REGULATION OF THE EHEC LEE PATHOGENICITY ISLAND BY BACTERIAL AND HOST SIGNALING

APPROVED BY SUPERVISORY COMMITTEE

Vanessa Sperandio, Ph.D. _____

Kevin McIver, Ph.D. _____

Kim Orth, Ph.D. _____

Eric Hansen, Ph.D. _____

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REGULATION OF EHEC LEE PATHOGENICITY ISLAND BY BACTERIAL AND HOST SIGNALING

by

MATTHEW S. WALTERS

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Matthew S. Walters, PhD.

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Supervising Professor: Vanessa Sperandio, PhD

Enterohemorrhagic *E. coli* O157:H7 (EHEC) causes outbreaks of bloody diarrhea and hemolytic-uremic syndrome throughout the world. The locus of enterocyte effacement (LEE) consists of five major operons (*LEE1* – *LEE5*) and is required for formation of attaching and effacing (AE) lesions that disrupt intestinal epithelial microvilli. We have previously reported that expression of EHEC LEE genes is regulated by the *luxS* quorum sensing system. The *luxS* gene in EHEC affects the production of autoinducer-3 (AI-3), which activates the LEE. Epinephrine and norepinephrine also activate the LEE in a manner similar to AI-3. The *luxS* mutant had diminished transcription from the LEE promoters during mid-exponential growth phase, decreased levels of the LEE-encoded proteins EscJ, Tir, and EspA, and reduced secretion of EspA and EspB, encoded by *LEE4*. Epinephrine enhanced LEE expression in both wildtype (WT) and the *luxS* mutant, but WT still exhibited

greater LEE activation. The results suggest a possible synergistic relationship between AI-3 and epinephrine. The combined effects of these two signaling molecules may lead to greater LEE expression and a more efficient infection.

Given the virulence defects resulting from the *luxS* mutation, we next examined pathways which may be affected that lead to reduced AI-3 synthesis. We show that several species of bacteria synthesize AI-3, suggesting a possible role for AI-3 in inter-species bacterial communication. The LuxS enzyme produces the autoinducer-2 (AI-2) precursor 4,5-dihydroxy-2,3-pentanedione (DPD) and homocysteine. Homocysteine is required for the *de novo* synthesis of methionine in the cell. The *luxS* mutation leaves the cell with only one pathway for the synthesis of homocysteine, involving the use of oxaloacetate and L-glutamate. The exclusive use of this pathway appears to alter metabolism in the *luxS* mutant, leading to decreased production of AI-3. Addition of aspartate and increasing the cellular concentration of aromatic amino acids, such as tyrosine, restored AI-3-dependent phenotypes in a *luxS* mutant. The defect in AI-3 production, but not in AI-2 production, was also restored by expressing the *P. aeruginosa S*-adenosylhomocysteine hydrolase, which produces homocysteine directly from *S*-adenosylhomocysteine, in the *luxS* mutant. Furthermore, Phenotype MicroArrays (Biolog) revealed that the *luxS* mutation caused numerous metabolic deficiencies, while AI-3 signaling had little effect on metabolism. These studies examine the effects of the *luxS* mutation on LEE expression, how AI-3 production is affected by mutation of *luxS*, and explores the roles of the LuxS / AI-2 system in metabolism and QS.

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Walters, M., Sircili, M.P., and Sperandio, V. (*In Press*) AI-3 Synthesis is Not Dependent on *luxS* in *E. coli*. Journal of Bacteriology.

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LIST OF ABBREVIATIONS

86-24	Enterohemorrhagic *E. coli* wild-type strain 86-24
AE	Attaching and Effacing
AHL	Acyl homoserine lactone
AI	Autoinducer
AI-2	Autoinducer-2
AI-3	Autoinducer-3
CDC	Centers for Disease Control
CFU	Colony forming unit
CRP-cAMP	Cyclic AMP receptor protein-cyclic AMP complex
DAEC	Diffusely adherent *E. coli*
DMEM	Dulbecos modified Eagle's medium
DNA	Deoxyribonucleic acid
DPD	AI-2 precursor; 4,5-dihydroxy-2,3-pentanedione
E. coli	*Escherichia coli*
EAEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EnA-78	Epithelium-derived neutrophil-activating peptide 78
EPEC	Enteropathogenic *E. coli*
Esps	*E. coli* secreted proteins

ETEC	Enterotoxigenic *E. coli*
FAS	Fluorescence Actin Staining
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
g	Gram
Gb3	Globotriaosylceramide
GCP-2	Granulocyte Chemotactic Protein 2
gDNA	Genomic DNA
GI	Gastrointestinal
h	Hour(s)
HUS	Hemolytic Uremic Syndrome
IF-0	Inoculating fluid-0
IL	Interleukin
IPTG	Isopropyl-β-d-thiogalactopyranoside
IS	Insertion sequence
kDa	kiloDalton
LB	Luria-Bertani
LEE	Locus of Enterocyte Effacement
ler	*LEE*-encoded regulator
LPS	Lipopolysaccharide
Map	Mitochondrion-associated protein
MG1655	*E. coli* K-12

MGSA	Melanoma Growth Stimulatory Activity
MIP	Macrophage Inflammatory Protein
ml	Milliliter
Nle	Non-LEE-encoded
N-WASP	Neuronal Wiskott-Aldrich Syndrome Protein
OD	Optical Density
PBS	Phosphate-buffered saline
PC medium	Preconditioned medium
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PM	Phenotype MicroArray
PMN	Polymorphonuclear leukocytes
QS	Quorum sensing
qse	*quorum sensing E. coli regulator*
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel
SOC	Super optimal catabolite medium

SRH	S-ribosylhomocysteine
sRNAs	Small regulatory RNAs
Stx	Shiga toxin
Tir	Translocated intimin receptor
TNF-α	Tumor necrosis factor alpha
TTSS	Type III secretion system
UTR	Untranslated region
VS94	Enterohemorrhagic *E. coli luxS* mutant
WCL	Whole-cell lysates
WT	Wildtype
µl	Microliter

CHAPTER ONE

ESCHERICHIA COLI O157:H7 LITERATURE REVIEW

I. Classification of Enterohemorrhagic *Escherichia coli* O157:H7

Escherichia is a genus of gram negative, facultatively aerobic, rod-shaped intestinal bacteria. *Escherichia coli* (*E. coli*) belongs to the family of Enterobacteriaceae, whose name is derived from the Greek word *enterikos* that relates to the intestine. *E. coli* was first isolated and characterized in 1885 by the German pediatrician and bacteriologist Theodor Escherich and originally named *Bacterium coli*. In 1919, the name was changed to *Escherichia coli* after its discoverer. *E. coli* is a ubiquitous inhabitant of the gastroinstestinal (GI) tract of both humans and animals. Enterohemorrhagic *Escherichia coli* O157:H7, or EHEC, is a strain of *E. coli* that is responsible for outbreaks of hemorrhagic colitis and hemolytic-uremic syndrome (HUS) throughout the world and was first described by Riley *et al.* in 1983 (155). *E. coli* O157:H7 gets its name from the fact that it expresses the 157th identified somatic "O" antigen and the 7th flagellar "H" antigen.

II. Historical Perspective

E. coli is one of the most versatile microorganisms known. It is widely used as a cloning host in recombinant DNA technology, and it is the most abundant facultative

anaerobe of the normal intestinal microflora of humans and other mammals. Human infants are typically colonized within a few days after birth (55). *E. coli* is able to occupy a niche in the mucous layer of the mammalian intestine where it coexists with the host in a commensal relationship. Despite the large numbers of other bacteria at this site, *E. coli* is able to maintain its niche.

Several *E. coli* clones have acquired specific virulence factors that enable them to colonize new niches leading to a broad spectrum of diseases in humans. These virulence traits are oftentimes encoded on mobile genetic elements capable of horizontal gene transfer or on genetic elements that were once mobile and have now become a more stable part of the genome. The advantageous combinations of persistent virulence factors have led to the development of *E. coli* pathotypes capable of causing disease in healthy individuals. These pathotypes cause disease leading to three general clinical syndromes: enteric / diarrheal disease, urinary tract infections, and sepsis / meningitis (93). The intestinal *E. coli* pathogens are grouped into six well defined categories: EHEC, enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), and enteroinvasive *E. coli* (EIEC). This dissertation research focuses on EHEC, a pathogen responsible for causing disease throughout the world.

Enterohemorrhagic *Escherichia coli* O157:H7 is a bacterial pathogen responsible for outbreaks of bloody diarrhea and HUS. EHEC colonizes the large intestine where it forms attaching and effacing (AE) lesions and produces Shiga toxins (Stx), which are responsible for the development of HUS. HUS is a major contributor to the morbidity and mortality observed in children and elderly. EHEC was identified as an emerging pathogen in a report

by Riley *et al.* in 1983 (155). The authors investigated two outbreaks of "an unusual gastrointestinal illness" in which at least 47 people in Oregon and Michigan developed bloody diarrhea with little or no fever after eating undercooked hamburger meat from the same fast-food restaurants. Bacterial cultures from stools and contaminated beef did not reveal any known pathogens. The authors discovered a previously rare *E. coli* serotype, O157:H7, in the stools of patients and from a contaminated beef patty. Riley *et al.* concluded that *E. coli* O157:H7 was responsible for a clinically distinctive GI illness and transmitted by undercooked meat.

III. Epidemiology

E. coli O157:H7 is a major cause of illness throughout the world. Over 30 countries on six continents have reported human infections of EHEC. In the United States, EHEC is projected to cause 73,480 illnesses annually, leading to an estimated 2,168 hospitalizations and 61 deaths each year (153). It was not until 1993, after a large multistate outbreak linked to contaminated beef that affected over 700 people and resulted in the deaths of four children (15), that EHEC became recognized as an important emerging pathogen in the United States. The Centers for Disease Control (CDC) recognized EHEC as a notifiable infection in 1994 and required mandatory reporting of infections in the continental United States in 2000. Figure 1 illustrates the number of EHEC outbreaks from 1982-2002 (153). There were 350 reported outbreaks leading to 8,598 cases of EHEC infection between 1982 and 2002 (153).

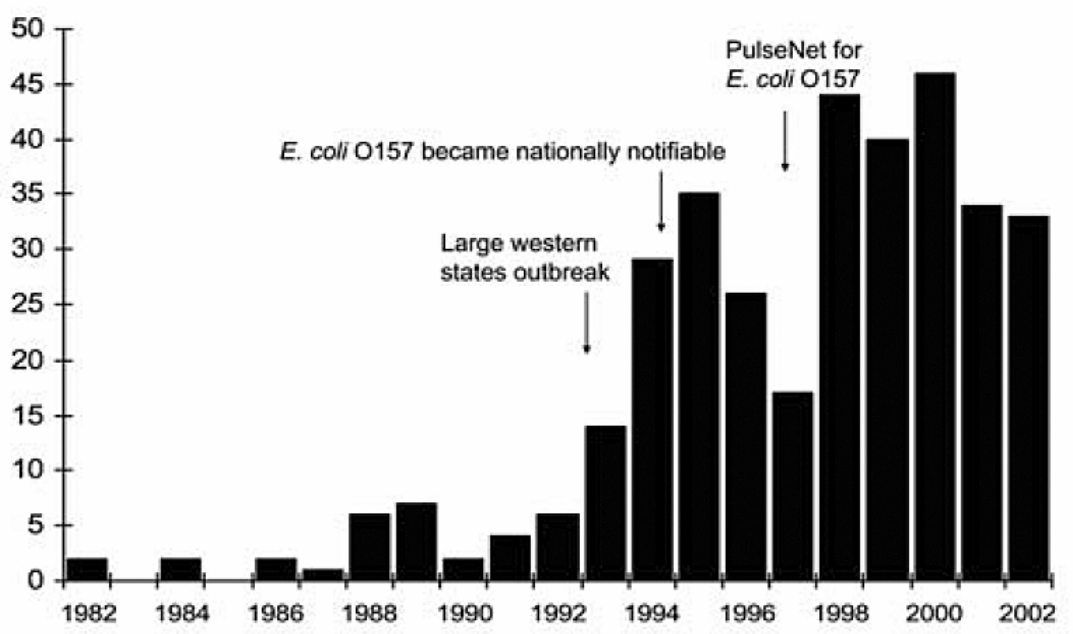


Figure 1: *Escherichia coli* O157:H7 outbreaks by year, 1982-2002 (153).

These cases resulted in 1,493 (17.4%) hospitalizations, 354 (4.1%) cases of HUS, and 40 (0.5%) deaths (153). Of the 40 deaths, 25 were the result of HUS and 15 were due to other causes. Age ranges of fatalities were between 1-4 years and 61-91 years (153), illustrating the increased risk of EHEC infection in the young and elderly.

Infected bovine species are the main reservoir of EHEC. It is thought that the colonization rates of EHEC in bovine populations are extremely high. One study in Bavaria found that 84%-90% of the cattle tested were colonized with Stx-producing EHEC (151). In addition to cattle, EHEC has been isolated from many other animals, including sheep, deer, goats, dogs, birds, horses, and flies (26, 77). EHEC infections can be transmitted by a number of different means, though in many cases the route of transmission is unclear.

Food is the principal transmission route leading to EHEC infection. The first outbreak of EHEC was reported in 1982 and linked to ground beef (155). Ground beef and beef products continue to be the most common vehicle of EHEC transmission. Of the 350 outbreaks reported between 1982 and 2002, 183 outbreaks involved the transmission of EHEC from contaminated food products (153). Of these foodborne outbreaks, the mean of transmission in 75 of the cases was ground beef, unknown in 42 cases, produce in 38 cases, other beef products in 11 cases, other food in 10 cases, and dairy products in 7 cases (153). The outbreaks involving ground beef peaked in the summer months and occurred most frequently at the community wide level (153). Other bovine products implicated in outbreaks of EHEC include roast beef, salami, and raw milk. The infectious dose of EHEC is very low. During a 1994 outbreak of EHEC infections from salami, the infectious dose was estimated to be fewer than 50 colony forming units (CFU) (205).

Transmission of EHEC infection can also occur from person-to-person contact, waterborne outbreaks, and animal contact. Person-to-person transmission is spread by the fecal-oral route. During the 20 year study by Rangel *et al.*, 80% of the person-to-person outbreaks occurred in child daycare centers and also peaked during the summer months (153). Many EHEC outbreaks have also been waterborne; either from recreational water or drinking water. Recreational water-associated outbreaks were first reported in 1991 and occurred mostly in lakes or ponds (153). During 1982 to 2002, outbreaks involving contaminated drinking water tended to result in a greater number of cases per outbreak. Drinking water outbreaks only constituted 3% of all outbreaks, but accounted for 15% of all outbreak-related cases (153). Several of the drinking water outbreaks occurred in municipal or local well water systems without proper chlorination. Animal contact is the newest reported mode of transmission for EHEC infections and was first reported in the United States in 1996. This mode of transmission has been recently publicized due to several outbreaks resulting from animal contact at farms, petting zoos, or county fairs. Case-fatality rates do not seem to vary significantly by transmission route (153).

Surveillance detects only a small proportion of actual EHEC infections, because not all infected individuals seek treatment. EHEC infections may also be misdiagnosed or laboratories may not screen for EHEC strains from stool samples. From 1982 to 2002, approximately 89% of the reported outbreaks occurred between the months of May to November. Minnesota reported the most outbreaks (43 outbreaks), followed by Washington (27 outbreaks), New York (22 outbreaks), California (18 outbreaks), and Oregon (18 outbreaks) (153). Griffin *et al.* also found that EHEC infections were more common in

northern states than southern states and that the highest incidence of infection occurred during the summer months (72). The mechanism behind these phenomena is currently not known.

In 1992, pulsed-field gel electrophoresis (PFGE) was developed as a method of subtyping *E. coli* O157 strains, leading to improved early detection by the national network PulseNet (194). Estimation of the overall incidence of EHEC infections and the spreading of EHEC serotypes is complicated by the lack of standard methodology among laboratories and the fact that stool culture is not commonly performed for EHEC infections (95). The continued molecular subtyping of EHEC strains will greatly increase the detection of outbreaks that are geographically dispersed and spread by contaminated commercial products.

IV. Clinical Presentation, Diagnosis, and Treatment

EHEC infection begins with ingestion of the organism, followed by a 3 to 4 day incubation period. During the incubation period, EHEC colonizes the large intestine and multiplies. After the typical incubation period, patients develop nonbloody diarrhea and abdominal cramping. One or two days later, patients develop bloody diarrhea. Approximately 94% of cases are resolved within 5 to 7 days after the onset of illness without the assistance of medical treatment. However, about 6% of patients develop HUS (94). EHEC is thought to cause 90% of all cases of HUS in industrialized countries (177). HUS is characterized by thrombocytopenia, acute renal failure, and microangiopathic hemolytic

anemia and is typically diagnosed 6 days after the onset of diarrhea (13). The development of HUS can lead to potentially fatal acute renal failure. Patients infected with EHEC are more likely to report having bloody diarrhea and abdominal cramps and less likely to report fever than patients infected with other intestinal pathogens that cause diarrhea.

Abdominal cramping and bloody diarrhea in the absence of fever may suggest EHEC infection, but culture and isolation of the organism from diarrheal stools is the definitive diagnosis. The agar plates most commonly used to screen for *E. coli* O157:H7 are sorbitol MacConkey and cefixime tellurite sorbitol MacConkey. EHEC does not rapidly ferment sorbitol, which differentiates it from most other *E. coli*. An alternative method of diagnosis of EHEC is directly testing stool samples for the presence of the Stx produced by the bacteria that leads to the development of HUS. The use of gene probes and polymerase chain reaction (PCR) can also be used to identify EHEC from stool samples. The primary targets for PCR detection are the *stx* genes. Arbitrarily primed PCR has been successfully used to discriminate among EHEC O157:H7 strains (81, 118). The results from these assays are arrays of DNA fragments that can serve as strain-specific fingerprints when separated by PFGE. This method is considered to be the "gold standard" of identifying and analyzing the pattern of *E. coli* strains during outbreaks and was crucial to the development of PulseNet. Confirmation of outbreaks can be obtained by isolation of EHEC of the same serotype from the incriminated food.

Treatment of EHEC infections is largely supportive. Patients are monitored for dehydration and symptoms such pallor, oliguria, and vomiting that may indicate HUS. The use of antibiotics in the treatment of EHEC infections is controversial. There has been no

evidence that antibiotics improve the course of disease. Certain antibiotics have been suggested to increase the amount of toxin released by the bacteria in the intestine and precipitate kidney complications leading to HUS (6, 148, 216, 231). Patients should also avoid the use of antidiarrheal agents, such as loperamide. These drugs have been suggested to worsen the clinical course of EHEC infection (28). Management of HUS involves fluid and electrolyte balance, treatment of anemia, control of hypertension, nutritional support, and treatment of azotaemia (177).

V. Pathology

EHEC infection produces the most severe pathology in the ascending and transverse colon (70, 97). Colonic tissue samples from infected patients range in appearance from normal histopathology to gross dilation with hyperemia leading to hemorrhage, submucosal edema, and an increase in the width of the bowel wall (97, 161). Cases can be so severe that the lumen of the ascending colon is almost completely destroyed. Microscopically the most common colonic pathology is submucosal hemorrhage, fibrin exudation, and edema, with hemorrhage, ulceration, mild neutrophil infiltration in the mucosa, and capillary thrombi being less common (70, 97). EHEC produces a characteristic histopathologic feature known as attaching and effacing (AE) lesions. These lesions are characterized by an extensive rearrangement of the intestinal epithelial cell cytoskeleton to produce a pedestal-like structure leading to an intimate adherence between the bacterium and epithelial cell (Figure 2). Infection leads to the effacement of intestinal epithelial cell microvilli and a mild to

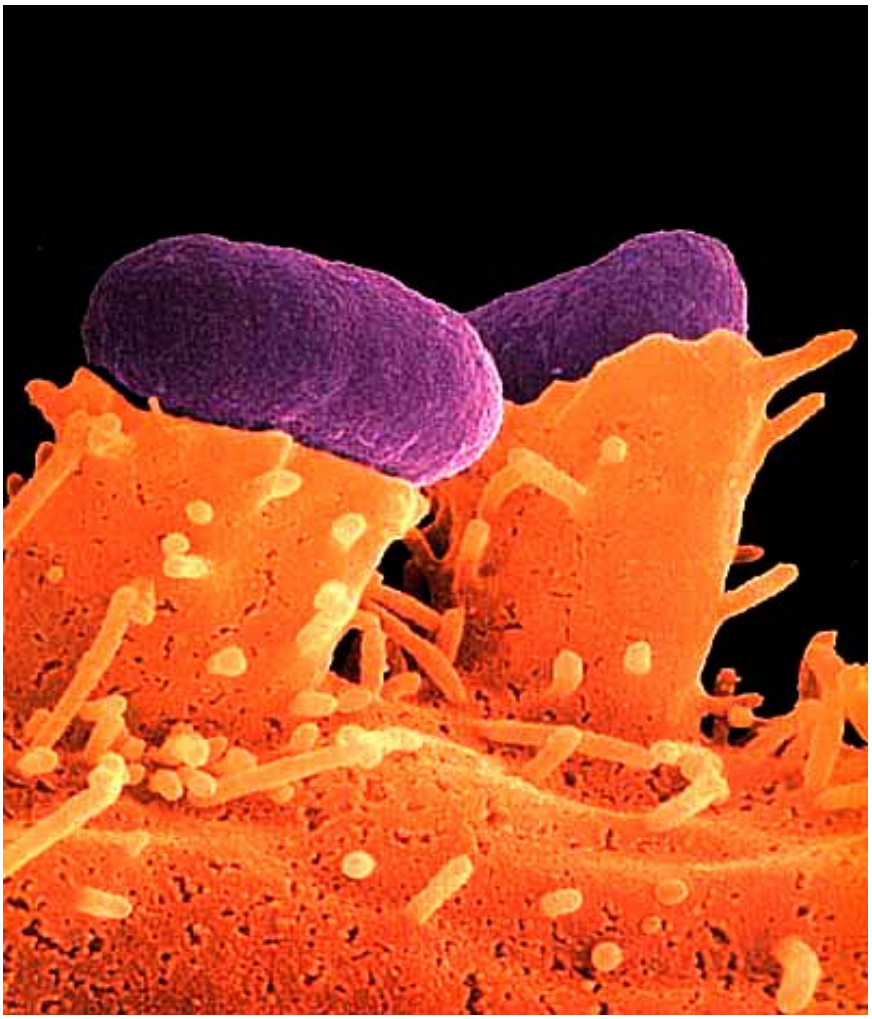


Figure 2: *E. coli* attaching and effacing lesions on epithelial cells. Figure from Rosenshine *et al.* 1996 (160).

moderate infiltration of neutrophils and other inflammatory cells in the lamina propria and epithelium. Dehydration and diarrhea are thought to result from the effacement and the destruction of the microvilli and an increase of intracellular calcium in the eukaryotic cells that leads to chloride secretion (12, 209).

VI. Genetic Content

Genome sequence analysis of two EHEC O157:H7 strains was completed in 2001, identifying many putative virulence factors and allowing for evolutionary analysis of EHEC O157:H7 (79, 150). The EHEC genome is approximately 5.5 Mb and contains 5,361 protein coding regions (79, 150). EHEC contains a 4.1 Mb sequence that is conserved in the *E. coli* K-12 MG1655 strain. This 4.1 Mb sequence represents the conserved chromosomal backbone present in most *E. coli* strains. The EHEC genome contains approximately 1.4 Mb of DNA that is not present in the MG1655 strain and are referred to as O islands. *E. coli* K-12 MG1655 contains 530 Kb of DNA that is absent in the EHEC genome and are referred to as K islands. The extra genomic material in EHEC encodes for several virulence factors that play a role in EHEC pathogenesis, including potential adhesins, iron uptake systems, Stx, and two type-III secretion systems.

E. coli O55:H7 strains are genetically the most closely related ancestor of EHEC O157:H7 (221). EHEC O157:H7 is thought to have evolved through a series of transitional steps from a nontoxigenic ancestor (Figure 3) (56, 222). The first step towards the development of the O157:H7 strain was the acquisition of Stx2 by ancestor 1 (A1, Figure 3),

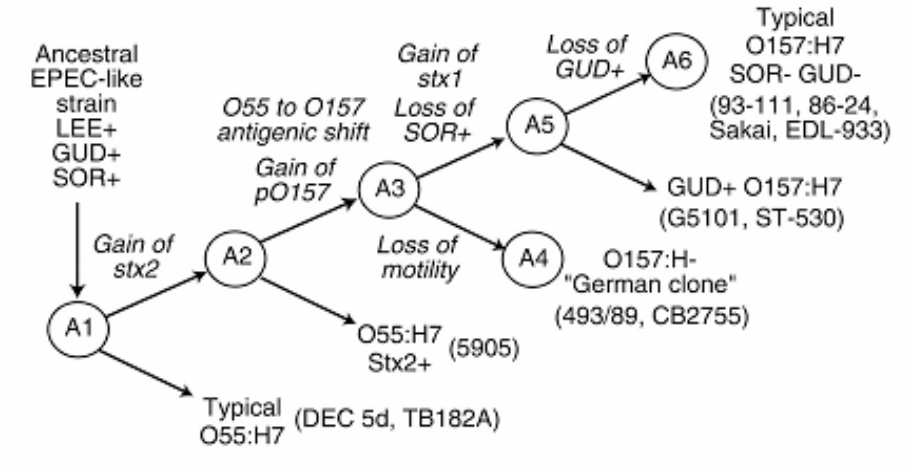


Figure 3: EHEC O157:H7 evolutionary genomic changes. Adapted from Wick *et al.* 2005 (222).

presumably by transduction, creating A2. The large pO157 virulence plasmid was acquired next. The shift of the somatic antigen from O55 to O157 is thought to have occurred at the same time as pO157 acquisition, leading to the development of A3. From this stage, two separate lines have evolved. One branch had a mutation in the flagellar operon leading to the loss of motility (A4). The other branch gained Stx1 and lost the ability to ferment sorbitol (A5). The modern EHEC O157:H7 strain was created by mutational inactivation of the *uidA* gene in the A5 progenitor leading to the non-sorbitol-fermenting, β-glucuronidase-negative phenotype typical of EHEC O157:H7 (A6).

Sequencing of the EHEC genome revealed the presence of many mobile genetic elements. Twenty types of insertion sequences (IS) and 18 prophages or phage remnants have been identified in the genome. Almost half (48.2%) of the EHEC specific sequences (those not present in MG1655) are of bacteriophage origin, suggesting that bacteriophages have played an important role in the evolution of EHEC O157:H7 (79). The genetic content of prophages among closely related EHEC O157:H7 strains has been shown to be extremely variable, suggesting that the phage genomes rapidly diversify (222). This process can lead to the erosion of the phages and loss of genes required for phage mobility. The majority of phage genes are gained and lost quickly, except for those retained by natural selection because they confer advantages to the bacterium (222). Bacteriophage and island acquisitions appear to have promoted the genetic change through a "trial-and-error" process that has led to the development of EHEC O157:H7 (222).

VII. Pathogenesis

A. Colonization of the Gut

The infectious dose of EHEC has been estimated to be on the order of 50 to 100 CFU (147, 205). This is several orders of magnitude lower than the infectious dose of other related enteric pathogens such as ETEC and EPEC. EHEC's resistance to the acidity of the stomach may play an important role in their ability to successfully colonize the human colon at such a low infectious dose. This acid tolerance response is mediated by the stationary-phase sigma factor RpoS. Exposure of EHEC to low pH induces an acid tolerance response, which increases survival in the human stomach and in mildly acidic foods (68, 110).

B. LEE Encoded TTSS

Once EHEC has passed through the stomach and small intestine, it reaches and colonizes the colon. It is thought that EHEC moves towards the intestinal epithelium using its flagella. A *fliA* mutant, which cannot produce flagella, has been shown to be less fit in a streptomycin-treated cattle model for colonization (183), underlying the importance of motility in EHEC colonization and infection. Once the bacteria are in close proximity to the intestinal epithelium, they must adhere to the target cells. Different EHEC strains, even those belonging to serotype O157:H7, display different patterns of adherence to epithelial cells in tissue culture models. Some strains adhere evenly over the surface of cultured

epithelial cells in a process known as diffuse adherence. Other strains use localized adherence in which microcolonies form at a limited number of sites on the epithelial surface. The cell line also influences the pattern of adherence of a given EHEC strain. The initial bacterial adherence process that occurs *in vivo* is not well defined.

The best characterized EHEC adherence phenotype is the AE lesion (Fig. 2). These lesions cause the loss of enterocyte microvilli and intimate attachment of the bacterium to the epithelial cell. Actin and other cytoskeletal components accumulate beneath the attached bacteria, creating a pedestal structure. The genes necessary for the formation of the AE lesions are located on a 35.5 kb pathogenicity island termed the locus of enterocyte effacement (LEE) that encodes a type III secretion system (TTSS) and several effector proteins (Figure 4). The LEE contains 41 genes, organized into five polycistronic operons (50, 51, 127). The LEE is highly conserved across pathogenic bacteria capable of forming AE lesions. It appears that is has been acquired multiple times during the evolution because the LEE is often found inserted into diverse chromosome loci among pathogenic EPEC and EHEC strains (223).

The first gene of *LEE1* encodes the *lee* encoded regulator (*ler*) that is required for expression of the LEE genes (22, 50, 61, 76, 127, 163, 185). The majority of the remaining genes in *LEE1,* as well as the *LEE2* and *LEE3* operons, encode structural and secondary proteins required for the formation of the TTSS (88). *LEE5* contains genes encoding an adhesin (Intimin) and its receptor which is translocated through the TTSS into the host cell (Tir) (89, 99). *LEE4* encodes several *E. coli* secreted proteins (Esps) that make up the

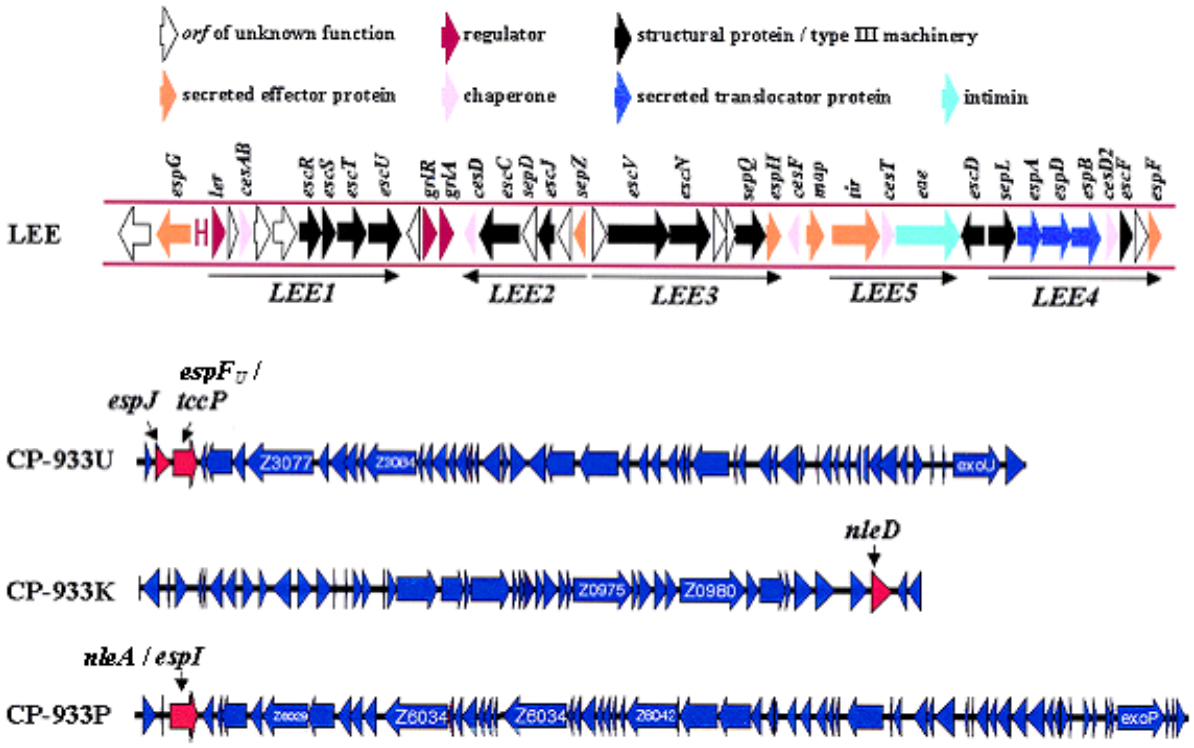


Figure 4: Genetic organization of the EHEC locus of enterocyte effacement (LEE) and prophages CP-933U, CP-933K, and CP933P. Adapted from Garmendia *et al.* 2005 (64).

translocon portion of the TTSS (51, 103).

Type III secretion systems are utilized by pathogenic bacteria to translocate virulence factors from the bacteria directly into the host cell. The EHEC TTSS consists of multiple constituents made from the products of approximately 20 genes (Figure 5). Many of these components are conserved in other TTSSs and assembly occurs in two distinct steps. Membrane bound components are first exported in a *sec*-dependent manner to form the base of the TTSS. Next, proteins which make up the distal portions of the TTSS apparatus are exported via the type III export machinery from the first step in a *sec*-independent manner. The energy for this process and secretion of effector proteins is thought to be provided by cytoplasmic ATPases (5). Much of the work studying formation and function of the LEE TTSS has been performed in EPEC. Given the high homology of the LEE between the bacteria harboring this pathogenicity island, it is generally assumed that the TTSS of each functions in a similar manner.

The main component of the inner membrane portion of the TTSS is made up of EscV, which is encoded within *LEE3*. EscV is directed to the membrane in a *sec*-dependent manner by a putative signal sequence. The product of *escJ*, part of the *LEE2* operon, is thought to span the periplasmic space and connect the inner and outer membrane protein rings (33). EscC, also encoded within *LEE2*, is the main component of the outer membrane portion of the TTSS. This protein belongs to the secretin family of proteins that are found in all known TTSSs. EscC forms a large homomultimeric annular complex that transports molecules across the outer membrane (83). The needle complex is comprised of a single protein encoded within *LEE4* named EscF (224). EscF is essential for the secretion of the

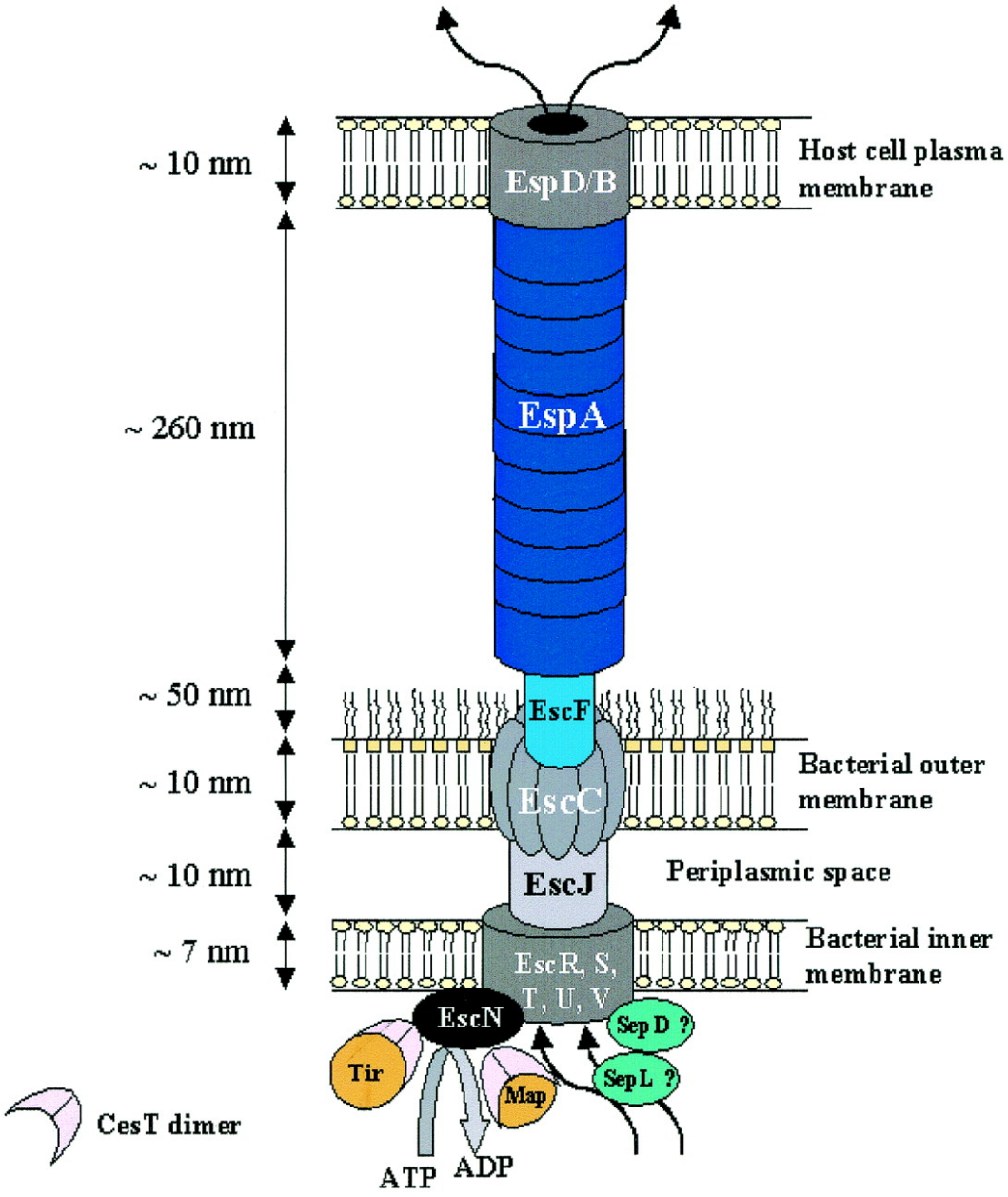


Figure 5: The EHEC type III secretion system. The basal body is composed of the outer membrane protein EscC, the inner membrane proteins EscR, EscS, EscT, EscU, EscV, and the lipoprotein EscJ. The needle complex is formed by EscF and the filament is made up of EspA subunits. EspB and EspD form a pore in the host cell membrane. EscN is the ATPase which provides the energy for protein translocation. SepD and SepL may constitute a switch that controls secretion. Figure from Garmendia *et al*. 2005 (64).

translocator and effector proteins (224). A filamentous extension of the needle complex is formed by the *LEE4* encoded protein EspA (103, 174). The EspA filament creates a channel through which the effector proteins are translocated and is unique to LEE encoded TTSSs. The EspA filament also plays a role in the adhesion of the bacterial cell to the target epithelial cell (45). Two other *LEE4*-encoded proteins, EspB and EspD, interact with each other to form a pore in the plasma membrane of the host cell that allows the translocation of effector proteins into the host cytoplasm (24, 35, 84, 107). The SepD and SepL proteins, encoded by the *LEE2* and *LEE4* operons respectively, interact with each other and may form a molecular switch from secretion of translocator proteins to secretion of effector proteins (31, 64).

