

ABSTRACT

Title of Dissertation / Thesis: EVALUATION OF THE ROLE OF BarA-UvrY
TWO-COMPONENT SYSTEM IN
ESCHERICHIA COLI.

Isha Patel, Masters, 2004

Dissertation / Thesis Directed By: Assistant Professor, Dr. Suman Mukhopadhyay,
Department of Veterinary and Medicine

Pathogenic strains of *Escherichia coli*, a gram-negative bacterium, colonize the gastric mucosa and urinary tracts of birds, animals and human beings causing diseases like chronic gastritis, diarrhea, peptic ulceration, and urinary tract infections. Pathogenic strains cause a worldwide problem affecting 20% population in the U.S. The BarA-UvrY is a two-component system involved in bacterial adaptation and survival. The *barA* (**b**acterial **a**daptive **r**esponse) gene, induced in uropathogenic *E. coli* upon contact with eukaryotic cell surface, plays a key role in attachment and colonizing urinary tract epithelia during infection and codes for the transmembrane sensor kinase, BarA. The UvrY protein, is a *barA*-regulated transcriptional modulator whose targets are yet to be determined.

Determination of genes under regulation of BarA-UvrY signaling cascade under various stress conditions will help in better understanding the overall role of this pathway in metabolic adaptation and pathogenesis. Microarray work done in the

laboratory shows that BarA-UvrY regulates several stress-response and membrane-transport genes. One such gene identified from the microarray results, *luxS*, is involved in the detoxification of the S-adenosyl methionine in *E. coli* and produces a furanone, essential for cell density-dependent bacterial quorum sensing. LuxS is known to regulate virulence in *E. coli*. We chose to study the regulation of the *luxS* gene expression as it has not been studied in great detail. Using physiological approaches, we validated part of the microarray results.

EVALUATION OF THE ROLE OF BarA-UvrY TWO-COMPONENT SYSTEM IN
ESCHERICHIA COLI

By

Isha Rameshbhai Patel

Thesis or Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
[Master of Sciences]
[2004]

Advisory Committee:
Assistant Professor Suman Mukhopadhyay
Professor Siba K. Samal
Assistant Professor Steve Hutcheson
Assistant Professor William E. Bentley

© Copyright by
Isha Rameshbhai Patel
2004

Dedication

I would like to dedicate this work to my grandfather Mr. Chhaganbhai Naik, my grandmother Mrs. Pushpaben Naik, my father Mr. Rameshbhai Patel, and my mother Mrs. Taraben Patel for their unconditional love and support and for always being there for me.

Acknowledgements

I would like to express my sincere gratitude to my major advisor Dr. Suman Mukhopadhyay, for his invaluable guidance and persistent support throughout this project. I thank all my committee members, Dr. Samal, Dr. Hutcheson, and Dr. Bentley for their invaluable time, input and support throughout my study.

My sincere appreciation to my colleagues Helina Tuminaro, Dr. Sahu and Mrs Sharmistha Acharya and Arindam for their support in my work. Special thanks goes to my friends in the department Dr. Subbaih, Govi, Erin and Subrat for all their help. My gratitude goes to Mr. Daniel Rockemann and Mr. Peter Voyage for their friendly encouragement and timely help during various phases of my work. I would also like to thank the faculty and staff of the department of Veterinary Medicine for their cooperation and support during my period of study especially Ireen for her motherly support. I would like to thank Dr. Inder K. Vijay and Edith Silvius for all their help from the Animal Sciences Department.

Finally, I will always be indebted to my parents, grandparents, family especially and friends especially Sumit Dangi, for their unconditional love, moral support and understanding.

Table of Contents

Dedication.....	ii
Acknowledgements.....	iii
Table of Contents.....	iv
CHAPTER 1	1
Introduction.....	1
1.1. Background.....	1
1.1.1. Escherichia coli, a pathogen	1
1.1.2. Two-component signal transduction system.....	2
1.1.3. BarA, a sensor kinase.....	5
Figure 2: Domain Organization of BarA sensor kinase.....	7
1.1.4. UvrY, the putative response regulator of BarA	8
1.1.5. Role of BarA-UvrY in stress response.....	12
1.1.6. Role of BarA/UvrY in virulence of Gram-negative organism.....	17
1.1.7. Quorum sensing and BarA/UvrY system	19
1.1.8. Effect of motility in infection: role of BarA/UvrY	22
1.2. Objective.....	26
CHAPTER 2	27
Materials and Methods.....	27
2.1 Construction of Bacterial Strains.....	27
2.1.1. Transformation:.....	27
2.1.2. Electroporation:.....	28
2.1.3. Transduction	28
2.1.4. Screening.....	29
2.1.5. Complementation.....	30
2.1.6. Insertional mutation of a chromosomal gene with help of phage λ - recombinase enzyme.....	31
2.1.7. Cloning.....	32
2.1.8. BarA mutant using suicide plasmid:.....	33
2.1.9. Cloning of luxS gene (into low copy number plasmid) for transcriptional fusion with lacZ.	34
2.1.10. β -galactosidase assay:.....	35
2.1.11. Moving the fusion into the chromosome:.....	36
Table 1.A: List of strains of Escherichia coli	39
Table 1.B: List of Plasmids.....	40
Table 1.C: List of Bacteriophages	41
Table 2: List of Primers used in this study	42
2.2. Motility assay.....	43
2.3. Flagellar gene expression studies using reporter gene fusion vector:.....	43
2.4. Microarray chip hybridization protocol.....	44
2.5. RT-PCR- Two tube two-step procedure:	47
2.6. Northern Blot Analysis	49
2.7. Primer Extension & Sequencing:.....	51

2.8. Survival Assays.....	54
2.9. Scanning Electron Microscopy	55
CHAPTER 3	57
Results.....	57
3.1. Genome wide approach: Microarray Analysis	57
Figure 5. Representative Experiment Result showing normal.....	60
distribution of genes.....	60
Table 2. Partial Table for differentially regulated genes	62
3.2. Objective: To determine if the BarA/UvrY system in E. coli differentially	
regulates the expression of luxS gene.....	63
3.2.1. RT-PCR.....	63
Figure 6. RT-PCR Results	66
Table 3. The BarA-UvrY two-component system regulates luxS and pfs mRNA as	
determined by quantitative RT-PCR.....	67
Figure 7. PCR.....	69
3.2.2. Northern Blot Analysis	71
Figure 8. Northern Blot Analysis.....	72
3.2.3. Primer Extension studies.....	73
Figure 9. Primer Extension Results	74
3.2.4. Reporter gene analysis.....	75
Figure 10. luxS::lacZ cloning and expression.....	76
Figure 11. Expression of chromosomal copy of luxS::lacZ	78
3.3. Objective: To determine if the barA/uvrY system in E.coli regulates motility	
via flagellar gene expression.....	80
3.3.1. Motility assay on TB media.....	81
Figure 12. Swimming motility in soft agar.....	82
Table 4. Swimming motility results.....	82
3.3.2. Flagellar gene expression.....	83
Figure 13. Flagellar gene expression	84
3.3.3. Electron Microscopy Studies	85
3.4. Stress Assays.....	87
Figure 15. Stress Assay Results	89
CHAPTER 4	92
Discussion & Summary	92
Figure 15. Methyl cycle and BarA/UvrY	95
4.2 Summary	113
CHAPTER 5	114
Bibliography	114

CHAPTER 1

Introduction

1.1. Background

1.1.1. *Escherichia coli*, a pathogen

Escherichia coli is a Gram-negative bacterium and is a very important component of the biosphere. It colonizes gastric mucosa and urinary tracts of aquatic, avian and mammalian species including human beings. It is a facultative anaerobe and survives in the natural environment, thus allowing widespread dissemination to new hosts. Pathogenic strains of *E. coli* cause chronic gastritis, colisepticaemia, diarrhea, pericarditis, peptic ulceration, urinary tract infection (UTI) and various other diseases, which are often fatal. Pathogenic strains cause a worldwide problem affecting 80% of the adult population in the developing countries and 20% in the United States. The effectiveness of an infection depends on the ability of the bacterium to adapt and survive within the unfavorable environment of the gastric lumen or urinary tract. Several factors are required for effective and successful adaptation to the altering physiological conditions of the host during infection. Some of these are virulence factors, including specific structural components on the surface of the bacteria which play an important role in (i) motility of the bacteria towards mucosal surface to find a proper ecological niche, (ii) adherence and colonization of the bacteria to the epithelial tissue of the host system, (iii) invasion of the bacteria within the host cells, (iv) development of resistance towards the host immune system and most importantly (v) production of bacterial toxins that cause severe damage to host system.

Genes encoding the bacterial virulence factors are mostly clustered at specific regions known as the Pathogenicity Islands (PAI)(38,100), of the bacterial chromosome. These virulence-associated genes are often controlled by various regulatory systems of the bacteria. In most cases, these adaptive genes are regulated by two-component signal transduction systems in bacteria.

1.1.2. Two-component signal transduction system

In their natural environment, bacteria are often challenged by constant changes in nutrient availability and exposure to various forms of stress. Their adaptation to these environmental changes depends largely upon two-component signal transduction systems that act as sensory and response regulatory systems. Such two-component systems (TCS) have a membrane associated sensor kinase and its cognate Response Regulator (RR)(83). The transmembrane sensor histidine kinase (HK), also called Histidine Protein Kinase (HPK), consists of an N-terminal periplasmic sensing domain and a C-terminal cytoplasmic kinase domain (35). The HPK exists in a dimeric form and on detecting specific environmental stimuli it first undergoes ATP-dependent trans-autophosphorylation at conserved histidine residues, whereby one HK monomer phosphorylates a second monomer within the HK dimer complex (83). Subsequently, the phosphoryl group is then transferred to a conserved aspartic acid residue on the specific cognate RR molecule or to a different domain within the same HPK molecule (83, 35). Upon phosphorylation, the RR undergoes conformational changes and functions as a transcriptional regulator. Thus, these conformational changes activate specific transcription activators or repressors and initiate a response by modulating gene transcription, resulting in changes in the

physiology and metabolism of the bacteria to cope with the external environment (Figure 1). In *E. coli*, the BarA protein, encoded by bacterial adaptive response or *barA* gene, encodes a novel conserved HK regulatory switch for adaptation and modulation of metabolism (84). Tripartite HKs consist of a conserved aspartate residue at a phosphor receiver domain to which the phosphate group is first transferred and relayed to a final histidine residue before being transferred to a cognate RR (84). The BarA protein is highly conserved in most gram-negative bacteria and the UvrY in γ -subdivision of proteobacter, including *E. coli*.

Figure 1: A basic two-component system consisting of sensor kinase and response regulator domains.

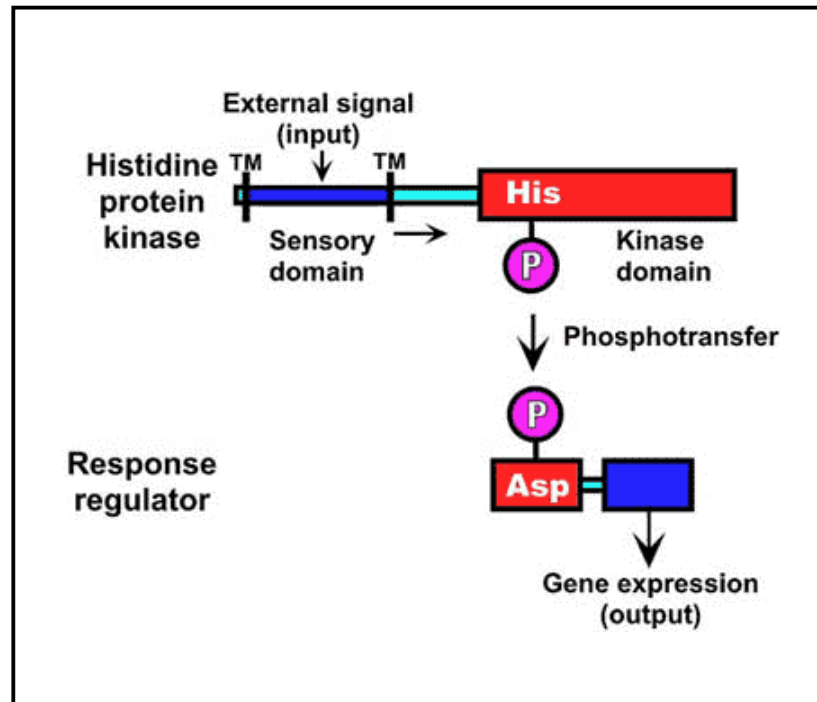


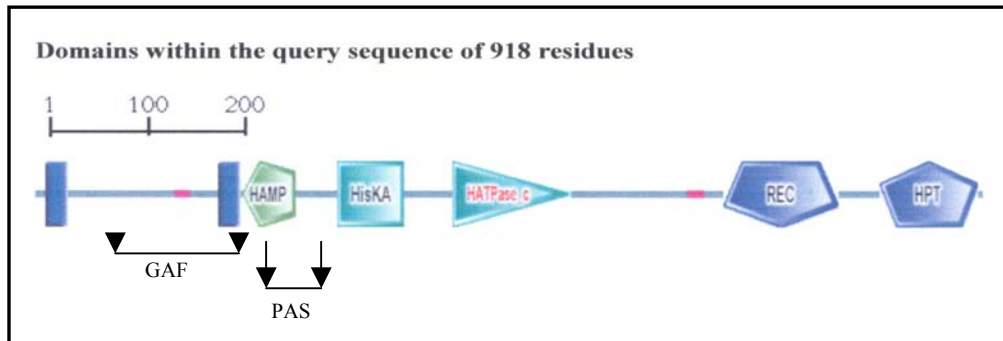
Fig.1 When the sensory domain of the protein kinase receives an external signal, it autophosphorylates at a conserved Histidine residue in its kinase domain. This phosphoryl group is transferred to a conserved Aspartate residue on the regulatory domain of the response regulator, which in turn modulates gene expression.

1.1.3. BarA, a sensor kinase

The BarA, a membrane associated protein, has been identified to have many orthologues and homologues in pathogenic yeast, fungi, moulds and plants. Some of them have been identified in *Salmonella spp* (BarA/SirA system) (4), *Pseudomonas spp* (GacA/GacS system) (18), *Bordetella pertussis* (BvgS virulence factor) (35), *Legionella pneumophila* (LetS/LetA) (39) and in some species of *Vibrio* (113). The *barA* gene, encoding BarA protein has been identified to phenotypically suppress the effect of a deletion mutation of *envZ*, which encodes the sensor domain of EnvZ-OmpR two-component signal transduction system. EnvZ-OmpR system regulates *ompC* and *ompF* gene expression, encoding major outer membrane porins to maintain osmotic potential within the *E. coli* cell (69). The length of BarA is 918 amino acids and the calculated map position is 62.79 minutes. The nucleotide sequence of *barA* reveals that it encodes a 102kDa protein, which has both the ‘sensor kinase’ as well as the ‘response regulator’ domains. These domains have been found to undergo *in vitro* phosphorylation by the characteristic three-step procedure of tripartite histidine kinases (Figure 2) (48). As BarA has a high degree of sequence similarity to both EnvZ and OmpR proteins, it has been implicated to be involved in bacterial adaptive response in *E. coli*. The *barA* gene is induced in uropathogenic *E. coli* upon contact with eukaryotic cell surface and plays a key role in the attachment and colonization of the urinary tract epithelia during infections (115). In *S. typhimurium*, BarA regulates the expression of invasion genes (4) and also regulates virulence functions of pathogenicity island II, essential for survival (79). BarA deficiency in *E. coli* has been

shown to impair catalase thus resulting in an oxidative stress sensitive phenotype (67).

Figure 2: Domain Organization of BarA sensor kinase



GAF	cGMP regulated cyclic phosphodiesterase, new class of cyclic nucleotide receptor for signaling in sensory proteins.
PAS	cytosolic signaling module, monitors changes in redox, light, small molecules, i.e., overall energy level of the cell.
HAMP	<u>H</u> istidine kinases, <u>A</u> denyl cyclases, <u>M</u> ethyl binding proteins, phosphatase domain.
HisKA	dimerization and phosphoacceptor domain.
HATPase c	histidine kinase like ATPases.
REC	Che-Y like h-t-h DNA binding domain.
HPT	Secondary Transmitter domain.

http://smart.embl-heidelberg.de/smart/show_motifs.pl

1.1.4. UvrY, the putative response regulator of BarA

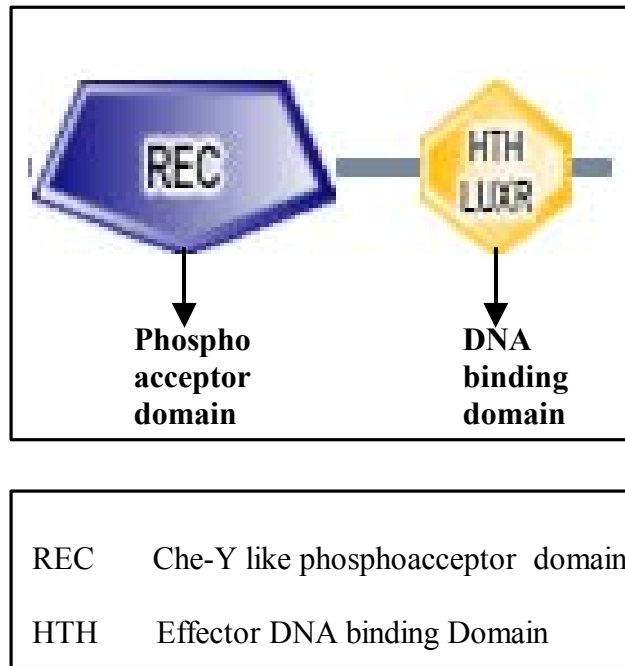
UvrY (and its orthologue SirA in *Salmonella*) was identified as the cognate response regulator of BarA in *E. coli* in an *invitro* phospho-transfer reaction study (76). This 23kDa (218 amino acid) protein has a calculated map position of 42.95 minutes. It has an N terminal phospho-acceptor domain with a conserved aspartic acid residue at position 54 followed by a helix-turn-helix DNA binding domain in the C terminal region (76) (Figure 3). The *uvrY* gene (615 bp) resides upstream to the *uvrC* gene, which encodes a DNA repair enzyme (66). UvrY orthologues are present in γ subdivision of proteobacteriae of the genera *Pseudomonas* (*gacA*), *Erwinia* (*expA*), *Escherichia* (*uvrY*), *Vibrio* (*varA*), and *Salmonella* (*sirA*) and belong to the FixJ family of regulators (64,76,34). These genes are not only encoded within an evolutionarily conserved region of the genome, but also have similar functions. UvrY orthologue, SirA, regulates bacterial motility in *S. enterica* (34,76). The complex regulatory network of the type III secretion system in *S. enterica* is required for modulating eukaryotic cellular physiology for uptake of bacteria and is, in part, regulated by BarA, probably by modulating the phosphorylation state of SirA (54). Whole genome transcription profiling in *E.coli* revealed that increased *sdiA* expression led to a 10-fold increase in *uvrY* transcription. This indicates the expression of *uvrY* gene is regulated by *sdiA* (107). The *sdiA* gene is involved in quorum sensing by monitoring small signaling molecules called autoinducers (61). Mouse infection assays indicate that a mutation in the *sirA* gene had a 10-fold attenuation of virulence compared to 4-fold attenuation by an isogenic *barA* mutant strain of *S. typhimurium* (54). These findings suggest a significant role for

BarA/UvrY system in establishing early infection in certain pathogenic gram-negative organisms.

Recently it has been shown that the BarA/UvrY system plays a role in biofilm formation through the complex regulatory network of CsrA/CsrB/CsrC system (98,108). CsrA is a RNA binding protein that represses gluconeogenesis and biofilm formation. CsrA activates glycolysis, motility and flagellum biosynthesis. CsrA has been shown to indirectly activate CsrB via the BarA/UvrY system (98). CsrC, similar to CsrB, binds to and antagonizes CsrA. Both CsrA and UvrY have been shown to activate CsrC. UvrY restored *csrC* expression in *csrA*- but CsrA could not restore the expression in *uvrY*- background. These studies indicate a CsrA/CsrB/CsrC independent role of UvrY in modulating cellular metabolism (Figure 4).

Previously it was thought that BarA/UvrY system is involved in the iron acquisition mechanism through siderophore system, by which enteric bacteria obtain the required amount of iron from the host system, in *E. coli* (77). However, later on it was established that BarA and not UvrY is directly involved in iron uptake of the same organism (75). Suzuki et al show that an autoregulatory loop exists between BarA and UvrY whereby UvrY positively autoregulates expression of *barA* (98).

Figure 3: Domain organization of UvrY



http://smart.embl-heidelberg.de/smart/show_motifs.pl

Figure 4: BarA/UvrY and CsrA/CsrB/CsrC regulatory systems

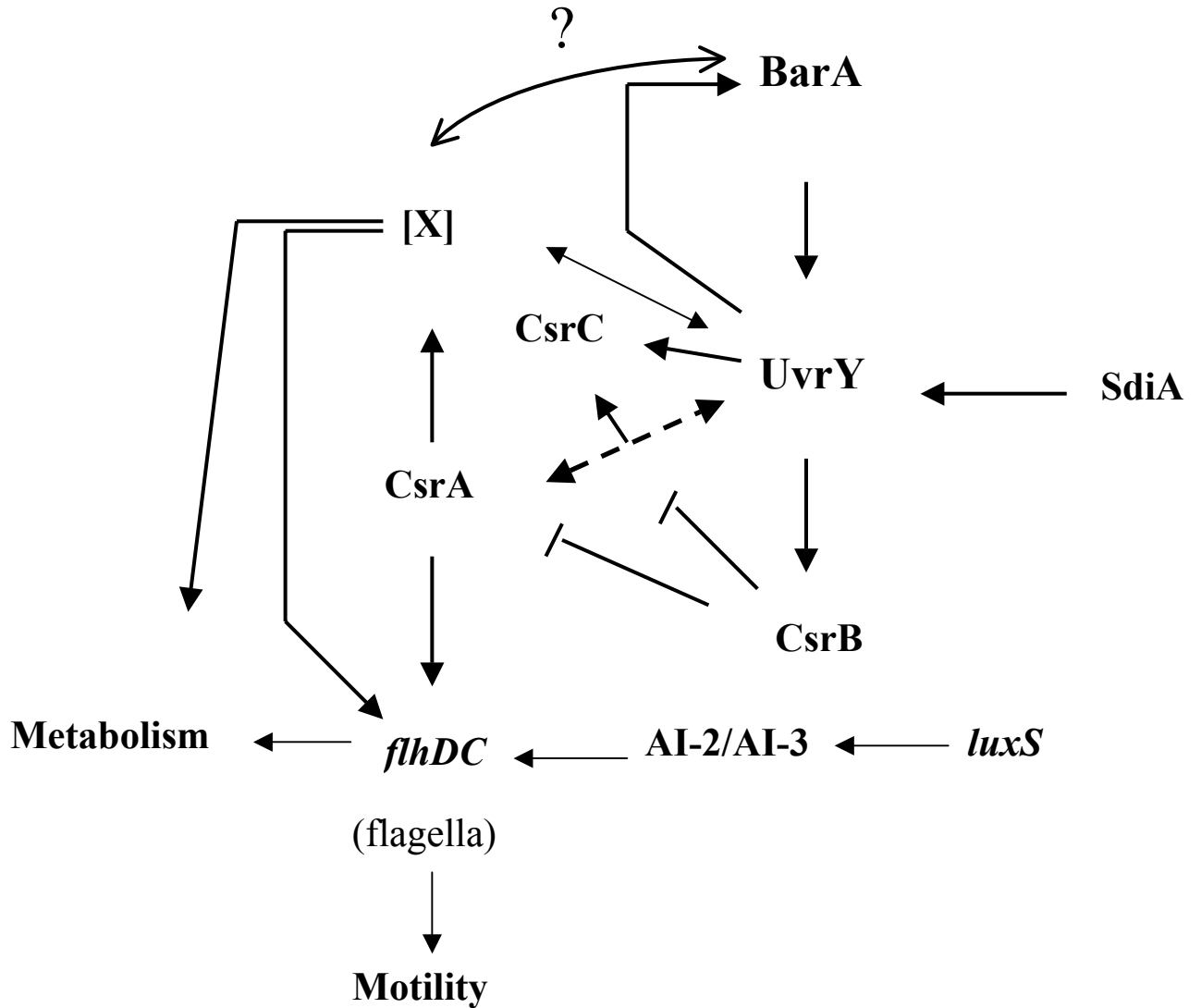


Fig. 4 Arrow heads show the direction of pathway, closed arrow indicate inhibitory effect, double head arrows show the pathway can go in either direction.

1.1.5. Role of BarA-UvrY in stress response

Pathogens have developed complex mechanisms to survive from normal host cellular processes, thereby inadvertently contributing to pathogenesis and disease outcome. To establish an infection within the host, enteropathogenic and enterohaemorrhagic bacteria must i) survive the acidic pH of the stomach, ii) progress into the small intestine and survive the bile salts, iii) adhere to intestinal epithelial cells, iii) finally enter into the blood stream and survive the oxidative stress faced during phagocytosis by macrophages. Similarly, in urinary tract infections, uropathogenic *E. coli* (UPEC) must modulate both their gene expression and metabolism in order to withstand the hostile condition in the host urinary tract. The bacteria have to i) withstand the fluid flow, ii) survive in the low nutrient environment and iii) survive the high osmotic stress and urea in the urinary tract. To adapt to and survive these potential stress situations, the bacteria require highly specific and temporal regulation of expression/repression of a number of sets of genes, which are probably governed by one or more global stress regulators. BarA protein, a sensor kinase of a two-component system, is one such potential global stress regulator. It has also been found that over expression of response regulators of bacterial two component systems confer drug resistance by controlling expression of some transporter genes (45).

Acid stress response

Membrane permeant organic acids not only endure stress upon the bacteria, but also their presence induces protective responses against increased acidic

environmental stress. *E. coli* and *Salmonella* can survive through the acidic pH of the stomach. Infact, they grow over a wide range of pH (4 to 9), and, depending on the availability of nutrients and electron acceptors, their metabolism shifts to compensate for an external pH change towards either extremity (28). Several regulatory proteins have been shown to play significant roles in acid mediated stress response e.g. alternate sigma factor RpoS, the iron regulatory protein Fur, a putative response regulator RssB that interacts with RpoS, a two-component signal transduction system PhoPQ, sodium/H⁺ transporter NhaA and its phosphorylated activator protein NhaR system etc. (28). BarA is also found to be a key factor in regulating oxidative stress response by enhancing catalase production through transcriptional activation of the *rpoS* gene. RpoS, the alternative sigma factor of *E. coli*, is involved in regulating gene expression in response to pH changes and changes in osmolarity, mainly carbon starvation. BarA along with its response regulator UvrY, may have an indirect or direct significant influence on these processes, as it is one of the transcriptional regulators of *rpoS*. BarA/UvrY system, being a putative global stress regulatory system might be a member of the acid-stress response regulon, an important regulon needed by all enteric pathogens for survival within the stomach and macrophages (88).

