THE CHARACTERIZATION OF THE FUCOSE SENSING KINASE (FUSK)

AND THE FUCOSE SENSING RESPONSE REGULATOR (FUSR)

AND THEIR ROLE IN VIRULENCE REGULATION

IN ENTEROHEMORRHAGIC *ESCHERICHIA COLI* O157:H7

APPROVED BY SUPERVISORY COMMITTEE

Vanessa Sperandio, Ph.D.

Neal Alto, Ph.D.

Kevin Gardner, Ph.D.

Michael Shiloh, Ph.D.

DEDICATION

I would like to thank my mentor Vanessa Sperandio, the members of my Graduate Committee, my husband Tiago Ramos, my parents Odette and Paulo Pacheco, my sisters Sandra, Renata and Rosana, my nephews Leonardo and Joao Paulo, my nieces Mareska, Rafaelly and Isabella, my aunt Lurdes and my dear longtime friend Mrs Benta. Special thanks for Mr. A., M., P.O. and Miss E.

THE CHARACTERIZATION OF THE FUCOSE SENSING KINASE (FUSK) AND THE FUCOSE SENSING RESPONSE REGULATOR (FUSR) AND THEIR ROLE IN VIRULENCE REGULATION IN ENTEROHEMORRHAGIC *ESCHERICHIA COLI* O157:H7

by

ALLINE ROBERTA PACHECO

DISSERTATION / THESIS

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

July, 2012,

THE CHARACTERIZATION OF THE FUCOSE SENSING KINASE (FUSK) AND THE FUCOSE SENSING RESPONSE REGULATOR (FUSR) AND THEIR ROLE IN VIRULENCE REGULATION IN ENTEROHEMORRHAGIC *ESCHERICHIA COLI* O157:H7

Alline Roberta Pacheco, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2012

Supervising Professor: Vanessa Sperandio, Ph.D.

EHEC causes outbreaks of bloody diarrhea worldwide, by colonizing the human large intestine, where it forms attaching and effacing (AE) lesions on the intestinal epithelium. AE lesion development requires the presence of the locus of enterocyte effacement (LEE) that encodes for a molecular syringe, a type three secretion system (T3SS), which translocates effectors to the host cell. Expression of the LEE is controlled by the AI-3/Epi/NE interkingdom signaling cascade. The

two-component systems QseBC and QseEF are at the core of the AI-3/Epi/NE signaling, controlling expression of flagellar motility genes, the LEE and type 3 secreted effectors in response to AI-3 and the catecholamine hormones Epi and NE. The network of regulatory proteins that form the AI-3/Epi/NE continues to expand, as shown by recent studies from our laboratory. Microarray analyses indicate that a putative two-component system (TCS), herein named FusKR, is repressed by QseBC and QseEF. FusK is the histidine kinase and FusR is the response regulator.

In this work, we started to unravel the role of FusKR in EHEC pathogenicity. We constructed isogenic knockouts of *fusK* and *fusR*, and investigated their participation in virulence gene regulation in EHEC. Microarray analysis shows that deletion of *fusK* and *fusR* alters transcription of virulence and metabolic genes. Phenotypic analyses show that *fusK*- and *fusR*- strains are hypervirulent *in vitro*, overexpress the LEE genes and produces higher amounts of the T3 secreted protein EspB. Nonetheless, the *fusK* mutant is attenuated for colonization of the mammalian intestine. Biochemical studies revealed that FusK senses fucose. Fucose is an important carbon source for commensal and pathogenic bacteria during intestinal colonization. Transcriptional analyses shows that FusKR signal transduction system regulates fucose utilization indirectly, through regulation of the predicted membrane transporter Z0461, that participates in optimal fucose uptake. Gut commensal *Bacteroides thetaiomicron* (*B.theta*)

degrades mucin, releasing free monosaccharides, including fucose, into the gut lumen. Co-culture of *B.theta* and EHEC on mucin indicates that this commensal supplies mucin-derived fucose to EHEC, reducing expression of the LEE. Our studies demonstrate that a novel TCS, FusKR, modulates intestinal colonization by EHEC, and it is involved in complex interactions with the microbiota during infection.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Pacheco A.R., Ritchie, J.M. Waldor, M.K., Curtis, M.M., Moreira, C.G., Sperandio V. Fucose sensing by a novel two-component system regulates bacterial intestinal colonization. *Nature*, under revision.

Pacheco, A.R. and Sperandio, V. Shiga Toxin in Enterohemorrhagic *E.coli*: regulation and novel antivirulence strategies. *Frontiers in Cellular and Infection Microbiology*, *in press.*

Pace, F., Nakazato, G., **Pacheco, A.,** Paiva, J.B., Sperandio, V., Silveira, W.D. The type VI secretion system contributes to pathogenesis of an avian pathogenic *Escherichia coli* (APEC) strain. 2010. *Infection and Immunity*. 78: 4990-4998.

Pacheco, A.R. and Sperandio, V. Interkingdom signaling: chemical language between bacteria and host. 2009. *Current Opinion in Microbiology* 2: 192-198.

Pacheco, A.R., Proença-Módena, J.L., Sales, A.I., Fukuhara, Y., da Silveira, W.D., Pimenta Módena, J.L., de Oliveira , R.B., Brocchi, M. Involvement of the *Helicobacter pylori* plasticity region and *cag* pathogenicity island genes in the development of gastroduodenal diseases. 2008. *European Journal of Clinical Microbiology and Infectious Diseases*. 27: 1053-1059.

LIST OF FIGURES

LIST OF TABLES

LIST OF DEFINITIONS

EHEC WT	Enterohemorragic *E.coli* wild-type strain 86-24
AE	Attaching and effacing lesion
AHL	Acyl homoserine lactone
AI	Autoinducer
AI-2	Autoinducer-2
AI-3	Autoinducer-3
ATP	adenosine triphosphate
bp	basepair
nt	nucleotide
CDC	Centers for Disease Control and Prevention
CFU	Colony-forming units
CNS	Central nervous system
DAEC	Diffusely adherent *E.col*i
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DPD	AI-2 precursor, 4,5-dihydroxi-2,3-pentanedione
E.coli	*Escherichia coli*
EAEC	Enteroaggregative *E.coli*
EDTA	Ethylenediamine tetraacetic acid
EHEC	Enterohemorragic *E.coli*
EIEC	Enteroinvasive *E.coli*
EMSA	Electrophoretic mobility shift assay
ENS	Enteric nervous system
EPEC	Enteropathogenic *E.coli*

Esp	*E.coli* secreted protein
ETEC	Enterotoxigenic *E.coli*
FAS	Fluorescent Actin Staining
FBS	fetal bovine serum
FITC	Fluorescein isothiocyanate
g	Gram
G	Gravity
Gb3	Globotriaosylceramide
gDNA	genomic DNA
GI	Gastrointestinal
h	Hour
HGT	Horizontal gene transfer
His	Histidine
HUS	Hemolytic Uremic Syndrome
IL	Interleukin
IPTG	Isopropyl-β-d-thiogalactopyranoside
kDa	kiloDalton
LB	Luria-Bertani
LEE	Locus of Enterocyte Effacement
ler	LEE-encoded regulator
LPS	Lipopolysaccharide
M	Molar
Map	Mitochondrial-associated protein
MG1655	*E.coli* K-12
mL	Milliliter
Nle	Non-LEE-encoded

N-WASP	Neuronal Wiskott-Aldrich Syndrome Protein
OD	Optical density
PAI	pathogenicity island
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PE	Phentolamine
PMSF	phenylmethylsulfonyl fluoride
QS	quorum sensing
qse	quorum sensing *E.coli* regulator
qRT-PCR	quantitative real time polymerase chain reaction
rpm	revolutions per minute
RT-PCR	Reverse-transcriptase PCR
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOC	super optimun catabolite medium
sRNAs	small RNAs
Stx	Shiga toxin
Tir	Translocated intimin receptor
TRITC	tetramethyl rhodamine isothiocyanate
T3SS	type 3 secretion system
UPEC	Uropathogenic *E.coli*
WCL	whole cell lysates
WT	Wild-type

X-gal	5-bromo-4-chloro-3-indolyl β-galactopyranoside
uL	microliter

CHAPTER ONE

LITERATURE REVIEW OF EHEC

Classification of *Escherichia coli*

E.coli was isolated from a child's fecal sample by the Austrian physician Theodor Escherich in the late nineteenth century, when it was described as *Bacterium coli* commune (312).

E.coli is a Gram-negative bacterium from the Enterobacteraceae family, which inhabits the intestine of healthy humans of all ages, where it establishes a lifelong commensal relationship with the host (Figure 1). The colonization site of *E.coli* is the mucus layer that overlays the intestinal epithelium. Colonization by *E.coli* occurs early in life, starting only a few hours after birth, probably from maternal origin; in fact, *E.coli* is among the first bacterial species to colonize the infant gut, before the proliferation of dominating anaerobes (24, 186, 226). The successful mutually beneficial relationship between *E.coli* and the human host is highlighted by the fact that *E.coli* is the predominant facultative anaerobe in the gut (134). *E.coli* does not cause harm to the immunocompetent host if it stays in its native colonization site, where it benefits from the continuous nutrient supply from the host, as well as stable environment. In turn, commensal *E.coli* provides a number of benefits to the mammalian host, such as protection against colonization

by pathogenic bacteria ("colonization resistance"), production of vitamin K, increased gut barrier and boost of intestinal immunity (151, 170, 288). Since it was first identified, *E.coli* has been isolated from the GI tract of mammalian (humans, monkeys, dogs, cats, horses, cows, mice, pigs, sheeps, rabbits) and non-mammalian (birds, turkey) hosts. Moreover, *E.coli* is used as an indicator of fecal pollution of the environment, as it can be isolated occasionally from water and soil (48, 53, 121, 199, 201).

E.coli is also well known as a classic model system in molecular biology, and it has contributed to development of the following fields: genetics, biochemistry, microbiology and molecular biology. The *E.coli* K-12 strain was isolated from stool of convalescent diphtheria patient in 1922 and is considered the workhorse of molecular biology for decades (312).

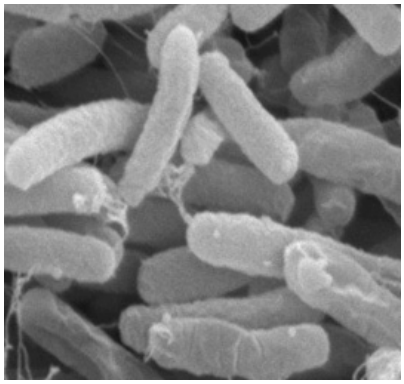

Figure 1.1. Electron micrograph of *E. coli*. Scanning E.M. Shirley Owens. Center for Electron Optics. Michigan State University.

In addition of being a beneficial bacterium in the human gut, there are pathogenic variants of *E.coli* that can cause a diverse range of intestinal and extra-intestinal diseases. These pathogenic variants have acquired virulence traits, commonly by mechanisms of horizontal gene transfer of mobile genetic elements, which allowed them to adapt to new niches and to cause disease. There are 6 major pathotypes of *E.coli* that cause diseases in humans: Enteropathogenic *E.coli* (EPEC), Enterohemorrhagic *E.coli* (EHEC), Enterotoxigenic *E.coli* (ETEC), Enteroaggregative *E.coli* EAEC, Enteroinvasive *E.coli* (EIEC) and Diffuse-Adherent *E.coli* (DAEC) (Figure 1.2). All these strains cause diarrhea by employing different strategies to adhere and to interact with host cells, subverting their normal function (Figure 1.2). EPEC forms biofilms and AE lesion. EHEC forms AE lesion and produces Stx, leading to the fatal sequela HUS. ETEC produces enterotoxins ST and LT. EAEC forms biofilms and produces toxins. EIEC is invasive of colonic enterocytes. DAEC induces long projections in the eukaryotic cell leading to bacterial engulfment (134). However, the strains uropathogenic *E.coli* (UPEC) and meningitis-associated *E.coli* (MNEC) cause extra-intestinal diseases; UPEC that causes urinary tract infections, and MNEC causes meningitis and sepsis (134, 202).

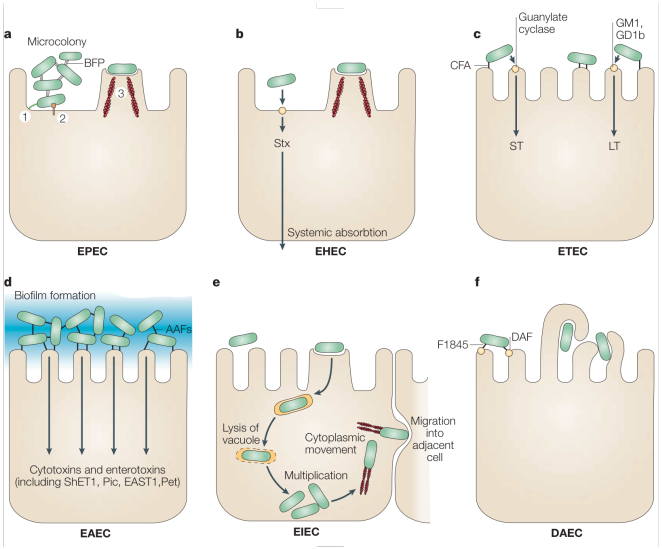

Figure 1.2. Pathogenic *E.coli* strains. EPEC, EHEC, ETEC, EAEC, EIEC and DAEC all cause diarrea, albeit by distinct mechanisms. EPEC is the causative agent of infantile diarrhea in developing countries; it form biofilms and AE lesion. EHEC causes bloody diarrea and produces Stx, leading to fatal sequela HUS. ETEC is the agent of "traveler' diarrea", by producing enterotoxins ST and LT. EAEC forms biofilms and produces toxins. EIEC is invasive of colonic enterocytes. DAEC induces long projections in the eukaryotic cell leading to bacterial engulfment. Source: (134).

Genomic Comparison of EHEC and *E.coli*: pathogenicity islands

DNA sequencing has facilitated the comparison among *E.coli* strains and has shed light into the evolution of *E.coli*. Comparison of the genome sequences of EHEC O157:H7 EDL933 and *E.coli* K-12 MG1655 showed that these strains share a backbone of 4.1 megabases. *E.coli* MG1655 K-12 contains 528 genes not

found in EHEC O157:H7, organized in K-12 specific “K-islands”, while EHEC harbors 1,400 EHEC-specific genes organized into “O-islands” that comprise 26% of the EHEC genome . The O-islands encode for several known and putative virulence factors, and correspond to prophages and transposons inserted into the genome (Figure 1.3) (227).

Recently, a broad comparison of 17 genomic sequences including commensal (HS), pathogenic (EHEC, EPEC, UPEC, ETEC and EAEC) and laboratory strains (K-12 and W3110) of *E.coli* has yielded valuable insight into the evolution of *E.coli* pathogenesis and identification of pathovar-specific genes. A model was proposed which the genetic content of *E.coli* strains is organized in a “pangenome” structure. This model estimated a total of 13,000 genes, with only 2,200 being conserved among all strains. Interestingly, the mosaic nature of the genome of the commensal *E.coli* HS suggests that commensal strains could act as a genetic repository for virulence traits, which can be spread by horizontal gene transfer and contribute to a continuing evolution of pathogenic *E.coli* species (233).

Recent analysis of EHEC’s genetic composition shows that EHEC O157:H7 has a high degree of genomic plasticity and it continues to evolve, gaining and adapting new virulence traits (68, 133, 172).

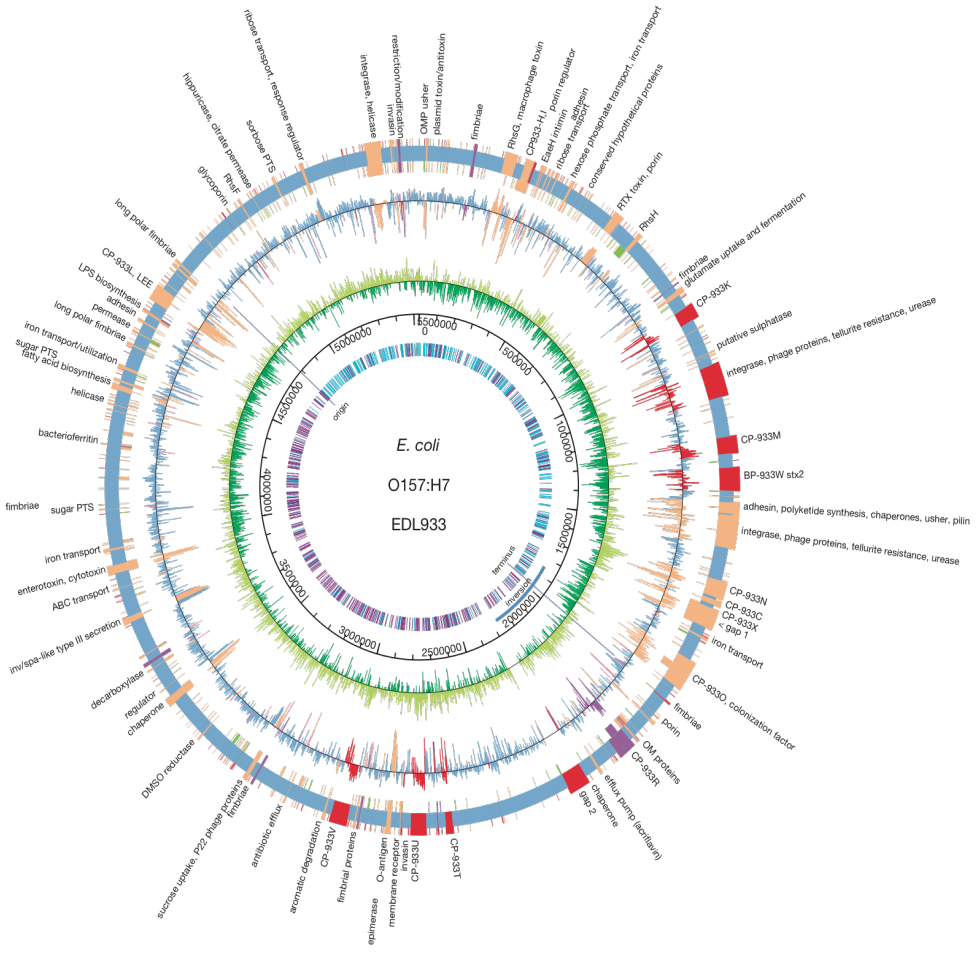

Figure 1.3. Genomic comparison of EHEC O157:H7 an *E.coli* K-12. The blue circle indicates the 4.1Mb of DNA shared by EHEC and *E.coli* K-12. Red boxes indicate EHEC OIs, green boxes indicate *E.coli* K- islands, orange boxes indicate K-12 and OIs occupying same position of genome. Source: (227).

Evolution of EHEC

E.coli strains have a clonal distribution, in which they can be organized in serogroups according to the O antigen of the lipopolysaccharide (LPS), or

serotypes, according to the O antigen combined to the flagellar (H) antigen. To date, 170 O antigens and 53 H antigens have been described (202, 308). EHEC O157:H7 is therefore a serotype of EHEC that is the leading cause of bloody diarrhea around the world. Genetic relationships among bacterial isolates is often determined by multilocus enzyme electrophoresis (MLEE), a method that compares electrophoretic mobility of housekeeping enzymes to determine enzyme polymorphism and consequently, genetic relatedness. Phylogenetic analysis based on MLEE profiles indicated genetic similarity between EHEC O157:H7 and EPEC O55:H7, the causative agent of infantile diarrhea, which is a non-toxigenic and less pathogenic *E.coli* strain, suggesting that both strains were derived from a common bacterial ancestor (316). Based on these evolutionary studies, it was proposed a model that suggests the emergence of EHEC O157:H7 from an EPEC O55:H7 precursor (71). The model states that EHEC evolved from an EPEC ancestor in a stepwise fashion, which involved a series of 4 consecutive events: (1) acquisition of the Shiga toxin 2 (*stx2*) bacteriophage; (2) acquisition of the plasmid pO157; (3) gain of the *stx1* bacteriophage; (4) then loss of the ability to ferment D-sorbitol and β-glucuronidase activity (GUD) (71, 317). MLST analyses indicate a parallel evolution of EHEC and EPEC, with acquisition of Stx and plasmid pO157 by EHEC and the EPEC adherence factor plasmid (pEAF) by EPEC. Further phylogenetic analysis of genetic contents of EHEC O157:H7 and EPEC O55:H7 corroborate the model. It was shown that 27 of the 33 genomic

islands specific to EHEC O157:H7, the O-islands, were also present in the EPEC O55:H7 (317).

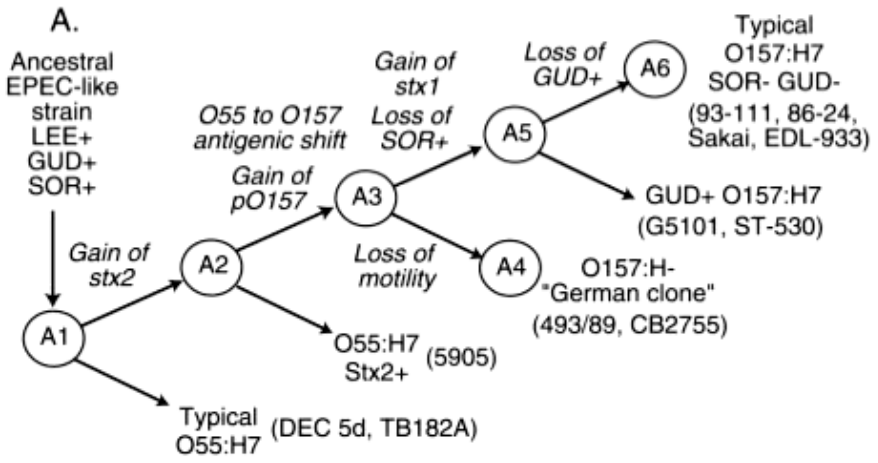

Figure 1.4. Evolution of EHEC O157:H7 from EPEC O55:H7 ancestor. Source: (71, 317).

The stepwise model of EHEC O157:H7 emergence from an EPEC O55:H7 ancestor predicts the existence of an intermediate clone unidentified or extinct. Recently, Jenke et al. claimed to have identified, via single-nucleotide polymorphisms (SNP) analyses, the elusive intermediate: a sorbitol-fermenting EHEC O157:H7 strain LSU-61 that colonizes deers (125). This findings complement the stepwise evolutionary model of EHEC (71, 317).

Phylogenetic analysis of EHEC O157 strains has revealed the existence of nine lineages or clades. Strains belonging to clade 8 are most frequently associated with hemolytic uremic syndrome (HUS) (172). Based on these

observations, it has been suggested that different lineages of EHEC O157 might differ in gene content or expression. In fact, genetic and phenotypic analyses of circulating outbreak clones have shown that highly virulent clones present higher number of Stx prophages or increased colonization *in vitro*. Transcriptional analyses comparing clade 8 and clade 2 strains showed increased expression of the LEE, particularly the type 3 secretion system (T3SS) proteins EspA and EspB, Stx2 and also plasmid-encoded virulence factors *stcE*, *toxB* and *hlyA*. Clade 8 strains also presented increased attachment ability compared to clade 2. These observations suggest that although gene content is conserved, evolution of increased gene expression leads to the emergence of more virulent strains and consequently, increased incidence of disease (2).

Epidemiology

EHEC O157:H7 is a deadly human pathogen that causes gastrointestinal (GI) infections worldwide. Annually in the United States, EHEC is responsible for an estimated 73,000 illnesses, 1800 - 3600 hospitalizations and from 61 - 541 deaths (231) (www.cdc.gov). EHEC is a foodborne pathogen, and most outbreaks (52%) occur through ingestion of contaminated food or water including: contaminated ground beef, steak, salami (20, 91, 124), dairy products (raw milk, cheese, butter, cookie dough) (122, 207) and vegetables (spinach, lettuce, sprouts) (89, 231, 265, 314). Moreover, EHEC infection can occur via other routes such as person-to-

person (14%) and animal to person (3%), the last usually resultant from contact with animals in petting zoos and farms (102, 231, 310). A remarkable feature of EHEC infection is the low infectious dose compared to other pathogenic *E.coli* species; it is estimated that 50 to 100 colony forming units (CFUs) of EHEC is sufficient to cause disease in healthy individuals (284).

Although EHEC is a human pathogen, this bacterium resides as a commensal in the GI tract of cattle (204, 252). EHEC presents tropism towards the rectum's lymphoid-follicle-dense mucosa or recto-anal junction (RAJ) of cattle, where it attaches forming the characteristic attaching and effacing (AE) lesion seen in other infected animals (204, 205). AE lesions are characterized by effacement of the microvilli and reorganization of the actin cytoskeleton of intestinal epithelial cells into a pedestal-like structure beneath the bacterium. Cattle can be transiently or long-term carriers of EHEC and this has major implications on disease transmission, given that fecal shedding from cattle represents an important source of EHEC contamination of foods (6, 49, 165). In fact, the first outbreaks of EHEC O157:H7 were linked to the consumption of contaminated ground beef, highlighting the importance of cattle as a reservoir (239, 313). Hence, many studies have focused on the eradication of EHEC from the GI tract of ruminants to reduce the infection rate of this enteric pathogen (243, 296). Several other animals such as sheeps, goats, pigs, buffalos, chickens and seagulls have also been pointed as EHEC reservoirs (153).

Diagnosis

EHEC O157:H7 expresses somatic antigen (O) 157 and flagellar antigen (H) 7, placing EHEC into specific serogroups O157 and H7. The display of these particular antigens allows serologic detection of EHEC by agglutination assays using specific antisera. In addition, a few metabolic features contribute to EHEC identification both in research and clinical laboratories: slow or inability to ferment D-sorbitol and lack of β-glucoronidase activity. These features can be visualized by plating fecal samples into Sorbitol MacConkey Agar supplemented with the β-glucuronidase artificial substrate MUG to help distinguish EHEC from other *E.coli* strains (283). The FDA recommends additional testing for confirmation of EHEC in foods, including screening for Stx production by ELISA and qRT-PCR (www.fda.gov).

EHEC and HUS

EHEC infection leads to a characteristic bloody diarrhea, which self-resolves. However, in approximately 5-7% of the cases, the patient develops a complication known as hemolytic uremic syndrome (HUS) (Figure 1.5) (212) (277) (136). HUS is the most serious sequelae of EHEC infection, and its incidence is higher in children and the elderly (28, 88, 218, 238, 277). The disease is characterized by

thrombocytopenia, hemolytic anemia and acute renal failure. In children infected by EHEC, HUS often develops soon after the onset of diarrhea (321). HUS is a life-threatening disease, contributing to a 5% mortality rate. The development of HUS is a direct result of the release of Shiga toxin (Stx), a very potent toxin encoded in a bacteriophage in the EHEC chromosome (134). Stx also contributes to the development of hemorrhagic colitis by lysing endothelial cells from the intestinal mucosa (277). Neurological manifestations of the central nervous system, including seizures and hemiplegia, have also been observed in EHEC patients (86, 87, 203).

Figure 1.5. Progress of EHEC infection. Modified from (277).

Stx is produced by the enteric pathogens *Shigella dysenteriae* serotype I and a collective group of *E. coli* strains called Stx-producing *E. coli* (STEC). Stx from *S. dysenteriae* serotype I was first identified by Kiyoshi Shiga (74). In 1983, it was first reported that EHEC O157:H7 could also produce Stx (214), providing the link between Stx production and the development of HUS (137). Initially, Stx was called Verotoxin due to its cytotoxicity against Vero cells in culture. Upon

the discovery that Verotoxin could be neutralized by an antitoxin against purified Stx from *Shigella*, the name Stx came into use and is the more common name to date (216). Stx and Verotoxin are used interchangeably, but for the purpose of this thesis, only Stx and the STEC strain EHEC will be discussed.

Pathogenesis of EHEC infection

EHEC implements two major virulence strategies: production of Stx and formation of AE lesions on enterocytes (134). EHEC contains a pathogenicity island termed the locus of enterocyte effacement (LEE), which is crucial for the development of AE lesions (176). The details of each virulence feature will be discussed in the following chapters.

Shiga Toxin

Stx inhibits protein synthesis and induces apoptosis. Stx is an AB_5 toxin, constituted by a catalytic subunit A bound non-covalently to a pentamer of B subunits (73, 274). The A subunit has N-glycosidase activity that cleaves an adenosine residue from the 28S ribosomal RNA of the 60S ribosomal subunit (66). As a result, it inhibits protein synthesis, causing cell death by apoptosis. The five B subunits form a structure that binds the globotriaosylceramide (Gb3) receptor on the surface of eukaryotic cells (120, 167). Gb3 is expressed by Paneth cells in the intestinal mucosa and by kidney epithelial cells [reviewed in (277)] (Figure 1.6). Stx enters systemic circulation through absorption by the epithelium

(4), enabling its access to the kidneys. The damage of the GI epithelium caused by EHEC likely aids in Stx systemic absorption (195). Upon receptor binding, Stx is endocytosed by the eukaryotic cell (247), bypasses the late endocytic pathway and undergoes retrograde transport from the trans-Golgi network to the endoplasmic reticulum (ER) where it encounters its target (171, 251).

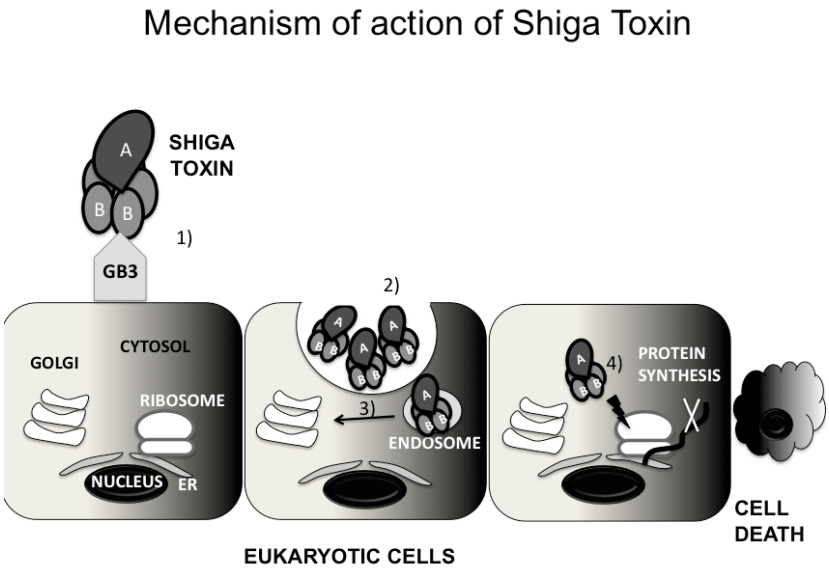

Figure 1.6. Mechanism of action of Shiga toxin (Stx). Stx is constituted by a pentamer of B subunits bound to a catalytic A subunit. The B subunits bind to globotriaosylceramide (Gb3) expressed by some eukaryotic cells (1). Stx is internalized by endocytosis (2). Subsequently, Stx undergoes retrograde transport to the trans-Golgi network (TGN) (3) and then to the endoplasmic reticulum (ER) (4). In the ER, Stx encounters its target, the ribosome, inactivating it (4). As a consequence, Stx inhibits protein synthesis, causing cell death by apoptosis. Source: (222).

The Stx family has two main groups, Stx1 and Stx2. The Stx1 family consists of the variants Stx1, Stx1c and Stx1d. The Stx2 family is composed of the variants Stx2c, Stx2c2, Stx2d, Stx2d$_{activatable}$, Stx2e and Stx2f (198). The Stx2 variants differ in their biological activity, immunological reactivity, and the receptor to which they bind. These binding properties allow the Stx variants to target different cell types (74). Although Stx1 and Stx2 share enzymatic activity and structural features, they are immunogically distinct. Stx1 is nearly identical to Stx from *Shigella*, with a difference in only a single amino acid in the catalytic A subunit. Conversely, Stx2 shares only 55% amino acid sequence similarity to Stx1 (74). Stx2 is more potent than Stx1 in humans (210), and it is commonly associated with hemorrhagic colitis and HUS (202) (27). The two subgroups of Stx can be found in different combinations in EHEC isolates. Epidemiological studies suggest that EHEC strains encoding Stx2 are more likely to cause severe disease than isolates that harbor only Stx1 or a combination of Stx1 and Stx2 (241).

Stx production is regulated by a multitude of pathways (reviewed by Pacheco and Sperandio, accepted). In EHEC O157:H7, Stx is encoded by two toxin-converting bacteriophages in the chromosome, 933W and 933J (215). The genes encoding *stx* are located in intact or partial genomes of prophages from the lambda family, which are integrated into the bacterial chromosome (128). Activation of the phage lytic cycle leads to Stx production (206), which is then

released upon bacterial cell lysis. The genes encoding Stx1 and Stx2 are located within the late phage genes, where expression of *stxAB* is under phage cycle control. Stx2 is only produced when the phage enters its lytic cycle (290), while Stx1 is regulated by phage cycle and an iron-regulated promoter (34, 303). The lambda phage remains quiescent due to the binding of the *c*I repressor to the right (O_R) and left (O_L) operator sites, in turn inhibiting the activity of the phage early promoters P_R and P_L (305). To enter its lytic cycle, the bacteriophage encoding *stxAB* takes advantage of the bacterial cell SOS response. Upon triggering of the SOS response, RecA is produced and activated (194). The activated form of RecA promotes cleavage of the *c*I repressor, de-repressing P_R. Absence of *c*I leads to the expression of the anti-terminators N and Q. The protein Q then binds to $P_{R'}$ to activate the late phage genes, including *stxAB*. Bacteria undergo lysis and release Stx into the environment (Figure 1.7). A study of the promoters and regulatory regions of the 933W phage provides evidence that *c*I removal is required for Stx production by the bacterial cell. A 933W derivative encoding a non-cleavable repressor cannot produce Stx (290).

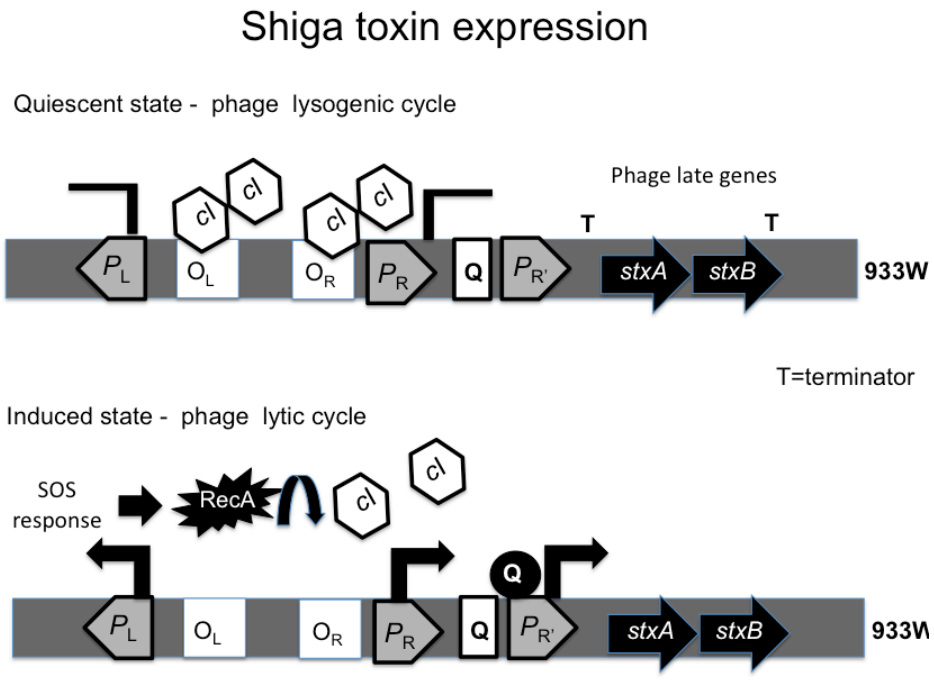

Figure 1.7. Regulation of Shiga toxin expression by the phage cycle. The *c*I repressor bound to operator sites O_L and O_R inhibitis transcription from the promoters P_L and P_R . The presence of terminator downtream of $P_{R'}$ inhibits transcription of *stx*AB and the phage remains quiescent. When the SOS response is triggered, RecA cleaves *c*I, relieving P_R repression and expression of antiterminators N and Q. Antiterminator Q binds $P_{R'}$ leading to transcription of the *stx*AB genes with the late phage genes. Source: (222).

Additionally, synthesis of Stx can be activated by antibiotics, which makes antibiotic treatment of STEC infection controversial (325). Quinolones and mitomycin C, commonly used in research for prophage induction for its ability to interfere with DNA replication, increase the production of Stx by activating the SOS response in EHEC (177, 325). Conversely, a recent study suggests that

inhibitors of cell wall such as fosfomycin do not trigger the SOS response, being potentially safer for treatment (325).

Additional mechanisms involved in Stx regulation include the RNA chaperone Hfq, nitric oxide, hydrogen peroxide (H_2O_2), iron, stationary phase of growth, and stress (295).

AE lesion

A hallmark of the pathogenesis of EHEC infection is the ability to form characteristic histopathological lesions, AE lesions, on enterocytes (134). AE lesions are characterized by intimate attachment of the bacterium to the apical enterocyte membrane, promoting cytoskeletal changes that results in effacement of the brush border microvilli, and extensive actin remodeling leading to the formation of a pedestal-like structure beneath the bacterium (Figure 1.8). The AE lesion is often referred to as a "pedestal". Other bacterial strains are also able to form this histopathology: the human pathogen EPEC, the rabbit pathogenic strain REPEC and the murine pathogen *Citrobacter rodentium* (14, 192, 253). It is of note that although EHEC is firmly attached, and the pedestals appear like rigid structures in pictures, these structures are very dynamic; pedestals are continuously formed and reformed as the bacteria undergoes cell division.

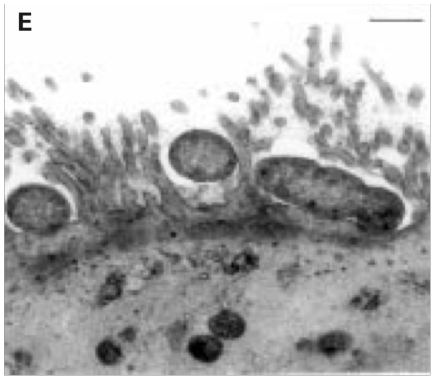

Figure 1.8. AE lesions formed by EHEC. Source: (228)

In 1983, Moon and colleagues were the first to observe AE lesions in the intestines of pigs and rabbits infected with diarreogenic EPEC O55:H7 (which is the EHEC ancestor) and REPEC, respectively (192). AE lesions by EPEC were also observed *in vivo* during infant infection (70). Later, Tzipori reported the same ultrastructural features in tissue cultured HEp-2 cells after infection by EHEC (292). Since then, EHEC has been known to form AE lesions in the intestinal mucosa of rabbits, pigs, cows and human intestinal explants (205, 301).

The mechanisms involved in AE lesion development are somewhat similar between EPEC and EHEC, being the most significant differences found in the recruitment of host cell factors that promote actin remodeling. In this chapter, we will focus on AE lesions caused by EHEC infection.

Locus of Enterocyte Effacement

Most of the genes required for AE lesion development are clustered in a 35Kb chromosomal pathogenicity island of EHEC, the locus of enterocyte effacement (LEE) (Figure 1.9) (McDaniel, 1995). The LEE comprises 41 genes, most of them organized in 5 major operons: *LEE1, LEE2, LEE3, LEE4* and *LEE5* (64). A minor variation among the LEE from EHEC, EPEC and *C.rodentium* exists, although the gene content and overall organization is well conserved in all AE-forming pathogens (320). The LEE from EPEC transplanted to *E.coli* K-12 was sufficient to confer AE-forming ability, while the EHEC LEE was not, indicating the involvement of additional factors required for pedestal formation in EHEC (65).

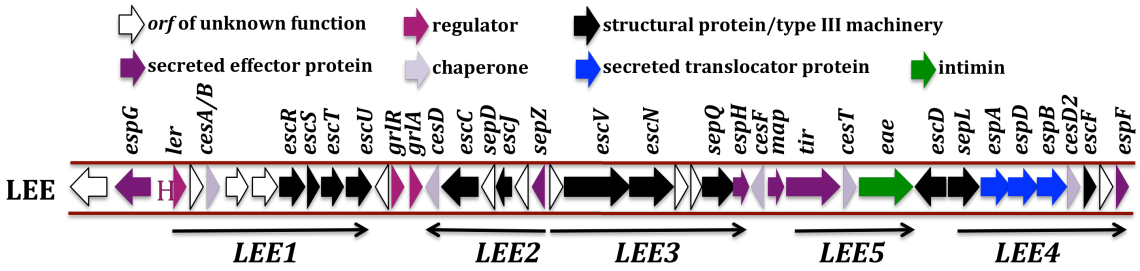

Figure 1.9. Organization of the Locus of enterocyte effacement (LEE). Adapted from Garmendia et al. (80). Figure courtesy of Meredith Curtis.

The LEE genes are directly activated by Ler, encoded by the first gene in the *LEE1* operon (63). Expression of *ler* is essential for AE lesion and pathogenesis, as a *ler* mutant strain does not present the AE phenotype *in vitro,* and it is attenuated during colonization of the infant rabbit intestine (327).

Genes in *LEE1, LEE2* and *LEE3* encode for the majority of the structural components of a T3SS apparatus, a needle-like struture that works as a molecular syringe, translocating bacterial-encoded effectors into the eukaryotic cell cytosol. An intact T3SS is required for EHEC pathogenicity, given that a non-functional T3SS EHEC strain present defects on colonization and AE lesion formation during infection of infant rabbits (242). The T3SS machinery is constituted by the product of nearly 20 genes: EscR, EscS, EscT, EscU (from *LEE1*) and EscV (*LEE3*) inserted into the inner membrane; EscJ (*LEE2*) in the periplasmic space connecting to inner ring to the outer membrane ring formed by EscC (*LEE2*); EscN is the ATPase that promotes effector translocation, through the narrow channel formed by EscF (*LEE4*), which is surrounded by the EspA filament (*LEE4*); the EspB and EspD proteins (*LEE4*) form the translocon that is inserted into the eukaryotic cell membrane (51, 83, 147, 318). Additionally, the T3SS is formed by the cytoplasmic components SepD (*LEE2*)- essential for secretion of translocator and effectors and AE lesion- and SepL (*LEE4*), which is required for EspA filament biogenesis and AE lesion formation (56, 217). The chaperones CesAB (*LEE1*), CesD (*LEE2*), CesD2 (*LEE4*) and CesMT (*LEE5*) and CesF are

important to assist secretion of their substrates T3S effectors, guaranteeing their efficient secretion and translocation (1, 61, 62, 304).