The LEE also encodes several chaperones that assist in the proper translocation of effector proteins. CesF, CesT, CesD, CesD2, and CesAB are the five LEE secretion chaperones that have been characterized. CesF is a class IA chaperone and interacts with the effector protein EspF (49). The effector proteins Tir and Map are chaperoned by CesT, another class IA chaperone (1, 30, 47). CesD is a class II chaperone that serves as a chaperone for both EspB and EspD (215). CesD2 is a second chaperone for EspD and is localized to the cytoplasm and inner membrane (139). The last chaperone, CesAB, is required for stabilization of cytoplasmic EspA and formation of the EspA filament (32).

C. EHEC Virulence Factors

1. LEE-Encoded Effectors

In addition to all of the components of the TTSS, the LEE also encodes a number of secreted effector proteins. These proteins are translocated into the host cell and alter eukaryotic cellular functions. EspF is one effector protein that functions to disrupt intestinal barrier function leading to the loss of transepithelial resistance, increased monolayer permeability, and disruption of tight junctions in cell culture models of infection (125). EspF contains four proline-rich repeats and the N-terminal region targets it to the host mitochondria where it disrupts the membrane integrity (137, 141). This interaction induces the release of cytochrome *c* into the cytosol and cleavage of caspases 9 and 3, inducing cellular apoptosis (137, 141).

Mitochondrion-associated protein (Map) also targets mitochondria via an N-terminal sequence when translocated into the target cell (101). Map induces the production of deformed mitochondria and mitochondrial damage by interfering with the cellular ability to maintain mitochondrial membrane potential (98, 101). Map also contributes to the disruption of the intestinal epithelium and tight junctions (39). A third role that has been described for Map is the formation of filopodium-like structures at the sites of bacterial attachment (100). This process is dependent on host Cdc42 and does not appear to be essential for colonization and disease, but it does seem to be advantageous in a competitive environment (44, 135).

EspG is another secreted effector that causes the destruction of microtubule

networks and the formation of actin stress fibers in fibroblasts (122). EspG is similar to the VirA protein of *Shigella flexneri*, which has also been shown to destabilize host microtubules. An *espG* mutant was slightly attenuated, but still able to cause disease in a rabbit model (48). EspB has also been shown to modulate the host cell cytoskeleton at the site of bacterial attachment, in addition to its role in translocation (200, 201). EspB binds to α-catenin, a molecule associated with the cell cytoskeleton, providing further evidence of the role of EspB in rearrangement of the cytoskeleton (104). SepZ is another recently identified secreted effector encoded by the LEE, although the exact function of SepZ inside the host cell remains unclear (90). The translocated intimin receptor (Tir) is encoded by *LEE5* and translocated into the host cell where it localizes to the plasma membrane (99). Tir forms a hairpin-like structure in the plasma membrane with both the N and C terminal ends inside the host cytoplasm leaving an exposed extracellular loop on the surface of the cell (37, 78). The extracellular loop interacts with Intimin, another protein encoded by *LEE5* and expressed on the bacterial surface (37, 78). The binding of Tir to Intimin creates an intimate attachment of the bacterial cell to the epithelial cell. Intimin has also been shown to bind β_1 integrin (58) and nucleolin (179, 180), suggesting that it may also play a role in the initial adherence of the bacterium to the epithelial cell. The amino and carboxy terminal ends of EHEC Tir interact with the phage-encoded protein $EspF_U$ (25, 65). The interaction between $EspF_U$ and Tir is essential for AE lesion formation and actin polymerization. Once associated with Tir, $EspF_U$ binds to neuronal Wiskott-Aldrich syndrome protein (N-WASP) and stimulates Nck-independent actin polymerization in conjunction with Arp2/3 (25, 65). These interactions

with Tir lead to the nucleation of actin, rearrangement of the host cell cytoskeleton, and formation of the pedestal structure beneath the bacterium.

2. Prophage-Encoded Effectors

Several other translocated effector proteins secreted by the LEE TTSS are carried on other pathogenicity islands or prophages (Fig. 4). NleA (also known as EspI (134)), or non-LEE-encoded effector A, is carried on the prophage CP-933P (73). NleA colocalizes with the Golgi apparatus inside of the target cell (73) and was found to be present in 86% of EHEC clinical isolates also containing the LEE (133). The presence of *nleA* was more prevalent in strains from patients with symptomatic EHEC infections (133), suggesting that it is an important EHEC virulence factor. The *nleD* gene is encoded on prophage CP-933K and has been shown to be translocated into host cells in a tissue culture infection model, although its role in pathogenesis remains to be discovered (120). The *espJ* gene encodes another effector protein and is located on the prophage CP-933U. Mutation of *espJ* resulted in EHEC being carried longer in a lamb model of infection (34). EspJ may influence the clearance of EHEC from the host's intestinal tract and play a role in pathogen transmission. The $EspF_U$ (also known as TccP (65)) protein that interacts with Tir is a second non-LEE encoded effector carried on the prophage CP-933U (25). $EspF_U$ is required for the development of AE lesions during EHEC infection.

3. pO157 Effectors

EHEC also contains a variety of plasmid-encoded virulence determinants. The vast majority of EHEC clinical isolates contain a large 90 kb plasmid that has been designated as pO157 (206). This plasmid encodes for a hemolysin that produces small turbid zones of hemolysis on blood agar plates. The genes responsible for production and secretion of the hemolysin, termed EHEC *hlyA-D*, are homologous to the alpha-hemolysin operon of other *E. coli* strains. pO157 also encodes a periplasmic catalase-peroxidase termed *katP* (21). An extracellular serine protease, EscP, is carried on the pO157 plasmid and widespread among serogroup O157 EHEC (20). EspP cleaves coagulation factor V, which may promote mucosal hemorrhage (20). The pO157 plasmid also contains a homologue of *toxB* from *Clostridium difficile* that seems to contribute to epithelial cell adherence (198). StcE is a metalloprotease encoded on pO157 (108). StcE has been shown to cleave C1 esterase inhibitor which leads to reduced complement-mediated lysis of host and bacterial cells (74, 108). StcE also has mucinase activity believed to be important in the early stages of infection, degrading the protective layer of mucins and glycoproteins on the host cells (74). In addition, pO157 has 13 genes that make up a type II secretion system that secretes StcE and may play a role in secretion of other proteins (170). The exact role of pO157 in EHEC virulence has been hindered by the lack of an animal model that manifests all clinical features of human infection.

4. Shiga Toxin

Many pathogenic strains of EHEC produce Stx1, Stx2, or both toxins. The *stx* genes are encoded on prophages in the EHEC genome. Stxs are highly toxic to human renal microvascular endothelial cells and can lead to HUS and acute kidney failure (114, 154). These toxins bind to globotriaosylceramide (Gb_3) receptors, which are highly expressed in the cortical region of the kidney, the main site of renal lesions in patients with HUS (18). Both Stx1 and Stx2 are A-B toxins and homologous to Stx from *Shigella dysenteriae* type 1. The B polypeptide forms a pentamer and binds to the Gb_3 receptor (87, 112). Once bound the entire receptor-holotoxin complex is endocytosed. The internalized toxin then undergoes retrograde transport via the Golgi apparatus and endoplasmic reticulum and is released into the cytosol of the target cell. The A subunit gets cleaved by trypsin and the product is reduced to two subunits, A_1 and A_2. The A_1 polypeptide has *N*-glycosidase activity that causes the depurination of a critical residue in the 28S rRNA of 60S ribosomes halting protein synthesis (52, 165). The A_2 polypeptide is required to bind the A_1 polypeptide to the B subunit (10).

The Stx family is composed of the Stx1 and Stx2 subgroups. The cytotoxic activity of these toxins cannot be neutralized by heterologous antisera (142). Stx1 only has 1 amino acid difference compared to Stx from *S. dysenteriae* type 1 and does not display antigenic diversity (191, 192). On the contrary, Stx2s display significant antigenic divergence from Stx and Stx1 and include Stx2, Stx2c, Stx2d, and Stx2e (149, 171). The variability occurs mainly in the B subunit and can alter receptor binding (171). Stx2 is about 1,000-fold more

toxic to human renal microvascular endothelial cells than Stx1 (114). Epidemiologic studies also indicate that Stx2 EHEC strains are more frequently associated with HUS than EHEC containing Stx1 alone or both Stx1 and Stx2 (143, 173). Stxs may also play a role in the destruction of enterocytes in the lumen and diarrhea. A recent study used a human intestine *in vitro* organ culture system to demonstrate Stx2-induced epithelial cell damage in the absence of Gb_3 (172), although the exact mechanism of this damage is not clear. The divergence of Stx2 may alter binding specificity, leading to a broader range of target cells.

VIII. Animal Models

Numerous animals, including mice, rabbits, pigs, ferrets, cows, baboons, and macaques, have been used as model systems to study EHEC infection, but no single animal model has been able to reproduce all aspects of EHEC disease in humans. Mice have been used to study the effects of Stx administered parenterally, as well as orally administered EHEC infection. Intraperitoneal injection of Stx1 or Stx2 caused colonic mucosal necrosis and hemorrhage, lymphoid necrosis in several tissues, and renal toxic tubular necrosis (144, 203). Oral and intragastric inoculation of EHEC requires that the mice be treated with streptomycin, which depletes the normal intestinal flora and allows for the colonization of a streptomycin-resistant strain of EHEC (214). Mice infected with EHEC in this manner develop lesions in the kidneys consisting of tubular necrosis. However, EHEC do not form AE lesions in mice and neither induce diarrhea nor colitis (214). The use of ferrets as an animal model produced similar results. EHEC was able to colonize streptomycin-treated

ferrets in a manner which was enhanced by the presence of the intimin adhesin and infection resulted in renal histologic damage consistent with human cases of EHEC-mediated HUS (228). As with the mouse model, AE lesions and diarrhea did not occur in the ferret model.

Infant rabbits have also been used as a model of EHEC infection. The infant rabbit model uses rabbits between 3 and 10 days old. This was the first model in which diarrhea was induced by infection with EHEC (54). Intragastric inoculation of EHEC in infant rabbits induces the formation of AE lesions and attachment of EHEC to the colonic mucosal. EHEC infection also induces mucosal epithelial apoptosis, crypt dilation, neutrophil infiltration, and mucin depletion in infant rabbits (145). These effects are thought to be mainly due to Stx, as intragastric inoculation with Stx produces the same pathology. The infant rabbit model has also been used to demonstrate that *eae* and *tir* are required for colonization, consistent with what is predicted to happen in human infection (156). One limitation of the infant rabbit model is that rabbits do not develop microangiopathy or HUS. This may be due to the absence of the Stx receptor, Gb3, which is not expressed in rabbits until they are 16 days old.

The gnotobiotic pig model has been used successfully to demonstrate that EHEC forms AE lesions in the large intestine and causes the effacement of the microvilli near the site of attachment (57, 211). This model has demonstrated the essential role of *eae* in AE lesion formation and the greater toxicity of Stx2-producing strains than Stx1-producing EHEC strains (42, 123, 210). Gnotobiotic pigs also occasionally develop central nervous system vascular lesions caused by Stx2, but do not demonstrate renal lesions as in human infections. The gnotobiotic pig model requires specialized animal facilities and is more expensive than other animal models, limiting its use.

EHEC does not produce disease in adult cattle. However, neonatal calves do develop diarrhea as a result of EHEC infection. This has led to the development of the neonatal calf model of infection in which calves are infected with EHEC between the ages of 36 hours old and as old as 5- to 10-days in some cases (119, 164). Infection of calves with EHEC via suckled milk results in the formation of AE lesions in the large and small intestines, enterocolitis, and diarrhea. Long exposure to EHEC can cause calves to display extensive intestinal hemorrhage, neutrophil infiltration, and pseudomembrane formation (38). Weaned calves have also been used to study asymptomatic EHEC infections to discover factors important for the colonization of cattle and potential ways to reduce EHEC carriage in cattle, reducing the risk of EHEC disease in humans. As with the gnobiotic pig model, the calve model of infection is cost and facility prohibitive.

Nonhuman primates have also been used as model systems for HUS (199) and EHEC infection (92). Intravenous injection of Stx in baboons produced classical HUS with renal failure, thrombocytopenia, schistocytosis, anemia, and melena. Infection of macaques with EHEC produced diarrhea, acute colitis, AE lesions, and disruption of the intestinal epithelium. While nonhuman primates seem to replicate the EHEC pathogenesis observed in humans, they are expensive and difficult to manage for large scale studies, limiting their use.

IX. Host Immune Response to EHEC Infection

The host immunological response to EHEC infection has not been as well characterized as with other bacterial pathogens. The lack of a good animal model of EHEC

infection has hampered efforts to determine host immune responses. Most studies involve tissue culture models of infection or examining serum from patients diagnosed with an EHEC infection. Infected patients exhibit a strong serum antibody response to the O lipopolysaccharide (LPS) antigen. Several other EHEC virulence factors also produce an antibody response from the host. Antibodies against Stx (17, 131, 132), the secreted proteins EspA and EspB (111, 119), enterohemolysin (169), the secreted effector protein Tir (111), and the outer membrane protein Intimin (111) have all been detected in serum from patients infected with EHEC.

EHEC LPS has been shown to produce an imbalance between proinflammatory and anti-inflammatory cytokine levels in blood from patients previously infected with EHEC (220). Patients infected with EHEC have been shown to have decreased levels of interleukin 10 (IL-10), resulting in an increased tumor necrosis factor alpha (TNF-α) / IL-10 ratio (220). The changes in the TNF-α / IL-10 ratio create an imbalance in cytokine levels leading to inflammation. Flagellin and Stx also exacerbate inflammation by activating several CXC chemokines in intestinal epithelial cells (159, 204). Interleukin 8 (IL-8), granulocyte chemotactic protein 2 (GCP-2), melanoma growth stimulatory activity (MGSA), epithelium-derived neutrophil-activating peptide 78 (EnA-78), macrophage inflammatory protein 2α (MIP-2α), and MIP-2β are all potent neutrophil chemoattractants and members of the CXC chemokine family. The CXC chemokines play an important role in the recruitment of polymorphonuclear leukocytes (PMN) to the lamina propria and intestinal lumen during infection. PMNs are able to bind Stx and release the toxin when in contact with target cells expressing the Gb3 receptor (202). They may contribute to the pathogenesis of EHEC by

transporting Stx to target tissues and promoting inflammation leading to the destruction of the intestinal epithelial barrier, allowing Stx into the bloodstream.

X. Vaccinology

Currently, there are two main immunization strategies being investigated to reduce the incidence of EHEC infections in humans. The first option is to vaccinate livestock to reduce EHEC colonization in these animals and lessen the risk of entry into human food products. This strategy requires vaccination of a very large animal population and determination of EHEC factors required for colonization of the animal host. The factors required for carriage in animal populations are not well studied and little is known. Cattle are a major reservoir for EHEC and many human infections are caused by contact with contaminated bovine products. One potential animal vaccine used the proteins secreted by EHEC to induce an immune response in cattle. Although this vaccine did not block colonization, the prevalence and duration of EHEC shedding was diminished in the vaccinated animals compared to controls (152). They found Tir to be an essential component to this vaccine, but Tir alone was unable to protect as well as total secreted protein, suggesting that immunity against colonization was multifactorial (152). The vaccine in this study administered three doses during the clinical trial. This may create a compliance problem with feedlot operators because of the need to repeatedly vaccinate a large number of cattle. In a follow up study that only used two doses of vaccine, there was no significant difference between vaccinated and control animals in the prevalence of fecal EHEC (213).

An animal vaccine may reduce the risks of human EHEC infections, but more work is needed to create an effective and cost-efficient livestock vaccine.

The second vaccine option is to block EHEC transmission to human hosts or interrupt the pathogenic process leading to disease. One area of EHEC vaccine research has focused on creating vaccines against Stx. Vaccines based on Stx are expected to be effective in the prevention of HUS associated with EHEC infections. Many Stx1 and Stx2 vaccines have been created and tested in animals. One problem with vaccinating with the toxin is the adverse effects on the host. To circumvent these effects, several methods have been devised. One technique creates an amino acid substitution in Stx2 in the active-site region of the A subunit. This toxoid has been shown to protect piglets from edema disease after oral challenge with Stx2+ EHEC (17). Another alternative means of avoiding toxicity is to just use the B subunit of the toxin as an immunogen. Several groups have shown that animals produce neutralizing antibodies when immunized with the toxin B subunit (19, 43, 192). More recently, the vaccine potential of live-attenuated enteric bacteria expressing Stx antigens has been examined. The Stx1 B-subunit has been expressed in a *Vibrio cholera* strain and used to immunize rabbits. Sera from these rabbits contained considerable amounts of Stx1 B-subunit specific antibodies capable of neutralizing Stx1 *in vitro* (3).

While Stx-based vaccines may help reduce the risk of patients developing HUS and the associated serious systemic complications, they will not be able to prevent colonization of the gut and transmission of the disease to new hosts. Vaccines against colonization factors required for EHEC virulence may prevent or lessen infection. Intimin has been proposed as a potential vaccine candidate. This protein plays an essential role in the formation of AE

lesions and the intimate attachment between the bacterial cell and epithelial cell. Inactivation of the gene *eae*, which encodes Intimin, attenuated AE lesion formation in new born piglets (42). Butterton *et al*. were able to elicit a strong serum antibody response to Intimin using a *V. cholera* strain carrying a chromosomal copy of *eae* (23). However, cross-protection of vaccines based on Intimin may be limited due to the distinct amino acid sequence heterogeneity among EHEC strains (113). Other vaccines have been developed against LPS from EHEC. These vaccines have been shown to be effective at preventing infection and safe in clinical trials (4, 106). These vaccines will only provide serotype-specific protection, which limits the overall efficacy of the vaccine. More recently, a bivalent conjugate vaccine containing the Stx1 B subunit as a carrier for the O157 O antigen has been used in an attempt to broaden coverage and protection (105). Mice injected with the bivalent vaccine developed neutralizing antibodies against Stx1 and bactericidal antibodies against EHEC O157:H7 (105). Multivalent vaccines may prove useful in the development of a successful broad spectrum vaccine.

XI. Quorum Sensing

Quorum sensing (QS) is a cell-to-cell signaling mechanism that involves the ability of bacteria to regulate gene expression in response to chemical molecules, termed autoinducers (AI), in a dose-dependent manner. When an AI concentration reaches a critical threshold level, the bacteria sense this and respond by altering gene expression. QS was first characterized as controlling bioluminescence in the marine bacterium *Vibrio fischeri* (138).

V. fischeri colonizes the light organs of marine animal hosts where it grows to high cell densities and produces light by expressing the *luxCDABEGH* operon (166). Transcription of this operon, and ultimately production of light, only occurs at high densities of *V. fischeri* and is repressed at low cell densities. LuxI was found to be responsible for the production of an AI that diffuses across the membrane into the environment or back into the bacterial cytoplasm. Once the AI enters back into the cytoplasm, it is bound by LuxR. The LuxR-AI complex then becomes an active transcription factor that promotes the transcription of the *lux* operon and light production.

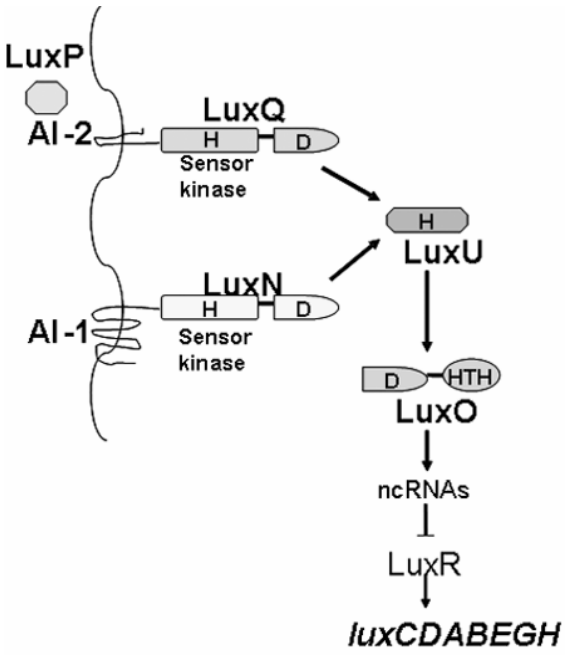
QS has now been attributed to the regulation of an extensive range of functions in diverse bacteria. These activities include antibiotic production by *Erwinia carotovora*, sporulation and competence in *Bacillus subtilis*, plasmid transfer and plant tumor induction by *Agrobacterium tumefaciens*, competence for DNA uptake in *Streptococcus pneumoniae*, and virulence gene expression in many human pathogens, such as *Vibrio cholerae*, *Staphylococcus aureus*, EPEC, and EHEC. To date, three major QS mechanisms have been described. The first system is the LuxIR system, used by gram-negative bacteria and described first in *V. fisheri*. The AI in this system is an acyl homoserine lactone (AHL). More than 70 bacterial species have been found to contain LuxIR homologues (62, 146). This system exhibits high specificity and AIs produced by one bacterial species rarely interact with LuxR homologues from other species of bacteria. The second QS system uses small oligopeptides as AI signaling molecules and is present in gram-positive bacteria. The peptides are synthesized as precursor peptides in the cytoplasm and are cleaved, modified,

and exported. Two-component systems detect the secreted peptide signals and modulate gene transcription.

The last major QS mechanism that has been described is the LuxS / AI-2 system. This system has been found in over 55 bacterial species composed of both gram-negative and gram-positive members (130, 229). The widespread nature of this system has led to the hypothesis that AI-2 may be a universal signal for interspecies bacterial communication (129). The LuxS / AI-2 system was initially characterized as part of the bioluminescence regulatory cascade in *Vibrio harveyi* (167). AI-2 is produced from *S*-adenosylmethionine (SAM) through a series of enzymatic steps. SAM acts as a methyl donor and creates a toxic intermediate *S*-adenosylhomocysteine (SAH) which is hydrolyzed by the enzyme Pfs to *S*-ribosylhomocysteine (SRH) (167). The LuxS enzyme catalyzes the cleavage of SRH to form homocysteine and the AI-2 precursor; 4,5-dihydroxy-2,3-pentanedione (DPD) (167). DPD is an unstable compound that spontaneously cyclizes to form several furanone ring formations, including AI-2.

The structure of AI-2 for *Vibrio* spp. has been determined to be a furanosyl-borate diester (27). The AI-2 signal is detected by the periplasmic protein LuxP that binds to LuxQ. At low cell densities, when a small amount of AI-2 is present, a phosphorylation cascade involving LuxQ, LuxU, and LuxO leads to the expression of small regulatory RNAs (sRNA) that, along with the chaperone Hfq, destabilize the mRNA that encodes LuxR, the protein required for transcription of the luciferase genes (109) (Fig. 6A). When a high concentration of AI-2 is present, LuxQ becomes a phosphatase and the system becomes dephosphorylated,

A.



B.

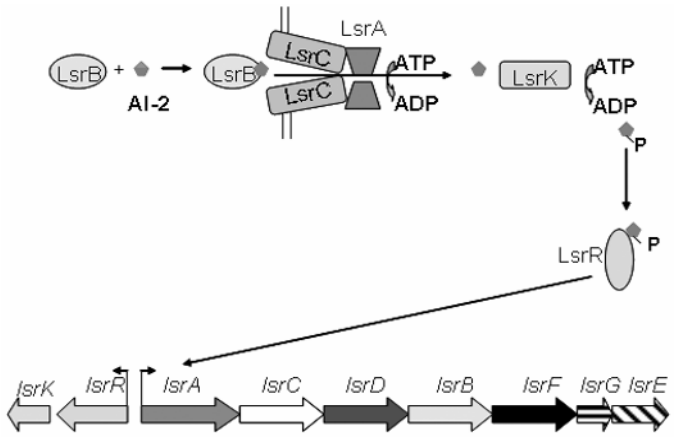


Figure 6: AI-2 signaling pathways in *V. harveyi*, *E. coli*, and *Salmonella*. (A) In *V. harveyi*, AI-2 is bound by LuxP. This signals LuxQ to become a phosphatase leading to LuxU and LuxO dephosphorylation, allowing LuxR expression and activation of the luciferase operon. (B) Uptake of AI-2 by the Lsr ABC transporter system in *E. coli* and *Salmonella*. The *lsrACDBFGE* genes are transcribed as an operon, while *lsrK* and *lsrR* are transcribed divergently. Once AI-2 is bound, it is transported into the cell through the Lsr ABC transporter, phosphorylated by LsrK, and is then thought to interact with LsrR and relieve repression of the *lsr* operon.

allowing for expression of LuxR and activation of the luciferase operon. Homologues of this system have been found only in other *Vibrio* species.

Salmonella enterica serovar Typhimurium and *E. coli* both contain the *luxS* gene and produce AI-2 capable of activating bioluminescence in *V. harveyi*. In *Salmonella* and *E. coli*, the genes that comprise the *lsr* operon are the only genes demonstrated to be directly regulated by AI-2 (197, 230) (Fig. 6B). The *lsr* operon encodes an ABC transporter, where the periplasmic protein LsrB binds a chemically distinct form of the AI-2 signal, (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF), that does not contain boron (130). Directly upstream of the *lsr* operon are two divergently transcribed genes, *lsrR* and *lsrK*. The *lsrR* gene encodes a repressor of the *lsr* operon, while *lsrK* encodes a kinase which phosphorylates internalized AI-2. Phosphorylated AI-2 is then hypothesized to indirectly induce expression of the *lsr* operon by binding to the LsrR repressor and inactivating it, leading to higher expression of the *lsr* operon and increased uptake of AI-2 from the environment (196).

The mutation of *luxS* has pleiotropic effects on the production of autoinducer-3 (AI-3), which serves as the QS signal activating EHEC virulence genes (188). AI-3 increases the transcription of the LEE and activates motility in EHEC (188). This compound was shown to be chemically distinct from AI-2. AI-2 is a very polar furanone that does not bind to C_{18} columns. In contrast, the AI-3 signaling molecule does bind to C_{18} columns and can only be eluted with methanol (188). Electrospray mass spectrometry analysis of AI-3 revealed a major peak at 213.1 Da and minor peaks at 109.1, 164.9, 176.1, 196.1, 211.1, 214.1, and 222.9 Da (188). These masses are different than those of AI-2 (27). Chemical stress tests

have also revealed that AI-3 is an aromatic compound and does not contain a furanone structure like AI-2 (Falck and Sperandio, unpublished data).

XII. Bacteria-Host Signaling

Mammalian cells, as well as bacteria, communicate with each other through the use of hormone or AI-like chemical compounds. The mammalian hormones epinephrine and norepinephrine cross-talk with the AI-3 QS system (188). These catecholamine hormones activate transcription and expression of the LEE and motility genes in a manner similar to AI-3 (188). AI-3 and epinephrine / norepinephrine are agonists, and the action of both signals can be blocked by α and β-adrenergic receptor antagonists such as propranolol and phentolamine (188). Norepinephrine has been previously shown to increase bacterial growth and may act as a siderophore (60). In addition to activation of the LEE and motility genes (188), norepinephrine has also been shown to increase Stx production in EHEC by an unknown mechanism (117).

Both epinephrine and norepinephrine are present in the intestine (46). Epinephrine is synthesized by the central nervous system and the adrenal glands. Since epinephrine is released by the adrenal medulla into the bloodstream, it acts in a systemic manner, including the GI tract. Norepinephrine is synthesized within the adrenergic neurons that are part of the enteric nervous system (63). The physiological role of epinephrine and norepinephrine in the mammalian intestine is to modulate intestinal smooth muscle contraction, chloride and potassium secretion, and submucosal blood flow (82). There are nine known human

adrenergic receptors divided into three subclasses: α1, α2, and β. The structure of the human β2 adrenergic receptor was predicted based on the structure of bovine rhodopsin (the only atomic-level structure of a G protein-coupled receptor currently available) by Freddolino *et al*. (59). The predicted ligand-binding pockets for epinephrine and norepinephrine are largely similar suggesting that both catecholamines are recognized by the same receptor. Based on the fact that epinephrine and norepinephrine activate EHEC virulence genes in a manner strikingly similar to AI-3, these two eukaryotic hormones may also be recognized by the same EHEC receptor as AI-3. EHEC would be able to modulate virulence gene expression based on a bacterial quorum sensing system (AI-3) and a eukaryotic signaling system (epinephrine / norepinephrine), allowing for proper production of virulence factors at the different stages of infection.

XIII. Quorum Sensing in EHEC

Quorum sensing has been shown to regulate several EHEC phenotypes. EHEC cannot synthesize the AHL signaling molecules produced by other bacteria because there is no LuxI homologue in the EHEC genome. However, EHEC does contain one functional LuxR transcriptional activator homologue, SdiA (80), suggesting that EHEC may be able to respond to AHL's produced by other bacteria. SdiA was initially characterized as an activator of the *ftsQAZ* operon that is involved in cell growth and division (7, 91, 218). It has been reported that SdiA may also act as a negative regulator of *espD*, *eae*, and *fliC* (91). However, many of these conclusions were based on over-expression of SdiA in EHEC and

may not represent actual physiological conditions. More work is needed to clearly define the role of SdiA as a QS regulator in EHEC pathogenesis. Indole has also been suggested to be a QS signal in *E. coli* (121, 217). The addition of indole activated transcription of *astD*, *gabT*, and *tnaB* genes (217). Indole is proposed to be produced by the bacteria at high cell densities by TnaAB (217), which is activated by the cyclic AMP receptor protein-cyclic AMP complex (CRP-cAMP) (40). The *astD* and *gabT* encode proteins that degrade amino acids to pyruvate or succinate acid (11). TnaA, which is also induced by indole, allows for the cell to catabolize cysteine, tryptophan, and serine to pyruvate (182). The ability to catabolize these substrates may give EHEC an advantage over other bacteria during stationary phase growth. It has also been reported that indole may act as a signal to control the expression of adhesion and biofilm factors (121).

The notion that QS may be involved in virulence gene regulation of enteric pathogens evolved from EHEC and EPEC. Sperandio, *el al*. found that cell-free supernatants from *E. coli* cultures activated expression of the LEE (187). Since EHEC contained the *luxS* gene and AI-2 was implicated as an important QS signal, an EHEC *luxS* mutant was created. Culture supernatants from the EHEC *luxS* mutant were unable to activate expression of the LEE. An array containing the *E. coli* K-12 genome was used to compare wildtype (WT) EHEC and the *luxS* mutant (187). The microarray analysis revealed that approximately 10% of the genes common between EHEC an *E. coli* K-12 were differentially regulated in the WT and *luxS* mutant.

The array study led to the identification of several QS regulators in the *E. coli* genome that control EHEC virulence factors. These regulators were named *qse* for quorum

sensing *E. coli* regulator. So far, six regulatory factors have been implicated in the QS regulatory cascade in EHEC. QseBC is a two-component system that was found to be regulated by QS (190). QseBC activates the flagellum regulon in response to QS, as well as autoactivating itself (29). QseC is a sensor kinase that responds to the AI-3 and epinephrine QS signals (Clarke *et al*., *Proc. Natl. Acad. Sci. USA.* In Press). A *qseC* mutant is unable to respond to these QS signals and exhibits reduced motility. However, the *qseC* mutant has normal LEE expression, suggesting that another sensor is responsible for detecting the QS compounds and activating the LEE. The microarray analysis revealed a second two-component system, named QseEF, that was differentially regulated in the *luxS* mutant. This two-component system regulates the expression of $EspF_U$, a protein required for AE lesion formation, and may detect the AI-3 and epinephrine / norepinephrine signals (Reading and Sperandio, unpublished data).

QseA is a LysR family member that was found to be regulated by QS (184). QseA is activated by QS and plays a role in the regulation of the LEE. QseA binds to and activates the expression of *ler*, which in turn activates transcription of the *LEE2*, *LEE3*, and *LEE5* operons. A *qseA* mutant has reduced expression of the LEE, but does not exhibit a motility defect (184); therefore, QseA seems to activate expression of the LEE and plays no role in regulation of the flagellum operon. In addition to direct activation of *LEE1*, QseA also seems to indirectly regulate *LEE1* by activating the transcription of the *grlRA* operon located between the *LEE1* and *LEE2* operons (Russell, Sharp, and Sperandio, unpublished data). GrlR has been shown to repress the transcription of the LEE, while GrlA acts as an activator (41). Another regulator of the LysR family, QseD, was also regulated by QS and modulates

expression of the LEE and flagellum genes (Sharp, Walters, and Sperandio, unpublished data). A *qseD* knockout has increased transcription from the *LEE1* promoter and is less motile, suggesting that QseD may be important in the switch from flagellar expression to LEE transcription and formation of the TTSS.

The current model of QS regulation of EHEC virulence gene expression is illustrated in Figure 7. The AI-3, epinephrine / norepinephrine, and AI-2 signaling molecules are imported into the periplasmic space. AI-2 has been shown to activate expression of the LsrABC transporter that is responsible for the uptake of AI-2, but it does not seem to play a role in the regulation of EHEC virulence. AI-3 and epinephrine / norepinephrine interact with the sensor kinases of two two-component systems, QseC and possibly QseE. QseC autophosphorylates and transfers the phosphate to QseB, the cognate response regulator. Phosphorylated QseB activates expression of the *flhDC*, the flagellum master regulator, leading to increased motility. It is hypothesized that QseE also autophosphorylates in response to AI-3 and epinephrine / norepinephrine signaling. QseF, the response regulator, is then phosphorylated and plays a role in the activation of $espF_U$ and possibly other genes required for proper TTSS function and AE lesion formation. The interaction of AI-3 and epinephrine / norepinephrine with at least two sensor kinases may be important in the timing of expression of EHEC virulence genes. In this manner, EHEC could avoid producing both flagella and the LEE TTSS at the same time, a condition that would not be energetically favorable to the cell. It is hypothesized that EHEC first activates expression of the flagellum regulon via QseBC and activates the genes necessary for AE lesion formation through QseEF later.

