG -3’. The mutations were transduced into fresh background and characterized for known phenotypes associated with both *uvrY* and *luxS* mutations.

Construction of chromosomal *luxS::lacZ* transcriptional fusion.

A 469 bp fragment encompassing 290-bp upstream regulatory sequences region and 59 codons of *luxS* gene was PCR amplified with Tgo Polymerase from MG1655 chromosomal DNA using primers OSM-53- 5’-CCCGTCGACATAGCATTTGCAGAAGCCTACCGTA-3’(SalI, 139 bp within 3’end of the *gsh* gene) and OSM54-5’CCCGGGCCCATACAAACAGGTGCTCCAGGGTATG3’(SmaI, 179 bp within the *luxS* gene). The amplified fragment was cloned within the SalI-SmaI site of promoterless *lacZ* transcriptional fusion vector pSP417, a modified pRS415 vector with extended multiple cloning sites. The clones were sequenced to check the integrity of the amplified fragment and the fusion junction. The plasmid-borne fusion was transferred to λRS45. The resulting recombinant phage, λP*luxS-lacZ* (λSM001) was used to transfer the fusion into MG1655Δ*lac*, creating a merodiploid *luxS*$^+$ *luxS-lacZ* fusion (SM105). Single-copy fusions were isolated and verified by a Ter assay followed by measurement of β-galctosidase activity.

Autoinducer Bioassay

The detection of AI-2 in cell-free supernatant was performed using a *Vibrio harveyi* reporter system. *Vibrio harveyi* BB120 was the wild type for this assay. The reporter strain BB170, a *luxN* mutant of BB120 was sensitive to AI-2 but not to AI-1. *V. harveyi* was cultured in autoinducer bioassay (AB) medium. The positive controls were either BB152 (AI-1$^-$, AI-2$^+$) or BB120 (AI-1$^+$ AI-2$^+$) and the negative control was *Escherichia coli* DH5α, a *luxS* mutant which was unable to synthesize AI-2.

Relevant strains were grown in 5ml AB media overnight for 16 hrs in duplicates at 30°C. The cells were pelleted by centrifugation at 10,000g for 2 min. The cell-free culture supernatant so obtained was then passed through a 0.22 mm filter (Millex-GS). The overnight reporter was diluted 2500 times in AB media typically, 10µl reporter in 25ml AB media, to cancel out the background luminescence due to the reporter itself. The assay was performed in white 96 well microwell plate (Nunc, Denmark). The ratio of cell-free supernatant to that of diluted reporter was 1:9 and typically 20µl of cell-free supernatant was used with 180µl of diluted reporter. Higher volume/well ratio was used to reduce fluctuations in luminescence and light scattering. The plates were incubated at 30°C with mild shaking. Bioluminescence was monitored every 30 or 60 minutes in either mediators PhL luminescence microplate reader or by a VICTOR^{3}™V Multilabel Counter (PerkinElmer). Each cell-free culture supernatant was assayed at least three times and the mean values were reported.

Saturation Assay

For this assay, an increasing volume of cell free supernatant was used to achieve light saturation. Cell-free supernatant starting from 5, 10, 25, 50, 60 and 70 µl was added to a fixed volume (50µl) of diluted reporter (1:2500). The ratio of diluted reporter to CFS would vary throughout the saturation curve. The various ratio of reporter to CFS would be 50:5 or 10:1, 50:10 or 5:1, 50:25 or 2:1, 50:50 or 1:1 (Saturation occurs at 1:1) , 50:60 or 1:1.2 and 50:70 or 1:1.4. The Kd for saturation curve was defined as “one-half the volume of cell free supernatant to reach light saturation”.

For the AI-2 kinetics assay, we used a ratio of diluted reporter (180μl) to cell free supernatant (20μl) as 18:2 or 9:1. Hence the ratio that would be closest between the two assays would be the first one of saturation to that of the kinetics assay respectively. i.e. 10:1 to 9:1. Thus for comparing the first dilution the reported value of AI-2 in kinetics assay should be multiplied by 10/9 to compare with the saturation curve, given the ratio of reporter to CFS works linear at all volumes.

RNA stability assay

Total RNA was isolated at an OD_{600} at which CsrA activity is optimally expressed. Rifampicin (Sigma Aldrich) was then added to the culture medium at a final concentration of 500 μg/ml to inhibit transcription initiation. Rifampicin prevents initiation of new transcripts by binding to the β subunit of RNA polymerase. Samples were then removed at 2.5, 5, 7.5 and10 minutes after addition of rifampicin. Amount of remaining *luxS* mRNA was calculated from the intensities of the bands by normalizing with intensities of *icd*, housekeeping gene. The cells were harvested at 14,000 rpm in a microcentrifuge and frozen in solid CO_2-ethanol, with no more than 2 min allowed to elapse between sampling and freezing.

β−galactosidase activity

Strains were grown overnight in TB media with appropriate antibiotics. The overnight cultures were subcultured 1:100 in fresh TB media with antibiotics and were allowed to grow at 37^{o}C water bath until an $O.D_{600}$ of 0.4-0.6 is reached. The subculture is then diluted in fresh TB media so that starting $O.D_{600}$ of 0.05 is achieved. 100µl of the cultures were aliquoted periodically, vortexed and stored in 900µl of Z buffer at 4°C. 200µl of ONPG was added to the aliquots to initiate the reaction, mixed and starting time was noted. Once a sufficient yellow color develops, the reaction was stopped by adding 0.5ml of a 1M sodium carbonate solution and finishing time recorded. The solution is then centrifuged at 11,000 rpm for 2min and $O.D_{420}$ was measured. The β-galacatosidase activity was reported as follows:

$$\text{Units of } \beta\text{-galactosidase} = \frac{1000 \;\text{X}\; O.D_{420}}{\text{Time X Volume X } O.D_{600}}$$

Gel Shift Assay

Interaction between *luxS* transcripts with CsrA protein was demonstrated by gel retardation assay. Briefly, templates were prepared by PCR amplification of the leader region of *luxS* using primers OSM317 and OSM318 such that the transcripts contain a minimal T7 promoter sequence upstream to the transcription start site. The PCR amplified products were gel purified and quantitated by A_{260}/A_{280} and visualized on 0.7% agarose gel in TE buffer. 50ng of template was used for in vitro transcription in a total volume of 20µl. In vitro transcription was performed in accordance with MAXIscript (Ambion, CA) protocol. The transcripts so generated were gel purified and dephosphorylated prior to end labeling with [γ- ^{32}P] ATP using T4-polynucleotide kinase. The labeled transcript was then heated at 80°C and slowly allowed to cool at room temperature to permit formation of secondary structures. Binding reaction condition employed 30 pm labeled RNA with increasing concentration of CsrAHis-Tagged Purified protein at 0, 10,20,40,50,160 and 320 nM.

The binding buffer for this reaction includes 10mM Tris-HCl (pH 7.5), 10mM $MgCl_2$, 100mM KCl, 32.5ng yeast RNA, 10mM DTT, 10% glycerol, 4 U of RNase inhibitor in a 10μl volume. Cold RNA was also included in the reaction mix. The reaction mix was allowed to incubate at 37^{o}C for half an hour. The samples were fractionated in 6% native polyacrylamide gels. Gels were dried and radioactive bands were visualized using a phosphorimager.

Results

Growth rate impaired on *barA*, *uvrY* and *luxS* mutants

The *barA* and *uvrY* genes were disrupted in *E. coli* MG1655 using λRed recombinase system. The *barA::kan* insertion mutants probably did not have polar effect on the *relA* gene by their ability to growth in M9 suplemented medium. However, the colony morphology of both the mutants were smaller than the wild-type strain when grown in TB agar plates or minimal media, but not so much on LB agar plates. The growth rate defect was more in the *uvrY* mutant (G = 34.5 min) than the *barA* mutants (G = 29.5 min). The defect was observed in the exponential phase and amplified when grown in minimal medium or nutrient poor medium (TB) (*barA*= 40.2 min and *uvrY* = 53.1 min compared to 35.2 min of the wild-type strain) as compared to DH5α (*luxS*$^{-}$ *relA*$^{-}$) (G = 35.1 min and 40.2 min), a known slow growing strain as a reference control (Figure 6). The growth rate defect of *csrA* was minimal. Thus this defect could be due to nutritional utilization deficiency or could be due to accumulation of toxic metabolites or both.

Accumulation of exogenous AI-2 on mutation of *barA, uvrY* and *csrA* genes

The synthesis of AI-2 is dependent on LuxS enzyme catalyzed reaction from S-ribosyl homocysteine to homocysteine in methyl cycle (Figure 5). Each cycle generates one AI-2 molecule and thus AI-2 activity could be a potential indicator of cellular metabolism. Several cues indicated potential involvement of the BarA/UvrY/Csr system in *luxS* based AI-2 signaling. Firstly, UvrY also have a LuxR type domain commonly associated with binding of autoinducers (Figure 3) Secondly, several studies have also indicated that expression of small RNAs CsrB and CsrC increases with cell population density [192, 203]. Thirdly, *E. coli* is not known to have LuxI type homologue which synthesizes autoinducer-1. Finally cell-cell communication plays an important role in virulence and efficient adaptation inside host. These have led us to assess whether BarA-UvrY TCS had an effect on AI-2 signaling in *E. coli*. *Vibrio harveyi* reporter BB170 was used to detect AI-2 activity from cell-free culture supernatant. This reporter has a mutation in *luxN*, and impaired in AI-1 detection but a fully functional *luxQ* which specifically detects AI-2 activity.

Our results indicated accumulation of AI-2 in cell-free culture supernatants grown in LB is growth phase dependent as reported previously [204]. Mutation in the *barA*, *uvrY*, or both genes reduced exogenous AI-2 accumulation in *E. coli* MG1655 (Figure 7). Compared to isogenic *luxS::kan* mutant strain, the wild type strain produced 300-fold higher AI-2 at late exponential phase. The accumulation of AI-2 in the *barA* or *uvrY* mutant background was several fold (~3 fold) lower than wild type strain in mid-exponential phase and in early stationary phase. The defect could be complemented in the mutant with ectopic expression of *barA* or *uvrY* from a plasmid. Although the relative amount of AI-2 accumulation in the complemented stain was similar to the wild-type, the accumulation of AI-2 in supernatants was slightly delayed. The effect was more severe in an *uvrY* mutant with lower AI-2 accumulation in mid-exponential phase and in early stationary phase. In the complemented strain, the extracellular AI-2 accumulation was similar to the mutant mid-exponential phase indicating that over-expression of UvrY may be initially limiting AI-2 accumulation. However, the complemented strain exhibited higher level of AI-2 accumulation than AI-2 wild type strain during late exponential-phase.

Effects of BarA/UvrY/Csr signalling cascade on *luxS::lacZ* transcriptional fusion

Since a disruption of the *luxS* gene caused a gowth-defect, we constructed a merdiploid strain with a single-copy *luxS::lacZ* transcriptional fusion with 290-bp upstream sequence from the *luxS* ATG codon. A single copy fusion integrated within the λ *att* site of the *E. coli* chromosome was selected to study *luxS* expression under various experimental conditions in LB and TB medium.

The *luxS::lacZ* transcriptional fusion exhibited a growth-phase dependent expression similar to the extracellular AI-2 accumulation. The expression of the *luxS::lacZ* fusion in a *barA* mutant was found to be 2-fold lower in mid-exponential phase and about in early-stationary phase as compared to the wild type strain. The level of expression could be complemented to a large extent but not similar to the wild type, with a plasmid-borne copy of the *barA* gene. The basal level of expression of the fusion was higher in an *uvrY* mutant and it was only 2.5-fold lower than wild type in mid-exponential and stationary-phase. These results suggest that the BarA-UvrY two-component system, in part, regulates growth phase dependent *luxS* expression, more so in the exponential phase than in the stationary phase. The moderately higher level of basal transcription of the fusion in absence of UvrY indicates that there may be additional factors involved in the regulation of *luxS* expression in stationary phase.

Regulatory interaction of CsrA with luxS transcripts

On the other hand, mutation in the downstream global regulator, *csrA* showed approximately 4-fold upregulation of AI-2 activity and 6-fold upregulation of *luxS::lacZ* chromosomal reporter activity which could be restored to wild-type level upon complementation (Figures 7 and 8). The repression of CsrA takes place at the entry into the stationery phase, and is coincident at which the BarA/UvrY TCS shows induction of *luxS* expression. The repression of AI-2 by CsrA is growth phase dependent and entry into the stationery phase displayed a sharp decrease of AI-2 in extracellular milieu. This suggested a probable post-transcriptional regulation of *luxS* by the Csr system.

The regulatory role of CsrA on *luxS* transcript is further explored by assaying *luxS* transcript stability assay. A *csrA* mutant displays an increase in transcript stability as compared to isogenic wild type. Mutation in *csrA* increases *luxS* mRNA half-life as by more than three minutes (Figure 9A). Secondary structure prediction of *luxS* leader by RNA fold, generated two stem loop region, one of which have a GGA sequence in a hairpin and also occlude the Shine-Dalgarno sequence (Figure 9B).

CsrA is known to bind leader of various transcripts having multiple binding sites for CsrA (such as *glgC*, *pgaA*) and thereby inhibit translation efficiency by occluding the Shine-Dalgarno sequence. CsrA is also known to bind *hfq*, which have a single binding site. Furthermore the direct interaction between CsrA protein and *luxS* leader is also displayed by gel shift assay. A shift was observed between 80 to 160nM of CsrA protein (Figure 9C).

Effect of mutation of *barA, uvrY* and *csrA* on Lsr transporter

In *E. coli* the rapid disppareance of AI-2 from the extracellular milieu was due to an ATP-binding cassette, Lsr transporter which is induced upon entry into stationery phase. Glucose is known to repress *lsr* and as the level of nutrients decreases, *lsr* is induced resulting in a concomitant decrease in AI-2 level from the extracellular supernatant. The role of BarA/UvrY/Csr signaling cascades in the uptake of AI-2 by an, Lsr was investigated. As expected, the *lsr* activity was minmal until the mid log phase and as stationery phase is approached the operon is induced. The *barA* and *uvrY* mutants showed a slightly higher level of *lsr* activity as compared to the parent strain. The *csrA* mutant in contrast, showed a 4-fold reduction in *lsr* activity as compared to the wild type strain, typically at the entry into stationery phase. Real time RT-PCR also demonstrated a sharp increase in expression in *lsrK*, *lsrA*, and *lsrR* upon complementation or over expression of CsrA (Table 2). Hfq, a RNA chaperone, facilitates the base pairing between transcripts and regulates message stability. A loss of *hfq* also reduced the *luxS* expression which could be plasmid-complemented, suggesting possible involvement of Quorum regulatory RNAs (Figure 10).

Effects of the mutation of *uvrY* on swarming motility

Bacterial motility is a complex phenomenon regulated by flagellum regulated by a hierarchical cascade starting with the *flhDC* master operon that encodes tetrameric DNA binding regulatory proteins. Since *in vitro* studies indicate UvrY does not directly bind to *flhDC* promoter, the effect may be either, in part, through the post-transcriptional activation of the *flh*DC genes via the BarA→UvrY→CsrB/C→CsrA system, or it could be in part via a Csr-independent mechanism.

Swarming, a population dependent flagellar motility is characterized by rapid and coordinated group migration over solid surfaces. The ability to swarming is considered a virulence factor and associated with biofilms in several species like *Proteus mirabilis* and *Salmonella typhimurium*. Swarming requires cell-cell communication for migration over a wet solid surface as a group. A loss of *uvrY* also demonstrated reduced swarming motility in semi-solid agar media in presence of glucose. The defect could be restored upon complementation (Figure 11). However, *E. coli* K-12 does not show good swarming partly because of a lack of fully functional O-antigen. A *luxS* mutant also displayed a reduction in swarming ability (not shown).

Discussion

The BarA/UvrY TCS has been shown to regulate central carbon metabolism via regulating the CsrB/C/A system in *E. coli*. BarA-UvrY system also acts as a metabolic switch between glycolytic and gluconeogenic pathways. BarA-UvrY orthologues are conserved in the γ-subdivision of proteobacteria, and plays a role in regulating secondary metabolism in *E. coli*, *Pseduomonous fluorescens*, *Azotobacter vinelandii*, and *Vibrio fischeri*. This study shows that in *E. coli*, AI-2 sysnthesis and uptake is controlled by the BarA/UvrY/CsrA signaling cascade at transcriptional and post-transcriptional level for efficient utilization of carbon flow into the cell. The BarA/UvrY/Csr system regulates *luxS* expression and AI-2 activity.

Autoinducer 2 (AI-2) is generated as a by product of activated methyl cycle. The methyl-cycle is an important metabolic detoxification-recycling loop for s-adenosylmethionine (SAM). The cycle detoxifies s-adenosylhomocysteine (SAH), formed post-methyl donation from SAM, by breaking it down by PfS to generate S-ribosylhomocysteine. LuxS then recycles homocysteine back into the cycle for generation of SAM, in the process generating 4,5-dihydroxy-2,3-pentanedione (DPD), a precursor of AI-2. It is assumed that AI-2 crosses the outer-membrane and accumulates to a threshold concentration before they trigger a cellular QS response via one or more receptors, including the QseB-C system. The role of BarA-UvrY in QS has not been tested. The exact nature of signal detected by the BarA-UvrY TCS is presently unknown, even though it seems to be pH dependent. However, recent findings in *Pseudomonas*, and *Vibrio fisheri* indicated that it is highly possible that this two-component system may be one of the regulating factors for *luxS*-mediated QS in *E. coli*. Both the autoinducers, AHL and AI-2, positively regulate luminescence in marine *Vibrio* spp. (*V. fischeri* and *V. harveyi*), closely related γ-proteobacteria similar to *E. coli*. A *gacA* (*uvrY*) deletion mutant of *V. fischeri* exhibit no detectable luminescence in liquid culture. Addition of known inducers of luminescence, specific AHL (AI-1), marginally complemented the defect. The defect was neither due to reduced synthesis of AHL indicating that the defect in luminescence in the *gacA* (*uvrY*) mutant was affected by AHL-independent mechanism. However, unlike *V. fischeri*, *E. coli* does not have a known functional AHL synthesis pathway which UvrY orthologues are known to regulate. Secondly, we also detect impairment in the ability of an *uvrY* mutant to swarm in semisolid agar. Swarming motility is dependent on coordination among members of a group of bacterial species and is dependent on QS. Thirdly, UvrY also have a characteristics LuxR type domain commonly present in proteins involved in Quorum Sensing. Thus, we hypothesized that, UvrY (GacA) and BarA may be regulating AI-2 synthesis.