Osmoregulation in presence of bile salts

If bacteria survive the acidic environment of the stomach, they move to the small intestine where they face the challenge from the bile salts. Bile salts are surfactants and are potent antimicrobial fluids causing osmotic imbalance (36). These pathogens survive the intestinal bile salt - stress by altering a wide range of

properties, including motility, biofilm formation (80), toxin and pili production (37), and the ability to invade eukaryotic cells (79). In response to change in temperature and osmolarity, bacteria can vary the pore diameter of their outer membrane porins to accommodate larger molecules (nutrients) or to exclude inhibitory substances. Membrane porins play this significant role in the regulation of osmotic balance by restricting the flow of different ions through the membranes (88). In *E. coli*, the role of BarA in regulation of the osmotic stress response has been shown (69). *S. enterica* serovar Typhimurium, when grown in the presence of bile salts, showed severely reduced ability to invade epithelial cells probably by down regulating *sirC*. The invasion gene *sirC*, is controlled by SirA, the cognate response regulator of BarA sensor in *Salmonella* (79). This indicates that BarA, a putative global stress regulator, plays a highly significant role in the adaptation to the osmotic stress situation in *Salmonella* spp. and *E. coli*, by regulating the expression of the outer membrane proteins that are important in osmoregulation or by modifying the ability of the bacteria to invade the host cell during infection.

Oxidative stress response

Once the pathogens cross the epithelial barrier in both intestinal and urinary tract, they encounter macrophages. Macrophages engulf the pathogens and challenge them with a burst of reactive oxygen species, low pH, lysozymes and proteases within the phagosomal complex (103). Bacteria survive this oxidative stress response mainly by two regulatory systems viz. OxyR system that is required for hydrogen peroxide stress and SoxRS system that is required for superoxide stress (33). There are two super oxide dismutases (SOD) in *E. coli*, one is Mn-Fe SOD that acts outside the

bacteria and the other is Cu-Fe SOD that acts inside the bacteria. As a part of the antioxidant regimen, *E. coli* and *Salmonella* possess two catalases, Hydroperoxidase I and Hydroperoxidase II (86,88). The first, encoded by the *katG* gene, exhibits dual catalase and peroxidase activity. The expression of *katG* is induced by OxyR (an autoregulator) at the level of transcription in response to exposure to the sub-lethal levels of hydrogen peroxide *in vitro* (68). Hydroperoxidase II (HPII), encoded by *katE* gene, has catalase activity and constitutes a major anti-oxidative stress response system. Both HPI and HPII show a growth phase dependent expression, 10 fold higher expressions in stationary phase than in the exponential phase. This is largely dependent on RpoS, the alternative sigma factor for the RNA polymerase in the stationary phase of growth (86). The oxidative stress response mediated by hydroperoxidases is primarily regulated by the alternative sigma factor RpoS at the transcriptional level. BarA functions as a transcriptional regulator of *rpoS* (67). In fact it has been shown that *barA* is maximally expressed in early exponential phase immediately before the transcriptional induction of *rpoS*. The regulation of RpoS itself is very complex process known to be controlled by multiple two-component systems and is not fully understood (72). In summary, BarA/RpoS system plays a major role in regulating oxidative adaptive stress response mainly mediated by hydroperoxidases. This signifies the importance of this system in the adaptation to potential oxidative stress challenges faced during the intracellular growth within the macrophage, during the course of infection.

Iron is required as a cofactor for almost all of the antioxidant responsive enzymes including catalases. In the absence of functional BarA, the level of active

HP I peroxidase is very low, but the activity can be restored to a significant level by addition of exogenous iron to the culture medium (88). This suggests that besides BarA there are several other factors that may be involved in oxidative stress response. However, BarA may regulate not only the transcription of the major antioxidant genes via the RpoS pathway, but also Fe-transport and/or metabolism (88). UvrY does not appear to be involved in *barA* mediated activation of *rpoS* (43). Thus, BarA does this probably with the help of a yet unidentified response regulator. However, UvrY has been shown to negatively regulate *rpoS* (72).

Other stress responses

In *E. coli*, other potential stress situations in which BarA is involved include response to common preservatives like weak acids and common oxidative stress (68) and switching between gluconeogenic and glycolytic carbon sources (75). During macrophage-mediated killing, the bacteria are challenged with both NADPH oxidase and inducible nitric oxide synthase (iNOS). These challenges generate antimicrobial reactive oxygen and nitrogen intermediates (104), resistance against which in *S. enterica* serovar Enteritidis is controlled by a global regulator ArcA (57). A number of *E. coli* transcriptional regulators have been implicated in modulating gene expression in response to reactive nitrogen species. Under aerobic conditions, MetR (methionine biosynthesis transcriptional regulator) dependent NO-induction of the *hmpA* gene was reported (60). Reactive oxygen and nitrogen species share chemical properties. Two regulators of *E. coli* responses to oxidative stress are modified by reactive nitrogen species. The iron-sulfur cluster containing protein SoxR as well as the OxyR transcription factor can be activated by both reactive oxygen and nitrogen

generating compounds (74). This suggests, and also it has been shown that in *E. coli*, there is a common link between hydrogen peroxide, superoxide and nitric oxide mediated stress. Thus, being an oxidative stress regulator, BarA along with its response regulator UvrY may be involved in nitric oxide-mediated stress too (33, 22), as BarA is suggested to be a potential global stress regulator.

In summary, it can be suggested that BarA/UvrY system probably acts as a global sensor and regulatory mechanism, in a wide variety of stress situation, under both extracellular and intracellular growth condition in *E. coli* and *Salmonella* spp.

1.1.6. Role of BarA/UvrY in virulence of Gram-negative organism

Bacteria disrupt the normal host cell function and utilize the cellular machinery in five major stages, for their own benefit. These are i) adhesion or specific attachment of the bacteria to the host cell surface, ii) invasion or bacteria induced entry into the host cell by a modified phagocytosis mechanism, iii) survival of the bacteria inside the host macrophage cell and/or bacterial defense against the host immune system, iv) extracellular colonization on the host tissue and v) cell toxication by bacterial toxins and other products (27). Type I pili, curli fimbriae, extracellular polysaccharides, flagella and several other factors have been implicated in adhesion and invasion of different bacteria and hence their virulence properties (7, 78, 59). A number of these virulence properties are regulated by two-component signaling systems such as the BarA/UvrY system (4, 3, 98).

In *Pseudomonas* spp, association traits and virulence are globally controlled by the GacS/GacA system. The BarA/UvrY system has similarity to the virulence factors of GacS/GacA system in *Pseudomonas* spp (82) and BvgS of *B. pertussis*

(101). It is known that the GacS/GacA system and their homologues are involved in several stages of infection, regulating important properties related to virulence in different species of *Pseudomonas* and other microorganisms. These include regulation of toxin production (82), regulation of type III secretion system (46), regulation of iron acquisition through siderophore system (56), alginate synthesis (16), biofilm formation (73), resistance to different antibiotics (73) and invasion (49). Previous studies in UPEC have demonstrated that *barA* (or *airs*) gene is activated only upon attachment of the pathogen to the urinary tract epithelial cells, suggesting its role in adhesion of the pathogen to the urinary tract against the flow of the urine (115). In *Salmonella* spp., the *barA* gene plays an important role in the invasion of the cultured epithelial cells as a deletion in the *barA* gene leads to a reduction in invasion (4). *S. enterica* serovar Typhimurium harbors two pathogenicity islands, SPI-1 and SPI-2, which encode the type III secretion system (47). The genes encoded by SPI-1 have been shown to be essential for the invasion of epithelial cells (30) and for inducing cellular apoptosis (52, 65) whereas SPI-2 genes are required for systemic infection (88, 92, 44). A complex regulatory network of the type III secretion system is required by the bacteria for modulating eukaryotic cellular physiology during different stages of infection especially invasion. Therefore, the assumption that some global regulators might play significant role in modulating different sets of genes involved in a common network, is logical. In fact, in *Salmonella* it was found that SirA, a response regulator of BarA belonging to the FixJ family of RR, is involved in inducing the expression of type III secretion system and invasion of the epithelial cells to elicit bovine gastroenteritis (34). OmpR, known to be regulated by BarA in

absence of EnvZ in osmoregulation of *E. coli* has also been described to regulate another two-component system SsrA-SsrB in *Salmonella* SPI-2 (55). Moreover, the expression of *hila*, a gene encoded by the *Salmonella* SPI-1, is found to be regulated by BarA/SirA and EnvZ/OmpR systems. In *S. enterica* serovar Typhimurium, HilA, a member of OmpR/ToxR family of transcription factor, is required for the expression of most of the genes that are integral components of the type III secretion system and are important for the invasion. Thus, BarA might have a very important and crucial role in regulating genes of both SPI-1 and SPI-2 in *Salmonella*. Hence, it may play a significant role in the formation and assembly of the type III secretion system, which is required for appropriate invasion into the eukaryotic epithelial cells during infection by *Salmonella* and other gram-negative organisms.

The BarA and UvrY independently are involved in the regulation of RpoS, the alternative sigma factor for the stationary phase of growth, regulating transcription of *rpoS* gene. RpoS, in turn modulates the expression of the *spv* genes present in the virulence plasmid of *S. typhimurium* required for lethal systemic infection (111, 26, 70, 53). These findings indicate that BarA, and UvrY like its orthologues, may have an important role in the virulence mechanism, especially in the invasion of the organisms belonging to the γ -subdivision of proteobacteriae. Hence, BarA/UvrY probably affects the infection process as a whole. However, the specific and direct influence of BarA/UvrY in the pathogenesis is yet to be identified.

1.1.7. Quorum sensing and BarA/UvrY system

In bacteria, cell density-dependent gene regulation is known as Quorum sensing (10). Quorum sensing, also known as cell-cell signaling allows cells to

communicate using molecules that are produced, dispersed and then received. There are two types of quorum sensing systems in bacteria, intra-species and inter-species. The quorum sensing molecules, also known as autoinducers (AI), interact with bacterial transcriptional regulators once they reach a certain threshold concentration, thereby regulating gene expression. In *V. harveyi*, AI-1 is used for intraspecies and AI-2 for inter-species communication (8, 9, 97). The AI-1 has been purified and identified as acyl homoserine lactone (HSL) and its synthesis depends on *luxL* and *luxM* (97). In AI-2 synthesis, the enzyme Pfs converts *S*-adenosylhomocysteine (SAH) to *S*-ribosylhomocysteine (SRH). LuxS is responsible for the conversion of SRH to homocysteine and DPD. DPD is predicted to spontaneously rearrange into various furanones. The furanone predicted to lead to the formation of *V. harveyi* AI-2 is the only one shown and is termed pro-AI-2. Borate adds to pro-AI-2 to form the active signaling molecule AI-2. The chemical structure of AI-2 has been suggested to be a furanosyl borate diester (17). These hormone-like compounds interact with regulatory proteins, which regulate the transcription of several genes that are involved in a variety of phenotypes including production of antimicrobial agents, flagellation, motility (94). A reporter-fusion based assay indicated that the expression of the *luxS* gene is constitutive while the *pfs* expression is co-related to growth-phase dependent AI-2 production in *Salmonella* (12). Proteomic studies have shown that under low pH and in the presence of acetate, the LuxS protein is induced (96). The *luxS* family of genes has wide spread distribution in both pathogenic and non-pathogenic species of both gram-positive and gram-negative bacteria (92). The ecological role of *luxS* in bacteria is still poorly characterized, but one of its putative functions is to allow

bacteria to optimize gene expression in response to the density of all *luxS*-containing species occupying the same niche. There has been no homology reported of the *luxS* genes to any other gene known to be involved in AI production.

Moreover, there is clear indication that BarA is important for virulence, especially the invasion and the attachment of the bacterial pathogen. Whole genome transcription profiling in *E. coli* K-12 revealed that increased *sdiA* expression led to a 10-fold increase in *uvrY* transcription, the potential response regulator of BarA (107). The *sdiA* gene encodes a LuxR family of transcription activator involved in sensing and responding by quorum sensing mechanism to a mixed microbial population (103).

In *Pseudomonas*, GacA/GacS regulate AI-1 synthesis (109). In *V. fischerii*, when GacA was mutated, AI-1 was not affected but the luminescence was severely reduced. On providing exogenous AI-1, the luminescence was not restored to its original level. Probably this was due to GacA regulating luminescence via AI-2. In *E. coli*, AI-1 has not been reported. So, we hypothesized that the GacA homologue of *E. coli*, UvrY, may regulate bioluminescence via AI-2.

Quorum sensing regulates the expression of flagella and motility (34, 93) through a novel two-component system in *E. coli* (94). Quorum sensing has also been shown to regulate the transcription of genes regulating the type III molecular syringe system (92) and protein secretion in EHEC and EPEC (93). Sperandio *et al* recently have shown that *E. coli* also produces AI-3, which in fact regulates flagellar driven motility and secretion of proteins into host cells using the type III secretion system. Like AI-2, the synthesis of AI-3 depends on the presence of a functional *luxS* gene

(111). Partially purified AI-3 when provided to a *luxS* mutant restored type III dependent protein secretion and motility, whereas *in vitro* synthesized AI-2 had no effect. BarA/UvrY, a putative global regulatory system, might have a role in regulating one or more of the above-mentioned physiological properties by regulating *luxS* expression through quorum sensing mechanism. Comparing transcripts of wt versus *barA*-, *uvrY*- and *barA-uvrY*- by Microarray hybridization shows that the *ygaG* (*luxS*) gene is down regulated in the mutants compared to the wild-type *E. coli* strain. This led us to further study how the BarA/UvrY system may influence the *luxS* gene expression, if it does. Our other preliminary chemiluminescence studies show that BarA regulates AI-2 accumulation (data not shown).

1.1.8. Effect of motility in infection: role of BarA/UvrY

The role of bacterial flagella driven motility in the virulence and pathogenesis has been reported (32). Adhesion and invasion possess a very complicated mechanism. The mechanism may involve regulation of a number of genes located in or outside the pathogenicity islands. In *E. coli*, flagella produced by EPEC contribute to the adherence properties of the bacteria and that a molecule secreted by eukaryotic cells induces their expression, which incidentally is epinephrine (32,111). In *H. pylori*, for colonization of mucous layer of the human stomach, a two-component system involving two CheY response regulators and a histidine kinase sensor CheA are essential for motility and chemotaxis (29). In *Vibrio cholerae*, the phosphorylation of the flagella regulatory protein FlrC that belongs to the FlrB/FlrC two component system is necessary for motility and colonization of the infant mouse small intestine (19). In *P. aeruginosa* the adhesion process is under the control of the

rpoN gene, which also regulates pilin synthesis and flagellum formation. Another two component system FleS/FleR controls both motility and adhesion acting downstream of RpoN although it remains unclear whether the specific adhesin is a flagellar protein or another protein that uses flagellar export apparatus for localization (81). Another gene *fliO* is also identified, which is involved in flagellar biosynthesis and non-pilus mediated adherence (89). Lateral flagella play an important role in swarming motility, biofilm formation, adhesion to and the invasion of the Hep-2 cells in *Aeromonas* species (31). Some researchers have demonstrated that in *S. enterica* serotype Enteritidis, both fimbriae and flagella are important for the association and the invasion of the cultured epithelial cells (23). Other groups have shown that the functional flagella and the flagella associated motility, but not the fimbriae, are important for the adherence of the *S. enterica* serotype Enteritidis to chick gut explants (2). The significance of flagellin gene *fliC* in invasion of the Caco-2 cells had also been demonstrated, although this gene is not important for adherence (102). Expression of *E. coli* flagella transcriptional cascade starts with the flagellar master operon *flhD* and *flhC*. The mutation in the *flhD* gene in *S. enterica* serovar Typhimurium has a differential effect on mouse model and cultured epithelial cells (87). It has been found that neither the presence of flagella nor the synthesis of flagellar export machinery is required for the pathogenicity of the organism in the mouse. However, the presence of flagella is required for the full invasive potential of the bacterium in the tissue culture and flagellar secretory apparatus is also required for the maximum fluid secretion in the enterocolitis model (87). In EPEC, the flagellin gene has been shown to be essential for adherence and microcolony

formation on the cultured cells and the synthesis of flagella, in response to the presence of the eukaryotic cells. Flagellin is regulated by type IV pili, type III secretion pathways and quorum sensing (32). Recently it has been argued that in EPEC, besides the interaction between the major outer membrane adhesin intimin and its receptor on the host cell, other interactions are equally important for effective adhesion and effacing effect on the epithelial cells. These include the interactions between the bundle forming pilus or flagella and specific receptors on the host cell surface (71).

The role of BarA/UvrY and their homologues in the motility and virulence has been studied in a few organisms. In *L. pneumophila*, it has been found that the strain displayed poor expression of flagella when it had mutation in a two-component system LetA/LetS, which is homologous to GacA/GacS in *Pseudomonas* and SirA/BarA in *Salmonella*. This defect was also manifested when there is a mutation in the flagella sigma factor FliA and stationary phase sigma factor RpoS (39). The FliA is involved in motility, cytotoxicity and the ability of the organism to infect macrophages (39). The mutation in the orthologues of SirA, the potential response regulator of BarA, in various organisms including *E. coli*, *V. cholerae*, *P. fluorescence* and *P. aeruginosa* causes defect in motility suggesting the that the control of flagellar regulons may be an evolutionarily conserved function of SirA orthologues (34). In *P. fluorescence* F113, GacA, the SirA homologue, regulates both motility and virulence property during colonization on the alfalfa roots (85). GacA/GacS system, which is similar to BarA/UvrY, is also involved in the swarming motility, characterized by a dendritic pattern on semisolid agar plates, of *P. syringae*

(50). However, the virulence factor BvgAS of *B. pertussis*, which is similar to BarA, is found to have a repressing effect on the synthesis of flagella when expressed in *E. coli* (40). HilA is the major regulator of the invasion genes in *Salmonella enterica* serovar Typhimurium. HilA, encoded by SPI, is found to be regulated by several genes in SPI-1 (*hilC/sirC/sprA* and *hilD*) and outside the SPI-1 (*phoP/phoQ*, *sirA/barA* two component systems) (4, 25, 11). It has recently been shown that the flagellar genes like *flhD*, *flhC* and *fliA* exert their regulatory effect, in an independent way, on the expression of *hilA* in *S. enterica* serovar Typhimurium (58). These findings relate the regulatory effect of BarA/SirA and flagellar function to virulence gene expression. Recently, SirA was reported to directly activate virulence expression via *hilA* and *hilC* and repress the flagellar regulon indirectly via *csrB* (99). BarA/UvrY and their homologues may have regulatory roles in flagella related virulence properties. Thus, it can be summarized that functional flagella are involved in various stages of infection, particularly adhesion and invasion, in different organisms and are potent virulence determinants.

1.2. Objective

The literature discussed in the previous chapter has suggested that BarA/UvrY two-component system of *Escherichia coli* may be involved in some of the important physiological properties related to adaptation and virulence determination during infection.

Thus the main objective of this research is

“ Evaluation of the role of BarA/UvrY two-component system in adaptation in *Escherichia coli* ”

- 1) **A genome wide molecular approach: Microarray Analysis:** To directly identify genes differentially expressed by mutation in the *barA*, *uvrY* and *barA/uvrY* genes compared to the wild type *Escherichia coli*.
- 2) **Molecular biological approach:** To study the effect of *barA* and *uvrY* mutation on the expression of *luxS* gene.
- 3) **Physiological approach:** To directly study the effect of mutation in the *barA* and *uvrY* genes on motility and survival under stress in *Escherichia coli*.

CHAPTER 2

Materials and Methods

2.1 Construction of Bacterial Strains

2.1.1. Transformation:

Preparation of competent cells for transformation: Bacterial cells were grown overnight at 37 °C in 5 ml LB (Luria Bertani) media with appropriate antibiotic if required. The cells were then subcultured (1:100 dilution) in 100 ml LB media with antibiotic and allowed to grow up to an O.D₆₀₀ of 0.7-0.9. The cultures were then kept on ice for 15 minutes after which they were centrifuged at 7000 rpm (rotations per minute) at 4 °C. The supernatant was discarded and the bacterial pellet was resuspended in half (50 ml) of chilled Magic Solution (60 mM CaCl₂, 15% glycerol, 10 mM MOPS (N-Morpholino propanesulfonic acid)). The cells were kept on ice for 45 minutes and again centrifuged at 7000 rpm at 4 °C. The supernatant was discarded and the pellet was resuspended in fifteenth fraction (6.6 ml) of Magic Solution. 100 µl of these cells were aliquot into sterile appendorf tubes and stored immediately at –80 °C for further use.

Transformation: 100 µl competent cells were thawed on ice. 2-3 µl (10-100 ng) DNA was added to the cells and allowed to stand on ice for 30 minutes. Heat shock was given at 42 °C for 90 seconds and then immediately cells are kept on ice for 2 minutes. Then 200 µl of LB was added to the cells and allowed to grow at 37 °C for 1 hour. The cells were then plated on agar plates containing antibiotic and

incubated at 37 °C overnight. Next day, the transformants were restreaked for further purification.

2.1.2. Electroporation:

Competant cell preparation: Cells were grown overnight in SOC (2% Bactotryptone, 0.5% Bacto yeast extract, 0.05% NaCl, 1% 250 mM KCl, after autoclaving-0.5% 2 M MgCl₂) media. When subculturing, 1mM arabinose was added and the cells were allowed to grow upto O.D₆₀₀ of 0.7-0.9. The cultures were then allowed to cool on ice for 15 mins. The cells were then centrifuged at 4000 rpm for 20 mins at 4°C. The supernatant was discarded and the pellet was resuspended in 10% chilled sterile glycerol. The cells were washed thrice each time using fresh 10% glycerol. Finally the pellet from the last was resuspended in 100th volume (of starting culture) of 10% glycerol. The now electrocompetant cells were stored at -80 °C or used immediately.

Electroporation: 50 µl electrocompetant cells were thawed on ice. 100 ngm DNA to be electroporated was added to the cells and sit on ice for 1 minute. Immediately this was transferred to a prechilled cuvette and cells were shocked with 1700-1900 mVolts. Immediately, 1 ml of SOC was added to the cells and they were allowed to grow for 1 hour before plating on respective antibiotic agar plates.

2.1.3. Transduction

The details of all the strains used in this study are listed in the Table 1. *E. coli* transductants were constructed by using P1 phage lysate of *E. coli* AKP014 (*barA::kan-lacZ*) to transduce *E. coli* MG1655Δ*lac* (*wt* K12). Wild type (*wt*) bacteria were

grown in 5 ml of Luria-Bertani (LB) media overnight with 0.005 M CaCl₂ and 0.1 M MgSO₄. The cells were subcultured (1:100) and allowed to grow till late log phase, pelleted down and re-suspended in 1 ml of MC buffer (0.1 M MgSO₄, 0.005 M CaCl₂). 200 µl of the re-suspended bacteria were incubated with 50 µl of the phage lysate of AKP014 for 20 minutes at 37 °C. The infection was stopped by adding 200 µl of citrate buffer (0.1 M sodium citrate pH 5.5) and washed once with 200 µl of citrate buffer. Then 100 µl of the supernatant was disposed and the bacteria were re-suspended in the remaining and plated on LB-kanamycin (50 µg/ml) plates along with the mock-infected cells. The plates were incubated at 37 °C overnight and the kanamycin resistant colonies were further purified. Purified colonies were then checked for proper deletion of the gene by PCR verification as mentioned later on.

Similarly, *barA*- was transduced with P1 lysate of *uvrY*- to make *barA-uvrY*-.

2.1.4. Screening

The selected mutants were grown in micro-titer plates and replica plating was done on respective antibiotic agar plates. Only those colonies were selected which were resistant to the marker antibiotic, which was suspected to be moved into the strain. As *barA::kan* is related to *relA*-, it was screened on M9 minimal medium. Further, by conducting a 10% H₂O₂ sensitivity test, the probable mutants were screened.

Also, overnight cultures of the wt and probable mutants were sub-cultured in LB-antibiotic and grown at 37 °C till mid-log phase. 500 µl of each culture was added to 5.0 ml of R-top Agar (0.01 gm/ml Bacto-tryptone, 0.001 gm/ml Bacto yeast

extract, 0.008 gm/ml NaCl, 0.002 M CaCl₂, 0.1% Glucose and 0.8% Bacto-agar), individually. This was mixed and poured onto R-agar plates (0.01 gm/ml Bacto-tryptone, 0.001 gm/ml Bacto yeast extract, 0.008 gm/ml NaCl, 0.002 M CaCl₂, 0.1% Glucose and 1.2% bactoagar). After allowing to solidify, a whattman paper-disc saturated with 10% H₂O₂ was placed on the center of each plate. The plates were incubated at 37 °C overnight. The zone of inhibition of the growth of the bacteria created by H₂O₂ would be more for the mutants than that for the wt. Only those mutants were selected that had zones of inhibition comparable to that of respective transducing strains.

2.1.5. Complementation

Competent cells of the mutants were prepared (as described earlier) and transformed with a plasmid carrying a functional wt copy of the respective mutated gene to complement the mutation in the mutant by following the same procedure as described earlier. The transformed cells selected on respective antibiotic agar plates.

Screening: The selected transformants were grown in micro-titer plates and replica plating was performed on respective antibiotic agar plates to select for only resistant colonies. The complemented cells were screened also by performing a H₂O₂ sensitivity test as described above. The zone of inhibition of the growth of the bacteria created by H₂O₂ would be more for mutants than the wt and complemented transformants. The transformants, whose zones of inhibition were comparable to the wt, were selected as effective complemented cells. All the bacterial strains that were used in the research are listed in Table 1.