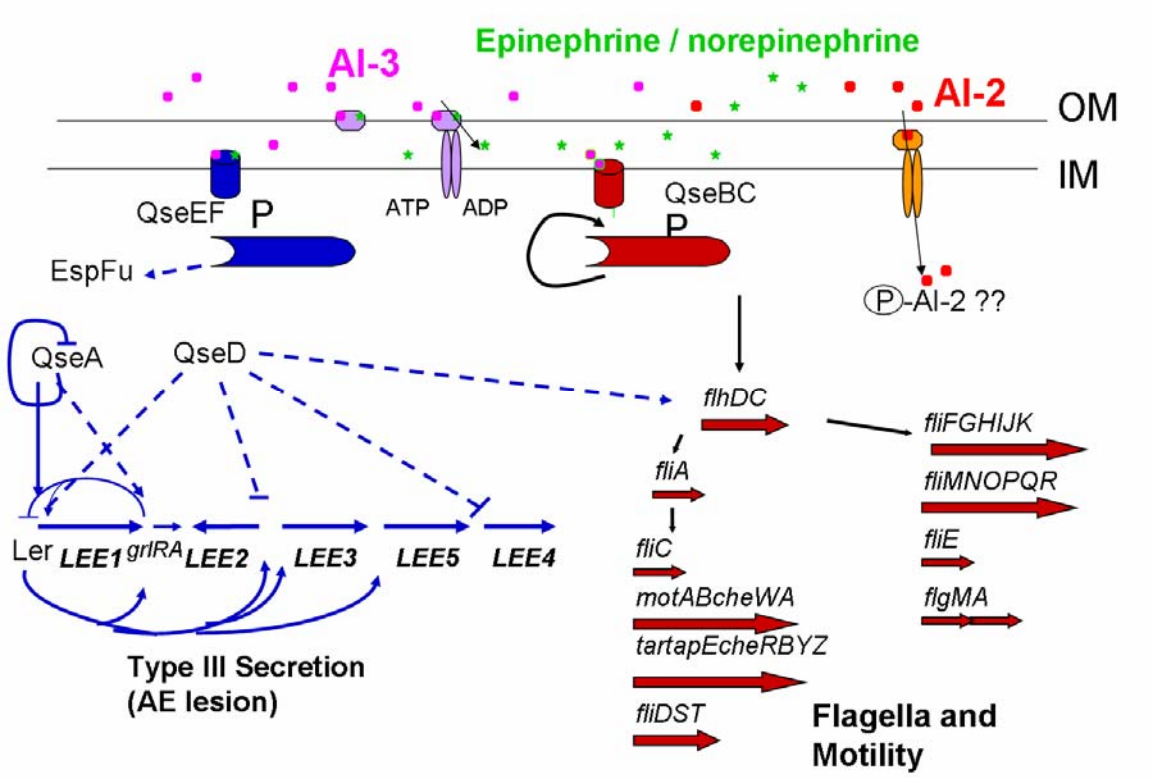


Figure 7: Model of AI-3 / epinephrine / norepinephrine signaling cascade in EHEC. AI-3 and epinephrine / norepinephrine are thought to be recognized by the same outer membrane receptor(s). QseC and QseE interact with these signals and autophosphorylate. These sensor kinases then transfer the phosphate to their cognate response regulators, which modulate virulence gene expression. The QseBC system activates the flagella and motility genes in EHEC in response to AI-3 and epinephrine / norepinephrine. QseEF appears to regulate AE lesion formation. A *qseF* mutant is unable to form AE lesions, because it does not express the effector $EspF_U$. QseA is a positive regulator of the LEE and auto-represses its own transcription. QseD seems to be a negative regulator of the LEE genes and a positive regulator of the flagella and motility genes, suggesting it may provide a link between the LEE and motility genes.

CHAPTER TWO

OVERALL OBJECTIVE AND SYNOPSIS

EHEC is a bacterial pathogen that colonizes the large intestine, resulting in bloody diarrhea and hemolytic uremic syndrome in affected patients. Once in the GI tract of humans, EHEC uses QS to recognize that it is within the host and activate virulence gene expression. AI-3 is produced by the resident GI flora, as well as EHEC, and is one of the signals that activates EHEC virulence gene expression (188). Epinephrine and norepinephrine present in the GI tract also activate expression of EHEC virulence genes in a similar manner (188). Once in close proximity to the epithelial cells, the LEE-encoded TTSS is expressed, leading to AE lesion formation. The LuxS / AI-2 QS system regulates expression of the LEE and motility genes (186, 187). LuxS in directly involved in the production of AI-2 and indirectly affects AI-3 production (167, 188). Mutation of *luxS* disrupts the synthesis of AI-3 and results in decreased LEE gene expression and motility (188). The overall objective of this dissertation has been to characterize the effects of a *luxS* mutation on the kinetics of LEE expression, as well as begin to examine how inactivation of *luxS* diminishes AI-3 production.

Previous studies of LEE gene expression in an EHEC *luxS* mutant strain VS94 (referred to as the *luxS* mutant in this work) utilized a *LEE1*::*lacZ* reporter gene on the *E. coli* K-12 chromosome (187, 188). Using this reporter system, it was shown that AI-3-dependent regulation of the LEE occurred during late-exponential growth in the *E. coli* K-12 background (186, 188). However, a major drawback of this system is that not all of the regulators of the LEE are present in the *E. coli* K-12 genome. There are several known

factors that regulate the LEE genes that are specific to EHEC. To this end, studies were performed to extensively examine the kinetics of LEE gene expression directly in an EHEC background for the first time.

LEE gene transcription was examined in an EHEC background using real-time RT-PCR. The transcription of *ler* (*LEE1*), *escC* (*LEE2*), *escV* (*LEE3*), *eae* (*LEE5*), and *espA* (*LEE4*) were measured during early-, mid-, and late-exponential growth in WT EHEC, an isogenic *luxS* mutant, and a *luxS* complemented strain. It was found that transcription of all five LEE operons was significantly downregulated in the *luxS* mutant during mid-exponential growth, differing from what had been previously observed using the *E. coli* K-12 reporter system. This difference is likely the result of EHEC-specific regulators not present in the *LEE1*::*lacZ* reporter system in *E. coli* K-12, highlighting the importance of studying LEE expression in the native background. Complementation of the *luxS* mutation from a multicopy plasmid containing *luxS* resulted in increased transcription of the LEE genes during early-exponential growth and restored LEE transcription during mid-exponential growth. Addition of epinephrine increased transcription *ler*, *escC*, and *escV* in both WT and the *luxS* mutant. However, WT still exhibited greater transcription during mid-exponential growth, suggesting that the combination of AI-3 and epinephrine leads to greater LEE transcription.

The expression of three proteins encoded by *LEE2* (EscJ), *LEE5* (Tir), and *LEE4* (EspA) during early-, mid-, and late-exponential growth were examined and compared to the transcriptional data. Analysis of whole-cell lysates (WCL) from WT, the *luxS* mutant, and the *luxS* complemented strain revealed that the *luxS* mutant had decreased expression of all

three proteins during mid-exponential growth (compared to WT). This decrease in protein production was not observed in the *luxS* complemented strain. The amounts of EspA and EspB proteins, encoded by *LEE4* and secreted by the TTSS, were also studied during early-, mid-, and late-exponential growth. The *luxS* mutation resulted in reduced amounts of EspA and EspB secretion as compared to WT, while complementation of *luxS* led to earlier and greater secretion of these proteins. Epinephrine increased secretion of EspA and EspB in WT and the *luxS* mutant, but WT still secreted more protein. Ultimately, the decreased expression of the LEE in the *luxS* mutant resulted in a delay in the mutant's ability to form AE lesions on cultured epithelial cells.

The *luxS* mutation resulted in decreased virulence phenotypes, but the underlying mechanism leading to decreased AI-3 production is not known. It is known that the LuxS enzyme is not directly involved in the production of AI-3. Studies were undertaken to begin to characterize pathways that may be affected by the *luxS* mutation resulting in decreased AI-3 synthesis. The *luxS* mutation leaves the cell with only one pathway, involving oxaloacetate and L-glutamate, for *de novo* synthesis of homocysteine, and exclusive use of this pathway seems to alter cellular metabolism. Complementing homocysteine production using two methods restored AI-3 production in the *luxS* mutant as measured by *ler* transcription. Tyrosine is an amino acid essential for the production of epinephrine and norepinephrine. Given the similarities between AI-3 and epinephrine / norepinephrine signaling, it was hypothesized that tyrosine may be important for AI-3 synthesis. Indeed, increasing the concentrations of tyrosine restored transcription of *ler* in the *luxS* mutant.

The results from these studies further characterize the effects of the *luxS* mutation by examining LEE transcription in an EHEC background and also provides for the first time evidence of a synergistic relationship between AI-3 and epinephrine / norepinephrine. The work presented here also distinguishes the role of AI-3 signaling from that of AI-2 signaling and begins to explore how the *luxS* mutation affects AI-3 production. A better understanding of the signals that activate EHEC pathogenesis will help to direct new therapeutic approaches.

CHAPTER THREE

MATERIALS AND METHODS

I. Bacterial strains, plasmids, and cultivation

A list of *E. coli* stains and plasmids used is shown in Table 1. Overnight cultures of *E. coli* were typically grown in Luria-Bertani (LB) broth shacking at 37˚C with the appropriate antibiotic. Antibiotics for selection were used at the following concentrations: ampicillin, 100 μg/ml; streptomycin, 50 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 25 μg/ml. *Vibrio harveyi* strain BB170 was grown in AB medium (0.05 M $MgSO_4$, 0.2% w/v casamino acids, 0.01 M KPO_4, pH 7.0, 1 mM l-arginine, and 2% v/v glycerol) shaking at 30˚C. Recombinant protein expression was induced with 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG). *E. coli* DH5α was used as host for all plasmid constructions and protein purifications. All bacterial strains were stored in LB broth supplemented with 20% glycerol at -80˚C.

II. Transformation of *E. coli*

A. Preparation of chemically competent *E. coli* DH5α

Preparation of chemically competent *E. coli* DH5α was performed using standard

Table 1: Bacterial strains and plasmids used in this study

Strain/Plasmid	Description	Reference
Strain		
86-24	Stx2+ EHEC (serotype O157:H7)	(71)
DH5α	F^- 080d*lacZ* ΔM15 Δ (*lacZYA-argF*)*U169 deoR* *recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* 1- *thi-1 gyrA96 relA1*	Promega
BB170	*V. harveyi* (sensor 1^-, sensor 2^+)	(193)
E2348/69	EPEC (serotype O127:H6)	James B. Kaper
EHEC O26:H11	EHEC clinical isolate	Luis R. Trabulsi
EPEC O111lac:H9	EHEC clinical isolate	Luis R. Trabulsi
E. coli commensal	*E. coli* clinical isolate; 1 strain tested	Hospital Sao Paulo
Shigella sp.	*Shigella* clinical isolate; 5 strains tested	Hospital Sao Paulo
Salmonella sp.	*Salmonella* clinical isolate; 1 strain tested	Hospital Sao Paulo
Klebsiella pneumonia	*Klebsiella pneumonia* clinical isolate; 17 strains tested	Hospital Sao Paulo
Enterobacter cloacae	*Enterobacter cloacae* clinical isolate; 1 strain tested	Hospital Sao Paulo
Citrobacter diversus	*Citrobacter diversus* clinical isolate; 1 strain tested	Hospital Sao Paulo
TEVS232	*LEE1*::*lacZ* reporter strain	(186)
VS94	86-24 isogenic *luxS* mutant	(187)
VS102	E2348/69 isogenic *luxS* mutant	(181)
VS104	VS102 p*luxS*	(181)
lsr mutant	86-24 isogenic Δ*lsrR* mutant	This Study

MW90	VS94 p*luxS*	This Study
MW192	VS94 p*aroP*	This Study
MW196	VS94 p*sahH*	This Study
MW199	VS94 p*tyrP*	This Study
PA01	*Pseudomonas aeruginosa*. *algU*$^{+}$ *algD*$^{+}$ *algW*$^{+}$ *mucD*$^{+}$ Alg2^{-wt}	Type Strain
Plasmid		
pACYC177	Cloning Vector	New England Biolabs
pQE30	Cloning/Expression Vector	Qiagen
pVS212	*luxS* cloned in pQE30	(188)
pVS214	*pfs* cloned in pQE30	(188)
pMW191	*aroP* cloned in pQE30	This study
pMW195	*sahH* from *P. aeruginosa* cloned in pACYC177	This study
pKD3	λRed template plasmid	(36)
pKM201	λRed helper plasmid	(136)
pCP20	λRed resolvase plasmid	(36)
pBAD33	Low copy number expression vector	(75)
pRS551	*lacZ* reporter gene fusion vector	(178)
pd2EGFP	*gfp* vector driven by the *lac* promoter	Clontech

techniques with slight modifications (162). A 1:100 dilution of an overnight DH5α culture was made in LB and grown to OD_{600} 0.4 shaking at 37˚C. Cells were cooled on ice for 15 minutes and then pelleted by spinning at 2,000 revolutions per minute (rpm) for 10 minutes at 4˚C. The supernatant was discarded and the cells were resuspended in Tfb I (30 mM KOAc, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerol, pH 5.8) and incubated on ice for 15 minutes. The cells were then centrifuged at 2,000 rpm for five minutes. The supernatant was removed and the cells were resuspended in Tfb II (10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15% glycerol, pH 6.5). The cells were incubated 15 minutes on ice and then snap frozen and stored at -80˚C.

To perform chemical transformation of *E. coli* DH5α, an aliquot of chemically competent *E. coli* DH5α was thawed on ice. The DNA to be transformed was then added to the cells. Following a 30 minute incubation on ice, the cells were placed at 42˚C for 45 seconds and then placed on ice for two minutes. The cells were then transferred to a 14 ml tube containing 200-1000 µl of super optimal catabolite (SOC) medium and incubated one hour shaking at 37˚C. Cells were then plated on LB-agar plates containing the appropriate antibiotic for selection of the transformed DNA.

B. Electroporation of EHEC

All transformations of EHEC were performed by electroporation. Preparation of electrocompetent EHEC was performed using standard techniques (162). An overnight culture of EHEC was diluted 1:100 in LB broth and grown to OD_{600} 0.5. The cells were then

centrifuged 6,000 rpm for 10 minutes at 4˚C. The supernatant was discarded and the cells were resuspended in 100 ml cold, sterile dH_2O. The cells were centrifuged again at 6,000 rpm for 10 minutes at 4˚C. The supernatant was discarded and the pellet was resuspended in 35 ml cold, sterile dH_2O. The cells were centrifuged for 8,000 rpm for 5 minutes at 4˚C. The pellet was resuspended in 2 ml 10% glycerol and centrifuged again at 8,000 rpm for 5 minutes at 4˚C. The cells were then resuspended in 300 µl 10% glycerol. Forty microliter aliquots were snap frozen and stored at -80˚C.

To perform electroporation of EHEC, an aliquot of electrocompetent EHEC was thawed on ice and the DNA was added. After a minute incubation, the DNA / EHEC mixture was transferred to a 2 mm gap electrocuvette (MBP Molecular Bioproducts). Cells were electroporated using a Biorad GenePulser II set at 25 µF, 200 Ω, and 2.50 kV. The cells were then transferred to a 14 ml tube containing 1 ml SOC medium and incubated at 37˚C for one hour. Cells were then plated on LB-agar plates containing the appropriate antibiotic for selection of the transformed DNA.

III. DNA and RNA Isolation

Standard methods were used to perform plasmid purification (Sigma GenElute Plasmid Kit). Genomic DNA used for PCR and verification of real-time RT-PCR primers was purified from an overnight 50 ml culture of EHEC using standard techniques. Briefly, the cells were centrifuged 8,000 rpm for 20 minutes at 4˚C. The pellet was then resuspended in 4.75 ml TE. One hundred twenty-five microliters of 10% SDS and 12.5 µl proteinase K

were added. After the mixture was incubated for one hour at 37˚C, 600 µl 5 M NaCl was added. Next, 375 µl of pre-warmed (65˚C) CTAB-NaCl (10% CTAB in 0.7 M NaCl) was added and the solution was incubated at 65˚C for 20 minutes. Six milliliters of chloroform-isoamyl alcohol (24:1) was added, mixed, and centrifuged at 8,000 rpm for 25 minutes at room temperature. The top aqueous layer was transferred to a new tube and the previous step was repeated. Genomic DNA was precipitated by the addition of 0.6 volumes of ethanol. A glass tube was use to remove the precipitated DNA which was then washed with 70% ethanol. The DNA was transferred to a new tube, dried under vacuum, and resuspended in 1 ml TE-0.1 (10 mM Tris-Cl, pH 7.5; 0.1 EDTA, pH 8.0). The DNA was then treated with an RNase for 1 hour at 37˚C and then ethanol precipitated. The precipitated DNA was washed with 70% ethanol and resuspended in 1 ml TE-0.1.

RNA was extracted from three biological replicate cultures of 86-24, VS94, MW90, MW192, MW196, MW199, VS94 + 0.5 mM aspartate dipeptides (BACHEM), VS94 + 50 mM sodium fumarate dibasic, and VS94 + 0.2% ammonium sulfate grown in DMEM (Invitrogen) aerobically at 37˚C. RNA was extracted using the RiboPure – Bacteria RNA isolation kit (Ambion) following manufacturer's guidelines. Briefly, cultures were grown to an OD_{600} of 0.2, 0.5, and 1.0 and centrifuged 4,000 rpm for 10 minutes at 4˚C. The cells were then resuspended in RNAWhiz (Ambion), lysed with silica beads, and centrifuged 13,000 rpm for 5 minutes at 4˚C to remove cell debris. The supernatant was then transferred to a new tube, treated with chloroform, centrifuged 13,000 rpm for 5 minutes at 4˚C, supernatant removed, and RNA precipitated by addition of ethanol. The RNA was then further purified on a column, treated with DNase I (Ambion), and stored at -20˚C.

IV. Recombinant DNA Techniques

PCR, ligation, restriction, and DNA gel electrophoresis were performed using standard methods (162). PCR reactions were performed using either *Taq* (Invitrogen) or *Pfx*-proofreading enzymes (Invitrogen) according to manufacture's guidelines. The primers used in these studies are listed in Table 2. DNA sequence analysis was carried out at the University of Texas Southwestern Medical Center Sequencing Core Facility using an ABI automated sequencer.

Strain MW192 was created by amplifying *aroP* from 86-24 genomic DNA (gDNA) with *Pfx* polymerase (Invitrogen) using primers AroPF1 and AroPR1, sub-cloned into pCR-Blunt II-TOPO (Invitrogen), digested with *KpnI* and *BamHI* restriction enzymes, cloned into pQE30 (Qiagen), and transformed into VS94. Strain MW196 was created by amplifying *sahH*, including its native promoter, from *Pseudomonas aeruginosa* PA01 gDNA with *Taq* polymerase (Invitrogen) using primers SAHFA and SAHRA, sub-cloned into pCR 2.1-TOPO (Invitrogen), digested with *HindIII* and *EcoRV* restriction enzymes, cloned into pACYC177 (New England Biolabs), and transformed into VS94. Strain MW199 was created by amplifying *tyrP* from 86-24 gDNA with JumpStart KlenTac Lr polymerase (Sigma) using primers tyrP F1 and tyrP R1, sub-cloned into pCR 2.1-TOPO (Invitrogen), digested with *HindIII* and *PstI* restriction enzymes, cloned into pQE30 (Qiagen), and transformed into VS94.

Table 2: Primers used in this study

Primer	Sequence
lsrR P1l Red	ATAAATGCGCAAGAACTGAACAATTGCATTAAAGATTTAAA TATGTTCAAGTGTAGGCTGGAGCTGCTTC
lsrR P2l Red	TCTGTTCCTCTATACGTTCTCCATCATTCCCGGTAATAAGGT CTGCAAACATATGAATATCCTCCTTA
AroP F1	CGGGCACCCGCATTATTCTTGATCTG
AroP R1	GGGGTACCCCGGCGTAGAGAGATTA
SAHFA	CGCTATAATCGCCCGCTCAG
SAHRA	DTGGTTGTAGTGATCGGCGA
ler RT F1	CGACCAGGTCTGCCCTTCT
ler RT R1	GCGCGGAACTCATCGAAA
tyrP F1	CAGGACAGAAGAAAGCGTGA
tyrP R1	CGTTAATTCTGGCACCCAAT
escC RT F1	GCGTAAACTGGTCCGGTACGT
escC RT R1	TGCGGTAGAGCTATTAAAGGCAAT
escV RT F1	TCGCCCCGTCCATTGA
escV RT R1	CGCTCCCGAGTGCAAAA
eae RT F1	GCTGGCCTTGGTTTGATCA
eae RT R1	GCGGAGATGACTTCAGCACTT
espA RT F1	TCAGAATCGCAGCCTGAAAA
espA RT R1	CGAAGGATGAGGTGGTTAAGCT
rpoART F1	GCGCTCATCTTCTTCCGAAT
rpoART R1	CGCGGTCGTGGTTATGTG

V. Protein Expression and Purification

All protein expression was performed in *E. coli* DH5α using the pQE30 (Qiagen) 6-histidine vector. Typically, an overnight *E. coli* culture was diluted 1:100 in 100 ml LB broth containing the appropriate antibiotic for strain selection. Cells were grown shaking at 37˚C to OD_{600} 0.6, at which time 1 mM IPTG was added to the culture to induce protein expression. Cultures were further grown shaking at 37˚C for three hours. The cells were then pelleted, supernatant discarded, and frozen at -80˚C overnight. The next day, the pellet was thawed on ice, 8 ml Lysis buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 10mM imidazole, and 1 mg/ml lysozyme) was added, and incubated on ice for 30 minutes. Cells were then lysed by passing the solution through a French press two times. Cell debris was pelleted by centrifuging 10,000 rpm for 10 minutes. One milliliter of Ni-NTA mixture (Qiagen) was added to the lysate and mixed at 4˚C for one hour. The lysate-Ni-NTA mixture was then loaded onto a column. The flow-through was passed through the column twice, washed with Wash buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 40 mM imidazole) twice, and eluted with Elution buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 250 mM imidazole) in eight 500 µl fractions. Purity of the fractions was assessed by SDS-PAGE and Bio-Safe Coomassie (Biorad) staining.

VI. SDS-PAGE and Immunoblotting

For blots using whole cell lysates, total proteins were extracted from strains 86-24, VS94, and MW90 grown in DMEM to an OD_{600} of 0.2, 0.5, and 1.0. Briefly, 3 ml of culture was pelleted (13,000 rpm for 5 min at 4˚C), resuspended in 300 µl lysis buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 5% glycerol, 1 mM DTT, and 30 mM PMSF), lysozyme added to a final concentration of 300 µg/ml, incubated at 4˚C for 4 hours, DNase I treated for 45 min at 4˚C, cell debris pelleted (13,000 rpm for 10 min at 4˚C), and supernatant containing whole cell protein removed. SDS-PAGE and immunoblotting procedures were performed as previously described (162) , and probed with polyclonal antisera against either EscJ (kindly provided by Dr. Gad Frankel, Imperial College London), EspA, EspB, or Tir (kindly provided by Dr. James Kaper). Primary antibody dilutions of 1:5000 and secondary antibody dilutions of 1:25,000 were used in these studies. Proteins were detected using enhanced chemiluminescence (ECL) (Biorad). Equal amounts of whole cell lysate protein were determined using the Lowry assay (162) and verified by probing blots with a monoclonal antibody against RpoA (Neoclone).

VII. Preparation of Secreted Proteins

Secreted proteins from 86-24, VS94, and MW90 were harvested as previously described by Jarvis *et al*. (88). Briefly, 80 ml cultures of bacteria were grown aerobically in

DMEM at 37˚C and collected at early-exponential (OD_{600} 0.2), mid-exponential (OD_{600} 0.5), and late-exponential (OD_{600} 1.0) growth. The cultures were then centrifuged 8,000 rpm at 4˚C for 10 minutes. The supernatants were then passed through a 0.22 μM filter to remove any remaining bacteria. Next, 400 μl phenylmethanesulfonyl fluoride (PMSF) (10 mg/ml), 800 μl EDTA, and 20 μl aprotinin (2 mg/ml) were added. Trichloroacetic acid to a final concentration of 10% was added to precipitate proteins. The solution was incubated at 4˚C overnight. The solution was next centrifuged 20,000 rpm at 4˚C for one hour. The pellet containing the secreted proteins was resuspended in 40 μl PBS. The samples were then subjected to SDS-PAGE and immunoblotting with rabbit polyclonal antisera to EspA and EspB (kindly provided by Dr. James Kaper) and visualized with ECL.

VIII. Real-time RT-PCR

The primers used in the Real-Time assays were designed using Primer Express v1.5 (Applied Biosystems) (Table 2). Real-Time RT-PCR was performed in a one-step reaction using an ABI 7500 sequence detection system (Applied Biosystems). For each 20 μl reaction, 10 μl 2X SYBR master mix, 0.1 μl Multi-scribe reverse transcriptase (Applied Biosystems), and 0.1 μl RNase inhibitor (Applied Biosystems) were added. Amplification efficiency of each of the primer pairs (detectors) was verified using standard curves of known genomic DNA concentrations. Detectors were considered acceptable if they had a slope of -3.3 ± 0.3 for the standard curve, representing a reaction efficiency of 90-100%. Melting curve analysis was used to ensure template specificity by heating products to 95˚C for 15

seconds, followed by cooling to 60˚C, and heating to 95˚C while monitoring fluorescence. Once amplification efficiency and template specificity were determined for each detector, relative quantification (RQ) analysis was used to analyze the unknown samples using the following conditions for cDNA generation and amplification: 1 cycle at 48˚C for 30 minutes, 1 cycle at 95˚C for 10 minutes, 40 cycles at 95˚C for 15 seconds and 60˚C for 1 minute. The *rpoA* (RNA polymerase subunit A) transcript was used as the endogenous control.

Data collection was performed using the ABI Sequence Detection 1.3 software (Applied Biosystems). Data were normalized to levels of *rpoA* and analyzed using the comparative critical threshold (C_T) method previously described (9). Briefly, ΔC_T was calculated by subtracting the C_T of the endogenous *rpoA* from the C_T of the target gene to normalize the samples. Next, $\Delta\Delta C_T$ was found by subtracting the ΔC_T value of WT from the ΔC_T of other strains/conditions. The RQ of target transcript was determined by $2^{-\Delta\Delta CT}$. Standard deviations (SD) were determined by the following formula: $SD=\sqrt{(\text{target } SD^2+\text{endogenous } SD^2)}$. RQMin and RQMax were calculated by $2^{-(\Delta CT \pm SD)}$. Real-time data is presented as fold change compared to 86-24 (WT). Error bars represent the RQMax standard deviation.

IX. β-galactosidase assays

The TEVS232 reporter strain containing a chromosomal transcriptional fusion between the *LEE1* promoter and *lacZ* was used to assay AI-3 dependent transcription of

LEE1. TEVS232 was grown in fresh medium or in medium supplemented with preconditioned (PC) culture supernatants and grown to an OD_{600} of less than 0.1. The cultures were then diluted 1:10 in Z buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $Na_2HPO_4 \cdot H_2O$, 10 mM KCl, $MgSO_4 \cdot 7H_2O$, 50 mM β-mercaptoethanol) and assayed for β-galactosidase activity using o-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate as previously described (128).

X. *In vitro* synthesis of AI-2

In vitro synthesis of AI-2 was carried out as previously described (168). His-tagged Pfs and LuxS were purified from pVS212 and pVS214 (Table 1) by using a nickel resin (Qiagen) according to the manufacturer's protocol. *In vitro* synthesis of AI-2 was performed with 1 mM SAH (Sigma), 1 mg/mL His-LuxS, and 1 mg/mL His-Pfs in 10 mM sodium phosphate buffer pH 7.5 at 37˚C for 1 hour. The AI-2 was separated from the Pfs and LuxS proteins by a Centrifuge Biomax-5 size-exclusion column (Millipore). The amount of AI-2 was indirectly quantified by measuring homocysteine production using Ellman's test for the sulfhydryl group as previously described (167).

XI. *V. harveyi* bioluminescence assay

AI-2 activity in PC media, enzymatically derived AI-2, and chemically synthesized DPD AI-2 precursor (a gift from Dr. Michael Meijler and Dr. Kim D. Janda, The Scripps Research Institute (115)) were assayed by using the *V. harveyi* BB170 reporter strain (193), which responds only to AI-2. The assays were performed as previously described (193). An overnight culture of BB170 was diluted 1:5000 in AB medium. Ninety microliters of diluted culture was then transferred to wells in an opaque 96-well plate. The volume of each well was then brought to 100 µl with the addition of either 10 µl fresh medium (to serve as a blank), 1 µl PC medium or AI-2 + 9 µl fresh medium, or 10 µl PC medium or AI-2. The cultures were then grown at 30˚C and shaking at 200 rpm. The bioluminescence of each well was read with a BioRad Lumimark microplate reader. Samples were performed in triplicate for each condition.

In order to test the EHEC Δ*lsrR* mutant, the following protocol was used. 86-24 and the Δ*lsrR* mutant were grown in LB to an OD_{600} of 1.0, pelleted by centrifugation, washed 3 times with LB, and then incubated with synthetic AI-2 for 1h at 37˚C. The bacteria were again pelleted, and the supernatants were filter sterilized and assessed for the amount of remaining AI-2 using the *V. harveyi* bioluminescence assay, as previously described by Taga *et al* (197).

XII. Fluorescent actin staining (FAS) test

FAS assays were performed as previously described by Knutton *et al.* (102). In brief, overnight bacterial cultures grown aerobically in LB at 37˚C were diluted 1:100 and used to infect confluent monolayers of HeLa cells grown on glass coverslips at 37˚C and 5% CO_2. Cells were grown for 6 hours at 37˚C and 5% CO_2 with samples being removed each hour. At the specified time points, two coverslips were removed and washed three times with PBS pre-warmed to 37˚C. Cells were fixed with 2% formaldehyde in PBS for 20 minutes with gentle shaking. The cells were again washed three times with PBS, permeabilized with 0.1% Triton X-100 in PBS for six minutes, and washed three times with PBS. Actin was stained with FITC-phalloidin (1 μg/ml) in PBS, incubated in the dark at 37˚C, and washed three times with PBS.

Bacteria were then stained with propidium iodide using the following protocol. The cells were washed twice with 2X SSC and then treated with RNase A (100 μg/ml) in 2X SSC for 20 minutes at 37˚C protected from light. The samples were then washed three times with 2X SSC, two minutes per wash. Nucleic acids were stained by the addition of 500 nM propidium iodide for five minutes, followed by three washes with 2X SSC. Coverslips were then mounted on slides face down with 2 μl Anti-fade solution (50% glycerol, 1X PBS, 0.1% p-phenylenediamine, pH 8) and sealed using clear nail polish. Samples were visualized by immunofluorescence using a Zeiss Axiovert microscope. The entire field of two coverslips from each time point per strain was examined and images taken of AE lesions.

XIII. Immunostaining of bacteria on HeLa cell monolayers

Immunofluorescence studies were performed as previously described (103, 227) using bacteria expressing GFP from the pd2EGFP vector (Clontech). Overnight EHEC strains containing the pd2EFGP vector were diluted 1:100 in wells of a 12-well plate containing HeLa cells grown on glass coverslips. Expression of GFP from pd2EGFP was induced by the addition of 1 mM IPTG and the cells were grown at 37˚C and 5% CO_2 for 6 hours with samples being removed each hour. Cells were fixed with 2% formalin for 30 minutes at 25˚C and washed three times with PBS. Next, the coverslips were incubated for one hour with 100 µl of a 1:80 dilution of the primary antibody in fetal calf serum (FCS) (α-EspA, kindly provided by Dr. Gad Frankel, Imperial College London and Dr. Stuart Knutton, University of Birmingham, or α-Intimin, kindly provided by Dr. James Kaper, University of Maryland School of Medicine). After incubation with the primary antibody, three 10-mintue washes with PBS were performed to remove unbound antibody and then incubated with 100 µl of a 1:3,000 dilution of the secondary anti-rabbit IgG conjugated to TRITC (Sigma) in FCS. Samples were visualized and photographed with a Zeiss Axiovert microscope. The TRITC filter was used to excite fluorescence of the secondary antibody. The orange TRITC color was changed to red, in order to better contrast with the green bacteria, using the Zeiss Axiovert software.

XIV. *lsr* isogenic mutant construction

The isogenic *lsr* mutant was constructed as previously described (36). Plasmid pKM201 encoding the lambda Red recombinase was introduced into 86-24 and made electrocompetent. An *lsr* PCR product was produced by using the lsr P11 Red and lsr P21 Red primers (Table 2) and plasmid pKD3 as template DNA. The PCR product was purified using the Qiagen PCR purification kit following manufacture's guidelines. The *lsr* PCR product was then electroporated into 86-24 containing pKM201. Expression of the lambda Red recombinase was induced and the cells were grown shaking at 37˚C for two hours with IPTG. The cells were then plated on LB agar plates containing chloramphenicol and incubated at 37˚C overnight. Resulting colonies were then patched onto LB agar plates containing either ampicillin or chloramphenicol. Positive clones were chloramphenicol resistant and ampicillin sensitive, indicating that plasmid pKM201 had been lost. PCR was used to verify *lsr* gene replacement with the chloramphenicol cassette.

XV. Biolog Phenotype MicroArrays

Strains 86-24 and VS94 were used in these assays. Four conditions were compared and performed in duplicate: VS94 vs. 86-24, VS94 plus enzymatically synthesized AI-2 vs. VS94, 86-24 plus 5 μM epinephrine vs. 86-24, and VS94 plus 5 μM epinephrine vs. VS94. All assays were performed by Biolog, Inc. (Hayword, California) as previously described

(16). Briefly, bacteria were cultured on R2A Agar (per liter: yeast extract 0.5 g; proteose peptone 0.5 g; casein hydrolysate 0.5 g; glucose 0.5 g; soluble starch 0.5 g; sodium pyruvate 0.3 g; dipotassium hydrogenphosphate 0.3 g; magnesium sulphate 0.05 g; agar-agar 12.0 g) overnight at 37˚C. Cell suspensions were prepared by removing bacterial cells from the agar surface with a sterile cotton swab and gently transferring them to a glass tube containing inoculating fluid-0 (IF-0) (Biolog). Turbidity was then adjusted to 85% by the addition of either more cells or IF-0. Next, 100 µl of cell suspension was added to each well of the PM array. PM tests were performed in 96-well microplates with each well containing a different nutrient source or inhibitor. The PM arrays were incubated at 37˚C in a humidified incubator for 24-48 hours. Cell respiration was measured using a tetrazolium dye which produces a strong purple color when cells are actively respiring. The absorbance of the tetrazolium dye was then measured and compared between conditions. In some PM arrays, such as those designed to measure nitrogen, phosphorus, and sulfur utilization, sodium succinate was added to the IF-0 as a carbon source because no other carbon sources were present.

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CHAPTER FOUR

AI-3 / EPINEPHRINE SIGNALING IN THE KINETICS OF LEE GENE EXPRESSION IN EHEC

I. Introduction

The LEE is composed of 41 genes, the majority of which are organized into five polycistronic operons (*LEE1-5*) (50, 51, 127). The TTSS encoded by the LEE facilitates translocation of the LEE-encoded effector proteins Tir, EspH, EspG, EspF, SepZ, and Map (90, 99, 101, 124, 207) as well as several non-LEE-encoded (nle) effectors such as NleA, NleB, NleC, NleD, NleE, NleF, NleG, and $EspF_U$ (25, 41, 73, 133) into eukaryotic target cells.