The AI-2 synthesis was maximum in early stationary-phase and declined thereafter as reported earlier even though the *luxS* expression remained constant at a basal level. In the *barA::kan* mutant, there was a ~ 10-fold reduction of AI-2 in mid-exponential phase and ~ 6-fold reduction in early stationary phase. The AI-2 accumulation could be restored by carrying the *barA* gene *in trans*. Similar result was obtained in an *uvrY::cm* mutant strain, with ~8-fold and 3-fold reduction of AI-2 in mid-exponential and early early stationary phase respectively. The background levels of AI-2 was slightly higher than the in the *barA* mutant. The level of AI-2 was upregulated atleast 4-fold at the entry into stationery phase which suggested a possible post-transcriptional regulation. CsrA affects stability of various target transcripts and here we showed that mutation of *csrA* indeed increased transcript stability of *luxS* mRNA. Furthermore, computational prediction of *luxS* leader indicated a stem loop occluding the RBS site and contains a conserved GGA bindite site in the loop of the hairpin. Gel shift analysis furthermore demonstrated that the effect of CsrA is direct. The involvement of small RNA in this regulation is also another possibility as an *hfq* mutant displayed reduced expression of *luxS*.

This study shows in *E. coli*, the BarA/UvrY/CsrA signaling cascade regulates *luxS* expression and consequently AI-2 accumulation in extracellular environment in a growth phase dependent manner. The BarA-UvrY TCS regulate carbon metabolism and switches between gluneogenic pathways for efficient adaptation through the activity of CsrA. Here we demonstrate a similar effect by the BarA/UvrY TCS in regulation of *luxS* expression and AI-2 accumulation. The regulation of *luxS* by the signaling cascade suggests a balance between synthesis and uptake of AI-2 (Figure 12A). AI-2 is a 5-carbon moiety furanone, which we propose to be efficiently utilized by the Csr system at the onset of stationery phase. In this model, at low cell density CsrB and CsrC is not optimally expressed and increasing free CsrA leads to tight repression of *luxS*. The expression of small RNA CsrB and CsrC is under positive control of UvrY. However with increasing cell population density, there is an increase in transcription of small noncoding RNA CsrB and CsrC, which titrates free CsrA in the cell. This in turn relieves the repression of *luxS* and consequently AI-2 accumumulation is increased at exponential phase when cells grow rapidly and peaks at the entry of stationery phase. Once cells reach into the stationery phase, Lsr transporter is induced which internalizes the AI-2 from extracellular environment. CsrA also stimulates Lsr transporter activity starting from entry into stationery phase. CsrD an endonuclease which facilitates RNAse E mediated decay of CsrB and CsrC small RNA. Once these small RNAs are decayed, the level of free CsrA once again increases in the cell, presumably deep into the stationery phase. Thus once the cells enter into the stationery phase, CsrA represses *luxS*, and thereby reducing the synthesis of AI-2 while simultaneously induces Lsr transport system and thereby increases uptake of AI-2 into the cell. Thus, in a *csrA* mutant higher level of AI-2 is detected in extracellular environment which falls exponentially deep into the stationery phase due to induction of Lsr transporter. This could mean that AI-2 could be used as a nutrient once the cell enters in the stationery phase while balancing the flow of carbon by the global regulatory protein, CsrA. Alternatively, AI-2 induced genes have to be controlled in a population dependent manner and may not necessarily remain induced deep into the stationery phase. In nutshell, the BarA-UvrY TCS alongwith Csr system regulates *luxS* expression both at transcriptional and post-transcriptional level. This suggests a complex interplay of the BarA/UvrY/Csr in regulation of *luxS* expression, AI-2 synthesis and uptake in *E. coli* (Figure 12B).

Metabolic adaptation is achieved via a network of different signals at different stages of growth and adaptation modulating gene expression. These signals might be interacting with one or more sensor kinases that direct gene transcription for adaptation in a given niche efficiently. Although further experiments are needed to understand exact basis and nature of BarA/UvrY and CsrA in the methyl cycle-regulation, one central outcome of these studies indicate that BarA/UvrY TCS regulates *E. coli* metabolism, communication activities, and nutrient acquisition, an underlying basis of bacterium-host signaling recognition and pathogenesis.

Figures and Tables

Figure 6. Growth curve of the *barA*, *uvrY*, *csrA* and *luxS* mutants. The mutants displayed growth defect which could be restored upon *trans* complementation. Mutation in *luxS* displayed a marked reduction in growth rate. The growth defect was more pronounced in TB or minimal media.

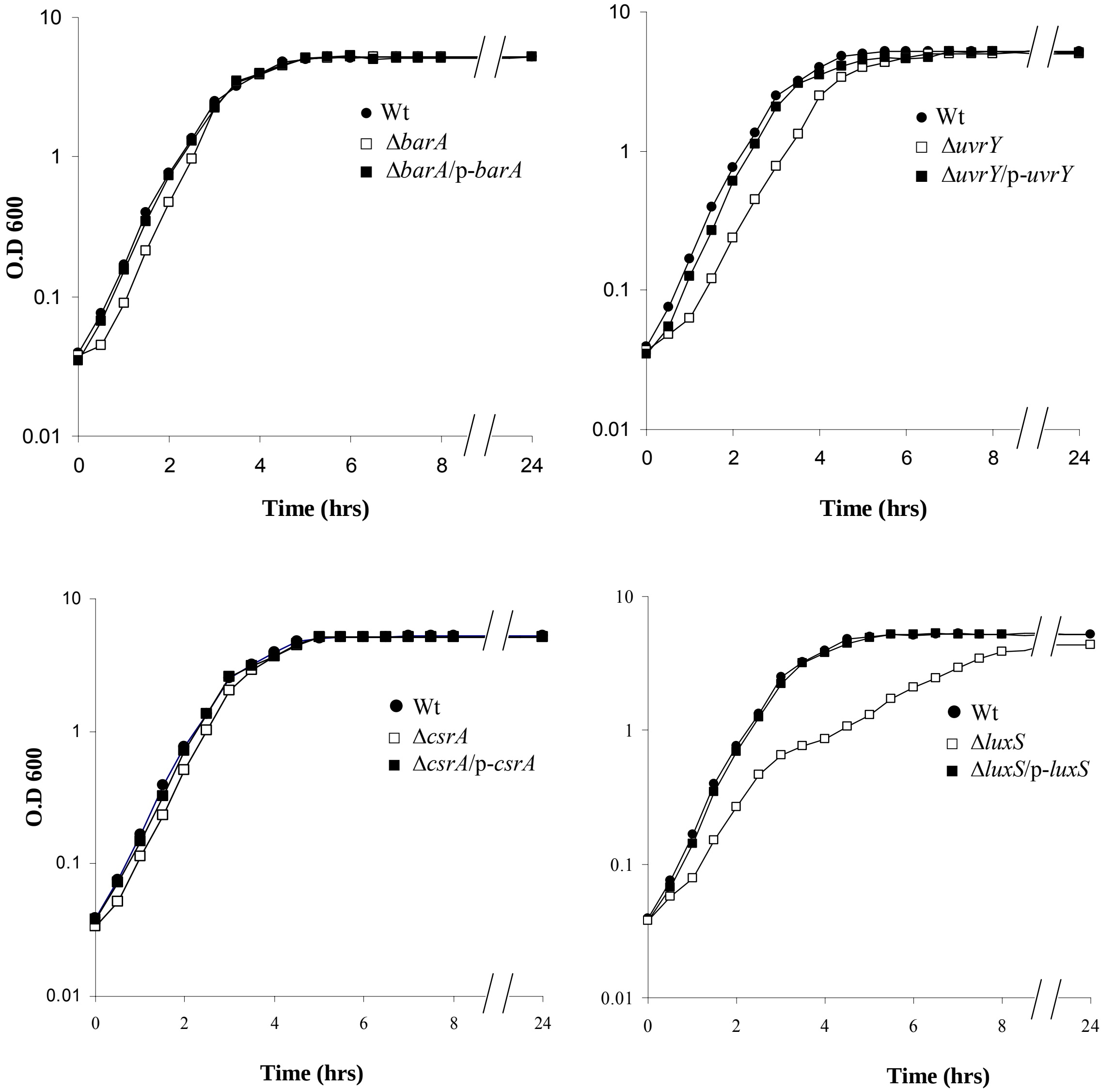


Figure 7. Exogenous accumulation of AI-2 in *barA*, *uvrY*, *csrA* and *luxS* mutants. The *barA* or *uvrY* mutants displayed a reduced accumulation of AI-2 whereas the *csrA* mutant displayed a conincidental increase in AI-2 activity at the entry of stationery phase. Furthermore, Kd of *uvrY* and *csrA* mutant was also reported and defined as one half-the volume of cell free supernatant to reach light saturation.

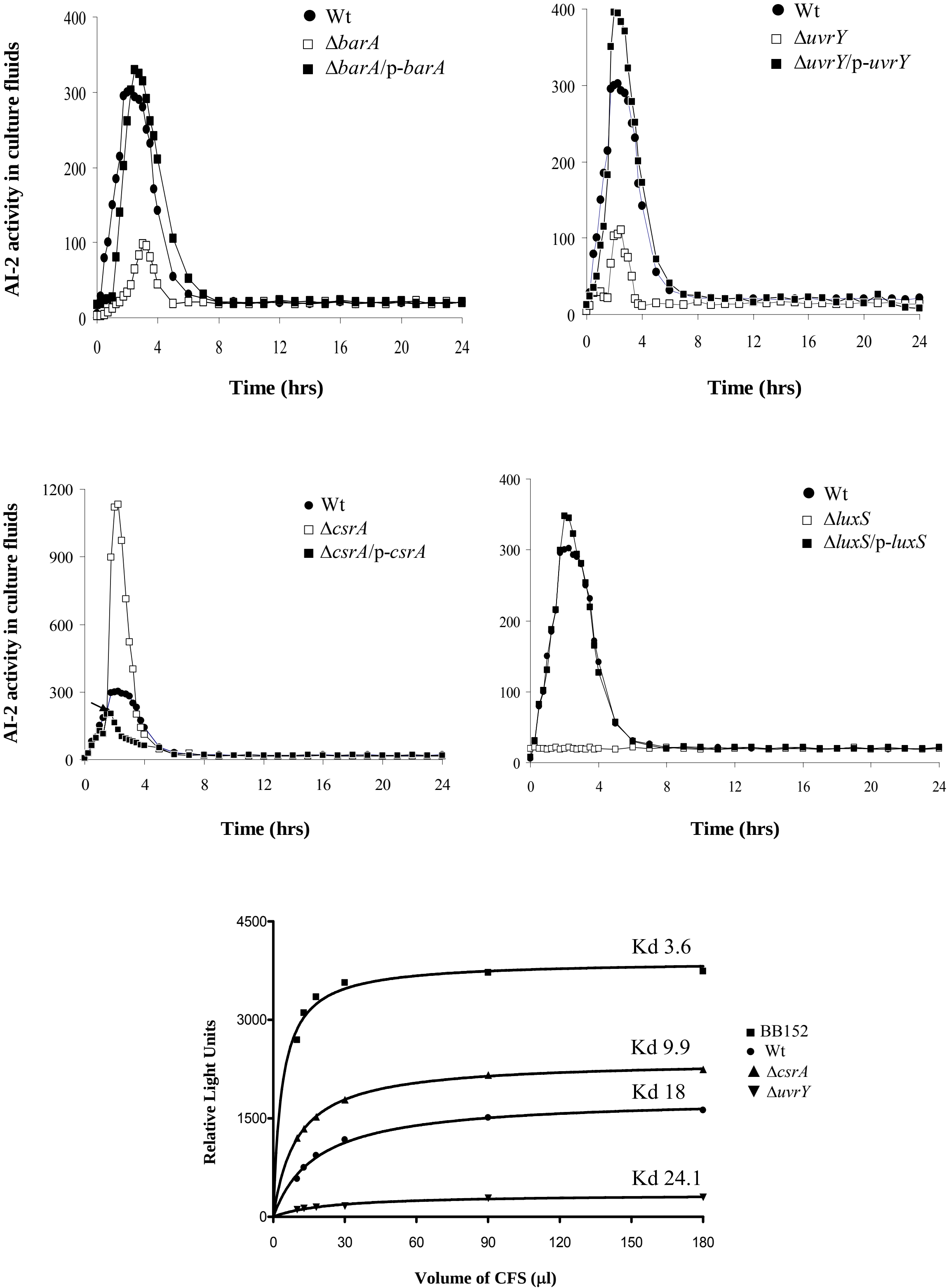


Figure 8. Effect of mutation of the *barA*, *uvrY* or *csrA* genes on the activity of single copy *luxS::lacZ* transcriptional fusion. Mutation in *barA* or *uvrY* demonstrated reduced activity of *luxS::lacZ* reporter activity, whereas *csrA* mutant displayed an increased reporter activity, both of which could be restored to wild type level upon complementation.

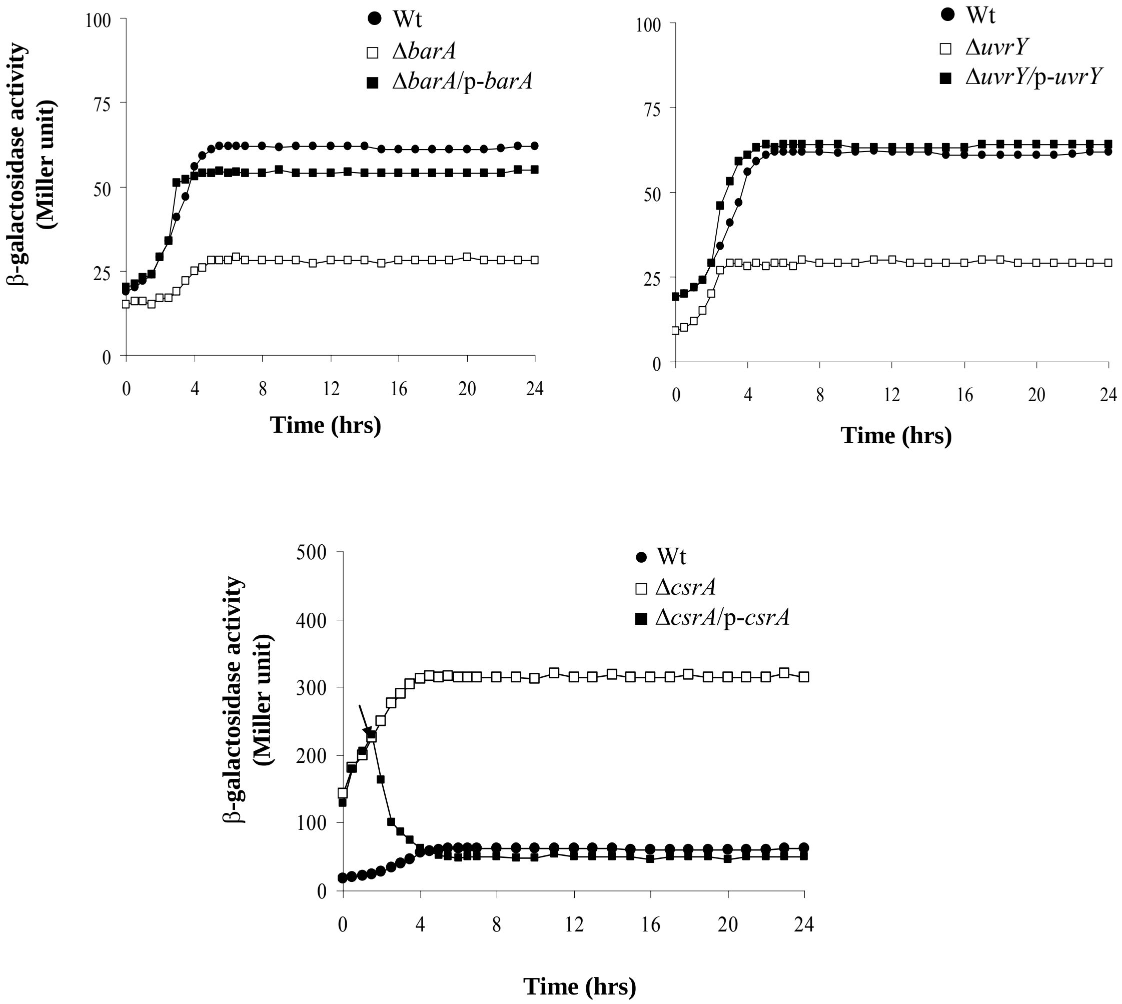
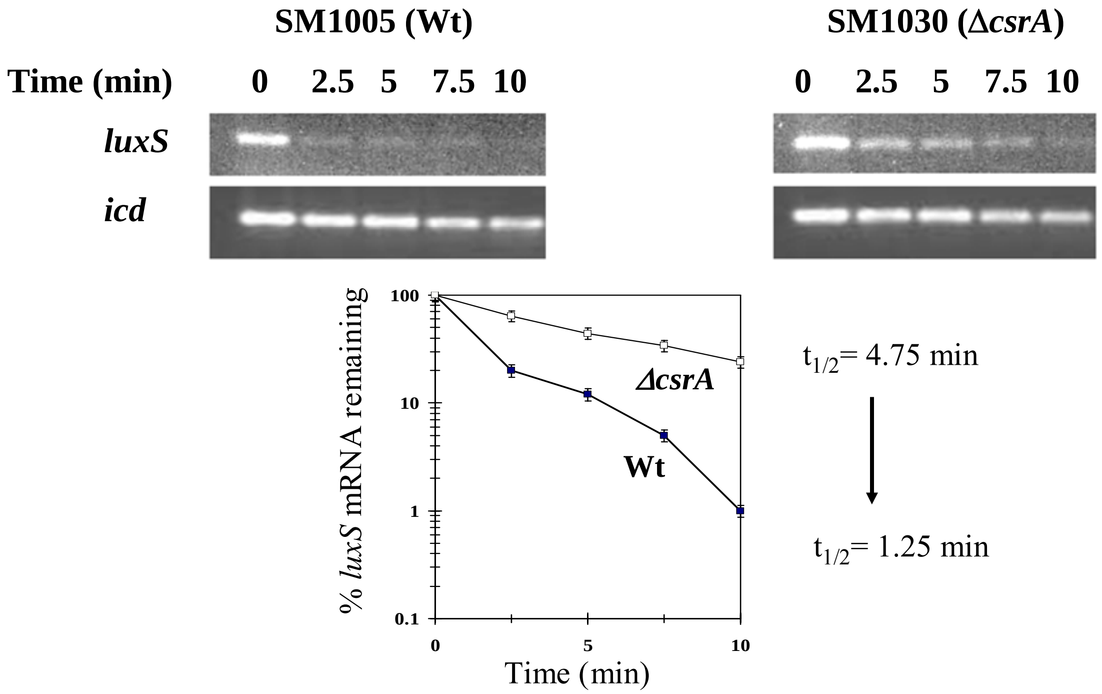
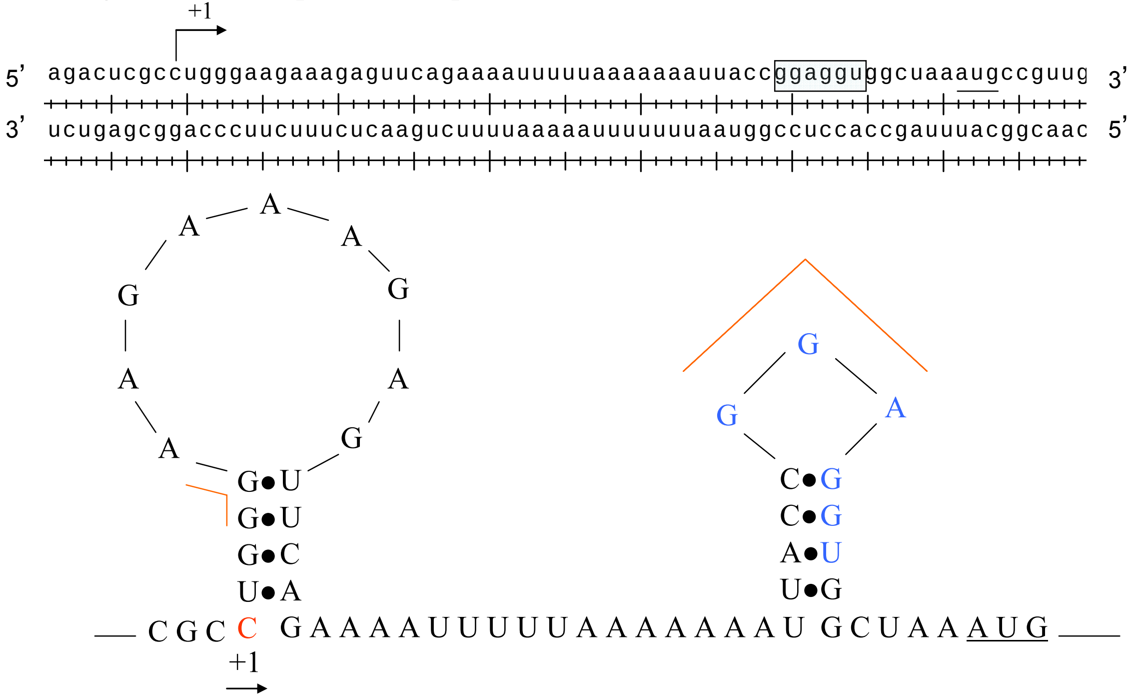