2.1.6. Insertional mutation of a chromosomal gene with help of phage λ -recombinase enzyme

Competent cells of *E. coli* were transformed with pKD46. The ampicillin resistant colonies were purified at 30 °C and stored. These cells were used to prepare electro-competent cells (as described above). The *kan* gene was PCR amplified using pairs of primers with ~36 nucleotide homology extensions of *barA* gene flanked by ~20-nt priming sequences for the template plasmids pKD3 or pKD4 with FRT (FLP recognition target) sites. The FRT sites are included so that after selection, the resistance gene can be eliminated from the mutants by using a helper plasmid expressing the FLP recombinase, which acts on the directly repeated FRT sites. Using Tgo DNA polymerase (Roche Diagnostics, Germany) along with the above mentioned primers and template, the PCR products were generated. Template pKD3 gave a 1.1-kbp fragment while the pKD4 gave a 1.6-kbp fragment, which was seen on a 1% agarose gel by staining with ethidium bromide in 1X TAE buffer. The respective 1.1- or 1.6-kbp PCR products were purified with help of QIAquick PCR purification kit (Qiagen). Then the samples were digested with DpnI. This DNA (10-100 ng) was then electroporated into electro-competent cells of bacteria (50 μ l). The electro-competent cells harbored the Red recombinase gene in trans on a low copy number plasmid (pKD20). The plasmid harbors bacteriophage λ -Red recombination system under an arabinose inducible promoter that promotes recombination. Shocked cells were added to 1 ml of SOC media and incubated at 37 °C for one and half hour. 100 μ l of these cells were plated onto respective antibiotic agar plates to select for Kan^R or Cm^R transformants. If none grew within 24 hours at 30 °C, the remainder

was spread on larger plates. Mutants were selected on basis of Kan^R or Cm^R at 30 °C. After this first selection, the mutants were plated on medium without antibiotic. The Red (and FLP) helper plasmids are temperature sensitive replicons and could be cured by growing the cultures at 37 °C. Therefore, the mutants were colony purified non-selectively at 37 °C and then tested for ampicillin sensitivity to test for loss of the helper plasmid. If the plasmid was not lost, then the mutants were colony-purified at 43 °C and tested again similarly. Cm^R or Km^R mutants, which were ampicillin sensitive, were then stored and used for further verification. The mutants were verified for having a correct mutation within the gene of interest (*barA* or *uvrY*) by PCR amplification.

PCR verification: Probable mutants were grown overnight with respective antibiotic and used for genomic DNA isolation. A PCR reaction was set up using this DNA and test primers for the gene to be knocked out. Tgo DNA polymerase enzyme was used and as a control the DNA from wild-type strain was used. The PCR products were run on a 1% agarose gel in 1X TAE buffer for 1 hour at 100 volts. If the gene had been knocked out, compared to the wild-type size, the size of the band was 1.1- or 1.6- kbp larger. This confirmed that the antibiotic marker had insert in between the gene and knocked the gene out. These strains were used for all further experiments.

2.1.7. Cloning

Cloning of genes in TOPO vector: The gene of interest (*barA*, *uvrY*, *luxS*) was amplified using specific primers (Table 2), wild type MG1655 genomic DNA as template and Tgo DNA polymerase. The amplified product was checked by running

on a 1% agarose gel. The confirmed product was then cleaned using Qiagen PCR purification kit or by cutting the fragment from the agarose gel and extracting the DNA. This was then cloned into TOPO vector using the pCR2.1 TOPO-TA cloning kit for sequencing, Invitrogen Corporation, USA.

Ligation: TOPO TA cloning kit was used. The PCR amplified DNA is first cleaned free of reagents by any appropriate method. 3' A overhangs were then added to the PCR reagents free DNA with help of Taq polymerase at 72°C for 15 minutes. 4 µl of this DNA reaction was mixed with 1 µl salt solution provided in the kit and 1 µl of pCR[®] 4-TOPO vector (kit) in 0.5 µl microcentrifuge tube. The solutions were mixed by carefully pipetting up and down and incubated at room temperature for 15-45 minutes for ligation and then placed in ice. 2 µl of the ligated products were used to transform one vial of one shot[®] TOPO10 chemically competent *E. coli* cells. A control reaction was also set up with pUC19 control plasmid provided in the kit. Transformation was followed same way as mentioned above and transformants were selected on appropriate (Kan/Amp) antibiotic agar plates.

Isolation of the plasmids and sequencing: The clones were grown in LB kanamycin overnight and the plasmids containing the inserts were isolated by using QIAGEN[®] Plasmid Mini kit, QIAGEN, USA). They were sent to University of Maryland Sequencing Core facility for sequencing. The sequences were blasted using the BLAST tool at the NCBI home page <http://www.ncbi.nlm.nih.gov>.

2.1.8. BarA mutant using suicide plasmid:

The *barA* gene was first amplified using primers (Table 2, OSM1, OSM2) and cloned into pCR[®] 4-TOPO vector of the TA cloning kit, Invitrogen as mentioned

earlier. To disrupt the gene, a *kan-gfp* gene fusion (39) was introduced in *Cla*I site of the *barA* gene. Then the *barA::kan-gfp* gene was sub cloned into pCVD422 with help of restriction enzymes. pCVD422 is a suicide vector constructed to engineer mutations in host strains via allelic exchange (22). This vector can only grow in strains that have the *pir* gene encoding the Pi protein, which is necessary for replication of R6K plasmids. The *pir* gene is usually supplied in trans by a lambda lysogen, λ -*pir*. The vector with the *barA::kan-gfp* gene was transformed into competent cells and transformants were confirmed to carry this vector by cutting the gene with specific restriction enzymes and mapping its size on a 1% agarose gel. Since pCVD422 cannot multiply in a strain lacking *pir* protein, the only ampicillin resistant strains that arise have a chromosomally integrated copy of the plasmid. Only kan^r and GFP expressing colonies that were amp^r were selected. Further kan^r , GFP expressing, amp^s colonies were selected that indicate the loss of the suicide vector. These putative mutants were confirmed by PCR amplification of the retained *kan-gfp* gene.

2.1.9. Cloning of *luxS* gene (into low copy number plasmid) for transcriptional fusion with *lacZ*.

The *luxS* gene was PCR amplified with primers (Table 2., OSM53, 54) containing specific restriction enzyme sites and using Tgo DNA polymerase. The amplified DNA was run on a 1.0% agarose gel and the ~500 bp fragment was gel extracted. This DNA was digested with *Sma*I and *Sal*I and ligated into the vector pSP417 digested with the same enzymes. The pSP417 is a plasmid containing a promoterless *lacZ*. The ligation mix was transformed into DH5 α competent cells

using standard transformation procedure mentioned in materials and methods. The Amp^R colonies are selected and further purified by restreaking on ampicillin containing agar plates. The transformants are then inoculated for plasmid isolation, and the isolated plasmids are then checked for presence of insert (*luxS*). The transformants containing the inserts are used for further experiments.

To check the functionality of the *luxS-lacZ* fusion: The β -galactosidase activity was measured in the newly constructed fusion strains, using normal pSP417 without insert, as a control. The clones that showed activity were used for further study. The plasmid with a functional gene was named pIRP046 and used for future studies.

2.1.10. β -galactosidase assay:

Bacterial cells are grown overnight in 5 ml of LB containing required antibiotic. The cells are diluted 1:100 the next day and allowed to grow to an OD₆₀₀ \approx 0.5. Then the cells were resubcultured into 50 ml media with antibiotic such that the OD₆₀₀ at time zero would be 0.05. Then at every 20-minute time interval, sample was collected into cuvettes and the time of sample collection was noted. At each time point, the OD₆₀₀ was measured and 100 μ l of cells were added to pre aliquot tubes containing 900 μ l Z-buffer, 25 μ l chloroform and 25 μ l 0.1% SDS. The tubes were vortexed to break the cell wall and then 200 μ l of 4 mg/ml ONPG (o-Nitrophenyl β -D-Galactopyranoside) was added to the tubes. The tubes were vortexed again and incubated in 28 °C water bath till pale yellow color developed. Then 500 μ l of 1 M Na₂CO₃ was added to the tubes to stop the reaction. The start time- time at which the

ONPG was added and the stop time- time at which Na_2CO_3 was added is noted too. The tubes were centrifuged at 11,000 rpm for 5 mins and then the OD_{420} was measured.

The calculations were done as per the following formula

$$\beta\text{-gal activity in Miller Units} = \frac{\text{OD}_{420} \times 1000}{\text{OD}_{600} \times \text{Aliquot (0.1 ml)} \times \text{Reaction time in minutes}}$$

2.1.11. Moving the fusion into the chromosome:

Amplification of the lambda phage: *E. coli* host strain LE392 was grown in TBMM (Bacto tryptone 10 gm/lt, NaCl 8 gm/lt, 0.2% Maltose, 10 mM MgSO_4) till saturation and 200 μl of these cells were added to 2.5 ml of top agar (with 0.01 M MgSO_4 , 0.2% Maltose, 0.05 M CaCl_2) and overlaid on TB plates. With help of a sterile wire loop, lambda phage was streaked onto this plate after the top agar solidified. The plates were incubated at 37 °C overnight. The next day, clear zones are seen at the area where the phage was streaked. With help of a micropipette one plaque was picked and added to 0.1 ml overnight grown LE392 cells and incubated at 37 °C for half hour. This was then added to 5 ml of LB (with 0.01 M MgSO_4) and allowed to grow on rotor at 37 °C until lysis was seen (~5 hrs). Then a few drops of CHCl_3 were added and after vortexing thoroughly the tube was allowed to sit at room temperature for a few minutes. Then the cells were centrifuged at 5000 rpm for 10 mins and the supernatant was saved as phage lysate at 4 °C for future use.

Titer of the lysate: The lysate was first diluted serially in TMG (0.05 M Tris-Cl pH 7.5, 0.01 M MgSO₄, 5 ml 2% gelatin/lit of TM). 0.1 ml of the diluted lysate was added to 0.2 ml of LE392 and mixed. This mix was added to 2.5 ml of top agar (with 0.01 M MgSO₄, 0.05 M CaCl₂) and poured onto TB agar plate and incubated at 30 °C overnight. The plaques were counted the next day, which are indicative of the titer.

Moving the fusion: The host strain IRP046 (pSP417-*luxS-lacZ*) was grown in TBMM till saturation. The phage was diluted as per required in TMG. Then 0.1 ml of the phage was mixed with 0.2ml of the host and kept at 37°C for half hour. This was added to 5 ml LB and kept on rotor till lysis was seen (4 hrs). Then 50 µl CHCl₃ was added and the mixture was vortexed and centrifuged at 5000 rpm for 10 mins. The supernatant was used as lysate. This lysate was diluted and mixed with 0.2 ml of LE392 cells and kept at 37°C for half hour. Then the cells were mixed with 2.5 ml of H-Top agar (with 0.01 M MgSO₄, 0.05 M CaCl₂) with X-Gal (40 mg/ml) and overlaid on TB plates and incubated at 37 °C overnight. Blue plaques were picked with micropipette and resuspended in 1 ml of Tris-Mg (0.01 M) the next day. This was diluted and used to infect LE392 cells as mentioned above and plated on TB plates with X-Gal. Blue plaques were purified till all the plaques were blue indicating the lysate was pure.

To prepare lysogens: 0.1 ml of the above purified *lac*⁺ lysate mixed with 0.1 ml saturated culture of host strains-wt and mutants. This was allowed to stand for 20 minutes at 25°C and added to 2ml of TBMM. The culture was rotated at 37°C for two hours and the cells were then washed twice with TB and finally resuspended in 100µl TBMM and plated on TB-Xgal. Both light and dark blue plaques were seen which

were streaked out onto respective antibiotic agar plates. The next day the stable lysogens were further purified by restreaking. The pure colonies were stored as well as screened for selecting monolysogens.

Ter assay or Immunity i^{434} assay to screen monolysogens: λ -DJ140 (λi^{434}), which shows plaques with multiple lysogens and not monolysogens, was grown in TBMM. LE392 was used as a control. Saturated cultures of the above lysogens were used to make lysate with λ -DKC170 (lysate from NIH) following the same procedure as mentioned above. The lysates were then diluted and used to infect the DJ140 and LE392 cells. Using the same method as above, the cells were finally overlaid on TB plates and incubated at 37°C. The phage population, which formed plaques on λ -DJ140 were multiple lysogens with more than one cos site and were not used for later studies. Only the ones with no plaques on DJ140 but with plaques on LE392 were selected for later use and stored.

The wt, isogenic *barA*- mutant, isogenic *uvrY*- mutant and the double mutant now carrying the fusion were used for further *luxS* expression studies. The strains were named as shown in the (Table 1). The mutant strains were complemented with wild type copy of the gene and the galactosidase assay was performed to verify the mutants.

Table 1.A: List of strains of *Escherichia coli*

Strains	Genotype	Sources/ references
MG1655Δlac	<i>wt</i> K-12 λ - <i>rph-1</i> Δlac	D. J. Jin
MC4100	F- <i>araD139</i> Δ (<i>araF-lac</i>) U169 λ - <i>flh</i> Δ5301 <i>fruA25 rpsL150 relA1 deoC1 ptsf25 rbsR22</i> <i>flb5301</i>	<i>E.coli</i> genetic stock center
DH5α	<i>SupE44 [lacU169</i> Φ (80Δ <i>lacZ</i> 58(M15)) λ - <i>rfbD1</i> <i>gyrA96 recA1 endA1 thi-1 hsdR17</i>	Laboratory collection
LE392	F- <i>hsdR514 supE44 supF58</i>	Laboratory collection
HS703	MC4100 <i>barA::λplacMu53</i> [Φ (<i>barA-lacZ</i>)1010]	(84)
HS8100	MC4100 <i>rpoS::λplacMu53</i> [Φ (<i>rpoS-lacZ</i>)143]	(84)
MD-AI2	W3110 F- λ - IN (<i>rrnD-rrhE</i>) <i>rph-1 luxS::Tc^R</i>	W. E. Bentley
IRP011	MG1655 Δlac <i>barA::kan</i> , P1 (AKP014)	This study
IRP014	MG1655 Δlac <i>uvrY::cm</i>	This study
IRP015	MG1655Δlac <i>barA: kan, uvrY:: cm</i>	This study
IRP016	MG1655Δlac <i>luxS::cm</i>	This study
IRP035	<i>pluxS</i> in IRP011	This study
IRP041	<i>pluxS</i> in IRP014	This study
IRP042	pANA001 in IRP014	This study
IRP046	pSP417- <i>luxS-lacZ</i> in DH5 α	This study
IRP050	<i>luxS</i> in pCR2.1 Topo vector	This study
SM1005	<i>luxS-lacZ</i> in MG1655Δlac	This study

SM1006	<i>luxS-lacZ</i> in IRP011	This study
SM1007	<i>luxS-lacZ</i> in IRP014	This study
SM1009	<i>luxS-lacZ</i> in IRP015	This study

Table 1.B: List of Plasmids

Plasmids	Genotype	Sources/ references
PANA001	pBR322 containing <i>wt barA</i> gene	(87)
<i>PluxS14</i>	pCR 2.1 containing <i>wt luxS</i> gene	This Study
pMM <i>rpoS2</i>	pBR322 with <i>wt rpoS</i> gene	Laboratory collection
pIRP046	<i>luxS-lacZ</i> in pSP417	This Study
pSP417	<i>lacZYA'</i> operon fusion vector (pBR322 origin, Ap ^R)	Podkovyrov and Larson (1995)
pKD3	Ap ^R Kan ^R oriR _γ	Datsenko et al. (2000)
pKD4	Ap ^R Cm ^R oriR _γ	Datsenko et al. (2000)
pKD46	Ap ^R , containing the Red recombinase of λ phage	Datsenko et al. (2000)
pVS182	<i>flhD::lacZ</i> in pRS551	(93)
pVS183	<i>fliAehK12::lacZ</i> in pRS551	(93)
PFDCZ6	<i>flhDC::lacZ</i> in pMBL1034	(93)

Table 1.C: List of Bacteriophages

Phages	Genotype	Sources/ references
lambda	Temperate phage	Campbell, A. (1961)
P1	Temperate phage, generalized transducer	Laboratory collection

Table 2: List of Primers used in this study

Primer Name	Sequence
OSM 1	^a GCATACGCCAAAATGAGGACAG ^b
OSM 2	GAAACCAGCGTCATAAAAAGCC
OSM 17	GTGCCAGATGGGATTAGCTAGTAG
OSM 18	GTCGAGTTGCAGACTCCAATCC
OSM 19	CCCGGATCCCATATGATCAACGTTCTACTTGTTGATGACCACG
OSM 34	GTGAAGCTTGTTTACTGACTAGAT
OSM 35	GTGTCTAGAAAAACACGCCTGACAG
OSM 43	TGGTGC GCGCAGGGATACGACGCATTCTGGAAGTTGCATATGAATATCCTCCTTAGT
OSM 44	CATTGTTGAGCGATGTCAGAAGCAATGTAACGCTGACCGTGTAGGCTGGAGCTGCTTC
OSM 46	CCCTTCGAAATAATTTTCATCGTAGGGCTTACTGTGA
OSM 47	CCCCTGCAGATGCACGCCTGGCTGGCTGGTTAC
OSM 48	CCCCTGCAGATGCATGCCGATTGCTACTCGACA
OSM 49	TGCGCTTCTGCGTGCCGAACAAAGAAGTGATGCCAGTTGCATATGAATATCCTCCTTAGT
OSM 50	CACGCTGCTCATCTGGCGTACCAATCAGACTCATATACTGTGTAGGCTGGAGCTGCTTCG
OSM 51	CCCCCGGGATAGCATTTCGAGAAGCCTACCGTA
OSM 52	CCCCATGGATACAAACAGGTGCTCCAGGGTATG
OSM 53	CCCGTCGACATAGCATTTCGAGAAGCCTACCGTA
OSM 54	CCCGGGCCCATACAAACAGGTGCTCCAGGGTATG
OSM 64	CCCGAATTCATAATTTTCATCGTAGGGCTTACTGTGA
OSM 74	AGTACTGAACTATTACAGAGGCGTAATGTGTCTGGCATATGAATATCCTCCTTAG
OSM 75	ATCTACACGTCAAGGACGTTGAAGAGAAAGCCGTCACATATGAATATCCTCCTTAG
OSM 76	GGATAAAGACCTCTATAAGGAAACTAGTTGCAAGATTGTGTAGGCTGGAGCTGCTT
OSM 77	CAATGTAACGAAGACTATAGCGAGTTGTTTACCGCATGTGTAGGCTGGAGCTGCTT
OSM 78	CAACAATCTATCGAAGTGTCAGCTAGTATGGGCCTATGTGTAGGCTGGAGCTGCTT
OSM 82	CTGGAGATATTCCTTTGATCAACGTTCTAC
OSM 83	ACGCTTTTGC GTCAA ACTGATCACTCACTG
OSM 170	TGCAATGGAAGAAGAAGTTAC
OSM 171	AGCGTTAAGATTCAGTTCG

^a 5' end of Primer

^b 3' end of Primer

2.2. Motility assay

Assay for motility on soft agar: Overnight grown cultures of strains of *E. coli* wt MG1655 Δ *lac*, isogenic mutants and mutants complemented with wt copy of gene on plasmid, were tested for their ability to swarm. Cultures were diluted (1:100) in TB with the required antibiotic and grown at 37 °C for 4-5 hrs. The density of all the cultures was made approximately the same by bringing the O.D₆₀₀ of all the cultures to ~0.20 by diluting with fresh TB. Motility agar plates with rich media (0.25 % Tryptone broth or 0.25 % LB) were prepared in 150 X 15 mm petri-plate. 5 μ l of each of the bacterial cultures of adjusted O.D. was stabbed halfway through the agar in the plates after the plates were allowed to solidify. The plates were incubated at room temperature (~18-22 hours) or until the measurable motility zones were formed.

2.3. Flagellar gene expression studies using reporter gene fusion vector:

MG1655 Δ *lac*, the isogenic *barA*-, *uvrY*- and *barA-uvrY*- were transformed with a reporter gene fusion vector pVS182, pVS183 and pFDCZ6. Bacterial transformants were selected on the basis of antibiotic resistance. The transformants were grown overnight at 37 °C, diluted in fresh nutrient media and were allowed to grow. Throughout the growth cycle, at different time points, OD₆₀₀ was noted and the sample cultures were collected the same way as explained earlier. The cultures were diluted 1:10 in Z-buffer and assayed for β -galactosidase activity as described previously (Miller, 1972).

2.4. Microarray chip hybridization protocol

RNA isolation: The bacterial strains were grown overnight at 37 °C in 3 ml of LB without antibiotic. For each strain, 20 ml of Phenol-Ethanol solution (1.0 ml Acidic phenol, pH 4.3 + 19 ml 100 % Ethanol) was prepared. 9 ml was used in 41 ml of bacterial culture to arrest the RNA. All other steps of RNA isolation by Qiagen kit for MIDI column were followed. The RNA quality was checked by running a 1 % agarose gel and quantitated with help of NanoDrop spectrophotometer (version 1.0, NanoDrop Technologies Inc., Delaware, USA). The RNA was treated with DNase I to remove the contaminating DNA.

Removal of contaminating DNA from total RNA isolations: 250 µl of DNase I cocktail (154 µl water + 88 µl 10X RQ1 buffer + 11 µl RNAsin-Rnase inhibitor) was added to each RNA sample of ~550 µl. The sample was mixed gently by flicking the tubes several times and then spun briefly. This was incubated at 37 °C for 30 mins. The RNA sample was then purified by phenol chloroform extraction.

Phenol: chloroform extraction for purification of DNase treated RNA: DNase reaction tubes were kept on ice for 2 mins. 700 µl of acidic phenol was added to each tube and vortexed for 2 mins, and kept on ice for 1 min. The samples were then spun at room temperature for 3 mins. The top aqueous layer was collected in a new appendorf tube and another 700 µl of acidic phenol was added and the step was repeated. The aqueous layer was again taken to a new tube and 700 µl of chloroform was added and vortexed and spin was repeated. 35 µl of 5 M NaCl was then added

and vortexed and spun again. Then 700 μ l of 100% Isopropanol was added to each tube and vortexed to mix and the tubes were then placed at -20 $^{\circ}$ C for 2 hrs atleast to precipitate RNA. The tubes were centrifuged at 4 $^{\circ}$ C for 30 mins at maximum speed. The supernatant was discarded and the pellet was washed with 750 μ l of ice-cold 70% Ethanol. Tubes were again centrifuged at 4 $^{\circ}$ C for 15 mins and pellet dried in Speed-Vac for \sim 5 mins. The pellet was resuspended in 200 μ l of 1X TE (pH 7.4) and vortexed briefly and incubated at room temperature for \sim 10 mins. The concentration was checked using NanoDrop spectrophotometer.

Labelling and Pre-hybridization: Then this RNA was labeled for the Microarray. 10 μ g Random hexamers were annealed at 65 $^{\circ}$ C to 100 μ g of total RNA. Labeling master mix cocktail was prepared-3.8 μ l RNAsin (Rnase inhibitor) + 30.0 μ l 5x first strand buffer + 15.0 μ l 0.1 M DTT + 7.5 μ l 20X dNTPs (low-T because RT replaces dUTP for T). This master mix was briefly spun and kept on ice. 8 μ l Cy-3 dUTP was added to the control (wild type) RNA-hexamer mix and 8 μ l Cy-5 dUTP was added to the mutant RNA-hexamer mix. The tubes were now covered to maintain dark environment, as the dyes are light sensitive. 10 μ l Superscript II Enzyme reverse transcriptase was used for cDNA preparation. The reaction was initiated by adding 26 μ l of the master mix to each RNA sample and incubated at 42 $^{\circ}$ C for 1 hr. Then additional 2 μ l enzyme was added to each tube and incubated again for 1 hr under same conditions. 6 μ l of 1 M NaOH was added to degrade RNA and after 10 mins at 65 $^{\circ}$ C, 6 μ l of 1 M HCl was added to neutralize the reaction. This labeled cDNA was purified using Qiagen Qiaquick PCR purification kit. NanoDrop Spectrophotometer was used for the analysis of purified Cy-labeled DNA. The cDNA was then

concentrated in a Speed-Vac for ~15 mins. The sample volume was measured (should be around 20 μ l).

Pre-hybridization buffer- 12.5 ml Formamide + 12.5 ml 20X SSC + 500 μ l 10% SDS + 167 μ l 30 mg/ml BSA + 24.3 ml nanopure water, was prepared in a 50 ml Falcon tube and kept at 42 °C for ~5 mins. A single Microarray slide was placed into the tube and kept at 42 °C for one hour with intermittent mixing every 5 mins. The slide was then removed and placed into another fresh Falcon tube with 45 ml nanopure water. This was allowed to rock gently for 30 secs and the slide was removed and placed in an empty Falcon tube. The slide was dried by centrifuging for 5 mins at 1500 X g at room temperature.

Hybridization: 40 μ l of Hybridization buffer (23 μ l Formamide + 25 μ l 20X SSC + 1 μ l 10% SDS + 1 μ l 10 mg/ml Salmon Sperm DNA) was added to a combination of 20 μ l Cy3-cDNA and 20 μ l Cy5-cDNA in a 0.5 ml microfuge tube. The probe solution was incubated at 95 °C for 5 mins and then briefly spun and allowed to cool to room temperature. The array was placed in Corning Hybridization Chamber and 80 μ l of probe was added onto the surface of the printed side of the slide without forming bubbles. Carefully a cover slip was placed on the slide and the chamber was assembled as described in its package. The chamber was then submerged in a 60 °C water bath overnight. The after overnight incubation, the array slides were carefully removed from the hybridization chamber and placed into 2X SSC, 0.1% SDS at 42 °C to wash off the coverslip. Then the slide was again washed with fresh 2X SSC, 0.1% SDS at 42 °C for 5 mins. The slide was then transferred into 0.1X SSC, 0.1% SDS and with gentle shaking was kept at room temperature. The

next post-hybridization wash was with 0.1X SSC and this was repeated 4 times, using fresh buffer each time. Finally the slides were placed into a 50 ml Falcon screw cap tube without any buffer and spun for 5 mins at 2500 X g to dry. The slide was then placed in a new 50 ml Falcon screw cap tube and covered with foil. The slide was then scanned with ScanArray 4000XL (ScanArray Express, CT, USA).

Analysis: Signal intensities for each spot representing a gene, were determined from the resulting pseudocolor image using the adaptive circle method for ScanArray Express. Quantitation is performed locally in a spot patch and pixels on each channel are selected separately. The software uses Lowess Normalization method and calculates the logarithmic ratio of Cy5 to Cy3 for each spot on the array. A representative experimental result is presented in the scatter plot in Fig. 5. The X-axis and Y-axis represent gene expression in the wt and mutant strain respectively, as determined from the intensities of the Cy dyes.