Regulation of the LEE involves factors present in both *E. coli* K-12 and EHEC, as well as several EHEC-specific regulators. An overview of LEE regulation is shown in Figure 8. H-NS is a global regulator involved in the thermoregulation of the LEE, repressing transcription of *LEE1* at 27^{o}C, but not at 37^{o}C (212). It also represses transcription of the *LEE2*, *LEE3*, and *LEE5* operons by binding to the target promoter and preventing promoter recognition by the transcription machinery (22, 76, 127, 163, 185). IHF, another global regulatory factor, binds to the *LEE1* promoter and activates transcription of *ler* (61). The nucleoid-associated protein Fis has been shown to modulate LEE expression in EPEC (66), but its role in EHEC LEE expression remains to be examined. Hha, and its homologues, are environment-dependent regulators of gene expression that act as a negative regulator by

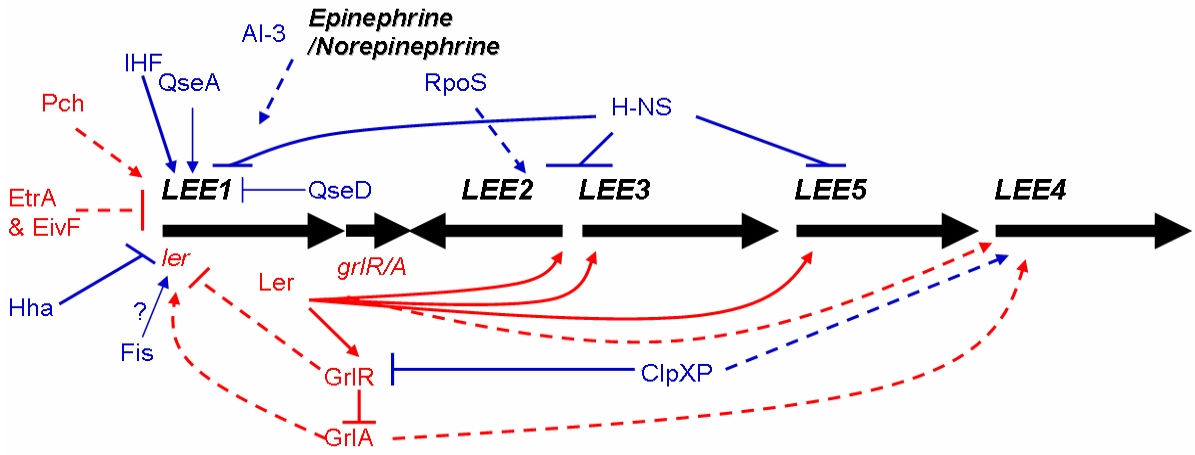


Figure 8: Model of LEE regulation. Factors shown in blue are present in both *E. coli* K-12 and EHEC, while regulators shown in red are specific to EHEC. Solid lines represent regulators whose direct interactions with the target promoter have been biochemically defined and dashed lines represent interactions which occur indirectly or have not been shown to bind biochemically to the target. H-NS is a global regulator that binds to the promoters of the *LEE1*, *LEE2*, *LEE3*, and *LEE5* operons and represses transcription. Ler activates *grlR/A*, *LEE2*, *LEE3*, and *LEE5* by binding to their promoters displacing H-NS and allowing for the transcription of these operons. IHF also activates transcription of *LEE1*. Hha represses *LEE1* by either oligomerizing with H-NS or binding directly to the promoter sequence. The ClpXP protease regulates LEE expression through interactions with RpoS and GrlR. Fis has been shown to activate the LEE in EPEC, but its role in EHEC has not been examined. QseA and QseD are two LysR-type regulators. QseA seems to activate LEE expression, while QseD represses the LEE. AI-3 and epinephrine / norepinephrine signal through unknown receptors to activate transcription of the *LEE1* operon and *ler*. GrlR and GrlA, two LEE-encoded regulators, repress and promote transcription of the *LEE1* operon respectively. EtrA and EivF are two regulators encoded on a second non-functional TTSS in EHEC which negatively influence expression of *LEE1*. The *pch* genes are another set of EHEC specific regulators that activate transcription of *LEE1* and consequently the entire LEE.

either binding to a specific DNA sequence in the target promoter (53) or by oligomerizing with H-NS and then binding the target DNA (140). In EHEC, Hha is a negative regulator of *ler* and consequently the entire LEE (175). QseA is a member of the LysR family of transcription factors and activates transcription of *ler*, thereby promoting expression of the other LEE genes (184). QseD, another LysR family member, seems to repress expression of the LEE genes (Sharp, Walters, and Sperandio, unpublished data). The ClpXP protease degrades damaged and incomplete proteins and also affects LEE expression (85). ClpXP is thought to regulate the LEE through interactions with RpoS and an EHEC-specific regulatory factor, GrlR (85). RpoS is a stationary-phase sigma factor and has been shown to positively regulate transcription of the *LEE3* operon in an *E. coli* K-12 background (186).

A number of regulatory pathways and factors are limited to EHEC such as the *pch* genes, *etrA*, *eivF*, *ler*, and *grlR/A*. The *pch* genes, which are not present in *E. coli* K-12, positively regulate expression of the LEE, and are necessary for full virulence of EHEC (86). The five *pch* genes are encoded outside of the LEE and are homologous to *perC* in EPEC (86). EtrA and EivF are encoded within a pathogenicity island that contains a second, nonfunctional type III secretion system in the EHEC genome (232). Both of these proteins have been shown to be negative regulators of the LEE (232). Ler, encoded by *LEE1*, is able to overcome the H-NS mediated repression and activate the transcription of the *LEE2, LEE3,* and *LEE5* operons (76, 163, 185). GrlR and GrlA, also encoded by the LEE, repress and activate transcription of *ler*, respectively (41). Ler can also activate transcription of the *grlR/A* operon, creating a regulatory loop (14, 50). The specific role of this regulatory loop has not been defined, but it has been suggested that it is necessary to maintain the balance of

regulatory factors that help achieve optimal expression of the LEE in the host environment (14).

The numerous factors that control gene expression suggest that LEE regulation is highly complex. In this study, we sought to examine a possible role for AI-3 and epinephrine / norepinephrine in activation of the LEE in wildtype (WT) EHEC and an isogenic EHEC *luxS* mutant (defective in AI-3 synthesis). Previous work has only examined transcription of the LEE genes in an *E. coli* K-12 background using PC media from EHEC cultures. Herein, we directly examined the effects of AI-3 / epinephrine on LEE transcription in WT EHEC and a *luxS* mutant. We found that the kinetics of LEE expression were different from those previously observed with the *E. coli* K-12 reporter strain, highlighting the importance of examining the LEE regulation in a native EHEC background. Moreover, the results from these studies indicate a synergistic effect between AI-3 and epinephrine. This relationship may allow EHEC to mount a more efficient infection than if only responding to one signal alone.

II. Results

A. EHEC LEE gene transcription is reduced in a *luxS* mutant during mid-exponential growth

Expression of the LEE in EHEC is induced by both a bacterial signal, AI-3, and two eukaryotic hormones, epinephrine and norepinephrine (188). The LuxS enzyme, which is

involved in the metabolism of *S*-adenosylmethionine to produce AI-2, is also required for efficient production of the AI-3 autoinducer (188). Previous studies assessing AI-3 / epinephrine / norepinephrine activation of LEE gene transcription were performed using a LEE::*lacZ* transcription reporter system in an *E. coli* K-12 background with pre-conditioned (PC) media from WT and a *luxS* mutant (186, 188).

Given the array of regulatory factors specific to EHEC (Fig. 8), we sought to examine LEE transcription in WT and a *luxS* mutant in native EHEC backgrounds. For this purpose, we used real-time RT-PCR. Real-time RT-PCR avoids many of the drawbacks of plasmid-based reporter systems, such as copy-number issues and coiling effects, and quantifies the amount of target transcripts. Real-time RT-PCR is also more sensitive than plasmid-based reporter systems, allowing for subtle changes in gene transcription to be detected.

The amount of *ler* (*LEE1*), *escC* (*LEE2*), *escV* (*LEE3*), *eae* (*LEE5*), and *espA* (*LEE4*) transcription was measured at early-exponential (OD_{600} 0.2), mid-exponential (OD_{600} 0.5), and late-exponential (OD_{600} 1.0) growth points for WT, an isogenic *luxS* mutant, and a *luxS* complemented strain grown aerobically in DMEM, conditions known to activate LEE expression. All values are represented as fold-expression with respect to 86-24 (WT) at early-exponential growth phase. Transcription of *ler* in the *luxS* mutant was not significantly different than WT at the early-exponential growth (Fig. 9A). Expression of the LEE at the early-exponential growth phase is likely low because autoinducer levels are not sufficient to activate the LEE. In the *luxS* complemented strain, transcription of *ler* was increased during early-exponential growth almost 10-fold over WT, implying that IPTG induced expression of LuxS from a plasmid during early-exponential growth leads to higher AI-3 levels. During

mid-exponential growth, transcription of *ler* in WT increased 4-fold compared to the early-exponential growth. Transcription of *ler* at mid-exponential growth in the *luxS* mutant was reduced 2.2-fold compared to WT at the same growth phase ($P \leq 0.025$). The mutant's inability to synthesize sufficient amounts of AI-3 most likely led to the reduced amounts of *ler* transcript. Transcription of *ler* was restored in the *luxS* complemented strain during mid-exponential growth. At late-exponential growth phase, *ler* transcription was the same in both WT and the *luxS* mutant. These results suggest that AI-3-dependent regulation of *ler* occurs during mid-exponential growth. AI-3 dependent regulation does not appear to play as important of a role in *ler* transcription during early- and late-exponential growth. There was not a significant difference in the transcription of genes within the LEE between WT and the *luxS* mutant at these growth phases, suggesting other factors are controlling LEE expression. When LuxS is expressed from a plasmid, transcription of *ler* is increased. The greater amounts of LuxS seem to enhance the production of AI-3 through an unknown pathway, resulting in earlier activation of the LEE.

The other LEE operons displayed transcription patterns similar to *ler* (Fig. 9B-E). There was not a significant difference in the transcription of *escC* between WT and the *luxS* mutant at the early-exponential growth phase (Fig. 9B). Transcription of *escC* in the *luxS* mutant was down-regulated almost 5-fold compared to WT at mid-exponential growth ($P \leq 0.006$), and there was no significant difference observed at late-exponential growth. Transcription of *escC* in the complemented strain was induced approximately 26-fold over WT during early-exponential growth (Fig. 9B). Similar to *ler* and *escC* transcription, the *luxS* mutant had significantly decreased transcription of *escV* (13-fold, $P \leq 0.0001$), *eae* (13-

fold, $P \leq 0.0001$), and *espA* (18-fold, $P \leq 0.0002$) at the mid-exponential growth phase compared to WT at the same growth phase (Fig. 9C-E). Transcription of *escV*, *eae*, and *espA* was not significantly different between the *luxS* mutant and WT during late-exponential growth, analogous to the results observed for *ler* and *escC*. The complemented strain again had higher levels of *escV* (20-fold), *eae* (120-fold), and *espA* (374-fold) transcription compared to WT during early-exponential growth (Fig. 9C-E).

IPTG-induced expression of LuxS led to earlier activation of genes within each of the five LEE operons at the early-exponential growth phase. Normalization with the constitutively transcribed *rpoA* revealed that transcription of all genes tested increased with growth of the WT strain (Fig. 9A-E). This trend was not observed in the *luxS* mutant. We consistently observed significantly lower levels of transcription by the *luxS* mutant during mid-exponential growth, suggesting that AI-3-dependent regulation plays a major role in LEE transcription during mid-exponential growth (when bacteria are rapidly dividing). Growth curves did not reveal any difference in growth between the three strains (data not shown), indicating that these results are not due to differences in growth kinetics.

B. TTSS protein expression is decreased in a *luxS* mutant

To establish a relationship between our transcriptional results and LEE protein expression, we isolated bacterial whole cell lysates of WT, *luxS* mutant, and *luxS* complemented strains from early, mid, and late-exponential growth stages. We examined the major components of the TTSS by performing immunoblot analysis using rabbit polyclonal antisera directed

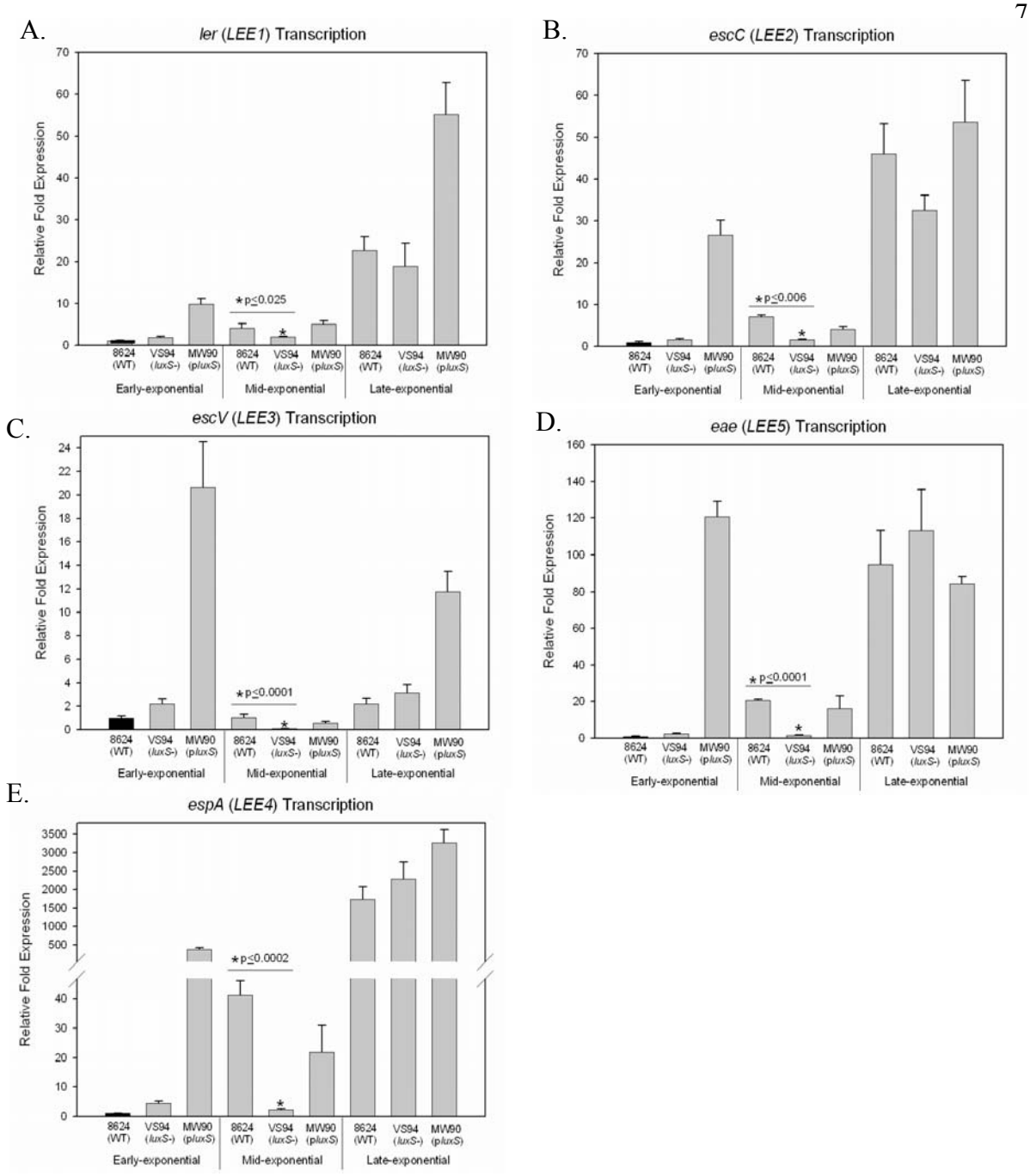


Figure 9: WT EHEC, an isogenic *luxS* mutant, and a *luxS* complemented strain LEE transcriptional profile during early-, mid-, and late-exponential growth as measured by real-time RT-PCR. (A) *ler* from the *LEE1* operon. (B) *escC* from the *LEE2* operon. (C) *escV* from the *LEE3* operon. (D) *eae* from the *LEE5* operon. (E) *espA* from the *LEE4* operon. Relative fold expression represents the fold change in transcription compared to the 86-24 (WT) early-exponential sample for each gene (black bar, value=1.0). Results are means and standard deviations from triplicate experiments. The levels of *rpoA* transcript were used to normalize the C_T values to account for variations in bacterial numbers.

against a structural component of the TTSS (EscJ), a translocated effector protein (Tir), and the outer filament of the TTSS (EspA). Hence, we were able to examine expression of proteins that compose three distinct portions of the TTSS machinery (Fig. 10A). A mouse monoclonal antibody to the constitutively expressed *E. coli* RNA polymerase alpha subunit (RpoA) was used to verify that equal amounts of proteins were loaded.

Figure 10B shows that expression of EscJ was decreased in the *luxS* mutant during mid-exponential growth, in agreement with the transcription data of *escC* (Fig. 9B). An antibody to EscJ was used to examine protein expression of the *LEE2* operon because generation of an antibody against EscC was unsuccessful. Since both *escJ* and *escC* are encoded in the same operon, it is expected that they would be transcribed together and share similar expression patterns. There appears to be no significant difference in the expression of EscJ at the late-exponential growth phase, further supporting the transcriptional data for *escC*. Surprisingly, although transcription of *LEE2* was highly upregulated in the complemented strain during early exponential-growth, we did not observe an increase in EscJ expression at this growth phase in this strain. The reason for this disparity between transcription of *LEE2* and protein levels of EscJ is unknown, and future experiments will further examine this phenomenon.

Similar results were observed when examining the expression of Tir (*LEE5*) in bacterial whole cell lysates. Our transcription data showed a significant decrease in the amount of *LEE5* transcription (Fig. 9D) at mid-exponential growth in the *luxS* mutant when compared to either WT or the complemented strain. Indeed, the levels of Tir in the WCL were decreased during mid-exponential growth (Fig. 10C). Although the levels of *tir*

transcription were high during late-exponential growth, we observed a decrease in the amount of Tir present in whole cell lysates during the same growth phase. The difference between transcript and protein levels may have resulted from the secretion of Tir by the TTSS into the culture medium. Hence, lower amounts of Tir will be present within the bacterial whole cell lysates used for immunoblot analysis. In accordance, we have previously reported significant secretion of Tir during late-exponential growth (188).

EspA protein expression was decreased in the *luxS* mutant during both mid- and late-exponential growth (Fig. 10D). Interestingly, despite there being no significant difference in the transcription of *espA* between the WT and *luxS* mutant at the late-exponential growth phase (Fig. 9E), there was less EspA protein produced by the *luxS* mutant during this phase of growth (Fig. 10D). This may be a result of differences in the post-transcriptional regulation of *espA* in the WT and *luxS* mutant. Roe *et al.* have demonstrated that EspA secretion is phase variable and controlled at the post-transcriptional level through an uncharacterized mechanism (158). A constraint seems to be placed on the *espADB* transcript so that it is only translated when the appropriate signals are present. The *luxS* mutant may not be capable of producing these signals to allow for the *espADB* transcript to be translated, causing the observed decrease in the levels of EspA protein present in the whole cell lysate. Transcription of *espA* was much higher at the late-exponential growth phase than during mid-exponential growth phase (Fig. 9E), but a comparable increase in EspA expression was not observed in whole cell lysates (Fig. 10D). Cellular levels of EspA were also influenced by its secretion, similar to Tir. Indeed, we found that the greatest amount of EspA and EspB secretion occurred during late-exponential phase in the WT, *luxS* mutant, and complemented

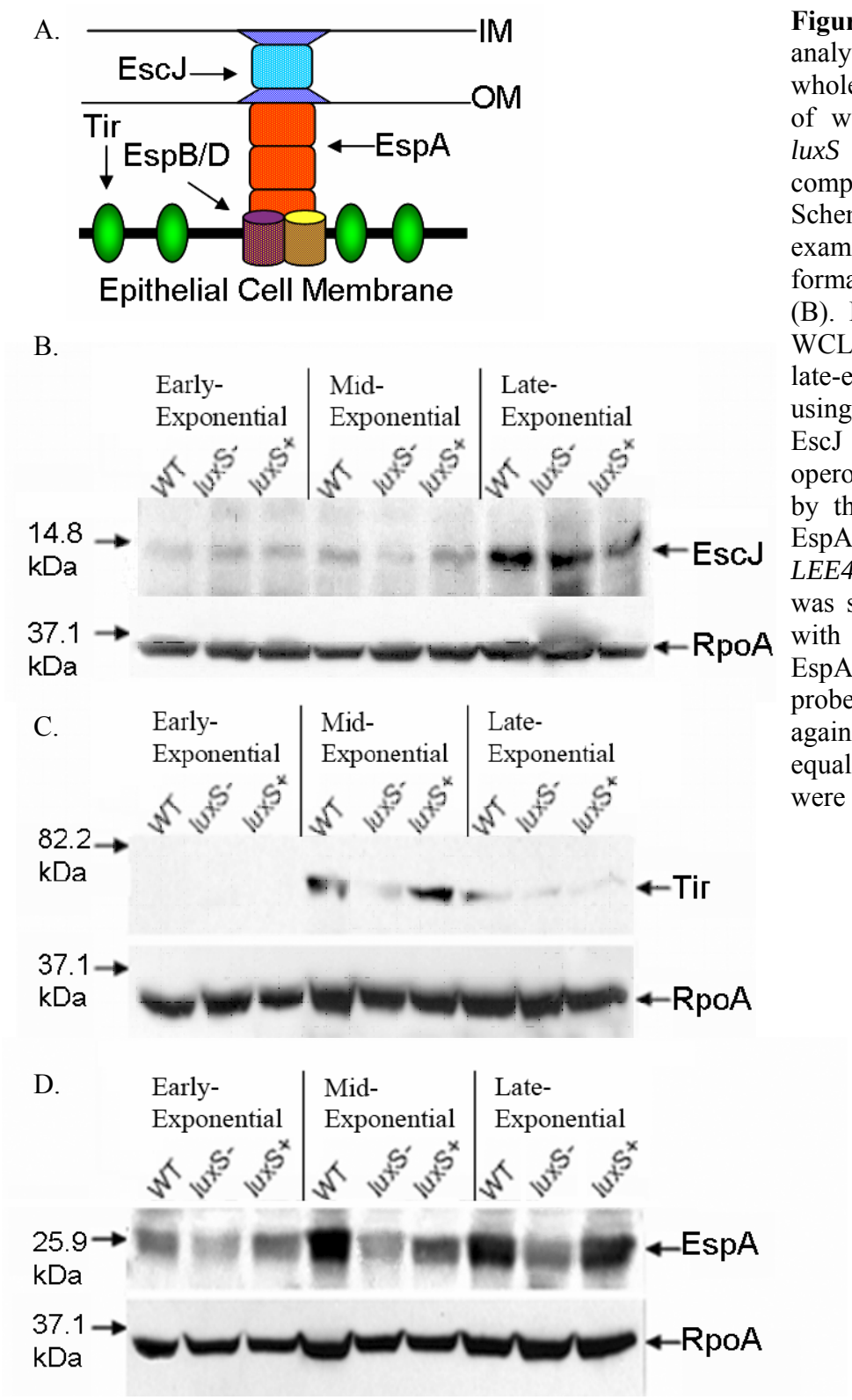


Figure 10: Immunoblot analysis of LEE proteins in whole-cell lysates (WCL) of wild-type, an isogenic *luxS* mutant, and *luxS* complement. (A) Schematic of the proteins examined and their role in formation of the TTSS. (B). Protein expression in WCL at early-, mid-, and late-exponential growth using an antibody against EscJ encoded by the *LEE2* operon. (C). Tir encoded by the *LEE5* operon. (D) EspA encoded by the *LEE4* operon. Each blot was stripped after probing with the EscJ, Tir, and EspA antibodies and re-probed with an antibody against RpoA to verify that equal amounts of protein were loaded.

strains (Fig. 12A). Transcription of *LEE5* (*tir*) and *LEE4* (*espA*) were also up-regulated in the complemented strain at early exponential growth. However, the levels of Tir and EspA in WCLs in this strain were comparable to WT at this growth phase (Figs. 10B and 10C). Since the complemented strain is already secreting these proteins through the TTSS at early-exponential growth (Fig. 12A), our inability to observe an increase in the levels of Tir and EspA in the complemented strain's WCL was again likely due to the fact that they have been secreted into the medium.

C. Epinephrine increases transcription of the LEE

To examine the effects of epinephrine in an EHEC background, we performed real-time RT-PCR analysis of genes within the *LEE1*, *LEE2*, and *LEE3* operons in WT and an isogenic *luxS* mutant in the presence and absence of epinephrine. The addition of epinephrine to a final concentration of 50 μM at time 0 (previously shown to induce maximal signal in *E. coli* K-12 (188)) increased transcription of *ler* (*LEE1*) in both the WT and *luxS* mutant nearly 100-fold more than medium alone at early-exponential growth (Fig. 11A). At the early-exponential phase, it is likely that there is not sufficient AI-3 to endogenously activate *LEE1* transcription (Fig. 9A). Hence, epinephrine increases transcription of *LEE1* in both WT and the *luxS* mutant to the same extent. By mid-exponential growth, transcription of *ler* in the *luxS* mutant with epinephrine is significantly less than WT with epinephrine ($P \leq 0.05$) (Fig. 11A). Since the *luxS* mutant cannot readily produce AI-3, epinephrine is the main signal present to activate expression of the LEE. In WT, both AI-3 and epinephrine are

present to activate *LEE1* transcription. The combination of these two signals results in increased expression of *ler* and the rest of the LEE. These results suggest that there may be an agonistic relationship between AI-3 and epinephrine to activate *LEE1* transcription, inasmuch as significant additive effects are observed in the WT plus epinephrine condition during mid-exponential growth. During late-exponential growth, *ler* transcription was activated over 1000-fold in the WT and *luxS* mutant with epinephrine than the respective strains without epinephrine at the same growth phase. There was not a significant difference in the levels of *ler* transcription during late-exponential growth between WT and the *luxS* mutant.

We also examined the effect of epinephrine on the transcription of the other downstream LEE operons. Specifically, we measured the transcription of the *escC* (*LEE2*) and *escV* (*LEE3*) in response to epinephrine (Figs. 11B and 11C). Epinephrine increased the transcription of *escC* only during late-exponential growth in both the WT and *luxS* mutant (Fig. 11B). When epinephrine was present, transcription of *escC* during late-exponential growth was significantly higher in WT than in the *luxS* mutant ($P \leq 0.01$). The delay in increased *escC* transcription suggests that the effect of epinephrine on *LEE2* transcription may be indirect and a result of the increased amounts of Ler over time. During early-exponential growth, epinephrine decreased transcription of *escC*. By the mid-exponential growth phase, epinephrine did not significantly affect the transcription of *escC* in WT or *luxS* mutant compared to cultures without epinephrine. The *escV* gene (*LEE3*) revealed a transcriptional pattern similar to the *escC* gene of *LEE2* (Fig. 11C). At early-exponential growth, addition of epinephrine resulted in a decrease in the amount of *escV* being

transcribed in both WT and the *luxS* mutant. By the mid-exponential phase of growth, addition of epinephrine resulted in an increase of 2- and 7-fold for the WT and *luxS* mutant respectively (when compared WT and *luxS* mutant with no epinephrine at the same growth phase). WT displayed significantly higher transcription than the *luxS* mutant in response to epinephrine during mid-exponential growth ($P \leq 0.02$). This result is similar to the transcription without epinephrine and further suggests that AI-3 is responsible for the increased transcription observed in WT during mid-exponential growth. Epinephrine addition resulted in over a 100-fold increase of *escV* transcription during late-exponential growth for both the WT and *luxS* mutant as compared to WT and *luxS* mutant (with no epinephrine at the same growth phase). There was no significant difference in the transcription of *escV* between WT with epinephrine and *luxS* mutant with epinephrine in late-exponential growth.

D. The *luxS* mutation reduces EspA and EspB protein secretion

To examine the function of the LEE-encoded TTSS as a whole in the WT and *luxS* mutant, we assessed the amounts of EspA and EspB actively secreted from cultures grown in the presence and absence of epinephrine. EspA composes the filament of the TTSS (103), while EspB helps to form a pore in the eukaryotic membrane that is necessary to translocate effector proteins into the eukaryotic cell (174, 176, 219) (Figure 10A). Both of these proteins are required for virulence and formation of AE lesions on the intestinal epithelium (2). Previous studies examining EspA and EspB secretion in WT and the *luxS* mutant used a

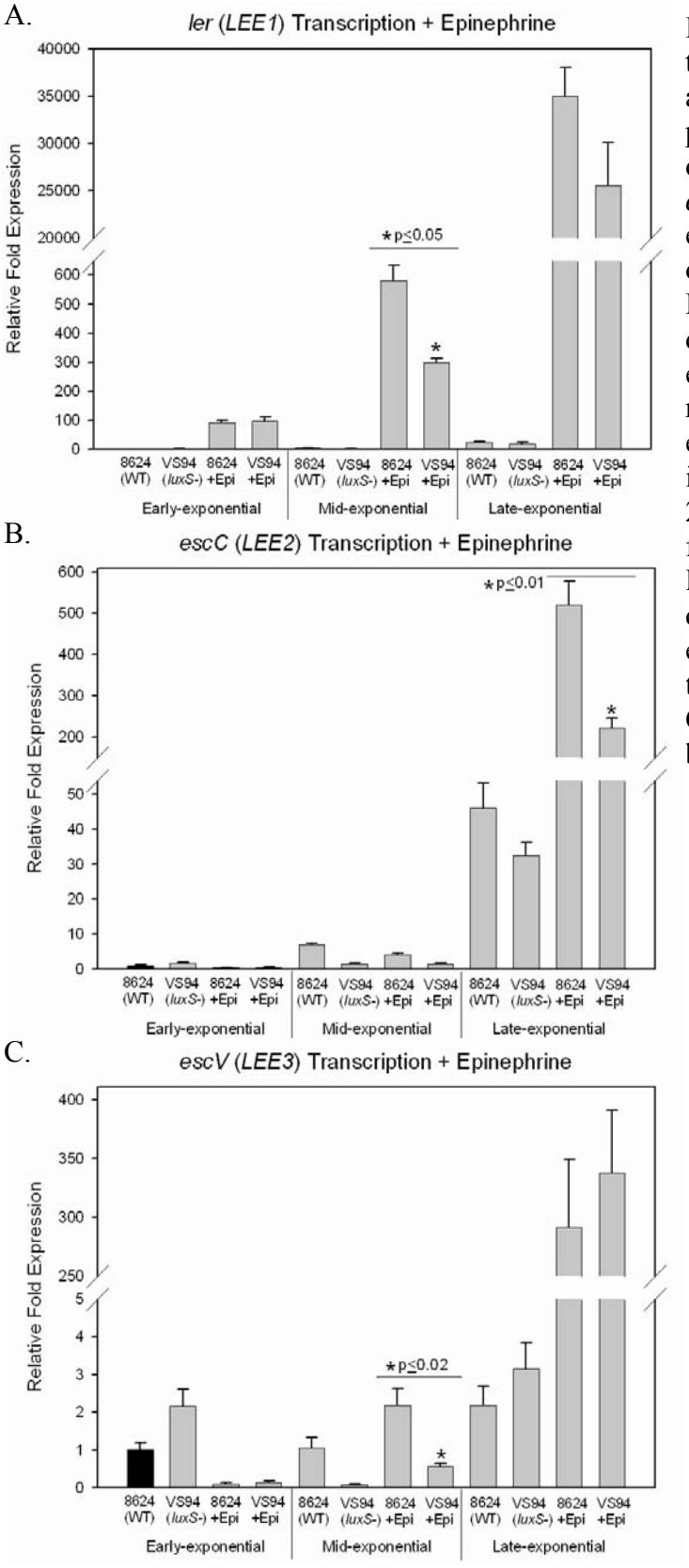


Figure 11: Epinephrine increased the transcription of the *LEE1*, *LEE2*, and *LEE3* operons. Transcriptional profile of: (A) *ler* from the *LEE1* operon ± 50 μM epinephrine, (B) *escC* from the *LEE2* operon ± 50 μM epinephrine, (C) *escV* from the *LEE3* operon ± 50 μM epinephrine for WT EHEC and an isogenic *luxS* mutant during early-, mid-, and late-exponential growth as measured by real-time RT-PCR. Relative fold expression represents the fold change in transcription compared to the 86-24 (WT) early-exponential sample for each gene (black bar, value=1.0). Results are means and standard deviations from triplicate experiments. The levels of *rpoA* transcript were used to normalize the C_T values to account for variations in bacterial numbers.

primary antiserum against total secreted proteins (188). Protein secretion in the *luxS* mutant could not be detected with these antisera against total secreted proteins. The studies presented here employ a specific anti-EspA antibody and a specific anti-EspB antibody. The specific antisera allow for more sensitive detection of secreted EspA and EspB in culture supernatants, resulting in detection of secreted proteins from the *luxS* mutant.

Secretion of EspA and EspB occurred in the early growth phase in the *luxS* complemented strain when expression of *luxS* was induced with 1 mM IPTG (Fig. 12A), linking the early increase in transcription of the 5 LEE operons observed by real-time RT-PCR (Fig. 9A-E) with earlier TTSS activity. At mid-exponential growth, the WT strain secreted more EspA and EspB protein than the *luxS* mutant. A more pronounced difference was observed at the late-exponential growth phase. Despite transcription of the LEE being significantly lower in the *luxS* mutant only during mid-exponential growth, a defect in TTSS activity is most prominently observed during late-exponential growth. Addition of epinephrine increased the amount of EspA and EspB secreted by WT and the *luxS* mutant (Fig. 12B), in agreement with previous findings (188). WT secreted more EspA and EspB than the *luxS* mutant did in response to epinephrine. The greater amount of protein secreted by WT again suggests that there may be a synergistic relationship between epinephrine and AI-3 since the *luxS* mutant is deficient in AI-3 production. Epinephrine did not appear to result in increased EspA and EspB secretion during mid-exponential growth, consistent with the transcription data from the *LEE2* and *LEE3* operons in response to epinephrine.

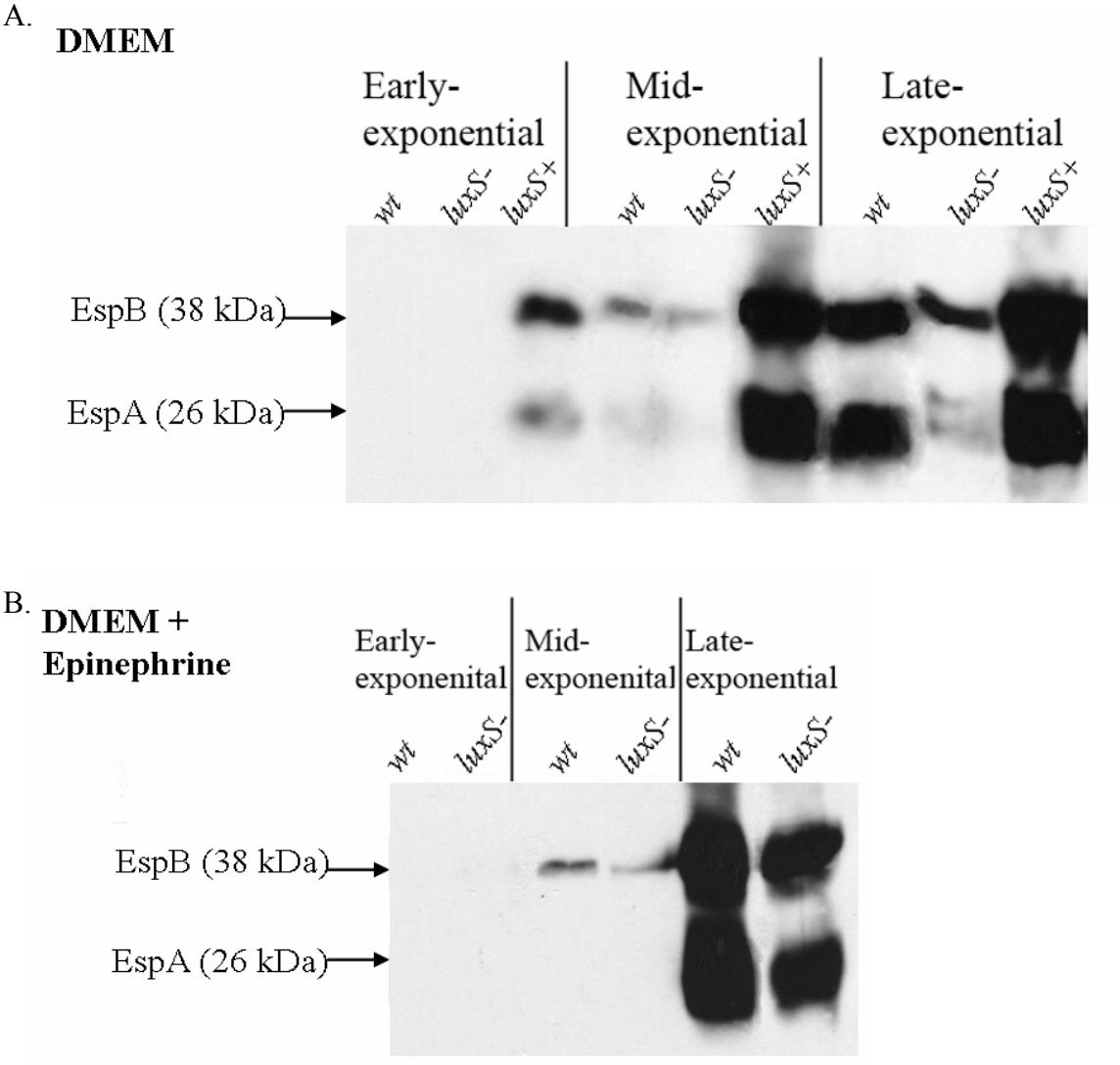


Figure 12: Total protein secreted in equal culture volumes was TCA precipitated and examined by SDS-PAGE and immunoblot. Immunoblots of secreted EspB and EspA proteins from WT EHEC and *luxS* mutant culture supernatants: (A) without epinephrine and (B) with the addition of 50 μM epinephrine.