LEE5 encodes for the adhesin intimin and its own receptor, transmembrane intimin receptor (Tir). Intimin is an outer-membrane adhesin (126). Tir is translocated into the host epithelial cell via the T3SS, forms a homodimer and binds to the surface-exposed intimin to promote intimate attachment of the bacteria to the enterocyte. The interaction between Tir and intimin is critical for intestinal colonization, as *in vivo* studies show that mutant strains unable to produce either of these factors are defective for colonization of the mammalian intestine (141, 241).

The host cell factors recruited by EHEC Tir are the main difference between AE lesion formation in EHEC and EPEC. EHEC Tir interacts with the I-BAR family host factors IRSp53 and IRTKS, which in turn recruit and bind to the prophage-encoded T3S effector EspFU (also known as TccP) (37, 81). EspFU binds to and activates NWASP, which in turn activates the actin-nucleting factor Arp2/3, triggering actin polymerization underneath the attached bacterium (37, 81, 298, 311) (Figure 1.10). Tir and EspFU are the only effectors in EHEC required for pedestal formation (36).

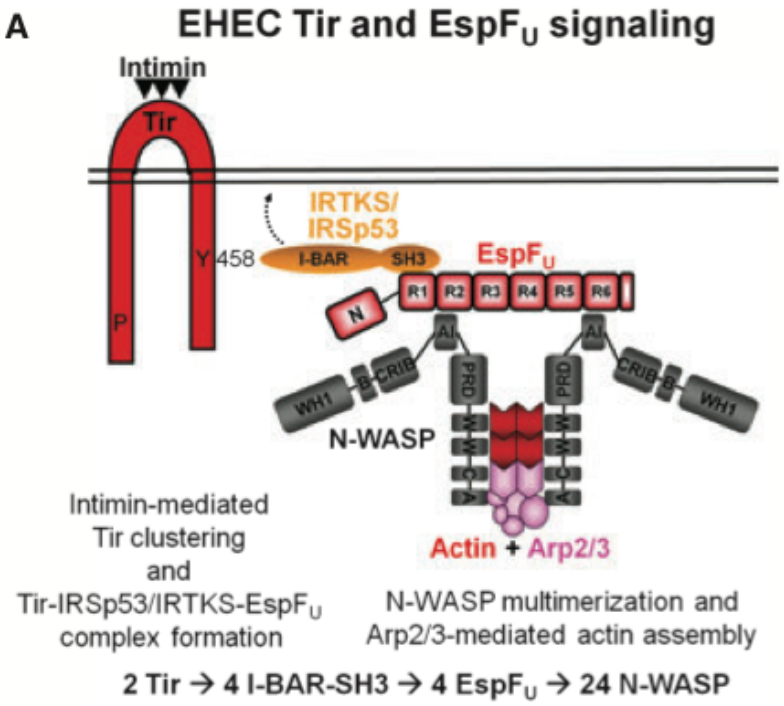

Figure 1.10. Pedestal formation by EHEC. Source: (35)

In vivo studies indicate that EspFU is important to sustain EHEC colonization of infant rabbits and gnotobiotic piglets intestine but not lambs or calves (299); *espFU* mutant strains can initially colonize the mammalian gut, despite their decreased ability to form pedestals on epithelial cells, but they present impaired proliferation (240).

Type 3 Secreted Effectors

In addition to Tir and EspFU, EHEC encodes a vast arsenal of T3S effectors. It is estimated that nearly 51 effectors are translocated by this machinery into the host cell, subverting the host cell architecture contributing to epithelial damage and the onset of diarrhea. The production of T3S effectors is tightly regulated and their secretion and translocation is likely a hierarchical process (55). In the past 10 years, there has been a remarkable increase in the knowledge of T3S effectors function. T3S effectors are encoded within the LEE or outside of the LEE (non-LEE-encoded or Nle) in cryptic prophages (286). Encoded in the LEE are Map, EspF, EspG, EspH, EspZ and Tir (141) (5, 54, 115, 148, 179, 258, 289).

Not encoded in the LEE but also translocated by the LEE-encoded T3SS: NleA (or EspI), NleB, NleC, NleD and NleE, NleG/NleI, NleH, EspJ, EspK, EspL, EspM, EspN, EspO, EspR, EspV, EspW, EspX, EspY, and EspFU, which collectively target multiple eukaryotic factors [reviewed in (320)].

NleA interferes with intracellular trafficking by binding to Sec24, and consequently inhibiting the COPII-dependent protein secretion pathway, which causes disruption of epithelial cell tight junctions, a mechanism that has been implicated in the development of diarrhea. Infection studies using mice infected with *C.rodentium* showed the importance of NleA for pathogenesis, given that *nleA* mutant is severely attenuated (92, 143, 197).

NleB, NleC, NleD, NleE and NleH together influence the inflammatory response generated in the initial stages of EHEC infection, by targeting individual components of the NF-κB signaling cascade, and consequently preventing EHEC elimination by the innate immune response (320). NleB inhibits NF-κB activation triggered by TNF, while NleE prevents NF-κB activation in response to both TNF and IL-1β, decreasing production of IL-8 (208). NleC is a metalloprotease that targets the p65 subunit of NF-κB, and likely acts in concert with NleE and NleB to inactivate NF-κB, which causes immune suppression during EHEC infection (15). NleD is also a protease that cleaves the MAP kinases JNK and p38, inactivating the AP-1 signaling pathway that regulates, among other cellular processes, the inflammatory response (15). NleH1 and NleH2 are Ser/Thr protein kinases that exert anti-apoptotic and anti-inflammatory effects by interacting with BAX-1 inhibitor and inhiting NF-κB activation, respectively (79, 101).

Plasmid pO157

Most EHEC O157:H7 strains harbors a 92Kb virulence plasmid, pO157, which encodes few characterized and several putative virulence factors (32). The role of pO157 in EHEC virulence comes from studies that show that loss of pO157 increases acid resistance, by increasing expression of the *gad* system, while it impairs RAJ colonization in a cattle model of infection (166, 262). pO157 is also involved in biofilm formation, by promoting exopolysaccharide (EPS)

production (164). Among the known virulence genes found in this plasmid are: StcE protease, Ehx enterohemolysin, the potential adhesin ToxB, lymphostatine LifA, Ehx, MsbB acetyltransferase, and the catalase-peroxidase KatP (3, 16, 30, 144, 254, 278). *In vivo*-induced antigen technology (IVIAT) showed that the genes *stcE* and *msbB2*, encoded in pO157 are expressed *in vivo* during human infection (130).

The pO157 plasmid encodes a type-2 secretion system (T2SS) (255). Deletion of the T2SS decreases EHEC adhesion to epithelial cells *in vitro*, and also intestinal colonization in an infant rabbit model of infection (103).

StcE is a zinc metalloprotease that displays mucin-degradation activity and inhibits C1-esterase (C1-INH) (94, 157). StcE is secreted by the plasmid-encoded Etp T2SS, and contributes, but it is not required, to intimate attachment of EHEC to the intestinal epithelium *in vitro* (93). Cleavage of C1-INH by StcE diminishes the inflammatory response and bacterial lysis via complement, likely contributing to EHEC persistence during infection (156). Moreover, StcE cleaves the salivary proteins gp340 and MUC7, which has been suggested as a mechanism used by EHEC to avoid entrapment in the mucus and consequently, prevent bacterial elimination (93).

EspP is an autotransporter with serine protease activity that contributes to intestinal colonization of cattle (60). EspP cleaves the complement fragments C3/C3b and C5, likely affecting the immune response to EHEC infection (220).

Also, EspP was shown to cleave human coagulation factor 5, suggesting a role for EspP in development of hemorrhagic colitis (29).

Regulation of the LEE in EHEC

The regulation of the LEE in AE pathogens is complex and hierarchical, and multiple environmental factors, as well as regulatory proteins, act to correctly time gene transcription according to subsequential stages of infection [reviewed in (180)]. Regulation of the LEE involves a complex network of multiple activators and repressors, encoded within and outside of the LEE (Figure 1.11).

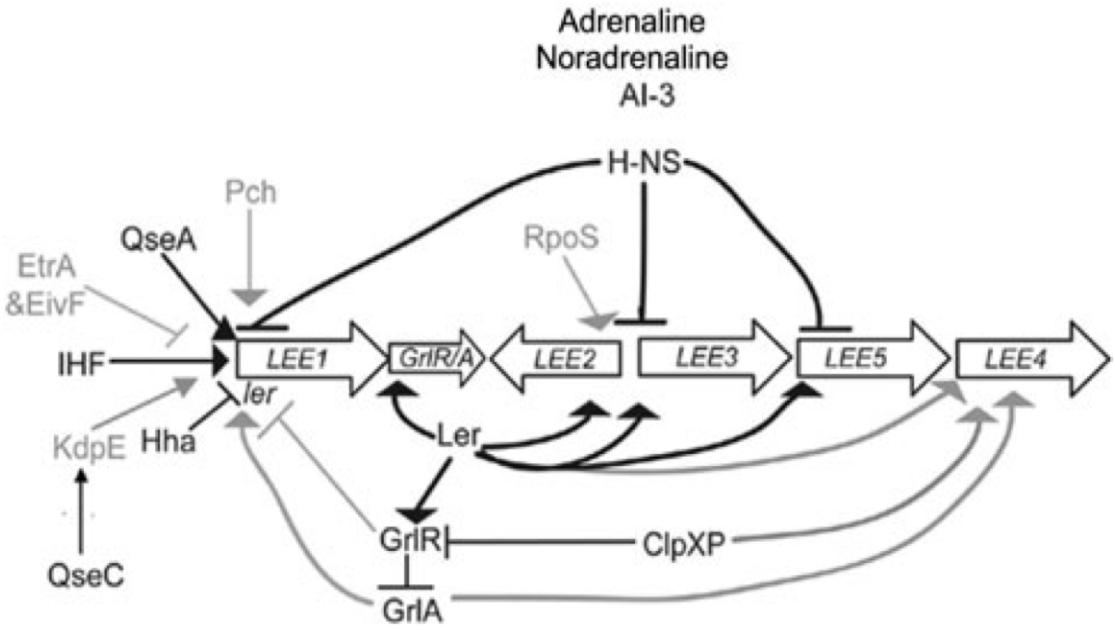

Figure 1.11. Network that regulates LEE transcription. Black arrows indicate proved direct interactions with the promoter region, while grey arrows represent indirect or not yet proved direct regulation. Source: (140)

Regulators encoded within the LEE

The *LEE1* operon encodes for a positive regulator, the LEE-encoded regulator (Ler) (182). Ler is the first gene in the *LEE1* operon. Ler overcomes H-NS-mediated repression to activate transcription of *LEE2*, *LEE3*, *LEE5* directly, and of *LEE4* indirectly (33, 76, 96, 182, 267). Ler shares similarity to H-NS (267), and it has higher binding affinity to DNA than H-NS. Its mechanism of action involves formation of high-order oligomers in solution, of 5000 KDa, constituted of 100-250 copies of the monomer (15.1KDa) (181). Ler may autoregulate its own expression by directly binding to the *LEE1* promoter; however, this observation remains controversial (12, 21). Ler also activates expression of the LEE-encoded genes *grlRA*, *map* and *espG* (63, 162, 250, 294). Together, Ler, GrlA and GrlR form an autoregulatory loop that governs expression of the LEE. GrlA activates *ler* transcription while GrlR represses it by interacting with and inactivating GrlA (12, 56, 109, 116, 127, 248).

Ler also plays a regulatory role over genes encoded outside of the LEE. Recently, it has been suggested that Ler regulates expression of *nleH1* and *nleH2*, given that the transcript levels of these two effectors was lower in *ler* mutant strains (104). Ler also activates expression of the long polar fimbriae 1 (*lpf1*) (246). Therefore, *ler* is a global regulator of virulence in AE forming pathogens.

Global regulators of LEE transcription

In addition to H-NS, which acts as a "silencer" of the *LEE1-LEE5* operons, another nucleoid-binding protein, integration host factor (IHF) acts as a positive regulator of LEE transcription by activating expression of *ler*. Fis is also a positive regulator of the LEE that acts through *ler* The environmental-dependent regulator Hha negatively regulates *ler* transcription by interacting directly with the *LEE1* promoter (259, 260).

Post-transcriptional control

Post-transcriptional regulation also controls expression of the LEE genes. The RNA chaperone Hfq modulates LEE transcription differently in EHEC O157:H7 strain EDL933, and EHEC O157:H7 86-24; Hfq is a negative regulator of the LEE in the strain EDL933, while it positively regulates the LEE in 86-24. The non-coding RNA DsrA triggers LEE expression through *ler* in a RpoS-dependent manner. CsrA has a dual role in controlling LEE transcription, behaving as a positive or negative regulator according to its concentration. RnaseE participates in post-transcriptional processing of the *LEE4* transcript (25, 100, 138, 154, 168, 257, 300).

Regulators encoded in mobile genetic elements

In addition to *ler* and *grlRA*, transcriptional control of the LEE by other horizontally acquired genetic elements has been reported. The *pch* genes, which are homologous to the EPEC plasmid-encoded *perC*, act as activators of *LEE* transcription (119). The regulatory factors EtrA and EivF, encoded in the cryptic TTSS of EHEC chromosome ETT2, function as repressors of the LEE (324). GrvA and the phosphorelay RscBCD also regulate transcription of *ler* (211, 285).

Regulation of the LEE by interkingdom signaling and two component systems

Interkingdom signaling in EHEC: communication between bacteria and the host via the AI-3/Epi/NE signaling cascade

Eukaryotic organisms utilize several hormones to regulate different aspects of their physiology and maintain homeostasis. Prokaryotic cells signal through autoinducers (AIs), which are hormone-like compounds that allow microorganisms to coordinate gene expression involved in a multitude of processes. Co-evolution of prokaryotic species and their respective eukaryotic host has exposed bacteria to host hormones and eukaryotic cells to the bacterial-derived molecules during host colonization. Therefore, it is not surprising that

some pathogenic species have high-jacked these signaling systems to promote disease (111). It has been demonstrated that bacteria can modify gene expression upon sensing molecules produced by the host. Conversely, a few bacterial chemicals have been shown to cause effects in host cells. This crosstalk among bacteria and eukaryotic cells is named inter-kingdom signaling and it participates in both symbiotic and pathogenic bacteria-host relationships (221).

LEE transcription is triggered by the interkingdom signaling cascade comprising the microbiota-produced autoinducer 3 (AI-3), and the host stress hormones epinephrine (Epi) and norepinephrine (NE) (AI-3/Epi/NE signaling cascade) (268). Epi/NE are catecholamine hormones that are part of the stress response in mammals. NE is synthesized by neurons of the enteric nervous system (ENS) while Epi is synthesized in the adrenal medulla, and reaches the intestine via the bloodstream. Both molecules are present in the GI tract where they modulate important physiological roles such as smooth muscle contraction, submucosal blood flow and potassium/chloride secretion. EHEC senses Epi and NE, activating LEE transcription (269, 306). This was the first study to show evidence of crosstalk between prokaryotic and eukaryotic signaling systems involving Epi/NE (269). The Epi/NE response is mediated by the sensor histidine kinase QseC, and could be antagonized in the presence of the α-adrenergic antagonist phentolamine. Therefore, QseC was the first functional analog of an adrenergic sensor identified in bacteria (41). The integration of AI-3 sensing and

stress hormones detection indicates that the AI-3/Epi/NE is an inter-kingdom communication mechanism of EHEC (Figure 1.12). Since it was firstly described, the adrenergic sensor QseC (99) has been linked to virulence and metabolic gene regulation in non-pathogenic *E.coli* (271), EHEC, EPEC, UPEC (67, 149, 150), *Salmonella* (17, 18, 183, 193, 196, 229, 230, 232, 266), *Francisella tularensis* (189, 232), *Francisella novicida* (59), *Aeromonas hydrophila* (142, 152), *Actinobacillus pleuropneumoniae*, periodontal pathogen *Aggregatibacter actinomycetemcomitans* (213) and the fish pathogen *Edwardsiella tarda* (309).

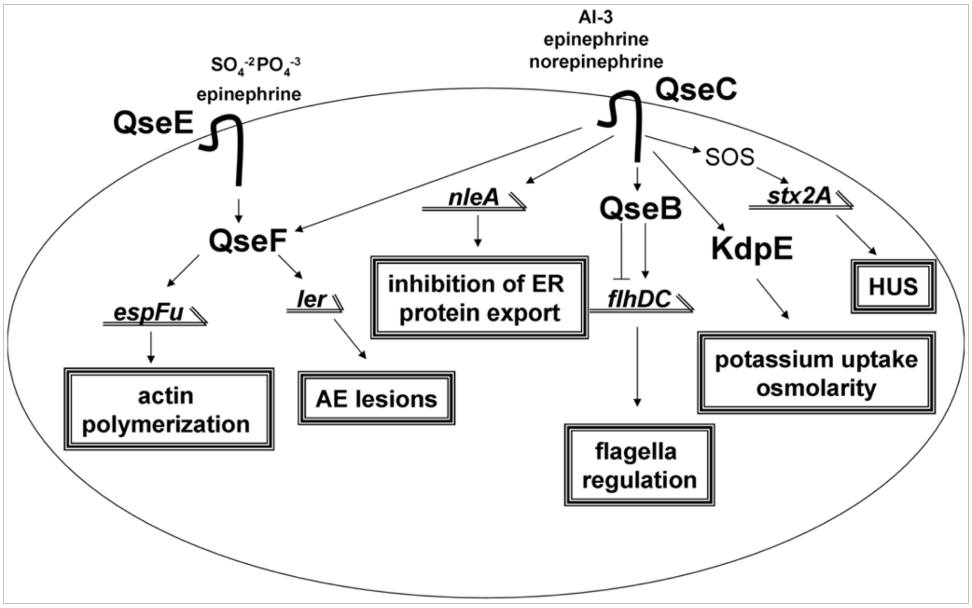

Figure 1.12. The AI-3/Epi/NE inter-kingdom signaling cascade of EHEC. For explanations, see text. Source: (110).

Regulation of the LEE by TCS of the AI-3/Epi/NE signaling cascade

The AI-3/Epi/NE cascade employs at least in part the coordinated action of two component systems. The prototypic and simplest TCS is constituted by two major proteins: a sensor histidine kinase (HK) and a response regulator (RR). These proteins are modular in nature, composed of functional domains, each one involved in one aspect of the signal transduction pathway. The HK contains a sensory (or input) domain and a catalytic (or kinase) domain, which contains the dimerization and conserved histidine domains. HKs can be membrane bound or soluble. The RR harbors a receiver domain and a DNA binding domain, and most RRs are transcription factors. Upon sensing stimuli, the HK autophophorylates at a conserved histidine (His) residue, creating a high-energy phosphoryl group. HKs function as dimers, therefore, what occurs is a trans-phosphorylation reaction in which one HK monomer phosphorylates the other monomer's His (275). Subsequently, the HK then transfers the phosphate to a conserved aspartate (Asp) in the received domain (REC) of the RR. Conformational changes in the effector domain, triggered by phosphorylation, activate the RR changing affinity of the DNA binding for the target, resulting in differential gene expression (Figure 1.13). Some HKs have a dual role, displaying also phosphatase activity over their cognate RR and therefore, HKs can control the response elicited by the RR via phosphorylation and dephosphorylation of the RR (78, 275). Typically, genes

encoding cognate HKs and RRs are found adjacent in the genome and many are co-transcribed constituting one transcriptional unit or operon (293).

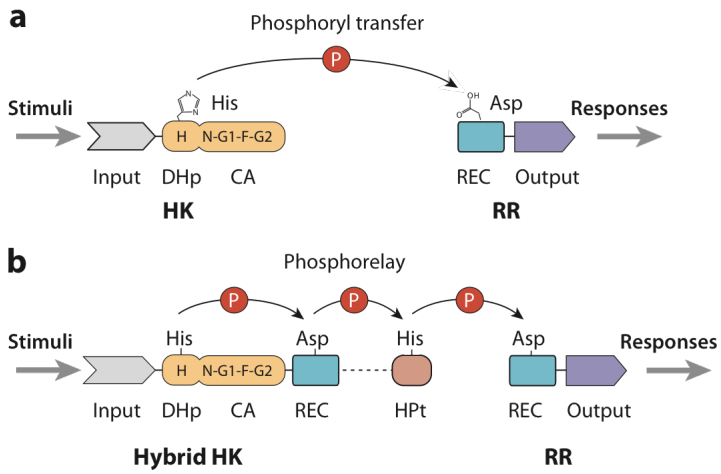

Figure 1.13. Signal sensing and transduction by TCSs. Two-component systems are constituted by a HK and a RR, forming a signal transduction pathway based on transfer of a phosphate group from the HK to the RR, resulting in RR activation and consequently, changes in gene expression. Source:(78).

The TCSs QseBC and QseEF are the major players in the AI-3/Epi/NEsignaling cascade of EHEC. Upon sensing AI-3, Epi or NE, the bacterial adrenergic sensor QseC phosphorylates the non-cognate response regulator KdpE, activating *LEE1* expression (110). Also part of this regulation of virulence, the LysR-type regulator QseA acts as an activator of *LEE1* transcription by directly binding to the *ler* promoter (140, 261). Additionally, EHEC employs the LuxR-homolog SdiA to control transcription of the LEE. SdiA interacts with the *ler*

regulatory region upon binding acyl-homoserine lactones (AHLs), decreasing expression of the LEE (112). AI-3 is sensed by the sensor kinase QseC, which is part of a two-component system with the cognate response regulator QseB. Studies have shown that QseC is the only sensor of AI-3 in EHEC. QseC is directly involved in EHEC pathogenesis, given that a *qseC* mutant strain is attenuated for colonization of the infant rabbit intestine (41). QseC phosphorylation of its cognate RR QseB leads to activation of the flagellar genes, triggering expression of flagella and motility (41).

QseE is also an adrenergic sensor of EHEC, and recent studied showed that QseC and QseE are the only epinephrine sensors in bacteria (211, 235). QseE alsoincreases its autophosphorylation in response to phosphate and sulphate sources (235). QseE is a sensor kinase that is part of a TCS together with the cognate RR QseF, which is a sigma-54 transcriptional regulator (236). QseEF TCS regulates iron acquisition, Shiga toxin and EspFU expression, although EspFU regulation is indirect.

Recently, a novel member of the QS regulation of virulence in EHEC was identified: QseD. QseD is a LysR-like transcriptional regulator ubiquitous in enterobacteria, that positively regulates motility in *E.coli* K-12 and negatively controls T3S in EHEC (98).

To date, the AI-3/Epi/NE interkingdom signaling continues to expand and appears to be even more complex. Recent studies from our laboratory have shown

that QseE negatively regulates the LEE and NleA by decreasing expression of the RcsB RR (211). QseEF regulation is downstream of QseBC, given that *qseE* expression is activated by QseC (236). Moreover, cumulative evidence indicates that QseC plays a central regulatory role in EHEC virulence, also being part of a crosstalk with the TCS QseEF and other proteins. QseC activates LEE transcription by phosphorylating the non-cognate RR KdpE, triggers Stx production via QseF and increases NleA expression indirectly (110, 211). QseF also participates in this crosstalk at a biochemical level, being phosphorylated by at least 4 HKs encoded by *E.coli* (323).

Animal models for EHEC infection and Stx

Many animal models to study the pathogenesis of EHEC infection *in vivo* have been developed. Some of these models are more suitable to study colonization, while others are more informative to evaluate the effects of Stx. Nonetheless, no animal model currently available is able to mimic all aspects of EHEC-mediated disease, including the infectious dose or development of hemorrhagic colitis and HUS (188). The animal models include: mice, rats, infant rabbits (223, 241), gnotobiotic piglets, gnotobiotic calves, and primates.

A mouse model is usually the choice for *in vivo* studies, due to lower cost, easier maintenance, availability of reagents and knockout strains. The first mouse model for EHEC infection was developed by Wadolkowski and colleagues (302),

and was based on the model developed by Myhal (200), who studied colonization by non-pathogenic *E.coli* strains. In this model, the animals receive antibiotics in their drinking water prior to EHEC infection to reduce the microbial flora and diminish competition with EHEC. EHEC stably colonizes the small and large intestines for about a month. However, the mice do not develop diarrhea or any other hallmark GI symptoms of EHEC infection. Because most of the mouse models developed to date require manipulation such as antibiotic treatment (302), dietary restrictions, or mitomycin C administration to improve Stx expression, Mohawk and colleagues developed a model that maintains an intact commensal flora (ICF) (187). They infected BALB/c mice with different inoculums and assessed bacterial colonization by CFU recovery from intestinal segments and stool. A high infectious dose (10^9 CFU) resulted in the highest colonization rate and bacterial recovery compared to lower ones (10^5, 10^6, 10^7 and 10^8 CFU). The highest levels of Stx are detected in the large intestinal contents. Histopathology analysis of kidney sections shows mild kidney damage, suggesting Stx systemic effects. This study suggests that by using a high infectious dose, mice can be colonized by EHEC without alteration to their commensal gut microbiota. This model represents an advance in the use of mice to study EHEC colonization and persistence. However, a disadvantage of this particular model is the absence of hemolytic anemia and thrombocytopenia, making the ICF mice more attractive for colonization studies than evaluation of systemic effects resulting from EHEC

infection. The reports using mice as a model for EHEC infection have to be interpreted with caution. Mice do not develop diarrhea, and AE lesions are not observed. Also, it is of note that the bacterial inoculum used in these experiments is many orders of magnitude higher than the estimated infectious dose of EHEC (50-100 CFU).

Gnotobiotic piglets are highly susceptible to EHEC infection. The gnotobiotic piglet model was developed by Tzipori in 1986 (292), and this model has been a useful tool to study Stx-mediated pathogenesis and the systemic effects of Stx. (195). Upon infection by EHEC, these animals develop watery diarrhea, colitis and inflammation, and EHEC forms the characteristic AE lesions in their intestinal epithelium (292). Although the gnotobiotic piglets do not develop HUS, they demonstrate several signs of systemic disease including convulsions, tremor, ataxia and brain hemorrhage. Formation of thrombotic microangiopathy, which causes acute kidney failure, was observed when gnotobiotic piglets were orally infected by 10^8-10^{10} CFU of EHEC (95). The gnotobiotic piglet model best represents the systemic effects of EHEC infection seen in humans, and it is an excellent model to study EHEC pathogenesis *in vivo*. However, a drawback of using this animal model is the requirement of a complex animal facility to perform the experiments.

The infant rabbit model is also used to study EHEC pathogenesis *in vivo*. Developed by Pai and colleagues in 1986 (223), 3-day old infant rabbits are

inoculated intragastrically with 10^8 CFU of EHEC. The rabbits develop diarrhea and colonic inflammation, and a portion succumbs to death. In 2003, Ritchie and colleagues investigated the contribution of individual virulence factors to the development of disease by using EHEC isogenic mutants to infect infant rabbits (241). The infant rabbit model allowed the evaluation of Stx2, as well as the receptor and ligand crucial for AE lesion formation, Tir and Intimin, respectively, in the pathogenesis of EHEC infection, by inoculating 3-day old New Zealand white rabbits with $5x10^8$ CFU/90g body weight of EHEC WT or the EHEC isogenic mutants *stx2*, *eae* or *tir*. When the infant rabbits are infected with EHEC wild type (WT), the animals develop diarrhea and intestinal inflammation but not HUS. The absence of the Stx receptor Gb3 in the rabbit kidneys likely accounts for the lack of HUS in these animals (329). Similarly to gnotobiotic piglets, infant rabbits infected with EHEC develop AE lesions on their intestinal epithelium (241). However, in the absence of Tir or Intimin, EHEC cannot colonize the intestine or cause disease, indicating the important role that AE lesion formation plays in pathogenesis. Stx2 promotes an inflammatory response at the intestinal mucosa but does not contribute to colonization, as shown by infection of rabbits with a *stx2* mutant of EHEC or administration of purified Stx2. The infant rabbit model is useful to study the intestinal pathogenesis of EHEC infection, and it is advantageous to the gnotobiotic piglet model in its cost effectiveness.

The use of ferrets as an animal model for EHEC infection was developed as an effort to obtain an alternative small animal model, given that mouse models present some restrictions. Ferrets pre-treated with streptomycin previous to oral EHEC infection do not develop colitis, similarly to mice; however, infected ferrets presented significant weight loss, destruction of renal glomeruli and thrombocytopenia, indicating that the ferret model is potentially relevant for the study of Stx-mediated disease (322).

Chickens can also be colonized by EHEC and develop the characteristic AE lesions, but not diarrhea (19, 272, 276). Orally inoculated EHEC was able to adhere to and colonize the ceca of chickens for 90 days, and even penetrate the cecal epithelium (19).

Infection of adult monkeys with EHEC O157:H7 has successfully reproduced some aspects of the human infection. Monkeys orally infected with EHEC develop diarrhea (non-bloody) and present AE lesions in the cecum and colon. Additional damage of the intestinal epithelium was also observed. However, it should be noted that the inoculum used was 10^{12} CFU, with is many orders of magnitude higher than the infectious dose for humans (132).

A cow model of EHEC infection has also been developed, and it has been employed to study bacterial colonization and shedding. EHEC can persist in the lower GI tract of calves for up to 2 months after oral inoculation (90). EHEC

presents tropism for the recto-anal junction of cows, where it forms AE lesions (204, 205).

CHAPTER TWO

OVERALL OBJECTIVES AND SYNOPSIS

EHEC is a human pathogen that causes outbreaks of bloody diarrhea worldwide, which can be fatal due to the HUS sequelae. The three major virulence traits harbored by EHEC is the production of flagella, Stx and the formation of the histopathological attaching and effacing lesions, characterized by destruction of the intestinal epithelium. The production of virulence factors in EHEC is controlled by the AI-3/Epi/NE interkingdom signaling cascade that comprises at its core, the two-component systems QseBC and QseEF. QseBC and QseEF are adrenergic sensory systems of EHEC. The QseBC signaling cascade governs flagellar motility and LEE expression, while QseEF controls AE lesion. Transcriptomic analyses indicated that both QseBC and QseEF repress expression of the putative TCS herein named FusKR. FusK is the sensor histidine kinase and FusR is the response regulator. FusK/FusR are encoded by genes clustered in the EHEC-specific PAI OI-20, together with the predicted MFS transporter Z0461. The overall objective of this dissertation was to characterize the participation of FusKR in EHEC pathogenicity. To pursue that, genetic and biochemical investigations were conducted. Additionally, the interplay between EHEC and the commensal bacterium *B.theta* was explored using *in vitro* approaches, in order to

obtain insight into the interplay between EHEC and the microbiota during infection.

Combining qRT-PCR and EMSAs, we discovered that FusKR is directly repressed by QseBC. QseB binds the *fusKR* promoter, reducing *fusK* expression. Additionally, AI-3 decreases *fusK* and *fusR* trancription. These results indicate that FusKR is a new member of the AI-3/Epi/NE signaling cascade of EHEC.

To begin examining the function of FusKR in EHEC virulence, we constructed the isogenic mutants *fusK*- and *fusR*-, and performed microarray studies. Deletion of *fusK* and *fusR* results in differential expression of nearly 1,000 genes including virulence and metabolic ones, indicating that FusKR plays a major regulatory role in EHEC. The *fusK*- and *fusR*- mutant strains overexpress the LEE genes. QRT-PCR confirms that FusKR act as negative regulators of the LEE.

We sought to obtain further insight into the role of FusKR in EHEC virulence. Therefore, we analyzed production of type 3 secreted effectors and AE lesion formation in tissue culture. FAS assay shows that *fusK*- and *fusR*- are hypervirulent *in vitro*, capable of forming more pedestals per cell compared to EHEC WT. Additionally, *fusK*- and *fusR*- secrete higher amounts of EspB, the translocon of the LEE-encoded T3SS.

At this point, we were able to demonstrate that FusKR participates in the regulation of virulence genes in EHEC, although the signal that triggers the

FusKR signaling pathway remained undefined. In an effort to determine the signal detected by FusK, we investigated the environmental cues that activated expression of the *fusKR* operon. It has been demonstrated that *fusKR* transcription in activated by the presence of mucus. We infected mucus-producing colonic HT29 cells with EHEC WT and measured the expression of *fusR* by qRT-PCR, confirming the *fusR* transcription is triggered by mucus. Mucus is 50% made of polysaccharides and fucose is one its major constituents, and is abundant in the intestine. We discovered that FusKR repress transcription of the fucose regulon that controls fucose utilization. By performing growth curves, we found that *fusK*- and *fusR*- mutants grow faster in fucose compared to EHEC WT. Based on these results, we hypothesized that FusK could sense fucose as a signal. We reconstituted full-length epitope tagged FusK into liposomes and measured autophosphorylation activity in the presence of fucose and other sugars. We were then able to show that FusK is a fucose sensor HK.

To investigate whether fucose sensing by FusK affected LEE transcription, we measured expression of *ler* in the presence of fucose by qRT-PCR. Our studies revealed that fucose-sensing by FusK mediated *ler* repression, confirming the function of FusK as a fucose sensor of EHEC.

Moreover, we determned that the regulation of the *fuc* genes by FusKR is indirect, given that our EMSA analyses show that FusR is unable to interact with the *fuc* promoter. Z0461 is a predicted membrane transporter encoded adjacent to

fusKR. QRT-PCR indicates that FusKR repress *z0461* transcription. Z0461 is involved in optimal fucose uptake, as shown by its slower growth on fucose and congruent reduced expression of the *fuc* regulon.

EHEC has tropism for the human large intestine, which is a complex ecosystem that harbors trillions of commensal bacteria. *Bacteroides thetaiotaomicron* is a predominat member of the intestinal microbiota. *B.theta* scavenges fucose from host glycans then uptake it to use as carbon and energy source. We hypothesized that *B.theta* degradation of mucin would supply fucose to EHEC, altering LEE expression. Co-culture of *B.theta* and EHEC on mucin as a sole carbon source results in reduced *ler* transcription, while *B.theta* does not impact *ler* expression during co-culture in the presence of free fucose.

The interplay between EHEC and *B.theta* and the effect of this human symbiont in *ler* expression prompted us to explore the role of FusKR in EHEC pathogenicity *in vivo*. Using the infant rabbit model, we discovered that *fusK*- is impaired for colonization of the mammalian intestine. Therefore, fucose sensing by FusK is important for successful establishment of EHEC in the mammalian gut.

The results obtained in this study contribute to elucidate the interactions between commensal and pathogenic bacteria, which are not well understood but nonetheless critical for the outcome of infection by enteric pathogens.

CHAPTER THREE

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Growth Conditions

Strains and plasmids are listed in Table 1. *E.coli* strains were grown aerobically at 37°C in DMEM (Gibco) or LB unless otherwise stated. For studies involving fucose utilization, bacterial cultures were grown in M9 minimal media containing 0.4% L-fucose or glucose (Sigma) as a sole carbon source. For the co-culture experiments between EHEC and *B. thetaiotaomicron*, these strains were grown anaerobically at 37°C in DMEM with or without mucin or free fucose, at a 1:9 ratio. Enumeration of EHEC was performed through serial dilution of these cultures in McConkey agar containing streptomycin (EHEC strain 86-24 is streptomycin resistant, while *B. thetaiotaomicron* is sensitive to this antibiotic). Enumeration of *B. thetaiotaomicron* was performed through serial plating in TYG medium supplemented with 10% horse blood in the presence of gentamycin (*B. thetaiotaomicron* is gentamycin resistant, while EHEC is sensitive to this antibiotic).

Table 1. Bacterial Strains.

Strain	Genotype	Source or Reference
86-24	Stx+ EHEC strain serotype O157:H7	Griffin et al, 1988
ARP01	86-24 *fusK* isogenic mutant	This work
ARP02	86-24 *fusR* isogenic mutant	This work
ARP03	pARP10 in TOP10	This work
ARP04	pARP11 in TOP10	This work
ARP05	pARP10 in *fusK*-	This work
ARP06	pARP11 in *fusR*-	This work
ARP09	*fusK*- complemented pARP12	This work
ARP10	*fusR*- complemented pARP13	This work
BL21	F- *ompT hsdSB(rB-, mB-) gal dcm* (DE3)	Invitrogen
TOP 10	Host *E. coli* strain for protein expression from pBADMycHis vector	Invitrogen
DH5alpha	*supE44* Δ*(argF-lac)U169 (␣80dlac␣(Z)M15) deoR hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Stratagene
VS138	*qseC* isogenic mutant	(270)
MC474	*qseB* isogenic mutant	(110)
NR01	*qseE* isogenic mutant	(236)
NR02	*qseF isogenic mutant*	(236)

Table 2. Plasmids.

Plasmids	Genotype	Source or Reference
pBADMycHis A	C-terminal Myc-His-Tag vector	Invitrogen
pACYC184	Cloning vector	New England biolabs
pKD3	pANTS derivative containing FRT-flanked chloramphenicol resistance	(52)
pKD46	λ red recombinase expression plasmid	(52)
pCP20	TS replication and thermal induction of FLP synthesis	(52)
TOPO PCR blunt	Commercial blunt end cloning vector	Invitrogen
pARP10	FusK in pBADMycHisA	This study
pARP11	FusR in pBADMycHisA	This study
pARP12	*fusK* in pACYC184	This study
pARP13	*fusR* in pACYC184	This study
pARP05	*fusK* in TOPO	This study
pARP06	*fusR* in TOPO	This study
TOPO fusR-up	326 bp upstream region of fusR in TOPO	This study
pVS154	QseB in pBADMycHis	(270)
pNR02	QseF in pBADMycHis	(236)

Table 3. Oligonucleotides.

	Primers used for mutagenesis and cloning
Z0462lambdaredP1	TTAATGTCGCGCCAATCTTCGTCATTGGGTGATTTTGCTGTTTAT TGTGCTAGCCTGGGGAGTGTAGGTTGGAGCTTGCTTC
Z0462lambdaredP2	CGGGATACCTAAGACTTTTTCCTGGTTATCCGGCGATT GTTGCAAAAATGTGGGCAAGTCATATGAATATCCTCCTTA
Z0462F	AAGCTTATTCGCGCAATCTTCG Clone *fusK* in pBADMyc His
Cz0462rev	AAGCTTATTCCGGCGATTTGTTGC Clone *fusK* in pBADMycHis
Z0462for	GTCGACTCGGCTCGACCACCATCTGC Clone *fusK* in pACYC184
Z0462rev	TCTAGATGAACGCGCGTGCA Clone *fusK* in pACYC184
Z0463lambdared P1	ATTAGATAATAAGAGAAGAAAAGTATGATTCGGGTA GTGCTGGTGGATGACCATGTTGTGGTGTAGGCTGGAGCTGCTTC
Z0463lambdaredP2	TCCCCAGGCTAGCACAATAAACAGCAAAATCACCCAA TGACGAAGATTGCGCGACATTAACATATGAATATCCTCCTTA
z0463f2	AAGCTTCCATTCGGGTAGTGCTGG Clone *fusR* in pBADMyc His
Z0463r2	AAGCTTAATGCCCCGCCAGCAG Clone *fusR* in pBADMyc His
Z0463for	GTCGACGTTGATTGCCAGCGCCGCGC pACYC184 cloning
Z0463rev	TCTAGACCGCCTGTTGACCGTTATTG pACYC184 cloning
Z0461lambdaredP1	GCAATCAGAGTAGAGGTAAAGGTCATTTTCATTGTTTTATCCTT CGAGCGTGAGTGGTGGTGATGCCCGCTGTGTAGGCTGGAGCTGC TTCG
Z0461lambdaredP2	TTCATATAGTCAGCAACATGGAGACAACCATGCACGCGCGTTCA GCG AGAGAAATTAATCAATGCCATATGAATATCCTCCTTA
	Primers used for Real time PCR
Ler	(306)
escC	(306)
escV	(306)
Eae	(306)
espA	(306)
rpoA	(306)
Z0461-201F1	AACCCGCCGCAGCTTT
Z0461-262R1	TGGCCCACAAATGCTGATT
Z0463-343R1	CGGTGCCAGCGGGTATT
Z0463F	GCGCACCGCCTGTACTAACT
afuAF-887F1	TCGCCAAACGCTTTGGTT
afuB-1809F1	AGCCGGCATCGAAATAATGAC
afuB-1870R1	GGTCCGCCCGTTTCGT
afuC-894F1	CCAGACGCAAAATGGTGGTT
afuC-952R1	GCAAATGGTGACGCTGCTT
fucA-269F	GGCGCGCAAGGAATAGAA
fucA-328R	GATCCCCGCTATTCACTACATGA

fucP-1139F	CCAAATACGGTTCGTCCTTCA
fucP-1206R	ACCCATGACCGGAGTGACAA
fucR-260F	CGCTCGCGTGGATTGAA

Recombinant DNA techniques

Molecular biology techniques were performed as previously described (Sambrook et al., 1989). Primers used in qRT-PCR and cloning are listed in supplemental Table 2.

Isogenic mutant construction

Construction of isogenic *fusK*, *fusR* and *z0461* mutants was performed using a lambda- red mediated recombination method as previously described (Datsenko and Wanner, 2000). Briefly: a mutagenic PCR product was generated using primers containing homologous regions to sequences flaking *z0462* (for *fusK* mutant), *z0463* (*fusR* mutant), and *z0461* to amplify a chloramphenicol resistance gene from pKD3. 86-24 cells harboring pKD46 were electroporated using the mutagenic PCR product and selected for chloramphenicol (Cm) resistance. Nonpolar mutants were generated by resolving the Cm resistant clones with resolvase encoded by pCP20. For complementation of the mutants, *z0462* and *z0463* previously cloned in ZeroBlunt TOPO, digested with *BamHI* and *SalI* then cloned into pACYC184, generating the plasmid pARP12 and pARP13, respectively. pARP12 was electroporated into *fusK*- to generate ARP09 complemented strain; pARP13 was electroporated into *fusR*- to generate ARP10 complemented strain.