2.5. RT-PCR- Two tube two-step procedure:

Equal amount, 5 µg of freshly isolated RNA of the different strains was used to set up a RT. In the first tube, first strand cDNA synthesis was performed under optimal conditions required for SuperScript™ II Reverse Transcriptase (Invitrogen Inc., USA), using random hexamers, oligo (dT) primers (generating cDNA pool), or sequence specific primers. The primers were designed using PrimerSelect version 5.06 (DNASTAR Inc., USA) software. The RNA was first allowed to anneal with 2.0 ul random hexamers at 65 °C for 5 mins and immediately cooled on ice. Then the following cocktail was added to the RNA: 4.0 µl 5X RT buffer + 2.0 µl 0.1 M DTT + 0.5 µl RNAsin + 0.5 µl 20 mM dNTPs + 5.0 µl water + 1.0 µl SuperScript™ II RT.

The reaction was incubated at 42 °C for 1 hr and then additional 1.0 µl enzyme was added and incubated for an additional hour. The reaction was stopped by heating at 72 °C for 15 mins. Then 0.1 M NaOH (final concentration) was added to the reaction and incubated for 10 mins at 65 °C to denature any RNA if present and neutralized with 0.1 M HCl. The cDNA was then column purified using Qiagen PCR purification kit. The purified cDNA concentration was then measured using NanoDrop Spectrophotometer. A real-time reaction was performed in LightCycler (Roche, Idaho Technologies Inc., ID, USA). The LightCycler FastStart DNA Master SYBR Green kit (Roche Applied Biosystems) was used to set up the reaction. 10 ng cDNA was used as template for each of the strains in a 10 µl reaction volume. The MgCl₂ concentration was first titrated and so was the template concentration. This reaction was loaded onto a capillary and briefly spun at 3000 rpm. The capillary was set to run in the LightCycler with 35 cycles of denaturation, annealing and extension, with fluorescence measured at the end of each extension segment. An additional step of melting curve analysis was added at the end to circumvent primer dimer interference. Fluorescence was measured continuously during the melting curve, ensuring that only the fluorescence of the desired amplicon (~300 bp in length) is detected at the high melting temperature, as the primers are single stranded. Water was used as template for the negative control and wt genomic DNA for positive control. The same reaction using the same amount of template was set with *rrnA* primers to make sure the loading of the template was same.

The amplified product was run on a 1% agarose gel containing ethidium bromide to make sure that the product is of the right size. To quantitate, we used

comparative C_t method. The statistically significant increase in amount of amplicons is detected by choosing a fluorescence threshold level such that for all the samples the amplicons are in their linear range. The threshold cycle (C_t) is determined by drawing a line perpendicular to the set threshold fluorescence (relative light units). This method involves comparing the C_t values of the samples of interest (mutants) with a control (wt). The C_t values of both the control and the samples of interest are normalized to an appropriate endogenous housekeeping gene, we use the *rrnA*.

The comparative C_t method is also known as the $2^{-[\Delta][\Delta]C_t}$ method, where

$$[\Delta][\Delta]C_t = [\Delta]C_{t,\text{sample}} - [\Delta]C_{t,\text{reference}}$$

Here, $[\Delta]C_{T,\text{sample}}$ is the C_t value for any sample normalized to the *rrnA* housekeeping gene and $[\Delta]C_{t,\text{reference}}$ is the C_t value for the wild type also normalized to the endogenous housekeeping gene. For the $[\Delta][\Delta]C_t$ calculation to be valid, the amplification efficiencies of the target and the endogenous reference must be approximately equal.

2.6. Northern Blot Analysis

RNA was isolated as mentioned above. Equal concentrations of RNA from each sample was mixed with RNA loading dye (Invitrogen Inc., USA) and the volume of the loading mixture for each sample was adjusted to 45 μ l. This was heated at 70 °C for 10 mins, cooled on ice for 5 mins and was loaded on a 1% formaldehyde gel (1.2% Agarose, 1X MOPS running buffer, 15 ml Formaldehyde). The gel was run at 100 volts in 1X MOPS buffer till position of dye was almost at the end of the gel (~2 hrs). The gel was then stained with ethidium bromide to check for presence of

RNA. The gel was washed twice with water and then with 0.05 N NaOH for 20 mins. Then the gel was soaked in 20X SSC buffer for half hour.

Transfer of RNA from gel to hybrid nylon membrane: All apparatus were thoroughly cleaned with RNA zap. A tip box was placed in a tray and a glass slide was placed on top of it. Blotting paper was placed forming a cross sign as it would be acting as wicks. This wick was soaked with 2X SSC buffer and some 20X SSC buffer was added to the tray. One more blotting paper of the size of the glass plate was placed on top of the wicks. Then the gel was placed such that the topside faced the glass slide side. The nylon membrane was then soaked in water after cutting one edge of the membrane as a mark. The membrane was carefully placed onto the gel. To prevent leaking, Para film was aligned on all sides of the set apparatus. To make sure there was no air bubble between the membrane and gel, a glass rod was lightly rolled on. More 20X SSC buffer was poured onto this membrane. A filter paper was placed onto the membrane and also a thick stack of whattman paper was placed on top. Heavy weight was placed on top of this to create pressure. This apparatus was left overnight to allow transfer after making sure there was enough SSC buffer in the tray. To minimize evaporation whole apparatus was covered with saran wrap.

After 18 hours, the membrane was placed onto a filter paper and allowed to cross link in a UV chamber twice at $12,000 \text{ Js}^{-1}$ for 15 sec. The membrane was then placed in a special BRL bag and sealed for storing at $-20 \text{ }^{\circ}\text{C}$.

Probe synthesis and Hybridization: To label the probe, GIBCO BRL RadPrime DNA labeling System was used. The DNA was denatured by heating for 5 mins in boiling water and immediately cooling on ice. Then to the tube 1 μl of 500 μM (dATP,

dGTP, dTTP) mix was added along with 20 μ l 2.5X random primers solution, 5 μ l $\alpha^{32}\text{P}$ -dCTP, and water to make final volume 49 μ l and 1 μ l klenow fragment. The tube was centrifuged for 15 to 30 sec and kept at 37 °C for 10 mins. Then 5 μ l of stop buffer was added. This mixture was loaded onto a sephadex G-25 column the same way as was done for the primer extension to remove unlabeled RNA. The membrane was prehybridized in 20 ml of prehybridization buffer (2 X SSC, 0.1 % SDS, 0.5g BSA) for 3 and half hrs. Then 50 μ l of the labeled probe was added to the tube and allowed to hybridize at 42.5 °C overnight. The membrane was washed twice with 1% SDS the next day for 30 mins each time. Next the membrane was washed with 0.1% SDS for 15 mins and carefully take out onto saran wrap without allowing to dry. The membrane was then placed into a pre desensitized phosphoimager plate and left overnight at room temperature before scanning.

2.7. Primer Extension & Sequencing:

^{32}P / ^{33}P end Primer Labeling: Primer (Table 2.) was labeled with $\gamma^{32}\text{P}$ -ATP (3000 Ci/mmol), with help of T4 PNK polynucleotide kinase. The reaction was set up in 10 μ l volume at 37 °C for 20 minutes and then at 90 °C for 2 mins. Then 40 μ l of DEPC treated water was added. A G25 column was spun down in a plastic tube at 1000 rpm for 1 min and the drain was discarded. The column was spin again and now placed into a new holder tube. The labeled primer was now added to the packed column and centrifuged at 1000 rpm for 4 mins. The liquid flow through or drain is stored at -20 °C.

Primer Extension reaction: The labeled primer was mixed with total RNA and 2X AMV Reverse Transcriptase (RT) (Promega Corporation, USA) and incubated to anneal at 58 °C for 20 mins. This mixture was cooled to room temperature for 15 mins. Then AMV primer extension buffer, sodium pyrophosphate, AMVRT and water were added to the annealed sample and extension was allowed at 42 °C for 45 mins. Then 10 µl of loading dye was added to the tube and stored at –20 °C.

Sequencing reactions: Template DNA was mixed with labeled primer, sequencing buffer, *Taq* DNA polymerase (Invitrogen Inc., USA), water and nucleotide (A/T/G/C one in each tube). This reaction was denatured at 95 °C for 2 mins and 95 °C for 30 sec, annealed at 54 °C for 30 sec and extended at 70 °C for 1 min. Thirty cycles were preprogrammed in the PCR machine and at the end of the cycles; stop solution was added to each tube and stored at –20 °C.

Urea-polyacrylamide gel electrophoresis (6% Sequencing gel): The gel apparatus was first thoroughly cleaned and dried. Then the plates of the apparatus were fixed together after choosing required size comb and spacers, carefully to make sure there is no chance of leakage while pouring the gel. The sides of the glass plates were taped together if required. For a large gel, 80 ml of solution was required to pour a gel. 66 ml of 6% sequencing gel solution (SequaGel, National Diagnostics, GA, USA) was mixed with 14 ml of buffer reagent. Then ~300 µl of 10% APS (ammonium per sulfate) was added to this solution and mixed properly. Immediately this solution was poured into the gel apparatus with help of pipette carefully such that not bubbles were allowed to form. The gel was allowed to solidify before loading the samples.

The tubes containing the amplified samples were thawed. 15 μ l of the loading dye was added to each tube and heated at 85 °C for 5 mins. 5 μ l of this mixture was loaded on 6 % urea-polyacrylamide gel (SequaGel, National Diagnostics, GA, USA) and was run at 64 watt using 1X TBE (Tris-borate-EDTA, pH 7.0) as running buffer until the bromophenol blue dye front ran out of the gel. The gel was fixed in fixing solution (15 % ethyl alcohol, 5 % acetic acid) for 30 mins and dried in gel drier (Slab Gel Drier SGD4050 equipped with ThermoSavant UVS400 Universal vacuum systems, USA) for 2 hrs. The gel was then exposed to a phosphor imager film (Fuji film imaging plate, 35 X 43 cm) and kept in a closed cassette overnight. The film was scanned by a phosphor imager scanner (Fuji films FLA3000, Fuji medical systems, USA) and the gel was exposed to X ray film. The X ray film was developed after few days (depending upon the intensity of the radioactivity). Both the scanned image and the X ray plate were analyzed and band intensity was measured using Fuji Image Gauge 3.46 software.

Loading the gel: The gel was clamped onto a vertical gel apparatus and running buffer 1X TBE was poured into both the upper and lower chambers of the apparatus. The gel was checked for any leakages. Then the wells in the gel were washed to make free of any gel material. The samples, both of primer extension and sequencing, were heated at 95 °C for 3 mins and cooled on ice and then loaded into the wells of the gel. The gel was prerun at 35 watts and then actually run at 64 watts using 1X TBE (Tris-borate-EDTA, pH 7.0) as running buffer until the bromophenol blue dye front ran out of the gel. The gel was fixed in fixing solution (15 % ethyl alcohol, 5 % acetic acid) for 30 mins and dried in gel drier (Slab Gel Drier SGD4050

equipped with ThermoSavant UVS400 Universal vacuum systems, USA) for 2 hrs. The gel was then exposed to a phosphor imager film (Fuji film imaging plate, 35 X 43 cm) and kept in a closed cassette overnight. Using a phosphor imager scanner (Fuji films FLA3000, Fuji medical systems, USA), the film was scanned and the gel was exposed to X ray film. The X ray film was developed after few days (depending upon the intensity of the radioactivity). Both, the scanned image and the X ray plate were analyzed and with the help of Fuji Image Gauge 3.46 software, the band intensity was measured.

2.8. Survival Assays

The strains to be studied were transformed with pBR322 and purified. The IRP011 was additionally transformed with pANA001 (plasmid with wild-type copy of the *barA* gene) and *luxS14*. The transformed strains were grown overnight in LB media with pH 7.0 and respective antibiotics. Subcultured samples were allowed to grow to midlog phase and then re-subcultured ($O.D_{600} \sim 0.05$) into media with neutral pH. Then the cells were allowed to grow till an $O.D_{600} \sim 0.2$ and then they were split into different flasks. One set of flasks was with pH 7.0 (control) and the other set of flasks were with media containing different stress challenges- pH 5.0, pH 5.0 + $NaNO_2$, 5 % SDS and 1 M NaCl. At different time intervals, samples were collected, serially diluted and plated on their respective antibiotic plates. Plates were incubated overnight at 37 °C and the next day colonies were counted to determine bacterial concentration. The data were plotted on a graph to compare the percent survival with and without stress in the wild type compared to the mutants.

Sensitivity assays were also performed in microtiter plates. The bacterial cultures were grown overnight with respective antibiotics and subcultured and allowed to grow to an $O.D_{600} \sim 0.5$. Then the cultures were diluted such that the $O.D_{600} \sim 0.05$ when adding to the microtiter plate wells. Each plate had one strain. The different concentrations of the stress agent, SDS, bile salts or ethidium bromide was made in the plate by serial dilutions. The highest dilution was in column 1 and the least in 11. After diluting the stress agent in LB in the plate wells, equal aliquot of cells of equal O.D were added to each well. Column 12 was used as the negative control with neither stress agent nor any culture added. The plate was then read at both nm and nm in a micro-titer plate reader (Mediators PHL, Mediators diagnostic systems, Austria, Europe) in the chemi-luminescence intergral mode. The percentage survival was calculated. Plates were incubated at 30 °C shaker, and cell density was monitored similarly by following the absorption at 600_{nm} every 6 hours. Concentrations required to inhibit 50 % of the growth (LD_{50}) were determined.

2.9. Scanning Electron Microscopy

The surface physiology of the wt and mutants IRP011, IRP011, IRP014 and IRP015 was determined with the help of SEM. The strains were grown in LB media without shaking with two subcultures. Then the cells were washed at very low speed with 1X PBS (phosphate buffered saline) twice and handled on ice. The cells were then fixed with 2% gluteraldehyde. The cells were then post-fixed with osmium tetraoxide, sequentially dehydrated in ethanol, critical point dried, and coated with gold palladium. A Hitachi S4700 FESEM electron microscope (University of Maryland) was used to view the cell surface appendages. Individual cells were

carefully viewed at X 25,000 magnification where as clustered cells were viewed at X 15,000 magnification to see the inter-cellular communicating linkages.

CHAPTER 3

Results

3.1. Genome wide approach: Microarray Analysis

Objective: Global profiling of gene expression affected by mutations in the *barA/uvrY* genes in *E. coli*.

Introduction and Rationale: To understand microbial adaptation, the molecular details of interaction between a pathogen and its host are of prime importance because this is the basis of establishment of an infection. Global profiling of gene expression is thus one approach to assess gene expression in organisms like *E. coli*, whose complete genomic sequence is known. DNA microarray technology can measure the expression of vast number of genes in a biological sample. DNA microarrays, under any given condition with a given control, use primary sequence data to measure differentially expressed transcript levels for every gene. A gene involved in adaptation is usually transcribed only when and where its function is required. Therefore, determining conditions under which a gene is expressed would allow one to predict its function in the adaptive process. This would be true for both known genes and yet uncharacterized open reading frames (ORF's). Thus, by monitoring microbial gene expression, one can probe physiological adaptations made under various environmental stress situations, identify virulence genes, and test the effects of drugs (20).

The key principle of all microarray experiments follows hybridization of labeled nucleic acid molecules in solution, to complementary genomic sequences

immobilized on a solid surface, with high sensitivity and specificity. This facilitates parallel quantitative measurement of many different sequences in a complex mixture (91). Although several methods for building microarrays have been developed (105), in this study the experiments were done using *E. coli* MG1655 DNA Microarray glass slides obtained from Frederick R. Blattner, *E. coli* Genome Project, University of Wisconsin-Madison. These arrays are constructed by physically spotting PCR amplified gene fragments representing the entire genome of *E. coli* MG1655 strain on a microscope slide. These arrays provide a rapid method to compare the expression of all represented genes (4405 genes in duplicate), in two given sets of conditions, in a single hybridization experiment.

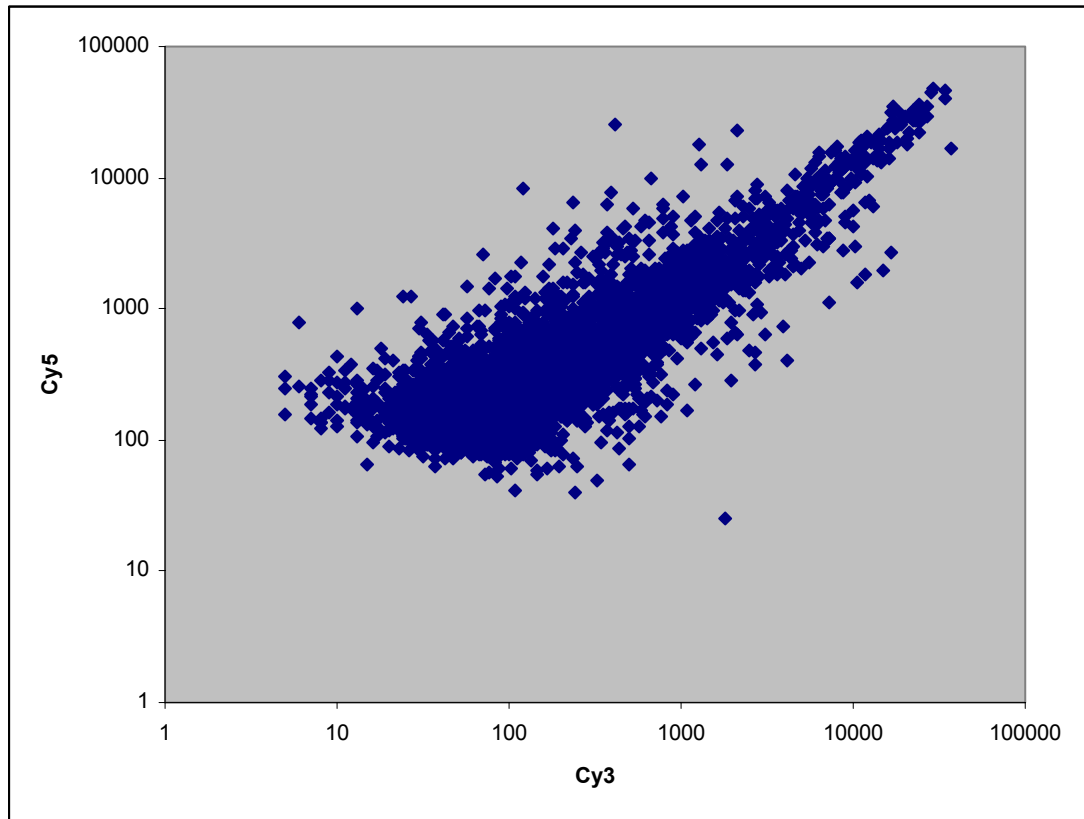
Under laboratory conditions of growth, one can study adaptive gene expression using DNA microarrays. Gene expression studies may reveal key regulatory differences that lead to differing virulence between closely related pathogen strains. For example, variations in virulence of *L. monocytogenes* serotypes have been correlated with differential transcription of PrfA-regulated virulence genes. Also microarrays have been used to identify regions of variations within the *E. coli* species from the wild type *E. coli* K12 background (5).

Microarray Hybridization and analysis: Total RNA was prepared from the wt *E. coli* MG1655 strain and the isogenic *barA*-, *uvrY*- and *barA-uvrY*- double mutants as described in materials and methods. Equal amount of RNA was reverse transcribed using different nucleotide conjugated dCTP cyanine fluorophores (dyes Cy3 and Cy5). Cy3 (green) was used in each experiment to label the wt cDNA whereas Cy5 (red) was used to label the mutant cDNA. The two-labeled cDNA mixtures were

hybridized simultaneously to one glass slide Microarray, washed and scanned using ScanArray 4000 XL scanner (Perkin Elmer).

Signal intensities for each spot representing a gene, were determined from the resulting pseudocolor image using the adaptive circle method for ScanArray Express. Quantitation is performed locally in a spot patch and pixels on each channel are selected separately. The software uses Lowess Normalization Method (locally weighted scatterplot smoothing) and calculates the logarithmic ratio of Cy5 to Cy3 for each spot on the array. A representative experimental result is presented in the scatter plot in Figure 5, which shows the average gene distribution was normal. The X-axis and Y-axis represent gene expression in the wt and mutant strain respectively, as determined from the intensities of the Cy dyes. The results indicate that the average gene expression was normal except for a subset of genes that indicated more than 2 fold induction/repression in the presence (wt)/absence (mutant) of a gene. Our preliminary results thus indicate that there is a considerable amount of difference in the level of genome wide expression between a wild type and its isogenic *barA*-, *uvrY*- and *barA-uvrY*- double mutant strains. The table shows a few of the many genes that were found to be differentially expressed. The positive values are genes over expressed in the mutant compared to the wild type, where as the negative values are the genes repressed in mutants compared to the wild type.

Figure 5. Representative Experiment Result showing normal distribution of genes



X-axis represents the wild-type MG1655 genes
Y-axis represents the mutant genes

Our aim was now to determine the effect of BarA and its response regulator UvrY on potential virulence determining genes observed to be differentially regulated in the microarray experiments. We decided to choose the *luxS* gene because I) it plays a role in regulating virulence in EPEC and EHEC (92). II) In *E. coli*, the *luxS* gene is involved in quorum sensing, a very important phenomenon playing a major role in occurrence of pathogenicity. The signaling molecule AI-2/AI-3 has been reported to regulate virulence, but who regulates AI-2/AI-3 expression is still under investigation. Both *luxS* and *pfs* genes are required for synthesis of an active AI-2 molecule. They work towards metabolic detoxification and at the same time produce the signaling molecule. We decided to verify the results from the Microarray data analysis, which show down regulation of the *luxS* and *pfs* gene in the BarA mutant, UvrY mutant and double mutant. Firstly we adopted a Real Time PCR method to examine the expressional profile of *luxS* gene in different mutant strains and to provide information on differential expression patterns. Secondly, we decided to use Northern Blot analysis to verify differential gene expression in wild type *E. coli* and its isogenic *barA*-, *uvrY*- and *barA-uvrY*- mutants. We further performed primer extension studies to determine the location and the level of transcripts of the *luxS* gene.

Table 2. Partial Table for differentially regulated genes

Gene	ID	BarA-	UvrY-	BarA-	Function	
Virulence determinants						
Pfs	b0159	-2.4 ^a		-2.0	enzyme,Al-2 sysnthesis	S-adenosylhomocysteine nucleosidase
YgaG	b2687	-5.0		-1.5	enzyme,Al-2 sysnthesis	autoinducer 2 synthase
flhDC regualted						
MglA	b2149	-2.8	-8.0	-4.5	transport; small molecules: Carbohydrates, organic acids, alcohols	ATP-binding component of methyl-galactoside transport
MreD	b3249	-8.9	-4.7	-2.7	structural component; Murein sacculus, peptidoglycan	rod shape-determining protein
NrfA	b4070	-2.4		-7.5	carrier; Energy metabolism, carbon: Electron transport	periplasmic cytochrome c(552): nitrite reduction
Pta	b2297	-1.8	-4.3	-4.9	enzyme; Degradation of small molecules: Carbon compounds	phosphotransacetylase
OppA	b1243	-3.2	-2.2	-2.2	transport; Protein, peptide secretion	oligopeptide transport; periplasmic binding protein
OmpT	b0565	7.4 ^b	2.0	2.0	enzyme; Outer membrane constituents	outer membrane protein 3b (a), protease VII
Male	b4034	4.1	12.5	5.4	transport;small molecules: Carbohydrates, organic acids, alcohols	periplasmic maltose-binding protein; transport chemotaxis
Cell surface and outer membrane determinants						
OmpT	b0565	7.4	2.0	4.0	enzyme; Outer membrane constituents	outer membrane protein 3b (a), protease VII
OmpF	b0929	3.0		3.9	membrane; Outer membrane constituents	outer membrane protein 1a (1a;b;F)
FhuA	b0150	5.5		13.9	membrane; Outer membrane constituents	omp receptor - ferrichrome, colicin M, phages T1, T5, phi80
LpxD	b0179	5.2		1.6	enzyme; Surface polysaccharides & antigens	UDP-3-O-(3-hydroxymyristoyl)-glucosamine transferase
FlgL	b1083	3.7		2.0	structural component; Surface structures	flagellar biosynthesis; hook-filament junction protein
Transport proteins						
AccB	b3255	-2.4	-4.0	-2.0	carrier; biotin carboxyl carrier protein (BCCP)Biosynthesis of cofactors	acetylCoA carboxylase, BCCP subunit; carrier of biotin
Male	b4034	4.1		6.5	transport;small molecules: Carbohydrates, organic acids, alcohols	periplasmic maltose-binding protein; transport,chemotaxis
Other metabolic pathways						
Fbp	b4232	-4.0	-7.7	-5.6	enzyme; Central intermediary metabolism: Gluconeogenesis	fructose-bisphosphatase
PckA	b3403	-2.7	-2.0	-2.0	enzyme; Central intermediary metabolism: Gluconeogenesis	phosphoenolpyruvate carboxykinase
FadA	b3845	-2.4	-5.4	-2.0	enzyme; Degradation of small molecules: Fatty acids	thiolase I; 3-ketoacyl-CoA thiolase; acetyl-CoA transferase
NirC	b3367	-1.7		-2.4	enzyme; Energy metabolism, carbon: Anaerobic respiration	nitrite reductase activity
MurE	b0085	-5.7		-2.6	enzyme; Murein sacculus, peptidoglycan	meso-diaminopimelate-adding enzyme

Negative numbers indicate (^a) genes downregulated, and positive (^b) genes upregulated in mutant compared to the wild type.

3.2. Objective: To determine if the BarA/UvrY system in *E. coli* differentially regulates the expression of *luxS* gene.

Introduction and rationale: Recent studies with DNA arrays have implicated AI-2 in regulation of a large number of genes in *E. coli* (94, 20). BarA/UvrY, a putative global regulatory system, might have a role in regulating one or more physiological properties by regulating *luxS* expression through quorum sensing mechanism. Preliminary results from the Microarray hybridization show that the *ygaG* (*luxS*) gene is down regulated in the mutants compared to the wild-type *E.coli* strain. This led us to further study if at all then how the BarA/UvrY system may influence the *luxS* gene expression. Our other preliminary chemiluminescence studies done in the lab (data not shown) show that BarA regulates AI-2 accumulation, required for quorum sensing.