E. Intimin and EspA filament of WT and the *luxS* mutant on infected HeLa cells

In order to assess expression of LEE-encoded proteins in the WT and *luxS* mutant during growth on HeLa cells, immunofluorescence studies involving surface expressed proteins were performed. EHEC expressing GFP from the *lac* promoter were used to infect HeLa cells and the expression of EspA and Intimin was examined using immunofluorescent antibodies. Both the WT and *luxS* mutant expressed EspA on their surface and secreted it into the medium at one hour post infection (Figure 13). EspA expression was observed at each subsequent time tested with no apparent difference between WT and the *luxS* mutant. The other surface protein studied was intimin, encoded by *LEE5* and expressed on the bacterial outer membrane. No detectable difference in Intimin expression was observed between the *luxS* mutant and WT (Figure 14). Intimin was observed at one hour post-infection and at all subsequent times tested.

F. The *luxS* mutant is delayed in AE lesion formation on infected HeLa cells

EHEC is able to produce AE lesions on eukaryotic epithelial cells. The LEE encodes the factors necessary to induce the formation of these AE lesions. To assess the entire production and expression of the LEE, the abilities of WT, an isogenic *luxS* mutant, and the

complemented *luxS* strain to form AE lesions were observed using fluorescent actin staining (FAS) assays (Fig. 15). EHEC (red) were stained with propidium iodide while actin (green) was visualized with FITC- phalloidin. WT and *luxS* complemented bacteria formed

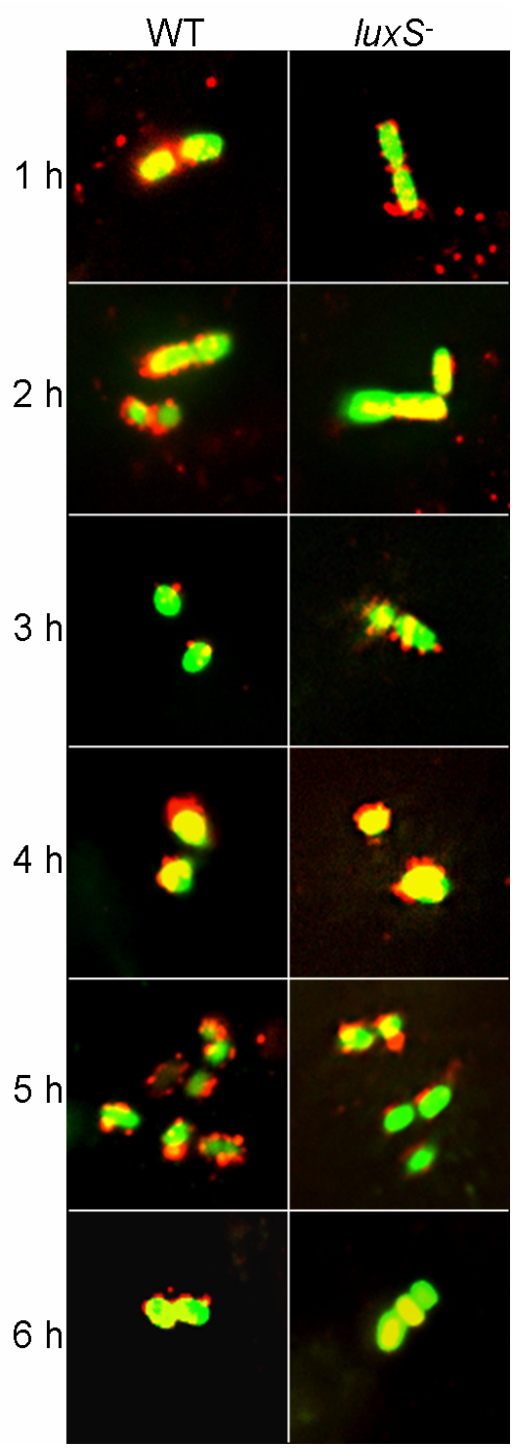


Figure 13: EspA expression during HeLa cell infection. Immunofluorescence of EspA filaments on the surface of the WT and *luxS* mutant was observed over 6 hours in a HeLa cell infection model. Bacteria (green) are expressing GFP from a plasmid containing the *lac* promoter and EspA filaments (red) were stained using fluorescent antibodies.

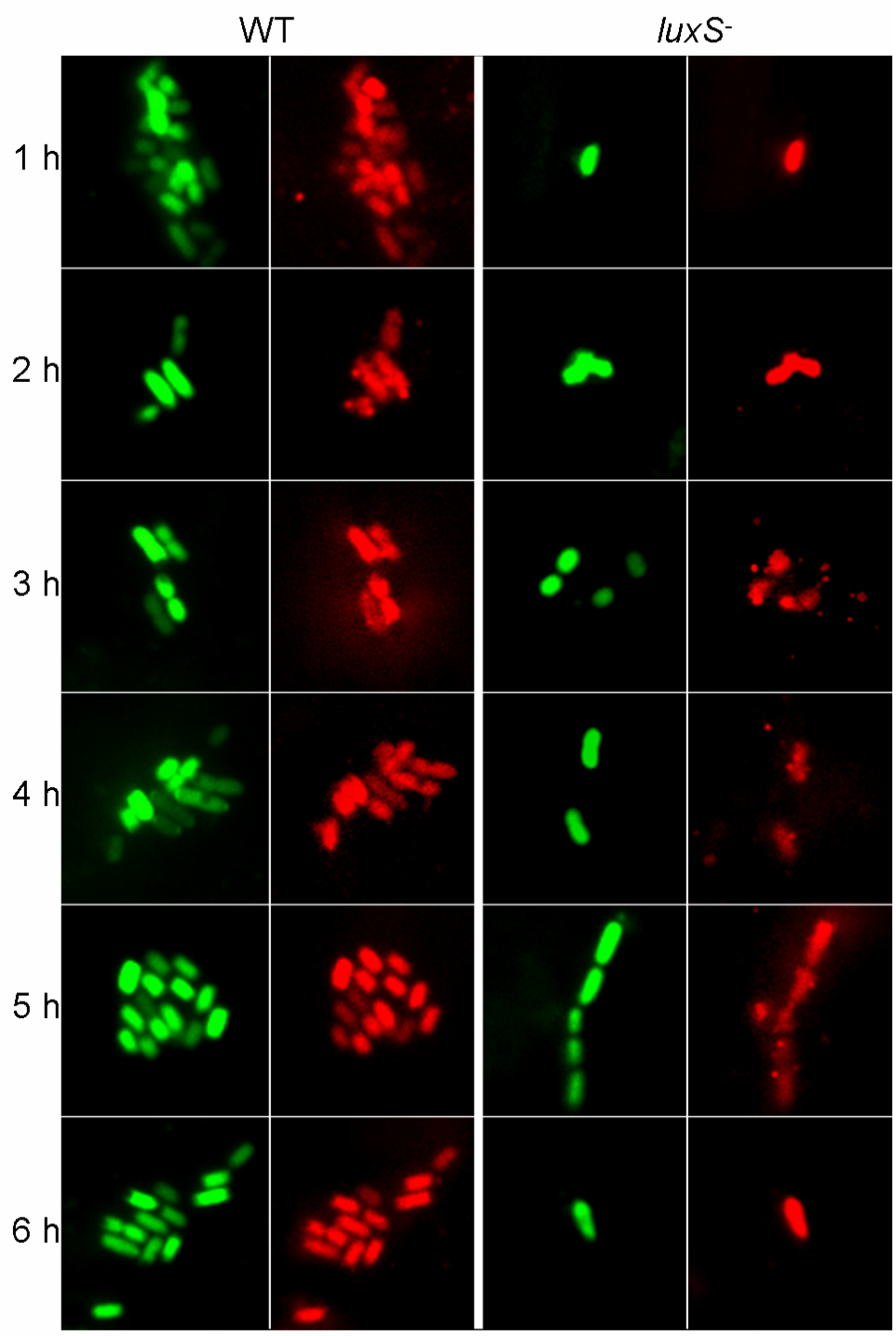


Figure 14: Expression of Intimin on the bacterial surface as observed by immunofluorescence in the WT and *luxS* mutant strains. Bacteria are shown in green and Intimin is stained red.

AE lesions at 3 hours post-infection, between early and mid-exponential growth phases. The *luxS* mutant was delayed 2 hours in AE lesion formation in this tissue culture model and did not display the AE phenotype until 5 hours post-infection corresponding to a time between mid and late-exponential growth phases used in the transcriptional studies. In previous studies using these strains, only the late-exponential (6 hr) growth phase was examined for the presence of AE lesions and no difference between WT and the *luxS* mutant was detected (188). This work provides the first phenotypic difference in AE lesion formation between the WT and *luxS* mutant.

III. Discussion

The *luxS* gene is necessary for efficient production of the AI-3 quorum sensing signal (188). However, the *luxS* mutation does not affect the ability of EHEC to respond to the AI-3 and epinephrine / norepinephrine signals (188). In our *in vitro* studies, the only AI-3 present is produced by the bacteria. The *luxS* mutant allows us to study the relationship of LEE activation and AI-3 production *in vitro*. We have shown here that the *luxS* mutation leads to decreased transcription of the LEE promoters during mid-exponential growth. This is in contrast to the late-exponential activation of the LEE promoters by this signaling system previously observed in an *E. coli* K-12 background (186). The difference in the kinetics of activation between the two backgrounds can most likely be attributed to the additional regulators of the LEE present in EHEC, but not in *E. coli* K-12 (Fig. 8). Pch, EtrA, EivF, GrlR/A, and Ler are several of the known LEE regulators present in EHEC and not *E. coli*

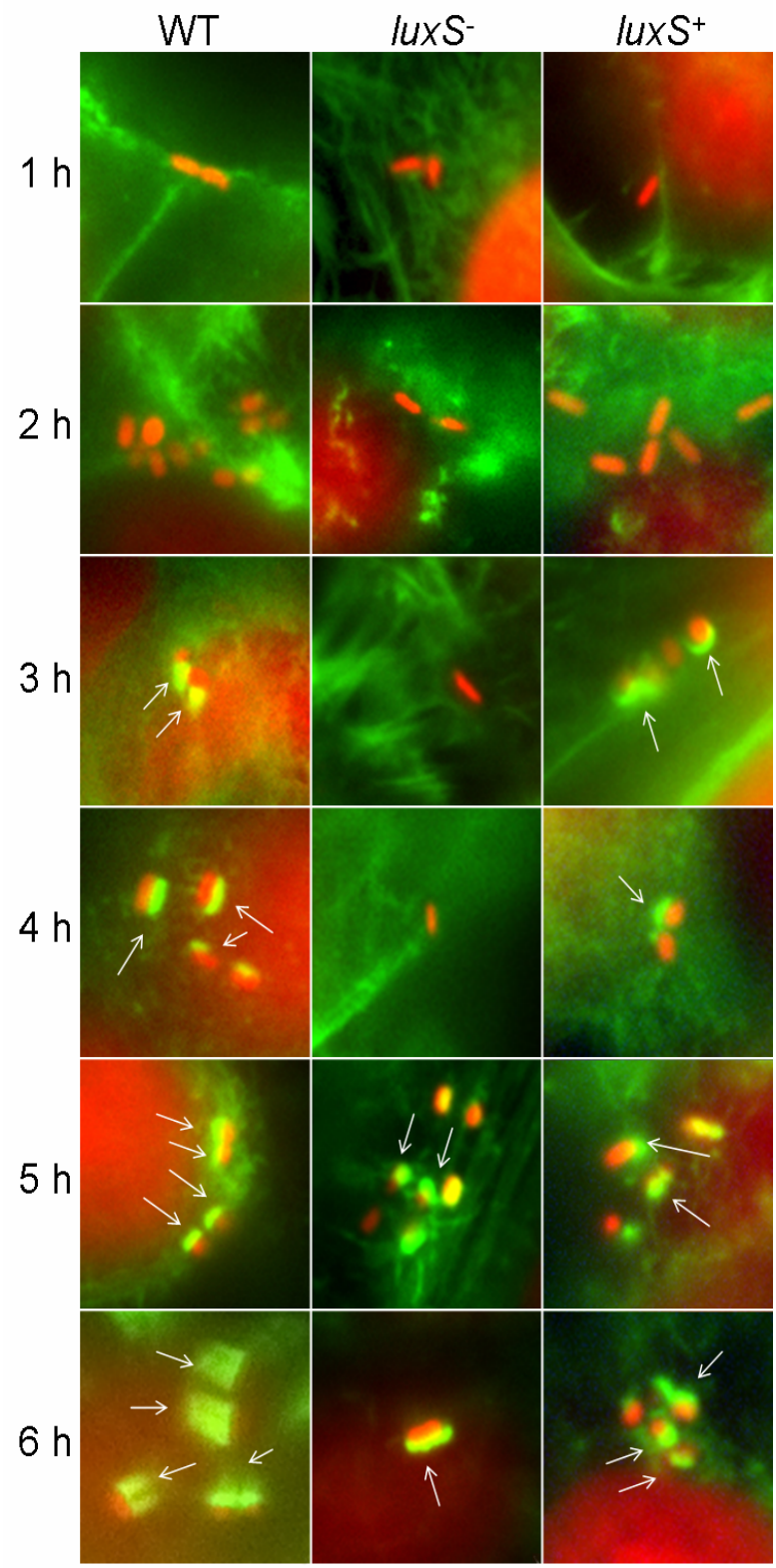


Figure 15: Fluorescent actin staining (FAS) to measure AE lesion formation of the WT, an isogenic *luxS* mutant, and the *luxS* complement in a HeLa cell infection model. Two hours post-infection corresponds with early-, 4 hours corresponds with the mid-, and 6 hours corresponds with the late-exponential growth. EHEC is stained red with propidium iodide, and the actin cytoskeleton is stained in green with FITC-phalloidin. AE lesions are indicated by arrows.

K-12. Furthermore, there may be additional uncharacterized regulatory factors specific to EHEC that influence LEE expression. The reduced LEE transcription led to a reduction in protein expression in the *LEE2*, *LEE5*, and *LEE4* operons. EspA and EspB protein secretion by the LEE TTSS was also reduced in the *luxS* mutant. The *luxS* mutant exhibited reduced amounts of EspA and EspB secretion compared to WT at the late-exponential growth (Fig. 12A), although no difference in transcription was observed (Fig. 9E). This may be a result of the *luxS* mutant's decreased ability to properly assemble functional TTSS machinery due to the decreased transcription of the LEE genes during mid-exponential growth or a result of the post-transcriptional regulation of the *espADB* operon, which has been described previously (158). Complementation of the *luxS* mutation restored the transcriptional activity of the LEE promoters, as well as cognate protein production and secretion.

When WT and the *luxS* mutant were grown in the presence of epinephrine, transcription of *LEE1*, *LEE2*, and *LEE3* increased. The *ler* gene is the only gene examined that shows a direct increase in transcription at the early-exponential growth phase in response to epinephrine addition. WT bacteria exhibited a greater increase in the transcription of *ler* (*LEE1*) at the mid-exponential growth phase, presumably because WT's ability to produce the AI-3 signal. Epinephrine and AI-3 seemed to signal in a synergistic fashion to activate the transcription of *ler*. We did not observe AI-3-dependent regulation during early- and late-exponential growth (Fig. 9A), and the effect of epinephrine appeared comparable for both the WT and the *luxS* mutant during these growth phases (Fig. 11A).

Both *escC* and *escV* transcription increased during late-exponential growth in response to epinephrine (Figs. 11B and 11C). Interestingly, addition of epinephrine to WT

only resulted in a larger increase of *escC* transcription (compared to the *luxS* mutant plus epinephrine) during late-exponential growth. There was not a significant difference in the transcription of *escV* between these two strains at this growth phase. AI-3 may influence the expression of another repressor that diminishes transcription of the *LEE3* operon, but not the *LEE2* operon at this growth phase. Addition of epinephrine also increased EspA and EspB protein secretion in the WT and *luxS* mutant, in agreement with previous data (188). Epinephrine and AI-3 increased the secretion of these proteins to a larger extent in the WT than was observed for the *luxS* mutant, again suggesting a synergistic relationship between these signals. The end result of the decreased transcription and expression of the LEE was a delay in the formation of AE lesions by the *luxS* mutant on cultured epithelial cells.

It is important to note that the *luxS* mutation does not abolish LEE expression, and the mutant is still able to respond to exogenous activating signals such as epinephrine. AI-3 and epinephrine / norepinephrine appear to play a large role in the proper expression and possibly the coordinated production of the LEE to yield a functional TTSS. The work presented here for the first time reveals the effects of the *luxS* mutation on the transcription of the LEE in a native EHEC background (containing all regulators of LEE expression). The disruption of *luxS* leads to a defect in the production of AI-3 (188) and to lower transcription of the LEE operons in EHEC.

EHEC infects the colon and has a very low infectious dose, estimated to be as few as 10-100 organisms. Because so few organisms are able to cause an infection, it is unlikely that EHEC relies on the small amount of self-produced AI-3 early during infection to activate expression of the LEE. The more likely scenario is that EHEC uses both the AI-3 produced

by the normal flora of the colon (188) and epinephrine / norepinephrine naturally present in the intestine (46) to recognize that it is within the host. The precise epinephrine / norepinephrine concentrations in the GI tract are not known, though substantial amounts of both epinephrine and norepinephrine have been shown to be present in the intestine (46). Epinephrine from the blood stream may spill out from enterocytes or may reach the lumen after the first round of infection and the resultant disruption of the intestinal epithelium and blood entering the colon. Norepinephrine is produced in the GI tract by adrenergic neurons in the enteric nervous systems. The concentration of norepinephrine in the lumen may also increase after destruction of the intestinal epithelium.

The data from this study suggest that there is a synergistic effect between AI-3 and epinephrine. Such combined signals then likely activate LEE expression in the same manner. This relationship would allow for a more efficient infection than responding to one signal alone. During the initial infection, the first wave of EHEC would sense the AI-3 produced by the normal flora, as well as any epinephrine / norepinephrine that may be present in the intestinal lumen, resulting in activation of the LEE. As the intestinal epithelium becomes more disrupted, more epinephrine / norepinephrine will be released into the GI tract. This increased amount of epinephrine / norepinephrine, as well as the AI-3 synthesized by the escalating EHEC population, would be detected by EHEC, leading to increased activation of the LEE and another wave of infection.

In summary, this study further characterizes the effects of the *luxS* mutation by examining LEE transcription in an EHEC background and also provides for the first time evidence of a synergistic relationship between AI-3 and epinephrine / norepinephrine. A

better understanding of the signals that activate EHEC pathogenesis will help to direct new therapeutic approaches.

CHAPTER FIVE

AI-3 SYNTHESIS IS NOT DEPENDENT ON LUXS IN E. COLI

I. Introduction

LuxS is an enzyme involved in the production of the AI-2 precursor and homocysteine. The LuxS / AI-2 system has been identified in over 55 bacterial species (130, 229). AI-2 regulates light production in *Vibrio spp.* (167) and activates expression of the *lsr* operon encoding an AI-2 ABC transporter in EHEC (230). *E. coli lsr* transporter mutants maintain the ability to slowly take up AI-2 from the environment, suggesting the presence of an additional low-affinity transporter involved in AI-2 uptake (230). It has been suggested that the *luxS*/AI-2 system may be more involved in cell metabolism than in QS signaling in enteric bacteria (225, 226). Winzer *et al*. have proposed that AI-2 may be toxic to the cell during exponential growth and internalized at a later stage of growth during which controlled amounts can be degraded (225). This process would be metabolically beneficial to the bacteria since they are no longer losing one 'ribose-equivalent' unit per methyl-group transfer (225). It remains unclear if the primary role of AI-2 uptake in enteric bacteria is central metabolism, or if it is a mechanism of regulating gene expression by monitoring cell population density as well as a method of inter-species communication.

Mutation of *luxS* affects the production of AI-3, as well as that of AI-2. LuxS does not produce AI-3 directly, suggesting that the *luxS* mutation disrupts another pathway

involved in AI-3 synthesis. LuxS is involved in the production of AI-2 and homocysteine from SAM through a series of enzymatic steps (Figure 16, Pathway 1). The *luxS* mutation renders the bacteria unable to convert SRH to homocysteine. Homocysteine is needed for *de novo* synthesis of methionine in the cell. Methionine is an essential nonpolar amino acid in living cells and required for the production of SAM, an important methyl donor used in many critical cellular functions. The two cellular pathways in *E. coli* that produce the homocysteine needed for *de novo* synthesis of methionine are depicted in Figure 16. The *luxS* mutant can only synthesize homocysteine from the pathway that involves the use of oxaloacetate (Pathway 2), which may disrupt normal amino acid synthesis and cellular metabolism.

A previous gene array study indicated that the *luxS* mutation resulted in altered transcription of genes involved in amino acid biosynthesis and metabolism, nucleotide biosynthesis and metabolism, and carbon compound catabolism in addition to the effects seen on the LEE and motility genes (187). One of the genes downregulated in the *luxS* mutant was *aroP*, which produces a protein that transports aromatic amino acids into the cell. The *luxS* mutant may be unable to efficiently transport aromatic amino acids into the cell, leading to further disruption of normal amino acid biosynthesis. In the present study, we examine the affected pathways leading to diminished AI-3 production and altered metabolism in the *luxS* mutant and further distinguish the roles of AI-2 and AI-3 in EHEC.

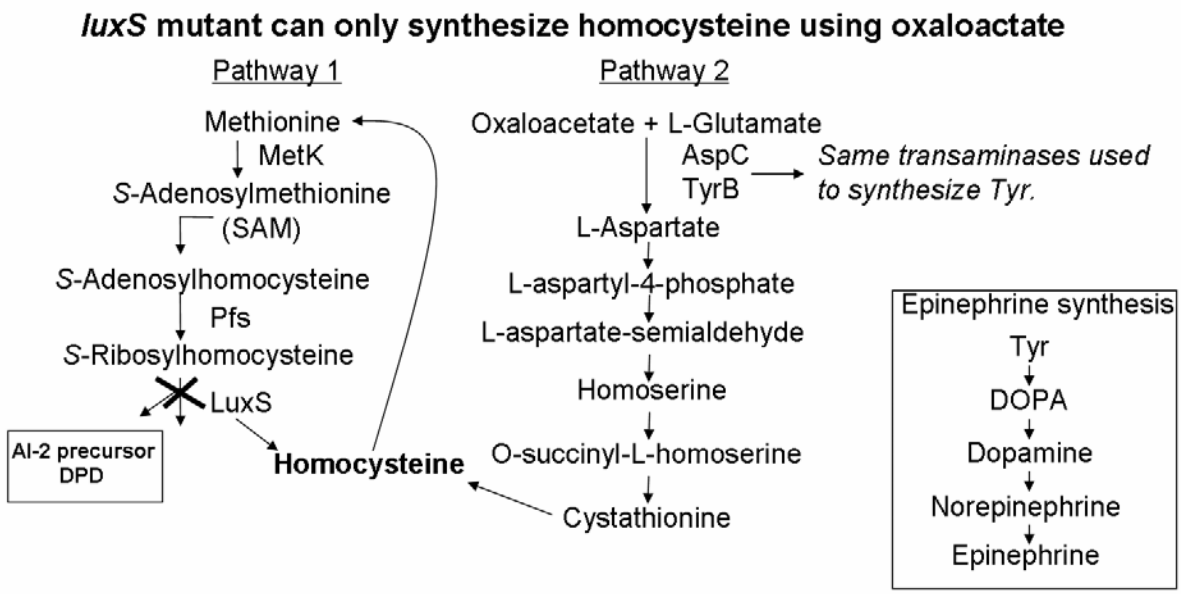


Figure 16: Pathways for homocysteine synthesis in *E. coli*. Homocysteine is needed in the cell for *de novo* synthesis of methionine, and methionine is required for the production of the vital metabolic enzyme *S*-Adenosylmethionine (SAM). SAM is an important methyl donor in the cell involved in the methylation of lipids, proteins, RNA and DNA. The *luxS* mutant cannot produce homocysteine through *S*-Ribosylhomocysteine (SRH) hydrolysis, leaving only one pathway involving the use of oxaloacetate to generate homocysteine. Oxaloacetate, L-glutamate, and the AspC and TyrB transaminases are used to produce aspartate, which can then proceed through a series of reactions resulting in the synthesis of homocysteine. Exclusive use of this pathway may lead to altered metabolism and amino acid content in the *luxS* mutant resulting in reduced AI-3 synthesis.

II. Results

A. The LuxS/AI-2 QS system does not activate the LEE genes

It has been previously shown that the *luxS* mutation leads to decreased LEE expression, and that LEE activity cannot be restored by addition of either purified or enzymatically synthesized AI-2 (188). LuxS is involved in converting SRH into DPD and homocysteine. To confirm that AI-2 does not play a role in LEE activation, we tested the ability of two different sources of AI-2 to activate *LEE1* transcription. The first form of AI-2, designated AI-2S, was generated using His-tagged purified Pfs and LuxS enzymes *in vitro* (Fig. 17). Chemically synthesized AI-2 precursor, designated DPD (126), was also tested for its ability to activate transcription from the *LEE1* promoter.

A β-galactosidase reporter system containing the *LEE1* promoter and a promoterless *lacZ* gene was used to assess the effect of AI-2 on LEE activation. Neither AI-2S nor DPD was able to activate transcription from the *LEE1* promoter (Fig. 18A). PC media from 86-24 (wildtype), containing AI-3, was able to activate transcription from the *LEE1* promoter (Fig. 18A). In order to demonstrate that both sources of AI-2 were biologically functional, we tested each source for its ability to activate bioluminescence in *Vibrio harveyi* strain BB170 (193). Supernatant from 86-24 (WT), AI-2S, and DPD were able to activate bioluminescence in *V. harveyi* (Fig. 18B). PC media from *E. coli* strain DH5-α, which does not produce AI-2 (193), was used as a negative control.

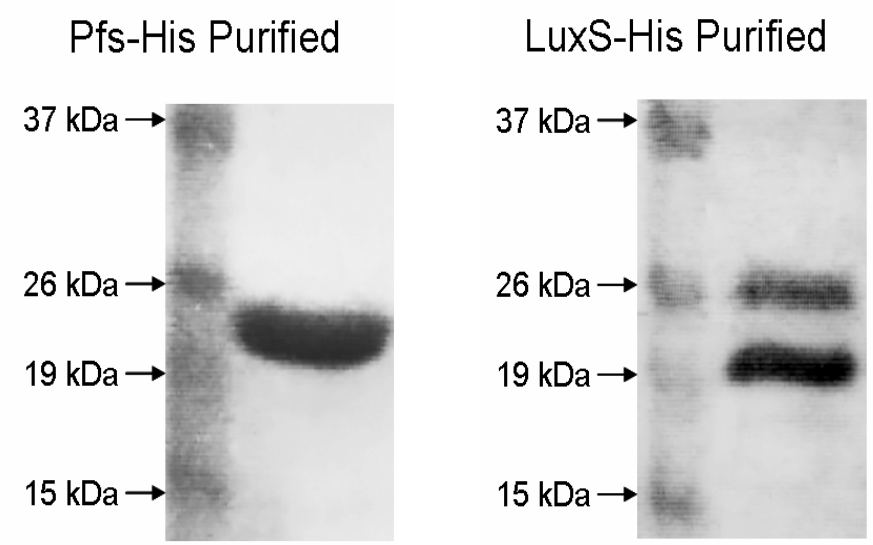
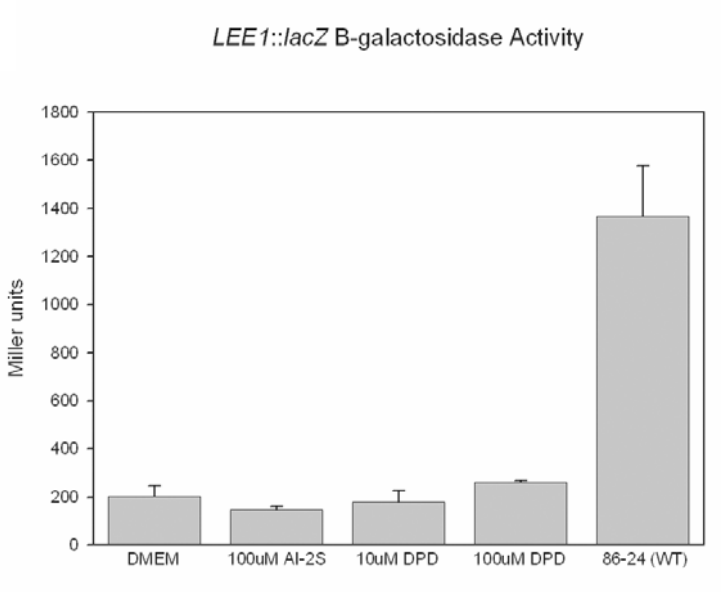


Figure 17: His-tagged Pfs and LuxS were purified on nickel affinity columns and separated by SDS-PAGE. Coomassie stains of the gels were performed to determine fraction purity. Fractions containing purified Pfs or LuxS were used to enzymatically synthesize AI-2 *in vitro*.

A.



B.

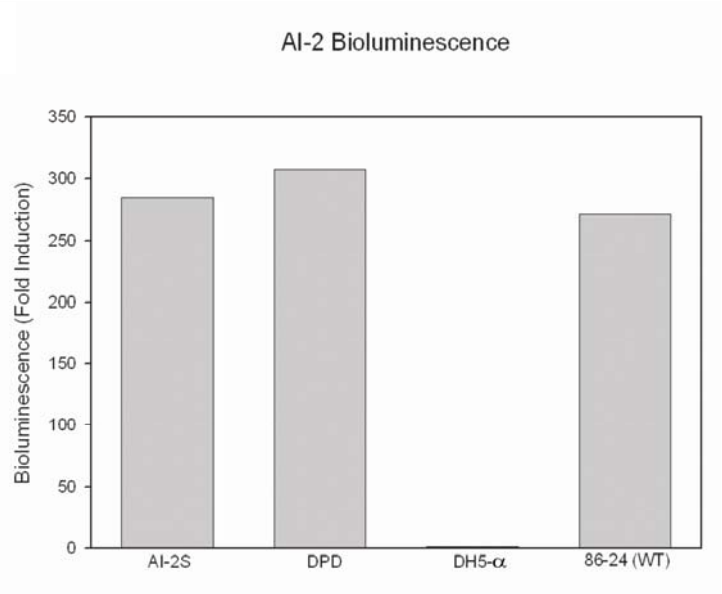
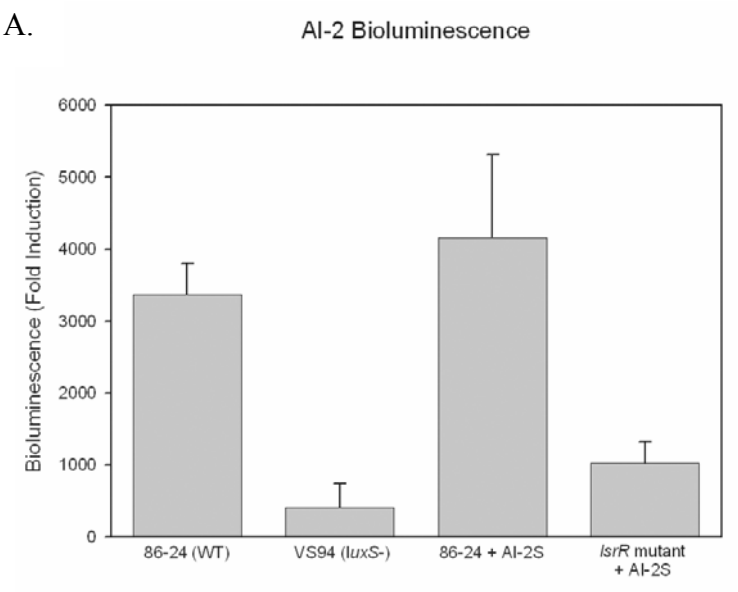


Figure 18: AI-2 does not activate the LEE. (A) Neither AI-2 produced enzymatically (AI-2S) nor chemically synthesized AI-2 (DPD) can activate the transcription of *LEE1* in the *luxS* mutant as shown by the β-galactosidase detection assay. (B) *V. harveyi* bioluminescence test to determine AI-2 production demonstrating that AI-2S, as well as DPD, activates bioluminescence. DH5-α does not produce AI-2 and was used as a negative control.

B. LsrR mutation does not affect LEE expression

An EHEC Δ*lsrR* deletion mutant was created in order to further examine if AI-2 plays a role in the pathogenesis of EHEC. Exogenous AI-2S was added to the wildtype (WT) and the Δ*lsrR* mutant and the supernatants examined for the AI-2 remaining in the supernatant. Taga *et al*. have previously demonstrated that a Δ*lsrR* mutant no longer represses transcription of the Lsr ABC transporter and that the mutant imports AI-2 from supernatants into the cell more efficiently than WT (196). As expected, the *lsr* mutant was found to import more AI-2 from the media than WT, thus leaving less AI-2 signaling molecule in the PC media (Fig. 19A). The Δ*lsrR* mutation caused higher expression of the Lsr ABC transporter, which led to less AI-2 present in the culture supernatant. Next, we assessed the effects of the *lsrR* mutation on the function of the LEE pathogenicity island. The EspA and EspB proteins are encoded by *LEE4* and secreted through the LEE type III secretion system. Proper expression of *ler* (*LEE1*) is required for transcription of the *espA* and *espB* genes and the secretion of these proteins through the type III secretion apparatus. To examine LEE function as a whole in the Δ*lsrR* mutant, we examined the amount of EspA and EspB secreted into culture supernatants by Western blot analysis. There was no detectable difference in secretion of these two proteins by the WT or the Δ*lsrR* mutant (Fig. 19B), further suggesting that AI-2 does not regulate the LEE.

A.



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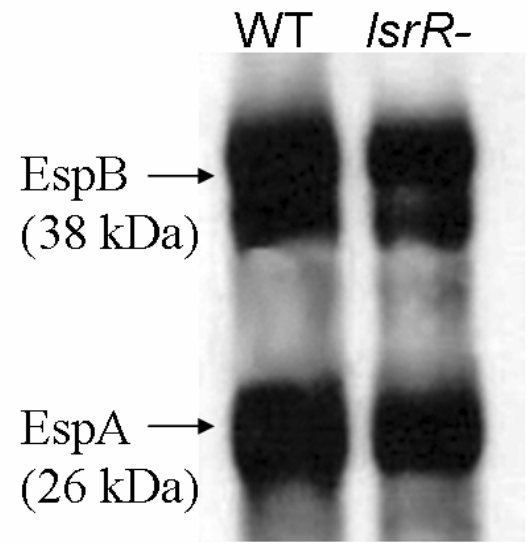


Figure 19: An EHEC *lsr* mutant imports more AI-2 from the supernatant, but displays normal LEE encoded type III secretion of EspA and EspB. (A) Enzymatically synthesized AI-2 was added to late-exponential cultures of either WT or an *lsr* EHEC mutant for 1 hr. The *V. harveyi* bioluminescence assay was used to determine the amount of AI-2 left in the supernatants. Less AI-2 was left in the *lsr* mutant supernatant, indicating increased AI-2 uptake as compared to WT. PC media from 86-24 (WT) and VS94 (Δ*luxS*) were used as controls. (B) Immunoblot analysis of the amount of EspA and EspB secreted into culture supernatants did not reveal any differences in LEE expression and function between the WT and *lsr* mutant.

C. Commensal bacteria and other pathogens synthesize both AI-2 and AI-3

The signaling cascade for AI-3 detection is present in many bacterial species. In order to examine which bacterial species are capable of producing AI-2 and AI-3, supernatants from many different bacterial cultures (strains and number tested listed in Table 1) were tested for their ability to activate *V. harveyi* bioluminescence and transcription of the *LEE1* promoter using the *LEE1*::*lacZ* β-galactosidase reporter system. All of the strains tested, except for strains without a functional *luxS* gene (DH5-α and *luxS* mutant), were able to produce AI-2 (Fig. 20A). Supernatants from all species activated bioluminescence at least 10-fold higher than the *luxS* mutant and DH5-α. Supernatants from all species activated bioluminescence at least 10 fold higher than the *luxS* mutant and DH5-α. Many bacterial supernatants were also able to activate transcription from the AI-3 dependent *LEE1* promoter, suggesting that these bacterial species also make AI-3 (Fig. 20B). Commensal *E. coli*, as well as several other intestinal bacterial species (EPEC E2348/69, EHECO26:H11 a clinical isolate, EPECO111lac:H9 a clinical isolate, *Klebsiella pneumoniae*, *Shigella sp.*, *Salmonella sp.*, and *Enterobacter cloacae*), were found to produce both AI-2 and AI-3. The wide variety of enterobacteria able to produce AI-3 suggests that it may serve as another inter-species QS signal.