Figure 9. Regulatory interaction of CsrA with *luxS* transcript.

A. Transcript stability of *luxS* mRNA. Rifampicin was added to block transcription and *luxS* message stability was assayed for 10 minutes. Mutation in *csrA* showed relatively stable message (4.75 minutes) as compared to the wild type (1.25 minutes).



B. Predicted structure of *luxS* mRNA leader. The most stable predicted structure indicated two hairpins, one of which occludes the ribosome binding site with a GGA binding site in the loop of the hairpin.



C. Gel Mobility shift analysis of CsrA-*luxS* leader interaction. 5'- end labeled *luxS* leader transcript was incubated with CsrA at concentration as shown below each lane. Positions of free and bound RNA are shown.

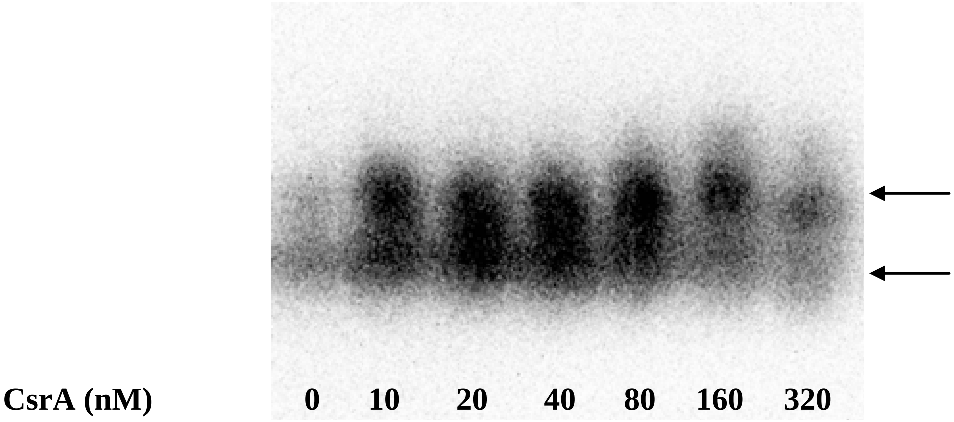


Table 2. Effect of mutation of the BarA/UvrY/CsrA signaling system on Lsr transporter activity. Mutation in *csrA* showed approximately four-fold repression of *lsr* promoter activity and reduced expression of *lsrR* and *lsrA*.

Relevant Genotype	Relative mRNA level			β - galactosidase (Miller units)
	lsrk	*lsrR*	*lsrA*	p*lsr::lacZ*
wt	100	100	100	40.8 ± 3.5
barA::kan	98.0 ± 2.0	95.0 ± 1.5	96.0 ± 1.0	55.1 ± 3.0
uvrY::cm	83.0 ± 1.5	34.0 ± 1.5	90.0 ± 1.0	58.6 ± 3.5
csrA::kan	83.0 ± 1.5	45.0 ± 1.5	44.0 ± 1.5	10.3 ± 1.5

Figure 10. Effect of mutation of *hfq* on *luxS::lacZ* reporter activity. Mutation in *hfq* reduced reporter activity at the entry of stationery phase and subsides once deep into stationery phase. The effect could be restored on complementation**.**

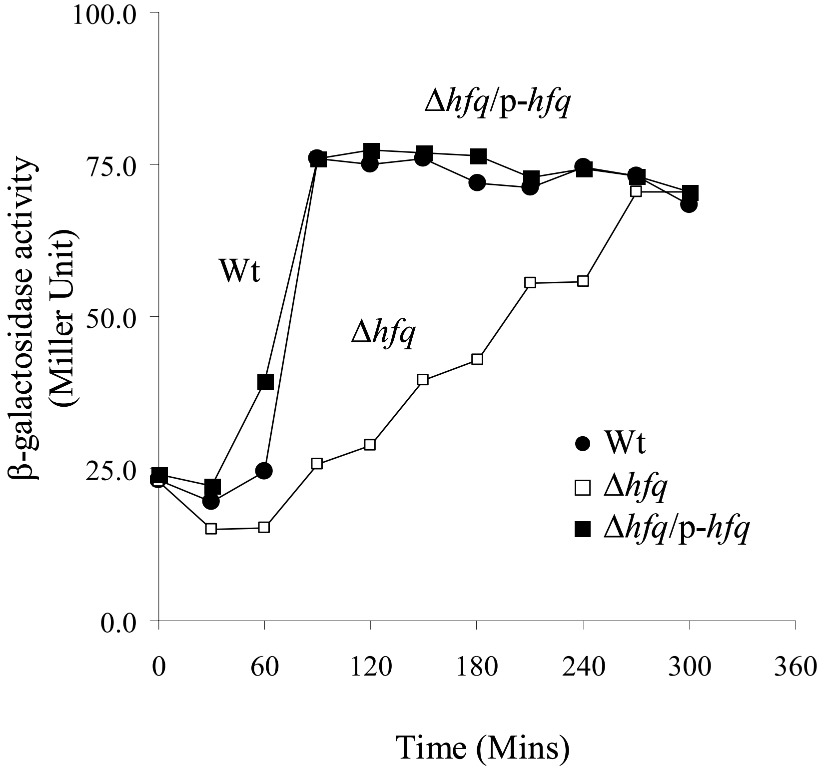


Figure 11. Impairment of swarming motility upon loss of *uvrY* on semisolid agar. The diameter of the swarming colony was represented by the underlined bar.

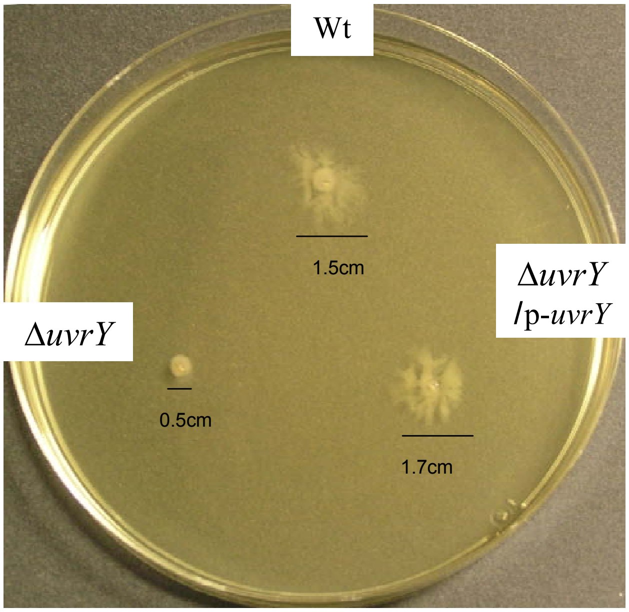
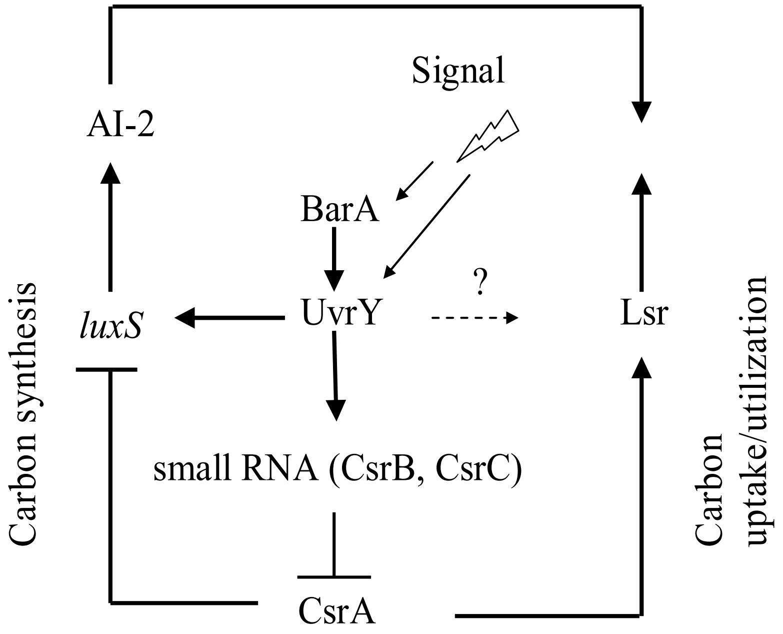
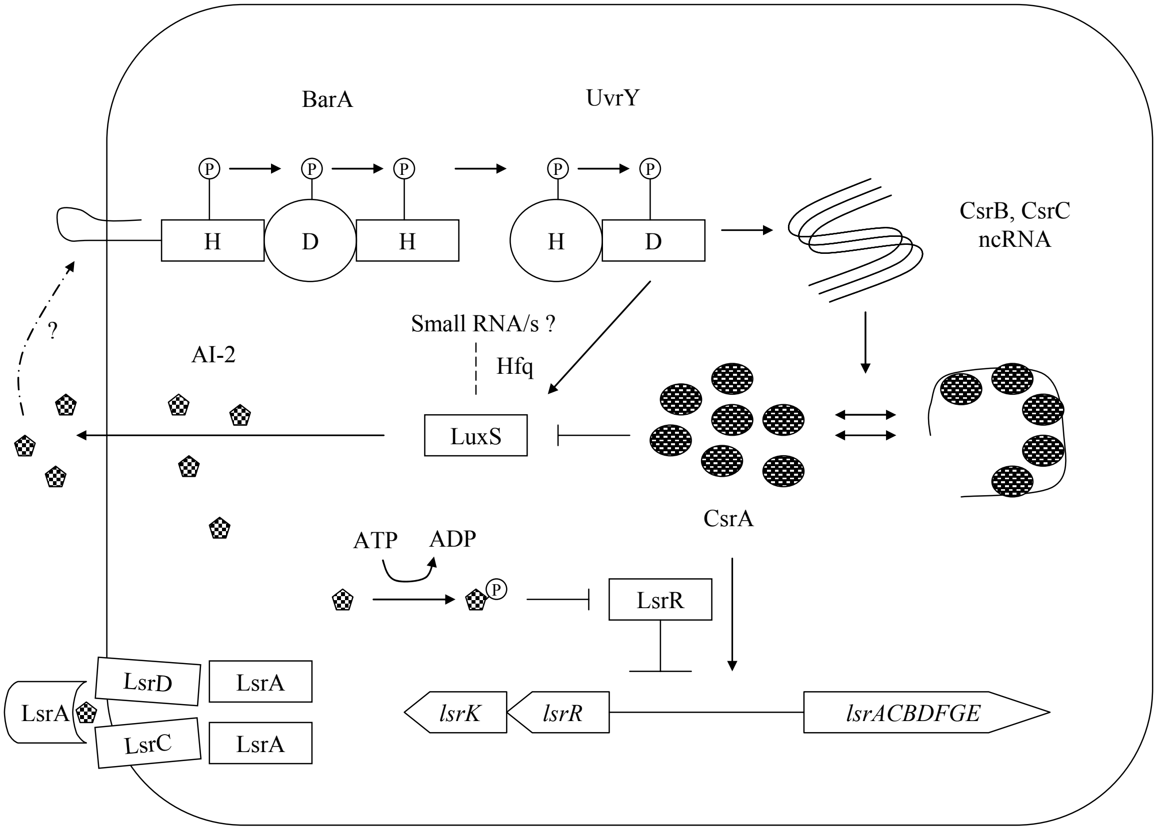


Figure 12. Proposed regulatory circuit of AI-2 synthesis and uptake in *E. coli*.

A. Regulation of AI-2 activity by balance of carbon flow. At low cell density the CsrB and CsrC is not optimally expressed leading to a tight repression by CsrA on *luxS*. However expression of CsrB and CsrC increases with population density and consequent titration of free CsrA leads to derepression of *luxS* and more accumulation of AI-2 in the extracellular environment. CsrA also induces the Lsr transporter involved in AI-2 uptake and thereby balances the flow of carbon at the entry into stationery phase.



B. QS circuit in *Escherichia coli*. The BarA/UvrY TCS regulates *luxS* expression positively at transcriptional level whereas CsrA negatively regulates *luxS* post-transcriotionally. A possible role of quorum sensing regulatory RNA exists in the circuit. The sensing stimulus of the BarA/UvrY TCS is unknown at the moment.



Chapter IV: Biofilm formation in Uropathogenic *Escherichia coli* is influenced by the transcriptional regulator, *uvrY* in a type 1 pilus dependent manner

Abstract

Biofilm formation is an important virulent determinant in Urinary Tract Infections. Uropathogenic *E. coli* are the principal causative agent in community and hospital acquired UTI. Several factors contribute to biofilm formation among which Type 1 pili, Pap pili, production of exopolysaccharides, flagellar associated motility are critical in *E. coli*. We studied the role of transcriptional regulator *uvrY* in biofilm formation in Uropathogenic *Escherichia coli*. Absence of *uvrY* cause reduced expression of *fimA* and *papA,* fimbrial major subunit of Type 1 Pilus and Pap pilus respectively. Using PCR Inversion Assay we demonstrate that *uvrY* regulates phase variation of Type 1 pilus. Furthermore, acidic exopolysaccharide accumulation and the ability to swarm are also being impaired by deficiency of the regulator. Finally, *uvrY* mutants demonstrate a lack of colonization in kidneys and bladders in an ascending model of UTI. Overall, the effect of *uvrY* on biofilm formation seems to be multi-factorial and might play a critical role in adaptation and colonization of UPEC.

Introduction

The ability of bacteria to adapt and colonize is critical for survival and persistence of bacteria in a dynamically changing environment. Bacterial adaptation to new environment relies on a signaling cascade called two-component regulatory systems (TCS). A two-component system consists of a sensory protein kinase (HPK) and a cognate response regulator (RR). The sensor kinase is involved with detection of environmental cues which is transduced to the response regulator. The response regulator, in turn, responds by appropriate modulation of gene expression.
Around thirty TCS have been recognized in *Escherichia coli* out of which the BarA-UvrY two-component regulatory system have been shown to be strongly linked with virulence. In this system, BarA is the sensor kinase and UvrY is the cognate response regulator. Several orthologues of this TCS are present in diverse species of γ–division of proteobacteria including BarA-SirA of *Salmonella*, GacS-GacA of *Pseudomonas*, VarS-VarA of *Vibrio* and ExpS-ExpA of *Erwinia,* all of which have been demonstrated to be strongly involved with virulence.