3.2.1. RT-PCR

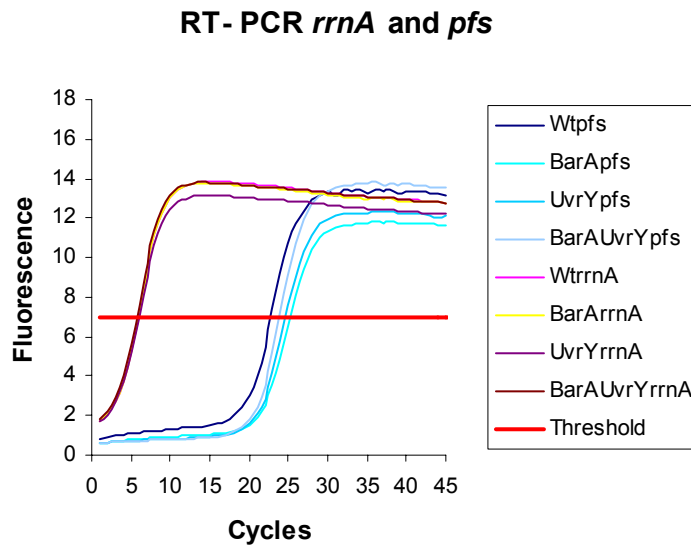
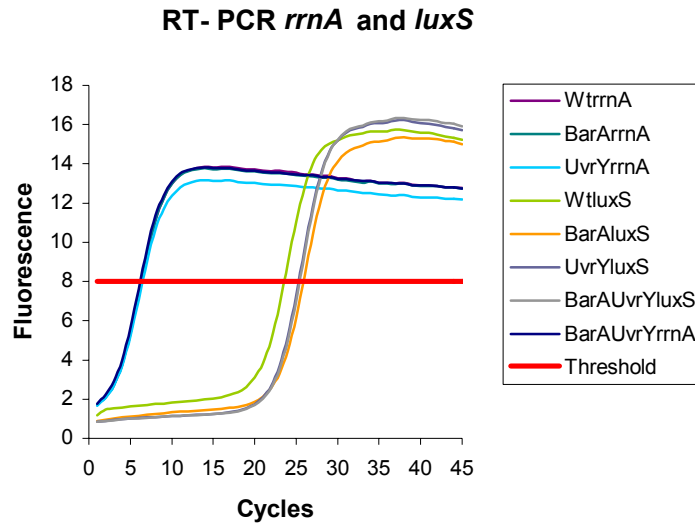
Sensitive methods for detection and analysis of small rare mRNA transcripts or other RNA present in low abundance are important aspect of most cell/ molecular biology studies. RNA cannot serve as a template for PCR, so it must be reverse transcribed into cDNA. Quantification of transcripts can be achieved either by real-time (RT)-PCR monitoring of product formation or competitive RT-PCR followed by gel analysis. The LightCycler (Roche Applied Science, Germany) allows detection of the PCR product during the entire course of amplification. Thus, sequence-specific detection is ensured by the use of internal hybridization probes and the kinetics obtained during the exponential phase of PCR is used for quantification. As a control, a ubiquitously expressed internal housekeeping gene is usually quantified at the same time, and the

number of copies of the gene of interest is normalized against the number of copies of the housekeeping gene. To determine temporal gene expression in bacteria, quantification of the 16S rRNA is often used as a reference. The advantage of the LightCycler is that several samples (upto 32) can be run at a time and the results are calculated directly by the integrated software. However, sources of error may include unspecific amplification or additional amplicons that do not hybridize to the fluorescence-labeled probes but compete with the specific PCR. Therefore, the PCR products have to be analyzed on agarose gels and, in cases of multiple bands; the PCR conditions should be optimized to avoid false priming. This method is useful for experiments where multiple transcripts have to be analyzed from the same RT reaction or for specific applications such as differential display reverse transcription (DDRT) or for Rapid amplification of cDNA Ends (RACE).

The standard protocol for quantitative LightCycler PCR utilizing 35 cycles of denaturation, annealing and extension, with fluorescence measured at the end of each extension segment was used. An additional step of melting curve analysis was added at the end to circumvent primer dimer interference. Fluorescence was measured continuously during the melting curve, ensuring that only the fluorescence of the desired amplicon is detected at the high melting temperature, as the primers are single stranded. After the run was over the PCR product was run on a 1 % agarose gel to make sure the product was of the correct size. The fold difference in *luxS* and *pfs* transcript levels were calculated using the formula described in materials and methods. The threshold value was selected in the linear range of all the samples. The calculated differences are shown in

Table 3. Compared to the wt, *barA*-, *uvrY*- and double mutants have more than 3 fold less transcripts.

Figure 6. RT-PCR Results



Real time PCR results using LightCycler.
 X-axis represents PCR cycle number
 Y-axis represents Fluorescence in RLU (Relative light units)

Table 3. The BarA-UvrY two-component system regulates *luxS* and *pfs* mRNA as determined by quantitative RT-PCR.

Strain	Relevant Genotype	C _t values ^a			Fold ^b Difference
		<i>rrnA</i>	<i>luxS</i>	<i>pfs</i>	
MG1655 <i>Δlac</i>	wild type	6.5 ± (0.5) ^c	23.5 ± (0.6)	23.0 ± (0.5)	1.0
IRP011	<i>barA::kan</i>	6.5 ± (0.5)	26.0 ± (0.5)	25.5 ± (0.6)	5.6 / 5.6 ^d
IRP014	<i>uvrY::cm</i>	6.5 ± (0.5)	25.5 ± (0.6)	25.5 ± (0.6)	4.0/ 4.7
IRP015	<i>barA::kan, uvrY::cm</i>	6.5 ± (0.5)	25.5 ± (0.6)	24.5 ± (0.6)	4.0/ 2.8

^a C_t values are the threshold values of PCR cycles where the SYBR-Green fluorescence was detected above the background in the linear range, taken at 7.0 Relative Light Units.

^b The fold down-regulation is calculated as $2^{-\Delta\Delta C_t}$. Where $\Delta\Delta C_t = \Delta(C_{t \text{ sample}} - C_{t \text{ rrnA}}) - \Delta(C_{t \text{ reference}} - C_{t \text{ rrnA}})$.

^c Standard Deviation of three independent experiments.

^d Fold-difference of the *luxS* transcript / *pfs* transcript normalized with *rrnA* levels and compared to the wild type strain

We also PCR amplified the cDNA's mentioned above and stopped the amplification of one tube of each sample at 10, 12, 14, 16, 18 and 20 cycles. The amplified product was run on a 1% agarose gel. Equal quantity of template was used to start the reaction and this was confirmed by using *rrnA* primers. The gel was stained with ethidium bromide and the pixel intensities of the amplified product were calculated. This method is less reliable than the real-time LightCycler method and more time consuming too.

However, the gel picture demonstrates (Figure 7) that indeed the *luxS* and *pfs* gene transcripts are lower in abundance in absence of the BarA/UvrY system.

Figure 7. PCR

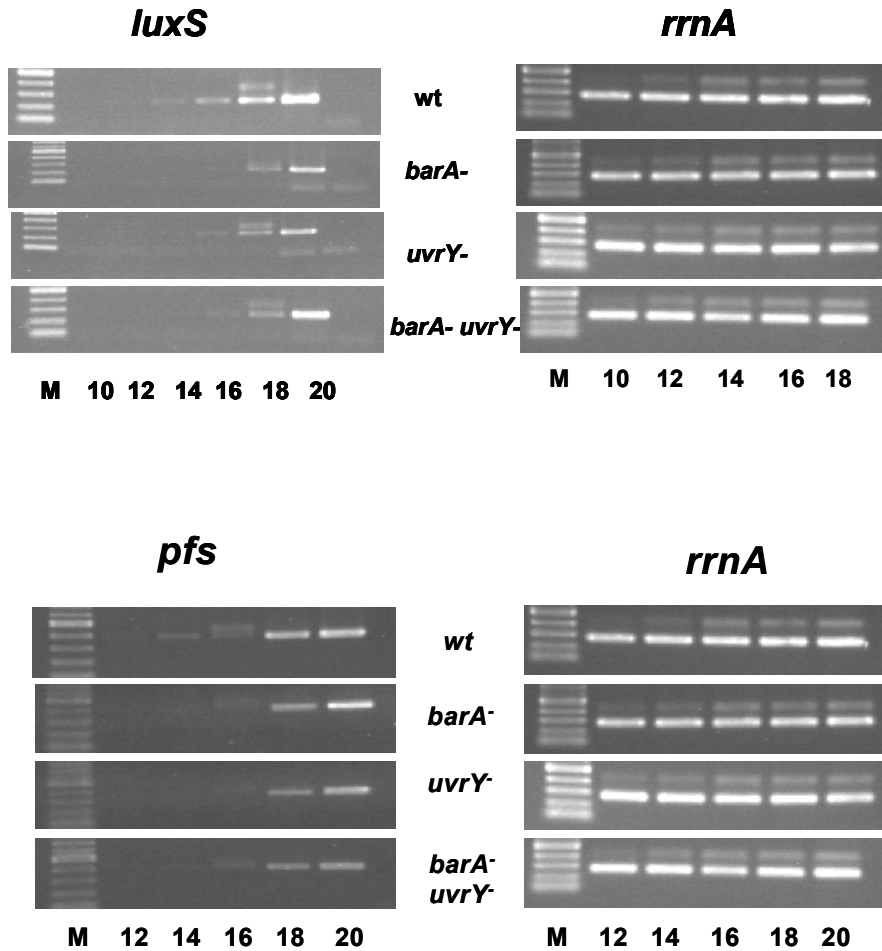


Figure represents agarose gel images, equal amount of PCR product was loaded in each lane.

Upper gels show that the *luxS* transcript starts amplifying at earlier cycles than the mutants. The lower gels show that the *pfs* transcripts start amplifying earlier in the wild type than the mutants.

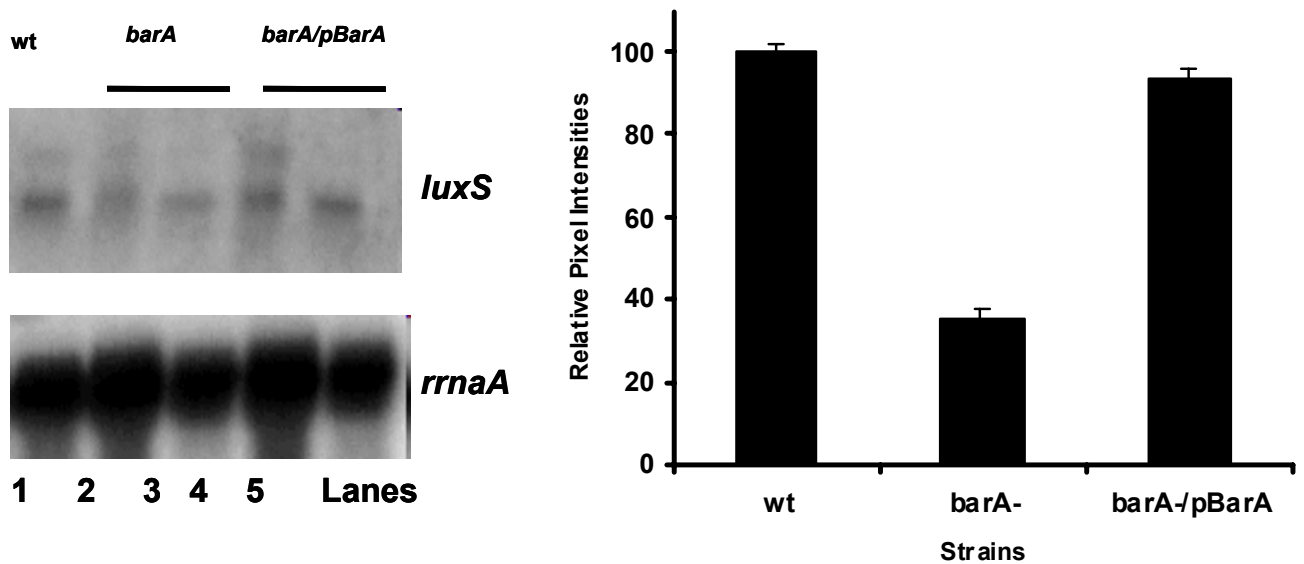
The numbers below the gels are the PCR cycle numbers and M is the marker.

3.2.2. Northern Blot Analysis

RNA was freshly isolated from wild type, isogenic *barA*- mutant and mutant complemented with a plasmid borne copy of *barA*. The RNA was run on a formaldehyde gel with MOPS buffer, transferred to a hybond nylon membrane, and hybridized with $\alpha^{32}\text{P}$ labeled *luxS* probe overnight as explained in Chapter 2. The membrane was then washed thoroughly with SDS and placed over a pre-desensitized phosphorimager plate and left overnight at room temperature.

Next day the plate was scanned to get a digitized image. The bands were then quantified similarly as in primer extension using FUJIFILM Image Gauge version 3.46 under the quant mode. The wt strain displayed a three fold higher mRNA expression than the *barA*- mutant. In other words, compared to the wt, in the *barA*-mutant, a 3 fold down regulation of the *luxS* transcript was observed, which was complemented by wild-type copy of the *barA* gene on a plasmid.

Figure 8. Northern Blot Analysis



a. The digitized image from the northern blot. Upper spots represent the *luxS* transcript and the lower spots are the internal loading control *rrnA* transcripts.

b. Represents the quantitation results from the digitized image as calculated by relative pixel intensities.

3.2.3. Primer Extension studies

Primer extension is used to map the 5' ends of mRNA fragments and determine the putative transcription start sites. It is done by annealing a specific oligonucleotide primer to a position downstream of 5' end as explained in materials and methods. This is extended with reverse transcriptase, which can copy RNA template, making a fragment that ends at the 5' end of the template molecule. Sequencing reactions as well as the primer extension reactions were run on a 6 % sequencing gel.

The gel was exposed to phosphor-imager plates for 24 hours and the plates were scanned to see the digitized image of the gel. The *barA*- mutant showed 2 fold fewer *luxS* transcripts than the wt visually. The bands from the digitized image were quantitated using FUJIFILM Image Gauge version 3.46 under the quant mode. The *luxS* transcript was found to be expressed two to three folds higher in the wt compared to the mutants as seen from the quantitation. We observed more than one putative transcription start sites, shown marked in the figure below. We quantitated the relative strengths for the putative transcription start sites from the digitized image. The results have to further be confirmed by using different sets of primers. One of the four may be a constitutive promoter and the others may be inducible.

Figure 9. Primer Extension Results

Figure 9.A. Relative promoter strengths

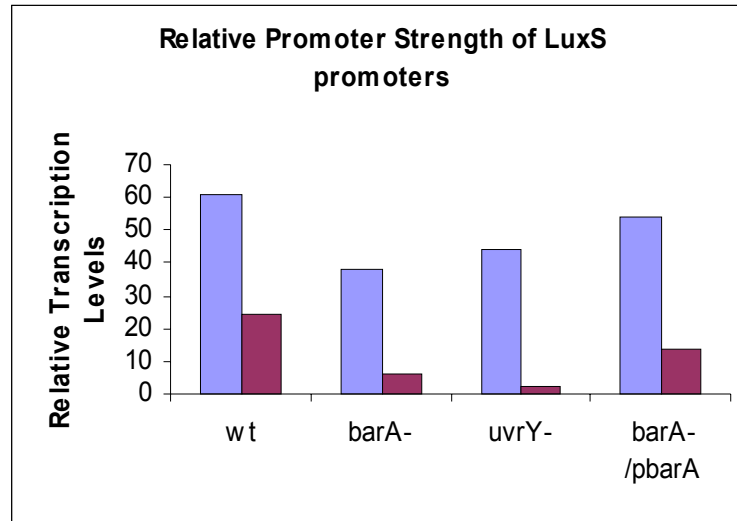


Figure 9.B. Putative transcriptional start sites in sequence

GCATTTGCAGAAGCCTACCGTAATCTGCTGCGTGAAGAGCCGCTGGAAATTCTGC
 GCGAAGAGGATTTTGTAGCCGAGCGCGAGGCGTCTGAACGCCGTCAGCAGGAAA
 TGGAAAGCCGCTGATACCGAACGTTTTCGCGTGTGGCTGGAAAAACACGCCT**GAC**
AGAAAAGAAAAAGGCCACTCGTGAGTGGCCAAAATTTTCATCTCTGGAATTCAGGG
 AT **GATGATAA** CAAATGCGCGTCTTT **CATATA** CT **CAGACTCGC** CTGGGAAGAAAG
 -10
AGTTCAG AAAATTTTAAAAAA **ATTACCGGA** GGTGGCTAA **ATGCCGTTGTTAGA**
 TAGCTTCACAGTCGATCATACCCGGATGGAAGCGCCTGCAGTTCGGGTGGCGAAA
 ACAATGAACACCCCGCATGGCGACGCAATCACCGTGTTTCGATCTGCGCTTCTGCG
 TGCCGAACAAAGAAGTGATGCCAGAAAGAGGGATCCATACCTGGAGCACCTGTT
 TG

9.A. Relative promoter strengths calculated from the digitized image represented in pixel intensities.

9.B. Promoter region of *luxS*, putative transcription start site are shown as boxes.

3.2.4. Reporter gene analysis

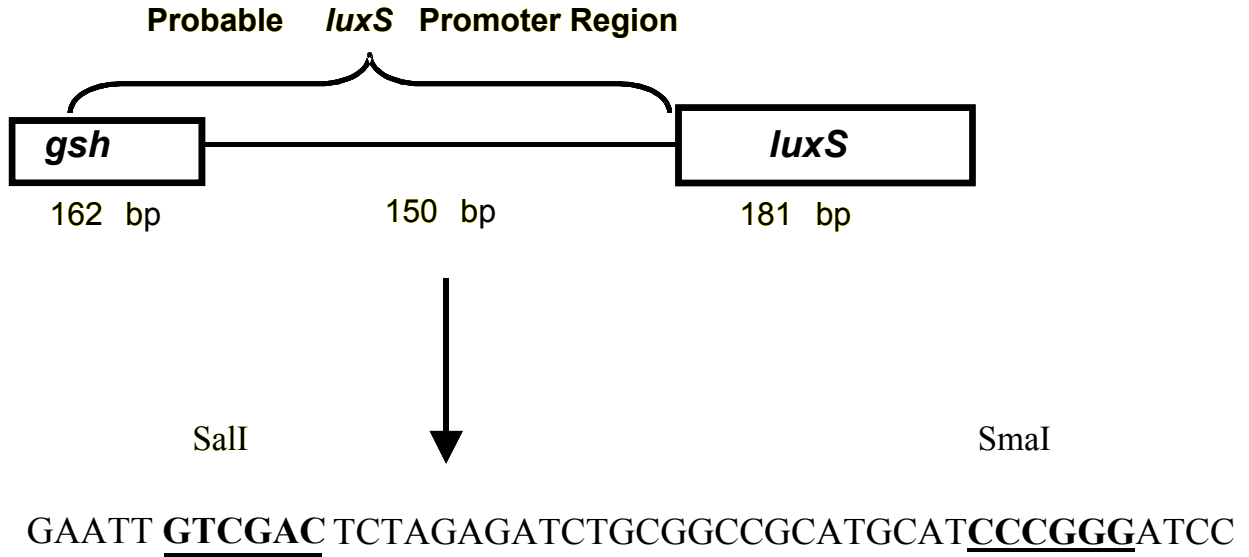
To validate the above results obtained from RT-PCR, Northern blot studies, and primer extension, we adopted a genetic approach by creating a plasmid borne transcriptional fusion of the *luxS* and *lacZ* gene and moved this construct to the chromosome of the wt, isogenic *barA*-, *uvrY*- and *barA-uvrY*- double mutants. The chromosomal copy of the *luxS* fusion with the reporter gene would allow us to compare the difference in amount of *luxS* transcript formed in wt and the mutants with help of expression studies.

The strategy used for preparing the *luxS::lacZ* fusion is shown in Figure 10.A. The *luxS* gene was cloned into the multiple cloning site of vector pSP417 using *SalI* and *SmaI* sites before a promoterless reporter gene, *lacZ*. This construct was transformed into competent cells. The plasmid was isolated from the transformants and cut with the same enzymes to make sure the ~500bp insert was ligated into the vector.

As shown in the Figure 10.B, the transformants harboring the construct showed ~2 log fold more regulation of the reporter gene. The plasmid DNA was then sequenced to check for mutations and to further confirm the *luxS* gene insert.

Figure 10. *luxS::lacZ* cloning and expression

A.



Multiple Cloning Site in pSP417 (Podkovyrov & Larson, 1995)

B.

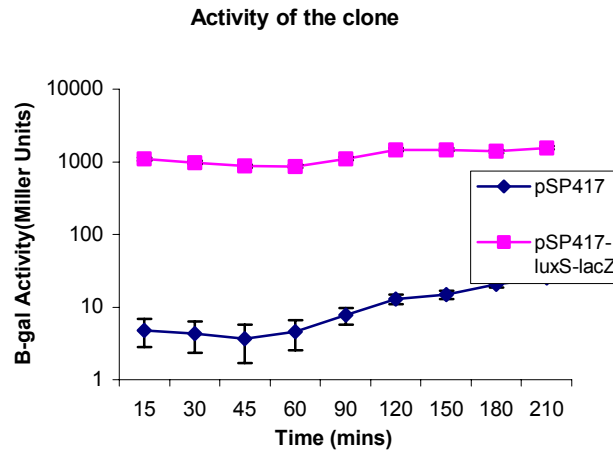


Fig 10A. Multiple cloning site in pSP417 used to clone the promoter region of *luxS* in a promoterless *lacZ* vector.

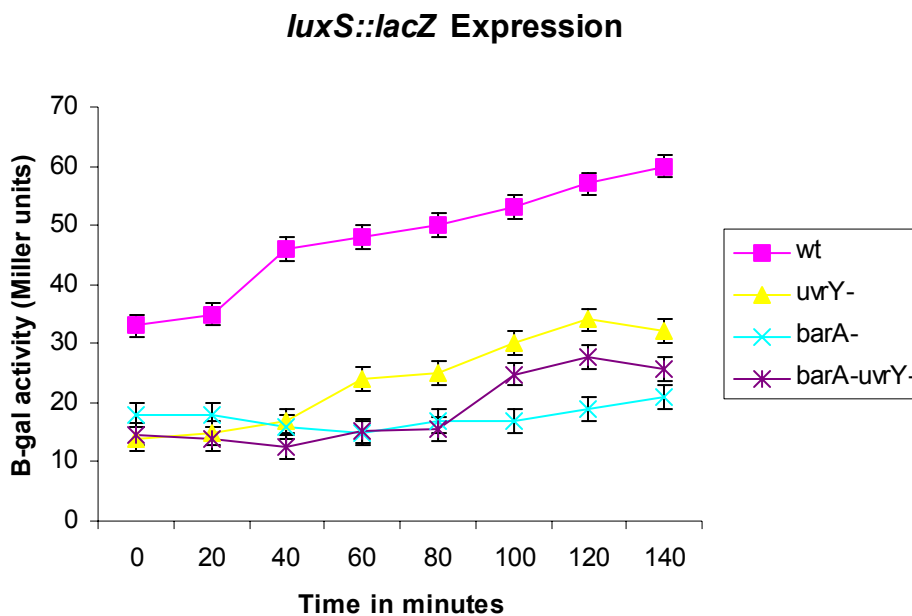
10B. β -galactosidase assay performed to check the activity of the clone.

This construct was then moved into the chromosome of the wt and mutant strains with help of λ -phage system as described in materials and methods. The fusion would have integrated at the *att* site in the *E. coli* chromosome. The pure merodiploid colonies were stored as well as screened for selecting monolysogens. Ter assay or Immunity i^{434} assay was used to screen monolysogens. DJ140 (λi^{434}), which shows plaques with lysogens with more than one *cos* site (multiple lysogens) and not monolysogens, was used in this assay as control and LE392 cells were used as host. The lysogens, which showed colonies with both DH140 and LE392, were not used for further purification. The monolysogens were screened and stored for further use. The wt, isogenic *barA* mutant, isogenic *uvrY*- mutant and the double mutant now carrying the fusion were used for further *luxS* expression studies.

The growth of the strains carrying chromosomal *luxS-lacZ* fusion were studied under various media conditions. The expression of the *luxS* gene was studied in presence and absence of 0.1 %glucose, 60 mM acetate and 500 mM NaCl in the SM1005 (wt) and isogenic mutants SM1006, SM1007, SM1009. This was done to study if BarA/UvrY play a role in regulating *luxS* expression in presence of different metabolic regulator. The mutant strains were complemented with wild type copy of the gene and the β -galactosidase assay was performed to verify the mutants (data not shown).

Figure 11. Expression of chromosomal copy of *luxS::lacZ*

a.



β -galactosidase activity to study the difference in *luxS::lacZ* expression between wild type and mutant strains.

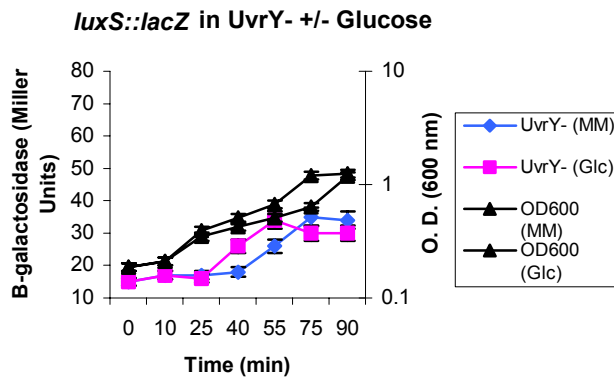
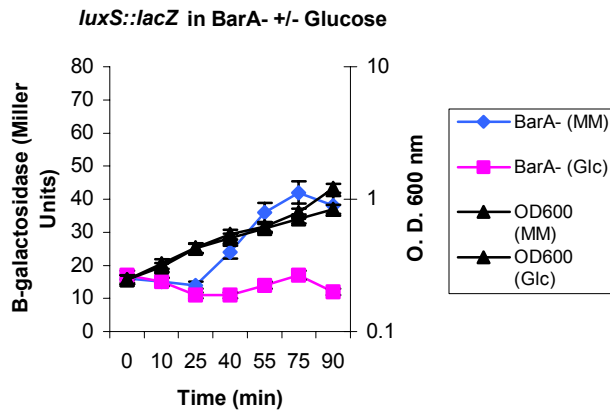
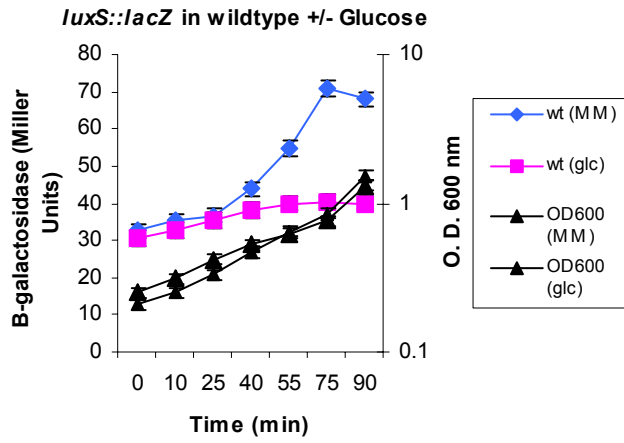
b. Expression of *luxS::lacZ* in wild type compared to mutants in presence of metabolic regulators.