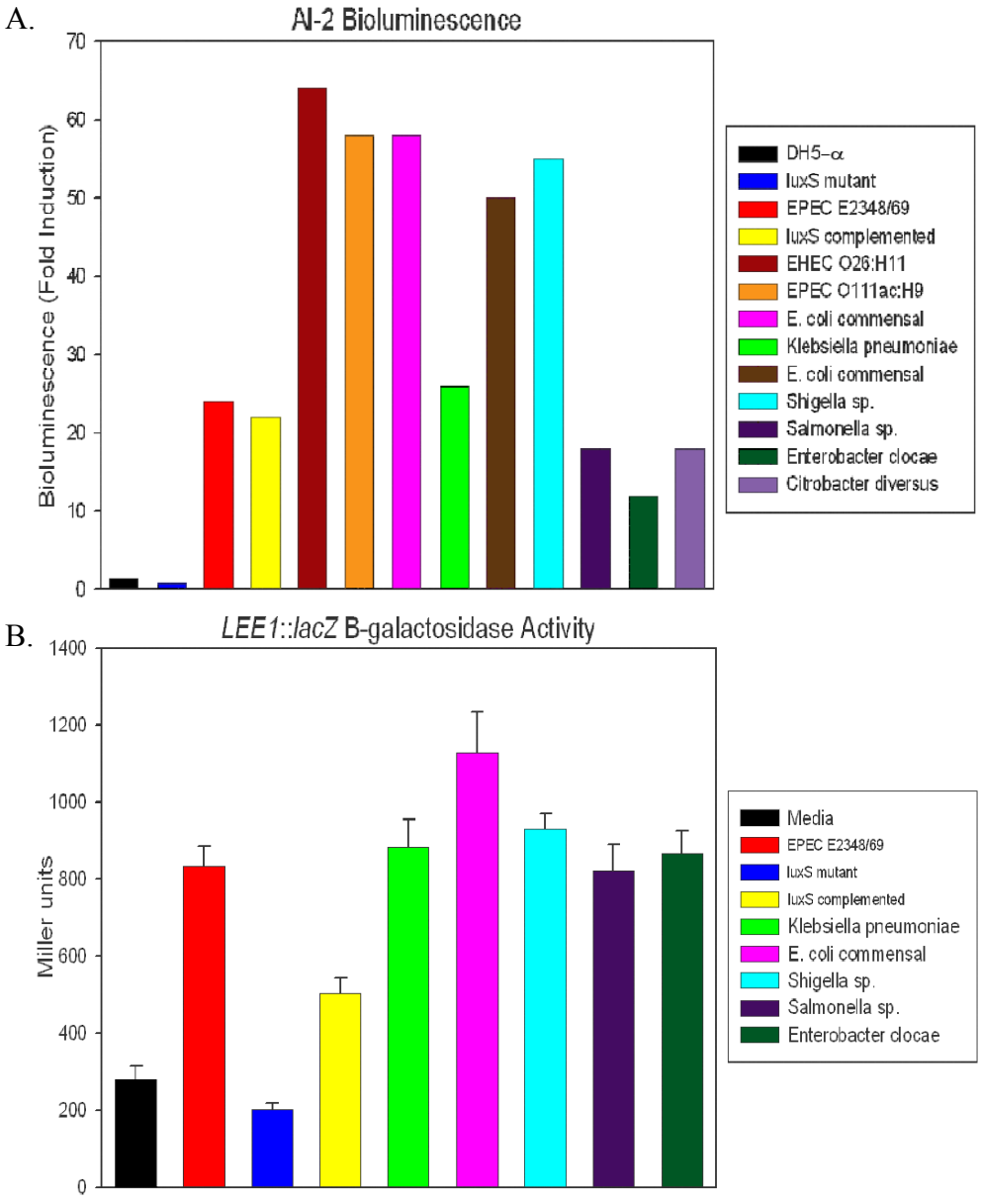


Figure 20: Many commensal and pathogenic bacterial strains produce both AI-2 and AI-3. (A) *V. harveyi* bioluminescence test to determine AI-2 production. All strains containing a functional *luxS* gene produced AI-2 and culture supernatants from these strains activated bioluminescence in *V. harveyi*. DH5-α and the *luxS* mutant do not produce AI-2. (B) A *LEE1*::*lacZ* β-galactosidase assay was used detect AI-3 in PC media. All strains tested produced AI-3 which activated transcription from the *LEE1* promoter, except the *luxS* mutant.

D. Aspartate restores *LEE1* transcription and protein secretion in the *luxS* mutant

In order to explore the hypothesis that the *luxS* mutation causes a metabolic shift and exclusive use of the oxaloacetate pathway may lead to decreased AI-3 synthesis, we first studied the effects addition of aspartate to the growth media. The DMEM medium used for EHEC growth in these assays did not contain aspartate, thus all aspartate must be synthesized endogenously by the cell. L-aspartate is the second product in the pathway that utilizes oxaloacetate to produce homocysteine (Fig. 16). This reaction involves the AspC and TyrB transaminases, which are also required for tyrosine production. By adding exogenous aspartate to the DMEM growth media, we attempted to decrease the requirement of AspC and TyrB transaminases to synthesize aspartate, allowing them to play other roles in cellular metabolism. Restoration of AI-3 synthesis was assessed by monitoring AI-3 dependent phenotypes, such as transcription of *LEE1* and secretion of EspA and EspB.

Addition of 0.5 mM aspartate dipeptide, a concentration similar to the other amino acids present in DMEM, restored transcription from the *LEE1* promoter in the *luxS* mutant to near WT levels using a *LEE1*::*lacZ* reporter system (Fig. 21A). These results were further characterized by measuring the amount of *ler* (*LEE1*) transcription in response to aspartate by real time RT-PCR. The *luxS* mutation resulted in a decrease of *ler* transcription, which was complemented when *luxS* is expressed from a plasmid (Fig. 21B). The addition of aspartate restored *ler* transcription in the *luxS* mutant to greater than WT (Fig. 21B). Growing the *luxS* mutant in the presence of aspartate also increased the secretion of the EspB and EspA proteins, which is diminished in the *luxS* mutant (188) (Fig. 21C). The addition of

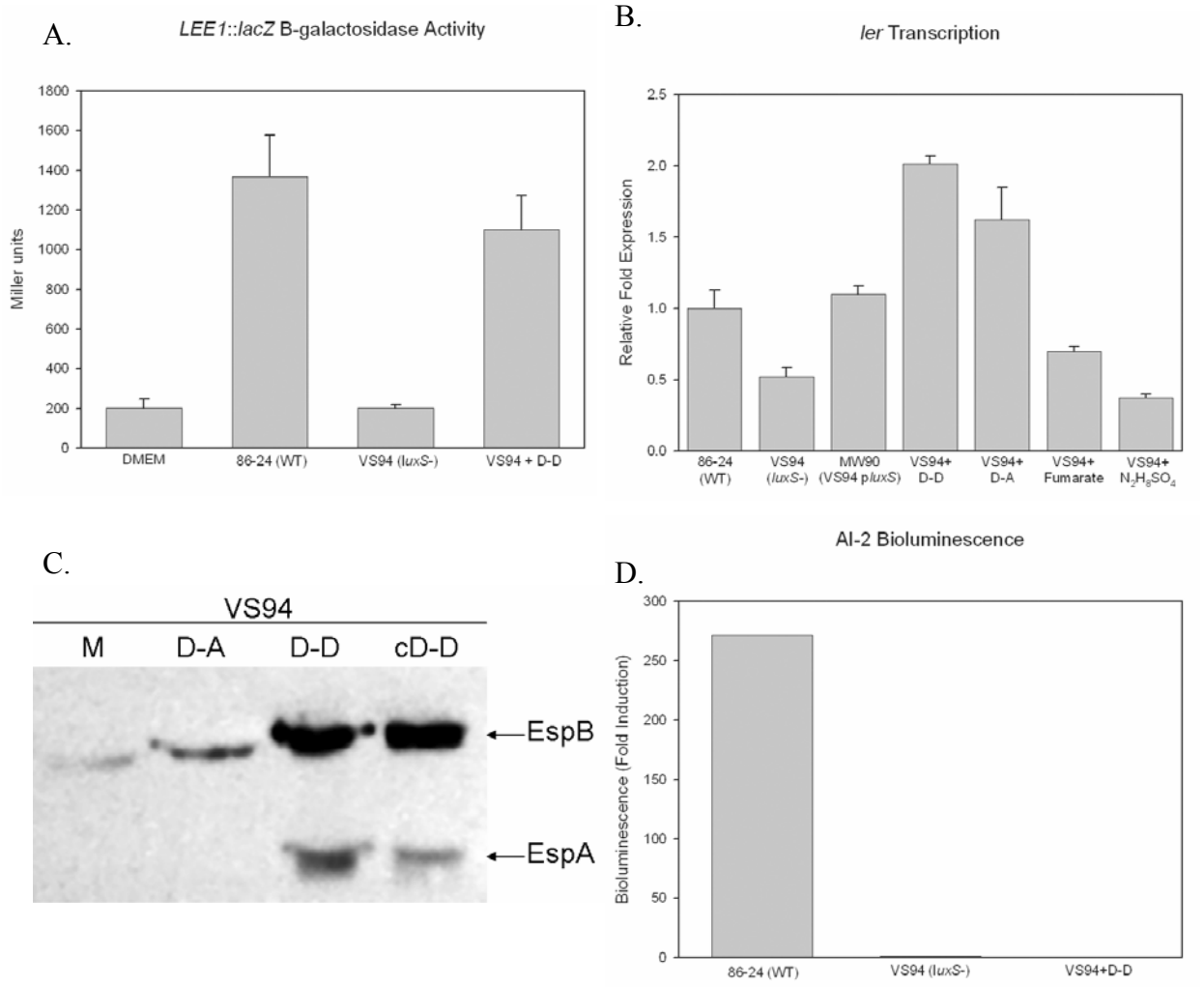


Figure 21: The addition of aspartate restores *LEE1* transcription and EspA and EspB secretion in the *luxS* mutant. (A) The addition of 0.5 mM aspartate dipeptide to the *luxS* mutant restored the AI-3 dependent activation of *LEE1* in an *E. coli* K-12 background. Only supernatants from WT and *luxS* mutant with the addition of aspartate were able to activate transcription from the *LEE1* promoter in this system. (B) Real time RT-PCR revealed *ler* transcription in *luxS* mutant was restored to greater than WT by the addition of aspartate. Complementing *luxS* on a plasmid also restored *ler* transcription levels. (C) Aspartate increased secretion of the LEE encoded EspA and EspB proteins in the *luxS* mutant as seen by immunoblot. (D) *V. harveyi* bioluminescence assay showing that the addition of aspartate to the *luxS* mutant does not restore the mutant's ability to produce AI-2.

aspartate complemented a defect in the *luxS* mutant, restoring transcription of *ler* and function of the LEE type III secretion system.

In order to test if the effects of aspartate addition were due to an increase in the cellular nitrogen levels, we supplemented the DMEM growth media with 0.2% ammonium sulfate to increase nitrogen levels in the cell. The addition of 0.2% ammonium sulfate did not restore *ler* transcription in the *luxS* mutant, suggesting that nitrogen limitation was not responsible for the decrease in AI-3 production (Fig. 21B). We also explored the idea that the decreases in *ler* transcription and AI-3 production may result from altered carbon metabolism in the *luxS* mutant. The addition of 50 mM fumarate, which will increase available carbon, may have partially restored transcription of *ler*, although the increase in transcription was not significantly different from that of the *luxS* mutant (Fig. 21B).

The effect of aspartate on AI-2 production was assessed using culture supernatants from WT, *luxS* mutant, and *luxS* mutant plus the addition of aspartate dipeptides in the *V. harveyi* bioluminescence assay for AI-2. As expected, it was found that addition of aspartate to the *luxS* mutant had no effect on AI-2 production and the *luxS* mutant did not produce AI-2 (Fig. 21D).

E. SahH restores *ler* transcription, but not AI-2 production in the *luxS* mutant

When SAM is used as a methyl donor in the cell, SAH is formed. SAH is a potent feedback inhibitor of SAM-dependent methyltransferases and its hydrolysis is necessary to avoid toxic effects on the cell. Organisms utilize one of two pathways to further process

SAH and inhibit its lethal effects on the cell. *E. coli* uses a 5'-methylthioadenosine/ SAH nucleosidase (Pfs) and a SRH cleavage enzyme (LuxS) to convert SAH to homocysteine (167) (Fig. 22). *P. aeruginosa* does not contain Pfs/LuxS, and uses a SAH hydrolase to convert SAH to homocysteine in a single step reaction (Fig. 22) (225). Low concentrations of homocysteine added to minimal media, such as DMEM, have been shown to be inhibitory to growth of *E. coli* (157, 208). To increase homocysteine levels in the cell while avoiding cell toxicity and interference with growth, we complemented the EHEC *luxS* mutant's inability to produce homocysteine through SAM detoxification by expressing *sahH* (SAH hydrolase) from *P. aeruginosa* in the EHEC *luxS* mutant.

Expression of the *P. aeruginosa* SahH in the EHEC *luxS* mutant restored the ability of the *luxS* mutant to produce AI-3. AI-3 was present in PC media from WT, the *luxS* mutant expressing *sahH*, and the *luxS* complemented strain (Fig. 23A). SahH also restored the AI-3 dependent-transcription of *ler* as measured by real-time RT-PCR to greater than WT levels (Fig. 23B). The SAH hydrolase restored the *luxS* mutant's ability to produce homocysteine from SAM, restoring normal metabolism in the cell, and AI-3 production. To confirm that expressing SahH in the *E. coli* background had no effect on AI-2 production, we tested this strain's ability to produce AI-2 using the *V. harveyi* bioluminescence assay. As expected, SahH expression did not restore AI-2 production in the *luxS* mutant (Fig. 23C).

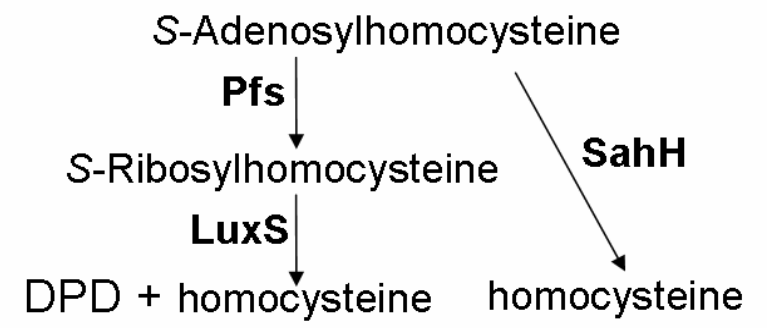


Figure 22: SAH hydrolysis in bacteria. Bacteria utilize either 1-step (i.e. *P. aeruginosa*) or 2-step (i.e. *E. coli*) method to hydrolyze *S*-adenosylhomocysteine (SAH). SAH is both a product and an inhibitor of SAM-dependent methyltransferases that play essential roles in the methylation of lipids, proteins, and nucleic acids. SAH hydrolysis is required to maintain active methylation. Hydrolysis of SAH in *E. coli* produces the AI-2 precursor DPD and homocysteine. In *P. aeruginosa*, SAH hydrolysis leads to the production of adenosine and homocysteine.

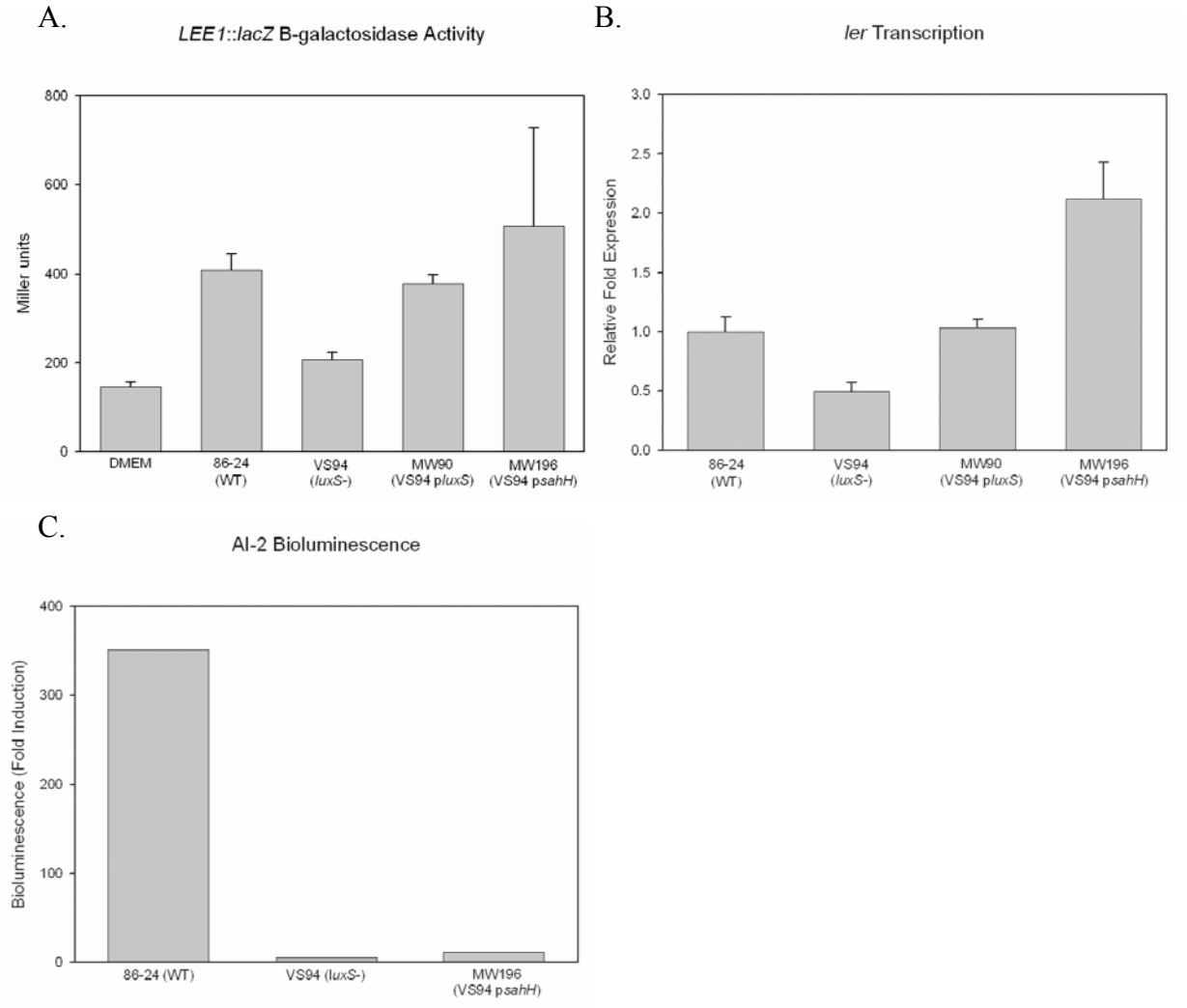


Figure 23: SahH restores transcription of *ler* in the *luxS* mutant. (A) The expression of *sahH* in the *luxS* mutant restored AI-3 dependent activation of the *LEE1* promoter in an *E. coli* K-12 background. PC media from WT, the *luxS* mutant expressing *sahH*, and the *luxS* complemented strain activated transcription from the *LEE1* promoter as measured by β-galactosidase activity. (B) Real time RT-PCR was used to demonstrate that *ler* transcription is restored in the *luxS* mutant by expressing *P. aeruginosa sahH* from a plasmid. (C) Expression of *P. aeruginosa sahH* did not restore the EHEC *luxS* mutant's ability to produce AI-2 as determined by the *V. harveyi* bioluminescence assay.

F. AroP and TyrP complement the AI-3 defect of the *luxS* mutant

The results of the previous experiments suggest that the decreased AI-3 production could occur as a result of exclusive use of the oxaloacetate pathway to produce homocysteine. Under normal cell metabolism, the major biosynthetic pathway to aspartate is through transamination between oxaloacetate and L-glutamate involving the AspC and/or TyrB amino acid transaminases. These are the same transaminases involved in the biosynthesis of tyrosine. Increased use of this pathway to produce homocysteine could lead to altered amino acid levels in the cell, including tyrosine since the AspC and TyrB transaminases would be used to synthesize aspartate and not tyrosine (Fig. 16). Tyrosine is a component in the DMEM growth medium at a concentration of 0.398 mM. AroP is responsible for transporting aromatic amino acids, such as tyrosine into the cell. However, a gene array revealed that *aroP* is downregulated in the *luxS* mutant (187). A decrease in AroP production may impair the ability of the *luxS* mutant to import aromatic amino acids.

To verify the results of the array study indicating *aroP* downregulation in the *luxS* mutant, real time RT-PCR was used to measure *aroP* transcript levels in the WT and *luxS* mutant. The transcription of *aroP* was significantly reduced in the *luxS* mutant as compared to WT (Fig. 24). To further study the effects of *aroP* on AI-3 production and LEE activation, we expressed *aroP* in the *luxS* mutant under an IPTG inducible promoter and measured the amount of AI-3 in culture media using the *LEE1*::*lacZ* reporter assay. Inducing the expression of AroP, and presumably increasing the intracellular concentration of aromatic

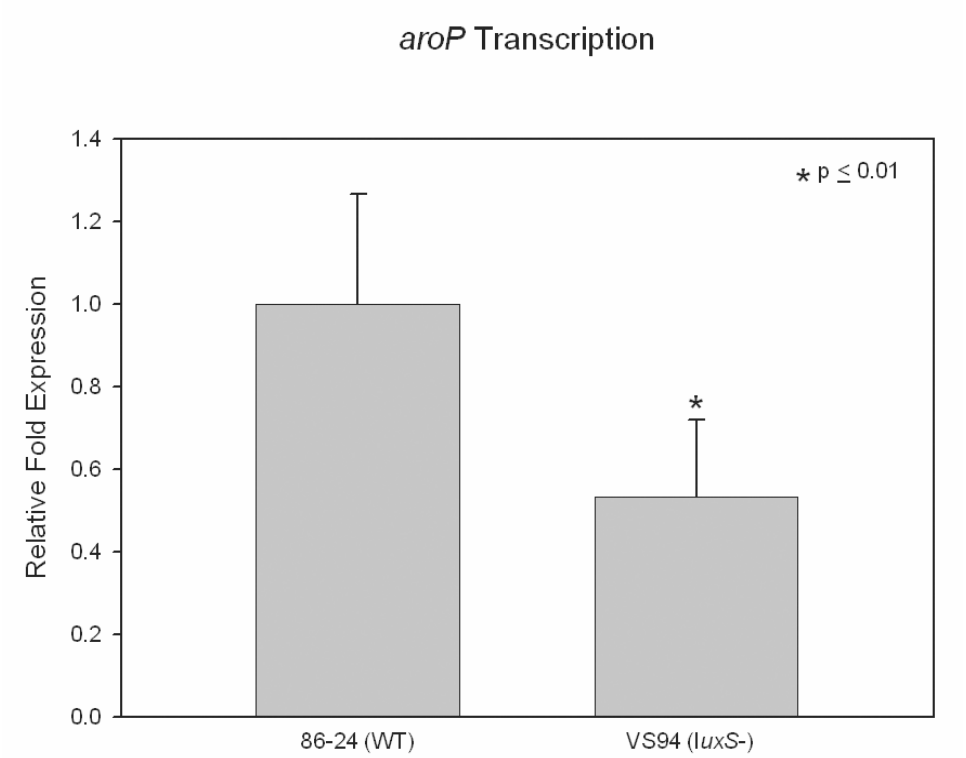


Figure 24: *aroP* is downregulated in the *luxS* mutant. Real-time RT-PCR analysis of *aroP* transcription in WT and the *luxS* mutant. AroP is important for importing aromatic amino acids, such as tyrosine, into the cell.

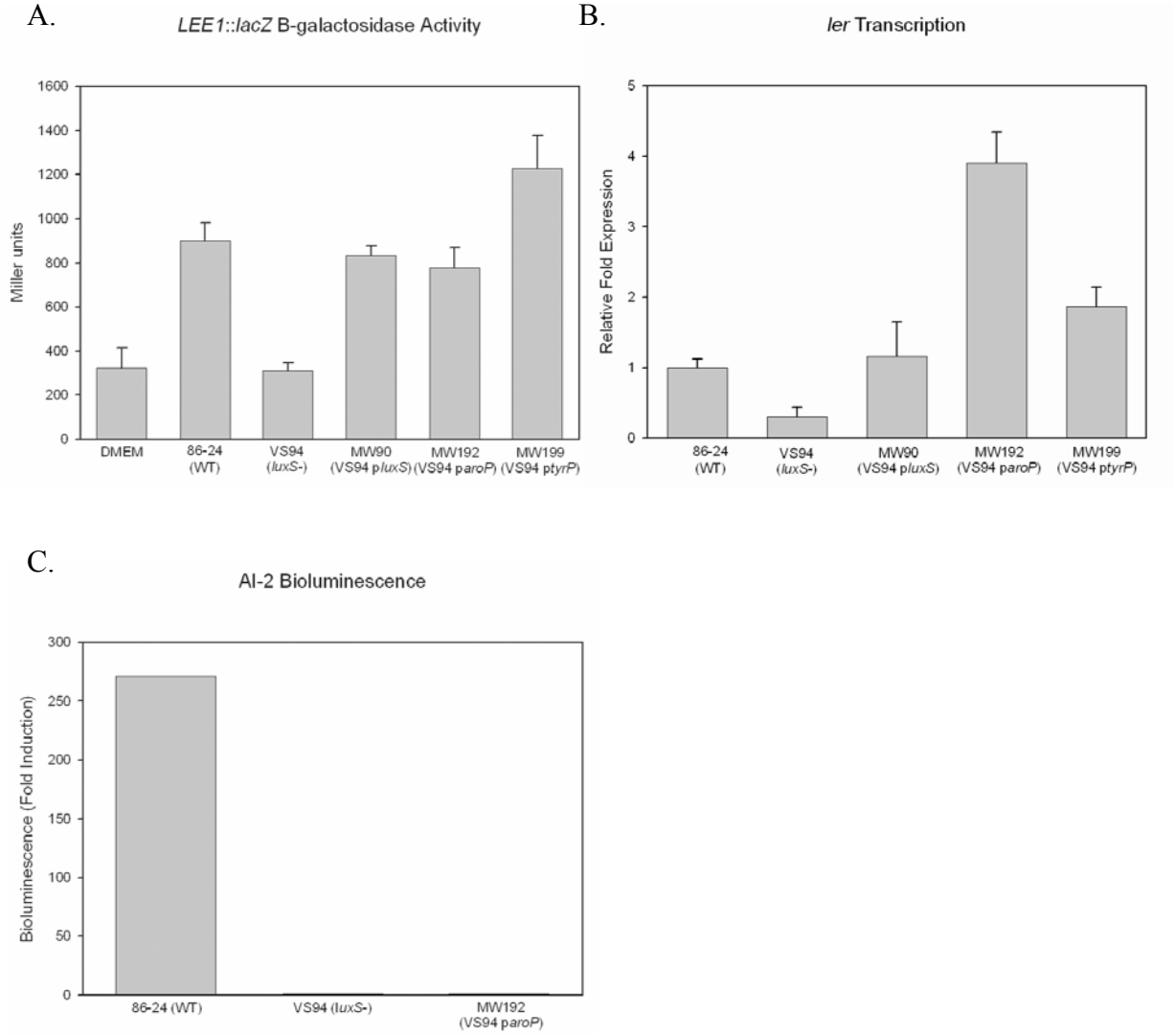


Figure 25: The *luxS* mutant *ler* transcriptional defect can be complemented by overexpressing *aroP* and *tyrP*. (A) The expression of *aroP* in the *luxS* mutant restored AI-3 dependent activation of the *LEE1* promoter in an *E. coli* K-12 background. PC media from WT, the *luxS* mutant expressing *sahH*, and the *luxS* complemented strain activated transcription from the *LEE1* promoter as measured by β-galactosidase activity. (B) Expressing *aroP* or *tyrP* on an inducible plasmid in the *luxS* mutant restored transcription of *ler* as measured by real time RT-PCR. (C) The expression of *aroP* did not affect AI-2 production in the *luxS* mutant as determined by the *V. harveyi* bioluminescence test.

amino acids, complemented the AI-3 defect observed in the *luxS* mutant (Fig. 25A). When *aroP* was expressed in the *luxS* mutant, transcription of *ler* was also restored (Fig. 25B). To more specifically address the role of tyrosine in AI-3 synthesis, the tyrosine-specific transporter TyrP was expressed from an IPTG inducible promoter in the *luxS* mutant and the amount of AI-3 in culture supernatants was determined using the *LEE1*::*lacZ* reporter assay. Inducing *tyrP* expression in the *luxS* mutant restored AI-3 activity in culture supernatants to WT levels (Fig. 25A). TyrP also restored transcription of *ler* in the *luxS* mutant to greater than WT levels as measured by real-time RT-PCR (Fig. 25B). The increased import of aromatic amino acids and tyrosine from the growth media appears to have allowed for more AI-3 production, suggesting these molecules are important in AI-3 synthesis. As expected, expression of AroP had no effect on AI-2 production as measured by the *V. harveyi* bioluminescence test (Fig. 25C).

G. Phenotype MicroArray analysis

The exact roles of the *luxS* AI-2 QS in EHEC and other enteric bacteria remain unclear. The previous results from this study suggested that the reduced AI-3 production by the *luxS* mutant was a result of altered cellular metabolism. In order to examine the metabolic roles of the *luxS*/AI-2 QS system, Phenotype MicroArrays (PM) were used to globally examine the effects of the *luxS* mutation on metabolism. These arrays screen nearly 2,000 cellular phenotypes (16). We examined four different conditions in duplicate comparing the WT and *luxS* mutant and the effects of adding in the quorum sensing signals

AI-2S and epinephrine (which can substitute for AI-3). Pure AI-3 was not used due to the difficulty in obtaining sufficient quantities of purified AI-3 needed for these studies. A summary of results obtained from the PM's for all four conditions is shown in Table 3.

The first condition compared the *luxS* mutant to WT (Appendix A). The *luxS* mutant gained 45 phenotypes when compared to WT. Of these 45 phenotypes, 37 were related to increased antimicrobial resistance, most likely the result of the efflux pump encoded by the tetracycline cassette that was used to inactivate the *luxS* gene in this strain. The *luxS* mutant lost 172 growth phenotypes when compared to WT. Forty-two of these conditions involved the utilization of nitrogen sources. The *luxS* mutant also lost the ability to utilize 15 carbon sources, 5 phosphate sources, and 5 sulphur sources. Ninety-four phenotypes involved nutrient stimulation. All of these nutrient stimulation phenotypes occurred on the same PM array plate in a minimal media with strict metabolic sources. These results may suggest that minimal media does not support efficient growth of the *luxS* mutant. The effects observed may not be due to the different compounds in each well, but rather the inability of the *luxS* mutant to grow in this medium.

We next examined the effects of the addition of enzymatically synthesized AI-2 to the *luxS* mutant (Appendix B). AI-2 synthesis was performed for 1 hour at 37˚C using conditions previously described (167). Carrying out this reaction for 1 hour allows for the oxidation of the homocysteine produced by the synthesis reaction (167). Homocysteine levels were undetectable using Ellman's test for the sulfhydryl group (data not shown). It was found that 62 growth phenotypes were gained by the addition of synthesized AI-2 to the *luxS* mutant. Many of these involved the utilization of metabolic compounds such as: 26

nitrogen sources, 16 phosphate sources, and 10 carbon sources. Twenty-two of these phenotypes were the same ones lost in the *luxS* mutant when compared to WT. Addition of AI-2 resulted in 17 phenotypes being lost, including the ability to utilize 7 sulphur sources. Three of the phenotypes lost by the addition of AI-2 were gained by the *luxS* mutant when compared to WT.

The effects of adding 5 μM epinephrine to the WT strain versus WT without the addition of epinephrine were also tested (Appendix C). Seven phenotypes were gained by the addition of epinephrine. Three were involved in antimicrobial resistance, while 3 others were involved in nitrogen metabolism. Four phenotypes were lost due to the addition of epinephrine. The last condition examined was the addition of 5 μM epinephrine to the *luxS* mutant versus the *luxS* mutant with no epinephrine added (Appendix D). No phenotypes were lost in this condition. Four phenotypes were gained when epinephrine was added. These phenotypes involved cell wall modifications which resulted in increased antimicrobial resistance. Consensus PM arrays and correlation between replicates are shown in Appendix E and Appendix F, respectively.

III. Discussion

In the present study, we have addressed the role of the *luxS* gene in the production of the AI-2 and AI-3 QS signals produced by EHEC. Several EHEC virulence factors, such as motility and the LEE, are under QS control (186, 187). Quorum sensing relies on signals that are secreted by bacteria and regulate gene expression when a critical threshold is reached.

Table 3: Phenotype MicroArray Results

Phenotype Gained	VS94 vs. 86-24	VS94+AI-2 vs. VS94	86-24+epi vs. 86-24	VS94+epi vs. VS94
chelator, lipophilic	1	-	1	-
cholinergic antagonist	1	-	-	-
C-source	-	10	-	-
cyclic nucleotide phosphodiesterase	1	-	-	-
DNA intercalator	1	-	-	-
DNA polymerase	1	-	-	-
DNA topoisomerase	5	1	-	-
folate antagonist	1	-	-	-
ion channel, K+	1	-	-	-
membrane, detergent	3	-	-	-
membrane, transport	1	-	-	-
N-source	-	26	3	-
phenothiazine	-	1	-	-
protein synthesis	15	1	2	-
P-source	-	16	-	-
RNA polymerase	-	1	1	-
wall, cephalosporin	5	2	-	1
wall, lactam	9	2	-	3
Phenotype Lost				
anti-capsule, anti-inflammatory	-	1	-	-
anti-tuberculosic	1	-	-	-
C-source	15	-	-	-
DNA polymerase	-	1	-	-
DNA topoisomerase	-	1	1	-
folate antagonist	1	-	-	-
fungicide	-	-	1	-
membrane	2	-	-	-
membrane, detergent	-	-	1	-
N-source	42	1	1	-
nutrient stimulation	94	-	-	-
oxidizing agent	1	2	-	-
pH, deaminase	2	-	-	-
protein synthesis	1	1	-	-
P-source	5	-	-	-
respiration	-	2	-	-
S-source	5	7	-	-
transport, toxic anion or cation	3	-	-	-
wall, cephalosporin	-	1	-	-

The greatest density of signaling molecules occurs at high bacterial densities, and the largest population of bacterial species in the human body occurs in the GI tract.

The human GI flora produces both AI-2 and AI-3 (189) and this study specifically demonstrates that many other commensal and enteric pathogens are also capable of producing both AI-2 and AI-3. Given the large numbers of bacteria in the GI tract, and the ability of many different species to produce both AI-2 and AI-3 (Fig. 20), it seems possible that EHEC may use one or both of these signals to recognize that it is within a host. The QS signal which has been shown to activate motility and the LEE is AI-3 (188). The low infectious dose of EHEC, estimated to be as few as 50-100 organisms, may be a result of its ability to detect the high concentration of autoinducers in the GI tract and regulate its virulence genes accordingly. This may be advantageous to EHEC because it could activate expression of the virulence genes required for infection quickly without the need to grow to a high cell density and produce its own autoinducers.

The existence of QS gene regulation in EHEC was initially observed in an EHEC *luxS* mutant (186). It was originally assumed that the lack of AI-2 produced by the *luxS* mutant was responsible for the reduced virulence phenotypes, but the decrease in virulence was later shown to be a result of the absence of another autoinducer termed AI-3 (188). AI-3 is chemically distinct from AI-2. It is less polar, binds to C-18 columns, and only elutes with methanol, while AI-2 is a polar furanone that does not bind C-18 columns and elutes with buffer alone (188). To date, the only *E. coli* and *Salmonella* genes known to be regulated in response to AI-2 are in the *lsr* operon (197, 230). This study further demonstrates that AI-2 does not activate the transcription of *ler* and expression of the LEE using both enzymatically

and chemically synthesized AI-2. Our previous work used enzymatically synthesized AI-2 to demonstrate that AI-2 does not affect *LEE1* transcription (188). We have shown that chemically synthesized DPD (126), which is more pure than enzymatically prepared AI-2, also does not affect transcription of *LEE1*. LsrR has been suggested to be the transcription factor that interacts with AI-2 (230). Here, we have demonstrated that a Δ*lsrR* mutant displays normal expression and function of the LEE-encoded type III secretion system, despite this mutant's ability to import AI-2 more efficiently into the cell (Fig. 19).