Biofilms contribute up to 80% of chronic inflammatory diseases including urinary tract infections (UTI), cystic fibrosis, otitis media, colitis, conjunctivitis, dental plaque, endocarditis, peridontitis, and prostatitis [88]. Presence of biofilms in indwelling medical devices (such as urinary catheters) and other devices in healthcare settings often results increase incidence of nosocomial infections. Uropathogenic *E coli* (UPEC) are commonly associated with community and hospital acquired Urinary Tract Infections (UTI). A critical determinant of successful colonization of UPEC is the ability to form biofilms. In *E. coli* several adhesins such as Type 1, exopolysaccharide accumulation and flagellar associated motility is critical for biofilm formation. In this study, we decided to investigate the contribution of the response regulator *uvrY* in Uropathogenic *Escherichia coli* by evaluating pilus expression, exopolysaccharide accumulation and flagellar motility that are critical for biofilm development.

Materials and Methods

Bacterial Strains, Plasmids, Primers

All bacterial strains, plasmids, primers are listed in Tables 6 and 7.

Cloning of functional *uvrY* gene

Relevant genes were disrupted in UPEC CFT073 using λ Red recombinase system. The *uvrY* gene was amplified with 178 bp 5' sequence just before the divergent *yec*F promoter using primers OSM 64 5' CCCGAATTCATAATTTCATCGTAGGGCTTACTGTGA 3' and OSM 65 5' CCCCTGCAGATGCACGCCTGGCTGGGTTAC 3'. The amplified product was cloned using TOPO-TA cloning method into vector pCR2.1 (Invitrogen). Few clones were sequenced to confirm intact amplification. A 700-bp BamH1-EcoRV fragment was cloned within the BamH1-EcoRV site of pBR322, with the open reading frame of the *uvrY* gene oriented in the same direction as the *tet* gene in the vector.

Cloning of *uvrY* gene for over expression and purification of the UvrY protein

The *uvrY* gene was cloned at BamH1-Pst1 site of the multiple cloning sites in pQE30 (N- terminal 6x His) vector. The clones were sequenced and checked for the presence of His-tag sites and subsequently transformed in pREP4 for expression studies and protein purification. The 6His-Tag UvrY have been purified on a small scale.

Biofilm Assays (Growth Conditions)

Overnight cultures of *Escherichia coli* in LB broth with appropriate antibiotics were subcultured (1:100) in 50 ml LB broth with necessary antibiotic and grown at 37^{o}C for 1 hour. The cultures were then transferred to Petri-plates (Falcon, 150X15m) containing 8-12 sterile borosilicate cover slips and in microtiter plates. The plates were incubated at room temperature. Media was periodically removed every 24 hours, washed with 20 ml of 1X Phosphate buffer saline (PBS) (pH 7.4) and fresh LB media with antibiotics were added.

Crystal Violet Staining

Coverslips were taken out of the Petri-plate and washed thoroughly by dipping in 1X PBS (pH 7.4) buffer. They were taken in fresh Petri-plate (96X16mm) and dry-fixed for 1 hour at 60°C. 10 ml of 0.1% CV (SIGMA Chemicals, MO, USA) in isopropanol: ethyl alcohol: PBS (pH 7.4) (1:1:18) were added to the coverslips in the Petri-plates and were allowed to stand at room temperature for 15 minutes. Excess crystal violet was then removed by washing cover slips at least twice with 10 ml of 1X PBS (pH 7.4) or till the washings were clear. The coverslips were allowed to dry, broken with a glass cutter and taken in 1.5ml microfuge tubes. 1 ml of 33% acetic acid was added to each tube to dissolve the crystal violet dye and the O.D. was measured at 570 nm with required dilution. The same assay was also done in glass and PVC microtiter plates.

Ruthenium Red Staining

The bacteria were grown under the same conditions as previously described in 150X15mm Petri-plates with sterile cover slips at the bottom of the Petri-plates. Two cover slip per plate was removed carefully, washed by dipping in 1X PBS (pH 7.4) buffer in a beaker, fixed at 60°C for 1 hour and placed in a well of a 6 well tissue culture plate. 1 ml of stain I (0.15% ruthenium red-0.5% glutaraldehyde dissolved in 0.1M cacodylate buffer) was added to each of the wells and allowed to stand at room temperature for 1 hour. The stain I was removed and 1 ml of stain II (0.05% ruthenium red-0.5% glutaraldehyde dissolved in 0.1M cacodylate buffer) was added and allowed to stand at room temperature for 2 hours. Stain II was removed, washed five times with 1 ml of 0.1M cacodylate buffer and observed under light microscope at either 40X or 100X magnifications.

Assays for *fim* switch orientation

The assays for orientation of *fim* switch was done as earlier described . In brief, after isolation of chromosomal DNA, equal amount of genomic DNA was used as a template to determine the “ON” phase and “OFF” phase respectively by using two sets of primers. The amplified fragments were then run in 2% agarose gels and visualized by ethidium staining. The “ON” and “OFF” population were represented by lower band and upper band respectively. The primers were listed in Table 6.

Hemagglutination Assay

Relevant strains were grown in LB with appropriate antibiotic as necessary (ampicillin 100, kanamycin 50, and chloramphenicol 20) without shaking for three passages (48 hrs each). The assay was carried in a 96 well round bottom plates (Costar, Corning, NY) in triplicates. Briefly, 50µl of PBS were added with and without 50mM Mannose in separate lanes of the plate and serial dilution of each culture is attained by addition of 100 µl of each cultures, carefully mixing, and then transferring 100 µl of the mix into the next well. The dilution is performed in a similar manner till the second last lane. 100µl of mix from the last lane were removed from the last lane to achieve appropriate dilution ratio. Finally, 50µl of human erythrocytes (1%) were added to each lane from higher to lower concentration of cells to permit more time to visualize a clear agglutination. The resultant final volume of each lane then becomes 100 µl. The assays were tested on type O^+ human, sheep and gunieapig blood (Lampire Biological Laboratories, Pipersville, PA). The maximum dilution of cells that gives a visible agglutination is reported as the titer. The plates were incubated on ice and appropriate titers were visualized on a mirror and photographed.

RNA stability assay

Total RNA was isolated at an OD_{600} at which CsrA is maximally expressed. Rifampicin (Sigma Aldrich) was then added to the culture medium at a final concentration of 500 µg/ml to inhibit transcription initiation. Rifampicin prevents initiation of new transcripts by binding to the β subunit of RNA polymerase. Samples were then removed at 2.5, 5, 7.5 and 10minutes after addition of rifampicin. Amount of remaining *luxS* mRNA was calculated from the intensities of the bands by normalizing with intensities of *icd*. The cells were harvested at 14,000 rpm in a microcentrifuge and frozen in solid CO_2-ethanol, with no more than 2 min allowed to elapse between sampling and freezing. The level of *fimA* mRNAs declined relatively quickly in a *csrA* mutant strain as compared to a wild type.

Swarming Assay

Strains were grown under static conditions in LB broth with relevant antibiotics for three passages of 48 hours each. The media for swarming were LB with 0.6% Agar (wt/vol) with 0.5% (wt/vol) glucose. Each experiment was conducted in triplicates. Equal number of cells as adjusted by optical density and by colony forming units was used for inoculation into the middle of the soft agar plates. Strains were incubated overnight in 37^{o}C. The diameter of the spread of colonies was measured and photographs were taken with an Olympus C765 Ultra Zoom camera.

Confocal Scanning Laser Microscopy (CSLM)

Coverslips were taken out, washed by dipping in 1X PBS (pH-7.4). They were taken in 35 X 10mm tissue culture dish and gently covered with SYTO 9, a fluorescent nucleic acid stain that is a part of LIVE/DEAD® BacLight™ Bacteral Viability Kits (Molecular probes Inc, Eugene, OR), after diluting the dye four times with water. They were incubated in dark at room temperature for 15 minutes. The coverslips were washed thrice with 1ml of 1X PBS (pH-7.4), and mounted on slides. The biofilms were viewed using a 40X dry objective using a confocal scanning laser microscope (CSLM) which is a Zeiss inverted microscope, and a dual laser-scanning confocal imaging system equipped with a 100mW argon laser and a 5mW krypton argon laser. The thickness of the biofilms was measured from the orthogonal sections of the images formed by Z-stack scanning.

cDNA synthesis, quantitative PCR, and quantitative RT-PCR

Quantitative polymerase chain reaction (qPCR) and quantitative real-time polymerase chain reaction (qRT-PCR) were performed as per the manufacturers' recommendations. Total RNA was isolated using 4ml of liquid culture is stopped with 0.9ml stop solution. (Phenol::EtOH - 1::19). Total RNA was isolated in accordance with RNeasy mini protocol (Qiagen, CA) in a final 50µl volume in water. Lyzozyme was used in a final concentration of 1mg/ml. The integrity of RNA as well as possible DNA contamination was checked in 1.5% formaldehyde gels and spectrophotometrically determined. Total RNA was subjected to a rigorous DNase treatment to remove any possible DNA contamination (Turbo DNA *free*, Ambion). A second visual inspection in 1.5% formaldehyde gels ensures RNA is free from any possible trace contamination DNA and OD260/280 ratio were determined for subsequent cDNA preparation. For qPCR, the first-strand cDNA was synthesized from 5µg of total RNA using Moloney Murine Leukemia Virus reverse transcriptase, Superscript II RnaseH$^-$ (Invitrogen, Carlsbad, CA) and 50 ng of random hexamers (Invitrogen, Carlsbad, CA) as primers according to manufacturer's instructions. The quality of cDNA synthesis was determined by electrophoresis in 1.2 % agarose gels and quantitation using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Relevant Internal gene-specific primer pairs were designed with a control 16S *rrnA* gene-specific primers in a 25 µl total reaction volume with Taq polymerase in a series of tubes, using a Biometra T-Gradient PCR instrument (Biometra, Horsham, PA) for 30 cycles. At various cycle intervals, a gene-specific and a control reaction tube was removed. Five µl of the reaction products were resolved separately in a 1.2% agarose gel, visualized by ethidium bromide staining, and the double-stranded DNA (ds-DNA) product intensities quantitated using a BioRad Gel Documentation system (BioRad, Hercules, CA). The linear range of amplification for the *rrnA* gene was from 5-15 cycles in all backgrounds, while that of the *luxS* and the *pfs* genes were from 12-22 cycles in the wild-type strain, and appeared much later cycles for the mutants. The amplification product produced only a distinct 300 bp ds-DNA band. A qRT-PCR reaction was performed on the above set of samples under identical reaction conditions in a Light Cycler (Roche, Indianapolis, IN) with SYBER Green-1 PCR Master Mix. The fluorescence signal from SYBER Green intercalation was monitored to quantify double-stranded DNA product formed after each PCR cycle.

Mouse Challenges

CBA/J mice were anaesthetized in a chamber by isofluorane (a halogenated volatile anaesthetic which induces and maintains general anaesthesia by depression of the central nervous system and resultant loss of consciousness). The innoculum volume was carefully adjusted so that there is no forceful inoculation into the kidneys. Transurethral inoculation (Harvard pump) involved administration of catheter all the way to the lumen of the urinary bladder. Bacterial strains were grown on 2 large agar slants and resuspended in total of 5ml PBS ~ $2x10^9$ CFU/ml. One- half bladder and kidney used for histological purposes and one half for spiral plating. After 3 days the mouse were sacrificed, and harvested for the presence of bacteria in kidney and bladder. The mice were handheld and pressed in their neck and abdomen region for collection of urine. The urine so collected was weighed, and dissolved in appropriate volume of PBS.

Results

Effect of *uvrY* on biofilm production in UPEC

A lack of *uvrY* shows marked decrease in biofilm production in UPEC CFT073 on glass and PVC surfaces (Figure 13). Part of the downstream effect of the regulator *uvrY* in biofilm formation is mediated by the global regulator, CsrA which represses PGA, a basic adhesin. However, complementation of CsrA does restore the *uvrY* mutant phenotype indicated that the effect of *uvrY* in mediating attachment to abiotic surfaces could be due to additional regulation that might be affected independently of CsrA.

Effect of *uvrY* on expression of Type 1 and Pap pilus

Type 1 pilus is absolutely critical for biofilm formation in *Escherichia coli.* Importantly, Type 1 pili have been implicated in the colonization of the bladder in UTI. On the other hand, Pap pili (Pyelonephritis associated pilus) commonly associated with UPEC are important for colonization in the kidneys. To determine whether *uvrY* have an effect on expression of these adhesins, we tested the expression of *fimA* and *papA*, the major fimbrial subunit of Type 1 and P pilus respectively by semi-quantitative and Real Time RT-PCR. We have observed both *fimA* and *papA* being down regulated in the process (Figure 14). This corresponds to an ability to colonize the bladder and kidneys respectively.

Effect of *uvrY* on *fim* switch orientation of Type 1 pilus

Expressions of fimbrial genes are carefully coordinated as it utilizes a lot of cellular resources. An important attribute of Type 1 fimbrial expression is its ability to switch between “ON” and “OFF” phase characterized by fimbriated and afimbriated phase respectively. While the ON phase mediate attachment to host cells by interaction with surface receptors, the OFF phase shows cell-surface receptors, the afimbriated OFF phase may be equally advantageous and might aid invasion through the viscous mucus layer that envelop the intestinal epithelium or evasion from phagocytosis by macrophages. The switch between OFF and ON is mediated by a 314-bp invertible element flanked by 9bp long inverted repeats located immediately upstream to *fimA*, encoding the major fimbrial subunit. The invertible element contains the *fimA* promoter element and the orientation of the promoter switches ON or OFF the transcription of the Type 1 fimbriae. Two recombinase termed *fimB* and *fimE*, apart from other regulators are involved in the regulation of the genetic switch. The FimB can switch from between ON and OFF in either direction, FimE preferentially switches from ON to OFF position. However, environmental signals and DNA topology also plays a role in this orientation of the switch. Mutation in *uvrY* switches OFF the fimbrial population and restores the ON population upon complementation. Furthermore, both the recombinase *fimB* and *fimE* have reduced expression on loss of *uvrY*. This could be due to a change in DNA topology upon interaction with the upstream regulatory region. However the downstream effect by CsrA also switches OFF the circuit, but unlike *uvrY* doesn't restore the ON population and instead the OFF population is even further improved. The *fimA* message seems to be unstable in absence of CsrA, suggesting post-transcriptional regulatory mechanisms affecting type 1 pilus (Figure 15).

Effect of *uvrY* on exopolysaccharide accumulation

The effect on exopolysaacharide accumulation is further demonstrated by Ruthenium Red dye staining. Ruthenium Red Stain stains the acidic exopolysaccharides. A lack of *uvrY* shows reduced accumulation of exopolysaccharides which could be complemented (Figure 16). Exopolysaccharide promote adhesion to solid surfaces, cell-cell adherence, and stabilization of biofilms structure in *E. coli* [49, 85, 194, 205]. Ruthenium red stain, a stain specific for polysaccharides and often used in EPS detection, was used to stain biofilms formed on glass slides [206]. It is known that

BarA-UvrY TCS regulates the expression of CsrA protein, a major player in regulating biofilms formation and EPS production [194]. However, the role of LuxS in the process is not clear; although it appeared that something in the culture free supernatant regulated by LuxS was contributing to the adhesion and biofilms formation. Ectopic expression of *uvrY* led to ruthenium red stainable nuclei in the biofilms, indicating that EPS, among other factors, contributed to enhanced biofilms formation. However, over expression of *luxS* did not exhibit similar intense ruthenium red-stainable nuclei, although there was considerable EPS production as seen under a microscope. Interestingly, the average depths of the films were over 40 μm in either case (not shown).

Regulation of genes involved in attachment

Attachment and biofilms formation in a △*fim* background indicated factors other than type 1 fimbriae as initiating biofilms. Apart from EPS, type 1 fimbriae and antigen 43 have been implicated in initial attachment and biofilms formation [207-210]. Neither did ectopic expression of *luxS* did not exhibit EPS producing nucleated bacterial clusters. Since global gene expression in *E. coli* biofilms is known [208], the expression of *flu* encoding (Ag 43) assisting biofilms formation, was determined in various background. Using quantitative RT-PCR on total RNA isolated from various cultures, the level of *flu* mRNA was down regulated in both *barA* and *uvrY* mutants. The level was 26% less than wild type in the *barA* mutant and 34% less (more than 2 fold lower than wild type) in the *uvrY* mutant (data not reported).

Confocal Image Analysis

Mutation in *uvrY* reduces thickness of biofilms significantly. The thickness of the biofilms in the wild type and *uvrY* complemented strains were approximately 60 µm in depth whereas that in an *uvrY* mutant cells are almost as in a monolayer as average *E. coli* length is 2-3 µm (Figure 17). As a control, *uvrY* also restores biofilm formation in a △*fim* strain suggesting that *fim* independent pathways also controlled by *uvrY*. Loss of *uvrY* was also marked with poor microcolony formation and reduced thickness. Scanning electron micrographs of mutants indicated that a deletion of either *barA* or *uvrY* led to a decreased visible cell surface appendages and extracellular coatings traditionally seen on a *E. coli* saturated culture or taken from solid surfaces (not shown). The surface architecture of the mutant bacteria indicated that the adhesion defect may be due to a defect in the pili and surface adhesins.