FusR purification

FusR was cloned into ZeroBlunt TOPO, digested using *XhoI* and *HindIII* restriction sites then cloned into pBADMycHisA, generating pARP11. pARP11 was subsequently transformed into TOP10 cells, generating the ARP04 strain. ARP04 strain was grown in LB to OD_{600} 0.6 at 37°C, at which point protein expression was induced by addition of a final concentration of 0.2% arabinose and growth overnight at 25°C. *FusR* was then purified using nickel columns (Qiagen).

FusK purification and Reconstitution into Liposomes

FusK was cloned into ZeroBlunt TOPO, digested using *XhoI* and *HindIII* restriction sites then cloned into pBADMycHisA. This plasmid was subsequently transformed into TOP10 cells, generating the ARP03 strain. ARP03 strain was grown LB at 37°C until OD_{600} 0.5 then protein expression was induced by addition of a final concentration of 0.2% arabinose and growth for 5 hours at 30°C. Cells were collected, resuspended in 50mL of Lysis buffer (50mM phosphate buffer pH 8.0, 1% Deoxycholic acid, 10mM imidazol, 300mM NaCl, 15% Glycerol, 5mM DTT, 100uL protease inhibitor cocktail), then lysed using emulsiflex. Lysates were incubated for 1 hour for solubilization then cleared by centrifugation at 18,000 rpm for 30 minutes. The soluble fraction was collected by ultracentrifugation at 45,000 rpm for 1 hour to obtain membrane fractions, then

membranes were resuspended in lysis buffer and incubated with Nickel beads for 1 hour at 4°C with gentle agitation. Membrane suspension and clear lysates were loaded into nickel–NTA columns, washed with Wash Buffer (50mM phosphate buffer pH 8.0, 20mM Imidazol, 300mM NaCl, 5mM DTT, 0.1% Deoxycholic acid) and eluted in three steps with elution buffer (250mM Imidazol, 300mM NaCl, 1mM DTT, 0.1% Deoxycholic Acid). Protein was concentrated using centricons with a molecular cutoff of 30,000KDa, then its concentration was determined by Bradford assays. Liposomes were loaded with *FusK* at a ratio of 20:1. Liposomes were reconstituted as previously described (Janausch et al., 2004). The presence of FusK into liposomes was confirmed by western blot using anti-Myc antibody (Invitrogen).

Autophosphorylation and Phosphotransfer Assays

Autophosphorylation assays were performed as described previously (41). To 100uL of FusK or QseC- loaded liposomes, 5mM $MgCl_2$ and 1mM DTT was added, and divided in 10uL aliquots. To each aliquot (in triplicate) it was added 2uL of 500uM monosaccharide (final concentration of 100uM) or deionized water as negative control. Liposome aliquots were frozen in liquid nitrogen three times then incubated statically at room temperature for 1 hour after the last freeze to allow reformation of the liposomes and incorporation of signals. After the incubation, phosphorylation reactions were initiated by addition 0.3uL of [γ^{32}P]

ATP and incubated at RT for 30 minutes (FusK) or 10 minutes (QseC). For time course experiments of FusK autophosphorylation, reactions were carried for 10, 20, 30, 45 and 60 minutes. In phosphotransfer reactions, 10ug of response regulators (FusR, QseB or QseF) were added, and reactions were let to proceed for 10, 30 and 60 minutes. All autophosphorylation reactions were stopped by addition of 3uL of 4X Laemmli buffer and run in a 12% polyacrylamide gel. Gels were dried at 80°C under vacuum for 1 hour, then exposed to the Phosphorimager overnight. The bands were quantitated using IMAGEQUANT version _ software (Amershan Pharmacia) and phosphorylation activity was stated as phosphorimager units. An average of the phosphorimager units was calculated for each replicate, and normalized to the average of the samples to which no signal was added. The error bars reflect the variation among the replicates. Data is expressed as fold change relative to no signal control. A Student's t test was used to determine statistical significancy. A *P* value of less than 0.05 was considered statistically significant. A concentration of 100uM L-fucose or D-glucose was used. The bands were quantitated using IMAGEQUANT version _ software.

Electrophoretic Mobility Shift Assay (EMSA)

EMSAs were performed using purified FusR-Myc-His and radiolabelled probes. Primers were end-labelled using [γ^{32}P]ATP and T4 polynucleotide kinase (NEB), and subsequently used on a PCR to generate radiolabelled probes. End-labelled

amplicons were run on a 6% polyacrylamide gel, excised, and purified using Qiagen Gel Extraction kit. To test the ability of *FusR* to directy bind to its target promoters, increasing amounts of FusR (0 to 4.35uM) were incubated with end-labelled probe (10 ng) for 20 minutes at 4°C in binding buffer (500ug/mL BSA, 50ng poly-dIdC, 6-mM HEPES pH 7.5, 5mM EDTA, 3mM DTT, 300mM KCl and 25mM $MgCl_2$). A sucrose solution was used to stop the reaction (249). The reactions were run on a 6% polyacrylamide gel for 6 hours and 30 minutes at 180V. The gels were dried under vacuum and EMSAs were visualized by autoradiography.

DNAseI Footprinting

DNAseI footprinting was performed as previously described (267). Briefly: primer Ler-18FP-R (Table 2) was end-labeled using [γ^{32}P]ATP and T4 polynucleotide kinase (NEB) and used in a PCR with cold primer Ler-299FP-F (Table 2) to generate probe LerFP. The resulting end-labeled probe was used in a binding reaction (described above in EMSA) for 20 min at room temperature. At this time, 1:100 dilution of DNAseI (NEB) and the manufacturer-supplied buffer were added to the reaction and digestion proceeded for 7 min at room temperature. The digestion reaction was stopped by addition of 100uL of stop buffer (200mM NaCl, 2mM EDTA and 1% SDS). Protein extraction was performed by phenol-chloroform and DNA was precipitated using 5M NaCl,

100% ethanol and 1uL glycogen. The DNAse reactions were run in a 6% polyacrylamide-urea gel next to a sequencing reaction (Epicentre). Amplicon generated using primers Ler-299FP-F and Ler-18FP-R (radiolabeled) was used as a template for the sequencing reaction. Footprint was visualized by autoradiography.

Nested Deletion Analysis

Transcriptional fusions of the *ler* promoter with promoterless *lacZ* were described before (261) and are listed in Table 1. To integrate the transcriptional fusions into the chromosome, *E.coli* MC4100 was lysogenized with phage λ45 and generating strains FS14 and FS16, respectively.

β-galactosidase activity assays

The bacterial strains FS14, FS15 and FS16 were transformed with p*FusR* or empty vector (pBADMycHisA) and grown in aerobically in DMEM containing 0.2% arabinose at 37°C to an OD_{600} of 0.8. The cultures were diluted 1:100 in Z buffer (60mM $Na_2HPO_4.7H_2O$, 50mM β-mercaptoethanol) and assayed for β-galactosidase activity by using o-nitrophenyl-β-D-galactopyranoside as substrate as previously described (184).

RNA extraction and Real-Time PCR

Bacterial cultures were grown aerobically overnight in LB at 37°C, diluted to 1:100 in appropriate media and incubated as described earlier. RNA from three biological replicates of each strain was extracted using TriZol followed by purification using RiboPure kit according to manufacturer's instructions. RNA concentration was determined using NanoDrop. Primers used in real time PCR were designed using software Primer Express v1.5 (Applied Biosystems). Primer validation was performed by verifying amplification efficiency and template specificity as described previously (307). Briefly: amplification efficiency of each primer was determined using standard curves of known RNA concentrations. Template specificity for each primer was determined by melting curve analysis performed as follows: products were heated at 95°C for 15s, followed by cool at 60°C and heating at 95°C while monitoring fluorescence. Real time RT-PCR was performed in a one-step reaction using Applied Biosystems ABI 7500 sequence detection system. Reactions of 20uL contained 10uL 2X Power Syber Green master mix (AB), 0.1uL multiscribe reverse transcriptase (AB), 0.1uL RNAse inhibitor (AB) and 0.25uM primer and 100ng of RNA. The analyses of unknown samples used the following conditions for cDNA generation and amplification: 1 cycle at 48°C for 30 min, 1 cycle at 95°C for 10 min, and 40 cycles at 95°C for 15s and 60°C for 1 min. The RNA polymerase subunit A, *rpoA*, was used as endogenous control. Data was collected using ABI Sequence Detection 1.3

Software (AB). Data was normalized to *rpoA* levels and analyzed using the Comparative critical threshold (Ct) method described previously. The expression level of each target gene was compared using the relative quantification method. Gene expression is represented as fold differences compared to the EHEC wild type strain 86-24. The error bars indicate the standard deviations of the $\Delta\Delta C_T$ values. The Student t-test was used to determine statistical significance using GraphPad Prism software. A *P*-value of less than 0.05 was considered significant.

Microarrays

Microarrays and analysis were performed as previously described (139). The GeneChip *E. coli* Genome 2.0 array system of the Affymetrix system was used to compare the gene expression in strain 86-24 to that in strains *fusK*- and *fusR*-. The GeneChip E. coli Genome 2.0 array includes approximately 10,208 probe sets for all 20,366 genes present in the following four strains of E. coli: K-12 lab strain MG1655, uropathogenic strain CFT073, O157:H7 enterohemorrhagic strain EDL933, and O157:H7 enterohemorrhagic strain Sakai (http://www.affymetrix.com/products/arrays/specific/ecoli2.affx). These chips were used to compare the transcriptome of EHEC 86-24 WT to the fusK- and fusR- strains. The RNA processing, labeling, hybridization, and slide-scanning procedures were preformed as described in the Affymetrix Gene Expression

Technical Manual (http://www.affymetrix.com/support/technical/manual/expression_manual.affx).

Microarray Data Analysis

The output from scanning a single replicate of the Affymetrix GeneChip *E. coli* Genome 2.0 array for each mutant strain was obtained using GCOS v 1.4 according to the manufacturer's instructions. Data were normalized using Robust Multiarray analysis at the RMAExpress website (http://rmaexpress.bmbolstad.com/). The resulting data were compared to determine features whose expression was increased or decreased in response to inactivation of the *fusK* and *fusR* genes. Custom analysis scripts were written in Perl to complete multiple array analyses. Expression data can be accessed using accession number GSE34991 at the NCBI GEO database.

Preparation of Secreted Proteins, Whole Cell Lysates and Immunoblotting

Secreted proteins from strains 86-24 WT, *fusK-, fusK+, fusR-* and *fusR+* were harvested as previously described (Walters and Sperandio). Briefly: Bacterial strains were grown at 37°C in DMEM until OD_{600} of 1.0. Cells were collected by centrifugation at 4,000rpm for 10 minutes and pellets were spared to prepare whole cell lysates (WCL). Supernatant fractions were filtered using

0.22um filters (Millipore) and treated with protease inhibitors (PMSF, aprotinin and EDTA); 10ug BSA was added as a control for precipitation. Total secreted proteins from culture supernatants were precipitated overnight at 4°C using 10% TCA and followed by ultracentrifugation at 28,000rpm for 2 hours. Precipitated proteins were collected and resuspended in 100uL of 1X PBS. For preparation of WCLs, spared pellets were resuspended in lysis buffer (50mM Tris-HCL, pH 7.5, 50 mM NaCl, 5% glycerol, 1 mM dithiothreitol, and 30 mM phenylmethylsulfonyl fluoride), treated with 30ug/mL lysozyme for 2 hours at 4C followed by treatment with DNaseI for 30 minutes at 4°C. Cell lysate suspensions were centrifugated at 13,000 rpm for 30 minutes for removal of cell debris and supernatants containing whole cell proteins were collected. Sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were completed as previously described (229). Secreted protein and WCLs were analyzed by Western blot using anti-EspA or anti-EspB antibody. As a loading control for secreted proteins samples was subjected to SDS-PAGE follwed by Coomassie staining; a major 60KDa band correspond to the BSA control. As a loading control for WCL, anti-rpoA antibody (Neoclone) was used to probe WCL samples. Western Blots were then visualized by enhanced chemiluminescence ECL system (BioRad).

Culture of HeLa and HT29 cells and PAS staining

Hela and HT29 cells were routinely grown in 75 cm^2 culture flasks (Corning) DMEM supplemented with 10% heat-inactivate fetal bovine serum (FBS) (Invitrogen) and 50ug/mL penicillin-streptomycin-glutamine (Invitrogen). Cells were stored in DMEM containing 10% FBS and 10% DMSO. HT29 cells were differentiated by addition of sodium butyrate into the culture medium every 2-3 days for 30 consecutive days (7). Mucus production was detected by periodic acid Schiff staining (163). Briefly: HT-29 cells were fixed with 4% paraformaldehyde for 20 minutes and permeabilized with 1% Triton X-100. Fixed cells were treated with 1% periodic acid solution for 30 minutes before application of Schiff's reagent. Stained cells were visualized by brightfield microscopy.

Fluorescent Actin Staining (FAS) Assay

Fluorescein actin staining (FAS) assays were performed as previously described (Knutton et al., 1989). Pedestal enumeration was performed in 600 infected cells. The Student t- test was used to determine statistical significance. A *P*-value of less than 0.05 was considered significant.

Adhesion assays

Bacterial strains were grown for 18 hours in LB at 37◦C and 1 X 10^8 cfu were added to HeLa cells and incubated for 6 hours at 37◦C 5% CO_2. For quantification of adherence, after a 6 hour incubation, the non-adherent bacteria were removed by washing with PBS and the HeLa cells lysed with 1% triton. Serial dilutions of the bacterial cells were plated in LB agar plates and colony forming unites (cfu) counted.

Infant rabbit infection studies

Litters of 3-day old infant rabbits were infected as described previously (Ritchie et al 2003). Individual rabbits were oro-gastrically inoculated (approx. 5 x 10^8 cfu per 90g) with 1:1 mixtures of wild type (*lacZ-)* EHEC and the *fusK*-mutant. The animals were necropized 2 days post-inoculation and colonic tissue samples removed and homogenized prior to microbiological analysis. The number of wild type and *fusK* mutant cells present in the tissue homogenate was determined by serial dilution and plating on media containing Sm and bromo-chloro-indoyl-galactopyranoside (X-gal) as previously described (Ritchie et al 2003). Competition indexes (CI) were calculated as the ratio of *fusK* to wild type in tissue homogenates divided by the ratio of *fusK* to wild type in the input. The CI was compared to the CI value obtained when otherwise isogenic *lacZ*+ and *lacZ*- wild type strains were given to rabbits. Differences in CIs were compared

using the Mann-Whitney test, where a P-value of less than 0.05 was considered significant.

Statistical Analysis

To determine statistical significant for real time PCR experiments, it was performed Two-way ANOVA with Bonferroni's multiple comparison test using GraphPad Prism Software. For *in vivo* assays, the Mann-Whitney test was employed to compare differences in competitive index. For microarray analysis, a *P* value of less than 0.05 was considered statistically significant.

CHAPTER FOUR

Fucose Sensing by a Novel Two-Component System Regulates Virulence Gene Expression and Bacterial Intestinal Colonization

INTRODUCTION

The mammalian gastrointestinal (GI) tract provides a complex and competitive environment for the resident microbiota, which is ordinarily composed of trillions of bacteria cells, from over 1,000 species (105, 106). Successful intestinal colonization by pathogenic bacteria is thought to depend on their scavenging nutrients, sensing chemical signals present in the intestine, competing with the resident bacteria for space and nutrients, and precisely regulating expression of virulence genes (72). The interactions between enteric pathogenic bacteria and the commensal microbiota that occur when pathogens colonize the intestine have not been well characterized.

The human pathogen EHEC colonizes the colon. Besides diarrhea, clinical manifestations of EHEC can include hemorrhagic colitis and hemolytic uremic syndrome (HUS), which result from EHEC production of Shiga toxins (134). EHEC intestinal colonization is dependent on the locus of enterocyte effacement (LEE) pathogenicity island (PAI) (134). This PAI encodes a master regulator for its own expression, *ler*, a type 3 secretion (T3S) apparatus, translocator proteins

EspA and EspB, an adhesin, intimin, and its own receptor, the translocated intimin receptor (Tir) (Kaper et al., 2004). Expression of LEE genes leads to the formation of attaching and effacing (AE) lesions on enterocytes. AE lesions are characterized by extensive remodeling of the host cell cytoskeleton, leading to effacement of the microvilli and formation of a pedestal-like structure beneath the bacteria (37, 82, 291) (55, 297).

Expression of LEE genes and Shiga toxin is regulated by an inter-kingdom chemical signaling system involving the host hormones epinephrine and/or norepinephrine and the microbial flora-produced signal autoinducer 3 (AI-3) (269). These signals are sensed by two bacterial adrenergic receptors, QseC (41) and QseE (235)that are histidine sensor kinases. Upon sensing these signals, QseC and QseE autophosphorylation increases, initiating a signaling cascade that promotes virulence gene expression.

During colonization of the human colon, pathogenic bacteria encounter two major barriers: the mucus layer that overlays the intestinal epithelium and the abundant and diverse intestinal microbiota (105, 106). The mucus layer has a complex composition that harbors most of the carbohydrate content in the intestine; these carbohydrates are a source of nutrients for commensal bacteria (178). Fucosylated glycans are abundantly expressed on the surface of intestinal epithelial cells (26, 40, 47), and are scavenged by the microbiota for use as an energy source (47). Pathogens, like commensals, can utilize carbohydrates from

the mucus layer (38, 185, 225). Bacterial pathogens such as EHEC can grow on mucus (302), and previous studies showed that carbohydrates from the mucus can support EHEC colonization of mice (185).

Here, we identified a new EHEC two component system (TCS) that is repressed by the EHEC QseC/B and QseE/F signaling systems. This TCS, here named FusK/R, represses expression of LEE genes as well as genes for fucose utilization. FusK, the sensor component of this TCS, autophosphorylates in response to fucose, thus revealing for the first time a signal transduction mechanism that senses fucose to regulate expression of the LEE as well as EHEC intestinal colonization. In aggregate, our findings suggest that EHEC uses fucose, a host-derived signal made available by the microbiota, to modulate EHEC pathogenicity and metabolism.

RESULTS

A novel two-component system acquired by horizontal gene transfer in EHEC

Bacteria-host communication in the gut is important to modulate expression of virulence genes by pathogenic bacteria (Hughes and Sperandio, 2008). Two-component systems (TCS) play a major role in bacterial signal sensing and transduction. TCS represent a widespread sensory mechanism in bacteria, and consist of a histidine sensor kinase (HK) and a response regulator (RR). Upon sensing a signal, the sensor HK autophosphorylates at a conserved histidine residue and then transfers its phosphate to a conserved aspartate residue in the RR (323). Subsequently, most RRs bind DNA, promoting changes in gene expression (275). The cognate RR for QseC is QseB, and for QseE is QseF (Fig 4.1A). In a microarray analysis comparing differential gene expression between *qseC*, *qseB* (110), *qseE* and *qseF* (234) mutants to WT EHEC, expression of the genes *z0462* and *z0463* was increased in all of these four mutants. To confirm the transcriptomic data, quantitative real time PCR (qRT-PCR) was performed to assess *z0462* expression in the *qseB*, *qseC*, *qseE* and *qseF* mutants. *Z0462* expression was significantly increased in each of the mutant strains compared to the WT, confirming that *z0462* is repressed by the QseBC and QseEF systems (Figure 4.1B). These data suggested that Z0462/Z0463 could be part of the

signaling cascade initiated by the QseBC and QseEF TCSs. Because QseC is the sensor for the AI-3 signal involved in inter-species communication (41), we tested whether addition of AI-3 affected expression of *z0462* and *z0463*. Congruent with AI-3 activation of QseC, and QseC's repression of Z0462/Z0463 transcription, addition of AI-3 decreased transcription of these genes (Fig. 4.1D). To investigate whether QseB/QseF regulation of Z0462 expression was direct, eletrophoretic mobility shift assays (EMSA) were performed and showed that QseB directly binds to the z0462/z0463 promoter, while QseF did not (Figure 4.1E). These data show that QseB repression of *z0462*/*z0463* expression is direct, while QseF-mediated repression is likely indirect. This finding is in agreement with the fact that QseF is a sigma 54-dependent transcriptional regulator, and these proteins act as activators of gene transcription (13). QseF likely activates the expression of a repressor of *z0462*/*z0463* transcription. Thus, QseB, QseC, QseE and QseF repress expression of *z0462*/*z0463*.

The genes encoding Z0462 and Z0463 are clustered in a pathogenicity island (PAI) [EHEC O-island 20 (OI-20)] (227) which is found in EHEC O157:H7 strains and enteropathogenic *E. coli* strains exclusively from the 055:H7 serotype (which gave rise to the O157:H7 EHEC serotype), but absent in all other *E. coli* strains. The remaining genes in OI-20 encode for a putative ferric iron ABC transporter (AfuABC) and a putative permease (Z0461) that belongs to the major facilitator superfamily (MFS) of transporters (224) (Fig.4. 1F). Analyses of

the operonic structure of OI-20 by reverse transcriptase PCR (RT-PCR) suggest that the genes in this PAI are organized in three transcriptional units: *z0462*/*z0463*, *z0461* and *afuABC* (Fig. 4.1F). The genes *z0462/z0463* encode for a putative TCS: *z0462* encodes a sensor HK, and *z0463* encodes a RR. Z0462 is a predicted membrane-bound HK of 513 amino acids (aa) (molecular weight of 57.98 KDa) that has 8 transmembrane domains. It is predicted to be a hexose phosphate sensor kinase, and it shares low levels of sequence similarity to a glucose-6-phosphate sensor, UhpB (~30%). Like UhpB, Z0462 has a membrane-associated sensing domain (MASE1), which are found in HKs, diguanylate cyclases and other bacterial signaling proteins in γ-proteobacteria (209). Z0462 also harbors a conserved dimerization-histidine phosphotransfer domain (DHp) and an ATPase domain. Z0463 is a predicted RR of 209 aa (molecular weight 22.8 KDa). Domain prediction (Smart) indicated that Z0463 has a conserved receiver domain (REC, CheY-like) at its N-terminus and a LuxR-type DNA binding domain at its C-terminus (Figure 4.1B).

Z0462/Z0463 comprise a functional and cognate TCS.

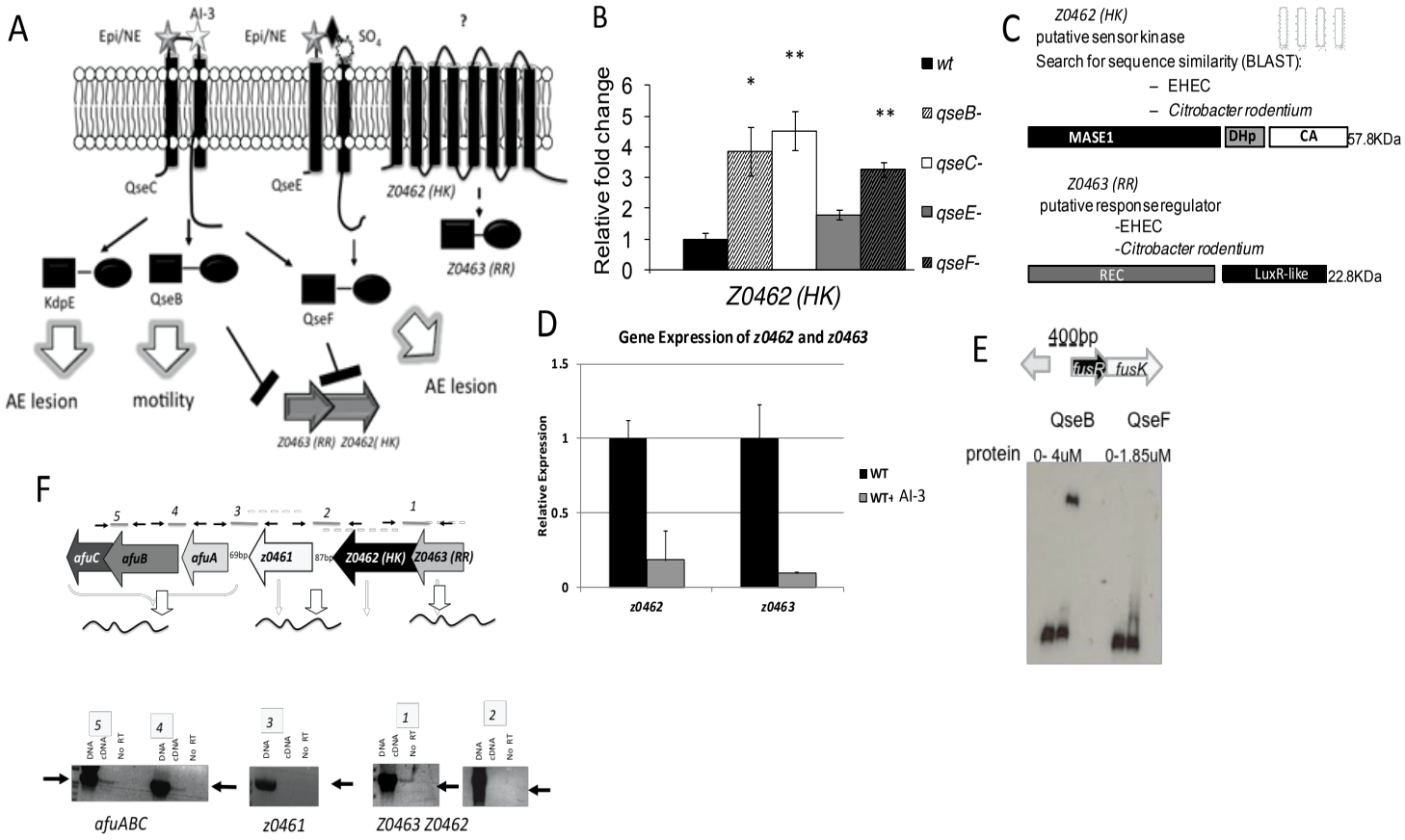
A
AI-3
Epi/NE
Epi/NE
SO4
?
QseC
QseE
Z0462 (HK)
Z0463 (RR)
KdpE
QseB
QseF
AE lesion
motility
AE lesion
Z0463 (RR) Z0462(HK)
B
Relative fold change
wt
qseB-
qseC-
qseE-
qseF-
Z0462 (HK)
C
Z0462 (HK)
putative sensor kinase
Search for sequence similarity (BLAST):
– EHEC
– Citrobacter rodentium
MASE1
DHp
CA
57.8KDa
Z0463 (RR)
putative response regulator
-EHEC
-Citrobacter rodentium
REC
LuxR-like
22.8KDa
D
Gene Expression of z0462 and z0463
Relative Expression
WT
WT+ AI-3
z0462
z0463
E
400bp
fusR
fusK
QseB
QseF
protein
0- 4uM
0-1.85uM
F
afuC
afuB
afuA
69bp
z0461
87bp
Z0462 (HK)
Z0463 (RR)
DNA
cDNA
No RT
afuABC
z0461
Z0463 Z0462

Figure 4.1. The novel TCS FusKR of EHEC.

A) Schematic representation of the QseC/QseE signaling cascade. QseC senses AI-3, epinephrine (Epi) and NE. QseE senses Epi/NE, SO_4 and PO_4. QseC transfers its phosphate to QseB, which activates transcription of the flagellar genes; KpdE activates the LEE promoting formation of AE lesion; and QseF. QseE only phosphorylates QseF. Microarray analysis comparing differential gene expression of *qseC*- and *qseF*- compared to WT EHEC revealed that an unknown TCS, Z0462 (HK) and Z0463 (RR), was repressed by QseBC and QseEF.

B) qRT-PCR analysis of EHEC WT, *qseB*, *qseC*, *qseE* and *qseF* strains for *z0462* expression grown in DMEM. Relative gene expression is represented as fold differences normalized to EHEC WT strain 86-24. Each sample was normalized to *rpoA* as an internal control. The error bars indicate the standard deviations of ΔddCt values. Significance is indicated as follows: one asterisk, $P \leq 0.01$; two asterisks, $P \leq 0.001$.

C) Domain organization of Z0462 and Z0463.

D) Expression of *z0462* and *z0463* in WT in the absence and presence of AI-3.

E) EMSA of QseB and QseF with the regulatory region of *z0463*.

F) Schematic diagram of the genetic organization of OI-20. Numbers flanking *z0461* represent amount of base pairs separating *z0461* from upstream and downstream genes in OI-20. Analysis of the operonic structure of OI-20 genes by RT-PCR. The genes in OI-20 are organized in three transcriptional units: *z0462z0463*, *z0461* and *afuABC*.

To determine if Z0462 is a functional HK, we reconstituted epitope-tagged Z0462 into liposomes, and assessed its capacity for autophosphorylation as previously described (Clarke et al., 2006; Reading et al., 2009). Time-course assays show that Z0462 autophosphorylation was detectable at 10 minutes and continued to increase until at least 60 minutes (Figure 4.2A). Typically, genes encoding for cognate TCS are co-transcribed, constituting one operon. Because *z0462* and *z0463* are co-transcribed (Figure 4.1F), we tested whether Z0463 could be the cognate RR for the HK Z0462. Phosphotransfer assays using liposome-reconstituted Z0462 and soluble Z0463, showed that Z0462 transferred its phosphate to Z0463 starting at 10 minutes, and phosphotransfer rates increased over time (Figure 4.2B). Together these *in vitro* reconstitution experiments suggest that Z0462 and Z0463 constitute a cognate TCS.

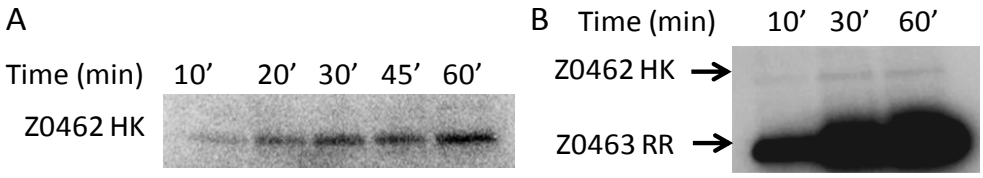

Figure 4.2. Z0462/Z0463 is a cognate TCS.

A) Autophosphorylation assay of Z0462 histidine kinase in liposomes.

B) Phosphotransfer assay from Z0462 HK (in liposomes) to Z0463 RR (ratio 1 HK: 4 RR).

Z0462/Z0463 represses the expression of the type III secretion system and AE lesion formation.

To gain a global view of gene regulation by Z0462 and Z0463, microarray analyses comparing gene expression in *z0462*- and *z0463*- strains to WT were carried out. In these experiments, bacteria were grown in DMEM until late logarithmic phase, conditions known to induce the expression of genes in the LEE PAI. In the *z0462*- strain, 785 genes exhibited increased expression relative to WT, while 433 genes had decreased expression. In the *z0463*- strain, a total of 660 genes had increased expression and 273 genes had decreased expression (Table 4). Thus, Z0462 and Z0463 appear to mainly act as repressors of gene transcription in EHEC, since a greater number of differentially regulated genes in *z0462*- and *z0463*- mutants had increased expression.

Table 4. Summary of genes activated or repressed upon gene deletion of *z0462* and *z0463*.

	NUMBER OF GENES WITH ALTERED EXPRESSION IN THE ISOGENIC MUTANT STRAINS	
	MUTANT STRAIN	
	86-24 *Z0462-*	**86-24 *Z0463-***
INCREASE	**785**	**660**
MARGINAL INCREASE	**452**	**386**
NO CHANGE	**8279**	**8004**
MARGINAL DECREASE	**433**	**885**
DECREASE	**259**	**273**
TOTAL	**10208**	**10208**

Notably, both Z0462 and Z0463 repress the expression of genes in the LEE PAI, including *ler*, the master regulator of LEE gene expression (Figure 4.3). Also, additional OI-20 genes are differentially regulated in the *z0462* and *z0463* mutant strains. qRT-PCR analyses confirmed that LEE genes are upregulated in

the mutant strains. These assays revealed that transcripts from all 5 of the principal LEE operons (*LEE1-5*; (180)) were more abundant in the *z0462* (Figure 4A) and *z0463* (Figure 4C) mutants. Complementation *in trans* was able to restore the expression of *ler* to wild type levels (Figures 4B and 4D). These results indicate that Z0462/Z0463 repress the expression of the LEE.

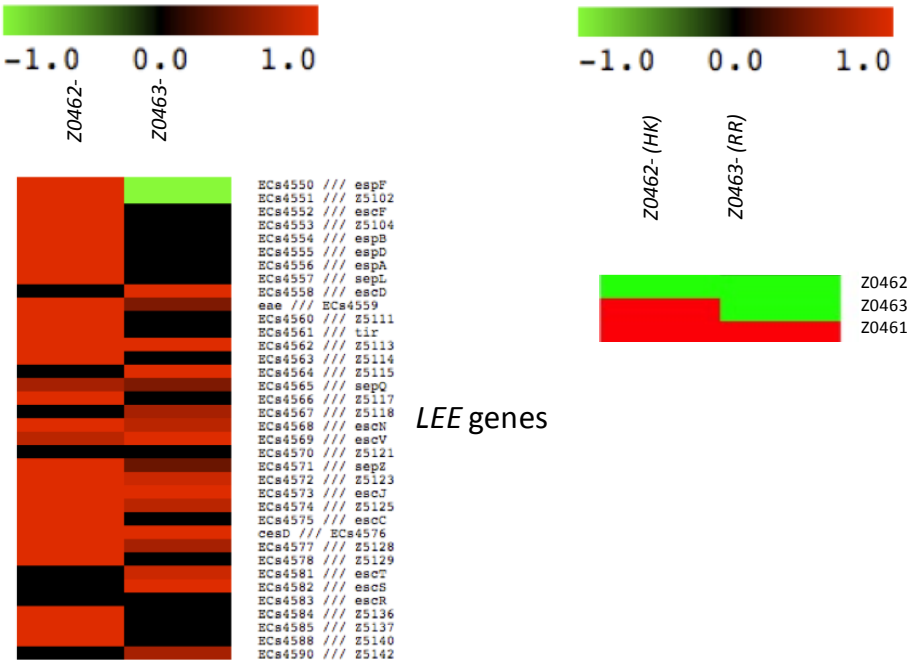

Figure 4.3. Heat map from microarray analysis representing genes with altered expression in the *z0462*- and *z0463*- compared to EHEC WT. Fold differences are represented in a $\log_2$ scale. Genes whose transcription was decreased or increased (at least 2-fold) are represented by green and red spots, respectively.

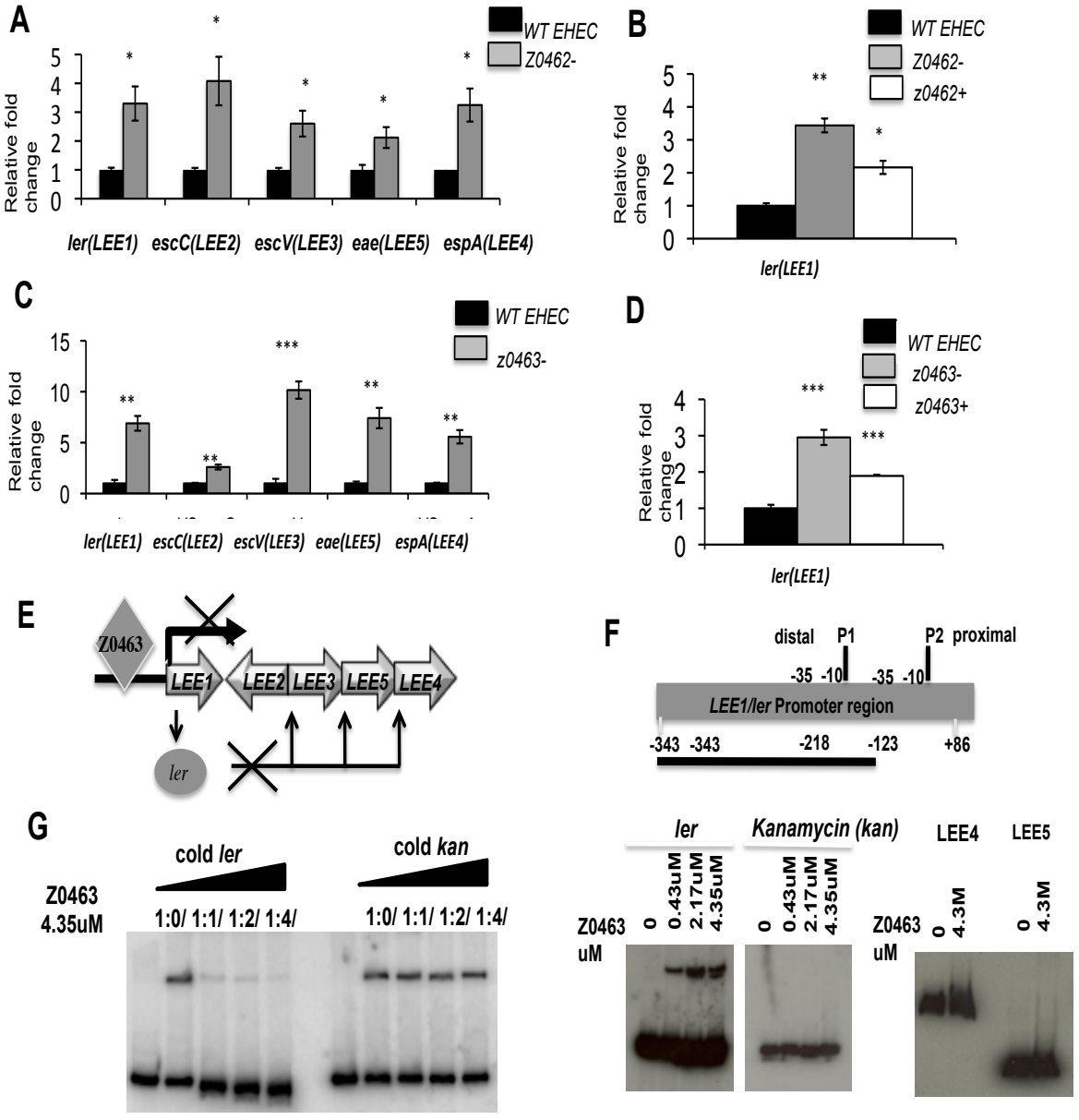
A
WT EHEC
Z0462-
Relative fold change
ler(LEE1) escC(LEE2) escV(LEE3) eae(LEE5) espA(LEE4)
B
WT EHEC
Z0462-
z0462+
Relative fold change
ler(LEE1)
C
WT EHEC
z0463-
Relative fold change
ler(LEE1) escC(LEE2) escV(LEE3) eae(LEE5) espA(LEE4)
D
WT EHEC
z0463-
z0463+
Relative fold change
ler(LEE1)
E
Z0463
LEE1
LEE2
LEE3
LEE5
LEE4
ler
F
distal
P1
P2
proximal
-35 -10
-35 -10
LEE1/ler Promoter region
-343 -343
-218
-123
+86
G
cold ler
cold kan
Z0463 4.35uM
1:0/ 1:1/ 1:2/ 1:4/
1:0/ 1:1/ 1:2/ 1:4/
ler
Kanamycin (kan)
LEE4
LEE5
Z0463 uM
0
0.43uM
2.17uM
4.35uM
0
0.43uM
2.17uM
4.35uM
Z0463 uM
0
4.3M
0
4.3M

Figure 4.4. Z0462/z0463 regulates expression of the LEE.

A) Relative transcript abundance of LEE genes in EHEC WT and *z0462-* grown in DMEM, measured by qRT-PCR. Expression of each of the major 5 LEE operons (*LEE 1- 5)* is represented by analysis of a single gene. Significance is indicated as follows: one asterisk, P<0.001; two asterisks, P<0.0001.
B) Relative transcript abundance of *ler* in WT, *z0462-* and *z0462+* complemented strains based on qRT-PCR analysis of strains grown in DMEM. Significance is indicated as follows: one asterisk, P≤0.001; two asterisks, P≤0.0001.
C) Transcriptional profile of LEE genes in EHEC WT and *z0463-,* measured by real time PCR in DMEM. Significance is indicated as follows: two asterisks, P <0.001; three asterisks, P≤0.0001.
D) qRT-PCR analysis of expression of *ler* in WT, *z0463-* and *z0463+* complemented strains in DMEM. Significance is indicated as follows: one asterisk, P≤0.001; two asterisks, P≤0.0001.
E) Schematic representation of the LEE operons and regulation through Ler and Z0463. F) EMSAs of ler, kan, LEE4 and LEE5 with Z0463.
G) Competition EMSA of ler with Z0463 with ler and kan cold probes.

Because transcription of the LEE genes is activated by Ler (180), and *ler* transcription is repressed by Z0462 and Z0463, we reasoned that the RR Z0463 directly repressed transcription of *ler*, and consequently the other LEE operons in a cascade fashion (Figure 4E). Congruent with this hypothesis, Z0463 bound to the *ler* (*LEE1*) regulatory region and not to *LEE4*, *LEE5* or the *kan* gene (negative control) (Figure 4F). Addition of cold *ler* probe competed Z0463 binding to the

ler promoter, but addition of cold *kan* did not, suggesting that this binding is specific (Figure 4G). Phosphorylation of the RR in a conserved aspartate residue in the receiver domain causes conformational changes in the RR, what promotes binding to a target DNA sequence (275). To assess whether phosphorylation of Z0463 affects binding to the *ler* promoter, we performed EMSAs in the presence or absence of acetyl phosphate (AcP), used as phosphodonor. As shown in this figure 4.5, in the presence of acetyl phosphate, Z0463 binds the *ler* promoter in nanomolar amounts, while in the absence of acetyl phosphate, micromolar amounts of Z0463 are necessary to shift the *ler* probe (Figure 4.5A-B). These results indicate that phosphorylation of Z0463 increases the affinity for its target region. Finally, nested deletion analyses, followed by more EMSAs and footprinting experiments identified the binding region of Z0463 to the *ler* promoter. The *ler* gene has two promoters (P2 proximal, and P1 distal), and Z0463 binds in between these promoters (Figure 4.6C), decreasing *ler* transcription.