These observations lead to the question of why the EHEC *luxS* mutant has decreased AI-3 production and subsequently decreased activation of the LEE and motility genes. This study examines the possible metabolic defects present in the *luxS* mutant which lead to reduced AI-3 synthesis. The *luxS* mutation leaves only one pathway to produce homocysteine. The *luxS* mutant can only use the pathway involving oxaloacetate to generate homocysteine (Fig. 16). Homocysteine is an important compound in the cell and is required for the *de novo* synthesis of methionine. The *E. coli* MetK enzyme uses methionine to produce SAM. SAM is a multipurpose essential growth compound playing a role in many key metabolic aspects of the cell such as polyamine biosynthesis and serving as a primary methyl donor in many biosynthetic reactions such as the methylation of DNA, RNA, lipids, and proteins (116, 195).

To examine if the reduced AI-3 production by the *luxS* mutant was due to altered metabolism, we assessed restoration of AI-3 dependent phenotypes by complementing the defects in the *luxS* mutant at different levels in the oxaloacetate-homocysteine pathway. The homocysteine biosynthesis pathway thought to be employed by the *luxS* mutant uses

oxaloacetate and L-glutamate to generate L-aspartate that is converted to homocysteine in a series of reactions (Fig. 16). The medium used in all of our virulence assays does not contain aspartate. Addition of aspartate to the *luxS* mutant was able to restore production of AI-3, transcription of *LEE1*, and secretion of EspA and EspB (Fig. 21). The addition of aspartate to the growth medium could change the nitrogen and carbon levels in the *luxS* mutant. When free aspartate is available in the growth medium, the need for aspartate biosynthesis in the cell will diminish. L-glutamate and oxaloacetate will no longer be required for synthesis of aspartate, leading to increased availability of these compounds within the cell. L-glutamate is an important factor in the nitrogen assimilation cycle, and an increase in the levels of L-glutamate may lead to an increase in the nitrogen levels in the cell. It is possible that the restoration of tyrosine synthesis may have resulted from the higher nitrogen levels or precursor molecules from the aspartate/glutamate pathways in the cell. Altering nitrogen levels with the addition of ammonium sulfate did not restore transcription of *ler* in the *luxS* mutant, suggesting that the aspartate-induced transcription of *ler* was not a result of altered nitrogen levels within the cell.

If the exclusive use of the oxaloacetate pathway to produce more homocysteine in the *luxS* mutant is responsible for the decreased AI-3 production, correcting the defect of the pathway leading to homocysteine production from SAM should restore normal AI-3 synthesis. *E. coli* uses the two-step reaction involving Pfs and LuxS to hydrolyze SAH and SRH respectively to produce DPD and homocysteine, while *P. aeruginosa* uses the SahH enzyme that produces adenosine and homocysteine as a result of SAH hydrolysis in a one-step reaction. Accordingly, *P. aeruginosa* is not capable of producing DPD, and

consequently AI-2. We were able to restore production of AI-3 in the *luxS* mutant without restoring AI-2 production by expressing *sahH* from *P. aeruginosa*. These experiments suggest that SahH expression in the *luxS* mutant lessens the need for oxaloacetate to be used for homocysteine synthesis, restoring some metabolic defects in the *luxS* mutant and resulting in AI-3 production. These experiments allowed us to uncouple AI-2 and AI-3 production in *E. coli*. The *luxS* mutation seems to alter cellular metabolism leading to decreased AI-3 production, possibly by reducing the tyrosine levels in the cell.

The eukaryotic hormones epinephrine and norepinephrine are able to activate transcription from the *LEE1* promoter, restore type III secretion of the EspB and EspA proteins in the *luxS* mutant, and restore the motility of the *luxS* mutant to WT levels (188). The synthesis of both hormones begins with a tyrosine molecule (67). The effects of epinephrine/norepinephrine on LEE activation can be blocked by the use of adrenergic receptor antagonists, such as propranolol and phentolamine (188). These adrenergic receptor antagonists also decrease *LEE1* transcription and secretion of EspB and EspA in the WT strain, both of which are controlled by AI-3 signaling (188). AI-3 and epinephrine / norepinephrine are recognized by the QseC sensor kinase (Clarke *et al*., *Proc. Natl. Acad. Sci. USA.* In Press), suggesting they share many similar structural features. Increased use of the oxaloacetate-homocysteine pathway in the *luxS* mutant may lead to higher production of aspartate (Fig.16). This could lead AspC and TyrB to be engaged in the synthesis of aspartate, making them less available for the production of tyrosine. If AI-3 synthesis begins with a tyrosine molecule, as with epinephrine and norepinephrine, a decrease of tyrosine in

the cell would lead to decreased synthesis of AI-3 and the virulence defects observed in the *luxS* mutant.

Tyrosine is present in the DMEM medium used in all of our assays, but the *luxS* mutant may be unable to import tyrosine as efficiently as WT because of a decrease in *aroP* transcription. AroP is a transporter protein responsible for transporting aromatic amino acids, such as tyrosine, into the cell. Increasing AroP levels in the *luxS* mutant, by expressing AroP from an IPTG inducible promoter, was able to restore transcription from the *LEE1* promoter. These results further suggest that aromatic amino acids, including tyrosine, are important for AI-3 synthesis. In order to examine the effect of tyrosine in a more direct manner, the tyrosine-specific TyrP transporter was expressed from a multicopy plasmid in the *luxS* mutant. Induction of *tyrP* expression with IPTG restored *ler* transcription in the *luxS* mutant to above WT levels and restored AI-3 levels in culture supernatants. Increasing cellular tyrosine levels seems to have allowed for more AI-3 to be produced, complementing the defects in LEE transcription observed for the *luxS* mutant.

The decreased AI-3 production in the *luxS* mutant seems to be the result of metabolic defects created by the mutation. Phenotype MicroArray (PM) studies were performed to gain an understanding of the role that *luxS* and autoinducers play in cell metabolism. We used epinephrine to study the effects of AI-3 signaling on cell growth because of the difficulty in purifying large enough amounts of AI-3 required for the PM's. Very few phenotypes in both the WT and *luxS* mutant were altered by the addition of 5 μM epinephrine. Several of the phenotypes affected by the addition of epinephrine in the WT and all of those in the *luxS*

mutant involved cell wall modifications which resulted in increased antimicrobial resistance. Metabolism was not greatly affected by the addition of epinephrine.

The *luxS* mutation resulted in numerous metabolic changes compared to WT. Sixty-seven of the phenotypes lost involved the inability to use carbon, nitrogen, phosphate, and sulfur sources that the WT strain could utilize for growth. The *luxS* mutant lost the ability to use 42 nitrogen sources, suggesting that nitrogen metabolism is significantly altered by the *luxS* mutation. However, the altered nitrogen metabolism does not seem to affect AI-3 dependent phenotypes, as increased nitrogen levels did not restore AI-3-dependent transcription of *ler* in the *luxS* mutant (Fig. 21B). The results from the PM array suggest that the *luxS* mutation drastically alters the metabolism of the cell. These growth phenotype assays revealed that although the *luxS* mutant is able to grow at the same rate as WT in laboratory medium, it exhibits a variety of defects when grown in minimal media with select compounds available to the cell. This work helps to establish that the *luxS* mutation not only results in the loss of AI-2 production, but also significantly alters the metabolism of the cell.

In *E. coli*, AI-2 has only been shown to regulate the expression of the *lsr* operon that controls uptake of AI-2 (230). To try to further understand the function of AI-2, we assessed the effects of adding enzymatically synthesized AI-2 to the *luxS* mutant and examining the consequences using PM's. The addition of AI-2 resulted in 62 phenotypes being gained when compared to the *luxS* mutant with no AI-2 present. The majority of the phenotypes gained involved the ability to use different carbon, nitrogen, and phosphate sources. It is unclear if the ability to use these compounds is a result of AI-2 being metabolized or a product of AI-2 signaling. AI-2 addition also resulted in several phenotypes being lost when

compared to the *luxS* mutant. These phenotypes may represent metabolic pathways that were active in the *luxS* mutant and are no longer required when AI-2 is present. In addition, three of these phenotypes lost by the addition of AI-2 were phenotypes gained by the *luxS* mutant when compared to WT. All three phenotypes involved antibiotic resistance, with the addition of AI-2 making the *luxS* mutant more sensitive. The addition of AI-2 will activate the Lsr ABC transporter which imports AI-2 into the cell. One possible explanation for the increased antibiotic sensitivity is that the expression of the Lsr ABC transporter may disrupt the expression of other efflux pumps in the cell that normally remove the antibiotics from inside the cell or the Lsr transporter may bring antibiotics into the cell.

In summary, our results suggest that the *luxS* mutation affects the production of AI-3 by altering cellular metabolism. The *luxS* mutation leaves the cell with only one pathway to produce homocysteine, which is required for *de novo* synthesis of methionine. Exclusive use of this pathway may change metabolism and alter amino acid levels in the cell, possibly leading to reduced tyrosine levels and decreased AI-3 production based on the assumption that epinephrine and AI-3 share similar structures and synthesis pathways. The PM array studies revealed that the *luxS* mutation alters many metabolic aspects of the cell and that addition of AI-2 to the media can affect different growth phenotypes, either by signaling or being metabolized. The work presented here further distinguishes the role of AI-3 signaling from that of AI-2 signaling and begins to explore how the *luxS* mutation affects AI-3 production.

CHAPTER SIX

CONCLUSIONS AND FUTURE DIRECTIONS

EHEC is a worldwide pathogen responsible for producing hemorrhagic colitis and HUS in infected individuals. Although EHEC infections are not among the most frequently reported diseases, the statistics likely do not represent the true incidence of the disease. Many of the reported cases involve patients who have developed clearly identifiable symptoms of EHEC infections, such as profuse diarrhea with visible blood. Less severe cases may go unreported because the patient is less likely to seek medical attention. The limited therapy and danger of chronic renal sequelae highlight the risk this pathogen poses for public health. As a result, a great deal research has focused on understanding the mechanisms of virulence gene regulation in EHEC. Accordingly, the underlying objective of this dissertation was to examine signals involved in activation of EHEC pathogenic factors.

Based on previous data from our laboratory, we sought to examine the effects of the *luxS* mutation on the kinetics of LEE gene expression in EHEC, as well as begin to unravel the pathways involved in the production of AI-3, a signal responsible for EHEC virulence gene activation. It was first reported seven years ago that the expression of the LEE-encoded TTSS was regulated by QS and that mutation of *luxS* resulted in lower LEE expression (186). Initially, it was thought that the AI-2 QS signal produced by *luxS* was responsible for the observed effects. However, purified AI-2 was unable to restore LEE expression, while pre-conditioned media could. These results suggested that another signal may be produced by

EHEC and is responsible for activation of its virulence genes. It was not until 2003 that the activating signal was purified from culture supernatants and named AI-3 (188).

Previous studies concerning LEE transcription had been performed using a LEE promoter linked to a reporter gene on the *E. coli* K-12 chromosome. These results revealed that AI-3 regulation of the LEE occurred maximally during late-exponential growth phase in this system (186, 188). One of the main focuses of this dissertation was to examine the kinetics of LEE expression in an EHEC background (Chapter 4). To this end, we extracted RNA from WT, *luxS* mutant, and *luxS* complemented cultures during early-, mid-, and late-exponential growth phases. Real-time RT-PCR was then used to measure the amount of gene transcripts present from each of the five LEE operons. It is important to note that the *luxS* mutant allows us to study the relationship of LEE activation and AI-3 production *in vitro* because of the strain's inability to produce AI-3 in quantities sufficient for LEE activation.

The real-time RT-PCR studies revealed that the *luxS* mutation decreased transcription of the LEE promoters during mid-exponential growth, in contrast to the late-exponential activation of the LEE promoters observed in the *E. coli* K-12 background. These results demonstrate the importance of examining LEE transcription in the native EHEC background. The most likely explanation for the differences in LEE transcription observed between the two systems is the number of EHEC-specific LEE regulators (Fig. 8). During late-exponential growth, there was not a significant difference in LEE gene transcription between the WT and *luxS* mutant, suggesting that other AI-3 independent factors regulate the LEE during these growth phases. Future studies in LEE gene regulation could attempt to identify

factors important for LEE gene expression during late-exponential and stationary growth. One possible way to elucidate factors important for LEE expression during stationary phase growth may be to perform transposon mutagenesis of EHEC strains containing a chromosomal reporter gene fused to the different LEE promoters. The resulting mutants could then be screened and selected for clones with normal LEE expression during early- and mid-exponential growth, but have altered LEE expression during stationary growth phase. RpoS, a stationary-phase sigma factor, appears to regulate expression of the *LEE3* and *LEE5* operons and has been identified as being important for the transcription of *LEE3* (186). The precise factors responsible for regulation of the remaining LEE operons during stationary growth remain unknown. Further work in the LEE regulation during late-exponential and stationary growth may elucidate mechanisms by which EHEC is able to switch from a period of high LEE expression (observed in mid- to late- exponential phase growth) to a period of relatively low LEE gene expression (observed in stationary phase growth).

We also examined expression of LEE-encoded proteins that comprise the three major portions of the TTSS and compared the expression of proteins to the observed gene transcription during early-, mid-, and late-exponential growth. It was found that expression of all three proteins was diminished in the *luxS* mutant during mid-exponenital growth, the same growth period when the *luxS* mutant had decreased LEE transcription. When we examined the expression of EspA, a portion of the extracellular TTSS, we observed that the *luxS* mutant produced less protein than WT during all three growth phases, even though transcription of *espA* was only significantly reduced during mid-exponential growth. In addition, the *luxS* complemented strain exhibited protein expression similar to WT during

early-exponential growth, despite the large increases in the transcription of genes within *LEE2, LEE5*, and *LEE4* observed at this growth phase. The mechanisms responsible for these observed patterns remain unclear.

The WCL protein expression studies reveal that the *luxS* mutant does produce less LEE-encoded proteins during mid-exponential growth. The fact that the transcriptional and protein expression data do not always match, suggest that there may be posttranscriptional regulation of the expression of these genes. Posttranscriptional regulation of another TTSS protein YopQ of *Yersinia enterocolitica* has been previously described (8), and it has been reported that secretion of EspA is controlled at the posttranscriptional level, although the mechanism behind this regulation is unknown (158). Our results suggest that it is likely that posttranscriptional regulation involved in LEE expression. Posttranscriptional mechanisms usually involve intracellular signals that interact with an RNA element in the 5' untranslated region (UTR). These signals are typically a protein, a metabolite, or small regulatory non-coding RNAs that lead to posttranscriptional regulation of genes. The promoter sequences for the genes in question could be examined for the presence of predicted UTRs *in silico*. If any UTRs are predicted, the sequence could be mutated and the effects on the transcription of the gene and expression of the protein could be performed. More than 50 sRNAs have been identified in *E. coli* and the majority of them use the Hfq RNA chaperone (69). An *hfq* knockout could be constructed and changes in the transcription and expression of the LEE may reveal if sRNAs are important in LEE regulation. Stability of the mRNA may also play a role in the expression of these genes and explain the difference observed between transcription and protein levels. The half-lives of the various mRNAs could be measured by

a pulse-chase experiment in which the bacteria are grown in the presence or [^{3}H]uridine and then chased with unlabelled uridine and cytidine. One final explanation of the disparity between gene transcription and protein expression may be that translation of effector proteins may be coupled with translocation through the TTSS, similar to what has been described for TTSS and production of flagella in *Salmonella* (96).

The addition of epinephrine to EHEC cultures increased expression of the LEE in both WT and the *luxS* mutant (Chapter 4). However, WT consistently had significantly higher levels of LEE transcription during mid-exponential growth and greater protein secretion in response to epinephrine (compared to the *luxS* mutant). Our previous data suggested that AI-3 transcriptional regulation occurred to the greatest extent during mid-exponential growth, the same growth phase that WT had elevated LEE transcription in response to epinephrine. It appears that both the AI-3 and the epinephrine signals are able to work in a coordinated fashion to activate LEE transcription and protein secretion. The work described in this dissertation demonstrates a synergistic relationship between AI-3 and epinephrine for the first time. Future work will be needed to demonstrate that these two signals are in fact working cooperatively to activate the LEE and if this signaling occurs through the same receptor, as suggested by the fact the adrenergic receptor antagonists block the effects of both AI-3 and epinephrine (188). Addition of AI-3 and epinephrine in varying amounts and measuring LEE transcription would further characterize this relationship. At this time, purification of AI-3 from culture supernatants yields very little AI-3 making these assays difficult to perform. Solving the structure of AI-3 will be paramount to these studies. Once a structure is known, it may be possible to chemically synthesize AI-3 and generate

sufficient quantities to perform stoichiometric studies of AI-3 / epinephrine activation of the LEE.

The lower LEE activity in the *luxS* mutant ultimately leads to reduced TTSS phenotypes. The mutant secretes less protein *in vitro*, suggesting that the TTSS is not as active. When TTSS activity is examined in the presence of cultured epithelial cells, there was a delay in the ability of the *luxS* mutant to form AE lesions. This is the first time a phenotype differing from WT has been described using this assay. Previous studies examined AE lesion formation only during late-exponential growth, the growth phase that AI-3 appeared to be most active using the *E. coli* K-12 reporter system. While the *luxS* mutant is still able to form AE lesions on epithelial cells, its inability to produce AI-3 appears to result in a delay when compared to strains capable of AI-3 synthesis. It is important to note that the tissue culture medium (DMEM + 5% FBS) used in these assays contained approximately 36 μM epinephrine. The *luxS* mutant is able to respond to this signal *in vitro* and it is likely that the epinephrine influences the formation of AE lesions. Future experiments examining the formation of AE lesions by the WT and the *luxS* mutant could be performed with ITS synthetic culture media (Invitrogen), which does not contain epinephrine. This media may uncover a more profound delay or the inability of the *luxS* mutant to form AE lesions.

It is apparent that many factors are involved in LEE regulation and expression throughout the different growth phases. Future studies aimed at dissecting which regulators are active during specific growth phases would facilitate a better understanding of LEE expression. These studies may include the use of an EHEC transposon mutant library. The

library could be screened during the different growth phases for mutants that display altered LEE regulation. This method may allow new regulators to be identified and at what stage of growth they are active.

The other half of this dissertation focused on understanding the mechanism through which the *luxS* mutation disrupts AI-3 production (Chapter 5). These studies suggest that mutation of *luxS* greatly alters the metabolism of the cell. This was reflected in the PM analysis of WT and the *luxS* mutant. The *luxS* mutant lost the ability to utilize numerous metabolic sources compared to WT. The *luxS* mutant does not display any growth defects when grown in normal laboratory medium, such as LB broth. However, the PM studies used a minimal media that revealed the many growth defects caused by mutation of *luxS*. Many phenotypes in various bacterial species have been attributed to AI-2 through the use of *luxS* mutants. The results from the PM array study indicate that the *luxS* mutation disrupts many metabolic pathways in the cell, suggesting that some phenotypes previously attributed to the lack of AI-2 may need to be re-evaluated with an emphasis on the metabolic effects of the *luxS* mutation.

The results from Chapter 5 indicate that it is the metabolic effects of the *luxS* mutation, not AI-2 production, which leads to diminished LEE expression as a result of decreased AI-3 production. The *luxS* mutation leaves the cell with only one pathway to produce homocysteine (Figure 16). Homocysteine is important for the *de novo* synthesis of methionine in the cell. Since the *luxS* mutant can only produce homocysteine from one pathway involving the use of oxaloacetate and L-glutamate, the use of this pathway may be increased to compensate for the loss of the LuxS pathway. We were able to restore

expression of the LEE in the *luxS* mutant by complementing homocysteine production. The addition of an aspartate dipeptide restored transcription of *ler* and secretion of EspA and EspB through the TTSS. Aspartate is the first product in the oxaloacetate pathway for homocysteine production and is not present in the media used for our virulence assays, requiring the cell to synthesize aspartate. When aspartate is present in the medium, the need to synthesize this amino acid will be reduced. This allows the AspC and TryB transaminases, which are involved in aspartate synthesis, to synthesize other amino acids, such as tyrosine. Production of homocysteine from the LuxS pathway was also restored by expressing *P. aeruginosa sahH* in the *luxS* mutant. SahH converts the toxic intermediate SAH into adenosine and homocysteine. Expression of SahH in the *luxS* mutant restored the transcription of *ler* to greater than WT levels. Given the similarities between AI-3 and epinephrine / norepinephrine LEE activation and that tyrosine is required for the synthesis of epinephrine / norepinephrine, we hypothesized that tyrosine may be important for AI-3 synthesis. Previous data from a microarray study (187) revealed the *aroP* was downregulated in the *luxS* mutant. AroP is important for transporting aromatic amino acids, such as tyrosine, into the cell. After confirming the *aroP* microarray data, we expressed *aroP* from a plasmid in the *luxS* mutant and restored AI-3 production as measured by transcription of *ler*. The overexpression of the tyrosine-specific TyrP transporter in the *luxS* mutant also restored *ler* transcription to greater than WT levels. The increased availability of tyrosine in the cell may have provided a key amino acid for the synthesis of AI-3 and resulted in greater AI-3 production. These studies also helped to distinguish the role of AI-3 from that of AI-2 in LEE activation. The addition of aspartate dipeptides, expression of

sahH, and complementation with *aroP* were all able to restore AI-3, but not AI-2, production in the *luxS* mutant.

These data suggest that homocysteine and tyrosine are important for AI-3 production. Future experiments could examine the effects of the addition of homocysteine on LEE transcription. Homocysteine is toxic to *E. coli* (157) and a concentration not inhibitory to growth, but still able to reduce the need of the cell to synthesize homocysteine would need to be used. Additionally, future experiments could address the affects of tyrosine dipeptides on AI-3 production. If tyrosine is important for the synthesis of AI-3, then providing more tyrosine may increase AI-3 production. Tyrosine dipeptides are insoluble in water. It would be important to find a solvent that does not affect the cell or to use a dipeptide made up of one tyrosine and another amino acid that is soluble in water. Both homocysteine and tyrosine levels in the medium would have to be monitored to ensure that these compounds are being imported into the bacterial cells. Most likely, the structure of AI-3 will need to be determined before the exact pathway leading to its synthesis can be elucidated.

It was found that many pathogenic and commensal bacterial isolates produce both AI-2 and AI-3. These data, as well as previously published data demonstrating that fecal filtrates from volunteers contain AI-3 (188), suggest that there is abundant AI-2 and AI-3 in the human intestine. The PM studies indicate that AI-2 affects the metabolism of EHEC. At this time, it is unknown whether AI-2 is being directly metabolized or if it signals through unknown pathways that allow the bacteria to utilize different metabolic sources. The more obvious conclusion is that AI-2 is imported back into the cell and used as an energy source. AI-2 contains a ribose sugar background that may provide energy for the cell. Indeed,

Winzer *et al*. (226) have speculated that AI-2 may be toxic to the cells at high levels, such as during exponential phase growth. However, they propose that during later stages of bacterial growth, smaller amounts of AI-2 are imported into the cell and metabolized. This would create a metabolically favorable condition. AI-2 may play dual roles in that it is able to serve as a signaling molecule and provide a nutrient source for bacteria during stationary phase growth when nutrients become limited. The addition of epinephrine to either WT or the *luxS* mutant resulted in very few phenotypic differences, suggesting that AI-3 and epinephrine / norepinephrine do not play an important role in metabolism of the cell. AI-2 does not seem to play a role in the regulation of EHEC virulence genes, while AI-3 and epinephrine / norepinephrine play a significant role in the activation of the LEE and motility genes. The combined effects of AI-3 produced by the normal intestinal flora, as well as host epinephrine / norepinephrine, may lead to greater EHEC virulence gene activation. The synergistic effects between these signaling compounds leads to greater LEE transcription and increased TTSS activity, and may allow for a more efficient infection than responding to one signal alone.

In conclusion, the *luxS* mutation indirectly affects the production of AI-3 and alters the kinetics of LEE expression in EHEC. We were able to demonstrate that AI-3 plays an important role in the regulation of EHEC LEE expression during mid-exponential growth (Chapter 4). We were able to monitor AI-3 regulation directly in the native EHEC background using real-time RT-PCR and the *luxS* mutant. Furthermore, our studies were the first to demonstrate a synergistic relationship between AI-3 and epinephrine. When the two signals are present together, they lead to greater LEE expression and function. Ultimately,

the *luxS* mutation resulted in a delay in AE lesion formation on cultured epithelial cells. The normal metabolism of EHEC is interrupted by the *luxS* mutation. This metabolic disruption leads to decreased AI-3 production in the *luxS* mutant. Complementation of pathways leading to homocysteine production, by either addition of aspartate dipeptides or expression of *sahH*, restored the AI-3 dependent transcription of *ler* and increased TTSS activity in the *luxS* mutant (Chapter 5). It was also found that tyrosine appears to be important in the synthesis of AI-3, similar to epinephrine / norepinephrine synthesis. Increasing the intracellular concentration of tyrosine resulted in increased transcription of *ler* in the *luxS* mutant. PM studies revealed that the *luxS* mutation greatly alters the metabolism of the cell and demonstrated that AI-2 affects metabolism, although the exact mechanism is unknown. Through the use of transcriptional and expression assays, as well as global phenotypic studies, we have achieved a comprehensive analysis of the effects of the *luxS* mutation on LEE expression, AI-3 production, and cell metabolism.

APPENDIX A

Phenotype MicroArray: *luxS* mutant vs. WT

Test	Difference	Mode of Action
5,7-Dichloro-8-Hydroxyquinoline	130	chelator, lipophilic
Orphenadrine	150	cholinergic antagonist
Promethazine	184	cyclic nucleotide phosphodiesterase
Acriflavine	153	DNA intercalator
Phleomycin	135	DNA polymerase
Oxolinic acid	147	DNA topoisomerase
Nalidixic Acid	110	DNA topoisomerase
Lomefloxacin	197	DNA topoisomerase, quinolone
Ofloxacin	192	DNA topoisomerase, quinolone
Enoxacin	140	DNA topoisomerase, quinolone
Trimethoprim	114	folate antagonist, dihyldrofolate reductase
Cetylpyridinium Chloride	143	membrane, detergent, cationic
Lauryl sulfobetaine	91	membrane, detergent, zwitterionic
Amitriptyline	151	membrane, transport
Puromycin	162	protein synthesis
Cinoxacin	146	protein synthesis
Lincomycin	107	protein synthesis
Josamycin	196	protein synthesis, macrolide
Oleandomycin	143	protein synthesis, macrolide
Spiramycin	140	protein synthesis, macrolide
Chlortetracycline	185	protein synthesis, tetracycline
Penimepicycline	181	protein synthesis, tetracycline
Demeclocyline	174	protein synthesis, tetracycline
Rolitetracycline	169	protein synthesis, tetracycline
Tetracycline	157	protein synthesis, tetracycline
Doxycycline	154	protein synthesis, tetracycline
Cefuroxime	170	wall, cephalosporin
Cefoxitin	168	wall, cephalosporin
Cefamandole	155	wall, cephalosporin
Cetoperazone	137	wall, cephalosporin
Cefotaxime	108	wall, cephalosporin
Ampicillin	194	wall, lactam
Penicillin G	191	wall, lactam

Test	Difference	Mode of Action
Piperacillin	174	wall, lactam
Phenethicillin	159	wall, lactam
Nafcillin	158	wall, lactam
Cloxacillin	148	wall, lactam
Oxacillin	127	wall, lactam
Carbenicillin	119	wall, lactam
Amoxicillin	109	wall, lactam
Tylosin	89	protein synthesis
Dequalinium Chloride	87	ion channel, K+
Dodecyltrimethyl Ammonium Bromide	79	membrane, detergent, cationic
Chloramphenicol	74	protein synthesis
Erythromycin	74	protein synthesis, macrolide
Ethionamide	-132	anti-tuberculosic
D,L-a-Glycerol- Phosphate	-72	C-source
D-Lactic Acid Methyl Ester	-79	C-source
m-Tartaric Acid	-86	C-source
L-Malic Acid	-87	C-source
D,L-Malic Acid	-88	C-source
Bromo-Succinic Acid	-89	C-source
D-Fructose	-89	C-source
D-Xylose	-89	C-source
D-Mannose	-98	C-source
D-Melibiose	-100	C-source
Fumaric Acid	-108	C-source
N-Acetyl-Neuraminic Acid	-115	C-source
N-Acetyl-D-Galactosamine	-120	C-source
Dulcitol	-125	C-source
Mucic Acid	-174	C-source
Sulfadiazine	-76	folate antagonist
p-Cresol	-123	membrane
Procaine	-100	membrane, anaesthetic
Met-b-Ala	-70	N-source
b-Ala-Ala	-73	N-source
Met-Leu	-73	N-source
Leu-Glu	-74	N-source
Gly-Leu	-75	N-source
Gly-Lys	-75	N-source
Ile-Met	-75	N-source
Arg-Met	-76	N-source
Asp-Leu	-76	N-source
Trp-Asp	-76	N-source
Leu-Asp	-77	N-source

Test	Difference	Mode of Action
L-Ornithine	-78	N-source
Trp-Lys	-78	N-source
Leu-Tyr	-80	N-source
Lys-Phe	-82	N-source
Gly-Cys	-83	N-source
Leu-Gly-Gly	-85	N-source
L-Cysteine	-87	N-source
Trp-Phe	-88	N-source
Phe-Met	-89	N-source
Lys-Val	-90	N-source
Leu-Gly	-91	N-source
Trp-Trp	-92	N-source
Ile-Trp	-94	N-source
Lys-Gly	-94	N-source
His-Leu	-97	N-source
Leu-Met	-98	N-source
Adenine	-99	N-source
Trp-Tyr	-101	N-source
Leu-Leu	-103	N-source
Leu-Leu-Leu	-104	N-source
Tyr-Leu	-104	N-source
Leu-His	-107	N-source
Trp-Val	-107	N-source
Ile-Leu	-111	N-source
Leu-Trp	-114	N-source
Gly-Gly-Leu	-117	N-source
Leu-Phe	-122	N-source
Trp-Leu	-122	N-source
His-Asp	-133	N-source
Val-Lys	-135	N-source
D-Serine	-136	N-source
L-Cysteine	-98	nutrient stimulation
N-Acetyl D-Glucosamine	-105	nutrient stimulation
Adenosine	-107	nutrient stimulation
Glutathione (reduced form)	-110	nutrient stimulation
Inosine	-111	nutrient stimulation
Nicotinamide	-112	nutrient stimulation
Thymine	-115	nutrient stimulation
(5) 4-Amino-Imidazole-4(5)-Carboxamide	-117	nutrient stimulation
Adenine	-117	nutrient stimulation
D-(+)-Glucose	-117	nutrient stimulation
Hypoxanthine	-117	nutrient stimulation

Test	Difference	Mode of Action
Inosine + Thiamine	-118	nutrient stimulation
D-Glutamic Acid	-119	nutrient stimulation
Folic Acid	-119	nutrient stimulation
Hematin	-119	nutrient stimulation
Nicotinic Acid	-119	nutrient stimulation
b-Nicotinamide Adenine Dinucleotide	-120	nutrient stimulation
D-Pantothenic Acid	-120	nutrient stimulation
Pyrrolo-Quinoline Quinone	-120	nutrient stimulation
b-Alanine	-121	nutrient stimulation
D-Alanine	-121	nutrient stimulation
Guanosine-3',5'-Cyclic Monophosphate	-121	nutrient stimulation
2`-Deoxy-Inosine	-122	nutrient stimulation
D,L-Diamino-Pimelic Acid	-122	nutrient stimulation
L-Tryptophan	-122	nutrient stimulation
Pyridoxine	-122	nutrient stimulation
Thiamine	-122	nutrient stimulation
Deferoxamine Mesylate	-123	nutrient stimulation
Pyridoxal	-123	nutrient stimulation
Pyridoxamine	-123	nutrient stimulation
Thiamine Pyrophosphate	-123	nutrient stimulation
D-Aspartate	-124	nutrient stimulation
Guanine	-124	nutrient stimulation
Spermidine	-124	nutrient stimulation
Spermine	-124	nutrient stimulation
Uridine	-124	nutrient stimulation
Chorismic Acid	-125	nutrient stimulation
Shikimic Acid (-)	-125	nutrient stimulation
Cytidine	-126	nutrient stimulation
d-Amino-Levulinic Acid	-126	nutrient stimulation
L-Citrulline	-126	nutrient stimulation
L-Methionine	-126	nutrient stimulation
p-Amino-Benzoic Acid	-126	nutrient stimulation
Riboflavin	-126	nutrient stimulation
Uracil	-126	nutrient stimulation
Cyano-Cobalamine	-127	nutrient stimulation
Cytosine	-127	nutrient stimulation
L-Glutamic Acid	-127	nutrient stimulation
L-Isoleucine + L-Valine	-127	nutrient stimulation
Menadione	-128	nutrient stimulation
m-Inositol	-128	nutrient stimulation
4-Hydroxy L-Proline (trans)	-129	nutrient stimulation

Test	Difference	Mode of Action
Adenosine-3',5'-Cyclic Monophosphate	-129	nutrient stimulation
D-Biotin	-129	nutrient stimulation
L-Arginine	-129	nutrient stimulation
L-Serine	-129	nutrient stimulation
Glycine	-130	nutrient stimulation
L-Histidine	-130	nutrient stimulation
L-Homoserine Lactone	-130	nutrient stimulation
L-Phenylalanine	-130	nutrient stimulation
Thymidine	-130	nutrient stimulation
Choline	-131	nutrient stimulation
Guanosine	-131	nutrient stimulation
L-Leucine	-131	nutrient stimulation
L-Lysine	-131	nutrient stimulation
L-Valine	-131	nutrient stimulation
2`-Deoxy-Adenosine	-132	nutrient stimulation
L-Asparagine	-132	nutrient stimulation
D,L-Mevalonic Acid	-133	nutrient stimulation
L-Aspartate	-133	nutrient stimulation
D,L-a-Lipoic Acid (oxidized form)	-134	nutrient stimulation
L-Alanine	-134	nutrient stimulation
L-Threonine	-134	nutrient stimulation
L-Tyrosine	-134	nutrient stimulation
L-Proline	-135	nutrient stimulation
D,L-Carnitine	-136	nutrient stimulation
2`-Deoxy-Guanosine	-137	nutrient stimulation
L-Isoleucine	-137	nutrient stimulation
Quinolinic Acid	-137	nutrient stimulation
2`-Deoxy-Uridine	-138	nutrient stimulation
Oxaloacetic Acid	-139	nutrient stimulation
Caprylic Acid	-140	nutrient stimulation
Putrescine	-140	nutrient stimulation
2`-Deoxy-Cytidine	-142	nutrient stimulation
a-Keto-Butyric Acid	-142	nutrient stimulation
L-Glutamine	-142	nutrient stimulation
Tween 40	-142	nutrient stimulation
Tween 20	-143	nutrient stimulation
L-Ornithine	-144	nutrient stimulation
Tween 60	-144	nutrient stimulation
Tween 80	-145	nutrient stimulation
Negative Control	-146	nutrient stimulation
Butyric Acid	-148	nutrient stimulation

Test	Difference	Mode of Action
D,L-a-Hydroxy-Butyric Acid	-149	nutrient stimulation
Plumbagin	-84	oxidizing agent
pH 9.5 + Agmatine	-71	pH, deaminase
pH 9.5 + L-Lysine	-152	pH, deaminase
Geneticin (G418)	-121	protein synthesis, aminoglycoside
D-3-Phospho-Glyceric Acid	-75	P-source
Thymidine- 5'-Monophosphate	-94	P-source
Thiophosphate	-119	P-source
2-Deoxy-D-Glucose 6-Phosphate	-125	P-source
Dithiophosphate	-132	P-source
Lanthionine	-97	S-source
L-Cysteinyl-Glycine	-100	S-source
Cystathionine	-107	S-source
Hypotaurine	-112	S-source
L-Djenkolic Acid	-133	S-source
Sodium Tungstate	-108	transport, toxic anion, molybdate analog
Chromium Chloride	-80	transport, toxic cation
Aluminum Sulfate	-115	transport, toxic cation