Effect of *uvrY* on swarming motility

One commonly surface associated behavior controlled in a population dependent manner is Swarming Motility, a process of flagellar dependent locomotion. In *E. coli* K-12, flagellum is critical for initial attachment and overcoming repulsion between similarly charged bacterial and inert surfaces [211]. In *E. coli* K-12, the transcription of flagella genes and exopolysaccharides are oppositely regulated. It is thought that flagella plays a role in motility and when bacteria become associate with a surface, flagella genes are shut off while exopolysaccharide synthesis were up regulated. Mutation of *uvrY* impairs the ability to swarm on a semi-solid agar plates in presence of glucose (Figure 18). The swarming ability is restored upon complementation. Semi-quantitative RT-PCR and Real time PCR displayed reduced expression of both *flhD* and *flhC*, the expression of both could be increased upon complementation.

Mutation of *uvrY* exhibits poor colonization in ascending model of UTI

The ability of *uvrY* mutants were also tested in an ascending mouse model of UTI. Lack of *uvrY* displayed a reduced ability to colonize either in kidneys or bladder (Figure 19). The ability to persist in urine is also significantly impaired.

Discussion

Biofilm formation requires a modulation of gene expression facilitating initiation, attachment and subsequent maturation. In *E. coli*, biofilm development is governed by several factors including Type 1 pilus and flagellar motility. The initial process of attachment is mediated by several adhesins of which Type 1 and Pap Pili play a critical role in colonization in Urinary bladder and Kidneys respectively. We tested the involvement of a model two-component regulatory system, the BarA-UvrY TCS in Uropathogenic *E. coli* in an ascending model of Urinary Tract Infections. The ability to cause urinary tract infections by UPEC relies on its ability to form intrabacterial biofilms in the bladder, in the form of pods in a polysaccharide based matrix. Such intrabacterial communities (IBC) have been demonstrated to express type 1 pilus, Ag43 and polysaccharides. We have seen *uvrY* mutants do not persist very well in bladder or kidneys as compared to the corresponding wild type.

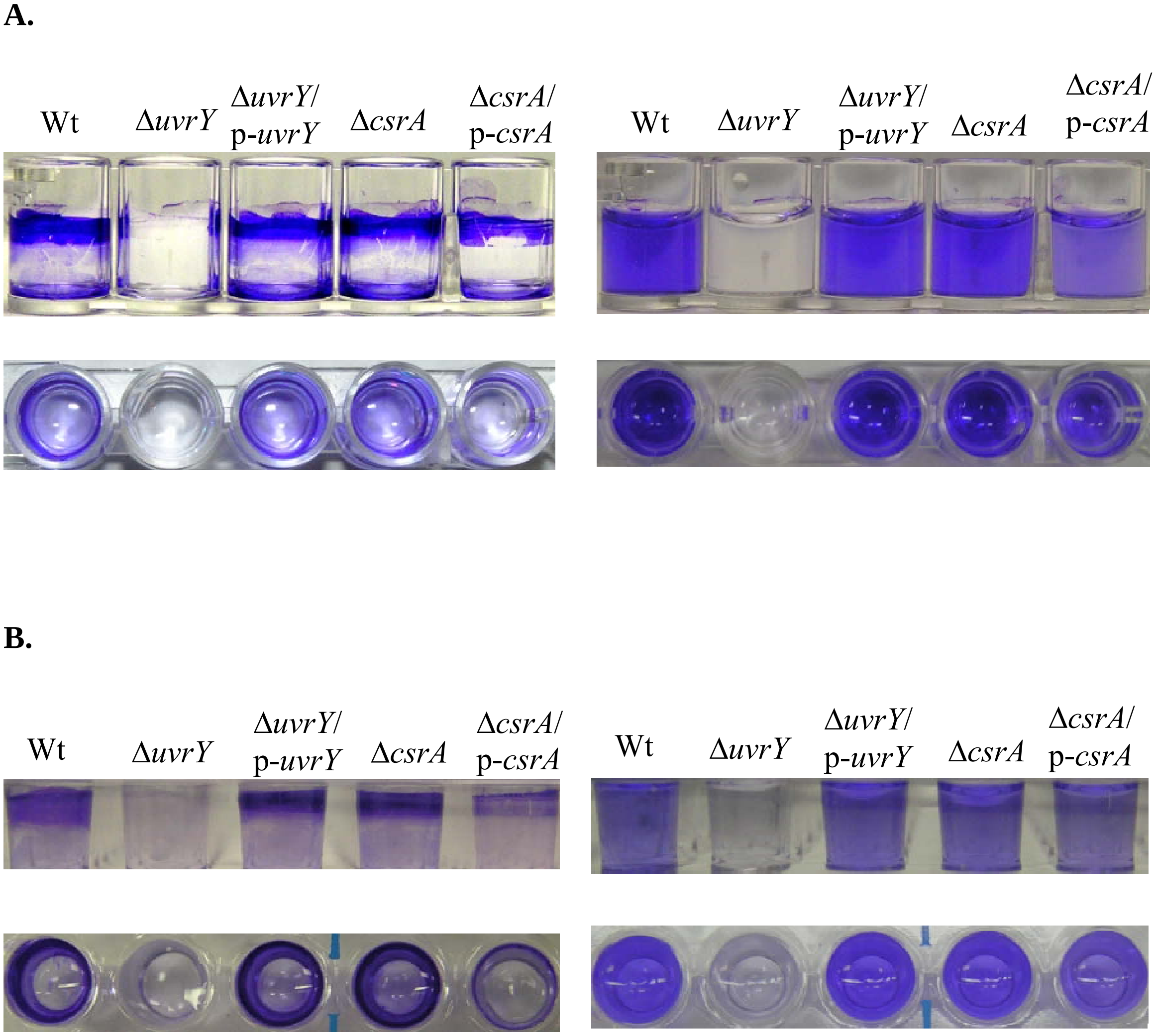
Hence a mutation in *uvrY* might affect persistence in bladder/kidney in several possible ways: Down regulation of pilus, both type 1 and pap pilus would affect adhesion in bladder and kidney respectively and the ability to form biofilms in these organs. Specifically, bladder mucosal cells express Tamm Horsefall protein, which interacts with Type 1 pilus and other adhesins of UPEC for internalization. A mutation in *uvrY* predisposes the *fim* switch to OFF phase further indicate that afimbriated bacteria are not able to colonize the bladder as well as the fimbriated wild type.

Initial stages of biofilms for successful colonization in the bladder could thus be prevented. Secondly, a mutation in *uvrY* in UPEC would result in hypersensitivity to hydrogen peroxide. Even biofilms that are formed by an *uvrY* mutant might be subsequently cleared due to the oxidative burst by the PMN and subsequent phagocytosis. Wild type biofilms (IBC) in contrast would be difficult to penetrate due to polysaccharide based matrix and protective uroplakin. Thirdly, Quorum Sensing might be inhibited which would block cooperation, coordination and appropriate gene expression among members of the biofilm community (unpublished results). This would result in alteration of biofilm phenotype, if not a weaker biofilm. Interestingly, biofilms formed by QS mutant display a greater sensitivity to hydrogen peroxide in *Pseudomonas* spp. Finally, flagellar motility might also play a key role in ascending model of UTI, even though flagellar motility may not be absolutely critical for virulence. In fact, studies have shown down regulation of flagella in UPEC during infection, most likely to avoid triggering of TLR-5 type mediated innate immune responses resulting in IL-8 production. However, transient expression of flagellar motility is thought to be important for initial colonization of UPEC in urinary tract. Co-challenge experiments with wild type UPEC and flagellar mutants have demonstrated that flagellar motility is important for colonization against a strain which lacks such traits and thereby contribute to fitness of UPEC. Thus, mutation in *uvrY* might affect stages in colonization/intracellular biofilm formation (IBC)/fitness/persistence of UPEC in the urinary tract.

On the other hand, Hospital acquired UTI are widespread due to the ability of bacteria to adhere and form biofilms on the abiotic surface of indwelling medical devices such as catheters, renal dialysis shunts and prosthetic valves. A significant proportion of UTI (more than 90%) under clinical settings is catheter related and designated as "Catheter-Associated Urinary Tract Infection" (CAUTI). CAUTI have been reported to increase mortality and correlated with increased mortality in immunocompromised, debilitated and diabetic patients. With that in mind, in *vitro* test for biofilm formation with UPEC strains on abiotic surfaces such as glass and PVC were performed.

Figures and Tables

Figure 13. Effect of mutation of *uvrY* in biofilm formation on abiotic surfaces. Figures A and B represent biofilm formation as detected by crystal violet staining after 24 hours in glass and PVC microtiter plates respectively. Figure C shows biofilm biomass production over a period of 48hours.



C.

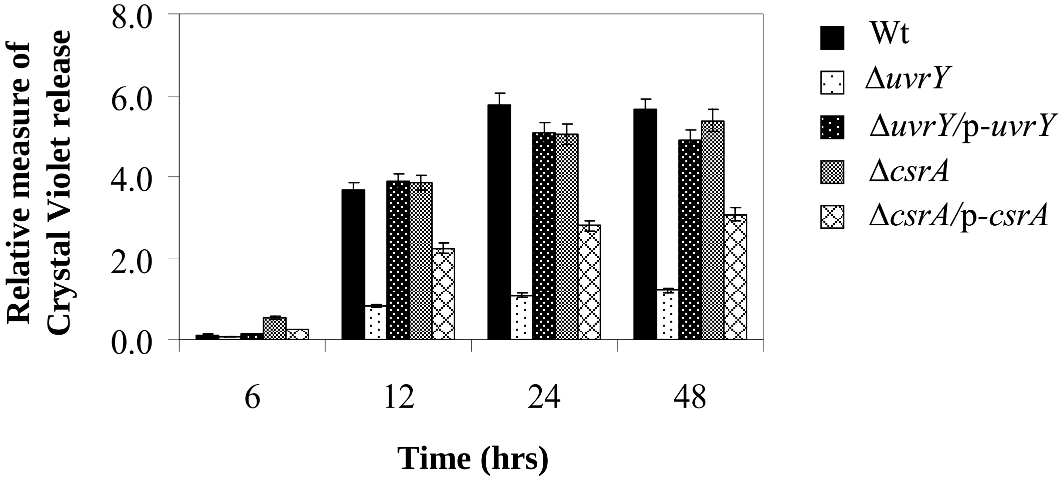


Figure 14. Mutation in *uvrY* reduced expression of *fimA* and *papA*, major fimbrial subunit of type 1 and pap pilus respectively. Semi-quantitative RT-PCR exhibiting reduced expression of *fimA* and *papA* upon loss of *uvrY*. The expression could be restored upon complementation.

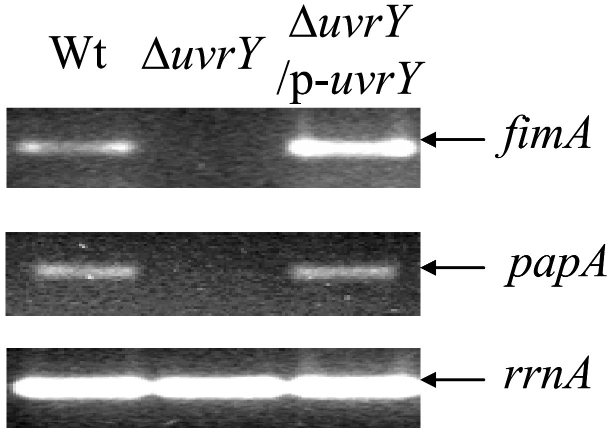
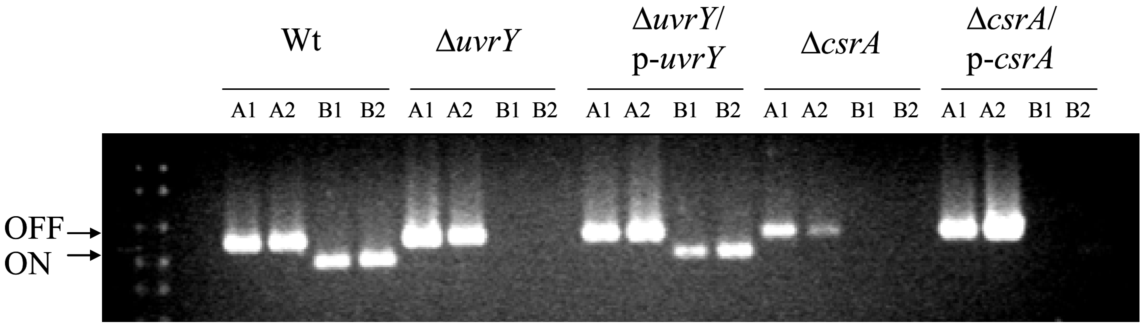
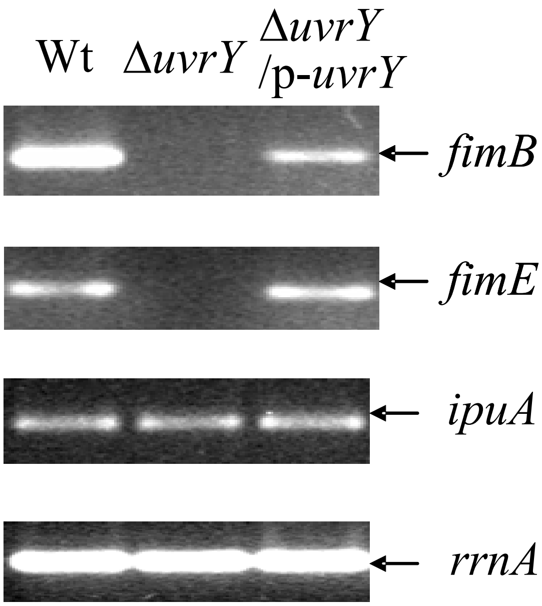


Figure 15. Mutation of *uvrY* affects fimbrial switch orientation and expression.

A. Inverse PCR to determine orientation of the "*fim switch*". Two independent genomic DNA isolates from each strain is used for amplification reaction for determining switch orientation.



B. RT-PCR of the recombinase *fimB*, *fimE* and *ipuA* demonstrating that while the expressions of *fimB* and *fimE* recombinases were downregulated *ipuA* doesn't have much change in expression on mutation of *uvrY*.



C. CsrA stabilizes *fimA* transcript. Mutation in *csrA* decreases *fimA* mRNA half life by 5 minutes. Rifampicin was added when CsrA was optimally expressed.

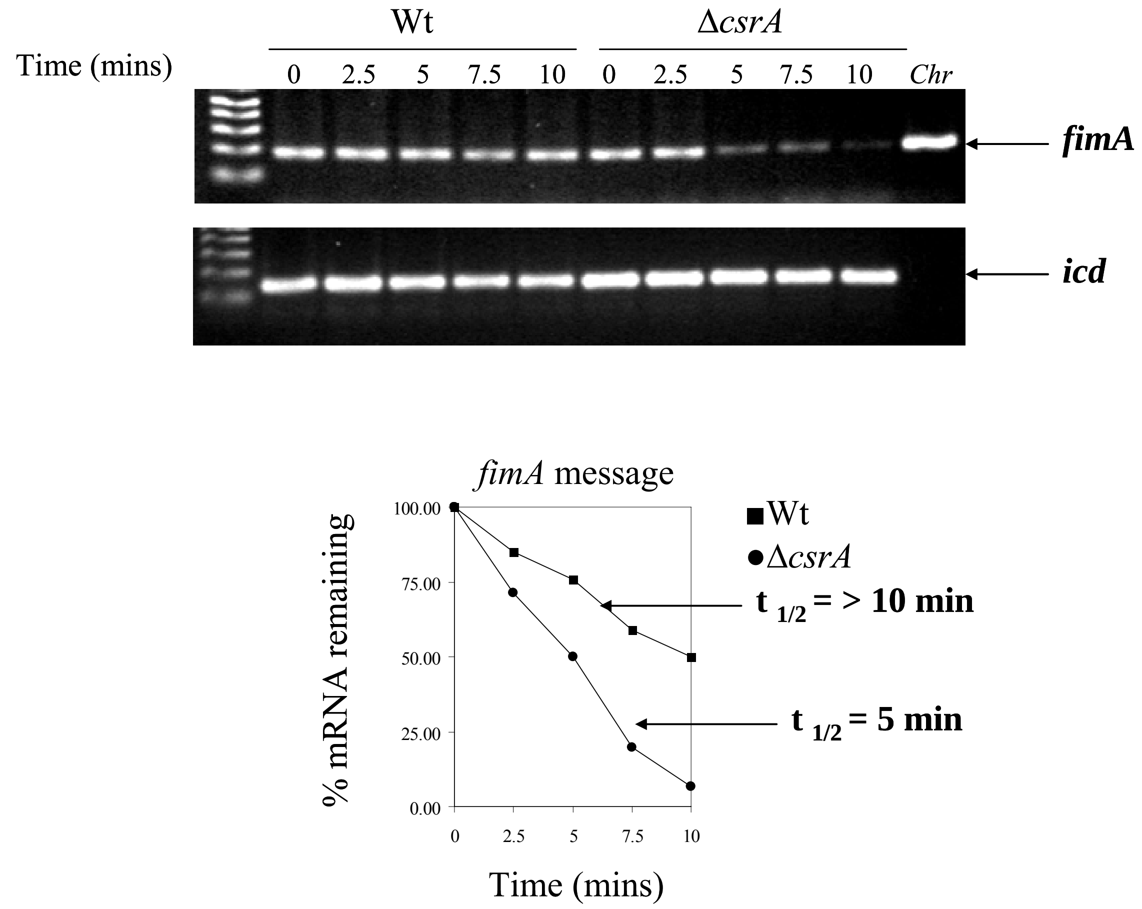
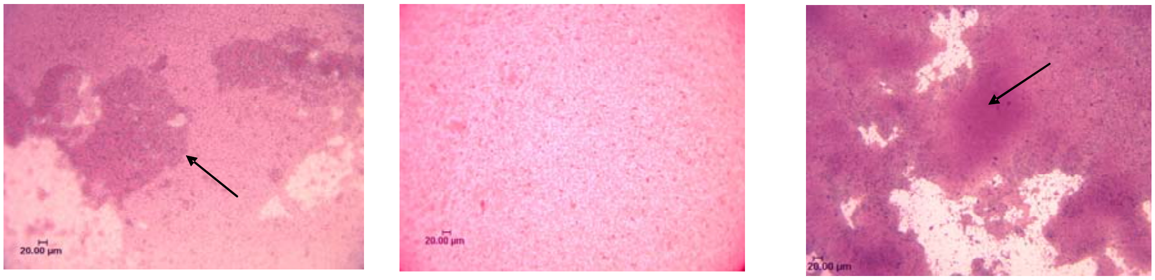


Figure 16. Mutation of *uvrY* reduces acidic exopolysaccharide accumulation. The arrow head indicate the accrual of acidic exopolysaccharide.