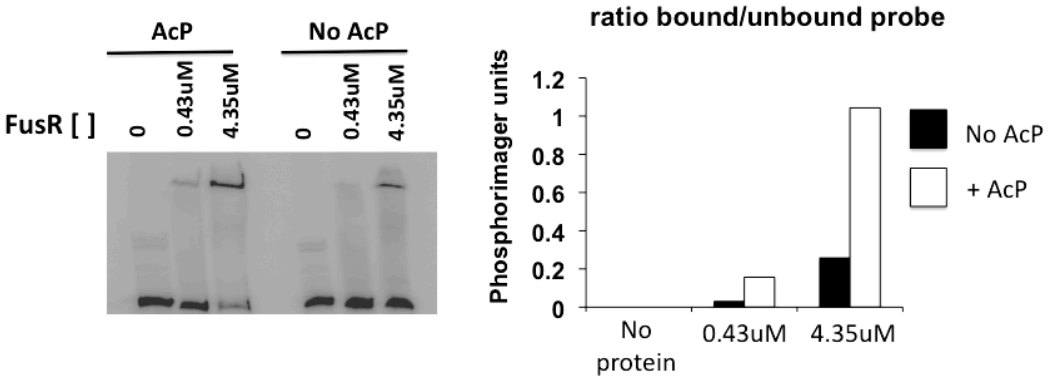

Figure 4.5. Phosphorylation of Z0463 improves binding to *LEE1* promoter. A) EMSA of *ler* with Z0463 in the absence and presence of acetyl phosphate (AcP). B) Quantification of bound probe shown in (A) using ImageQuant. Graph represents ratio of bound/unbound probe measured in phosphorimager units, in reactions with (+ AcP) and without acetyl phosphate (No AcP).

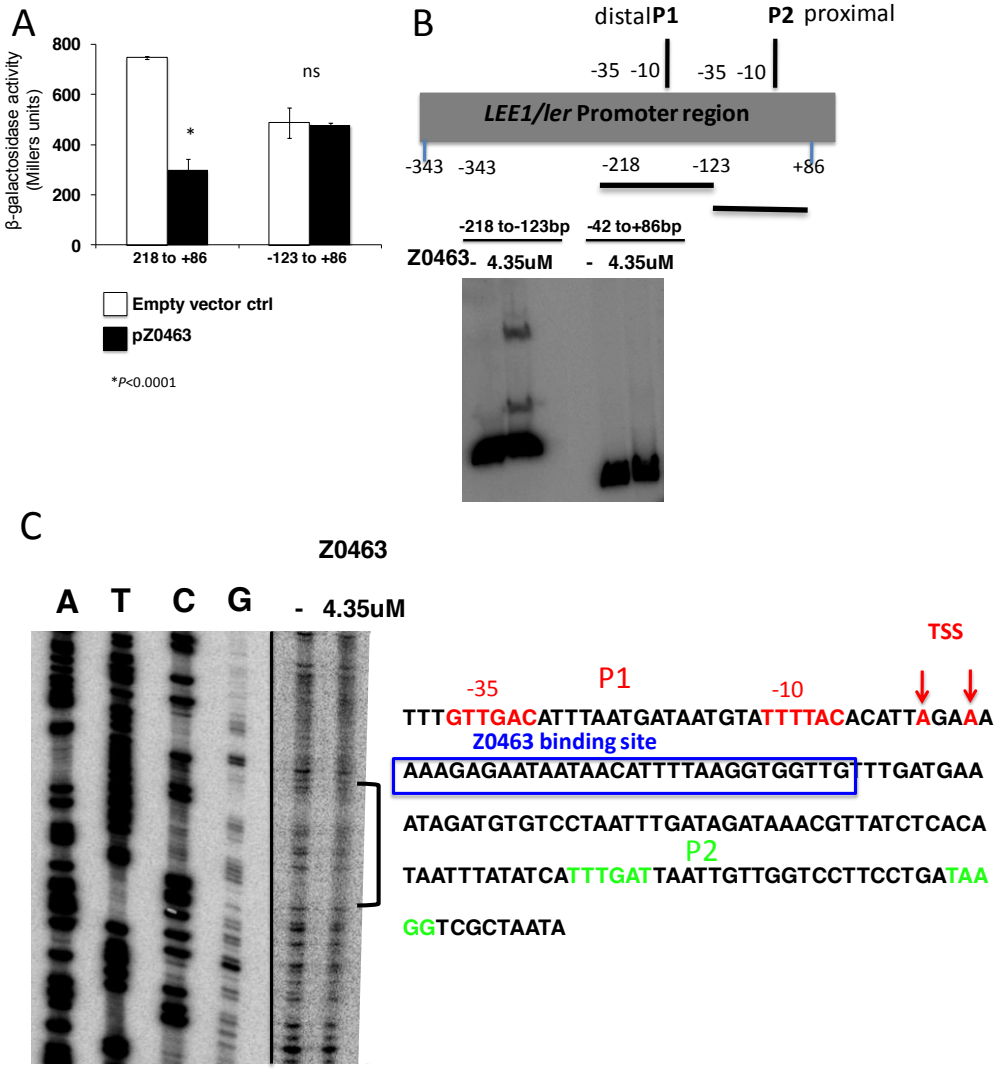

Figure 4.6. Z0463 binding site on *LEE1* promoter.

A) Nested deletion analyses of the *LEE1* (*ler*) promoter region. Transcriptional fusions of different fragments from the *LEE1* regulatory region (-218 to +86bp and -123 to +86bp) were generated and introduced into the K-12 MC4100 strain genome (does not have the *z0463*), and assayed for β-galactosidase activity in the presence of empty vector or Z0463. B) EMSA of smaller fragments of the *LEE1* promoter with Z0463.

C) DNA footprinting of the *LEE1* promoter with Z0463. The two *LEE1* promoters are depicted in red (P1) and green (P2), and the Z0463 binding region is boxed in blue. One asterisk, P<0.001.

Congruent with the increased expression of the LEE in the *z0462*- and *z0463*- strains, both mutants secreted more EspB, a T3SS translocon component, into the supernatant (Figure 4.6A), and formed more pedestals per cell compared to the WT (Figures 4. 6B-C). The *z0462*- (68.9%, P<0.0001) and *z0463*- (61.4%, P<0.0001) strains often presented more than 10 pedestals per cell compared to the WT (26.1%) (Figures 4.6B and 4.6C). Complementation *in trans* restored pedestal formation to levels similar to WT (Figures 4.6B-C). Therefore, we concluded that Z0462 and Z0463 repress AE lesion formation on tissue-cultured cells. It is worth noting that this effect is on AE lesion formation, and not adhesion, given that adhesion of all five strains to epithelial cells is similar (Figure 4.8).

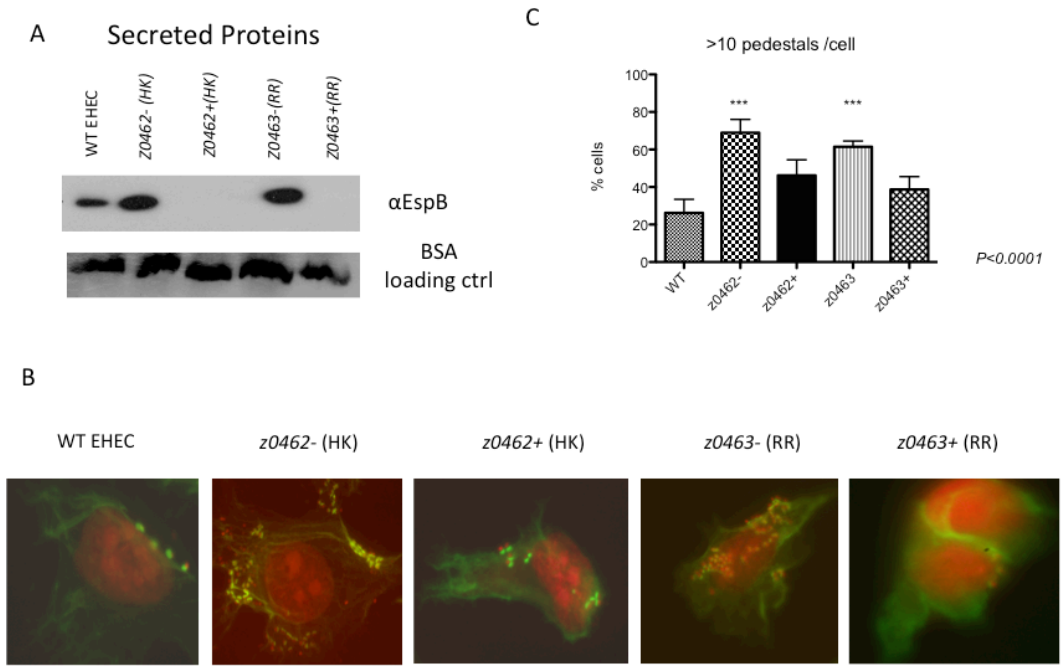

Figure 4.7. Z0462 and Z0463 repress T3S and A/E formation.

A) Western blot of the secreted protein EspB detected in the supernatants of EHEC WT, *z0462-*, *z0462+*, *z0463-* and *z0463+* strains. BSA was added to supernatants to be used as a control for protein precipitation of supernatants and loading.

B) FAS assay. HeLa cells were infected with EHEC WT, *z0462-*, *z0462+*, *z0463-* and *z0463+*. After 6 hours, cells were fixed, stained with FITC-phalloidin (actin) and propidium iodide (bacteria and HeLa DNA) and observed with fluorescence microscopy.

C) Quantification of FAS assay. Infection of HeLa cells with each bacterial strain was performed in triplicate. A hundred cells were counted in 3 separate coverslips (triplicate). Significance is indicated as follows: three asterisks, P<0.0001.

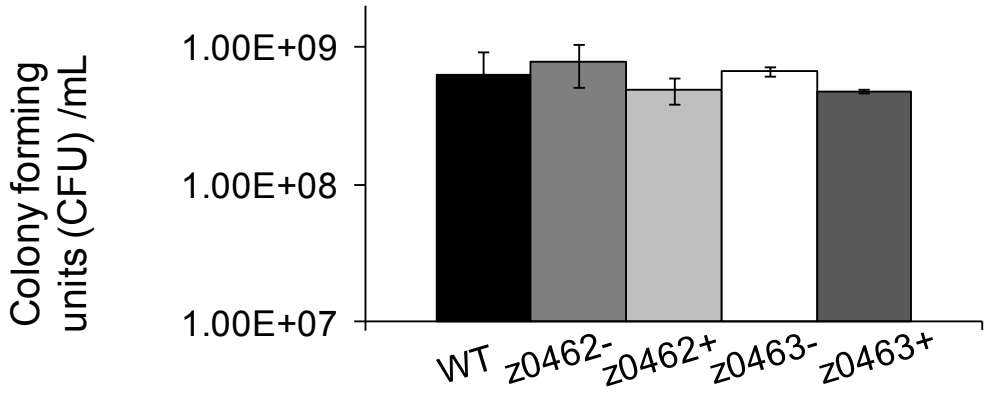

Figure 4.8. **Adhesion assay.** HeLa cells were infected with EHEC WT, *z0462*-, *z0462*+, *z0463*- and *z0463*+ strains. After 6 hours, bacteria were serially diluted and plated for CFU count.

Z0462 (FusK) senses fucose and regulates fucose utilization

It has previously been demonstrated that *z0462* and *z0463* are highly expressed when EHEC was grown in bovine mucus (9). To investigate whether *z0462*/*z0463* expression is regulated by the presence of mucus, we infected differentiated mucus producing HT29 cells with WT EHEC and measured expression of *z0463*. EHEC (WT) infected undifferentiated HT29 cells were used

as controls, since they do not produce mucus. We observed a remarkable (>16-fold) increase in *z0463* expression in the presence of differentiated mucus producing HT29 cells compared to undifferentiated non- mucus producing HT29 (Figure 4.9A). These results suggest that *z0463* expression is activated by the presence of mucus produced by the HT29 cell line.

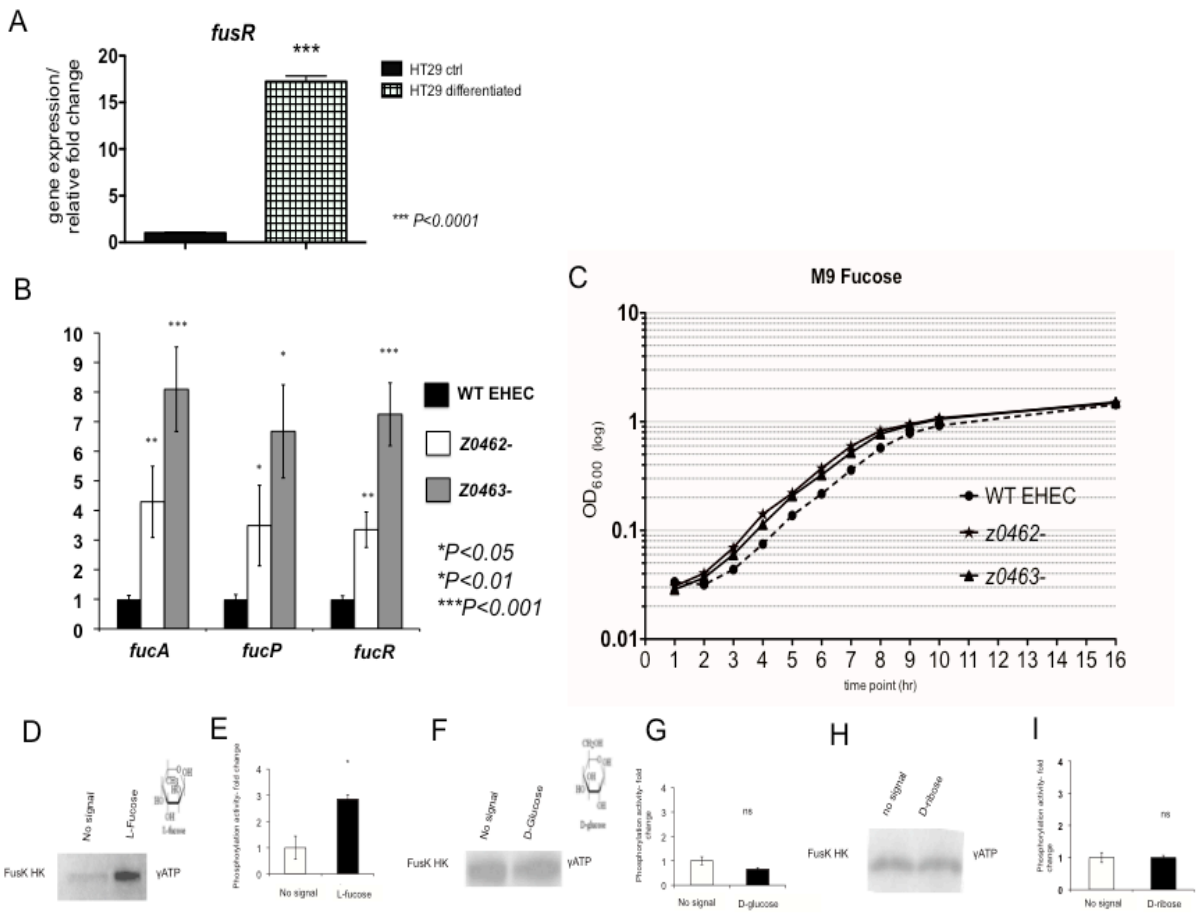
A
fusR
gene expression/ relative fold change
HT29 ctrl
HT29 differentiated
*** P<0.0001
B
WT EHEC
Z0462-
Z0463-
*P<0.05
*P<0.01
***P<0.001
fucA
fucP
fucR
C
M9 Fucose
OD600 (log)
WT EHEC
z0462-
z0463-
time point (hr)
D
No signal
L-Fucose
FusK HK
γATP
E
No signal
L-fucose
F
No signal
D-Glucose
FusK HK
γATP
G
ns
No signal
D-glucose
H
no signal
D-ribose
FusK HK
γATP
I
ns
No signal
D-ribose

Figure 4.9. Z0462/Z0463 is a fucose sensing TCS.
A) qRT-PCR analysis of expression of *z0463* RR in WT EHEC in the presence of undifferentiated non-mucus producing HT29 (not treated with sodium butyrate) or differentiated HT29 mucus-producing cells (treated with sodium butyrate). RNA samples were extracted from cell culture monolayers infected with WT EHEC as for FAS assay. The error bar indicates the standard deviations of ΔddCt values. Significance is indicated as follows: three asterisks, P≤0.0001.
B) Transcriptional profile of fucose utilization genes in EHEC WT, *z0462*- and *z0463*- strains grown in DMEM to an OD_{600} 1.0, measured by qRT-PCR. Significance is indicated as follows: one asterisk, P≤0.05; two asterisks, P≤0.01; three asterisks, P≤0.001.
C) Growth curve analysis of EHEC WT, *z0462*- and *z0463*- strains in M9 minimal media with L-fucose as a sole carbon source.
D) Autophosphorylation assay of the FusK histidine kinase (in liposomes) in the presence of L-fucose. Significance is indicated as follows: one asterisk asterisk, P≤0.01. E) Quantification of triplicates of autophosphorylation assays of FusK in the presence of L-fucose. Phosphorylation activity is represented at fold change compared to autophosphorylation in the absence of signal. Error bar indicates the standard deviation of fold change values. Quantification performed using ImageQuant.
F) Autoradiograph of autophosphorylation assay of FusK histidine kinase in the presence of D-glucose.
G) Quantification of triplicates of autophosphorylation assays of FusK in the presence of D-glucose.
H) Autoradiograph of autophosphorylation assay of FusK histidine kinase in the presence of D-ribose.
I) Quantification of triplicates of autophosphorylation assays of FusK in the presence of D-glucose.

Z0462 has a predicted sugar sensor (hexose-phosphate-sensor) domain. Hence, we hypothesized that Z0462 may sense sugars present in the intestinal mucus and modulate carbohydrate utilization. Fucose is a major component of mucin glycoproteins, and it is highly abundant in the intestine (244). Furthermore, recent reports have suggested that EHEC fucose utilization is important for the pathogen to colonize the intestine (69, 263). In *E.coli,* L-fucose utilization involves an inducible pathway mediated by a permease (*fucP*), an isomerase (*fucI*), a kinase (*fucK),* an aldolase (*fucA*), an oxidoreductase (*fucO*), and an activator protein (*fucR*) (39). We investigated whether Z0462/Z0463 influenced the expression of the fucose utilization genes in EHEC using qRT-PCR. In the *z0462*- and *z0463*- strains, expression of *fucA*, *fucP* and *fucR* was increased relative to the WT strain, suggesting that Z0462/Z0463 repress the expression of the fucose utilization system (Figure 4.9B). The repression of *fuc* genes by Z0462/Z0463 was more pronounced in the *z0463*- strain. This pattern of gene regulation by RRs and HKs is commonly found, as small intracellular phosphodonors such as acetyl-phosphate could phosphorylate the RR at basal levels independently of the HK (175). Additionally, cross- phosphorylation of the RR by non-cognate HKs could account for the increased effect of deletion of the RR vs the HK (323). Congruent with the qRT-PCR data, the *z0462*- and *z0463*- mutants grew faster in the presence of L-fucose as a sole carbon source in M9 medium compared to the WT strain. The generation times are of 92.4 minutes for

the WT, 64 minutes for *fusK*- and 84 minutes for *fusR*- (Figure 4.9C). Therefore, Z0462/Z0463 regulate fucose utilization genes. It is worth noting that this response is specific to fucose, with the mutants and WT growing at similar rates when other sugar sources (D-glucose, D-galactose, L-rhamnose, D-xylose) are provided as the sole carbon source (Figure 4.10A-D).

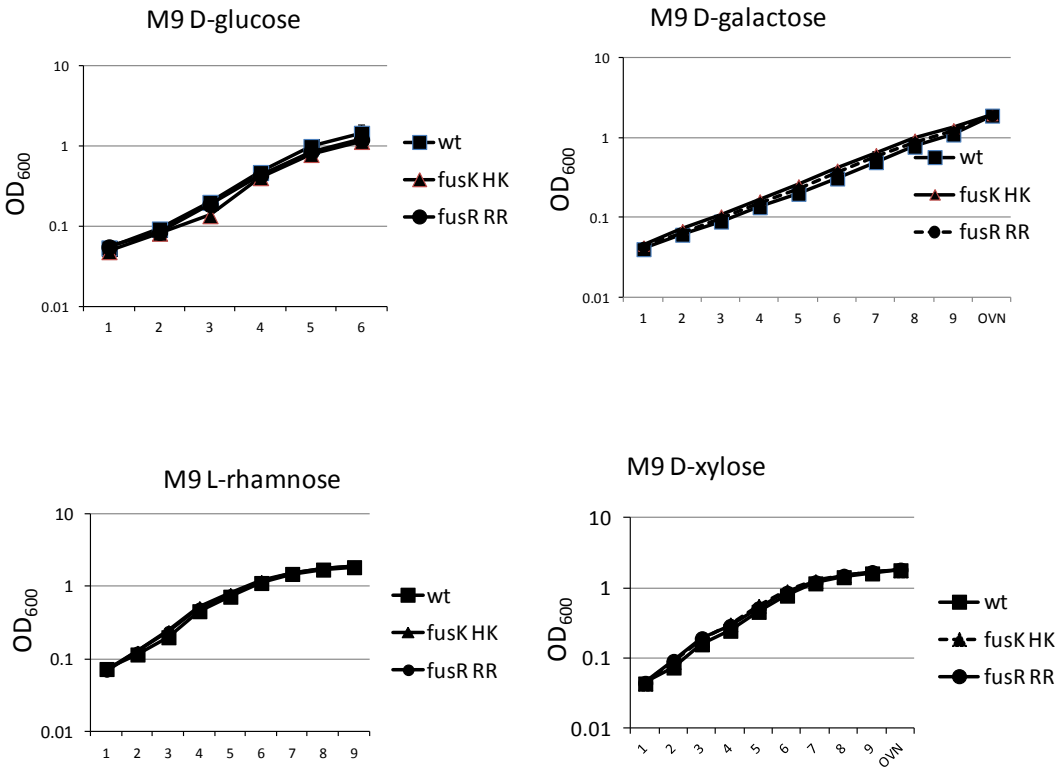

Figure 4.10. Growth curves in M9 minimal media with different carbon sources.

Since the Z0462/Z0463 TCS appears to regulate fucose utilization, we hypothesized that the Z0462 HK senses fucose. Autophosphorylation assays were performed using Z0462 reconstituted into liposomes, in the presence of 100uM fucose or glucose. The concentration of fucose used in these experiments is physiologically relevant, according to fucose measurements within the mammalian intestine (123). Z0462 autophosphorylation increased a significant 3-fold in the presence of L-fucose (Figures 4.9D,E), but did not increase in the presence of glucose or D-ribose (Figures 4.9F-I). Hence we concluded that Z0462 is a fucose sensing HK, and renamed this protein FusK for fucose sensing HK, and its cognate RR (Z0463) FusR for fucose sensing RR. Taken together, our results indicate that the FusKR TCS senses fucose as an environmental cue to regulate expression of the LEE and fucose utilization genes.

Allosteric regulation of fuc expression through altered fucose transportation

FusKR shares low levels of homology to the UhpAB two-component system. UhpAB senses glucose-6-phosphate and activates expression of the *uhpT* gene that encodes a hexose-phosphate MFS transporter (117, 315). Because the gene encoded downstream of *fusKR*, *z0461*, encodes a predicted MFS (Figure 4.11A), and our data indicated that FusKR strongly repressed transcription of *z0461* (Figure 4.11B and Figure 4.3), we assessed whether *z0461* played any role

in growth in fucose and fucose-mediated regulation. An EHEC *z0461* mutant has decreased growth in M9 medium with fucose as a sole carbon source (generation times for WT is 88.2 minutes and for the *z0461* mutant is 96.6 minutes) (Figure 4.11C), but grows similar to WT with glucose as the sole carbon source (Figure 4.11D). These data suggest that *z0461* may be involved in optimal transport of fucose to the bacterial cell, but does not play a significant role in the import of glucose. Transcription of the *fuc* operons is linked to fucose uptake, given that upon its uptake, fucose yields fuculose-1-phosphate that is the inducer of the FucR transcription factor that activates the *fuc* operons (39, 328). Transcription of *fucA*, *fucP*, and *fucR* is decreased in the *z0461* mutant, in agreement with this mutant being impaired in optimal fucose transport. These data suggest that FusKR regulation of the *fuc* genes occurs through its regulation of the Z0461 MFS transporter. Upon induction of FusKR by fucose, this two-component system represses expression of *z0461*, probably decreasing the import of fucose, and decreasing the intracellular levels of the fuculose-1-phosphate inducer of FucR (Figure 4.11G). In further support of this indirect regulation of the *fuc* genes, FusR does not interact directly with the *fucPIKUR* promoter region (Figure 4.11F), which is in contrast to the direct repression of *ler* by FusR (Figure 4.4F).

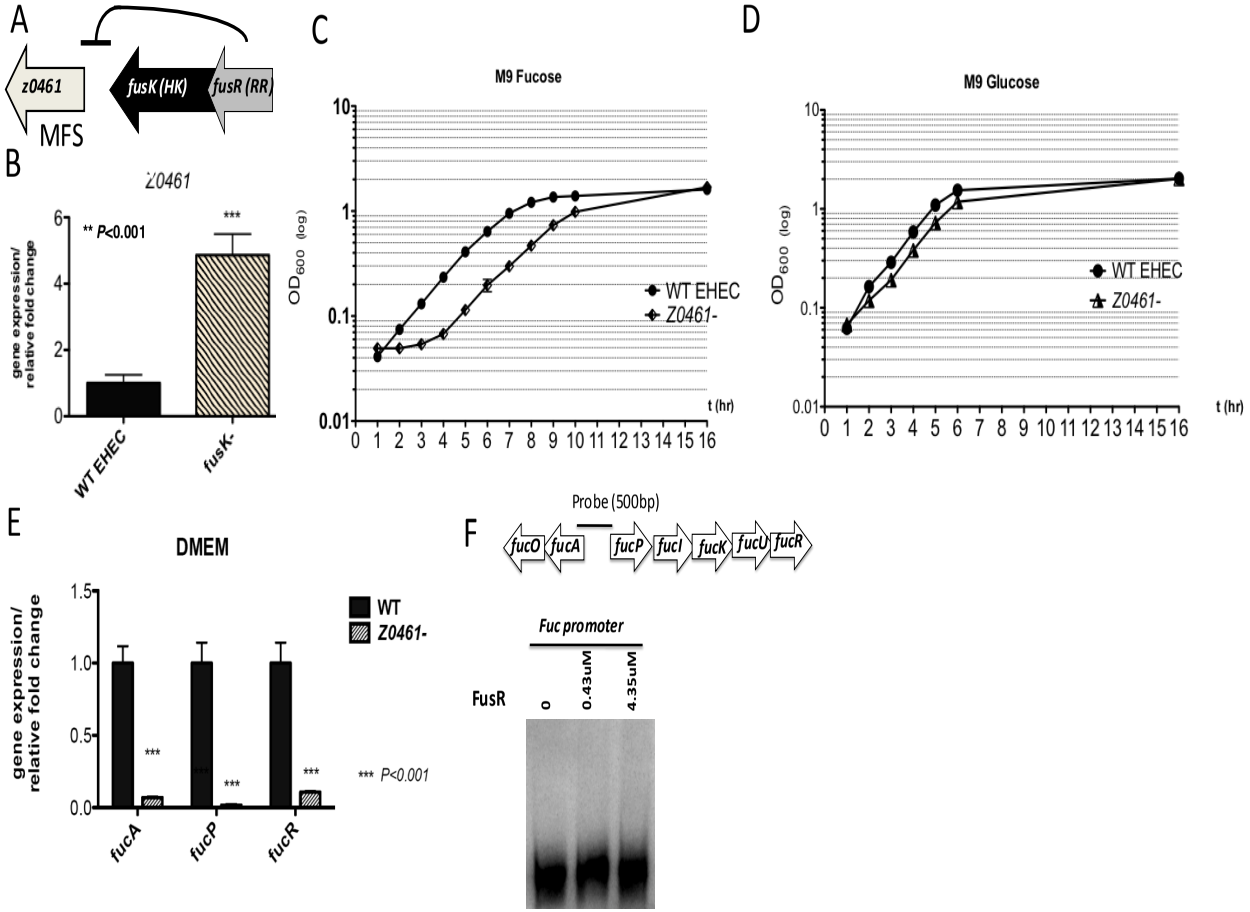
A
z0461
fusK (HK)
fusR (RR)
MFS
B
Z0461
** P<0.001
***
gene expression/ relative fold change
WT EHEC
fusK-
C
M9 Fucose
OD600 (log)
t (hr)
WT EHEC
Z0461-
D
M9 Glucose
OD600 (log)
t (hr)
WT EHEC
Z0461-
E
DMEM
gene expression/ relative fold change
WT
Z0461-
*** P<0.001
fucA
fucP
fucR
F
Probe (500bp)
fucO
fucA
fucP
fucI
fucK
fucU
fucR
Fuc promoter
FusR
0
0.43uM
4.35uM

Figure 4.11. Indirect regulation of *fuc* genes.

A) Schematic representation of the *fusKR* operon to *z0461*.

B) qRT PCR of *z0461* expression in WT and the *fusK* mutant.

C) Growth curves of WT EHEC and the *z0461* mutant in M9 medium with fucose as a sole carbon source.

D) Growth curves of WT EHEC and the *z0461* mutant in M9 medium with glucose as a sole carbon source.

E) qRT PCR of *fucA*, *fucP* and *fucR* expression in WT and the *z0461* mutant.

F) EMSA of the *fucPIKUR* promoter with FusR.

Fucose sensing by FusK represses the LEE

Because FusK senses L-fucose, we investigated whether FusK-dependent LEE regulation is influenced by this sugar. WT and *fusK*- EHEC were grown in M9 minimal media with L-fucose or D-glucose as a sole carbon source and qRT-PCR was performed to measure the expression of the gene encoding the LEE transcriptional activator Ler. Expression of *ler* in the WT strain grown in fucose was reduced a significant 2- fold ($P<0.02$) compared to growth in glucose (Figure 4.12A). Also, increased *ler* expression was observed in the *fusK*- mutant compared to the WT, demonstrating that FusK also represses *ler* in M9 minimal media. In contrast, expression of *ler* in the *fusK*- mutant did not significantly differ during growth in fucose vs glucose (Figure 4.12A). Together these observations suggest that fucose-dependent repression of LEE expression is mediated by FusK, and that the *fusK*- mutant is "blind" to fucose and introduce the possibility that fucose sensing and *ler* regulation by FusKR play a role in intestinal colonization by EHEC.

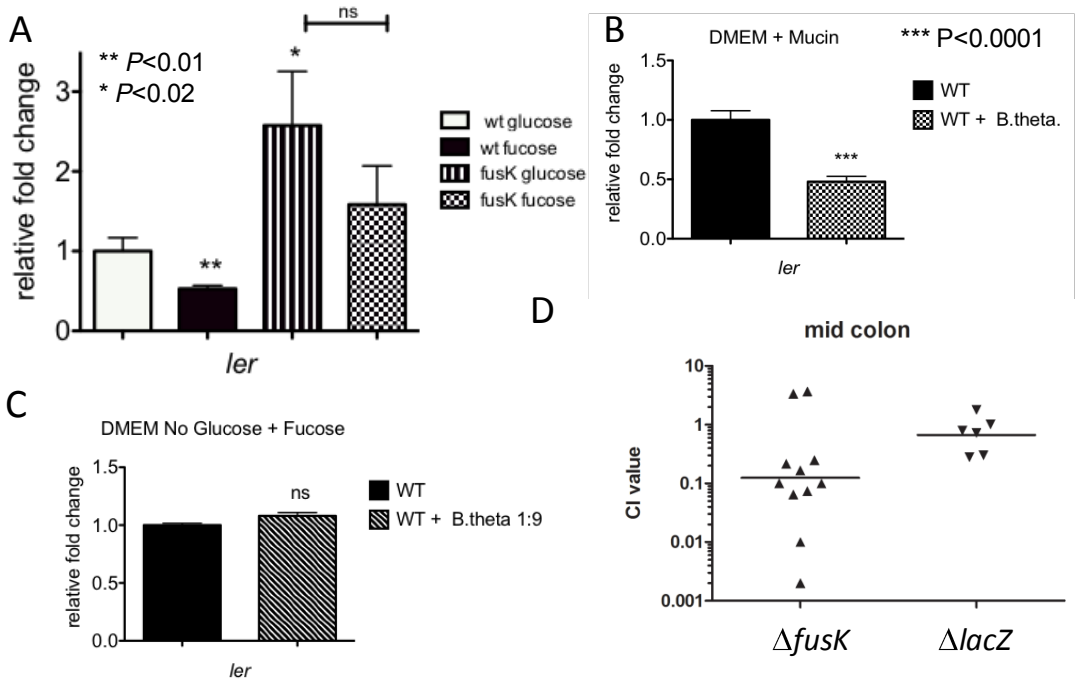

Figure 4.12. Role of FusK in the interplay among the pathogen, microbiota and host. A) qRT-PCR analysis of expression of *ler* in WT or *fusK*- strains. RNA samples were extracted from bacterial cultures grown in M9 with either D-glucose or L-fucose as sole carbon sources. The error bar indicates the standard deviations of ΔddCt values. Significance is indicated as follows: three asterisks, P≤0.0001.

B) Relative abundance of *ler* transcripts in WT EHEC alone or in the presence of *B.thetaiotaomicron*, assessed using qRT-PCR. RNA samples were extracted from

bacterial cultures grown in DMEM containing mucin. The error bar indicates the standard deviations of ΔddCt values. Significance is indicated as follows: three asterisks, P≤0.0001.

C) Relative abundance of *ler* transcripts in WT EHEC alone or in the presence of *B.thetaiotaomicron*, assessed using qRT-PCR. RNA samples were extracted from bacterial cultures grown in DMEM containing L-fucose.

D) Competition assays to assess intestinal colonization of *fusK* mutant. 1:1 mixtures of *fusK* and WT EHEC or *lacZ*- and *lacZ*+ EHEC were intragastrically inoculated into infant rabbits. CFU in the mid colon for each strain were determined 2 days post-inoculation. Difference between CIs was compared using the Mann-Whitney test.

Primary fermenters such as *Bacteroides* are the gateway for the entrance of carbohydrates in the network of syntrophic links in the microbiota. The *Bacteroides* are a prominent glycophagic species, degrading complex carbohydrates into monosaccharides that can then be consumed by other members of the gut microbiota (72). The fucosidase activity of commensal bacteria (47, 107) cleaves fucose from host glycans, resulting in high fucose availability in the gut lumen. *Bacteroides thetaiotaomicron* (*B. theta*) produces multiple fucosidases that can cleave fucose from host glycans (72). Consequently, we hypothesized that *B. theta* might supply mucin-derived fucose to EHEC, influencing FusKR-mediated repression of the LEE. To explore this possibility, EHEC alone, or in

combination with *B. theta* was grown anaerobically in DMEM containing mucin (access to free fucose in this condition requires mucin degradation) or free fucose and the expression of the FusKR-dependent gene *ler* was measured. As shown in figure 4.12, co- culture with *B. theta* in the presence of mucin reduced *ler* expression by 2-fold ($P<0.0001$), whereas in the presence of free fucose no change in *ler* expression was observed in the presence of absence of *B. theta* (Fig. 4.12C). Together, these observations suggest that during growth in mucin, *B. theta* contributes to *ler* regulation by cleaving fucose from mucin, thereby activating the FusKR signaling cascade that leads to repression of *ler*. When free fucose is available in the growth media, fucose-dependent regulation of *ler* is no longer dependent on the presence of *B. theta*. To test whether *B. theta* contributes to EHEC growth in the presence of mucin, growth curves of EHEC and *B. theta* alone or in co-cultures were investigated in DMEM containing mucin. It was observed that *B. theta* decreased the initial growth of EHEC (Figure 4.13A), and that *B. theta* grows faster when EHEC is present throughout (Figure 4.13B).

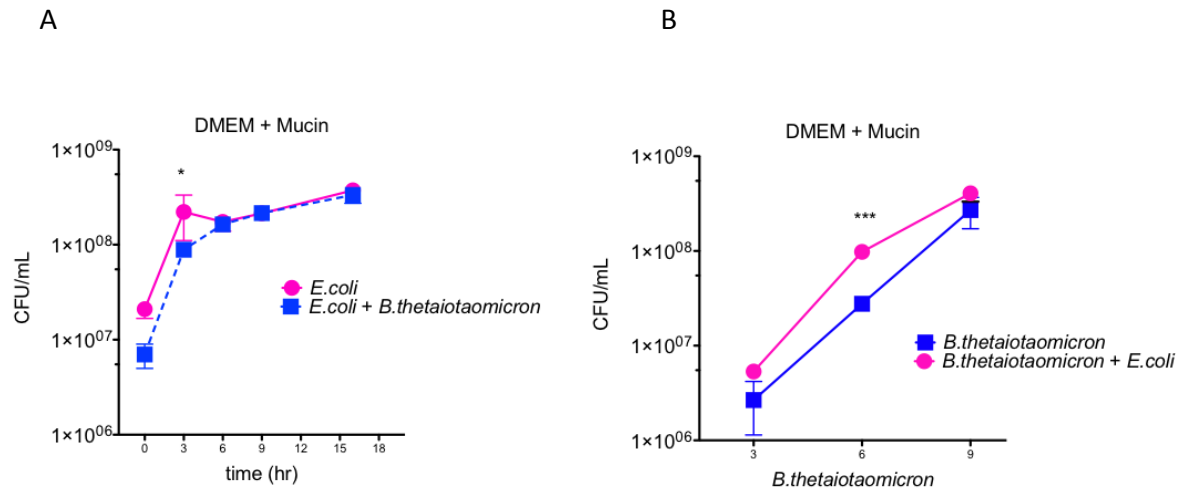

Figure 4.13. Growth curves of EHEC WT (A) and *B.thetaiotaomicron* (B) in DMEM containing mucin. Statistical significance was determined using Two-way ANOVA with multiple comparison of Bonferroni, and it is indicated as follows: one asterisk, P<0.001; three asterisks, P<0.0001.

The intricate role of FusK in the regulation of EHEC metabolism and virulence, potentially at the interface of the pathogen and the microbiota within the host intestine, suggested that FusK might play a role in intestinal colonization by EHEC. To address this question, we investigated the role of FusK in EHEC colonization of the mammalian colon using the infant rabbit model (Clarke et al., 2006; Ritchie et al., 2003). Competition assays were carried out in which three

day-old infant rabbits were inoculated with WT and *fusK*- EHEC strains at 1:1 ratio. Two days after inoculation, the WT strain outcompeted the *fusK* mutant nearly 10-fold. The mean of the competitive index from three litters was 0.12, a value that is statistically different (P=0.039) from a control competition assay, where the WT (*lacZ+)* strain was competed against a *lacZ* mutant (mean competitive index 0.7) (Fig 4.12D). Hence, a functional FusK is necessary for robust EHEC intestinal colonization, suggesting that exquisite regulation of expression of both virulence (LEE) and fucose metabolism genes during infection is crucial for EHEC to establish itself in its mammalian host.

DISCUSSION

The mammalian GI tract harbors trillions of indigenous bacteria (85) whose co-existence relies on differential utilization of limiting resources by the members of this community. Infection by enteric pathogens can cause dysbiosis, inflammation, and result in long term changes in the composition of the microbiota and the well being of the host (169, 273). Complex interactions between host, microbiota and enteric pathogens occur during infection, but relatively little is known of the factors and mechanisms that mediate these interactions. EHEC, which has a very low infectious dose (284), likely relies on sensing multiple signals to exquisitely coordinate the expression of its virulence and metabolic genes to successfully colonize the human gut. EHEC exploits the AI-3/epinephrine/norepinephrine inter-kingdom signaling cascade to trigger virulence gene expression (269). Here, we found that the AI-3/epinephrine/norepinephrine signaling cascade represses expression of the *fusKR* operon. FusK is the first sensor HK shown to sense L-fucose. Sensing of this host derived sugar by the FusKR TCS represses the expression of LEE genes as well as genes involved in L-fucose utilization, albeit through different mechanisms, with repression of the LEE occurring through direct binding of FusR to the *ler* promoter (Figure 4.4F), while expression of the *fuc* genes is indirectly affected by repression of the Z0461MFS transporter (Figure 4.11). Thus, in aggregate, our

findings suggest that FusKR uses host signals to couple EHEC metabolism and virulence gene expression to modulate intestinal colonization. Notably, our observations also suggest that indirect interactions between EHEC and commensal colonic microbiota, such as *Bacteroides*, modulate EHEC intestinal colonization; commensals cleave fucose from host mucins, thereby generating the free fucose that can be sensed by EHEC in the intestinal lumen.

The genes encoding *fusKR* are clustered in a PAI (OI-20) only present in EPEC O55:H7 (the *E. coli* lineage that gave rise to EHEC O157:H7) (317), EHEC O157:H7 and *C. rodentium*, AE GI pathogens that colonize the colon. Horizontal acquisition of PAIs contributes to virulence of an organism, allowing exploitation of other niches and hosts for colonization (219). Our results suggest that the interplay between ancient and recent evolutionary acquisitions has shaped EHEC pathogenicity. Interestingly, EHEC's ancestor, EPEC O55:H7(317), is the only other serotype of *E. coli* to harbor *fusKR*, suggesting that acquisition of these genes is recent. The recent acquisition of OI-20 on EHEC evolution provided this pathogen with a novel signal transduction system. OI-20 genes are up-regulated when EHEC is grown in the presence of bovine mucus (9), and during infection of the colonic mucus-producing cell line HT29 (145). Furthermore, we found that expression of *fusR* is increased in the presence of mucus producing HT29 cells (Fig. 4.9A), suggesting that expression of this response regulator in mucus facilitates EHEC adaptation to the mammalian intestine. Thus, it is tempting to

speculate that acquisition of OI-20 enhances EHEC's capability to successfully compete for a niche in the colon.