Strain	Relevant Genotype	Fold Induction ^a	
		60mM Na-acetate	500mM NaCl
SM1005	wild type, <i>luxS-lacZ</i>	2.3 ± (0.2) ^b	2.0 ± (0.2)
SM1006	<i>barA::kan</i> <i>luxS-lacZ</i>	1.5 ± (0.3)	1.7 ± (0.6)
SM1007	<i>uvrY::cm</i> <i>luxS-lacZ</i>	1.5 ± (0.5)	1.9 ± (0.4)
SM1009	<i>barA::kan</i> , <i>uvrY::cm</i> <i>luxS-lacZ</i>	1.8 ± (0.6)	1.9 ± (0.2)

^a Fold induction calculated by formula, $\frac{\text{induced}}{\text{uninduced}}$

^b Standard deviation of two independent experiments

c.



3.3. Objective: To determine if the *barA/uvrY* system in *E.coli* regulates motility via flagellar gene expression

Introduction and rationale: Earlier literature indicates flagellar synthesis and motility are regulated by type IV pili, type III secretion pathways and quorum sensing (32, 94). The BarA/UvrY two-component system has been shown to have important roles in motility and virulence of different organisms including *Salmonella* species (BarA/SirA) (34), *P. aeruginosa* (GacS/GacA) (85) and *L. pneumophila* (LetA/LetS) (39). These systems being homologous to BarA/UvrY, may suggest that the BarA/UvrY two-component system may have an important role in motility. The direct role of BarA in the motility of *E. coli* is yet unknown. We observed from the preliminary data of the microarray analysis that certain flagellar genes and stress response genes in the *barA* mutant shows a difference in expression profile compared to the wild-type. LuxS mediated quorum sensing has a regulatory effect on motility (94). Our preliminary chemiluminescence studies show that BarA regulates AI-2 accumulation, required for quorum sensing. Also we have seen in our laboratory that BarA has a regulatory role in biofilm formation in *E. coli* K-12 by altering overall surface properties. Thus, it may be reasonable to hypothesize that BarA may have a regulatory effect on motility by modulating quorum sensing and surface properties. Our aim was thus to first determine the effect of BarA and its response regulator UvrY on motility of *E. coli* physiologically by measuring the diameter of swimming of the bacteria on soft agar. Secondly we adopted another physiological approach coupled to a molecular approach to study the flagellar gene expression by measuring reporter gene activity on a fusion vector. Thirdly, we decided to study the presence of

cell-surface appendages on the surface of wild type *E. coli* and isogenic *barA*⁻, *uvrY*⁻ and *barA-uvrY*⁻ mutants.

3.3.1. Motility assay on TB media

We studied the swimming motility of the *E. coli* MG1655 Δ lac (wild type), *barA*⁻, *uvrY*⁻, *barA*⁻/pANA001, *barA*⁻/*luxS* *uvrY*⁻/*puvrY*, *uvrY*⁻/*luxS* and *barA-uvrY*⁻ strains on 0.25 % Tryptone broth (TB) agar plate. The experiment was repeated thrice under similar conditions and comparable results were obtained.

After incubation at room temperature for 20 hours, the wt *E. coli* cells were found to swim through the soft agar creating a zone of cloudy growth, the diameter of which was measured with a ruler. The zone of growth created by swimming motility of wt was observed to be larger than *barA*⁻, *uvrY*⁻ and *barA-uvrY*⁻ (Figure 12). In the isogenic *barA*⁻ mutant, the zone of swimming was decreased by ~2 times, which was complemented when wild type *barA* gene or *luxS* gene was expressed from a plasmid in trans. This suggests *barA* may regulate motility in a *luxS* independent or dependent way.

In the isogenic *uvrY*⁻ mutant, the zone of swimming was also decreased by ~1.5 times and was infact further decreased when wild type *uvrY* or *luxS* gene was expressed from a plasmid. These results support the fact that UvrY doesnot have a direct role in binding and regulating flagellar gene expression (101). Thus, *uvrY* may regulate motility via both a CsrA/CsrB/CsrC independent and dependent pathway.

Figure 12. Swimming motility in soft agar

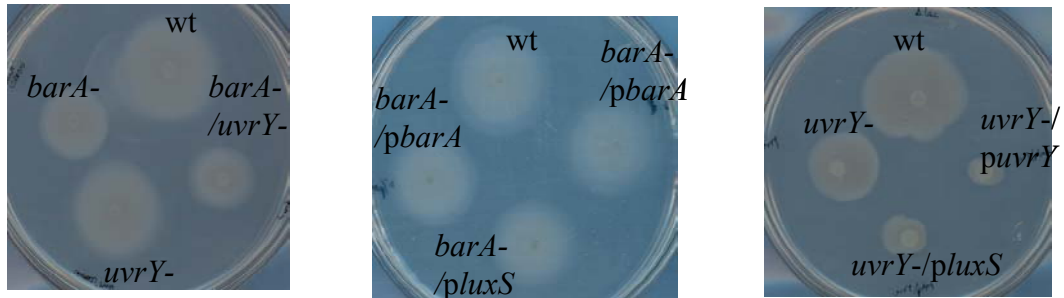


Table 4. Swimming motility results

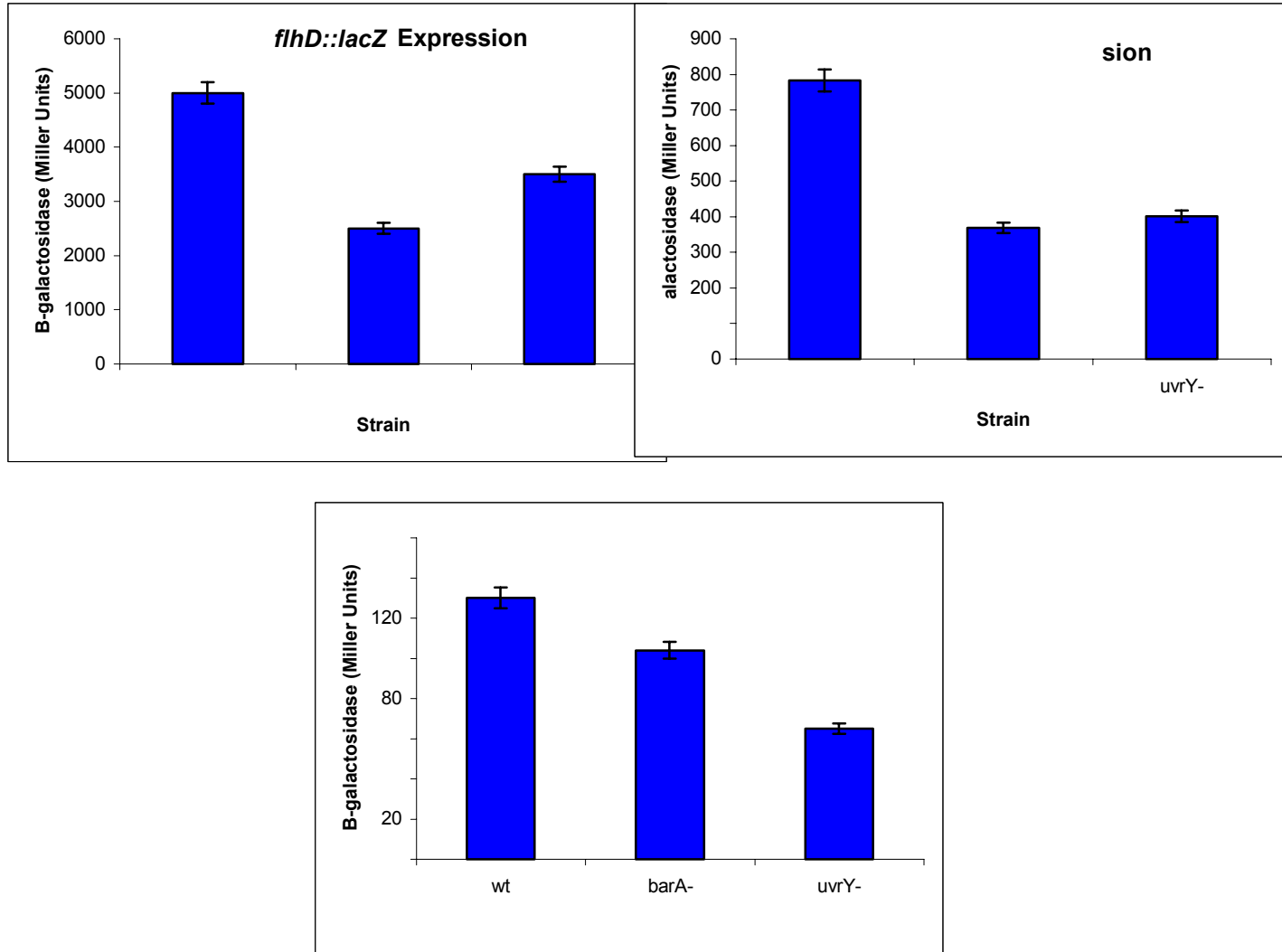
Strains	Average Diameter of growth region due to swimming (mm)
Wild type	34.0 ± 0.5
<i>barA</i> -	16.5 ± 0.5
<i>uvrY</i> -	28 ± 0.05
<i>barA-uvrY</i> -	15.5 ± 0.5
<i>barA</i> -/ <i>pbarA</i>	32.5 ± 0.5
<i>barA</i> -/ <i>pluxS</i>	33 ± 0.5
<i>uvrY</i> - / <i>puvrY</i>	0.5 ± 0.25
<i>uvrY</i> -/ <i>pluxS</i>	18.0 ± 0.5

3.3.2. Flagellar gene expression

Introduction and rationale: We transformed *E. coli* MG1655 Δ *lac* (wt), with a reporter gene fusion vector pVS182, pVS183 and pFDCZ6 (a kind gift from Vanessa Sperandio). Amp^R transformants were selected and restreaked to purify for future use. The strains were grown overnight and subcultured twice before sampling. The experiment was repeated thrice under similar conditions and comparable results were obtained.

The overnight grown strains were subcultured twice and then samples were collected at different time intervals and the β -galactosidase activity was measured as explained in materials and methods. The *flhD::lacZ* expression, determined from the β -galactosidase activity, was found to be reduced by approximately 2.0 folds in the *barA*-, 1.5 fold in *uvrY*-, mutants as compared to the wild type *E. coli* cells (Figure 13). The *flhDC::lacZ* expression was found to be reduced 2.5 folds in *barA*- and 2 fold in *uvrY*-. The *fliA::lacZ* expression was seen to be reduced 1.5 fold in all the mutants as determined from the β -galactosidase activity. These results support the motility assay results, showing *uvrY*- swarming more than the *barA*-. The master regulator of flagellar operon is seen down-regulated supporting BarA/UvrY play a role in flagellar driven motility.

Figure 13. Flagellar gene expression

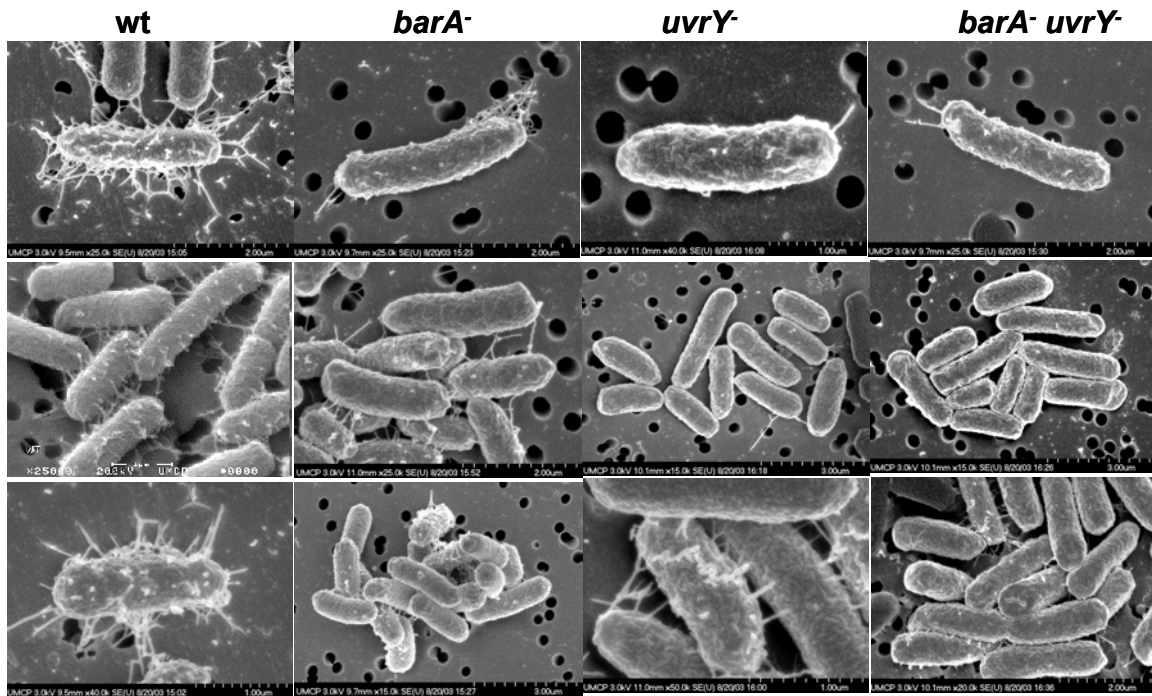


LacZ expression results to study the expression of the flagellar genes in wild type and respective mutants.

3.3.3. Electron Microscopy Studies

The *E. coli* MG1655 Δ lac (wild type), its isogenic *barA*- mutant, *uvrY*- mutant and *barA-uvrY* double mutant were grown at 37°C with very mild shaking, to avoid rupture of flagella. We used an AMRAY 1820D Scanning Electron Microscope to examine the cell surfaces of the bacteria. The SEM pictures (Figure 14) showed that the numbers of cell surface appendages on the surface of the isogenic *barA*-, *uvrY*- and *barA-uvrY*- mutants are lower compared to the wild type bacteria. Although the nature of these appendages was not characterized in this study, the results demonstrate that the scarcity of cell surface appendages may be a cause of impaired motility in the *barA*-, *uvrY*- and *barA-uvrY*- mutants of *E. coli*.

Figure 14. Electron Microscopy Study



Upper lane pictures of individual cells at X 25,000 magnification and other lanes of clustered cells at X 15,000 magnification using Hitachi S4700 FESEM electron microscope (University of Maryland) to see the intercellular communicating linkages.

3.4. Stress Assays

Objective: To determine if the *barA-uvrY* system in *E.coli* alters surface properties and plays a role in stress response.

To adapt to and survive potential stress situations, the bacteria require highly specific and temporal regulation of expression/repression of a number of sets of genes, which are probably governed by one or more global stress regulators. Studying some of these genes that may be involved in regulating survival directly or indirectly under any of the stress challenges may be highly informative. BarA protein, a sensor kinase of a two-component system, is one such potential global stress regulator. It has also been found that over expression of response regulators of bacterial two component systems confer drug resistance by controlling expression of some transporter genes (45).

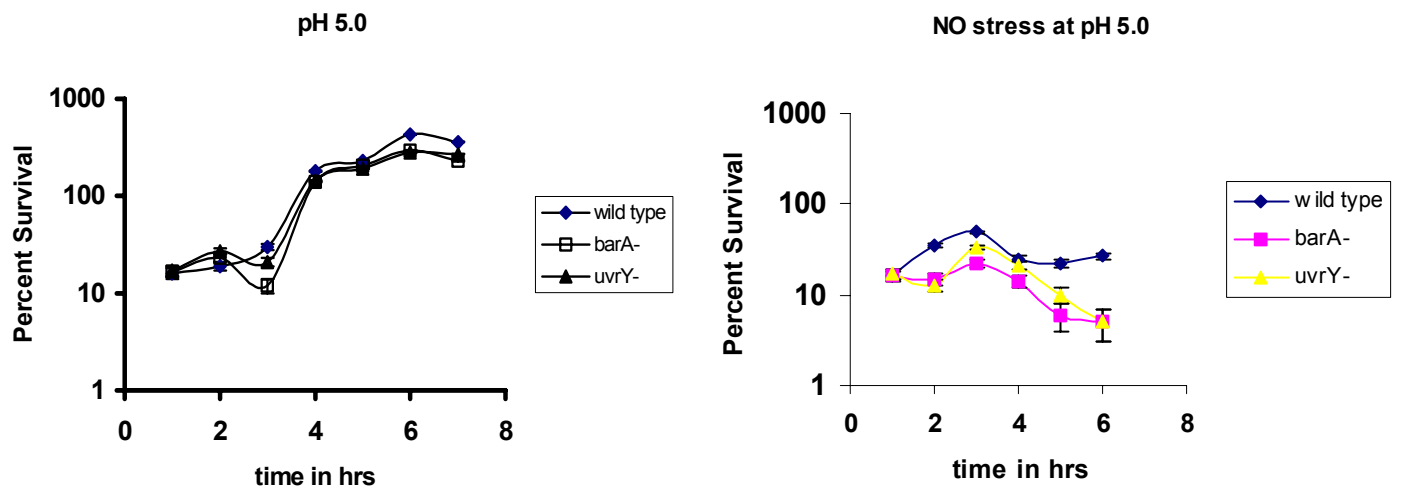
The strains were challenged with acidic pH 5.0 as that is the environment faced in the host macrophages. Along with low pH, the bacteria are challenged with reactive nitric oxide species. Therefore, we challenged the wt, *barA*-, *uvrY*- strains to NaNO₂ at low pH to see if survival under stress is affected by deletion of the *barA* or *uvrY* genes. First we titrated the minimum concentration of NaNO₂ that the *barA*-mutant would survive. We also found out that in absence of either BarA or UvrY, the strains grow as well as the wt at pH 5.0. But, when challenged with 15 mM NaNO₂ (from the titration) at that pH, the mutants are more sensitive to the NaNO₂ than the wild type.

The strains were also challenged with osmotic stress and other stressors like NaCl, Bile salts, SDS, and Ethidium bromide. NaCl was used as a charged osmotic stressor,

bile salts are uncharged osmotic stressors, SDS as a general membrane stressor and Ethidium Bromide to study charged molecules membrane transport.

Figure 15. Stress Assay Results

a.



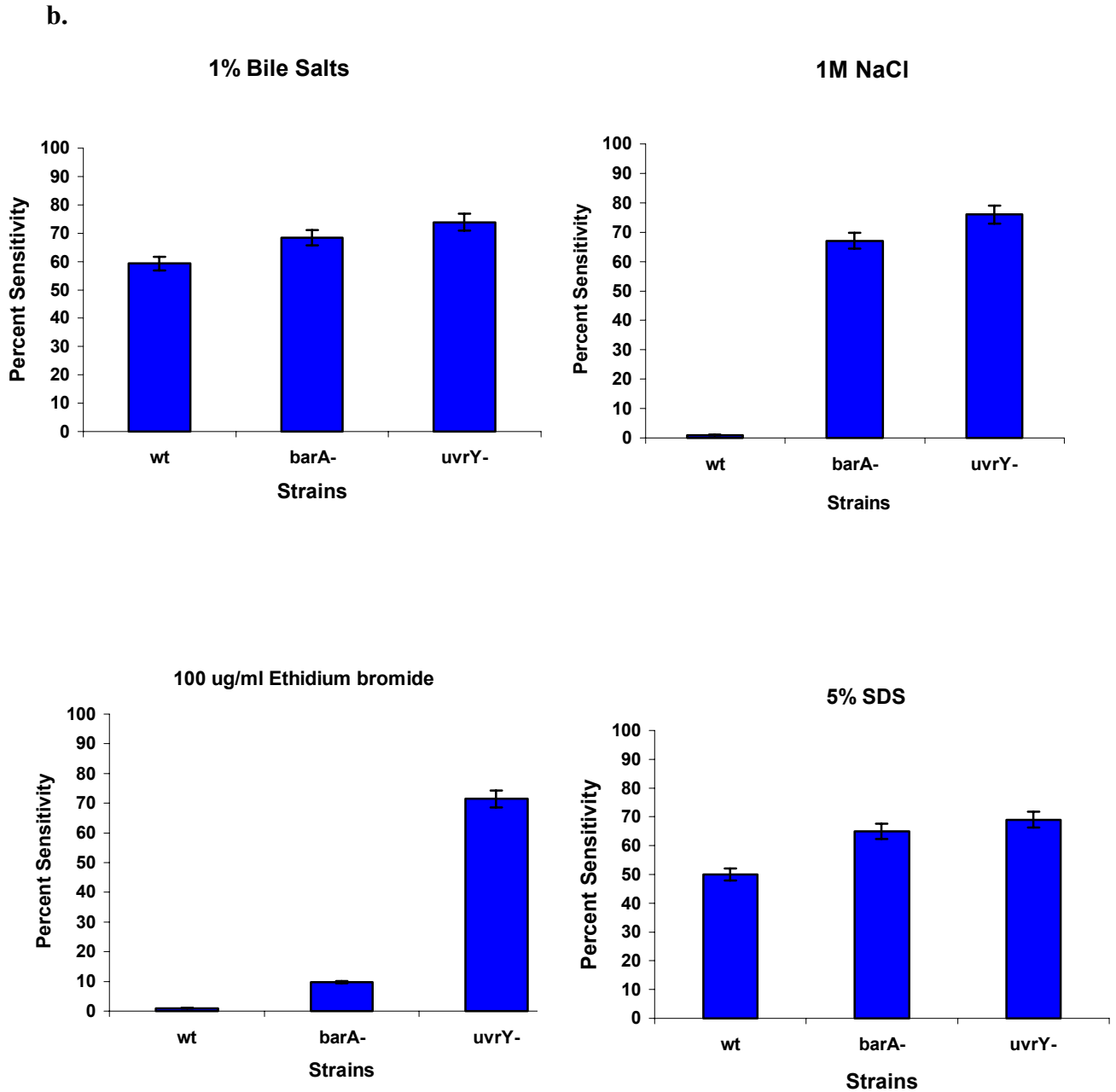


Fig.15.a. Percent survival of the different strains when challenged with pH 5.0 and pH 5.0 + NaNO₂ calculated by the formula,

$$\frac{\text{number of bacteria (at different time interval)} \times 100}{\text{number of bacteria before adding the stressor}}$$

b. Percent sensitivity of the different strains when challenged to different stressors calculated by,

$$\frac{\text{decrease in number of bacteria (at different time interval)} \times 100}{\text{number of bacteria before adding the stressor}}$$

As seen from the graphs, the *barA*- and *uvrY*- are sensitive to NaCl stress. The resistance to this osmotic stress is recovered in the *barA*- partly when wt copy of the gene is provided in trans and fully when *luxS* gene is provided in trans on a plasmid (data not shown). Thus, *barA* and *uvrY* may both play a role in providing resistance against charged osmotic stress. The *barA*- and the *uvrY*- are almost as resistant to 5 % SDS challenge as wt. BarA and UvrY may not play a significant role in resistance to general membrane stress. Further, Ethidium Bromide as seen 24 hrs post challenge, has a greater effect on the *uvrY*- mutant than the wt. UvrY may play a significant role in charged molecule membrane transport as suggested by these results. We see that bile salts do not have a great effect on the growth or survival of the bacteria in presence or absence of the BarA/UvrY.

CHAPTER 4

Discussion & Summary

BarA/UvrY two-component signal transduction system has been found in *E. coli*. Biochemical and genetic studies demonstrate direct phosphotransfer from BarA to UvrY (76) demonstrating UvrY to be the cognate response regulator for BarA. BarA in *E. coli* was reported to activate transcription of the *rpoS* gene, which encodes a sigma factor involved in the expression of stationary phase and stress response genes. UvrY, independent of BarA, has been shown to negatively regulate *rpoS* (72). The regulation of *rpoS* is very complex, but BarA regulates it probably with the help of a yet unidentified response regulator (43).

Two-component systems homologous to BarA/UvrY have been identified in other gram-negative species like BarA/SirA in *S. enterica*, ExpS/ExpA or GacS/GacA in *E. carotovora*, and GacS/GacA in *Pseudomonas* (41). Some of these TCSs have been shown to be involved in regulation of virulence traits. SirA was recently shown to regulate motility and virulence through independent pathways in *S. enterica* (99). The hypothesis tested in this study was that BarA/UvrY TCS plays a role in adaptation in *E. coli*. The studies were done using wild type *E. coli* MG1655 *in vitro*.

The BarA/UvrY system is shown to be needed for metabolic switching between glycolytic and gluconeogenic carbon sources, a function for adaptation to new environments (75). UvrY recently is reported to autoregulate the expression of *barA* gene. BarA/UvrY plays a role in biofilm formation via the CsrA/CsrB/CsrC system (98). The global regulator CsrA (carbon storage regulator) activity in *E. coli* is

antagonized by CsrB and CsrC. UvrY and CsrA play a role in activation of CsrC. Their studies also indicate a CsrA/CsrB/CsrC independent role of UvrY in modulating cellular metabolism. The SdiA regulates expression of the *uvrY* gene (106). It has been shown that *Salmonella* can detect AHL produced by other species with help of SdiA (90). *E. coli* is not yet known to synthesize AHL. In *Pseudomonas*, UvrY homologue GacA regulates production of AHLs for QS.

The QS molecule AI-2 has been suggested to have a role in virulence in *E. coli*. Type III secretion apparatus of EPEC and EHEC, which lead to formation of attachment and effacing lesions, have been shown to be dependent on *luxS* (94). DNA microarray analysis in *E. coli* suggested that 5-10% of the genome is affected by AI-2 signaling (21). In *V. cholerae*, both AI-2 and AI-1 are used to control virulence gene expression and biofilm formation. The hypothesis of part of this study was that the BarA/UvrY TCS may regulate AI-2 production in *E. coli* via *luxS*.

We used Microarray technology, which allows global comparative analyses of gene content among different bacterial strains, to study differential gene expression in the wt and *barA*-, *uvrY*- and *barA-uvrY*- strains. From analysis of the microarray results, we see several membrane transport genes and genes related to stress response to be differentially regulated. We also see that the *ygaG* gene and *pfs* genes involved in synthesis of the active AI-2 molecule are down regulated in *barA*- and *barA-uvrY*-. We chose to study the *luxS* gene not only because there is no known regulatory mechanism for it, but also because it plays a role in regulating virulence in EPEC and EHEC (94,102,32).