APPENDIX B

Phenotype MicroArray: *luxS* mutant + AI-2 vs. *luxS* mutant

Test	Difference	Mode of Action
Mucic Acid*	119	C-source
Dulcitol*	89	C-source
Capric Acid	83	C-source
m-Inositol	66	C-source
L-Rhamnose	59	C-source
Gelatin	58	C-source
L-Lyxose	58	C-source
L-Glutamic Acid	56	C-source
N-Acetyl-b-D-Mannosamine	56	C-source
Formic Acid	53	C-source
Oxolinic acid	181	DNA topoisomerase
L-Cysteine	272	N-source
Lys-Phe*	103	N-source
Lys-Val*	97	N-source
Gly-Cys*	91	N-source
Trp-Val*	86	N-source
Leu-Met*	85	N-source
Ile-Trp*	81	N-source
Cys-Gly	76	N-source
Lys-Gly*	75	N-source
Leu-Glu*	74	N-source
Val-Lys*	73	N-source
Gly-Gly-Leu*	71	N-source
Ile-Met*	69	N-source
Leu-Asp*	69	N-source
L-Tryptophan	63	N-source
Arg-Met*	62	N-source
L-Glutamic Acid	62	N-source
Asp-Leu*	61	N-source
His-Asp*	61	N-source
Lys-Met	60	N-source
Gly-Leu*	59	N-source
Phe-Met*	58	N-source
L-Alanine*	55	N-source
Trp-Arg	54	N-source
Ala-Lys	53	N-source

Test	Difference	Mode of Action
Leu-Gly*	51	N-source
Chlorpromazine	129	phenothiazine
Vancomycin	65	protein synthesis
Methylene Diphosphonic Acid	188	P-source
Adenosine- 3',5'-Cyclic Monophosphate	184	P-source
Negative Control	184	P-source
Triethyl Phosphate	177	P-source
Phosphono Acetic Acid	175	P-source
Thymidine 3',5'- Cyclic Monophosphate	175	P-source
Uridine- 3',5'- Cyclic Monophosphate	162	P-source
Guanosine- 3',5'-Cyclic Monophosphate	157	P-source
Cytidine- 3',5'-Cyclic Monophosphate	155	P-source
2-Deoxy-D-Glucose 6-Phosphate*	125	P-source
Hypophosphite	121	P-source
Inositol Hexaphosphate	88	P-source
Guanosine- 3'-Monophosphate	65	P-source
Uridine- 3'- Monophosphate	64	P-source
Uridine- 5'- Monophosphate	61	P-source
Uridine- 2',3'- Cyclic Monophosphate	56	P-source
Rifampicin	83	RNA polymerase
Cefuroxime	102	wall, cephalosporin
Cefmetazole	67	wall, cephalosporin
Ampicillin	186	wall, lactam
Azlocillin	156	wall, lactam
Ketoprofen	-41	anti-capsule, anti-inflammatory
Phleomycin**	-180	DNA polymerase
Ofloxacin**	-87	DNA topoisomerase, quinolone
His-His	-80	N-source
Plumbagin	-67	oxidizing agent
Methyl viologen	-84	oxidizing agent
Fusidic Acid	-92	protein synthesis
Ruthenium red	-89	respiration, mitochondrial Ca++ porter
Menadione	-85	respiration, uncoupler
Taurine	-54	S-source
D-Methionine	-62	S-source
L-Methionine Sulfoxide	-64	S-source

Test	Difference	Mode of Action
Glycyl-L-Methionine	-65	S-source
N-Acetyl-D,L-Methionine	-74	S-source
L-Methionine	-83	S-source
Lanthionine	-112	S-source
Cefoxitin**	-125	wall, cephalosporin

* - phenotypes lost by *luxS* mutation (Table 1) and gained back by addition of AI-2

** - phenotypes gained by *luxS* mutation (Table 1) and lost by addition of AI-2

APPENDIX C

Phenotype MicroArray: WT + epi. vs. WT

Test	Difference	Mode of Action
8-Hydroxyquinoline	297	chelator; lipophilic
Tyr-Tyr	71	N-source
Phe-Pro	62	N-source
Tyr-Trp	61	N-source
Troleandomycin	300	protein synthesis; macrolide
Penimepicycline	79	protein synthesis; tetracycline
Rifamycin SV	123	RNA polymerase
Ciprofloxacin	-118	DNA topoisomerase; quinolone
Dichlofluanid	-98	fungicide; phenylsulphamide
Poly-L-Lysine	-32	membrane; detergent; cationic
Trp-Leu	-111	N-source

APPENDIX D

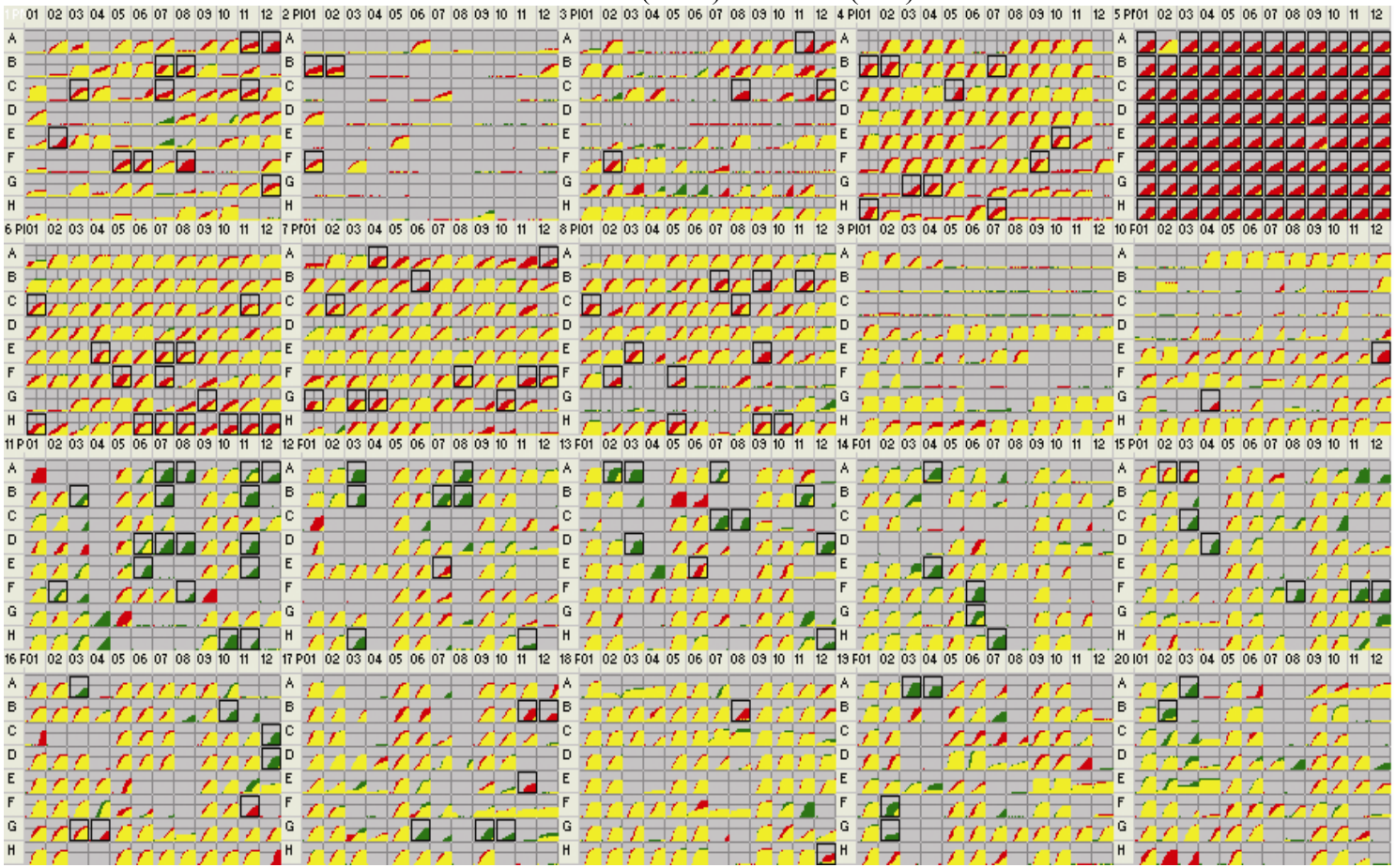
Phenotype MicroArray: *luxS* mutant + epi. vs. *luxS* mutant

Test	Difference	Mode of Action
Cefuroxime	204	wall; cephalosporin
Ampicillin	215	wall; lactam
Azlocillin	208	wall; lactam
Monalactam	204	wall; lactam

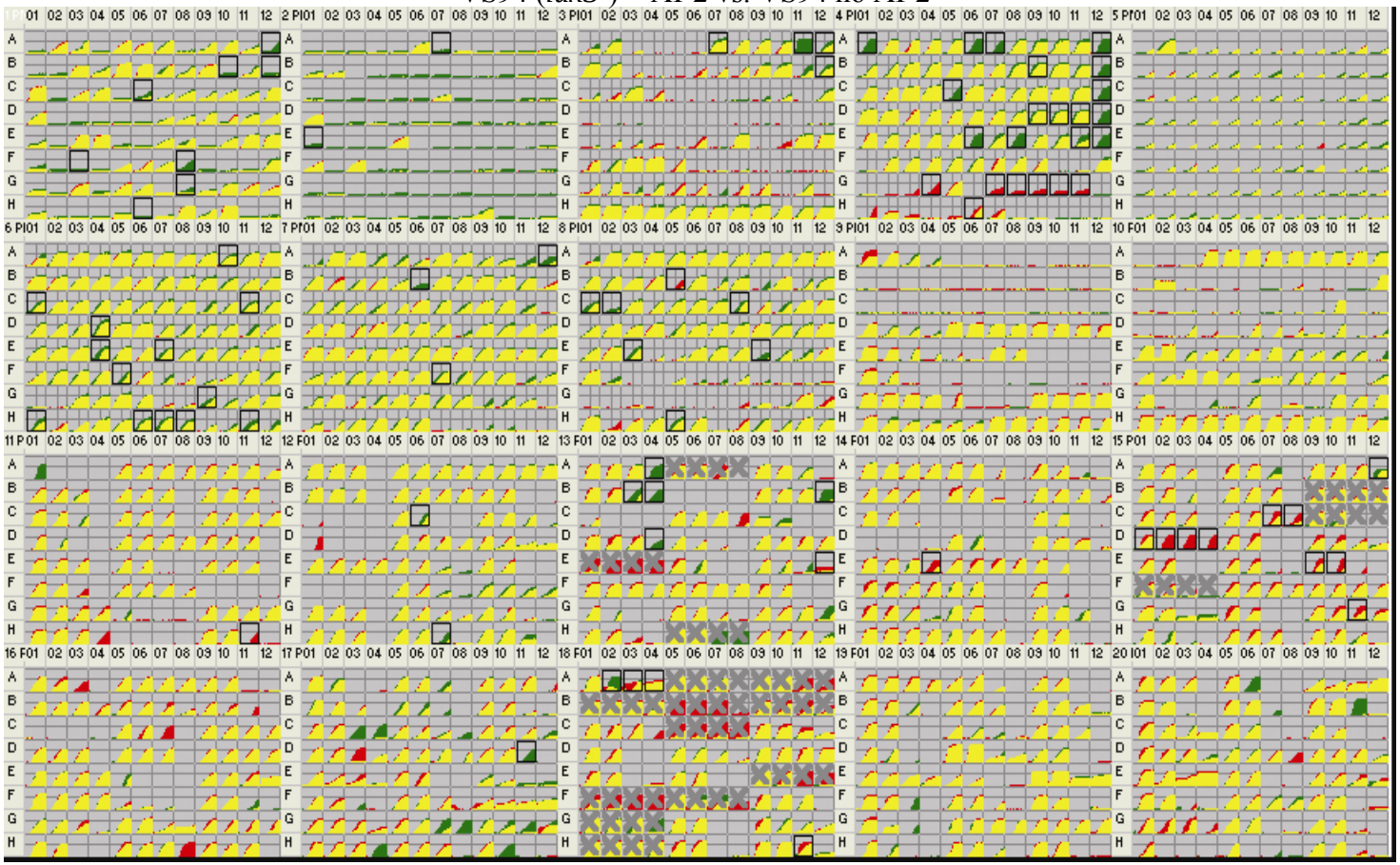
APPENDIX E

Phenotypic changes in Phenotype MicroArray assays

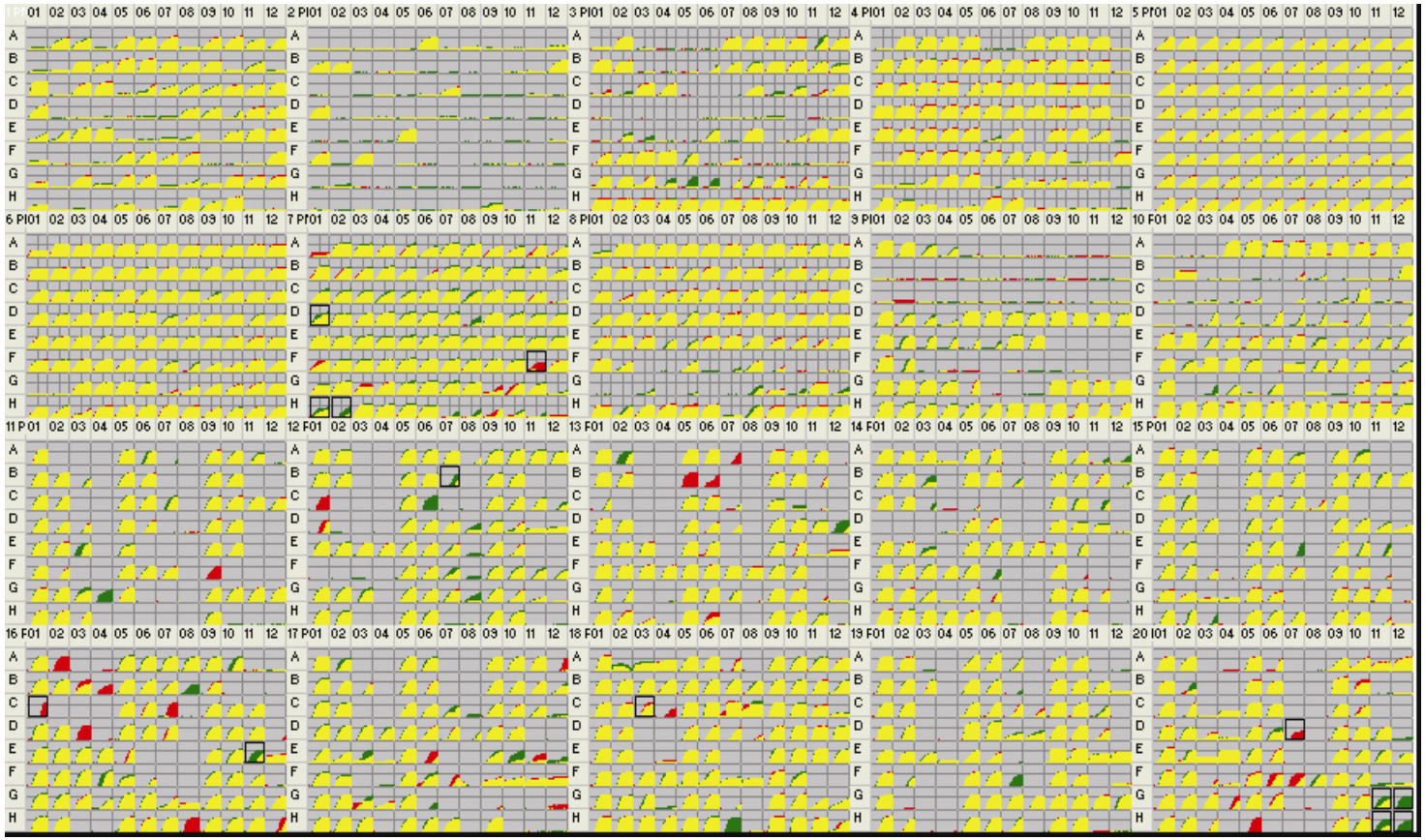
VS94 (*luxS*-) vs. 86-24 (WT)



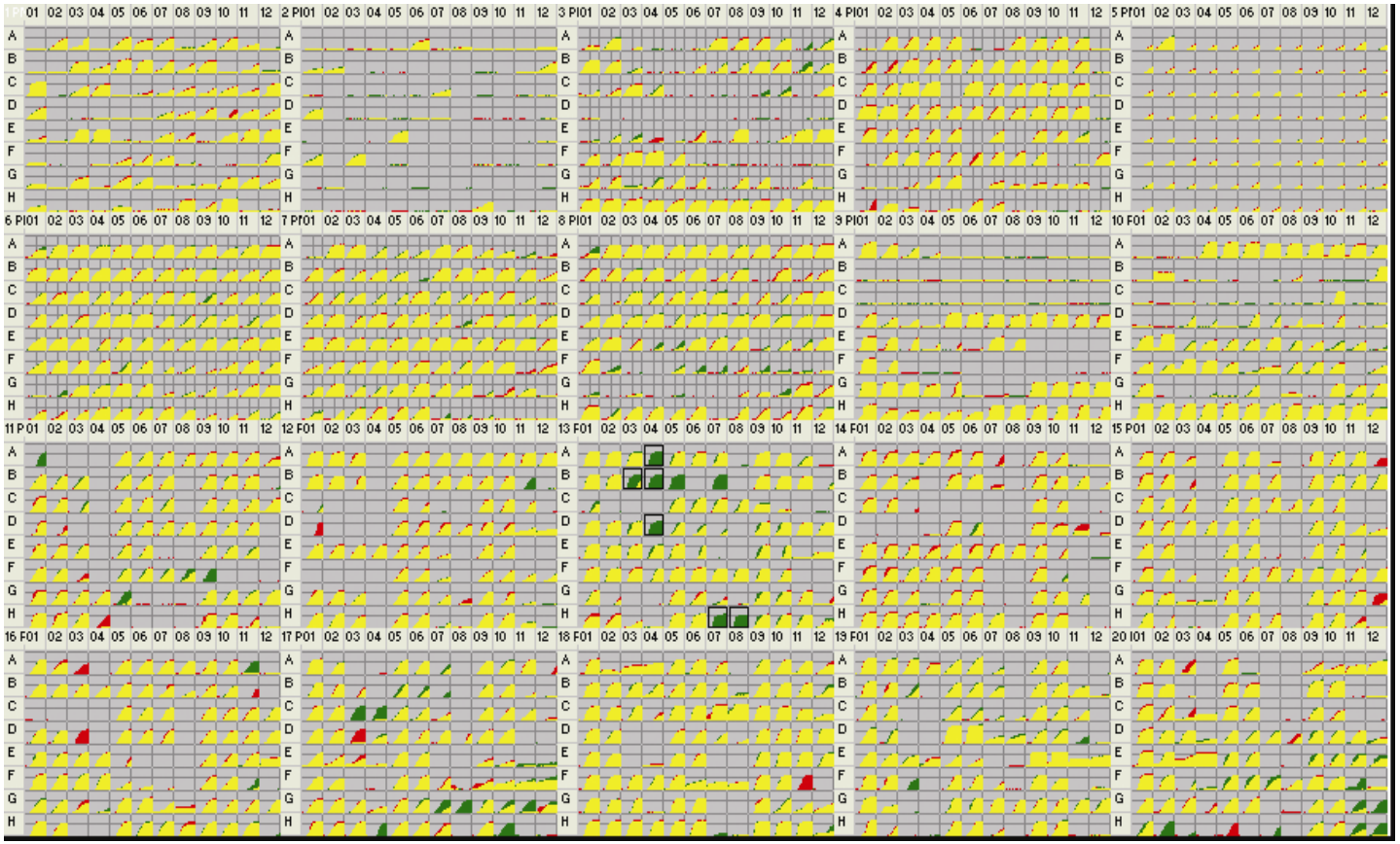
VS94 (*luxS*-) + AI-2 vs. VS94 no AI-2



86-24 (WT) + epi vs. 86-24 no epi



VS94 (*luxS*-) + epi vs. VS94 no epi

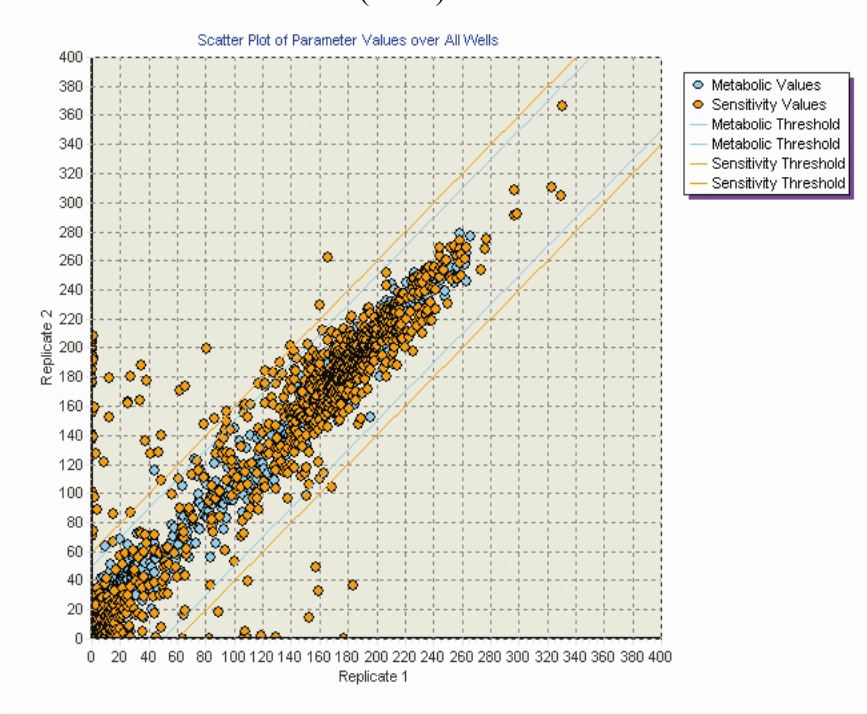


APPENDIX F

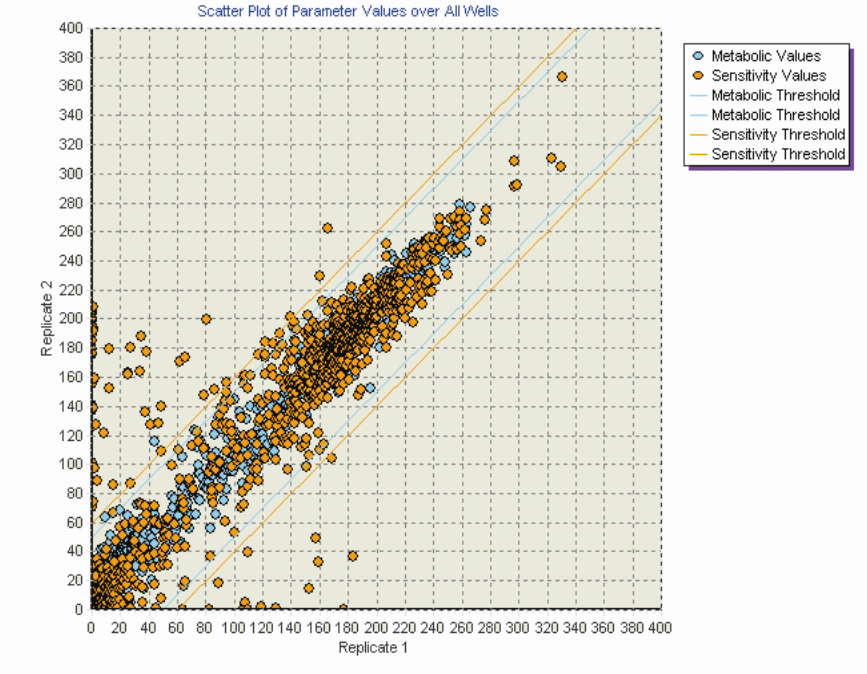
Correlation between Phenotype MicroArray replicates

PM 1: *luxS* mutant vs. WT

VS94 (*luxS*-)

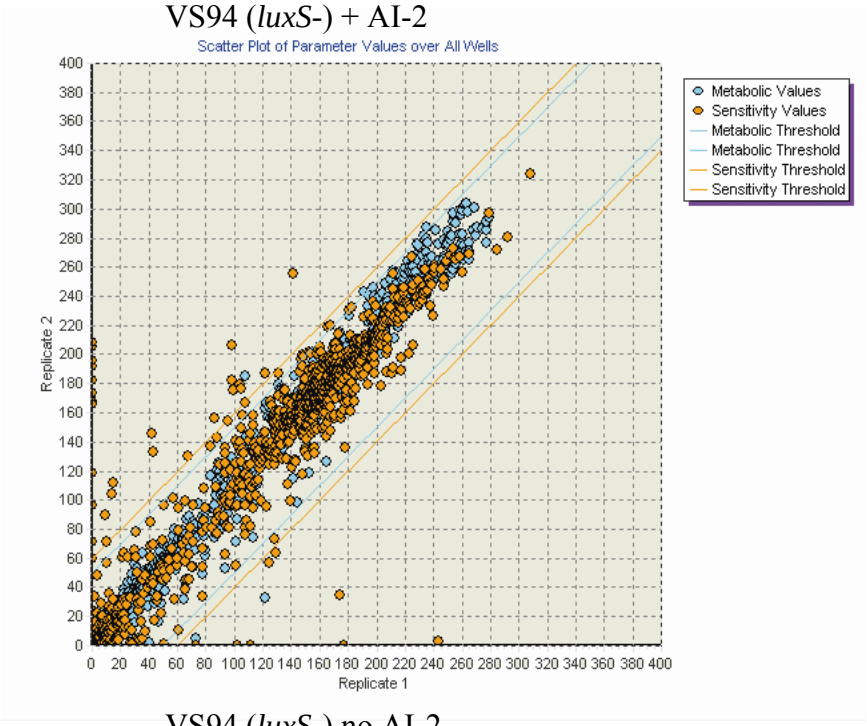


86-24 (WT)

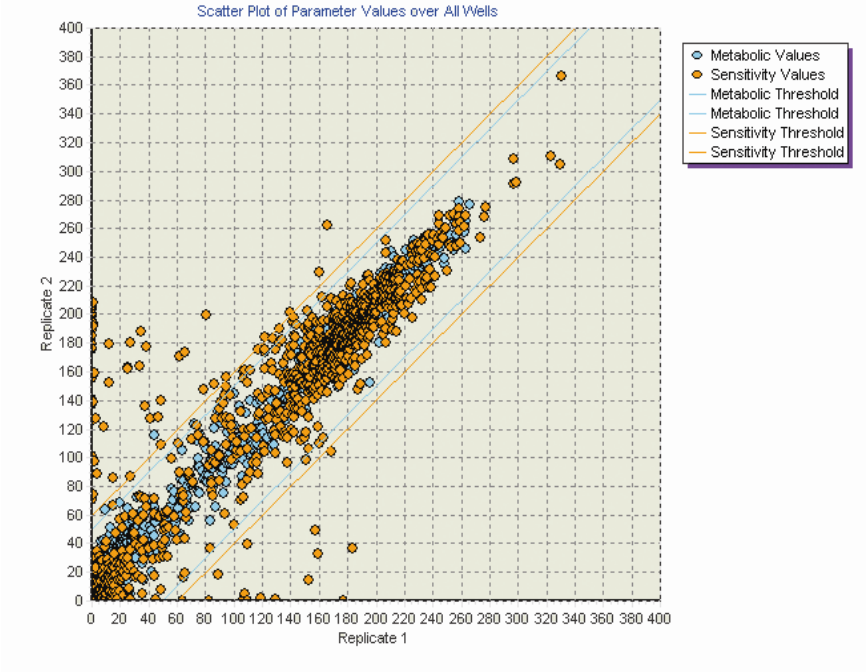


PM 2: *luxS* mutant + AI-2 vs. *luxS* mutant

VS94 (*luxS*-) + AI-2

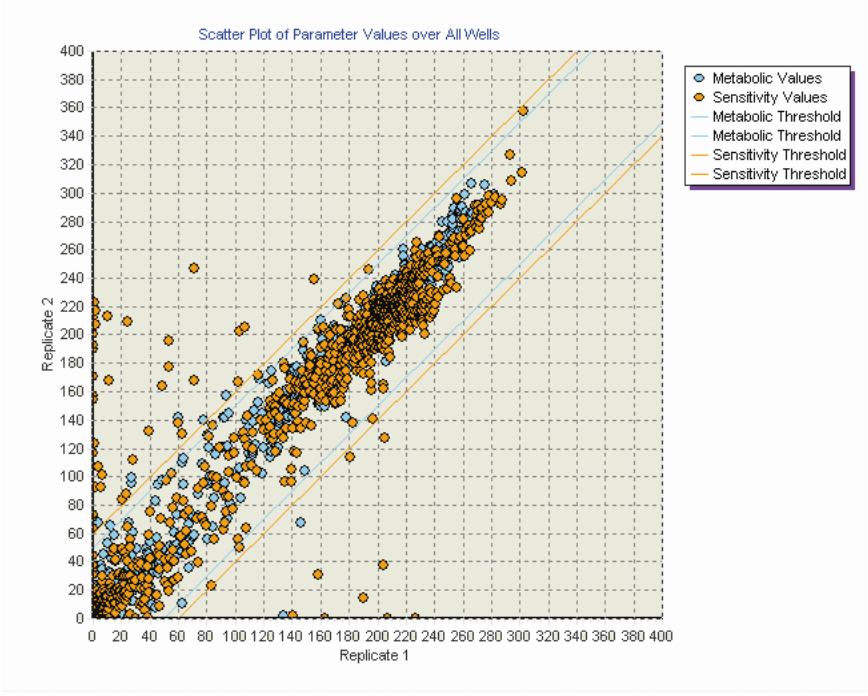


VS94 (*luxS*-) no AI-2

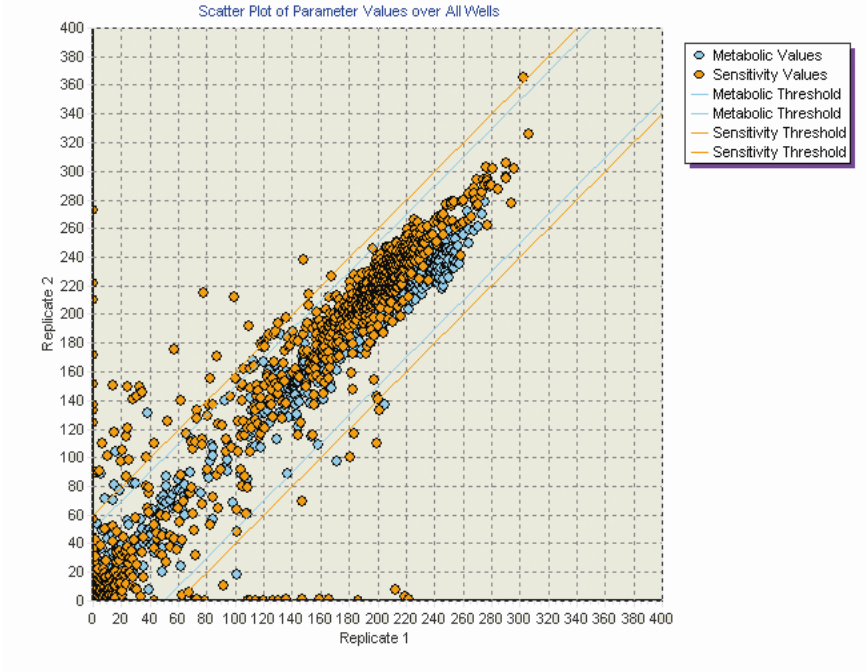


PM 3: WT + epi. vs. WT

86-24 (WT) + epi

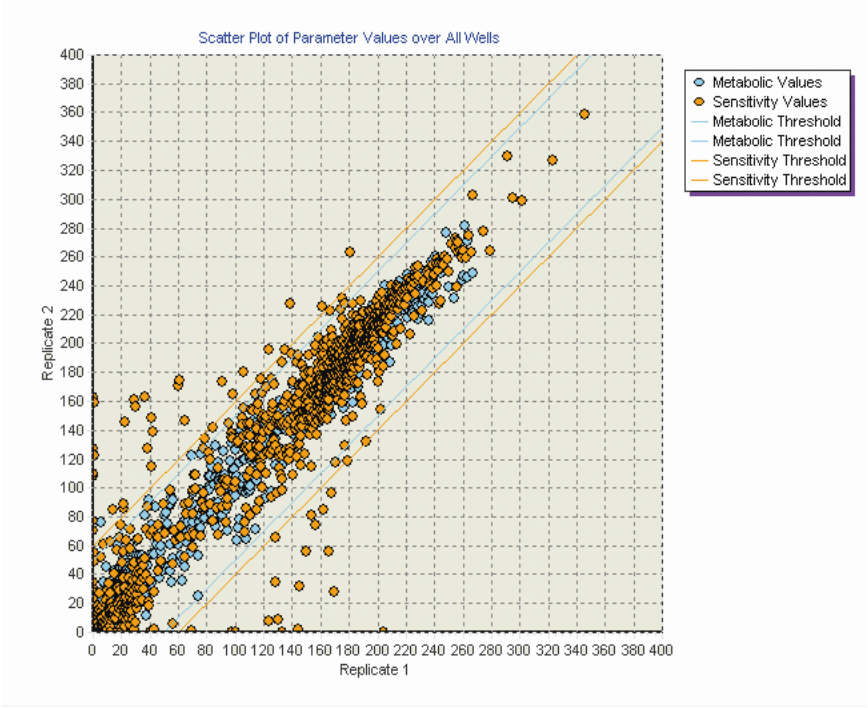


86-24 (WT) no epi

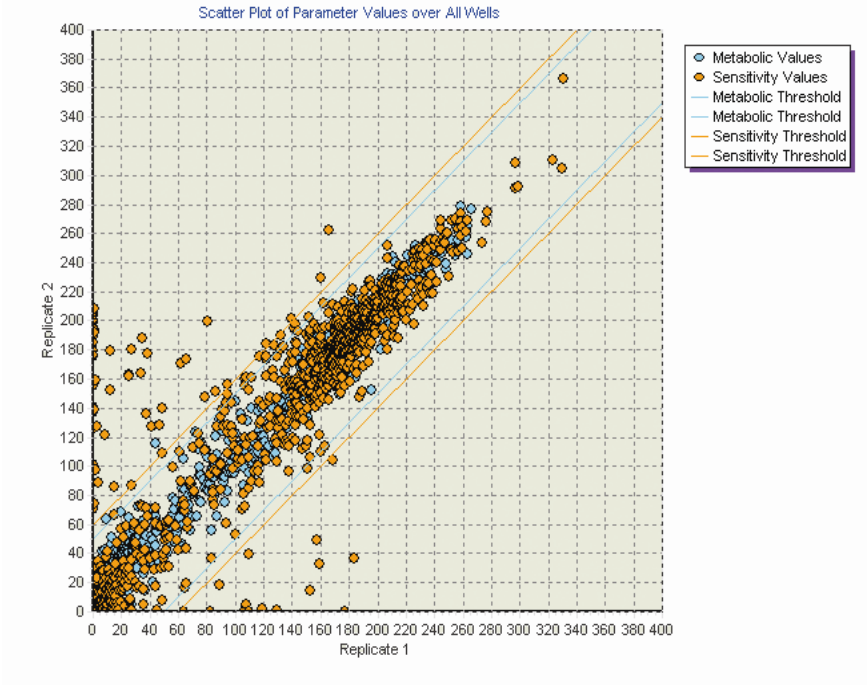


PM 4: *luxS* mutant + epi. vs. *luxS* mutant

VS94 (*luxS*-) + epi



VS94 (*luxS*-) no epi



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VITAE

Matthew Steven Walters was born in Kearney, Nebraska on December 24, 1978, the son of Kathryn Ann Morrison Walters and Steven Mark Walters. Matthew was a member of the National Honor Society and graduated in the top 10% of his class from Kearney Senior High in Kearney, Nebraska in 1997. He was admitted to The University of Nebraska, Kearney in August, 1997 on a full academic scholarship. As an undergraduate, Matthew was awarded two grants as a principal researcher and presented his research during the annual meeting of the Society for Neuroscience in 2000. He was awarded the Great Plains States Society for Molecular Biology and Genetics Merit Award for his poster presentation. During his undergraduate studies, Matthew was also a Certified Master Tutor in Biology and Chemistry. In May 2001, Matthew received his Bachelor of Science degree in Biology with an emphasis in Molecular Biology. In August of 2001, he entered the Graduate School of Biomedical Sciences at The University of Texas Southwestern Medical Center at Dallas where he joined the Molecular Microbiology program. During his time at UT Southwestern, he has been awarded the Outstanding Journal Club Presentation Award, a travel grant from the American Society of Microbiology (ASM) for the 2005 Meeting on Beneficial Microbes, and the NIH Doctoral Training Fellowship Award. Additionaly, he presented his work at several ASM meetings and at the 2005 Molecular Mechanisms of Microbial Adhesion Gordon conference. He was conferred the degree of Doctor of Philosophy in June, 2006. He will be joining the lab of Harry Mobley, Ph.D. at the University of Michigan as a post-doctoral fellow in July, 2006. In June, 2002 he married Heather Marie Juel Walters.

Permanent Address: 3919 Avenue K
Kearney, NE 68847