Linking metabolism to the precise coordination of virulence expression is a key step in the adaptation of pathogens towards niche recognition of suitable sites for colonization. It is known that the invading enteric pathogen *C*. *rodentium* (a murine pathogen that models the enteric infection of the human pathogen EHEC, and also harbors FusKR) causes inflammation within the gut that in turn diminishes the overall numbers of bacteria in the microbiota, acting as an initial competitive advantage to the pathogen (169, 273). Additionally, infection with *C*. *rodentium* also causes significant changes in the structure of the microbial community, decreasing the number of anaerobes (such as Bacteroidetes), and increasing the numbers of γ-Proteobacteria (169). γ-Proteobacteria normally constitute a minute portion of the microbiota in healthy individuals, but this scenario quickly changes in pathogen-induced inflammation (256). This has important consequences towards niche competition. EHEC does not compete significantly with *B*. *theta* for nutrient utilization during growth in mucus (Figure 4.13), but competes with commensal *E*. *coli* (the predominant species within the γ-Proteobacteria) for the same carbon sources during growth within the mammalian intestine (8, 38, 69, 185). One such carbon source is fucose, which is released into the lumen by glycophagic bacteria such as *B*. *theta*, and then can be utilized by *E*. *coli*, which cannot hydrolyze the complex mucus carbohydrates (38,

69, 185). Because both EHEC and commensal *E. coli* compete for fucose utilization within the lumen (8, 69), it would be counter-productive for EHEC to invest a lot of resources in the utilization of this carbon source in this compartment, where commensal *E. coli* are present. However, EHEC can efficiently use other carbon sources such as: galactose, hexorunates, mannose and ribose, which are not used by commensal *E. coli* within the intestine (69). Additionally, in contrast to commensal *E. coli* that are restricted to the lumen and mucus layer, EHEC is found closely associated with the intestinal epithelium (185). Therefore, EHEC may be able to utilize nutrients exclusively available at the surface of the epithelial cells. The exclusivity of such nutrients to EHEC can be further enhanced by the damage EHEC causes to the membranes in the intestinal epithelium. A remarkable example is the ability of enteric pathogens to utilize ethanolamine as a nutrient source. Ethanolamine is a major component of the mammalian cell membrane (10, 58), and the rapid turnover and exfoliation of intestinal cells releases ethanolamine into the intestine (46, 264). The ability to use ethanolamine as a non-competitive source of carbon and/or nitrogen aids pathogens such as *Salmonella enterica* and EHEC in colonizing the host (282) (23). Consequently, the decreased expression of the *fuc* operon mediated through fucose sensing by FusKR (Figures 4.9 and 4.10), may prevent EHEC from expending excessive amounts of resources in fucose utilization in the lumen, where it competes with the commensal *E. coli* for this resource, and focus on

utilizing other carbon sources, not used by this competitor, to gain a growth advantage during growth in this intestinal compartment. It is also worth noting that repression of LEE expression in the lumen is likely advantageous for EHEC to establish colonization within the host. Expression of the LEE- encoded T3SS in this GI compartment would constitute a superfluous expenditure of energy. Hence it is desirable that upon sensing fucose, released in the lumen by the glycophagic Bacteroidetes within the microbiota, FusKR represses LEE expression. Once in close contact to the epithelial surface, the QseCE adrenergic sensing system, would be triggered by the host hormones epinephrine and norepinephrine and activate virulence both directly through the QseCE cascade, as well as indirectly by repressing expression of *fusKR* (Figure 4.14). This model is supported by the observation that upon growth in mucus *B. theta* releases fucose to repress LEE expression, and that an EHEC *fusK* mutant is defective for colonization of the mammalian GI tract (Figure 4.14). Thus, the colonization defect of the *fusK* mutant may result from its inability to correctly time virulence expression.

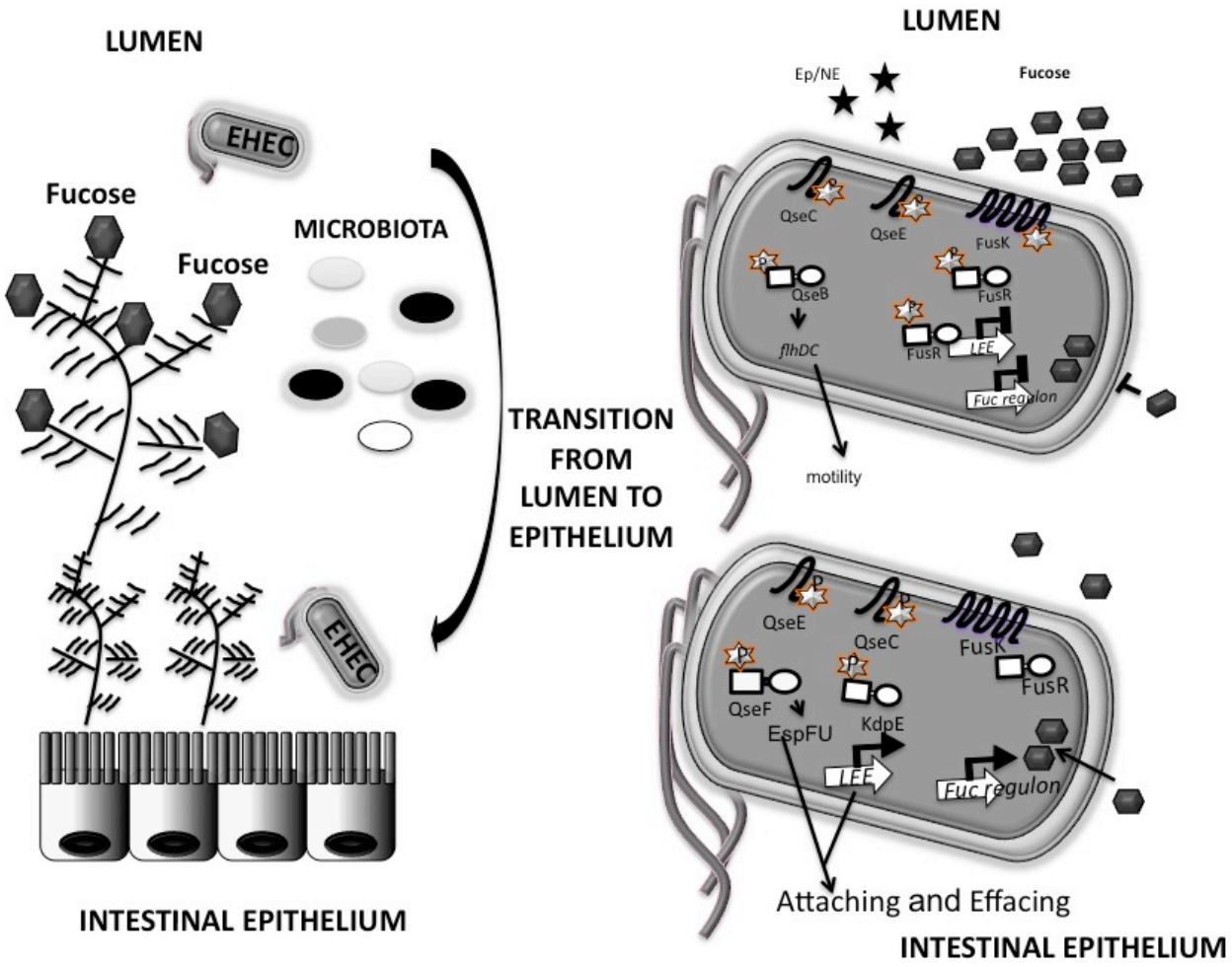

Figure 4.14. Model of fucose sensing modulating intestinal colonization by EHEC. Fucose available in the lumen through Bacteroidetes degradation of host glycans inhibits LEE expression through FusKR. In proximity to the epithelial the QseCE adrenergic sensors repress FusKR derepressing LEE expression, and also activate LEE expression through the KdpE RR.

The FusKR TCS modulation of EHEC intestinal colonization provides another compelling and complex example of how inter-kingdom signaling governs EHEC pathogenicity. In this case, the polysaccharide degrading activity (173) of the commensal flora (eg. *Bacteroides*) (44) results in the release of fucose from host glycans; this sugar can serve both as an available carbon source and as a signal to coordinate nutrient acquisition and virulence gene expression by the pathogen. Inter-kingdom signaling in the intestine is proving to be a major mechanism that shapes bacteria-host relationships (111). In the GI tract environment, where ~1,000 different bacterial species co-exist, the exquisite integration of varied nutritional and chemical cues to regulate gene expression is essential for pathogens to successfully compete (72).

CHAPTER FIVE

Crosstalk among Two-Component Systems in EHEC

INTRODUCTION

EHEC employs the QseBC and QseEF TCSs to sense and adapt to the mammalian host. The QseBC system senses AI-3, which is produced by EHEC itself and by members of the intestinal microbiota. QseBC also detects the stress hormones Epi and NE produced by the mammalian host. Upon sensing these signals, a signaling cascade is activated leading to activation of expression of flagellar motility genes and the LEE. The QseEF TCS, which is activated by the QseBC system, senses Epi/NE, triggering expression of EspFU and pedestal formation in EHEC. Because motility and AE lesion formation need to happen in different phases of colonization of the intestine, EHEC likely needs to exquisitely time the expression of the virulence genes involved in this processess. The ability to sense multiple signals and subsequently translate this information into changes in gene expression is essential for pathogenic bacteria, because it could lead to niche colonization or elimination by the host. Therefore, it is likely that EHEC utilizes and merges additional signal transduction systems to control hierarchical production of virulence traits and metabolic features.

The 33 TCSs of *E.coli* have been characterized *in vitro*, and phosphotransfer profiling analyses have determined the degree of crosstalk among them (3% out of 692 possible combinations). Nine HKs (BarA, DcuS, EnvZ, RtsB, UhpB and YedV) can crossphosphorylate non-cognate RRs. For instance, UhpB can phosphorylate 9 non-cognate RRs. On the other hand, 9 RRs (AtoC, CheY, CusR, HydG, KdpE, NarL, NarP, NtrC and YfhA or QseF) can be phosphorylated by non-cognate HKs. It has been suggested that crosstalk among TCS is important to regulate gene expression by integrating multiple signals (323). Nonetheless, TCS crosstalk has received criticism (158). The possibility of crosstalk have important implications for signal integration and tight control of gene expression in bacteria, which is of major importance for quick bacterial adaptation or survival during host colonization. Phenotypic analyses of deletion strains indicate that some TCS regulate expression of multiple phenotypes. Deletion of ArcAB, CpxRA, OmpR-EnvZ and NtrBC results in pleiotropic effects (326). ArcAB, composed by the ArcB HK and the ArcA RR, regulate genes involved in respiratory and fermentative metabolism by responding to oxygen limitation and quinones (77, 84, 118). A number of TCS have been shown to play a role in virulence regulation in enteric pathogens.

Our laboratory has provided evidence of crosstalk among TCS involved in the regulation of virulence traits. QseC can phosphorylate its cognate RR (QseB) and non-cognate RRs (KdpE and QsEF), thereby controlling flagellar motility and

LEE expression by likely sensing a common triggering signal. QseE regulates expression of several TCS, and therefore, it likely plays a major role in EHEC adaptation and differential gene expression upon sensing multiple environmental cues. In this chapter, we show that FusKR crosstalks with QseBC and QseEF via distinct regulatory mechanisms. FusK phosphorylates the non-cognate RR QseF. On the other hand, FusR can be phosphorylated by non-cognate HKs QseC and QseE. Crosstalk at the transcriptional levels was also observed, as FusKR activates expression of *qseEF*. Data presented herein suggest that crosstalk among TCS related within the AI-3/Epi/NE signaling cascade appears to be common, and it might play a significant role in control of pathogenic traits of EHEC.

RESULTS

Crosstalk of TCSs FusK/FusR, QseBC and QseEF

Although many reports suggest that crosstalk among TCSs is minimal or occurs only under specific conditions, we have shown extensive crosstalk between QseBC and QseEF in EHEC (110, 211, 236). Firstly, we investigated whether *fusK/fusR* regulate *qseB*, *qseC*, *qseE* and *qseF* by measuring the expression of these genes by qRT-PCR in *fusK*- and *fusR*- strains. FusK/FusR do not regulate *qseB* or *qseC* transcription, while this TCS activates the expression of *qseE* and *qseF* (Figure 5.1A).

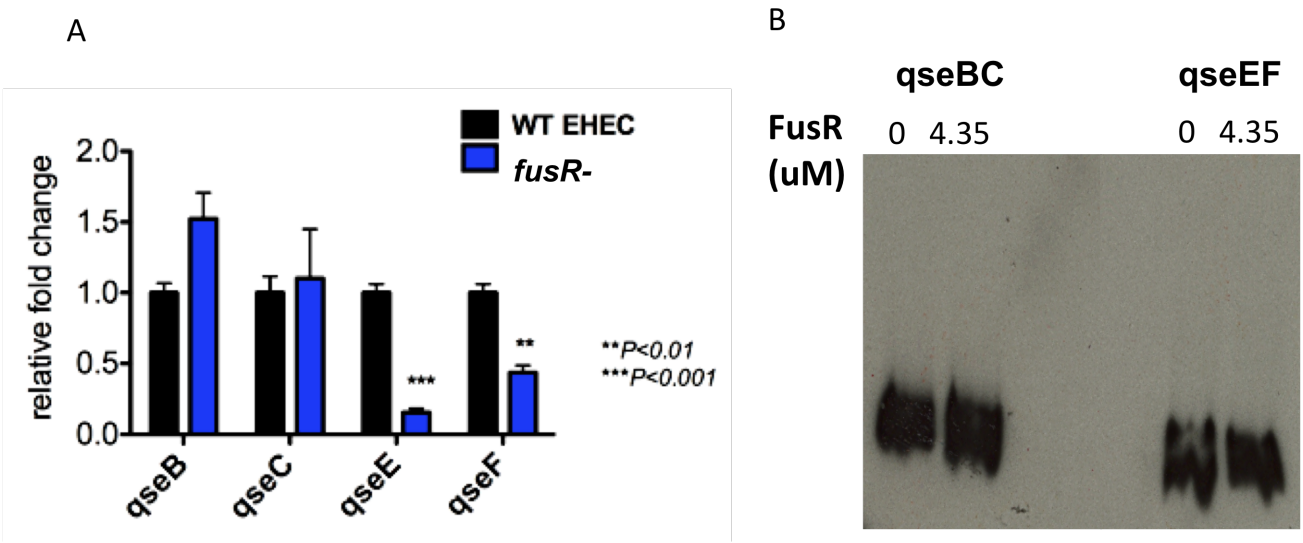

Figure 5.1. Crosstalk among FusKR, QseBC and QseEF. A) qRT-PCR shows that FusR activates *qseE* and *qseF* expression, but not *qseB* or *qseC*. Statistical significance was determined using the Two-way ANOVA test with multiple

comparison of Bonferroni, and it is indicated as follows: two asterisks, $P<0.01$; three asterisks, $P<0.001$. B) EMSA shows that FusR does not interact with the *qseBC* or *qseEF* promoters.

To investigate whether FusR regulation of *qseEF* was direct, we performed EMSAs, where FusR was tested for its ability to interact with the promoter regions of *qseBC* and *qseEF*. FusR does not bind to either *qseBC* or *qseEF* regulatory regions, indicating that regulation of *qseEF* by FusKR is not direct (Figure 5.1B). Congruent with our findings, QseEF has a sigma 54 promoter, and FusR is a putative sigma 70 transcriptional regulator, activation of *qseEF* by FusK/FusR is likely indirect. It is possible that FusK/FusR represses the expression of an activator of QseEF.

Evidence of crosstalk can also be observed at the biochemical level, where a HK may be able to crossphosphorylate non-cognate RRs, or a single RR may be targeted by more than one HK (323). We have shown that the sensor kinase QseC is able to phosphorylate its cognate (QseB) and non-cognate RRs such as QseF and KdpE (110). We then investigated the possibility of crosstalk among TCS FusK/FusR, QseBC and QseEF. QseC was tested for its ability to phosphorylate FusR, and QseB served as a positive control. QseC was able to cross-phosphorylate FusR, although to a lesser extent compared to its cognate RR QseB (Figure 5.2).

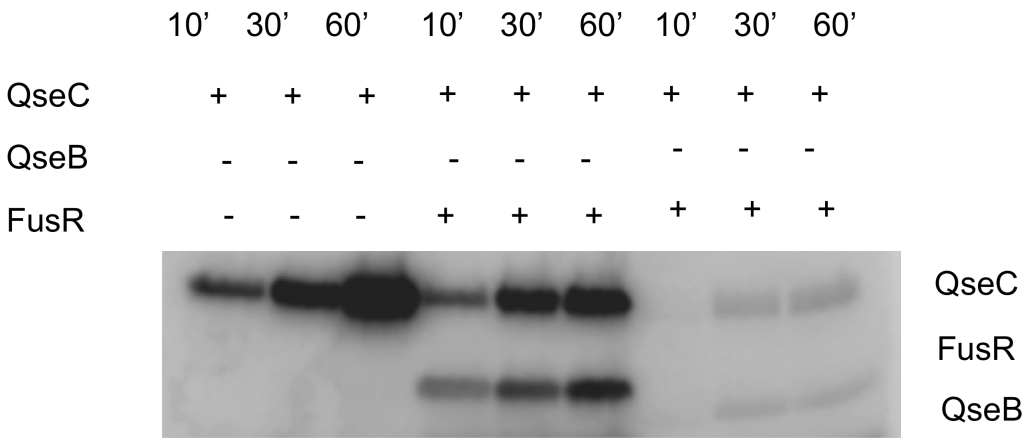

Figure 5.2. Crosstalk between FusKR and QseBC. QseC reconstituted into liposomes phosphorylates non-cognate RR FusR. QseB is used as a positive control.

We also investigated the ability of QseE to phosphorylate FusR. We used a truncated form of QseE comprised only by the catalytic domain for phosphotransfer, and QseF as positive control. QseE was able to cross-phosphorylate FusR (Figure 5.3). Similarly to phosphorylation by QseC, FusR is phosphorylated to a lesser extent by QseE compared to the cognate RR QseF. Our results suggest that FusR is a promiscuous RR, targeted by at least 3 HKs tested: QseC, QseE and FusK. This profile of cross-phosphorylation could also indicate a major regulatory point in EHEC, given that FusR acts as a negative regulator of the LEE, which is critical for EHEC pathogenicity.

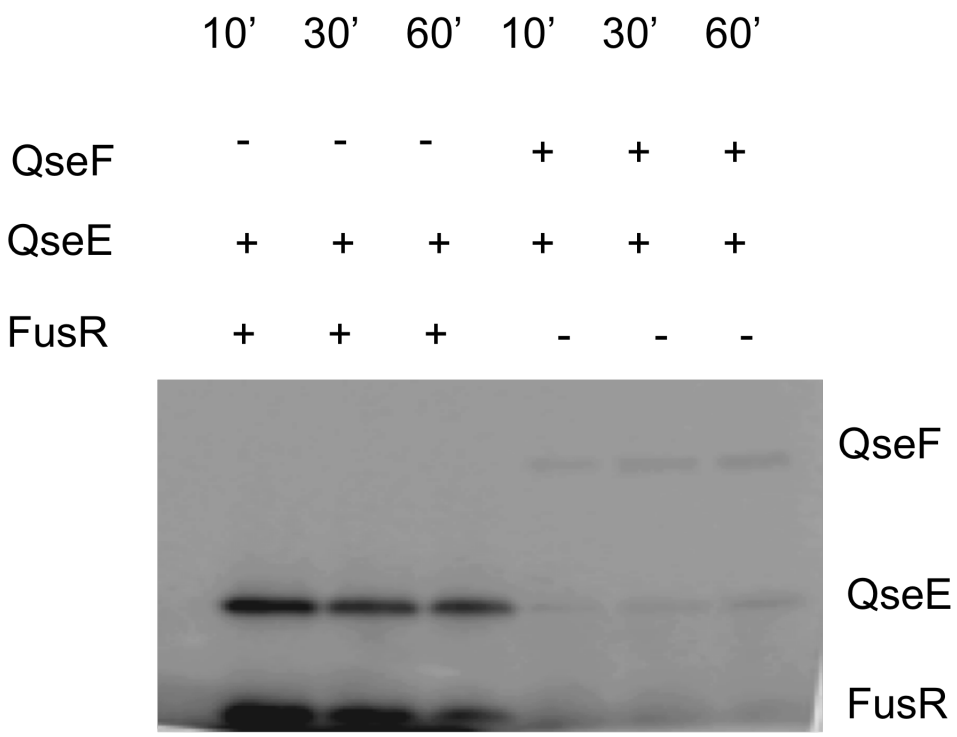

Figure 5.3. Crosstalk between FusKR and QseEF systems. QseE kinase domain phosphorylates non-cognate RR FusR.

We also investigated whether FusK was able to phosphorylate the non-cognate RR QseF. Our results show that FusK phosphorylates QseF. It is of note that autophosphorylation of FusK is higher in the presence of QseF (Figure 5.4). QseF is known to be phosphorylated by at least 6 HKs: QseE, QseC, BaeS, EnvZ, UhpB and RtsB (323).

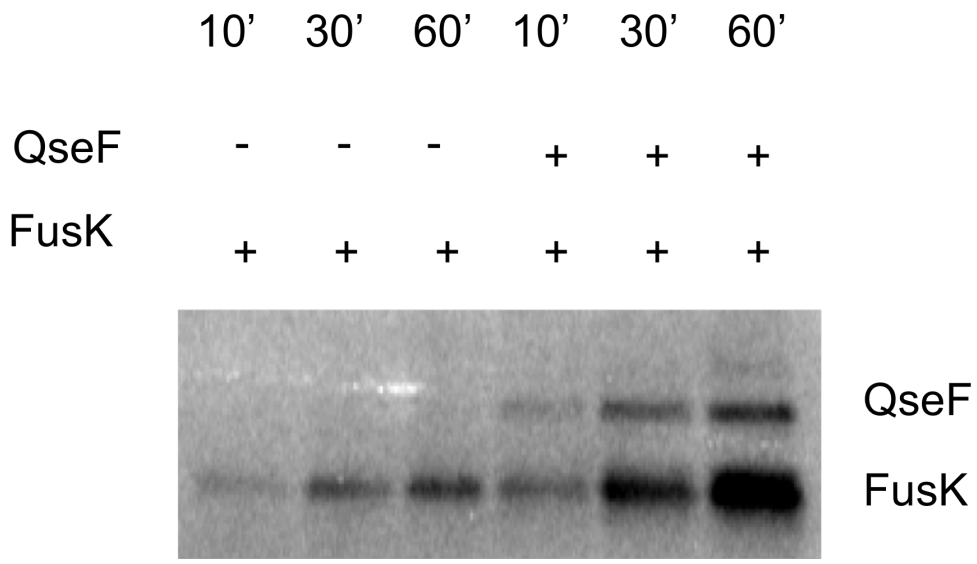

Figure 5.4. Crosstalk between FusKR and QseEF (part 2). FusK phosphorylates non-cognate RR QseF. Also, QseF increases FusK autophosphorylation.

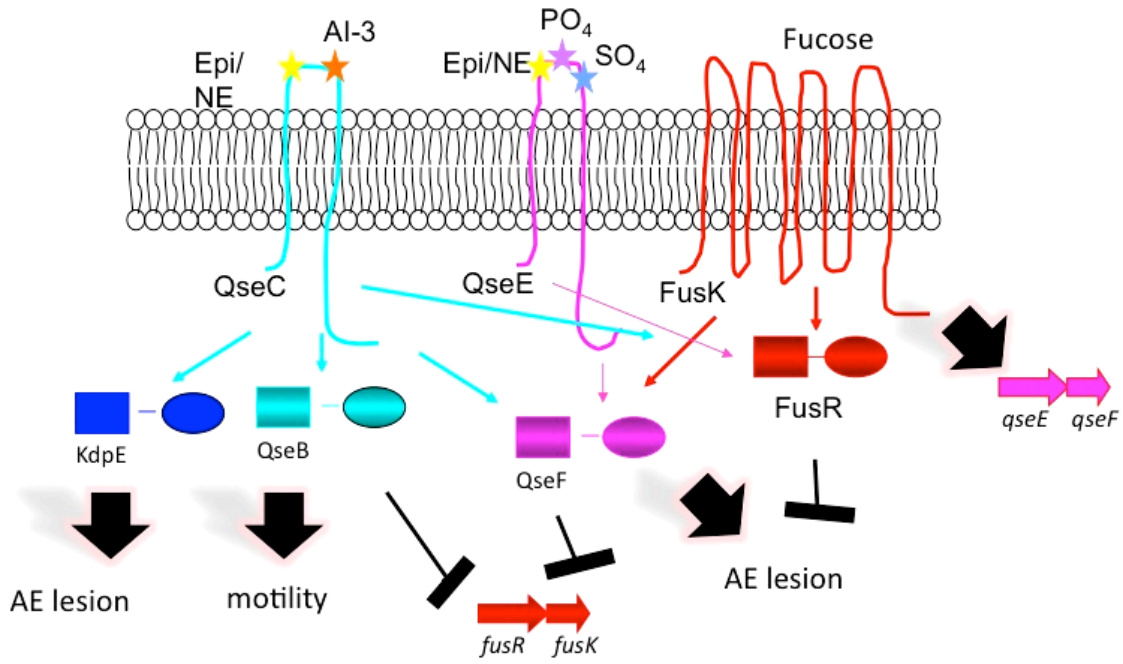

FIGURE 5.5. Model of crosstalk among TCSs in the AI-3/Epi/NE signaling cascade of EHEC. QseC phosphorylates QseB, activating expression of flagellar motility genes; QseC cross-phosphorylates the non-cognate RR KdpE, promoting AE lesion formation by activating transcription of the LEE. QseE phosphorylates QseF, which activates EspFU expression contributing to AE lesion formation. QseC and QseE cross-regulate FusKR by repressing *fusKR* transcription and cross-phosphorylating FusR. FusK phosphorylates its cognate RR FusR, repressing the LEE, decreasing AE lesion. FusKR activates QseEF expression; also, FusK cross-phosphorylates non-cognate RR QseF.

DISCUSSION

Cell-to-cell communication among bacteria in the intestine is a major mechanism that shapes bacteria-host relationships. Pathogenic bacteria such as EHEC can also cross-communicate with the host by detecting mammalian hormones (269). In virtue of its remarkably low infectious dose (50 CFU) (284), successful colonization of the human colon by EHEC relies largely on sensing multiple signals to coordinate the expression of virulence genes. EHEC exploits the AI-3/Epi/NE inter-kingdom signaling cascade to trigger expression of motility genes and the AE lesion, which are two pathogenic traits that are crucial for colonization but required at different time points of the infection (269). The AI-3/Epi/NE is a complex signal transduction system that comprises the TCSs QseBC and QseEF, and along with the transcription factors QseA, QseD and KdpeE. In this work we describe a novel member of this signaling cascade, the TCS FusKR. FusKR is constituted by FusK sensor HK and FusR RR. FusK/FusR is a negative regulator of LEE transcription, non-LEE encoded effectors and AE lesion formation. Our results indicate that FusK/FusR is an additional regulatory circuit of EHEC virulence expression that controls factors involved in pathogenicity.

Activation of flagellar genes and the LEE likely occurs at different time points during EHEC infection. Once EHEC reaches the intestinal lumen, it will

face two major barriers, represented by the distal gut microbial flora and the mucus layer protecting the intestinal epithelium. AI-3 produced by commensal bacteria activates QseC, triggering flagella expression by QseB (110). Flagellar motility allows EHEC to swim across the mucus layer that overlays the intestinal epithelium. We hypothesize that FusK/FusR repression of the LEE might be important during luminal localization of EHEC, since expression of the LEE is not desired during transition from the lumen to the epithelium. Incorporation of FusK/FusR into the AI-3/Epi/NE signaling cascade allows EHEC to fine-tune regulation of virulence gene expression and hence, guarantee a successful outcome during infection of the mammalian colon. EHEC colonization of the human gut involves formation of the characteristic histopathological AE lesions on enterocytes, a feature that requires the LEE-encoded T3SS and the prophage encoded effector EspFU. In proximity with the intestinal epithelium, EHEC senses the mammalian hormones Epi/NE, activating the LEE. During pedestal formation, expression of flagellar genes is not longer required.

We performed phosphotransfer assays to investigate the possibility of crosstalk amongst FusKR, QseBC and QseEF. We provide evidence that FusKR crosstalks with both the QseBC and QseEF systems. FusK can phosphorylate the non-cognate RR QseF (Figure 5.4). FusR can be phosphorylated by the non-cognate HKs QseC and QseE (Figure 5.2 and 5.3). There is previous evidence of crosstalk among TCSs of the AI-3/Epi/NE adrenergic sensory system (110, 211).

Yamamoto et al (323) also observed cross-signaling among TCSs, Although it has been suggested that crosstalk at the phosphotransfer level might be more an exception than a rule or only likely to occur in certain conditions (159), it has also been significantly documented by other investigators (158, 323). Most *in vitro* assays to investigate crosstalk among non-cognate HKs and RRs do not utilized full-length HKs, using truncated versions of the HKs that lack a sensory domain (158, 323). It has been suggested that the sensory domain is an important regulator of HK autophosphorylation, not only by conferring signal binding but also for transferring information from the sensory to the transmitter domain (174). Therefore, studies of crosstalk *in vitro* using truncated sensor kinases might no reflect the level of crosstalk that could happen *in vivo*.

Here we show that FusR is phosphorylated by the non-cognate HKs QseC and QseE. Cross-phosphorylation of one RR by multiple kinases could be an important evolutionary trait that allows a bacterium to quickly adjust gene expression by channeling various input signals. Although both QseC and QseE sense Epi, each one of these proteins also senses other environmental cues. Only QseC senses AI-3, while QseE can respond to phosphate and sulphate sources. Crosstalk can also occur when a HK is able to transfer its phosphate to more than one RR. Our results indicate that the ability of a sensor kinase to activate multiple RRs might play a global regulatory role in fine tuning and coordinating

hierarchical expression of multiple target genes in response to the integration of different signals.

The events that culminate in colonization of the intestinal epithelium by EHEC required precise coordination of the expression of virulence genes. Flagellar motility and the LEE-encoded T3SS are regulated by the AI-3/Epi/NE cell-to-cell signaling, in which the TCSs QseBC and QseEF are major players. The LEE is the target of an exquisitely complex regulatory network. However, a negative regulator downstream of QseBC/QseEF signaling cascade has not been described until now. The data presented here show that the TCS FusKR is a novel member of the AI-3/Epi/NE signaling cascade. Taken together, our results suggest that FusKR is an important system that regulates virulence factors in EHEC. Fine-tune regulation of virulence genes mediated by FusKR is important for EHEC colonization of the mammalian intestine. Signal-sensing by FusK should be further explored to elucidate how the FusKR signaling cascade is triggered in EHEC. The discovery of FusKR as a novel TCS that is part of the AI-3/Epi/NE signaling cascade indicates that coordination of virulence gene expression in EHEC by inter-kingdom signaling is exquisitely complex and hierachical, and it likely plays an extensive role in EHEC pathogenicity.

CHAPTER SIX

Modulation of EHEC virulence gene expression by carbohydrate utilization

INTRODUCTION

In chapter 4, we demonstrated that the FusKR signaling cascade is triggered by the presence of fucose. Fucose sensing by EHEC regulates expression of *ler*. We demonstrated that EHEC growth on fucose as a sole carbon source activates the FusKR pathway, which culminates in FusR binding to the *LEE1* promoter, repressing transcription of the LEE. We have also shown that mucin-derived fucose supplied by *B.theta* in co-culture with EHEC on mucin results is decreased *ler* transcription.

EHEC colonizes the colonic epithelium by forming AE lesions characterized by intimate attachment of the bacterium to the apical portion of enterocytes. To reach the epithelial lining, EHEC must cross the gel-like mucus layer that overlays the intestinal epithelial cells, shielding the colonic epithelium from bacteria (129).

The mucus layer is in dynamic state, being constantly synthesized and secreted by specialized goblet cells, and degraded to a large extent by indigenous intestinal microbes (108, 155). The mucus is composed by mucin, antimicrobial

peptides, glycoproteins, glycolipids and epithelial cell debris but is 50% comprised of polysacharides (22). The major structural component of the mucus is mucin, which is a glycoprotein constituted by a protein backbone connected to hydrophilic and hygroscopic oligosaccharide side-chains, which form a gel-like tridimensional structure (146). The O-linked glycans make up for 80% of the total weight of the mucins, and constitute a major nutrient source for bacteria, and it provides attachment sites for commensal and pathogenic bacteria (129, 245). A diverse collection of 13 monossacharides is part of the mucus composition: arabinose, fucose, galactose, gluconate, glucuronate, galacturonate, mannose, glucosamine, N-acetyl-glucosamine, galactosamine, N-acetyl-galatosamine, N-acetylneuramic acid and ribose. All of these sugars are made available to pathogenic bacteria due to the turnover of host epithelial cells and the polysaccharide-degrading activity of commensal anaerobes. Hence, the mucus layer represents a habitat, and a source of nutrients for bacterial communities that colonize mucosal surfaces. Both pathogenic and commensal bacteria can consume carbohydrates from the mucus as a carbon and energy source (38, 185, 191, 225).

Carbon utilization has been implied to play a major role during colonization of the intestine by commensal and pathogenic bacteria. Bacterial growth on mucus has been assessed *in vitro* and *in vivo*, suggesting that the ability to use sugar sources present in the mucus represents a competitive advantage for

enteric bacteria such as EHEC. EHEC likely colonizes the large intestine by growing on mucus (185, 302).

Gene systems involved in the catabolism of 7 of the 13 sugars in the mucus are induced when EHEC is grown in mucus: fucose, gluconate, glucoronate, galacturonate, GalNAc, NANA and ribose. There is evidence that catabolism of mucin-derived carbohydrates have an impact on colonization by EHEC (263). *E.coli* mutant strains unable to use fucose as a carbon source present colonization defects in mice (38) and cattle models of infection (263). Commensal and pathogenic *E.coli* strains differ in the types of carbon sources they utilize *in vivo*.

In this chapter, we show that EHEC utilization of other monosaccharides found in the mucin also affects *ler* expression. Catabolism of D-galactose and D-mannose by EHEC results in reduced *ler* transcription, albeit likely independent of FusKR. We also demonstrate that FusK responds to D-galactose increasing its autophosphorylation activity. Conversely, FusK presents lower autophosphorylation activity in the presence of GalNac and GlucNac. Moreover, FusK detects L-rhamnose, which is a dideoxy hexose that presents a configuration similar to L-fucose. In aggregate, our findings suggest that multiple carbon sources impact EHEC virulence gene expression, and it might constitute a link between metabolism and virulence during competition for a niche in the mammalian colon.

RESULTS

FusK senses other carbohydrates present in mucus

Because the mucus contains a varied repertoire of monosaccharides, we tested the possibility that FusK also senses other sugars present in the mucus. Autophosphorylation assays were performed using FusK reconstituted into liposomes in the presence of the following major mucin carbohydrates: D-galactose, D-mannose, N-acetyl-glucosamine (GlucNac), N-acetyl-galactosamine (GalNac) and sialic acid. FusK presented increased autophosphorylation (3-fold) in the presence of D-galactose (Figure 6.1A-B). Addition of GalNac and GlucNac caused a significant 2-fold decrease in FusK autokinase activity (Figure 6.1C-D). On the other hand, FusK autophosphorylation did not change significantly upon addition of D-mannose or sialic acid (Figure 6.1E-F).

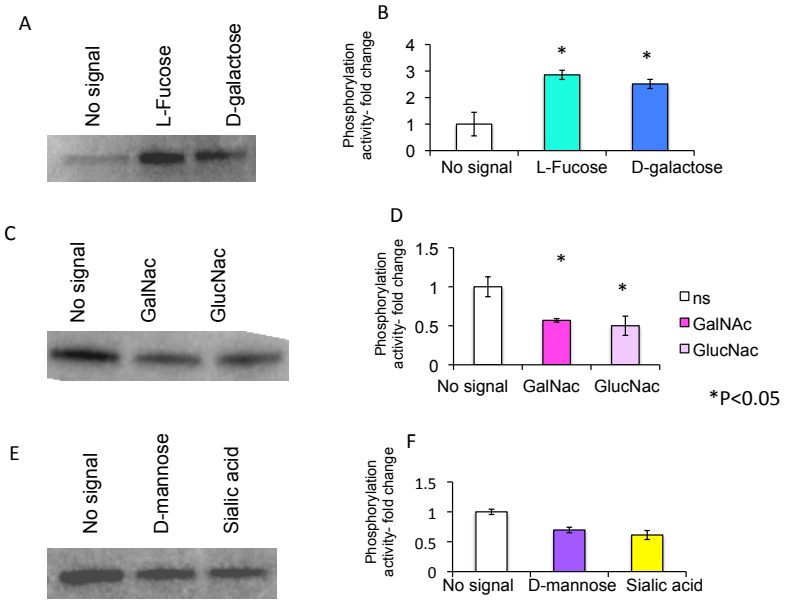

Figure 6.1 A-F. FusK also senses D-galactose, GalNac and GlucNAc. Autophosphorylation assays using FusK reconstituted into liposomes. Statistical significance is indicated as follows: one asterisk, P<0.05. FusK increases autophosphorylation in the presence of D-galactose, while decreases phosphorylation in the presence of GalNac and GlucNac. Addition of mannose or sialic acid did not alter FusK autophosphorylation significantly.

FusK responds to L-rhamnose

Response of FusK to L-rhamnose was also pursued, given the several similarities to L-fucose: both L-fucose and L-rhamose are methyl pentoses, with natural occurrence in the L-form rather than the D-form (287), which is the common isomer of all other naturally occurring sugars; their dissimilation in bacteria converges to the same substrates: L-lactaldehyde and dihydroxyacetone phosphate, after cleavage by their respective aldolases (Figure 6.2) (11). Autophosphorylation using FusK+ membranes and FusK- membranes (originated from the fusK- strain complemented with empty vector – pBADMycHis) in the presence of 100uM L-rhamnose increased FusK autophosphorylation (Figure 6.3). However, when FusK reconstituted into liposomes was used, no response to L-rhamnose was observed. Therefore, investigation of whether FusK senses L-rhamnose should be further confirmed.

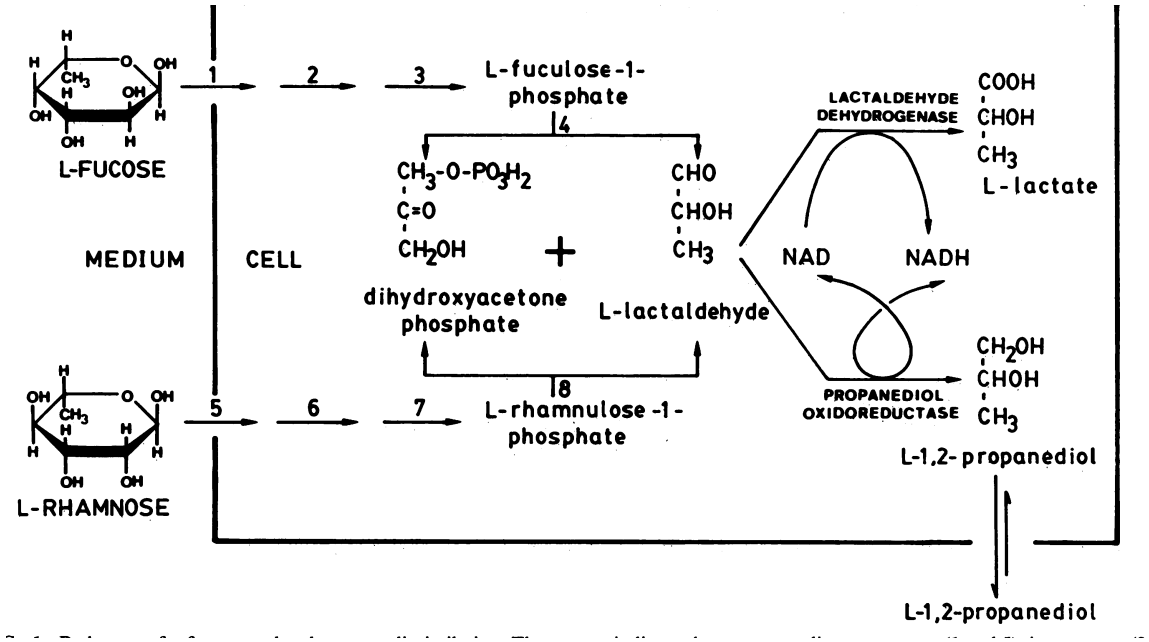

Figure 6.2. Assimilation of L-fucose and L-rhamnose in *E.coli*. Source: (11)

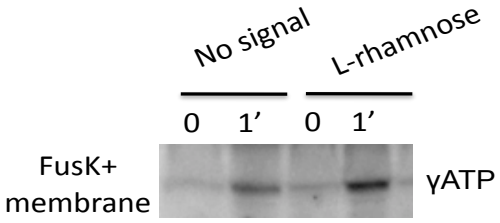

Figure 6.3. FusK senses L-rhamnose. Autophosphorylation assays using membranes of fusK- strain overexpressing FusK shows that FusK increases autophosphorylation in the presence of L-rhamnose.

D-galactose and mannose repress ler transcription

The role of FusK in mucus-sugar sensing prompted us to assess whether other carbohydrates abundant in the mucus affected *ler* expression via FusK. FusK also senses D-galactose, increasing its autokinase activity (Figure 6.1 A-B). Therefore, we sought to determine whether D-galactose could alter *ler* expression, in a FusK-dependent manner. WT EHEC and *fusK*- strains were grown in M9 with glucose or D-galactose as sole carbon source and *ler* expression was analyzed by qRT-PCR. D-galactose resulted in a 2-fold decrease in *ler* expression in EHEC WT; however, *fusK*- still responded to the presence of D-galactose by reducing *ler* transcription a significant 5-fold compared to *fusK*- grown on glucose (Figure 6.4). Similarly to the response to D-galactose, EHEC WT grown

on D-mannose presented 2-fold reduction in *ler* expression, and the *fusK*- strains still sensed this sugar, repressing *ler* transcription 5-fold compared to *fusK*- grown on glucose (Figure 6.4). These results suggest that although FusK detects D-galactose, it might not be the primary sensor of D-galactose involved in *ler* regulation. Given that FusK autophosphorylates in the presence of D-galactose, and a fusK mutant can still sense this sugar, fusK is likely a secondary sensor of D-galactose or FusK represses the expression of a D-galactose sensor, which is activated in the absence of D-galactose. Additionally, D-galactose sensing by FusK could be involved in the regulation of other genes and not *ler*. Therefore, the role of FusK in D-galactose sensing is not well understood at this moment and it should be further explored. Our results also indicate that FusK is not a mannose sensor, although it might regulate a mannose sensor given that mannose causes a 10-fold decrease in *ler* transcription in the *fusK*- strain compared to EHEC WT (Figure 6.4).