To validate the results from the microarray, we used three independent genetic experiments. The RT-PCR results using LightCycler indicate that both the *luxS* and *pfs* gene transcripts are lower in fold abundance in the mutant strains compared to the wt. We also performed reverse transcription followed by PCR and ran the samples on agarose gels. The pixel quantitation using this method, though crude, also shows a difference in the transcript level when comparing the wt and the mutants. As seen in the results section, we saw ~6-fold less *luxS* transcript in *barA*-, and 4-fold in *uvrY*-

and *barA/uvrY*- compared to the wild type. We also checked if expression of *pfs* gene, which is also involved in AI-2 synthesis like *luxS*, is affected by the BarA/UvrY system. Results show that the *pfs* gene is ~6, 5 and 3-fold less expressed in the *barA*, *uvrY* and *barA/uvrY* mutants respectively compared to the wild type *E. coli*. Further, the results from quantitation of the image of the Northern blots indicate a ~2.5 fold abundance in *luxS* transcript in the wt compared to the *barA* mutant. The transcript level is comparable again to the wild type when a wild type copy of the *barA* gene is complemented on a plasmid in the mutant. We did not perform the northern blot analysis for the *uvrY* mutant and the double mutant due to lack of time. The results from the RT-PCR and Northern blot encouraged us to check if the promoter strength of the *luxS* gene is actually affected by the BarA/UvrY system. To do this, we performed Primer Extension studies. Both the RT-PCR and Northern blot studies support the microarray data that suggest that the BarA/UvrY TCS indirectly or directly play a role in regulation of *luxS*. The primer extension results show more than one transcription start sites. This may be a real result but to confirm that it is not an artifact of non-specific primer annealing, experiments with different sets of primers have to be performed in future. Also the altered levels of sequence-specific basal transcription factors may contribute to the understanding of the role of BarA/UvrY in affecting *luxS* expression. As we see the strengths of the putative promoters are different in the wt and mutants, we suspect that only one promoter must be constitutive, and the others inducible. But, as we could not standardize this experiment, we moved to a more suitable genetic approach.

To validate the above results obtained from RT PCR, northern blot studies, and primer extension, we adopted another genetic approach by creating a plasmid borne transcriptional fusion of the promoter of *luxS* and promoter-less *lacZ* gene and moved this construct to the chromosome of the wt, isogenic *barA*- and *uvrY*-. The chromosomal copy of the *luxS* fusion with the reporter gene would allow us to compare the difference in amount of *luxS* transcript formed in wt and the mutants with help of expression studies *in vitro*. From the results we see that the *luxS* expression as determined from the *lacZ* activity, is reduced in the wild type *E. coli* compared to its isogenic *barA*, *uvrY* and double mutants ~2 folds as shown in the graphs. It is not completely absent which indicates that this BarA/UvrY play a role above the basal level. This result encouraged us to check whether this regulatory effect of the BarA/UvrY on *luxS* is also growth phase dependent like AI-2. Surette and Bassler (1999) have shown that AI-2 is produced in growth media rich in glucose. As *luxS* is involved in AI-2 synthesis, we decided to check the effect of deletion of *barA* and *uvrY* on the expression of *luxS* in presence of glucose. Further, it has been reported that when high levels of acetate are provided exogenously to growth medium, the LuxS protein is up regulated (51). Entry into stationary phase coincides with the loss of glucose and accumulation of acetate. Therefore, we decided to also compare the effect of such metabolic regulators, acetate, glucose and NaCl upon expression of *luxS* in the wt versus the mutants. Our results show that in presence of acetate in the media, the expression of the *luxS::lacZ* is induced in the wt as well as the *barA*- and *uvrY*- strains. This suggests that acetate mediated expression of *luxS* is independent of BarA/UvrY. In presence of osmotic shock (500 mM NaCl),

we see BarA/UvrY independent regulation, reducing the expression of the *luxS* (data not shown). In presence of glucose, we see repression of *luxS::lacZ* in exponential phase in the wt strain. This repression was relieved in the *uvrY*- strain and not in the *barA*- strain suggesting a stronger role of UvrY in affecting expression of the *luxS::lacZ* fusion.

The role of BarA in the regulation of flagella biosynthesis and motility has yet not been clearly defined in *E. coli*. The orthologues/homologues of BarA are involved not only in regulation of the flagellar biosynthesis but also in attachment/invasion process during infection. In *E. coli*, quorum sensing through signaling molecule AI-2 is found to be involved in motility and flagellar gene expression. The microarray results also showed some flagella assembly genes to be differentially regulated in the mutants compared to the wt. Our preliminary chemiluminescence studies on quorum sensing had shown that BarA regulates AI-2 accumulation which is required for quorum sensing. We performed some physiological studies to see if swimming motility of *E. coli* in 0.25 % TB Agar is altered in the *barA* -, *uvrY*- and *barA-uvrY*- strains. The isogenic *barA*- mutant showed a 2.0 fold, *uvrY*- mutant 1.5 fold and *barA-uvrY*- double mutant 2.5 fold decrease in motility when compared to the wild type. This defect in swimming motility in *barA*- is restored when we express the wt copy of *barA* gene on a plasmid. Also, the defect of the *barA*- is restored when the mutant is complemented with *luxS* on a plasmid. Thus, BarA probably induces the production of the AI-2 in the medium, by transcriptionally activating *luxS* gene expression. This increase in the AI-2 production modulates the quorum sensing mechanism of the bacteria and that in turn regulates the motility through some other

two-component system. Surprisingly, when *uvrY* is transformed on a plasmid in *uvrY*⁻, the strain does not swim at all. Moreover, *luxS* gene does not restore the motility in *uvrY*⁻ either. Thus, from the swimming results we see that UvrY probably regulates motility partially by a BarA independent pathway, with yet unidentified intermediates.

The *flhDC* flagellar gene expression studies in the wt and isogenic mutant strains also show that in the *barA* and *uvrY* mutants, the *flhDC::lacZ* expression is less than that in the wt. Results show that *barA*, more than *uvrY* affects motility and flagellar gene expression of the *flhDC* operon. The array results support the expression studies. From the array we see genes like *mglA* and *oppA*, which are repressed by FlhD/FlhC, are found to be repressed in the mutants. The over expression of the *flhDC* may probably due to over expression of *csrA*, in absence of *barA/uvrY*, which activates the *flhDC* operon (100). It has been shown that the *uvrY* does not directly bind to and affect flagellar gene expression via *flhDC* (101). Probably *uvrY* has an effect on *flhDC* via another pathway independent of the *csrA/csrB/csrC* system. Another important observation from studies in the lab suggests that the regulatory effect of BarA is not mediated through RpoS, the response regulator of BarA-mediated signal transduction under several stress conditions including oxidative stress.

This observation was confirmed by studies done using culture supernatant of the wild type bacteria from a specific phase of growth, which is rich in AI-2 molecules. All the strains showed greater extent of motility than that when the culture media was collected from a *luxS* deficient strain. Moreover, when the percentage of

motility is compared with the wild type, it was observed that *barA* mutant had restored its motility defect to a considerable extent when AI-2 rich media was used instead of nutrient media.

Thus, it can be concluded that BarA/UvrY system is involved in the swimming motility of the *E. coli* and it exerts its effect by modulating *luxS* gene expression and thereby quorum sensing. However, further work on the regulatory effect of BarA/UvrY on the specific subunits of flagella or its motor apparatus will clearly define the exact role of BarA in the flagella derived swimming motility of *E. coli*. The role of quorum sensing mechanism in the motility and its link with the BarA mediated signal transduction also needs to be extensively studied in the future.

Since our swimming motility and flagellar gene expression results suggest that BarA/UvrY is involved in regulating motility of *E. coli*, we speculated that it might alter those properties by altering cell surface appendages. Thus, we proceeded to study the presence of flagellar appendages on the surface of wild type and *barA*-, *uvrY*- and *barA-uvrY*- double mutant of *E. coli* by SEM. The SEM images demonstrate that *barA*-, *uvrY*- and *barA-uvrY*- mutants have fewer cell surface appendages than the wild type bacteria. We, however, do not have an idea about the type of cell surface appendages, i.e. the actual identity of those cell surface appendages that showed a decrease in the appearance upon mutation in the *barA* gene. We also did not find out whether *barA/uvrY* system regulates the number of the cell surface appendages by modulating their biosynthetic genes or their assembly process. Further studies using immuno-electron microscopy with help of antibodies specific to one or more of the subunits of the different cell surface appendages may

reveal their identities. Thus the site of action of BarA/UvrY in regulating the appearance of cell surface appendages needs to be studied.

Further, experiments were done to study the difference in survival and growth of MG1655 wt *E. coli* and its isogenic *barA*- and *uvrY*- mutants under different potential stresses found within the host. Nitric oxide (NO), an intermediate in microbial denitrification, is a free radical with multiple and diverse biological functions. NO, secreted by host immune cells, also serves as broad-spectrum antibiotic, anti-viral and anti-tumor agent. Nitric oxide reductases and dioxygenases in many pathogenic bacteria convert NO to N₂O or nitrate respectively. *E. coli* and related organisms contain *norR* orthologues which may control defense against NO and its reactive nitrogen intermediates (RNI's). Microarray data showed *nirC*, a membrane protein affecting nitrite reductase to be down regulated in mutants. We therefore challenged the strains with NaNO₂ stress as the NO generator, to study the effect physiologically. Results show that overall the NO stress reduces the survival of all the strains when pH of the growth medium is acidic. The mutants are more sensitive to this stress environment than the wt. We also challenged the wt and mutants to osmotic stress using 1M NaCl. As seen from the results, the *barA*- and *uvrY*- are sensitive to NaCl stress, whereas the wild type is resistant. The resistance to this osmotic stress is recovered in the *barA*- partly when wt copy of *barA* gene is provided and fully when *luxS* gene is provided in trans on a plasmid.

Pathogenic bacteria are resistant to bile salts, probably due to both low permeability of outer bilayer membrane as well as due to active efflux mechanisms. Cholates and Deoxycholates are the most abundant bile salts in humans. We therefore

tried to determine if the mutants were sensitive to these bile salts once the *barA* and *uvrY* are deleted. It is seen from the results that the wt and the mutants have almost the same survival rate in presence of bile salts. This suggests that the bile salt resistance of *E. coli* is independent of the BarA/UvrY system.

Multidrug resistance (Mdr) proteins have drawn attention of many researchers in the past few years. Till date, the basics of transport related Mdr are still not clear. Some bacterial Mdr proteins recognize antibiotics that are uncharged in physiological solutions as well as cationic drugs. Ethidium Bromide, which represents charged substrates, has been used in this study. Mutations that cause alteration in outer membrane permeability of the cells, result in higher susceptibility to hydrophobic antibiotics and detergents (13). This is probably due to leakage of periplasmic proteins into the medium Ethidium bromide, as seen 24 hrs post challenge, has a greater effect on the *uvrY*- and *barA-uvrY*- mutants than the wt. The sensitivity of the mutants to SDS was studied to examine the permeability of the outer membrane to hydrophobic agents. The *barA*- is more sensitive than the *uvrY*- and wt to SDS challenge.

Our array results show that several genes encoding membrane transporters like the *malE*, *dppA*, *ptsG*, were over expressed in the mutants compared to the wild type bacteria. This may mean that the *barA* and *uvrY* system directly or indirectly regulate some transporters which may help in resistance towards stress. From the survival assays we see that the sensitivity to certain types of stressors is increased in mutants, which may be partly due to the over expression of some of the transport proteins and other regulators involved in the adaptation process. Thus probably

BarA/UvrY may affect membrane transport and thereby overall metabolism of the organism. The mechanism is still illusive and further details of the genetic players have to be delineated. This studies help give an idea that the BarA and UvrY individually as well as together could be potential target to study bacterial stress resistance mechanisms.

The QS molecule AI-2 has been suggested to have a role in virulence in *E. coli*. Type III secretion apparatus of EPEC and EHEC, which lead to formation of attachment and effacing lesions, have been shown to be dependent on *luxS* (94). DNA microarray analysis in *E. coli* suggested that 5-10% of the genome is affected by AI-2 signaling (21). In *V. cholerae*, both AI-2 and AI-1 are used to control virulence gene expression and biofilm formation. The hypothesis of part of this study was that the BarA/UvrY TCS may regulate AI-2 production in *E. coli* via *luxS*.

We used Microarray technology, which allows global comparative analyses of gene content among different bacterial strains, to study differential gene expression in the wt and *barA*-, *uvrY*- and *barA-uvrY*- strains. From analysis of the microarray results, we see several membrane transport genes and genes related to stress response to be differentially regulated. We also see that the *ygaG* gene and *pfs* genes involved in synthesis of the active AI-2 molecule are down regulated in *barA*- and *barA-uvrY*-. We chose to study the *luxS* gene not only because there is no known regulatory mechanism for it, but also because it plays a role in regulating virulence in EPEC and EHEC (94,102,32).

To validate the results from the microarray, we used three independent genetic experiments. The RT-PCR results using LightCycler indicate that both the *luxS* and

pfs gene transcripts are lower in fold abundance in the mutant strains compared to the wt. We also performed reverse transcription followed by PCR and ran the samples on agarose gels. The pixel quantitation using this method, though crude, also shows a difference in the transcript level when comparing the wt and the mutants. As seen in the results section, we saw ~6-fold less *luxS* transcript in *barA*⁻, and 4-fold in *uvrY*⁻ and *barA/uvrY*⁻ compared to the wild type. We also checked if expression of *pfs* gene, which is also involved in AI-2 synthesis like *luxS*, is affected by the BarA/UvrY system. Results show that the *pfs* gene is ~6, 5 and 3-fold less expressed in the *barA*, *uvrY* and *barA/uvrY* mutants respectively compared to the wild type *E. coli*. Further, the results from quantitation of the image of the Northern blots indicate a ~2.5 fold abundance in *luxS* transcript in the wt compared to the *barA* mutant. The transcript level is comparable again to the wild type when a wild type copy of the *barA* gene is complemented on a plasmid in the mutant. We did not perform the northern blot analysis for the *uvrY* mutant and the double mutant due to lack of time. The results from the RT-PCR and Northern blot encouraged us to check if the promoter strength of the *luxS* gene is actually affected by the BarA/UvrY system. To do this, we performed Primer Extension studies. Both the RT-PCR and Northern blot studies support the microarray data that suggest that the BarA/UvrY TCS indirectly or directly play a role in regulation of *luxS*. The primer extension results show more than one transcription start sites. This may be a real result but to confirm that it is not an artifact of non-specific primer annealing, experiments with different sets of primers have to be performed in future. Also the altered levels of sequence-specific basal transcription factors may contribute to the understanding of the role of BarA/UvrY in

affecting *luxS* expression. As we see the strengths of the putative promoters are different in the wt and mutants, we suspect that only one promoter must be constitutive, and the others inducible. But, as we could not standardize this experiment, we moved to a more suitable genetic approach.

To validate the above results obtained from RT PCR, northern blot studies, and primer extension, we adopted another genetic approach by creating a plasmid borne transcriptional fusion of the promoter of *luxS* and promoter-less *lacZ* gene and moved this construct to the chromosome of the wt, isogenic *barA*- and *uvrY*-. The chromosomal copy of the *luxS* fusion with the reporter gene would allow us to compare the difference in amount of *luxS* transcript formed in wt and the mutants with help of expression studies *in vitro*. From the results we see that the *luxS* expression as determined from the *lacZ* activity, is reduced in the wild type *E. coli* compared to its isogenic *barA*, *uvrY* and double mutants ~2 folds as shown in the graphs. It is not completely absent which indicates that this BarA/UvrY play a role above the basal level. This result encouraged us to check whether this regulatory effect of the BarA/UvrY on *luxS* is also growth phase dependent like AI-2. Surette and Bassler (1999) have shown that AI-2 is produced in growth media rich in glucose. As *luxS* is involved in AI-2 synthesis, we decided to check the effect of deletion of *barA* and *uvrY* on the expression of *luxS* in presence of glucose. Further, it has been reported that when high levels of acetate are provided exogenously to growth medium, the LuxS protein is up regulated (51). Entry into stationary phase coincides with the loss of glucose and accumulation of acetate. Therefore, we decided to also compare the effect of such metabolic regulators, acetate, glucose and NaCl

upon expression of *luxS* in the wt versus the mutants. Our results show that in presence of acetate in the media, the expression of the *luxS::lacZ* is induced in the wt as well as the *barA*- and *uvrY*- strains. This suggests that acetate mediated expression of *luxS* is independent of BarA/UvrY. In presence of osmotic shock (500 mM NaCl), we see BarA/UvrY independent regulation, reducing the expression of the *luxS* (data not shown). In presence of glucose, we see repression of *luxS::lacZ* in exponential phase in the wt strain. This repression was relieved in the *uvrY*- strain and not in the *barA*- strain suggesting a stronger role of UvrY in affecting expression of the *luxS::lacZ* fusion.

The role of BarA in the regulation of flagella biosynthesis and motility has yet not been clearly defined in *E. coli*. The orthologues/homologues of BarA are involved not only in regulation of the flagellar biosynthesis but also in attachment/invasion process during infection. In *E. coli*, quorum sensing through signaling molecule AI-2 is found to be involved in motility and flagellar gene expression. The microarray results also showed some flagella assembly genes to be differentially regulated in the mutants compared to the wt. Our preliminary chemiluminescence studies on quorum sensing had shown that BarA regulates AI-2 accumulation which is required for quorum sensing. We performed some physiological studies to see if swimming motility of *E. coli* in 0.25 % TB Agar is altered in the *barA* -, *uvrY*- and *barA-uvrY*- strains. The isogenic *barA*- mutant showed a 2.0 fold, *uvrY*- mutant 1.5 fold and *barA-uvrY*- double mutant 2.5 fold decrease in motility when compared to the wild type. This defect in swimming motility in *barA*- is restored when we express the wt copy of *barA* gene on a plasmid. Also, the defect of the *barA*- is restored when the

mutant is complemented with *luxS* on a plasmid. Thus, BarA probably induces the production of the AI-2 in the medium, by transcriptionally activating *luxS* gene expression. This increase in the AI-2 production modulates the quorum sensing mechanism of the bacteria and that in turn regulates the motility through some other two-component system. Surprisingly, when *uvrY* is transformed on a plasmid in *uvrY*⁻, the strain does not swim at all. Moreover, *luxS* gene does not restore the motility in *uvrY*⁻ either. Thus, from the swimming results we see that UvrY probably regulates motility partially by a BarA independent pathway, with yet unidentified intermediates.

The *flhDC* flagellar gene expression studies in the wt and isogenic mutant strains also show that in the *barA* and *uvrY* mutants, the *flhDC::lacZ* expression is less than that in the wt. Results show that *barA*, more than *uvrY* affects motility and flagellar gene expression of the *flhDC* operon. The array results support the expression studies. From the array we see genes like *mglA* and *oppA*, which are repressed by FlhD/FlhC, are found to be repressed in the mutants. The over expression of the *flhDC* may probably due to over expression of *csrA*, in absence of *barA/uvrY*, which activates the *flhDC* operon (100). It has been shown that the *uvrY* does not directly bind to and affect flagellar gene expression via *flhDC* (101). Probably *uvrY* has an effect on *flhDC* via another pathway independent of the *csrA/csrB/csrC* system. Another important observation from studies in the lab suggests that the regulatory effect of BarA is not mediated through RpoS, the response regulator of BarA-mediated signal transduction under several stress conditions including oxidative stress.

This observation was confirmed by studies done using culture supernatant of the wild type bacteria from a specific phase of growth, which is rich in AI-2 molecules. All the strains showed greater extent of motility than that when the culture media was collected from a *luxS* deficient strain. Moreover, when the percentage of motility is compared with the wild type, it was observed that *barA* mutant had restored its motility defect to a considerable extent when AI-2 rich media was used instead of nutrient media.

Thus, it can be concluded that BarA/UvrY system is involved in the swimming motility of the *E. coli* and it exerts its effect by modulating *luxS* gene expression and thereby quorum sensing. However, further work on the regulatory effect of BarA/UvrY on the specific subunits of flagella or its motor apparatus will clearly define the exact role of BarA in the flagella derived swimming motility of *E. coli*. The role of quorum sensing mechanism in the motility and its link with the BarA mediated signal transduction also needs to be extensively studied in the future.

Since our swimming motility and flagellar gene expression results suggest that BarA/UvrY is involved in regulating motility of *E. coli*, we speculated that it might alter those properties by altering cell surface appendages. Thus, we proceeded to study the presence of flagellar appendages on the surface of wild type and *barA*-, *uvrY*- and *barA-uvrY*- double mutant of *E. coli* by SEM. The SEM images demonstrate that *barA*-, *uvrY*- and *barA-uvrY*- mutants have fewer cell surface appendages than the wild type bacteria. We, however, do not have an idea about the type of cell surface appendages, i.e. the actual identity of those cell surface appendages that showed a decrease in the appearance upon mutation in the *barA*

gene. We also did not find out whether *barA/uvrY* system regulates the number of the cell surface appendages by modulating their biosynthetic genes or their assembly process. Further studies using immuno-electron microscopy with help of antibodies specific to one or more of the subunits of the different cell surface appendages may reveal their identities. Thus the site of action of BarA/UvrY in regulating the appearance of cell surface appendages needs to be studied.

Further, experiments were done to study the difference in survival and growth of MG1655 wt *E. coli* and its isogenic *barA*- and *uvrY*- mutants under different potential stresses found within the host. Nitric oxide (NO), an intermediate in microbial denitrification, is a free radical with multiple and diverse biological functions. NO, secreted by host immune cells, also serves as broad-spectrum antibiotic, anti-viral and anti-tumor agent. Nitric oxide reductases and dioxygenases in many pathogenic bacteria convert NO to N₂O or nitrate respectively. *E. coli* and related organisms contain *norR* orthologues which may control defense against NO and its reactive nitrogen intermediates (RNI's). Microarray data showed *nirC*, a membrane protein affecting nitrite reductase to be down regulated in mutants. We therefore challenged the strains with NaNO₂ stress as the NO generator, to study the effect physiologically. Results show that overall the NO stress reduces the survival of all the strains when pH of the growth medium is acidic. The mutants are more sensitive to this stress environment than the wt. We also challenged the wt and mutants to osmotic stress using 1M NaCl. As seen from the results, the *barA*- and *uvrY*- are sensitive to NaCl stress, whereas the wild type is resistant. The resistance

to this osmotic stress is recovered in the *barA*- partly when wt copy of *barA* gene is provided and fully when *luxS* gene is provided in trans on a plasmid

Pathogenic bacteria are resistant to bile salts, probably due to both low permeability of outer bilayer membrane as well as due to active efflux mechanisms. Chololate and Deoxychololate are the most abundant bile salts in humans. We therefore tried to determine if the mutants were sensitive to these bile salts once the *barA* and *uvrY* are deleted. It is seen from the results that the wt and the mutants have almost the same survival rate in presence of bile salts. This suggests that the bile salt resistance of *E. coli* is independent of the BarA/UvrY system.

Multidrug resistance (Mdr) proteins have drawn attention of many researchers in the past few years. Till date, the basics of transport related Mdr are still not clear. Some bacterial Mdr proteins recognize antibiotics that are uncharged in physiological solutions as well as cationic drugs. Ethidium Bromide, which represents charged substrates, has been used in this study. Mutations that cause alteration in outer membrane permeability of the cells, result in higher susceptibility to hydrophobic antibiotics and detergents (13). This is probably due to leakage of periplasmic proteins into the medium Ethidium bromide, as seen 24 hrs post challenge, has a greater effect on the *uvrY*- and *barA-uvrY*- mutants than the wt. The sensitivity of the mutants to SDS was studied to examine the permeability of the outer membrane to hydrophobic agents. The *barA*- is more sensitive than the *uvrY*- and wt to SDS challenge.

Our array results show that several genes encoding membrane transporters like the *malE*, *dppA*, *ptsG*, were over expressed in the mutants compared to the wild

type bacteria. This may mean that the *barA* and *uvrY* system directly or indirectly regulate some transporters which may help in resistance towards stress. From the survival assays we see that the sensitivity to certain types of stressors is increased in mutants, which may be partly due to the over expression of some of the transport proteins and other regulators involved in the adaptation process. Thus probably BarA/UvrY may affect membrane transport and thereby overall metabolism of the organism. The mechanism is still illusive and further details of the genetic players have to be delineated. This studies help give an idea that the BarA and UvrY individually as well as together could be potential target to study bacterial stress resistance mechanisms.

4.2 Summary

In summary, this study demonstrates that BarA/UvrY TCS in *Escherichia coli* K-12 is involved in several important processes that are required for adaptation of the organism to unfavorable environments. BarA/UvrY regulates these processes by altering genes involved in adaptive processes. These genes not only alter the cellular metabolism but also are genes involved in several biosynthetic pathways. However, this study did not specifically identify the actual signaling mechanisms or the downstream signaling molecules involved in these pathways. Figure 16 shows a schematic representation of probable signal transduction pathways based on this and previous research knowledge. BarA/UvrY may act independently or together to partially regulate genes involved in adaptive response. Motility is found to be regulated both independent of each other as well as together by BarA/UvrY. The BarA/UvrY may probably play a role in methyl cycle by regulation SAM (S-adenosyl methionine) breakdown, specifically via *luxS*. Thus this TCS may help in detoxification of methyl cycle intermediates and in turn synthesis of auto inducers AI-2/AI-3 required for quorum sensing. UvrY plays a role in regulation of *luxS* expression in the presence of glucose. Future research should focus on finding what other genes are involved with this system in this process. In short, the actual identity of up/downstream signaling molecules should be determined to verify the direct regulatory effect of BarA/UvrY on them. The work should also be extrapolated in pathogenic strains of *E. coli* to extensively study the infective process.

CHAPTER 5

Bibliography

1. **Adams J. L. and McLean R. C.** 1999. Impact of *rpoS* deletion on *Escherichia coli* biofilms. **Appl. Environ. Microbiol.** **65(9):** 4285-4287.
2. **Allen-Vercoe E. and Woodward M. J.** 1999. The role of flagella, but not fimbriae, in the adherence of *Salmonella enterica* serotype Enteritidis to chick gut explant. **J. Med. Microbiol.** **48(8):** 771-80.
3. **Altier C., Suyemoto M. and Lawhon S. D.** 2000. Regulation of *Salmonella enterica* serovar typhimurium invasion genes by *csrA*. **Infect. Immun.** **68(12):** 6790-7.
4. **Altier C., Suyemoto M., Ruiz A. I., Burnham K. D. and Maurer R.** 2000. Characterization of two novel regulatory genes affecting *Salmonella* invasion gene expression. **Mol. Microbiol.** **35:** 635-646.
5. **Anjum M.F., Lucchini S., Thompson A., Hinton J.C.D., and Woodward M.J.** 2003. Comparative genomic indexing reveals the phylogenomics of *Escherichia coli* pathogens. **Inf and Imm.** **71(8):** 4674-4683
6. **Anriany, Y. A., Weiner R. M., Johnson J. A., DeRezende C. E. and Joseph S. W.** 2001. *Salmonella enterica* serovar Typhimurium DT104 displays a rugose phenotype. **Applied and Environ. Microbiol.** **67:** 4048-4056.