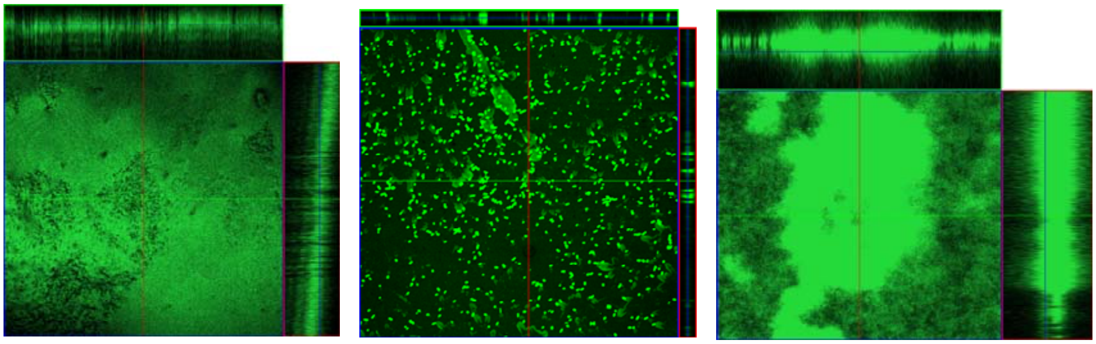


Wt

ΔuvrY

ΔuvrY/
p-*uvrY*

Figure 17. Confocal images exhibit reduced biofilm thickness in *uvrY* mutant. Mutation of *uvrY* leads to a significant reduction in biofilm thickness which could be restored upon complementation. The experiment is done in triplicates for each strain.



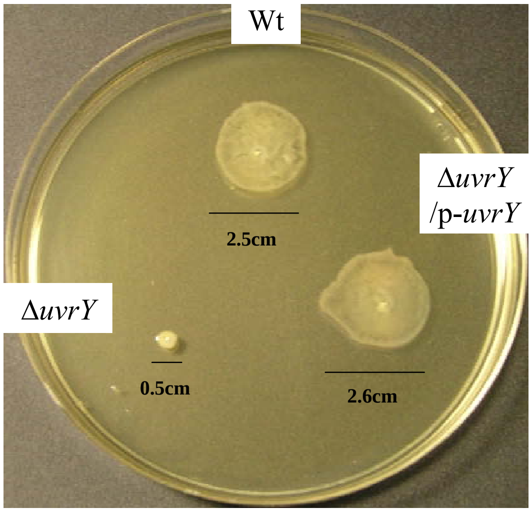
Wt
(63.0±1.5μm)

ΔuvrY
(4.0 ± 1.5 μm)

ΔuvrY/p-*uvrY*
(75.0 ± 2.5 μm)

Figure 18. Effect of mutation of *uvrY* on swarming motility.

A. Impairment of swarming motility upon loss of *uvrY* in CFT073. Swarming attributes were restored on complementation.



B. Semi-quantitative and real time RT-PCR demonstrating reduction in *flhD* expression upon mutation of *uvrY* in CFT073. *flhC* also displayed a similar change in gene expression (not shown)

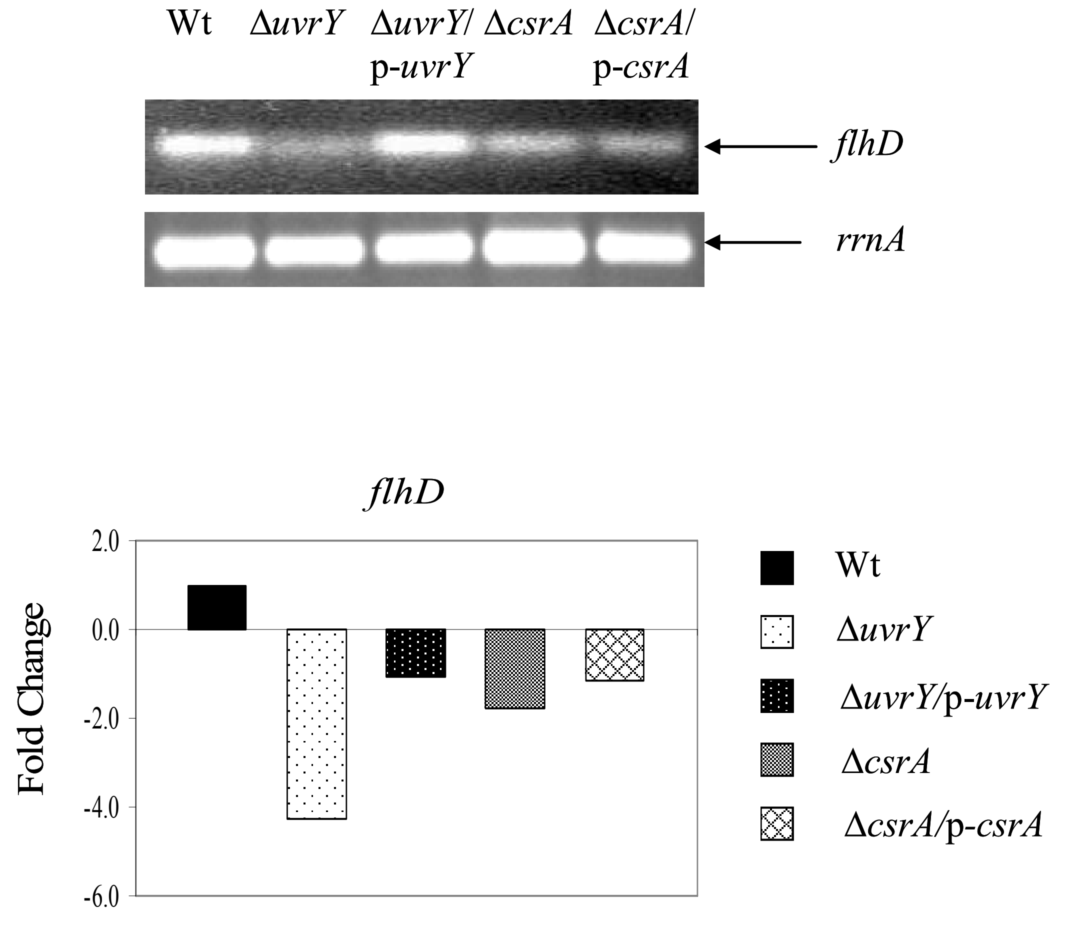


Figure 19. Mutation of *uvrY* reduces colonization in an ascending model of UTI. Mutation in *uvrY* displays poor colonization in an ascending model of Urinary Tract Infection. The open symbol represents the wild type whereas the filled symbols indicate the *uvrY* mutant.

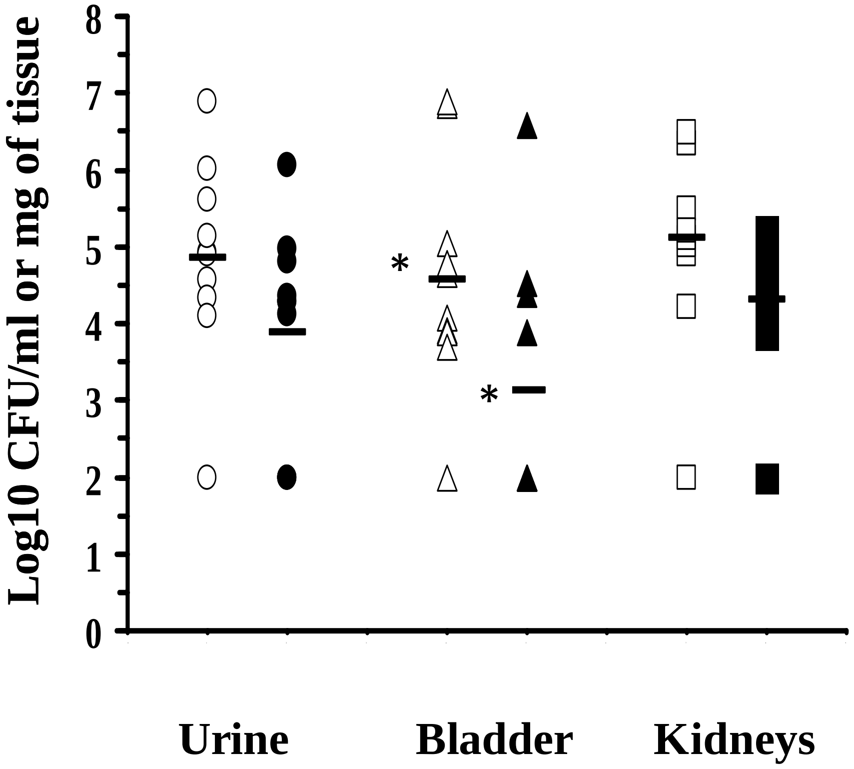


Table 3. Mutation in the BarA/UvrY TCS in APEC strain χ7122 leads to lower pilus expression, exopolysaccharide production, and increased susceptibility to oxidative stress. For real-time RT-PCR, Threshold cycle (C_T) values were determined for various amplification products. The ΔC_T values between samples were normalized to those for the *rrnA* product, ΔC_T = (C_T of mutant _ C_T of *rrnA*) _ (C_T of wild type _ C_T of *rrnA*) and fold difference in the initial concentration of each transcript is determined as $2^{-\Delta\Delta CT}$. The values are the means with standard deviations of the mean for two independent experiments in triplicates. The wild type was assigned a value of 1.0. The downward arrow indicates down regulation compared to the wild type.
The hydrogen peroxide sensitivity was measured by putting a sterile filter paper disc soaked with 1% hydrogen peroxide on top of freshly overlaid bacteria (5 $\log_{10}$ CFU bacteria) in soft agar. The results are mean diameters of inhibition after 18 h of incubation at 37°C with standard deviations of the means.
For EPS determination, bacteria were grown on LB agar overnight at 37°C, harvested by scraping, and resuspended in 2.5 ml of PBS. The cell number was determined from the turbidity at 600 nm. EPS was separated from the bacteria by vortexing each sample for 10 min, followed by ultracentrifugation of the bacterial suspension at 160,000 g for 60 min at 4°C. The supernatant was removed and dialyzed in double-distilled water for 3 h in a membrane with a 6-kDa cutoff. Uronic acids are common constituents of bacterial EPS. Uronic acid produced by various bacterial strains was determined by a colorimetric method, using pure uronic acid as a standard, and expressed as units per milligram of protein

Relevant Genotype	Fold change in mRNA levels detected by quantitative RT-PCR ($2^{-\Delta\Delta Ct}$ values)		Hydrogen peroxide sensitivity	Exopolysaccharide (uronic acid)
	fimA	*papA*	zone of inhibition (mm)	(units/ mg protein)
Wt	1.0	1.0	32.3 ± 0.2	2.0 ± 0.5
Δ*barA*	1.8 ± 0.2 (↓)	3.1 ± 0.2 (↓)	35.3 ± 0.3	0.9 ± 0.3
Δ*uvrY*	1.8 ± 0.3 (↓)	3.2 ± 0.5 (↓)	42.0 ± 0.4	0.8 ± 0.3

Table 4. Mutation in *barA* and *uvrY* exhibits a reduction in mannose resistant hemagglutination to chicken erythrocytes. The values are mean log2 of inverse dilution at which hemagglutination (HA) was observed with chicken blood. The standard deviation was <0.05 in all cases. The bacterial cultures were grown with two passages of 48 h each in static LB broth with appropriate antibiotics at 37oC to maximize type 1 fimbria expression. The assay was done on ice in duplicate in 96-well microtiter plates. Each bacterial culture was diluted twofold, before blood was added to study agglutination. The experiment was repeated twice with essentially similar results. The highest reciprocal of the dilution at which 50% of the erythrocytes sedimented to the bottom of the plate is taken as the HA titer.

Strain	Genotype	Hemagglutination of 1% chicken erythrocyte $\log_2$ [1/dilution] (50 mM mannose*)*	
		--	+
χ7122	*Wt*	4	4
AAEC072/ p-Type-1	Δ fim/ p-*fimA-H* operon	1	<1
AAEC072/ p-Pap G1	Δ fim/ p-*pap* operon	5	<1
AAEC072/ pSM1	Δ fim/ */p-barA*	4	4
AAEC072/ pSM2	Δ fim/ *p-uvrY*	4	2
SM3000	*barA-*	5	5
SM3001	*uvrY-*	1	1
SM3002	*barA*$^-$ *uvrY*$^-$	1	<1
SM3003	*barA*$^-$ /p-*barA*	3	2
SM3004	*uvrY*$^-$ /p-*uvrY*	4	3

Chapter V: *Escherichia coli* stress response as a tool for detection of toxicity

Introduction

The advent of microarray has opened new avenues for toxicologists to collect and interpret data [212-215]. It usually involves a comparison of global gene expression between normal and drug treated cells under *in vitro* conditions. The incorporation of genomics, bioinformatics and large-scale sequencing information have resulted in the construction of gene chips, which enable speedy screening of new targets for important cellular processes including toxicity. The emerging branch of toxicogenomics integrates application of functional genomics technologies and offers several advantages to that of conventional toxicology in terms of cost and time effectiveness, sensitivity and enhanced correlation between experimental models and human. Potential applications of this discipline are mechanistic insight of metabolic or biological pathways leading to toxicity, specially metabolic processes (at the level of transcription) affected by chemical, environmental or xenobiotic treatments, screening of probable drug candidates, facilitating the prediction of toxicity of unknown compounds, and improving interspecies and *in vitro-in vivo* extrapolations [216, 217].

The evolution of toxicogenomics has matured over the years with several series of developments in toxicological sciences. Previously, animal toxicity was assessed by traditional methods such as tissue pathology, system-level toxicity and overall mortality. However, animal bioassay was often lengthy, labor-intensive, expensive and limited in information [218-220]. Screening of more than fifty-thousand known chemicals for toxicity would be unfeasible using conventional methods; hence, newer alternative strategies are needed.

In recent times, the focus has shifted towards understanding toxicity at the molecular level. In the last thirty years, evaluation of toxicity underwent a remarkable transformation from assessing a single molecule change to the effect on entire genome. Genomic information plays a key role in understanding of the molecular attributes of toxicity, for example, the genetic background of an individual could influence metabolism, absorption, excretion or susceptibility of a metabolite or a chemical entity. The integration of genomics into the field of toxicological research will significantly advance our knowledge of molecular toxicity and key regulatory pathways that affect such processes (Fig. 1). Potential usefulness of genomics could be immensely important and often involves approaches that utilize candidate targets which are affected by environmental stimulants. Conversely, meticulous approach must be followed while analyzing genomic data and experimentation for validation must be integrated within such studies [221, 222].

Escherichia coli, a type bacterial type species of the family *Enterobacteriaceae*, is naturally distributed in the intestinal microbial flora of homeothermic animals including birds and humans [223]. Strains of *E. coli* are broadly categorized in three groups: commensals, intestinal pathogenic and extraintestinal pathogenic. The recently-added third group, termed as extraintestinal pathogenic *E. coli* (ExPEC), has been formed based on the presence of specific virulence factors and the ability to cause organ infection outside the intestine [18, 26, 27]. Typically, ExPEC characteristic virulence factors aid in invasion and colonization of the microbe which lead to infection in extraintestinal sites. Some ExPEC-specific virulence factors include adhesins (e.g., Type 1 fimbriae or P fimbriae), factors that evade defense mechanisms (e.g., capsules, lipopolysaccharides), toxins (e.g., hemolysins), and factors to acquire nutrient availability (e.g., siderophores) [28].

ExPECs are a growing concern, as evidenced by being causative agents of a plethora of diseases, including urinary tract infections (UTI), neonatal meningitis, pneumonia, septicemia, osteomyelitis and other extraintestinal infections [224-226]. Among the ExPEC, uropathogenic *E. coli* (UPEC) and avian pathogenic *E. coli* (APEC) cause significant morbidity and/or mortality in humans and poultry respectively. UPEC is the leading cause of urinary tract infections in the United States. Every year in the United States, UPEC associated-UTI results in 6-8 billion cases of uncomplicated cystitis with a healthcare cost of $1 billion, 250,000 cases of uncomplicated pyelonephritis with a direct cost of $175 million, and 250,000 to 525,000 cases of catheter-associated UTI healthcare, the cost of which is $170-350 million dollars [30]. APEC, on the other hand, is the leading cause of avian colibacillosis characterized by air sacculitis, pericarditis, peritonitis, salpingitis, polyserositis, septicemia, synovitis, osteomyelitis and yolk sac infection [227, 228]. In the US, cellulitis caused by APEC is the second leading cause of condemnation of broiler chickens and resulted an estimated loss of $40 million/year [229]. The underlying mechanisms of pathogenesis and toxicity of *Escherichia coli* have become more apparent with the application of genomics, bioinformatics and molecular biology.

Unlike commensals, many pathogenic bacteria were demonstrated to switch between free-living and host-associated states. Apart from extraintestinal sites, EXPECs have been reported to asymptotically colonize in intestinal sites like commensals [226, 230]. In contrast, the intestinal pathogenic strains are not capable of asymptomatic colonization in the intestine. The environments in which EXPECs thrive vary and must endure different stress conditions within the host. Often, the pathogenic bacteria have developed a complex signaling system that turns on specific sets of genes in a given environment and switch off those that are not required in that milieu. Multiple physiochemical cues, such as pH, osmolarity, temperature, and oxygen concentration might affect such change in gene expression. Interestingly, the gene expression pattern might be altered due to the presence of different environmental stimuli including those of various toxic chemicals. Regulatory mechanisms which affect such changes are complex and take place at the levels of transcription and translation. The overall effect of such changes in genome might be envisioned by the incorporation of genomics into this emerging field of toxicology.