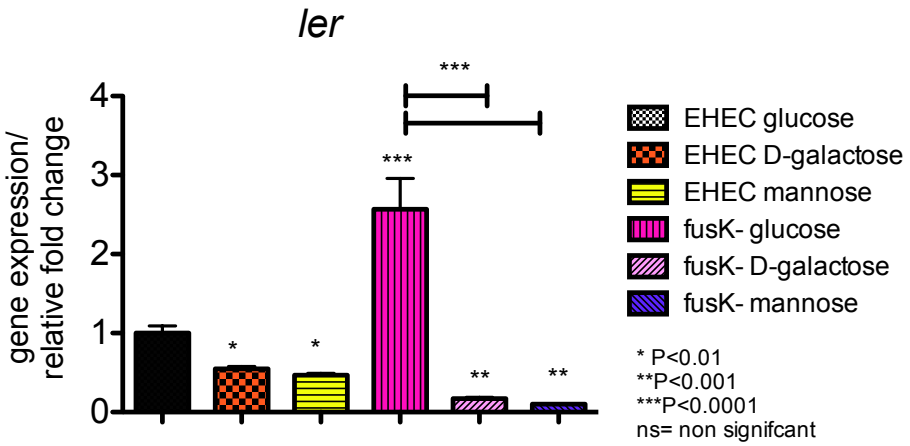

Figure 6.4. Carbohydrates from mucus alter *ler* transcription. QRT-PCR shows that EHEC WT grown in M9 minimal medium containing D-galactose or mannose as sole carbon source presents reduced *ler* expression. RNA levels were normalized to endogenous *rpoA* levels. Similarly, *fusK*- has reduced *ler* levels, suggesting that FusK may not mediate D-galactose or mannose- regulation of *ler* in EHEC. Statistical significance was determined using Two-way ANOVA with Bonferroni's multiple comparison test, and it is indicated as follows: one asterisk, $P<0.01$; two asterisks, $P<0.001$; three asterisks, $P<0.0001$.

DISCUSSION

In addition to expressing virulence factors to subvert the host, pathogenic bacteria's major task in the intestine is to find suitable nutrients. Efficient use of nutrient sources in the gut has an important impact in colonization by pathogenic bacterial species because of the high number of commensal bacteria highly adapted to live in the gut. According to Freter hypothesis, the ability of a pathogen to thrive during intestinal colonization is dependent upon the ability to efficiently use nutrients sources to compete with the microbiota and find a suitable niche for colonization (75). This theory also predicts that two bacterial strains that compete for the same limiting nutrient can still coexist if the less efficient one is attached to the intestinal wall. EHEC grows from remarkably low numbers (infectious dose is 50-100CFU) to high numbers (106-108 CFU/g feces) during infection of the human large intestine (135), which indicates that EHEC successfully persists despite of the commensal microbiota, finds a niche and utilizes and competes for nutrients very efficiently.

Commensal *E.coli* strains and pathogenic strains such as EHEC differ in the types of carbohydrates they utilize *in vivo* as carbon sources. EHEC can grow on mucus. Actually, pathogenic species that colonize the gut take advantage of the glycosidases produced by anaerobes from the microbiota to obtain

monossacharides as carbon sources (45). Studies indicate that EHEC can grow *in vitro* on cecal mucus prepared from mice but cannot grow in the luminal content, suggesting that bacteria colonize the mouse intestine by growing in the mucus layer that overlays the cecal epithelium. In addition, other studies provide evidence that carbohydrate from the mucus can support of *E.coli* during colonization of mice (38, 185, 191, 225).

In this work, we show that catabolism of D-galactose and D-mannose by EHEC decreases expression of *ler*. Although autophosphorylation assays show that FusK can detect D-galactose, it is likely not the primary sensor of this sugar. Moreover, we demonstrate that FusK shows lower levels of autophosphorylation in the presence of GalNac and GlucNac, also present in the mucus. It would be important to determine wheter GalNac and GlucNac reduce FusK autophosphorylation or increase FusK dephosphorylation. Given that FusK responds differently to sugars commonly found in mucin, it is important to investigate the effect of mucin of FusK activity, to determine the collective effect of mucin-derived monosaccharides in FusK autophosphorylation.

Our findings suggest that carbon sources are not only important as energy sources but they have a dual role as modulators of virulence gene expression (Figure 6.5). EHEC grown on D-galactose and D-mannose presents diminished expression of *ler*. EHEC may encode for a primary D-galactose sensor that mediates *ler* repression. Similarly, an unknown mannose sensor might be

involved in *ler* repression, given that FusK does not responded to mannose, and *ler* expression being imperative to promote AE lesions, it is important to further investigate the effect of D-galactose and D-mannose on AE lesion formation by FAS assays. FusK showed decreased phoshorylation in the presence of GalNac and GlucNac. It would be important to investigate by qRT-PCR whether GalNac and GlucNac alter *ler* transcription in EHEC mediated by FusK. It has also been demonstrated that the sugar N-acetyllactosamine (LacNac) inhibits EPEC adhesion to host cells and reduces transcription of *LEE1* (113). Hence, carbohydrates might play a major role in controlling expression of the LEE genes. Additionally, it should be further explored whether other mucin-derived carbohydrates affect expression of additional virulence genes, such as flagellar motility genes, which are crucial for EHEC swimming through the mucus layer.

Therefore, we provide an initial assessment of the role of mucin-derived carbohydrates in EHEC virulence gene expression. Further investigations of the effect of different monosaccharides on EHEC motility, adhesion and AE lesion should be conducted to elucidate the significant of EHEC to interpret the availability of multiple carbon sources and to coordinate expression of metabolic and virulence genes during infection.

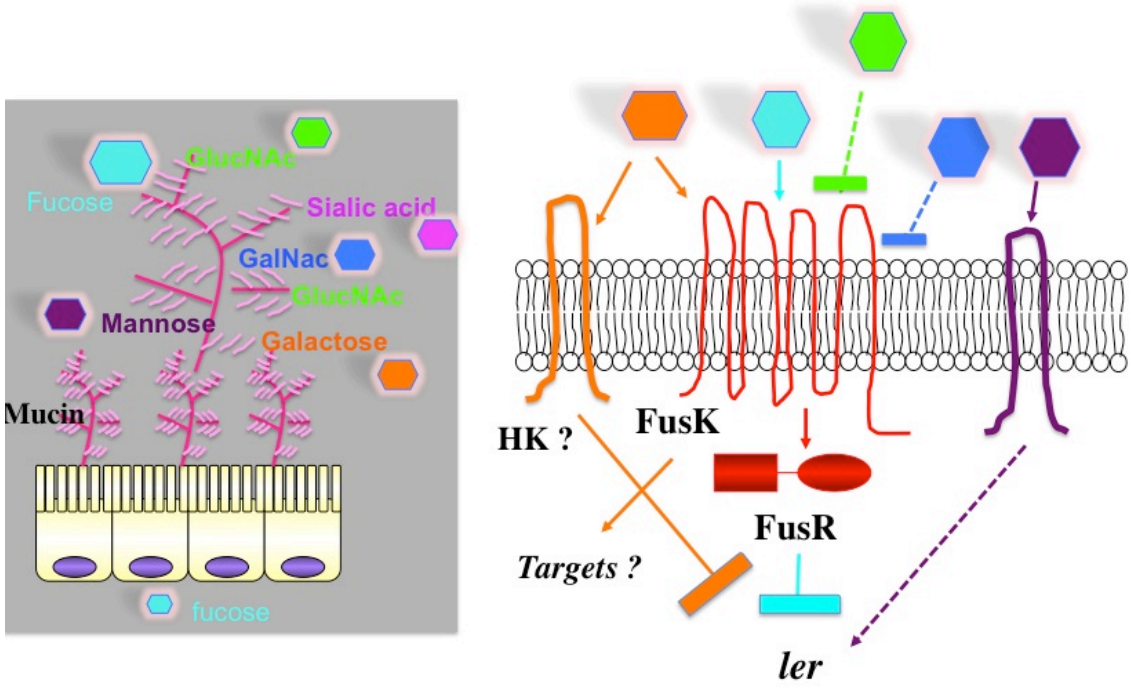

Figure 6.5. Model of sugar sensing by FusK and virulence regulation in EHEC.

CHAPTER 7

CONCLUSIONS AND FUTURE PERSPECTIVES

Colonization of the mammalian intestine by EHEC O157:H7 requires precise coordination of metabolic and virulence factors. The infectious dose of EHEC is remarkably low compared to other enteric pathogens, suggesting important adaptations of EHEC to the human intestine. The human distal intestine is one the most complex ecosystems on earth. EHEC must expand its population to high numbers and find a niche in the colon, which is a major challenge considering the immense number of residing commensal bacteria selected to live in the colon during millions of years of co-evolution with the human host (160).

The AI-3/Epi/NE cell-to-cell signaling cascade is an ancient evolutionary acquisition of EHEC that plays a fundamental role in the regulation of virulence gene expression. Commensal-bacteria produced AI-3 is an important environmental cue that primes EHEC for colonization of the intestine. Detection of AI-3 activates transcription of genes involved in flagellar motility, allowing EHEC to swim across the viscous mucus layer that separates the intestinal epithelium from the luminal contents and the microbiota. The mammalian stress hormones Epi and NE are also important signals that trigger expression of the

T3SS required for intimate attachment and AE lesion development on the intestinal epithelium and Stx production. The adrenergic sensors QseC and QseE are the only Epi sensors harbored by EHEC and, therefore, constitute the core of the AI-3/Epi/NE inter-kingdom signaling. QseC and QseE are the sensory component of the QseBC and QseEF TCSs, respectively. The QseBC system is a pleiotropic regulatory pathway that coordinates expression of flagellar motility genes, T3SS and Stx, by activating cognate (QseB) and non-cognate (QseF and KdpE) transcriptional regulators (110). QseEF together with the outer membrane protein QseG, controls the activity of the T3SS and pedestal formation by activating expression of EspFU (236). QseE also regulates transcription of several TCSs, hence it might be involved in coordination of multiple sensory pathways in EHEC (234).

We discovered a novel member of the AI-3/Epi/NE inter-kingdom signaling in EHEC, the TCS FusKR. FusK is a sensor HK and FusR is a RR (Figure 4.15). By employing biochemical and genetic approaches, we started the phenotypic characterization of FusKR. Isogenic mutants of *fusK* and *fusR* genes were constructed in order to investigate the regulatory role of FusKR in EHEC. Transcriptional analyses by qRT-PCR revealed that FusKR act as a negative regulator of the LEE (Figure 4.4) and AE lesion formation (Figure 4.7).

Congruent with the LEE transcriptional profile, *fusK* and *fusR* mutants over secrete EspB, the pore-forming T3SS effector that is involved in

translocation of effectors across the needle-like apparatus of EHEC into the host cell cytosol (Figure 4.7A). Interestingly, levels of EspA secreted by *fusK*- and *fusR*- strains is similar to levels detected in EHEC WT. Nonetheless, EspA levels in the *fusK*+ and *fusR*+ strains, which overexpress *fusK* and *fusR* from an inducible plasmid, is lower compared to EHEC WT, indicating the possibility of post-transcriptional regulation of *LEE4* mediated by FusKR, resulting in different levels of EspA and EspB transcripts. The *LEE4* operon is subject to post-transcriptional regulation involving RNAseE, resulting in heterogeneous population of *esp* transcritps (168). We performed northern blots for *LEE4* operon in EHEC WT, *fusK*- and *fusK*+ strains. Our results show increased levels of all *LEE4* transcripts in the *fusK*- strain compared to EHEC WT (Figure 7.1). These observations suggest FusKR regulates expression of small RNAs involved in processing of the *LEE4* transcripts, and differential processing of the EspA and EspB RNAs might be underlying the contrasting secretion of EspA/EspB by *fusK*-. To investigate the role of FusKR in small RNA regulation, RNAseq could be performed, allowing enrichment of small RNAs and further identification of the ones targeted by FusKR regulation.

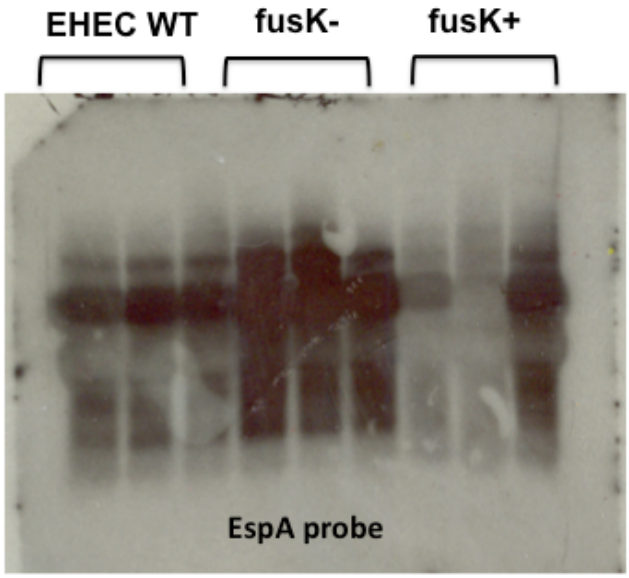

Figure 7.1. **Northern blot of *LEE4***. Northern blot analysis of LEE4 using probe for EspA shows increased levels of EspA-containing transcripts undergoing post-transcriptional processing.

Additionally, FusK/FusR might regulate production of other T3SS effectors. A preparation of secreted proteins obtained from *fusK*- strain showed increased levels of many proteins compared to EHEC WT, as visualized with Coomassie staining (Figure 7.2). The secreted profile of *fusK*- could be further explored by more powerful techniques such as electrospray ionization mass spectrometry (ESI-MS) or bidimentional electrophoresis, which can allow identification of new targets of FusK regulation.

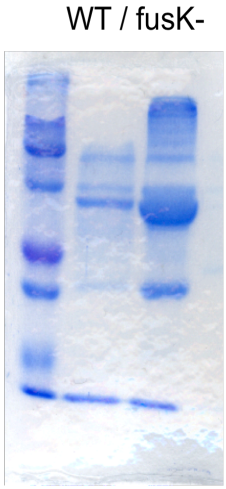

Figure 7.2. Secreted proteins. Secreted proteins collected from EHEC WT and *fusK*- culture supernatants.

We also conducted *in vitro* and *in vivo* infection experiments to elucidate the role of FusKR in EHEC pathogenicity. Infection of HeLa cells by *fusK*- and *fusR*- strains resulted in the formation of significantly higher numbers of AE lesions, indicating that *fusK*- and *fusR*- strains are hypervirulent *in vitro*. The phenotype displayed by *fusK*- was also reproduced during infection of polarized HT-29 cells (Figure 7.3). *In vivo* studies using the infant rabbit model, performed in collaboration with Jennifer Ritchie and Matthew Waldor, contributed to a better understanding of the importance of FusKR for EHEC pathogenicity.

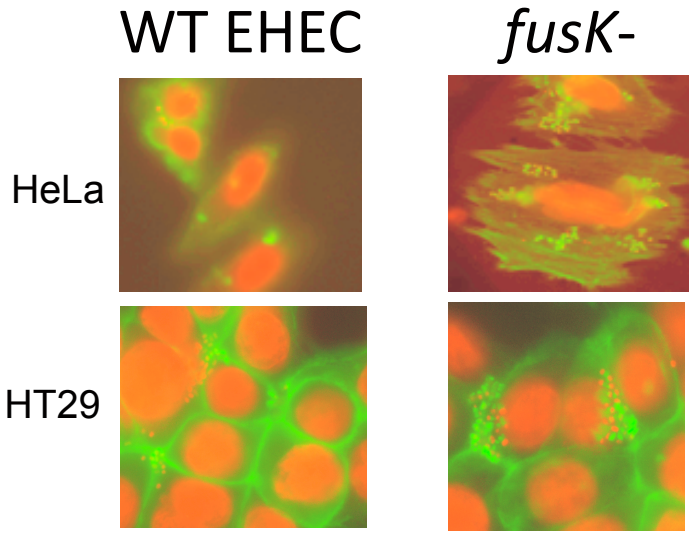

Figure 7.3. Infection of HT29 cells by EHEC. FAS of HT29 cells were infected with EHEC WT and *fusK*-.

In contrast to the hypervirulent phenotype seen during infection *in vitro*, *fusK*- was attenuated for colonization of the infant rabbit gut (Figure 4.12D). These observations suggest that overexpression of the T3SS might be deleterious, and result in EHEC elimination from the mammalian gut. Also, it could indicate that FusK/FusR controls *in vivo* production of other important virulence factors required for successful establishment of EHEC infection. We pursued the investigation of additional virulence genes in *fusK*- and *fusR*- strains. Our global transcriptional analyses by microarray indicate that *fusK*- and *fusR*- strains

presented altered levels of non-LEE encoded effectors as well as other putative effectors (Figure 4.3). We confirmed by qRT-PCR that FusK/FusR activate the expression of NleA, NleD and NleE, which are type 3 secreted effectors involved in epithelial cell damage by EHEC (Figure 7.4). Moreover, genes encoding the *gad* system, involved in acid resistance, presented decreased expression in *fusK*- and *fusR*- strains. A FusR binding site was identified upstream of *gadB*, the gene encoding one isoenzyme of the acid resistance gad complex. Hence, the poor colonization displayed by *fusK*- could be the results of global misregulation of virulence gene expression, suggesting that signal transduction by FusKR is a fundamental pathway governing EHEC colonization of the mammalian intestine. Regulation of acid resistance by FusKR could be further explored, by performing growth curves in acidified media and CFU measurement.

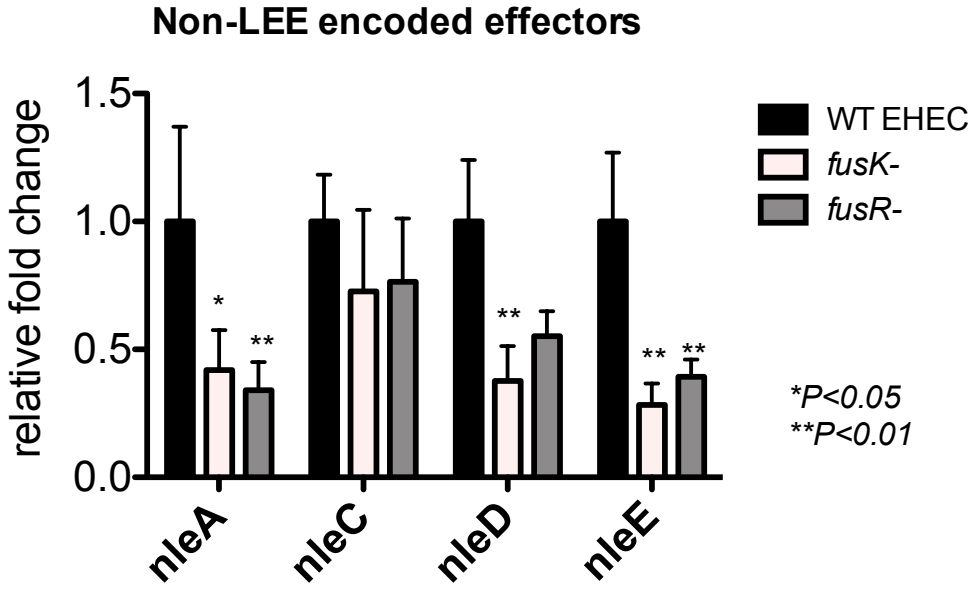

Figure 7.4. FusKR regulation of Non-LEE encoded effectors. FusK/FusR activate expression of NleA, NleD and NleE. RNA levels are normalized to endogenous *rpoA*. Statistical significance was determined using Two-way ANOVA with Bonferroni's multiple comparison test, and it is indicated as follows: one asterisk, $P<0.05$; two asterisks, $P<0.01$.

We have begun to elucidate the mechanisms underlying FusR-mediated repression of the LEE, by performing nested deletion analyses, EMSAs and footprinting. Investigation of the regulatory mechanism of FusR revealed that this RR targets the *LEE1* promoter, interacting with a nucleotide sequence localized between the P1 and P2 promoters (Figure 4.6). Being an essential determinant of EHEC pathogenesis, the LEE is targeted by a network formed by multiple

transcriptional regulators, including transcriptional activators that interact with the *LEE1* promoter in close proximity to the FusR binding site. Hence, it should be investigated whether FusR interacts with other LEE-regulators via protein-protein interactions. GrlA and GrlR are regulators of LEE transcription whose mechanism involves heterodimer formation GrlA/GrlR (50). The QseA binding site is located only 30 bp farther from the FusR binding site. QseA is a positive activator of the LEE and is part of the AI-3/Epi/NE signaling cascade (140). Yeast-two hybrid assays using recombinant FusR could lead to the identification of possible partners. Additionally, ongoing investigations in our laboratory indicate that the LuxR-homolog SdiA interacts with the *LEE1* regulatory region comprising both P1 and P2 promoters, similarly to FusR (Y Nguyen, data not published).

In the work presented herein, we employed biochemical techniques that allowed us to discover that FusK senses L-fucose as a cue. Fucose-sensing by a HK has never been demonstrated before. We successfully produced recombinant full-length FusK and reconstituted into liposomes. This task was a major challenge, in virtue of the 8-membrane spanning domains harbored by FusK (Figure 4.1). Predicted topology of FusK indicates the formation of an extended loop in the periplasmic domain that located immediately before the last transmembrane domain, similarly to UhpB topology (117). Future studies should investigate amino acids required for fucose detection by FusK. Alanine-scanning mutagenesis could be employed to generate point mutations in FusK, then

liposome reconstitution of FusK mutants would allow assessment of autophosphorylation in response to fucose. Additionally, the orientation of FusK in the bacterial membrane could be pursued, through construction of *phoA* and *lacZ* fusions to determine, respectively, the localization of domains facing the periplasm or cytoplasm.

Based on the discovery that FusKR senses fucose to regulate expression of *ler* (Figure 4.12), and that EHEC and *B.theta* interact to modulate this feature (Figure 4.12B), an interesting research area was opened and could be further explored. There are few reports about the interactions that occur between commensal and pathogenic bacteria (131, 190, 319). Among the several follow up projects that can be envisioned would be to investigate the impact of *B.theta* consumption of complex polysaccharides in virulence gene expression by EHEC. We performed qRT-PCR to measure expression of *ler* during co-culture of EHEC and *B.theta* in SHIME medium. The SHIME medium has a complex composition and it has been used to mimic the intestinal environment. We observed increased expression of *ler* in the presence of *B.theta* during co-culture with EHEC in SHIME medium, what contrasts with our results from co-cultures of EHEC and *B.theta* on mucin (Figure 7.5). SHIME medium contains, among other carbon sources, starch. Starch is a polymer of glucose that is efficiently consumed by *B.theta*, which harbors a cluster of genes required for starch utilization (Sus)

(237). Investigations on the role of starch in EHEC gene expression are in progress.

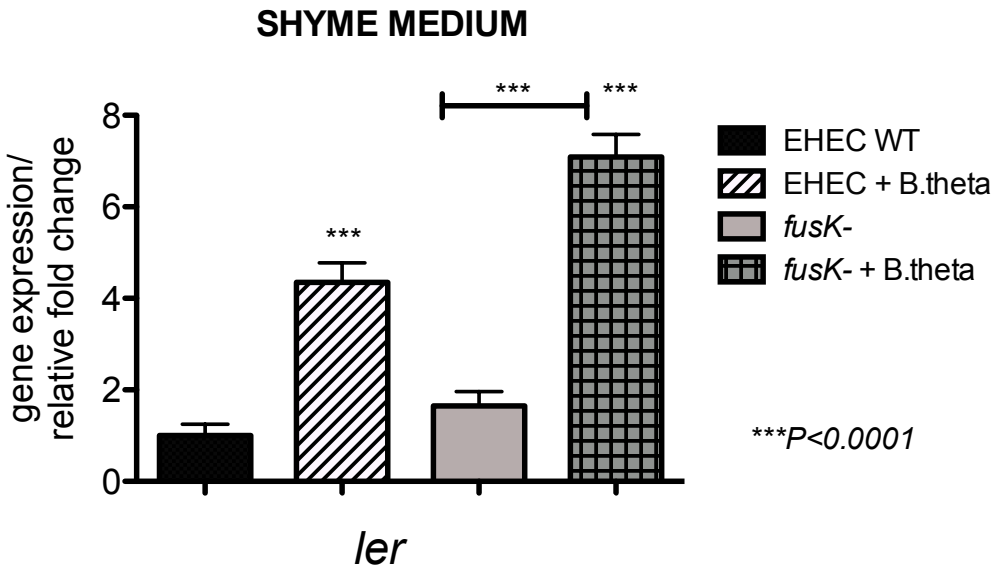

Figure 7.5. Co-culture of EHEC with *B.theta* in SHYME medium increases *ler* expression. Statistical significance was determined using One way ANOVA with Bonferroni's multiple comparison test, and it is indicated as follows: three asterisks, $P<0.001$.

Our co-culture experiments of *B.theta* and EHEC on mucin suggest that *B.theta* degrades mucin, likely supplying monosaccharides to EHEC (Figure 4.12). We also provide evidence that the presence of *B.theta* changes expression of *ler* upon growth in different culture media. One could hypothesize that degradation of other complex polysaccharides by *B.theta* could also modulate

EHEC virulence gene expression. It has been demonstrated that when *B.theta* is supplied a diet poor in complex glycans, it switches its metabolic activity towards host-derived glycans, indicating that the diet can affect the metabolic state of the microbiota. It is also known that *B.theta* is a primary fermenter capable to degrade a variety of complex polysaccharides, releasing simpler carbon sources that can be utilized by other members of the microbiota. Therefore, the effect of sugars sources introduced in the mammalian host by the diet impacts *B.theta*'s metabolism, which can consequently change the profile of carbohydrate sources available to other intestinal bacteria, commensal or pathogenic. Few studies have focused on the indirect effect of diet on bacterial pathogenesis, mostly involving probiotic bacteria or human milk polysaccharides. Manipulation of bacterial pathogenesis by a diet that could favor a "healthy microbiota" could represent a novel anti-virulence approach against infection by enteric bacteria. Fucoidan is a fucose polymer derived from algae. *B.theta* degradation of fucoidan would release free fucose into the medium in the absence of other carbohydrate sources, like during growth in the complex mucin. Co-culture of EHEC and *B.theta* using fucoidan is in progress.

In virtue of the importance of fucose for EHEC virulence and metabolism, it could be investigated whether EHEC changes fucosylation of the intestinal epithelium. EHEC can utilize fucose as carbon source *in vivo*. Inability to use fucose *in vivo* results in a colonization defect mainly in the later stages of

infection (69, 263). We show here that FusK/FusR represses the fucose regulon involved in fucose catabolism (Figure 4.9) indirectly, by decreasing transcription of Z0461 predicted transporter involved in fucose uptake (Figure 4.11). According to our model the FusK/FusR system is likely repressed in close proximity to the intestinal epithelial lining, relieving repression of the *fuc* regulon and consequently promoting fucose uptake (Figure 4.15). One could hypothesize that EHEC could use fucose at this stage. Given that fucose is usually a terminal-linked sugar in mucin glycoproteins, and AE lesion causes destruction of microvilli releasing cell membrane debris, fucose could still be accessible to EHEC during intimate attachment to the intestinal epithelium. Therefore, it could be tested whether EHEC induces fucosylation of the host glycans in order to coordinate a carbon supply. Fucosylation induction in eukaryotic hosts by bacteria has precedent (31). *B.theta* induces fucosylation in the small intestine by production of an unknown signal, and then harvest free fucose to use as carbon and energy source (105). Fucosylation could be tested by infecting HT-29 cells with EHEC and using the lectins *Ulex europaeus* agglutinin-1 (UEA-1) or *Aleuria aurantia* agglutinin (AAA), which has affinity for α-linked fucose, to detect fucosylated glycans (114, 279).

Regulation of the availability of fucosylated glycans might modulate the extent of AE lesion formation by EHEC. Studies on the interaction between EHEC and enterocytes might provide insights on how the fucose utilization by

bacteria can protect or promote EHEC infection. There is no known fucosidases produced by EHEC. However, this pathogen produces proteases, such as StcE, that contribute to mucin degradation (93, 157).

Increasing evidence points that infection by pathogenic bacteria affects microbiota composition (97, 131, 161, 319). On the other hand, little is known of the effects of pathogenic bacteria on the gene expression profile of commensals. Our studies suggest that *B.theta* degradation of mucin likely release monosaccharidic fucose into the culture media. Genomic analysis indicates that *B.theta* encodes four fucosidases that scavenge fucose from fucosylated glycans. Due to the close relationship between EHEC and *B.theta* involving fucose, it would be important to investigate whether EHEC alters fucosidase expression in *B.theta* during growth in mucin.

FusK/FusR appears to be more complex than the prototypic TCS composed by a HK and a RR. Data presented herein demonstrate that FusK/FusR represses transcription of Z0461, a putative MFS involved in efficient fucose utilization by an undefined mechanism (Figure 4.11). The participation of the putative membrane transporter Z0461 indicates that signal transduction mediated by FusK/FusR might involve another point of regulation: substrate transport. There is evidence of the participation of ABC or MFS transporters in regulation of autophosphorylation activity, a phenomenon recently referred to as "co-sensing" (280). The lysine transporter LysP regulates CadC activity; UhpC alters

UhpB response to the signal glucose-6-phosphate (281). To further explore the role of Z0461 in FusK/FusR signaling cascade, it should be evaluated the role of Z0461 as a fucose transporter. To this end, a recombinant Z0461 should be generated and inserted into liposomes. Moreover, Z0461 could affect FusK response to fucose. This possibility could be tested *in vitro* by reconstituting both FusK and Z0461 into liposome vesicles, then performing autphosphorylation assays in the presence of fucose.

FusK/FusR is repressed by QseBC. Given that *fusK* expression does not change in the presence of epinephrine, *fusK* repression mediated by QseB could be activated by AI-3. In this study, we provide the foundation for future studies that will dissect regulation of FusKR expression. We showed by EMSA that phosphorylated QseB directly represses *fusK* transcription by interacting with the *fusKR* regulatory region (Figure 4.1). Similarly, we showed that FusR is capable to repress its own expression by interacting with the *fusKR* promoter in the presence of acetyl phosphate as a phosphodonor. Using primer extension analysis, we have identified the promoters that control *fusK/fusR* expression. The regulatory region of *fusKR* comprises two overlapping promoters, one sigma 28 and one sigma 70 (Figure 7.6). It has been reported that QseB binds both classes of promoters: the *flhDC* promoter (sigma 28) and the *qseBC* promoter (sigma 70) (42, 43). On the other hand, FusR is a sigma 70 transcriptional regulator. The regulation of the *fusKR* promoter could be further investigated to better

understand the importance of the crosstalk among QseBC and FusK/FusR transduction systems. Nested deletion analysis could be conducted, using transcriptional fusions of the *fusKR* regulatory region that contain solely the sigma 28 or the sigma 70 promoters. QseB regulates motility directly while FusR regulates AE lesion. The presence of such a complex promoter region suggests that FusKR might be a critical system participating in the switch between the swimming motility and intimate attachment to the epithelium.

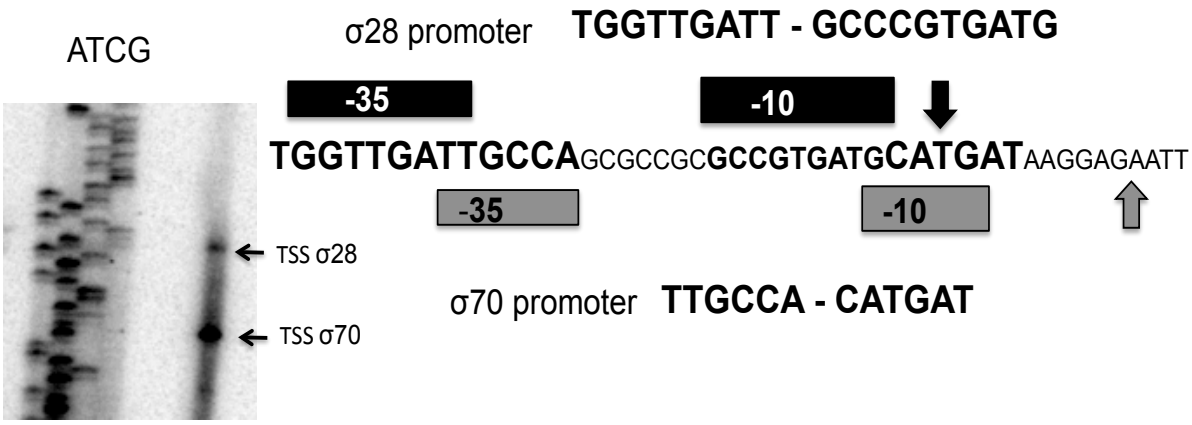

Figure 7.6. Identification of the *fusKR* promoter. Primers extension analysis was performed with RNA from EHEC WT grown at 37°C to an OD_{600} of 1.0. Two transcriptional start sites were mapped, leading to the identification of two overlapping sigma 28 (black box) and sigma 70 (grey box) promoters in the regulatory region of *fusKR*.

Only a few bacterial species harbor *fusK/fusR* or the OI-20 that cluster these genes: EHEC O157:H7, EPEC O55:H7, *Citrobacter rodentium* and *E.coli* ED1a. The *E.coli* ED1a strain is the only commensal *E.coli* that encodes FusK/FusR. It has been proposed that *E.coli* ED1a is a human-specific clone. In this work we show that FusK/FusR regulates virulence and metabolic genes. The role of FusK/FusR in commensal *E.coli* ED1a should be investigated to evaluate if fucose sensing can also be involved in host commensal-host relationships. Otherwise, the presence of *fusK/fusR* in *C. rodentium* would allow future animal work using mice. Infection by the natural murine pathogen *C. rodentium* has been used as a model for human infection by EPEC and EHEC, as they are also capable to form AE lesions in the murine intestine (57). The use *of C.rodentium* to study FusK/FusR *in vivo* is convenient given the possibility use germ-free and knockout mice strains. *C. rodentium* encodes the whole OI-20 therefore could be a relevant model for investigations of FusK/FusR implications in pathogenicity. Attempts to generate a *fusK*- in *C.rodentium* have failed but are still ongoing.

In summary, the findings obtained in this study revealed a novel signal transduction system of EHEC, the FusK/FusR TCS. While the signal that activate many HKs are elusive, we successfully identified that fucose is the signal that triggers the FusKR signaling cascade. FusKR is a major regulatory feature of

EHEC, controlling expression of the T3SS and secreted effectors. Fucose-sensing by FusKR is involved in complex interactions between EHEC and the human symbiont *B.theta* during growth on mucin. Regulation of the LEE by FusKR is crucial for EHEC pathogenicity, and lack of the FusK HK renders EHEC unable to maximally colonize the mammalian intestine. Therefore, the results generated in this investigation represent a major step towards a better understanding of the mechanisms underlying EHEC pathogenicity at the microbiota-host interface.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my mentor, Dr. Vanessa Sperandio, for her support and encouragement throughout my graduate studies. Thank you, Vanessa, for allowing me to work in such an exciting and challenging project that kept me mentally stimulated for all these years. It has been a privilege to work with you. Thank you also for believing in me and for helping me to explore my potential to the fullest; you've helped me to become a better and more confident scientist. I would also like to thank the members of my graduate committee, Dr. Neal Alto, Dr. Kevin Gardner and Dr. Michael Shiloh, for their guidance and productive criticism that helped me to evolve my critical skills and to improve my research.

I also would like to thank the past and current Sperandio lab members for their support and stimulating discussions. Thank you David Hughes, Nicola Reading, Regan Russell, Melissa Kendall, Charley Gruber and Christopher Parker. Special thanks for Cristiano Moreira and Meredith Curtiss for actively contributing to the completion of this project. It was a privilege to work with such brilliant scientists.

Most importantly, I would like to thank my family for their love and support, and for have always encouraged me to pursue my dreams, even when that meant to loose me for a far away land. I would like to thank my mom Odette

and my father Paulo, for all your support, I love you very much. Thank you for your unconditional love throughout my whole life. I would like to thank my sisters Rosana, Sandra and Renata, for always encouraging me to keep going even when things seemed so difficult. Thank you my nephews Mareska, Rafaelly, Isabella, Joao Paulo and Leonardo, for reminding me of how loved I am and give me the strength to complete my journey here.

Specially, I would like to thank my husband Tiago Ramos for his love and support. Thank you, my love, for leaving everything behind just to be by my side while following my dream. Thank you for being there, for the best and for the worst in graduate school. I would not have done without you. I love you, always.

BIBLIOGRAPHY

1. **Abe, A., M. de Grado, R. A. Pfuetzner, C. Sanchez-Sanmartin, R. Devinney, J. L. Puente, N. C. Strynadka, and B. B. Finlay.** 1999. Enteropathogenic Escherichia coli translocated intimin receptor, Tir, requires a specific chaperone for stable secretion. Mol Microbiol **33:**1162-75.
2. **Abu-Ali, G. S., L. M. Ouellette, S. T. Henderson, D. W. Lacher, J. T. Riordan, T. S. Whittam, and S. D. Manning.** 2010. Increased adherence and expression of virulence genes in a lineage of Escherichia coli O157:H7 commonly associated with human infections. PloS one **5:**e10167.
3. **Abu-Median, A. B., P. M. van Diemen, F. Dziva, I. Vlisidou, T. S. Wallis, and M. P. Stevens.** 2006. Functional analysis of lymphostatin homologues in enterohaemorrhagic Escherichia coli. FEMS Microbiol Lett **258:**43-9.
4. **Acheson, D. W., R. Moore, S. De Breucker, L. Lincicome, M. Jacewicz, E. Skutelsky, and G. T. Keusch.** 1996. Translocation of Shiga toxin across polarized intestinal cells in tissue culture. Infect Immun **64:**3294-300.
5. **Alto, N. M., F. Shao, C. S. Lazar, R. L. Brost, G. Chua, S. Mattoo, S. A. McMahon, P. Ghosh, T. R. Hughes, C. Boone, and J. E. Dixon.** 2006. Identification of a bacterial type III effector family with G protein mimicry functions. Cell **124:**133-45.
6. **Arthur, T. M., X. W. Nou, N. Kalchayanand, J. M. Bosilevac, T. Wheeler, and M. Koohmaraie.** 2011. Survival of Escherichia coli O157:H7 on Cattle Hides. Applied and Environmental Microbiology **77:**3002-3008.
7. **Augeron, C., and C. L. Laboisse.** 1984. Emergence of permanently differentiated cell clones in a human colonic cancer cell line in culture after treatment with sodium butyrate. Cancer Res **44:**3961-9.
8. **Autieri, S. M., J. J. Lins, M. P. Leatham, D. C. Laux, T. Conway, and P. S. Cohen.** 2007. L-fucose stimulates utilization of D-ribose by Escherichia coli MG1655 DeltafucAO and E. coli Nissle 1917 DeltafucAO mutants in the mouse intestine and in M9 minimal medium. Infect Immun **75:**5465-75.
9. **Bai, J., S. P. McAteer, E. Paxton, A. Mahajan, D. L. Gally, and J. J. Tree.** 2011. Screening of an E. coli O157:H7 Bacterial Artificial Chromosome Library by Comparative Genomic Hybridization to Identify Genomic Regions Contributing to Growth in Bovine Gastrointestinal Mucus and Epithelial Cell Colonization. Frontiers in microbiology **2:**168.

10. **Bakovic, M., M. D. Fullerton, and V. Michel.** 2007. Metabolic and molecular aspects of ethanolamine phospholipid biosynthesis: the role of CTP:phosphoethanolamine cytidylyltransferase (Pcyt2). Biochem Cell Biol **85:**283-300.
11. **Baldoma, L., and J. Aguilar.** 1988. Metabolism of L-fucose and L-rhamnose in Escherichia coli: aerobic-anaerobic regulation of L-lactaldehyde dissimilation. J Bacteriol **170:**416-21.
12. **Barba, J., V. H. Bustamante, M. A. Flores-Valdez, W. Deng, B. B. Finlay, and J. L. Puente.** 2005. A positive regulatory loop controls expression of the locus of enterocyte effacement-encoded regulators Ler and GrlA. J Bacteriol **187:**7918-30.
13. **Barrios, H., B. Valderrama, and E. Morett.** 1999. Compilation and analysis of sigma(54)-dependent promoter sequences. Nucleic Acids Res **27:**4305-13.
14. **Barthold, S. W., G. L. Coleman, R. O. Jacoby, E. M. Livestone, and A. M. Jonas.** 1978. Transmissible murine colonic hyperplasia. Vet Pathol **15:**223-36.
15. **Baruch, K., L. Gur-Arie, C. Nadler, S. Koby, G. Yerushalmi, Y. Ben-Neriah, O. Yogev, E. Shaulian, C. Guttman, R. Zarivach, and I. Rosenshine.** 2011. Metalloprotease type III effectors that specifically cleave JNK and NF-kappaB. Embo J **30:**221-31.
16. **Bauer, M. E., and R. A. Welch.** 1996. Characterization of an RTX toxin from enterohemorrhagic Escherichia coli O157:H7. Infect Immun **64:**167-75.
17. **Bearson, B. L., and S. M. Bearson.** 2008. The role of the QseC quorum-sensing sensor kinase in colonization and norepinephrine-enhanced motility of Salmonella enterica serovar Typhimurium. Microb Pathog **44:**271-8.
18. **Bearson, B. L., S. M. Bearson, I. S. Lee, and B. W. Brunelle.** 2010. The Salmonella enterica serovar Typhimurium QseB response regulator negatively regulates bacterial motility and swine colonization in the absence of the QseC sensor kinase. Microb Pathog **48:**214-9.
19. **Beery, J. T., M. P. Doyle, and J. L. Schoeni.** 1985. Colonization of chicken cecae by Escherichia coli associated with hemorrhagic colitis. Appl Environ Microbiol **49:**310-5.
20. **Bell, B. P., M. Goldoft, P. M. Griffin, M. A. Davis, D. C. Gordon, P. I. Tarr, C. A. Bartleson, J. H. Lewis, T. J. Barrett, J. G. Wells, and et al.** 1994. A multistate outbreak of Escherichia coli O157:H7-associated bloody diarrhea and hemolytic uremic syndrome from hamburgers. The Washington experience. Jama **272:**1349-53.