7. **Bahrani-Mougeot F. K., Buckles E. L., Lockett C. V., Hebel J. R., Johnson D. E., Tang C. M. and Donnenberg M. S.** 2002. Type 1 fimbriae and extracellular polysaccharides are pre-eminent in uropathogenic *Escherichia coli* virulence determinants in the murine urinary tract. **Mol. Microbiol.** **45**: 1079-1093.
8. **Bassler B. L., Greenberg E. P., Stevens A.M.** 1997. Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. **J. Bacteriol.** **179(12)**: 4043-5.
9. **Bassler B. L.** 1999. How bacteria talk to each other: regulation of gene expression by quorum sensing. **Curr Opin Microbiol.****2(6)**: 582-7. Review.
10. **Bassler B. L.** 2002. Small talk. Cell-to-cell communication in bacteria. **Cell.** **109(4)**: 421-424.
11. **Baxter M. A., Fahlen T. F., Wilson R. L. and Jones B. D.** 2003. HilE interacts with HilD and negatively regulates *hilA* transcription and expression of the *Salmonella enterica* serovar Typhimurium invasive phenotype. **Infect. Immun.** **71(3)**: 1295-305.
12. **Beeston A. L. and Surette M. G.** 2002. *pfs*-dependent regulation of autoinducer-2 production in *Salmonella enterica* serovar Typhimurium. **J. Bacteriol.** **184(13)**: 3450-3456.
13. **Beja O, Bibi E.** 1996. Functional expression of mouse Mdr1 in an outer membrane permeability mutant of *Escherichia coli*. **Proc Natl Acad Sci U S A.** **11;93(12)**:5969-74.

14. **Brian K Hammer, Eliko Tateda, Michele Swanson,** 2002. A two-component regulator induces the transmission phenotype of stationary-phase *Legionella pneumophila*. **Mol. Microbiol.** 44(1):107-18.
15. **Brown P.O., Botstein D.** 1999. Exploring the new world of the genome with DNA microarrays. **Nat Genet.** 21: 33-7.
16. **Castaneda M., Guzman J., Moreno S. and Espin G.** 2000. The GacS sensor kinase regulates alginate and poly-beta-hydroxybutyrate production in *Azotobacter vinelandii*. **J. Bacteriol.** 182: 2624-2628.
17. **Chen X., Schauder S., Potier N., Van Dorsselaer A., Pelczar I., Bassler B. L. and Hughson F. M.** 2002. Structural identification of a bacterial quorum-sensing signal containing boron. **Nature.** 415(6871): 545-549.
18. **Corbell N., and Loper J.E.** 1995. A global regulator of secondary metabolite production in *Pseudomonas fluorescens* Pf-5. **J. Bacteriol.** 177(21): 6230-6.
19. **Correa N. E., Lauriano C. M., McGee R. and Klose K. E.** 2000. Phosphorylation of the flagellar regulatory protein FlrC is necessary for *Vibrio cholerae* motility and enhanced colonization. **Mol. Microbiol.** 35(4): 743-55.
20. **Cummings C. A., Relman D. A.** 2002. Genomics and microbiology. Microbial forensics--"cross-examining pathogens". **Science.** 14; 296(5575): 1976-9.
21. **DeLisa M.P., Wu C.F., Wang L., Valdes J.J., and Bentley W.E.** 2001. DNA Microarray-based identification of genes controlled by autoinducer 2-stimulated quorum sensing in *Escherichia coli*. **J. Bacteriol.** 183: 5239-5247.

22. **Demple B.** 1999. Genetic responses against nitric oxide toxicity. **Braz. J. Med. Biol. Res.** **32(11):** 1417-1427.
23. **Dibb-Fuller M. P., Allen-Vercoe E., Thorns C. J. and Woodward M. J.** 1999. Fimbriae- and flagella-mediated association with and invasion of cultured epithelial cells by *Salmonella enteritidis*. **Microbiology.** **145(5):** 1023-31.
24. **Donnenberg, M.S. and J.B.Kaper.**1991. Construction of an eae deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. **Infect Immun.** **59(12):** 4310-7.
25. **Fahlen T. F., Mathur N. and Jones B. D.** 2000. Identification and characterization of mutants with increased expression of *hilA*, the invasion gene transcriptional activator of *Salmonella typhimurium*. **FEMS Immunol. Med. Microbiol.** **28(1):** 25-35.
26. **Fang F. C., Libby S. J., Buchmeier N. A., Loewen P. C., Switala J., Harwood J. and Guiney D. G.** 1992. The alternative sigma factor KatF (RpoS) regulates *Salmonella* virulence. **Proc. Natl. Acad. Sci. USA.** **89:** 11978-11982.
27. **Finlay B. B. and Cossart P.** 1997. Exploitation of mammalian host cell functions by bacterial pathogens. **Science** **276:** 718-725.
28. **Foster J. W.** 2000. Microbial Response to acid stress. Bacterial Stress Response, ed: Storz G., Hengge-Aronis R. **Amer. Soc. Microbio. Press, Washington DC.**

29. **Foynes S., Dorrell N., Ward S. J., Stabler R. A., McColm A. A., Rycroft A. N. and Wren B. W.** 2000. *Helicobacter pylori* possesses two CheY response regulators and a histidine kinase sensor, CheA, which are essential for chemotaxis and colonization of the gastric mucosa. **Infect. Immun.** **68(4):** 2016-23.
30. **Galan J. E.** 2001. *Salmonella* interactions with host cells: type III secretion at work **Ann. Rev. Cell. Dev. Biol.** **17:** 53-86.
31. **Gavin R., Merino S., Altarriba M., Canals R., Shaw J. G. and Tomas J. M.** 2003. Lateral flagella are required for increased cell adherence, invasion and biofilm formation by *Aeromonas spp.* **FEMS Microbiol. Lett.** **224(1):** 77-83.
32. **Giron J. A., Torres A. G., Freer E. and Kaper J. B.** 2002. The flagella of enteropathogenic *Escherichia coli* mediate adherence to epithelial cells. **Mol. Microbiol.** **44:** 361-379.
33. **Gonzalez F. B. and Demple B.** 2000. Genetic responses to free radicals. Homeostasis and gene control. **Ann. N.Y. Acad. Sci.** **899:** 69-87.
34. **Goodier R. I. and Ahmer B. M.** 2001. SirA orthologs affect both motility and virulence. **J. Bacteriol.** **183:** 2249-2258.
35. **Grebe T. W. and Stock J. B.** 1999. The histidine protein kinase superfamily. **Adv. Microb. Physiol.** **41:** 139-227.
36. **Gunn, J. S.** 2000. Mechanisms of bacterial resistance and response to bile. **Microbes Infect.** **2:** 907-913.

37. **Gupta S., and Chowdhury R.** 1997. Bile affects production of virulence factors and motility of *Vibrio cholerae*. **Infect. Immun.** **65**: 1131-4.
38. **Hacker J., and Kaper J.B.** 2000. Pathogenicity Islands (PAIs) and the evolution of microbes. **Annu Rev Microbiol.** **54**:641-79.
39. **Hammer B. K., Tateda E. S. and Swanson M. S.** 2002. A two-component regulator induces the transmission phenotype of stationary-phase *Legionella pneumophila*. **Mol. Microbiol.** **44(1)**: 107- 18.
40. **Han Y. W., Uhl M. A., Han S. J. and Shi W.** 1999. Expression of *bvgAS* of *Bordetella pertussis* represses flagellar biosynthesis of *Escherichia coli*. **Arch. Microbiol.** **171(2)**: 127-30.
41. **Heeb S. and Haas D.** 2001. Regulatory roles of the GacS/GacA two component system in plant-associated and other Gram-negative bacteria. **Mol. Plant-Microbe Interact.** 14
42. **Heim R, Cubitt AB, Tsien RY.** 1995. Improved green fluorescence. **Nature**, **373(6516)**: 663-4.
43. **Hengge-Aronis.** 2002. Signal transduction and regulatory mechanisms involved in control of the σ^S (RpoS) subunit of RNA polymerase. *Microbiol and Mol. Biol. Rev.* 66(3): 373-395.
44. **Hensel M., Shea J. E., Waterman S. R., Mundy R., Nikolaus T., Banks G., Vazquez-Torres A., Gleeson C., Fang F. C. and Holden D. W.** 1998. Genes encoding putative effector proteins of the type III secretion system of *Salmonella* pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. **Mol. Microbiol.** **30**:163-674.

45. **Hirakawa H, Nishino K, Hirata T, Yamaguchi A.** 2003. Comprehensive studies of drug resistance mediated by overexpression of response regulators of two-component signal transduction systems in *Escherichia coli*. **J. Bacteriol.** **185 (6):**1851-6.
46. **Hirano S. S., Charkowski A. O., Colmer A., Willis D. K, and Upper C. D.** 1999. Role of Hrp type III secretion system in growth of *Pseudomonas syringae* pv. *syringae* B728a on host plants in the field. **Proc. Natl. Acad. Sci. USA.** **96:** 9851-9856.
47. **Hueck C. J.** 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. **Microbiol. Mol. Biol. Rev.** **62:** 379-433.
48. **Ishige K., Nagasawa S., Tokishita S. and Mizuno T.** 1994. A novel device of bacterial signal transducers. **EMBO J.** **13:** 5195-202.
49. **Johnston C., Pegues D. A., Hueck C. J., Lee A. and Miller S. I.** 1996. Transcriptional activation of *Salmonella typhimurium* invasion genes by a member of the phosphorylated response-regulator superfamily. **Mol. Microbiol.** **22:** 715-727.
50. **Kinscherf T. G. and Willis D. K.** 1999. Swarming by *Pseudomonas syringae* B728a requires *gacS* (*lemA*) and *gacA* but not the acyl-homoserine lactone biosynthetic gene *ahII*. **J. Bacteriol.** **181(13):** 4133-6.
51. **Kirkpatrick C., Maurer L. M., Oyelakin N. E., Yoncheva Y. N., Maurer R., Slonczewski J. L.** 2001. Acetate and formate stress: opposite responses in the proteome of *Escherichia coli*. **J. Bacteriol.** **183(21):** 6466-77.

52. **Knodler L. A. and Finlay B. B.** 2001. *Salmonella* and apoptosis: to live or let die? **Microbes Infect.** **3:** 1321-1326.
53. **Kowarz L., Counault C., Robbe Saule V. and Norel F.** 1994. The *Salmonella typhimurium katF (rpoS)* gene: cloning, nucleotide sequence, and regulation of *spvR* and *spvABCD* virulence plasmid genes. **J. Bacteriol.** **176:** 6852-6860.
54. **Lawhon S. D., Maurer R., Suyemoto M. and Altier C.** 2002. Intestinal short-chain fatty acids alter *Salmonella typhimurium* invasion gene expression and virulence through BarA/SirA. **Mol. Microbiol.** **46(5):** 1451-64.
55. **Lee A. K., Detweiler C. S. and Falkow S.** 2000. OmpR regulates the two-component system SsrA-SsrB in *Salmonella* pathogenicity island 2. **J. Bacteriol.** **182:** 771-781.
56. **Liao C. H., McCallus D. E., Wells J. M., Tzean S. S. and Kang G. Y.** 1996. The *repB* gene required production extracellular enzymes and fluorescent siderophores in *Pseudomonas viridiflava* is an analogue of the *gacA* gene in *Pseudomonas syringae*. **Can. J. Microbiol.** **42:** 177-182.
57. **Lu S., Killoran P. B., Fang F. C. and Riley L. W.** 2002. The global regulator AcrA controls resistance to reactive nitrogen and oxygen intermediates in *Salmonella enterica* serovar Enteritidis. **Infect. Immun.** **70:** 451-461.
58. **Lucas R. L., Lostroh C. P., DiRusso C. C., Spector M. P., Wanner B. L. and Lee C. A.** 2000. Multiple factors independently regulate *hilA* and

- invasion gene expression in *Salmonella enterica* serovar typhimurium. **J. Bacteriol.** **182(7)**: 1872-82.
59. **Martinez J. J., Mulvey M. A., Schilling J. D., Pinkner J. S. and Hultgren S. J.** 2000. Type 1 pilus-mediated bacterial invasion of bladder epithelial cells. **EMBO J.** **19(12)**: 2803-12.
60. **Membrillo-Hernandez J., Coopamah, M.D., Channa, A., Hughes, M.N. & Poole, R.K.** 1998. A novel mechanism for upregulation of the *Escherichia coli* K-12 hmp (flavo-haemoglobin) gene by the 'NO releaser', S-nitrosoglutathione: nitrosation of homocysteine and modulation of MetR binding to the glyA-hmp intergenic region. **Mol. Microbiol** **29(4)**: 1101-12.
61. **Michael B, Smith J.N., Swift S., Heffron F., Ahmer B.M.** 2001. SdiA of *Salmonella enterica* is a LuxR homolog that detects mixed microbial communities. **J Bacteriol.** **183 (19)**: 5733-42.
62. Michael g surette, bassler, 1999
63. **Miller M. B. and Bassler B. L.** 2001. Quorum sensing in bacteria. **Ann. Rev. Microbiol.** **55**:165-99.
64. **Mizuno T.** 1997. Compilation of all genes encoding two-component phosphotransfer signal transducers in the genome of *Escherichia coli*. **DNA Res.** **4(2)**: 161-168.
65. **Monack D. M., Navarre W. W. and Falkow S.** 2001. *Salmonella*-induced macrophage death: the role of caspase-1 in death and inflammation. **Microbes Infect.** **3**:1201-1212.

66. **Moolenaar G. F., van Sluis C. A., Backendorf C. and van de Putte P.** 1987. Regulation of the *Escherichia coli* excision repair gene *uvrC*. Overlap between the *uvrC* structural gene and the region coding for a 24 kD protein. **Nucleic Acids Res.** **15(10):** 4273-89.
67. **Mukhopadhyay S., Audia J. P., Roy R. N. and Schellhorn H. E.** 2000. Transcriptional induction of the conserved alternative sigma factor RpoS in *Escherichia coli* is dependent on BarA, a probable two-component regulator. **Mol. Microbiol.** **37(2):** 371-81.
68. **Mukhopadhyay S. and Schellhorn H. E.** 1994. Induction of *Escherichia coli* hydroperoxidase I by acetate and other weak acids. **J. Bacteriol.** **176:** 2300-2307.
69. **Nagasawa S., Tokishita, Aiba H. and Mizuno, T.** 1992. A novel sensor-regulator protein that belongs to the homologous family of signal-transduction proteins involved in adaptive responses in *E. coli*. **Mol. Microbiol.** **6(6):** 799-807.
70. **Norel F., Robbe Saule V., Popoff M. Y. and Coynault C.** 1992. The putative sigma factor KatF (RpoS) is required for the transcription of the *Salmonella typhimurium* virulence gene *spvB* in *Escherichia coli*. **FEMS Microbiol. Lett.** **78:** 271-276.
71. **Nougayrede J. P., Fernandes P. J. and Donnenberg M. S.** 2003. Adhesion of enteropathogenic *Escherichia coli* to host cells. **Cell. Microbiol.** **5(6):** 359-72.

72. **Oshima T., Aiba H., Masuda Y., Kanaya S., Sugiura M., Wanner B. L., Mori H. and Mizuno T.** 2002. Transcriptome analysis of all two-component regulatory system mutants of *Escherichia coli* K-12. **Mol. Microbiol.** **46:** 281-291.
73. **Parkins M. D., Ceri H. and Storey D. G.** 2001. *Pseudomonas aeruginosa* GacA, a factor in multihost virulence, is also essential for biofilm formation. **Mol. Microbiol.** **40:** 1215-26.
74. **P. Mukhopadhyay, Zheng M., Bedzyk L.A., LaRossa R.A., Storz G.** 2004. Prominent roles of the NorR and Fur regulators in the *Escherichia coli* transcriptional response to reactive nitrogen species. **Proc. Natl. Acad. Sci. U.S.A.** 101(3): 745-50.
75. **Pernestig, A., Georgellis D., Romeo T., Suzuki K., Tomenius H., Normark S. and Melefors O.** 2003. The *Escherichia coli* BarA-UrY two-component system is needed for efficient switching between glycolytic and gluconeogenic carbon sources. **J. Bacteriol.** **185:** 843-853.
76. **Pernestig A. K., Melefors O. and Georgellis D.** 2001. Identification of UvrY as the cognate response regulator for the BarA sensor kinase in *Escherichia coli*. **J. Biol. Chem.** **276(1):** 225-31.
77. **Pernestig A. K., Normark S. J., Georgellis D. and Melefors O.** 2000. The role of the AirS two-component system in uropathogenic *Escherichia coli*. **Adv. Exp. Med. Biol.** **485:**137-142.
78. **Prigent-Combaret C., Prensier G., Le Thi T. T., Vidal O., Lejeune P. and Dorel C.** 2000. Developmental pathway for biofilm formation in curli-

producing *Escherichia coli* strains: role of flagella, curli and colanic acids. **Env. Microbiol.** **2(4)**: 450-464.

79. **Prouty A. M. and Gunn J. S.** 2000. *Salmonella enterica* serovar Typhimurium invasion is repressed in the presence of bile. **Infect. Immun.** **68**: 6763-6769.
80. **Prouty, A. M., Schwesinger W. H. and Gunn J. S.** 2002. Biofilm formation and interaction with the surfaces of gallstones by *Salmonella* spp. **Infect. Immun.** **70**: 2640-2649.
81. **Ramphal R., Arora S. K. and Ritchings B. W.** 1996. Recognition of mucin by the adhesin-flagellar system of *Pseudomonas aeruginosa*. **Am. J. Respir. Crit. Care Med.** **154(4 Pt 2)**: S170-4.
82. **Rich J. J., Kinscherf T. G., Kitten T. and Willis D. K.** 1994. Genetic evidence that the *gacA* gene encodes the cognate response regulator for the *lemA* sensor in *Pseudomonas syringae*. **J. Bacteriol.** **176**: 7468-7475.
83. **Robinson V. L., Buckler D. R. and Stock A. M.** 2000. A tale of two components: A novel kinase and regulatory switch. **Nat. Str. Biol.** **7**: 626-633.
84. **Sahu, S. N., Acharya, S., Tuminaro, H., Patel, I., Dudley, K., LeClerk, E., Cebulla, T. and Mukhopadhyay S.** 2003. The bacterial adaptive response gene *barA*, encodes a novel histidine kinase regulatory switch for adaptation and modulation of metabolism in *E. coli*. **Mol. Cell. Biochem.** **(253(1/2))**: 167-77

85. **Sanchez-Contreras M., Martin M., Villacieros M., O'Gara F., Bonilla I. and Rivilla R.** 2002. Phenotypic selection and phase variation occur during alfalfa root colonization by *Pseudomonas fluorescens* F113. **J. Bacteriol.** **184(6):** 1587-96.
86. **Schellhorn H. E.** 1995. Regulation of hydroperoxidase (catalase) expression in *Escherichia coli*. **FEMS Microbiol. Lett.** **131:** 113-119.
87. **Schmitt C. K., Ikeda J. S., Darnell S. C., Watson P. R., Bispham J., Wallis T. S., Weinstein D. L., Metcalf E. S. and O'Brien A. D.** 2001. Absence of all components of the flagellar export and synthesis machinery differentially alters virulence of *Salmonella enterica* serovar Typhimurium in models of typhoid fever, survival in macrophages, tissue culture invasiveness, and calf enterocolitis. **Infect. Immun.** **69(9):** 5619-25.
88. **Shea J. E., Hensel M., Gleeson C. and Holden D. W.** 1996. Identification of a virulence locus encoding a second type III secretion system in *Salmonella typhimurium*. **Proc. Natl. Acad. Sci. USA** **93:** 2593-2594.
89. **Simpson D. A., Ramphal R. and Lory S.** 1995. Characterization of *Pseudomonas aeruginosa* *fliO*, a gene involved in flagellar biosynthesis and adherence. **Infect. Immun.** **63(8):** 2950-7.
90. **Smith J.N., Ahmer B.M.** 2003. Detection of other microbial species by *Salmonella*: expression of the SdiA regulon. **J Bacteriol.****185(4):**1357-66.
91. **Southern E., Mir K., Shchepinov M.** 1999. Molecular interactions on microarrays. **Nat. Genet.** **21:**5-9.

92. **Sperandio V., Mellies J. L., Nguyen W., Shin S. and Kaper J. B.** 1999. Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic *Escherichia coli*. **Proc. Natl. Acad. Sci. USA.** **96(26):** 15196-201.
93. **Sperandio V., Torres A. G., Giron J. A. and Kaper J. B.** 2001. Quorum sensing is a global regulatory mechanism in enterohemorrhagic *Escherichia coli* O157:H7. **J. Bacteriol.** **183(17):** 5187-5197.
94. **Sperandio V., Torres, A. G. and Kaper, J. B.** 2002. Quorum sensing *Escherichia coli* regulators B and C (QseBC): a novel two-component regulatory system involved in the regulation of flagella and motility by quorum sensing in *E. coli*. **Mol. Microbiol.** **43(3):** 809-821.
95. **Sperandio V., Li C.C., Kaper J.B.** 2002. Quorum-sensing *Escherichia coli* regulator A: a regulator of the LysR family involved in the regulation of the locus of enterocyte effacement pathogenicity island in enterohemorrhagic *E. coli*. **Infect Immun.** **70(6):** 3085-93.
96. **Stancik L. M., Stancik D. M., Schmidt B., Barnhart D. M., Yoncheva Y. N. and Slonczewski J. L.** 2002. pH-dependent expression of periplasmic proteins and amino acid catabolism in *Escherichia coli*. **J. Bacteriol.** **184(15):** 4246-4246.
97. **Surette MG, Bassler BL.** 1999. Regulation of autoinducer production in *Salmonella typhimurium*. **Mol Microbiol.** **31(2):**585-95.
98. **Suzuki K., Wang X., Weilbacher T., Pernestig A. K., Melefors O., Georgellis D., Babitzke P., Romeo T.** 2002. Regulatory circuitry of the

- CsrA/CsrB and BarA/UvrY systems of *Escherichia coli*. **J. Bacteriol.** **184(18)**: 5130-40.
99. **Teplitski M., Goodier R.I., Ahmer B.M.** 2003. Pathways leading from BarA/SirA to motility and virulence gene expression in *Salmonella*. *J Bacteriol.* 185(24): 7257-65.
100. **Torres, A.G., and Kaper J.B.** 2002. PAIs of intestinal *E. coli*. **Curr Top Microbiol Immunol.** **264(1)**:31-48.
101. **Uhl M. A. and Miller J. F.** 1996. Integration of multiple domains in a two-component sensor protein: the *Bordetella pertussis* BvgAS phosphorelay. **EMBO J.** **15**: 1028-36.
102. **Van Asten F. J., Hendriks H. G., Koninkx J. F., Van der Zeijst B. A. and Gaastra W.** 2000. Inactivation of the flagellin gene of *Salmonella enterica* serotype enteritidis strongly reduces invasion into differentiated Caco-2 cells. **FEMS Microbiol. Lett.** **185(2)**: 175-9.
103. **Vazquez-Torres A. and Fang F. C.** 2000. Cellular routes of invasion by enteropathogens. **Curr. Opin. Microbiol** **3**: 54-59.
104. **Vazquez-Torres A., Jones-Carson J., Mastroeni P., Ischiropoulos H. and Fang F. C.** 2000. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental Salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages *in vitro*. **J. Exp. Med.** **192**: 227-236.

105. **Watson A., Mazumder A., Stewart M., Balasubramanian S.** 1998. Technology for microarray analysis of gene expression. **Curr. Opin. Biotechnol.** **9**:609-14.
106. **Wei B. L., Brun-Zinkernagel A., Simecka J. W., Pruß B. M., Babitzke P. and Romeo T.** 2001. Positive regulation of motility and *flhDC* expression by the RNA-binding protein CsrA of *Escherichia coli*. **Mol. Microbiol.** **40(1)**: 245-256.
107. **Wei Y., Lee J. M., Smulski D. R. and LaRossa R. A.** 2001. Global impact of *sdia* amplification revealed by comprehensive gene expression profiling of *Escherichia coli*. **J. Bacteriol.** **183(7)**: 2265-72.
108. **Weilbacher T., Suzuki K., Dubey A.K., Wang X., Gudapaty S., Morozov I., Baker C.S., Georgellis D., Babitzke P., Romeo T.** 2003. A novel sRNA component of the carbon storage regulatory system of *Escherichia coli*. **Mol. Microbiol.** **48(3)**: 657-70.
109. **Whiteley, M., M. R. Parsek, and E. P. Greenberg.** 2000. Regulation of quorum sensing by RpoS in *Pseudomonas aeruginosa*. **J. Bacteriol.** **182**:4356-4360.
110. **Williams V. and Fletcher M.** 1996. *Pseudomonas fluorescens* adhesion and transport through porous media are affected by lipopolysaccharide composition. **Appl. Environ. Microbiol.** **62**: 100-104.
111. **Wilson J. A., Doyle T. J. and Gulig P. A.** 1997. Exponential-phase expression of *spvA* of the *Salmonella typhimurium* virulence plasmid:

induction in intracellular salts medium and intracellularly in mice and cultured mammalian cells. **Microbiology** **143**: 3827-3839.

112. **Winzer K., Williams P.** 2003. *Escherichia coli* gets the message. **Nat. Med.** **9**: 1118-1119.
113. **Wong, S.M., Carroll,P.A. et al.**1998.Modulation of expression of the ToxR regulon in *Vibrio cholerae* by a member of the two-component family of response regulators.*Inf and Imm.*
114. **Xavier K. B. and Bassler B. L.** 2003. LuxS quorum sensing: more than just a numbers game. **Curr. Opin. Microbiol.** **6(2)**: 191-977.
115. **Zhang J. P. and Normark S.** 1996. Induction of gene expression in *Escherichia coli* after pilus-mediated adherence. **Science.** **273**: 1234-1236.
116. **Zhu J., Miller M.B., Vance R.E., Dziejman M., Bassler B.L., Mekalanos J.J.** 2002. Quorum-sensing regulators control virulence gene expression in *Vibrio cholerae*. **Proc.Natl.Acad.Sci. U.S.A.** **99(5)**: 3129-34.