Stress Response

The effect of various stress responses on *E. coli* has been studied in greater details [231-237]. Molecular oxygen, for example, plays a crucial role in cellular metabolism; however the effects of reactive oxygen species (ROS), such as superoxide radical, hydrogen peroxide and hydroxyl radical can be deleterious and may even cause apoptosis of the aerobic cells. Various strategies, including enzymatic and non enzymatic defenses have been employed to prevent such damages [238]. Enzymatic defense systems, such as superoxide dismutase, catalase and peroxidase, scavenge superoxide radicals and hydrogen peroxide and convert them into less reactive species. Non-enzymatic antioxidants include Vitamin C and E, glutathione and β-carotene. Usually a balance exists between ROS and antioxidants under normal conditions of the cell. A disruption in this critical balance could lead to oxidative stress either due to excess accumulation of ROS or depletion of antioxidants [239]. These, in turn, either damage cell components or trigger specific cell signaling pathways leading to modulation of various cellular processes, improving the health of the cell or leading to cell death [240].

Release of ROS changes the oxidation reduction potential within the cell, leading to oxidative stress. The generated ROS molecules can carry out nucleophilic attacks on any electron-deficient group including biomolecules such as DNA, protein and lipids leading to the formation of adduct, covalent binding of ROS to macromolecule and disruption of cellular functions. The basic mechanisms to remove ROS involve chemical reactions that generate a non-reactive compound by altering gene expression to activate gene products that are designated to deal with toxic insults and turn off those that are not required. Cellular oxidative stresses are controlled either by direct or indirect alteration of gene expression. Chemicals or ROS may activate intracellular receptors that directly regulate transcription of target genes. Alternatively, ROS may interact with other molecules within the cell, which carries on the signal and elicits coordinated responses to cellular toxicity.

Bacteria have developed adaptive responses while shifting from anaerobic to aerobic growth conditions to counteract reactive oxygen species [241]. Usually, these responses are mediated in a coordinated manner by groups of genes termed regulons, each group under a common regulator. One key system is based on the *oxyR* system which acts in response to hydrogen peroxide and induces at least eight genes to counteract oxidative stress, including *ahpFC* encoding alkyl hydroperoxidase, glutathione reducatase encoded by *gor*, *katG* encoding catalase hydroperoxidase and *dps*, a DNA binding protective protein. OxyR protein is thought to act by binding and stimulating transcription from various promoters upon receiving signals. Many of the OxyR regulon genes are also regulated by the stationery phase starvation response system programmed by *rpoS*, a sigma 38 protein. The stationery phase alternative sigma factor *rpoS* controls the expression of several genes involved in cell survival and is essential for expression of various stress resistances [175, 242, 243]. Under laboratory conditions, *rpoS* mutants are sensitive to oxidative and osmotic stress as well as temperature and acid shift. On the other hand, the SoxRS system induces many genes to combat the superoxide-generating agents and nitric oxide. The SoxRS response is initiated in two stages. Upon activation, the *soxR* sensor molecule induces *soxS* which, in turn, activates the transcription of *soxRS* regulon. *The stationery phase alternative sigma factor* σ^S *is present in many bacterial species belonging to* γ *subdivision of proteobacteria. The regulation of sigmaS is complex and regulated at* *the level of transcription, post transcription and protein stability [244]. In E. coli rpoS transcription is regulated by cAMP-CRP complex as well as by several two-component signaling systems, including the BarA/UvrY system whose role is illustrated* [173, 245-247].

Genomics are increasingly more useful in exploring pathways and mechanisms underlying oxidative stress response. DNA microarrays have been used to characterize genes involved in oxidative stress responses. Interestingly, the patterns of gene expression altered in mammary cells in the presence of hydrogen peroxide, menedions, and t-butyl hydroperoxide were found to be quite similar regardless of the ROS source [248]. Another study showed that the effect of DMNQ, 2, 3-dimethoxy-1,4-naphthoquinone, a ROS-generating chemical, in HepG2 cells was comparable to that of heavy metal toxicity [249]. Such studies have substantiated the notion that different stimuli can lead to generation of ROS and oxidative stress (Fig. 2.). Hence, production of ROS and oxidative stress might be considered as a general stress response.

Two-component as signal transducers

Wide range of toxic insults often alters gene expression profiles in microbes specific to the nature of chemicals tested. Adaptation to toxic compounds by bacterial species often enables that species to better cope in that environment. This response, appropriately called adaptive response, refers to the ability of bacteria to withstand harmful-damaging effects of the given stress provided if it is previously exposed to the similar stress environment at a lower dose. Several types of agents induce an adaptive response, including alkylating agents, heat stress, oxidative stress and radiation among others. Adaptive response usually involves modulation of a plethora of genes in a coordinated manner. In bacteria, adaptation to a new environment largely relies on a signal transduction system called the two-component system. There is no common pathway for adaptation; however there exists quite a few common themes. In *E. coli* adaptation to a new environment often involves use of several two-component systems that plays a crucial role for survival in an ever-changing environment. Two-component systems (TCS), comprises of a membrane-bound sensor histidine kinase (HPK) and a cognate response regulator (RR). The sensor kinase undergoes autophosphorylation at a conserved histidine residue upon reception of an appropriate environmental signal, and subsequently, this phosphate group is transferred to a conserved aspartate residue on the cognate response regulator. Upon phosphorylation, the response regulatory protein undergoes structural modification and acts as a gene transcription factor and often regulates gene expression or cellular responses, enabling the organism better adapt in new environment [1, 2, 250]. Approximately 60 such TCS are present in *E. coli* and have been shown to be involved in adaptation, including intracellular metabolism, biofilm formation, global stress response and virulence. One such system is the BarA-UvrY TCS involved in various physiological functions including oxidative stress, sigmaS expression, biofilm formation and carbon metabolism.

The BarA (Bacterial Adaptive Response Gene A) sensor kinase was first identified for its ability to suppress a deletion *envZ* mutant by controlling expression of outer membrane proteins [166, 251]. BarA is a member of tripartite sensor kinase having three domains: an N-terminal transmitter domain with a conserved histidine residue (H1), a central receiver domain with a conserved aspartate residue (D1) and a C-terminal transmitter domain with a conserved histidine residue H2, also called Hpt domain. Triggering of this system seems to be mediated in an ATP- dependent manner via His-Asp-His-Asp phosphorelay cascade. UvrY is a member of the FixJ family and has been recently shown to be a cognate regulator of the sensor kinase, BarA [176]. It has an N terminal phosphoacceptor domain with a conserved aspartic acid residue at position 54, followed by a LuxR type helix-turn-helix DNA binding domain in the C-terminal region. It also has a close linkage with *uvrC*, a bicistronic mRNA, even though *uvrY* has no known role in DNA repair system. Apparently this system seems to be induced in response to a pH change.

The BarA/UvrY system plays a crucial role in carbon metabolism and biofilm formation. This TCS has also been implicated in hydrogen peroxide resistance. Both the *barA* and *uvrY* mutants were hypersensitive to hydrogen peroxide. It has been reported that the expression of the sensor kinase, *barA*, could be induced in the presence of weak acids, possibly indicating the significance of this TCS in survival of acid onslaught in stomach and inside macrophages. Additionally, this TCS could be induced in the presence of food preservatives such as benzoate or bile salts, implying the importance of this TCS in adaptation to various stress responses and persistence.

Bacterial Biosensors as a tool for detection of toxicity

Presence of environmental stimulants or toxic chemicals often elicits variety of stress responses in bacteria. Compounds demonstrating similar toxicities would ideally induce a specific pattern in gene expression. It is hypothesized that compounds that exhibit similar changes in gene expression might have similar mechanisms of action or act in similar biological processes or pathways. Thus, toxicity-induced alteration of gene expression might be used as a signature for classification and characterization of unknown chemicals. Genomic insults due to toxin-induced stimulation induce several stress responses, with alteration in gene expression that are often associated with diverse biological pathways. Once within the host, pathogenic bacteria often deal with diverse stress responses such as pH, nutrient deprivation, high osmolarity and oxidative stress. Inflammatory cells or phagocytes possess enzymes that are capable of generating ROS in response to invasion of pathogens. However, excess production of ROS also might affect the phagocytes and the surrounding tissue. Chronic renal scarring in pyelonephritis has been directly correlated with phagocytic oxidative damage. Hence, virulence genes involved in colonization or survival inside the host often have common genes that are affected by stress responses. Such genes have often been used as a sensor for detection and quantization of toxic chemicals in the environment. These sensors have the potential to be a warning system for toxicity detection and thereby reduce harmful effect on the environment.

Whole-cell bacterial biosensors detect gene products of reporter genes that are either naturally present or artificially introduced into the relevant bacterial strain.

Commonly used reporter genes include *lacZ* encoding β-galactosidase *(E. coli), lux* encoding bacterial luciferase, *luc* encoding firefly luciferase and *gfp* encoding green fluorescent protein. In the case of general biosensors, the reporter gene is placed downstream to a constitutively expressed promoter, and a decrease in intensity of signal indicates a decrease in metabolic activity. On the other hand, semi-specific biosensors involve placing a reporter gene downstream to a stress-responsive promoter and an increase in reporter activity indicates an increase in stress (e.g., SOS or heat shock response). Furthermore, specific biosensors incorporate a reporter gene being placed downstream to a regulated promoter or regulatory protein, either activator or repressor. Even though general biosensors are most popular due to their simplicity, they are non-specific and could lead to false-positives. In contrast, stress responsive biosensors offer several advantages over that of general biosensors. As different stimulants often lead to common stress response, such sensors can be good indicators of toxicity and stress inducing conditions such as DNA and protein damage, oxidative stress and membrane damage. Their simplicity, selectivity and sensitivity have made them extremely useful and popular. Specificity of such sensors might be increased by incorporating several different types of semi-specific biosensors to determine type and variety of toxicity. The stress promoter-reporter could be present in separate strains, or two reporters could be incorporated in the same strain. Identification of such stress-related genes for such sensors involves scanning through the transcription profile of the genome. Numerous stress gene promoter including *sulA*, *katG, recA* and *uvrA*, have been fused with a reporter to construct biosensors for detection of compounds that cause DNA damage [252, 253].

Panels of stress-responsive biosensors are also on the rise. Oxidative stress sensitive cell array chip have been employed for identification of putative targets in the entire genome [254]. Sensitivity of such sensors could be significantly improved by fine-tuning the promoter and modification of host strains. Challenges for improvement of such sensors would encompass identification of strong promoters that are sensitive to a given stimuli, knowledge of gene regulatory network, designing of instruments that are easy to use and inexpensive, refinement of older reporters and creation of new reporter genes [255-258].

Global gene expression profiling of the BarA/UvrY TCS

To further identify downstream targets and pathways that are affected by the BarA/UvrY two-component system, we have begin to study the effect of mutation of either *barA* or *uvrY* and compare it with a wild-type or a mutant expressing the UvrY protein from a low copy plasmid-borne vector p-*uvrY* in UPEC CFT073. At first, the raw digitalized intensity of Affymetrix single-color slides was internally normalized using Microarray Suite version 5 (MAS 5.0, Affymetrix). The universally 'absent' genes from the normalized data were then eliminated. The noise generated due to chip-chip non-biological variance was minimized through interchip-LOWESS normalization between the wild-type and individually treated samples using GeneSpring v6 (Agilent, Inc., CA). The resultant genomic regulation was determined as the ratio of the individual gene intensity of treated samples to that of the control samples. The normalized genes of the treated ensemble showing at least 1.15 fold difference (up or down regulation) from that of wild-type were accepted for the remaining analysis. Approximately 1400 genes from the selected genome showed a similar regulatory trend between *uvrY* and *barA* strain, of which around 570 genes were from CFT segment and about 200 genes were from intergenic region. Similar analysis identified roughly 900 genes, including ~270 and ~100 entries from CFT and intergenic segment respectively that are expressed oppositely between *uvrY* and p-*uvrY* strains. Apparently, about 170 regulated genes according to the aforesaid null hypothesis showed similar regulation between *barA* and *uvrY* strain while simultaneously exhibited reverse regulation between *uvrY* and p-*uvrY* strain. This last genome contained ~50 CFT genes and ~20 intergenic entries. Unsupervised heretical clustering was performed for each of the three genomes independently using a standard correlation algorithm. To conclude, the biological, molecular and cellular functions of each gene, part of the abovementioned three genomes, were mined using NetAffix GeneOntology (GO) analysis tool (Affymetrix, Inc., CA), and the genome was segmented according to their primary functions.

Several groups of genes have been annotated based on their function. Genes involved in metabolism, biosynthesis, cell adhesion, transcription and translation, catalysis, membrane and many genes of unknown functions were significantly affected by the mutation. Representative genes that are affected at least two-fold by the mutation were reported (Table 1). This TCS, by virtue of its role in virulence, stress response, carbon regulation, and other key regulatory pathways in *E. coli,* could be a potential target for toxicity detection studies in the future.

Conclusion

Toxicogenomics now evolves into a multi-disciplinary field by integrating several branches of biology including toxicology, genetics, molecular biology, bioinformatics, functional genomics, transcript profiling, proteomics, metabolomics and pharmacogenomics. With ongoing whole genome sequencing efforts, the potential for identifying candidates for toxicity testing or pathways has been significantly accelerated using available high throughput and inexpensive molecular genetic tools. An important strategy towards identification of novel toxic chemicals involves employing potential targets that are susceptible to various stresses in the presence of deleterious compounds. Genomics enable pinpointing such potential candidates by scanning through an entire genome in a high throughput fashion. Identification, validation and categorical classification of such targets will enhance future toxicity detection studies.

Figures and Tables

Figure 20. Principle of evaluation of toxicity in toxicogenomics.

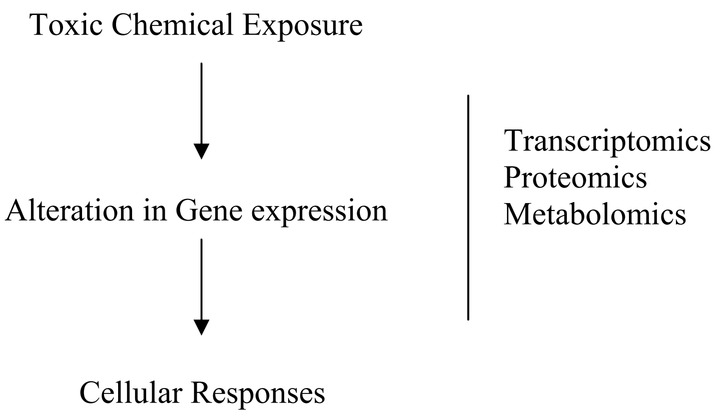


Figure 21. Changes in gene expression profile on increased reactive oxygen species levels.

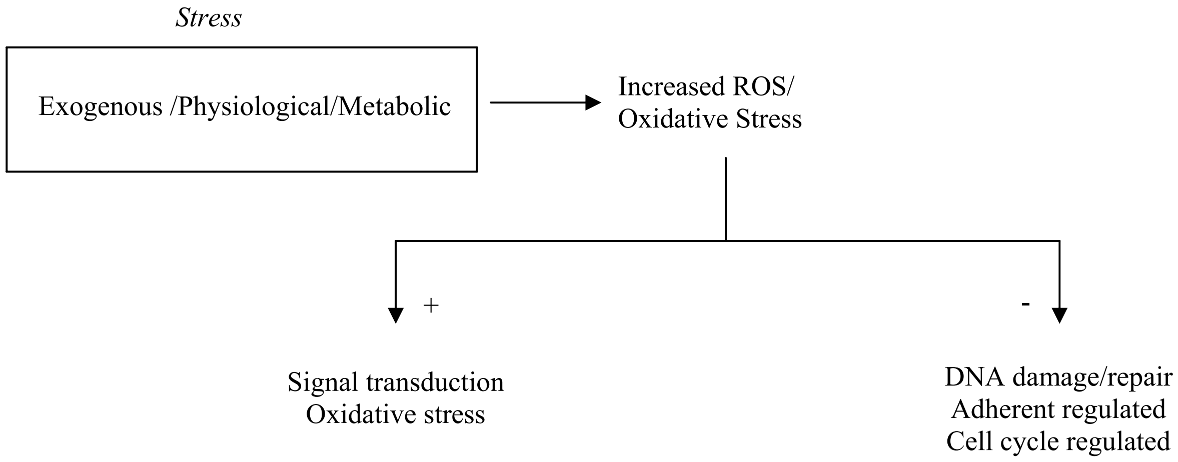


Table 5. Microarray analysis of BarA/UvrY TCS in Uropathogenic *Escherichia coli*.