21. **Berdichevsky, T., D. Friedberg, C. Nadler, A. Rokney, A. Oppenheim, and I. Rosenshine.** 2005. Ler is a negative autoregulator of the LEE1 operon in enteropathogenic Escherichia coli. J Bacteriol **187:**349-57.
22. **Bergstrom, K. S., V. Kissoon-Singh, D. L. Gibson, C. Ma, M. Montero, H. P. Sham, N. Ryz, T. Huang, A. Velcich, B. B. Finlay, K. Chadee, and B. A. Vallance.** 2010. Muc2 protects against lethal infectious colitis by disassociating pathogenic and commensal bacteria from the colonic mucosa. PLoS pathogens **6:**e1000902.
23. **Bertin, Y., J. P. Girardeau, F. Chaucheyras-Durand, B. Lyan, E. Pujos-Guillot, J. Harel, and C. Martin.** 2011. Enterohaemorrhagic Escherichia coli gains a competitive advantage by using ethanolamine as a nitrogen source in the bovine intestinal content. Environ Microbiol **13:**365-77.
24. **Bettelheim, K. A., and S. M. Lennox-King.** 1976. The acquisition of Escherichia coli by new-born babies. Infection **4:**174-9.
25. **Bhatt, S., A. N. Edwards, H. T. Nguyen, D. Merlin, T. Romeo, and D. Kalman.** 2009. The RNA binding protein CsrA is a pleiotropic regulator of the locus of enterocyte effacement pathogenicity island of enteropathogenic Escherichia coli. Infect Immun **77:**3552-68.
26. **Bjork, S., M. E. Breimer, G. C. Hansson, K. A. Karlsson, and H. Leffler.** 1987. Structures of blood group glycosphingolipids of human small intestine. A relation between the expression of fucolipids of epithelial cells and the ABO, Le and Se phenotype of the donor. J Biol Chem **262:**6758-65.
27. **Boerlin, P., S. A. McEwen, F. Boerlin-Petzold, J. B. Wilson, R. P. Johnson, and C. L. Gyles.** 1999. Associations between virulence factors of Shiga toxin-producing Escherichia coli and disease in humans. J Clin Microbiol **37:**497-503.
28. **Brandt, M. L., S. O'Regan, E. Rousseau, and S. Yazbeck.** 1990. Surgical complications of the hemolytic-uremic syndrome. J Pediatr Surg **25:**1109-12.
29. **Brunder, W., H. Schmidt, and H. Karch.** 1997. EspP, a novel extracellular serine protease of enterohaemorrhagic Escherichia coli O157:H7 cleaves human coagulation factor V. Mol Microbiol **24:**767-78.
30. **Brunder, W., H. Schmidt, and H. Karch.** 1996. KatP, a novel catalase-peroxidase encoded by the large plasmid of enterohaemorrhagic Escherichia coli O157:H7. Microbiology **142 (Pt 11):**3305-15.
31. **Bry, L., P. G. Falk, T. Midtvedt, and J. I. Gordon.** 1996. A model of host-microbial interactions in an open mammalian ecosystem. Science **273:**1380-3.

32. **Burland, V., Y. Shao, N. T. Perna, G. Plunkett, H. J. Sofia, and F. R. Blattner.** 1998. The complete DNA sequence and analysis of the large virulence plasmid of Escherichia coli O157:H7. Nucleic Acids Res **26:**4196-204.
33. **Bustamante, V. H., F. J. Santana, E. Calva, and J. L. Puente.** 2001. Transcriptional regulation of type III secretion genes in enteropathogenic Escherichia coli: Ler antagonizes H-NS-dependent repression. Mol Microbiol **39:**664-78.
34. **Calderwood, S. B., and J. J. Mekalanos.** 1987. Iron regulation of Shiga-like toxin expression in Escherichia coli is mediated by the fur locus. J Bacteriol **169:**4759-64.
35. **Campellone, K. G.** 2010. Cytoskeleton-modulating effectors of enteropathogenic and enterohaemorrhagic Escherichia coli: Tir, EspFU and actin pedestal assembly. The FEBS journal **277:**2390-402.
36. **Campellone, K. G., H. C. Cheng, D. Robbins, A. D. Siripala, E. J. McGhie, R. D. Hayward, M. D. Welch, M. K. Rosen, V. Koronakis, and J. M. Leong.** 2008. Repetitive N-WASP-binding elements of the enterohemorrhagic Escherichia coli effector EspF(U) synergistically activate actin assembly. PLoS pathogens **4:**e1000191.
37. **Campellone, K. G., D. Robbins, and J. M. Leong.** 2004. EspFU is a translocated EHEC effector that interacts with Tir and N-WASP and promotes Nck-independent actin assembly. Developmental cell **7:**217-28.
38. **Chang, D. E., D. J. Smalley, D. L. Tucker, M. P. Leatham, W. E. Norris, S. J. Stevenson, A. B. Anderson, J. E. Grissom, D. C. Laux, P. S. Cohen, and T. Conway.** 2004. Carbon nutrition of Escherichia coli in the mouse intestine. Proc Natl Acad Sci U S A **101:**7427-32.
39. **Chen, Y. M., Y. Zhu, and E. C. Lin.** 1987. The organization of the fuc regulon specifying L-fucose dissimilation in Escherichia coli K12 as determined by gene cloning. Mol Gen Genet **210:**331-7.
40. **Chow, W. L., and Y. K. Lee.** 2008. Free fucose is a danger signal to human intestinal epithelial cells. Br J Nutr **99:**449-54.
41. **Clarke, M. B., D. T. Hughes, C. Zhu, E. C. Boedeker, and V. Sperandio.** 2006. The QseC sensor kinase: a bacterial adrenergic receptor. Proc Natl Acad Sci U S A **103:**10420-5.
42. **Clarke, M. B., and V. Sperandio.** 2005. Transcriptional autoregulation by quorum sensing Escherichia coli regulators B and C (QseBC) in enterohaemorrhagic E. coli (EHEC). Mol Microbiol **58:**441-55.
43. **Clarke, M. B., and V. Sperandio.** 2005. Transcriptional regulation of flhDC by QseBC and sigma (FliA) in enterohaemorrhagic Escherichia coli. Mol Microbiol **57:**1734-49.

44. **Comstock, L. E., and M. J. Coyne.** 2003. Bacteroides thetaiotaomicron: a dynamic, niche-adapted human symbiont. Bioessays **25:**926-9.
45. **Corfield, A. P., S. A. Wagner, J. R. Clamp, M. S. Kriaris, and L. C. Hoskins.** 1992. Mucin degradation in the human colon: production of sialidase, sialate O-acetylesterase, N-acetylneuraminate lyase, arylesterase, and glycosulfatase activities by strains of fecal bacteria. Infect Immun **60:**3971-8.
46. **Cotton, P. B.** 1972. Non-dietary lipid in the intestinal lumen. Gut **13:**675-81.
47. **Coyne, M. J., B. Reinap, M. M. Lee, and L. E. Comstock.** 2005. Human symbionts use a host-like pathway for surface fucosylation. Science **307:**1778-81.
48. **Craven, J. A., and D. A. Barnum.** 1971. Ecology of intestinal Escherichia coli in pigs. Can J Comp Med **35:**324-31.
49. **Cray, W. C., Jr., and H. W. Moon.** 1995. Experimental infection of calves and adult cattle with Escherichia coli O157:H7. Appl Environ Microbiol **61:**1586-90.
50. **Creasey, E. A., R. M. Delahay, S. J. Daniell, and G. Frankel.** 2003. Yeast two-hybrid system survey of interactions between LEE-encoded proteins of enteropathogenic Escherichia coli. Microbiology **149:**2093-106.
51. **Crepin, V. F., S. Prasannan, R. K. Shaw, R. K. Wilson, E. Creasey, C. M. Abe, S. Knutton, G. Frankel, and S. Matthews.** 2005. Structural and functional studies of the enteropathogenic Escherichia coli type III needle complex protein EscJ. Mol Microbiol **55:**1658-70.
52. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A **97:**6640-5.
53. **David, D. E., A. M. Lynne, J. Han, and S. L. Foley.** 2010. Evaluation of virulence factor profiling in the characterization of veterinary Escherichia coli isolates. Appl Environ Microbiol **76:**7509-13.
54. **Dean, P., and B. Kenny.** 2004. Intestinal barrier dysfunction by enteropathogenic Escherichia coli is mediated by two effector molecules and a bacterial surface protein. Mol Microbiol **54:**665-75.
55. **Deng, W., Y. Li, B. A. Vallance, and B. B. Finlay.** 2001. Locus of enterocyte effacement from Citrobacter rodentium: sequence analysis and evidence for horizontal transfer among attaching and effacing pathogens. Infect Immun **69:**6323-35.
56. **Deng, W., J. L. Puente, S. Gruenheid, Y. Li, B. A. Vallance, A. Vazquez, J. Barba, J. A. Ibarra, P. O'Donnell, P. Metalnikov, K. Ashman, S. Lee, D. Goode, T. Pawson, and B. B. Finlay.** 2004.

Dissecting virulence: systematic and functional analyses of a pathogenicity island. Proc Natl Acad Sci U S A **101:**3597-602.

57. **Deng, W., B. A. Vallance, Y. Li, J. L. Puente, and B. B. Finlay.** 2003. Citrobacter rodentium translocated intimin receptor (Tir) is an essential virulence factor needed for actin condensation, intestinal colonization and colonic hyperplasia in mice. Mol Microbiol **48:**95-115.
58. **Dowhan, W.** 1997. Molecular basis for membrane phospholipid diversity: why are there so many lipids? Annu Rev Biochem **66:**199-232.
59. **Durham-Colleran, M. W., A. B. Verhoeven, and M. L. van Hoek.** 2010. Francisella novicida forms in vitro biofilms mediated by an orphan response regulator. Microb Ecol **59:**457-65.
60. **Dziva, F., A. Mahajan, P. Cameron, C. Currie, I. J. McKendrick, T. S. Wallis, D. G. Smith, and M. P. Stevens.** 2007. EspP, a Type V-secreted serine protease of enterohaemorrhagic Escherichia coli O157:H7, influences intestinal colonization of calves and adherence to bovine primary intestinal epithelial cells. FEMS Microbiol Lett **271:**258-64.
61. **Elliott, S. J., S. W. Hutcheson, M. S. Dubois, J. L. Mellies, L. A. Wainwright, M. Batchelor, G. Frankel, S. Knutton, and J. B. Kaper.** 1999. Identification of CesT, a chaperone for the type III secretion of Tir in enteropathogenic Escherichia coli. Mol Microbiol **33:**1176-89.
62. **Elliott, S. J., C. B. O'Connell, A. Koutsouris, C. Brinkley, M. S. Donnenberg, G. Hecht, and J. B. Kaper.** 2002. A gene from the locus of enterocyte effacement that is required for enteropathogenic Escherichia coli to increase tight-junction permeability encodes a chaperone for EspF. Infect Immun **70:**2271-7.
63. **Elliott, S. J., V. Sperandio, J. A. Giron, S. Shin, J. L. Mellies, L. Wainwright, S. W. Hutcheson, T. K. McDaniel, and J. B. Kaper.** 2000. The locus of enterocyte effacement (LEE)-encoded regulator controls expression of both LEE- and non-LEE-encoded virulence factors in enteropathogenic and enterohemorrhagic Escherichia coli. Infect Immun **68:**6115-26.
64. **Elliott, S. J., L. A. Wainwright, T. K. McDaniel, K. G. Jarvis, Y. K. Deng, L. C. Lai, B. P. McNamara, M. S. Donnenberg, and J. B. Kaper.** 1998. The complete sequence of the locus of enterocyte effacement (LEE) from enteropathogenic Escherichia coli E2348/69. Mol Microbiol **28:**1-4.
65. **Elliott, S. J., J. Yu, and J. B. Kaper.** 1999. The cloned locus of enterocyte effacement from enterohemorrhagic Escherichia coli O157:H7 is unable to confer the attaching and effacing phenotype upon E. coli K-12. Infect Immun **67:**4260-3.
66. **Endo, Y., K. Tsurugi, T. Yutsudo, Y. Takeda, T. Ogasawara, and K. Igarashi.** 1988. Site of action of a Vero toxin (VT2) from Escherichia coli

O157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins. Eur J Biochem **171:**45-50.

67. **Eom, J. S., B. Y. Hwang, J. W. Sohn, W. J. Kim, M. J. Kim, S. C. Park, and H. J. Cheong.** 2002. Clinical and molecular epidemiology of quinolone-resistant Escherichia coli isolated from urinary tract infection. Microb Drug Resist **8:**227-34.
68. **Eppinger, M., M. K. Mammel, J. E. Leclerc, J. Ravel, and T. A. Cebula.** 2011. Genomic anatomy of Escherichia coli O157:H7 outbreaks. Proc Natl Acad Sci U S A **108:**20142-7.
69. **Fabich, A. J., S. A. Jones, F. Z. Chowdhury, A. Cernosek, A. Anderson, D. Smalley, J. W. McHargue, G. A. Hightower, J. T. Smith, S. M. Autieri, M. P. Leatham, J. J. Lins, R. L. Allen, D. C. Laux, P. S. Cohen, and T. Conway.** 2008. Comparison of carbon nutrition for pathogenic and commensal Escherichia coli strains in the mouse intestine. Infect Immun **76:**1143-52.
70. **Fagundes-Neto, U., E. Freymuller, M. S. Gatti, L. G. Schmitz, and I. Scaletsky.** 1995. Ultrastructural study of enteropathogenic Escherichia coli O111ab:H2 infection in an infant with acute diarrhea. Arq Gastroenterol **32:**152-7.
71. **Feng, P., K. A. Lampel, H. Karch, and T. S. Whittam.** 1998. Genotypic and phenotypic changes in the emergence of Escherichia coli O157:H7. J Infect Dis **177:**1750-3.
72. **Fischbach, M. A., and J. L. Sonnenburg.** 2011. Eating for two: how metabolism establishes interspecies interactions in the gut. Cell host & microbe **10:**336-47.
73. **Fraser, M. E., M. M. Chernaia, Y. V. Kozlov, and M. N. James.** 1994. Crystal structure of the holotoxin from Shigella dysenteriae at 2.5 A resolution. Nat Struct Biol **1:**59-64.
74. **Fraser, M. E., M. Fujinaga, M. M. Cherney, A. R. Melton-Celsa, E. M. Twiddy, A. D. O'Brien, and M. N. James.** 2004. Structure of shiga toxin type 2 (Stx2) from Escherichia coli O157:H7. J Biol Chem **279:**27511-7.
75. **Freter, R., H. Brickner, M. Botney, D. Cleven, and A. Aranki.** 1983. Mechanisms that control bacterial populations in continuous-flow culture models of mouse large intestinal flora. Infect Immun **39:**676-85.
76. **Friedberg, D., T. Umanski, Y. Fang, and I. Rosenshine.** 1999. Hierarchy in the expression of the locus of enterocyte effacement genes of enteropathogenic Escherichia coli. Mol Microbiol **34:**941-52.
77. **Fu, H. A., S. Iuchi, and E. C. Lin.** 1991. The requirement of ArcA and Fnr for peak expression of the cyd operon in Escherichia coli under microaerobic conditions. Mol Gen Genet **226:**209-13.

78. **Gao, R., and A. M. Stock.** 2009. Biological insights from structures of two-component proteins. Annu Rev Microbiol **63:**133-54.
79. **Gao, X., F. Wan, K. Mateo, E. Callegari, D. Wang, W. Deng, J. Puente, F. Li, M. S. Chaussee, B. B. Finlay, M. J. Lenardo, and P. R. Hardwidge.** 2009. Bacterial effector binding to ribosomal protein s3 subverts NF-kappaB function. PLoS pathogens **5:**e1000708.
80. **Garmendia, J., G. Frankel, and V. F. Crepin.** 2005. Enteropathogenic and enterohemorrhagic Escherichia coli infections: translocation, translocation, translocation. Infect Immun **73:**2573-85.
81. **Garmendia, J., A. D. Phillips, M. F. Carlier, Y. Chong, S. Schuller, O. Marches, S. Dahan, E. Oswald, R. K. Shaw, S. Knutton, and G. Frankel.** 2004. TccP is an enterohaemorrhagic Escherichia coli O157:H7 type III effector protein that couples Tir to the actin-cytoskeleton. Cellular Microbiology **6:**1167-83.
82. **Garmendia, J., A. D. Phillips, M. F. Carlier, Y. Chong, S. Schuller, O. Marches, S. Dahan, E. Oswald, R. K. Shaw, S. Knutton, and G. Frankel.** 2004. TccP is an enterohaemorrhagic Escherichia coli O157:H7 type III effector protein that couples Tir to the actin-cytoskeleton. Cell Microbiol **6:**1167-83.
83. **Gauthier, A., J. L. Puente, and B. B. Finlay.** 2003. Secretin of the enteropathogenic Escherichia coli type III secretion system requires components of the type III apparatus for assembly and localization. Infect Immun **71:**3310-9.
84. **Georgellis, D., O. Kwon, and E. C. Lin.** 2001. Quinones as the redox signal for the arc two-component system of bacteria. Science **292:**2314-6.
85. **Gill, S. R., M. Pop, R. T. Deboy, P. B. Eckburg, P. J. Turnbaugh, B. S. Samuel, J. I. Gordon, D. A. Relman, C. M. Fraser-Liggett, and K. E. Nelson.** 2006. Metagenomic analysis of the human distal gut microbiome. Science **312:**1355-9.
86. **Goldstein, J., C. F. Loidl, V. P. Creydt, J. Boccoli, and C. Ibarra.** 2007. Intracerebroventricular administration of Shiga toxin type 2 induces striatal neuronal death and glial alterations: an ultrastructural study. Brain research **1161:**106-15.
87. **Goldwater, P. N.** 2007. Treatment and prevention of enterohemorrhagic Escherichia coli infection and hemolytic uremic syndrome. Expert Rev Anti Infect Ther **5:**653-63.
88. **Gould, L. H., L. Demma, T. F. Jones, S. Hurd, D. J. Vugia, K. Smith, B. Shiferaw, S. Segler, A. Palmer, S. Zansky, and P. M. Griffin.** 2009. Hemolytic uremic syndrome and death in persons with Escherichia coli O157:H7 infection, foodborne diseases active surveillance network sites, 2000-2006. Clin Infect Dis **49:**1480-5.

89. **Grant, J., A. M. Wendelboe, A. Wendel, B. Jepson, P. Torres, C. Smelser, and R. T. Rolfs.** 2008. Spinach-associated Escherichia coli O157:H7 outbreak, Utah and New Mexico, 2006. Emerg Infect Dis **14:**1633-6.
90. **Grauke, L. J., I. T. Kudva, J. W. Yoon, C. W. Hunt, C. J. Williams, and C. J. Hovde.** 2002. Gastrointestinal tract location of Escherichia coli O157:H7 in ruminants. Appl Environ Microbiol **68:**2269-77.
91. **Greig, J. D., and A. Ravel.** 2009. Analysis of foodborne outbreak data reported internationally for source attribution. Int J Food Microbiol **130:**77-87.
92. **Gruenheid, S., I. Sekirov, N. A. Thomas, W. Deng, P. O'Donnell, D. Goode, Y. Li, E. A. Frey, N. F. Brown, P. Metalnikov, T. Pawson, K. Ashman, and B. B. Finlay.** 2004. Identification and characterization of NleA, a non-LEE-encoded type III translocated virulence factor of enterohaemorrhagic Escherichia coli O157:H7. Mol Microbiol **51:**1233-49.
93. **Grys, T. E., M. B. Siegel, W. W. Lathem, and R. A. Welch.** 2005. The StcE protease contributes to intimate adherence of enterohemorrhagic Escherichia coli O157:H7 to host cells. Infect Immun **73:**1295-303.
94. **Grys, T. E., L. L. Walters, and R. A. Welch.** 2006. Characterization of the StcE protease activity of Escherichia coli O157:H7. J Bacteriol **188:**4646-53.
95. **Gunzer, F., I. Hennig-Pauka, K. H. Waldmann, R. Sandhoff, H. J. Grone, H. H. Kreipe, A. Matussek, and M. Mengel.** 2002. Gnotobiotic piglets develop thrombotic microangiopathy after oral infection with enterohemorrhagic Escherichia coli. Am J Clin Pathol **118:**364-75.
96. **Haack, K. R., C. L. Robinson, K. J. Miller, J. W. Fowlkes, and J. L. Mellies.** 2003. Interaction of Ler at the LEE5 (tir) operon of enteropathogenic Escherichia coli. Infection & Immunity **71:**384-92.
97. **Haag, L. M., A. Fischer, B. Otto, R. Plickert, A. A. Kuhl, U. B. Gobel, S. Bereswill, and M. M. Heimesaat.** 2012. Intestinal microbiota shifts towards elevated commensal Escherichia coli loads abrogate colonization resistance against Campylobacter jejuni in mice. PloS one **7:**e35988.
98. **Habdas, B. J., J. Smart, J. B. Kaper, and V. Sperandio.** 2010. The LysR-type transcriptional regulator QseD alters type three secretion in enterohemorrhagic Escherichia coli and motility in K-12 Escherichia coli. J Bacteriol **192:**3699-712.
99. **Hadjifrangiskou, M., M. Kostakioti, S. L. Chen, J. P. Henderson, S. E. Greene, and S. J. Hultgren.** 2011. A central metabolic circuit controlled by QseC in pathogenic Escherichia coli. Mol Microbiol **80:**1516-29.

100. **Hansen, A. M., and J. B. Kaper.** 2009. Hfq affects the expression of the LEE pathogenicity island in enterohaemorrhagic Escherichia coli. Mol Microbiol **73:**446-65.
101. **Hemrajani, C., C. N. Berger, K. S. Robinson, O. Marches, A. Mousnier, and G. Frankel.** 2010. NleH effectors interact with Bax inhibitor-1 to block apoptosis during enteropathogenic Escherichia coli infection. Proc Natl Acad Sci U S A **107:**3129-34.
102. **Heuvelink, A. E., C. van Heerwaarden, J. T. Zwartkruis-Nahuis, R. van Oosterom, K. Edink, Y. T. van Duynhoven, and E. de Boer.** 2002. Escherichia coli O157 infection associated with a petting zoo. Epidemiol Infect **129:**295-302.
103. **Ho, T. D., B. M. Davis, J. M. Ritchie, and M. K. Waldor.** 2008. Type 2 secretion promotes enterohemorrhagic Escherichia coli adherence and intestinal colonization. Infect Immun **76:**1858-65.
104. **Holmes, A., C. S. Lindestam Arlehamn, D. Wang, T. J. Mitchell, T. J. Evans, and A. J. Roe.** 2012. Expression and regulation of the Escherichia coli O157:H7 effector proteins NleH1 and NleH2. PloS one **7:**e33408.
105. **Hooper, L. V., L. Bry, P. G. Falk, and J. I. Gordon.** 1998. Host-microbial symbiosis in the mammalian intestine: exploring an internal ecosystem. Bioessays **20:**336-43.
106. **Hooper, L. V., T. Midtvedt, and J. I. Gordon.** 2002. How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu Rev Nutr **22:**283-307.
107. **Hoskins, L. C.** 1993. Mucin degradation in the human gastrointestinal tract and its significance to enteric microbial ecology. European Journal of Gastroenterology & Hepatology **5**.
108. **Hoskins, L. C., M. Agustines, W. B. McKee, E. T. Boulding, M. Kriaris, and G. Niedermeyer.** 1985. Mucin degradation in human colon ecosystems. Isolation and properties of fecal strains that degrade ABH blood group antigens and oligosaccharides from mucin glycoproteins. J Clin Invest **75:**944-53.
109. **Huang, L. H., and W. J. Syu.** 2008. GrlA of enterohemorrhagic Escherichia coli O157:H7 activates LEE1 by binding to the promoter region. J Microbiol Immunol Infect **41:**9-16.
110. **Hughes, D. T., M. B. Clarke, K. Yamamoto, D. A. Rasko, and V. Sperandio.** 2009. The QseC adrenergic signaling cascade in Enterohemorrhagic E. coli (EHEC). PLoS pathogens **5:**e1000553.
111. **Hughes, D. T., and V. Sperandio.** 2008. Inter-kingdom signalling: communication between bacteria and their hosts. Nature reviews. Microbiology **6:**111-20.

112. **Hughes, D. T., D. A. Terekhova, L. Liou, C. J. Hovde, J. W. Sahl, A. V. Patankar, J. E. Gonzalez, T. S. Edrington, D. A. Rasko, and V. Sperandio.** 2010. Chemical sensing in mammalian host-bacterial commensal associations. Proc Natl Acad Sci U S A **107:**9831-6.
113. **Humphries, R. M., T. P. Griener, S. L. Vogt, G. L. Mulvey, T. Raivio, M. S. Donnenberg, P. I. Kitov, M. Surette, and G. D. Armstrong.** 2010. N-acetyllactosamine-induced retraction of bundle-forming pili regulates virulence-associated gene expression in enteropathogenic Escherichia coli. Mol Microbiol **76:**1111-26.
114. **Hurd, E. A., J. M. Holmen, G. C. Hansson, and S. E. Domino.** 2005. Gastrointestinal mucins of Fut2-null mice lack terminal fucosylation without affecting colonization by Candida albicans. Glycobiology **15:**1002-7.
115. **Iizumi, Y., H. Sagara, Y. Kabe, M. Azuma, K. Kume, M. Ogawa, T. Nagai, P. G. Gillespie, C. Sasakawa, and H. Handa.** 2007. The enteropathogenic E. coli effector EspB facilitates microvillus effacing and antiphagocytosis by inhibiting myosin function. Cell host & microbe **2:**383-92.
116. **Islam, M. S., L. E. Bingle, M. J. Pallen, and S. J. Busby.** 2011. Organization of the LEE1 operon regulatory region of enterohaemorrhagic Escherichia coli O157:H7 and activation by GrlA. Mol Microbiol **79:**468-83.
117. **Island, M. D., and R. J. Kadner.** 1993. Interplay between the membrane-associated UhpB and UhpC regulatory proteins. J Bacteriol **175:**5028-34.
118. **Iuchi, S., and E. C. Lin.** 1988. arcA (dye), a global regulatory gene in Escherichia coli mediating repression of enzymes in aerobic pathways. Proc Natl Acad Sci U S A **85:**1888-92.
119. **Iyoda, S., and H. Watanabe.** 2004. Positive effects of multiple pch genes on expression of the locus of enterocyte effacement genes and adherence of enterohaemorrhagic Escherichia coli O157 : H7 to HEp-2 cells. Microbiology **150:**2357-571.
120. **Jacewicz, M., H. Clausen, E. Nudelman, A. Donohue-Rolfe, and G. T. Keusch.** 1986. Pathogenesis of shigella diarrhea. XI. Isolation of a shigella toxin-binding glycolipid from rabbit jejunum and HeLa cells and its identification as globotriaosylceramide. J Exp Med **163:**1391-404.
121. **Jacks, T. M., and P. J. Glantz.** 1967. Virulence of Escherichia coli Serotypes for Mice. J Bacteriol **93:**991-5.
122. **Jaeger, J. L., and D. W. Acheson.** 2000. Shiga Toxin-Producing Escherichia coli. Curr Infect Dis Rep **2:**61-67.

123. **Jaswal, V. M., H. S. Babbar, and A. Mahmood.** 1988. Changes in sialic acid and fucose contents of enterocytes across the crypt-villus axis in developing rat intestine. Biochem Med Metab Biol **39:**105-10.
124. **Jay, M. T., V. Garrett, J. C. Mohle-Boetani, M. Barros, J. A. Farrar, R. Rios, S. Abbott, R. Sowadsky, K. Komatsu, R. Mandrell, J. Sobel, and S. B. Werner.** 2004. A multistate outbreak of Escherichia coli O157:H7 infection linked to consumption of beef tacos at a fast-food restaurant chain. Clin Infect Dis **39:**1-7.
125. **Jenke, C., S. R. Leopold, T. Weniger, J. Rothganger, D. Harmsen, H. Karch, and A. Mellmann.** 2012. Identification of intermediate in evolutionary model of enterohemorrhagic Escherichia coli O157. Emerg Infect Dis **18:**582-8.
126. **Jerse, A. E., J. Yu, B. D. Tall, and J. B. Kaper.** 1990. A genetic locus of enteropathogenic Escherichia coli necessary for the production of attaching and effacing lesions on tissue culture cells. Proc Natl Acad Sci U S A **87:**7839-43.
127. **Jimenez, R., S. B. Cruz-Migoni, A. Huerta-Saquero, V. H. Bustamante, and J. L. Puente.** 2010. Molecular characterization of GrlA, a specific positive regulator of ler expression in enteropathogenic Escherichia coli. J Bacteriol **192:**4627-42.
128. **Johansen, B. K., Y. Wasteson, P. E. Granum, and S. Brynestad.** 2001. Mosaic structure of Shiga-toxin-2-encoding phages isolated from Escherichia coli O157:H7 indicates frequent gene exchange between lambdoid phage genomes. Microbiology **147:**1929-36.
129. **Johansson, M. E., J. M. Larsson, and G. C. Hansson.** 2011. The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. Proc Natl Acad Sci U S A **108 Suppl 1:**4659-65.
130. **John, M., I. T. Kudva, R. W. Griffin, A. W. Dodson, B. McManus, B. Krastins, D. Sarracino, A. Progulske-Fox, J. D. Hillman, M. Handfield, P. I. Tarr, and S. B. Calderwood.** 2005. Use of in vivo-induced antigen technology for identification of Escherichia coli O157:H7 proteins expressed during human infection. Infect Immun **73:**2665-79.
131. **Kamada, N., Y. G. Kim, H. P. Sham, B. A. Vallance, J. L. Puente, E. C. Martens, and G. Nunez.** 2012. Regulated Virulence Controls the Ability of a Pathogen to Compete with the Gut Microbiota. Science.
132. **Kang, G., A. B. Pulimood, R. Koshi, A. Hull, D. Acheson, P. Rajan, G. T. Keusch, V. I. Mathan, and M. M. Mathan.** 2001. A monkey model for enterohemorrhagic Escherichia coli infection. J Infect Dis **184:**206-10.
133. **Kaper, J. B., and M. A. Karmali.** 2008. The continuing evolution of a bacterial pathogen. Proc Natl Acad Sci U S A **105:**4535-6.

134. **Kaper, J. B., J. P. Nataro, and H. L. Mobley.** 2004. Pathogenic Escherichia coli. Nature reviews. Microbiology **2:**123-40.
135. **Karch, H., H. Russmann, H. Schmidt, A. Schwarzkopf, and J. Heesemann.** 1995. Long-term shedding and clonal turnover of enterohemorrhagic Escherichia coli O157 in diarrheal diseases. J Clin Microbiol **33:**1602-5.
136. **Karmali, M. A.** 2004. Prospects for preventing serious systemic toxemic complications of Shiga toxin-producing Escherichia coli infections using Shiga toxin receptor analogues. J Infect Dis **189:**355-9.
137. **Karmali, M. A., B. T. Steele, M. Petric, and C. Lim.** 1983. Sporadic cases of haemolytic-uraemic syndrome associated with faecal cytotoxin and cytotoxin-producing Escherichia coli in stools. Lancet **1:**619-20.
138. **Kendall, M. M., C. C. Gruber, D. A. Rasko, D. T. Hughes, and V. Sperandio.** 2011. Hfq Virulence Regulation in Enterohemorrhagic Escherichia coli O157:H7 Strain 86-24. J Bacteriol **193:**6843-51.
139. **Kendall, M. M., D. A. Rasko, and V. Sperandio.** 2007. Global effects of the cell-to-cell signaling molecules autoinducer-2, autoinducer-3, and epinephrine in a luxS mutant of enterohemorrhagic Escherichia coli. Infect Immun **75:**4875-84.
140. **Kendall, M. M., D. A. Rasko, and V. Sperandio.** 2010. The LysR-type regulator QseA regulates both characterized and putative virulence genes in enterohaemorrhagic Escherichia coli O157:H7. Mol Microbiol **76:**1306-21.
141. **Kenny, B., R. DeVinney, M. Stein, D. J. Reinscheid, E. A. Frey, and B. B. Finlay.** 1997. Enteropathogenic E. coli (EPEC) transfers its receptor for intimate adherence into mammalian cells. Cell **91:**511-20.
142. **Khajanchi, B. K., E. V. Kozlova, J. Sha, V. L. Popov, and A. K. Chopra.** 2012. The two-component QseBC signalling system regulates in vitro and in vivo virulence of Aeromonas hydrophila. Microbiology **158:**259-71.
143. **Kim, J., A. Thanabalasuriar, T. Chaworth-Musters, J. C. Fromme, E. A. Frey, P. I. Lario, P. Metalnikov, K. Rizg, N. A. Thomas, S. F. Lee, E. L. Hartland, P. R. Hardwidge, T. Pawson, N. C. Strynadka, B. B. Finlay, R. Schekman, and S. Gruenheid.** 2007. The bacterial virulence factor NleA inhibits cellular protein secretion by disrupting mammalian COPII function. Cell host & microbe **2:**160-71.
144. **Kim, S. H., W. Jia, R. E. Bishop, and C. Gyles.** 2004. An msbB homologue carried in plasmid pO157 encodes an acyltransferase involved in lipid A biosynthesis in Escherichia coli O157:H7. Infect Immun **72:**1174-80.

145. **Kim, Y., S. Oh, S. Park, and S. H. Kim.** 2009. Interactive transcriptome analysis of enterohemorrhagic Escherichia coli (EHEC) O157:H7 and intestinal epithelial HT-29 cells after bacterial attachment. Int J Food Microbiol **131:**224-32.
146. **Kim, Y. S., and S. B. Ho.** 2010. Intestinal goblet cells and mucins in health and disease: recent insights and progress. Curr Gastroenterol Rep **12:**319-30.
147. **Knutton, S., I. Rosenshine, M. J. Pallen, I. Nisan, B. C. Neves, C. Bain, C. Wolff, G. Dougan, and G. Frankel.** 1998. A novel EspA-associated surface organelle of enteropathogenic Escherichia coli involved in protein translocation into epithelial cells. Embo J **17:**2166-76.
148. **Kodama, T., Y. Akeda, G. Kono, A. Takahashi, K. Imura, T. Iida, and T. Honda.** 2002. The EspB protein of enterohaemorrhagic Escherichia coli interacts directly with alpha-catenin. Cell Microbiol **4:**213-22.
149. **Kostakioti, M., M. Hadjifrangiskou, C. K. Cusumano, T. J. Hannan, J. W. Janetka, and S. J. Hultgren.** 2012. Distinguishing the contribution of type 1 pili from that of other QseB-misregulated factors when QseC is absent during urinary tract infection. Infect Immun.
150. **Kostakioti, M., M. Hadjifrangiskou, J. S. Pinkner, and S. J. Hultgren.** 2009. QseC-mediated dephosphorylation of QseB is required for expression of genes associated with virulence in uropathogenic Escherichia coli. Mol Microbiol **73:**1020-31.
151. **Kozakova, H., J. Kolinska, Z. Lojda, Z. Rehakova, J. Sinkora, M. Zakostelecka, I. Splichal, and H. Tlaskalova-Hogenova.** 2006. Effect of bacterial monoassociation on brush-border enzyme activities in ex-germ-free piglets: comparison of commensal and pathogenic Escherichia coli strains. Microbes Infect **8:**2629-39.
152. **Kozlova, E. V., B. K. Khajanchi, V. L. Popov, J. Wen, and A. K. Chopra.** 2012. Impact of QseBC system in c-di-GMP-dependent quorum sensing regulatory network in a clinical isolate SSU of Aeromonas hydrophila. Microb Pathog.
153. **La Ragione, R. M., A. Best, M. J. Woodward, and A. D. Wales.** 2009. Escherichia coli O157:H7 colonization in small domestic ruminants. FEMS Microbiol Rev **33:**394-410.
154. **Laaberki, M. H., N. Janabi, E. Oswald, and F. Repoila.** 2006. Concert of regulators to switch on LEE expression in enterohemorrhagic Escherichia coli O157:H7: interplay between Ler, GrlA, HNS and RpoS. Int J Med Microbiol **296:**197-210.
155. **Larson, G., P. Falk, and L. C. Hoskins.** 1988. Degradation of human intestinal glycosphingolipids by extracellular glycosidases from mucin-degrading bacteria of the human fecal flora. J Biol Chem **263:**10790-8.

156. **Lathem, W. W., T. Bergsbaken, and R. A. Welch.** 2004. Potentiation of C1 esterase inhibitor by StcE, a metalloprotease secreted by Escherichia coli O157:H7. J Exp Med **199:**1077-87.
157. **Lathem, W. W., T. E. Grys, S. E. Witowski, A. G. Torres, J. B. Kaper, P. I. Tarr, and R. A. Welch.** 2002. StcE, a metalloprotease secreted by Escherichia coli O157:H7, specifically cleaves C1 esterase inhibitor. Mol Microbiol **45:**277-88.
158. **Laub, M. T., E. G. Biondi, and J. M. Skerker.** 2007. Phosphotransfer profiling: systematic mapping of two-component signal transduction pathways and phosphorelays. Methods Enzymol **423:**531-48.
159. **Laub, M. T., and M. Goulian.** 2007. Specificity in two-component signal transduction pathways. Annu Rev Genet **41:**121-45.
160. **Ley, R. E., C. A. Lozupone, M. Hamady, R. Knight, and J. I. Gordon.** 2008. Worlds within worlds: evolution of the vertebrate gut microbiota. Nature reviews. Microbiology **6:**776-88.
161. **Li, E., C. M. Hamm, A. S. Gulati, R. B. Sartor, H. Chen, X. Wu, T. Zhang, F. J. Rohlf, W. Zhu, C. Gu, C. E. Robertson, N. R. Pace, E. C. Boedeker, N. Harpaz, J. Yuan, G. M. Weinstock, E. Sodergren, and D. N. Frank.** 2012. Inflammatory Bowel Diseases Phenotype, C. difficile and NOD2 Genotype Are Associated with Shifts in Human Ileum Associated Microbial Composition. PloS one **7:**e26284.
162. **Li, M., I. Rosenshine, S. L. Tung, X. H. Wang, D. Friedberg, C. L. Hew, and K. Y. Leung.** 2004. Comparative proteomic analysis of extracellular proteins of enterohemorrhagic and enteropathogenic Escherichia coli strains and their ihf and ler mutants. Appl Environ Microbiol **70:**5274-82.
163. **Li, W. C., K. L. Ralphs, J. M. Slack, and D. Tosh.** 2007. Keratinocyte serum-free medium maintains long-term liver gene expression and function in cultured rat hepatocytes by preventing the loss of liver-enriched transcription factors. Int J Biochem Cell Biol **39:**541-54.
164. **Lim, J. Y., H. J. La, H. Sheng, L. J. Forney, and C. J. Hovde.** 2010. Influence of plasmid pO157 on Escherichia coli O157:H7 Sakai biofilm formation. Appl Environ Microbiol **76:**963-6.
165. **Lim, J. Y., J. Li, H. Sheng, T. E. Besser, K. Potter, and C. J. Hovde.** 2007. Escherichia coli O157:H7 colonization at the rectoanal junction of long-duration culture-positive cattle. Appl Environ Microbiol **73:**1380-2.
166. **Lim, J. Y., H. Sheng, K. S. Seo, Y. H. Park, and C. J. Hovde.** 2007. Characterization of an Escherichia coli O157:H7 plasmid O157 deletion mutant and its survival and persistence in cattle. Appl Environ Microbiol **73:**2037-47.

167. **Lindberg, A. A., J. E. Brown, N. Stromberg, M. Westling-Ryd, J. E. Schultz, and K. A. Karlsson.** 1987. Identification of the carbohydrate receptor for Shiga toxin produced by Shigella dysenteriae type 1. J Biol Chem **262:**1779-85.
168. **Lodato, P. B., and J. B. Kaper.** 2009. Post-transcriptional processing of the LEE4 operon in enterohaemorrhagic Escherichia coli. Mol Microbiol **71:**273-90.
169. **Lupp, C., M. L. Robertson, M. E. Wickham, I. Sekirov, O. L. Champion, E. C. Gaynor, and B. B. Finlay.** 2007. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. Cell host & microbe **2:**119-29.
170. **Magerl, M., V. Lammel, F. Siebenhaar, T. Zuberbier, M. Metz, and M. Maurer.** 2008. Non-pathogenic commensal Escherichia coli bacteria can inhibit degranulation of mast cells. Experimental dermatology **17:**427-35.
171. **Mallard, F., C. Antony, D. Tenza, J. Salamero, B. Goud, and L. Johannes.** 1998. Direct pathway from early/recycling endosomes to the Golgi apparatus revealed through the study of shiga toxin B-fragment transport. J Cell Biol **143:**973-90.
172. **Manning, S. D., A. S. Motiwala, A. C. Springman, W. Qi, D. W. Lacher, L. M. Ouellette, J. M. Mladonicky, P. Somsel, J. T. Rudrik, S. E. Dietrich, W. Zhang, B. Swaminathan, D. Alland, and T. S. Whittam.** 2008. Variation in virulence among clades of Escherichia coli O157:H7 associated with disease outbreaks. Proc Natl Acad Sci U S A **105:**4868-73.
173. **Martens, E. C., H. C. Chiang, and J. I. Gordon.** 2008. Mucosal glycan foraging enhances fitness and transmission of a saccharolytic human gut bacterial symbiont. Cell host & microbe **4:**447-57.
174. **Mascher, T., J. D. Helmann, and G. Unden.** 2006. Stimulus perception in bacterial signal-transducing histidine kinases. Microbiol Mol Biol Rev **70:**910-38.
175. **McCleary, W. R., and J. B. Stock.** 1994. Acetyl phosphate and the activation of two-component response regulators. J Biol Chem **269:**31567-72.
176. **McDaniel, T. K., K. G. Jarvis, M. S. Donnenberg, and J. B. Kaper.** 1995. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. Proc Natl Acad Sci U S A **92:**1664-8.
177. **McGannon, C. M., C. A. Fuller, and A. A. Weiss.** 2010. Different classes of antibiotics differentially influence shiga toxin production. Antimicrob Agents Chemother **54:**3790-8.

178. **McGuckin, M. A., S. K. Linden, P. Sutton, and T. H. Florin.** 2011. Mucin dynamics and enteric pathogens. Nature reviews. Microbiology **9:**265-78.
179. **McNamara, B. P., A. Koutsouris, C. B. O'Connell, J. P. Nougayrede, M. S. Donnenberg, and G. Hecht.** 2001. Translocated EspF protein from enteropathogenic Escherichia coli disrupts host intestinal barrier function. J Clin Invest **107:**621-9.
180. **Mellies, J. L., A. M. Barron, and A. M. Carmona.** 2007. Enteropathogenic and enterohemorrhagic Escherichia coli virulence gene regulation. Infect Immun **75:**4199-210.
181. **Mellies, J. L., G. Benison, W. McNitt, D. Mavor, C. Boniface, and F. J. Larabee.** 2011. Ler of pathogenic Escherichia coli forms toroidal protein-DNA complexes. Microbiology **157:**1123-33.
182. **Mellies, J. L., S. J. Elliott, V. Sperandio, M. S. Donnenberg, and J. B. Kaper.** 1999. The Per regulon of enteropathogenic Escherichia coli : identification of a regulatory cascade and a novel transcriptional activator, the locus of enterocyte effacement (LEE)-encoded regulator (Ler). Mol Microbiol **33:**296-306.
183. **Merighi, M., A. N. Septer, A. Carroll-Portillo, A. Bhatiya, S. Porwollik, M. McClelland, and J. S. Gunn.** 2009. Genome-wide analysis of the PreA/PreB (QseB/QseC) regulon of Salmonella enterica serovar Typhimurium. BMC Microbiol **9:**42.
184. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
185. **Miranda, R. L., T. Conway, M. P. Leatham, D. E. Chang, W. E. Norris, J. H. Allen, S. J. Stevenson, D. C. Laux, and P. S. Cohen.** 2004. Glycolytic and gluconeogenic growth of Escherichia coli O157:H7 (EDL933) and E. coli K-12 (MG1655) in the mouse intestine. Infect Immun **72:**1666-76.
186. **Mitsuoka, T., and K. Hayakawa.** 1973. [The fecal flora in man. I. Composition of the fecal flora of various age groups]. Zentralbl Bakteriol Orig A **223:**333-42.
187. **Mohawk, K. L., A. R. Melton-Celsa, T. Zangari, E. E. Carroll, and A. D. O'Brien.** 2010. Pathogenesis of Escherichia coli O157:H7 strain 86-24 following oral infection of BALB/c mice with an intact commensal flora. Microb Pathog **48:**131-42.
188. **Mohawk, K. L., and A. D. O'Brien.** 2011. Mouse models of Escherichia coli O157:H7 infection and shiga toxin injection. J Biomed Biotechnol **2011:**258185.
189. **Mokrievich, A. N., A. N. Kondakova, E. Valade, M. E. Platonov, G. M. Vakhrameeva, R. Z. Shaikhutdinova, R. I. Mironova, D. Blaha, I.**

V. Bakhteeva, G. M. Titareva, T. B. Kravchenko, T. I. Kombarova, D. Vidal, V. M. Pavlov, B. Lindner, I. A. Dyatlov, and Y. A. Knirel. 2010. Biological properties and structure of the lipopolysaccharide of a vaccine strain of Francisella tularensis generated by inactivation of a quorum sensing system gene qseC. Biochemistry (Mosc) **75:**443-51.

190. **Momose, Y., K. Hirayama, and K. Itoh.** 2008. Competition for proline between indigenous Escherichia coli and E. coli O157:H7 in gnotobiotic mice associated with infant intestinal microbiota and its contribution to the colonization resistance against E. coli O157:H7. Antonie Van Leeuwenhoek **94:**165-71.
191. **Montagne, L., R. Toullec, and J. P. Lalles.** 2000. Calf intestinal mucin: isolation, partial characterization, and measurement in ileal digesta with an enzyme-linked immunosorbent assay. J Dairy Sci **83:**507-17.
192. **Moon, H. W., S. C. Whipp, R. A. Argenzio, M. M. Levine, and R. A. Giannella.** 1983. Attaching and effacing activities of rabbit and human enteropathogenic Escherichia coli in pig and rabbit intestines. Infect Immun **41:**1340-51.
193. **Moreira, C. G., D. Weinshenker, and V. Sperandio.** 2010. QseC mediates Salmonella enterica serovar typhimurium virulence in vitro and in vivo. Infect Immun **78:**914-26.
194. **Muhldorfer, I., J. Hacker, G. T. Keusch, D. W. Acheson, H. Tschape, A. V. Kane, A. Ritter, T. Olschlager, and A. Donohue-Rolfe.** 1996. Regulation of the Shiga-like toxin II operon in Escherichia coli. Infect Immun **64:**495-502.
195. **Mukherjee, J., K. Chios, D. Fishwild, D. Hudson, S. O'Donnell, S. M. Rich, A. Donohue-Rolfe, and S. Tzipori.** 2002. Human Stx2-specific monoclonal antibodies prevent systemic complications of Escherichia coli O157:H7 infection. Infect Immun **70:**612-9.
196. **Mulder, D. T., C. A. Cooper, and B. K. Coombes.** 2012. Type VI Secretion System-Associated Gene Clusters Contribute to Pathogenesis of Salmonella enterica Serovar Typhimurium. Infect Immun **80:**1996-2007.
197. **Mundy, R., L. Petrovska, K. Smollett, N. Simpson, R. K. Wilson, J. Yu, X. Tu, I. Rosenshine, S. Clare, G. Dougan, and G. Frankel.** 2004. Identification of a novel Citrobacter rodentium type III secreted protein, EspI, and roles of this and other secreted proteins in infection. Infect Immun **72:**2288-302.
198. **Muthing, J., C. H. Schweppe, H. Karch, and A. W. Friedrich.** 2009. Shiga toxins, glycosphingolipid diversity, and endothelial cell injury. Thromb Haemost **101:**252-64.
199. **Myers, L. L.** 1975. Characterization of Escherichia coli obtained from newborn calves with diarrhea. Infect Immun **11:**493-6.

200. **Myhal, M. L., D. C. Laux, and P. S. Cohen.** 1982. Relative colonizing abilities of human fecal and K 12 strains of Escherichia coli in the large intestines of streptomycin-treated mice. Eur J Clin Microbiol **1:**186-92.
201. **Nakazato, G., C. Gyles, K. Ziebell, R. Keller, L. R. Trabulsi, T. A. Gomes, K. Irino, W. D. Da Silveira, and A. F. Pestana De Castro.** 2004. Attaching and effacing Escherichia coli isolated from dogs in Brazil: characteristics and serotypic relationship to human enteropathogenic E. coli (EPEC). Vet Microbiol **101:**269-77.
202. **Nataro, J. P., and J. B. Kaper.** 1998. Diarrheagenic Escherichia coli. Clin Microbiol Rev **11:**142-201.
203. **Nathanson, S., T. Kwon, M. Elmaleh, M. Charbit, E. A. Launay, J. Harambat, M. Brun, B. Ranchin, F. Bandin, S. Cloarec, G. Bourdat-Michel, C. Pietrement, G. Champion, T. Ulinski, and G. Deschenes.** 2010. Acute neurological involvement in diarrhea-associated hemolytic uremic syndrome. Clin J Am Soc Nephrol **5:**1218-28.
204. **Naylor, S. W., J. C. Low, T. E. Besser, A. Mahajan, G. J. Gunn, M. C. Pearce, I. J. McKendrick, D. G. E. Smith, and D. L. Gally.** 2003. Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic Escherichia coli O157 : H7 in the bovine host. Infection and Immunity **71:**1505-1512.
205. **Naylor, S. W., A. J. Roe, P. Nart, K. Spears, D. G. Smith, J. C. Low, and D. L. Gally.** 2005. Escherichia coli O157 : H7 forms attaching and effacing lesions at the terminal rectum of cattle and colonization requires the LEE4 operon. Microbiology **151:**2773-81.
206. **Neely, M. N., and D. I. Friedman.** 1998. Functional and genetic analysis of regulatory regions of coliphage H-19B: location of shiga-like toxin and lysis genes suggest a role for phage functions in toxin release. Mol Microbiol **28:**1255-67.
207. **Neil, K. P., G. Biggerstaff, J. K. Macdonald, E. Trees, C. Medus, K. A. Musser, S. G. Stroika, D. Zink, and M. J. Sotir.** 2012. A Novel Vehicle for Transmission of Escherichia coli O157:H7 to Humans: Multistate Outbreak of E. coli O157:H7 Infections Associated With Consumption of Ready-to-Bake Commercial Prepackaged Cookie Dough--United States, 2009. Clin Infect Dis **54:**511-8.
208. **Newton, H. J., J. S. Pearson, L. Badea, M. Kelly, M. Lucas, G. Holloway, K. M. Wagstaff, M. A. Dunstone, J. Sloan, J. C. Whisstock, J. B. Kaper, R. M. Robins-Browne, D. A. Jans, G. Frankel, A. D. Phillips, B. S. Coulson, and E. L. Hartland.** 2010. The type III effectors NleE and NleB from enteropathogenic E. coli and OspZ from Shigella block nuclear translocation of NF-kappaB p65. PLoS pathogens **6:**e1000898.

209. **Nikolskaya, A. N., A. Y. Mulkidjanian, I. B. Beech, and M. Y. Galperin.** 2003. MASE1 and MASE2: two novel integral membrane sensory domains. Journal of molecular microbiology and biotechnology **5:**11-6.
210. **Nishikawa, Y., Z. Zhou, A. Hase, J. Ogasawara, T. Cheasty, and K. Haruki.** 2000. Relationship of genetic type of Shiga toxin to manifestation of bloody diarrhea due to enterohemorrhagic Escherichia coli serogroup O157 isolates in Osaka City, Japan. J Clin Microbiol **38:**2440-2.
211. **Njoroge, J., and V. Sperandio.** 2012. Enterohemorrhagic Escherichia coli virulence regulation by two bacterial adrenergic kinases, QseC and QseE. Infect Immun **80:**688-703.
212. **Noris, M., and G. Remuzzi.** 2005. Hemolytic uremic syndrome. J Am Soc Nephrol **16:**1035-50.
213. **Novak, E. A., H. Shao, C. A. Daep, and D. R. Demuth.** 2010. Autoinducer-2 and QseC control biofilm formation and in vivo virulence of Aggregatibacter actinomycetemcomitans. Infect Immun **78:**2919-26.
214. **O'Brien, A. D., T. A. Lively, T. W. Chang, and S. L. Gorbach.** 1983. Purification of Shigella dysenteriae 1 (Shiga)-like toxin from Escherichia coli 0157:H7 strain associated with haemorrhagic colitis. Lancet **2:**573.
215. **O'Brien, A. D., J. W. Newland, S. F. Miller, R. K. Holmes, H. W. Smith, and S. B. Formal.** 1984. Shiga-like toxin-converting phages from Escherichia coli strains that cause hemorrhagic colitis or infantile diarrhea. Science **226:**694-6.
216. **O'Brien, A. O., T. A. Lively, M. E. Chen, S. W. Rothman, and S. B. Formal.** 1983. Escherichia coli O157:H7 strains associated with haemorrhagic colitis in the United States produce a Shigella dysenteriae 1 (SHIGA) like cytotoxin. Lancet **1:**702.
217. **O'Connell, C. B., E. A. Creasey, S. Knutton, S. Elliott, L. J. Crowther, W. Luo, M. J. Albert, J. B. Kaper, G. Frankel, and M. S. Donnenberg.** 2004. SepL, a protein required for enteropathogenic Escherichia coli type III translocation, interacts with secretion component SepD. Mol Microbiol **52:**1613-25.
218. **Oakes, R. S., R. L. Siegler, M. A. McReynolds, T. Pysher, and A. T. Pavia.** 2006. Predictors of fatality in postdiarrheal hemolytic uremic syndrome. Pediatrics **117:**1656-62.
219. **Ochman, H., J. G. Lawrence, and E. A. Groisman.** 2000. Lateral gene transfer and the nature of bacterial innovation. Nature **405:**299-304.
220. **Orth, D., S. Ehrlenbach, J. Brockmeyer, A. B. Khan, G. Huber, H. Karch, B. Sarg, H. Lindner, and R. Wurzner.** 2010. EspP, a serine protease of enterohemorrhagic Escherichia coli, impairs complement

activation by cleaving complement factors C3/C3b and C5. Infect Immun **78:**4294-301.

221. **Pacheco, A. R., and V. Sperandio.** 2009. Inter-kingdom signaling: chemical language between bacteria and host. Current Opinion in Microbiology **12:**192-8.
222. **Pacheco, A. R. S., V.** 2012. Shiga Toxin in Enterohemorrhagic *E.coli*: regulation and novel antivirulence strategies. Frontiers in Cellular and Infection Microbiology **in press**.
223. **Pai, C. H., J. K. Kelly, and G. L. Meyers.** 1986. Experimental infection of infant rabbits with verotoxin-producing Escherichia coli. Infect Immun **51:**16-23.
224. **Pao, S. S., I. T. Paulsen, and M. H. Saier, Jr.** 1998. Major facilitator superfamily. Microbiol Mol Biol Rev **62:**1-34.
225. **Peekhaus, N., and T. Conway.** 1998. What's for dinner?: Entner-Doudoroff metabolism in Escherichia coli. J Bacteriol **180:**3495-502.
226. **Penders, J., C. Thijs, C. Vink, F. F. Stelma, B. Snijders, I. Kummeling, P. A. van den Brandt, and E. E. Stobberingh.** 2006. Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics **118:**511-21.
227. **Perna, N. T., G. Plunkett, 3rd, V. Burland, B. Mau, J. D. Glasner, D. J. Rose, G. F. Mayhew, P. S. Evans, J. Gregor, H. A. Kirkpatrick, G. Posfai, J. Hackett, S. Klink, A. Boutin, Y. Shao, L. Miller, E. J. Grotbeck, N. W. Davis, A. Lim, E. T. Dimalanta, K. D. Potamousis, J. Apodaca, T. S. Anantharaman, J. Lin, G. Yen, D. C. Schwartz, R. A. Welch, and F. R. Blattner.** 2001. Genome sequence of enterohaemorrhagic Escherichia coli O157:H7. Nature **409:**529-33.
228. **Phillips, A. D., S. Navabpour, S. Hicks, G. Dougan, T. Wallis, and G. Frankel.** 2000. Enterohaemorrhagic Escherichia coli O157:H7 target Peyer's patches in humans and cause attaching/effacing lesions in both human and bovine intestine. Gut **47:**377-81.
229. **Pullinger, G. D., S. C. Carnell, F. F. Sharaff, P. M. van Diemen, F. Dziva, E. Morgan, M. Lyte, P. P. Freestone, and M. P. Stevens.** 2010. Norepinephrine augments Salmonella enterica-induced enteritis in a manner associated with increased net replication but independent of the putative adrenergic sensor kinases QseC and QseE. Infect Immun **78:**372-80.
230. **Pullinger, G. D., P. M. van Diemen, F. Dziva, and M. P. Stevens.** 2010. Role of two-component sensory systems of Salmonella enterica serovar Dublin in the pathogenesis of systemic salmonellosis in cattle. Microbiology **156:**3108-22.

231. **Rangel, J. M., P. H. Sparling, C. Crowe, P. M. Griffin, and D. L. Swerdlow.** 2005. Epidemiology of Escherichia coli O157:H7 outbreaks, United States, 1982-2002. Emerg Infect Dis **11:**603-9.
232. **Rasko, D. A., C. G. Moreira, R. Li de, N. C. Reading, J. M. Ritchie, M. K. Waldor, N. Williams, R. Taussig, S. Wei, M. Roth, D. T. Hughes, J. F. Huntley, M. W. Fina, J. R. Falck, and V. Sperandio.** 2008. Targeting QseC signaling and virulence for antibiotic development. Science **321:**1078-80.
233. **Rasko, D. A., M. J. Rosovitz, G. S. Myers, E. F. Mongodin, W. F. Fricke, P. Gajer, J. Crabtree, M. Sebaihia, N. R. Thomson, R. Chaudhuri, I. R. Henderson, V. Sperandio, and J. Ravel.** 2008. The pangenome structure of Escherichia coli: comparative genomic analysis of E. coli commensal and pathogenic isolates. J Bacteriol **190:**6881-93.
234. **Reading, N. C., D. Rasko, A. G. Torres, and V. Sperandio.** 2010. A transcriptome study of the QseEF two-component system and the QseG membrane protein in enterohaemorrhagic Escherichia coli O157 : H7. Microbiology **156:**1167-75.
235. **Reading, N. C., D. A. Rasko, A. G. Torres, and V. Sperandio.** 2009. The two-component system QseEF and the membrane protein QseG link adrenergic and stress sensing to bacterial pathogenesis. Proc Natl Acad Sci U S A **106:**5889-94.
236. **Reading, N. C., A. G. Torres, M. M. Kendall, D. T. Hughes, K. Yamamoto, and V. Sperandio.** 2007. A novel two-component signaling system that activates transcription of an enterohemorrhagic Escherichia coli effector involved in remodeling of host actin. J Bacteriol **189:**2468-76.
237. **Reeves, A. R., J. N. D'Elia, J. Frias, and A. A. Salyers.** 1996. A Bacteroides thetaiotaomicron outer membrane protein that is essential for utilization of maltooligosaccharides and starch. J Bacteriol **178:**823-30.
238. **Reiss, G., P. Kunz, D. Koin, and E. B. Keeffe.** 2006. Escherichia coli O157:H7 infection in nursing homes: review of literature and report of recent outbreak. J Am Geriatr Soc **54:**680-4.
239. **Riley, L. W., R. S. Remis, S. D. Helgerson, H. B. McGee, J. G. Wells, B. R. Davis, R. J. Hebert, E. S. Olcott, L. M. Johnson, N. T. Hargrett, P. A. Blake, and M. L. Cohen.** 1983. Hemorrhagic colitis associated with a rare Escherichia coli serotype. N Engl J Med **308:**681-5.
240. **Ritchie, J. M., M. J. Brady, K. N. Riley, T. D. Ho, K. G. Campellone, I. M. Herman, A. Donohue-Rolfe, S. Tzipori, M. K. Waldor, and J. M. Leong.** 2008. EspFU, a type III-translocated effector of actin assembly, fosters epithelial association and late-stage intestinal colonization by E. coli O157:H7. Cellular Microbiology **10:**836-47.

241. **Ritchie, J. M., C. M. Thorpe, A. B. Rogers, and M. K. Waldor.** 2003. Critical roles for stx2, eae, and tir in enterohemorrhagic Escherichia coli-induced diarrhea and intestinal inflammation in infant rabbits. Infect Immun **71:**7129-39.
242. **Ritchie, J. M., and M. K. Waldor.** 2005. The locus of enterocyte effacement-encoded effector proteins all promote enterohemorrhagic Escherichia coli pathogenicity in infant rabbits. Infect Immun **73:**1466-74.
243. **Rivas, L., B. Coffey, O. McAuliffe, M. J. McDonnell, C. M. Burgess, A. Coffey, R. P. Ross, and G. Duffy.** 2010. In Vivo and Ex Vivo Evaluations of Bacteriophages e11/2 and e4/1c for Use in the Control of Escherichia coli O157:H7. Applied and Environmental Microbiology **76:**7210-7216.
244. **Robbe, C., C. Capon, B. Coddeville, and J. C. Michalski.** 2004. Structural diversity and specific distribution of O-glycans in normal human mucins along the intestinal tract. Biochem J **384:**307-16.
245. **Robbe, C., C. Capon, C. Flahaut, and J. C. Michalski.** 2003. Microscale analysis of mucin-type O-glycans by a coordinated fluorophore-assisted carbohydrate electrophoresis and mass spectrometry approach. Electrophoresis **24:**611-21.
246. **Rojas-Lopez, M., M. M. Arenas-Hernandez, A. Medrano-Lopez, C. F. Martinez de la Pena, J. L. Puente, Y. Martinez-Laguna, and A. G. Torres.** 2011. Regulatory control of the Escherichia coli O157:H7 lpf1 operon by H-NS and Ler. J Bacteriol **193:**1622-32.
247. **Romer, W., L. Berland, V. Chambon, K. Gaus, B. Windschiegl, D. Tenza, M. R. Aly, V. Fraisier, J. C. Florent, D. Perrais, C. Lamaze, G. Raposo, C. Steinem, P. Sens, P. Bassereau, and L. Johannes.** 2007. Shiga toxin induces tubular membrane invaginations for its uptake into cells. Nature **450:**670-5.
248. **Russell, R. M., F. C. Sharp, D. A. Rasko, and V. Sperandio.** 2007. QseA and GrlR/GrlA regulation of the locus of enterocyte effacement genes in enterohemorrhagic Escherichia coli. J Bacteriol **189:**5387-92.
249. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, second ed. Cold Spring Harbor Laboratory Press.
250. **Sanchez-SanMartin, C., V. H. Bustamante, E. Calva, and J. L. Puente.** 2001. Transcriptional regulation of the orf19 gene and the tir-cesT-eae operon of enteropathogenic Escherichia coli. J Bacteriol **183:**2823-33.
251. **Sandvig, K., O. Garred, K. Prydz, J. V. Kozlov, S. H. Hansen, and B. van Deurs.** 1992. Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum. Nature **358:**510-2.

252. **Savageau, M. A.** 1983. Escherichia-Coli Habitats, Cell-Types, and Molecular Mechanisms of Gene-Control. American Naturalist **122:**732-744.
253. **Schauer, D. B., and S. Falkow.** 1993. Attaching and effacing locus of a Citrobacter freundii biotype that causes transmissible murine colonic hyperplasia. Infect Immun **61:**2486-92.
254. **Schmidt, H., L. Beutin, and H. Karch.** 1995. Molecular analysis of the plasmid-encoded hemolysin of Escherichia coli O157:H7 strain EDL 933. Infect Immun **63:**1055-61.
255. **Schmidt, H., B. Henkel, and H. Karch.** 1997. A gene cluster closely related to type II secretion pathway operons of gram-negative bacteria is located on the large plasmid of enterohemorrhagic Escherichia coli O157 strains. FEMS Microbiol Lett **148:**265-72.
256. **Sekirov, I., S. L. Russell, L. C. Antunes, and B. B. Finlay.** 2010. Gut microbiota in health and disease. Physiol Rev **90:**859-904.
257. **Shakhnovich, E. A., B. M. Davis, and M. K. Waldor.** 2009. Hfq negatively regulates type III secretion in EHEC and several other pathogens. Mol Microbiol **74:**347-63.
258. **Shames, S. R., W. Deng, J. A. Guttman, C. L. de Hoog, Y. Li, P. R. Hardwidge, H. P. Sham, B. A. Vallance, L. J. Foster, and B. B. Finlay.** 2010. The pathogenic E. coli type III effector EspZ interacts with host CD98 and facilitates host cell prosurvival signalling. Cell Microbiol **12:**1322-39.
259. **Sharma, V. K., S. A. Carlson, and T. A. Casey.** 2005. Hyperadherence of an hha mutant of Escherichia coli O157:H7 is correlated with enhanced expression of LEE-encoded adherence genes. FEMS Microbiol Lett **243:**189-96.
260. **Sharma, V. K., and R. L. Zuerner.** 2004. Role of hha and ler in transcriptional regulation of the esp operon of enterohemorrhagic Escherichia coli O157:H7. J Bacteriol **186:**7290-301.
261. **Sharp, F. C., and V. Sperandio.** 2007. QseA directly activates transcription of LEE1 in enterohemorrhagic Escherichia coli. Infect Immun **75:**2432-40.
262. **Sheng, H., J. Y. Lim, H. J. Knecht, J. Li, and C. J. Hovde.** 2006. Role of Escherichia coli O157:H7 virulence factors in colonization at the bovine terminal rectal mucosa. Infect Immun **74:**4685-93.
263. **Snider, T. A., A. J. Fabich, T. Conway, and K. D. Clinkenbeard.** 2009. E. coli O157:H7 catabolism of intestinal mucin-derived carbohydrates and colonization. Vet Microbiol **136:**150-4.

264. **Snoeck, V., B. Goddeeris, and E. Cox.** 2005. The role of enterocytes in the intestinal barrier function and antigen uptake. Microbes Infect **7:**997-1004.
265. **Sodha, S. V., M. Lynch, K. Wannemuehler, M. Leeper, M. Malavet, J. Schaffzin, T. Chen, A. Langer, M. Glenshaw, D. Hoefer, N. Dumas, L. Lind, M. Iwamoto, T. Ayers, T. Nguyen, M. Biggerstaff, C. Olson, A. Sheth, and C. Braden.** 2011. Multistate outbreak of Escherichia coli O157:H7 infections associated with a national fast-food chain, 2006: a study incorporating epidemiological and food source traceback results. Epidemiol Infect **139:**309-16.
266. **Spencer, H., M. H. Karavolos, D. M. Bulmer, P. Aldridge, S. R. Chhabra, K. Winzer, P. Williams, and C. M. Khan.** 2010. Genome-wide transposon mutagenesis identifies a role for host neuroendocrine stress hormones in regulating the expression of virulence genes in Salmonella. J Bacteriol **192:**714-24.
267. **Sperandio, V., J. L. Mellies, R. M. Delahay, G. Frankel, J. A. Crawford, W. Nguyen, and J. B. Kaper.** 2000. Activation of enteropathogenic Escherichia coli (EPEC) LEE2 and LEE3 operons by Ler. Mol Microbiol **38:**781-93.
268. **Sperandio, V., J. L. Mellies, W. Nguyen, S. Shin, and J. B. Kaper.** 1999. Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic Escherichia coli. Proc Natl Acad Sci U S A **96:**15196-201.
269. **Sperandio, V., A. G. Torres, B. Jarvis, J. P. Nataro, and J. B. Kaper.** 2003. Bacteria-host communication: the language of hormones. Proc Natl Acad Sci U S A **100:**8951-6.
270. **Sperandio, V., A. G. Torres, and J. B. Kaper.** 2002. Quorum sensing Escherichia coli regulators B and C (QseBC): a novel two-component regulatory system involved in the regulation of flagella and motility by quorum sensing in E. coli. Mol Microbiol **43:**809-21.
271. **Sperandio, V., A. G. Torres, and J. B. Kaper.** 2002. Quorum sensing *Escherichia coli* regulators B and C (QseBC): a novel two-component regulatory system involved in the regulation of flagella and motility by quorum sensing in *E. coli*. Mol. Microbiol. **43:**809-821.
272. **Stavric, S., B. Buchanan, and T. M. Gleeson.** 1993. Intestinal colonization of young chicks with Escherichia coli O157:H7 and other verotoxin-producing serotypes. J Appl Bacteriol **74:**557-63.
273. **Stecher, B., and W. D. Hardt.** 2008. The role of microbiota in infectious disease. Trends Microbiol **16:**107-14.

274. **Stein, P. E., A. Boodhoo, G. J. Tyrrell, J. L. Brunton, and R. J. Read.** 1992. Crystal structure of the cell-binding B oligomer of verotoxin-1 from E. coli. Nature **355:**748-50.
275. **Stock, A. M., V. L. Robinson, and P. N. Goudreau.** 2000. Two-component signal transduction. Annu Rev Biochem **69:**183-215.
276. **Sueyoshi, M., and M. Nakazawa.** 1994. Experimental infection of young chicks with attaching and effacing Escherichia coli. Infect Immun **62:**4066-71.
277. **Tarr, P. I., C. A. Gordon, and W. L. Chandler.** 2005. Shiga-toxin-producing Escherichia coli and haemolytic uraemic syndrome. Lancet **365:**1073-86.
278. **Tatsuno, I., M. Horie, H. Abe, T. Miki, K. Makino, H. Shinagawa, H. Taguchi, S. Kamiya, T. Hayashi, and C. Sasakawa.** 2001. toxB gene on pO157 of enterohemorrhagic Escherichia coli O157:H7 is required for full epithelial cell adherence phenotype. Infect Immun **69:**6660-9.
279. **Terahara, K., T. Nochi, M. Yoshida, Y. Takahashi, Y. Goto, H. Hatai, S. Kurokawa, M. H. Jang, M. N. Kweon, S. E. Domino, T. Hiroi, Y. Yuki, Y. Tsunetsugu-Yokota, K. Kobayashi, and H. Kiyono.** 2011. Distinct fucosylation of M cells and epithelial cells by Fut1 and Fut2, respectively, in response to intestinal environmental stress. Biochem Biophys Res Commun **404:**822-8.
280. **Tetsch, L., and K. Jung.** 2009. The regulatory interplay between membrane-integrated sensors and transport proteins in bacteria. Mol Microbiol **73:**982-91.
281. **Tetsch, L., C. Koller, I. Haneburger, and K. Jung.** 2008. The membrane-integrated transcriptional activator CadC of Escherichia coli senses lysine indirectly via the interaction with the lysine permease LysP. Mol Microbiol **67:**570-83.
282. **Thiennimitr, P., S. E. Winter, M. G. Winter, M. N. Xavier, V. Tolstikov, D. L. Huseby, T. Sterzenbach, R. M. Tsolis, J. R. Roth, and A. J. Baumler.** 2011. Intestinal inflammation allows Salmonella to use ethanolamine to compete with the microbiota. Proc Natl Acad Sci U S A **108:**17480-5.
283. **Thompson, J. S., D. S. Hodge, and A. A. Borczyk.** 1990. Rapid biochemical test to identify verocytotoxin-positive strains of Escherichia coli serotype O157. J Clin Microbiol **28:**2165-8.
284. **Tilden, J., Jr., W. Young, A. M. McNamara, C. Custer, B. Boesel, M. A. Lambert-Fair, J. Majkowski, D. Vugia, S. B. Werner, J. Hollingsworth, and J. G. Morris, Jr.** 1996. A new route of transmission for Escherichia coli: infection from dry fermented salami. Am J Public Health **86:**1142-5.

285. **Tobe, T., H. Ando, H. Ishikawa, H. Abe, K. Tashiro, T. Hayashi, S. Kuhara, and N. Sugimoto.** 2005. Dual regulatory pathways integrating the RcsC-RcsD-RcsB signalling system control enterohaemorrhagic Escherichia coli pathogenicity. Mol Microbiol **58:**320-33.
286. **Tobe, T., S. A. Beatson, H. Taniguchi, H. Abe, C. M. Bailey, A. Fivian, R. Younis, S. Matthews, O. Marches, G. Frankel, T. Hayashi, and M. J. Pallen.** 2006. An extensive repertoire of type III secretion effectors in Escherichia coli O157 and the role of lambdoid phages in their dissemination. Proceedings of the National Academy of Sciences of the United States of America **103:**14941-6.
287. **Tonetti, M., L. Sturla, A. Bisso, D. Zanardi, U. Benatti, and A. De Flora.** 1998. The metabolism of 6-deoxyhexoses in bacterial and animal cells. Biochimie **80:**923-31.
288. **Trebichavsky, I., I. Splichal, V. Rada, and A. Splichalova.** 2010. Modulation of natural immunity in the gut by Escherichia coli strain Nissle 1917. Nutr Rev **68:**459-64.
289. **Tu, X., I. Nisan, C. Yona, E. Hanski, and I. Rosenshine.** 2003. EspH, a new cytoskeleton-modulating effector of enterohaemorrhagic and enteropathogenic Escherichia coli. Mol Microbiol **47:**595-606.
290. **Tyler, J. S., M. J. Mills, and D. I. Friedman.** 2004. The operator and early promoter region of the Shiga toxin type 2-encoding bacteriophage 933W and control of toxin expression. J Bacteriol **186:**7670-9.
291. **Tzipori, S., J. Montanaro, R. M. Robins-Browne, P. Vial, R. Gibson, and M. M. Levine.** 1992. Studies with enteroaggregative Escherichia coli in the gnotobiotic piglet gastroenteritis model. Infect Immun **60:**5302-6.
292. **Tzipori, S., I. K. Wachsmuth, C. Chapman, R. Birden, J. Brittingham, C. Jackson, and J. Hogg.** 1986. The pathogenesis of hemorrhagic colitis caused by Escherichia coli O157:H7 in gnotobiotic piglets. J Infect Dis **154:**712-6.
293. **Ulrich, L. E., and I. B. Zhulin.** 2010. The MiST2 database: a comprehensive genomics resource on microbial signal transduction. Nucleic Acids Res **38:**D401-7.
294. **Umanski, T., I. Rosenshine, and D. Friedberg.** 2002. Thermoregulated expression of virulence genes in enteropathogenic Escherichia coli. Microbiology **148:**2735-44.
295. **Vareille, M., T. de Sablet, T. Hindre, C. Martin, and A. P. Gobert.** 2007. Nitric oxide inhibits Shiga-toxin synthesis by enterohemorrhagic Escherichia coli. Proc Natl Acad Sci U S A **104:**10199-204.
296. **Vilte, D. A., M. Larzabal, S. Garbaccio, M. Gammella, B. C. Rabinovitz, A. M. Elizondo, R. J. C. Cantet, F. Delgado, V. Meikle, A. Cataldi, and E. C. Mercado.** 2011. Reduced faecal shedding of

Escherichia coli O157:H7 in cattle following systemic vaccination with gamma-intimin C(280) and EspB proteins. Vaccine **29:**3962-3968.

297. **Vingadassalom, D., K. G. Campellone, M. J. Brady, B. Skehan, S. E. Battle, D. Robbins, A. Kapoor, G. Hecht, S. B. Snapper, and J. M. Leong.** 2010. Enterohemorrhagic E. coli requires N-WASP for efficient type III translocation but not for EspFU-mediated actin pedestal formation. PLoS pathogens **6:**e1001056.
298. **Vingadassalom, D., A. Kazlauskas, B. Skehan, H. C. Cheng, L. Magoun, D. Robbins, M. K. Rosen, K. Saksela, and J. M. Leong.** 2009. Insulin receptor tyrosine kinase substrate links the E. coli O157:H7 actin assembly effectors Tir and EspF(U) during pedestal formation. Proc Natl Acad Sci U S A **106:**6754-9.
299. **Vlisidou, I., F. Dziva, R. M. La Ragione, A. Best, J. Garmendia, P. Hawes, P. Monaghan, S. A. Cawthraw, G. Frankel, M. J. Woodward, and M. P. Stevens.** 2006. Role of intimin-tir interactions and the tir-cytoskeleton coupling protein in the colonization of calves and lambs by Escherichia coli O157:H7. Infect Immun **74:**758-64.
300. **Vogel, J., and B. F. Luisi.** 2011. Hfq and its constellation of RNA. Nature reviews. Microbiology **9:**578-89.
301. **Wada, Y., M. Nakazawa, and M. Kubo.** 1994. Natural infection with attaching and effacing Escherichia coli (O15) in an adult cow. J Vet Med Sci **56:**151-2.
302. **Wadolkowski, E. A., J. A. Burris, and A. D. O'Brien.** 1990. Mouse model for colonization and disease caused by enterohemorrhagic Escherichia coli O157:H7. Infect Immun **58:**2438-45.
303. **Wagner, P. L., J. Livny, M. N. Neely, D. W. Acheson, D. I. Friedman, and M. K. Waldor.** 2002. Bacteriophage control of Shiga toxin 1 production and release by Escherichia coli. Mol Microbiol **44:**957-70.
304. **Wainwright, L. A., and J. B. Kaper.** 1998. EspB and EspD require a specific chaperone for proper secretion from enteropathogenic Escherichia coli. Mol Microbiol **27:**1247-60.
305. **Waldor, M. K., and D. I. Friedman.** 2005. Phage regulatory circuits and virulence gene expression. Curr Opin Microbiol **8:**459-65.
306. **Walters, M., and V. Sperandio.** 2006. Autoinducer 3 and epinephrine signaling in the kinetics of locus of enterocyte effacement gene expression in enterohemorrhagic Escherichia coli. Infect Immun **74:**5445-55.
307. **Walters, M., and V. Sperandio.** 2006. Quorum sensing in Escherichia coli and Salmonella. Int J Med Microbiol **296:**125-31.
308. **Wang, L., D. Rothemund, H. Curd, and P. R. Reeves.** 2003. Species-wide variation in the Escherichia coli flagellin (H-antigen) gene. J Bacteriol **185:**2936-43.

309. **Wang, X., Q. Wang, M. Yang, J. Xiao, Q. Liu, H. Wu, and Y. Zhang.** 2011. QseBC controls flagellar motility, fimbrial hemagglutination and intracellular virulence in fish pathogen Edwardsiella tarda. Fish Shellfish Immunol **30:**944-53.
310. **Warshawsky, B., I. Gutmanis, B. Henry, J. Dow, J. Reffle, G. Pollett, R. Ahmed, J. Aldom, D. Alves, A. Chagla, B. Ciebin, F. Kolbe, F. Jamieson, and F. Rodgers.** 2002. Outbreak of Escherichia coli 0157:H7 related to animal contact at a petting zoo. Can J Infect Dis **13:**175-81.
311. **Weiss, S. M., M. Ladwein, D. Schmidt, J. Ehinger, S. Lommel, K. Stading, U. Beutling, A. Disanza, R. Frank, L. Jansch, G. Scita, F. Gunzer, K. Rottner, and T. E. Stradal.** 2009. IRSp53 links the enterohemorrhagic E. coli effectors Tir and EspFU for actin pedestal formation. Cell host & microbe **5:**244-58.
312. **Welch, R. A.** 2006. The genus Escherichia. Prokaryotes **6:**60-71.
313. **Wells, J. G., B. R. Davis, I. K. Wachsmuth, L. W. Riley, R. S. Remis, R. Sokolow, and G. K. Morris.** 1983. Laboratory investigation of hemorrhagic colitis outbreaks associated with a rare Escherichia coli serotype. J Clin Microbiol **18:**512-20.
314. **Wendel, A. M., D. H. Johnson, U. Sharapov, J. Grant, J. R. Archer, T. Monson, C. Koschmann, and J. P. Davis.** 2009. Multistate outbreak of Escherichia coli O157:H7 infection associated with consumption of packaged spinach, August-September 2006: the Wisconsin investigation. Clin Infect Dis **48:**1079-86.
315. **Weston, L. A., and R. J. Kadner.** 1987. Identification of uhp polypeptides and evidence for their role in exogenous induction of the sugar phosphate transport system of Escherichia coli K-12. J Bacteriol **169:**3546-55.
316. **Whittam, T. S., M. L. Wolfe, I. K. Wachsmuth, F. Orskov, I. Orskov, and R. A. Wilson.** 1993. Clonal relationships among Escherichia coli strains that cause hemorrhagic colitis and infantile diarrhea. Infect Immun **61:**1619-29.
317. **Wick, L. M., W. Qi, D. W. Lacher, and T. S. Whittam.** 2005. Evolution of genomic content in the stepwise emergence of Escherichia coli O157:H7. J Bacteriol **187:**1783-91.
318. **Wilson, R. K., R. K. Shaw, S. Daniell, S. Knutton, and G. Frankel.** 2001. Role of EscF, a putative needle complex protein, in the type III protein translocation system of enteropathogenic Escherichia coli. Cell Microbiol **3:**753-62.
319. **Winter, S. E., P. Thiennimitr, M. G. Winter, B. P. Butler, D. L. Huseby, R. W. Crawford, J. M. Russell, C. L. Bevins, L. G. Adams, R.**

M. Tsolis, J. R. Roth, and A. J. Baumler. 2010. Gut inflammation provides a respiratory electron acceptor for Salmonella. Nature **467:**426-9.

320. **Wong, A. R., J. S. Pearson, M. D. Bright, D. Munera, K. S. Robinson, S. F. Lee, G. Frankel, and E. L. Hartland.** 2011. Enteropathogenic and enterohaemorrhagic Escherichia coli: even more subversive elements. Mol Microbiol **80:**1420-38.
321. **Wong, C. S., S. Jelacic, R. L. Habeeb, S. L. Watkins, and P. I. Tarr.** 2000. The risk of the hemolytic-uremic syndrome after antibiotic treatment of Escherichia coli O157:H7 infections. N Engl J Med **342:**1930-6.
322. **Woods, J. B., C. K. Schmitt, S. C. Darnell, K. C. Meysick, and A. D. O'Brien.** 2002. Ferrets as a model system for renal disease secondary to intestinal infection with Escherichia coli O157:H7 and other Shiga toxin-producing E. coli. J Infect Dis **185:**550-4.
323. **Yamamoto, K., K. Hirao, T. Oshima, H. Aiba, R. Utsumi, and A. Ishihama.** 2005. Functional characterization in vitro of all two-component signal transduction systems from Escherichia coli. J Biol Chem **280:**1448-56.
324. **Zhang, L., R. R. Chaudhuri, C. Constantinidou, J. L. Hobman, M. D. Patel, A. C. Jones, D. Sarti, A. J. Roe, I. Vlisidou, R. K. Shaw, F. Falciani, M. P. Stevens, D. L. Gally, S. Knutton, G. Frankel, C. W. Penn, and M. J. Pallen.** 2004. Regulators encoded in the Escherichia coli type III secretion system 2 gene cluster influence expression of genes within the locus for enterocyte effacement in enterohemorrhagic E. coli O157:H7. Infect Immun **72:**7282-93.
325. **Zhang, X., A. D. McDaniel, L. E. Wolf, G. T. Keusch, M. K. Waldor, and D. W. Acheson.** 2000. Quinolone antibiotics induce Shiga toxin-encoding bacteriophages, toxin production, and death in mice. J Infect Dis **181:**664-70.
326. **Zhou, L., X. H. Lei, B. R. Bochner, and B. L. Wanner.** 2003. Phenotype microarray analysis of Escherichia coli K-12 mutants with deletions of all two-component systems. J Bacteriol **185:**4956-72.
327. **Zhu, C., S. Feng, T. E. Thate, J. B. Kaper, and E. C. Boedeker.** 2006. Towards a vaccine for attaching/effacing Escherichia coli: a LEE encoded regulator (ler) mutant of rabbit enteropathogenic Escherichia coli is attenuated, immunogenic, and protects rabbits from lethal challenge with the wild-type virulent strain. Vaccine **24:**3845-55.
328. **Zhu, Y., and E. C. Lin.** 1986. An evolvant of Escherichia coli that employs the L-fucose pathway also for growth on L-galactose and D-arabinose. J Mol Evol **23:**259-66.

329. **Zoja, C., D. Corna, C. Farina, G. Sacchi, C. Lingwood, M. P. Doyle, V. V. Padhye, M. Abbate, and G. Remuzzi.** 1992. Verotoxin glycolipid receptors determine the localization of microangiopathic process in rabbits given verotoxin-1. J Lab Clin Med **120:**229-38.