Gene Name or ID	**Category or Function**	**Fold Induction**	**Description**
rfaJ	Biosynthesis	2.2	Lipopolysaacharide 1, 2-glycosyltransferase
serB		2.1	Phosphoserine phosphotase
hemA		2.2	Glutamyl tRNA reducatse
hisB		2.5	Histidine biosynthesis bifunctional protein
aroC		2.4	Chorismate synthase
dsdA	Metabolism	2.3	D-serine dehydratase
bglA		2.5	6-phospho-beta-glucosidase
ldcC		2.6	Lysine decarboxylase
c5039		2.4	Putative lactate dehydrogenase
ucpA		2.2	Oxidoreductase
kpsT	Transport	2.4	ATP binding transporter
kpsM		2.1	ATP binding
sitC		2.5	ABC transporters
iroN		2.4	Siderophore receptor
papC		2.5	Fimbrial usher protein
malK		2.2	Maltose transporter
focG	Adhesion	2.5	F1C minor fimbrial subunit protein
c4209		2.3	Putative minor fimbrial subunit precursor
c4214		2.7	Putative major fimbrial subunit precursor
csgA		2.1	Major curli subunit precursor
papH		2.5	Fimbrial protein
papI	Transcription	2.2	Fimbrial protein transcriptional regulators
flhC		2.6	Flagellar transcriptional activator
ymfL		2.4	Hypothetical protein
c2411		2.1	DNA-binding protein H-NS
pcnB		2.2	Poly (A) Polymerase
yhiH		2.3	Hypothetical ABC transporter
fimB	Binding	2.7	Type 1 fimbriae regulatory protein
zntA		2.4	Lead, Cadmium, Zinc transporting ATPase
dppD		3.1	Dipeptide transport ATP binding protein
rseB		2.4	Sigma E factor regulatory protein
c0934		2.7	Hypothetical Protein
dsdA	Catalysis	2.3	D-serine dehydrates
nrdD		2.1	Anerobic ribonucleoside triphosphate reductase
trpB		2.5	Trytophan synthase beta chain
agp		2.4	Glucose 1-phosphatase precursor
ydjQ		2.7	Hypothetical protein
mtr	Membrane	2.4	Tryptophan specific transport protein
ompC		3.1	Outer membrane protein C precursor
ompA		2.4	Outer membrane protein A precursor
pitB		2.3	Probable low affinity inorganic phosphate transfer
yjaN	Unknown	2.6	Hypothetical Protein
yfgJ		2.4	Hypothetical Protein
ycjX		2.7	Hypothetical Protein

Chapter VI: Conclusions and Future Directions

The BarA/UvrY/Csr system and its homologues are present in many γ-division of proteobacteria. The BarA/UvrY/Csr system regulates diverse physiological processes in adaptation of *Escherichia coli*. This work demonstrates two population-dependent physiological processes affected by this signaling cascade, namely a process of cell-cell communication termed quorum sensing which employs small molecules called autoinducers and the cooperative ability of bacterial species to form biofilms. The study here demonstrates the role of BarA-UvrY TCS in regulation of adhesion mediated biofilm formation and cell-cell communication formation in *Escherichia coli*. The fine control of processes affecting Quorum sensing, Biofilm formation and Stress responses all of which require careful coordination and environmental adaptation serves as an important strategy for survival of bacteria in a varying milieu. The regulation of such processes at the level of transcription and post transcription by the signaling cascade suggest tighter control and coordination needed for efficient bacterial adaptation in a changing environment.

Quorum sensing regulates diverse physiological processes like biofilm formation, antibiotic production, and virulence in many gram negative bacterial species. Quorum sensing involves population dependent control of gene expression by the utilization of autoinducers. In *E. coli*, the autoinducer AI-2 is synthesized as a by product of methyl-cycle. The specific reaction involves LuxS, which breaks down S-ribosyl homocysteine to homocysteine, while generating the DPD. DPD undergoes spontaneous cyclization to form AI-2. LuxS is present in diverse bacterial species and is thought to be involved in inter-species quorum sensing. Mutation in *barA* or *uvrY* displayed reduced expression of *luxS* and AI-2 levels while mutation in *csrA* displayed an opposite effect, both at the entry into stationery phase. Transcript stability, computational prediction of *luxS* leader region, and direct regulatory interactions suggest that CsrA play a major role in regulation of *luxS*. CsrA most likely bind to the predicted GGA- conserved stem loop region of luxS leader and inhibits translation initiation. The known AI-2 uptake system, Lsr transporter, also displayed an interesting observation in *E. coli.* While a loss of *barA* or *uvrY* genes displayed an increase in expression of Lsr transporter, loss of *csrA* on the other hand displayed an opposite effect. This suggests a potential balance of carbon flow, as AI-2 is a 5-carbon moiety, at the entry of sationery phase indicating that CsrA while repressing *luxS* expression it's reducing the synthesis of AI-2, while utilizing the furanone (AI-2) by upregulating the lsr transporter. Thus saving on energy utilization for synthesis of carbon at the onset of stationery phase seems to be the basis for regulation of luxS by the BarA/UvrY/Csr system. The involvement of small RNA in this regulation is also likely. Interestingly, CsrA alongwith three small RNA also regulate Quorum sensing in *Vibrio cholerae*.

Earlier studies have shown that mutation in *barA* or *uvrY* in Avian Pathogenic *E. coli* reduced expression of virulence in chicken embryo model and also demonstrate poor attachment in chicken fibroblasts and macrophages. Downregulation of Type1 and Pap pilus and reduced exopolysaccharide accumulation was attributed for poor colonization and reduced virulence. In Uropathogenic *Escherichia coli*, the ability to form biofilms in *invitro* on abiotic surface such as catheters contributes greatly towards persistent UTI. *In vivo*, UPEC colonize bladder and kidneys by Type 1 and Pap pilus respectively. The ability to form intrabacterial biofilm like pods also adds to the ability of the UPEC to persist in harsh conditions of the host. The BarA/UvrY/Csr pathway also displayed a dual control at the level of transcription and post-transcription for biofilm formation in UPEC. Both type 1 and pap pilus displayed reduced expression upon mutation of *uvrY.* Both the recombinase *fimB* and *fimE* expression which controls the fim promoter switch was also downregulated. Additionally *uvrY* also displayed an ability to turn fim switch ON, but not *csrA*. This suggests that even though the BarA/UvrY TCS have a known downstream effect via CsrA, there also seems to be direct regulatory role in biofilm formation via regulation of pilus in UPEC. Mutation of *uvrY* also displayed reduced exopolysaccharide accumulation and showed a swarm defective phenotype, both of which contribute to biofilm development. Finally, *uvrY* mutants also demonstrated poor colonization in bladder, kidneys and urine in an ascending model UTI. These suggest that *uvrY* might play a crucial role in adaptation, colonization and virulence in UPEC.

Two-component regulatory systems have been utilized as a novel therapeutic strategy particularly those systems involved with virulence. ExPECs cause significant economic loss in poultry and humans. A vaccine towards ExPEC could help in reducing that financial burden. This work demonstrates that phosphorelay signaling cascade through the BarA/UvrY two-component system is critical for adhesion, colonization and population dependent behavior namely quorum sensing. These social behaviors in microbes, particularly for processes affecting adaptation, may be targeted for potential novel therapeutic strategies and this becomes relevant in recent years when antibiotic resistance is increasingly prevalent. Targeting such pathways, could offer a fresh approach for therapeutic strategy.

Table 6. List of bacterial strains and plasmids used in the study.

Bacterial Strains	Relevant Genotype	Reference or source
Bacterial Strains		
MG1655dlac	Wt K-12 *λ- rph*-1 *Δlac*	D. J. Jin
SM1005	MG1655Δlac *luxS::lacZ*	Lab Stock
SM1006	MG1655Δlac *luxS::lacZ barA::kan*	Lab Stock
SM1007	MG1655Δlac *luxS::lacZ uvrY::cam*	Lab Stock
SM1009	MG1655Δlac *luxS::lacZ barA::kan uvrY::cam*	Lab Stock
SM1010	MG1655Δlac *luxS::lacZ rpoS::Tn10*	Lab Stock
SM1011	MG1655Δlac *luxS::lacZ barA::kan*/p-*barA*	This study
SM1012	MG1655Δlac *luxS::lacZ uvrY::cam*/p-*uvrY*	This study
SM1014	MG1655Δlac *luxS::lacZ rpoS::*Tn*10*/p-*rpoS*	This study
SM1020	MG1655Δlac *luxS::lacZ cya::kan*	This study
SM1021	MG1655Δlac *luxS::lacZ uvrY::cam cya::kan*	This study
SM1030	MG1655Δlac *luxS::lacZ csrA::kan*	This study
SM1031	MG1655Δlac *luxS::lacZ csrA::kan*/p-*csrA*	This study
SM1032	MG1655Δlac *luxS::lacZ csrB::cam*	This study
SM1050	MG1655Δlac *luxS::lacZ hfq::cam*	This study
SM1051	MG1655Δlac *luxS::lacZ hfq::cam*/p-*hfq*	This study
SM1052	DH5α/p-*hfq*	This study

Bacterial Strains	Relevant Genotype	Reference or source
SM1053	TRMG1655 *csrA::kan*/p-*hfq*	This study
SM1060	MG1655Δlac *luxS::lacZ/*p-*sraD*	This study
SM1061	DH5α/ p-*sraD*	This study
AM1001	MG166Δlac *barA::kan*	Lab Collection
AM1002	MG166Δlac *uvrY::cam*	Lab Collection
AM1003	MG166Δlac *barA::kan uvrY::cam*	Lab Collection
AM1004	MG1655Δlac *barA::kan/*p-*barA*	This study
AM1005	MG1655Δlac *uvrY::cam/*p-*uvrY*	This study
AM1006	MG166Δlac *luxS::cam*	Lab Collection
AM1007	MG166Δlac *luxS::cam/*p-*luxS*	This study
AM1008	MG166Δlac *luxS::cam/*p-*uvrY*	This study
AM1009	MG166Δlac *uvrY::cam/*p-*luxS*	This study
RGB1655	MG1655 *csrB::cam*	T. Romeo
TR1-5 MG1655	MG1655 *csrA::kan*	T. Romeo
BB120	Wild type *Vibrio harveyi* (AI-1+; AI-2+*)*	B.L. Bassler
BB170	BB120 *luxN::*Tn*5* (sensor-1- sensor-2+)	B.L. Bassler
BB152	BB120 *luxL::*Tn*5* (AI-1-; AI-2+)	B.L. Bassler
JJ055	Nonpiliated K-12	J. R. Johnson
JJ014	Nonpiliated K-12/ p-*fimA-H* operon; Cm^r	J. R. Johnson
JJ015	Nonpiliated K-12/ p-*papG*III; Ap^r	J. R. Johnson

Bacterial Strains	Relevant Genotype	Reference or source
AAEC189	K-12 Δ*fim* Δ*lac*	William R. Schwan
χ7122	APEC O78:K80: H9 *gyrA*::Nal^r	R. Curtiss (III)
SM3000	χ7122 *barA::kan*	Lab Stock
SM3001	χ7122 *uvrY*::cam	Lab Stock
SM3002	χ7122 *barA*::*kan*/p-*barA*	This study
SM3004	χ7122 *uvrY*::cam/p-*uvrY*	This study
SM3005	χ7122 *luxS*::cam	Lab Stock
SM3006	χ7122 *luxS*::cam/p-*luxS*	Lab Stock
CFT073	Wt Uropathogenic *E. coli*	H. L. Mobley
SM3007	CFT073 *luxS*::cam	Lab Stock
SM3008	CFT073 *luxS*::cam/p-*luxS*	Lab Stock
SM3009	CFT073 *barA*::cam	Lab Stock
SM3010	CFT073 *uvrY*::cam	Lab Stock
SM3011	CFT073 *csrA*::cam	Lab Stock
SM3012	CFT073 *barA*::cam/p-*barA*	Lab Stock
SM3013	CFT073 *uvrY*::cam/ p-*uvrY*	Lab Stock
SM3014	CFT073 *csrA*::cam/p-*csrA*	Lab Stock

Bacterial Plasmids	Relevant Genotype	Reference or source
pBR322	Cloning Vector	Invitrogen
pAN001	pBR322 containing *barA* gene; Ap^r	Lab collection
pAM001	pBR322 containing *uvrY* gene; Ap^r	This Study
pCA114	*csrA* under P_{araBAD} control on pBAD18; Ap^r	Craig Altier
pLuxS	PCR2.1 containing *luxS* gene; Apr	Lab Collection
pFZY1	*galK*'-*lacZYA* transcriptional fusion vector; Ap^r	W. E. Bentley
pLW11	pFZY1 derivative, containing *lsrACDBFG* promoter region; Ap^r	W. E. Bentley
pPP2-6	pPR274 with MCS	William R. Schwan
pBB2-1	*fimA-lacZYA* on pPR274	William R. Schwan
pWS124-17	*fimA-lacZYA* locked on on pPP2-6	William R. Schwan
pJLE4-3	*fimE-lacZYA* on pPP2-6	William R. Schwan
pJB5A	*fimB-lacZYA* on pPP2-6	William R. Schwan
P1-vir bacteriophage	Transducing Phage	Lab collection

Table 7. List of primers used in this study

Primer Designation	Sequence (5'-3')	Gene/target sequence
OSM79	TGATCCTGCACTTTCAGCAC	*luxS*
OSM80	CAATCACCGTGTTCGATCTG	
OSM250	AGCGTTCTGTAAGCCTGTGAAGGT	*rrnA*
OSM251	TAACGTTGGACAGGAACCCTTGGT	
OSM252	GGCACATTCTGGCAGCAAGTTGTA	*lsrK*
OSM253	TTTCTTCGGCACAGAAAGCATCGC	
OSM254	TGCGCCCTTACTCATAACCTTCGT	*lsrA*
OSM255	CAATACTTGCGGCGAAGCTTCCAA	
OSM256	AACCACAACAGATGCTGGCGATTG	*lsrR*
OSM257	TTAAGCTGCCCGATTCCCGTCATA	
OSM258	ACTGTACATGGTACACGCACTGGAT	*flu*
OSM259	TTCAGGGTGACATTCGTGGCTGTA	
OSM260	ACCGTTCAGTTAGGACAGGTTCGT	*fimA*
OSM261	TCTGCAGAGCCAGAACGTTGGTAT	
OSM271	GGAATCGGTGTAGATGTAACCCC	*icd*
OSM272	CGTCCTGACCATAAACCTGTGTGG	
OSM275	ATGCCGCAGGTATCCCGATG	*manA*
OSM276	GCGCGGGATTTTTCTTCACC	
OSM277	AGCCCGTTCAATGCTGCCAG	*manX*
OSM278	GTTGGAGCCGCTTTTGGTGC	
OSM279	TCGCACTGGCAATCCCTCTG	*manY*
OSM280	CATCAGGTAGCCAGCACGCA	
OSM281	AGTTCGTCAGGGTCTGGCGA	*galU*
OSM282	CAACGCCATATGCGGTCACA	

Primer Designation	**Sequence (5’-3’)**	**Gene/target sequence**
OSM283	TTGTGGGGCGCAGAAAATGT	*rcsD*
OSM284	CGACCGTTGCCAGATGTCCT	
OSM285	AACCTGCCGAAACTGGATGC	*rcsB*
OSM286	AGCTTTCGGCAGATCGGTCG	
OSM287	GCTCGTCACGGTCGCAACAA	*lrhA*
OSM288	ACATCCAGCGCTAATTTCGG	
OSM289	AACGGCAGAGGGCGATTTGT	*wcam*
OSM290	AGCGTGGCTAACGGTCAGGT	
OSM291	CCATGATGCAGGCGGTTTGT	*fimE*
OSM292	GCACGTTCCTGGGTCCACAT	
OSM293	CCGGTGGCGCTTTATTTGAC	*fimH*
OSM294	AGAAACATCGCAGCCGCCAG	
OSM295	CAGTAATGCTGCTCGTTTTGCCG	*fim* promoter
OSM296	GACAGAGCCGACAGAACAACG	
OSM297	CGACAGCAGAGCTGGTCGCTC	*fim* switch orientation
OSM298	GTAAATTATTCTCTTGTAAATTAATTTCACATCACCTCCGC	
OSM299	GCGGAGGTGATGTGAAATTAATTTACAATAGAAATAATTTAC	
OSM309	ACTCTGCGGACCACTTGGGA	*papA*
OSM310	CCAACTATTCCTCAGGGGCA	
OSM311	AACTCAACGGCACTGGCTGC	*papH*
OSM312	CTCAGAATTGTGCGAAACGG	
OSM313	CAGCAACTCAGCACCAGGAC	*glmU*
OSM314	CTTACTCACGGGCGCGATGT	
OSM315	GATGAAACCGCAGAAGGCTT	*kpsE*
OSM316	GCGATGCGATGTGACATCTC	

Primer Designation	Sequence (5'-3')	Gene/target sequence
OSM317	TAATACGACTCACTATA**G**GGAGA*GGCTGGAAAAACAC*	T7-*luxS*
OSM318	CGCTTCCATCCGGGTATGATCG	
OSM345	TATTCCGAGCCATCAGGGTG	*hlyC*
OSM346	TTCGTGCTTTGTCCTGCTGA	
OSM347	CAAGGGCGCTGGTGAACAAC	*hlyB*
OSM348	AACAGGAACTCGCTGAACCC	
OSM349	CTTACTCACGGGCGCGATGT	*glmU*
OSM350	CAGCAACTCAGCACCAGGAC	
OSM351	AGTTCGTCAGGGTCTGGCGA	*galU*
OSM352	CAACGCCATATGCGGTCACA	
OSM353	GTACGGCGATGGCATTACCT	*rcsB*
OSM354	ACCGTAACCACCAGCACTGA	
OSM355	ACGACCGTTGCCAGATGTCC	*rcsD*
OSM356	TTGTGGGGCGCAGAAAATGT	
OSM357	CCATGATGCAGGCGGTTTGT	*fimE*
OSM358	CCACGGCTTCACGCTCATCA	
OSM359	GCCAAAGCAAAACCACACGA	*fimB*
OSM360	AACGCACCCGCTATTGAACA	
OSM361	TGCACGTTTTCCAGCCTCAC	*ipbA*
OSM362	TGATGGCTTTCATTCACGGT	
OSM363	TTTCATGGTCTGCGTGTTAGTG	*ipuA*
OSM364	TTACCCGCAGCAGAAACTATGT	
OSM365	CCCCTGCAAAAAGAAACTGT	*ipuB*
OSM366	TAGCTAAAGCATACCCACAACC	

Abbreviations

APEC	Avian Pathogenic *Escherichia coli*
AHL	N-acyl homoserine lactone
AIP	Autoinducing Peptide
AI-2	Autoinducer 2
BarA	Bacterial Adaptive Response gene A
Csr	Carbon Storage Regulator
ExPEC	Extraintestinal Pathogenic *Escherichia coli*
HPK	Histidine Protein Kinase
HTH	Helix-turn-helix
PBS	Phosphate Buffered Saline
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RR	Response Regulator
TCS	Two-component regulatory system
UPEC	Uropathogenic *Escherichia coli*
UTR	Untranslated Region
Uvr	UV-resistance

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